Evaluating the role of social attention in the causal path to Autism Spectrum Disorder

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Anna Gui

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Department of Psychological Sciences
Birkbeck, University of London

ORIGINALITY STATEMENT

I, Anna Gui, hereby declare that, except where explicit attribution is made, the work presented in this thesis is entirely my own.

My PhD was based on analyses of longitudinal data from the Phase 1 and 2 cohort of the British Autism Study of Infant Siblings (BASIS) and from the Studying Autism and ADHD Risk (STAARS, also known as BASIS Phase 3), which have been collected and pre-processed by the BASIS team members as detailed below. My contribution to data collection was to coordinate the BASIS – Genome (or gBASIS) project for which I collected DNA and behavioural data from family members of children who participated in BASIS Phase 1 and 2. For what specifically concerns this thesis, I cured ethical amendment approval, recruitment and data collection for all salivary DNA samples (N=498), computerized task (N=249) and parent-report questionnaires (N=347). Further, I prepared DNA samples for extraction and carried out the necessary quality-check steps of genotype data following imputation. I also supervised the data entry and performed t-score calculation of the all the parent-report questionnaires collected as part of gBASIS.

The following contributions to the work presented in this thesis are acknowledged:

The BASIS team conceived and collected data for the infant and toddler data which have been used in all experimental chapters of this thesis: Mullen Scales for Early Learning behavioural assessment, Autism Observation Scale for Infants behavioural assessment, Face/Noise EEG task, face pop-out eye-tracking task, gaze following eye-tracking task, gap-overlap video-coded and eye-tracking task, all parent-report questionnaires and DNA samples from cheek-swabs for genotype and DNA methylation analyses. Specifically,

Jannath Begun Ali supervised the data entry and t-score calculation of the parent-report questionnaire and behavioural assessment measures collected at T1 and T2, used in Chapters 2, 3 and 6.

Greg Pasco supervised the data entry and t-score calculation of the parent-report dimensional outcome measures at T3 and T4 used in Chapter 2, 3, 4, 5 and 6.

Mayada Elsabbagh pre-processed the BASIS Phase 1 Face/Noise EEG data used in Chapter 2, the Phase 1 gap-overlap video-coded data used in Chapter 3 and the Phase 1 face-popout eye-tracking data used in Chapter 3, 4 and 5.

Charlotte Tye pre-processed the BASIS Phase 2 EEG data used in Chapter 2 and extracted mean amplitudes of the Nc component from combined sample.

Emily Jones pre-processed the Phase 2 gap-overlap eye-tracking data used in Chapter 3.

Racheal Bedford pre-processed the Phase 1 gaze following data used in Chapter 3 and 4.

Janet Parsons pre-processed the Phase 2 gaze following data used in Chapter 3 and 4.

Alex Hendry pre-processed the Phase 2 face-popout eye-tracking data used in Chapter 3, 4, 5 and 6.

Luke Mason pre-processed the face pop-out eye-tracking data used in Chapter 5.

Sarah Lloyd-Fox pre-processed the fNIRS data used in Chapter 6.

For genetic and epigenetic data,

Hamel Patel supervised and performed SNP genotyping, quality control and imputation of the gBASIS DNA samples used in Chapters 4 and 5.

Rebecca N. S. Harrison performed principal component analysis in related samples on the familial genotype data used in Chapter 4.

Chloe Y.Y. Wong and Baocong Xia performed DNA methylation data generation and quality control (Chapter 6).

For gBASIS,

Laurel Fish prepared the DNA samples for genotyping and coordinated part of the gBASIS questionnaires data entry done by volunteer BSc students (Chapter 4 and 5).

Matt Danvers contributed to gBASIS questionnaire data entry and prepared a comprehensive pedigree file for genotype data analysis (Chapter 4 and 5).

Zeta Konstantinidi performed data pre-processing for the Gaze Monitoring Test data used in Chapter 4.

Emily Parr, Alice S. Zacharia, Laura Lennuyeux-Comnene, Naomi Wainer and Laura Collazos contributed to questionnaire data entry (Chapter 4 and 5).

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ABSTRACT

This thesis evaluated the evidence for the hypothesis that early disruptions in social attention are involved in the causal pathway to Autism Spectrum Disorder (ASD). The sample included infants at high and low familial risk for neurodevelopmental disorders participating in a prospective longitudinal study, and their family members. Five studies were conducted to test whether social attention atypicalities precede the onset of behavioural symptoms and whether they are related to familial, genetic and epigenetic burden for ASD.

***Chapter 2** examined neural correlates of attention measured with multi-channel electro-encephalography in 8-month-old infants attending to faces and non-social stimuli, in relation to outcomes at age 3. **Chapter 3** used structural equation modelling to investigate whether disruptions in neural response have cascading effects on learning from the environment via looking behaviour. Next, to further understand whether disruptions in social attention lie between genetic risk and ASD phenotype, **Chapter 4** examined the association between ability to detect eye-gaze direction in a familial sample, severity of ASD symptoms and polygenic risk for ASD. **Chapter 5** explored these patterns earlier in development, looking at the relationship between social attention at 14 months of age and familial burden, polygenic risk and parent-report traits of ASD and ADHD. Finally, **Chapter 6**, leveraging DNA methylation data, explored whether epigenetic signals were associated with early neural and behavioural correlates of social attention as well as developmental change leading to atypical outcome.*

Taken together, this work examined in depth the multifaceted nature of social attention, pointing to neural and behavioural atypicalities at critical time points as promising targets for cognitive and affective interventions. Furthermore, it pioneers future work integrating genetics, epigenetics and early neurocognitive measures of social attention in large prospective longitudinal studies of individuals at increased vulnerability for neurodevelopmental disorders, to shed light on the developmental mechanisms underlying the emergence of ASD.

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List of Abbreviations

5mC – 5-methyl cytosine base
ADHD – Attention Deficit and Hyperactivity Disorder
ADI-R – Autism Diagnostic Interview – Revised
ADOS-2 – Autism Diagnostic Observational Schedule – second edition
ADOS-G – Autism Diagnostic Observational Schedule - Generic
AIC – Akaike Information Criterion
AOSI – Autism Observation Scale for Infants
AQ – Autism-Spectrum Quotient
ASD – Autism Spectrum Disorder
BASIS – British Autism Study of Infant Siblings
BAP – Broader Autism Phenotype
BIC – Bayesian Information Criterion
bp – base pairs
BDNF – Brain-Derived Neurotrophic Factor gene
CBCD – Centre for Brain and Cognitive Development (Birkbeck College)
CBQ – Children's Behavior Questionnaire
CFI – Comparative Fit Index
CGI – CpG Island
CNV – Copy Number Variant
CSS – Comparison Severity Score (ADOS)
DAWBA – Development and Well-Being Assessment
DNA – Deoxyribonucleic acid
DNAm – DNA methylation
DSM-4/DSM-5 – Diagnostic and Statistical Manual of Mental Disorders, fourth/fifth edition
ECBQ – Early Childhood Behavioral Questionnaire
EEG – Electro-Encephalography
eQTL – Expression Quantitative Trait Locus
ERP – Event-Related Potential
EWAS – Epigenome-Wide Association Study
FA – Face with Averted Gaze (EEG task)
FD – Face with Direct Gaze (EEG task)
FDR – False Discovery Rate (also indicating q-values)
fMRI – Functional Magnetic Resonance Imaging
fNIRS – Functional Near Infrared Spectroscopy
gBASIS - British Autism Study of Infant Siblings – Genome
GFP – Global Field Power
GWAS – Genome-Wide Association Study
h^2– heritability
HR – High-Risk
HR-ASD – HR children who received a diagnosis of ASD at age 3
HR-Aty – HR children with signs of atypical development but subthreshold ASD symptoms at age 3
HR-noASD – HR-Aty+HR-TD
HR-TD – HR children with typical development at age 3
HWE – Hardy-Weinberg equilibrium
IBD – Identity-By-Descent
IQ – Intelligence Quotient

iPSYCH – Lundbeck Foundation Initiative for the Integrative Psychiatric Research
LD – Linkage Disequilibrium
LR – Low-Risk
M4 – Microstate 4
Mb – Mega base
ME – Module Eigenvalue
Ms – Microstate (referring to microstate 4 identified in Chapter 2)
MSEL– Mullen Scales of Early Learning
mQTL – Methylation Quantitative Trait Locus
MR – Mendelian Randomization
Nc – Negative central
NHS – National Health System in England
OXTR – Oxytocin receptor gene
PC – Principal Component
PGC – Psychiatric Genomics Consortium
PGS – Polygenic Score
QTL – Quantitative Trait Locus
RAGU – Randomization Graphical User Interface
REC – Research Ethics Committees (reference number for ethical approval)
RMSEA - Root-Mean-Square Error Adjusted
RNA – Ribonucleic Acid
RRB – Restricted and Repetitive Behavior (SRS)
RT – Reaction Time
SASI – Scales for Assessment of Social Intelligence
SCI – Social Communication Impairment (SRS)
SCDQ – Social and Communication Disorders Checklist
SCQ – Social Communication Questionnaire
SEM – Structural Equation Modelling
SES – Socio-Economic Status
SFARI – Simons Foundation Autism Research Initiative
SGDP – Social, Genetic & Developmental Psychiatry centre (King's College London)
SNP – Single Nucleotide Polymorphism
SNV – Single Nucleotide Variant
SRS – Social Responsiveness Scale
STAARS – Studying Autism and ADHD Risk
St.β – Standardised beta coefficient
T0 – BASIS/STAARS visit occurred at around 5 months of age (age range: 3-6 months)
T1 – BASIS/STAARS visit occurred at around 8 months of age (age range: 6-11 months)
T2 – BASIS/STAARS visit occurred at around 14 months of age (age range: 11-18 months)
T3 – BASIS/STAARS visit occurred at around 2 years of age (age range: 21-35 months)
T4 – BASIS/STAARS visit occurred at around 3 years of age (age range: 25-48 months)
TANOVA – Topographic Analysis Of Variance
TCT – Topography Consistency Test
VABS – Vineland Adaptive Behavior Scale
VABS Soc. – VABS Socialization domain Standard Score
VABS Mot. – VABS Motor Skills domain Standard Score
WGCNA – Weighted Gene Co-methylation Network Analysis
WES – Whole Exome Sequencing
WGS – Whole Genome Sequencing

CHAPTER 1

GENERAL INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder of great public interest. This fame likely arises from the high uncertainty surrounding its causal mechanisms and from limited evidence of effective interventions for the 'cure' of ASD, which ignited debates at the level of social media and non-scientific community. In fact, although research is abundant in this topic, science has not provided the public with a clear explanation of the causal pathways leading to the emergence of disrupted social interactions and behavioural atypicalities. The advances in our understanding of ASD, and some of the most successful methods to address these complex questions, are reviewed in this chapter. The PhD work presented in this thesis aims to make a step further in trying to understand the mechanisms underlying the emergence of ASD by investigating the role of early atypicalities in social attention in shaping developmental trajectories. A multidisciplinary approach testing the evidence that early social attention is involved in the causal pathway to ASD, as a risk or protective factor, could help evaluating whether it is a good candidate target for intervention.

1.1 STUDYING ASD

In the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), the American Psychiatric Association (APA, 2013) defines the core domains of symptoms for ASD: impairments in social interaction and communication, and restricted interests and repetitive behaviours (RRBs), which includes sensory hyper- or hyposensitivity. Deficits in both domains are required for diagnosis of ASD, according to the DSM-5. ASD now unifies four diagnostic categories that identified separate, though highly related, conditions under the previous DSM-4: autistic disorder (autism), Asperger's disorder, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS). A diagnosis of ASD is assigned following clinical behavioural assessment if symptoms show onset during early childhood.

1.1.1 Epidemiology

While in the early 1990s prevalence estimates of ASD were in the order of 1 per 1,000, a controversial record revealed that in 2014 one in 68 (i.e. 14.7 per 1,000) American children under 8 years of age received a diagnosis of ASD (Baio et al., 2018). On average epidemiological studies reveal that ASD now affects between 1 and 2% of the population in Western countries (Idring et al., 2015; Russell, Rodgers, Ukoumunne, & Ford, 2014; Xu, Strathearn, Liu, & Bao, 2018). Younger ages at diagnosis, differential migration, changes in diagnostic criteria and inclusion of milder cases do not fully explain the observed increases in ASD rate (Hertz-Picciotto & Delwiche, 2009). Observation of the prevalence in developing countries suggests that broadening of diagnostic boundaries, increased service availability and awareness of the heterogeneous manifestations of the condition in both families and professional public might contribute to the observed prevalence increase (Elsabbagh, Divan, et al., 2012; Samadi & McConkey, 2011; Taha & Hussein, 2014).

Importantly, the ratio between male and female individuals with ASD is around 4:1. Possible explanations for this prevalence difference between sexes are sex-related social behaviours which might help female individuals with ASD to seemingly mask their symptoms (Dean, Harwood, & Kasari, 2017; Milner, McIntosh, Colvert, & Happé, 2019) or show a milder symptomatology compared to males (Baron-Cohen, Knickmeyer, & Belmonte, 2005). On the other hand, females who do receive a diagnosis of ASD appear to be more impaired than ASD males, suggesting that they may require additional disorder burden to cross the threshold for ASD diagnosis (Robinson, Lichtenstein, Anckarsater, Happe, & Ronald, 2013; Werling & Geschwind, 2015).

The average age of diagnosis of ASD is typically between 4 and 5 years (Baio et al., 2018; Brett, Warnell, McConachie, & Parr, 2016), although parents tend to report they first became concerned about their child's behaviour when they are around 2- to 3-year-old (Crane, Chester, Goddard, Henry, & Hill, 2016) and researchers observed signs of structural change in the brain and atypical development in the first two years of life (Jones, Gliga, Bedford, Charman, & Johnson, 2014; Shen & Piven, 2017).

1.1.1.1 A unitary construct for a heterogeneous condition

ASD is frequently accompanied by the presence of other features which can have a negative impact on development. For example, epilepsy is present in 20% of the cases and has often onset after 10 years of age (Bolton et al., 2011). Sleep (Won, Feldman, & Huffman, 2019) and

gastrointestinal problems (Coury et al., 2012) might also co-occur with ASD. Motor deficits, such as problems with coordination, balance, locomotion, object control, manual dexterity and fine and gross motor abilities, have a prevalence rate ranging from 33 to 100% depending on the study (Van Damme, Simons, Sabbe, & Van West, 2015).

A great part of the heterogeneity of the ASD phenotype is associated with levels of intelligence. On the one hand, intellectual disability is observed in ~35% of individuals with ASD, who can also show no verbal language skills (Matson & Shoemaker, 2009). On the other hand, a subset of individuals with ASD, considered "high-functioning", have average to exceptional skills in some areas of intelligence (Siegel, Minshew, & Goldstein, 1996). Importantly, similar levels of cognitive ability do not often correspond to comparable symptom profiles (Brunsdon & Happé, 2014).

Additionally, overlap with phenotypes observed in other neuropsychiatric disorders, such as attention-deficit/hyperactivity disorder (ADHD), obsessive-compulsive disorder and schizophrenia is reported in 35% of ASD cases (Morgan, Roy, & Chance, 2003). Comorbidity with ADHD in particular has been widely recognized. Some cases might switch from ASD to ADHD and vice-versa in different periods of life. In fact, 20 to 50% of the children with ADHD meet criteria for ASD and 30 to 80% of ASD children meet criteria for ADHD (Rommelse, Franke, Geurts, Hartman, & Buitelaar, 2010). Anxiety symptoms are also often experienced by children with ASD and may be manifestations of concurrent anxiety syndromes like separation anxiety or social anxiety (Renno & Wood, 2013).

While on the one hand individuals with ASD differ significantly in severity of the core symptoms and also on other cognitive and behavioural dimensions, on the other hand multiple plausible causal mechanisms might lead to similar symptoms presentation (Geschwind, 2008; Johnson, 2017). The rapid advances in genetics have allowed researchers to study large populations with the aim to disentangle the heterogeneity of pathobiological pathways underlying ASD (Jeste & Geschwind, 2014)

BOX 1 Glossary of genetic terms

- **DNA**: deoxyribonucleic acid, a double-stranded helix of alternating phosphate residues and a five-carbon sugar residue (deoxyribose) which has a nitrogenous bases attached to it. There are four types of bases: adenine, thymine, cytosine and guanine. The two strands of the DNA are held together by hydrogen bonds which connect complementary bases: in DNA, adenine always bonds to thymine, while cytosine always bonds to guanine.
- **RNA**: a nucleic acid with a structure similar to DNA but with a ribose sugar. In RNA, the thymine base found in the DNA is replaced by an uracil base. RNA molecules can be distinguished into coding and non-coding RNA (see below).
- **Protein**: combination of amino acids attached to one another in long chains and arranged in a 3-dimensional structure. The amino acid sequence determines the protein function for the body. Examples of proteins are antibodies, enzymes, structural components, messenger proteins like some types of hormones, proteins which transport or store atoms or small molecules throughout the body.
- **Coding RNA or mRNA**: RNA molecule which contains a coding sequence from DNA which is involved in the protein synthesis machinery.
- **Non-coding RNA**: RNA molecules that do not serve as templates for coding amino acids but are instead involved in assisting gene expression. Those include: small interfering RNAs (siRNAs), micro RNAs (miRNAs), and PIWI-interacting RNAs (piRNAs), which regulate the activity of mRNA primarily through RNA interference, and long non-coding RNAs (lncRNAs), which act in interaction with epigenetic factors.
- **Transcription**: DNA is used as a template for the synthesis of RNA.
- **Translation**: mRNA is decoded to make proteins.
- **Gene expression**: transcription of DNA into mRNA.
- **Genome**: all the DNA sequences of an organism. The human genome contains about 3 billion DNA base pairs (bps).
- **Epigenome**: epigenetic events throughout the genome.
- **Gene**: a region of genome that codes for a protein or RNA product. There are around 24,000 genes in the human genome.
- **Exon**: DNA sequence transcribed into mRNA and translated into protein.
- **Intron**: DNA sequence within a gene that is transcribed into mRNA but spliced out before being transcribed into protein.
- **Exome**: all the genome which codes for proteins (less than 2% of the entire genome).
- **Allele**: a version, or alternative form, of a gene or DNA sequence. At each locus, or position on the DNA sequence, each individual has two alleles, one inherited from the father and one from the mother.
- **Genotype**: an individual's combination of alleles at a particular locus.
- **Microarray**: glass slide the size of a postage stamp which contains short DNA sequences called probes. The individual's DNA is cut into small fragments, amplified through polymerase chain reaction, made single stranded and washed over the probes of the microarrays. The individual's DNA will hybridize to probes if they find exact matches and consequently fluoresce.
- **SNP genotyping array:** a type of microarray designed to genotype hundreds of thousands of SNPs across the genome per individual in a single reaction. The availability of SNP genotyping arrays enabled genome-wide association studies of large numbers of individuals.

1.1.2 Human genetic studies

Until the 1970s, ASD was believed to be environmentally caused by cold, rejecting parents or by brain insults (Bettelheim, 1967). However, researchers found out that the probability that both members of a twin pair have ASD is much higher for identical twins (monozygotic, or MZ), who share 100% of their DNA sequence, than for fraternal twins (dizygotic, or DZ), who on average only share 50% of their segregating genes. Working under the assumption that pre-natal and post-natal environmentally caused similarity is equivalent for both types of twin pairs, genetic influence in ASD risk can be inferred. While twin studies index the extent of genetic contributions to ASD, they do not identify specific DNA sequence variants associated to the disorder; identification of individual DNA sequence variants (or genes) that contribute to phenotypic differences is the focus of molecular genetics (Plomin, DeFries, Knopik, & Neiderhiser, 2013). In this section, I will provide an overview of the most popular approaches that have been used in the study of genetic aetiology of ASD. Main findings of ASD genetic research are reported of **section 1.2.2**.

Box 1 contains concise explanations of the genetic terms, methods and approaches that will aid navigation of the following pages (terms are derived from Plomin, DeFries, Knopik, & Neiderhiser, 2013; Strachan & Read, 2011).

1.1.2.1 Twin studies

Genetic effects at the origins of individual differences can be inferred by contrasting the phenotypic and genotypic similarity between related individuals; if DNA sequence influences expressivity of a trait, the resemblance between pairs of relatives is expected to increase with increasing genetic relatedness. Familial resemblance could be due to shared family environmental experiences as well as shared genes. One way to tease apart the relative contribution of genetic (variation in DNA sequence) and environmental (variation in environmental experiences) factors to a trait is to compare MZ and DZ twins, who share the same family environmental experiences but differ in their genetic similarity. Genetic influence can be quantified by the extent to which phenotypic correlation is higher in MZ twins than in DZ twins (Plomin et al., 2013; Thomas, Kovas, Meaburn, & Tolmie, 2015).

Heritability (or h^2) is defined as the proportion of phenotypic variation that can be explained by genetic variation, and provides an index of relative magnitude of genetic influence on complex traits and disorders such as ASD. Twin heritability estimates are obtained by doubling the difference between MZ and DZ correlations. To date, twin studies consistently show that ASD

has a strong genetic component. A recent meta-analysis of nine ASD twin studies that included 6,413 twin pairs reported MZ correlation of 0.98, with DZ correlations ranging from 0.53 to 0.67 (depending on the prevalence rate assigned for the estimation), and substantial heritability estimates (0.64–0.91) (Tick, Bolton, Happé, Rutter, & Rijsdijk, 2016).

In summary, twin studies indicate that ASD is heritable and have motivated molecular genetic investigations (i.e. the study of DNA directly) of ASD that are focused on the identification of the specific genetic causes and the biological mechanisms by which they contribute to psychological and cognitive traits (Plomin, DeFries, Knopik, & Neiderhiser, 2016).

1.1.2.2 Molecular genetic studies

In 1990, the Human Genome Project was launched with the aim of sequencing all three billion base pairs (bp*s*) of the human genome (Green, Watson, & Collins, 2015). Following its successful completion in 2003 (Collins, Morgan, & Patrinos, 2003), the 1000 Genomes Project was set up five years later with the aim of cataloguing DNA sequence variants in the human genome reconstructed from the genomes of 2,504 individuals from 26 populations (Campbell et al., 2015). Coupled with the development of new technologies that are able to measure many hundreds of thousands of genetic variants in large numbers of individuals quickly and cost effectively (using microarrays, see **Box 1**), researchers now know where the vast majority of common genetic variation resides and are able to test individual variants for association with disease in large numbers of individuals (Kruglyak, 2008).

Because each variant tested has been mapped to a specific position on a chromosome, identification of a statistically significant signal immediately allows researchers to query associated biological pathways and functions (Bill & Geschwind, 2009). Crucially, this approach requires no prior hypotheses or assumptions about the chromosomal location or biological function of the DNA variants with respect to the phenotype of interest. The main association approaches used in ASD research are focused around DNA variation data generated by 1) whole-genome sequencing (WGS); a read-out of the entirety of the genetic code of an individual (though parts of the genome eludes even high-quality sequencing at present, Jarvik & Evans, 2017), 2) deep exome sequencing (WES), which reads DNA sequence of the ~2% of the protein coding regions of the human genome and 3) SNP genotyping arrays; for obtaining genotypes for more than one million common genetic variants, or single nucleotide polymorphisms (SNPs), distributed throughout the human genome (De La Torre-Ubieta, Won, Stein, & Geschwind, 2016). Whilst the WGS approach is the most informative as it generates a more complete set of

genetic information for an individual (including rare and common variation), it remains prohibitively expensive to perform on large samples. Consequently, the majority of research has applied WES and/or SNP array approaches to family or population-based samples. **Box 2** indicates the main types of DNA sequence variations identified by WES and SNP-array approaches, based on De La Torre-Ubieta et al. (2016) and Sudmant et al. (2015).

BOX 2 Types of genetic variations

- **SNP**: Single-nucleotide polymorphism— a locus with two or more alleles, where each form is common (>1%) in the population.
- **SNV**: Single-nucleotide variant—a rare (<1%) or common single-bp change in the genome.
- **CNV**: Copy-number variation—sub-microscopic deletion or duplication of large genomic regions leading to changes in the number of copies of the genetic elements encoded within those regions.
- **Structural variants**: a change in DNA bp sequence, which can be heritable or *de novo* (i.e. present in the offspring but that was not inherited from either parent).
  Types of structural variants (Sudmant et al., 2015):
  - Deletion (biallelic)
  - Duplication (biallelic)
  - Multi allelic CNV (mCNV)
  - Inversion
  - Mobile element insertion (MEI)
  - Nuclear mitochondrial insertion (NUMT)

The application of high-resolution, high-throughput technologies such as SNP arrays and/or so-called next-generation sequencing (WGS and WES) approaches had led to a greater understanding of the relative importance of both common and rare genetic variation, respectively. Broadly speaking, common variants each contribute a small effect on ASD susceptibility, and rare genetic variation, whilst not accounting much of the heritability of ASD, have a larger effect on the development of ASD (Jeste & Geschwind, 2014).

Studying rare DNA variations

Next-generation sequencing designs aim to identify rare variations in the DNA which might disrupt the production of proteins needed for the functioning of the organisms (pathogenic variants, Jarvik & Evans, 2017).

The main statistical method used on sequencing data is to compare the frequency of the observed pathogenic variants in affected individuals to the expected rate at which null variations would occur in that gene, therefore significance of an association between a pathogenic variant

and a disease or disorder depends on the number of variants found in cases and controls, as well as the overall number of cases and controls (De La Torre-Ubieta et al., 2016). Because the observed events are rare, large populations are needed to enhance the chances of discovering novel candidates.

Using next-generation sequencing approaches and family-based designs, many putative ASD risk genes have been identified, among which rare variations that are considered "de novo" as they are found in the affected child but not in her healthy parents (Gilman et al., 2013; Iossifov et al., 2014, 2012; Levy et al., 2011; Sanders et al., 2011). However, rare recessive variations that are inherited have also been found at a higher rate in ASD probands compared to controls (Brandler et al., 2018; Krumm et al., 2015; Leppa et al., 2016; Levy et al., 2011). These findings will be described in more details in **section 1.2.2**, where I present candidate causal mechanisms for ASD.

Studying common genetic variants

Genome-wide association studies (GWAS) using SNP genotyping arrays have also demonstrated that common variation in the genome contribute to ASD risk, either individually or in combination. The GWAS approach involves testing more than one million common variants (typically SNPs) distributed throughout the genome for association with a trait or disease using regression; namely, the phenotype is considered the dependent variable in a linear regression model, and the genotype (i.e. whether the individual carries 0, 1 or 2 of the reference alleles) is the independent variable (Plomin et al., 2013)[1]. Due to the multiple testing issue, a stringent p-value threshold of $p \leq 5 \times 10^{-8}$ has been established as the threshold for statistical significance (Pe'er, Yelensky, & Daly, 2008).

GWASes have shown that neurodevelopmental disorders and psychiatric conditions, including ASD, are highly polygenic, with each common variant having only a tiny effect on the phenotype (Dick et al., 2018; Grove et al., 2019). Researchers quickly realised that GWAS data could be interrogated in other ways, and methods were developed that moved beyond the identification of individual trait-associated DNA variants to explore the genetic architecture of complex traits and measure the aggregate effect of common variants on ASD liability. For example, Linkage Disequilibrium score regression provides a measure of genetic correlations between different

[1] Of note, this type of analysis is restricted to SNPs with two possible variations (or haplotypes) observed in the population, therefore multiallelic SNPs and mutations are not included in this analysis. Additionally, typically SNPs with minor allele frequency below 0.01 (i.e. found in less than 1% of the population) are also excluded.

phenotypes using the summary results of previous GWASes (Bulik-Sullivan et al., 2015); Genome-wide Complex Trait Analysis (GCTA, Yang, Lee, Goddard, & Visscher, 2011), based on Genetic Relatedness Estimation through Maximum Likelihood (GREML, Lee, Yang, Goddard, Visscher, & Wray, 2012), estimates total variance of liability by assessing the genetic relatedness of cases and controls across all the SNPs measured on a genotyping array.

Another key method recently developed is the construction of genetic 'scores' for individuals using the summary statistics from GWASes of very large samples and has become very popular to predict psychological as well as health traits (Dudbridge, 2013). A polygenic score is calculated for each individual as the sum of risk alleles identified in an independent GWAS weighted by the effect size which was identified in the GWAS (Wray et al., 2014). The polygenic score provides an individual-specific score for genetic predisposition that can be used like any other variable in a dataset.

Studies on the contribution of common genetic variants to ASD revealed some important findings: 1) cognitive, psychological and neurological features of ASD are under genetic control and this effect is likely to be due to many genetic variants of small effect (Hagenaars et al., 2016; The Brainstorm Consortium, 2018; Warrier et al., 2018), 2) there is high genetic correlation between most psychiatric disorders (Hong et al., 2013; Smoller et al., 2013), 3) phenotypic heterogeneity of ASD does not seem to necessarily map onto different genetic architectures (Chaste et al., 2015; Hong et al., 2013). That is, multiple genetic risk factors can produce similar phenotypes (De La Torre-Ubieta et al., 2016; Geschwind, 2008).

The described advances in statistical methods allowed heritability estimates to be derived directly from the DNA of large samples of unrelated individuals (Plomin, DeFries, Knopik, & Neiderhiser, 2016; Wray et al., 2018), which currently represent the upper limit for polygenic prediction of categorical ASD. SNP-heritability estimates, which quantify the additive contribution to a trait's heritability of common genetic variants, explain between 11 and 19% of the liability to ASD (Hong et al., 2013). The assumptions and limitations of DNA-based heritability estimates differ from twin study estimates in important ways (see Manolio et al., 2009), but broadly speaking the results provide heritability estimates that are typically lower than those obtained from twin studies (so called 'missing heritability' issue) (Plomin, 2013). One possible explanation for it is that part of the genetic effects on a trait depends on the pattern of gene expression such that SNP heritability would be higher if developmental timing and gene function are taken into account (Trerotola, Relli, Simeone, & Alberti, 2009). Studying possible disfunctions of the machinery regulating gene expression might also provide insights on the genetic contribution to ASD (Geschwind & Konopka, 2009).

1.1.2.3 Epigenetics

While polygenic scores derived from large-scale GWASes can capture a significant proportion of genetic liability for ASD, the mechanisms through which genetic and non-genetic risk factors influence early brain development and result in autistic symptoms are not fully understood. Advances can be made by using complementary functional genomic approaches profiling gene expression and epigenetic regulation (Meaburn, Saffari, & Dudbridge, 2017). In this section I focus on the study of epigenetic marks on the DNA that, regulating genome function and consequently gene expression, contribute to inter-individual variability variation in complex phenotypes (Meaburn & Schulz, 2012).

The term "Epigenetics" literally means "above the genome" and was originally defined as the field that studies a whole complex of developmental processes connecting genotype and phenotype (Waddington, 1942). Epigenetics accounts for chemical modifications to chromosome structure, inherited during cell division, that modify gene expression (Wolffe & Matzke, 1999). Within the nucleus of each cell, DNA is wrapped around histone proteins, forming a complex called chromatin. Modifications of the chromatin structure determine the accessibility of genes to be transcribed, while leaving the DNA code intact (Rudenko & Tsai, 2014). Thus, epigenetic factors are responsible for changes in the genome function (i.e. how and when the information in the DNA is accessed) without a change in the DNA sequence and necessitate the study of how genes and environment interact throughout development (Wiers, 2012).

The most popular approaches to study epigenetic modifications in human studies of complex traits such as ASD is DNA methylation (DNAm), which will be described in details in **section 1.2.3**. In this summary of the molecular genetic approaches used in ASD research, a brief overview of the method is provided.

DNAm consists in the addition of a methyl group to a cytosine base located at specific locations in the DNA sequence, predominantly associated with gene promoters. This reaction results in downregulation of gene expression, typically by blocking transcription factor binding or recruiting other proteins with regulatory functions (Strachan & Read, 2011).

DNAm arrays are used to quantify genome-wide DNAm patterns, and consist of oligonucleotide probes that are specific (i.e., they have a different sequence) for methylated and unmethylated sites. DNA is treated first to make detection of methylated probes 'sequence' change, causing bisulfite-induced modifications of genomic DNA that convert cytosines to uracil while methylated cytosines remain nonreactive (Frommer et al., 1992). Following PCR amplification,

it is possible to detect (through fluorescence) and quantify the presence of methylated cytosines, to obtain a quantitative measurement of the proportion of methylated probes. One of the most commonly used DNAm platforms is the Illumina Infinium HumanMethylation450k BeadChip, which interrogates more than 450,000 individual sites (estimated to be less of 2% of all possible methylated cytosines) genome-wide (Dedeurwaerder, Defrance, & Calonne, 2011).

In epigenetic research, association between DNAm levels and ASD has been analysed by averaging signals at all sites and obtaining a measure of global methylation level, or by testing the effect of individual probes in relation to variations of the phenotype (epigenome-wide association study, or EWAS). Although research has initially focused on candidate genes, more recently a data-driven approach has been preferred by conducting EWASes in larger samples (Dall'Aglio et al., 2018). ASD EWASes to date have not identified significant signals in case-control studies (Andrews et al., 2018; Hannon et al., 2018). Critically, epigenome-wide changes in DNAm have been found in relation to prenatal and post-natal exposure to environmental factors and emerging evidence suggests that DNAm could play a mediating role in the relation between environmental risk and psychopathology (Barker, Walton, & Cecil, 2018). Recent examinations of typical and atypical DNAm modifications in critical periods point towards the possibility that causal contributions of ASD risk factors (including genetic burden and early exposure to adverse events) might leave early traces and affect brain development (Hannon et al., 2018; Spiers et al., 2015; Wong et al., 2018).

Genetic and epigenetic research used large samples to investigate common and specific gene-mediated biological pathways associated with ASD. Overall, one of the fundamental discoveries for the field was the pleiotropic nature of the genes involved in ASD (Geschwind, 2011). That is, the majority of the genes involved in ASD are not ASD specific. Many of the ASD risk genes have a function in the development of the brain, in transcription regulation or in the immune system (De La Torre-Ubieta et al., 2016). Clustering individuals by their phenotypic manifestations does not produce stronger signals in genetic studies (Chaste et al., 2015). On the contrary, heterogeneous phenotypes seem to emerge as a result of pleiotropy of combinations of individual loci, whose effects on complex traits are detectable only by mean of large datasets. Expanding aetiologic research where exposure data can be captured prospectively during potentially relevant critical windows and where outcomes are characterized in detail is a research approach that is increasingly advocated to take the current molecular genetic findings a step further in the study of causal mechanisms underlying ASD (Dick, 2018; Newschaffer et al., 2012).

1.1.3 The infant-sibling design

In order to understand how genetic/epigenetic factors contribute to ASD and autistic traits, a powerful approach is to study early postnatal development to better understand causal paths mediating the gene-ASD link, and the emergence of ASD traits (Messinger et al., 2013). This approach is based on the idea that by studying defined components of cognition in infants at genetic risk we could identify developmental features associated with candidate biological pathways (Johnson & Pasco Fearon, 2011).

Siblings of children with ASD are considered at high risk for ASD. In fact, the recurrence rate of ASD outcome in younger siblings of children with ASD is nearly 20% (Ozonoff et al., 2011), that is impressively higher than the population rate of 1% (Baird et al., 2006). Moreover, first-degree relatives of individuals with ASD are more likely to share some phenotypic features with their affected relatives, suggesting that common familial factors might influence behavioural traits (Dalton, Nacewicz, Alexander, & Davidson, 2007; Lyall et al., 2014; Scheeren & Stauder, 2008; Wallace, Sebastian, Pellicano, Parr, & Bailey, 2010; Wheelwright, Auyeung, Allison, & Baron-Cohen, 2010). The clever idea at the base of the infant-sibling design is to recruit families with an older child with ASD who also have a newborn child, and follow up her development with a series of lab-based assessments and parent interviews until she reaches an age at which stable diagnosis of ASD can be made (Ozonoff et al., 2015). Thus, the study of siblings of children with ASD offers opportunities to understand why behavioural symptoms of a neurodevelopmental disorder emerge in some cases and not in others and to investigate protective and risk factors at a genetic, neural and behavioural level (Elsabbagh & Johnson, 2010).

1.1.3.1 From risk to outcome and from outcome to risk

Prospective longitudinal studies of infants with an older sibling with ASD (high-risk infants, HR) have examined how behavioural symptoms unfold over developmental time. Such studies typically followed infants from close to birth to age 3, when they underwent diagnostic assessment with a team of experienced research clinicians. During the multiple lab visits which are carried out over the first three years of life, measures of infants' developmental features are usually obtained from parent reports, researcher-administered standardised behavioural assessments and eye-tracking and neuroimaging recording during experimental tasks. Data is then analysed retrospectively based on the child's diagnostic status at the outcome visit (Elsabbagh & Johnson, 2010).

Perhaps surprisingly, infant-sibling designs revealed that 6- to 8-month-old infants who later receive a diagnosis of ASD appear to be typically developing at the behavioural level (Jones et al., 2014). However, relative to infants with a neurotypical outcome, in the second year of life infants with emerging ASD show gradual declines in social interest and delayed or slower communication development that gradually accumulate (Ozonoff et al., 2014). By 14 months, behavioural measures of early signs of ASD begin to show some predictive validity for a later diagnosis (Bussu et al., 2018), and by 24 months a diagnosis is often possible (Szatmari et al., 2016). Thus, the study of infant siblings provided two fundamental insights about ASD: 1) the period between 8 and 24 months is particularly critical for identifying the causal processes involved in ASD, and 2) the use of dimensional neurocognitive measures as opposed to categorical diagnosis might be strategic to deeply understand the dynamic interaction between different functions characterising typical and atypical trajectories.

1.1.3.2 Uncovering early neurocognitive pathways to ASD

The use of neuroimaging, eye-tracking, measures of physiological response such as heart-rate and skin conductance in infant-sibling designs have been motivated by the need to have more direct measurements of cognitive and neural function, which might signal the onset of divergence developmental trajectories in children with ASD before overt social behaviours difficulties (Elsabbagh & Johnson, 2010).

One important discovery of infant-sibling studies is that patterns of early neural and behavioural atypicalities observed in the HR children might vary not only between individuals, but also within the same child across the first two years of life, possibly reflecting a complex interplay of risk and resilience mechanisms (Szatmari, 2018). In fact, HR infants who do not receive a diagnosis of ASD at three years can show, at earlier ages, an atypical (i.e. different from the low-risk – LR – control group, in the direction of the group with emerging ASD) response or an intermediate phenotypic manifestation (Gliga, Bedford, Charman, & Johnson, 2015a; Hendry et al., 2018; Lloyd-Fox et al., 2013; Wass et al., 2015). This observation suggests that some of these signs may be precursors of the disorder emerging as a result of vulnerabilities related to genetic or environmental risk factors, while others might represent compensatory responses which in some cases have protective value against the core ASD symptoms and in others, perhaps in combination with additional risk factors, lead to 'cascading' effects on different developmental features (Johnson, Gliga, Jones, & Charman, 2014).

Of interest, 10% of the HR infants who do not receive ASD diagnosis at 3 years manifest signs of mild to moderate developmental delay and 30% of them have elevated levels of autistic symptoms (compared to 3 and 15% of the LR children, respectively, Charman et al., 2017). These

children presumably carry some risk factors for ASD or neurodevelopmental disorder, but are, for still unknown reasons, resilient to developing the full syndrome (Szatmari, 2018).

Importantly, carefully following up developmental trajectories also allowed researchers to observe very early indicators of atypical pathways, such as age-specific differences in neural responses to social stimuli and progressive decline in social engagement at the level of subtle pattern of looking behaviour (Jones et al., 2016; Jones & Klin, 2013). However, another important consideration that arose from infant sibling studies is that early signs of atypical behaviour are not limited to the social domain but rather involve domain-general functions, such as attentional control and sensory processing (Gliga, Jones, Bedford, Charman, & Johnson, 2014). Accordingly, atypical characteristics of the brain of HR infants with later ASD can be seen in the first year of life and involve the primary visual cortex as well as sensorimotor areas (Hazlett et al., 2017; Lewis et al., 2017). This raised the possibility that ASD symptoms, particularly in the social domain, might emerge in the second year of life as a consequence of altered experience-dependent neuronal development due to disrupted sensorimotor and attentional experience (Piven, Elison, & Zylka, 2017).

Thus, mapping developmental trajectories of individuals at high risk to disturbances in brain development with a wide range of neurocognitive measures is likely to uncover what functions are more vulnerable and what elements of resilience play a role in the path taken.

1.1.4 The contribution of this PhD work

Behavioural symptoms of ASD emerge gradually over the first few years of life, such that a stable diagnosis can often be made by age 3 to 5 years (Zablotsky et al., 2017). Identifying the mechanisms that underpin behavioural symptoms is important for understanding the aetiology of ASD, and for designing new focused intervention strategies. Since genetic and environmental risk factors for ASD can act prenatally and in the first stages of postnatal life (Lasalle, 2013), to identify the potential mechanisms that lead to symptoms we need to study early development.

As described, two main methods have provided substantial advances in our understanding of pathophysiological mechanisms underlying atypical developmental trajectories. On the one hand, genetic large-scale studies have identified suites of genes implicated in ASD, which typically show peak expression profiles in foetal and infant development (Grayson & Guidotti, 2015). They importantly contributed to the field by identifying biological factors related to complex psychological traits, as well as highlighting and attempting to address the heterogeneity of potential causal mechanisms for ASD. On the other hand, one fruitful approach

has been the prospective longitudinal study of infants with an older sibling with ASD, who have about a 20% chance of developing the neurodevelopmental disorder themselves (Ozonoff et al., 2011). Such studies have provided insights into the early behavioural and neurocognitive profiles that precede later symptomatology (Elsabbagh & Johnson, 2010; Jones, Gliga, Bedford, Charman, & Johnson, 2014). To date these efforts have proceeded largely in parallel, making it difficult to integrate insights from genetics and developmental cognitive neuroscience to provide a full picture of how genetic risk leads to neurocognitive vulnerability and produces behavioural symptoms. The work presented in this thesis aimed to close this gap by combining these two approaches to examine genetic and familial risk factors in relation to measures collected during prospective longitudinal studies of high-risk infants.

Importantly, this attempt posed important challenges. First, obtaining DNA and phenotypic information from all members of families who already contributed so generously to research by participating in a longitudinal infant-sibling study might risk to overburden them. Moreover, collecting DNA from clinical and/or young population is not trivial and families might be unwilling to participate in a potentially unsuccessful or disrupting procedure. A large number of withdrawals should be expected and accepted for ethical reasons, especially given that these families often face important challenges and high levels of stress due to the children's disability (Crane et al., 2016). Second, longitudinal studies collecting a noticeable amount of neurocognitive measures are necessarily more limited in terms of sample sizes compared to case-control genetic studies. Analyses where complete datasets are needed for both phenotypic and genotypic data will require the exclusion of participants with missing data, limiting the power of detecting significant genetic effects. Further, observing the relationship between genetic factors and developmental trajectory requires good quality experimental data at multiple time points from an infant population as well as usable DNA information from the collected samples. This step too necessarily leads to a reduction of the number of participants and enhances the risk of selection bias which in turn might affect generalizability of results (Munafò, Tilling, Taylor, Evans, & Davey Smith, 2017).

Despite the acknowledged difficulties, the potential of such approach to provide novel insights on the mechanisms underlying the emergence of ASD motivated the work presented in this thesis. Besides, investigations of effect sizes, power and possible functional significance of the observed signals are needed in order to successfully plan the application of this approach to larger cohorts. **Figure 1.1** illustrates the multidisciplinary approach that would support the investigation of neurobiological mechanisms underlying cognitive functions, as proposed by Rueda, Pozuelos, & Cómbita (2015). Their model has been adapted to illustrate the contribution

of this PhD work, which was devoted specifically to uncovering the causal pathway leading to the emergence of the ASD phenotype.

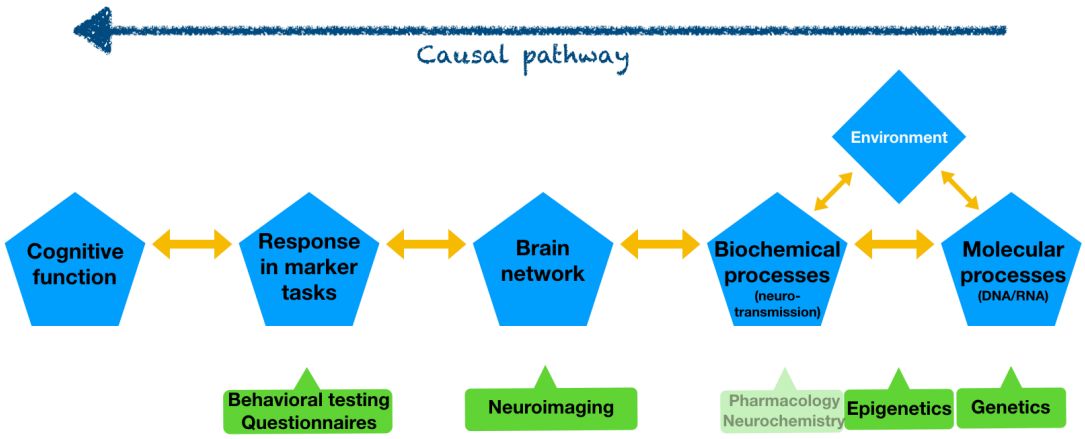

Figure 1.1 *Studying the gene-behaviour pathway underlying the development of cognitive functions*, adapted from Rueda, Pozuelos & Combita (2015). Blue pentagons represent elements of the causal pathway that can be objects of study. Yellow double-headed arrows refer to the concept of "circular causality" or "probabilistic epigenesis" of developmental processes, in which interactions between genes, structural brain changes, and psychological function are considered bi-directional and dynamic (Gottlieb, 2007; Johnson, 2011, see section 1.3.4). Green rectangles indicate the disciplines for the different levels of analysis presented in blue pentagons ("Epigenetics" has been added to the original model). Bright green indicates the disciplines involved in the present thesis. With respect to the original diagram by Rueda et al. (2015), a blue arrow has been added to indicate the direction of the causal relationship we are interested in studying. Moreover, a rhombus representing a possible effect of the environment in relation to molecular processes (arrow representing gene x environment interaction, Plomin et al., 2013, see section 1.3.4.2) and biochemical processes (arrow representing mechanisms of adaptation, Johnson, 2017, see section 1.3.4.2) was added by myself in the model.

1.2 CAUSAL MECHANISMS

ASD is one of the most heritable disorders in psychiatry. However, identified causal pathways between genetic disruptions and ASD phenotype account for less than 25% of the cases (Fernandez & Scherer, 2017). For the other 75% of cases, genetic aetiology is less understood and likely due to the combined influence of common and rare inherited as well as de novo variations and environmental risk factors (that might act through epigenetic processes) (van Loo & Martens, 2007).

1.2.1 ASD risk genes

1.2.1.1 Syndromic ASD

There are situations in which the causal mechanisms leading to the emergence of ASD are known and clinically recognizable, and this is the case of syndromic ASD. ASD is defined as being syndromic when it emerges as part of a condition characterised by a clinically defined pattern of somatic abnormalities and a neurobehavioral phenotype including autistic symptoms. Syndromic ASD has known genetic causes and the diagnosis is typically confirmed by targeted genetic testing. The disruptive events underlying syndromic ASD, obtained from Fernandez & Scherer (2017) and De La Torre-Ubieta et al. (2016), can be Chromosomal (as for Down syndrome or isocentric 15q leading to Prader-Willi/Angelman and Klinefelter syndrome), at the level of single gene mutations, as in the case of Neuro-Fibromatosis 1 (NF1), Tuberous Sclerosis (TSC1 or TSC2), PTEN-associated macrocephaly syndrome, Fragile-X syndrome (some males with full FMR1 mutation), CHD8 truncating mutations, Rett syndrome (MECP2), Timothy syndrome (CACNA1C) or syndromes caused by CNVs (for example, the Microdeletion 22q11.2 syndrome or SYT1) (Baker et al., 2018; De La Torre-Ubieta et al., 2016; Fernandez & Scherer, 2017). The Simons Foundation Autism Research Initiative (SFARI), that cures a scientific literature-based database listing the most up-to-date information on known human genes showed to be associated with ASD (www.gene.sfari.org, see Banerjee-Basu & Packer, 2010), reports 171 genes causing syndromic ASD. The present thesis does not include data from infants with syndromic ASD, whose developmental trajectories of psychological traits are often influenced by syndrome-specific characteristics (Glennon, Karmiloff-smith, & Thomas, 2017).

1.2.1.2 Rare variations: Genetic variants of large effect

The study of developmental trajectories of syndromic forms of ASD for known genetic disruptions have been highly informative with respect of potential pathogenic mechanisms leading to the ASD phenotype (Baker et al., 2018; Baker, Scerif, Astle, Fletcher, & Raymond, 2015). It is plausible that some of these pathways might be shared by individuals with a less etiologically defined form of ASD (Sztainberg & Zoghbi, 2016). Based on observations from the study of known disease-causing variants, the major gene/oligogenic risk model has been proposed as a framework for ASD pathogenesis (De La Torre-Ubieta et al., 2016). This model postulates that a causative genetic factor contributes a large risk, and often is considered sufficient for developing ASD (Zhao et al., 2007). Importantly, this account finds support in findings from WGS and WES studies, which identified disruptive single-gene/region variations

associated with ASD (Fernandez & Scherer, 2017). Examples of the high-confidence ASD risk variants of large effect identified via WGS and WES approaches are: 16p11.2 deletion/duplication, 15q13.3 deletion, 15q11.2 duplication, NRXN1 deletions, loss-of-function or missense variants located in ASD-risk genes (e.g. ADNP, ARID1B, ANK2, DYRK1A, GRIN2B, OPHN1, SCN2A, SHANK3, SYNGAP1, TBR1) (the SFARI Gene database, Banerjee-Basu & Packer, 2010; De La Torre-Ubieta et al., 2016; Fernandez & Scherer, 2017).

At a molecular level, many of these rare mutations have been characterized in terms of their functional downstream effects thanks to mice and in vitro models (De La Torre-Ubieta et al., 2016). Specifically, these ASD susceptibility genes appear to have many distinct roles in neural development and neuronal function, ranging from basic metabolism, synaptic transmission, and RNA splicing to neuronal migration as well as transcriptional regulation (De Rubeis et al., 2014; Gilman et al., 2013; Krumm, O'Roak, Shendure, & Eichler, 2014; Parikshak et al., 2014; Pilarowski et al., 2018). For example, they have been associated with synaptogenesis (NRXN1, SYNGAP1), neuronal migration (CNTNAP2, OPHN1), dendritic development (SHANK3, FMR1), various developmental processes required for brain development (ADNP, TBR1, DYRK1A, ARID1B), brain structure abnormalities (ARID1B), propagation of neural signals (SCN2A, GRIN2B), neurodegeneration (ANK2) (Geschwind & Levitt, 2007; Iossifov et al., 2012; the SFARI Gene database, Banerjee-Basu & Packer, 2010). Clustering genes by their functions revealed that chromatin regulation and synaptic function were the common pathways impacted by ASD-risk variants (De Rubeis et al., 2014; Sanders et al., 2015).

Reverse phenotyping studies, that aim to clinically characterise individuals with known genetic variations (Schulze & McMahon, 2004), revealed that ASD is found in a significant percentage of cases, although not all the individuals carrying a pathogenic variation manifest the core autistic symptoms. For example, 15% of cases with 16p11.2 deletion (Zufferey et al., 2012), 31% with 15q13.3 deletion (Ziats et al., 2016) and NRXN1 deletions (Lowther et al., 2017) have comorbid ASD. This on the one hand suggests that these genes do play a role in shaping developmental trajectories and that disruption of their function is causally implicated in the pathway to autistic traits. However, on the other hand these percentages also show that mutations of these genes do not always lead to ASD.

Accordingly, studies comparing the rate of de novo rare variations (CNVs and SNVs, see **Box 2**) in individuals with ASD and their siblings without ASD found that, although the carrier rate was almost twice in individuals with ASD compared to their unaffected siblings, the latter carried a number of mutations too (Iossifov et al., 2012; Sanders et al., 2011). Genetic models that incorporate information of functional pathways of protein-protein interactions that map to de

novo CNVs and SNVs indicated that most of the observed de novo events are unconnected to ASD; an increase in liability to ASD is observed when many variants that are incompletely penetrant (i.e. often not sufficient for disease) are detected in the same individual (Krumm et al., 2015; Neale et al., 2012).

Similar results were obtained by Leppa et al. (2016), who in addition examined the difference in the number of de novo and inherited rare variations between ASD families where there is more than one affected member (multiplex) and single-incidence (simplex) families. Interestingly, they found that the contribution of de novo risk-variants to ASD was lower for probands of multiplex families than for probands of simplex families (Leppa et al., 2016), where de novo events account for 10% of the ASD liability (Sanders et al., 2015). These findings highlighted the degree of heterogeneity of genetic risk factors to ASD. Of note, several studies have demonstrated a higher incidence of rare events in female probands (Iossifov et al., 2014, 2012; Neale et al., 2012; Sanders et al., 2015), suggesting that this category of genetic risk might be more penetrant when present in females. In girls from simplex families, there is high overlap between risk variants for ASD and low IQ (Iossifov et al., 2014).

Recently, Gaugler and colleagues examined the genetic architecture of ASD in a familial sample of 3046 individuals (466 ASD cases and 2580 controls) to understand what type of genetic contribution underlies idiopathic (i.e. non-syndromic) ASD (Gaugler et al., 2014). They found that more common genetic variants in aggregate account for up to 50% of the liability of ASD, but each individual variant has only a subtle effect, individually explaining <0.5% of the variance in liability in the population-based samples. Results of the estimates of genetic contribution to ASD liability from Gaugler et al. (2014) are represented on **Figure 1.2**.

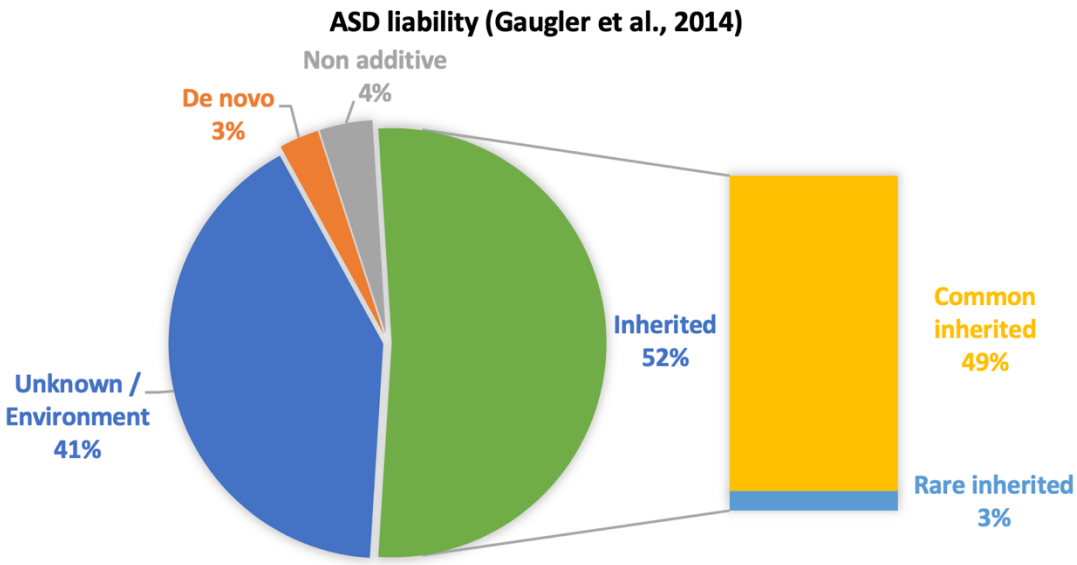

Figure 1.2 *The genetic architecture of non-syndromic ASD from Gaugler et al. (2014)*. Of note, 52% of the liability to ASD is inherited, and might therefore contribute to familial risk factors which might underlie differences in developmental trajectories observed by the infant sibling studies. Additionally, genetic association models are based on the assumption of additive effects, whereby they consider the genetic effect on a phenotype as the sum of independent contributions of single loci or alleles. Non-additive effects (in grey) might also contribute to risk via the mechanism of allele dominance (effect on phenotype of the dominant allele masks the contribution of the recessive allele at the same locus) or epistasis (the effect of one allele is dependent on the presence of one or more modifier genes).

In sum, rarer sub-microscopic chromosomal structural variations (i.e. CNVs and SNVs) account for a small proportion of idiopathic autism, and the frequency of these mutations is lower in multiplex families than in simplex families (Geschwind, 2011). Importantly, the most recent view is that the genetic architecture of ASD is based on the interplay between rare and common variants (Bourgeron, 2016; Weiner, Wigdor, Ripke, Walters, Kosmicki, Grove, Samocha, Goldstein, et al., 2017).

1.2.1.3 Common risk variants: many risk factors of small effect

The findings on the genetic architecture of ASD illustrated in **Figure 1.2** (Gaugler et al., 2014) are consistent with a polygenic risk model, where many genetic risk factors are expected to contribute to a small additive risk for the clinical phenotype (De La Torre-Ubieta et al., 2016). Under this model, common variants, in conjunction with rare variants, act as susceptibility or causal alleles and contribute in differing proportions to ASD risk in individual subjects (Weiner, Wigdor, Ripke, Walters, Kosmicki, Grove, Samocha, Robinson, et al., 2017). To test this hypothesis, large case-control association studies have been the preferred approach, as they more efficiently allow to detect small effects of common genetic variation.

GWAS using categorical ASD phenotype have been less successful than expected, so far. The larger of such studies is a meta-analysis carried out thanks to coordinated international effort of the ASD Working Group of the Psychiatric Genomics Consortium (PGC). This study included a discovery sample of 7,387 ASD cases versus 8,567 controls, followed by meta-analysis of summary statistics from two replication sets of 7,783 ASD cases versus 11,359 controls and 1,369 ASD cases versus 137,308 controls (Grove et al., 2019). No significant SNPs were found to be associated with ASD at a genome-wide significance threshold of $p \leq 5 \times 10^{-8}$ in this GWAS. The top hit signals did not overlap between cohorts. However, among the most SNPs most highly associated with ASD, specific loci previously shown to be involved in neurodevelopment and differentially expressed in the brains of ASD people were identified (Anney et al., 2017). The estimated heritability from this cohort was 33.4%, lower than expected based on Gaugler et al. (2014) possibly due to the presence of familial samples which might have led to an underestimation of heritability (Anney et al., 2017).

Comparing the sample size of this GWAS with other GWASes for psychiatric disorders which obtained genome-wide significant effects (for example, 36,989 cases versus 113,075 controls for schizophrenia, Ripke et al., 2014, or 135,458 cases versus 344,901 controls for major depression, Wray et al., 2018), a generally accepted explanation for the null results of this study is that a larger samples size is required to detect significant signals of very small effects (Anney et al., 2017). The fact that many of the top-hits seemed to be potentially meaningful for ASD motivated the use of summary statistics of this or previous ASD GWASes to test whether the aggregate effect of many variants associated with ASD at a lenient GWAS p-value threshold could explain differences in autistic traits as well as other psychological phenotypes.

Indeed, Clarke et al. (2015) found that ASD polygenic score explained 28% of the variance in liability to ASD in a smaller, independent cohort. Moreover, it explained a small (0.5%) but significant proportion of the variance in cognitive tests for logical memory, vocabulary and verbal fluency (Clarke et al., 2015). ASD polygenic score also predicted 7.5% of the variance in autistic traits in individuals with childhood-onset schizophrenia, defined by the onset of psychotic symptoms before age 13 (Ahn, An, Shugart, & Rapoport, 2016) and 0.11% of the phenotypic variance in an independent data set of individuals with Obsessive-Compulsive Disorder (Guo et al., 2017). These findings indicated that a contribution of many common genetic risk variants is, at least in part, responsible for the observed comorbidity between ASD and other neurodevelopmental disorders.

Genetic overlap

As mentioned earlier (**section 1.2.1**), another method to investigate the role of common genetic variants in neurodevelopment is to look at the genetic correlation between traits. Findings from a very large study (265,218 patients and 784,643 control participants) from the Brainstorm Consortium, which examined genetic correlation between 17 phenotypes including cognitive measures, neurologic and psychiatric conditions, confirm the high pleiotropy of the ASD risk genes. In fact, ASD showed significant genetic correlation with schizophrenia, intelligence, years of education and college attainment (The Brainstorm Consortium, 2018). In another study, significant genetic correlation was found between ASD and cognitive functions such as verbal-numerical reasoning and reaction times in a computerized game in which participants had to press a button as quickly as possible when seeing matching stimuli on the screen (Hagenaars et al., 2016). Importantly, evidence for shared genetic links between ADHD and ASD has been found throughout development from 8 to 17 years (Stergiakouli et al., 2017). However, no genetic overlap between these two conditions was found in an adult population (Hong et al., 2013).

Robinson et al. (2016) estimated genetic correlation between ASD and ASD traits using a continuous measure, the Social and Communication Disorders Checklist (SCDC) in 5,628 8-year-old children who participated in the Avon Longitudinal Study of Parents and Children (ALSPAC). They found, and replicated in an independent sample, that approximately one quarter of common genetic variants' influences on ASD shared that influence with the SCDC in the general population (Robinson et al., 2016). Importantly, St Pourcain et al. (2018) extended these findings by showing an age-specific effect of ASD-related polygenic influence of common variants on social and communication difficulties, which was stronger at 8 years and decreased with age throughout adolescence (St Pourcain et al., 2018). In sum, polygenic contribution to ASD predicts continuous ASD traits as well as other cognitive and psychological traits and comorbid conditions. Genetic correlation between ASD and ADHD is high during childhood, suggesting that a pleiotropic effect of common risk variants might be responsible for behavioural traits shared by both developmental disorders.

The studies of common variants reported thus far examine genomic variation which is responsible for increased risk of ASD in the general population. Family studies can be used to study the portion of common genetic variation that is inherited alongside autistic traits, constituting the largest part of ASD liability (Gaugler et al., 2014, see **Figure 1.2**). Common variants explaining variability in levels of autistic traits within families have been investigated by Lowe, Werling, Constantino, Cantor & Geschwind (2015). They tested whether inherited

common genetic variants were transmitted together with social impairment (measured with the Social Responsiveness Scale questionnaire, SRS, Constantino, 2002) in 590 multiplex families from the Autism Genetic Resource Exchange (N=1,480 individuals). They found robust evidence for an association between autistic traits and two loci on chromosome 8, involved in neuron survival and differentiation. In line with the expectations, they reported null evidence for common variants to be associated with SRS scores in 1,652 nuclear families (where it was not possible to ascertain whether they were multiplex or simplex families). These results provide suggestive evidence that inherited common risk factors underlie the increased risk of ASD traits in multiplex families.

1.2.2 Familial risk

As mentioned, research on the association between the effect of common polygenic factors as well as de novo and inherited rare variations on dimensional autistic traits in the general population demonstrated that indeed multiple types of genetic risk factors for ASD influence a continuum of behavioural traits (Niemi et al., 2018; Robinson et al., 2016). Evidence for a continuum polygenic model for ASD is also provided by the increased predisposition to behavioural atypicalities of family members of individuals with ASD which are less affected by de novo mutations (De La Torre-Ubieta et al., 2016).

1.2.2.1 The Broader Autism Phenotype (BAP)

Research consistently demonstrating that first- and second-degree relatives of people with ASD had higher probability to also show some of the social difficulties characterising ASD dates back to 20 years ago (Pickles et al., 2000; Piven, Palmer, Jacobi, Childress, & Arndt, 1997). The first studies based their investigations on family history interviews. They found higher rates of autistic symptoms in parents and grandparents of children with ASD within selected multiplex families (Piven et al., 1997) and in a large population sample of extended pedigrees (Lord et al., 2000), compared to individuals with no family history of ASD. Of note, the rate of autism-like phenotypic expression diminished with increasing genetic distance from the ASD proband (Pickles et al., 2000). The presence of characteristics similar to ASD but less severe in relatives of people with ASD had been conceptualized as a Broader Autism Phenotype (BAP)(Pisula & Ziegart-Sadowska, 2015).

Subsequently, the study of BAP evolved through the use of questionnaires devoted to the characterisation of ASD phenotype as a range of dimensional traits. The SRS (Constantino, 2002)

and the Autism-Spectrum Quotient (AQ, Baron-Cohen, Wheelwright, Skinner, Martin, & Clubley, 2001) were among the first instruments which served this purpose. Constantino & Todd (2005) conducted a population-based study where they collected the SRS in 285 ascertained twin pairs and their parents. They found that high SRS score, reflecting more autistic traits, of either parent was associated with elevated SRS score of the offspring. Moreover, when both parents manifested subthreshold autistic traits, their children exhibited a shift in the distribution of scores for impairment in reciprocal social behaviour toward the pathological end (Constantino & Todd, 2005). Lyall et al. (2014) found similar results when testing the co-occurrence of autistic traits in parents and offspring in an independent cohort of 1,649 families of children with and without a community diagnosis of ASD. Autistic traits were measured through the SRS, completed by mothers for the children's and fathers' SRS, and by spouse or relative for the mothers' SRS. They found that mean parent scores were higher among ASD-families than control families. In particular, fathers' scores were more similar to their children's in ASD-families, while no significant association was found for mothers' elevated scores (Lyall et al., 2014). Wheelwright et al. (2010) used the AQ scale for the identification of BAP among parents of children with ASD. Testing a sample of 1,582 ASD-families and 666 control families, they found that ASD-parents had significantly higher AQ scores, indicating more autistic traits, with 33% of fathers and 23% of mothers scoring above the BAP statistical cut-off (Wheelwright et al., 2010). Hasegawa et al. (2014) confirmed these findings on a Japanese population. They found higher scores in two AQ subscales (social skills and communications) in ASD parents. In particular, they reported an association between ASD children's SRS score (especially in the cognition and communication subscales) and mothers' AQ scores in the Imagination and Attention Switching subscales. Thus, consistent evidence showed that there is a strong heritability of social difficulties also in family members who do not present core ASD symptomatology.

1.2.2.2 BAP in multiplex versus simplex families

Individuals in multiplex families have higher liability for ASD traits than individuals in simplex families (Piven et al., 1997). Losh et al. (2008) tested 25 multiplex, 40 simplex and 30 Down syndrome families to see whether BAP traits are recurrent within families. They found a graded pattern of expression such that individuals of multiplex families had more symptoms than individuals from multiplex families and relatives of individuals with Down syndrome for personality behaviours, friendship and language. Multiplex fathers usually had more severe impairments in these areas. Within families, BAP expression in both parents was most likely in multiplex families, whereas in simplex families equal chance of BAP expression was found between one, both or neither parent (Losh, Childress, Lam, & Piven, 2008).

Gerdts, Bernier, Dawson, & Estes (2013) extended these findings by investigating what aspects of behaviour were particularly affected in individuals from multiplex families. They administered the Broader Phenotype Autism Symptom Scale (BPASS), which is a combination of behavioural observation and clinical interview (Dawson et al., 2007), to members of 87 multiplex families, with at least 2 children with ASD (117 unaffected children and 184 affected children), and 47 simplex families, with at least 2 unaffected siblings. The BPASS includes four domains: Social Interest, Expressiveness, Conversation and Flexibility/Restricted Interest. Simplex family members showed increased scores in the Social Interest and Expressiveness domains compared to multiplex. Clinical observations revealed that multiplex individuals less frequently used integrated eye-gaze, social smiling, directed facial expressions and typical vocal prosody. Multiplex mothers and multiplex fathers were significantly more likely to demonstrate decreased interest in interacting with peers. Multiplex siblings more consistently presented decreased social motivation, lower observed verbal and non-verbal communication skills and impairing repetitive patterns of behaviour (Gerdts et al., 2013). Further insights on the incidence of BAP in families came from a study of ASD symptoms in over 5,500 siblings of children with ASD. In this study, Frazier, Youngstrom, Hardan, Georgiades, & Constantino (2015) examined ASD traits expression in multiplex versus simplex families using the SRS and the Social Communication Questionnaire (SCQ, Rutter, Bailey, & Lord, 1993). When comparing multiplex and simplex siblings, they found that multiplex siblings had more severe traits, even when considering only children with no sign of language delay (which might emerge as a consequence of increased genetic liability for neurodevelopmental disorder) (Frazier et al., 2015).

The BAP concept is in agreement with genetic findings showing that that there might be familial risk effects underlying the pattern of higher incidence of ASD symptoms in multiplex families. However, it is still unclear whether these effects are all accounted for by genetic inheritance. A large proportion of ASD liability (41% according to Gaugler et al., 2014) consists in unidentified or environmental factors. At a molecular level, epigenetic mechanisms are good candidate mediators between genetic architecture and environmental exposure (Barker et al., 2018). Disruptions of DNA methylation, a well-studied epigenetic marker, have been proposed as a candidate mechanism shaping early neurodevelopmental trajectories towards an ASD phenotype (Ciernia & Lasalle, 2016). The study of DNA methylation profiles might advance our understanding of the role of biological mechanisms for BAP/ASD.

1.2.3 Epigenetic mechanisms: DNA methylation

The best widely studied mechanism of epigenetic regulation in mammalian genomes is DNA methylation (DNAm), which consists in the addition of a methyl group to a cytosine nucleotide to create a 5-methyl cytosine base (5mC). A methyl group is a basic unit in organic chemistry formed by one carbon atom attached to three hydrogen atoms (-CH3). In humans, methylation of cytosines typically occurs when the 5'-cytosine is linked by one phosphate to a 3'-guanine (i.e., at CpG dinucleotides). When a methyl group attaches to a CpG site, various proteins bind to the 5mC and recruit other effector proteins to methylated loci to typically result in a transcriptionally repressed state. This mechanism is important also to inactivate intergenic, non-coding regions which represents 45% of the mammalian genome and consist of potentially harmful transposable and viral elements (Schultz et a., 2006). Within genes, stretches of DNA where CpG sites occur with higher frequency are called CpG islands (CGIs) and typically populate promoter regions. Methylation of CGIs results in silencing of gene expression, due to limitation of DNA accessibility and transcription factor binding. CpG sites in other regions of the gene, such as the gene body (i.e. the region of the gene past the first exon), can also be methylated but how this mechanism contributes to gene regulation is still unclear (Moore, Le, and Fan 2013).

DNAm can occur in the context of non-CpGs as well (5mCH, where H = A, G and T). This epigenetic marker can be found in different tissue types (Schultz et al., 2015) and especially in embryonic cells and neurons (Lister et al., 2013). Additionally, another, less common type of methylation marker is formed when another chemical group is attached to the 5mC group, resulting in 5-hydroxymethylated C bases (5hmC). Taken together, DNAm plays a variety of roles in the regulation of genome expression, such as chromatin modification, transcription promotion or inhibition, long-term gene silencing, transposable element suppression, genomic imprinting, X-chromosome inactivation and maintenance of genomic stability (Strachan & Read, 2011).

A complex enzymatic machinery grants a certain stability of DNAm for the maintenance of the same patterns of gene expression through cell division and at the same time regulates dynamic modifications throughout the lifespan (Geiman & Muegge, 2010). This incredible degree of regulated epigenetic flexibility makes DNAm particularly relevant in neurodevelopment, which depends on activity-dependent plasticity underlying cognitive and behavioural processes (Qureshi & Mehler, 2014).

1.2.3.1 DNA methylation programming during development

DNAm plays a crucial role in the development of the organism, and also specifically in the formation of the brain. The majority of the current knowledge on developmental mechanisms involving DNAm comes from the study of 5mC, therefore in this thesis I will refer to DNAm mainly considering this marker. It is worth noting however, that 5mCH and 5hmC have been also recently suggested to play a relevant role in development, and neural development specifically. For example, levels of 5mCH increase during postnatal and adolescent prefrontal cortex development in mice and humans (Lister et al., 2013), and are therefore thought to dynamically regulate experience-dependent shaping of the neuronal transcriptome (Ciernia & Lasalle, 2016). Until recently 5hmC was believed to only serve as intermediate tag for 5mC bases which had to be demethylated. However, there is now evidence showing that it is enriched particularly in brain tissue (Kriaucionis & Heintz, 2009) and likely plays a role in foetal brain development (Spiers et al. 2017; Lister, Mukamel, Nery, Urich, Puddifoot, Nicholas, et al. 2013).

The fastest changes in DNAm occur during the foetal period (Numata et al., 2012). In fact, in the early phases of foetal life, two major waves of epigenetic reprogramming take place that allow the two individual germ cells epigenetic patterns to be removed and a new epigenetic profile to be established in the developing embryo (Geiman & Muegge, 2010). Subsequent phases of demethylation and re-methylation occur in embryogenesis, with dramatic demethylation as the embryo progresses from the morula to the blastocyst stage (Ciernia and Lasalle 2016). After the embryo implantation, changes in DNAm serve to regulate cell differentiation, especially for the differentiation of cells of the mammals' central nervous system (Moore, Le, and Fan 2013). DNAm remodelling for cell differentiation occurs mainly between 9 and 18 weeks of gestational age, while it remains more stable from 18 weeks to adulthood (Slieker, Roost, Van Iperen, & Suchiman, 2015). Thus, the transition from foetal life to early childhood often goes from demethylation prenatally to increased methylation postnatally (Numata et al., 2012). Non-gamete cells, such as the cells in the brain and all the other tissue types, maintain a quite stable level of 5mC methylation following implantation and establishment of tissue-specific methylation patterns (Ciernia & Lasalle, 2016). However, prenatal and post-natal environment can influence DNAm profiles in the brain as well as in other cells of the body (Barker et al., 2018).

Environmental exposures

Although global levels of methylation do not change drastically after birth (Slieker et al., 2015), animal work indicates that DNAm patterns can change postnatally in interaction with the environment. In mice, extracellular signals and neuronal electrical activity concur in influencing neurons' DNAm levels, inducing long-lasting methylation changes and related behavioural

modifications (Guo et al. 2012; Lister, Mukamel, Nery, Urich, Puddifoot, Johnson, et al. 2013). Human studies too have demonstrated that environmental exposures impact DNAm levels of specific genes and are associated to behavioural changes in the individual (see Mitchell, Schneper, and Notterman 2015 and Barker, Walton, and Cecil 2018 for review). For example, maternal psychopathology, criminal behaviours and substance use have been related to greater DNAm at birth of oxytocin receptor gene (OXTR), a gene related with prosocial behaviour; maternal folic acid intake before and during pregnancy was associated to DNAm and expression changes Insulin-like growth factor 2 (IGF2); DNAm patterns of the glucocorticoid receptor gene (NR3C1) in the brain and other tissues is influenced by childhood maltreatment (Mitchell et al., 2015). Prenatal exposure to bisphenol A (BPA), an endocrine disruptor and ubiquitous environmental toxicant, has been shown to be related to modification in BDNF DNAm at birth, which in turn predicted emotion regulation problems and aggressive behaviours in 3 to 5-year-old children (Kundakovic et al., 2015). Victimized monozygotic twins had greater levels of DNAm of the SLC6A4/SERT gene (a serotonin transporter linked to the hypothalamic–pituitary–adrenal axis regulating stress response) and lower levels of cortisol (a hormone considered a marker of the reaction to stress) than their co-twins who did not suffer this adverse event (Ouellet-Morin et al., 2013). These are only a few examples suggesting that environmental risk exposure can affect DNAm of genes that are important for brain function and development and consequently be involved in the causal pathway to later child psychopathology (Barker et al., 2018).

DNA methylation and human brain activity

Importantly, changes in DNAm have also been linked to change in brain activity not only in the rodents brain (Guo et al., 2012; Lister et al., 2013; Numata et al., 2012) but also in peripheral tissues in humans. Jack, Connelly, & Morris (2012) observed that higher DNAm levels in the OXTR gene were associated with enhanced brain activity evoked by perception of animacy (scenes of interactions between animated geometric shapes) in two brain regions known to play a role in social perception, namely the temporal parietal junction and the dorsal anterior cingulate cortex. Frodl et al. (2015) provided evidence for a significant association between the DNAm of SLC6A4/SERT, which transports serotonin from synaptic spaces into presynaptic neurons, and fMRI Blood-Oxygen-Level Dependent (BOLD) signal during emotional attention processing. Exposure to adverse environmental experiences (i.e. greater childhood maltreatment) was associated with higher percentage of SLC6A4/SERT methylation. Ursini et al. (2011) tested the relationship among life stress, peripheral DNAm in the COMT gene, responsible for inactivation of prefrontal dopamine, working-memory performance and prefrontal activity measured with fMRI in healthy adult subjects. They found that DNAm levels interacted with stress, modulating

prefrontal activity during the working-memory task such that greater stress and lower methylation were correlated with more inefficient prefrontal activity in the individuals who carried the Val allele (Ursini et al., 2011). Similarly, Hass et al. (2015) explored associations between network-level epigenetic changes in target gene sets, working-memory and brain measures in schizophrenic patients and healthy controls. They reported a significant interaction between working-memory performance and DNAm levels in Ephrin-A4, a gene implicated in mediating developmental events particularly in the nervous system, including axon guidance and synaptic long-term potentiation (Hass et al., 2015). Thus, even though relatively few studies have been conducted, and only on adults, there are preliminary indications of associations between peripheral DNAm and brain activity underpinning cognitive functions.

1.2.3.2 DNA methylation and ASD

DNAm is essential for typical neurodevelopment and alterations in methylation patterns in critical phases for brain development have been proposed as candidate mechanism for the development of ASD phenotype as a result of the action of and interaction between genetic and environmental risk factors (Ciernia & Lasalle, 2016). Historically, the fact that disruptions in DNAm lead to specific neurodevelopmental abnormalities that emerge as autism-like symptoms came from the study of Rett syndrome. Rett syndrome is a severe neurodevelopmental disorder characterized by brain abnormalities and gradual emergence of autistic features as well as motor and language regression (Neul et al., 2011). Indeed, this disorder is caused by mutations of the MeCP2 gene, a transcriptional modulator which binds to CpG dinucleotides and recruits other repression factors (Rudenko & Tsai, 2014). More recently, rare de novo mutations in the DNMT3a gene, involved in the regulation of embryonic methylation, have been found in people with ASD (Sanders et al., 2015). Additionally, age-related methylation changes in the human cortex during foetal and postnatal development are enriched within genes implicated in schizophrenia and ASD (Jaffe et al., 2016; Numata et al., 2012; Spiers et al., 2015). These findings suggest that disruptions in the epigenetic machinery could disturb the formation of brain circuits in these disorders.

Attempts to identify specific biological pathways through which DNAm could influence neurodevelopment have initially been carried on as candidate-gene studies (Flanagan, 2015). However, the absence of replication of candidate-gene studies comparing DNAm levels in ASD cases and controls, potentially explained by the heterogeneity of tissue types, analyses and age ranges, and by very small sample sizes, did not allow to identify credible candidate genes for the association of DNAm and ASD (see Dall'Aglio et al., 2018 for a review). Two recent large EWASes failed to find ASD-related differences at the level of single probes (Andrews et al., 2018; Hannon

et al., 2018). However, differences in DNAm in newborn blood samples were associated with increased genetic risk for ASD (Hannon et al., 2018). Differently, Wong et al. (2013) found that there was a significant variability within ASD-discordant MZ twin pairs in many DNA loci, revealing that (possibly environmentally-driven) DNAm patterns account for phenotypic differences also in individuals carrying identical DNA sequence. Taken together, developmental epigenetic research suggests that epigenetic marks are likely to be traces of developmentally relevant interplays between genes and environment, which might well explain part of the unknown liability to ASD (Ciernia & Lasalle, 2016; Lasalle, 2013). Prospective critical period-specific investigations accurately capturing temporal order of the association between changes in DNAm levels and behavioural signs of emerging ASD is a promising avenue to shed light on this mechanism.

1.3 MEASURES OF SOCIAL ATTENTION AS ENDOPHENOTYPES OF ASD

As emerged from the previous sections of this introduction, there is high heterogeneity of molecular factors which have been found to increase the liability of disruptions of neurodevelopment. Observing the effect of genetic and familial risk factors on emerging psychological functions in the first years of life might help identifying the origins of divergent developmental trajectories and possible indicators of resilience. Correlates of emerging components of cognition which are linked to genetic effects have been defined as developmental endophenotypes (Johnson & Pasco Fearon, 2011). An endophenotype is a quantitative biological trait that reliably reflects a heritable function of a biological system, and as such is potentially more closely related to the root cause of the disorder than a broad clinical phenotype (Gottesman & Gould, 2003). The concept of endophenotype in psychiatry has been introduced as a potential tool to help resolving questions about etiological models, because it provides a genetically tractable target lying in the gap between genetic burden and disorder process (Flint & Munafò, 2007).

A continuously quantifiable trait can be considered an endophenotype when: it is highly linked with the genetics of the disease and involved in a biological plausible mechanism of pathogenesis, it co-segregates with a psychiatric illness, it can be present when the disease is not, only at certain ages or under certain conditions, and it is more commonly observed in non-affected family members than in the normal population (Gottesman & Gould, 2003). Endophenotype measures can be neurophysiological, biochemical, endocrinological, neuroanatomical, cognitive, and neuropsychological (including self-report data) quantitative phenotypes. **Figure 1.3** re-proposes the diagram illustrating how to study biological mechanisms

underlying the emergence of cognitive phenotypes, presented as **Figure 1.1**, and highlights which of the factors of the causal pathway can be studied as endophenotypes.

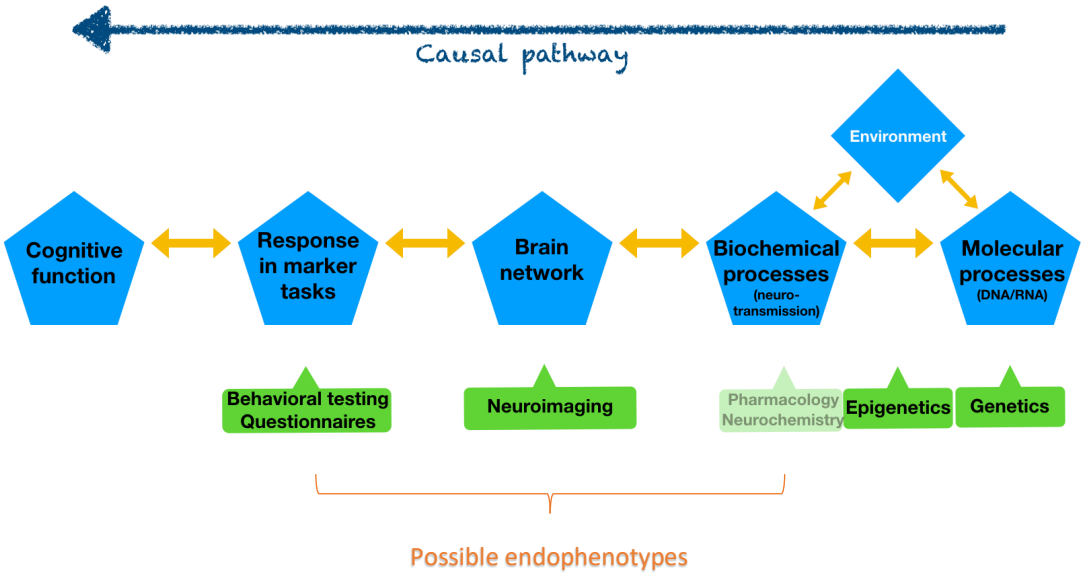

Figure 1.3 *Studying endophenotypes to shed light on the gene-behaviour pathway underlying the development of cognitive functions*, adapted from Rueda, Pozuelos & Combita (2015) as described for Figure 1.1. Possible endophenotypes are: biochemical processes, measures reflecting the activity of brain networks and measures of the response in specific marker tasks.

In this section I evaluate findings in favour of considering measures of social attention as possible endophenotypes of ASD. I start reviewing the evidence indicating that visual attention can be disrupted by effect of ASD risk genes influencing early development of the whole brain network which is activated during attentive states (Hellyer, Clopath, Kehagia, Turkheimer, & Leech, 2017). Indeed, infant-sibling designs have shown that altered sensory processing and atypical attentional control are detectable since infancy in children with ASD (Gliga et al., 2014) and might be precursors of social communication disorders later in development (Bedford et al., 2012; Jones, Venema, Earl, Lowy, & Webb, 2017; Keehn, Wagner, Tager-Flusberg, & Nelson, 2013). In particular, anomalies in the allocation of attentional resources to social stimuli have been found in prospective studies of children who later received a diagnosis of ASD (Elsabbagh et al., 2013, 2011; Gliga, Bedford, Charman, & Johnson, 2015b). I report accounts for considering the construct of social attention in a developmental perspective, where the integration of the different aspects of social attention as well as brain areas underpinning them is observed in typical development (Salley & Colombo, 2016). I then summarise which of the requirements for being used as endophenotypes of ASD are met by various measures of social attention and what aspects are still to be explored.

1.3.1 Glossary of visual attention

Attention (visual attention specifically, for this thesis) is a domain-general cognitive function which serves to prioritize processing of certain stimuli over others in order to select environmental information for learning and memory (Amso & Scerif, 2015; Scerif, 2010). This selection is complex, as it depends on the one hand on different subsidiary processes, including detection, localization, and probably some form of recognition, on the other hand on the nature of how inputs are selected (i.e. the type of task, saliency of the stimulus, environmental context) (Colombo & Cheatham, 2006). In early development, attention changes reflect the gradual development of the interaction between neurocognitive control mechanisms and salient perceptual characteristics of the sensory world (Scerif, 2010). Thus, the development of attention is influenced by the organization of the maturing brain, which relies on the interaction between feedforward (from lower to higher cortical regions) and feedback networks (from higher to lower cortical regions, Amso & Scerif, 2015).

In newborns, looking behaviour towards environmental stimuli depends on limitations both at the level of the peripheral sensory system (i.e. the structure of the eyes or eye muscles) and at the level of brain connectivity (Johnson & De Haan, 2015). During the first year of life, as cortical control of the eyes and head movement becomes accessible, infants start to integrate these functions to build the ability to direct or inhibit attention on a volitional basis (endogenous attention, Colombo & Cheatham, 2006). From the fourth month of age, infants start to be more competent in attention orienting shifts; they learn to suppress distracting information from the previously attended location and become faster at disengaging from the attended stimulus (Blaga & Colombo, 2006; Johnson, Posner, & Rothbart, 1991). Top-down executive control functions, deriving from a more mature connectivity pattern which allows recruitment of frontal and prefrontal areas, start to regulate attention processes in the presence of competing or conflicting stimuli from 4 to 8 months of age (Amso & Scerif, 2015). Thus, attention emerges as an ensemble of distinguishable but highly related processes, which become increasingly efficient in dealing with endogenous and exogenous influences and continue to develop until adolescence (Rueda et al., 2004). **Box 3** summarises some of the concepts that have been used in the attention literature and the underlying neural systems (based on: Amso & Scerif, 2015; Colombo & Cheatham, 2006; Johnson & De Haan, 2015; Petersen & Posner, 2012; Rueda, Pozuelos, & Cómbita, 2015).

BOX 3 Glossary of terms for visual attention

- **Saccade:** rapid movement of the eyes towards a stimulus.
- **Disengagement**: inhibiting fixation from a stimulus to make a saccade in another location. Around 1 month of age, typical infants show difficulties in disengagement ("sticky fixation") as a result of an unregulated tonic inhibition of the automatic exogenous saccade elicited on the peripheral visual field in newborns.
- **Smooth visual tracking (or pursuit)**: movement of the eyes to closely follow a moving object. This ability starts to develop from two months of age and improves around three months thanks to strengthening of connection between visual areas and other areas, in particular the frontal eye fields.
- **Overt shifts of attention**: target-driven saccades.
- **Covert shifts of attention**: shift of attention without movement of the eyes or other sensory receptors, and maintenance of focused attention.
- **Exogenous attention**: process through which attention is automatically drawn towards the stimuli, objects or events.
- **Endogenous attention**: process through which allocation of attention to stimuli, objects, or events is controlled as a function of events that are internal to the organism.
- **Alerting**: process involved in maintaining a vigilant attentive state which does not affect the build-up of information in the sensory or memory systems but does affect the rate at which the individual can respond to that stimulus. Neurotransmitter: norepinephrine.
- **Orienting**: Process of shifting attention towards a stimulus, usually by performing a visual saccade towards it. Neurotransmitter: acetylcholine.
- **Executive attention**: Process of top-down regulation of attention including sustainment of the focus of attention, inhibition of shifts to distractors, attention switching and initiation, performance-monitoring and adjustments within trials in real time. Neurotransmitter: dopamine, serotonin.
- **Sustained attention**: ability to maintain the direction of attention toward a target stimulus, even in the presence of distractors.
- **Dorsal visual pathway**: pathway of Posner's posterior attentional system (Posner & Petersen, 1990) which connects the lateral geniculate nucleus of the thalamus to the occipital cortex and involves both lower-order brainstem structures such as the superior colliculus and higher cortical areas in the parietal lobe. It is responsible for orienting to a stimulus in the visual field; monitoring the visual field; disengaging attention from its current focus, shifting attention to the new target, and engaging it there (also called the "where" system).
- **Ventral visual pathway**: pathway of Posner's posterior attentional system (Posner & Petersen, 1990) which arises from the geniculo-striate visual stream, but branches from the occipital lobe to visual areas along the temporal lobe, including the inferotemporal and fusiform cortices, which reside under the temporal cortex. It codes for orientation and colour of objects and it is responsible for perception and recognition of complex visual objects or stimuli, including faces (also called the "what" system).

1.3.1.1 Measuring components of attention in infancy

Originally, the attention system of the brain was conceptualised as composed of networks of anatomical areas which perform different but interrelated cognitive functions: maintaining a vigilant or alert state, orienting to sensory events and detecting signals for focal (conscious) processing (Posner & Petersen, 1990). These sets of attentional processes are carried out by different neural pathways, which have been defined as the alerting, orienting, executive networks, respectively (Petersen & Posner, 2012, see **Box 3**). The Attention Network Task (ANT) have been created combining traditional experimental paradigms to derive efficiency of the attention networks by calculating three scores from the task performances (Rueda et al., 2015). However, the ANT requires active collaboration and is therefore not adequate to explore developmental trajectories of the different components of attention in infants.

Instead, a series of marker screen tasks evoking oculomotor responses have been designed to assess the acquisition of visual orienting and executive attention (Holmboe, Pasco Fearon, Csibra, Tucker, & Johnson, 2008; Johnson & De Haan, 2015). Moreover, habituation paradigms have been used to determine processing load and attention engagement based on measurement of looking time (Colombo & Mitchell, 2009). Recording responses during behavioural tasks requesting attention flexibility have been specifically used to assess executive attention skills in infancy (Conejero & Rueda, 2017). Eye-tracking technologies have also been widely used as they allow researchers to obtain a precise measure of looking behaviour and also to obtain a measure of pupil dilation (Falck-Ytter, Bölte, & Gredebäck, 2013). Measures of pupil dilation have been shown to be regulated by the norepinephrine system, which underpin alerting (Einhauser, Stout, Koch, & Carter, 2008), and provides a measure of cognitive processing during attention engagement (Kang, Huffer, & Wheatley, 2014).

In order to understand the neural biology underlying attention, electroencephalography (EEG) recorded simultaneously with the presentation of the stimuli has been used to study, in adults and infants research, the electrical activity of the brain generated during the elicited attentional process (Rueda et al., 2015). In particular, event-related potentials (ERPs) result from the averaged EEG signal with respect to the stimulus presentation and provide information about timing and intensity of brain responses to stimuli (Michel, Koenig, Brandeis, Gianotti, & Wackermann, 2009). Being EEG a non-invasive technique that can be used in awake infants (differently from fMRI, for example), ERPs provide ideal measures of brain activity underlying infant attention (Richards, Reynolds, & Courage, 2010).

1.3.1.2 Genetic influences on attention

Attention components and their impairment have been shown to have high heritability. A small twin study found stronger concordance in MZ compared to DZ twin pairs for executive attention (h^2=0.72) and alerting (h^2=0.18), but not orienting (h^2=0) correlates measured with the ANT (Fan, Wu, Fossella, & Posner, 2001). A large longitudinal twin study revealed that genetic effects explain 75% of the variance in attention problems (measured with the parent-report questionnaire Child Behavior Checklist) in children and that heritability estimates were not different at 3, 10 and 12 years (Rietveld, Hudziak, Bartels, van Beijsterveldt, & Boomsma, 2004).

Candidate genes

At a molecular level, evidence for genetic influences on attention comes primarily from candidate-gene studies and genomics investigations on ADHD. Candidate genes that have been shown to play a role on the attention system are those involved in the catecholaminergic system, including dopamine (see **Box 3**). In line with the evidence for a prevalent role of the catecholaminergic system on executive attention, genotypic variations of the DAT1 (or SLC6A3, sodium- and chloride-dependent dopamine transporter), DRD2 and DRD4 (encoding for the D2 and D4 subtypes of dopamine receptor) and DBH gene (dopamine beta hydrolase) have been associated with sustained attention. For orienting skills, there is evidence for associations between APOE-ε4 genotype and speed of attentional reorienting and between variation in an alpha-4 cholinergic receptor gene (CHRNA4) and spatial attention (Bellgrove & Mattingley, 2008).

From a developmental perspective, DRD4 genotype was found to be associated with right frontal EEG asymmetry at 9-months of age and with parent-report temperamental issues such as difficulties in focusing and sustaining attention at 48-months of age (Schmidt, Fox, Perez-edgar, & Hamer, 2009). Of interest in the context of early neurodevelopment, an effect of CHRNA4 genotype on correct anticipatory gaze behaviour and later effortful control was found in 6- to 7-month-old infants (Sheese, Voelker, Posner, & Rothbart, 2009). Moreover, COMT (catechol-O-methyltransferase involved in monoamine synthesis) genotype was found to be significantly associated with distractibility, a precursor of executive attention, in 9 month old infants (Holmboe, Nemoda, et al., 2010). A role of genes involved in the serotonin system in mediating infant alerting in directing attention in the context of social stimuli has also been reported (Papageorgiou & Ronald, 2013). For example, DRD4 was associated with reduced sustained attention to familiar live facial stimuli (shorter looking time and shorter latencies to the first look away from the target) and this effect was enhanced by the interaction with the serotonin transporter (5-HTTLPR/SLC6A4) genotype (Auerbach, Faroy, Ebstein, Kahana & Levine,

2001). Moreover, attention disengagement and shifting to neutral and affectively salient stimuli at 7 months of age are mediated by allele differences in the TPH2 (tryptophan hydroxylase isoform 2) gene, which is involved in serotonin synthesis in the brain. Infants with the risk genotype showed increased difficulty in disengaging from the affective salient stimulus to direct their attention towards a peripheral task (Leppänen et al., 2011).

Polygenic contributions

Polygenic influences on dimensional phenotypes of attention difficulties (combined items from the mother-report Strengths and Difficulties Questionnaire, SDQ) have been observed in the general population throughout development, with a drop in early adolescence (Stergiakouli et al., 2017). Accordingly, a GWAS meta-analysis of 20,183 individuals diagnosed with ADHD and 35,191 controls identified 12 independent common genetic variants surpassing genome-wide significance p-value threshold. Polygenic scores accounted for 5.5% of the variance in categorical diagnosis (for comparison, in ASD the proportion of accounted variance is 2.5%, Grove et al., 2019) and SNP-heritability was estimated as 0.21, with enrichment for variants located in central nervous system specific regulatory elements (Demontis et al., 2019). Elevated genetic correlation of ADHD with other comorbid developmental conditions such as ASD was observed in female but not male individuals with ADHD. Moreover, the siblings of affected females have been found to be at higher familial risk for ADHD than the siblings of affected males, suggesting a higher burden of risk in female cases (Martin et al., 2018).

1.3.1.3 Atypical infant attention in ASD

Suggestive evidence for the possibility that the effect of polygenic risk factors on the developing brain might result in alterations of a domain general skill such as attention comes essentially from infant-sibling designs (Gliga et al., 2014). In a seminal study, Zwaigenbaum et al., (2005) reported results from a systematic longitudinal investigation of atypical looking behaviour in HR infants using different techniques. They conducted a prospective longitudinal study on infant siblings followed up from 6 to 24 months of age, for whom measures of looking behaviour were collected through parent-report questionnaire, behavioural assessment and a computerised visual task. As questionnaire, the Infant Behavior Questionnaire, which is used as a measure of early temperament (Garstein & Rothbart, 2003), was administered to parents. At 12 months of age, parents reported longer durations of orienting to objects; that is, a tendency to fixate on particular objects in the environment at the expense of more active visual explorations. As behavioural assessment, a semi-structured play session, the Autism Observation Scale for

Infants (AOSI, Bryson, Zwaigenbaum, McDermott, Rombough, & Brian, 2008), was conducted. Among the individual 12-month AOSI items predicting ASD at 24 months of age there were: atypical eye contact, visual tracking and disengagement of visual attention. As computerised task, infants were tested with the 'gap-overlap' task, which was designed to test the latency required for a child to disengage fixation from a central stimulus to shift the gaze to a peripheral competitor stimulus. In this task, once the child is engaged on the central fixation stimulus, a second (comparable) stimulus appears on either the left or right side. Reaction times to move the eyes to the peripheral stimulus are measured. The critical manipulation in this task consists on the fact that the central stimulus in some trials remains on the screen and in others disappears during presentation of the peripheral stimulus. The difference between the latency required to simply shift the gaze to the new lateral target when the central stimulus disappears (gap phase) is typically compared to the latency for disengaging from the central stimulus to perform a gaze shift when it remains on the screen (overlap phase). Twelve-month-old infants with emerging ASD showed difficulties in disengaging from one of the two competing stimuli and prolonged disengagement were predictive of higher ASD severity measured with the Autism Diagnostic Observational Schedule (ADOS, Lord et al., 2000) at 24 months (Zwaigenbaum et al., 2005).

Other research work followed, examining looking behaviour using different experimental setups including eye-tracking paradigms (Falck-Ytter et al., 2013; Jones et al., 2014). Atypicalities in HR infants, compared with LR infants with no familial history of ASD were found in all three of the Posner's attention systems. For orienting, Elsabbagh et al. (2013) replicated Zwaigenbaum et al. (2005) in an independent cohort and found that disengagement was impaired at 14 months, but not at 7 months, in infants who later received a diagnosis of ASD. Atypical orienting was observed also in an independent sample of 9- to 10-month-old HR infants compared to LR when examining duration required to disengage from a distractor stimulus in the Freeze-Frame task. This task tests infants' ability to maintain their attention at a central stimulus while inhibiting gaze shifts to the peripherally presented distractors, therefore is specifically designed to examine executive attention skills (Holmboe et al., 2008). HR infants showed higher proportion of looking time at the distractors. Further, this effect was not reduced, as it was in LR, in the condition presenting more interesting stimuli such as animated human or animal figures, compared with 'boring' geometrical shapes (Holmboe, Elsabbagh, et al., 2010). This finding revealed that atypicalities in the executive attention system are also associated with familial risk for ASD.

Moreover, a recent study reported differences in the alerting system associated with increased familial risk. Nine- to ten-month-old HR infants who received an ASD diagnosis at 36 months

have been recently found to have larger constriction of the pupillary light reflex compared with typically developing infants at high and low familial risk. Of note, HR infants who did not develop ASD symptoms later in childhood had on average shorter relative constriction than the ASD group, but larger responses compared to the LR group, indicating that the alerting component of ASD might be influenced by familial risk factors (Nyström et al., 2018). Interestingly, one study revealed that visual search performances were atypically good in 7-month-old infants with later ASD, demonstrating that in non-social contexts the endogenous orienting component of attention was not impaired (Gliga et al., 2015a). Thus, there is evidence for visual attention to be under genetic control and atypical in infants with emerging ASD or high familial loading. These atypicalities might play a disruptive role towards learning in social contexts by affecting information intake from stimuli with a social content.

1.3.2 Conceptualization of social attention

The term "social attention" has been used in the literature with a wide variety of meanings which have been reviewed and clustered into coherent categories by Salley & Colombo (2016). With the aim of providing a framework to the concept of social attention and characterizing the current uses of the term, they first described three types of conceptual approaches for this construct:

1) social attention as a social behavior which is based on coordinating attention allocation with other people. In this line of thoughts, social attention corresponds closely to joint attention. It includes sharing the focus of attention on an object or event with another human being and using non-verbal social communication behaviors such as eye-gaze and pointing;

2) social attention as a proxy for social motivation. The term has been used to characterize a series of clinically relevant differences in looking behavior towards and brain responses to social stimuli between individuals with and without ASD.

3) social attention as basic visual attention skill in a system primarily biased towards human stimuli. This research line includes a majority of papers examining attention shifting and accuracy in following gaze cues or screen-based tasks presenting faces in isolation.

Salley & Colombo (2016) concluded the review suggesting to consider the social attention construct in a developmental perspective. Attention regulation capacity may be equal for social and non-social stimuli until about 8-12 months. As brain networks develop, social attention becomes an independent function expressed by social interaction behaviors, including joint attention. They also acknowledged that, although social visual attention, social motivation and

social behavior may be distinct functions until about 18 months, they then merge into an unitary process in typical development (Salley & Colombo, 2016, see **Figure 1.4b**).

1.3.2.1 Interactive Specialization of the social attention network

Basic orienting mechanisms supporting rapid attention to salient, face-like stimuli are present since birth and assist in the tuning of cortical areas for face processing and social cognition more broadly (Johnson, 2005). In fact, a 'fast-track modulation' subcortical route, including the superior colliculus, pulvinar and amygdala, biases human newborns to directing their attention to face-like stimuli (Senju, Johnson, & Tomalski, 2014), particularly in the presence of eye contact (Senju & Johnson, 2009). Thus, from 3 to 12 months of age the developing visual areas, temporal areas such as the superior temporal sulcus and the fusiform face area, and frontal areas (in particular the dorsolateral pre-frontal cortex, and orbitofrontal cortex) become increasingly responsive to low-spatial frequency patterns characteristic of faces (Johnson et al., 2005).

Spontaneous gaze following emerges from 3 to 6 months of life as the ability of the infant to follow the direction of another person's eyes and/or head towards a visible target (Del Bianco, Falck-Ytter, Thorup, & Gredebäck, 2018). This is arguably a requirement for the development of the ability to respond to joint attention, that is to follow the direction of attention of other people (Mundy, 2018). At 6 to 10 months of age, the typically developing brain is already sensitive to changes in eye-gaze directions (Senju, Csibra, & Johnson, 2008), differently from infants with emerging ASD (Elsabbagh et al., 2009). **Figure 1.4b** represents the model of early development of social attention proposed by Salley & Colombo (2016), in which a typical brain progressively learn to extract key information from the social environment by orienting toward important individuals and subsequently to the objects of their attention.

This conceptualization is in line with the Interactive Specialization framework of functional brain development (Johnson, 2011). This framework postulates that during postnatal development cortical regions progressively change their computational abilities and structural characteristics, specialising their function as a result of the interaction and competition with each other. At the same time, the human cerebral cortex undergoes a process of re-organization of patterns of inter-regional interactions between brain regions (Johnson, 2011). Evidence from the study of 6- to 12-month-old typically developing infants confirmed that a progressive selectivity to social versus non-social stimuli is observed in EEG theta activity, which becomes more pronounced and widespread at the end of the first year (Jones, Venema, Lowy, Earl, & Webb, 2015). Thus, the first year of life appears to be critical for the infant's brain to tune a wide network of specialised and interconnected areas that, initially biased towards social cues, gradually learns

to select relevant information from these stimuli and to use them to actively engage in joint attention.

At the brain level, various functions, regulated by different but interconnected systems, are involved in social attention (Klein, Shepherd, & Platt, 2009). A posterior system, mainly implicated in orienting and perceptual attention, develops in the first months of life (Mundy & Newell, 2009). This network is highly interconnected with a network that plays a role in engaging in social aspects of attention behaviour such as coordinating the gaze with another person's gaze. The superior temporal sulcus, posterior parietal cortex and lateral intra-parietal area are among the important nodes that respond to dynamic features of facial expression, including eye-gaze direction, and orienting to socially-relevant stimuli. An anterior, goal-directed attention allocation system, including the frontal eye fields, the prefrontal association cortex, the orbital frontal cortex and the anterior cingulate is involved in the reward aspect of social attention (Klein et al., 2009). It underlies the integration between one's internal monitoring of attention control and the external monitoring of the relation between others' gaze direction and behaviour (Mundy & Newell, 2009).

Figure 1.4a is taken from Klein et al. (2009) and represents these pathways, which are all involved in different but related aspects of social attention. **Figure 1.4b** has been adapted from Salley & Colombo (2016) by adding circled colours which match the colour coding system of **Figure 1.4a**, to highlight the fact that different aspects of social attention become progressively integrated reflecting the process of interactive specialization of the underlying brain networks.

It is then clear that social attention is a complex cognitive process requiring coordinated interactions of large numbers of neurons distributed within and across different specialized brain areas (Uhlhaas & Singer, 2006). As such, it is likely to be more vulnerable to even minimal dysfunctions of local and long-range connectivity in critical periods for the tuning of an integrated brain network (Amso & Scerif, 2015).

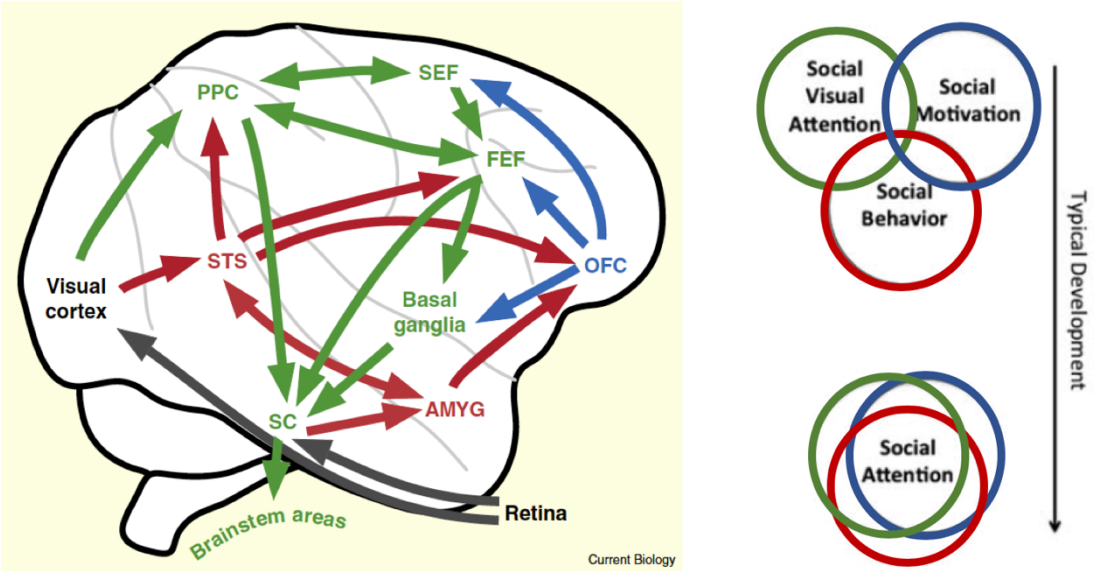

Figure 1.4 *Interactive specialization of neural networks underlying social attention.* a The social (red), reward (blue), and orienting (green) networks governing social attention as defined by Klein et al., 2009, including posterior parietal cortex (PPC), superior temporal sulcus regions (STS), SEF, supplementary eye fields (SEF), frontal eye fields (FEF), orbitofrontal cortex (OFC), amygdala (AMYG) and superior colliculus (SC). b Typical developmental trajectory of social attention from three only partly overlapping functions to a unitary construct (adapted from Salley and Colombo, 2016).

1.3.3 Social attention in the path to ASD

The mechanisms that underpin behavioural trajectories leading to the emergence of ASD remain unclear. However, one particularly strong candidate which has been suggested to contribute to the emergence of ASD are disruptions in how the child's brain engages in focused attentive states during social interaction (Klin, Shultz, & Jones, 2015). A long history of research has identified robust neurocognitive correlates of the state of focused attention that enhances learning in the infant brain.

When infants are focally attentive, they tend to show longer epochs of looking with minimal movement (Colombo & Cheatham, 2006; Colombo & Mitchell, 2009). Further, when infants direct their attention towards a visual stimulus, averaged profiles of brain activity captured with EEG show distinct posterior (P1, N290 and P400) and frontocentral (Nc) components after a stimulus is presented (De Haan, Johnson, & Halit, 2003; Luyster, Powell, Tager-Flusberg, & Nelson, 2014; Richards et al., 2010). When look durations and event-related components to social and non-social stimuli have been examined, infants with later ASD showed profiles consistent with diminished attention engagement in the first eight months of life (Elsabbagh, Mercure, et al., 2012; Jones et al., 2016; Jones & Klin, 2013). Thus, it is possible that genetic or environmental risk factors for ASD impact the brain systems necessary to maintain focused

attention to social stimuli. This process may reduce the child's ability to learn from people around them, which gradually makes the social world less comprehensible as it becomes more complicated (Klin et al., 2015). Infants may then gradually withdraw from the people in their environment as an adaptive response, producing subsequent symptoms of ASD (Johnson, 2017).

Evidence supporting this account could be obtained through linking processes like social engagement and other early developmental trajectories in infants developing ASD to the biological processes that have been associated with clinical diagnosis in large samples. One way to make progress in this area is through genetics. Indeed, twin studies indicate that social engagement (based on eye-tracking measures of face and eye looking) may be under genetic control (Constantino et al., 2017). A recent twin study showed that social attention profiles between 18 and 24 months, measured with eye-tracking, are highly heritable. In fact, MZ twins (41 pairs, N=82) exhibited high probability of shifting their eyes at the same time and fixating the same social content at the same moments, while watching the same or different scenes of social interactions. Twin–twin concordance for looking at the eyes or at the mouth of face stimuli, was significantly greater for MZ than DZ twins (42 pairs, N=84) or sex-matched non-siblings randomly paired (N=84). Importantly, these heritable correlates of social visual engagement, i.e. eyes- and mouth-looking behavior, were consistently reduced in 2 cohorts of age-matched toddlers with ASD (N=88) (Constantino et al., 2017). Thus, there is suggestive evidence that some components of social attention are heritable and that atypicalities can be observed at the neural and behavioral level before the emergence of social difficulties. Those elements provide some support for the possibility that early social attention difficulties might serve as intermediate phenotypes (or endophenotypes) between ASD risk factors and the emergence of social difficulties.

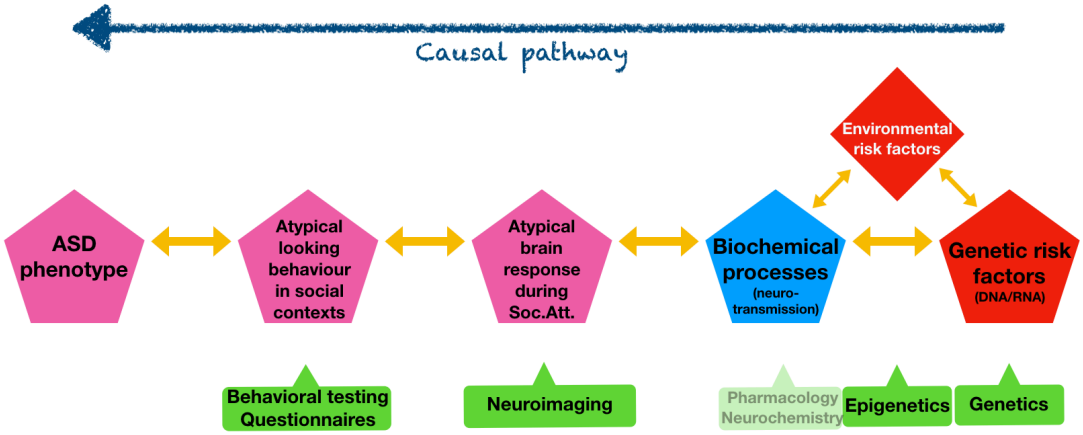

Figure 1.5 *Studying the gene-behaviour pathway underlying the development of ASD*, adapted from Rueda, Pozuelos & Combita (2015) as described for Figure 1.1. Elements of the pathway have been coloured in red when a "risk" value has been added to them, and in pink when the content has been adapted to the specific purpose of investigation of ASD social traits. Specifically, in this thesis I test the hypothesis that genetic and environmental risk factors interact and increase vulnerability in the developing brain, which show early signs of atypical response when engaged in social attention. This disruption occurring in critical periods influences looking behaviour in social contexts, thus limiting the opportunity of learning. This atypical developmental trajectory ultimately results in the ASD core symptoms of social difficulties.

Table 1.1 *Evidence for considering social attention an endophenotype of ASD*, based on the criteria for endophenotypes defined by Gottesman & Gould (2003).

The endophenotype…	Measure of social attention	N	Reference
(1) … is associated with illness in the population	Reduced looking at the eyes region	9 ASD vs. 9 rel. vs. 9 LR	Dalton et al., 2007
	Eye-gaze direction detection	26 ASD vs. 22 rel. vs. 26 LR	Wallace et al., 2010
	Eye-gaze direction detection	33 ASD vs. 38 LR	Forgeot D'Arc et al., 2017
	Looking at the eyes and mouth region of the face in toddlerhood (eye-tracking)	88 ASD vs. 250 LR	Constantino et al., 2017
(2) … is heritable	Looking at the eyes and mouth region of the face in toddlerhood (eye-tracking)	82 MZ vs. 84 DZ	Constantino et al., 2017
(3) … is primarily state-independent (manifests in an individual whether or not illness is active)	6 months: Proportion of looking time to the face during gaze following videos (eye-tracking). Latency of peak look to faces in habituation task (eye-tracking) (Jones et al., 2016). Nc amplitude and latency, P400 latency to faces in response to faces vs. objects (EEG) (Jones et al., 2016). 7 months P400 latency between gaze shifts away and toward the infant (EEG) 9 months: Interest in faces, shifts attention to person, response to name from parent-report (POEMS) 12 months: Interest in faces, waiting, shifts attention to person from parent-report (POEMS). Initiating Joint Attention in semi-standardized play-based assessment (ADOS). Gaze to faces and social smiling in semi-standardized assessment (MSEL). IJA and RJA structured assessment of social communication behaviors (ESCS). Behaviourally coded attention to social targets during distress condition in play-based assessment. Attentiveness to parent predicted (PCI). Eye Contact (AOSI). 13 months: Proportion of time to the referenced object during gaze following videos (eye-tracking). Proportion of looking time at the face in a face pop-out task (eye-tracking). 14 months: Interest in faces, shifts attention to person in semi-standardized play-based assessment (AOSI). Peak-look at the face in a face pop-out task (eye-tracking) (Hendry et al., 2018). IJA and RJA in assessment of social communication behaviours (CSBS DP). 15 months: RJA during live interaction (clinical assessment) 18 months: Attention and affective response to distress. Eye contact, social smiling in semi-standardized assessment (MSEL).		Evidence summarised from review on findings on infant siblings: (Jones et al., 2014). A table with all the listed studies can be found in this paper. More recent findings are added with their reference.
(4) Within families, endophenotype and illness co-segregate	Use of the eyes region of the face for emotion recognition	15 HR-BAP vs. 27 HR-noBAP vs. 20 LR	Adolphs et al., 2008
(5) … is found in nonaffected family members at a higher rate than in the general population	Reduced looking at the eyes region	9 ASD vs. 9 rel. vs. 9 LR	Dalton et al., 2007
	Eye-gaze direction detection	26 ASD vs. 22 rel. vs. 26 LR	Wallace et al., 2010

LR: low-risk individuals with no family history of ASD; MZ: monozygotic twins; DZ: dizygotic twins; EEG: electro-encephalography; POEMS: Parent Observation of Early Markers Scale, ADOS: Autism Diagnostic Observational Schedule; MSEL: Mullen Scales of Early Learning; PCI: Parent-Child Interaction; AOSI: Autism Observation Scale for Infants; IJA: Initiating Joint Attention; RJA: Responding to Joint Attention, CSBS DP: Communication and Symbolic Behavior Scales Developmental Profile, BAP: Broader Autism Phenotype.

1.3.3.1 Unsolved questions

Table 1.1 summarizes evidence from the literature suggesting what social attention measures might be good candidate endophenotypes of ASD. Main findings of key studies are described in the following chapters, but this table is useful to point out that there is already suggestive evidence supporting the hypothesis that social attention might be involved in the path to ASD. One aspect that can be noticed is that, while the majority of the adults' literature considers attention to the eyes region of the face as key measure, the infants' literature (**Table 1.1**, row (3)) includes different measures, derived from parent-report, behavioural, neurophysiological and eye-tracking assessments, often capturing atypical development when looking at faces in tasks with an array of objects (face-popout) or behaviours in interaction with people and objects. What aspects of early atypical looking behavior in social contexts are going to contribute more to the final ASD phenotype remains to be verified (and is the focus of **Chapter 2** and **3** of this thesis).

Moreover, the relationship between infants' early markers (3) and the components of social attention which meets requirements (1), (2), (4) and (5) (i.e. mainly attention to the direction of eye-gaze and visual scanning of the eyes region of the face) is not known. For example, it might be that findings listed in row (3) reflect domain-general difficulties, rather than social attention issues in attention, in infants. Or it might be that research accounting for (1), (2), (4) and (5) simply observed behaviors that are part of the core ASD social symptoms in adults, rather than refer to an independent construct as conceptualized by Salley & Colombo (2016). In sum, it is possible that that the different components disrupted in infants and adults are unrelated. This account is not implausible, especially considering that in atypical development the integration of the different aspects of social attention during the second year of life (see **Figure 1.4b**) might not happen in the same way as in typical development (Salley & Colombo, 2016). Observing whether the criteria for being considered endophenotypes are met in the same individuals, followed-up longitudinally, as in **Chapter 4**, might help to verify whether there is continuity between the measures used as endophenotypes.

Another concern that might arise after reading **Table 1.1** relates to the small number of participants of the studies listed as evidence for requirement (4) and (5). Although some studies might have been missed, evidence for these requirements might need confirmation in larger familial studies. During my PhD I collected measures of eye-gaze direction detection and autistic traits from all family members of infants who participated in the British Autism Study of Infant Siblings (BASIS). **Chapter 4** addresses the issues of uncertain continuity between infant and child measures and between different measures used in the adult literature. Specifically, it attempts to replicate requirements (4) and (5) in a larger sample than in earlier studies (Adolphs, Spezio,

Parlier, & Piven, 2008; Dalton et al., 2007; Wallace et al., 2010) with a measure of social attention previously associated with genetic variants (Skuse et al., 2014, see **section 4.1.2**).

If social attention is involved in the causal pathway for ASD we would expect that genetic variants increasing the liability of ASD also predict differences in social attention (Iacono, Malone, & Vrieze, 2017). This aspect is tested in **Chapter 4** in the familial sample, to see whether common biological mechanisms are responsible for the core symptoms and for social attention atypicalities. However, just as we need to take a developmental approach to studying the neurocognitive processes underpinning ASD, we need to take a developmental approach to genetics. Traditional behavioural or molecular genetics does not account for the fact that gene expression patterns are not developmentally static; rather, they change over developmental time and there are substantial individual differences in these changes (Moore, 2016). Understanding the emergence of ASD requires us to study dynamic changes in both neurocognitive systems and genome function in parallel. To this aim, **Chapter 5** explores the relationship between aggregate genetic risk and infants' social attention and **Chapter 6** examines the relationship with epigenetic profiles.

As mentioned, another source of doubt for considering social attention in the path between genetic risk factors and ASD phenotype is that infant measures collected prior the emergence of ASD symptoms might reflect disruptions of domain-general functions which are not specific to ASD. As the construct depends on the attention system, it might well be that the observed measures reflect risk loading for neurodevelopmental disorder in general or for the comorbid ADHD (Johnson et al., 2014). Thus, **Chapter 5** will examine to what extent early measures of social attention are accounted for by polygenic risk for ASD and ADHD.

In sum, the following questions will be addressed in the series of studies proposed in this thesis:

- Which of the candidate early markers of ASD contribute to developmental trajectories towards the emergence of the ASD behavioural phenotype?
- To what extent atypicalities of social attention identified in the adult literature map onto atypicalities observed before the emergence of ASD traits?
- Are previous findings of social attention atypicalities in family members of individuals with ASD replicable?
- Do atypicalities of social attention share genetic variance with ASD traits?

The various pieces of evidence collected in this thesis will allow us to evaluate whether there is substantive evidence for considering atypicalities in social attention as reflecting an intermediate step from ASD risk towards developmental trajectories leading to ASD.

1.3.4 A note on causality

In **section 1.3.2**, I referred to the Interactive Specialization as a conceptual framework to understand how disruptions in early brain development due to ASD risk factors might impact the efficiency of social attention networks with cascading effects on social and cognitive development (Johnson, 2011). This account is based on the circular causality/probabilistic epigenesis approach, which postulates the possibility of bidirectional causal relationships between genetic profiles, the brain and the behavioural outcome (see **Figure 1.1**) (Gottlieb, 2007). Under this model, it becomes very complex to establish neat paths of causality, especially when observations which would help to clarify relationships are not available. Many research works prefer to stay agnostic with respect to possible causal links, talking about observed associations instead. However, as I mentioned in the very first pages of this chapter, I believe it is time for scientists to try to provide evidence-based explanations of developmental mechanisms leading to ASD, if the design and methods allow them to. The use of longitudinal designs and statistical models which are adequate to test hypotheses on the direction of associations (like structural equation models) are among the tools that have been used to this aim in the presented PhD work.

The general direction of causality (blue arrow on **Figure 1.5**) is determined by the concept of endophenotype, which is defined as an intermediate phenotype between genetic factors and emerging ASD. In this thesis, I defined three direction-criteria to establish that a factor had a "causal" relationship with another:

- from early to later ages (temporal causality)
- from genes to behaviours (biological causality)
- from parents to offspring (transmission causality).

I acknowledge, however, that there are limitations to these assumptions, and I am going to briefly discuss them below.

1.3.4.1 Temporal causality

The idea underlying the 'temporal causality' criterion is that if a trait is observed when another trait has not emerged yet, and a significant relationship is found among the two variables, one (the "early marker") is more likely to have had an effect on the other. Of interest, Johnson and colleagues (2014) provided a distinction among different types of early markers of neurodevelopmental disorder and clarified that, while some of them might be considered 'antecedent' and as such they might be involved in the causal path, others might also be

'precursors', i.e. markers that simply indicate the approach of the disorder. In their paper, the authors list reduced social attention in infants with ASD among the examples of possible precursors, as it is conceptually related to the core domains. A proposed method to disentangle whether an early marker is considered an antecedent or a precursor of the emergent disorder would be to evaluate downstream effects of early interventions targeting the underlying function of such marker and using it as a measure of efficacy of the intervention (Johnson et al., 2014). Early parent-delivered social interventions have been shown to improve social attention performances as well as severity of social traits (Green et al., 2017; Jones, Dawson, Kelly, Estes, & Webb, 2017). However, these findings do not allow us to define whether social attention also has cascading consequences on later behaviour and or whether it is simply the reflection of the disorder process. Combining longitudinal observations and the study of associations with molecular processes might allow us to make a step further in understanding the evidence for social attention as a candidate intervention target (Green et al., 2015).

1.3.4.2 Biological causality

The deterministic epigenesis concept, as opposed to probabilistic epigenesis, states that there is a clear direction of causality which goes from genes to behaviour (see Gottlieb, 2007). While this account is justified by the fact that DNA sequence is there since the beginning of the individual's life and does not change, the study of epigenetics provided insights on the dynamic nature of gene expression changes and their functional role in relation to phenotypes. Of note, the fact that many genetic mutations have been associated with chromatin remodelling and DNA methylation regulation (as reviewed in **sections 1.2.1** and **1.2.3**) suggests that epigenetic mechanisms might be atypical in some individuals with ASD, making it even harder to study possible interactions. Thus, environmentally driven changes in gene expression patterns, especially in critical periods for brain development, are likely to play an important, individual-specific role in the way in which genetic variations act on the entire system. As proposed by Johnson and colleagues (2015, 2017), individual developmental trajectories leading to ASD core features likely emerge as a result of adaptive (i.e. devoted to increase fitness, see Frankenhuis, Panchanathan, & Nettle, 2016) choices of each neural system based on its degrees of processing limitations. One of these adaptive choices is the selection and construction of an environment that best suits the individual brain's processing style. This process critically determines the infant's possibilities for learning and, at the brain level, shapes the reorganization of connectivity patterns among areas, leading to structural changes (Johnson et al., 2014, Johnson, 2017).

This concept partly overlaps with the concept of gene-environment interaction (GxE), which describes that different genotypes respond to environmental variation in different ways and the

genetic influences become stronger with age as the individual selects the environment that better suits his or her genetic background (Plomin et al., 2013). Thus, a dynamic, bidirectional interaction exists between genetic factors and behavioural/computational choices based on a system level in interaction with the environment.

1.3.4.3 Transmission causality

As exposed earlier (see **section 1.2.2**) the study of the relationship between parental and offspring measures have informed the scientific community on the possibility that familial factors influence neurodevelopment (Pisula & Ziegart-Sadowska, 2015). Because, especially in the very earlier stages, parents' nurturing efforts represent the newborn individual's environment, it is plausible that parental genotype and phenotypic characteristics have an impact on early behaviour. At a molecular genetics level, the direction of influence can be established by determining which genetic variants are inherited and which ones are de novo. However, tracking down the complexity of interactions between factors within a familial environment is not straightforward. Two possible confounders are highlighted here, both potentially related to the concept of GxE: 1) the extent to which the context of a complex familial system might confound the relationship between measures within the parent-offspring dyad; and 2) the contribution of the child's behaviour in shaping parenting style, and consequently environmental exposure. Of note, the second aspect is included in the first, as the target infant too is part of the family system.

Familial context as a whole significantly contributes to child psychological development (Mathijssen, Koot, Verhulst, De Bruyn, & Oud, 1997). Specifically, familial context influencing human development depends on the interaction of genetics and environment in family processes, as well as the external influences affecting the family (Bronfenbrenner, 1986; Rowe & Plomin, 1979). Thus, the relationship between a parent's characteristic and the child's phenotype might be highly confounded by the two individual's susceptibility threshold to external events. Accordingly, twin studies have informed us that there is little effect of shared environment on social impairments, communication impairments, restricted repetitive behaviours both at the extreme and as measured on a continuum in the population (Ronald et al., 2006), suggesting that familial environment might be perceived differently by siblings. Of interest, the unique relationship of the parents with each of their children has been shown to play a role on the sibling relationship, which is part of the non-shared environmental influences (Jenkins, Rasbash, Leckie, Gass, & Dunn, 2012).

Additionally, when examining the role of parenting behaviour towards an individual child, the effect of the entire environmental context on the parent cannot be ignored. In fact, especially

in families of individuals with ASD, levels of stress and/or the feeling of being less effective as parents might significantly impact parental sensitivity and responsiveness (Crowell, Keluskar, & Gorecki, 2019). Moreover, children's genetic predispositions and negative parenting have been shown to be closely interrelated (Maccoby, 2000; Pasco Fearon et al., 2015; Soukup-Ascençao, D'Souza, D'Souza, & Karmiloff-Smith, 2016). Thus, on the one hand the association between parents' measures and their infants' trajectory are likely to be confounded by other unaccounted elements influencing the family system as a whole, on the other hand the child's condition is likely to have a direct effect on parenting style and consequent learning opportunities (Soukup-Ascençao et al., 2016).

In sum, by establishing criteria for the definition of causality I do not try to deny the complex dynamic interaction between genetic, environmental and familial factors that can shape human development. On the contrary, I acknowledge the importance of considering multiple influences in the causal pathway towards ASD. With my PhD work I aimed to test whether social attention meets basic criteria to be attributed a suggestive causal role. If those associations exist, this research will pioneer further work on deep investigations on the nature of causal relations, including measures of environmental factors, which are highly underrepresented in the present work.

1.4 OVERVIEW OF THE PRESENT WORK

An discussed, because social attention has been proposed as a key mediating mechanism underlying later emergence of social communication difficulties (Dawson, Bernier, & Ring, 2012; Klin et al., 2015), understanding how it is related to genetic and environmental risk in families with multiple affected children can shed light on its role as an endophenotype of ASD (Gliga et al., 2014; Jones, Venema, Earl, Lowy, & Webb, 2017). Importantly, familial risk is composed of both genetic and environmental contributions, and to the interaction between these two elements. For this reason, in the present project I aimed to investigate the specific contributions of genes, familial behavioural characteristics and epigenetic markers to neurodevelopmental outcomes. Moreover, looking at developmental trajectories in infants at high risk for ASD with different developmental trajectories could indicate whether social attention skills serve as a protective factor, by providing access to critical social experiences in early development (Chawarska, Macari, Powell, DiNicola, & Shic, 2016; Szatmari, 2018).

In **Chapter 1** I provided a general introduction to what genetics studies and infant-sibling designs have taught us about ASD, and explained that the aim of this thesis was to combine these two fields to investigate causal pathways leading to the development of ASD traits. I then presented social attention as a candidate endophenotype for ASD.

In **Chapter 2** I examine early signs of atypical brain states during social attention (6-11 months of age) as predictors of ASD and later difficulties in social behaviour (3 years of age). This research tests evidence for a possible causal association between disrupted social attention in critical stages for the development of social cognition and later outcome.

In **Chapter 3** I use structural equation modelling to study how neural (from **Chapter 2**) and behavioural correlates of social attention over the first two years of life contribute to developmental pathways of social behaviour, autistic traits, language skills, executive function. By investigating the relationship between different early markers of ASD, I also aim to shed light on the contribution of various components of social attention to different aspects of the ASD phenotype.

In **Chapter 4** I enquire to what extent social attention is affected by genetic and familial risk factors by looking at the relationship between social attention skills, autistic traits in the social domain and ASD polygenic score in family members of children with and without ASD. I also explore possible effects of parental polygenic risk factors and phenotypes on the infant measures which came out as meaningful for the trajectory to ASD symptoms in **Chapter 3**. Further, I discuss the evidence for protective and risk value of social attention in individuals at familial risk.

In **Chapter 5** the aim is to understand whether shared causal mechanisms are observed between infant social attention and neurodevelopmental disorders. To do this, I report the effect of polygenic risk for ASD and ADHD and older siblings' ASD and ADHD traits, as a proxy of familial burden, on infant social attention. These analyses, coupled with the investigation of a link with later symptoms of the two neurodevelopmental disorders, provide information on whether pathways from risk factors to later outcome through social attention are disorder-specific or general for neurodevelopmental disorders.

In **Chapter 6** I look at possible epigenetic mechanisms which might underlie individual variation in ASD phenotype expression and earlier markers of social attention atypicalities, trying to understand whether there is suggestive evidence for an epigenetic regulation of candidate risk genes/pathways influencing early social attention phenotypes.

Last, in **Chapter 7** I discuss how the work of this thesis provides insights into the mechanisms underlying developmental trajectories of ASD and the role of social attention in this pathway. This chapter also highlights how this work relates to and extends previous work in the field of developmental neuroscience. The limitations of the studies presented are also acknowledged. Moreover, I suggest possible directions for future research.

Figure 1.6 graphically represents how the work presented in each chapter of this thesis investigates different relationships which constitute the casual pathway towards ASD.

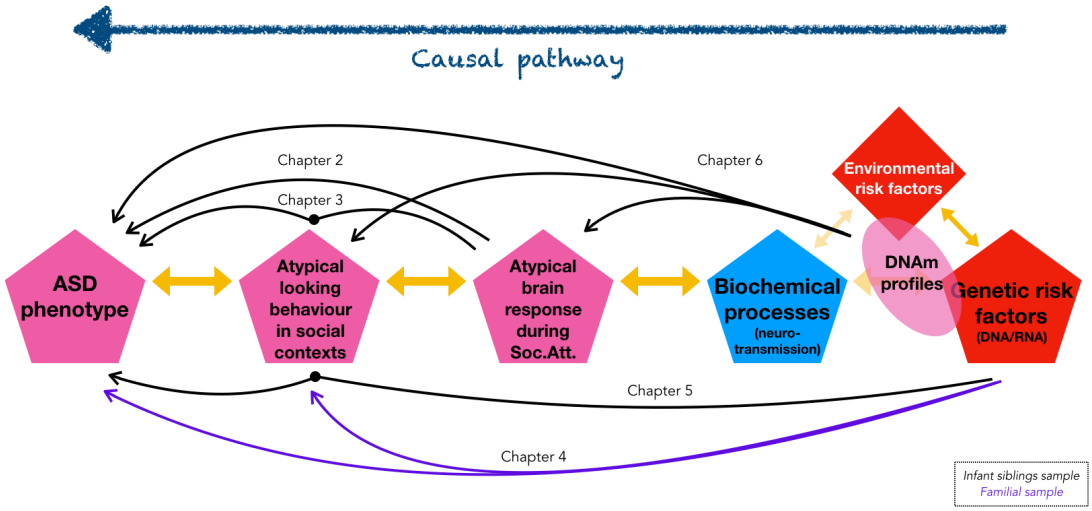

Figure 1.6 *Studying the role of social attention in the causal pathway of ASD*, adapted from Rueda, Pozuelos & Combita (2015) as described for Figure 1.1, and relationships studied in each chapter of the PhD work presented in this thesis.

Abbreviations: DNAm: DNA methylation; Soc.Att.: social attention.

CHAPTER 2

DIMINISHED ENGAGEMENT OF ATTENTIVE BRAIN STATES TO FACES PRECEDES THE EMERGENCE OF ASD

2.1 INTRODUCTION

Although ASD is highly heritable and alterations of the brain have been recognized before the first year of age in children who later exhibit core symptoms (Shen & Piven, 2017), little is known about the mechanisms leading to ASD symptomatology (De La Torre-Ubieta, Won, Stein, & Geschwind, 2016). Indeed, genetic or environmental risk factors, or probably a combinations of both, might lead to disturbances in the whole-brain organization, causing neural processing atypicalities during critical periods for learning (Johnson, 2017). In this chapter, I tested the possibility that early altered brain responses during social attention might be part of the early developmental path to ASD by investigating whether these precede and predict the development of social difficulties in infants at familial risk for ASD.

As mentioned in **Chapter 1**, prospective longitudinal studies of brain development in at-risk populations followed up from infancy provide promising opportunities to study the emergence of neurodevelopmental disorders (Elsabbagh & Johnson, 2010; Piven, Elison, & Zylka, 2017). Progress requires revealing the developmental processes that canalise the diverse set of identified genetic and environmental risk factors (Grayson & Guidotti, 2015) towards a coherent phenotypic profile that can be reliably recognized at the categorical level by trained clinicians (Jeste & Geschwind, 2014; Klin, Shultz, & Jones, 2015). Prospective studies of infants with an older sibling with ASD showed that behavioural differences in social attention, broadly defined as allocating attentional resources towards stimuli carrying a social content (see **section 1.3.2**), emerge over the first two years of life in infants who later receive diagnosis of ASD (Chawarska, Macart, & Shic, 2013; Jones et al., 2016; Jones & Klin, 2013; Ozonoff et al., 2010; Shic, Macari, & Chawarska, 2014). Looking at what might underpin these emerging changes in behaviour might be critical to understand the underlying biological mechanisms of ASD. In this study I focussed on a candidate process which might be involved in the pathway to ASD social difficulties: engagement of attentive brain states in response to social stimuli.

One leading hypothesis suggests that failure to develop experience-dependent cortical control

of attention to people might disrupt the development of social cognition (Klin et al., 2015). Indeed, in toddlers with ASD altered neural attention responses to faces relate to broader delays in socialization skills (Webb et al., 2011). Further, stronger brain responses during attention to faces are associated with an improvement of social symptoms following behavioural intervention in toddlers with ASD (Dawson, Jones, et al., 2012). If disrupted cortical social attention is involved in the pathway to ASD, atypical neural responses to people should be seen between 6 and 12 months, when particular brain areas or networks become increasingly tuned to respond to social cues (Jones, Venema, Lowy, Earl, & Webb, 2015). Prospective studies of infants who later received a diagnosis of ASD have reported altered social brain development, including reduced cortical responses to social videos at 4 to 6 months (Jones et al., 2015), reduced neural sensitivity to gaze shifts at 6 to 9 months (Elsabbagh et al., 2012), and altered neural responses to faces versus objects (Jones et al., 2016). In the present study, I specifically asked whether early atypicalities in brain activation when attending to faces compared to a control stimulus reflected later difficulties in the social domain in a larger group of children at familial risk for ASD.

2.1.1 Event-related potentials and attention

Neural responses during attention engagement have been largely explored using the analysis of averaged, event-related potentials (ERPs). ERPs represent the synchronous activation of electrical fields associated with the activity of a large population of neurons. The series of positive and negative deflections (i.e. the components) reflects changes in the brain's electrical activity in response to a discrete stimulus or event, and can be interpreted as different cognitive operations (Nelson & McCleery, 2008). ERPs have informed the study of infant perception and processing and the inference on the neural generators underlying these processes. In particular, four visual components have been principally investigated to examine infants' response to social stimuli: P1, which reflects the information propagation through the visual system underlying perceptual analysis of a general visual stimulus, N290 and P400, which are face-sensitive components (De Haan, Johnson, & Halit, 2003), and the Nc, considered an index of allocation of attentional resources to interesting or salient stimuli (Richards, Reynolds, & Courage, 2010).

The latter is of particular interest in this research because it has been extensively validated as a neural correlate of attention to interesting stimuli in infants, using simultaneous heart-rate recording, association with looking-time and careful experimental manipulation (Luyster, Powell, Tager-Flusberg, & Nelson, 2014; Reynolds, Courage, & Richards, 2010; Richards, 2003). The Nc ("negative central") component is a negative deflection measured around 300 to 500 ms

after the stimulus onset, and is detectable over frontal regions by 4-6 months of age (De Haan et al., 2003). This component is elicited by the activation of anterior temporal lobe and prefrontal cortex especially during social attention in infants from 4.5 months of age (Guy, Zieber, & Richards, 2016; Reynolds & Richards, 2005). As brain regions become more organized and specialised (Johnson et al., 2005), active cortical areas are detected more along the midline by 7.5 months, when the Nc is largely generated by the superior and posterior regions of the prefrontal cortex (Reynolds et al., 2010).

This ERP component is modulated by cognitive and/or emotional content of visual stimuli (De Haan et al., 2003). In particular, the Nc mean amplitude was found to be correlated with the magnitude of novelty preference measured in looking-time and it is larger during periods of high attentiveness to the stimulus identified with heart-rate (Reynolds et al., 2010). Research has shown that enhanced Nc is observed in infants in response to salient, rather than frequent, stimuli. For example, larger Nc is observed in 6-month-old infants looking at familiar versus unfamiliar faces (De Haan & Nelson, 1999). These findings led to the interpretation that this negative activation recorded over the frontal areas reflects the initial stages of endogenous attention orienting towards arousing stimuli (Richards et al., 2010) and mean amplitude of the Nc has been considered a neural correlate of attention engagement (De Haan et al., 2003; Jones et al., 2016).

The Nc component was used by Dawson at al. (2012) as an early marker of atypical neural function underlying social attention difficulties in children with ASD. They found that 18- to 30-months-old children with ASD, tested after receiving a comprehensive developmental behavioural intervention focused on improving social attention and social engagement, showed the same neural responses as age-matched typically developing children. On the contrary, the ASD children who did not receive the experimental intervention showed slower Nc when looking at faces, compared to toys. Importantly, these ERPs were correlated with social skills after the intervention, showing that the normalization of brain activity was also associated with an improvement of autistic symptoms (Dawson, Jones, et al., 2012). Jones et al. (2016) looked at the Nc component to show preliminary evidence that atypical neural processing when attending to faces is detectable at 6 months of age, significantly earlier than the emergence of ASD symptoms. In the present study, I built on these preliminary signals to examine neural correlates of attentional engagement to faces in a larger cohort of infants with and without family history of ASD.

2.1.2 Aims of the study

A less negative mean amplitude and shorter latency of the Nc when attending to faces was found in infants who later received diagnosis of ASD (N=6), compared with typically developing infants (N=25, Jones et al., 2016). The first aim of the present study was to replicate and extend this finding in a larger, independent sample of infants with (N=19) and without (N=112) later ASD. Infants underwent an EEG task where faces with the gaze directed towards or away from them and a control non-social stimulus were presented. Based on Jones et al., (2016), I hypothesized that infants with emerging ASD would show less negative amplitude and shorter peaks of the Nc component while looking at faces with direct gaze, which are typically highly salient social stimuli for young infants (Rigato, Farroni, & Johnson, 2010), compared to a non-social condition (visual noise). The EEG task design allowed me also to look at whether this effect is specific to direct gaze or not, and to better control for some of the visual properties of faces in the non-social condition, compared to Jones et al. (2016) who used images of brightly coloured toys. To test whether early neural response during social attention is involved in the pathway to the development of social skills, I assessed whether it predicted socialization skills measured at 3 years of age through a parent-report questionnaire, the Vineland Adaptive Behavior Scales (VABS).

Traditional approaches to ERP analysis depend on the position of the recording electrodes and the selection of the electrodes of interest is made a priori to assess the activity of the underlying neural population (Michel, Koenig, Brandeis, Gianotti, & Wackermann, 2009). In the last 10 years, research has shifted to view attention as reflecting a state of the brain spread over a network connecting multiple areas across the entire brain (Amso & Scerif, 2015; Hellyer et al., 2014; Klein, Shepherd, & Platt, 2009; Petersen & Posner, 2012; Richards et al., 2010). If structural and functional abnormalities can be found in infants with later ASD diagnosis from the sixth month of age (Piven et al., 2017), different spatial configurations of the scalp field should be observed in those infants, compared with typical infants, when attending to stimuli. In addition to examining the Nc component, the second aim of the study was to extend the ERPs analysis to the investigation of the entire scalp, to see whether infants with later ASD showed atypical scalp field topographies of neural activity during attention engagement.

The third aim of the current study was to conduct a more fine-grained analysis of the spatio-temporal characteristics of ERP data to further investigate whether increased vulnerability for ASD is manifested as atypical brain processing during social attention in infancy. In fact, the topography of the brain's electric field on the scalp results from the activities of underlying neuronal populations. Therefore, differences in map configurations over time indicate that

different functional processes are activated (Michel & Murray, 2012). The topography analysis, as well as previous research, indicated that different phases of the information processing might underlie the neural signal captured in the Nc time-window (Guy et al., 2016; Reynolds & Richards, 2005). Importantly, there has been a recent shift in the approach to the study of attention from looking at averaged indices to looking at how long a particular brain state lasts (King et al., 2018) and what is the strength of the connectivity pattern underpinning it (Hellyer et al., 2014). I further analysed group differences in the characteristics of the brain state during social attention using microstates. Microstates analysis provides information on the sequence of stable configurations of the scalp field potential, representing subsequent blocks of the information processing (Michel et al., 2009). "Typical" microstate maps were estimated from infants without a family history of ASD when attending to faces with direct gaze. We then tested whether duration and strength of the microstate reflecting social attention engagement were atypical in infants with emerging ASD and specifically predictive of social skills at three years in infants at high familial risk.

2.2 METHODS

2.2.1 Participants

2.2.1.1 BASIS Phase 1 and 2

A total number of 247 children participated in the British Autism Study of Infant Siblings (BASIS, www.basisnetwork.org). Fifty-four younger siblings of children with ASD (high-risk infants, HR) and 50 LR infants were recruited in the initial phase of BASIS, Phase 1. Subsequently, 116 HR and 27 LR participated in Phase 2. All LR infants, recruited from a volunteer database at the Birkbeck Centre for Brain and Cognitive Development, had gestational age between 37 and 42 weeks, except one born prior to 37 weeks, and no first or second relatives with ASD. For the HR infants, who had an older sibling with a community clinical diagnosis of ASD (hereafter proband), parents completed the Development and Well-Being Assessment (DAWBA, Goodman, Ford, Richards, Gatward, & Meltzer, 2000) and/or the Social Communication Questionnaire (SCQ, Rutter, Bailey, & Lord C., 1993) which were used by research clinicians in the BASIS team (T. Charman, G. Pasco) for confirmation of local clinical diagnosis. Screening for possible ASD in the older siblings of the LR infants was undertaken using the SCQ, with no child scoring above the instrument cut-off for ASD. Medical history review confirmed a lack of ASD within first-degree relatives of the LR participants.

At enrolment, all children in the HR group had an older sibling who received a diagnosis of ASD from a UK clinician. Enrolled children took part in research assessments when they were around 8-month-old (T1), 14-month-old (T2), 2-year-old (T3) and 3-year-old (T4). At T1 and T2, infants received a two-day assessment during which a series of measures were collected, such as parent-report questionnaires, behavioural assessments, eye-tracking and EEG experiments. Behavioural assessments and parent-report questionnaires were collected at T3 and T4. Recruitment and testing of these children were carried out by researchers of the BASIS Team working at Birkbeck College (T1 and T2) and King's College London (T3 and T4). Adaptive behaviour and developmental level were assessed at each visit using the VABS and the Mullen Scales of Early Learning (MSEL), respectively. **Table A2.1** (see **Appendix of Chapter 2**) summarises information on the Phase 1 and 2 samples, age range, adaptive skills (VABS Composite score) and cognitive abilities level (MSEL Composite score) at each of the four visits. [Of note, some of the children enrolled in Phase 2 also participated in a preliminary phase involving functional Near Infrared Spectroscopy (fNIRS) and/or functional Magnetic Resonance Imaging (fMRI) at around 5 months (T0). Data collected at T0 have only been used in exploratory analyses in **Chapter 6** of this thesis but do not constitute the research dataset for this PhD].

At T4, a clinical assessment was provided by an independent team to determine whether the child had developed ASD. 239 children participated in the follow-up visit. Of the 8 children who dropped out, 4 HR (across Phases) were excluded from all analyses presented in this thesis testing outcome group differences, while 4 LR children were included in these analyses in the control group. Experienced clinical researchers administered, or closely supervised the administration of, a battery of clinical research measures to the 36-month-old children and determined the clinical outcome by reviewing all the available measures. Among these, the Autism Diagnostic Observation Schedule-Generic (ADOS-G, Lord et al., 2000) is a semi-structured observational assessment used to determine the presence of autistic behaviours and the severity of ASD symptoms. ADOS-2 severity scores, reported in **Tables 2.1, 2.4** and **A2.1**, were calculated using the relevant raw item scores from the original ADOS-G assessment to re-calculate subscale and total scores as per ADOS-2. Comparison Severity Scores (CSS) were obtained from the new ADOS-2 overall total. Additionally, parents were interviewed using the Autism Diagnostic Interview – Revised (Lord, Rutter & Le Couteur, 1994), a detailed interview covering early development and autism diagnostic features, and required to fill the parent-report questionnaire VABS and Social Responsiveness Scale (SRS). Of the 166 HR infants who participated in BASIS Phase 1 and 2, 34 (20%) met criteria for an ASD diagnosis (HR-ASD) using ICD-10 criteria (Phase 1) or DSM-5 (Phase 2), 88 (53%) were classified as typically developing (HR-TD) and 44 (27%) were identified as showing signs of atypical development (HR-Aty) by scoring above the autism spectrum threshold on the ADOS-G, and/or scoring above the ASD

threshold on the ADI-R, and/or scoring below –1.5 standard deviations on the MSEL Early Learning Composite, Visual Reception, Receptive Language or Expressive Language subscales. Informed consent was obtained from parents of all the infants taking part in the study. Ethical approval for BASIS Phase 1 and 2 data collection was obtained from NHS Health Research Authority (REC reference number 08/H0718/76 and 06/MRE02/73).

2.2.1.2 Sample for the current study

Figure 2.1 provides information on the original and final number of participants and reasons for exclusion from the current study (see **Table 2.1** for comparison of the characteristics of the sample for the current study with the excluded participants). One hundred-thirty-one infants (40 LR, 91 HR) were included in the current study as they provided sufficient minimal artifact EEG data.

Categorical outcome

The 91 HR children were classified into three outcome groups following the 3-year visit: 19 were identified as HR-ASD; 48 as HR-TD and 24 as HR-Aty. Of the children in the HR-Aty group, 14 met ADOS-G criteria only, 3 met both ADOS-G and ADI-R criteria, 3 met both ADOS-G and MSEL criteria, and 4 met MSEL criteria only.

Dimensional outcome: social adaptive skills

As a measure of social skills at three years, the standard score of the Socialization Domain from the second edition of the Vineland Adaptive Behavior Scales (VABS) was used (Sparrow, Cicchetti, & Balla, 2005). VABS is a semi-structured interview measuring adaptive functioning in everyday life. It has been extensively used to capture variability in early difficulties in adaptive behaviour and differences in developmental trajectories in infants at high-risk for ASD (Bussu et al., 2018; Estes et al., 2015). It is designed to measure adaptive behaviour of individuals from birth to 90 years of age. Raw scores can be aggregated to form scores of five domains: Communication, Daily Living Skills, Socialization, Motor Skills and Maladaptive Behavior. Standard scores of each domain and a Composite score representative general adaptive behaviour were calculated by adjusting raw scores based on normative distributions for each specific age range, such that scores below 70-80 would represent borderline to severe impairment in adaptive skills for that age and higher scores indicated better adaptive skills.

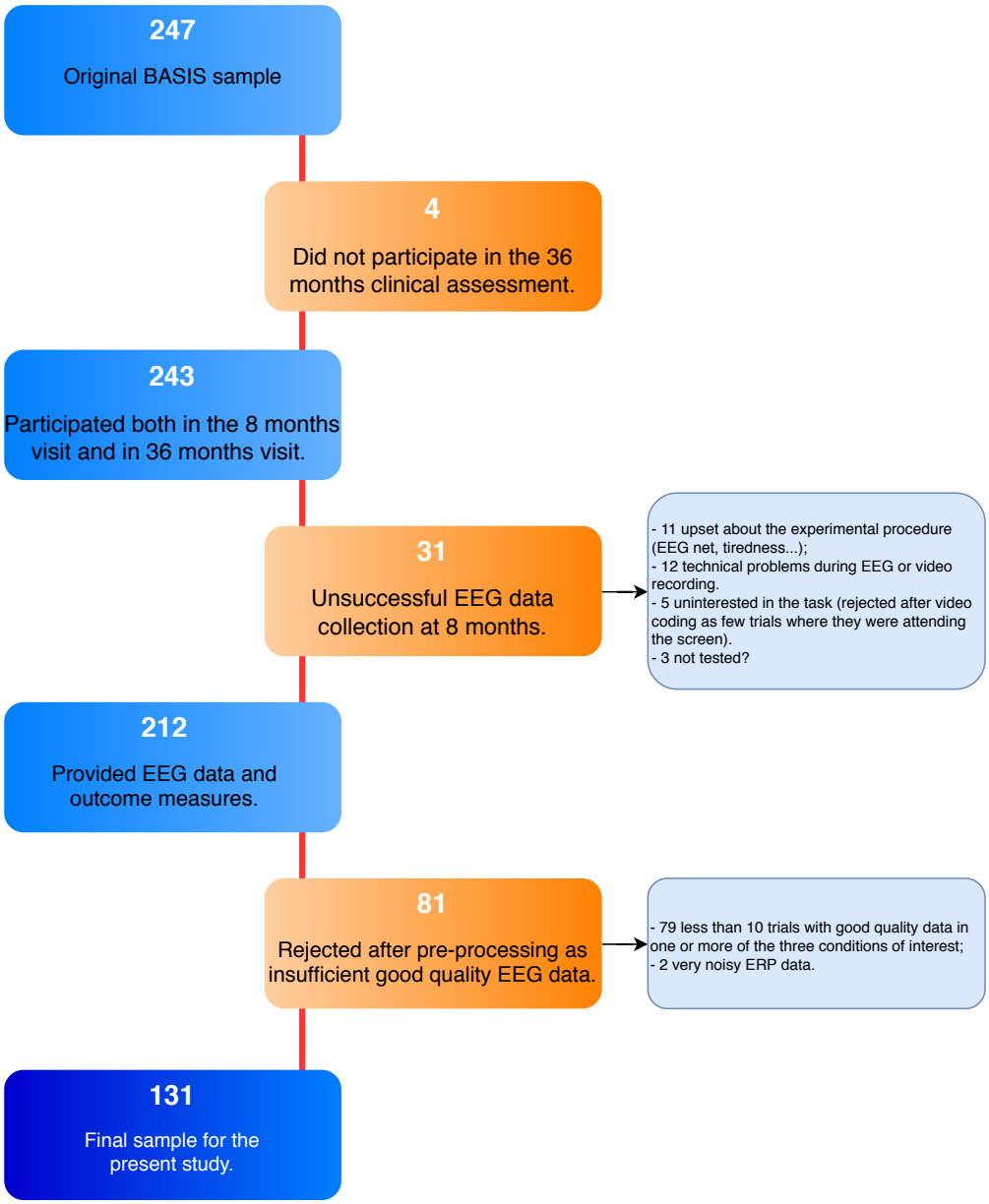

Figure 2.1 *Diagram showing the number of participants initially recruited as part of the British Autism Study of Infant Siblings* (original BASIS sample, top cell) and reasons for subsequent exclusion, leading to the actual sample for the current study (bottom cell). Orange cells contain information on the number of participants who were excluded at various steps.

Table 2.1 *Demographic characteristics and mean scores of behavioural measures for the study participants*, collected at T1 (8 months) and T4 (3 years). The group of excluded participants (N=116) is compared with the sample for the present study (N=131).

Participants	**Excluded**	**Present Study**		
Males/Females	55/61	65/66		
Phase 1/ Phase 2	42/74	62/69		
N LR	37	40		
N HR-TD	40	48		
N HR-Aty	20	24		
N HR-ASD	15	19		
	Mean (s.d.) Min - Max		**p**	**Cohen's D**
Age at T1 (months)	8.36 (1.11) 6 - 11	7.92 (1.27) 6 - 11	0.06	0.36
T1				
MSEL Composite Score	104.13 (15.69) 64 - 143	103.80 (14.42) 70 - 139	0.86	0.02
VABS Composite Score	95.59 (13.55) 49 - 144	95.58 (13.31) 66 - 150	1.00	<0.001
T4				
MSEL Composite Score	107.41 (24.54) 49 - 147	107.79 (20.99) 49 - 147	0.72	0.02
VABS Composite Score	98.85 (13.50) 52 - 121	98.70 (12.41) 57 - 131	0.93	0.01
ADOS Severity Score	2.83 (2.39) 1 - 10	3.06 (2.41) 1 - 10	0.38	0.10

LR: Low-Risk infants, HR-TD: High-Risk infants with Typical Development, HR-Aty: High Risk infants with Atypical development but not ASD, HR-ASD: High-Risk infants with Autism Spectrum Disorder; N: number of subjects; Mean: mean number of valid trials; s.d.: standard deviation; p: p-value of the independent samples t-test comparing the group of excluded participants with the sample for the present study, Cohen's D: measure of the effect size. MSEL: Mullen Scales of Early Learning; VABS: Vineland Adaptive Behavior Scales; ADOS: Autism Diagnostic Observation Schedule.

2.2.2 Electrophysiological recording and processing

Infants sat on their parents laps 60-cm from a 40 x 29-cm computer screen while brain activity was continuously recorded with a 128-channel Hydrocel Sensor Net. 50 trials were presented continuously for as long as the child remained attentive. Each block started with a static colourful fixation stimulus presented for a variable duration of 800-1,200 ms, followed by 4 colour pictures of a female model whose gaze was directed either toward (**Figure 2.2a1**) or away from the infant (**Figure 2.2a2**). Faces were presented in a pseudorandom order. Additionally, approximately one third of the blocks consisted in the control stimuli, called "Noise", constructed by randomizing the phase spectra of the face stimulus while keeping the amplitude and colour spectra constant (**Figure 2.2a3**) (Halit, Csibra, Volein, & Johnson, 2004). The trial duration was 800 ms, followed by a 500-ms interval with no visual stimulus.

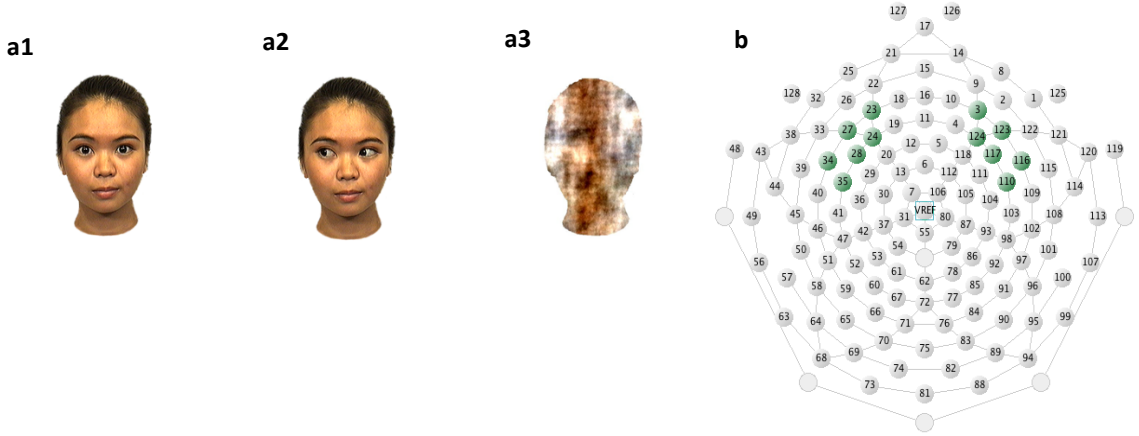

Figure 2.2 *Experimental stimuli and EEG montage*. **a1** Face with Direct Gaze, **a2** Face with Averted Gaze, **a3** Noise. **b** Left and right frontal electrodes, selected based on previous research investigating the Nc component in response to social stimuli in infants (Jones et al., 2016).

EEG data pre-processing was carried out by M. Elsabbagh (Phase 1) and C. Tye (Phase 2). The EEG signal was digitized with a sampling rate of 500 Hz and band-pass filtered between 0.1-100 Hz. The vertex has been used as reference (Cz in the conventional 10/20 system). Data were stored and analysed offline in EGI NetStation 4 (for Phase 1) and 5 (for Phase 2) using the same protocol as Elsabbagh et al. (2012). The EEG recording was segmented into 1000 ms (-200 to 800 ms peri-stimulus window for the Face with Direct Gaze condition, -200ms to 795 ms for Face with Averted Gaze and Noise).

Video-coding procedure was used to exclude those segments where the infant displayed gaze shifts, looked away from the screen or was crying during or 100 ms before stimulus presentation. Valid data were re-segmented and baseline corrected, with baseline from -195 ms till the stimulus onset. Segments with significant artifact were identified and removed through automatic detection. Specifically, for each segment, channels with EEG signal >400 μV were removed as bad signal recording; continuous data where the signal reached amplitudes >400 μV for 1,000 ms were removed as likely representing eye-blinks and for 160 samples were removed as eye movements. Channels were marked as bad if more than 75% of the data was detected as artifact. Following this automatic procedure, individual trials were visually inspected by experienced EEG researchers (M. Elsabbagh, C. Tye) and any channels showing artifacts were excluded. Single trials were excluded if they had more than 12 bad channels, while missing data from 12 or fewer channels were interpolated. Infants were excluded if there were less than 10 artifact-clean trials in any condition. Data were then re-referenced to the average. For each participant with good data obtained for a minimum of 10 trials per condition, stimulus-locked epochs were averaged for the following conditions: Face with Direct Gaze (FD), Face with

Averted Gaze (FA), Noise. **Table 2.2** shows the mean number of valid trials per group for each condition.

Table 2.2 *Number of valid trials from the EEG recording per condition* (Face Direct Gaze, Noise, Face Averted Gaze), for each outcome group (LR, HR-TD, HR-Aty, HR-ASD). P-values and effect sizes of one-way ANOVAs comparing the number valid trials between outcome groups for each condition are reported.

Condition	Outcome Group	N	Mean trials	s.d.	p	η^2
Face Direct Gaze	LR	40	19.925	6.290	0.668	0.012
	HR-TD	48	18.125	5.841		
	HR-Aty	24	17.667	5.585		
	HR-ASD	19	20.368	7.166		
Noise	LR	40	26.450	8.019	0.626	0.014
	HR-TD	48	25.604	7.374		
	HR-Aty	24	25.500	9.108		
	HR-ASD	19	27.789	9.247		
Face Averted Gaze	LR	40	20.175	6.425	0.821	0.007
	HR-TD	48	18.542	5.739		
	HR-Aty	24	18.000	5.641		
	HR-ASD	19	20.421	8.221		

LR: Low-Risk infants, HR-TD: High-Risk infants with Typical Development, HR-Aty: High Risk infants with Atypical development but not ASD, HR-ASD: High-Risk infants with Autism Spectrum Disorder; N: number of subjects; Mean trials: mean number of valid trials; s.d.: standard deviation; p-value: p-value of the one-way ANOVA with outcome groups as a between-subjects factor, η^2: partial eta-squared.

2.2.3 Analyses

2.2.3.1 Event-related potentials

As in previous research (Jones et al., 2016; Webb et al., 2011), Nc amplitude was defined as the mean amplitude of the negative deflection between 300 and 800 ms after stimulus onset across left and right frontal regions (**Figure 2.2b**). Mean amplitudes were extracted between 300 and 550 ms (early Nc) and between 550 and 800 ms (late Nc) using NetStation statistic extraction tool, following the approach used by Jones et al. (2016).

Of note, in the present study segments of averaged EEG data were 100 ms shorter than in the previous study, so dividing the components in two equal bins resulted in a different time-window compared to Jones et al. (2016). Moreover, visual inspection of the individual infants' data and averaged ERPs revealed that for many infants there was not a clear presence of two subsequent negative waves of activation within the Nc time window (**Figure 2.6**). A four-way, type-III sum of squares ANOVA was initially used to verify whether the Nc component significantly interacted with any of the variables of interest. Mean amplitude difference

between FD and Noise was entered as dependent variable, outcome (LR, HR-TD, HR-Aty and HR-ASD), Phase (1 and 2), sex (male and female) were entered as between-subjects factors, Nc (early and late) as within-subjects factor and age (in months) as a covariate. There was no main effect of Nc component ($F(1,115)=0.472$, $p=0.494$) and no interaction between outcome and the Nc ($F(3,115)=87.5$, $p=0.119$). None of the other interactions was significant (Nc X phase: $F(1,115)=0.923$, $p=0.339$; Nc X sex: $F(1,115)=2.717$, $p=0.102$; outcome X sex X Nc: $F(3,115)=1.137$, $p=0.337$; outcome X Nc X phase: $F(3,115)=0.651$, $p=0.584$; sex X Nc X phase: $F(1,115)=0.005$, $p=0.941$; outcome X phase X Nc X sex: $F(3,115)=0.309$, $p=0.819$). These results indicated that splitting the Nc into two components would not provide additional information to the analysis of mean amplitude effects. This observation corroborated the decision guided by visual inspection of the ERP shape of considering the Nc component as a whole. In order to further verify that this preliminary decision did not influence the pattern of results observed when considering the Nc as a whole, the multilevel mixed-effects models used as main analysis (see below), were re-run for the two components separately. Reassuringly, both analyses revealed the same pattern of results observed when considering the Nc as a whole (**Tables A2.2** and **A2.3**).

Therefore, mean amplitude was considered between 300 and 800 ms following the stimulus onset. Nc peak latencies were extracted using the 'erp.easy' package in R (R Core Team, 2013) by computing the latency of the most negative point within the same time-window. Our key dependent variables were the difference in Nc amplitude and latency, respectively, between the FD and Noise stimuli, to reflect attention engagement processes specific to social content. The same analyses were performed on the FA versus Noise contrast.

Differences in ERPs between the four outcome groups were assessed via multilevel mixed-effects linear models ('lme' function of the 'lmne' package in R, Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2018). To account for the fact that region was a repeated measure for each participant, "participant ID" was defined as a random factor with random intercept, and region as a nested variable (Field, Miles, & Field, 2012). Analysis of the model fit revealed that the model setting random intercepts for the participants significantly improved the model fit for both mean amplitude ($\chi^2(1)= 35.47$, $p<0.0001$) and latency ($\chi^2(1)=11.57$, $p<0.0001$), compared with baseline models with no random effects.

The multilevel approach allowed me to explicitly model the dependency of residuals of the repeated measures for the left and right region, and to control for the relationship between covariates and dependent variable which might vary across groups (Field et al., 2012; Hothorn, Bretz, & Westfall, 2019). For example, the infants' age in our sample ranged from 6 to 11 months

and previous research suggests that the effect of age on these ERP features could be different for children with later ASD (Johnson et al., 2005; Jones et al., 2016; Lewis et al., 2017; Webb et al., 2011). Thus, in addition to subject-specific random effects, the models could control for any of the following: proportion of attended trials to the Face versus Noise stimuli, age (184 – 351 days), age x outcome, sex (female or male), sex x outcome, study Phase (1 or 2), region (left or right), and all the interactions between phase, outcome and region. All models were evaluated, and significant improvement of fit of the multilevel model was tested using a chi-square likelihood ratio test (**Tables A2.4**, **A2.8**). For significant effects of groups, post-hoc contrasts were obtained with Tukey test using the 'glht' function of the 'multcomp' package in R (Hothorn et al., 2019), and p-values adjusted for multiple comparisons are reported below (**section 2.3.1**). Estimates and significance of the group differences assessed within the model with the lowest Akaike information criterion value (Posada & Buckley, 2004), as was done in similar research (Lewis et al., 2017), are reported in the **Appendix** (**Tables A2.5** and **A2.9**). T-statistic tested the difference between LR and each of the HR group to evaluate "atypicality" of the group responses.

Linear regressions were used to test whether mean amplitude and peak latency difference between the social and non-social stimuli predicted later socialization difficulties measured with VABS Socialization domain Standard Score (VABS Soc.) at 36 months. This questionnaire was selected as a dimensional measure of adaptive skills with minimal skew and associated with genetic burden (Robinson et al., 2016). Of note, VABS Soc. was preferred over other measures of social skills used in ASD research as it seemed to better capture variation in the study group. For example, the Social Affect score of the ADOS or the Social Communication Impairment (SCI) t-score of the Social Responsiveness Scale (SRS, Constantino & Gruber, 2012), showed very skewed distributions, with the majority of the children represented in the non-pathological end of the distribution (see **Figure A2.1**). These measures would not be adequate to capture individual differences as sensitively as VABS and were therefore not tested for association with the infants' neural measures in this chapter (but see **Chapter 3**).

For regression analyses, the ERP features were averaged across regions given that the multilevel mixed-effects models revealed no model fit improvement when adding region as predictor (see **Tables A2.4** and **A2.8**). Phase and sex were used as covariates. The total number of participants for this analysis was 123 (VABS Soc. scores were not available for 8 participants). In order to verify the specificity of the relationship for the social domain, when a significant result was found I also tested the association with a different domain of the same questionnaire which is not directly influenced by ASD social symptoms (VABS Motor Skills domain Standard Score, or VABS Mot.).

As a follow-up analysis, I examined whether effects were specific to faces with direct gaze by testing whether similar effects were seen for the FA versus Noise contrast.

2.2.3.2 Scalp Field Topography

To compare scalp field distributions of LR and HR-ASD infants in response to the faces and Noise stimuli, mean ERPs for each channel at all the time-points were analysed through the program Randomization Graphical User Interface (RAGU, Koenig, Kottlow, Stein, & Melie-García, 2011). Randomization statistics was used to test for whole-brain topographic differences between groups and conditions across time (Topographic Analysis of Variance or TANOVA, see Michel, Koenig, Brandeis, Gianotti, & Wackermann, 2009).

a) Topography Consistency Test

Before testing group by condition differences in the spatio-temporal characteristics of multichannel ERPs individual data, it is recommended to assess the quality of EEG recordings in terms of signal-to-noise ratio, and identify time periods where the temporal pattern of scalp field activation is consistent across subjects (Koenig et al., 2011). This preliminary analysis can be done performing a Topography Consistency Test (TCT). Of note, randomization statistics applied to multichannel ERP data considers significance of a moving average of the time series of t statistics. As an effect is considered significant when it lasts as long as the width of the moving average, this approach leads to more robust results in situations where multiple time-points and multiple channels are tested simultaneously.

Global Field Power (GFP) is reference free and can be used as a measure of strength of the neural response across all channels. It corresponds to the standard deviation of the potential field across channels at a given moment in time (Michel & Murray, 2012), as in **Equation 2.1**:

$$GFP = \sqrt{\frac{\sum_{j=1}^{n}(v_j - \bar{v})^2}{n}}$$

(Equation 2.1)

where *j* is the channel index, v_j is the potential (in microVolts) measured at channel *j*, $\bar{v}$ is the mean potential value across all channels and *n* is the number of channels.

As GFP of an event-related response depends on the single channels' potentials that are averaged across trials, the average GFP is expected to be larger than a noise distribution created

by randomly shuffling measurements at each channel in each point in time (Michel et al., 2009). TCT identifies periods of non-consistent topographies in which this assumption is not met.

Epochs-segmented artifact-clean data from 124 electrodes were entered into RAGU (channels 125, 126, 127 and 128 were excluded because not recorded in Phase 1). Separate datasets for the FD, FA and Noise conditions were imported for each subject. ERP data were average-referenced, baseline corrected, filtered between 2 and 20 Hz (recommended for TANOVA and microstate analysis, T. Koenig, personal communication) and normalised. To perform the TCT, the algorithm computed, for each subject, the GFP of the grand mean of time-point measurements that had been randomly shuffled across channels. By repeating this operation many times, a distribution of GFP under the null hypothesis of randomly occurring topographies was created. 1,000 randomization runs were selected for an estimate of significance at 5% (Koenig et al., 2011). Thus, the probability of the null hypothesis was given by the proportion of cases where the randomized GFP was equal or larger than the GFP of the observed data, computed from the (non-shuffled) grand mean across subjects at each time-point (Michel et al., 2009).

b) Topographic ANalysis Of Variance

TANOVA of multichannel ERP data in the period of consistent topography was implemented using non-parametric randomization tests with multi-factorial designs. To test whether the spatial configuration of the scalp field potential was modulated by the experimental design, differences in the maps between factor levels (i.e., groups or conditions, as in a classic ANOVA design) were calculated. These difference maps corresponded to the variance of factors grand-mean maps from which the grand-mean map across all conditions was subtracted, as in **Equation 2.2**, and quantified the strength (GFP) of difference maps between factor levels.

$$dGFP = \sqrt{\frac{\sum_{i=1}^{c}\sum_{j=1}^{n}(\overline{v_{ij}} - \overline{\overline{v}_j})^2}{n}}$$

(Equation 2.2)

where *c* is the number of factor levels to test main effects or combinations of factor levels to test an interaction effect, *n* is the number of channels, $\bar{v}_{ij}$ is the grand-mean across subjects of the potential of factor level *i* at channel *j*, and $\bar{\bar{v}}_j$ is the grand-mean across subjects and factor levels of the potential at channel *j*. Thus, dGFP represented the measure, or effect size, of the differences between conditions and groups, which would be large if the conditions/groups were different and small if they were similar (and therefore similar to the grand-mean across conditions).

For within- and between-group factors, RAGU randomly shuffled the data across conditions and groups and computed "null" grand-means of the randomized data, respectively. dGFP (**Equation 2.2**) under the null hypothesis was then calculated and the percentage of randomizations when this was equal or larger than dGFP obtained from the observed data represented the critical threshold to reject the null hypothesis (Michel et al., 2009). RAGU offers different options to control for false positives due to simultaneous multiple testing of many channels and many time points, which are based on the Fisher' test, a false positive count and a duration threshold, respectively. For the method based on the Fisher's test, a 5% p-threshold for the overall significance was chosen such that an effect was considered significant when the set of all p-values obtained using Fisher's method for testing significance of classification (Fisher, 1922) was larger than 95% of the false positive count obtained in the random data (Habermann, Weusmann, Stein, & Koenig, 2018). Further, a p-value based on false discovery count is obtained, as RAGU also estimates the distribution of false positives by counting, for each randomization run, the number of time-points with p-values below the 0.05 critical threshold (Koenig et al., 2011; Michel et al., 2009). Last, to avoid false positives due to multiple testing across time, RAGU computes duration threshold statistics to identify periods of significance exceeding the critical duration (Koenig et al., 2011). Results from all those methods to assess overall significance of the performed TANOVAs are reported in **Table 2.5**.

A two-by-two TANOVA was performed for the time-window between 318 and 794 ms after the stimulus onset as the TCT analysis revealed that this was the time-window of consistent topography across groups. Consistent topographies are a requirement for TAVOVA, as group comparison is possible only under the assumption that the event elicited the activation of a common set of sources across groups (Habermann et al., 2018). For the main analysis, group (LR versus HR-ASD) was set as a between-subjects factor and stimulus (FD versus Noise) as a within-subjects factor. **Figures 2.6** and **2.7** graphically represent the results for this TANOVA and are presented to give a concrete visualization of randomization statistics hypothesis testing.

The same test was performed for the FA versus Noise contrast. Additionally, LR and HR-TD were compared for the same two condition contrasts.

2.2.3.3 Microstates

Microstate analysis tracks the changes of brain functional states, defined by specific distributions of simultaneously active brain regions (Michel et al., 2009). This has been extensively done in the adults' literature by assigning each time period of the ERP to exactly one

of the spatially defined microstate templates obtained from clustering the data into predetermined numbers of topographies (microstate maps or prototypes, see Koenig, Stein, Grieder, & Kottlow, 2014; Pascual-Marqui, 1995).

Identification of the typical microstates

Periods of stable topographies of the ERP scalp field data, called microstates, were clustered using a randomization-based method (Koenig et al., 2014). The entire procedure is summarized in **Figure 2.3**. To understand whether HR infants showed an "atypical" neural response, it was first necessary to identify "typical" microstate maps associated with social processing. Thus, I first extracted maps in the LR group using ERP data for the FD condition (**Figure 2.10**). Microstates cross-validation was performed by RAGU (Koenig et al., 2011). Cross-validation was applied 250 times, each time randomly splitting the LR sample into 20 training datasets (i.e. individual data) and 20 test datasets. Between 1 and 10 microstate classes were estimated using an AACH algorithm. Briefly occurring microstates were suppressed using a segmentation smoothing algorithm (Pascual-Marqui, 1995), with penalty term for non-smoothness of 0.3 and a window-size for smoothing of 10 samples (Koenig et al., 2014). The mean correlation coefficient representing the amount of explained variance obtained in the test set (around 0.7, see **Figure 2.4**) is comparable to values obtained with the same technique for estimation of microstates from adults' ERPs data (see Koenig et al., 2014 and Habermann, Weusmann, Stein, & Koenig, 2018 for comparison).

A general linear model ('glm' function of the 'stats' R-package) was used to test whether changing the number of microstate maps, constructed with the training datasets, significantly increased the amount of explained variance in the test datasets across 250 cross-validation runs ($F(9,2490)=5621$, $p<0.001$). Bonferroni-corrected one-tailed pairwise t-tests were performed comparing explained variances obtained with 1 to 10 microstate maps. As observed in **Figure 2.4**, the results of the pairwise t-tests revealed that the amount of explained variance first increased with increasing number of microstates to reach a plateau where no further improvement was obtained by using models with more four maps (**Table 2.3**).

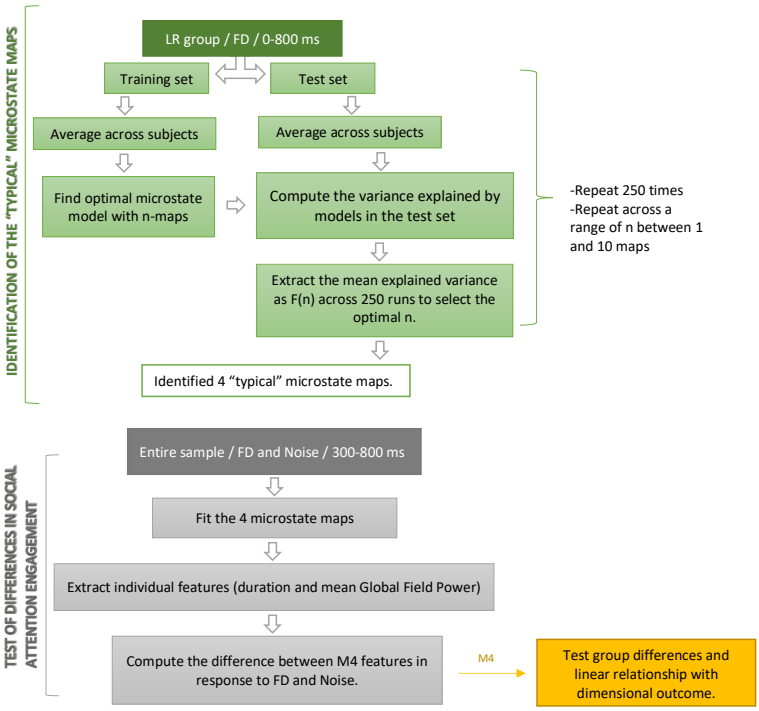

Figure 2.3 *Flow-chart illustrating the procedure for the microstate analysis*. The first part is adapted from Koenig at al., 2014 (Koenig et al., 2014).

LR: Low-Risk; FD: Face with Direct Gaze; M4: microstate 4.

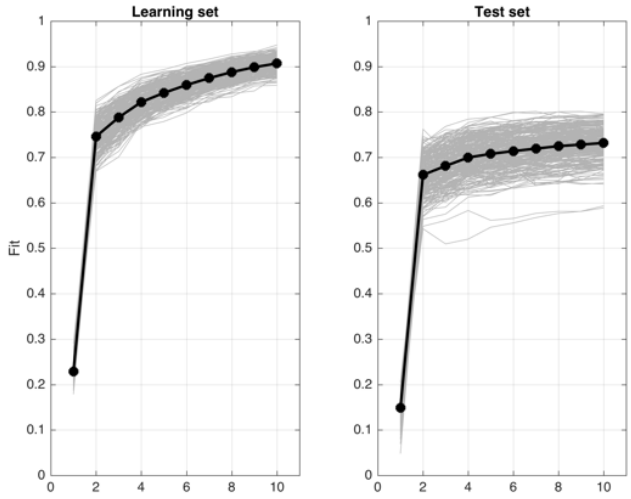

Figure 2.4 *Variance in the scalp field potentials explained by the microstate maps* estimated from the learning set (on the left) and tested on the test set (on the right). On the x-axis, the number of microstate maps used to fit the data is displayed. Each of the grey lines represents the performance of the models estimated from one of the 250 randomly created learning sets. The black solid line represents the mean variance explained across 250 cross-validation runs.

Table 2.3 *Results of the pairwise t-tests to select the optimal number of microstate maps*, showing that a significantly higher amount of variance in the ERP data in the test set across 250 cross-validation runs was obtained with four microstate maps.

Microstate models	Exp. Var. mean	s.e.	comparisons N of maps	Adj. p
1 microstate map	0.149	0.002		
2 microstate maps	0.662	0.003	2>1	<0.0001*
3 microstate maps	0.681	0.003	3>2	<0.0001*
4 microstate maps	0.700	0.003	4>3	<0.0001*
5 microstate maps	0.708	0.003	5>4	0.382
6 microstate maps	0.714	0.003	6>5	1.000
7 microstate maps	0.719	0.003	7>6	1.000
8 microstate maps	0.724	0.003	8>7	1.000
9 microstate maps	0.728	0.003	9>8	1.000
10 microstate maps	0.731	0.003	10>9	1.000

Exp.Var mean: mean proportion of explained variance of the ERPs data in the test dataset over 250 repetitions; s.e: standard error; comparisons N of maps: number of microstate maps compared in the post-hoc pairwise one-tailed t-tests; Adj. p-value: Bonferroni-corrected p-values of the indicated contrast.
* $p<0.05$.

Subsequently, I examined whether there were differences in the degree to which these "typical" brain states were expressed across the HR sample in relation to diagnostic outcome. To do this, the 4 "typical" maps, reflecting brain states of LR infants in the FD condition, were identified in the individual ERP data for the study sample in the FD and Noise conditions, between 300 and 794 ms, corresponding to the Nc time window. Of note, as Noise segments ended 795 ms after the stimulus onset, the period between 300 and 794, instead of 800 ms, was chosen for this analysis, given that for microstate analyses all individual recordings should include the same number of samples.

For each subject, the four microstates GFP, corresponding to the standard deviation of the voltage of all channels at a given moment in time interpreted as a measure of the strength of the scalp field (Michel et al. 2009), and duration, i.e., period assigned to the same microstate map within the time-window of 300-794 ms, were selected a priori among the output features resulting from RAGU (onset, offset, duration, area under the curve, centre of gravity, mean GFP). This choice was made as other investigations of brain states in attention and ASD research have revealed that strength of the connectivity pattern is associated with increased attention (Hellyer et al., 2014) and group differences in duration of the connectivity configurations have been found between ASD cases and controls (King et al., 2018). The other features were not examined.

M4: the attentive microstate

Since microstate map 4 (M4) most closely represented the fronto-central negative deflection corresponding to the Nc component (see **Figure 2.10a**), the difference in the duration and mean GFP of M4 in response to FD and Noise was extracted for each subject in the time window between 300 and 794 ms. Type-III sum of squares ANOVAs, with sex, Phase and age (in days) as covariates, were used to test differences between the three HR groups in M4 duration and mean GFP difference between FD and Noise. The LR infants were excluded from these analyses given that the microstates were tuned on their data.

When testing the predictive value of microstate features towards later social skills, I wanted to verify whether the same mechanisms were observed in infants with and without ASD. If an association between brain states and later outcome was observed only for the HR-ASD group, this would provide evidence that this early marker is very close to the emergence of ASD symptoms, therefore probably reflecting a precursor rather than an antecedent of ASD (Johnson, Gliga, Jones, & Charman, 2014). To test this, "ASD" was introduced as dummy variable to test whether different interactions were observed between the two diagnostic groups. Robust linear regression using Huber's M-estimator ('rlm' function of the 'MASS' R-package) was used to estimate the coefficients for the association between the M4 features (FD-Noise difference in duration and GFP) at T1 and VABS Socialization domain scores at T4. This approach was preferred to the standard linear regression after observing the residuals distribution for these models, in order to obtain robust estimates against extreme values which might significantly influence the results if not down-weighted. Thus, predictors of the models included: sex, Phase and microstate feature interacting with a binary variable which reflected affection status (0=no-ASD, 1=ASD). Significance of the coefficients was tested with robust F-test (Wald test for multiple coefficients, 'f.robftest' of the R-package 'sfsmisc'). P-values reported in the **Tables A2.16-18** refer to this statistic. As for the ERP analyses, a comparable non-social outcome measure (VABS Mot.) was used as independent variable to test whether observed significant associations were specific to the social domain.

Comparing microstates in the Nc time-window

To complement the hypothesis-driven approach to only look at the M4 component, control analyses testing for differences between the FD and N condition in microstates 1 (M1), 2 (M2) and 4 were performed. These analyses had the double objective to verify whether microstate maps estimated on the FD condition fit equally well the Noise data (by comparing duration in the two conditions) and to explore the data relative to other brain states in the Nc time-window (namely, M1 and M2). M3 was not included because it was only found in a subset of the infants

in the period between 300 and 800 ms following the stimulus presentation (N=72). The multivariate approach was chosen because microstate features were expected to depend on each other (e.g., a longer duration in one state was necessarily related to a shorter duration of the following state, see **Figure A2.6** for correlation coefficients among all the microstate features in response to FD in the entire sample). Multivariate ANalyses Of Co-VAriance (MANCOVAs) were performed using IBM SPSS® software. Two MANCOVAs were run on the LR data, with duration and mean GFP as dependent variables, respectively. Microstates (M1, M2 and M4) and condition (FD and Noise) were entered as within-subjects variables, and Wilks's Lambda test was used to test their effects (Field, 2013), while controlling for differences in age (in days), sex and recruitment phase.

The same analyses were conducted on the HR group, where outcome group (HR-TD, HR-Aty and HR-ASD) was added as between-subjects variable interacting with microstate and condition. Post-hoc tests were conducted using Bonferroni correction for multiple testing.

Table 2.4 *Demographic characteristics and scores of the behavioural measures of the study participants* who provided data for the present study, divided into outcome groups.

Participants	**LR**	**HR-TD**	**HR-Aty**	**HR-ASD**		
N current study	40	48	24	19		
Phase (1/2)	31/9	16/32	6/18	9/10		
Males/Females	13/27	20/28	14/10	15/4		
	Mean (s.d.) Min - Max				**p**	**E.S.**
Age in days	244.97 (40.65) 186 - 346	256.58 (37.14) 189 - 351	270.83 (34.47) 184 - 332	251.21 (31.64) 184 - 309	0.06	0.06
T1						
MSEL Composite Score	106.33 (11.54) 86 - 132	104.19 (15.71) 70 - 134	101.08 (13.84) 77 - 130	100.17 (16.75) 77 - 139	0.355	0.03
VABS Composite Score	100.67 (12.74)[a] 78 - 130	94.45 (14.13) 66 - 150	91.67 (12.35)[a] 68 - 114	92.72 (10.83) 71 - 113	0.028*	0.07
VABS Socialization Score	103.23 (12.78) 81 - 132	98.66 (13.76) 70 - 152	99.21 (11.54) 77 - 126	98.22 (10.03) 81 - 118	0.32	0.03
VABS Communication Score	101.88 (13.03) 66 - 123	96.21 (17.04) 55 - 143	91.83 (16.17) 55 - 118	94.84 (11.51) 70 - 112	0.061	0.06
VABS Daily Living Skills Score	100.55 (15.25) 54 - 122	101.44 (14.64) 54 - 143	99.50 (11.54) 77 - 111	97.74 (13.51) 77 - 117	0.796	0.01
VABS Motor Skills Score	97.45 (14.11)[a,b,c] 73 - 127	87.50 (16.19)[a] 56 - 144	81.75 (15.83)[b] 56 - 113	84.16 (13.69)[c] 56 - 106	<0.001*	0.14
T4						
MSEL Composite Score	115.50 (15.06)[a,b] 80 - 147	113.79 (16.21)[c,d] 79 - 138	97.71 (24.26)[a,c] 63 - 145	92.39 (26.19)[b,d] 49 - 142	<0.001*	0.19
VABS Composite Score	107.26 (9.17)[a,b,c] 93 - 131	101.21 (9.45)[a,d,e] 67 - 121	93.65 (9.93)[b,d,f] 78 - 111	84.26 (12.66)[c,e,f] 57 - 109	<0.001*	0.38
VABS Socialization Score	105.79 (7.11)[a,b] 94 - 122	100.96 (9.43)[c,d] 70 - 116	94.57 (10.71)[a,c,e] 72 - 114	80.16 (13.50)[b,d,e] 61 - 110	<0.001*	0.43
VABS Communication Score	107.94 (11.05)[a,b] 85 - 139	102.32 (8.98)[c] 83 - 125	97.75 (13.72)[a,d] 76 - 125	87.83 (15.12)[b,c,d] 52 - 112	<0.001*	0.25
VABS Daily Living Skills Score	109.00 (7.65) [a,b] 91 - 127	105.84 (8.63)[c] 91 - 125	101.55 (7.29)[a,d] 87 - 115	86.67 (14.80)[b,c,d] 62 - 119	<0.001*	0.39
VABS Motor Skills Score	100.55 (13.09)[a,b] 61 - 121	96.52 (10.84)[c,d] 78 - 124	88.10 (7.50)[a,c,e] 78 - 104	84.61 (10.71)[b,d,e] 64 - 100	<0.001*	0.23
ADOS-2 CSS	2.50 (1.86)[a,b,c] 1 - 7	1.60 (1.01)[a,d,e] 1 - 6	4.88 (2.05)[b,d] 1 - 8	5.47 (2.99)[c,e] 1 - 10	<0.001*	0.50

LR: Low-Risk infants, HR-TD: High-Risk infants with Typical Development, HR-Aty: High Risk infants with Atypical development but not Autism Spectrum Disorder (ASD), HR-ASD: High-Risk infants with ASD. s.d.: standard deviation; Min: minimum value; Max: maximum value; p: p-value of the one-way ANOVA with outcome group as between-subject factor for age, MSEL and VABS scores, and Kruskal-Wallis non-parametric test for ADOS scores; E.S.: effect size (η^2 for effects of outcome groups on age, MSEL and VABS scores. For ADOS-2 Severity Scores, effect sizes of each of the significant post-hoc contrasts were calculated with the following formula: $r = |Z|/\sqrt{N}$, where Z values were obtained using Mann-Whitney U test and N represents the total number of samples. The largest effect size, from comparison between HR-TD and HR-Aty, is reported. Effect sizes of the significant contrasts for this analysis ranged from 0.21 to 0.5); MSEL: Mullen Scales of Early Learning, Early Composite Score; VABS: Vineland Adaptive Behavior Scales; ADOS: Autism Diagnostic Observation Schedule; CSS: Calibrated Severity Scores, calculated as explained in section 2.2.1.

[a,b,c,d,e,f] Different superscript letters denote that groups are significantly different from each other based on Tukey's Honest Significant Difference post-hoc analyses with 95% family-wise confidence level for age, MSEL and VABS scores and pairwise comparisons using Mann-Whitney U test with Bonferroni correction for multiple comparisons for ADOS-2 CSS.

* p<0.05.

2.3 RESULTS

Table 2.4 reports information on the outcome groups demographic characteristics and scores at the five domains of the VABS, as well as a measure of global developmental level obtained with the MSEL Composite Score, at the time of EEG testing (8 months) and outcome assessment (36 months). ADOS calibrated severity scores are also reported as a measure of autistic traits.

2.3.1 Speed and depth of attention engagement: event-related potentials

EEG data collected from 131 8-month-old infants were used for these group analyses, comparing 40 LR infants with no family history of ASD with HR infants who had an older sibling with ASD, divided into three outcome groups: 48 HR-TD; 19 HR-ASD and 24 HR-Aty. Based on a previous study (Jones et al., 2016), less negative amplitudes and shorter latencies when attending to faces than to Noise were expected in the HR-ASD infants, suggesting that neural correlates of reduced social attention engagement precede the development of difficulties in socialization. **Figure 2.5** illustrates the ERPs for each of the outcome groups.

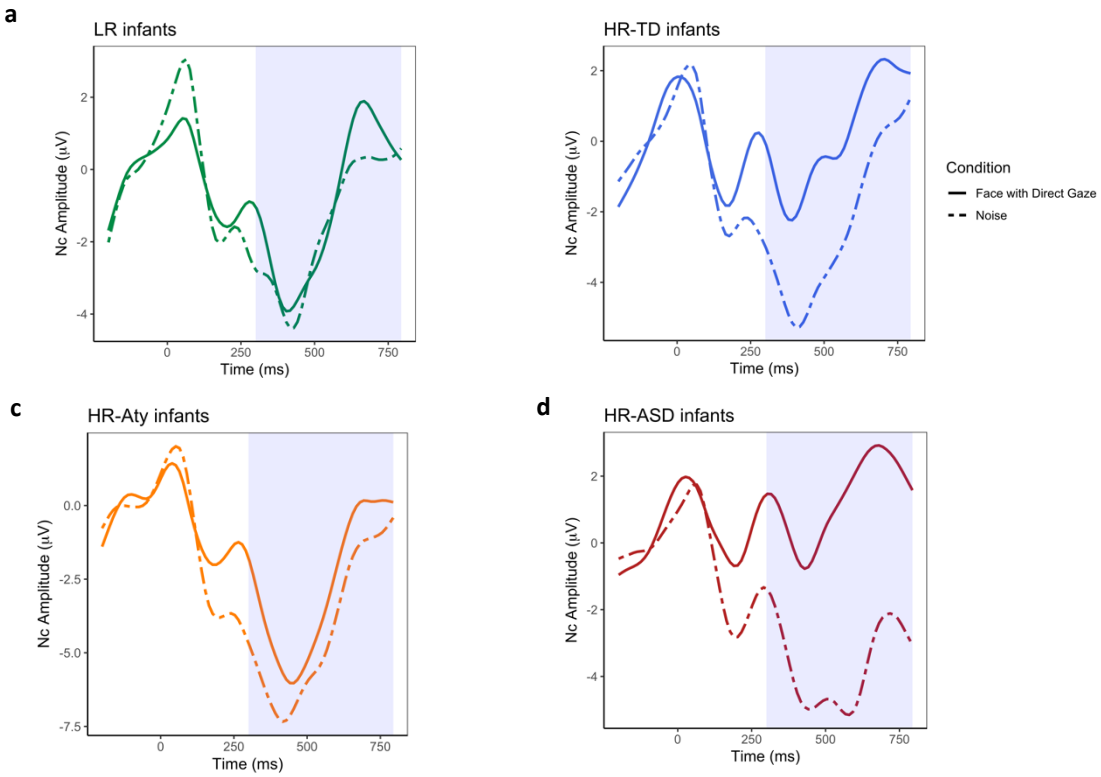

Figure 2.5 *Illustration of the grand average ERPs over the lateral frontal electrodes for the four outcome groups*, with violet shade highlighting the Nc time window. **a** illustrates ERPs for the LR group, **b** for the HR-TD group, **c** for the HR-Aty group, **d** for the HR-ASD group. ERP data have been smoothed for representation purposes using the 'gam' function of the 'ggplot2' package in R (R Core Team, 2013).

Linear mixed-effects analyses revealed that differences in Nc amplitude ($\chi^2(15)=13.31$, p=0.004, **Figure 2.6a**) and latency ($\chi^2(15)=9.6$, p=0.022, **Figure 2.6b**) between the FD and Noise condition were largely explained by ASD outcome status. For amplitudes, Tukey post-hoc contrasts revealed that HR-ASD infants showed reduced engagement (i.e. less negative amplitudes) to FD than to Noise compared to LR (p=0.004). The HR-TD group showed a similar but smaller effect (p=0.055), while no significant difference was found between LR and HR-Aty (p=0.82). Interestingly, HR-TD versus HR-ASD (p=0.32) and HR-Aty versus HR-ASD (p=0.116) contrasts were non-significant. Moreover, there was no difference between HR-TD and HR-Aty (p=0.754). For latency, HR-ASD showed a faster Nc peak to FD versus Noise than LR group (p=0.029), HR-TD (p=0.018) and HR-Aty (p=0.027). The other contrasts were not significant (LR versus HR-TD: p=0.988, LR versus HR-Aty: p=0.928, HR-TD versus HR-Aty: p=0.982).

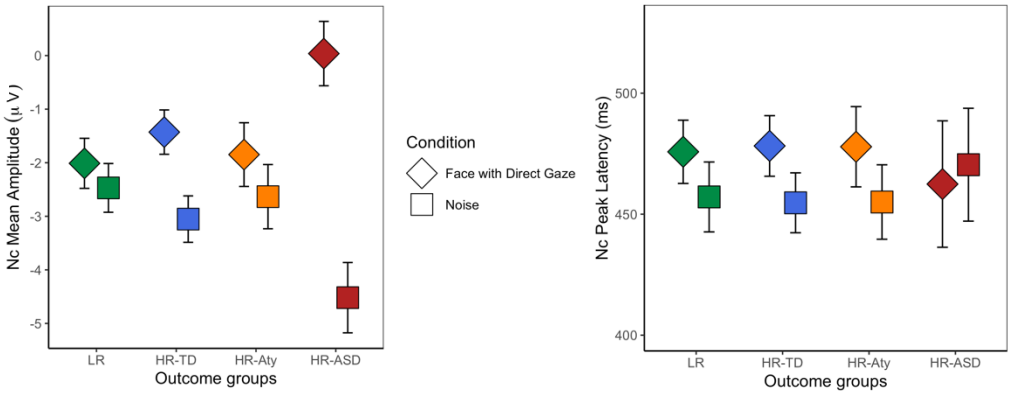

Figure 2.6 *Mean Nc amplitude and latency by condition for the four outcome groups.* **a** Mean amplitude of the Nc component in response to FD (represented by rhombuses) and Noise (represented by squares) in the four outcome groups. **b** Mean latency of the Nc component in response to FD and Noise in the four outcome groups. All bars represent ± standard error.

As reported in **Table A2.5** and illustrated in **Figure 2.7a**, the higher-order models with lower AIC revealed that age had a different effect for the outcome groups on Nc amplitude difference between FD and Noise. Specifically, the LR group showed a non-significant association with age (β=0.016, s.e.=0.024, p=0.512). Differently, for the HR groups more enhanced responses to FD than to Noise emerged with age. For HR-TD (β=-0.076, s.e.=0.033, p=0.023) and HR-ASD (β=-0.150, s.e.=0.049, p=0.003), but not for the HR-Aty group (β=0-0.036, s.e.=0.043, p=0.397) the relationship between Nc amplitude difference and age was significantly different from the LR group.

The higher-order models with lower AIC for Nc latency difference between FD and Noise (**Table A2.9**, see also **Figure 2.7b**) showed that there was no significant association with age for the LR

group (β=-0.001, s.e.=0.453, p=0.998) and the same trend of relationship was found in HR-TD (β=-0.126, s.e.=0.613, p=0.838) and HR-Aty children (β=-0.415, s.e.=0.788, p=0.600). On the contrary, slower Nc peaks to FD than to Noise was observed in younger infants within the HR-D group (β=2.346, s.e.=0.917, p=0.012

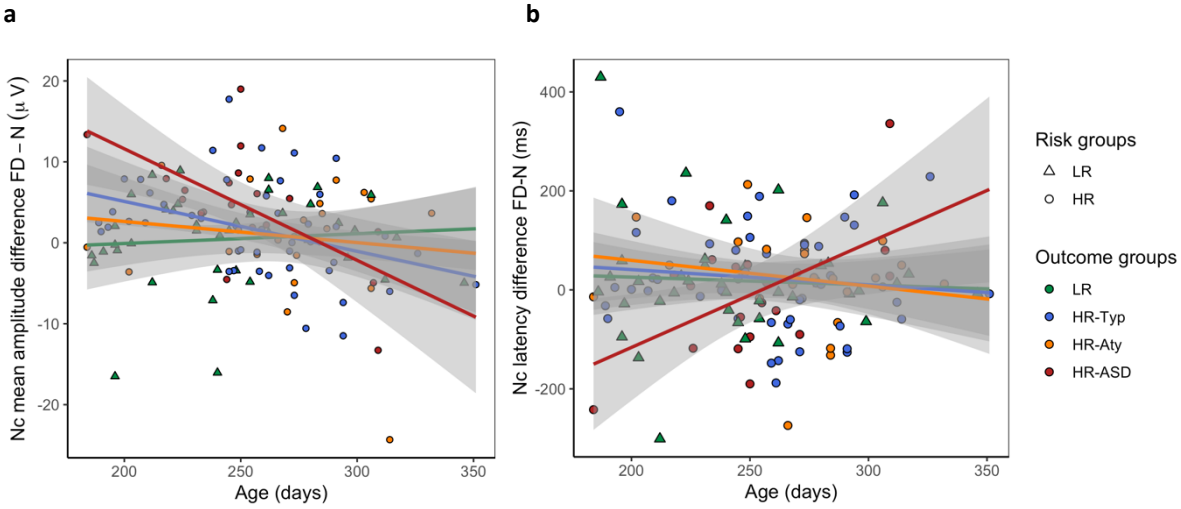

Figure 2.7 *Outcome-by-age interaction for the Nc features*. **a** Scatter plot depicting the relationship between age at the time of EEG testing, and the difference between Nc mean amplitude in response to Face with Direct Gaze (FD) and Noise (measured in microVolts). Positive values indicate more enhanced response to Noise than to FD, while negative values indicate more enhanced response to FD. **b** Scatter plot depicting the relationship between age at the time of EEG testing and the difference between Nc peak latency in response to FD and Noise (measured in milliseconds). Positive values indicate shorter response to Noise than to FD, while negative values indicate a shorter response to FD. In both figures, regression lines are displayed for each group, with grey shadows representing standard errors.

Across the whole cohort, a smaller Nc to FD than Noise (i.e. more positive values of the mean amplitude difference between the two conditions) at T1 predicted poorer social skills, measured by the VABS Soc. at T4 (β=-0.418, t(3)=-2.41, p=0.018, **Figure 2.8**). I selectively tested the association with VABS Mot. as a non-social related measure of parent-reported adaptive behaviour, but found no significant association (β=-0.147, t(3)=-0.954, p=0.342), suggesting relative specificity. Peak latency differences between FD and Noise at T1 did not predict social adaptive skills at T4 across the whole cohort (β=0.016, t(3)=1.68, p=0.096). Further details of all results can be found in the **Tables A2.4-A2.9** and **Figure A2.2**. Control analyses showed no significant group differences in the FA versus Noise contrast, nor association with later adaptive skills, suggesting the importance of direct gaze (all *ps*>0.19, **Tables A2.11-A2.14**, **Figure A2.3**).

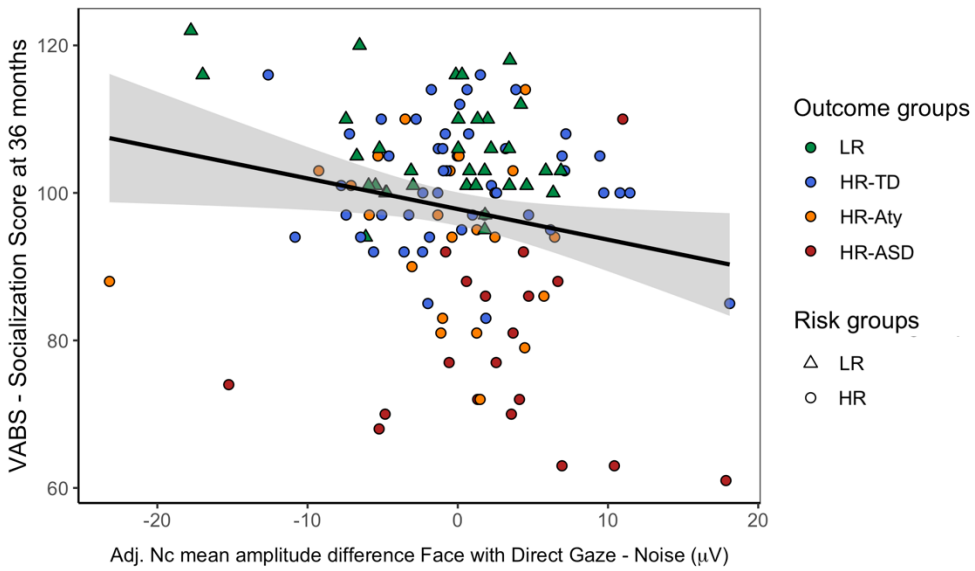

Figure 2.8 *Relation between Nc difference score and later socialization skills*. Nc mean amplitude difference between FD and Noise at 8 months, on the x-axis, and standard scores in the VABS Soc. collected at 36 months, on the y-axis. Negative values indicate higher attention engagement with FD, while positive values indicate higher attention engagement with the Noise stimulus. The regression line for the entire group of HR infants is displayed as a black line, with grey shadows representing standard errors.

2.3.2 Scalp field topography

Group analysis of ERPs is based on the, rarely tested, assumption that all subjects of a defined experimental group activate common processing resources, indicating that the event elicits the activation of a common set of sources (Habermann et al., 2018). This requirement of TANOVA was verified in the current study with the TCT (see **section 2.2.3**). Topographies were consistent across all subjects between 318 and 794 ms after the stimulus onset in all conditions. HR-Aty subjects had inconsistent topographies between 49 and 52 ms, and between 236 and 250 ms in the FD condition and between 28 and 58 ms after the stimulus onset in the FA condition. HR-ASD infants showed inconsistent topographies from 250 to 317 ms in the FD condition, and in the time windows from 14 to 30 and from 270 to 314 ms in the Noise condition.

The TANOVA comparing scalp field configurations of LR and HR-ASD groups in the FD and Noise conditions was therefore calculated in the period of consistent topography within the Nc time window (318-794 ms). The TANOVA using the Fisher's p method for adjusting for multiple testing (explained in **section 2.2.3**) revealed that there was a main effect of condition (p=0.001, **Figure 2.9a** depicts the result with respect to the empirical null distribution) and an interaction between condition and group (p=0.024, **Figure 2.9c**) in determining the difference in

configuration of the scalp field. Specifically, there was a main effect of condition between 374 and 508 ms and between 632 and 794 ms after the stimulus onset (all electrode-wise t-tests p*s*<0.05 for periods which were longer than the duration threshold of 56 ms, applied to control for multiple testing, see **Figure 2.10a**). The longest period of significant effect of the interaction between condition and group was significant between 560 and 606 ms (all p*s*<0.05), observed for a shorter period (46 ms) than the duration threshold of 56 ms established with the randomization procedure, see **Figure 2.10c**). There was no significant effect of group (p=0.092, **Figure 2.9b**). Thus, the two groups did not show the same topography of the electric signal recorded over the whole brain in response to FD and Noise between 560 and 600ms after stimulus presentation, although the time window where the effect was observed was not longer than what expected by chance.

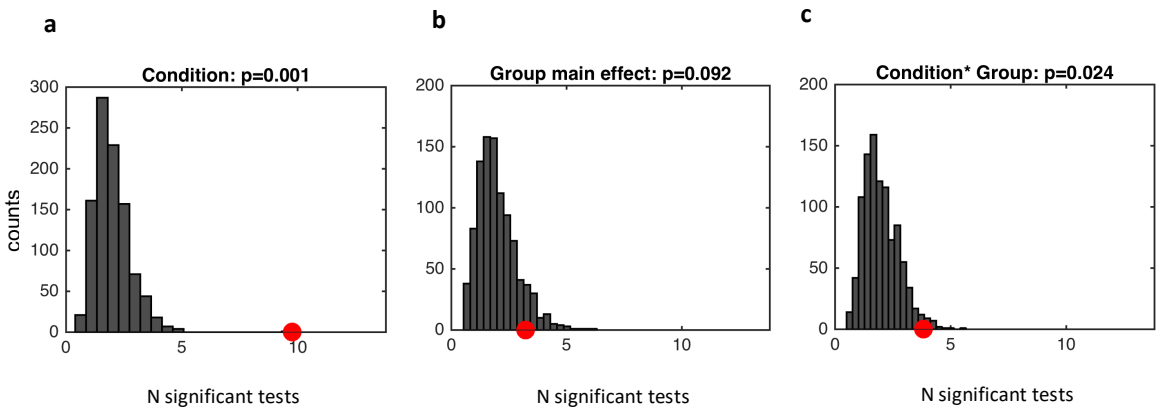

Figure 2.9 *Results of the TANOVA analysis using the Fisher's test method to control for multiple testing*. To control for false positives due to multiple testing of the signal recorded from different channels at many time points, an effect was considered significant when the set of all p-values obtained using Fisher's test was larger than 95% of the false positive count obtained in random data where channels and time-point data have been shuffled (Habermann et al., 2018), as explained in section 2.2.3. In this figure, the distribution of false positives at the Fisher's test under the null hypothesis (i.e. for all the randomization runs) is displayed for the main effect of **a** condition (Face with Direct Gaze vs. Noise), **b** group (Low-Risk vs. High-Risk with ASD) and **c** the interaction between condition and group. The x-axis represents the number of Fisher's test significant results, while the y-axis represents the number of randomizations in which the result in the x-axis was observed. Thus, bars represent the frequency of significant results under the null hypothesis as a function of randomization runs. The red dot represents the count of significant time-points obtained in the real data.

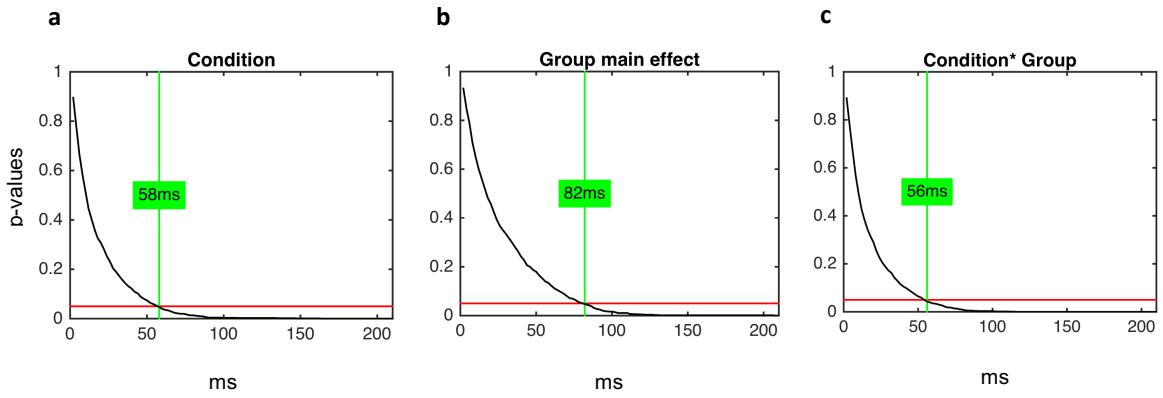

Figure 2.10 *Results of the TANOVA analysis using the duration threshold method to control for multiple testing.* This approach implemented by RAGU using randomization statistics to control for simultaneous tests performed at multiple time-points consists in establishing a duration threshold such that consequent significant results obtained for shorter periods should be considered as possibly occurred by chance (section 2.2.3). In the three figures, the x-axis presents time in milliseconds. On the y-axis, the probability of the null hypothesis being rejected is indicated, with a red line indicating a p-value of 0.05. The probability of the null hypothesis as a function of time is displayed as a black line for the main effect of **a** condition (Face with Direct Gaze vs. Noise), **b** group (Low-Risk vs. High-Risk with ASD) and **c** the interaction between condition and group. The red line represents the p-value threshold of 0.05. The green lines show the duration threshold for the three effects.

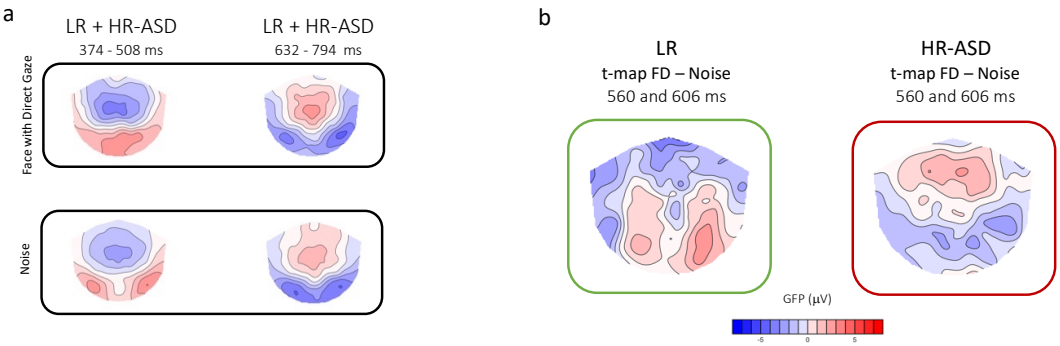

Figure 2.11 *Topographic maps representing the significant effects of the TANOVA*. **a** Main effect of stimulus between 374 and 508 ms and between 632 and 794 ms. Topographies of the scalp field potentials in response to FD (top) and Noise (bottom) for all the subjects involved in the analysis (LR and HR-ASD) are displayed. Maps represent the positive (red) and negative (blue) Global Field Power (GFP, in microvolts) at the lowest p-values testing the main effect of stimulus (424 ms and 684 ms, respectively). **b** Interaction between group and stimulus between 560 and 606 ms. T-maps represent the difference in neural activation (GFP) in response to FD and Noise for the LR group (left) and for the HR-ASD group (right). Red values represent higher positivity in the FD than in Noise condition. The HR-ASD group showed a more positive activation of the fronto-central areas, suggesting that the negative wave corresponding to the Nc might has ended earlier in response to FD.

The TANOVA comparing LR and HR-ASD group topographies in the Face with Averted Gaze and Noise conditions revealed a significant main effect of stimulus between 368 and 512 ms, and between 602 and 794 (ps<0.05 for a duration longer than the duration threshold of 54 ms).

There was a significant difference between LR and HR-ASD when looking at FA (p=0.012, all electrode-wise p*s*<0.05 between 556 and 630 ms, which is, however, just shorter (74ms) than the duration threshold of 76 ms identified to correct for simultaneous testing of ERPs at multiple time-points). No interaction between condition and group survived correction for multiple testing (duration threshold: 50 ms).

A TANOVA comparing LR and HR-TD was also performed as a control analysis to observe whether the suggestive effects obtained in the LR versus HR-ASD contrast were common to HR infants with subsequent typical development. A significant effect of condition emerged between 362 and 496 ms and between 520 and 794 ms (all p*s*<0.05). There was no effect of group nor significant interaction between group and condition. The same pattern of results was obtained when comparing FA and Noise within the same two groups (main effect of condition between 362 and 494 and between 614 and 794 ms, with all p*s*<0.05). These results suggested that neural signal in each of the conditions had the same spatio-temporal characteristics in the HR-TD and in the LR infants. All overall effects p-values adjusted for multiple testing with the three methods proposed by RAGU are shown in **Table 2.5**.

Table 2.5 *Results of all Topographic ANOVAs (TANOVA) adjusted for multiple testing using three methods available in RAGU*, explained in section 2.2.3. Fisher's test p indicates the probability that the obtained set of significant Fisher's test would be equal or larger than the number of significant tests obtained from random data; false positive count p indicates the probability that the observed number of significant tests are false positives based on a distribution of random data; the duration method computes duration threshold, that is the duration of periods with p-value <0.05 that needs to be exceeded to consider an effect significant. The longer effect duration indicates the longer period of the consecutive tests with p-value <0.05 for each tested contrast.

		Main effect of condition				**Main effect of group**				**Condition x group effect**			
TANOVA contrasts		**Fisher test p**	**False pos. count p**	**Duration**		**Fisher test p**	**False pos. count p**	**Duration**		**Fisher test p**	**False pos. count p**	**Duration**	
				thres hold	**longer effect**			**thres hold**	**longer effect**			**thres hold**	**longer effect**
LR vs. HR-ASD	**FD vs. Noise**	0.001*	0.001*	58 ms	162 ms	0.092	0.177	82 ms	40 ms	0.024*	0.054	56 ms	46 ms
	FA vs. Noise	0.001*	0.001*	54 ms	172 ms	0.012*	0.026*	76 ms	74 ms	0.309	0.42	50 ms	16 ms
LR vs. HR-TD	**FD vs. Noise**	0.001*	0.001*	54 ms	134 ms	0.355	1	76 ms	0 ms	0.283	0.512	58 ms	10 ms
	FA vs. Noise	0.001*	0.001*	50 ms	108 ms	0.408	1	74 ms	0 ms	0.671	1	54 ms	0 ms

LR: Low-Risk infants, HR-TD: High-Risk infants with Typical Development, HR-Aty: High Risk infants with Atypical development but not Autism Spectrum Disorder (ASD), HR-ASD: High-Risk infants with ASD. FD: Face with Direct Gaze, FA: Face with Averted Gaze.
* p<0.05.

Figure 2.11 shows that a positivity over the fronto-central areas occurred in both FD and Noise conditions in the second half of the considered time window. Thus, more than one scalp field configuration seemed to contribute to the signal in the Nc time window, in accordance with Reynolds & Richards (2005). Spatial correlation matrices, highlighting periods of stable (highly correlated) topographies, can be used to further understand TANOVA results. **Figure 2.12** shows correlation matrices of the scalp field potentials for each time point for the LR and HR-ASD group. High correlation between scalp field topographies at two time-points is thought to indicate that the configuration of the underlying sources is similar (Michel et al., 2009). Accordingly, each correlation "square" around the diagonal should reflect one functional brain process activated during a defined time period. The group by condition interaction observed in the LR versus HR-ASD TANOVA for the FD versus Noise contrast could reflect differences in the temporal sequence between the two conditions in the transition from one stable scalp field configuration (correlation square) occurring around 300 ms after the stimulus onset to the following one.

This hypothesis is in line with previous research indicating that combined activity from separate sources is involved in information processing recorded over the pre-frontal and frontal areas in the Nc time-window (Reynolds & Richards, 2005) and that this pattern of neural activation can be different in infants with later ASD diagnosis (Jones et al., 2016). I used microstates analysis to conduct a more fine-grained examination of the spatio-temporal characteristics of the scalp field underlying social attention in the current sample.

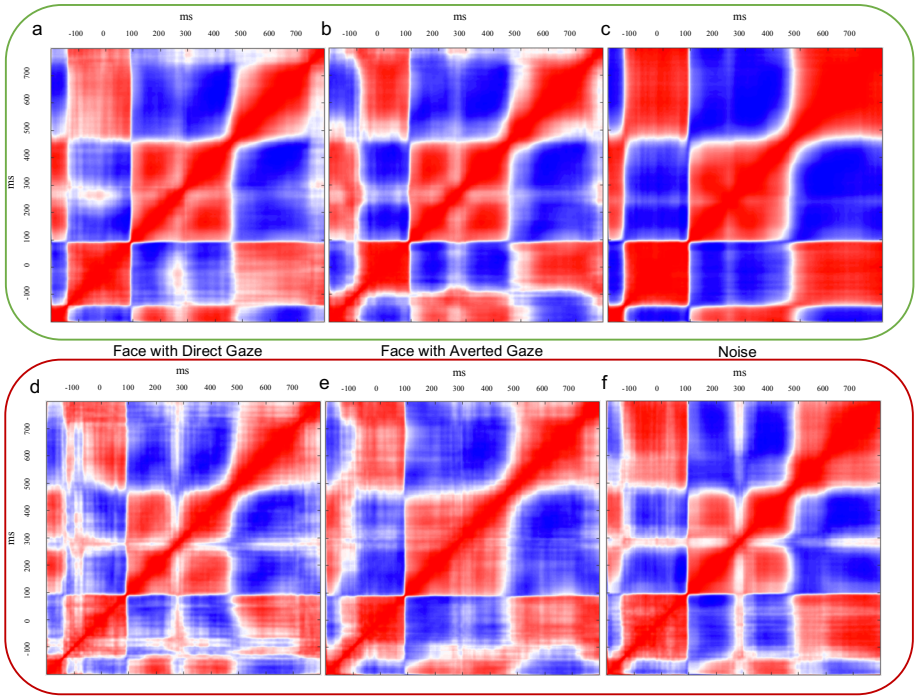

Figure 2.12 *Spatial correlation matrices* showing periods during which topographies are highly correlated, implying that similar generators are active (Michel et al., 2009). Vivid red indicates a correlation coefficient of +1, while vivid blue represents a correlation coefficient of -1. Figures **a**, **b** and **c** show matrices for the Low-Risk group, while **d**, **e** and **f** show matrices for the High-Risk infants who received diagnosis of ASD at age 3. **a** and **d** refer to the Face with Direct Gaze condition, **b** and **e** to the Face with Averted Gaze, **c** and **f** to the Noise condition.

2.3.3 States of attention: microstates

As explained in **section 2.3.3**, four "typical" microstates were extracted from the LR group in the time window between -200 and 794 ms in the FD condition. Their topographic maps and temporal sequence can be seen **Figure 2.13**. To test whether attention engagement to the stimulus was atypical in infants with emerging ASD, I selected the microstate map M4, whose spatial configuration and temporal profile of the scalp potential field correspond to the Nc (central negative deflection after 300 ms from the stimulus onset). Thus, M4 was identified in the three HR groups data between 300 and 794 ms in all stimuli conditions. The difference in duration of M4 between FD and Noise varied by HR outcome group ($F(2,75)=3.39$, $p=0.039$, $\eta^2=0.083$, **Table A2.16**, **Figure A2.4b**); the difference in mean GFP, representing the strength of the scalp field, did not ($F(2,72)=0.40$, $p=0.672$, $\eta^2=0.011$, **Table A2.15**, **Figure A2.4a**). Post-hoc comparisons of FD-Noise difference scores indicated that the HR-ASD group spent significantly less time than HR-Aty in M4 when attending the FD than to the Noise condition (Tukey post-hoc test, $p=0.036$, see **Figure 2.14a**); other comparisons between groups were not significant (HR-

TD versus HR-Aty: p=0.531, HR-TD versus HR-ASD: p=0.144). Socialisation scores at 3 years were significantly predicted by difference in mean GFP of M4 between the FD and Noise condition (β=20.94, t(73)=2.111, p=0.039, **Table A2.17**, **Figure 2.14b**) but not in duration (β=-0.005, t(74)=-0.29, p=0.77, **Table A2.19**). Of note, FD-Noise difference in mean GFP of M4 did not significantly predict later non-social adaptive skills (β=10.85, t(73)= 1.15, p=0.25, **Table A2.18**).

MANOVA assessing differences between conditions in the LR group showed no such effect in mean Global Field Power (GFP, F(1,35)=1.672, p=0.204, η^2=0.046) and duration (F(1,36)=0.249, p=0.621, η^2=0.017). Microstates were different in terms of their GFP (F(2,34)=4.392, p=0.020, η^2=0.205) and nearly so in their duration (F(2,35)=3.101, p=0.058, η^2=0.151). Post-hoc pairwise comparisons with Bonferroni adjustment for multiple testing revealed that mean GFP of M4 and, to a lesser extent, M1 was higher than M2 (p=0.007 and p=0.067, respectively). However, there was no difference in mean GFP between M1 and M4 (p=1) nor in duration of the microstates (all p*s* =1 in post-hoc comparisons). The latter result provided suggestive evidence for the fact that microstate maps extracted in the FD condition had a good fit on the Noise data too. This aspect could be considered a confirmation of the fact that they were indeed reflecting underlying general attentional processes in the LR group. All results can be seen in the Appendix of this chapter (**Tables A2.20**,**A2.21**, **Figure A2.7**).

In the MANOVA testing for an interaction effect between condition and outcome group, I found no significant effect of outcome in mean GFP (F(2,80)=2.374, p=0.100, η^2=0.056) and duration (F(2,85)=1.675, p=0.193, η^2=0.038) of the three microstates. Mean GFP appeared to be higher in the FD than in the Noise condition overall (F(1,80)=5.938, p=0.017, η^2=0.069), with no significant interaction between condition and outcome group (F(2,80)=0.882, p=0.418, η^2=0.022). There was no difference in duration between the two conditions (F(1,85)=5.938, p=0.424, η^2=0.008) and the interaction between condition and outcome was non-significant (F(2,85)=0.882, p=0.061, η^2=0.064). Post-hoc investigations of this effect through separate ANOVAs for each microstate revealed that this effect did not emerge as significant in any of the microstates (all p*s*>0.121). Of interest, microstates features were not influenced by age, sex or Phase (see **Tables A2.22**, **A2.23** for all results of the HR group MANOVAs).

Thus, overall HR outcome groups were not different in terms of microstate features. This result is reassuring with respect to the possible bias that would have occurred if the microstate maps, tuned on the FD data, fitted the data better in the FD condition (if significantly longer durations of the microstates were observed in the FD compared to the Noise condition).

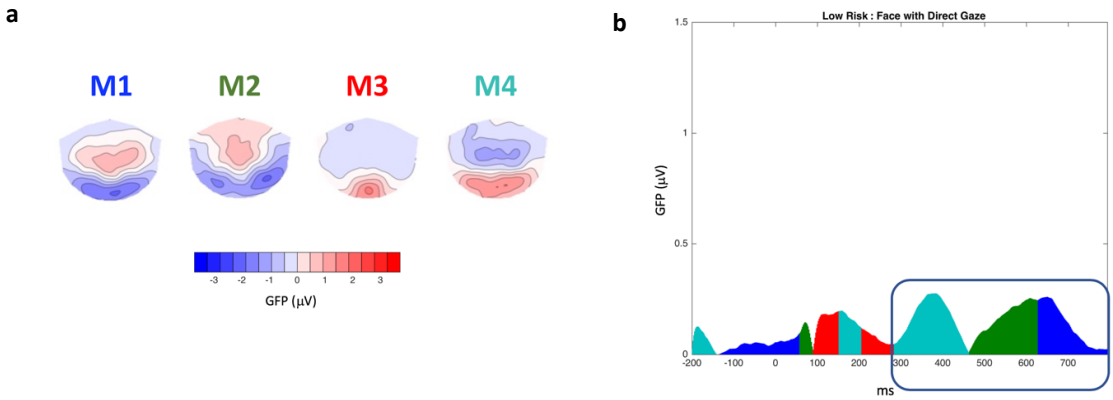

Figure 2.13 *The "typical" microstates during social attention*. **a** Scalp field topography of the four optimal microstate maps estimated from the Low-Risk (LR) infants in the Face with Direct Gaze condition. Global Field Power (GFP) in the microstate ranges from -3.5 (blue) to 3.5 (red) microVolts. **b** Sequence of microstates in the Face with Direct Gaze between -200 and 794 milliseconds (on the x-axis). The blue area indicates that the topography of the scalp field reflects microstate map 1 (M1), green reflects microstate map 2 (M2), red reflects microstate map 3 (M3) while cyan reflects microstate map 4 (M4). On the y-axis, absolute values of the mean GFP for each time-stamp, in microVolts, are indicated.

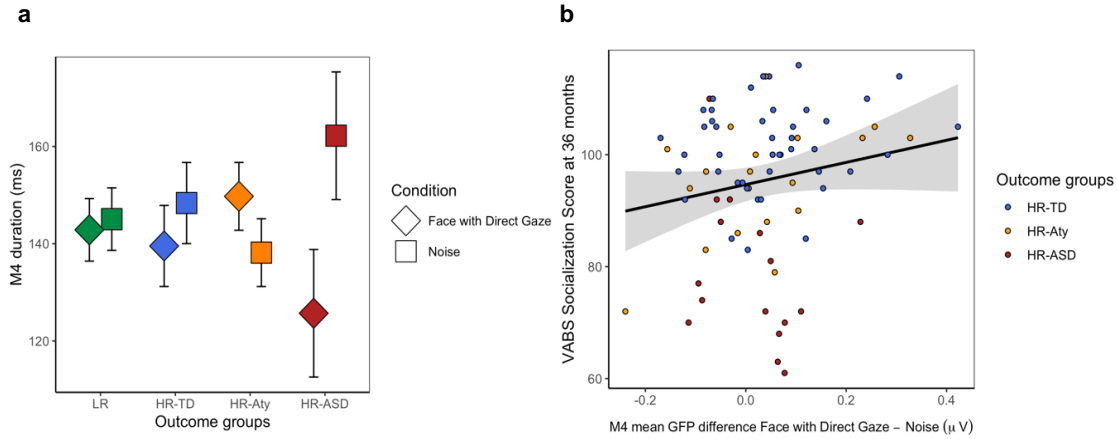

Figure 2.14 *Microstate 4 in relation to outcome*. **a** Mean microstate 4 (M4) duration, in milliseconds, in the Face with Direct Gaze (represented by rhombuses) and Noise (represented by squares) conditions for the four outcome groups. Bars represent ± standard error. **b** Scatterplot representing the relationship between M4 mean Global Field Power (GFP) difference between the FD and Noise conditions at 8 months (in microVolts), on the x-axis, and VABS Soc. at 3 years on the y-axis in the three high-risk groups. The regression line for the entire group of HR infants is displayed as a black line, with grey shadows representing standard errors.

2.4 DISCUSSION

Results presented in this chapter confirm the hypothesis that reduced brain response during attention engagement to faces in infancy is associated with a later ASD diagnosis and difficulties in socialization in childhood. The fact that atypical social attention precedes the emergence of core ASD symptoms is consistent with the idea that it might lie on the causal path to ASD, rather than be a secondary consequence of behavioural symptoms of the neurodevelopmental disorder. Further, this association is in response to faces gazing directly at the infant, but not in response to faces with averted gaze, confirming the importance of direct gaze for neural processing associated with early steps of face encoding (Rigato et al., 2010). Replicating and extending previous research in an independent sample, we found that infants who later received a diagnosis of ASD showed a smaller and shorter Nc to faces versus non-faces than infants who did not (Jones et al., 2016). Fine-grained analysis of the spatio-temporal characteristics of the signal over the entire scalp showed that the HR-ASD group spent significantly less time in the brain state (microstate) typically corresponding to attention engagement in response to social stimuli compared to HR infants with other developmental atypicalities. Mean GFP, indicating the strength of the scalp field response (Michel et al., 2009), during social attention significantly predicted dimensional variation in socialization in the HR children, consolidating evidence that reduced attentional engagement to social stimuli may play a role in the causal path to later poor social skills in ASD. Thus, these results consistently suggest that while the strength of the brain response to social stimuli (Nc amplitude and GFP), possibly reflecting the magnitude of attention engagement, is associated with later dimensional traits of social behaviour, the timing of this process (Nc latency and microstate duration) might indicate early atypicalities specifically associated with categorical ASD outcome.

2.4.1 Early atypical developmental trajectory of social cognition in ASD

The Nc findings replicate a smaller earlier study (Jones et al., 2016) showing atypical Nc responses to faces in 6-, but not 12-, month-old infants with subsequent ASD. In the present dataset, ranging from 6 to 11 months of age, response to faces became more enhanced and slower with age in infants with later ASD (**Tables A2.5** and **A2.9**, see **Figure 2.7**). A possible interpretation is that face processing might be delayed, rather than atypical, in children with ASD (Webb et al., 2011). This is in line with the idea that the brain of infants with ASD may undergo an individual process of adaptation (Johnson, 2017), such that transient delays at each

developmental stage can be partially overcome later in development. However, in the present sample the relationship between ERP metrics and age showed the opposite direction of association in the HR-ASD and the LR infants. These results emerge from a cross-sectional design and might be due to characteristics of the individual infants rather than to age differences. Longitudinal analyses similar to those conducted by Jones and colleagues (2016) but on a larger cohort might help to map developmental trajectories of neural responses to social and non-social stimuli.

Indeed, control multivariate analyses testing condition differences in all microstates (**Figure A2.7**, **Tables A2.22** and **A2.23**) showed that stronger GFP to the social then to the non-social stimulus was observed overall in the HR infants. M1 and M2 are characterised by a fronto-central positivity which could reflect the beginning of the Positive Slow Wave (PSW) observed after the Nc component (De Haan & Nelson, 1997; Richards & Hunter, 2006). This component has been associated with memory updating and recognition of the attended stimulus (De Haan et al., 2003; Richards, Reynolds, & Courage, 2010). Increased activity could indicate that in infants at high familial risk for ASD, processing resources are becoming devoted to static faces within the same age period (between 6 and 10 months) that typically developing infants start to specialise in more complex social stimuli, such as live scenes (Jones et al., 2015). Thus, delayed neural specialization for face processing in this critical period might be responsible for the onset of a divergent developmental pathway of behavioural correlates of social attention, such as looking at the eyes, which might have cascading effects on social learning (Klin et al., 2015). This hypothesis will be tested in **Chapter 3**.

The current findings, combined with those of the longitudinal study by Jones et al. (2016) showing that reduced Nc amplitude is a transient phenomenon in infants who later show ASD symptoms, and those of the intervention study by Dawson, Jones, et al. (2012) indicating that normalization of this marker is associated with improvement of behavioural symptoms, suggest that atypical brain processing during social attention might be an antecedent, rather than a precursor, of ASD, and have a causal relation to later symptoms (see the introduction of this thesis and Johnson, Gliga, Jones, & Charman, 2014, for further explainations of these concepts). Of note, infants at high familial risk who did not receive a diagnosis of ASD at 3 years did show early signs of atypicality in their neural response. In fact, their neural responses to FD versus Noise were not significantly different from HR-ASD infants in terms of Nc mean amplitude and M4 duration and GFP. Moreover, the robust results of the association between strength of the M4 in response to FD versus Noise and later socialization skills did not give evidence for a

different mechanism for HR children with and without ASD, failing to confirm that this feature simply reflected an early emergence of ASD core symptoms.

2.4.2 Functional states of the whole brain during attention engagement

Topographic information derived by multichannel EEG recording has several advantages compared to classic ERP analysis: it is less influenced by the reference electrode which could add additional bias to the data (Michel & Murray, 2012), it takes into consideration the orientation of the sources and controls for effects of overlapping scalp fields (Michel et al., 2009), it minimizes the dependence of findings by a priori choices, while maintaining statistical rigor (Maris, 2004). The TANOVA analysis revealed that there was suggestive evidence for an interaction between condition and groups between 556 and 630 ms when considering the LR versus HR-ASD contrast. Interestingly, t-maps depicted in **Figure 2.11b**, representing the difference in activation between the FD and Noise conditions, showed that the LR brain in response to the face was characterized by positive activations over the posterior regions for longer than in response to the non-social stimulus. On the contrary, in the HR-ASD brain the end of the negative frontal deflection (corresponding to the Nc) was observed earlier in the social than in the non-social condition. Topographic correlation matrices clearly revealed that two different arrangements of the brain activity could be detected in the Nc time window. This has been considered in previous research splitting the period between 300/400 and 800 ms into an early and a late Nc component (Jones et al., 2016). Critically, the beginning and end of an ERPs component is defined a priori. The temporal aspect of an ERP component is typically observed as peak latency, that does not provide information on the duration of a specific scalp field configuration. Topography analysis was critical to find out differences in the temporal sequence of neural activation of infants with emerging ASD in response to social versus non-social stimuli. Microstates analysis was used to further examine sequences of processing steps in the brain.

The microstate analysis confirmed that a brain state characterised by a frontal negativity when looking at faces with direct gaze was informative of categorical and dimensional ASD outcome. The microstates analysis provides a way to unify ERP analyses across scalp regions (Michel et al., 2009). Microstate 4, which had the spatio-temporal characteristics of the Nc, was characterised by a dipole that presented as a frontal negativity and occipital positivity (**Figure 2.10a**). The occipital positivity likely contributes to the P400 component, which is a positive ERP usually observed in infants between 300 and 800 ms after the stimulus onset in the posterior regions of

the scalp (Nelson & McCleery, 2008). Within the current dataset and other samples, analysis of the P400 and other posterior event-related potentials has indicated that P400 responses to faces vs. non-social stimuli are also altered in infants with later ASD (Elsabbagh et al., 2012; Jones et al., 2016). Previous studies have argued that, in infants, P400 and Nc are largely generated by the same dipole sources (Guy et al., 2016) and highly correlated during attention engagement with static faces (Jones et al., 2016). Studying brain states underlying attention allows us to unify these fields of research and to recognize how functional processes might be affected by atypical connectivity characteristics in the whole brain (Lewis et al., 2017; Piven et al., 2017). Thus, this finer-grained analysis converges on the probability that infants with later ASD diagnosis do not engage attentive brain processes in response to faces with direct gaze to the same extent as typically developing infants.

2.4.3 Social specificity

For the ERPs and microstates analyses I focused on difference scores between faces with direct gaze and matched non-social (visual noise) stimuli. This contrast was selected to parse attentional processes associated with social content from domain-general changes, following similar research (Dawson, Jones, et al., 2012). However, alterations in difference scores could also reflect atypical processing of the non-social stimulus. All the groups showed larger mean Nc amplitudes in response to the Noise stimulus than to faces with direct gaze, probably due to the novelty of the unusual non-social stimulus (Richards et al., 2010). This is in line with Jones et al. (2016). Also in line with this study, I found no difference between the face and non-social condition in the LR group for both amplitude and latency of the Nc. Of note, the Nc component is considered a neural correlate of attention engagement which is not face-specific. In fact, Guy and colleagues (2016) tested typically developing infants at 4.5, 6 and 7.5 months of age and did not find significant differences in Nc amplitude between responses to mother's face and infant's favourite toy. Instead, Nc amplitude was larger in periods of attention compared to periods of inattention (identified through heart-rate recording) in both conditions (Guy et al, 2016). Thus, lack of discrimination between conditions in the Nc, replicated in three independent cohorts including the present study, suggests that in the first year of life infants with no familial history of ASD show a similar level of engagement when attending to static images of social and non-social content. Differently from the LR group, I found that the HR-ASD and HR-TD groups showed a larger Nc when looking at the Noise stimulus than at images of faces with direct gaze. This may be consistent with eye-tracking studies showing that the ability to disengage attention from a non-social stimulus is atypical in infants at risk for ASD (Elsabbagh et al., 2009; Holmboe et al.,

2010). It is also consistent with fNIRS auditory studies that showed enhanced responses to noise than to vocal stimuli (Lloyd-Fox et al., 2018).

Moreover, in the present study post-hoc analyses revealed that a stronger scalp field of microstate M4 in response to Noise added to the prediction of low socialization skills in HR infants. Specifically, stronger neural response to the Noise stimulus was associated with lower socialization skills at three years in the infants who did not show ASD symptoms during childhood (β=-23.58, s.e.=12.12, p=0.055), while the opposite pattern was observed in infants with emerging ASD (β=71.28, s.e.=20.75, p<0.001, see **Figure A2.5b**). Taken together, these results suggest that differences in processing non-social stimuli might also play a role in the development of ASD. This is consistent with other evidence that a combination of differences in attention style both towards social and non-social stimuli underlie atypical developmental trajectories (Bedford et al., 2014; Gliga, Jones, Bedford, Charman, & Johnson, 2014; Johnson et al., 2014; Jones, Gliga, Bedford, Charman, & Johnson, 2014). In fact, infants at familial risk for ASD have enhanced visual search abilities (Gliga, Bedford, Charman, & Johnson, 2015), shorter time intervals between fixations (Wass et al., 2015) and difficulties in disengagement during visual orienting (Elsabbagh et al., 2013). Those characteristics are predictive of more severe ASD symptoms at 2 and 3 years. Building models that incorporate different types of phenotypes might help to understand the risk and protective value of various components of early behaviour in contributing to the development of social cognition (Johnson & Pasco Fearon, 2011) and will be the focus of the next chapter.

2.4.4 Endophenotypes

In the ERP analysis, HR-TD infants showed a similar but diminished profile of smaller Nc to FD than to Noise as the HR-ASD group. [The HR-Aty group showed a similar direction of effect but this was not significant, possibly due to lower power (HR-TD: N=44, HR-Aty: N=24)]. This is consistent with previous research suggesting that social attention may be a trait marker of genetic susceptibility, or endophenotype, of ASD (see **section 1.3**, and Constantino et al., 2017; Jones, Venema, Earl, Lowy, & Webb, 2017; Klin et al., 2015; Wade, Prime, & Madigan, 2015). As mentioned in **Chapter 1**, endophenotypes are measures that are closer to the biological bases of the disease than clinical phenotypes; they must be reliably quantifiable, they are observed earlier than clinical symptoms, and they are found to a higher extent in relatives of affected individuals than in the typical population (Gottesman & Gould, 2003). The social attention measures analysed in this chapter are promising in this regard, since they represent direct

measures of brain activity; they have been replicated in independent cohorts (Jones et al., 2016); they emerge at 8 months, that is prior to clear behavioural symptoms; and they are present in other HR infants or infants with parents with autistic-like social traits at an intermediate level (Jones, Venema, et al., 2017). Indeed, Constantino and colleagues recently showed that eye-tracking measures of social attention, which are atypical in toddlers with ASD, are highly heritable (Constantino et al., 2017); such an approach should now be taken with neural measures.

Interestingly, the difference in M4 responses to FD in children with ASD was clearest in comparison to the HR-Aty group, who exhibited some developmental concerns (such as high scores on the ADOS or low cognitive abilities). Possibly, the enhanced brain activity to FD versus Noise seen in the HR-Aty group could represent a process through which the vulnerable brain compensates for an increased genetic risk to develop ASD (Kaiser et al., 2010). Strong attention to social stimuli could compensate for other vulnerabilities and buffer children against an ASD outcome. Indeed, enhanced social attention as a protective factor against genetic loading for ASD has been claimed for female infant siblings (Chawarska, Macari, Powell, DiNicola, & Shic, 2016). Of note, the present results do not seem to confirm the presence of a sex-specific protective mechanism, as no significant effect of sex nor interaction between sex and outcome group was found (see **Tables A2.4** for FD versus Noise Nc amplitude and **A2.8** for latency).

One possibility is that social attention may be a modifier of developmental outcome in the context of lower-level processing atypicalities (Jones, Venema, et al., 2017). Specifically, infants from families with a tendency towards less interest in other people may be more likely to develop ASD in the presence of genetic risk factors that compromise early brain development. Infants from families with a tendency towards more social interest may develop subthreshold symptoms, or other types of neurodevelopmental profile like ADHD. Evidence of a further contribution to prediction from non-social processing is consistent with this possibility (Bedford et al., 2014). Studying the actual genetic profile of these children and building statistical models of the relation between domains over developmental time will be critical to deepening our theoretical understanding of the emergence of ASD and will be the focus of the next chapters.

2.4.5 Limitations and future directions

Conventional event-related potential analysis, which critically replicated earlier data from an independent cohort, and examination of spatio-temporal characteristics of the entire scalp field

based on randomization procedures converged in showing that infants with later ASD have a diminished ability to maintain attentive brain states in social contexts at 6 to 11 months. This provides support for the idea, tested in the present thesis, that atypical brain response during social attention is involved in the causal path to ASD, as it precedes and predicts the emergence of socialization difficulties in infants at familial risk for neurodevelopmental disorder. In line with previous studies, we found that early characteristics of non-social processing also contribute to the development of social difficulties. However, this finding is not fully explored in the present study and the design and analyses only allow me to conclude that the combination of atypical brain states when attending to social and non-social scenes from infancy is likely to be responsible for differences in developmental trajectories. Further analyses using machine-learning could help to shed light on the individual contribution of neural responses to social or non-social stimuli to later outcome (Gui, Bussu et al., under review). I also acknowledge that in this study nearly 40% of the original sample was excluded from analyses due to insufficient EEG data for an ERP design. The clinical impact of the present findings is somewhat limited by the reduced sample size, especially for the HR-ASD group.

Randomization statistics is an excellent tool to obtain robust results from the analysis of differences in ERP topographies, where multiple channel-wise comparisons challenge the power of the study and reliability of the results (Maris, 2004). One limitation of this method is that from significant results no inference on the general population can be drawn, as the null hypothesis distributions are driven by randomly shuffled collected data (Michel et al., 2009). However, Maris (2004) argues that a similar limitation might apply to generalizable tests too. In fact, they assume that the participants are drawn completely at random from the general population while this is rarely the case in psychophysiological studies. Randomization methods used in the study of spatio-temporal ERP data increase statistical power and allow to detect effects in groups with fewer subjects and lower signal-to-noise ratio (Koenig et al., 2014). Therefore, for studies like the present one, randomization techniques are ideal as they reliably test the effects of the experimental design, exploiting the information contained in such valuable data. The combination of these with classical statistics provides reliability to the present results.

Using multichannel EEG allowed me to show that brain states of global stable connectivity reflecting periods of synchronized network activation underlying cognitive processes (Rieger, Hernandez, Baenninger, & Koenig, 2016) can be identified in the infants' brain. Importantly, although microstates have been widely used to identify atypical brain functioning in psychiatric conditions (Rieger et al., 2016), they have not been used in infancy research thus far. This approach in this sense is highly novel; replication of the "typical" maps estimation in a larger

sample is needed. Of note, the age-range of the study participants included infants from 6 to 11 months. Importantly, Jones and colleagues (2016) found significant changes in the Nc between 6 and 12 months of age. Additionally, the present study revealed that an effect of age on Nc amplitude and latency was found for some of the study groups. Future studies should use narrower age-ranges to evaluate critical period-specific effects associated with risk and resilience.

The study of microstates in infancy might allow us to make a step further in the study of brain responses in social context, moving from looking at static indices to measuring sequences of functional processes (Maris, 2004). Understanding the state the developing brain is in a social interaction is especially relevant for early intervention in children with ASD. In fact, EEG is a non-invasive neuroimaging technique that has been used to assess the effects of intervention in boosting social attention skills (Dawson, Bernier, & Ring, 2012; Jones, Dawson, Kelly, Estes, & Webb, 2017). The present study revealed that microstate features identified in infants during attention to social stimuli are predictive of later outcome and social skills. On the one hand this suggests that they can be used as valid targets for social communication interventions (Green et al., 2017). On the other hand, the present results encourage to consider the use of microstates to plan personalised interventions in infants at high vulnerability for atypical neurodevelopmental outcome. Microstates analysis has been successfully used with adults to examine information intake in real time (Michel & Murray, 2012; Rieger et al., 2016). Exploring brain states changes in response to live stimuli is a next, promising avenue to identify optimal windows, and consequently tailor opportunities, for learning in the real world.

2.5 SUMMARY OF FINDINGS

In line with previous literature (Bedford et al., 2014; Gliga et al., 2014), I found that early characteristics of non-social processing also contribute to the development of later social difficulties. A combination of atypical brain states when attending to social and non-social scenes from infancy is likely to be responsible for differences in developmental trajectories of social cognition. In the next chapter I explore the contribution of these neural measures in a developmental prospective, to verify the extent to which they are related to later behavioural signs of atypical social attention that emerge during the second year as early markers for neurodevelopmental disorders in infants at familial risk (Johnson et al., 2014).

CHAPTER 3

ROLES OF ATTENTIVE BRAIN STATE AND LOOKING BEHAVIOUR IN THE DEVELOPMENT OF SOCIAL COGNITION

3.1 INTRODUCTION

ASD is a neurodevelopmental disorder. This simple definition implies that ASD derives from disruptions in the development of the central nervous system. Core symptoms of ASD, like difficulties in social interactions and communication and restricted interests and repetitive behaviours, as well as sensory anomalies, emerge in early developmental periods as behavioural manifestations of such organic dysfunction (American Psychiatric Association, 2013). Frank behavioural symptoms do not become pronounced until around two years of age or even later (Szatmari et al., 2016). However, signs of atypical developmental trajectory in infants with emerging ASD have been detected before the manifestation of overt behavioural difficulties (Jones, Gliga, Bedford, Charman, & Johnson, 2014). In line with other studies (Elsabbagh, Mercure, et al., 2012; Jones et al., 2016), in the previous chapter I found that indeed atypicalities in neural processing of visual stimuli can be observed at 6 to 10 months of age in infants who later receive a diagnosis of ASD, and are predictive of low social adaptive skills at three years. Moreover, the study of looking behaviour, supported by non-invasive techniques such as eye-tracking and frame-by-frame video coding, have allowed researchers to learn about subtle differences in visual processing and physiological responses in infancy that could have cascading effects on learning (Falck-Ytter, Bölte, & Gredebäck, 2013). These studies suggested that anomalies in fixation duration at the stimulus (Hendry et al., 2018; Wass et al., 2015), patterns of visual scanning (Gliga, Bedford, Charman, & Johnson, 2015; Klin, Shultz, & Jones, 2015; Shic, Macari, & Chawarska, 2014) and saccadic reaction times (Elsabbagh et al., 2013; Zwaigenbaum et al., 2005) observed in infants at high familial risk for ASD might be the first signs of atypical developmental path resulting in differences in overt behaviour (Elsabbagh & Johnson, 2010).

In this chapter I investigate whether neural indicators of social attention relate to a range of behavioural domains over the first three years of life, and how these interrelations contribute to developmental trajectories of social and cognitive functions. One possibility is that atypical neural processing of social stimuli drives changes in behavioural social attention and that it has cascading effects on social communication skills (Klin et al., 2015); another is that domain-

general difficulties in attention lead to a variety of behavioural manifestations including atypical looking behaviour to social and non-social stimuli, each contributing to different aspects of the ASD phenotype (Elsabbagh & Johnson, 2016). Moreover, it can be that relatively separate networks and functions are impacted by risk factors to a different extent but they interact with each other. Specific behavioural signs during attention to social stimuli could reflect adaptive or protective mechanisms against neural vulnerability, genetic risk or sex-specific factors (Chawarska, Macari, Powell, DiNicola, & Shic, 2016; Johnson, Gliga, Jones, & Charman, 2014).

In the following sections I introduce measures of looking behaviours which have been reliably identified as early markers of later difficulties in different aspects of the ASD phenotype. I then summarise two possible frameworks to explain the relationship between neural and behavioural signs of atypical attention across the first two years of life, in relation to developmental trajectories of children at risk for ASD. I last define the analytic approach to use the data collected within BASIS at specific time points to evaluate the weight of evidence for the different conceptualisations of the models.

3.1.1 Early signs of atypical looking behaviour

Signs of atypical visual behaviour have been detected by the naked eye much earlier than the emergence of ASD traits (Jones et al., 2014). For example, Feldman et al. (2012) found that parents noticed reduced interest in faces and attention shifts from a toy to the person during play interactions in their 12-month-old children who later received a diagnosis of ASD. Similarly, less gazes to faces were reported at 12 months of age and less eye-contact at 18 months of age during experimenter-delivered standardized assessment (Ozonoff et al., 2010). These reports have encouraged researchers to deeply investigate subtle differences in looking behaviours in infants at high familial risk for ASD. The most robust findings are summarised below.

3.1.1.1 "Sticky fixation" style

As mentioned in **Chapter 1**, Zwaigenbaum and colleagues (2005) provided evidence for atypical looking behaviour underlying attention orienting from multiple sources of observation in 12-month-old infants with emerging ASD. Measures from parent-reports, researcher-administered behavioural assessment and the 'gap-overlap' task, a marker task for the ability of disengaging the gaze from a central stimulus to direct attention to a peripheral stimulus, converged in identifying a tendency to fixate on particular objects at the expense of a more active exploration in infants who later received a diagnosis of ASD. This 'sticky fixation' style has been shown to be

predictive of higher ASD severity measured with the ADOS (Lord et al., 2000) at 24 months (Zwaigenbaum et al., 2005).

In typical development, "sticky fixation" is the definition for a behaviour observed in 1-month-old babies who have difficulties in shifting their gaze away from a target of fixation. This behaviour emerges as a sign of the first, non-modulated cortical influences on oculomotor control (Johnson & De Haan, 2015). Following dendritic growth and myelination between the cortical visual cortex and other cortical areas, developmental changes occur in attention skills regulating disengagement in typical infants, mainly in the first 6 months of age (Colombo & Cheatham, 2006). Blaga and Colombo (2006) reported that after this age disengagement latencies measured with a gap-overlap task are not particularly affected by manipulations of the stimulus designed to affect visual processing. They argued that, in typical development, individual variability in look duration and cognitive concomitants is less influenced by differences in disengagement from the second half of the first year (Blaga & Colombo, 2006).

In atypical development, however, difficulties in disengagement might consolidate during the first year and re-emerge in the second year. For example, Elsabbagh et al. (2013) found that a "sticky fixation" attention style, characterised by difficulties in disengaging from the central stimulus in a gap-overlap task, was observed in HR infants with later ASD at 13 months, although not at 7 months. Similarly, Sacrey, Bryson, & Zwaigenbaum (2013) found that prolonged latency to disengage from a manipulated object during play was only observed from 12 months of age in HR infants who later received a diagnosis of ASD, and remained at 15, 18 and 24 months. Thus, the "sticky fixation" style emerges at the end of the first year of life in infants who will later develop core ASD symptoms. These disengagement difficulties have been shown to play a role in the developmental trajectory of ASD (Bedford et al., 2014). One possibility is that difficulties in the ability to disengage from distracting objects and look at a person's face or referent in joint attention situations might reduce possibilities for learning in social contexts. Bedford et al. (2016) found that latencies in disengagement were associated with later social communication difficulties in 13-month-old boys at risk for ASD. Differently, Keehn, Müller, & Townsend (2013) suggested that early impairments in disengagement, reflecting early inefficiencies in the orienting network in infants with emerging ASD, may have implications for the development of efficient executive control processes. This assumption was based on a study by Posner, Rothbart, Sheese, & Voelker (2012), who found that orienting to novel sensory information during infancy predicted effortful control at 3 and 4 years. Effortful control is a major form of self-regulation and consists in the ability to inhibit a dominant response to perform a subdominant response and to engage in action planning. Because monitoring and resolving conflicts between incompatible responses require voluntary and attentive control, this

construct is considered a function of executive attention (Rothbart & Rueda, 2009). Based on their results and on the literature on brain networks, neuromodulators and genetic contributions to attention orienting and executive attention, Posner and colleagues (2012) postulated that early orienting to novel stimuli may activate executive network functions necessary for self-regulation (Posner et al., 2012).

Thus, difficulties in disengagement have been consistently found to be early markers of ASD in at risk infants after the first year of life. To what degree the "sticky fixation" style is an early manifestation of the narrow-attention style characteristic of ASD or impact the development of social, communication and executive function acquisition has not been clarified yet.

3.1.1.2 Staring at faces

Atypicalities in looking behaviour in 1-year-old or older infants at familial risk for ASD have been also detected using eye-tracking during a face pop-out paradigm. This task consists in presentations of arrays of different objects including a face. It was originally designed to assess exogenous orienting towards a face stimulus such that if a typical orienting mechanism biased towards faces is in place, the infant is expected to direct her gaze to the face first. Elsabbagh, Gliga, et al. (2012) examined this performance in HR infants and found that infants who later received a diagnosis of ASD showed intact face orienting. Surprisingly, however, by 14 months of age these infants showed overall longer looking time at the face stimulus compared with typically developing infants.

Hendry et al. (2018) replicated this finding in an independent cohort, using a different measure which has been demonstrated to have high intra-individual consistency across tasks (Wass, 2014): the duration of the longest look (peak look) at the face. They found that HR infants who later received a diagnosis of ASD showed longer peak look durations at the face stimulus than LR controls. However, HR infants who did not receive an ASD diagnosis at age 3 also showed longer peak look durations at the face compared to LR infants. Of note, atypically long duration of the peak look was observed when infants were attending to faces specifically, while no group difference was found in peak look duration to non-social stimuli (Hendry et al., 2018). Interestingly, the rate of change in peak look duration from the first to the second year was predictive of effortful control but not of social difficulties at 3 years of age (Hendry et al., 2018).

Further, studies examining peak look durations to faces versus objects in habituation tasks found longer peak looks to faces in 12-month-old infants with emerging ASD (Jones et al., 2016; Jones, Dawson, Kelly, Estes, & Webb, 2017). In 18- to 30-month-old toddlers with ASD, longer peak

look durations during habituation to faces were associated with poorer social skills and lower verbal abilities, suggesting that indeed this looking behaviour might be tightly linked to ASD symptoms (Webb et al., 2010). In addition, evidence supporting a causal link between this early sign and later ASD traits comes from an intervention study showing that peak look duration at the face during habituation was reduced at 18 months following parent-delivered intervention aimed to improve social skills (Jones et al., 2017). Of note, this intervention had also the effect of normalising neural correlates of attention engagement (amplitude of the P400 ERP component) in response to faces at 12 months of age, suggesting that shallower neural processing during social attention might lead to slower learning and hence be reflected in slower habituation (Jones et al., 2017).

In sum, there is converging evidence that looking time at a static face stimulus might be involved in the path to ASD, although there are mixed findings with respect to which of the features of ASD might be more closely related to this early sign of atypicality: autistic social traits, communication difficulties or disrupted executive functioning.

3.1.1.3 Responding to joint attention

As reviewed in the **Chapter 1** (see **section 1.2.3**), social attention, considered as the allocation of attentional resources to conspecifics (Salley & Colombo, 2016), contributes to the development and partly correspond to the ability to direct our own attention in the direction of attention of other people (Mundy, 2018). This milestone of social cognition is called "responding to joint attention" (Mundy & Newell, 2009), and it has been studied widely in the ASD literature using "gaze following" paradigms (Salley & Colombo, 2016). The first signs of atypical processing of gaze direction in ASD have been observed between 6 and 10 months as failure to show neural signatures of gaze shift processing around 400 ms after the stimulus onset (P400 amplitude) unlike what observed in typically developing infants (Elsabbagh, Mercure, et al., 2012).

Additionally, using eye-tracking systems it has been possible to also study looking behaviour of infants when watching another person shifting the gaze towards an object. By 10 months, infants at high risk for ASD show reduced ability to direct their attention to the referent object (i.e. the gazed-at object) when only eyes shifts, without a concurrent head turn, are performed in naturalistic interactions (Thorup, Nyström, Gredebäck, Bölte, & Falck-Ytter, 2016). Similarly, Bedford et al. (2012) found that by 13 months infants at high familial risk for ASD show decreased attention engagement to the object to which the other person's gaze moved. This sign, which has been interpreted as difficulty in understanding the communicative relevance of

eye-gaze, was predictive of later social communication impairment in HR children (Bedford et al., 2012).

Importantly, attention engagement to the gazed-at object and disengagement difficulties have been shown to contribute to ASD in an additive manner, suggesting that they might reflect different manifestations of early diversions from the typical developmental trajectory (Bedford et al., 2014). As anticipated in the introduction, in neurodevelopmental disorders the development of the complex network underpinning attention, which includes circuits devoted to perceptual and memory processes, might be disrupted due to an inefficient combination of feedforward and feedback influences between visual areas and more-rostral cortical areas, including parts of the parietal, frontal and temporal cortices involved in visual attention (Amso & Scerif, 2015). In the case of the development of joint attention, atypical connectivity in neural networks affecting feedforward input from lower to higher cortical regions might lead to failure to direct attention to gaze shifts and in turn prevent the developing brain from obtaining important feedback information about the referent object (Amso & Scerif, 2015), with cascading effects on spontaneous learning in social context (Csibra & Gergely, 2011). Thus, it is possible that early neural disruption of the attention system in infants at risk for ASD lead to inefficient responding to joint attention at later stages, but this hypothesis has not been supported by data yet, to my knowledge. Importantly, the ability of infants to be sensitive to referential cues has been argued to be an important aspect of the human communication system what enables social learning (Csibra & Gergely, 2009). Thus, this looking behaviour in infancy is expected to have an impact on later social and communication skills.

In sum, this section summarises evidence showing that atypical looking behaviour emerges after the first year of life in HR infants who later develop core ASD symptoms. In particular, difficulties in disengagement precede later ASD (Bedford et al., 2014; Elsabbagh et al., 2013; Zwaigenbaum et al., 2005); longer looking time at static face stimuli has been shown to be predictive of social difficulties (Elsabbagh, Gliga, et al., 2012; Jones et al., 2016) as well as language impairment (Webb et al., 2010) and low executive function (Hendry et al., 2018)in childhood; reduced engagement with the object towards which a person directs her gaze or the person's face predicts later autism-like social difficulties and social adaptive behaviour (Bedford et al., 2012; Chawarska, Macart, & Shic, 2013). In the following section I review two frameworks for interpreting a possible role of atypical looking behaviour in the path to ASD.

3.1.2 Pathways from attentive brain states to ASD

In **Chapter 2** I identified atypical neural responses during attention engagement in infants at familial risk for ASD in the first year of life. In the previous section of this chapter I reviewed studies showing that atypicalities in looking behaviour when attending to stimuli become more evident during the second year of life in toddlers who will show difficulties in socialization, communication or executive functions. One big question is whether these changes in looking behaviour affect or reflect ongoing specialization of the social brain which will disrupt learning. I evaluate here two possible hypotheses which link attentive brain atypicalities, looking behaviour and later emerging affective and cognitive function.

One possibility is that changes in looking behaviours in response to faces reflect atypicalities in the "social brain". The social brain has been defined as a network of regions involved in social information processing, inferring others' mental state (also called "mentalising" or "theory of mind", Frith & Frith, 2006), and acting behaviours which are guided by the presence of other humans (Adolphs, 2009). In Klein, Shepherd, & Platt's model (2009), illustrated in **Chapter 1** (see Figure 1.4a), this network is represented in red. It includes primarily the fusiform gyrus, the superior temporal sulcus and temporo-parietal junction, the ventromedial prefrontal cortex, the mirror system in the premotor cortex, the amygdala and the orbitofrontal cortex (Adolphs, 2009; Frith, 2007). According to this account, atypicalities in social attention at the neural and behavioural level are both emerging as a consequence of early vulnerabilities which are specific or stronger for the social areas of the brain (Johnson, 2017).

Another possibility is that domain-general atypicalities end up creating socially specific effects as well as eliciting adaptive looking behaviours, all differently contributing to the way infants learn from the environment (Elsabbagh & Johnson, 2016). This would be confirmed if early neural atypicalities were predictive of non-social domains of ASD across development and if non-social aspects of looking behaviour contributed to the path towards social difficulties.

3.1.2.1 The "social first" account

In typical development, the cortical areas recruited exogenously by the salient social visual stimuli in the very first months of life (Senju, Johnson, & Tomalski, 2014) increase their specialization during early development, resulting in increasingly tuned patterns of brain activation in response to social versus non-social stimuli from 6 to 12 months of age (Jones, Venema, Lowy, Earl, & Webb, 2015). Early disruptions in social information processing in critical

periods might compromise the social attention network specialization and have cascading effects on social learning, shared attention and the acquisition of socio-communicative skills (Chevallier, Kohls, Troiani, Brodkin, & Schultz, 2012; Dawson, 2008; Johnson et al., 2005).

Using eye-tracking, Jones & Klin (2013) observed that preferential attention to the eyes region of the face in naturalistic social videos declined from 2 to 24 months in infants with emerging ASD. The degree of decline was significantly associated with ADOS Social Affect scores at 24 months of age. Although the developmental trajectory of looking behaviour started to be significantly different between groups from 12 months of age, a reduction in eyes fixation was observed since the second month of age in infants with later diagnosis of ASD (Klin, Shultz, & Jones, 2015). The authors argued that this decline might be the behavioural manifestation of an inefficient progression from a reflexive, subcortically mediated, response to social visual cues to a cortically mediated, experience-dependent endogenous response (Shultz, Klin, & Jones, 2018). Atypical social visual engagement identifiable from 12 months using eye-tracking in infants who later show core symptoms of ASD might results from early genetically-driven disruptions in the development of cortical circuits typically underlying face processing (Klin et al., 2015). In this view, difficulties in social attention detected before the emergence of overt social difficulties might be mediating the relationship between early disruptions in neural processing of social stimuli due to inefficiencies in the social brain and later autistic social symptoms.

3.1.2.2 The "domain-general" account

Atypical visual behaviour might emerge as an attempt to respond to environmental inputs in a condition of suboptimal neural processing capabilities (Johnson, 2017). For example, the narrowed focus of attention, characteristic of many children with ASD, could be an adaptive behaviour with the function of reducing confusion in a complex environment by concentrating all the resources on one attended stimulus. Accordingly, it is possible that the early behavioural signs of atypicality observed in infants at high risk for ASD are not early manifestations of social brain inefficiencies but rather responses to altered systems underpinning domain general functions (Elsabbagh & Johnson, 2016).

Delayed or atypical specialization of function or anomalous characteristics of the brain microstructures reducing the fidelity of processing can have significant consequences primarily for brain regions which rely on high temporal resolution integration to deal with complex, dynamic, and less predictable stimuli, such as social stimuli (Johnson, Jones, & Gliga, 2015). Infants at high brain vulnerability might develop looking behaviour strategies to cope with low signal-to-noise ratio (Rubenstein & Merzenich, 2003), which are independent from a path linking early difficulties in social attention with later social skills. Consequently, differences in the

sensorimotor and visual orienting skills observed in the first years of life between typically developing infants and infants with emerging ASD (Gliga et al., 2015; Wass et al., 2015) may alter experience-dependent neuronal development, which in turn leads to the development of autistic-like social deficits (Piven, Elison, & Zylka, 2017).

In line with this account, early atypicalities when attending to visual stimuli are not only affecting the social domain but also related to other aspects of cognition such as executive functions. Moreover, non-social looking behaviour anomalies, possibly emerging as individual adaptive responses, are expected to contribute to the path towards the core social symptoms of ASD.

3.1.2.3 Protective factors

Prospective longitudinal studies mapping developmental trajectories of individuals at risk offer a special opportunity to examine whether some aspects of behaviours can be early markers of resiliency instead of disease. Protective or resilience factors reflect individual, relational and contextual variables in the environment that facilitate the developmental path towards a 'good' outcome, despite the presence of some adversities (Szatmari, 2018).

Early enhanced attention engagement during a gaze following task has been attributed a protective value against social autistic traits. Specifically, Chawarska, Macari, Powell, DiNicola, & Shic (2016) proposed this mechanism to act in a sex-specific manner, having observed that, before the first year of life, HR girls showed longer looking times at the other person's face during social interactions including gaze shifts and child-directed speech. The proportion of looking time at the face from 6 to 12 months of age was predictive of autistic social traits as measured by the ADOS Social Affect severity score. Interestingly, the proportion of looking time at the entire scene during social video was associated with social adaptive skills at 2 years (Chawarska et al., 2016).

Evidence for sex-specific effects on looking behaviour in the gaze-following paradigm comes also from data collected on an independent cohort of 13-month-old infant siblings from Bedford et al. (2016). Differently from Chawarska and colleagues, they used looking time at the gazed-at object as variable of interest. They found that this measure was predictive of social communication impairment measured with the ADOS at three years only in boys but not in girls. This indicated that other sex-specific protective factors would act on both this component of social attention and ASD social symptoms (Bedford et al., 2016). Thus, it is still not clear whether the degree of early attention engagement during situations eliciting responses to joint attention might act itself as a protective factor in girls (Chawarska et al., 2016) or constitute a risk factor for the development of social skills in boys only (Bedford et al., 2016).

If an early observed biological or behavioural attribute or marker is positively associated with an outcome, the fact that individuals at the higher extreme tails of the distribution of the early marker end up with a better outcome is not sufficient to consider the underlying function a protective factor. This relationship might just indicate that the degree of impairment in that function is the result of increased risk. Importantly, protective factors are not just the opposite of risk factors. Rather, they are effect modifiers, which act by attenuating the impact of a risk factor associated with severity or a poor prognosis (Szatmari, 2018). One way to test whether a candidate protective factor is contributing to resilience against psychopathology is to evaluate whether it relates to later typical development across disorders (Johnson et al., 2014). Another method could be to evaluate whether individuals at risk who have 'better than expected outcome' show atypically high values of the marker measure for the candidate protective factor compared to individuals who are not at risk and end up with the same outcome. Prospective longitudinal studies of infant siblings offer the possibility to explore the presence of protective factors by observing precursors of typical developmental trajectories in individuals at risk, compared with individuals who are not at risk (Szatmari, 2018).

In conclusion, signs of atypical looking behaviour observed in infants at high genetic liability for ASD might reflect specific impairments in the brain networks devoted to select, process and use social information, which in turn inhibit the ability to interact and engage in more complex social situations during childhood (Klin et al., 2015). A mediation model linking brain responses during social attention, atypical looking behaviour in social tasks and later social traits would support this view.

Differently, it can be that children at risk adapt to an initial condition of neural processing inefficiency by showing different types of looking behaviours (Johnson, 2017). These might contribute to a different degree to various difficulties in cognitive and affective functions as a result of domain-general brain atypicalities (Piven et al., 2017). According to this model, the interaction between different aspects of early atypicalities, but not necessarily a mediation model, would predict later ASD symptoms in the social and non-social domain.

The relationship between neural measures at T1, looking behaviour at T2 and social, language and executive function outcome at T3 or T4 is investigated in this chapter to shed light on the contribution of the early signs of attention atypicalities to developmental trajectories of ASD.

3.1.3 Aims of the study

The study of ERPs allowed researchers to detect neural signs of social attention atypical development earlier than behavioural markers and overt communication and social symptoms of ASD (Elsabbagh, Mercure, et al., 2012; Jones et al., 2016). In accordance with these studies, results from **Chapter 2** confirmed that, at a neural level, different responses when attending to social and non-social stimuli may reflect the initial phases of atypical developmental trajectories in children with a network disorder such as ASD (Amso & Scerif, 2015; Webb et al., 2011). In this chapter, I aimed to test whether the atypicalities in the engagement of attentive brain states are directly associated to later signs of atypical looking behaviour which have been shown to have an effect on learning.

First, I looked at potential paths linking neural response to later social, communication and executive function skills and to ASD traits through candidate eye-tracking measures. Using structural equation modelling (SEM), I explored the relationship between attentive brain states at 6 to 10 months (see **Chapter 2**) and three aspects of looking behaviour which have been found to be atypical at around 14 months in infant with emerging ASD: latency at disengaging from a target non-social stimulus, looking duration at a static face stimulus and engagement with the object which is gazed-at by an interacting adult. These were evaluated in relation to social skills, ASD core symptoms, communication and executive function behaviour at 2 or 3 years, to verify the specificity of developmental pathways to the social domain. I tested the hypothesis that inefficient processing due to atypical attentive brain states predicts atypical looking behaviour, which in turn leads to difficulties in social and cognitive skills. If a mediation path involving looking behaviour in response to social stimuli exists between neural vulnerability and developmental social outcome, this could inform on specific targets for intervention aiming to improve social skills (Dawson, Bernier, & Ring, 2012). Alternatively, atypical looking behaviour might be an adaptive response of suboptimal nervous systems independently associated with ASD symptoms and contributing to different aspects of the phenotype (Johnson, 2017).

Second, I tested the potential role of social attention as protective factor against ASD in HR infants. To do this, I took a two-step approach: a) I tested whether HR infants with more atypical attentive brain state who did not develop ASD at three years showed enhanced social visual attention at 14 months (protective value of looking behaviour against suboptimal neural conditions), and b) I compared looking behaviour during social attention in LR and HR infants with no diagnosis of ASD at age 3 split by sex, to evaluate the extent to which social attention could be protective against familial risk and whether this effect was sex-specific. If the present data confirm the protective role of social attention for females proposed by Chawarska et al.,

2016, higher levels of social attention would be found in HR-TD and HR-Aty girls compared to LR girls.

3.2 METHODS

3.2.1 Participants

The participants for the current study were all infants who participated in BASIS phase 1 and 2, as described in **Chapter 2** (**section 2.2.1**). To briefly recapitulate, the sample included 247 infants (127 females). Of those, 170 (85 females) had at least one older sibling with a community diagnosis of ASD (HR) while 77 (42 females) were control infants with at least one older sibling and no family risk of ASD (LR) recruited from a volunteer database at the Centre for Brain and Cognitive Development (see **Table A2.1** for demographics and behavioural measures at T1, T2, T3 and T4 for the present sample, by recruitment Phase).

3.2.2 Measures

3.2.2.1 Neural response to face versus Noise

The neural measures calculated in **Chapter 2** were used as indicators of brain response during attention engagement to social versus non-social stimuli. Two were measures from the classic ERPs approach, i.e. the difference between the mean amplitude and peak latency values of the Nc component between the face (FD) and the Noise condition. The Nc is a validated neural correlate of attention engagement in infants (De Haan, Johnson, & Halit, 2003; Richards, Reynolds, & Courage, 2010) and its decreased amplitudes and shorter latencies to faces versus objects has been associated with later ASD (see **Chapter 2** and Jones et al., 2016). Additionally, in **Chapter 2** "typical" microstates were extracted from the LR group whole scalp ERP data while attending to the FD stimulus, and subsequently identified in the HR group data in the FD condition and in the entire group data from the Noise condition. Difference between FD and Noise for two features of M4, characterised by frontal negativity and posterior positivity between 300 and 800 ms after the stimulus onset, were considered: mean GFP and duration.

Thus, the difference between FD and Noise condition for four neural measures of attention engagement response to visual stimuli was considered in this study:

1) Nc mean amplitude: more negative values indicated more enhanced response in the face than in the Noise condition;
2) Nc peak latency: more positive values indicated longer times for neural processing of the face stimuli;
3) Microstate (Ms) mean GFP: higher values reflecting stronger field in response to faces than to Noise;
4) Ms duration: higher values indicating longer periods of attentive states in the face than in the Noise condition.

These four measures were included in the present analyses because in **Chapter 2** they were all found to be predictive of later ASD diagnosis (Nc latency and Ms duration) and socialization skills (Nc amplitude and Ms GFP). All four measures were included, because correlation coefficients demonstrated weak correlation among variables, suggesting that they were capturing different aspects of neural processing of the attended stimuli (see **section 3.3.1** and **Figure 3.3**). Because significant correlation was indeed observed between some of these variables, for SEM analyses the four neural variables were allowed to load into a unique 'attentive brain state' factor representing engagement of attention to social versus non-social stimuli.

The choice to use difference scores (i.e., FD-Noise) in **Chapter 2** was justified by the intention to parse attentional processes associated with social content from domain-general changes, following similar research (Dawson, Jones, et al., 2012). In **section 2.4.3**, however, I discussed the observed pattern of result, arguing that an atypical difference score reflected both higher neural engagement with the non-social stimulus and lower engagement with the social stimulus, which are both observed in the HR-ASD group. In this chapter, the 'attentive brain state' factor should be considered as representing the combination of atypical neural responses to social and non-social stimuli.

3.2.2.2 Looking behaviour at 14 months

Disengagement

For Phase 1, disengagement index (henceforth disengagement) was calculated from the gap-overlap task described by Elsabbagh et al. (2013). A 46-inch liquid crystal display monitor was used to present the stimuli to infants, while seated on their parent's lap at 60 cm distance. Looking behaviour was recorded using a video camera. A centrally presented animation which expanded and contracted to attract the infant to the centre of the screen was presented before the onset of each trial. Then, a rotating central fixation stimulus was displayed subtending 13.8° x 18.0°. In the baseline trials, once the infant looked to the centre of the screen, the central

fixation stimulus was extinguished and a peripheral target (a dynamic green balloon) appeared simultaneously, subtending 6.3° x 6.3°. In the overlap trials, the same animated peripheral target appeared while the central fixation stimulus remained displayed (but not animated, as to better match the relative attractiveness of the two competing stimuli). The peripheral stimulus was presented randomly either to the right or the left of the central fixation stimulus and remained displayed until the infant looked at it or for 2.5 s. Subsequently, an attractive animation of an animal with sound replaced the peripheral target and the next trial was presented.

Two conditions (one with a sun and the other with a clown as the central stimulus) were presented pseudo-randomly across two blocks which were identical except for the central fixation stimulus. The rate of trial presentation was controlled by the experimenter. Generally, more overlap trials were presented as they were less likely to yield valid reaction times (especially atypical infants were more likely to look away or become stuck on the central fixation, Elsabbagh et al., 2013). Trial presentation continued until the infant became fussy or until a maximum of 70 trials was reached.

Data pre-processing was supervised by M. Elsabbagh. Frame-by-frame video-coding was performed on the data by two independent raters, who established a reliability >0.9 (Cohen's K) for trial validity. Subsequently, saccadic reaction time (RT) for fixation shift from the central to the peripheral stimulus were extracted from all valid trials. Trials were considered valid when infants oriented towards the peripheral target between 100 and 1200 ms after the stimulus onset. If the infants failed to do so, and therefore could not disengage from the central stimulus, RTs could not be calculated and the trials were not analysed. A measure of disengagement was obtained by calculating the difference between the RT in overlap trials and the RT in the baseline trials.

For Phase 2, stimuli were presented on a Tobii 1750/TX120 eye-tracker, recording corneal reflection data of each eye by mean of an infrared light source and a camera mounted below the screen. A gaze-contingent stimulus presentation was performed using MATLAB and the Talk2Tobii toolbox. Infants were presented with five blocks. The first four blocks lasted 12 trials per block, with a short video (8 s) presented between trials 6 and 7. The last block continued until 12 usable trials per condition had been presented, until the infant became fussy or until 80 trials had elapsed. After the infant fixated the central fixation stimulus (a cartoon clock/balloon, subtending 4.5°), a peripheral stimulus (a cartoon cloud, subtending 3°) was presented to the

left or right at an eccentricity of 6° following a delay of 1.5 s. A brief audio-visual reward was played when the infant looked at the peripheral stimulus. RTs were assessed in the three types of trials as for Phase 1: (1) gap: peripheral stimulus presented 200 ms after the offset of the central fixation stimulus; (2) baseline: central stimulus offset simultaneous with peripheral stimulus onset; (3) overlap: the central stimulus remained on screen when the peripheral stimulus was presented. The trial onset and the reward were automatically triggered online when gaze was recorded in the relevant area of the screen, as custom routine in MATLAB/Psychtoolbox. RT was calculated as the time elapsed between the peripheral stimulus appearance and the reported position of gaze entering the peripheral stimulus Area Of Interest (AOI, a 9° box around the stimulus). Trials were excluded from analysis: if a period of 60 ms or more of continuous data loss was obtained between peripheral stimulus onset and the eyes entering the lateral target AOI; if the infant's eyes were not fixating the central stimulus at the time of peripheral stimulus onset; if the infant did not perform a saccade to the lateral target within 2 s of peripheral stimulus onset; or if the infant disengaged from the screen within this period without first saccading to the peripheral stimulus.

Subsequently, mean RTs for all conditions were calculated, excluding RTs less than 100 ms (thought to be less than the minimum latency required to program a saccade in response a stimulus appearing) and greater than 1200 ms (thought not to represent exogenously driven reactions to the stimulus presentation) as in Elsabbagh et al. (2013). A measure of disengagement was obtained as for Phase 1 by subtracting the RT in the baseline trials from the RT in overlap trials. Data pre-processing was carried out by E. Jones.

Peak look at the face

Average peak look duration at the face stimulus was calculated from an eye-tracking face pop-out task. In the face pop-out task, described in Elsabbagh, Gliga, et al. (2012) and Hendry et al. (2018), infants were seated on their caregivers' lap, at around 50 cm from a 17-inch flat-screen monitor. A Tobii eye tracker with a camera mounted below the screen was used to measure the eyes coordinates and extrapolate information on infants' looking behaviour. The height and distance of the screen were adjusted for each child to obtain good tracking of the eyes. A five-point calibration sequence was run and recording only started when at least four points were successfully calibrated for each eye.

Gaze data and pupil size were measured with a Tobii 1750 at a rate of 50 Hz (i.e. one data point every 20 ms) for Phase 1 or a Tobii 120 at a rate of 60 Hz (i.e. one data point every 16 ms) for Phase 2. Fourteen (Phase 1) or ten (Phase 2) different slides, containing arrays of five stimuli,

were presented for 15 s each. To ensure that the child's gaze was directed to the centre, a small animation was presented in the centre of the screen before each slide presentation. Visual presentation was accompanied by music to assist infants in maintaining attention throughout the task. Presentation of the given slide was manually interrupted if the infant looked away from the screen for more than 5 s.

Each of the 14 possible arrays presented a colour image of one of fourteen different faces, all with direct gaze, and different exemplars from each of the following categories: mobile phones, birds, and cars (**Figure 3.1**). Additionally, a non-social control stimulus, a visual 'Noise' image as in the EEG task presented in **Chapter 2**, was generated from the same face presented within the array, by randomizing the phase spectra of the faces while keeping the amplitude and colour spectra constant (Halit, Csibra, Volein, & Johnson, 2004). Similarity of visual saliency among faces and the other stimuli within each array was verified using Saliency Toolbox 2.2 (Walther & Koch, 2006). The slides' presentation was counterbalanced for sex, ethnicity, and vertical and horizontal location of the face stimulus within the array.

Figure 3.1 *Example of one slide containing an array of five stimuli presented during the face pop-out eye-tracking task* (from Elsabbagh, Gliga, et al., 2012).

Look target coordinates were calculated from an average of x and y gaze coordinates from both eyes or single-eye coordinates where data from one eye was missing. Seven rectangular AOIs were defined: centre of the screen, face, noise, car, bird, phone, and total (the entire slide). Fixations were obtained automatically from Tobii Studio for Phase 1 (T. Gliga) and using an automated procedure written in MATLAB for Phase 2 (A. Hendry). For Phase 2 only, periods of missing data for durations up to 150 ms within the same AOIs, caused by blinks and/or temporary failure of data capture, were interpolated. Where gaps occurred between different

AOIs, they were not interpolated. This automated look duration procedure was validated using hand coding on data from earlier visits. For both Phases, fixations <100 ms were removed.

Peak looks were defined as the longest look durations per stimulus per slide per participant. Three categories were defined for this analysis: face, scrambled face (Noise) and non-social (mobile, bird and car). For each slide for which at least two looks were available, the longest look in each category was identified. If no peak look was available for a particular category, the trial was excluded from the mean peak calculations for that category. Infants with fewer than three useable trials were excluded from analyses of peak look duration. An average of peak look durations across trials for the face category was calculated for each participant and used as variable in subsequent analyses. Peak looks were averaged across the trials to provide a more stable characterisation of individual differences (see Hendry et al., 2018).

Gaze following

For Phase 1, infants attending the gaze following eye-tracking task sat on their parents' lap at a distance of 50 cm from a 17-inch flat-screen monitor and a Tobii 1750 system recording corneal reflection data. As soon as they were positioned in front of the screen, a 5-point calibration sequence was run and the main experimental task was started when at least 4 points were marked as correctly calibrated for each eye. Gaze data were recorded at 50 Hz. Before the start of each trial, small colourful animations and beeping sounds appeared in the centre of the screen to attract the infant's attention where the model's face would appear. Subsequently, the experimenter pressed the key to start the trial. Each trial started with a scene with a female model, seated behind a table, facing down. Two toy objects were placed on the table, one to each side of the model. The videos consisted of three phases. In the 'looking down' phase (**Figure 3.2a**), the model remained still in the initial position for 3 s and then looked up such that both her head and eye-gaze were directed straight ahead. This was followed by the 'direct gaze' phase, which began as soon as the model's eyes were looking ahead, and finished 2 s later, when her head began to turn away (**Figure 3.2b**). The third phase, 'shift', was marked by the model's head turning to look at one of the objects, the congruent object, and finished at the end of the trial (**Figure 3.2c**). The non-gazed-at object was the incongruent object.

Twelve trials were presented to each infant using ClearView software. Six different pairs of objects were displayed. The objects' position with respect to the gaze was counterbalanced across trials, such that in different trials the same object would once be the congruent object and once the incongruent object. The direction of the model's gaze was fixed in the following pseudo-random order: RLLRLRRLRLR. Within each trial, look data were extracted as a total for the whole slide and for three rectangular AOIs defined around the face, congruent object and

incongruent object using ClearView software (face subtended 8° x 11.4° and objects by 3.7° x 4.5° for the smallest and 7.3° x 8.4° for the largest).

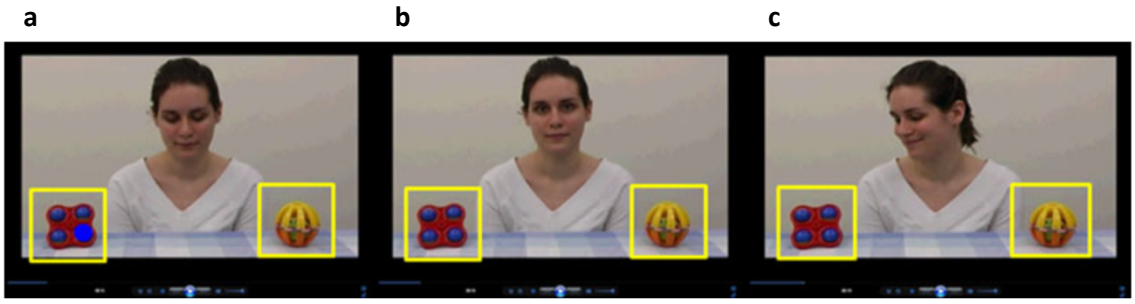

Figure 3.2 *Example of the stimuli in the three phases for the gaze following eye-tracking task* presented to Phase 1 infants (from Bedford et al., 2012). **a** The 'looking down' phase; **b** the 'direct gaze' phase, **c** the 'shift' phase.

For Phase 2, infants sat on their parents' lap at approximately 60 cm from a Tobii T120 eye-tracker screen. Data were recorded at 60 Hz. After a 5-point calibration run in which at least 4 points were marked as calibrated for each eye, the trial begun. The paradigm used in Phase 2 was different from Phase 1 for three main reasons: 1) the model talked to the infant during the entire duration of the trial; 2) eight, instead of twelve, trials were presented; 3) each trial was composed by six, instead of three, phases: three 'direct gaze' phases and three 'gaze shift' phases, with no initial 'looking down' phase. Specifically, the model started the trial with her eyes directed towards the observer ('direct gaze'), then performed one shift with her head and gaze towards the congruent object ('first shift'), returned to the 'direct gaze' position, performed a 'second shift' in the same direction to gaze at the congruent object, returned once more to the 'direct gaze' position and performed the 'third shift' in the same direction to gaze at the congruent object. The trial ended after the third shift. Head turns were accompanied by speech, as the model greeted the child ("hallo"), and named the gazed-at object every time she directed the gaze towards it.

Data pre-processing was carried out by R. Bedford for Phase 1 and by J. Parsons for Phase 2. For both Phases, a fixation filter of 60 ms was applied to exclude random noise unlikely to represent true fixations (Bedford et al., 2014). Trial exclusion criteria were: (1) no looking to the face during 'direct gaze', considered a prerequisite for gaze following behaviour; and (2) looking away from the computer screen for the entire 'shift' phase. One relevant difference between Phase 1 and 2 in the data processing was that, while for Phase 1 the proportion of looking time at the

congruent object was calculated only if the infant's first gaze was correctly directed towards the congruent object, in Phase 2 all trials where look data were available (indicating that the infant was attentive) were analysed. This choice was due to the fact that less trials were available (eight in Phase 2 versus twelve in Phase 1) and multiple gaze shifts were performed by the model during each trial. To exclude trials based on the first look in each of the trial's phase (first, second and third shift) would have significantly reduced the number of valid trials (J. Parsons, personal communication).

Gaze coordinates were extracted using ClearView. AOIs were defined separately around the face, referent object and the distractor using MATLAB. Samples were missing for <200 ms were interpolated if the coordinates before and after the missing data were in the same AOI. Looking time to the congruent object (out of total looking time to the slide, including looking to other parts of the screen such as face, torso and table) during the 'shift' phase was calculated.

3.2.2.3 Developmental outcome

Social adaptive skills

Social skills at T4 were measured using the standard score of the Socialization Domain from the second edition of the VABS (Sparrow, Cicchetti, & Balla, 2005), as in **Chapter 2** (see **section 2.2.1**).

Autistic traits

To measure autistic traits, the parent-report Social Responsiveness Scale (SRS) pre-school form collected at T4 was used (Constantino & Gruber, 2012). The SRS is a 65-item quantitative measure of autistic-like social impairment that has been extensively used in both clinically ascertained and population-based samples of subjects (Constantino & Gruber, 2012; Constantino & Todd, 2005; Frazier et al., 2014). Each item is rated on a scale from 1 (not true) to 4 (almost always true); the instrument usually requires 15–20 min to complete. Raw scores for specific items were summed up and converted into sex- and age-specific standardized scores to obtain two DSM-5 compatible domains: Social Communication Impairment (SCI) and Restricted Interests and Repetitive Behaviour (RRB). For this sample, the SRS was completed by parents.

Language abilities

The MSEL is a semi-standardized assessment used to evaluate cognitive and motor abilities in infants from birth to 68 months (Mullen, 1995). The assessment, carried out at T4, consisted in 25-35 minutes during which the experimenter administered the child with developmentally-appropriate tasks. The testing started from a basal level where a child scored at least 1 point on each of the 3 items, and continued until the ceiling level of 3 consecutive items with scores of 0 is reached. MSEL is composed of five scales: Gross Motor; Visual Reception; Fine Motor; Receptive Language and Expressive Language. Row scores were converted into standard scores (mean=50, s.d.=10) for each of the scales. Additionally, a total composite score was provided (mean=100, s.d.=15).

For the present study, the two Language scales from the 3-years assessment were used. The Receptive Language scale is composed of 33 items and measures a child's ability to process linguistic input. Specifically, it assesses auditory comprehension, auditory memory and auditory sequencing abilities. The Expressive Language scale is composed of 28 items testing the child's ability to use language productively, specifically tapping speaking ability, language formation and verbal conceptualization.

Executive function

The Early Childhood Behavior Questionnaire (ECBQ) is a parent-report questionnaire where caregivers are asked to answer about the relative frequency of occurrence of specific infant reactions in concrete situations referring to recent events. This instrument has been widely used to identify the early structure of temperament (Rothbart, Ahadi, Hershey, & Fisher, 2012). Differently from the other measures, which were collected at T3 and T4, ECBQ was only available at T3 for Phase 1 and 2 participants. The Effortful Control subdomain from the ECBQ (Putnam, Gartstein, & Rothbart, 2006) was used as a measure of executive function, as it has been shown to reflect early contributors to future executive control capabilities (Gartstein, Bridgett, Young, Panksepp, & Power, 2013; Rothbart et al., 2012). Lower effortful control scores indicate poorer executive attention functioning.

3.2.3 Analyses

3.2.3.1 Structural Equation Modelling

SEMs were used in order to analyse whether neural measures where directly associated with developmental outcome in childhood or through mediation of one of the looking behavioural measures. As a first step, I computed Pearson's correlation coefficients between neural (T1) and behavioural (T2) signs of attention and the outcome measures (T3 and T4), to examine how individual predictors and dimensional outcomes were related to one another, before controlling for the influence of other variables and assessing the specificity of associations. Shapiro-Wilk tests revealed that all variables were non-normally distributed (all p*s*<0.052, **Table A3.1**). Variables were not transformed for three reasons: 1) when attempted to do so, this procedure rarely led to non-significant Shapiro-Wilk tests, 2) using transformed scores would have affected interpretation of the results, 3) transforming the variable's value is often not an efficient method to obtain normality of the underlying residuals distribution, which does not closely mimic the distribution of the values themselves (Feng et al., 2014). Therefore, all continuous variables were scaled but not transformed. To deal with non-normality of the distributions, robust maximum likelihood estimator was used in all SEMs.

SEMs tested whether significant associations existed between experimental predictors and developmental cognitive outcomes and whether the path between neural response at 8 months and cognitive outcome at 2/3 years was a direct path or it was mediated by looking behaviour at 14 months. The four neural measures collected at T1 were constrained to load into a unique factor called "attentive brain state" as some of the variables were highly correlated (see **section 3.3.1** below). The three measures of looking behaviour collected at T2 were not set to load into a single factor as the previous correlation analysis revealed that their correlation was very low (rho*s*<0.11, see **Figure 3.3**).

Therefore, 4 SEMs were performed, one for each of the following socio-cognitive outcomes:

- social adaptive skills (VABS Soc. at T4)
- autistic traits (SRS SCI and RRB domain scores at T4)
- language abilities (MSEL receptive and expressive language scores at T4)
- executive functioning (ECBQ effortful control score at T3).

Additionally, to verify whether the same associations held when controlling for risk group (0=LR, 1=HR), I re-tested all models while including risk group as predictor of all variables and compared the model fit with the models without risk group. Results from the models with the best fit parameters are reported for each of the four socio-cognitive outcomes.

In all models, the attentive brain state factor and the three looking behaviour measures were modelled as predictors of the outcome variable/s, while Phase (1 and 2) was modelled as predictor for the attentive brain state factor, the three looking behaviour measures and MSEL receptive and expressive language. This choice was done to control for effect of Phase whenever there was the possibility that experimental conditions or data processing differed between Phases. Because language scores derived by a behavioural assessment which might have been dependent on setting and experimenter identify, Phase was included as predictor. On the contrary, I did not control for Phase for the parent-report questionnaires reflecting social adaptive skills, autistic traits and executive functioning. In the models including risk group, this binary variable (LR versus HR) was added as predictor for all other variables.

SEM analyses were performed using the 'sem' function of the 'lavaan' R-package. Missing data were considered missing at random and full information maximum likelihood approach (FIML) was used, where the likelihood is computed case by case, using all available data from that case. This method was chosen as it has been demonstrated to provide less biased parameter estimates than listwise deletion (Enders & Bandalos, 2001; Wildaman, 2006).

3.2.3.2 Testing the protective value of social attention

To evaluate the protective value of social attention I conducted two types of exploratory analyses:

a) Assuming that the attentive brain state factor reflected the extent to which early risk factors impacted the brain affecting processing during social and non-social attention, I tested whether HR infants with more atypical attentive brain state who did not receive diagnosis of ASD at age 3 showed enhanced visual attention skills at 14 months (protective value of looking behaviour against neural vulnerability);

b) I compared behavioural correlates of social attention in LR and HR boys and girls who did not develop core ASD symptoms in early childhood (sex-specific protective effect of looking behaviour against familial/genetic risk). This analysis allowed me to see whether HR children who have high familial/genetic burden for ASD but have a 'better than expected outcome' (Szatmari, 2018) showed unusually higher social attention at 14 months. If so, this would suggest that early social attention skills might have a protective value against familial/genetic risk. Moreover, this analysis allowed me to test whether such an effect was observed to the same extent in males and females. If exceptionally enhanced social attention was observed in HR females only, this would have confirmed the role of sex-specific effects in boosting social

attention. If higher social attention was observed in both HR males and females, this looking behaviour might have been interpreted as protective against risk independently from sex factors.

Protective effect of looking behaviour during social attention against neural vulnerability

For this analysis, I aimed to identify infants with inefficient engagement with social stimuli in the first year of life. The mean Nc amplitude and Ms duration difference between FD and Noise condition were selected as neural correlates of social attention because they were the two variables which mainly contributed to the attentive brain state factor in the SEMs (see **section 3.3.1**). As a first step, these two variables were scaled such that they both had mean=0 and s.d.=1. Of note, more negative values of Nc amplitude indicated more enhanced attention engagement. Consequently, more negative scores in the mean Nc amplitude difference between the face and the Noise condition indicated more engagement with the face stimulus. Thus, the mean Nc amplitude score were multiplied by -1 and a composite score for "neural vulnerability" was obtained by summing up the transformed Nc values with the Ms duration difference values. Subsequently, infants were divided into quartiles obtained by ranking all the scores and clustering them into four chunks. More negative values of the neural vulnerability composite score indicated enhanced (stronger and/or prolonged) neural response to the Noise than to the FD stimulus. Therefore, infants in the first two quartiles showed lower attention engagement to the FD than to Noise, while infants in the third and fourth quartiles showed higher attention engagement to the FD than to Noise.

To assess whether differences in looking behaviour were observed in infants at high brain vulnerability, only the HR infants were considered. HR infants in the top two quartiles were considered at "low neural vulnerability", while HR infants in the lower two quartiles were considered as "high neural vulnerability". Because I was interested in evaluating the protective role of social attention against ASD, the HR infants were divided into two ASD outcome groups: HR-ASD and HR-noASD, including infants from both the HR-TD and the HR-Aty group.

A two- (high versus low neural vulnerability) by-two (HR-noASD versus HR-ASD) ANOVA was performed to evaluate group and interaction effects for each of the two social attention looking behaviour measures: peak look at the face in the face pop-out task, and looking time at the gazed-at object in the gaze following task. Phase was added as covariate in all analyses. **Table 3.1** illustrates the number of participants per group for the two ANOVAs.

Table 3.1 *Number of participants for the ANOVAs testing the protective value of looking behaviour by neural vulnerability* (low versus high) and outcome group (HR infants without a diagnosis of ASD at three years, i.e. HR-noASD, versus HR-ASD).

Vulnerability groups	Outcome groups	Peak look at the face	Looking time at the gazed-at object
Low neural vulnerability quartiles	HR-noASD	32	34
	HR-ASD	5	5
High neural vulnerability quartiles	HR-noASD	32	31
	HR-ASD	10	8
Total		79	78

Sex-specific protective effect of looking behaviour during social attention against familial/genetic risk

To test whether atypical performance in social attention behaviour at 15 months could have a protective effect against ASD, I compared the three groups of infants with a non-ASD outcome, that is LR, HR-TD and HR-Aty. If any or both these HR groups showed significantly enhanced social attention compared to LR group, this would have pointed towards its possible protective effect against the risk of ASD. Because previous studies argued that such protective effect could be sex-specific (Bedford et al., 2016; Chawarska et al., 2016), I tested for a significant effect of sex in interaction with group. Thus, two 2-way ANOVAs were used to evaluate the effect of group (LR, HR-TD and HR-Aty), sex (males and females) and their interaction on the social attention eye-tracking measures: peak look duration at the face and proportion of looking time at the gazed-at object, respectively. Tukey's HSD method was used to correct of multiple comparisons in post-hoc analyses investigating significant main effects. As I was interested in the protective value of social attention specifically, disengagement was not investigated. Phase was used as a covariate in both ANOVAs. Number of participants per group for these analyses are shown in **Table 3.2**.

Table 3.2 *Number of participants for the ANOVAs testing the protective value of looking behaviour by familial/genetic risk,* assessing differences in peak look duration at the face from the face pop-out task and proportion of looking time at the gazed-at object from the gaze following task by group (LR, HR-TD and HR-Aty) and sex (males and females).

Outcome group	Sex	Peak look at the face	Looking time at the gazed-at object
LR	**Males**	27	26
	Females	37	28
HR-TD	**Males**	31	32
	Females	45	48
HR-Aty	**Males**	18	20
	Females	19	16
Total		177	170

3.3 RESULTS

3.3.1 Investigating developmental pathways

Figure 3.3 reports correlation coefficients among all the continuous variables for this study. Associations should be considered significant with a Bonferroni-corrected alpha level of 0.0006 (α=0.05/78=0.0006). Weak to moderate correlation was observed between Nc mean amplitude and the other three neural measures collected at 8 months (Nc latency and microstate features). In particular, correlation between Nc mean amplitude and Ms duration survived correction for multiple testing (rho=-0.36, p=2.08 x 10^{-5}). This result justified the choice of considering the four neural measures as loading into one 'attentive brain state' factor (see **section 3.2.2**). **Table 3.3** summarises outcome group differences for the three measures of looking behaviour collected at T2.

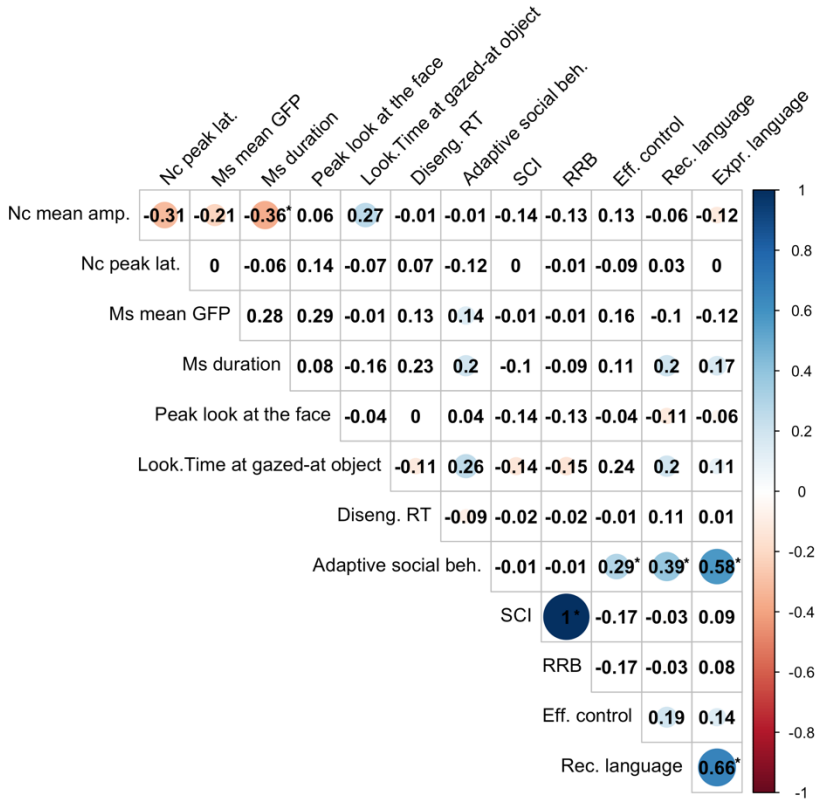

Figure 3.3 *Correlation coefficients (Pearson r) for associations between all variables included in SEMs*: neural correlates of social attention engagement at T1, looking behaviour at T2 and dimensional social and cognitive outcome at T3 or T4. Coloured circles highlight correlation coefficients for relationships that were statistically significant at a p-value<0.05, with blue indicating a positive correlation and red a negative correlation. * indicates significant correlations with Bonferroni correction for multiple testing (α=0.05/78=0.0006).

Nc mean amp.: Nc mean amplitude difference between FD and Noise; Nc peak lat.: Nc peak latency difference between FD and Noise, Ms mean GFP: microstate mean Global Field Power difference between FD and Noise; Ms duration: microstate duration difference between FD and Noise; Peak look at the face: peak look duration at the face in the face pop-out task; Look. time at gazed-at object: proportion of looking time at the gazed-at object in the gaze following task, Diseng. RT: disengagement reaction times in the gap-overlap task; Adaptive social beh.: adaptive social behaviour measured with VABS socialization domain standard score; SCI: SRS Social Communication Impairment domain score, RRB: SRS Restricted and Repetitive Behavior domain score, Eff. Control: effortful control score from the ECBQ questionnaire, Rec. language: receptive language scale from the MSEL, Expr. Language: expressive language scale from the MSEL.

Table 3.3 *Results of the ANOVAs testing differences in the measures of looking behaviour* collected at T2 between the four outcome groups.

	LR	HR-TD	HR-Aty	HR-ASD		
	Mean (s.d.) Min - Max	**Mean (s.d.) Min - Max**	**Mean (s.d.) Min - Max**	**Mean (s.d.) Min - Max**	**p**	**η^2**
Peak look at the face (ms)	1254 (646) 310 – 3950	1620 (777) 636 - 4436	1780 (1015) 448 - 5404	1713 (864) 760 - 4200	0.02*	0.04
Prop. looking time at gazed-at object	0.21 (0.10) 0.05 - 0.54	0.20 (0.14) 0.00 - 0.91	0.16 (0.12) 0.00 - 0.50	0.17 (0.11) 0.01 - 0.45	<0.001*	0.05
Disengagement RT (ms)	157 (103) -156 - 382	160 (105) -257 - 396	169 (110) -37 - 387	213 (133) 48 - 739	0.08	0.03

LR: Low-Risk infants, HR-TD: High-Risk infants with Typical Development, HR-Aty: High Risk infants with Atypical development but not ASD, HR-ASD: High-Risk infants with Autism Spectrum Disorder; s.d.: standard deviation; p: p-value of the ANOVA with outcome group as between-subject factor with four levels, with age (in months) at T2 and Phase as covariates, η^2: measure of the effect size of the effect of outcome group. RT: reaction time.

3.3.1.1 Adaptive social behaviour

The SEM for prediction of adaptive social behaviour did not provide a good fit to the data ($\chi^2(17)=39.024$, p=0.002, comparative fit index, CFI=0.877, root-mean-square error adjusted, RMSEA=0.068, 90% CI [0.040-0.105], Akaike Information Criterion, AIC=3845.220, Bayesian Information Criterion, BIC=3968.049). However, the model fit was improved by including risk group as explanatory variable ($\chi^2(20)=33.95$, p=0.044, CFI=0.946, RMSEA=0.048, 90% CI [0.008-0.078], AIC=3792.939, BIC=3933.315). Therefore, the latter model is further described.

Ms duration and Nc mean amplitude loaded more strongly on the attentive brain state factor (standardised β=0.98, p<0.001 and st.β=-0.38, p<0.001, respectively). Nc mean amplitude and latency showed significant covariance (st.β=-0.30, p<0.001). Consistent with the results of **Chapter 2**, differential attentive brain state to FD and Noise was directly associated with social adaptive skills at three years of age (st.β=0.22, p=0.009). Additionally, it was nearly significantly associated with looking time at the gazed-at object (st.β=0.17, p=0.055). The social adaptive outcome was also predicted by looking time at the gazed-at object (st.β=0.19, p=0.003) but not by the other behavioural measures (disengagement: st.β=-0.09, p=0.151; peak look at the face: st.β=-0.017, p=0.824). There was a significant covariance between disengagement difficulty and looking time at the gazed-at object (st.β=-0.16, p=0.040).

The diagram depicted on **Figure 3.4** shows significant relationships as black solid lines and trends of association (p<0.1) as dashed lines. All standardised and unstandardized estimates with robust standard errors are reported on **Table A3.2**. When examining the mediation path linking attentive brain state, looking time at the gazed-at object and adaptive social behaviour, there was a significant direct effect (β=0.213, s.e.=0.072, z=2.953, p=0.003), indicating that the relationship between neural measures and social outcome remained strong and was not

explained by the mediation effect of the looking behaviour measure. The indirect effect of the looking behaviour measure was nearly significant (β=0.028, s.e.=0.015, z=1.837, p=0.066), providing only suggestive evidence for partial mediation.

As in **Chapter 2**, to test whether the observed effects were specific to the social domain, the same model was tried using VABS Motor skills standard score as a measure of non-social adaptive skills at 3 years. However, the model optimizer could not find a solution after 1000 iterations. As the model did not converge, no fit measures and reliable estimates were provided.

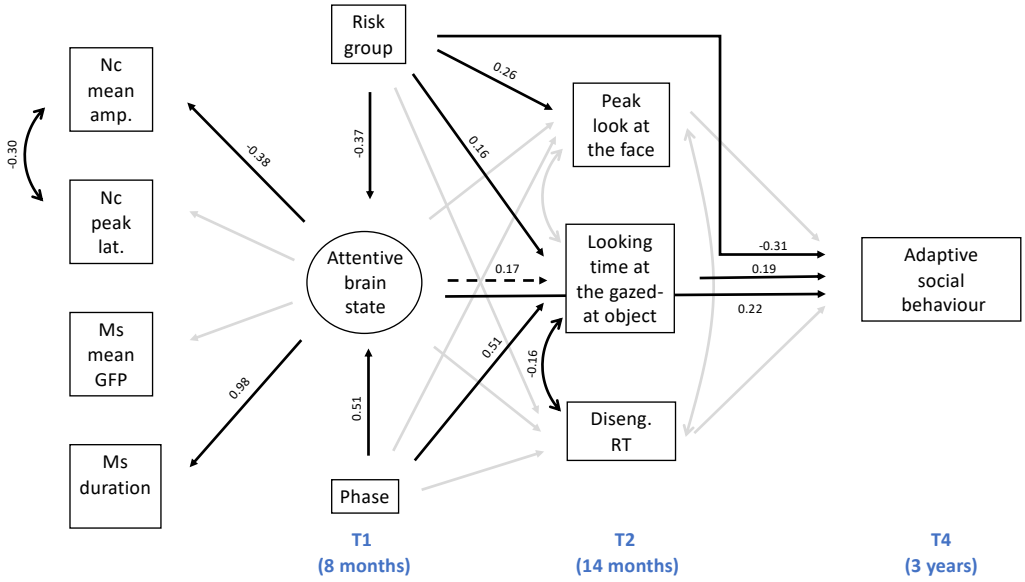

Figure 3.4 *Structural equation model predicting adaptive social behaviour* (VABS Soc.). Black arrows indicate significant relationships at p<0.05. Dashed arrows indicate the relationships that approached significance (p<0.1). Standardised betas are reported for these effects. Grey arrows indicate non-significant relationships.

3.3.1.2 Autistic traits

The SEM model evaluating the effect of experimental infant measures on autistic traits without controlling for the effect of risk group provided a discrete model fit, although significance of the chi-square statistics revealed that the estimates were dependent on the sample size ($\chi^2(21)=37.97$, p=0.013, CFI=0.992, RMSEA=0.053, 90% CI [0.024-0.079], AIC=2808.527, BIC=2955.921). A better fit was provided by the model including risk group ($\chi^2(24)=36.39$, p=0.050, CFI=0.994, RMSEA=0.044, 90% CI [0.000-0.071], AIC=2786.588, BIC=2955.038). This model revealed that the amount of looking time at the gazed-at object was negatively associated with both SCI and RRB, such that shorter looking times predicted more severe autistic traits (st.β=-0.15, p=0.018 and st.β=-0.16, p=0.015, respectively). In this model, the variance-covariance matrix was explained by a significant relationship between attentive brain state at

T1 and attention engagement with the gazed-at object at T2 (st.β=0.18, p=0.047). However, the neural measure did not predict social and non-social autistic traits at three years (st.β=0.12, p=0.285 and st.β=0.12, p=0.276, respectively). Significant covariances were observed between the following pairs of variables: Nc amplitude and latency (st.β=-0.31, p<0.001), SRS SCI and RRB scores (st.β=1, p<0.001) and disengagement and proportion of looking time at the gazed-at object (st.β=-0.16, p=0.043). Significant relationships as black solid lines and trends of association (p<0.1) as dashed lines are shown in **Figure 3.5**. All standardised and unstandardised estimates with robust standard errors are reported in **Table A3.3**.

The mediation model testing for the effect of attention engagement with the gazed-at object in the path between attentive brain state and autistic traits revealed that a direct effect was not observed between neural measure at T1 and dimensional outcome at T4 (SCI: β=0.072, s.e.=0.096, z=0.751, p=0.453, RRB: β=0.073, s.e.=0.096, z=0.761, p=0.446). However, indirect effects were also non-significant (SCI: β=-0.023, s.e.=0.017, z=-1.387, p=0.166, β=-0.024, s.e.=0.017, z=-1.398, p=0.162), therefore a mediation model was not supported by the present data.

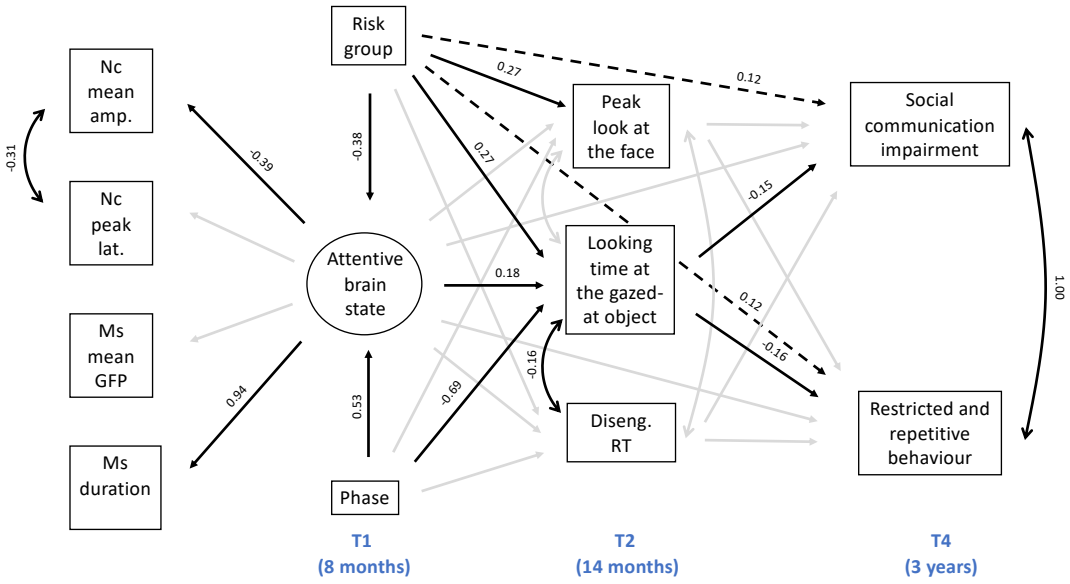

Figure 3.5 *Structural equation model predicting autistic traits* (SRS SCI and RRB). Black arrows indicate significant relationships at p<0.05. Dashed arrows indicate the relationships that approached significance (p<0.1). Standardised betas are reported for these effects. Grey arrows indicate non-significant relationships.

3.3.1.3 Language abilities

The model predicting language skills without accounting for the effect of risk ($\chi^2(27)=48.73$, p=0.006, CFI=0.95, RMSEA=0.055, 90% CI [0.029-0.079], AIC=4294.202, BIC=4452.124) fit the data less well than the model including risk group as a covariate, with exception of the BIC which was higher in the risk group model ($\chi^2(22)=32.33$, p=0.072, CFI=0.975, RMSEA=0.043, 90% CI [0.000-0.072], AIC=4290.008, BIC=4465.478).

The higher factor loading was observed again for Ms duration (st.β=0.99, p<0.001) and Nc mean amplitude (st.β=-0.38, p<0.001). The latter variable significantly covaried with Nc mean latency (st.β=-0.31, p<0.001). Receptive and expressive language variances at T4 showed highly significant covariance (st.β=0.72, p<0.001). The attentive brain state factor significantly predicted both receptive (st.β=0.26, p=0.023) and expressive language (st.β=0.27, p=0.03). Interestingly, peak look duration at the face was significantly associated with receptive language (st.β=-0.18, p=0.006) and, to a lesser extent, to expressive language (st.β=-0.14, p=0.06), such that shorter peak look durations predicted better language understanding. Trends towards significant relationships were observed between attention engagement to the gazed-at object and receptive (st.β=0.18, p=0.062) but not expressive language (st.β=0.14, p=0.11), and between disengagement and expressive (st.β=-0.10, p=0.083) but not receptive language (st.β=-0.05, p=0.429). Significant association between attentive brain state to FD versus Noise and looking time at the gazed-at object (st.β=0.19, p=0.018), and covariance between the latter variable and disengagement (st.β=-0.16, p=0.038) were observed, as in the other models. Significant paths are illustrated in **Figure 3.6**. All results, including standardised and unstandardised coefficients, are reported in **Table A3.4**.

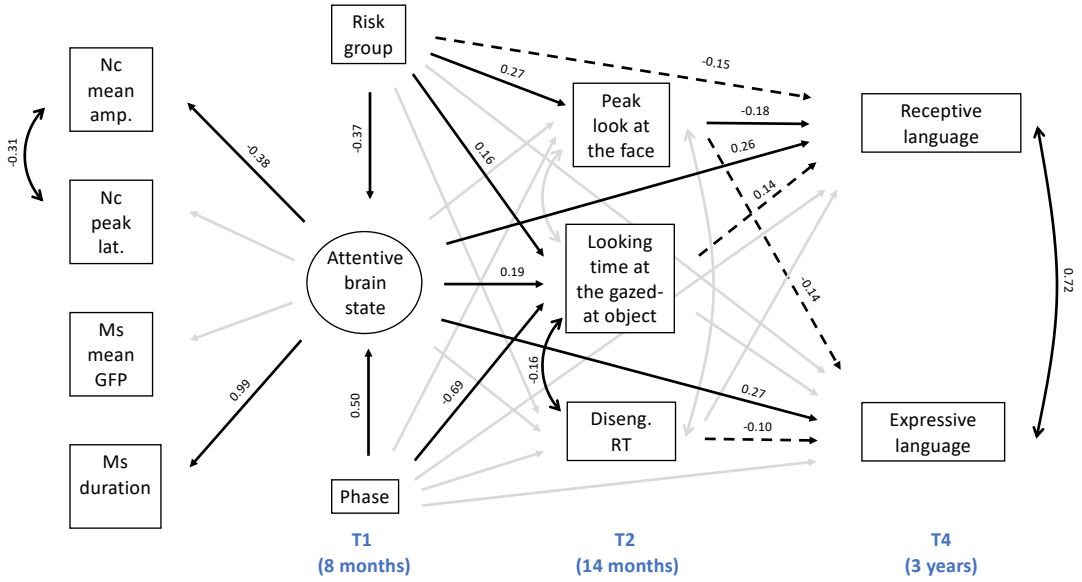

Figure 3.6 *Structural equation model predicting language abilities* (receptive and expressive language scores of the MSEL). Black arrows indicate significant relationships at p<0.05. Dashed arrows indicate the relationships that approached significance (p<0.1). Standardised betas are reported for these effects. Grey arrows indicate non-significant relationships.

3.3.1.4 Effortful control

Model fit was acceptable but influenced by sample size (significant chi-square statistics) when modelling the variance-covariance matrix for prediction of effortful control without introducing risk group as a covariate ($\chi^2(17)=44.87$, p=0.006, CFI=0.883, RMSEA=0.057, 90% CI [0.030-0.083], AIC=3787.190, BIC=3913.528). However, parameters for model fit evaluation improved, except the BIC, when controlling for risk group effect ($\chi^2(20)=27.96$, p=0.110, CFI=0.955, RMSEA=0.039, 90% CI [0.030-0.070], AIC=3779.640, BIC=3920.015). In this model too, differences between FD and Noise stimuli in Ms duration (st.β=0.99, p=0.002) and Nc mean amplitude (st.β=-0.37, p<0.001) were the highest contributors to the attentive brain state factor. Significant covariance was observed between Nc amplitude and latency (st.β=-0.30, p<0.001). Attentive brain state showed a trend for association with the executive function outcome at T3 (st.β=0.162, p=0.073). On the contrary, the looking behaviour phenotypes did not predict effortful control at T3 (peak look at the face: st.β=-0.07, p=0.437, looking time at the gazed-at object: st.β=0.12, p=0.117, disengagement: st.β=-0.02, p=0.751). In this model, attentive brain state significantly predicted looking time at the gazed-at object (st.β=0.17, p=0.039). A significant covariance between disengagement and looking time at the congruent object in the gaze following task was observed (st.β=-0.17, p=0.034). **Figure 3.7** illustrates significant paths for the estimated model. The model estimates and their significance can be found on **Table A3.5**.

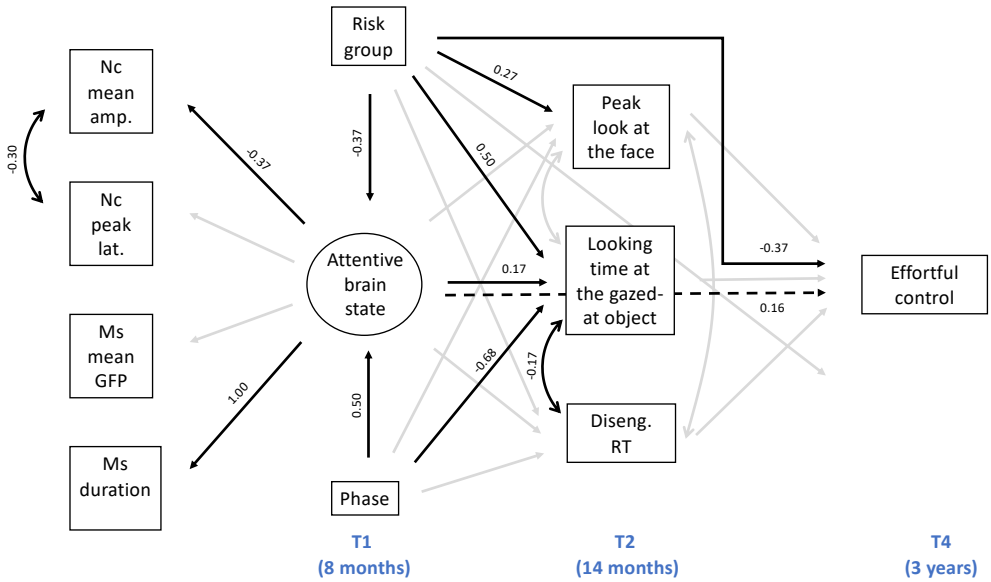

Figure 3.7 *Structural equation model predicting effortful control* (subscale of the ECBQ). Black arrows indicate significant relationships at p<0.05. Dashed arrows indicate the relationships that approached significance (p<0.1). Standardised betas are reported for these effects. Grey arrows indicate non-significant relationships.

3.3.2 Investigating the protective value of social attention

3.3.2.1 Protective effect of looking behaviour against neural vulnerability

To evaluate whether looking behaviour had a protective effect against brain vulnerability during social attention, I first computed a composite score between Ms duration and Nc mean amplitude, as previously explained (see **section 3.2.3**). I then divided the 'neural vulnerability composite' data for the entire sample in four quartiles, such that the first quantile included infants with reduced attention to faces than Noise and the fourth quartile included infants with more enhanced attention to the face. **Figure 3.8** illustrates the proportion of HR children in each neural vulnerability quartile for the three outcome groups.

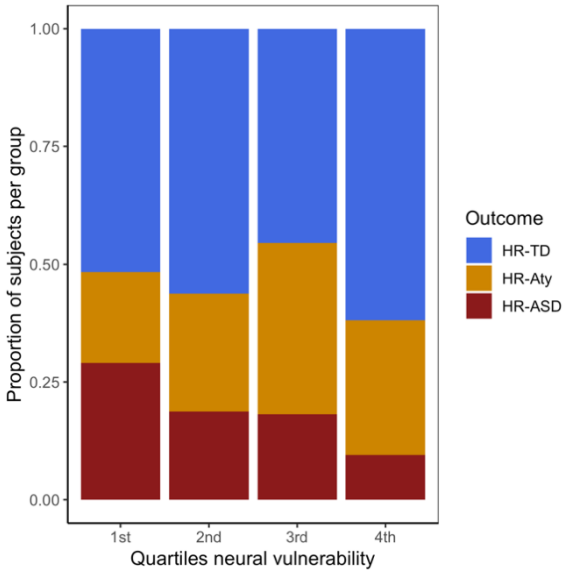

Figure 3.8 *Illustration of the proportion of HR children in each of the three outcome groups in the four quartiles of neural vulnerability*, estimated as the composite value of Nc mean amplitude and Ms duration difference between the FD and the Noise condition. The 1st quartile includes the infants with reduced attention engagement to faces with direct gaze than noise, while the 4th quartile includes the infants with enhanced neural responses to noise than to face (i.e. less social attention engagement). The blue part of the bars indicates the proportion of HR-TD children, the orange part of the bars indicates the proportion of HR-Aty children, the red part of the bars indicates the proportion of HR-ASD children.

When comparing HR children with and without ASD who showed reduced neural responses to the face compared to the non-social stimulus (high neural vulnerability) with those with enhanced neural responses to faces (low neural vulnerability), there was no significant effect of ASD group in peak look duration at the face ($F(1,74)=0.051$, $p=0.821$, $\eta^2=0.0001$) and looking time at the gazed-at object ($F(1,73)=2.773$, $p=0.100$, $\eta^2=0.097$). There was no effect of neural vulnerability on the peak look at the face ($F(1,74)=0.013$, $p=0.909$, $\eta^2=0.002$) while a significant effect was observed for the proportion of looking time at the gazed-at object in the gaze following task ($F(1,73)=9.946$, $p=0.002$, $\eta^2=0.001$). The interaction between outcome group and neural vulnerability was non-significant for both eye-tracking measures (peak-look at the face: $F(1,74)=0.046$, $p=0.831$, $\eta^2=0.0006$, looking time at the gazed-at object: $F(1,73)=0.064$, $p=0.802$, $\eta^2=0.0009$).

These analyses indicated that peak look duration at a static face stimulus during a face pop-out task at 14 months is not different between infants with and without later ASD, independently from their level of neural vulnerability defined as atypical brain responses to faces and non-social stimuli at 8 months. Differently, HR-ASD infants showed significantly reduced attention engagement with the gazed-at object compared to HR-noASD infants irrespective of neural vulnerability group. This indicated that looking behaviour during social attention at T2 did not

have a protective value for infants who showed more enhanced and prolonged neural responses when attending to faces than non-social stimuli at T1.

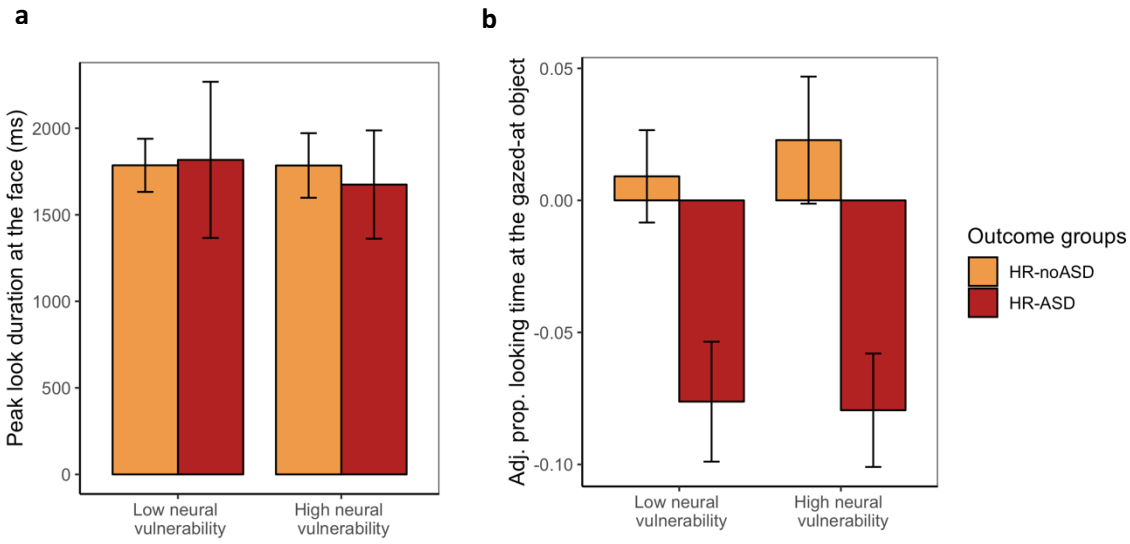

Figure 3.9 *Barplots indicating the interaction between neural vulnerability and ASD diagnosis in the HR children*. Differences in peak look duration at the face in the face pop-out task (**a**) and proportion of looking time at the gazed-at object in the gaze following task (**b**) are displayed by outcome group for HR infants considered at high and low neural vulnerability based on the difference in neural measures when looking at FD compared to Noise. In all plots, error bars represent standard errors.

3.3.2.2 Sex-specific protective effect of looking behaviour against familial/genetic risk

Another way to uncover potential protective factors is to compare individuals at risk who have 'better than expected outcome' with individuals with no risk factors (Szatmari, 2018). In this analysis, I examined whether exceptional attention in social contexts at 14 months was observed in infants at high risk who did not develop the core symptoms of ASD at three years (HR-TD and HR-Aty), compared to LR children. This effect was tested in interaction with sex, to uncover the presence of sex-specific mechanisms.

When examining the peak look duration at the face, I found a significant effect of group ($F(1,170)=6.207$, $p=0.003$, $\eta^2=0.048$). Post-hoc analyses revealed that HR-Aty ($p=0.004$) and HR-TD ($p=0.019$) stared for longer at the face than LR infants. No difference was observed between the two HR groups ($p=0.576$). There was no effect of sex ($F(1,170)=0.004$ $p=0.950$, $\eta^2=0.0002$) nor interaction between sex and group ($F(1,170)=1.062$ $p=0.348$, $\eta^2=0.012$), not confirming the idea of a protective value of this looking behaviour for girls at high familial risk for ASD. The effect of group on the proportion of looking time at the gazed-at object was non-significant ($F(1,163)=2.363$, $p=0.126$, $\eta^2=0.059$). There was a main effect of sex ($F(1,163)=2.363$, $p=0.126$,

η^2<0.0001) but no significant interaction between group and sex (F(1,163)=1.341, p=0.264, η^2=0.016).

In order to control whether results were influenced by lower cognitive abilities in the HR-Aty group, I performed the same analyses introducing MSEL composite score at T2 as covariate. The same pattern of result was observed, with a main effect of outcome for the peak look at the face (F(1,167)=6.178, p=0.002, η^2=0.047) and not for looking time at the gazed-at object (F(1,159)=1.977, p=0.142, η^2=0.063), and no effect of sex or interaction between outcome and sex (all p*s*>0.115, all η^2 <0.017). The relationship between cognitive abilities and behavioural correlates of social attention was not significant for the face pop-out (F(1,167)=0.006, p=0.938, η^2<0.0001) and approaching significance for gaze following measure (F(1,159)=3.377, p=0.068, η^2=0.021).

In sum, these analyses revealed that longer peak look durations at a static image of a face in a face pop-out task were associated with risk for ASD such that they are observed in infants who did not show core ASD symptoms at three years, differently from the LR group. Looking time at the object another person is gazing at seemed to be an early marker of ASD specifically. In fact, individuals at risk who underwent a typical developmental trajectory did show higher engagement to the gazed-at object compared to low-risk infants (see **Figure 3.10b**), but this effect was not significant probably due to the large standard errors. Overall, I found no evidence for sex differences in social attention measures suggesting a different resilience mechanism for male and female infants at high risk for ASD.

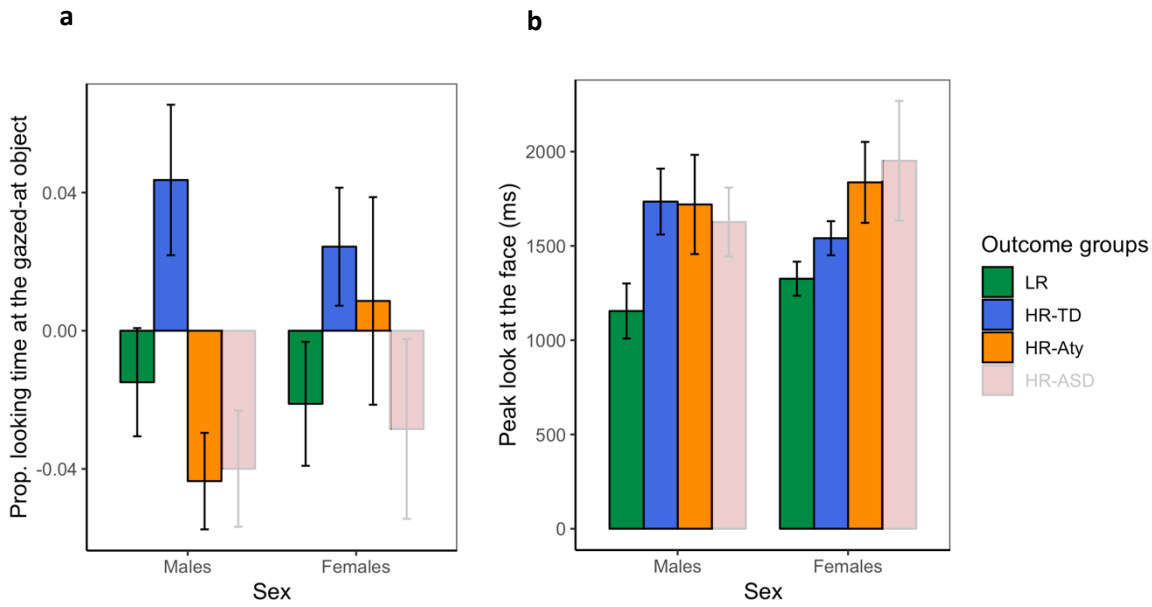

Figure 3.10 *Barplots indicating the interaction between sex and outcome group for the two eye-tracking measures of social attention*. Differences in peak look duration at the face in the face pop-out task (**a**) and proportion of looking time at the gazed-at object in the gaze following task, adjusted for the effect of phase, (**b**) are displayed for 14-month-old boys and girls at low familial risk (LR, in green), high familial risk for ASD with typical development at 3 years (HR-TD, blue) and high familial risk who showed features of atypical development at 3 years but no core ASD symptoms (HR-Aty, in orange). For comparison, mean scores for boys and girls with emerging ASD, not included in this analysis, are shown in transparent red. Error bars represent standard errors.

3.4 DISCUSSION

In this chapter, a series of analyses was conducted in order to understand the relationship between neural and behavioural measures previously indicated as early signs of atypical developmental trajectory in children at familial risk for ASD. In **Chapter 2** I identified correlates of atypical engagement of attentive brain states in response to faces with direct gaze versus non-social control stimuli (Noise) at around 8 months of age, which have been found to be predictive of later ASD in the HR group. I built on these results to explore the possibility that these neural atypicalities, possibly disrupting the development of neural circuits devoted to attention in general and social attention specifically, might lead to atypical looking behaviour at 14 months of age, which in turn has been proposed to precede the onset of social and cognitive difficulties associated with neurodevelopmental disorders. I used SEM to understand the possible role of prolonged peak look duration at face stimuli in a face pop-out task, reduced looking times at the gazed-at object in a gaze following task, and difficulties in disengagement from a central stimulus in developmental trajectories observed on 247 infants who participated

in BASIS Phase 1 and 2. I tested four different pathways to examine trajectories to four dimensional outcomes: social adaptive behaviour, autistic traits and language abilities at three years and effortful control at two years. Early attentive brain states were predictive of socialization difficulties but also other domains of the ASD phenotypes such as language and, to a lesser extent, effortful control. This suggests that early neural responses indicating reduced engagement when attending to faces and enhanced engagement to non-social visual stimuli are likely to reflect domain-general deficits rather than disruptions exclusively in the social domain. No evidence for a relationship between attentive brain state and peak look duration at the face or disengagement was found. Those two measures of atypical looking behaviour were associated with language skills at age 3 independently from the factor representing neural correlates of social attention. These findings might indicate that they do not play a major role in the path to ASD social traits. On the contrary, suggestive evidence for a partial mediation effect of the gaze following measure on the relationship between early attentive brain states to FD versus Noise and later social adaptive skills was observed. Moreover, this early marker seemed to be mainly associated with both social and non-social autistic traits. Thus, early disruptions in responding to joint attention might play a role in the path towards ASD symptoms and could be candidate targets for intervention.

A further investigation was conducted on the same measures to test the possible role of different aspects of looking behaviour as protective factors against brain vulnerability. I found no suggestive evidence for this hypothesis, although the analyses should be considered as preliminary given that they were limited by unbalanced group sizes. Last, the present study revealed no evidence for sex-specific mechanisms of resilience involving exceptionally high visual social attention behaviour protecting 14-month-olds at high genetic vulnerability from developing core ASD symptoms. However, descriptively I observed that this early marker might reflect protective mechanisms for HR siblings who have a typical outcome at three years.

3.4.1 Disengagement

A measure of disengagement was included in the models, although not directly reflecting social attention behaviour, as it has been argued that atypicalities in non-social aspects of visual orienting might result from early disruptions in the brain and contribute to the emergence of social traits (Piven et al., 2017). The observed lack of association between disengagement at T2 and neural correlates of attention at T1 might reflect the fact that individual differences in 'sticky fixation' style started to emerge earlier and independently from the inefficiencies in face processing reflected by the attentive brain state factor. Interestingly, disengagement during the

second year of life was not associated with later core ASD symptoms, in line with Blaga & Colombo (2006), who argued that after sixth month of age individual characteristics in disengagement contribute less to individual variability in cognitive skills. Of note, the fact that differences in disengagement were not associated with risk status nor were found to be specifically linked with later autistic traits suggests that individual differences in disengagement might not necessarily be causally linked to genetic ASD risk factors and ASD outcome. Instead, they might be farther from the biological causes of the neurodevelopmental disorder, and emerge instead as adaptive behaviour in response to specific environmental demands (Johnson, 2017).

It is also possible that attention resources recruited in the BASIS EEG task are different from those triggering disengagement difficulties. In fact, the ability to disengage from a central stimulus has been attributed to the dorsal stream in Posner's posterior attentional network (Colombo & Cheatham, 2006), which includes the intraparietal sulcus/superior parietal lobe and frontal eye fields (Petersen & Posner, 2012). While these circuits are necessarily involved in the orienting component of social attention (Klein et al., 2009), they might not have been recruited in the EEG Face/Noise paradigm, where a central stimulus was always presented in the same location.

In the SEM models, disengagement significantly covaried with looking time at the gazed-at object recorded during the gaze following task, such that more difficulties in disengagement were associated with less attention engagement with the referent object. This result is in line with Mundy & Newell (2009), who suggested that difficulties in disengagement might interfere with social orienting with cascading effects on joint attention and consequently social cognition. The present SEM results are in apparent disagreement with Bedford, Pickles, Gliga, Elsabbagh, Charman, & Johnson (2014), who claimed that the disengagement and gaze following measures independently and additively predicted ASD (of note, 84 of the 247 infants in the present study provided data for Bedford et al., 2014 too). Methodological choices can explain such differences. Bedford and colleagues' assumption of independent contribution was made a priori after verifying that the two variables were not significantly correlated (rho=-0.02 in LR and rho=-0.12 in HR-ASD). Correlation between these variables was comparable in the present study (rho=-0.11 in the entire group). However, the covariance of these two variables measured in the SEMs evaluated here was measured as correlation of the two scaled variables times the product of the variables' standard deviations (Hox & Bechger, 1998). A significant relationship indicated that the amount of individual variability in looking behaviour, after controlling for the other variables in the model, was correlated.

Given that the attentional engagement in the gaze following task was measured as proportion of looking time at the gazed-at object, staring for longer at the centre of the screen or at any other stimulus might indeed have caused a reduction of looking time to the target location. In everyday-life social context, infants who require longer latencies to disengage from one focus of attention might miss opportunities for learning about the world (Csibra & Gergely, 2011). In line with this hypothesis, the SEM revealed that there was a trend towards association between disengagement and expressive language, such that disengagement difficulties predicted lower abilities in speaking, language formation and verbal conceptualization.

Interestingly, Baranek et al. (2018) recently showed that the pathway between attention disengagement at 13 months and social orienting at 20-24 months is mediated by sensory seeking. The results of the present chapter are in agreement with the idea that infants who show a 'sticky fixation' style might be more prone to devote their resources to sensory stimulations and less open to engage in interactions with other people (Baranek et al., 2018).

3.4.2 Looking behaviour and effortful control

As reviewed in the Introduction of this chapter (**section 3.1.1**), signs of atypical looking behaviour have been proposed to play a role in effortful control (Hendry et al., 2018; Keehn et al., 2013). Among those, difficulties in disengagement and excessively long peak look durations when orienting towards a static face stimulus in a face pop-out task have been tested in the present study. Keehn, Müller, & Townsend (2013) suggested that early inefficiencies in regulating orienting responses in children who later receive a diagnosis of ASD, including difficulties in disengaging from a central stimulus to shift the gaze to another target, may impact the development of later executive control processes. The SEM tested in the present study does not support this view, as no association was found between disengagement at 14 months of age and effortful control at 2 years of age. However, it would be interesting to examine whether a relationship exists between disengagement and effortful control at three years of age, when this aspect of executive function might be more mature (Rothbart & Rueda, 2009).

Peak look duration at the face stimulus in the face pop-out task was also expected to be associated with later scores of the ECBQ domain effortful control based on Hendry et al. (2018). Indeed, the association found by Hendry et al. (2018) used latent change from the first to the second year of life as predictor, while in the present study peak look duration measured at 14 months of age was used. The choice of using the measure at T2 instead of latent change between T1 and T2 as predictor was done to obtain a coherent asset of early behavioural signs, and be

able to evaluate the various aspects of looking behaviours obtained at the same age. Additionally, it has been argued that looking time in the first and second year of life might actually reflect different underlying cognitive processes (Colombo & Cheatham, 2006), therefore measuring the change between 8 and 14 months would have required an additional level of interpretation. Another difference between the present study and Hendry et al. (2018) is that in the present work effortful control was measured at 2 instead of 3 years. In typical development, effortful control emerges specifically during the third year of life (Rothbart & Rueda, 2009), therefore measuring it at 2 years might not have allowed us to detect stable individual differences. Further, the executive function measure for the present study relies on a construct validated from of a temperament questionnaire, the ECBQ, designed for toddlers from 1.5 to 3 years (Putnam et al., 2006). Differently, Hendry et al. (2018) used a composite score from selected items from the CBQ, designed to capture differences in temperament from 3 to 8 years (Putnam & Rothbart, 2006). The CBQ was not available for participants non overlapping between the two studies (N=105 of the 247). This made it not appropriate for the SEM analysis, where a sample of at least 200 individuals is warranted (Kline, 2016).

3.4.3 Risk and peak look durations

In line with Hendry et al. (2018), no association was found between peak look duration to faces in infancy and later autistic traits measured with the SRS. However, a significant association between peak look duration at the face at T2 and language skills, and especially receptive language, at T4 was found. Webb et al. (2010) previously reported that longer looking times at the face stimulus in a habituation task predicted poorer verbal developmental scores in a sample of 18- to 30-month-old toddlers which included: children with ASD, HR children with typical development, children with developmental delay and typically developing controls. Importantly, longer looking times at the face in the face pop-out task were previously found to be atypical in the HR infants as a group, and not particularly so in those who received an ASD diagnosis at age 3 (Hendry et al., 2018). In the present cohort, partly overlapping with Hendry et al. (2018), I also found that HR infants who did not develop core ASD symptoms show atypically longer looking time at the face compared with LR 14-month-olds. Thus, this metric cannot be considered to have protective value against ASD, rather it seems to be closely linked with familial/genetic risk.

Lewis et al. (2017) recently conducted an fMRI prospective longitudinal study evaluating network inefficiencies in infants at risk for ASD from 6 to 12 months of age, and related those to outcome at 24 months. They found that brain network inefficiencies observed in HR infants, involving initially regions for auditory processing, included by 12 months additional areas such

as visual, somatosensory and motor areas, and regions involved in sensory integration and more abstract aspects of language processing (e.g., Broca's and Wernicke's areas) (Lewis et al., 2017). In the present study, the SEM revealed that peak look duration to faces in the face pop-out task was not predicted by earlier engagement in attentive brain states when processing social stimuli. It is possible that longer peak look duration at the social stimulus emerges as a sign of the low-level visual and sensory integration network inefficiencies identified by Lewis et al. (2017), rather than being associated to neural atypicalities during endogenous attention allocation. If integration of the sensory information with areas for language processing is disrupted in 1-year-old infants at high genetic liability for neurodevelopmental disorders, cascading effects might be observed on the acquisition of communication rather than social skills.

3.4.4 Eye-gaze

The present data support the idea of a developmental pathway where cascading effects of atypical attentive brain state in response to faces on social learning might be partially mediated by limited attention engagement in the social situations aimed at sharing information through gaze (and head) direction cues. Being able to follow the other person's gaze as communicative cue is a fundamental skills for joint attention, which requires the coordination of one's own attention with that of another person to share information (Mundy, 2018). As explained in **section 3.1.1**, the infants' ability to follow the direction of the gaze of others in order to share a common point of reference is referred to as "responding to joint attention". This ability is fully developed at 9 months of age (Senju, Csibra, & Johnson, 2008) and serves as self-organizing role in social information processing in early, unstructured social-learning situations (Mundy & Newell, 2009).

A significant relationship between attentive brain state in response to FD versus Noise at T1 and the amount of time spent looking at the gazed-at object at T2 resulted in all SEMs. This might suggest that higher and prolonged attention engagement to the face stimulus in the first year of life predicts higher attention engagement with the object cued by another person in the second year. The SEM analysing the relationship between these early signs of social attention engagement and social adaptive skills at 3 years of age revealed that the mediation effect of the gaze following measure was nearly significant. Moreover, the SEM using SRS domains as dimensional outcome revealed that a total effect of the relationship between early neural measures and autistic traits was no longer observed, as it was replaced by the indirect effect

through the eye-tracking metric at 14 months (although the indirect effect was non-significant too, probably due to variability and relatively small sample size with complete measures).

As mentioned in the introduction of this chapter (**section 3.1.1**), Elsabbagh, Mercure, et al. (2012) found that 6 to 10-month-old infants with later ASD showed reduced neural sensitivity to eye-gaze shifts measured 400 ms after the stimulus onset (P400 ERP component). Interestingly, the attentive microstate contributing to the neural correlate of social attention factor in the present study is characterised by posterior positivity in the scalp field after 300 ms from the onset of a face with direct gaze. Similarly, Nyström et al. (2017) used eye-tracking to reveal that infants at familial risk for ASD showed reduced looking time at the interacting person between 300 to 1000 ms after the she performed a gaze shift, which corresponds to the P400/Nc/microstate time window used in **Chapter 2**. Thus, both the neural and the behavioural correlate of social attention engagement seem to reflect information processing occurring from 300 ms after the visual stimulus presentation.

One possible explanation for the observed pathway is that atypicalities in perceptual processing of social information at 8 months might lead to a missed opportunity to tune specific 'social brain' circuits during critical periods for interactive specialization (Jones et al., 2015). Importantly, the SEM examining the path towards SCI and RRB revealed that the effect of attention engagement with the gazed-at object was not specific to the social domain. While covariance estimates suggested that the two scales of the SRS were not very sensitive in the present dataset (st.β=1, p<0.0001), this finding, together with the non-significant mediation models, does not allow me to conclude that I found evidence for the 'social first' account. However, the role of the gaze following measure as *predictor of* ASD symptoms and *predicted by* early atypicalities in attentive brain states suggests that responding to joint attention is a candidate antecedent of ASD (Johnson et al., 2014) and therefore possible target for early intervention (Mundy, 2018).

The importance of components of social attention in the path to ASD is confirmed by the two analyses on the possible protective value of looking behaviour. Among the infants with increased neural vulnerability at T1, enhanced attention to the gazed-at object, though non-significantly, was found in HR-noASD infants compared with HR-ASD (**Figure 3.9b**). This suggestive evidence for a mechanism of resilience is also supported by descriptive observation that HR-TD infants showed prolonged looking time at the gazed-at object compared to LR infants (**Figure 3.10b**).

In a sample partly overlapping with the one used in the present study (N=104 of the 247 participants), Bedford et al. (2016) found that attention engagement with the gazed-at object at 14 months, as well as AOSI scores and latency of disengagement, were predictive of ASD severity in boys but not in girls. In the present study, engagement with the gazed-at object during a gaze following task was descriptively observed in boys who showed later signs of atypical development, including high ADOS scores (see **Figure 3.10b**). Although HR females showed on average more social attention engagement than the LR group, in line with the hypothesis of a sex-specific protective effect (Chawarska et al., 2016), these results are not significant due to large standard errors. Thus, differently from Chawarska et al. (2016), the present study did not provide strong evidence for a sex-specific protective effect of social attention skills, which would have been revealed by enhanced attention engagement to the gazed-at object in HR girls compared to boys. One difference between the present study design and Chawarska and collaborators' design is that their key correlate of social attention was looking time when fixating the face of an interacting adult. On the contrary, in the present study the key measure of social attention was looking time at the referent object. Additionally, participants were younger in Chawarska et al. (2016) and the main effects were observed at 6 and 9 months but not at 12 months. Their results are not incompatible with those illustrated in the present study and might reflect age-sensitive sex-specific mechanisms underlying developmental trajectories of gaze following and joint attention.

In sum, the present findings allow me to conclude that the gaze following task represents an early marker of familial/genetic susceptibility for neurodevelopmental disorders, as lower values were observed in HR-ASD and HR-Aty boys with signs of atypical development, possibly reflecting an enhanced burden of risk factors for disruptions of neurodevelopment (Charman et al., 2017). The question whether sex-specific factors which moderate the developmental effects of this early marker are genetic will partly be addressed in the next chapters.

3.4.5 Limitations and future directions

The present chapter aimed to understand the relationship between different experimental measures which have been used to characterise profiles of attention and processing of social and non-social stimuli in infants with a familial history of ASD and low-risk controls. With a series of analyses, I explored the association between variables derived from eye-tracking and EEG tasks, obtained in two subsequent waves (i.e., Phases) of data collection of the British Autism Study of Infant Siblings, and dimensional measures of cognitive and social skills obtained with parent-report questionnaires and standardised assessments. As described, some of the

experimental paradigms were modified after Phase 1, reducing the comparability of the datasets. In fact, the results of this chapter showed that the gaze-following metric (proportion of looking time at the gazed-at object) was significantly different between Phase 1 and 2. Even when a non-significant difference between Phases emerged, changes in the experimental procedure and pre-processing pipeline of the data (which affected all eye-tracking tasks), the use of different devices and changes in the researchers who were responsible for data collection and processing (necessarily happening in a relatively large longitudinal study such as BASIS) might have affected the present results. In future studies, selecting variables that are homogeneous in terms of their characteristics of data collection, processing and distribution is recommended to eliminate sources of bias that are difficult to ascertain.

Another data-driven limitation of the current study emerged when considering the relatively low correlation between experimental measures that were theoretically expected to load onto a unique factor. For example, the original expectation for the SEMs was that the three eye-tracking measures collected at T2 could be constrained to load into a unique factor representing "looking behaviour at 14 months". However, exploratory factor analyses, correlation analyses (see **Figure 3.3** and **Table A3.1**) and associations with behavioural measures (AOSI items, see **Figure A3.1**) tested beforehand revealed that these measures could not be loaded onto a common factor. Although this does not represent a statistical issue for the SEMs, where the three measures were considered as separate variables, it raises important questions related to the interpretation and validity of eye-tracking measures, as well as on the reproducibility of results in an independent cohort.

Combining longitudinal data collected from infants at high and low familial risk for ASD to obtain larger datasets allow researchers to use sophisticated statistical tools such as SEM to understand the direction of association between measures, and possibly advance the current knowledge on the causal role of early neural response and looking behaviour for the development of psychological traits. A finding that consistently emerged from this study is that there was a significant association between risk group and experimental measures collected at T1 and T2 and often an association between risk and outcome. In larger samples, it would be interesting to specifically test whether the observed pathways can be observed in separate samples of LR and HR infants, in order to verify whether the developmental mechanisms involving social attention observed in this study are the same for the two risk groups. Quantifying 'risk' with continuous measures of environmental (i.e., parental sensitivity during interaction, parental stress, parental education or other measures of socio-economic status…) and/or genetic (i.e., polygenic score, CNV rate…) factors would be an interesting avenue to specifically test a mediation effects linking early social attention, familial risk and outcome.

3.5 SUMMARY OF FINDINGS

This chapter examined the relationship between early neural correlates of attention engagement to faces with direct gaze versus non-faces, looking behaviour at the beginning of the second year and socio-cognitive outcomes at 2-3 years of age. Neural response during attention engagement predicted enhanced engagement with the gazed-at object, which in turn predicted better social adaptive behaviours in early childhood. Peak look duration at the face was predictive of later language skills and could reflect an adaptive response to the environment from systems which show processing inefficiencies associated with increased familial/genetic liability for neurodevelopmental disorders. The extent to which these early signs of atypicality, and social attention in general, depend on the genetic and familial risk loading will be the focus of **Chapter 4** and **5**.

CHAPTER 4

FAMILIAL AND GENETIC RISK FOR ATYPICAL SOCIAL ATTENTION

4.1 INTRODUCTION

In the previous chapters I examined the evidence for atypical neural and behavioural correlates of social attention to precede the onset of autistic traits. Results of **Chapter 2** revealed that in infant siblings of children with ASD reduced neural responses during attention to faces with direct gaze and enhanced responses when attending to non-social stimuli preceded the onset of difficulties in social adaptive skills. In **Chapter 3** I found that these neural atypicalities predicted atypical looking behaviour at 14 months, especially reflecting reduced engagement with the object of shared attention in a gaze following task, which in turn is associated with later autistic symptoms. Another atypical looking behaviour, like the tendency to stare at static images of faces in a face pop-out array, predicted communication difficulties. These results indicated that early social attention skills are involved in the developmental path to later socialization abilities and that social attention might lie in the steps between ASD risk factors and the emergence of the disorder. As such, measures of social attention skills could be considered candidate endophenotypes of ASD (see **section 1.3**). An endophenotype is a marker which is linked with the biology of the disease and it is associated with genetic risk for the disorder (Gottesman & Gould, 2003). To validate candidate markers it is important to look at structural genetics and at whether the candidate marker runs in families (Iacono, Malone, & Vrieze, 2017).

If social attention is on causal path to ASD, it should show association with familial liability and genetic risk scores. In the present chapter, I evaluate evidence for this hypothesis by observing the relationship between social attention, social difficulties and polygenic score for ASD within families at low and high familial risk for ASD. If common genetic mechanisms are responsible for ASD and social attention, we should expect atypical social attention skills to be observed at an increased rate in family members of children with ASD, as part of the Broader Autism Phenotype (BAP, see **section 1.2.2**).

4.1.1 Familial influences on ASD traits

In **Chapter 1** I reviewed the literature indicating that family members of individuals with ASD are more likely to show sub-threshold symptoms of ASD, especially in the social domain (Pickles et al., 2000; Piven, Palmer, Jacobi, Childress, & Arndt, 1997). The presence of characteristics similar to ASD but less severe in relatives of people with ASD has been conceptualized as BAP (see Pisula & Ziegart-Sadowska, 2015 for a review). Research has consistently shown that autistic traits often co-occur in parents and offspring of individuals with ASD and that the presence of BAP in both parents increases the probability of ASD in the child (Constantino & Todd, 2005; Rubenstein et al., 2019; Xie et al., 2019). Furthermore, BAP is often observed in fathers of individuals with ASD (Losh, Childress, Lam, & Piven, 2008; Lyall et al., 2014; Wheelwright, Auyeung, Allison, & Baron-Cohen, 2010), and an association between mothers' difficulties in imagination and attention switching and their children's ASD traits has been reported in a Japanese population (Hasegawa et al., 2014).

Insights into the possible mechanisms underlying BAP came from the observation of differences in BAP manifestations between relatives at high-familial risk (HR) belonging to single- and multiple-incidence families. In single-incidence or simplex families (hereafter sHR), only one of the family members have been diagnosed with ASD, while in multiple-incidence or multiplex families (mHR) more than one member have ASD (Piven et al., 1997). As reviewed in **section 1.2.2**, studies have consistently found that members of mHR families have increased liability for lower social motivation and language skills compared to members of sHR families (Frazier, Youngstrom, Hardan, Georgiades, & Constantino, 2015; Gerdts, Bernier, Dawson, & Estes, 2013; Losh et al., 2008). A different genetic architecture is thought to identify ASD risk in sHR and mHR families, whereby an increased burden of rare pathogenic variants has been associated with ASD in sHR while mHR individuals share genetic predispositions based on a multifactorial etiology of common and rare variation (see **section 1.2.1**). These findings suggest that BAP might reflect different degrees of genetic burden for neurodevelopmental disorders due to inherited rare and common DNA variations. This hypothesis has been partly verified by molecular genetic studies: Specific inherited common genetic variants have been found to explain part of the variability in autistic traits in mHR families (Lowe, Werling, Constantino, Cantor, & Geschwind, 2015). On the other hand, aggregate effects of common genetic risk for ASD accounts for variation in sub-threshold ASD traits in the general population (Robinson et al., 2016). Interestingly, recent work suggests that the effect of ASD genetic risk factors on social and communication is higher in childhood than at later ages (St Pourcain et al., 2018). Taken together these findings point

towards the idea that an increased genetic burden for ASD inherited by mHR offspring might contribute to shape early development of social and communication skills

Two aspects of this account have not been deeply investigated, to my knowledge. First, patterns of familial associations can be due to genetic heritability, within-family environmental effect, and their interaction (Xie et al., 2019). The increased risk for atypical neurodevelopment in mHR family members might be also largely explained by non-genetic (environmental) factors, differently from the general population. Second, little is still known about what developmental mechanisms might underpin the relationship between genetic loading and shifts in the continuum of socio-communication traits towards the pathological end. In this chapter I combined behavioral measures of familial liability with estimates of genetic burden in families considered at different degrees of familial risk for ASD (low-risk, simplex and multiplex). In the previous chapter I found that atypical looking behaviour during social attention, especially in gaze following tasks, precedes the development of social difficulties. Verifying whether social attention skills are part of BAP would allow to make a step further in evaluating whether common genetic factors underlie autistic traits and social attention.

4.1.2 Social attention as BAP

Consistent with the idea of social attention as part of the BAP, previous studies as well as **Chapters 2** and **3** of this thesis have explored atypical social attention skills in infant siblings of children with ASD (Jones, Gliga, Bedford, Charman, & Johnson, 2014; Szatmari et al., 2016). In **Chapter 2** I found that HR infants with typical development at age 3 showed reduced attention engagement with the social versus non-social stimulus at the neural level by 8 months of age, although this pattern was less evident than in HR infants with emerging ASD. In **Chapter 3**, I showed that longer peak look duration when looking at the face in a face pop-out task is found in HR siblings who did not receive diagnosis of ASD at three (see also Hendry et al., 2018). Additionally, gaze-following skills were atypical at 14 months in HR male siblings with later atypical development but no ASD features, compared HR girls without ASD and low-risk (LR) controls (see also Bedford et al., 2012). These findings add to the evidence that some features of visual social attention might be directly reflecting increased genetic risk in the child or an indirect effect of parental genotype (Kong et al., 2018).

Other research tested the hypothesis that social attention, like autistic traits, is atypical in non-affected family members of people with ASD. Small studies investigating face processing abilities

during visual attention in family members of people with ASD provided preliminary evidence for social attention as a feature of BAP. For example, Wallace et al. (2010) tested 26 adults with ASD, 22 relatives (parents and adult siblings) from mHR families and 26 LR controls in a similar task testing discrimination of eye-gaze versus arrows directional cues as well as a facial expression versus object recognition task. They found that relatives performed worse than LR and better than ASD individuals in face recognition. Additionally, relatives as well as ASD cases did not show advantage for direction detection with direct compared to averted gaze, differently from LR controls (Wallace, Sebastian, Pellicano, Parr, & Bailey, 2010). Scheeren & Stauder (2008) showed that HR fathers (N=12) had decreased accuracy and slower reaction times when they had to evaluate eye-gaze direction, compared to LR fathers (N=14). No difference in processing the social cue was found between HR (N=13) and LR mothers (N=15) (Scheeren & Stauder, 2008). Accordingly, a study conducted by Adolphs, Spezio, Parlier, & Piven (2008) tested 15 HR parents considered "socially aloof" (BAP+) based on the Modified Personality Assessment Schedule-Revised (Piven et al., 1997), 27 "non-aloof" (BAP-) HR parents and 20 LR parents, in a face processing task in which participants were required to decide whether a partially hidden face was happy or fearful. Results revealed that the three groups had similar accuracy and reaction times, but they showed differences in their performance strategies. In fact, BAP+ parents showed a significant reduction of processing of the eyes region of faces, while BAP- and LR parents showed a substantial use of the eyes region. Also, BAP+ parents made more use of the mouth and less use of the eyes than the BAP- (Adolphs et al., 2008). These findings suggest that different aspects of face processing might be part of the BAP as they are atypical in HR family members of people with ASD.

Preliminary insights on the neural mechanisms underlying face processing difficulties in relatives of people with ASD are provided by Dalton et al. (2007), who used fMRI to investigate brain activation and eye-tracking to assess looking behaviour during a face recognition task. They compared 9 individuals with ASD, 9 unaffected siblings and 9 controls, all males, matched for IQ and age. They argued that reduced brain activation involved in face perception (right fusiform gyrus) as well as reduced visual engagement with the eyes region of the face might be responsible for atypical social attention in unaffected HR siblings (Dalton, Nacewicz, Alexander, & Davidson, 2007).

To understand whether increased genetic loading might be responsible for the social attention atypicalities, Oerlemans et al. (2015) compared affected and unaffected individuals from sHR and mHR families, assuming that sHR-mHR stratification identifies forms of ASD with a different genetic architecture. The within-family discrepancy between proband and unaffected sibling in identification of facial emotions and face recognition was larger for sHR than for mHR families.

On the contrary, there was no difference between groups in visual working memory, task switching and inhibition, suggesting that the main areas of difficulty for the HR groups were more related to social attention than to executive function (Oerlemans et al., 2015)**.** Further evidence for a genetic contribution to social attention and face processing performances comes from a study from Skuse and colleagues (2014), who tested the association between oxytocin-receptor gene (OXTR) polymorphisms and heritable social abilities. They used the Scales for Assessment of Social Intelligence (SASI) computerised tasks (Skuse, Lawrence, & Tang, 2005) to test face recognition memory, eye-gaze direction detection and facial emotion recognition in 112 families of people with ASD (N=340). In this familial sample, four OXTR SNPs (rs2301261, rs9860869, rs9878427, rs17049544) were nominally associated with performance in eye-gaze direction detection (Skuse et al., 2014).

Thus, there is suggestive evidence that some aspects of social attention are also impaired in non-affected family members of individuals with ASD. This could indicate that there are indeed genetic influences on social attention skills as there are in autistic traits. It does not clarify whether social attention is involved in a developmental path, though. Observing the relationship between parental characteristics and their children's social attention since infancy might be informative on whether BAP features emerge early on as atypicalities of attention in social contexts.

4.1.3 Familial influences on developmental trajectories

Genetic factors largely contribute to early looking behavior when attending to social stimuli (Constantino et al., 2017). Social attention performances in the first years of life are very dynamic and in critical periods might play crucial roles for shaping individual developmental trajectory (Klin, Shultz, & Jones, 2015). For example, in **Chapter 3** I exposed the hypotheses that some features of infant looking behavior might have a protective value against ASD (Chawarska, Macari, Powell, DiNicola, & Shic, 2016). Understanding whether early social attention atypicalities covary with familial risk for ASD is crucial to clarify the causal path between genetic factors and the development of social skills.

A relationship between parents' BAP and offspring's early neurocognitive profiles have been reported before**.** Jones and collaborators (2017) showed that brain activity underpinning social attention skills in infancy is associated with highly heritable ASD-related traits of the parents in the general population. Typically developing 6-month-old infants with parents with lower social motivation showed relatively greater amplitude of posterior ERPs (P400) to objects versus faces,

consistent with lesser engagement of social attention. Additionally, 6- and 12-month-old infants of parents with lower levels of social motivation showed smaller frontal theta oscillations, indicating reduced attention engagement at the neural level, while attending to naturalistic social scenes (Jones, Venema, Earl, Lowy, & Webb, 2017). Ronconi et al. (2014) found similar results on non-social visual attention performance. They reported an association between higher attention to details and communication difficulties in fathers and worse orienting skill in their 8-month-old infants. On the contrary, there was no significant association between mothers' autistic traits and their children's attentional indexes (Ronconi et al., 2014). Differently, Elsabbagh et al. (2014) found suggestive evidence that neural correlates of visual discrimination (the P100 ERP) when looking at gaze shifts at 8 months was related to maternal sensitivity during interactive play.

4.1.4 Aims of the study

Familial risk may be due either to genetic or to environmental contribution, or to the interaction between these two elements. In fact, features of the family environment and also associations between family environment and child outcomes are at least partly mediated by genetics (Ge et al., 1996; O'Connor et al., 2000; Plomin, 1994). In the project exposed in this chapter I aimed to investigate the specific contributions of genetic loading on social attention and social difficulties in family members at risk for ASD. I did this by a) evaluating the evidence for BAP and social attention atypicalities in first degree relatives, and b) testing whether this is explained by inherited genetic risk (polygenic score) for ASD. Additionally, I investigated to what extent parental phenotype and genotype influence infants' social attention skills.

As measure of social attention collected from each family member, the gaze monitoring task validated by Skuse et al. (2005) was selected. This choice was justified by theoretical and practical reasons: 1) various studies reported a possible effect of familial risk on eye-gaze direction detection (Adolphs et al., 2008; Scheeren & Stauder, 2008; Wallace et al., 2010); 2) there was suggestive evidence for a genetic contribution in the performance of this specific task (Skuse et al., 2014); 3) the same task could be administered to parents and children participants as it had been validated in both adult and young populations (from 6 years of age); 4) it could be administered online, thus allowing us to obtain a larger number of participants than with a researcher-supervised administration. The task is described in **section 4.2.2**.

This chapter presents three main parts. First, I evaluated the relationship between the selected measure of social attention and ASD/BAP. To verify whether social attention can be considered within the BAP construct, I examined performances in eye-gaze direction detection and autistic social traits in children (comparing LR children, HR siblings with no diagnosis of ASD and HR children who received a diagnosis of ASD) and in parents (comparing parents from LR, sHR and mHR families). Additionally, I tested whether worse social attention was related to more severe ASD traits using the SRS SCI t-score. Based on previous studies, I predicted lower SRS SCI scores in mHR fathers (Lyall et al., 2014; Wheelwright et al., 2010) and possibly mothers (Hasegawa et al., 2014) compared to sHR and LR parents. The same pattern was expected for the social attention measure, supposing that it was part of the BAP. I also examined sex differences to see whether better social/social attention skills were observed in females of the mHR families (mothers and unaffected girls) as a sign of sex-specific protective factor (Chawarska et al., 2016; Frazier et al., 2015).

Second, I assessed the contribution of ASD-associated genetic variants to individual differences in social attention (eye-gaze direction detection) and social autistic traits (SRS SCI) in individuals at low and high familial risk for ASD. Polygenic score (PGS) consists of the weighted sum of trait-associated alleles for a subset of top ranking genetic markers calculated on an initial training sample (Dudbridge, 2013) (see **section 4.2.3** for details). I hypothesized that ASD PGS explained not only some of the variance in ASD social traits, but also in social attention performance. If this pattern of results was observed, it would provide evidence that the same common genetic variants are responsible for social impairment and social attention difficulties, confirming a causality link between genetic risk variants for ASD and phenotypic traits of social attention.

Third, I tested whether infants' neurocognitive measures of social attention were predicted by parents' social attention performance, autistic traits and polygenic score. To this aim, I examined the relationship between parents' eye-gaze direction detection, SRS SCI and PGS and the infants' measures which were shown to be involved in developmental pathways towards later social and communication difficulties in **Chapter 3**. A direct effect of parental genetic loading on infants' social attention has not been studied yet to my knowledge, so this analysis was intended as exploratory with respect to the size of possible association effects. Based on the literature, lower SRS SCI scores in the parents were expected to predict reduced responses in the neural correlates of attention to faces in the infants' (Jones et al., 2017). I predicted the fathers' SRS and social attention skills to covary with their children's looking behaviour in infancy (Ronconi et al., 2014).

4.2 METHODS

4.2.1 Participants

The study cohort included family members of infants who participated in BASIS Phase 1 and 2 (described in **section 2.1.1**, see also **Table A2.1**). BASIS is a prospective longitudinal study following up the development of infants with (HR) and without (LR) an older sibling with ASD. Two recruitment phases lead to a comprehensive sample of 247 deeply phenotyped infant siblings (170 HR and 77 LR) from whom multiple experimental measures (EEG, eye-tracking, standardized behavioral assessments, parent-report questionnaires) have been collected during the first three years of life.

For the present study, I collected DNA from the family members of the BASIS infant siblings in addition to collecting questionnaires on autistic traits and measures of social attention. The phenotypic data collection was approved as an amendment of the BASIS project ethics approval (REC number 06/MRE02/73). The collection of human saliva and blood samples for DNA extraction and analysis received ethical approval as part of a project called BASIS – Genome, or gBASIS (REC number 15/LO/0468). As part of the project, blood samples for RNA extraction and gene expression analysis were also collected. The work presented in this chapter will focus on the salivary DNA samples collected for genetic analysis. I refer hereafter to the joint (genetic and phenotypic) family data collection performed for this study as gBASIS.

4.2.1.1 The gBASIS sample

Although the number of HR siblings in the combined Phase 1 and 2 samples was 170, five of the HR families had two children who participated in BASIS as infant siblings (3 pairs in two different Phases and 2 pairs in the same Phase), leaving a final sample of 165 HR families to be recruited for gBASIS. One additional HR family was recruited even if the infant sibling, initially enrolled, did not provide data for the longitudinal study.

The LR families were initally recruited from a pool of volunteer families who agreed to take part in research studies at the Centre for Brain and Cognitive Development (CBCD). Contact information for the Phase 1 LR families was provided by researchers who had previously contacted them for a follow-up BASIS study (Salomone et al., 2018). Of the 50 LR families, 13 were not retained in that study, therefore no contact information was available to recruit these families for gBASIS. Contact information for the Phase 2 LR families was obtained from the CBCD

databse. In total, 166 HR families and 64 LR familes were initially contacted by telephone and invited to participate in gBASIS. **Figure 4.1** summarises the available sample, reasons for attrition and final number of participants for genetic, behavioural and questionnaire data.

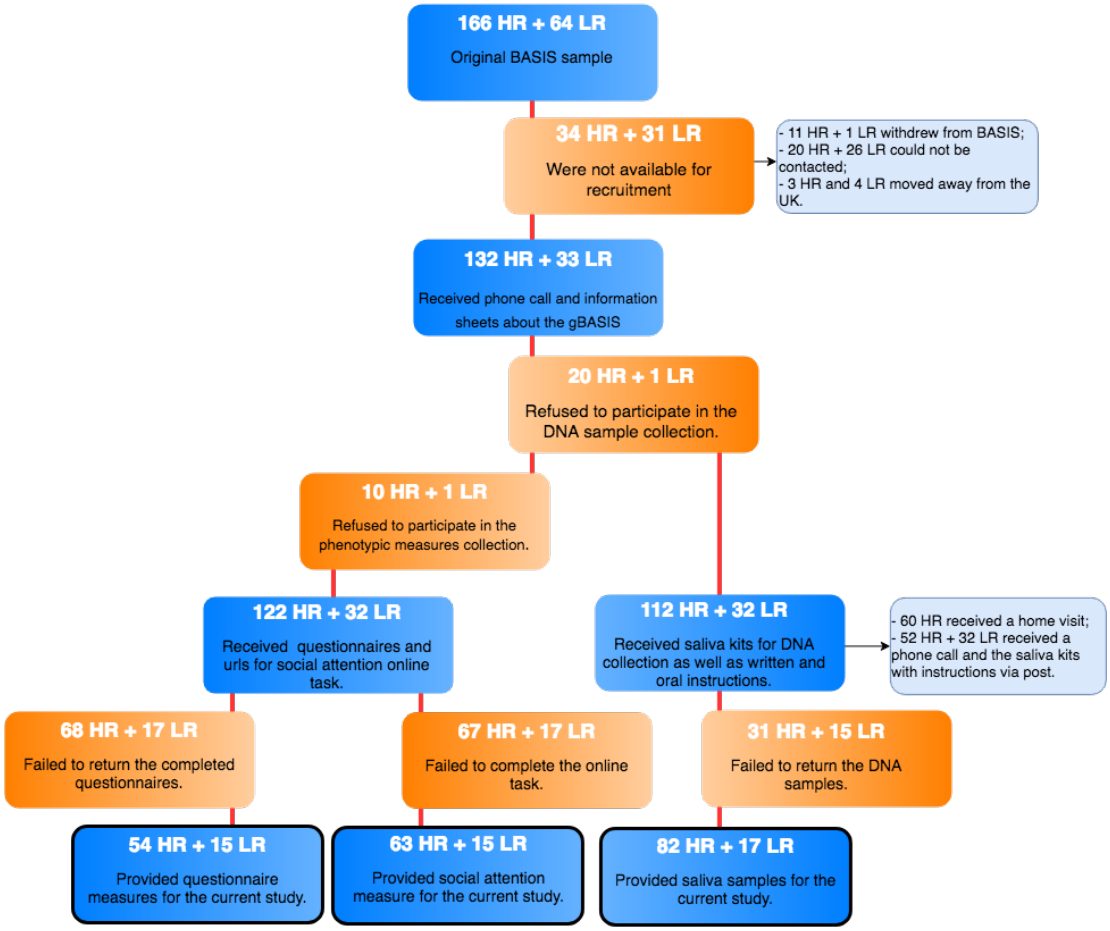

Figure 4.1 *Diagram showing the number of participants initially recruited as part of gBASIS* (original BASIS sample, top cell) and reasons for subsequent exclusion, leading to the actual sample for the current study (bottom cells) for 1) questionnaires (left), 2) online social attention task (middle) and 3) salivary DNA samples (right). Orange cells contain information on the number of participants who were excluded at various steps.

Children were classified into LR, high-risk with ASD (defined as 'ASD', including the older siblings who received a community diagnosis of ASD, or 'probands', and the BASIS target children who received diagnosis of ASD as part of the BASIS three-years visit assessment) and HR-noASD (including siblings without a formal diagnosis of ASD). Parents were assigned to 'familial risk' groups based on whether in their family there were no individuals with ASD (LR), one child with ASD only (sHR) or two or more individuals with ASD (mHR). To determine familial risk status, information about ASD diagnosis for all family members was obtained through the Medical and Psychiatric History Interview collected at the same time as the online task and combined with

database records of previous visits. When the concurrent Medical and Psychiatric History was not available (N=15), the same questionnaire collected at the BASIS T4 visit was used instead.

4.2.2 Phenotypic measures

4.2.2.1 The Social Responsiveness Scale questionnaire

The SRS, described in **section 3.2.2**, is a quantitative measure of autistic-like social impairment that has been extensively tested in both clinically ascertained individuals and population-based samples (Constantino & Gruber, 2012; Constantino & Todd, 2005; Frazier et al., 2014). For the present study, the SRS-2 was used, which consists of three versions: the School-Age Form (teacher or parent-report), for 4- to 18-year-olds, the Pre-school Form (teacher or parent-report), for 2.5- to 4.5-year-olds, and the Adult Form (self-report or relative/other-report), for 19- to 89-year-olds (Constantino & Gruber, 2012).

All three instruments can be completed by an adult informant who has regularly observed the subject in naturalistic social contexts over a period of at least 1 months (Bruni, 2014). Each item is rated on a scale from 1 (not true) to 4 (almost always true) and the instrument requires 15–20 min to complete. The raw scores can be converted into on gender- and age- specific standardized scores to obtain a total score that reflects the severity of ASD traits and, of interest for the present research, scores for two DSM-5 compatible domains: Social Communication and Interaction (SCI) and Restricted Interests and Repetitive Behaviors (RRB).

In our study, families received the School-Age form (parent report of children) and the SRS-Adult self-report form (self-report for mother and father). SRS questionnaires were completed by 69 families (54 HR and 15 LR). Of those, 38 HR and 13 LR families provided questionnaire data for all family members. For the remaining 16 HR and 2 LR families, data for at least one family member (typically the father) was missing. In total, 125 parents (69 mothers and 56 fathers) and 177 children (71 females and 106 males) provided valid SRS data (see **Tables 4.1** and **4.2**).

Table 4.1 *Number of gBASIS child participants who provided valid SRS data*, divided by group (based on risk and outcome status), split by sex. Children were divided into four groups: low-risk children (LR), children with a diagnosis of ASD (ASD), high-risk siblings without a diagnosis of ASD (HR-noASD), and high-risk siblings not yet assessed for ASD (HR-below3). Note that as the final category of siblings were younger than three they did not have a final outcome status and were excluded from this study.

Group	Sex	N	Total
LR	**Females**	9	18
	Males	9	
ASD	**Females**	11	65
	Males	54	
HR-noASD	**Females**	47	84
	Males	37	
HR-below3	**Females**	4	10
	Males	6	

Table 4.2 *Number of parent participants who provided valid SRS data*, divided by risk group and sex. Parents were divided into three groups based on their familial risk status: low-risk (LR), single-incidence (sHR) and multiple-incidence (mHR).

Group	Sex	N	Total
LR	**Females**	14	23
	Males	9	
sHR	**Females**	36	64
	Males	28	
mHR	**Females**	19	38
	Males	19	

4.2.2.2 The Gaze Monitoring online Task

The Gaze Monitoring Task (GMT) has been previously used to assess common DNA polymorphisms associated with human social recognition skills in probands with ASD, their parents and their siblings (Skuse et al., 2014). The GMT is part of the SASI (Skuse et al., 2005). As the computerized tasks of the SASI were no longer available, an online version of the GMT was re-created by a Birkbeck Informatics Technician thanks to the collaboration of D. Skuse's team, who shared the stimuli and instructions to exactly reproduce the original validated SASI version.

The first online version of the task was originally validated in 848 adults between 17.90 and 79.60 years of age (Skuse et al., 2005). Additionally, 271 adults and 477 children from 6 to 18 years of age were individually tested in person by trained psychologists (Skuse et al., 2005). External reliability between the online (unsupervised) and the experimenter-supervised versions of the GMT was assessed, by comparing the individuals' mean scores, on 48 pairs of adults matched by age and gender (Skuse et al., 2005). Given that the online version of the GMT was not originally validated with children, a pilot study was conducted previous administration of the online task to the gBASIS participants (see below).

Procedure

The task was run via computerized presentation and was administered over the Internet, by simply clicking on a url linked to it. General information about the task and aims of the study were displayed on the landing page, so that parents were able to evaluate whether they would like to try the task with their children. Two versions of the task were created, one for parents and one for children, which differed in the format of the information and instruction pages. In the child version, the participant was required to select whether he/she was able to read the instructions and complete the task without supervision or whether parental help was required to assist a non-verbal child in understanding the task. All parents supervising their children received written as well as oral (via phone call) instructions to be careful not to interfere with their children's responses by suggesting answers in either explicit or subtle ways.

Participants, or their supervising parents, were asked to read and complete a consent form, and enter their age and gender, before undertaking the task. Participants were then instructed to sit directly in front of the computer screen, with the face at the same high of the screen, and try to avoid to do the task in an environment containing sources of visual or audio interference. Information about the environment and time of day in which the task was completed was not recorded. Instructions were presented in both written form and illustrated with images. Additionally, if the participant was unable to read and understand the instructions, his or her parent was explicitly asked to read the instructions aloud for the participant. The task usually lasts approximately 5 minutes, depending on the participant's compliance.

The GMT assessed the accuracy with which gaze direction is ascertained from 30 colour photographs of faces (15 male and 15 female), with eyes directed toward the viewer. The direction of gaze was in some trials perpendicular to the screen, in others deviated between 5° and 20° in either direction (as displayed in **Figure 4.2**). If the participants thought that the person

in the photograph (see **Figure 4.3** for two examples) was looking straight at them (i.e. perpendicular to the screen), they clicked on the box that said, "Into my eyes", appearing in the middle of the screen just below the stimulus. If the participants thought that the person was looking to their left (deviated either 5° and 20°), they would have to click on the box that said "To my left", appearing on the left part of the screen. If the participants thought that the person was looking to their right (again deviated either 5° and 20°), they would have to click on the box that said "To my right", appearing on the right part of the screen. After two practice trials (with unique photographs not repeated during the test part of the task), participants completed a total of 30 test trials. The presentation of trials was random for each participant. RT was recorded as was accuracy of responses. RT was used in the pre-processing steps to exclude non-valid trails: specifically, individual answers with RT greater than 3 s.d. from the mean of the participants' answers to that item or <100 ms were removed (Whelan, 2008). Following this step, an accuracy score (ranging from 0 and 30) was calculated for each participant. The proportion of correct trials for each participant was calculated and used as a final measure of accuracy in eye-gaze direction detection (Skuse et al., 2014).

The only results feedback to the participants was whether they were more accurate with male or female faces. Following completion of the experiment, participants were fully debriefed and were provided with a link to download the debrief form.

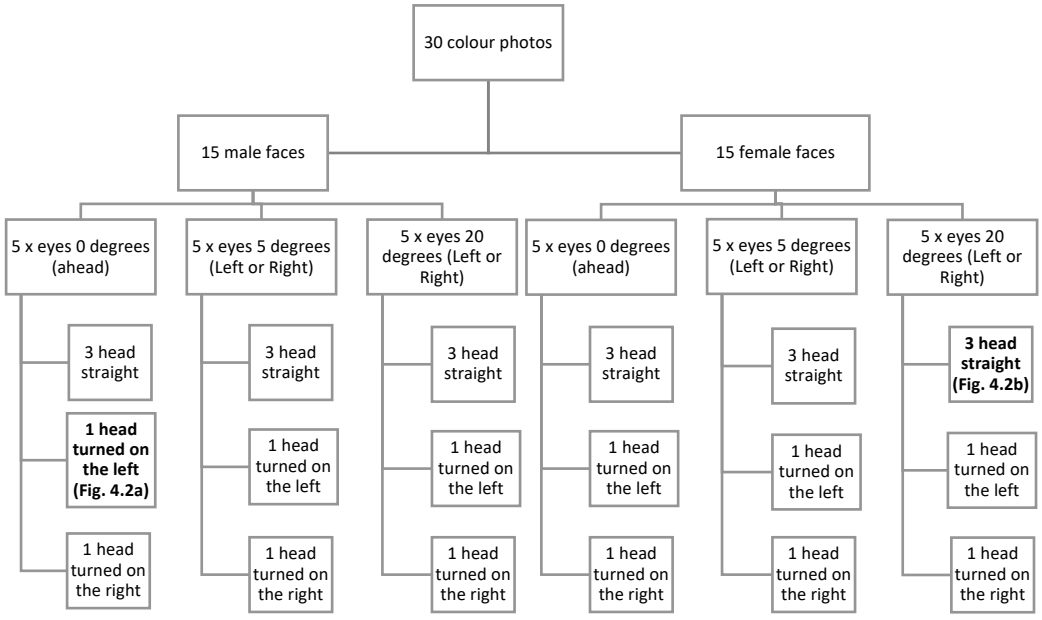

Figure 4.2 *Diagram illustrating the combinations of eye gaze and head directions* for the thirty photos displayed in the test phase of the Gaze Monitoring Task.

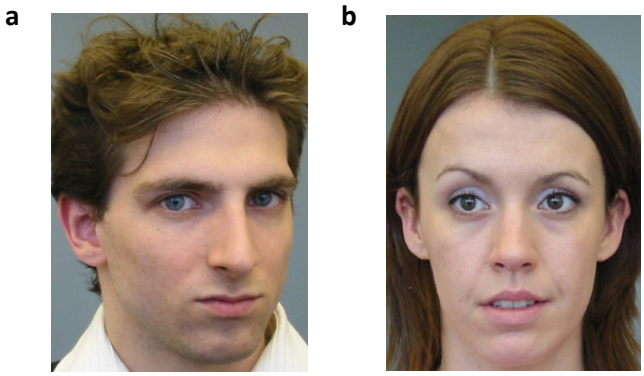

Figure 4.3 *Examples of the stimuli for the Gaze Monitoring Task*. **a** Male model with head turned on the left and eyes directed into the participants' eyes. **b** Female model with head straight and eyes directed 20 degrees to the participants' left.

Pilot study of child Gaze Monitoring Task

As mentioned earlier, the original online version of the GMT was only validated with adults (Skuse et al., 2005). As we aimed to collect researcher-unsupervised online measures from all family members of children that previously participated in gBASIS – including children-, I first conducted a pilot study in an independent sample of typically developing children. Ethical approval for this pilot study was obtained from the Birkbeck Ethics Committee (reference number 151645).

With the pilot study, I aimed to verify:

- whether the parents could supervise their children without a researcher present,
- whether (and at what age) children could complete the test part of the task without any direct help,
- whether the researcher-unsupervised task results were comparable with the standardized scores of the validated version (Skuse et al., 2005).

To these purposes, 49 children aged between 4 to 11 years were recruited. This age range was selected based on the age of the Phase 1 and 2 children at the time of recruitment for gBASIS. For the pilot study, urls to the task were sent via e-mail to parents who were currently enrolled in the CBCD Babylab database. Age and gender of each participant, as well as consent for the use of anonymized data for research purposes, were requested before accessing the task. No other demographic information was requested or recorded.

In addition to the task responses, the following feedback questions were asked to supervising parents:

- Was your child able to complete the entire quiz by him/herself? (yes/no)
- Do you think the quiz was too difficult for your child? (yes/no)
- Did you leave your child during the test part of the quiz? (yes/no)
- Did you have to assist your child during the test part of the quiz? (yes/no)

Feedback answers for children between 4 and 5 years of age (N=10, 6 females, mean age = 4.65, min: 4, max: 5.75) revealed that all parents had assisted their children during the test. Whilst only one parent reported that the task was too difficult for the child, 6 of the 10 children were unable to complete the task for themselves. Moreover, the data confirmed that the proportion of correct responses were just above chance level (i.e., accuracy proportion $\leq$0.33, given that there were three possible gaze directions, Skuse et al., 2005) for 6 of the 10 participants. For this reason - and because normative values provided by D. Skuse's collaborators were available only for children older than 6 (Skuse et al., 2005) -, I focused validation analyses on children aged from 6 to 12 years (N=39, 19 females, mean age=8.8, min: 6.3, max: 11.8).

Individual accuracy scores were compared with the age appropriate distribution of normative values collected with experimenter-delivered task by Skuse and colleagues (2005). 5% of the participants' scores fell below the 10th percentile and 10% of the participants' scores fell above the 90th percentile for their age. This confirmed that results from the online version of the GMT were comparable to those obtained with the experimenter-delivered version. All parents of children above 6 years confirmed that the task was doable by their children. Therefore, the online GMT seemed to be a valid measure to be used with the BASIS children older than 6 years of age.

Gaze Monitoring Task for gBASIS

For the online computerised GMT, an individualised url link was assigned to every gBASIS participant that contained their pseudonymized ID and enabled me to link participants to their GMT data. As gender and age information were requested before completing the quiz, I was able to exclude participants when there was a mismatch between task ID and expected participants' age or gender for that ID (N=6).

Written instructions and urls for the GMT were send by e-mail or post to 122 HR and 32 LR families who had previously been contacted by phone and sent information sheets about the gBASIS study (**Figure 4.1**). For 13 HR and 7 LR families, the GMT was completed by all family members, whilst for 50 HR and 8 LR families partial data was collected, for a total of 63 HR families (51.6% of the original sample) and 15 LR families (46.7%). Overall, 249 family members

of children who participated in BASIS provided valid data for the GMT. Of those, 120 were parents (68 mothers and 52 fathers) and 129 were children (56 females and 73 males). Children were aged between 6 and 19 years (mean=10.9, s.d.=2.87). There was a significant difference in children's age between groups ($F(2,123)=8.189$, $p=0.0005$), which was due to ASD probands being on average older than HR-noASD ($p=0.002$) and LR children ($p=0.002$). The effect of sex and the interaction between group and sex were non-significant ($F(1,123)=0.042$, $p=0.84$ and $F(2,123)=1.120$, $p=0.33$, respectively). **Tables 4.3** and **4.4** indicate the number of participants per group.

Table 4.3 *Number and age of child participants who provided valid Gaze Monitoring Test data*, divided by group and sex. Children were divided into three groups based on their risk and outcome status: low-risk controls (LR), children with a diagnosis of ASD (ASD), high-risk siblings without a diagnosis of ASD (HR-noASD). Mean age in years, standard deviation (s.d.) and minimum and maximum age are also reported.

Group	Sex	N	Total	Mean Age (s.d.) Min - Max
LR	**Females**	16	28	10.36 (1.87) 6.5 – 13.8
	Males	12		9.47 (2.31) 6.5 – 13.2
ASD	**Females**	8	44	11.16 (2.9) 6.3 – 14.8
	Males	36		12.49 (2.82) 6.8 – 19.6
HR-noASD	**Females**	32	57	10.32 (2.53) 6.1 – 16.5
	Males	25		10.35 (3.30) 6.3 – 17.6

Table 4.4 *Number of parent participants who provided valid Gaze Monitoring Test data*, divided by group and sex. Parents were divided into three groups based on their familial risk status: low-risk controls (LR), single-incidence families (sHR) and multiple-incidence families (mHR).

Group	Sex	N	Total
LR	**Females**	15	25
	Males	10	
sHR	**Females**	36	64
	Males	28	
mHR	**Females**	17	31
	Males	14	

4.2.3 Genetic data

Ninety-nine families (82 HR and 17 LR, total N of individuals=498) participated in the salivary DNA sample collection. DNA saliva samples were collected during home visits (N=60 HR families; total N=282), or via post (N=22 HR + 17 LR families; total N=216) using Oragene DNA OG-500 (for adults) or OG-575 (assisted collection for children) kits. DNA extraction and quantification for extraction were performed by LGC (https://www.biosearchtech.com) in accordance with standard procedures. Three individuals were re-contacted to donate a new saliva sample as the extraction of the original sample failed (DNA concentration <10 ng/μl).

In addition, 295 buccal-swabs DNA samples of BASIS infant siblings and infant siblings who participated in a subsequent phase of the longitudinal study, the Studying Autism and ADHD Risks, or STAARS (see next chapter, **section 5.2.1** and **Table A5.1**, for details), were included. These DNA samples were collected by the BASIS team and extracted at the Social, Genetic & Developmental Psychiatry (SGDP) Centre at King's College, London. As multiple cheek-swabs samples were collected for each infant longitudinally, the DNA sample with the highest concentration was selected for each infant to take forward for genotyping. If both a saliva and cheek-swab DNA sample(s) existed for an infant then both samples were genotyped.

4.2.3.1 Pre-processing

SNP genotyping, quality control and imputation were carried out at the SGDP Centre by the BRC core genomics team. A total of 796 DNA samples were genotyped using the Illumina Infinium Global Screening Array-24 v2.0 BeadChip.

The raw array data was subjected to standard quality control procedures to identify individuals and SNPs for exclusion (https://confluence.brc.iop.kcl.ac.uk:8493/display/ PUB/Production+ Version%3A+Illumina+Exome+Chip+SOP+v1.4). Specifically, samples were removed on the basis of sex mismatches (N=2), excessive or low genetic heterozygosity (±3 s.d. from the mean, N=21), individual call rate less than 99% (N=56, of which 28 were duplicates). Samples that passed all other quality control thresholds were retained during subsequent phasing and imputation to leave a final genetic sample of 717 DNA samples from 648 individuals. SNPs were removed if they had a minor allele frequency <0.01, a call rate <0.95, or deviated from Hardy-Weinberg equilibrium (HWE, $p<5 \times 10^{-7}$). This resulted in 768,142 genotyped SNPs. Imputation was performed through the Michigan Imputation Server, using 1000 Genomes reference haplotypes

(Version 5 Phase 3), phasing set to "eagle v3" and population set to "mixed". Built GRC37 was used for both the genotyped data and the reference panel.

Imputed SNPs were excluded from all further analyses if they had a minor allele frequency <0.01, an info score <0.9, call rate <0.99 and HWE p< 5 x 10^{-7}, which resulted in a total of 5,663,312 SNPs. Imputed data were excluded for duplicate and non-European individuals. Specifically, genome-wide Identity-By-Descent (IBD) estimation was performed to detect mistaken identities, following in-house pipeline developed at the Geschwind Lab, University College of Los Angeles. The full procedure is reported in the Appendix (**Figure A4.2**). Ancestry assignment was obtained using multidimensional scaling analyses compared with HapMap 3 (The International HapMap 3 Consortium, 2010). Criteria for assignment of the samples of unknown ancestry to populations were decided based on visual inspection, following the procedure for ancestry assignment utilized at the Geschwind Lab. **Figure A4.1b** represents the samples after color-coding based on assigned ancestry. HapMap 3-based ancestry assignment was manually verified by comparing it with parent-reported ancestry origins, which were available for 675 of the 717 gBASIS samples. **Table A4.1** shows the number of gBASIS samples by population after HapMap 3-based ancestry assignment. Following the quality control steps described, complete genotypic data were retained for 579 individuals (4,398,111 SNPs).

4.2.3.2 Polygenic score construction

A PGS is a cumulative measure of genetic risk for an individual based on the summed effects of many thousands of risk alleles (for the disorder or trait of interest) distributed throughout the genome. Polygenic scores are constructed in the following way: Firstly, SNPs from a 'base' GWAS (i.e., the original GWAS of the trait of interest) are ranked for evidence of association, usually by p-value. Then, in a second independent 'target' dataset, a PGS is calculated for each individual as a sum of each 'risk' allele (i.e. whose p-value in the GWAS is below a selected threshold) carried by the individual for the selected SNPs, with each SNP weighted by the effect size (e.g. log odds ratio (OR) for case – control studies) in the base GWAS (Wray et al., 2014).

$$PGS_{P_T,j} = \sum_{i=1}^{m} \beta_i G_{i,j}$$

(**Equation 4.1**)

Equation 4.1 indicates how a PGS for individual *j*, where *j*=1, 2, …, *n*, is calculated at a GWAS p-value threshold P_T. For a SNP *i*, where *i* = 1, 2, ..., *m*, a p-value P_i is calculated for the association between the SNP genotypes, $G_{i,j}$ = {0,1,2} for individual *j*, and the phenotype. Under an additive model, a corresponding effect size is estimated, by β_i, for the effect of a unit increase in

genotype, G_{ij}, on the phenotype. This process is usually repeated across different p-value thresholds P_T, and the model fit of the regression of the target phenotype on PGS is compared (Euesden, Lewis, & O'Reilly, 2015). While variance explained (R^2) is a well-defined concept for continuous trait outcomes, Nagelkerke R^2 is used as a conceptual proxy for R^2 for case-control outcomes (although this is possibly biased as it does not take into account the case population prevalence, Choi et al., 2018).

As standard procedure, the PGS is constructed using the GWAS p-value threshold that better distinguishes between cases and controls in the base sample, and PGS is then treated like any other continuous predictor variable in further analyses. In the current study, PRSice-2 software was used for PGS calculation (Euesden et al., 2015). This program offers the option of an 'high-resolution' scoring that identifies the best-fit PGS to a high degree of approximation. Of note, Euesden et al. (2015) performed additional permutation studies to evaluate adequate adjustment for multiple testing in the PRSice high-resolution approach. Based on their results, they recommend to apply a p-value threshold of 0.004 in order to ensure a false-positive rate below 0.05 when establishing whether the high-resolution best-fit PGS predicts the phenotype of interest in the target sample, (Euesden et al., 2015).

For ASD-PGS calculation, 43 individuals with missing phenotype data were excluded (infant siblings who were too young to undergo diagnostic assessment as part of the 3-year visit). Of the 536 individuals with known ASD phenotype, 111 were cases and 425 were controls. Cases included all BASIS older siblings with a community clinical diagnosis of ASD, parents who reported having received a diagnosis of ASD (through EU-AIMS Medical and Psychiatric History Interview, https://www.eu-aims.eu), and all BASIS infant siblings who were classified as having ASD following BASIS diagnostic assessment at three years, as describes in **section 2.1.1**. The control group included all LR parents and older siblings (screening for ASD in older siblings of the LR BASIS participants was obtained with the SCQ), all BASIS infant siblings who did not receive a diagnosis of ASD at 3 years by the BASIS research clinicians, and all the other available family members with no reported record of ASD (based on the Medical and Psychiatric History Interview).

Prior to PGS calculation, additional quality check was performed to exclude SNPs and individuals with no missing call rate (geno<0), minor allele frequency <0.05, significant HWE test at a p-value threshold $<1 \times 10^{-7}$, and SNPs with minor allele labelled as 0. Thus, the final target sample for PGS calculation consisted of 4,398,111 variants and 536 individuals.

In order to exclude further resilient population structure which might confound PGS results (Curtis, 2018), the first two principal components were added as covariates in downstream PGS analyses (Choi et al., 2018; Novembre et al., 2009). The 'PC-AiR' function of the 'GENESIS' R-package (Conomos, Miller, & Thornton, 2015) was used to calculate principal components (PCs) robust to possible familial relatives in the sample (R. Harrison). Prior to this analysis, clumping was performed to keep only one representative SNP per region of Linkage Disequilibrium (LD), such that 387,622 variants were included in the PC calculation. PC-AiR computes pairwise relatedness (kinship coefficients) and pairwise ancestry divergence to identify a subset of mutually unrelated individuals which is representative of the sample's ancestry composition. The kinship coefficient estimated by the program based on kinship matrix was 0.125, and it identified 280 unrelated and 256 related individuals within the sample. Subsequently, standard PCs were obtained for this subset of unrelated individuals, and PC values for the excluded individuals were predicted from genetic similarity.

ASD GWAS summary statistics from a meta-analysis of a Danish population-based case-control sample from the Lundbeck Foundation Initiative for the Integrative Psychiatric Research (iPSYCH) and a European ancestry sample from the Psychiatric Genomics Consortium (PGC) was used as a 'base' GWAS (Grove et al., 2019). This dataset reports results for a combined sample of 18,381 cases and 27,969 controls. The base GWAS dataset, the target gBASIS dataset and the two ancestry PCs as covariates were submitted to PRSice-2 for PGS calculation (Euesden et al., 2015). The following parameter settings were used for the analysis. LD was accounted for by selecting the SNP in the base phenotype dataset with the lowest GWAS p-value in a sliding window of 500kb, only retaining variants with a pairwise LD r^2 <0.1 within 250kb to both ends of the index SNP (default settings in PRSice-2). LD estimation for clumping was based on the 1000 Genomes reference panel, as recommended for samples of around 500 individuals (Euesden et al., 2015). PGSs for ASD were generated for the gBASIS individuals for a range of p-value thresholds ($0.001<P_T\leq1$) and the proportion of phenotypic variance explained by each PGS predictor reported as Nagelkerke R^2. The high-resolution best-fit PGS was extracted for each individual and used in downstream analyses.

4.2.4 Analyses

All analyses were performed in R (R Core Team, 2013).

4.2.4.1 Social attention as a trait

The first series of analyses aimed to verify whether the measure of social attention collected using the online GMT from the familial sample 1) reflected familial risk and ASD diagnosis, and 2) covaried with autistic traits. Separate analyses were conducted on the children's and parents' data.

Group analyses

Children were divided into three groups as explained in **section 4.2.1**: LR, ASD (including the probands and the BASIS target children who received diagnosis of ASD) and HR-noASD (including siblings without a formal diagnosis of ASD). As mentioned earlier (**section 4.2.2**), SRS data were available for 10 HR infant siblings enrolled in STAARS who were younger than 3, and therefore did not receive a diagnostic assessment for ASD. As ASD status could not be ascertained for these infants, they were excluded from analyses. A three-by-two ANOVA was performed on the children's GMT data, with group (LR, ASD and HR-noASD) and sex as between-subjects variables. Age (in years) and a binary variable indicating whether the child received parent's assistance to complete the task were added as covariates. Post-hoc pairwise comparisons with Bonferroni correction were used to further investigate the results.

Parents were divided into three groups based on familial risk: LR (no individuals with ASD in the family) (LR), sHR (single-incidence families) or mHR (multiplex families, where two or more family members received a diagnosis of ASD). To determine familial risk status, information about ASD diagnosis for all family members was obtained through the Medical and Psychiatric History Interview collected at the same time as the online task and combined with database records of previous visits. When the concurrent Medical and Psychiatric History was not available (N=15), the same questionnaire collected at the BASIS T4 visit was used instead.

A three-by-two ANOVA was performed on parents' data to investigate effects of familial risk group (LR, sHR and mHR) and sex, with age as a covariate.

Dimensional analyses

To investigate the relationship between social attention and autistic social traits, a general linear model was used with the proportion of correct answers at the GMT as dependent variable and SCI scores of the SRS interacting with sex as predictors. Age and parent help were added as covariates. To verify whether the observed effect was specific to the social domain of the SRS, RRB score was also used as independent variable in a similar model to test prediction of the

GMT results. The same approach was used for the parents' data (with the difference that no variable controlling for parent help during the online task was introduced). Additionally, 2 three-by-two ANOVAs were used to investigate differences in the SRS SCI and RRB domains, respectively, between LR, sHR and mHR families, for fathers and mothers respectively.

4.2.4.2 Effect of polygenic risk on social attention and ASD traits

Group analyses

To compare PGS in children with ASD, their siblings who were not diagnosed with ASD and LR controls, a two-by-three ANOVA testing for main effects of sex and group and their interaction was performed. Tukey HSD was used for post-hoc analyses. Similarly, for parents a two-by-three (LR, sHR and mHR) ANOVA testing group differences in PGS in interaction with sex

Dimensional analyses

Subsequently, children and parent data were combined to estimate the relationship between PGS for ASD and accuracy in eye-gaze direction detection. Multilevel mixed-effects linear models ('lme' function of the 'lmne' R package) were used as allowed me to enter all the data in the same models (N=208) while accounting for between-families differences (assumed to account for familial environmental factors) by setting family as a random effect, following similar research (Jenkins, Rasbash, Leckie, Gass, & Dunn, 2012; Oliver & Alison, 2018). Thus, the baseline model was defined by setting a random intercept per family and performance at the GMT (proportion of correct answers) as dependent variable.

Models including other possible explanatory variables were evaluated one at the time, and significant improvement of model fit was tested using a chi-square likelihood ratio test (Field, Miles, & Field, 2012). Specifically, the baseline model was compared with models each subsequently adding one of the following predictors: age when the GMT was completed, PGS, familial risk (mHR versus LR and sHR), the interaction between PGS and familial risk and a binary variable indicating whether GMT scores were obtained for all or some of the family members (N=20 families GMT data for all family members and N=43 families provided incomplete GMT data). In these models, the mHR group was set as referent for the linear regressions testing for the effect of familial risk because a stronger relationship between phenotype and PGS was expected for this group, as discussed in **section 1.2.1** and **4.1.1**. Estimates and significance of the independent variables were assessed within the higher-order models which significantly

improved the model fit (chi-square statistics reported on **Table A4.6**). All results of this model are reported in the appendix (**Table A4.7**).

In the same way, multilevel mixed models were used to assess whether PGS predicted social communication difficulties (SRS SCI domain) in interaction with familial risk (N=207, **Tables A4.8** and **A4.9**).

4.2.4.3 Familial risk and infant measures of social attention

I then explored whether the infant measures were predicted by parents' polygenic risk and behavioural characteristics. To this aim, I tested whether parents' social attention (GMT), autistic traits (SCI) and PGS predicted infant measures of social attention that have been shown to be involved in the pathways towards social and communication outcome in the previous chapter (Nc mean amplitude and microstate 4 duration difference between FD and Noise, peak look at the face and proportion of looking time at the gazed-at object, see **section 3.2.2** for more information on these measures).

Parental measures of social attention, severity of social impairment and polygenic risk were weakly correlated, as shown in **Figure A4.4** (all uncorrected p*s*>0.173). Additionally, there was no significant correlation between maternal and paternal measures [Of note, a complete dataset of all parental measures was obtained with only 23 couples]. The low correlation between measures and the noticeable reduction of sample size when all parental measures had to be considered together guided the choice to test the association between each parental measure and each infant phenotype independently. If a significant association was observed in the larger possible sample between one or more of the parents' measures and their child's early social attention markers, further analyses could have followed to disentangle these effects and further investigate the specificity of the significant relationship with respect to parental role or type of measure. Therefore, six linear regressions were conducted to test the effect of maternal and paternal GMT, SRS SCI and PGS, respectively, on each of the four infants' neurocognitive phenotypes. P-value correction for the six multiple testing was applied to results of the regressions using FDR method. Phase was included as covariate in all analyses, as it was shown to be significantly associated with the measures in the previous chapter.

4.3 RESULTS

4.3.1 Social attention as a trait

4.3.1.1 Eye-gaze direction detection and autistic traits in children

The ANOVA evaluating group differences in the children's social attention skills, measured as performance in the GMT, revealed that there was a main effect of group ($F(2,121)=4.83$, $p=0.01$, $\eta^2=0.074$) due to a significant difference between LR and ASD ($p=0.017$) and a trend of significant difference between HR-noASD and ASD ($p=0.092$). No difference was observed between LR and HR-noASD ($p=0.527$). There was a main effect of sex ($F(1,121)=4.49$, $p=0.036$, $\eta^2=0.040$) with females scoring higher than males (females' mean= 0.503, males' mean=0.453). There was also a significant group by sex interaction ($F(2,121)=4.2$, $p=0.0173$, $\eta^2=0.065$) with males performing worse than females in the HR-noASD group ($p=0.012$) but not in the other groups (LR: $p=0.999$, ASD: $p=0.839$), and HR females without ASD performing significantly better than females with ASD ($p=0.034$), while no such difference was observed between HR-noASD and ASD males ($p=0.988$). **Figure 4.4a** illustrates these results. Older children performed significantly better in this task ($F(1,121)=18.53$, $p<0.001$, $\eta^2=0.084$) but there was no effect of parents' help on the results ($F(1,121)=0.636$, $p=0.427$, $\eta^2=0.002$).

For SRS SCI scores, there was a main effect of group ($F(2,157)=$, $p<0.001$, $\eta^2=0.376$), due to a highly significant difference between ASD and LR ($p<0.001$) and between ASD and HR-noASD ($p<0.001$), while the LR and HR-noASD groups showed no significant difference ($p= 0.12$). There was no significant effect of sex ($F(1,157)= 0.806$, $p= 0.371$, $\eta^2=0.005$), as expected given that the SRS standardized scores account for sex differences (Constantino & Gruber, 2012), and role by sex interaction ($F(2,157)= 0.334$, $p=0.716$, $\eta^2=0.004$).

One-hundred children provided valid data for both SRS and GMT. When investigating the relationship between GMT and SRS SCI in children, I found, in line with the expectations, that higher social attention was associated with less social impairment ($\beta=-0.003$, s.e.=0.0007, $p=0.0006$). This effect was not different between boys and girls ($\beta=-9.05 \times 10^{-5}$, s.e.= 0.001, $p= 0.94$, see **Figure 4.4b**). Interestingly, GMT performance also, but to a lesser extent, predicted by RRB domain of the SRS ($\beta=-0.002$, $p=0.0007$, $p=0.004$), for both males and females ($\beta=-0.001$, s.e.=0.001, $p=0.402$). **Tables A4.2** and **A4.3** report the all estimates from these multiple regressions.

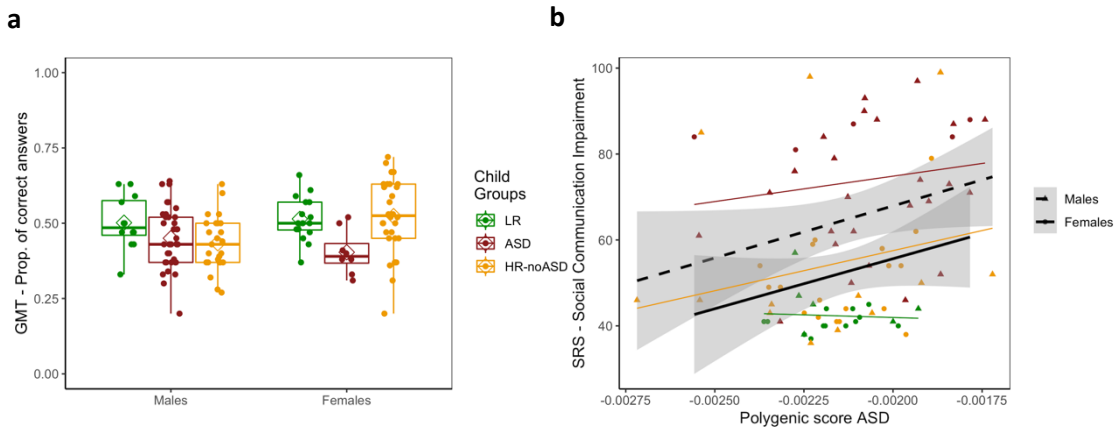

Figure 4.4 *Children's phenotypes in relation to outcome and polygenic score for ASD.* **a** Boxplots showing the proportion of correct answers at the Gaze Monitoring Task (GMT), in males and females in the three child groups: low-risk (LR), affected probands (ASD), and high-risk siblings without a diagnosis of ASD (HR-noASD). All individual data are represented by points. The lower and upper hinges of the boxplots correspond to the first and third quartiles (the 25th and 75th percentiles). The upper and lower whiskers extend from the hinges to the largest and smallest values, respectively, no further than 1.5 x inter-quartile range. Horizontal lines within boxplots indicate the median value, while rhombi represent mean values. **b** Relation between children's polygenic score for ASD, on the x-axis, and Social Responsiveness Scale, Social Communication Impairment domain t-scores, on the y-axis. Small triangles represent individual data points for males and dots for females, colour-coded in red for ASD children, yellow for HR-noASD children, and green for LR children. The dashed black line represents the regression line for males and the solid black line represents the regression line for females, with grey shaded areas depicting standard errors. Coloured lines represent the regression lines for the ASD (red), HR-noASD (yellow) and LR (green) groups.

In sum, the results of this section revealed that children with ASD performed worse than the other groups in the GMT task, and that girls overall performed better than boys. This effect was especially true for HR-noASD females, while the opposite was observed in the ASD group. The three groups were also different with respect to social impairment, with ASD group performing worse than the other two groups. A significant negative relationship between the GMT and SCI scores was found, such that worse performance at the GMT task was associated with more severe autistic symptoms.

4.3.1.2 Eye-gaze direction detection and autistic traits in parents

In parents, I examined whether there was a significant difference between performance in the GMT in LR, sHR and mHR parents. **Figure 4.5a** illustrates the results. ANOVA revealed that the effect of familial risk was not statistically significant ($F(2,114)=2.804$, $p= 0.065$, $\eta^2= 0.047$), with LR (mean=0.546, s.d.=0.065) performing only slightly better than mHR (mean=0.527, s.d.=0.076, p= 0.718) and worse than sHR (mean=0.57, s.d.=0.09, p=0.42). There was no effect of sex

(F(1,114)= 1.600, p= 0.209, η^2= 0.014) nor interaction between familial risk and sex (F(2,114)= 0.896, p= 0.411, η^2= 0.015).

The three groups of parents were significantly different in SRS SCI scores (F(2,119)=6.18, p=0.003, η^2= 0.094, **Figure 4.5b**), with LR (mean=43.74, s.d.=6.62) showing less social impairment than mHR (mean=53.58, s.d.=11.52, p=0.002) and sHR (mean= 49.41, s.d.=10.55, p=0.073). There was no significant difference between mHR and sHR (p=0.147), although sHR had lower impairment than mHR, as expected based on previous research (Frazier et al., 2015). There was also a significant effect of sex (F(1,119)=4.23, p=0.042, η^2= 0.026), with mothers having lower scores (mean= 51.71, s.d.=11.1), i.e. better social responsiveness, than fathers (mean= 47.94, s.d.=10.2), in line with what previously found in a larger population (Frazier et al., 2014). The interaction between sex and group was not significant (F(2,119)=0.944, p=0.392, η^2= 0.015).

There was a negative association between parents' SRS SCI scores and GMT performance (β=-0.003, s.e.= 0.002, p=0.049). Interestingly, there was also a significant interaction between SRS and sex, with mothers showing a positive relationship (β=0.006, s.e.= 0.002, p=0.004) indicating that in this group better performance at the GMT was associated with lower social responsiveness. The same pattern of results was observed when looking at the RRB domain of the SRS, with better social attention associated with less restricted and repetitive behaviours in fathers (β=-0.003, s.e.=0.002, p=0.048) but with higher RRB severity in mothers (β=0.006, s.e.= 0.002, p=0.005). All results can be found in the Appendix of this chapter (**Tables A4.4** and **A4.5**).

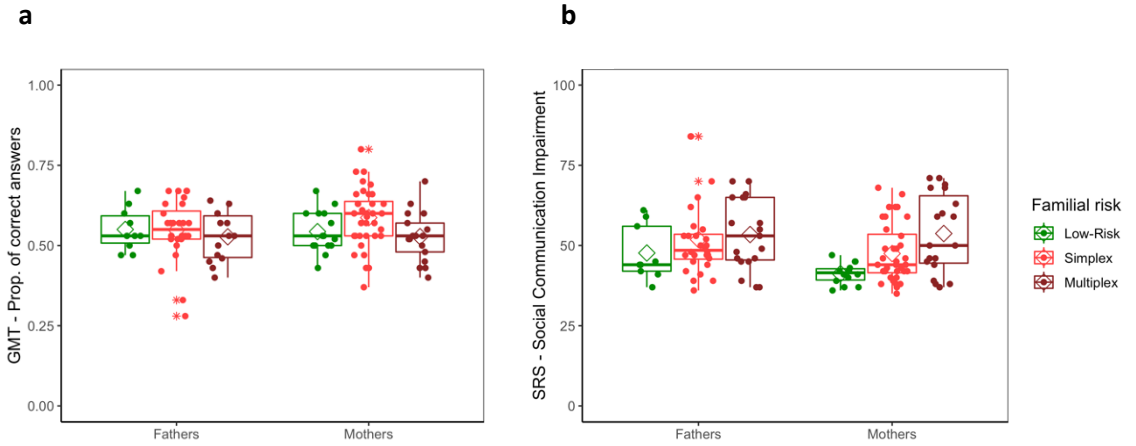

Figure 4.5 *Boxplots showing the parents' performance at the Gaze Monitoring Task* (GMT), measured as proportion of correct answers (**a**), and the Social Responsiveness Scale, Social Communication Impairment domain scores (**b**) in fathers and mothers in the three familial risk groups: low-risk (green), high-risk from simplex families (scarlet) and high-risk from multiplex families (burgundy). All individual data are represented by points. The lower and upper hinges of the boxplots correspond to the first and third quartiles (the 25th and 75th percentiles). The upper and lower whiskers extend from the hinges to the largest and smallest values, respectively, no further than 1.5 x inter-quartile range. Data beyond the end of the whiskers are plotted individually and represented by stars. Horizontal lines within boxplots indicate the median value, while rhombi represent mean values.

Thus, for parents' behavioural measures, LR parents had lower impairment than mHR parents in both the SRS social domain and the GMT, although for the latter measure the effect was non-significant. sHR parents showed reduced impairment compared with mHR. Mothers had overall less severe symptoms than fathers in the social domain. SRS and GMT scores were negatively associated, as expected, in fathers. However, the opposite effect was found in mothers, with better performances at eye gaze direction detection predicting more severe social difficulties and restricted and repetitive behaviours.

4.3.2 Effect of polygenic risk on social attention and ASD traits

4.3.2.1 Group differences in polygenic score

The PGS which best distinguished between ASD cases and controls in the gBASIS cohort, composed by 536 individuals (111 with and 425 without ASD), was obtained at a GWAS p-value threshold of 0.01605 (**Figure 4.6**). This high-resolution best-fit PGS was significantly higher for ASD cases than controls. The difference in PGS between cases and controls was obtained with p=0.00396 [just below the p-value threshold of 0.004 recommended in order to ensure a false-positive rate below 0.05 (Euesden et al., 2015)]. Nagelkerke's R^2 was 0.0249, indicating that 2.49% of the variance in ASD in the present cohort was explained by PGS, in line with previous estimates (2.45% in the largest ASD GWAS published thus far by Grove et al., 2019, which was also the 'base' GWAS for this analysis. **Tables 4.5** and **4.6** show means and standard deviations for ASD PGS by familial risk group in children and parents, respectively.

Table 4.5 *Polygenic score for children participants with good quality genetic data* divided by group (low-risk, LR, high-risk siblings without a diagnosis of ASD, HR-noASD, and affected probands, ASD) and familial risk: LR, single-incidence families (sHR) and multiple-incidence families (mHR).

Group	Familial Risk	N	Mean	s.d.
LR	**LR**	23	-0.00220	0.00016
HR-noASD	**sHR**	30	-0.00216	0.00021
	mHR	13	-0.00224	0.00018
ASD	**sHR**	19	-0.00209	0.00020
	mHR	17	-0.00208	0.00018

N: number of participants; s.d.: standard deviation.

Table 4.6 *Polygenic score for parent participants with good quality genetic data*, divided by group (low-risk, LR, and high-risk, HR) and specifically familial risk: LR, single-incidence families (sHR) and multiple-incidence families (mHR).

Group	Familial Risk	N	Mean	s.d.
LR	**LR**	18	-0.00213	0.00013
HR	**sHR**	44	-0.00212	0.00021
	mHR	25	-0.00213	0.00018

N: number of participants; s.d.: standard deviation.

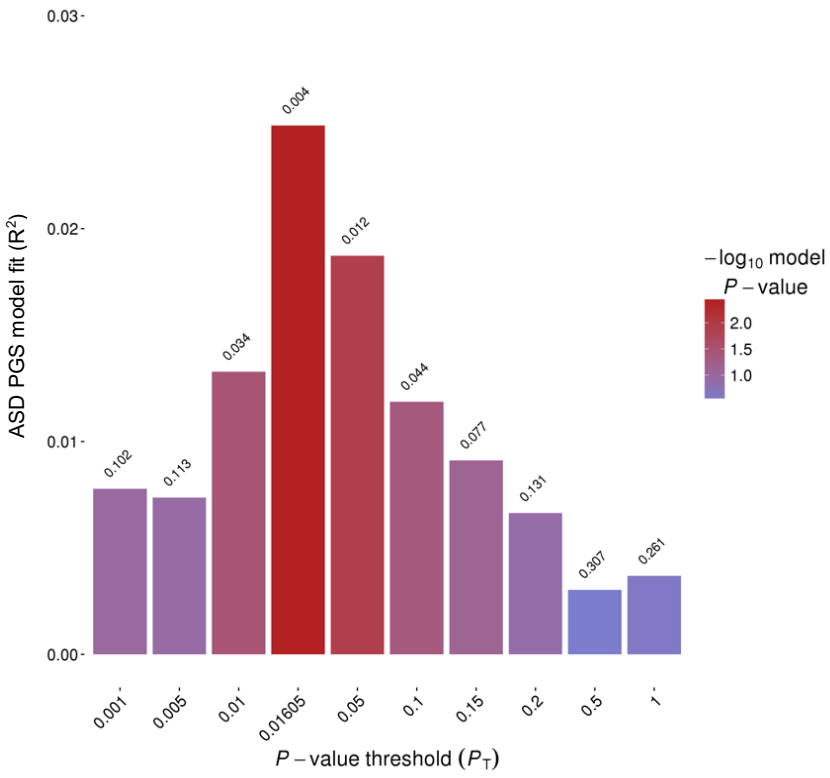

Figure 4.6 *Results of the polygenic score (PGS) for ASD at various p-value thresholds*. Height of bars (y-axis) represents the model fit (R^2). X-axis represents the 8 selected p-value thresholds and the p-value threshold selected for the high-resolution best-fit polygenic score. Numbers above bars represent p-values. Bars are coloured on a continuous scale from red (significantly higher in ASD cases than controls) to violet (no difference between cases and controls).

When comparing child groups (LR, HR-noASD and ASD) for PGS, I found a significant effect of group ($F(2,107)=3.723$, $p=0.027$, $\eta^2=0.084$, **Figure 4.7a**), which was due to a significant difference between HR-noASD and ASD ($p=0.047$) and a nearly significant difference between LR and ASD ($p= 0.069$). There was no significant difference between LR and HR-noASD ($p=0.951$). Moreover, the effect of sex on PGS ($F(1,107)=2.474$, $p= 0.118$, $\eta^2=0.023$) and interaction between group and sex ($F(2,107)=0.342$, $p= 0.711$, $\eta^2=0.006$) were non-significant.

Parents at low risk, parents of multiplex and parents of simplex families were not significantly different for their PGS ($F(1,89)= 0.008$, $p= 0.992$, $\eta^2=0.0002$, **Figure 4.7b**). Moreover, there was no significant difference between mothers and fathers ($F(2,89)=0.502$, $p= 0.480$, $\eta^2=0.006$) nor a significant interaction between familial risk group and sex ($F(2,89)= 1.176$, $p= 0.313$, $\eta^2=0.026$).

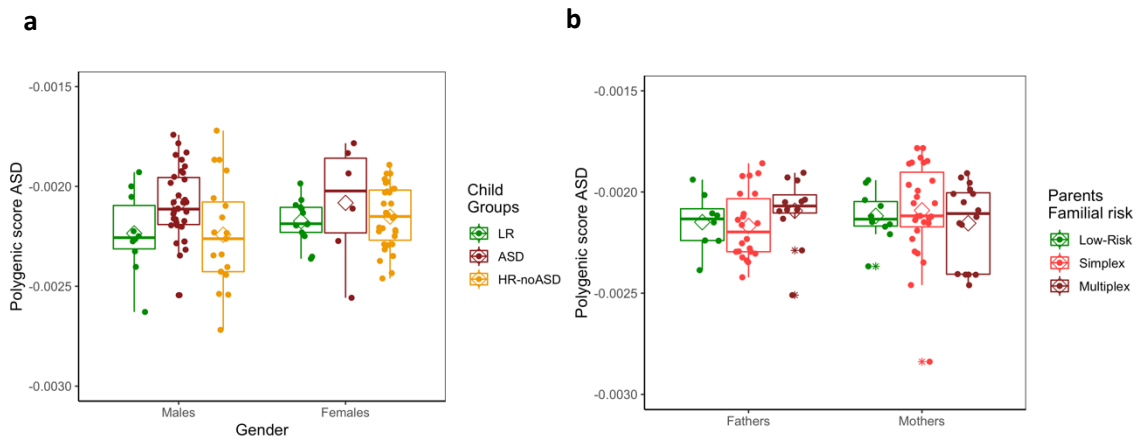

Figure 4.7 *Boxplots showing group differences in polygenic score for ASD.* **a** Boxplots showing polygenic score for ASD in males and females in the three child groups: low-risk (LR), high-risk siblings without a diagnosis of ASD (HR-noASD) and affected probands (ASD). **b** Boxplots showing polygenic score for ASD in fathers and mothers in the three familial risk groups: low-risk, high-risk from simplex families and high-risk from multiplex families. All individual data are represented by points. The lower and upper hinges of the boxplots correspond to the first and third quartiles (the 25th and 75th percentiles). The upper and lower whiskers extend from the hinges to the largest and smallest values, respectively, no further than 1.5 x inter-quartile range. Data beyond the end of the whiskers are plotted individually and represented by stars. Horizontal lines within boxplots indicate the median value, while rhombi represent mean values.

Thus, group analyses revealed that PGS was higher in children with ASD, indicating an increased presence of ASD risk variants in this group compared to non-affected individuals. However, PGS was not different between individuals at LR and family members of people with ASD. There was also no significant difference between males and females.

4.3.2.2 Polygenic score prediction of behavioural measures

The mixed-effects model testing the association between GMT performance and PGS in the entire group (N=208, 63 families) revealed that this was not significant (β=33.35, s.e.=35.25, p=0.346). This relationship was non-significant even when accounting for possible differences between multiple-incidence families, single-incidence families and low-risk families. Surprisingly, a trend towards a positive association was found between GMT and PGS (β=99.20, s.e.= 59.426, p= 0.097, **Figure 4.8a**), meaning that, differently from predictions, higher genetic risk for ASD was associated with better performance at the eye-gaze direction detection task. Adding the interaction between PGS and familial risk did not improve the model fit (χ^2 (9)=3.589, p=0.166, **Table A4.6**), but it revealed that the relationship between GMT and PGS was not positive in the members of the simplex families (β=-97.34, s.e.=73.73, p=0.189). **Table A4.7** reports all the results of the higher order significant model.

When testing the relationship between SRS SCI and PGS (N=207, 54 families), multilevel model fit analysis revealed that introducing PGS as an explanatory variable significantly improved the model fit (χ^2(4)=4.647, p=0.031, **Table A4.8**). In fact, there was a significant positive relationship between SRS SCI scores and PGS, indicating that higher polygenic risk for ASD was associated with more severe social difficulties (β=14843, s.e.=6873, p=0.032, **Figure 4.8b**). There was no significant improvement in the model fit when including familial risk in interaction with PGS (χ^2(8)=0.851, p=0.653). However, the effect of PGS on SRS was no longer significant in the highest order significant models, when taking into consideration differences in autistic social traits between members of families belonging to different familial risk groups (**Table A4.9**). Of interest, PGS was highly associated with RRB (β=17244, s.e.=6579, p=0.010) and the significant effect of such association was retained even when correcting SRS scores for differences in family risk (β=12339, s.e.=6093, p=0.045).

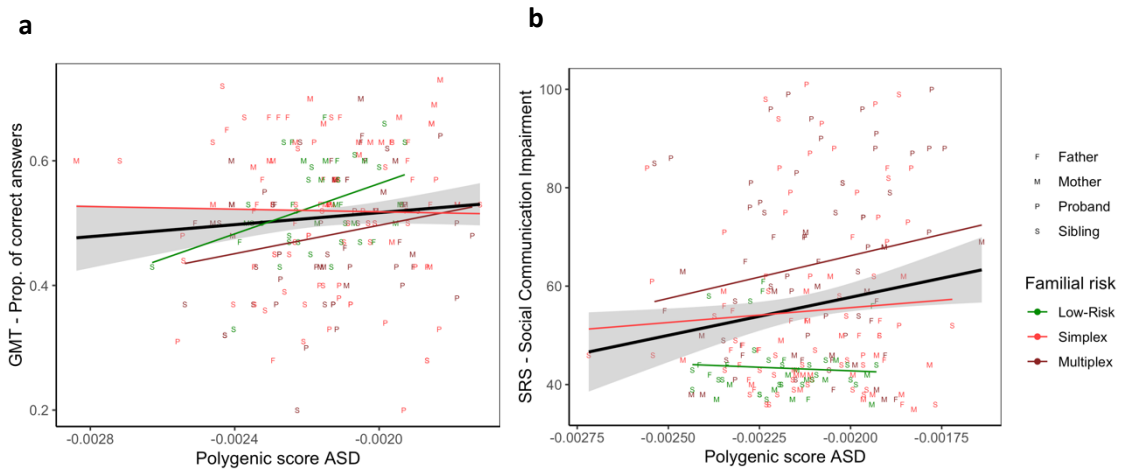

Figure 4.8 *Relationship between polygenic score for ASD and behavioural measures in the entire familial sample.* **a** Relation between polygenic score for ASD, on the x-axis, and performance at the Gaze Monitoring Task (GMT) measured as proportion of correct answers, on the y-axis in the entire gBASIS cohort. **b** Relation between polygenic score for ASD, on the x-axis, and Social Responsiveness Scale, Social Communication Impairment domain scores, on the y-axis, for the entire gBASIS cohort. Small letters represent individual data points for fathers (F), mothers (M), probands (P) and siblings (S), colour-coded by familial risk group: low-risk (green), high-risk from simplex families (scarlet) and high-risk from multiplex families (burgundy). Black lines represent the regression lines between the variables, with grey shaded areas depicting standard errors. Coloured lines represent the regression lines for the low-risk (green), simplex (scarlet) and multiplex (burgundy) families.

In line with the expectations, higher PGS predicted more severe ASD symptoms in the social, and, to a larger extent, non-social domain. On the contrary, PGS was not associated with performance in the eye-gaze monitoring task. Although only descriptively (see **Figures 4.8**), the relationship between PGS and behavioural measures seemed stronger for multiplex families. Unexpectedly, in this group and in the LR group increased genetic risk for ASD was associated with better eye-gaze direction detection.

4.3.3 Familial risk and infant measures of social attention

Tables A4.10-A4.13 show the results of the linear regressions testing the association between parents' measures (GMT, SRS SCI and PGS) and infants' neurocognitive measures of social attention. No relationship survived correction for multiple testing. There was one nominally significant association between maternal autistic social traits and infants' attention engagement with the gazed-at object in the eye-tracking gaze following task administered at 14 months (β=0.003, s.e.=0.001, p=0.024, FDR=0.144). This result was in the opposite direction than expected, and showed that more severe maternal difficulties in the social and communication domain were associated with more attention engagement with the gazed-at object in the infants

(**Figure 4.9a**). Similarly, a trend of association was observed between longer peak look durations at the face stimulus in a face-pop-out task at 14 months and better maternal performances in the GMT (β=2155, s.e.=1166, p=0.071, FDR=0.308). Of note, a negative association between paternal eye-gaze direction detection performance and looking time at the gazed-at object in the infants was observed too, although the effect was non-significant (β=-0.291, s.e.=0.169, p= 0.094, FDR= 0.250, Figure 4.9b). There was no significant association between the neural measures of attention engagement with faces with direct gaze versus non-social stimulus and parental SRS SCI (all p*s*>0.210, FDR*s*>0.917).

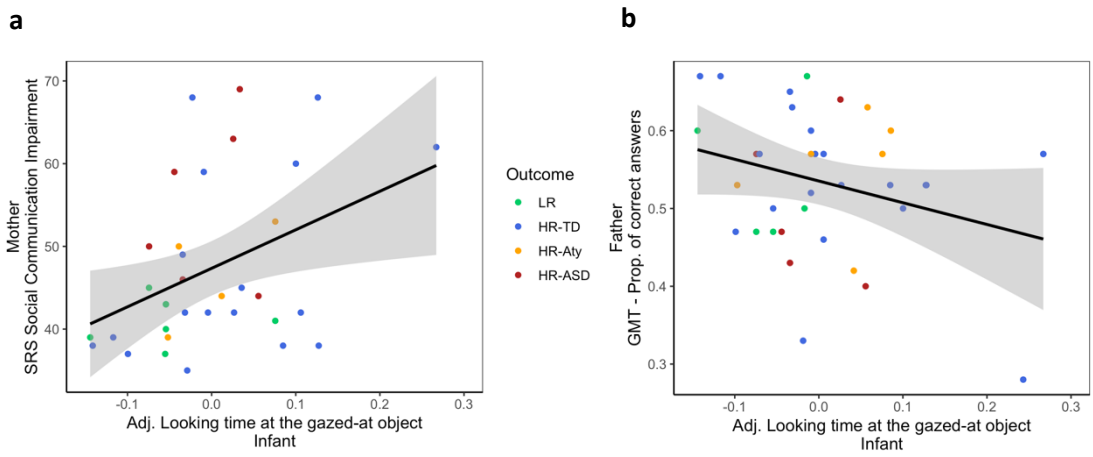

Figure 4.9 *Relationship between parental behaviour and infants' social attention.* **a** Relation between infants looking time at the gazed-at object in an eye-tracking gaze following task administered at 15 months, adjusted for the effect of phase, on the x-axis, and maternal scores at the Social Responsiveness Scale, Social Communication Impairment domain, on the y-axis. **b** Relation between infants looking time at the gazed-at object adjusted for the effect of phase, on the x-axis, and fathers' performance at the Gaze Monitoring Task (GMT) measured as proportion of correct answers, on the y-axis. In both figures, green dots represent low-risk controls (LR), blue high-risk infants with typical development at 3 years (HR-TD), orange high-risk infants with developmental concerns but no ASD traits (HR-Aty), and red high-risk infants who received clinical diagnosis of ASD at 36 months (HR-ASD). Black lines represent regression lines with grey shaded areas depicting standard errors.

4.4 DISCUSSION

In this chapter I aimed to verify some of the requirements for considering social attention an endophenotype of ASD. To do this, I explored the relationship between genotype data and measures of social attention and autistic traits in the social domain in family members of the infant siblings who participated in BASIS. With a series of analyses, I tested the hypothesis that common genetic variants increasing the risk of ASD also contributed to differences in social attention skills, providing evidence for a shared causal mechanism between ASD and social attention. I then evaluated the degree to which parental behavioural traits as well as polygenic loading were associated with early signs of atypical developmental trajectories in the infants, to

understand to what extent familial risk was accounted for by the direct effect of genetics or by direct and indirect contextual environmental components (Jones et al., 2017).

Results revealed that the selected measure of social attention, i.e. the proportion of correct answers in an online task assessing the eye-gaze direction detection (the GMT), was significantly associated with social difficulties in children and fathers (but not in mothers). Additionally, worse performances were observed in children with ASD compared with non-affected children (LR and HR-noASD). Descriptively, lower accuracy in eye-gaze direction detection was also observed in parents of multiplex families, who are considered more likely to carry more genetic risk variants for ASD (Oerlemans et al., 2015; Piven et al., 1997), than parents from simplex families. However, there was no statistically significant difference between HR and LR parents. Thus, this chapter provides mixed findings on whether accuracy in assessing eye-gaze direction detection as measured by the GMT should be considered an endophenotype of ASD. On the one hand, results of the analyses from the child sample confirmed that this measure of social attention met one of the tested requirements for being considered an endophenotype of ASD (see **Table 1.1** and **section 1.3.3**), namely "Within families, endophenotype and illness co-segregate". On the other hand, it did not meet another of the requirements, stating that "the endophenotype is found in non-affected family members at a higher rate than in the general population" (Gottesman & Gould, 2003).

I also found that HR girls and mothers performed better at the GMT than HR boys and fathers, respectively. Enhanced social attention skills of HR-noASD versus LR girls (though non-significant) and in sHR versus LR mothers (significant) were also observed. Thus, this finding is in agreement with the hypothesis of a protective value of social attention against familial risk. Of note, female girls performed generally better in the GMT than boys and mothers showed less social difficulties than fathers, possibly reflecting the effect of sex-specific protective factors.

GMT performance was not associated with ASD PGS, although an increased polygenic risk for ASD predicted more severe social communication difficulties. Thus, I found no evidence for an impact of common genetic variants in aggregation on eye-gaze direction detection. Of note, both social attention skills and ASD polygenic score were associated with autistic social traits. There was no evidence for a significant effect of parental genetic and behavioural loading on infants' neurocognitive correlates of social attention.

4.4.1 A Broader Social Attention Phenotype?

Practical as well as theoretical reasons discussed at the beginning of this chapter (**section 4.1.4**) guided the choice of using the online version of the GMT as a measure of social attention for this study. However, this measure captures a very specific ability, which is the detection of subtle changes in eye-gaze direction. Although inaccurate eye-gaze direction detection has been documented in people with ASD (see Forgeot D'Arc et al., 2017 for a review), this reflects only a minimal component of the social attention impairments documented in the ASD literature (Salley & Colombo, 2016). One preliminary step was, therefore, to evaluate whether this measure collected from the gBASIS participants did reflect the expected pattern of atypicality in children with ASD and their relatives at high familial risk.

4.4.1.1 Aspects of gaze processing

In line with the expectations, I found that children with ASD had lower performances in the GMT compared with LR controls and HR siblings without a diagnosis of ASD. However, this finding could be confounded by the fact that individuals with ASD could on average lower IQ. A measure of IQ was not included in the present design, as the available online versions of IQ assessment would have required the same or higher level of compliance and attention skills as the GMT, thus eliminating part of the phenotypic variability we were interested in. Additionally, this request would have risked to significantly overburden the gBASIS families. Inevitably, the GMT could only be performed by children with a minimal level of compliance and understanding, despite the fact that there was the possibility for non-verbal participants to be helped by their parents in understanding the task. Thus, individuals with severe cognitive impairment were naturally excluded.

Importantly, Skuse et al. (2014) found no correlation between full-scale IQ measured with the British Picture Vocabulary Scale, the Wechsler Abbreviated Scale of Intelligence, or the Wechsler Intelligence Scale for Children–Third and Fourth Editions and the GMT in 103 probands aged 4.5 to 20 years of age. Based on their result, the obtained pattern is less likely to be just driven by differences in IQ between groups. Further, the HR-noASD group included children who might have shown signs of developmental delay, low cognitive abilities or sub-threshold autistic traits, such as the BASIS infant siblings who were classified as HR-Aty at the three years follow-up visit (as explained in **section 2.1.1**). If IQ influenced the GMT performance, an effect on the HR-noASD would be observed too, perhaps leading to a less biased estimate of the ASD group only. The lack of a measure of IQ ability is acknowledged as a limitation of the present design although it

is plausible that the inclusion in the analyses of covariates such as age at the time of testing and whether the children received parental help to understand the instructions of the task might have accounted for part of the difference which would have been captured by a measure of IQ.

Of note, there was a highly significant effect of age on the children's score, indicating that different factors, some of which developmental, might have contributed to the GMT performance. Pantelis & Kennedy (2017) showed that eye-gaze direction detection ability, impaired in most individuals with ASD, is not one unique ability, but rather can be unpacked into a variety of fundamental components. They found that inaccurate detection of gaze direction toward a visual cue can arise from disruption of idiosyncratic aspects of gaze processing for different subsets of individuals with ASD. For example, some people can show noisier (i.e. more imprecise) processing of the sensory input, others have a mis-calibrated perception of the direction of the gaze direction, others rely more on salience of the gazed-at stimulus or on the expectations of the direction of gaze based on prior experience (Pantelis & Kennedy, 2017). As in Pantelis & Kennedy (2017), I also found individuals with ASD who did not show particular impairment in this task. In fact, the noticeable overlap of the groups' distributions of GMT scores (see **Figure A4.3**) suggests that the ability measured by this task was not completely disrupted in children with ASD.

In sum, although the observed results are in line with the expectations, this measure of social attention is possibly limited in terms of its ability to capture the various aspects of social attention assessed in the infants' literature. Moreover, test-retest reliability assessments were not performed to evaluate how stable the performance was within the same individual (although test-retest reliability and validity of the construct were confirmed by Skuse et al., 2005).

4.4.1.2 Familial risk

The fact that HR relatives did not perform significantly differently from LR participants in this task is in agreement with the results obtained by Skuse and colleagues (2014). In line with the idea of social attention as part of BAP, I found that HR siblings performed slightly worse than LR children, and that parents from mHR families, who are considered more likely to carry ASD risk genetic variants than parents from sHR and LR families (Piven et al., 1997), performed worse than sHR parents. Unexpectedly, LR parents showed similar performances as mHR, i.e. lower accuracy than sHR. This result might be due to selection bias for the small sample of LR parents who took part in the study. However, analyses on the SRS, which came largely from the same participants, showed that indeed the sample of LR parents participating in gBASIS had lower social communication impairment than the HR parents. Thus, the small sample of LR parents

does not seem to have unexpected high levels of autistic traits. A plan for the next future is to enlarge the sample of control parents to verify whether this result indicates a true finding, possibly pointing towards the idea of a protective role of social attention in sHR families.

4.4.1.3 Attention to details

The significant association between GMT performance and SRS SCI in both child and parent cohorts suggests that eye-gaze direction detection might reflect some aspects of the BAP. Interestingly, GMT performance was also associated with restricted and repetitive behaviors in both cohorts, although to a lesser extent. This indicates that this measure of social attention did not completely reflect differences in social skills. An interesting finding emerged from the analysis observing the relationship between GMT and PGS. Differently from the expectations, a trend towards a positive association between the two variables was observed, especially for the LR and mHR groups (**Figure 4.8a**), indicating that the presence of ASD genetic risk factors possibly increased performances in the detection of eye-gaze direction. Interestingly, PGS was highly associated with the RRB domain of the SRS, in line with the idea that genetic risk might increase vulnerability especially at the level of sensory processing skills (Elsabbagh & Johnson, 2016; Piven, Elison, & Zylka, 2017). Thus, if any genetic effect was observed on the selected measure of social attention, this was likely to influence the visual perception components contributing to GMT performance, such that enhanced attention to details was possibly advantageous in this task (Baron-Cohen, Ashwin, Ashwin, Tavassoli, & Chakrabarti, 2009).

4.4.2 Sex-specific effects

It has been argued that female individuals may require additional disorder burden to cross the threshold for ASD diagnosis (Szatmari, 2018; Werling & Geschwind, 2015). Accordingly, Frazier et al. (2015) found increased autistic traits in next-born male siblings from mHR female ASD-containing families, supporting the idea that female ASD-containing families may be at higher recurrence risk. Moreover, the fact that non-ASD females showed less impairment in social responsiveness than non-ASD males was interpreted as possibly reflecting the presence of protective factors in females from mHR families (Frazier et al., 2015). In the present study, I found that sHR mothers had enhanced accuracy in gaze direction detection than LR mother. Most interestingly, I found that HR-noASD girls performed better in the eye-gaze direction detection task than HR-noASD boys, while the opposite effect was observed in the ASD group. Although HR-noASD females had higher mean accuracy scores in the GMT than LR females, this

result was not significant. Given that the association between GMT performance and SRS was not different between boys and girls, better social attention performances during childhood/adolescence in girls might indeed reflect a sex-specific protective mechanism. In this section I discuss the present results in light of possible mechanisms that might underpin sex differences in social attention skills. I report, as an example, on mechanisms involving oxytocin, which have been studied for its sex-specific effects and previously associated with social attention.

No sex differences were observed in polygenic scores for ASD. This indicates that the possible female protective factor reflected in better social attention performance in females was not strongly deriving from the cumulative effect of common genetic variants. However, it is possible that individual genetic risk variants, whose effect is not detectable with a PGS approach, can be predominantly penetrant in males, such that if such variants are carried by females they do not manifest the phenotype as much as male carriers do (Werling & Geschwind, 2015). The present analysis is incomplete to rule out this possibility as I did not examine individual effects of genotype at single loci.

One candidate gene for this analysis would have been the OXTR gene, as GMT performance was shown to be nominally associated with a polymorphism located in this gene (Skuse et al., 2014). The oxytocin receptor (OXTR) gene is located on chromosome 3 and its functional SNPs have been repeatedly associated with increased risk for ASD (Vanya, Szucs, Vetro, & Bartfai, 2017). In fact, OXTR genetic variation, which regulates the number, organization, or functioning of oxytocin-receptors, has been suggested to influence the efficacy of the oxytocin signal in the brain (Yamasue, 2013), with cascading effect on the development of social attention. From findings on OXTR expression in primates, Freeman & Young (2016) suggested that oxytocin could mediate some aspects of the changes in eye movements and shifts in visual attention in response to social cues. Given that genetic data is available for the present cohort, one tempting possible future direction would be to observe the effect of individual OXTR SNPs in the present cohort, to test whether it is specifically associated with GMT in males and not in females. However, the current study is likely to be underpowered to find such effect, given that Skuse et al. (2014) could not perform powered analyses for sex differences in a sample of 112 families, and only 63 families provided GMT data as part of gBASIS.

4.4.3 Genetic risk in multiplex families

PGS is calculated for each individual to investigate cumulative influences of alleles which have been demonstrated to be more common in ASD cases than controls on a phenotype. Heritability estimates from large adult and children populations revealed that the combined effect of SNPs explains no more than 2.5% of variation in ASD (Grove et al., 2019) and autistic traits (St Pourcain et al., 2018). Reassuringly, the result of the ASD PGS calculation (Nagelkerke R^2=0.249) was in line with these previous findings. Despite the relatively small sample, a PGS significantly able to distinguish between cases and controls in the gBASIS population was obtained. However, the so-constructed PGS was not associated with the candidate endophenotype representing social attention skills.

Figure 4.8b graphically represents the results of the mixed effect model where I investigated whether the effect of polygenic score on ASD social symptoms was different based on familial risk. Using random effects, I allowed the model to set a random intercept for each family, therefore accounting for possible between-families differences which might have confounded the results (such as, for example, environmental influences which are specific for each family). Introducing the interaction between PGS and familial risk as fixed effects, I tested whether such association was specific for one group of families. A stronger effect of PGS on phenotypes was expected for mHR families while ASD in sHR families is more likely to be due to de novo genetic variations, which only affect one individual and are by definition not inherited from parents (Iossifov et al., 2014; Leppa et al., 2016). Although a trend toward a stronger effect of PGS on behavioural measures in mHR families can be noticed on **Figure 4.8**, this was not significantly significant[2]. This null result might be due to the combination of the small sample size for this analysis and the low explanatory power of ASD PGS (Grove et al., 2019). However, the lack of statistically significant difference in the association between PGS and autistic traits between sHR and mHR is in line with recent findings showing that common genetic variants contribute to variability in phenotypic manifestation to the same extent in individuals with and without rare variants of large effects (Niemi et al., 2018).

[2] Control analyses examining the interaction between family position and PGS in determining the social phenotype revealed that there was no stronger association in probands, mothers, fathers or siblings. These results do not provide reliable information to the study as they were likely to be underpowered. Therefore, they have not been reported in the thesis.

Although they reported a significantly lower rate of de novo events in mHR than in sHR families, Leppa et al. (2016) found that a higher burden of large, rare copy number variants (CNVs), including inherited events, was observed in individuals with ASD from multiplex families compared with their unaffected siblings. Thus, a large proportion of genetic risk which characterise multiplex families is not accounted for by PGS, which only considers risk factors from common genetic variants. Moreover, a recent study by Brandler et al. (2018) revealed that rare genetic structural variants (i.e. multi-allelic CNVs, deletions, duplications, insertions, and inversions, Sudmant et al., 2015), which are enriched in individuals with ASD compared with their unaffected siblings, are often inherited from the father. Integrating information on de novo and inherited variants to this analysis could provide novel insights on the genetic architecture of social attention and autism traits.

4.4.4 Complexity of environmental influences

One potential approach to investigate whether infant measures of social attention can be considered endophenotypes of ASD is to relate them to familial risk, as estimated from their parents' genetic and behavioural burden for ASD liability (Jones et al., 2017). Possibly for a combination of both limited power and reduced role of polygenic factors from common variants in determining familial risk, no association was found in the current study between parental PGS and infant measures. As pointed out in **section 4.2.4**, the initial idea for the analyses investigating the role of parental genetic and behavioural loading for ASD on children early social attention skills was to combine all parental measures in one model, to estimate the combined and independent effect of contextual influences and genetic factors. However, the plan for the analysis was revised based on the limited number of observations which would have resulted if only trios with no missing data in all variables were retained. I choose to evaluate single relationships between pairs of variables to have a first insight on relevant associations. Surprisingly, no significant association was observed after correcting p-values for multiple testing.

The analysis investigating an effect of parental polygenic score on infants' attention was probably underpowered. A recent study found an effect of PGS for educational attainment on parenting style in a sample of ~630 individuals (β=0.12-0.16) thus it is likely that a larger sample is needed for this type of studies (Wertz et al., 2019). For parents' phenotypic measures, the non-significant relationship with the infants' measures could be due to the fact that the latter were collected years later, so they might not properly reflect parental attitude experienced by

offspring in the first years of life. However, under the assumption that these measures are proxy for traits, they should not drastically change with time. Of note, both GMT and SRS had good test-retest reliability in adult populations (Bruni, 2014; Skuse et al., 2005), so multiple measurements from the same person are not expected to be very different from each other. On the other hand, it is worth noting that stress and adaptive behaviours of the parents in response to the emergence of ASD in their children might have contributed to increase the intra-individual variability in traits and parenting behaviours (Crowell, Keluskar, & Gorecki, 2019). This aspect is not measured in this design and this constitutes a limitation of the current study.

4.4.4.1 Effects of parental traits

A less conservative approach allows me to discuss potential pathways for the effects of parental phenotypes on infants' social attention, although the discussed results must be considered with caution given their limited statistical power. While there was no effect of parental genetic liability on the infants' measures, there was suggestive evidence for a trend of association between lower eye-gaze direction detection ability in the fathers and worse attention engagement with the gazed-at object in their 14-month-old children. This association is in line with findings from previous reports, indicating on the one hand lower gaze following abilities in fathers from HR families (Adolphs et al., 2008; Scheeren & Stauder, 2008), and on the other hand an effect of parental social skills on early social attention engagement and orienting skills (Jones et al., 2017; Ronconi et al., 2014). One way in which paternal social attention skills, but not PGS, might have influenced infants' attention is through an indirect effect of the parental genotype. Kong and colleagues (2018) modelled this phenomenon, that they called 'genetic nurture', based on a population familial sample. They found that a polygenic contribution of the non-transmitted alleles explained a small but significant proportion of individuals' phenotypic traits, including educational attainment, smoking behaviour and a range of physical and health characteristics. They also argued that an additional amount of variance in the individuals' phenotype is explained by assortative mating, which enhances the nurturing effects of specific genetic variants in the parents even when non transmitted (Kong et al., 2018). Indeed, the fact that assortative mating increases the contribution of additive genetic variance as well as explains genetic comorbidity in some psychiatric disorders such as ASD, ADHD and schizophrenia has been recently acknowledged (Plomin, Krapohl, & Reilly, 2016). In the present study, I found little evidence for correlation between social attention skills, autism social traits and polygenic score between fathers and mothers of the same families (**Figure A4.4**), although this analysis was based on a very small sample (23 couples). Nevertheless, this does not exclude the possibility that the relationship between parental phenotype and infants' social attention skills reflects

genetically-driven effects on environmental factors contributing to developmental trajectories (Crowell et al., 2019; Jones et al., 2017).

This account might help to explain the nominally significant positive association found between maternal social communication impairment and infants' engagement with the object, measured as proportion of looking time at the object in a gaze following task administered at 14 months. This result is surprising as enhanced, and not reduced, responsiveness in the mother was expected to be associated with better social attention skills in infants based on previous findings (Crowell et al., 2019; Elsabbagh et al., 2014; Jones et al., 2017; Mc Donald, Baker, & Messinger, 2016). Other factors mediating infant's susceptibility to maternal behaviour might have contributed to the observed results, possibly involving different susceptibility of the child to maternal attitude (Belsky et al., 2015; Brune, 2012). More simply, it is also possible that infants with less socially engaged mothers become more engaged with the object either as a behaviour learned through experiencing interactions where the attention of the adult is directed preferably to objects, or as a resilient strategy to exploit as much as possible the more limited opportunities for learning in situations of interaction with the mother. Further research in larger samples is needed to understand to what extent the observed results are reliable.

4.4.5 Limitations and future directions

A limitation of the current study is certainly that only 33% of the HR families returned questionnaire data for gBASIS, only 37% of them completed the online GMT task and 49% of them provided DNA samples for genetic analyses. Only 27% of the LR families provided questionnaire, GMT and genetic data for gBASIS. This study sample is unlikely to be representative of the population and even of the original BASIS sample itself. For discussion of the 'selection bias' issue across this thesis the reader is invited to refer to **Chapter 7** (**section 7.3.1**). As a consequence of the relatively unsuccessful data collection for gBASIS, not only the generalizability of the results should be questioned, but also the power of all analyses resulted substantially limited compared to the initial plan. Descriptively, some of the reasons for not returning the questionnaires and/or DNA sampled reported by families during a dissemination event held when data collection was ended, were: that they felt overwhelmed by the quantity of paperwork requested, that they had misplaced the questionnaires or the saliva kits, that they forgot and would have liked to receive several reminders or that they had not fully appreciated the importance of their participation in the research. Future research should take into account this important feedback. Public events aimed to motivate the potential participants before recruitment, as well as strategies to disseminate the results and keep the families engaged and

informed should be programmed if there is any plan to contact them again in the future (Abshire et al., 2017).

A further limitation of the present study is that ASD status, relevant for the child group analyses and polygenic score calculation performed in this chapter, was assigned using different approaches for the individuals involved in the study. On the one hand, for parents and older siblings a parent-report interview was used and combined with information recorded at the time of initial enrolment in BASIS. At the time of gBASIS data collection (namely three to nine years later), parents were requested to provide information on any medical or demographic information update concerning the family members. Notably, this approach, relying on parent report, could have led to imprecise classification of individuals into cases and controls. On the other hand, diagnostic assessment at the BASIS 3-year visit was used to categorise HR children who participated in BASIS as infant siblings (aged between four and ten at the time of data collection for this study). Of interest, a Baby Siblings Research Consortium study, including a subset of the BASIS participants (Phase 1), revealed that a minority of infants who were not diagnosed with ASD at the 3-year visit received a diagnosis of ASD by the same teams of research clinicians when assessed after 5 years of age (Ozonoff et al., 2018). A mid-childhood assessment is ongoing for the Phase 2 children who participated in BASIS. Once all children included in this study will receive the mid-childhood diagnostic assessment, it would be interesting to evaluate whether HR siblings with a late diagnosis, who were necessarily considered in the HR-noASD group in the present study, showed a polygenic score and social attention profile more similar to the ASD group.

As discussed above (**section 4.4.4**), the reduced sample size for the analysis of the association between parental measures and infant social attention questions whether non-significant results emerged due to lack of power or as a null result. Although it is important to recognise that larger samples would have provided more robust results, it should also be acknowledged that the present data simply do not provide any evidence for an association between infants' neurocognitive measures (Nc mean amplitude and microstate 4 duration difference between FD and Noise, peak look at the face in the face pop-out task and proportion of looking time at the gazed-at object in the gaze-following eye-tracking task) and parents' polygenic score, accuracy in eye-gaze direction detection and socio-communication difficulties. Researchers should be encouraged by these considerations to direct resources towards future investigations where the number of participants required for well-powered analyses can be estimated in advance, building on effect sizes of results from large samples (Fearon, 2019). This would allow them to prioritise studies that will be informative in the case of both a significant and a non-significant novel finding.

4.5 SUMMARY OF FINDINGS

Overall, no evidence was found supporting a direct or indirect (through parental characteristics) effect of polygenic risk for ASD on the infants' neurocognitive measures. The component of social attention tested in the present study, namely the accuracy in evaluating small variations in eye-gaze direction, is associated with socio-communication difficulties in children with and without ASD, their siblings and their fathers. However, it cannot be fully considered an endophenotype of ASD as non-affected relatives of children with ASD do not show worse performance than LR controls. In the next two chapters I focus on investigating direct polygenic and epigenetic effects on social attention in the first two years of life.

CHAPTER 5

SIGNS OF SOCIAL ATTENTION ATYPICALITY AS RISK MARKERS OF NEURODEVELOPMENTAL DISORDERS

5.1 INTRODUCTION

To understand mechanisms underlying the emergence of neurodevelopmental disorders and co-occurring conditions, we need to identify processes that mediate between genetic risk and later symptoms (Johnson & Pasco Fearon, 2011). The present thesis so far focused on social attention as a candidate mechanism for ASD specifically. In **Chapter 2** I observed that atypicalities in engaging attentive brain states when looking at social and non-social stimuli precede the onset of ASD and dimensional social outcome. In **Chapter 3** I reported that these neural atypicalities in the first year of life and reduced attention engagement with the referent object in joint attention situations in the second year are related and both contribute to the pathway to autistic symptoms and social traits. Another behavioural feature reflecting social attention, the duration of the longest look at a static face picture among other non-social stimuli, was predictive of later communication skills. These findings provided evidence for considering measures of social attention atypicalities as early markers of ASD traits. Results of the study described in **Chapter 4**, which examined social attention skills in a familial sample, demonstrated that, although these are associated primarily with difficulties in the social domain of ASD, they might be influenced by genetic factors which have an effect on domain-general attentional functions.

Problems in attention have been proposed as a shared endophenotype of ASD and ADHD (Amso & Scerif, 2015). In fact, shared genetic factors have been found to underlie difficulties in executive function such as inattention and attentional switching capacity in adults and children with co-occurrent signs of ASD and ADHD (Polderman et al., 2013; Sinzig, Morsch, Bruning, Schmidt, & Lehmkuhl, 2008). In infants, attenuated decline in peak look duration to faces from 8 to 14 months was found to be associated with lower effortful control at 3 years of age, suggesting that executive attention could be underpinning looking behaviour in a face pop-out task (Hendry et al., 2018). Indeed, more attentional control could be required to selectively orient away from the face towards competing non-social stimuli. In the present chapter I questioned the specificity of the contribution of social attention to the development of ASD traits by looking at peak look duration in a face pop-out task in relation with genetic and familial

loading as well as later dimensional traits of ASD and ADHD. I conducted the same analyses on peak look duration in response to social and non-social stimuli, to understand whether the observed relations were specific to the social content of the stimulus. These analyses were intended to make a step forward in the attempt to disentangle general mechanisms underlying the emergence of neurodevelopmental disorders and specific effects of early difficulties in social attention in the path to ASD.

5.1.1 Developmental roles of early markers

Behavioural symptoms of ASD start to emerge during the first years of life (American Psychiatric Association, 2013). As previously mentioned, prospective longitudinal studies of infants at high familial risk have identified a series of early signs which can be detected before the emergence of core symptoms and are predictive of later diagnostic outcome (Jones, Gliga, Bedford, Charman, & Johnson, 2014; Szatmari et al., 2016). These neural or behavioural indicators that predict change across the trajectory are defined "risk markers" of neurodevelopmental disorders (definition from the Research Domain Criteria Strategic Plan project of the National Institute of Mental Health, https://www.nimh.nih.gov/about/strategic-planning-reports/index.shtml). However, the role of these early signs in the path to later cognitive and affective traits might be different for different markers. In fact, while some of them indeed reflect vulnerabilities related to genetic or environmental risk factors, others can be manifestations of compensatory processes or secondary effects deriving from atypical interactions with the environment (Johnson, Gliga, Jones, & Charman, 2014). In this chapter, I will refer to these as "early markers", to highlight the fact that these measurements are thought to reflect phenotypes that emerge before a clinical diagnosis is made.

Social attention, defined as the allocation of attentional resources to conspecifics (Salley & Colombo, 2016), is a putative early marker of ASD. In fact, as reviewed in the general introduction of this thesis (see **Table 1.1**), studies on infant siblings at risk for and toddlers with ASD confirmed that, since the beginning of the second year of life, atypical behavioural correlates of social attention engagement can be detected in children who go on developing the core symptoms of this disorder. The majority of the studies on social attention skills measured with eye-tracking indicate that individuals with ASD demonstrate decreased looking time to social stimuli (usually faces) compared with controls when dynamic stimuli were presented (Chita-Tegmark, 2016). Of interest, while orienting behaviours towards static face pictures are not different between infants with later ASD and typically developing participants (Elsabbagh et al., 2012), when measuring fixation duration to faces, studies consistently found greater looking

duration in individuals who go on to develop ASD (Elsabbagh et al., 2012; Guillon, Hadjikhani, Baduel, & Rogé, 2014; Hendry et al., 2018). As discussed in **Chapter 3**, genetically driven atypicalities at the brain level might result in domain-general problems which affect sensory processing and attentional control (Gliga, Jones, Bedford, Charman, & Johnson, 2014). Thus, anomalies in looking behaviour reflecting attention engagement to social stimuli are candidate markers for ASD, lying in the path from risk factors to later outcome.

Importantly, atypicalities in the domain of attention constitute the core symptoms of another neurodevelopmental disorder, the Attention Deficit and Hyperactivity Disorder or ADHD. ADHD is a common neurodevelopmental disorder which affects between 5 and 15% of the general population (Rowland et al., 2015). It is defined through clinical observation of the emergence, typically before 6 years of age, of two types of behavioural problems: inattentiveness and/or hyperactivity and impulsiveness (American Psychiatric Association, 2013). Inattentiveness refers broadly to a phenotype which includes enhanced distractibility, difficulties in sustained attention, forgetfulness, poor planning and disorganization; the hyperactivity/impulsiveness behavioural phenotype is characterised by the inability to sit still or wait, excessive physical movement and fidgeting, little or no sense of danger. ADHD, as ASD, is highly heritable (h^2=0.88 estimated from twin studies, Larsson, Chang, D-Onofrio, & Lichtenstein, 2014) and first-degree relatives of people with ADHD are at increased risk of developing the disorder themselves, compared to the general population (odds ratios ranging between 11.4 and 13.5, Hidalgo-Lopez, Gomez-Alzate, Garcia-Valencia, & Palacio-Ortiz, 2019; Miller et al., 2019). Heritability accounted for by common genetic variance is estimated to be around 0.44 for inattention, 0.55 hyperactivity/impulsivity and 0.59 for total ADHD, with high genetic correlation between the two core domains (rho=0.86, Cinnamon Bidwell et al., 2017). Substantial overlap has been found between ADHD and other neurodevelopmental condition, in particular ASD. Importantly, between 20 and 50% of children who have received a diagnosis of ADHD also meet criteria for ASD and 30-80% of children who have been diagnosed with ASD also meet criteria for ADHD, indicating that common causal mechanisms might underlie behavioural manifestations of the two disorders (Rommelse, Franke, Geurts, Hartman, & Buitelaar, 2010).

5.1.2 Models for the development of ASD and ADHD traits

Symptoms of both ADHD and ASD likely result from a complex interaction between emerging neurodevelopmental vulnerabilities and aspects of the child's prenatal and postnatal environment (Geschwind, 2011; Thapar, Cooper, Eyre, & Langley, 2013). Twin studies estimated that 50-72% of the phenotypic similarity between ASD and ADHD cases might be due to shared

additive genetic factors (Rommelse et al., 2010). However, specific genetic and environmental influences have been found for the two disorders, suggesting that they are etiologically distinct and that co-occurrence of features, rather than complete comorbidity, is often observed in individuals who show both symptoms of ASD and ADHD (Ronald, Larsson, Anckarsater, & Lichtenstein, 2014). Molecular studies are in agreement with this account, as little overlap in genetic risk coming from common variants has been recently reported between diagnostic groups in the clinical population (The Brainstorm Consortium, 2018) and between disorders traits in the general population (Stergiakouli et al., 2017).

Differently, familial studies indicate that family members of people with ASD show an increased level of ADHD symptoms, compared to relatives of individuals with no neurodevelopmental disorder (Chien et al., 2017). This suggests that familial risk factors are likely to have a general effect on ASD and ADHD symptomatology, possibly due to shared attention-related problems linked to pleiotropic risk factors underlying attention and social difficulties (Sokolova et al., 2017). Thus, different genetic background might result in similar adaptive behavioural manifestations. Evaluating whether the pathway towards the emergence of behavioural traits is also condition-specific could not only shed light on the biological mechanisms underlying psychopathology but also inform on potential targets for effective intervention in populations at risk.

Preliminary work studying early derailments from typical developmental trajectories suggests that the co-occurrence of ASD and ADHD might be either due to the co-presence of condition-specific risk factors, or to common risk factors which lead to comorbid ASD and ADHD. Johnson et al. (2014) hypothesized four possible models for the distinct and shared mechanisms leading to the emergence of ASD and/or ADHD (see **Figure 5.1**). (A) The presence of condition-specific risk factors facilitates the emergence of condition-specific markers which precede core symptomatology of each of the two disorders. In this model, comorbidity emerges as a result of the effect of risk factors for both neurodevelopmental disorders or as a result of a specific set of markers which lead to the comorbid phenotype (B) Early markers are generally associated with symptoms of neurodevelopmental disorders and are caused by the combination of common and condition-specific risk factors. (C) Common risk factors generate common adaptive processes which, depending on the developmental periods when they are activated, produce condition-specific outcomes. (D) Risk factors for ASD and ADHD are condition-specific and so are early markers, but the relation between these and future outcome depends on the presence of general protective factors. Thus, ASD, ADHD and overlapping symptomatology might emerge based on the interaction with a domain-general protective factor.

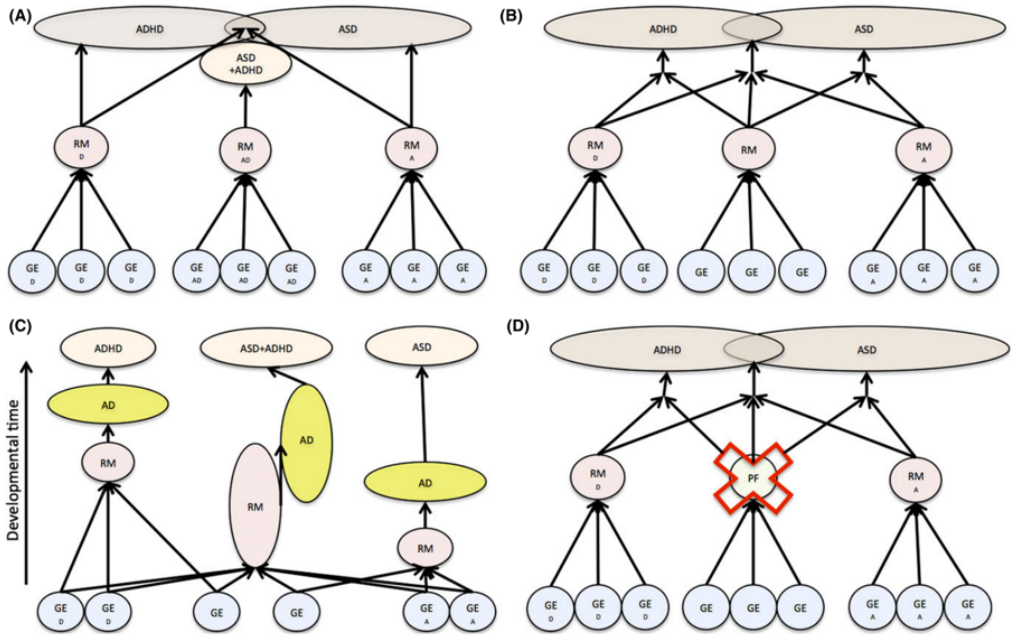

Figure 5.1 *Four possible models of the role of early markers in the developmental pathway of ASD and ADHD*, reproduced with permission from Johnson et al. (2014). Key: RM = Risk Marker; PF = Protective Factor; A = ASD; D = ADHD; AD = Adaptive response. GE = genetic and/or environmental risk factors.

As repeatedly mentioned in this thesis, early atypicalities in social attention could reflect difficulties in engaging with social stimuli specifically or difficulties in visual attention more generally. Early markers can be manifestations of distinct versus overlapping mechanisms underlying ASD and ADHD. Observing whether they precede the emergence of symptomatology of one neurodevelopmental disorder specifically or whether they reflect aspects of developmental paths that are shared between disorders might inform prognostic evaluations and approach to treatment (Johnson, 2017; Thapar, Cooper, & Rutter, 2016).

5.1.3 Aims of the study

The present chapter aimed to understand whether early signs of social attention atypicalities are specifically part of the causal pathway to ASD versus general neurodevelopmental issues through dissecting effects of ASD and ADHD at different levels: familial burden (symptoms in the older sibling with a formal diagnosis, defined as “the proband”); genetics; and later symptoms

in the child. Specifically, I tested whether longer peak look durations to faces lie between risk factors and neurodevelopmental outcome by looking at: 1) familial risk-group difference; 2) their association with quantitative familial risk for ASD and ADHD (probands' traits); 3) their association with polygenic scores; 4) their association with later ASD and ADHD dimensional symptoms.

Figure 5.2 depicts the four study hypotheses tested in the present chapters, based on the risk marker models conceptualized by Johnson et al. (2014) describe above. The analyses conducted in this study where intended to verify whether the relationship between risk factors, outcome and the selected early markers (peak look at the face and non-face stimuli in a face pop-out array) resemble more closely one of the illustrated pathways.

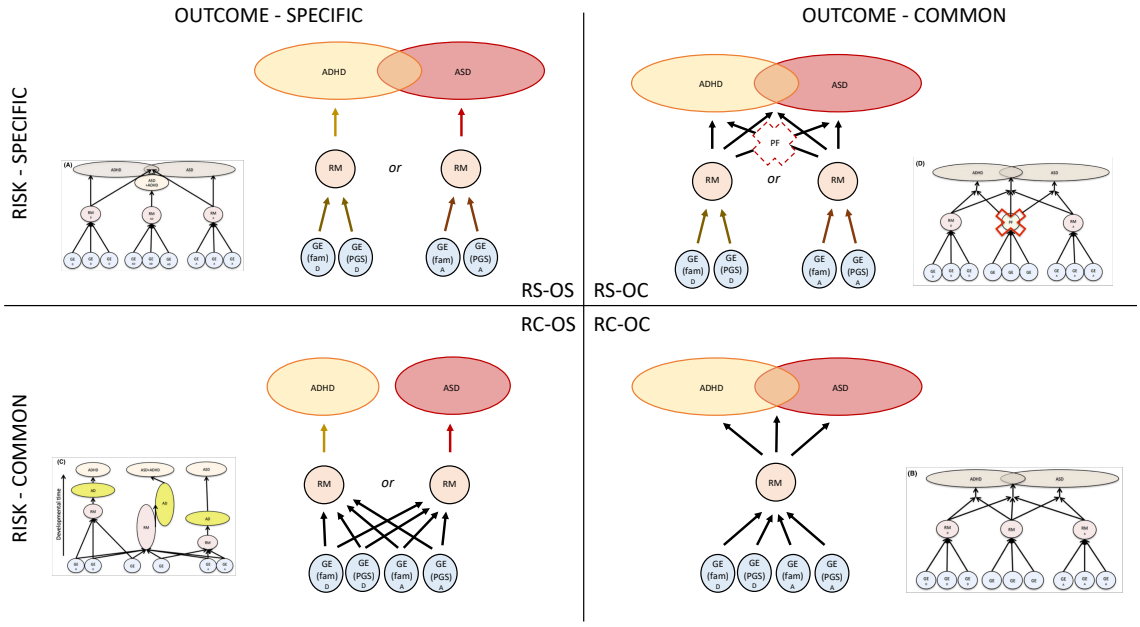

Figure 5.2 *Four possible models of the role of early markers in the developmental pathway from risk factors to behavioural symptoms of ASD and ADHD*. Light blue circles indicate genetic and/or environmental risk factors (GE), evaluated in their familial (fam, or older sibling's behavioural traits) and polygenic (PGS or polygenic score) specific contribution for ASD (A) and ADHD (D). Pink circles indicate the early risk marker (RM), which in this study corresponds to the peak look duration at the face or at the non-face stimuli in a face pop-out array, measured with eye-tracking technology at approximately 14 months of age. The yellow ellipse indicates ADHD dimensional traits, while the red ellipse indicates ASD dimensional traits. PF indicates possible protective factors. The figure is divided into four quadrants: the two at the top illustrate situations in which condition-specific risk factors predict variation in the early marker (risk-specific, RS) while the two at the bottom illustrate situations in which the early marker is predicted by common risk factors (risk-common, RC). The two models in the left-hand part of the figure illustrate situations in which the early marker predicts later symptoms of only one of the two neurodevelopmental conditions (outcome-specific, OS) while the models in the right-hand part illustrate situations in which the early marker predicts variation in dimensional domains of both ASD and ADHD (outcome-common, OC). Models A, B, C and D reported in the external parts of the figure are reproduced from Johnson et al. (2014) and represent the authors' original conceptualization.

According to two of the possible pathways, RS-OS and RS-OC, the early marker would be predicted by condition-specific genetic (polygenic score) and familial (older siblings' traits) risk factors. Therefore, under these models, risk factors either for ASD or for ADHD would be associated with the infants' looking behaviour. The difference between these two models is that, while in RS-OS the early marker would predict dimensional variation of one of the two neurodevelopmental disorders traits and not the other (outcome-specific), according to the RS-OC model it is possible that the condition-specific marker is predictive of both ASD and ADHD traits, depending on the effect of a general protective factor. Models RC-OS and RC-OC would be confirmed if the attention marker is predicted by genetic and/or familial risk factors for both neurodevelopmental disorders (risk – common). While in model RC-OS the risk marker is expected to predict only one of the two conditions (outcome-specific), in model RC-OC it is expected to be associated with variation in later behavioural traits characterising both the conditions.

Importantly, the models described in this thesis are simplifications of the original models conceptualized by Johnson et al. (2014), which have been made to allow testing specific associations with the measures available for the study sample. One major difference is that in the present study children did not undergo a diagnostic assessment for ADHD. Instead, dimensional measures of ASD and ADHD traits have been used to evaluate neurodevelopmental outcome in children at familial risk for ASD. Moreover, models RS-OS and RC-OC are incompletely defined here because no elements reflecting risk factors known to contribute to both disorders are tested in this thesis. Models RS-OC and RC-OS do not include specific hypothesis testing for protective factors and multiple measurements over time to identify timing of the emergence of adaptive behaviours, respectively (see **section 5.4** for further discussion).

The "risk" part of the model (light blue circles to pink circles in **Figure 5.2**) is tested by exploring whether social (and non-social) attention atypicalities in infants covary with the amount of ASD and/or ADHD traits in the probands (i.e. the older affected sibling of children who participated in BASIS) and whether a significant amount of variance in the early marker is predicted by polygenic score for ASD and ADHD. The "outcome" part of the model (pink circles to yellow and red ellipses in **Figure 5.2**) is tested by using parent-report questionnaires of disorder-relevant temperamental features at 2 years and core symptoms of the two neurodevelopmental disorders between 6 and 10 years (see **section 5.2.2** for details on the outcome measures).

In sum, in the present study I evaluated if differences in attention reflect distinct or shared perturbations and at what level (risk or outcome) they are specifically involved in the

developmental path to ASD. I do this by examining whether peak look duration at faces (hereafter, peak look at the face) and non-face stimuli are associated with ASD and ADHD risk factors and later dimensional traits in infants with and without a first degree relative with a diagnosis of a neurodevelopmental disorder. Observing whether the same model fit both measures of social and non-social attention would shed light on whether the observed pathway involves responses specifically elicited by the social content of the visual stimulus.

5.2 METHODS

5.2.1 Participants

The sample for the present study included the BASIS participants who took part in Phase 1 and 2, as described in chapter 2 of this thesis (**section 2.2.1**, see also **Table A2.1**). The combined sample included 247 infants (127 females); of those, 170 (85 females) were high-risk infants with at least one older sibling with a community diagnosis of ASD (HR) and 77 (42 females) were control infants with at least one older sibling and no family history of ASD (LR).

An additional cohort of 164 infant siblings (70 females) was included in the present study. This cohort is part of the Studying Autism and ADHD Risks (STAARS) project, which recruited infants with (N=134, 58 females) and without (N=30, 12 females) an older sibling with a community diagnosis of ASD and/or ADHD or a parent with ADHD. Thus, HR infants could be further distinguished into three risk groups: HR(ASD) if they had a first-degree relative (sibling or parent) with a diagnosis of ASD; HR(ADHD) if they had a first-degree relative with a diagnosis of ADHD; and HR(ASD/ADHD) if they had a first-degree relative with comorbid ASD and ADHD.

STAARS was planned as a continuation of BASIS after Phase 2, therefore I will refer to this study by calling it “Phase 3”. Phase 3 participants underwent a similar protocol as Phase 1 and 2 participants. They were recruited for an initial assessment at around 5 months of age (T0), then at around 10 months (T1), 14 months (T2), 2 years (T3) and 3 years of age (T4). Phase 3 study is still ongoing and data for visits T3 and T4 were not available for the current study. **Table A5.1** provides information relative to the number of male and female participants, age and composite scores of the MSEL and VABS as measures of developmental level and adaptive skills, respectively, at the first three lab visits, by risk group. **Table A5.2** reports the measures collected during the three Phases and gBASIS protocols that were available for the current study. **Table 5.1** shows the total number and age of participants involved in the present study because they provided valid face pop-out data at 14 months, divided by risk group and phase.

Table 5.1 *Number and age of the participants who provided valid eye-tracking data at the face pop-out task for each Phase of BASIS by risk group.*

	Phase 1		Phase 2		Phase 3		Total N
	N	Age (s.d.) Min - Max	N	Age (s.d.) Min - Max	N	Age (s.d.) Min - Max	
Low-risk	45	13.95 (1.58) 11 – 20	19	15.05 (0.85) 14 – 17	23	14.17 (0.65) 13 - 15	87
High-risk	49	13.80 (1.51) 11 - 18	97	14.82 (1.05) 13 - 18	101 HR(ASD):69 HR(ADHD): 25 HR(ASD/ADHD):7	14.27 (0.75) 12 - 16	247
Total N	94		116		124		334

N: number of participants; s.d.: standard deviation; HR(ASD): HR infants with a first-degree relative with a diagnosis of ASD; HR(ADHD): HR infants with a first-degree relative with a diagnosis of ADHD; HR(ASD/ADHD): HR infants with a first-degree relative with comorbid ASD and ADHD.

5.2.2 Measures

5.2.2.1 Early markers

The early markers used in the present study consisted of the average peak look duration at the face and non-face stimuli in the face pop-out task. In the face pop-out task, an array of five pictures, including a face with direct gaze, a mobile phone, a bird, a car and a control stimulus defined as "Noise" in **Chapter 2** and **3**, obtained by randomizing the phase spectra of the face stimulus while keeping the amplitude and colour spectra constant (Halit, Csibra, Volein, & Johnson, 2004). An example of the array is presented in **Figure 3.1**. Children were presented with multiple different arrays, each showing a different set of face and non-social stimuli, whose visual saliency similarity was verified using Saliency Toolbox 2.2 (Walther & Koch, 2006). Slides were counterbalanced for gender, ethnicity, and vertical and horizontal location of the face stimulus within the array (Elsabbagh et al., 2012).

Information on the protocol for data acquisition and key variable calculation for Phase 1 and 2 are provided in **Chapter 3** (**section 3.2.2**). For Phase 3, 6 of the 14 arrays created for Phase 1 were presented as part of a battery of eye-tracking tasks. Infants sat at approximately 60 cm from the screen on their parent's lap. Tobii TX-300 was used for recording gaze data at a sampling rate of 300 Hz. Stimuli were presented within a fixed 17-inches 5:4 virtual display

surrounded by black borders, in order to make it comparable in size to Phase 1 and 2 stimuli presentation.

Following calibration procedure, the slides were presented, each for 15 s, and accompanied by music to help maintaining the infants' attention. Look target and duration were calculated from gaze coordinates for seven rectangular AOIs, as for Phase 1 and 2 data: centre of the screen, face, noise, car, bird, phone, and total (the entire slide), using automated procedures written in MATLAB for the purpose by project investigators (pipeline developed by L. Mason for the Longitudinal European Autism Project, described in Loth et al., 2017). This automated procedure included interpolation of periods of missing data for durations up to 300 ms, possibly caused by blinks and/or temporary failure of data capture, if the previous and following coordinates fell within the same AOI. No interpolation was performed for gaps that occurred between different AOIs.

Peak looks were defined as the longest look durations per stimulus per slide per participant: look duration data were first divided into three categories: face, scrambled face (Noise) and non-face (mobile, bird and car). Subsequently, for each slide for which at least two looks were available, the longest look in each category was identified. For the present research, an average of peak look durations across slides was calculated for each participant for the face and non-face category. **Table 5.2** presents the elements of difference between data acquisition and processing procedures between the three consequent study Phases.

Table 5.2 *Experimental differences between Phases in the face pop-out eye-tracking task.*

	Phase 1	**Phase 2**	**Phase 3**
Eye-tracker	Tobii 1750	Tobii 120	Tobii TX-300
Sampling rate	50 Hz	60 Hz	300 Hz
Screen dimension	17 inches	17 inches	23 inches (but stimuli presented within a fixed 17-inches 5:4 display).
Number of trials	14	10	6
Assignment of gaze coordinates to AOIs	Tobii Studio e	MATLAB	MATLAB
Missing data interpolation within the same AOI	/	<150 ms	<300 ms

Peak look duration at the face and at the non-face stimuli (average between bird, car, phone) were used as key variables indicating the early marker for a series of analyses evaluating risk.

These two measures were independently tested for association with variables indicating risk and outcome of ASD and ADHD. **Table 5.3** lists the measures used for the various types of analyses. Of note, there was a weak but significant correlation between the two measures of infant attention in the entire sample (rho=0.11, p=0.042).

Table 5.3 *Information on the measures used for the analyses performed in Chapter 5* assessing the relationship between the early marker and risk factors (Risk) and later behavioural traits (Outcome).

		ASD	ADHD	N	Phase
Risk	**Familial risk**	ASD risk vs. others Probands' SCQ	ADHD risk vs. others Probands' SDQ Hyp./Inatt.	167 108	3 2
	Polygenic risk	ASD GWAS (Grove et al., 2019)	ADHD GWAS (Demontis et al., 2018)	Face: 197; Non-face: 207	1, 2, 3
Outcome	**2 years**	ECBQ Soc.	ECBQ Imp. ECBQ Inhib. ECBQ Att. Foc.	184	1, 2
	6-10 years	SRS SCI SRS RRB	C-3P Hyp. C-3P Inatt.	SRS: 54 C-3P: 45	1, 2 (gBASIS)

SCQ: Social Communication Questionnaire; SDQ: Strengths and Difficulties Questionnaire; Hyp.: hyperactivity/impulsivity; Inatt.: inattention; GWAS: Genome-Wide Association Study; ECBQ: Early Childhood Behavior Questionnaire; Imp.: impulsive behaviour; Inhib.: inhibitory control; Att. Foc.: attention focusing; Soc.: sociability; SRS: Social Responsiveness Scale; SCI: Social and Communication Impairment; RRB: Restricted and Rebetitive Behavior; C-3P: Conners 3-Parent.

5.2.2.2 Risk factors

Familial risk

The degree of severity of ASD and ADHD traits in the proband were used in the present study as a measure of familial burden for the neurodevelopmental disorder. Dimensional measures of probands' traits were collected using parent-report questionnaires administered at different stages of the longitudinal design in which their younger sibling was enrolled. Specifically, the Social Communication Questionnaire (SCQ) and Strengths and Difficulties Questionnaire (SDQ) were collected as part of the Phase 2 protocol at T4, as summarised in Table A5.1. The SCQ and SDQ were chosen as one-dimensional measures of ASD and ADHD burden, respectively.

The Social Communication Questionnaire

The SCQ is a screening instrument which was designed as a questionnaire version of the ADI-R primarily for individuals who had already been clinically referred because of concerns of ASD or who had already been diagnosed. Two versions of the questionnaire exist: the "Lifetime" version, which would be used to support a diagnosis, and the "Current" version, which would be used for an evaluation of current difficulties. In each version, the principal caregiver (typically the parent) is requested to complete 40 yes/no answer items about the characteristics of the child. The individual should have a mental age of at least 2 years. Otherwise, this measure can be used with individuals of any chronological age.

The Lifetime version yields a total score that is interpreted in relation to a specific cut-off. Specifically, individuals scoring higher than this cut-off are likely to have ASD. Three domains subscales (Social Relating, Communication, and Range of Interests) can also be scored, but their utility has not been widely researched (Rutter, Bailey, & Lord, 1993). In the current study, the total score of the parent-report SCQ-Lifetime has been used as a dimensional measure of ASD severity for 114 probands.

<u>The Strengths and Difficulties Questionnaire</u>

The SDQ is a self- or parent/teacher-report screening questionnaire which captures emotional and behavioural difficulties in children and young people aged from 4 to 16 (Goodman, Meltzer, & Bailey, 1998).

There are currently three versions of the SDQ: a short form composed of 25 items, a longer form with an impact supplement (which assesses the impact of difficulties on the child's life) and a follow-up form. The 25 items form comprises 5 scales of 5 items each: the Emotional Symptoms subscale, the Conduct Problems subscale, the Hyperactivity/Inattention subscale, the Peer Relationships Problem subscale and the Prosocial Behaviour subscale (Goodman, 1997). In the parent/teacher-report form, caregivers are requested to indicate whether the description in each of the items is "not true", "somewhat true" or "certainly true" when applied to the child's behaviour over the previous six months. In the present study, scores of the Hyperactivity/Inattention subscale obtained from the parent-report short version of the SDQ were used as key variable indicating a general burden for ADHD traits.

Genetic risk

The genetic data used in the present chapter were processed as explained in **Chapter 4** (**section 4.2.3**). Of the 579 individuals of European ancestry for whom good quality, imputed genotype data, 203 previously participated in the BASIS/STAARS infant sibling design as target children. Of those, good quality eye-tracking data for peak look at the face and non-face stimuli was obtained for 197 and 207 infants, respectively. PGS for ASD and ADHD, representing the aggregate genetic risk derived from common variants associated with the neurodevelopmental disorder, were constructed for the two measures of social and non-social attention. Therefore, four PGSs were calculated:

1) ASD PGS predicting peak look duration to faces
2) ADHD PGS predicting peak look duration to response to faces
3) ASD PGS predicting peak look duration to non-face stimuli
4) ADHD PGS predicting peak look duration to non-face stimuli.

Details on PGS definition and procedural steps for construction can be found in **section 4.2.3**. Following quality control (100% call rate, minor allele frequency <0.05, significant Hardy-Weinberg equilibrium test at $p<1 \times 10^{-40}$), 4,398,111 variants and 197 individuals with peak look at the face and 4,401,334 variants and 207 people with peak look at the non-face stimuli non-missing data, respectively, remained as target datasets for PGS calculation.

5.2.2.3 Outcome

As a preliminary step, group differences were evaluated based on categorical diagnostic assignment included in the BASIS T4 visit (as detailed in **section 2.2.1.1**). Subsequently, the association between risk markers and later dimensional outcomes of ASD and ADHD have been evaluated in two steps. First, the relationship with temperamental levels of sociability and executive attention at 2 years was estimated in a larger cohort including children from Phase 1 and 2. Second, dimensional outcome reflecting difficulties in the core domains of ASD and ADHD during childhood (6 to 10 years) was obtained as part of gBASIS for a subset of Phase 1 and 2 children who participated in the longitudinal study in early childhood.

The Early Childhood Behavior Questionnaire at age 2

The ECBQ is a parent-report questionnaire which has been described previously in this thesis (**section 3.2.2**). This questionnaire, designed to evaluate temperament traits in the first three years of life (Rothbart, Ahadi, Hershey, & Fisher, 2012), was completed for 184 of the Phase 1 and 2 participants. It includes 18 subscales whose scores are constructed by clustering items assessing different dimensions of temperament (Putnam, Gartstein, & Rothbart, 2006). For the

present research, the Sociability scores, reflecting the attitude of seeking and taking pleasure in interactions with other people, was used as a proxy of socialization skills. The Attentional Focusing scores, representing the ability to maintain the duration of orienting on an object of attention and resisting to distraction; the Impulsivity scores, reflecting the speed of response initiation, and the Inhibitory Control scores, measuring the capacity to stop, moderate or refrain from a behaviour under instruction, were used to explore the relationship with difficulties associated with ADHD but also with the executive functions, which is a domain of common vulnerability for the two neurodevelopmental disorders (Johnson, 2012).

The Social Responsiveness Scale at school age

The SRS has been described in **sections 3.2.2** and **4.2.2** of this thesis. The SRS-2 School Age version was administered to the parents, who were requested to complete it thinking about their child's behaviour in the previous month. This questionnaire was administered as part of gBASIS. To evaluate specific relation between infants' social attention and the two core domains of ASD, I used t-scores for two DSM-5 Compatible domains: Social Communication and Interaction (SCI) and Restricted Interests and Repetitive Behaviours (RRB). The total number of participants for this analysis was 54.

The Conners 3P at school age

The third edition of the Conners ADHD exists in two forms, the Parent and Teacher form, created to evaluate ADHD traits in school-age children aged between 6 and 18 years (Kao & Thomas, 2010). 110 and 115 items are presented to parents and teachers, respectively. The adults are requested to describe the child's behaviour or report how frequently situations have occurred in the previous month, using a Likert scale ranging from 0 ("not true at all") to 3 ("very much true"). The Conners 3-Parent (C-3P) includes two indexes: the Conners 3 ADHD Index and the Conners 3 Global Index, which can be used for a quick and more detailed examination, respectively, of ADHD and related features such as executive functioning, learning, aggression, hyperactivity/impulsivity, peer relations and inattention. The questionnaire also provides scores to screener items for anxiety, depression and severe conduct disorders. Additionally, C-3P offers the possibility to calculate refined t-scores for symptoms categories, including the DSM-4 Conduct Disorder, Oppositional Defiant Disorder and, of interest for the present research, ADHD Inattentive and Hyperactive/Impulsive categories.

T-scores convert the raw scores to reflect what is typical or atypical for the child's age and gender. All t-scores have a mean of 50 and a standard deviation of 10 for homogenous age and

gender groups in the general population. T-scores between 40 and 59 (e.g., ±1 standard deviation from the mean) are considered average scores, reflecting a typical level of parental concern. T-scores higher than 65 (e.g., 1.5–2 standard deviations above the mean) usually indicate significant concerns.

For this study, the DSM-4 ADHD Inattentive and the Hyperactive/Impulsive t-scores were calculated from the parent-report form of the C-3P and used as key variables indicating dimensional outcome for ADHD core symptoms. Parent-report measures of difficulties in the core dimensions of ADHD and ASD were obtained for BASIS participants whose families took part in gBASIS (N=45).

5.2.3 Analyses

5.2.3.1 Testing the risk – early marker relationship

This part of the model, evaluating the association between risk factors and peak look duration at 14 months, was tested on the combined cohort of infants who participated in BASIS Phase 1, 2 and 3. As a preliminary step, the scores distributions of the three Phases groups were compared to check whether differences in the protocol and pre-processing procedures might have led to systematic biases. Because the three Phases were different in the proportion of individuals at risk (see **Table 5.1**), group differences were assessed with an ANOVA where risk group was added as a covariate, to limit the fact that a significant effect of Phase was confounded by the effect of risk group. ANOVAs revealed that there was a main effect of Phase ($F(2,330)=17.44$, $p<0.0001$) which was due to a significant difference between Phase 1 and 3 (Tukey HSD-adjusted $p<0.0001$) and between Phase 2 and 3 ($p<0.0001$), while no significant difference between Phase 1 and 2 emerged ($p=0.257$). Similarly, for peak look at the non-face stimuli there was a main effect of Phase ($F(2,342)=12.719$, $p<0.0001$), driven by significant differences between Phase 1 and 3 ($p=0.002$) and between Phase 2 and 3 ($p<0.0001$), while Phase 1 and 2 were not significantly different ($p=0.547$). Of note, the same pattern of results was obtained using non-parametric Kruskal-Wallis test (K-W $\chi^2(2)=33.652$, $p<0.0001$ for peak looks at the face and K-W $\chi^2(2)=35.419$, $p<0.0001$ for the non-face stimuli). The distributions of peak look at the face and at the non-face stimuli for the three Phases are illustrated in **Figure 5.3a** and **Figure 5.4a**, respectively.

Part of the observed difference in distribution might be due to the fact that different eye-tracking, software and pre-processing procedures were used to obtain Phase 1, 2 and 3 data (see **Table 5.2**). To limit the confounding due to this bias, the difference between the median of

the combined Phase 1 and 2 values and the Phase 3 values was computed (401.23 ms for peak look duration at the face and 268.35 ms for peak look duration at the non-face stimuli) and added to all Phase 3 values. The adjusted distributions are reported in **Figures 5.3b** and **5.4b**, respectively. ANOVAs controlling for the effect of risk group revealed that there was no significant difference between Phases after this adjustment for peak look at the face (ANOVA controlling for the effect of risk: F(2,330)=1.512, p=0.222; Kruskal-Wallis test: $\chi^2(2)=3.708$, p=0.157) and non-face stimuli (ANOVA: F(2,342)=1.013, p=0.364; K-W: $\chi^2(2)=1.426$, p=0.490).

Adjusted data were used for all analyses on the combined Phase 1, 2 and 3 sample evaluating the effect of risk on the peak look measures.

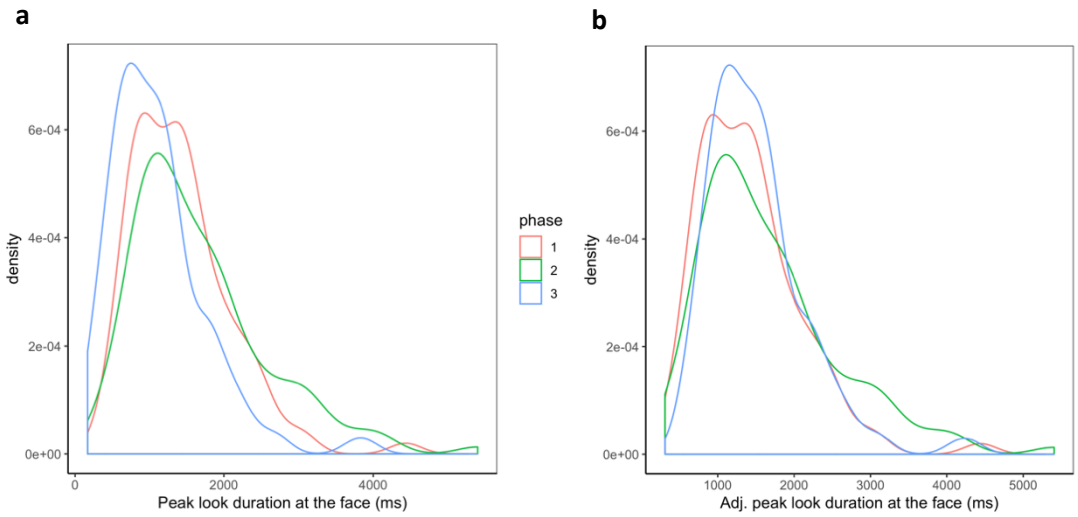

Figure 5.3 *Distribution of the peak look duration at the face for the three Phases of BASIS*: 1 in pink, 2 in green and 3 in blue. **a** Distribution of the average peak look duration per trial; **b** Distribution of the values for the three Phases after adjustment of Phase 3 values.

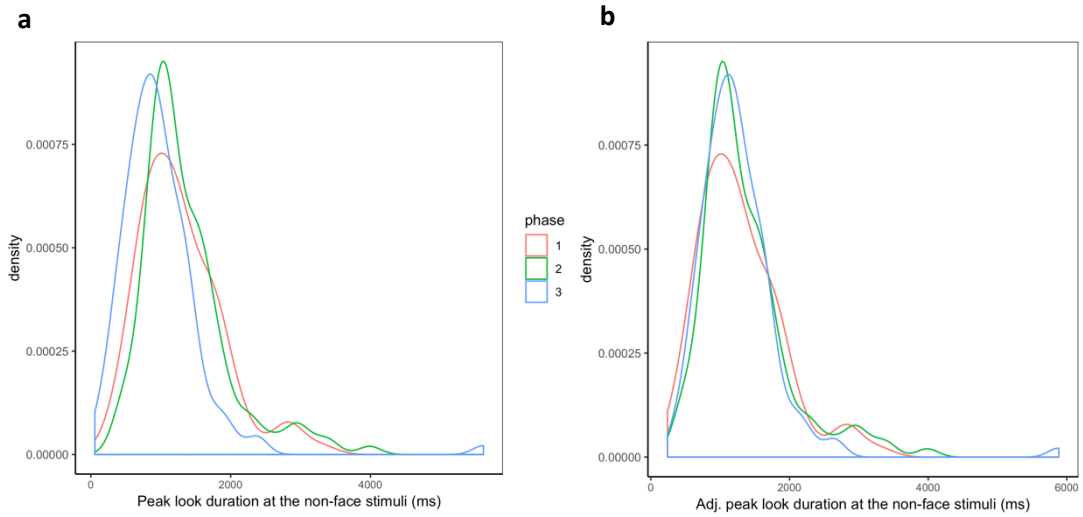

Figure 5.4 *Distribution of the peak look duration at the non-face stimuli for the three Phases of BASIS*: 1 in pink, 2 in green and 3 in blue. **a** Distribution of the average peak look duration per trial; **b** Distribution of the values for the three Phases after adjustment of Phase 3 values.

Risk-group differences

The first level of investigation aimed to evaluate whether there were group differences between infants are high and low familial risk for a neurodevelopmental disorder. Wilcoxon rank sum test was used to test differences in peak look durations between the HR and LR group. In these analyses, infants at high familial risk for ASD, ADHD and comorbid ASD/ADHD were grouped together (see **Table 5.1** for summary of the number of participants per group). Control ANOVA's were run to check the effect of age (in months) at the time of testing and sex and results are reported in the Appendix.

While Phase 1 and 2 HR infants were all recruited because younger siblings of children with ASD, Phase 3 also included infants who were at familial risk for ADHD or comorbid ASD/ADHD. To evaluate whether a difference in the early marker of attention was observed between groups at risk for different neurodevelopmental disorders, a multiple robust linear regression was performed on Phase 3 data only, testing for the effect of ASD (dummy=0 for LR and HR(ADHD), 1 for HR(ASD) and HR(ASD/ADHD)), ADHD (dummy=0 for LR and HR(ASD), 1 for HR(ADHD) and HR(ASD/ADHD)) and their interaction. Robust regression (Huber's M-estimator) was chosen to limit the effect of the most influential cases (see Phase 3 data distribution in Figures 5.3 and 5.4). Age (in months) at the time of testing and sex were added as covariates. Statistically significant difference of the robust estimates from 0 was tested using Wald F-test for multiple coefficients in robust linear regressions ('f.robftest' function of the 'sfsmisc' R-package).

Familial burden

One way to evaluate whether infant measures might reflect increased familial burden for a neurodevelopmental condition is to evaluate the severity of symptomatology in their older affected siblings (Frazier, Youngstrom, Hardan, Georgiades, & Constantino, 2015). I therefore tested whether the infants' marker of social attention was predicted by their older siblings' levels of ASD or ADHD symptomatology. These were measured with the total score of the SCQ (N=114) and the Hyperactivity/Inattention score of the SDQ (N=108), respectively. These probands measures of parent-reported traits were available only for the Phase 2 HR cohort.

As the dependent variables (peak look at the face and non-face stimuli) contained outliers, robust linear regression was used to test the relationship with the two outcome measures, using Huber's M-estimator to reduce the weight of the most influential (extreme) cases. Age (in months) at the time of eye-tracking testing and sex of the infant sibling were added as covariates in the analysis. P-values of the Wald F-test for multiple coefficients in robust linear regressions are reported as results, together with robust estimates.

Polygenic risk

Differently from the analysis presented in **Chapter 4**, where the high-resolution best fit PGS was estimated at a GWAS p-value threshold which allowed better distinction between ASD cases and non-ASD controls, in the present study a continuous variable was used as phenotype: the peak look duration. Because the selected phenotypes were not normally distributed (Shapiro-Wilk test: $W=0.896$, $p<0.0001$ for peak look at the face, $W=0.853$, $p<0.0001$ for non-face), logarithmic transformation was applied to the variables before PGS calculation. **Figure 5.5** and **5.6** present the distributions before and after logarithmic transformation for peak look at the face (S-W: $W=0.997$, $p=0.819$) and at the non-face stimuli (S-W: $W=0.991$, $p=0.032$).

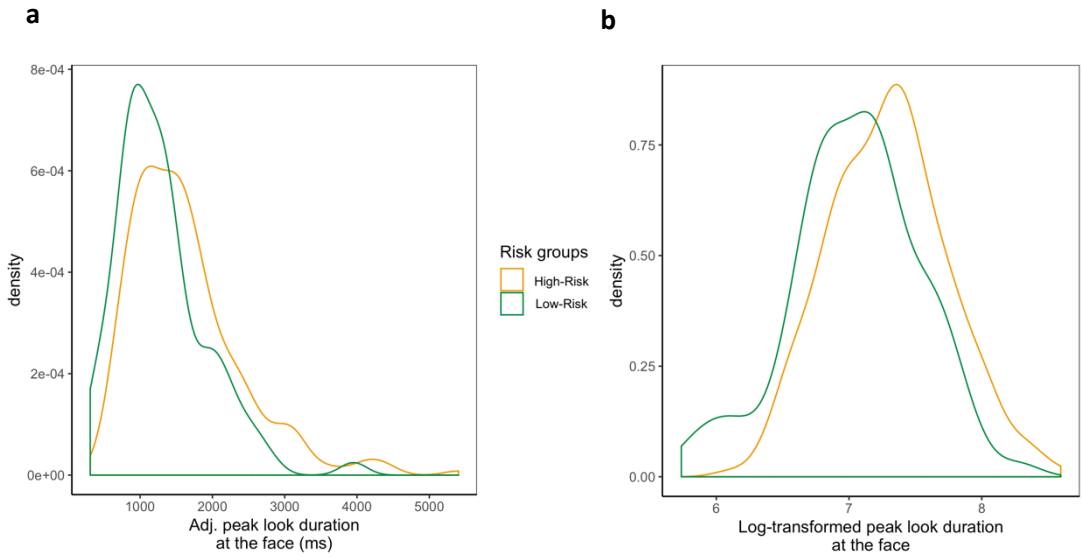

Figure 5.5 *Peak look at the face distributions before and after log-transformation*. **a** Distribution of the adjusted peak look duration at the face for the high-risk (yellow) and low-risk (green) group. **b** Distribution of the log-transformed values of peak look duration at the face for the high-risk (yellow) and low-risk (green) group.

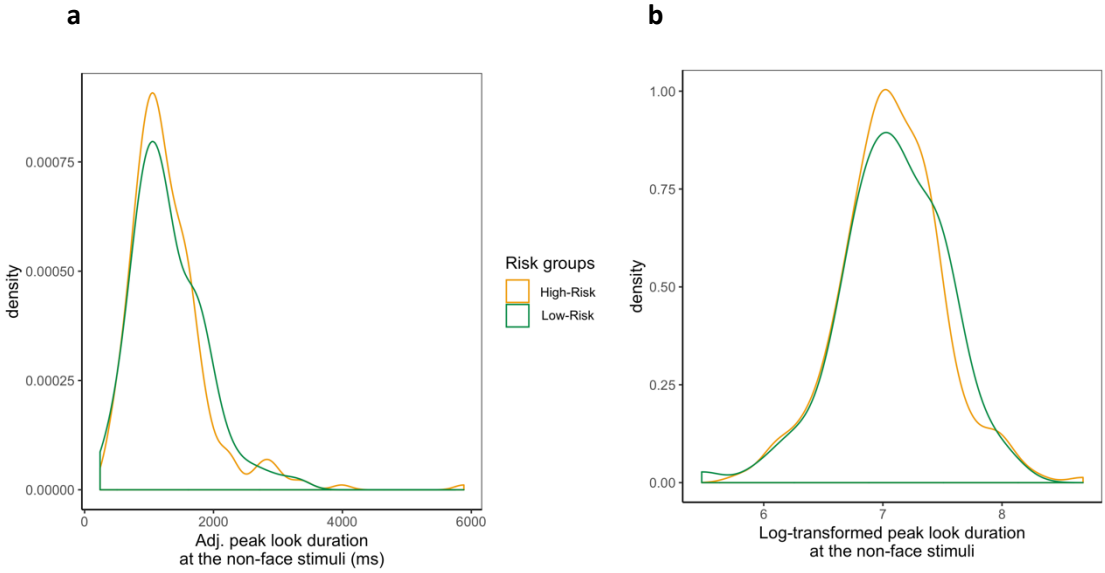

Figure 5.6 *Peak look at the non-face stimuli distributions before and after log-transformation*. **a** Distribution of the adjusted peak look duration at the face for the high-risk (yellow) and low-risk (green) group. **b** Distribution of the log-transformed values of peak look duration at the face for the high-risk (yellow) and low-risk (green) group.

The two base datasets used to compute PGS for ASD and ADHD consisted in summary statistics data obtained by the most recent and larger GWASes for the two disorders, obtained through meta-analyses of data collected by the iPSYCH and PGC consortia. Specifically, the ASD GWAS reports results from 18,381 ASD cases and 27,969 controls (Grove et al., 2019) while the ADHD GWAS includes 19,099 ADHD cases and 34,194 controls of European ancestry (Demontis et al., 2019). Five PCs (recommended procedure at the Geschwind Lab, UCLA), and age in months at the time of testing (T2) were added as covariates in the analyses. Sex was not added as covariate initially to avoid eliminating some of the variability of interest, especially given that the sex-difference in prevalence in neurodevelopmental disorders has been shown to affect liability of SNP-heritability (Martin et al., 2018). Control analyses estimating the best fit PGS associated with peak look at the face including sex as covariate too are reported in the Appendix (**Figures A5.1** and **A5.2**). LD clumping as in **section 4.2.3.2** was based on the 1000 Genomes reference panel data.

PRSice-2 calculated PGSs for each individual using a pre-defined range of p-value thresholds (0.001, 0.005, 0.01, 0.05, 0.1, 0.15, 0.2, 0.5, 1). Additionally, a high-resolution best fit PGS was automatically estimated from SNPs associated at the GWAS p-value threshold which better captures the variability of the continuous phenotypes (Euesden, Lewis, & O'Reilly, 2015). The amount of explained variance is calculated as R^2. Of note, high resolution best fit PGS should be considered as significantly predicting the phenotype as a p-value threshold <0.004 (Euesden et al., 2015).

5.2.3.2 Testing the early marker – outcome relationship

This part of the model was tested on Phase 1 and 2 participants, for whom outcome assessment was done.

Categorical outcome group differences

As first, preliminary step, differences in the early markers by categorical outcome group assigned at 3 years were tested for 207 children who participated in Phase 1 and 2 (group assignment for Phase 3 participants had not been finalised as many of the participants had not been assessed with the 3-year visit when this thesis was written). A Kruskal-Wallis non-parametric test was performed to compare peak look durations between four outcome groups: LR, HR-TD, HR-Aty and HR-ASD. Dunn's Test was used for post-hoc comparisons with FDR Benjamini-Hochberg p-value adjustment for multiple testing (Benjamini & Hochberg, 1995; Dunn, 1964). To control for a possible effect of age (in months), sex and Phase which might have

influenced the results, ANOVA was also performed with those variables as covariates and outcome group as a between-subjects factor with four levels.

Dimensional outcome at 2 years

In order to test the relationship between each of the two the early markers and dimensional outcomes measured by various domains of the ECBQ at 2 years, multiple linear regressions were used with scores of each of the four subscales (Sociability, Attention Focusing, Impulsivity and Inhibitory Control) as dependent variables and peak look duration as independent variable. Age at T2 and sex were included as covariates in the analyses.

For significant relationships between outcome measure and early marker, a further investigation interrogating whether the same association was observed in the high- and low-risk group was carried on. Here, the effect of risk group and the interaction between early marker and risk group was added in a linear regression model. The HR group was set as reference to evaluate whether the significant association was observed in this group in the first instance.

Dimensional outcome at school age

I next tested the association between outcome evaluated later in childhood (between 6 to 10 years of age). As data were positively skewed such that normality could not be reached by transforming the data, a Poisson distribution function with log link was used in the general linear models testing the association between each of the four outcome measures (the Hyperactive/Impulsive and the Inattentive t-scores of the Conners 3P for ADHD, the SCI and the RRB t-scores for ASD) and the infant marker of attention. As t-scores, calculated based on the age and sex of the individual subjects, were used as dependent variables, age and sex were not added as covariates. To correct for simultaneous testing of four different hypotheses, p-valued adjusted using FDR are also reported.

All the described analyses were first carried out for peak look at the face stimuli as early marker of social attention collected at 14 months. Subsequently, to check whether the observed effects were specific to social stimuli, the same analyses were conducted using peak look at the non-face stimuli (bird, car, phone).

5.3 RESULTS

In order to evaluate what conceptual model better explained the possible role early markers of social and non-social attention in the path to neurodevelopmental disorders (see **Figure 5.2** for illustration of the proposed models), a range of analyses were conducted testing 1) the association between risk factors and the early markers, and 2) the association between the early markers and later outcome. By assessing the significance of these relationships, I aimed to evaluate evidence for 1) an involvement of specific versus common risk factors for ASD and ADHD in affecting looking behaviour at 14 months, and 2) the degree to which these early signs of attention atypicality are specific to later ASD and ADHD or general for atypical neurodevelopmental outcome. As the primary focus of this thesis was to evaluate evidence for the role of social attention in early developmental trajectories, all analyses were first conducted for peak look at the face stimuli recorded with eye-tracking during a face pop-out task. As a second step, the same analyses were conducted using peak look at non-face stimuli, to evaluate whether the previously observed findings were specific to social contents of the visual target as in similar research (Elsabbagh et al., 2012; Hendry et al., 2018). Results are reported for peak look duration at the face and at the non-face stimuli separately.

The total sample size for the series of analyses aimed to evaluate the extent to which peak look duration to static visual stimuli in an array at 14 months were predicted by risk factors comprised measures collected from the children who participated in the longitudinal assessments as part of Phase 1, 2 and 3 of the BASIS project. Differences in the sample sizes in the specified analyses depended on the number of subjects with no missing data for each test. **Table 5.3** summarises the available data for each of the analyses.

5.3.1 Risk – early marker relationship

The following results were obtained for the analyses testing whether peak look durations 1) were different based on familial risk for ASD and ADHD, 2) were associated with familial burden of ASD and ADHD traits, estimated as the parent-report level of symptomatology for the two neurodevelopmental conditions in the HR children's older siblings who have received a community diagnosis of ASD, 3) were predicted by polygenic risk scores for ASD and ADHD.

5.3.1.1 Risk group differences

Peak look duration at the face

Wilcoxon rank sum non-parametric test revealed that there was a significant difference between high- and low-risk groups in the peak look duration at the face stimuli (W=13751, p=0.0001, see **Figure 5.7a**). Specifically, HR infants showed significantly longer peak-look duration at the face (mean=1627.42, s.d.=781.39, median=1490.36) compared with LR (mean=1285.05, s.d.=624.49, median=1188.54). Of note, this effect remained highly significant when including age at the time of testing, sex, Phase and the interaction between risk and Phase, which were all non-significant (all p*s*<0.124, see **Table A5.3**).

As the Phase 3 HR infants were recruited as having a family member with ASD, ADHD or comorbid ASD/ADHD, a further examination was carried out to observe effects in the presence of risk of ASD, ADHD and/or both. This analysis revealed that longer peak look duration at the face was found in infants at risk for ASD (β=259.24, s.e.=132.18, p=0.053) but not for ADHD (β=19.87, s.e.=159.69, p=0.902). **Figure 5.8** illustrates the pattern of results for each risk group (considering HR(ASD/ADHD) as a separate group, for illustration purposes). **Table A5.5** reports robust estimates and statistics for this analysis. [Of note, when considering Phase 3 infants only, there was no difference between HR group as a whole and LR group in peak look duration at the face: Wilcoxon rank sum test: W=1396, p=0.133).

Peak look duration at the non-face stimuli

As shown in **Figure 5.7b**, HR and LR groups were not different in terms of peak look duration to non-social stimuli (W=11152, p=0.583; HR group mean=1292.92, s.d.=632.37, median=1154.67; LR group mean=1304.64, s.d.=559.87, median=1230.74). The same result was observed when including covariates in the model, and there was no interaction between risk and phase (see **Table A5.4** for all results).

There was no significant difference between Phase 3 groups when dividing them into LR, HR(ASD), HR(ADHD) and HR(ASD/ADHD). All estimates and results of this analysis are reported in **Table A5.6**.

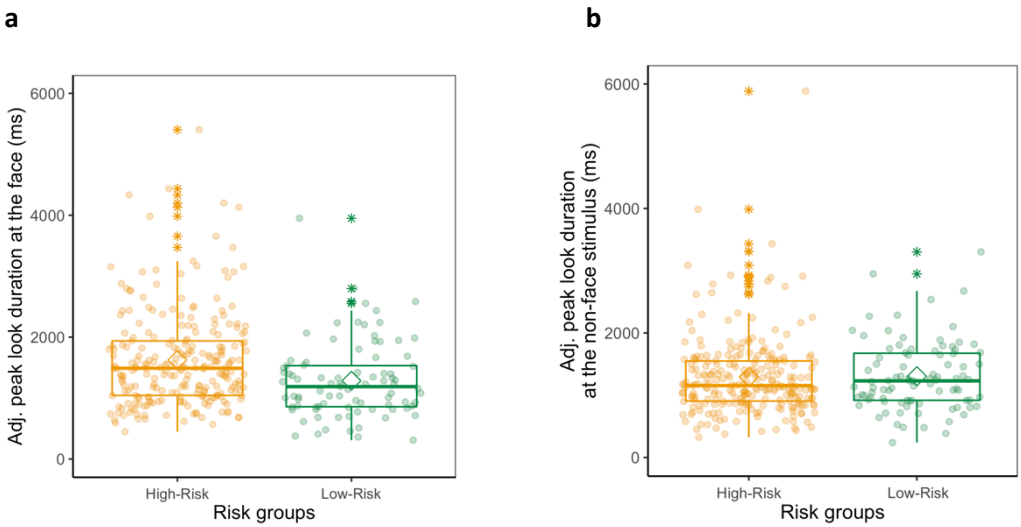

Figure 5.7 *Boxplots showing the peak look duration at the face (**a**) and at the non-face stimuli* (**b**), in milliseconds, in high-risk (yellow) and low-risk (green) 14-month-old infants. All individual data are represented by points. The lower and upper hinges of the boxplots correspond to the first and third quartiles (the 25th and 75th percentiles). The upper and lower whiskers extend from the hinges to the largest and smallest values, respectively, no further than 1.5 x inter-quartile range. Horizontal lines within boxplots indicate the median value, while rhombi represent mean values.

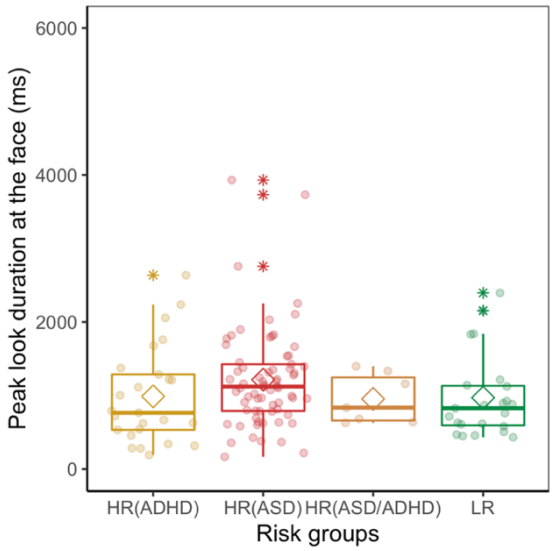

Figure 5.8 *Boxplots showing the peak look duration at the face, in milliseconds, in Phase 3 high-risk infants* at familial risk for ADHD (HR(ADHD), in ocher), ASD (HR(ASD), in red, comorbid ADHD and ASD (HR(ASD/ADHD), in beige) and low-risk infants (LR, in green). All individual data are represented by points. The lower and upper hinges of the boxplots correspond to the first and third quartiles (the 25th and 75th percentiles). The upper and lower whiskers extend from the hinges to the largest and smallest values, respectively, no further than 1.5 x inter-quartile range. Horizontal lines within boxplots indicate the median value, while rhombi represent mean values.

Thus, 14-month-old infants at high familial risk for neurodevelopmental disorders showed longer peak look duration at the face in a face pop-out task compared to LR infants, and this result seemed to be due mainly to familial risk for ASD. On the contrary, no group differences were observed in peak look at the non-face stimuli.

5.3.1.2 Familial burden

The effect of familial burden for ASD and/or ADHD on early signs of attention atypicality was evaluated by testing the relationship between the infant measures and their older siblings' phenotypic measures of ASD and ADHD, obtained from parent report questionnaire. The association with severity of the two neurodevelopmental disorders' symptomatology was tested using the SCQ total score for ASD and the SDQ Hyperactivity/Inattention subscale for ADHD.

Peak look duration at the face

A multiple robust regression was run to evaluate the amount of variance in the infants' measure explained by each of the two variables. This analysis (including 107 pairs of siblings with complete data) revealed that the probands' SCQ scores more significantly contributed to their younger siblings' social attention (β=10.99, s.e.=9.42, p=0.071) compared with the SDQ Hyperactivity/Inattention scores (β=56.36, s.e.=31.38, p=0.245).

Peak look duration at the non-face stimuli

For non-social attention, there was no significant association with probands' autistic dimensional traits measured with the SCQ scores (β=6.61, s.e.= 5.905, p= 0.258) nor with ADHD traits as measured by the SDQ Hyperactivity/Inattention scores (β=-17.38, s.e.=19.35, p=0.365).

In sum, longer peak look duration at the face in the infants were associated primarily with higher (though not significantly) levels of ASD symptomatology observed in their older siblings with ASD. Peak look duration at the non-face stimuli was not associated with familial burden for ASD and ADHD traits.

5.3.1.3 Polygenic risk

Polygenic score calculation was obtained using the PRSice-2 software (Euesden et al., 2015), which estimates the high resolution best fit PGS better explaining the variance in peak look durations at the face and non-face stimuli in 197 and 202 infant genotyped data, respectively.

Peak look duration at the face

33,124 SNPs associated with ASD at a p-value threshold <0.1507 in the base GWAS (Grove et al., 2019) were selected by PRSice-2 to compute the high resolution best fit PGS for prediction of the variation in peak look duration at the face at 14 months. This PGS predicted 0.76% of the total variance in the phenotype (R^2=0.0076, p=0.226, **Figure 5.9a**).

The best fit ADHD PGS included 805 SNPs which were associated with ADHD at a p-value threshold of p<0.0005 in the base GWAS (Demontis et al., 2019). The so-calculated PGS predicted 2.8% of the variance in peak look at the face (R^2=0.028, p=0.021, **Figure 5.9b**). Of note, the same pattern of results was observed when sex was added as covariate in the models for PGS construction (see **Figure A5.1**).

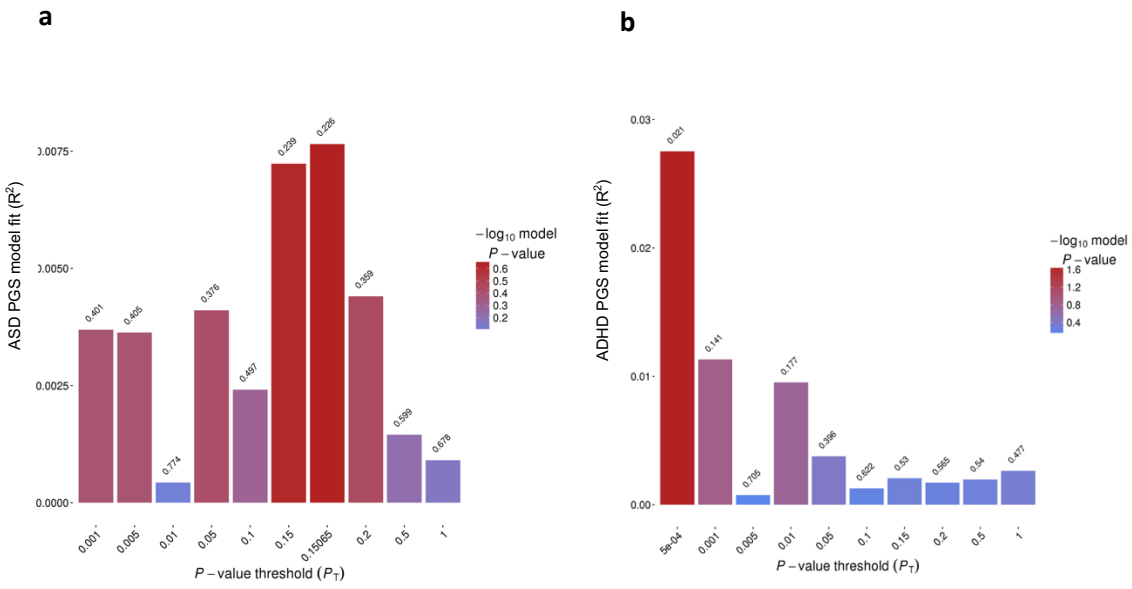

Figure 5.9 *Results of the polygenic score (PGS) for ASD (**a**) and ADHD (**b**) predicting peak look duration at the face* at various GWAS p-value thresholds. Height of bars (Y-axis) represents the model fit (R^2). X-axis represents the 9 selected p-value thresholds plus the p-value threshold selected for the high-resolution best-fit polygenic score. Numbers above bars represent p-values. Bars are coloured on a continuous scale from red (significantly higher for longer peak look durations) to light blue (lower for longer peak look durations).

In a follow-up regression analysis, I tested the relationship between log-transformed values of social attention and ADHD polygenic score by risk group. Results demonstrated that a significant association was observed in the HR group (β=96.70, s.e.=47.49, p=0.043) while no significant relation between polygenic risk and early attention to faces was found in the LR group (β=20.77, s.e.=114.38, p=0.856). **Figure 5.10** graphically illustrates the relationship with non-transformed phenotypic data.

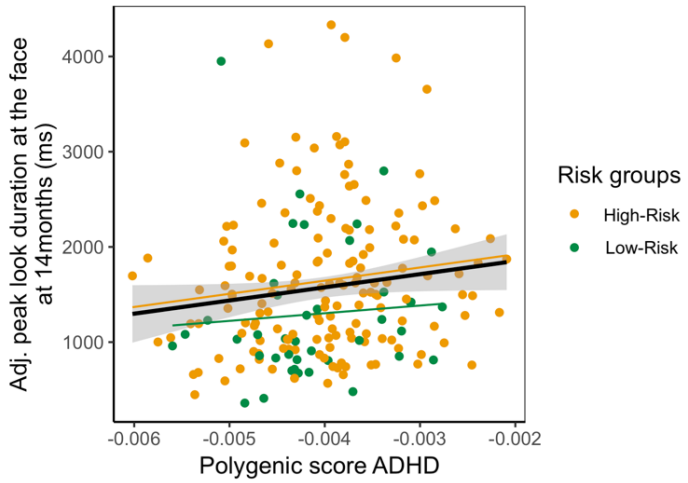

Figure 5.10 *Relation between polygenic score for ADHD, on the x-axis, and peak look duration at the face at 14 months*, in milliseconds, on the y-axis. Dots represent individual data points, colour-coded in yellow for high-risk infants and green for low-risk infants. The solid black line represents the regression line for the entire group, with grey shaded areas depicting standard errors. Coloured lines represent the regression lines for the high-risk (yellow) and low-risk (green) groups.

Peak look duration at the non-face stimuli

PGS obtained using the most associated SNPs with ASD at a p-value threshold of p<0.1357 in the base ASD GWAS by Grove et al. (2019) was not significantly predictive of peak look duration at the non-face stimuli (R^2=0.0075, p=0.215, **Figure 5.11a**). Similarly, the high resolution best fit PGS was obtained from SNPs associated with ADHD in the largest available GWAS for this condition (Demontis et al., 2019) at a p-value threshold of 0.0078. This ADHD PGS predicted 1.2% of the variance in the infant measure of non-social attention (R^2=0.012, p=0.107, **Figure 5.11b**).

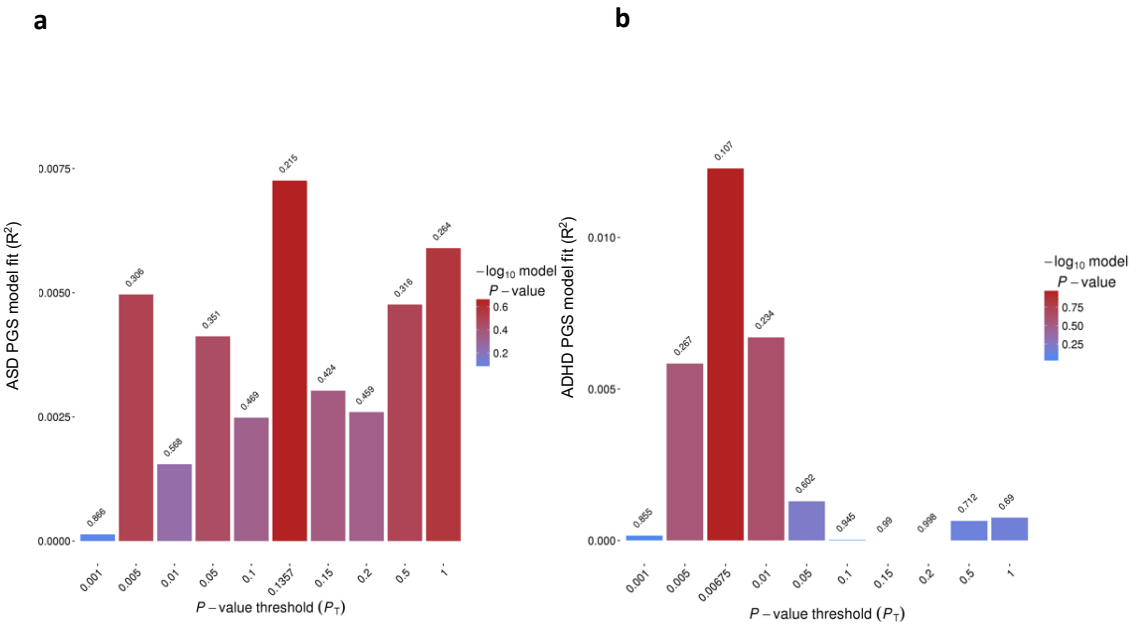

Figure 5.11 *Results of the polygenic score (PGS) for ASD (**a**) and ADHD (**b**) predicting peak look duration at non-face stimuli* at various GWAS p-value thresholds. Height of bars (Y-axis) represents the model fit (R^2). X-axis represents the 9 selected p-value thresholds plus the p-value threshold selected for the high-resolution best-fit polygenic score. Numbers above bars represent p-values. Bars are coloured on a continuous scale from red (significantly higher for longer peak look durations) to light blue (lower for longer peak look durations).

Overall, evidence for polygenic contributions of common genetic variants associated with ASD and ADHD to peak look durations in infancy was weak. Suggestive evidence that an aggregate score obtained by weighting the 800 variants which have been found to be most associated with ADHD in the largest ADHD GWAS of European ancestry to date (Demontis et al., 2019) predicts a small amount of variance in social attention at 14 months was found. The extent to which and reasons why these findings should be treated with caution will be discussed below.

5.3.2 Early marker – outcome relationship

I next tested whether the early markers of visual attention were predictive of later emergence of behavioural traits of ASD and/or ADHD. First, I examined group differences in peak look duration at the face and non-face stimuli at T2 between children who were categorised into one of the following four outcome groups by experienced clinicians as part of the T4 visit for BASIS: LR, HR-TD, HR-Aty and HR-ASD. Second, I tested the association between the early markers and

subscales of a parent-report temperament scale (the ECBQ) measuring children's social and attention functioning at two years, potentially reflecting early difficulties associated with emerging ASD and ADHD. Third, I tested whether the early markers were predictive of later dimensional measures of the DSM-defined core areas of difficulties for ASD (social communication; restricted interests/repetitive behaviour) and ADHD (hyperactivity/impulsivity; inattention) reported by parents of children aged between 6 and 10 years.

5.3.2.1 Outcome group differences

Peak look duration at the face

Kruskal-Wallis non-parametric test revealed that there was a significant effect of outcome on peak look duration at the face at 14 months (K-W $\chi^2(3)=13.06$, p=0.005, **Figure 5.12**). Post-hoc tests showed that this result was due to a significant difference between the LR and the three HR groups (LR versus HR-TD: p=0.020, LR versus HR-Aty: p=0.012, LR versus HR-ASD: p=0.020; all other corrected p*s*>0.865). An ANOVA was performed to verify the possible effect of covariates such as age at the time of testing (in months), sex and Phase, and testing for an interaction between Phase and outcome. This analysis revealed that a main effect of outcome group remained highly significant (F(3,197)=4.574, p=0.004) while all other effects were not (all p*s*>0.189, see **Table A5.7**).

Peak look duration at the non-face stimuli

There was no difference between the three outcome groups in terms of peak look duration at the non-social stimuli at 14 months (K-W $\chi^2(3)=2.34$, p=0.504). The same pattern of results was obtained from the ANOVA testing for the main and interaction effects of outcome group and Phase while controlling for the effect of age and sex (see **Table A5.8**). **Figure 5.12b** illustrates the observed results.

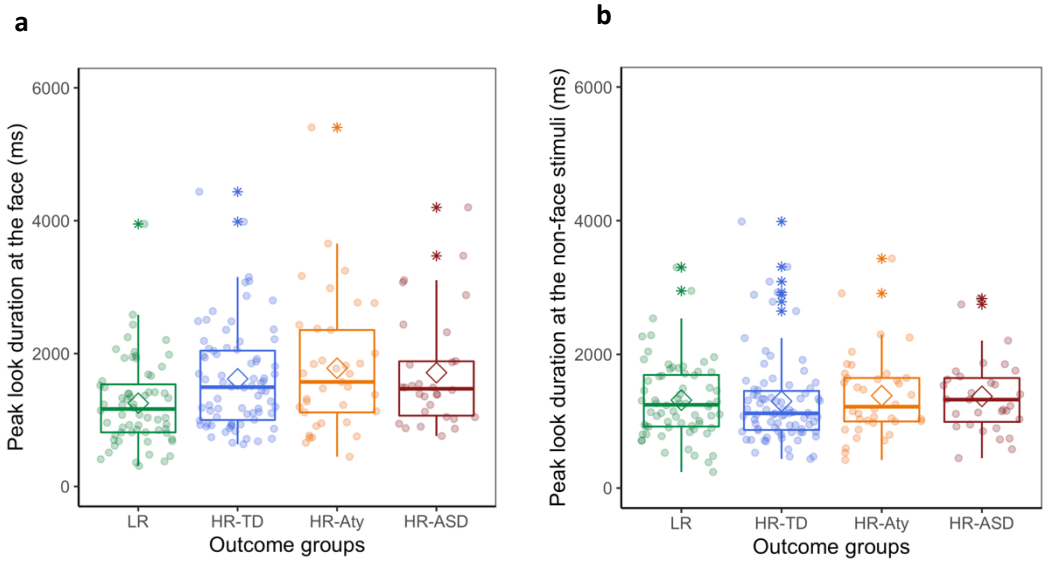

Figure 5.12 *Boxplots showing the peak look duration at the face (**a**) and at the non-face stimuli* (**b**), in milliseconds, in Phase 1 and 2 in low-risk infants (LR, in green); high-risk infants with typical development at 3 years (HR-TD, in blue); high-risk infants with developmental concerns who did not meet criteria for ASD (HR-Aty, in orange); and high-risk infants who received clinical diagnosis of ASD at 3 years (HR-ASD, in red). All individual data are represented by points. The lower and upper hinges of the boxplots correspond to the first and third quartiles (the 25th and 75th percentiles). The upper and lower whiskers extend from the hinges to the largest and smallest values, respectively, no further than 1.5 x inter-quartile range. Horizontal lines within boxplots indicate the median value, while rhombi represent mean values.

In conclusion, analysis of group differences in peak look duration at the face revealed that the LR infants were different from the three HR groups. However, no difference was observed between infants with emerging ASD, infants who showed a typical pattern of behaviour at 3 years and infants who developed some features of developmental concern or sub-threshold symptoms but did not meet the full criteria for a diagnosis. No differences between outcome groups were observed in peak look duration at the non-face stimuli at 14 months.

5.3.2.2 Dimensional outcome at 2 years

Peak look duration at the face

As measures of early signs of atypical developmental trajectory, the ECBQ Sociability subscale was used as a proxy of early difficulties in the social domain which characterise ASD while the Impulsive Behaviour, Attention Focusing, and Inhibitory Control subscales were used to detect

aspects of attention and executive functions mainly impaired in ADHD (although also arguably involved in the ASD phenotype). Linear regression revealed that there was no significant relationship between the infant measure of social attention and Sociability (β=68.94, s.e.=45.02, p=0.126), Attention Focusing (β=28.99, s.e.=52.23, p=0.589) and Impulsive Behaviour (β=-62.76, s.e.=73.77, p=0.400). Interestingly, however, peak look duration at the face at 14 months predicted Inhibitory Control scores at 2 years (β=-137.18, s.e.=46.51, p=0.004, see **Table A5.9** for all robust estimates).

To further interrogate whether the observed relationship was a general mechanism or whether it was specific to the risk group, an additional linear regression test was conducted adding the risk group dummy variable and testing its interaction with ECBQ Inhibitory Control scores. Interestingly, this analysis revealed that, while the observed negative relationship between the infant measure and inhibitory control at age 2 was highly significant in the HR group (β=-0.0004, s.e.=0.0001, p=0.003), a positive association between the two measures was observed in the LR group (β=0.0005, s.e.= 0.0002, p=0.030). This indicated that prolonged peak look duration at the face was found in HR infants with poorer inhibitory control at 2 years, while the opposite was found for the LR group (see **Figure 5.13**).

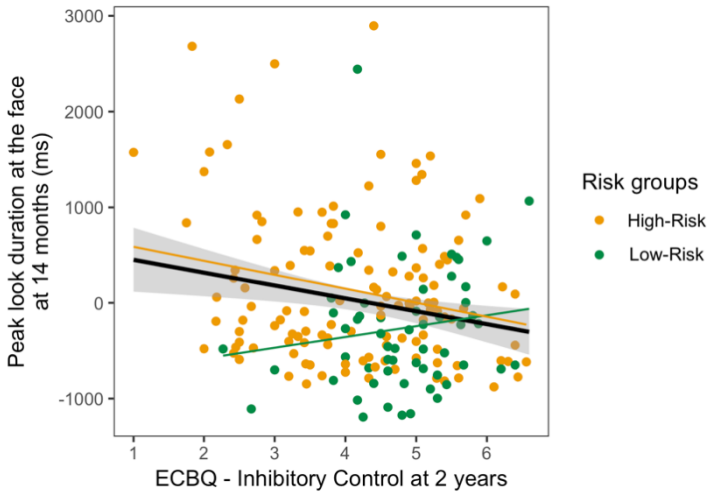

Figure 5.13 *Relation between Inhibitory Control scores of the Early Childhood Behavior Questionnaire (ECBQ) at 2 years, on the x-axis, and peak look duration at the face at 14 months*, in milliseconds, on the y-axis. Dots represent individual data points, colour-coded in yellow for high-risk infants and green for low-risk infants. The solid black line represents the regression line for the entire group, with grey shaded areas depicting standard errors. Coloured lines represent the regression lines for the high-risk (yellow) and low-risk (green) groups.

Peak look duration at the non-face stimuli

Peak look duration at the non-face stimuli at 14 months was not predictive of the ECBQ subscales scores (all p*s*>0.279, see **Table A5.10** for robust estimates and statistics).

In conclusion, longer peak look duration at the face at 14 months were found in HR infants who showed lower inhibitory control at 2 years. No relationship with later parent-report temperamental subscales was found for peak look at the non-social stimuli in the face pop-out array.

5.3.2.3 Dimensional outcome at school age

In order to evaluate whether atypicalities in looking behaviour when attending to social and non-social stimuli were predictive of ASD and ADHD traits in later childhood, I tested the relationship between peak look at the face or non-face stimuli and age and sex specific t-scores for each of the following subscales collected from parent-report questionnaires at 6 to 10 years: the Hyperactive/Impulsive and the Inattentive of the C-3P for ADHD, the SCI and the RRB of the SRS for ASD.

Peak look duration at the face

There was a significant association between peak look at the face at 14 months and SRS SCI (β=7.74 x 10^{-5}, s.e.=2.32 x 10^{-5}, p=0.0009, FDR=0.002) and RRB (β=8.89 x 10^{-5}, s.e.=2.32 x 10^{-5}, p=0.0001, FDR=0.0004) t-scores at 6 to 10 years of age, indicating that longer peak looks at static faces were predictive of higher levels of impairment in the social communication and restricted interests and repetitive behaviours domains, respectively, later in childhood. Differently, I found no evidence for association of the early marker of social attention atypicality and hyperactive/impulsive (β=-5.57 x 10^{-6}, s.e.=3.08 x 10^{-5}, p=0.857, FDR=0.857) and inattentive (β=3.178 x 10^{-5}, s.e.=2.30 x 10^{-5}, p=0.289, FDR=0.385) traits measured with the C-3P parent-report questionnaire at school age.

Peak look duration at the non-face stimuli

Peak look duration at the non-face stimuli was not predictive of later levels of SCI (β=1.57 x 10^{-5}, s.e.=3.31 x 10^{-5}, p=0.635, FDR=0.635) nor RRB (β=2.53 x 10^{-5}, s.e.=3.34 x 10^{-5}, p=0.449, FDR=0.599) measured with the DSM-5 core domains t-scores of the SRS. Similarly, the infant attention measure did not predict hyperactivity and impulsivity (β=6.16 x 10^{-5}, s.e.=3.68 x 10^{-5}, p=0.095, FDR=0.380), and inattention (β=2.98 x 10^{-5}, s.e.=3.70 x 10^{-5}, p=0.42, FDR=0.599) as measured by the DSM-4 subscales t-scores of the C-3P.

Thus, the selected early marker of social attention was predictive of severity of autistic traits at school age, while no evidence was found for an association with ADHD traits. Additionally, peak look duration at non-social stimuli were not associated with later ASD or ADHD traits.

Mediation role of inhibitory control

Intact executive functioning has been proposed to play a protective role against the development of neurodevelopmental disorders (Johnson, 2012). Additionally, the developmental change in peak look duration in the second year of life was found to be associated with later executive function in a subset of children included in the present study, suggesting that this early marker might reflect emerging divergence in the developmental trajectory of this domain-general ability (Hendry et al., 2018). To further explore the obtained results, I questioned whether the observed association between peak look duration at the face and later ASD traits was mediated by underlying individual differences in inhibitory control, which is a component of executive functioning.

Thus, as an exploratory analysis aimed to take a step further in understanding developmental mechanisms underlying the observed relationships between early social attention atypicalities, inhibitory control and autistic traits, a mediation path was estimated using the 'cfa' function of the 'lavaan' package in R. Given that the observed association between infant social attention and inhibitory control at 2 years was observed in the high-risk group only, low-risk individuals were excluded from the analysis. Thus, the final sample for the moderation analysis comprised 38 high-risk children from Phase 1 and 2 (which is admittedly small for this type of analysis). Bootstrapping method (N=1000 replications) was used to compute estimates and significance of the effects, as recommended for small samples and when normality of the variables distributions cannot be assumed (Hayes, 2009). All variables were scaled before being entered in the model. In the model, two moderation paths were estimated, one for SRS SCI t-scores and one for RRB t-scores at school age, both predicted by peak look duration the face at 14 months, inhibitory control at 2 years and the interaction between these two variables (**Figure 5.14**).

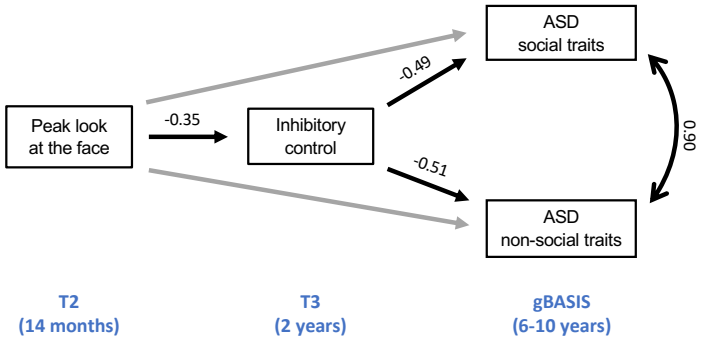

Figure 5.14 *Graphical representation of model testing the mediation effect of inhibitory control* measured with the Early Childhood Behavior Questionnaire (ECBQ) at 2 years on the relationship between peak look duration at the face at 14 months and ASD social and non-social ASD traits measured between 6 and 10 years of age using the Social Communication Impairment, and Restricted and Repetitive Behaviours t-scores of the Social Responsiveness Scales, respectively.

Results of the mediation analysis revealed that the association between peak look at the face and SRS RRB t-scores was mediated by inhibitory control (indirect effect through mediator: st.β=0.179, p=0.068; direct effect between peak look duration and RRB t-scores: st.β=0.193, p=0.412). To a lesser extent, inhibitory control also mediated the relationship between peak look duration at the face and SRS SCI scores (indirect effect: st.β=0.171, p=0.080; direct effect between peak look duration and SCI t-scores: st.β=0.129, p=0.515). Moreover, increased ability in inhibitory control at age 2 predicted lower severity in autistic traits (SCI: st.β =-0.485, p=0.004; RRB: st.β =-0.508, p=0.002). **Table A5.11** reports all estimates from the mediation model, which is graphically represented in **Figure 5.14**. Of note, although the model fit parameters seem to indicate excellent fit of the data (CFI=1, RMSEA=0, SRMS=0), the observed estimates are highly dependent on the examined sample and might not be generalizable (χ^2 (9)=119.4, p<0.001). This was expected given the limited sample size as expected. Therefore, this analysis should be considered exploratory and needs to be replicated in a larger sample.

In conclusion, the presented series of analyses revealed that peak look duration at the non-social stimuli did not show any association with early risk factors or outcome. Thus, the present study does not provide evidence for considering it an early marker of neurodevelopmental issues.

On the contrary, atypically long peak looks at static face images in 14-month-old infants are associated with increased familial loading for ASD, and only marginally with polygenic risk computed considering common genetic variants highly associated with ADHD (Demontis et al., 2019). In the HR group, better inhibitory control at 2 years of age was associated with more

typical looking behaviour at 14 months. This measure of social attention was also predictive of social and non-social autistic traits at school age, but this effect was found to depend on inhibitory control characteristics. **Table 5.4** summarises the results when testing the role of peak look duration at the face as an early marker of neurodevelopmental disorders.

Table 5.4 *Summary table of the results of the present study*, testing the relationships between peak look duration at the face in a face pop-out task at 14 months and measures of risk and behavioural outcome of ASD and ADHD. √ indicates significant associations; **~** indicates suggestive associations; **X** indicates no association.

		ASD	**ADHD**
Risk	**Familial risk**	ASD risk vs. others: √ Probands' SCQ: **~**	ADHD risk vs. others: **X** Probands' SDQ Hyp./Inatt.: **X**
	Polygenic risk	ASD PGS: **X**	ADHD PGS: **~**
Outcome	**2 years**	ECBQ Soc.: **X**	ECBQ Imp.: **X** ECBQ Inhib.: √ ECBQ Att. Foc.: **X**
	6-10 years	SRS SCI: √ SRS RRB: √	C-3P Hyp.: **X** C-3P Inatt.: **X**

SCQ: Social Communication Questionnaire; SDQ: Strengths and Difficulties Questionnaire; Hyp.: hyperactivity/impulsivity; Inatt.: inattention; PGS: polygenic score; ECBQ: Early Childhood Behavior Questionnaire; Imp.: impulsive behaviour; Inhib.: inhibitory control; Att. Foc.: attention focusing; Soc.: sociability; SRS: Social Responsiveness Scale; SCI: Social and Communication Impairment; RRB: Restricted and Rebetitive Behavior; C-3P: Conners 3-Parent.

5.4 DISCUSSION

The focus of this chapter was to shed light on the specificity of the role of social attention atypicalities in the path to ASD and ADHD. To this aim, a series of analyses were conducted to investigate the relationship between risk factors, early looking behaviour when attending to social and non-social stimuli and later symptoms of neurodevelopmental disorders. Results revealed that peak look duration at static images of a face, presented in an array of 5 elements matched in visual saliency, were longer for 14-month-old infants at high familial risk for ASD compared with infants at low risk and at risk for ADHD. When testing whether early differences in this measure of social attention was associated with familial burden for ASD and ADHD, I found that there was a trend toward a positive relationship between atypical peak look at the face in the infant and more parent-reported autistic (but not ADHD) traits in her older sibling

with ASD. Polygenic risk scores for ADHD predicted ~2.8% of the variance in peak look duration, which was higher than that seen for ASD polygenic scores (0.76%). With relation to outcome, early signs of social attention atypicality during infancy were associated with poorer inhibitory control as reported by parents when children were aged two. This relationship was observed only in the HR group, suggesting that the observed relationship might be specific to infants at increased familial liability for ASD. Importantly, longer peak look duration at the face in infancy significantly predicted increased severity of social and non-social autistic traits in the same child at school age, while no association was found with ADHD traits. Further exploring the relation between social attention, inhibitory control and ASD symptoms, suggestive evidence that individual differences in executive attention skills fully accounted for the relationship between peak look at the face and restricted and repetitive behaviour was found.

Of note, all these effects were observed only when examining peak look duration at the face stimuli; on the contrary, no significant relationships were observed when examining peak look duration at the non-face stimuli. Overall, these findings support the idea that that early disruptions in attention to social stimuli in infants at HR for ASD emerge from a general risk factors for neurodevelopmental disorders and their association with ASD symptoms might depend on individual differences in inhibitory control that emerge during toddlerhood.

5.4.2 The risk-to-marker path

In order to evaluate whether genetic or familial (genetic and environmental) burden for ASD and ADHD are associated with eye-tracking measures of face looking at the face at 14 months, I took two approaches: First, I examined the relationship between the infant marker and the severity of neurodevelopmental problems in the older sibling with a diagnosis of ASD; Second, I assessed whether the polygenic risk for ASD and ADHD explained a significant proportion of variance in the infant eye-tracking measure.

5.4.2.1 Familial risk

Results from the investigation of the relationship between familial risk factors and the selected early marker of atypical development revealed that there was a positive (nearly significant) association between infant social attention and his or her older sibling's autistic traits, measured as the total score of a parent-report screening instrument for ASD, the SCQ. However, no association between infant and proband's measures was found when looking at the SDQ Hyperactivity/Inattention subscale. Of note, these associations were examined in sibling pairs

enrolled in Phase 1 and 2, therefore all the probands, i.e. the older sibling, would have received a diagnosis of ASD but not necessarily for ADHD. Although there was a significant correlation between these two measures in the proband (rho=0.54, p<0.0001), variances were not homogeneous (Levene's test: F(1,220)=97.1, p<0.0001). By observing the scores distributions, it is possible to notice that SDQ scores were skewed towards the pathological end of the distribution (see **Figure A5.2b**). Thus, the majority of the probands showed increased levels of difficulties in hyperactivity and inattention (high scores in the SDQ scale). The observed pattern of results might indicate that probands with more severe autistic traits also showed more difficulties in the attentional domain. A more balanced design including older siblings diagnosed with ADHD only would be appropriate to test specificity of the risk factor to the early marker. Overall, this part of the analysis demonstrated that peak look duration at the face could represent one of the first signs of atypical developmental trajectory in infants with higher familial risk loading for ASD.

5.4.2.2 Polygenic risk

Altered looking behaviour when attending to faces might reflect disruptions in domain-general functions as a result of genetic risk factors which disturb brain development in critical periods (Elsabbagh & Johnson, 2016; Piven, Elison, & Zylka, 2017). To validate this early marker as an endophenotype of neurodevelopmental disorders, it is crucial to verify that it not only reflects increased liability for the disorder, but it is also under genetic influence (Iacono, Malone, & Vrieze, 2017). To this aim, I tested whether PGS for ASD and ADHD explains any of the variance seen in peak look duration at the face during early infancy.

PGSs, in which the risk effects of genotypes at many loci are summed, is a promising approach to obtain reliable results with reasonable sample sizes in the study of behavioural traits, where many genes of very small effect are responsible for heritability (Plomin, 2013). To date, to my knowledge, no PGS studies have investigated polygenic contributions to infant behavioural and neurophysiological endophenotypes. However, there is suggestive evidence that PGSs for neurodevelopmental disorders might be associated with markers of atypical neurodevelopment in infants (Cullen et al., 2019) and with continuous phenotypes relying on social attention skills (Warrier et al., 2018) and cognitive tests scores (Hagenaars et al., 2016). In the present study, in line with previous studies, a high resolution best fit approach was used for polygenic risk score construction, that selects the "optimal" threshold for building a predictive PGS (Euesden et al., 2015).

The PGS analyses in this chapter revealed that a predictor constructed using the top 800 SNPs associated with ADHD in the base GWAS predicted 2.8% of the variance in peak look duration at the face in the BASIS infants. This effect was not statistically significant after controlling for multiple testing of several polygenic scores for obtaining the "best fit" one (recommended threshold of significant: $p<0.004$, Euesden et al., 2015). However, it is worth noting that the amount of variance explained is higher than that observed in studies using ASD polygenic scores to predict autism-relevant continuous traits tested during childhood in large cohorts. For example, an ASD PGSs predicted 1.3% of the variance in autistic traits at 8 years of age (St Pourcain et al., 2018) in a sample of 5,553 children. A PGS for cognitive empathy predicted only 0.3% of the variance in the performance in a computerised test of 'theory of mind' abilities in 4,577 13-year-olds (Warrier & Baron-Cohen, 2018). Possible reasons for this discrepancy in effect sizes are numerous, and further work will be required to validate our results. More sobering still, the contribution of an ADHD PGS to individual phenotypic variability is very small in absolute terms; the PGS contributes to individual differences in the order of 142 ms in the present cohort, where one standard deviation corresponds to 758 ms.

It was unusual to note that the best fit ADHD PGS was constructed using SNPs associated with ADHD at a p-value threshold of 0.0005 and PGSs obtained at higher p-value thresholds were not predictive of the infant phenotype. This pattern of results is unexpected, as typically including a greater number of SNPs in the PGS construction either increases the predictive power of the PGS or leads to only a slight decrease due to the addition of a small proportion of "noisy" SNPs unrelated with the phenotype. One interpretation of the observed findings is that the genetic architecture of ADHD is such that only a small number of variants highly associated with ADHD contributes to social attention in infancy. Of note, a similar pattern of association was found in a study estimating the effect of an ADHD PGS on a computerised assessment of 'theory of mind' in ~4,000 adolescents, with an enhanced (though non-significant, $R^2=0.0009$) contribution of a PGS estimated at a more stringent p-value threshold ($P_T=0.01$) (Warrier & Baron-Cohen, 2018). Interestingly, in children ADHD difficulties in theory of mind are associated with deficits in executive function and attentional domain (Mary et al., 2015). In light of this observation, it is possible that an effect on attention in social contexts is observed only when considering the common genetic variants which are highly associated with ADHD. This might indicate that only variants with high expressivity for ADHD raise the risk for atypical social attention. It might also be that less strongly associated variants have variable expressivity across lifespan, with increased effects at later ages.

It is also possible that the results observed are an artefact of the sample. Given that the Warrier & Baron-Cohen (2018) study contained more than 4,000 individuals, the current sample size

cannot be blamed entirely for the observed pattern, and inspection of individual scores failed to identify outliers that might skew results. Other methods exist for construction of PGS, for example LDpred, (Vilhjálmsson et al., 2015) or lassosum, (Mak, Porsch, Choi, Zhou, & Sham, 2017). In the future a methodological comparison of different algorithm for PGS calculation might shed light on the origins of this pattern.

The study also showed that polygenic contribution to our early marker was highest when considering variants associated with ADHD. The base GWAS used for ADHD polygenic score calculation is larger (by ~2,000 cases and ~8,000 controls) than the one currently one available for ASD, so this might be simply a reflection of the greater statistical power of the ADHD GWAS rather than an indicator of genetic specificity. It will be interesting to see if (as predicted) the variance explained increases as larger and more powerful discovery GWASes for ASD emerge (Geschwind & Konopka, 2009).

Finally, PGSs can be built in PRSice using a categorical diagnosis of ASD in the target sample (as done in **Chapter 4** for ASD) and using this PGS as a predictor in a linear regression model with dimensional measures of peak look duration. Such an approach was not selected for two reasons: 1) not all the Phase 3 children received a diagnosis of ASD which would have reduced the available sample further to 81 infants; 2) a confirmation of ADHD diagnosis was not available for the BASIS participants and their family members, and therefore a PGS based on categorical diagnosis was not possible for this neurodevelopmental disorder.

In conclusion, longer peak look duration at the face in a face pop-out task was associated with more parent-reported social and attention difficulties in the older sibling with ASD, confirming that early differences in this measure of social attention might be associated with familial burden for both ASD and ADHD. Preliminary findings demonstrated that peak look duration was predicted by a PGS for ADHD to a larger extent than by a PGS for ASD, although these effects were small and need to be replicated in a larger sample (and possibly with more powerful discovery GWASes). Taken together, these results point toward the conclusion that environmental contributions as well as the effect of rare genetic variants, like CNVs, are likely to account for part of the observed familial influences (Miller et al., 2019; Stergiakouli et al., 2017; Thapar et al., 2016). According to the analyses on the relationship between proband's traits and infant's social attention, these effects seem to be shared between disorders.

5.4.3 The marker-to-outcome path

Atypical peak look duration at the face could represent an early sign of difficulties in the social area, as observed in response to a social stimulus, or in the attention function, specifically the infant's orienting and executive attention skills. A previous study, on a sample partly overlapping with this study sample, reported that longer looking duration was observed in children at familial risk for neurodevelopmental disorders independently from whether they ended up developing core ASD symptomatology, sub-threshold traits and/or other developmental atypicalities, or typical development at three years of age (Hendry et al., 2018). This finding was replicated here, as I found, in a larger sample than the original study, that all three HR groups showed significantly longer peak looks at the face than the LR group, but no differences among each other.

When examining the relation between the infant measure of social attention and short term dimensional skills I found that it was associated with inhibitory control but not with sociability, again in accordance with what Hendry et al. (2018) found in a subset of the present sample. At later ages (between 6 and 10 years of age), however, there was a significant association between peak look at the face and autistic traits in the social (SRS SCI) and non-social (SRS RRB) domain. Differently, there was no association between the infant marker and ADHD inattentive and hyperactive/impulsive traits measured with the C-3P.

5.4.3.1 Specific outcome?

Testing ADHD traits in children at risk for ASD

The fact that longer peak look at the face was associated with later ASD but not ADHD traits would support the models where an early marker is involved in a condition-specific developmental trajectory. However, as noted earlier, the present study does not fully allow me to demonstrate this hypothesis because the marker-to-outcome path was tested on infants at familial risk for ASD but not ADHD. Therefore, it is possible that the observed result is driven by the fact that more children were at increased liability for ASD traits specifically. Importantly, variances in the ADHD and ASD dimensional measures were highly comparable (Levene's Test: $F(1, 179)=4 \times 10^{-4}$, p=0.984), demonstrating that children showed a spread in ADHD symptoms similar to the variability in ASD symptoms. Moreover, traits from different scales showed some degree of correlation (see **Figure 5.15**), although all correlation tests were non-significant (SRS SCI – C-3P Inatt.: p= 0.087, SRS SCI – C-3P Hyp./Imp.: p= 0.334, SRS RRB – C-3P Inatt.: p= 0.150,

SRS RRB – C-3P Hyp./Imp.: p= 0.258). These results revealed that there was a high possibility of comorbidity, despite a wide variability in the combination of traits, in a relatively small sample of 45 individuals. Increasing the current sample size with children at risk for ADHD and comorbid ASD/ADHD who participated in Phase 3 will certainly point to more definite conclusions on whether peak look duration at the face in the second year of life is predictive of ASD traits also in individuals at enhanced familial risk for ADHD.

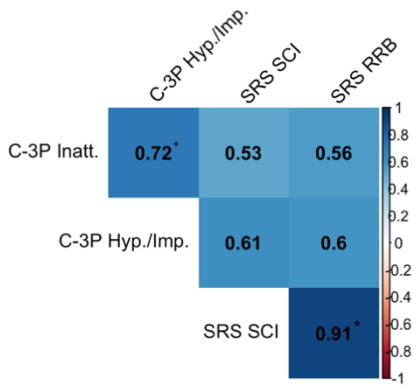

Figure 5.15 *Correlation coefficients for associations between ASD and ADHD traits at school age*. ASD traits were measured with the Social Responsiveness Scale Social Communication Impairment (SRS-SCI) and Restricted and Repetitive Behaviour (SRS-RRB) t-scores, which ADHD traits were measured with the Conners 3P Inattentive (C-3P Inatt.) and Hyperactive/Impulsive (C-3P Hyp./Imp.) t-scores in 47 children who participated in BASIS during infancy. Blue indicates a positive correlation. * indicates significant correlations ($p<0.05$), $^{+}$indicates $p<0.1$.

Reflections about measuring autistic traits across development

No association with the Sociability subscale at 2 years was found, suggesting that this marker did not reflect differences in social skills in early childhood. The choice of using ECBQ was guided by the fact that it provides subscales for both social and attention skills. However, ECBQ is an instrument which captures early differences in temperament (Putnam, Gartstein, & Rothbart, 2006) and was not created to assess autistic and ADHD traits. Thus, it is possible that it maps differently into ASD and ADHD symptoms. For this reason, relying on instruments that have been created to evaluate levels of symptomatology, like the SRS for ASD (Constantino & Gruber, 2012) and C-3P for ADHD (Kao & Thomas, 2010), might be more appropriate for this scope.

The study presented in this chapter is valuable as it examines prospectively the relationship between an early psychophysiological marker recorded at the beginning of the second year of life and outcome at school age in 54 children (32 HR and 15 LR). Interestingly, a significant association between the two measures was found. However, the relationship between the same measure of social attention and SRS SCI or RRB scores at 3 years was not significant, as revealed

by the SEM analysis in **Chapter 3**. It is possible that the association of the early marker with later ASD outcome becomes stronger later in childhood, as developmental trajectories diverge over time leading to more extreme SRS scores. This hypothesis is supported by the results of the mediation analysis, indicating that the changes in early attention might be related to later inhibitory skills, which in turn forms a risk factor for later ASD that accumulates over time.

Although promising, the described findings should be treated with caution due to the limited sample of children with available SRS scores during childhood. The fact that measures of dimensional outcome in mid-childhood were collected from children at very different ages (spanning between 6 and 10 years of age) is also not ideal to test developmental mechanisms, even if t-scores provide a standardised measure of traits for each age. A new wave of testing for the Phase 2 children is currently ongoing where SRS will be collected again; when available, scores for the children who are missing in the current sample should be incorporated in the present analysis; this might allow us to verify whether the present results are corroborated when increasing the sample size and obtaining a more homogeneous age range for the participants.

A path involving executive attention

In sum, results for this series of analyses demonstrated that atypically long peak look duration at the face at 14 months was not related to difficulties in sociability or in the ability of focusing attention at the end of the second year. It did not predict temperamental features of impulsive behaviour either, in agreement with previous findings on comorbidity of autistic features and ADHD-related traits (Polderman et al., 2013). However, it was associated with lower inhibitory control in high risk infants, in line with the possibility that early difficulties in face processing interact with domain-general executive attention processes also involved in the path to ASD (Hendry et al., 2018; Johnson, 2012).

When examining the relation with ASD and ADHD symptomatology in mid-childhood, I found that peak look duration at the face was highly associated with SRS scores reflecting difficulties in the social and non-social domain of ASD. In contrast, no association was found with C-3P scores for dimensional traits of the two types of behavioural problems characterising ADHD. Based on these results, the examined sign of atypical social attention at 14 months seems to be involved in the path of ASD symptoms, although larger samples and the inclusion of outcome measures from individuals at risk for ADHD will be needed to draw more definitive conclusions on the condition-specificity of this early marker.

5.4.4 A new model for the observed path

According to the results of the analyses summarised above, obtained by evaluating the relation between risk factors, early markers of atypical developmental trajectory and later ASD and ADHD traits, none of the four proposed models illustrated in **Figure 5.2** was completely validated. Overall, both ASD (familial) and ADHD (polygenic) risk factors were associated with atypically long peak look at the face at 14 months, in favour of one of the two RC models where a contribution of common risk factors is proposed. When examining outcome in children at high familial risk for ASD, evidence was found for relation between the early marker and ASD traits only, in favour of the OS models illustrating condition-specific paths. However, this argument is partly challenged by the following observations:

1) Infants who received a diagnosis of ASD at three years of age did not show more atypicalities in peak look duration at the face compared with children who underwent a typical development or who showed signs of atypical outcome but did not fully meet criteria for a diagnosis of ASD.
2) In **Chapter 3**, no association between the same early marker and social skills or autistic social and non-social symptoms at three years of age was found. Similar findings were obtained by Hendry et al. (2018) in a subset of the current sample, using different measures of autistic traits. Thus, peak look to faces at 14 months does not seem to be predictive of ASD in the following two years of life.
3) When examining the relation with short term outcome by looking at parent-report temperamental characteristics at age 2, an increased association between inhibitory control, but not sociability, was found for the selected measure of social attention. This result is also in line with Hendry et al. (2018), who reported a significant relationship between change in peak look duration between the first and the second year and measures of executive attention, but not core social symptoms of ASD, at age 3.

Examining the entire set of results in a developmental perspective, a new model might be more adequate to explain the observed pattern. This model unifies two of the possible scenario proposed by Johnson et al. (2014). Johnson (2012) theorised that executive function could have a protective value across neurodevelopmental disorders, as individuals with strong executive function skills might be better able to compensate for early neural atypicalities related to presence of risk factors. In this framework, symptoms associated with the risk marker would be expressed in the absence of such protective factor (model D reported in **Figure 5.1** and also, for convenience, in **Figure 5.16b**). Inhibitory control is one of the components of executive function and shorter fixation durations in infancy have been consistently associated with better inhibitory control at later ages in the typical population (see Conejero & Rueda, 2017, for a review). Of

note, in the present study such relation was observed only in the HR group. For these children, shorter ("typical", as comparable with the LR group) peak look durations to faces at 14 months were associated with better inhibitory control 10 months later. It could therefore be that infants with strong inhibitory control did not show signs of atypical looking behaviour despite being at high familial risk for neurodevelopmental disorders.

5.4.4.1 Inhibitory control as protective factor?

Inhibitory control has been shown to be intact in non-affected family members (parents and siblings) of children with ASD (McLean, Johnson, Zimak, Joseph, & Morrow, 2014; Wong, Maybery, Bishop, Maley, & Hallmayer, 2006), suggesting that this component of executive function could indeed play a role in preventing the development of the core symptomatology in individuals at increased familial risk. Moreover, results of the mediation analysis testing whether an effect of inhibitory control could contribute to the relationship between peak look at the face and later ASD traits are in line with this hypothesis. Thus, early social attention atypicalities might reflect enhanced vulnerability due to general risk factors that, in the absence of a resilient inhibitory control during toddlerhood and early childhood, will contribute to the consolidation of autistic behavioural features. It is also possible that peak look duration atypicalities reflect early-emerging components of inhibitory control. In typical development, shorter duration of fixations during infancy is related to better inhibitory control in adolescence (Sigman, Cohen, & Beckwith, 1997). Future well-powered studies should attempt to disentangle this matter, using neurocognitive marker tasks and longitudinal models to evaluate the relationship between emerging executive function and looking behaviour in the first years of life (Conejero & Rueda, 2017).

The new proposed model representing the observed results is illustrated in **Figure 5.16a**. This model is similar but extends model RC-OS, where common infant markers, possibly reflecting common mechanisms of brain adaptation or compensation emerge in the face of early disturbances to neurodevelopment (Johnson, 2017). It also acknowledges a possible interaction with a protective factor, which was part of the original model D from Johnson et al. (2014). Importantly, the model proposed here differs from model D, which was conceptualised to evaluate the common and specific developmental trajectories of children with a diagnosis of ASD and/or ADHD. In fact, in the present study it was not possible to define whether the effect of the protective factor was specific for one neurodevelopmental disorder or for a comorbid condition, given that diagnostic assessment for ADHD or comorbidity was not carried out in these children. The model illustrated in **Figure 5.16a** differs from model RS-OC too, as risk factors

for both neurodevelopmental disorders, and not specifically for one of the two, have been found to be associated with the early marker.

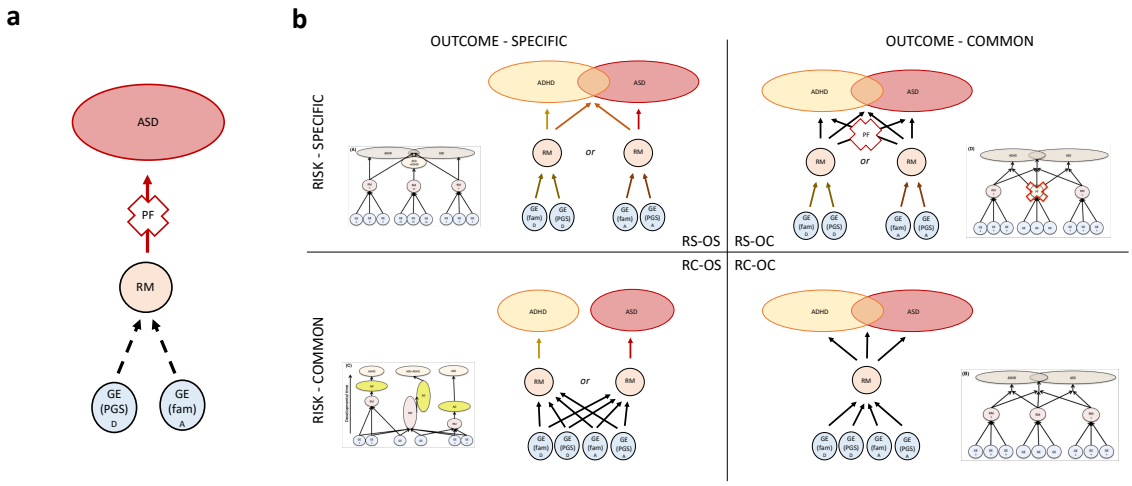

Figure 5.16 a *The new model for peak look duration at the face as an early marker of ASD supported by the results of the present study*. Dashed arrows represent suggestive evidence for association. **b** Reproduction of Figure 5.1, illustrating the four models initially proposed for the role of early markers in the developmental pathway from risk factors to behavioural symptoms of ASD and ADHD, and models A, B, C and D from Johnson et al. (2014). Light blue circles indicate genetic and/or environmental risk factors (GE), evaluated in their familial (fam, or older sibling's behavioural traits) and polygenic (PGS or polygenic score) specific contribution for ASD (A) and ADHD (D). Pink circles indicate the early risk marker (RM), which in this study corresponds to the peak look duration at the face or at the non-face stimuli in a face pop-out array, measured with eye-tracking technology at approximately 14 months of age. The yellow ellipse indicates ADHD dimensional traits while the red ellipse indicates ASD dimensional traits. PF indicates possible protective factors.

5.4.4.2 Non-social look duration

In the present study two early phenotypes reflecting looking behaviour at 14 months were observed: the duration of the peak look at the face and non-face stimuli, respectively, during a face pop-out eye-tracking task. No association of peak look duration at non-social stimuli with risk factors and future outcome of ASD and ADHD was observed. This demonstrated that the effects observed in the analyses considering peak look duration at the face were specific to the social content of the target stimulus. This result is in line with a previous fMRI study which reported that, when processing social stimuli during a task requiring inhibition of interference, individuals with ASD demonstrated reduced activation in key regions of the brain network typically involved in cognitive control (Ditcher & Belger, 2007). For the scope of this thesis, this is an important finding as it provides evidence that when considering peak look duration as early

marker, atypicalities in social attention, rather than in general attention to all types of visual stimuli, are specifically involved in the path to ASD.

5.4.5 Limitations and future directions

As discussed above (see **section 5.4.3.1**), the current study is strongly limited by the lack of an ADHD diagnostic assessment and of balanced groups of children with ASD and ADHD, which did not allow me to evaluate pathways to ADHD as a categorical outcome and to comorbidity, as originally auspicated by Johnson and colleagues (2014). This limitation also prevented me from constructing ASD and ADHD polygenic scores for prediction of case-control status, which has been done in other studies and would have produced results comparable with previous research. It should be added that different datasets were used to test the various hypotheses (see **Tables 5.3** and **A5.1**), arguably providing an incoherent picture of the relationship between variables. Phase 3 children have been added to the study sample to increase the power for evaluating the association between polygenic score and peak look at the face, and to observe the distribution of the early marker in participants with a familial risk for ADHD as well as ASD. However, these children could not be included in the analyses testing the marker-to-outcome path or the association with probands' traits, reducing the actual contribution of this cohort to the study hypotheses. Phase 3 is still ongoing therefore longitudinal and probands data were not available for these children, admittedly limiting the informative value of this cohort in understanding the involvement of social attention in the path to ADHD. It will be interesting to test the same hypotheses when Phase 3 data collection will be completed and the data made accessible to researchers.

Another consideration that has not been discussed yet concerns the consequences of dealing with non-normally distributed data. For example, estimates resulting from the analyses testing the association between infant early marker and dimensional traits in the child itself or in the proband produced large standard errors. This observation offers me the opportunity to reflect on the fact that linearity of the relationship between variables is an *assumption* of many of the most common statistical models used in the field of Psychology and Health Science. If this assumption is violated, estimates can be biased. Larger datasets and the use of data-driven approaches such as cluster analyses might allow researchers to explore whether different patterns of association between risk factors, early markers and outcome measures exist. In general, the distributions of the continuous variables examined in this study required the use of different statistical approaches to deal with the presence of extreme cases and skewed distributions. This type of variables often challenge developmental researchers and might affect

the interpretability of results. For example, in this study peak look duration was log-transformed to make it suitable for polygenic score calculation procedures. This type of transformation, though commonly used to deal with non-normality issues, establishes unverified assumptions on the nature of (i.e., function underlying) the relationship between variables (Feng et al., 2014). Additionally, results might not be directly comparable with results obtained from non-parametric analyses where the peak look durations were not log-transformed.

Another example of issues associated with methodological choices to deal with non-normally distributed data emerged in this chapter when considering that robust maximum likelihood was used in confirmatory factor analysis testing the mediation role of inhibitory control in the path to autism traits. When interpreting the results, one should consider that robust statistics down-weights the most extreme cases (i.e., those showing more difficulties in the social and non-social domain). Possibly, this analysis could be considered as mainly describing the association between variables in the "typical" range. These observations suggest that a collaboration between statisticians and developmental psychologists is advisable to avoid misinterpretation of the results. This is particularly important especially when studying neurodevelopmental disorders, where often the cases outside the normal range are those of interest.

5.5 SUMMARY OF FINDINGS

In conclusion, results of this chapter support the idea that early social attention atypicalities lie in the steps between general risk factors for neurodevelopmental disorders (ADHD polygenic risk and ASD traits in older siblings) and symptoms of ASD (but not ADHD) in mid childhood. Exploratory mediation analyses suggest that ASD symptoms at school age might result from the interaction between atypical looking behaviour towards faces and difficulties in inhibitory control emerging during toddlerhood. Thus, findings obtained triangulating data from affected relatives and genotyping, evaluating familial and polygenic burden, with longitudinal observations tracking the development of behavioural traits across toddlerhood and childhood, highlighted the importance of age-specific investigations of the interplay between risk factors and adaptive behaviours in shaping the path towards ASD. In the next chapter, this approach is undertaken observing epigenetic signatures associated with developmental changes in peak look duration at the face and adaptive skills, as well as emerging ASD.

CHAPTER 6

A PROOF-OF-PRINCIPLE STUDY OF DNA METHYLATION IN INFANTS AT RISK FOR ASD

6.1 INTRODUCTION

Identifying the mechanisms that underpin the emergence of behavioural symptoms is important for understanding the aetiology of ASD, and for designing new focused intervention strategies. The developmental processes involved in the path to ASD remain largely unclear. As exposed in **Chapter 1**, one particularly strong candidate which has been suggested to contribute to the emergence of ASD are disruptions in how the child's brain engages in focused attentive states during social interaction (Klin, Shultz, & Jones, 2015).

The work described in this thesis thus far identified robust neurocognitive correlates of the state of focused attention that enhances learning in the infant brain. When look durations and event-related components to social and non-social stimuli have been examined, infants with later ASD showed profiles consistent with diminished attention engagement in the first eight months of life (**Chapter 2** and **3**). Moreover, by 14 months infants at increased familial and polygenic burden for neurodevelopmental disorders showed longer looking time at a face image presented among other non-social stimuli (**Chapter 3** and **5**). Thus, it is possible that genetic or environmental risk factors for ASD impact the brain systems necessary to maintain attention engagement to social stimuli. This process may reduce children's ability to learn from people around them, which gradually makes the social world less comprehensible as it becomes more complicated (Klin et al., 2015). Infants may then gradually withdraw from the people in their environment as an adaptive response, producing subsequent symptoms of ASD (Johnson, 2017).

Evidence supporting this account could be obtained through linking processes like social engagement and other early developmental trajectories in infants developing ASD to the biological processes that have been associated with clinical diagnosis in large samples. One way to make progress in this area is linking neurocognitive phenotypes and genetics (see **Chapter 4** and **5**). However, this approach does not account for the fact that gene expression patterns are not developmentally static; rather, they change over developmental time and there are substantial individual differences in these changes (Moore, 2016). Understanding the

emergence of ASD requires us to study dynamic changes in both neurocognitive systems and genome function in parallel.

In this chapter, I present a proof-of-principle study that integrating epigenetics into prospective longitudinal studies of high-risk infants holds great potential for closing this gap. These insights will also be valuable to the broader community of researchers interested in the biological foundations of early developmental processes.

6.1.1 Dynamic developmental landscape of DNA methylation and ASD

As described in **Chapter 1** (see **section 1.2.3**), DNAm plays a crucial role in brain development. The fastest changes in DNAm occur during the foetal period (Numata et al., 2012). Although global levels of methylation do not change drastically after birth (Slieker, Roost, Van Iperen, & Suchiman, 2015), animal work indicates that DNAm patterns can change postnatally in interaction with the environment. In mice, extracellular signals and neuronal electrical activity concur in influencing neurons' DNAm levels, inducing long-lasting methylation changes (Guo et al. 2012; Lister, Mukamel, Nery, Urich, Puddifoot, Johnson, et al. 2013). Further, human studies have demonstrated associations between DNAm, environment and psychological phenotype (see Mitchell, Schneper, and Notterman 2015 and Barker, Walton, and Cecil 2018 for a review). Changes in DNAm have also been linked to change in brain activity in adults (e.g. Frodl et al. (2015), Ursini et al. (2011), Hass et al. (2015)). Thus, there are preliminary indications of associations between DNAm and brain activity underpinning cognitive functions.

Given that variability in DNAm is influenced both by genotype and environmental factors (Mitchell et al., 2015) disruption of the delicate interplay between genes and the environment during critical developmental phases might constitute a "perfect storm" which increases the risk of atypical neurodevelopment, as suggested by Ciernia and Lasalle (2016). There is emerging evidence that supports the idea that epigenetic variation between individuals is associated with complex traits and disorders, including ASD (Dall'Aglio et al., 2018). Studies based on single candidate genes or global methylation on small samples, might not have been able to capture insults to the dynamic developmental landscape of DNAm. Moreover, larger case-control EWAS studies (n<1,000) comparing DNAm levels obtained from blood collected at birth (Hannon et al., 2018) or during childhood (Andrews et al., 2018) did not show evidence of association with ASD at the single-probe level. However, differences in DNAm were associated with increased polygenic risk for ASD newborn blood samples (Hannon et al., 2018). Further, Spiers et al. (2015)

found that networks of probes involved in foetal brain development lie within genes associated with ASD. Taken together, these studies support the hypothesis that the DNAm machinery which regulates early development is particularly vulnerable to risk factors contributing to the aetiology of ASD (Ciernia & Lasalle, 2016; Lasalle, 2013). Critical period-specific investigations of the association between DNAm levels and developmental neurocognitive phenotypes may thus illuminate the causal pathway to ASD symptoms.

6.1.2 Limitations of previous studies that can be addressed in the infant-sibling design

Although highly promising, studying the relation between emergence of ASD and DNAm in humans poses practical, technical and theoretical challenges associated with data collection (Kato and Iwamoto 2014; Relton and Smith 2012). Since DNAm is responsible for cellular differentiation, epigenomic patterns vary across tissues. Selecting disease-relevant tissues is preferable in epigenetic studies (Michels et al., 2013). Brain tissue is considered the most relevant tissue for investigating mechanisms involved in neurodevelopmental disorders. Indeed, the study of epigenetic signatures on brain tissue have provided fundamental advances in our knowledge of biological mechanisms underlying the emergence of ASD (Sun et al., 2016; Wong et al., 2018). However, limited availability of human post-mortem brain tissue is a major issue, especially when studying early development and neurodevelopmental disorders. Moreover, DNAm in post-mortem brains will be affected by sample processing steps, donor characteristics (that are often not available), cause and timing of death (Lim et al., 2014), all of which will introduce noise and the possibility of confounding. Further, to really understand the causal nature of the relationship between DNAm and the emergence of ASD traits across development, multiple measurements from the same individual are required (Martino et al., 2013; Michels et al., 2013). This is clearly impossible when using post-mortem brain tissue.

The importance of tracking individual developmental trajectories of DNAm justifies the use of more available peripheral tissues, such as whole blood and buccal epithelial cells isolated from saliva (Barker et al., 2018). Saliva and cheek-swabs are easy to collect, especially from young children and individuals with ASD who might be distressed by a blood drawn procedure. Encouragingly, buccal epithelial cells are potentially more closely related to brain methylation patterns than blood, as they come from the same primary germ layer, ectoderm (Mitchell et al., 2015). For example, Smith et al., (2015) found that DNAm levels in saliva with a high number of epithelial cells appeared generally more similar to DNAm patterns extracted from various brain regions than from blood.

6.1.3 Aims of the study

Linking DNAm data to neurocognitive measures collected within a prospective longitudinal study of high-risk infants could enable unique insights into how epigenetics shape brain development. However, the exposed methodological and practical challenges mean this work is highly novel, expensive and risky, and thus the field is in need of proof-of-principle studies to determine its potential value. In this study, I explore the potential of using cheek-swab samples to analyse DNAm collected at multiple time points in a study of infants at high familial risk of ASD. As preliminary steps, I checked that the DNA isolated from cheek-swabs generated reliable DNA methylation data (as in Hannon et al., 2018). Subsequently, four main questions were addressed in this chapter.

First, I tested whether global DNAm levels (calculated by averaging DNAm levels of all probes across the microarray) are sensitive to either diagnostic categorical or dimensional ASD outcomes at 3 years of age. Studies with sample sizes comparable to the present one have produced mixed results when comparing ASD cases versus controls (Dall'Aglio et al., 2018). I was particularly interested in whether the use of a continuous measure of the ASD phenotype and/or of age and sex homogeneous samples could increase power for identifying relations with epigenetic variation, as previously suggested (Beauchaine & Constantino, 2017; Michels et al., 2013).

Second, I performed EWASes to identify potential significant associations between individual probes and categorical/dimensional ASD outcomes. I explored overlap of any identified probes with independent neurodevelopmental and ASD-related DNAm datasets. Further, I performed power analysis to estimate what sample size would be required to identify EWAS-significant probes at a genome-wide level of significance, thus aiming to determine whether this approach is feasible in the typical scale of infant-sibling studies. Previous estimates are based on adult samples from a wide age range and various tissue sources, and estimates might vary in an infant sample with a narrow age range (Saffari et al., 2018; Tsai & Bell, 2015).

Third, I performed a network-based analysis (Langfelder & Horvath, 2008) to look for associations between the DNAm profiles of suites of related probes and both categorical and dimensional outcomes and candidate endophenotypes. This method has been previously used to study developmental DNAm changes (Spiers et al. 2015) and relationships with dimensional ASD traits (Ginsberg, Rubin, Falcone, Ting, & Natowicz, 2012). The two measures selected have been previously associated with ASD outcome (**Chapter 2** and **3**, see also Hendry et al., 2018; Jones et al., 2016), and familial variation in ASD-related traits (**Chapter 5**, see also Jones, Venema, Earl, Lowy, & Webb, 2017): an eye-tracking measure of peak look at the face (the peak

look duration at the face in a face pop-out task) and a neural correlate of attention engagement (the Nc mean amplitude difference between to face with direct gaze and non-social control stimuli, the Noise stimulus presented in **section 2.2.2**). I used this information in association with upstream genetic regulatory information (developmental stage-specific methylation quantitative traits loci, or mQTL, Gaunt et al., 2016) to ask whether the analysis identified biologically relevant pathways potentially implicated in the early emergence of ASD phenotypes.

Finally, I explored the potential for joint longitudinal analysis of both epigenetic change and change in behaviour using two dimensional measures available at all timepoints (adaptive skills from parent-report, and peak look duration to faces).

Taken together, this chapter aimed to determine whether epigenetics from buccal epithelial cells provides a potentially valuable approach for the field of developmental cognitive neuroscience, and if so what analytic approaches and phenotypic associates are most appropriate to examine.

6.2 METHODS

6.2.1 Participants

The sample for the present study is derived from Phase 2 of BASIS, which consists of 143 children (65 females), as described in **Chapter 2** (see **section 2.2.1** and **Table A2.1** for summary demographic and developmental information for the entire Phase 2 sample). In this sample, 116 children were younger siblings of children with ASD (HR, 52 females) and 27 were infants with no first- or second-degree relatives with ASD (LR, 13 females). Genome-wide DNAm data from cheek-swabs DNA were available for a subset of 63 unrelated male infants from this cohort (49 HR, of whom 11 received a diagnosis of ASD at age 3) across various lab visits. Specifically, children took part in research assessments when they were around 8-month-old (T1), 14-month-old (T2), 2-year-old (T3) and 3-year-old (T4), as previously explained (**section 2.2.1**). Of note, for the subset of children included in this chapter the average age at T2 was 15 months and the average age at T3 was 25 months (see **Table 6.1**). Research assessments included collection of parent-report questionnaires, experimental tasks with eye-tracking and EEG and standardized behavioural assessments. At T4, experienced clinical researchers reviewed all the available measures to determine the clinical outcome.

For 33 participants (21 HR, of whom 5 had ASD), samples were collected at more than one visit. The total number of samples included in each of the analyses and at which time-point they were collected is detailed in **section 6.2.4** and summarised in **Table 6.3**.

Ethical approval for this study was obtained from the NHS Health Research Authority (REC reference number 06/MRE02/73).

6.2.2 DNA methylation

DNAm was quantified using the Illumina Infinium HumanMethylation450 BeadChip. DNA extraction, DNAm array data generation and quality control procedures were performed at King's College London (C. Y. Y. Y. Wong, B. Xia) following established guidelines. Specifically, all DNA samples were randomized with respect to phenotypic status to avoid batch effects throughout all experimental procedures. Genomic DNA (500ng) from each sample was treated with sodium bisulfite using the Zymo EZ DNA Methylation-Lightning Kit™ (Zymo Research, Irvine, CA, USA). Genome-wide DNAm was quantified using the Illumina Infinium® HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) and scanned on the HiScan System (Illumina, San Diego, CA, USA). All samples collected from the same individual across different early developmental stages were processed together on the same array to avoid potential batch effects. Illumina GenomeStudio software (Illumina, San Diego, CA, USA) was used to extract signal intensities for each probe, generating a final report that was imported into R (R Core Team, 2013) using the 'methylumi' package (Davis, Du, Bilke, Triche, & Bootwalla, 2012). Data quality control and pre-processing were performed using 'dasen' from the 'wateRmelon' package as described in Pidsley et al. (2013). Stringent filtering of the pre-normalized Illumina 450K data was performed. Cross-reactive probes and polymorphic CpGs as detailed in the Illumina annotation file and identified in recent publications were removed (Chen et al., 2013; Price et al., 2013). Polymorphic single nucleotide polymorphism control probes (N=65) located on the array were used to confirm individual identity for all longitudinal samples included in the final analysis. CpG sites with a detection p-value >0.05 in 5% of samples or a bead count <3 in 5% of samples identified by the 'pfilter' function within the 'wateRmelon' R package were also removed. Thus, the final analyses comprised of 402,971 probes. Relative methylation (β value) was calculated for each probe as the ratio of the normalized signal from the methylated probe to the sum of the normalized signals of the methylated and unmethylated probes, ranging from 0 (unmethylated) to 1 (fully methylated).

6.2.3 Phenotypes

6.2.3.1 Categorical outcome: ASD and atypical development

At age three, children underwent diagnostic assessment for ASD, including standardized and semi-standardized behavioural assessments, as described previously (**section 2.2.1**). Briefly, based on these assessments and unstructured observations, experienced clinicians assigned the HR children to one of the following three outcome groups: HR-TD, HR-Aty and HR-ASD. In this chapter, analyses using categorical groupings compared both outcome of ASD versus no-ASD (HR-TD, HR-Aty and LR); and of typical (LR and HR-TD) versus atypical (HR-Aty and HR-ASD) development. These groupings will be hereafter referred to as 'ASD' and 'atypical development', respectively.

Table 6.1 shows the number of participants, divided into the four final outcome groups, for whom good quality DNAm data were available. There was no significant difference in age between groups at T1 ($F(3,47)=0.83$, $p=0.48$), T2 ($F(3,20)=0.49$, $p=0.7$) and T3 ($F(3,28)=0.55$, $p=0.65$).

Table 6.1 *Numbers and age of the study participants per outcome group*, for the three visits.

Visit	Outcome group at age 3	N	Mean (SD) age	Total N	Mean (SD) age in months	Min – Max age in months
T1	LR	9	9 (0.9)	51	8.7 (0.8)	8 - 10
	HR-TD	19	8.8 (0.8)			
	HR-Aty	13	8.5 (0.8)			
	HR-ASD	10	8.8 (0.9)			
T2	LR	9	15 (0.9)	24	15.3 (1.1)	14 - 18
	HR-TD	3	15.7 (1.5)			
	HR-Aty	6	15.3 (1.5)			
	HR-ASD	6	15.7 (1.0)			
T3	LR	12	25.1 (1.1)	32	25 (1.7)	24 - 32
	HR-TD	13	25.4 (2.3)			
	HR-Aty	4	25 (1.2)			
	HR-ASD	3	24 (0.0)			

LR: Low-Risk infants; HR-TD: High-Risk infants with Typical Development; HR-Aty: High Risk infants with Atypical development who did not meet criteria for Autism Spectrum Disorder (ASD); HR-ASD: High-Risk infants who received diagnosis of ASD at age 3. N: number of participants; SD: standard deviation; Min: minimum age; Max: maximum age.

6.2.3.2 Dimensional outcome: adaptive skills

As a measure of dimensional outcome, the standard composite adaptive behaviour scores from the second edition of the VABS was used (Sparrow, Cicchetti, & Balla, 2005). VABS is a semi-structured interview measuring adaptive functioning in everyday life, as previously described (see **section 2.2.1**). The composite score is a measure of general adaptive functioning, it typically has mean of 100 and standard deviation of 15 in the general population, with scores between 70 and 80 considered borderline and scores below 70 as indicating deficient adaptive behaviour (Sparrow et al., 2005). In the present research, VABS was collected as a parent-report questionnaire at T1 and T2 and as a parent interview at T3 and T4. VABS composite scores at T4 were used as a measure of dimensional adaptive outcome in Analyses 1-3. VABS composite scores of the same scale at T1, T2 and T3 were used in Analysis 4.

6.2.3.3 Dimensional candidate endophenotypes

Peak look at the face

Infants completed the face pop-out task at T1, T2 and T3 months. Data from T2 were used for Analysis 3, as **Chapter 3** and **5** offered consistent evidence of the potential role of this social attention profile reflected by this measure in the path to ASD; in Analysis 4 data from all three visits were used to examine longitudinal change. The face pop-out task was created to measure infant's attraction to and preference for faces. The full paradigm and processing procedure has been described previously (**section 3.3.2.2**, see also Hendry et al., 2018). Briefly, at each visit, infants were presented with a series of visual arrays composed of five images (face, bird, car, mobile phone and a non-social control stimulus, called Noise, created by randomizing the phase spectra of the face stimulus while keeping the amplitude and colour spectra constant Halit, Csibra, Volein, & Johnson, 2004). In each visit, looking behaviour was recorded with an eye-tracker and the average duration of the longest look (peak look) at the face image was obtained at each time point (A. Hendry).

Nc mean amplitude to face versus Noise

At T1, infants' brain activity was measured during an EEG task as described in **Chapter 2** (see also Elsabbagh, Mercure, et al., 2012). Briefly, colour pictures of a female model whose gaze is either direct or averted, or the Noise control stimulus were presented on a screen. The trial

duration was 800 ms, followed by a 500-ms interval with no visual stimulus. Data pre-processing procedures, described in **section 2.2.2**, were carried out by C. Tye.

Following previous research (Webb, Jones, Merkle, Venema, et al. 2011; Jones et al. 2016; Richards, Reynolds, and Courage 2010), the Negative Central (Nc) ERP component was used as a neural correlate of attention engagement. Nc amplitude was defined as the mean amplitude of the negative deflection between 300 and 800 ms after stimulus onset across left and right frontal regions. Thus, the phenotypic measure of interest was the difference in Nc amplitude between the face with direct gaze and a non-face stimulus, Noise, to reflect attention engagement processes specific to social content. This measure, hereafter also called "Nc mean amp." for simplicity, has been consistently showed to be a precursor of later social difficulties seen in children with ASD (**Chapter 2** and **3**, see also Webb et al. 2011; Dawson et al. 2012; Jones et al. 2016).

Table 6.2 summarises the number of infant siblings for whom DNAm data at T1 and phenotypic information were available.

Table 6.2 *Number of participants who provided DNA methylation data at T1 as well as the phenotypic measures of interest*: ASD, atypical development, adaptive skills (as measured by the Vineland Adaptive Behavior composite standard score) at T4 (3 years of age), peak look at the face at T2 (15 months), and Nc mean amplitude difference between the face with direct gaze and the Noise in the EEG task described in Chapter 2 at T1 (8 months).

Phenotype	N Total	N LR	N HR-TD	N HR-Aty	N HR-ASD
ASD (T4)	51	9	19	13	10
Atypical development (T4)	51	9	19	13	10
Adaptive Skills (T4)	49	9	18	13	9
Peak look at the face (T2)	39	7	12	13	7
Nc mean amp. (T1)	26	4	9	7	6

N: number of participants. LR: Low-Risk infants; HR-TD: High-Risk infants with Typical Development; HR-Aty: High Risk infants with Atypical development who did not fully meet criteria for Autism Spectrum Disorder (ASD); HR-ASD: High-Risk infants who received diagnosis of ASD at age 3.

6.2.4 Analyses

6.2.4.1 Estimated age

DNA methylation (DNAm) dynamically changes with age and obtaining reliable data is fundamental to study developmental epigenetic effects. Estimation of chronological age for individual DNAm data, based on tissue-specific algorithms for age prediction defined as "the epigenetic clock" (Horvath, 2013), has been previously used as a validation analysis of samples collected at birth (Hannon et al., 2018). Similarly, the epigenetic clock algorithm was implemented on the entire dataset for the present study (N=107) to calculate estimated age in years (https://dnamage.genetics.ucla.edu, Horvath 2013). Pearson's correlation between the actual and estimated age was used, as previously done with paediatric cohorts (Hannon et al., 2018).

6.2.4.2 Analysis 1: Global methylation level

A global level of DNAm was calculated for each individual at each visit as a mean of methylation levels across all 402,971 probes. All 107 samples were included in this analysis, accounting for repeated measurements by modelling subjects as random effects. Two multi-level mixed models were used to explore the longitudinal trajectory of the change in global DNAm level in relation to categorical diagnosis of ASD or atypical development. The 'lme' function within the 'nlme' R-package (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2018) was used, with visit as a random factor nested within each subject. Fixed effects of group (ASD or atypical development), visit and their interaction were tested by comparing the overall fit of the multi-level models using a chi-square likelihood ratio test. The significant effect of visit was reported as linear model with T1 as reference. Finally, linear regressions were used to test associations with dimensional outcome measures (adaptive skills). To control for possible effects of age differences within each visit, batch and prenatal exposure to maternal smoking and alcohol intake on global methylation levels, all analyses have been repeated with these variables as covariates.

6.2.4.3 Analysis 2: Epigenome-Wide Association analyses (EWAS)

DNAm collected at 8 months was used for EWAS analyses (N=51). The choice to restrict this analysis to the first timepoint was motivated by the importance of narrow age ranges for DNAm analyses (Michels et al., 2013) and the fact that behavioural symptoms begin to emerge after this age (Szatmari et al., 2016).

A multiple regression was implemented through the 'cpg.assoc' R-package (Barfield et al. 2012) to test the association between DNAm levels and categorical (ASD and atypical development, N=51) and dimensional measures (adaptive skills, N=49) at age three. Since over 10% of the children in our cohort were not of European ancestry, I controlled for population stratification in DNAm data by including 10 PCs based on sets of CpG sites located within 50 bp of 1000 Genomes Project SNPs with minor allele frequency >0.01 (as indicated in Barfield et al. 2014). The location-based PCs showed some degree of correlation with age (participants ranged from 8 to 10 months), and covariates of prenatal exposure to smoking and array order, confirming that the majority of the unwanted variation in the methylome was accounted for. On the contrary, batch effect was not accounted for by the PCs and was thus included as a covariate in the EWAS models.

Two approaches were used to examine whether the associated probes (identified at a "discovery" p-value threshold of $p<5 \times 10^{-5}$, as in previous research on early DNAm and ASD (Andrews et al., 2018; Hannon et al., 2018) appear to be identifying potentially meaningful signals. Given the focus on ASD, the first approach was to examine the proportion of probes located in high-confidence ASD genes in the SFARI Gene database (www.gene.sfari.org, Banerjee-Basu & Packer, 2010, release Nov. 2018). This publicly available database includes all genes which have been previously associated with ASD in published scientific work or are candidate genes for their molecular or functional role (N=1,037 genes). The second approach was to examine the overlap with probes previously related to foetal brain development (Spiers et al., 2015, N=16,543 probes), given that the study involves neurocognitive development in young infants and epigenetic regulation occurs largely prenatally (see **section 2.1.3.4**). EWAS discovery-significant probes were annotated based on UCSC Genome Browser, build hg19 (Karolchik et al., 2004). The proportion of discovery-significant genes/probes with the SFARI and Spiers datasets, respectively, was compared with the proportion of such genes/probes which would have been identified by chance (4.1% for the SFARI genes and 7.1% for the Spiers probes) using chi-square statistics.

Power analyses were conducted to determine the power of the obtained results and what sample size would be required to identify EWAS-significant probes at a genome-wide level of significance. Although recognizing that effect sizes estimated in an underpowered study might not be reliable, I was interested in understanding whether, based on the current results, this approach is feasible in the typical scale of infant sibling studies. Previous estimates are based on adult samples from a wide age range and various tissue sources, and estimates might vary in an infant sample with a narrow age range (Saffari et al., 2018; Tsai & Bell, 2015). G*power (Erdfelder, Faul, Buchner, & Lang, 2009) was used to estimate the optimal sample size needed

to obtain significant results from the probe with the largest effect size and lower p-value with 80% confidence of avoiding the type II error. For sample size calculation based on the observed effect sizes, a p-value threshold criterion of α=2.4 x 10^{-7} was selected, as recently suggested by Saffari et al. (2018) for 450K arrays. Cohen's f^2 was calculated as measure of the effect size of the predictor of interest B (the phenotype) in multiple regressions where the effect of covariates A (the first 10 PCs and batch effect) was accounted for, using the formula in **Equation 6.1** as in Faul et al. (2009).

$$f_B^2 = \frac{R_{AB}^2 - R_A^2}{1 - R_{AB}^2}$$

(Equation 6.1)

6.2.4.4 Analysis 3: Weighted Gene Co-methylation Network Analysis

Identification of aberrant DNAm profiles associated with ASD might reflect either the cause or the consequence of atypical development. To investigate a potential causal link, I explored whether differentially methylated probes identified in our sample reflected known ASD-associated biological processes coupled to underlying genetic variations. Recent research has shown that genetic variation influences DNAm levels in a developmental-specific manner (mQTLs, Gaunt et al., 2016), and a relationship between known ASD-associated genetic variants and DNAm levels is observable at birth in peripheral tissue (Hannon et al., 2018). I therefore used available bioinformatic resources to investigate biological pathways through which genetic factors contribute to early epigenetic signatures associated with our phenotypes of interest.

Weighted Gene Correlation Network Analysis (WGCNA) is an approach used in functional genomic research to identify networks, also called modules, of probes whose expression or methylation levels are consistently correlated in all examined individuals (Zhang & Horvath, 2005). Subsequently, it tests the association between module eigenvalue (ME), representing the expression/methylation profile of the probes of a module, and the phenotype of interest (Langfelder & Horvath, 2008). In this analysis, I used WGCNA to identify modules of probes whose DNAm levels are consistently correlated across infants at T1 (Weighted Gene Co-methylation Network Analysis). A colour was assigned to each of the modules identified using a signed block-wise network construction at a soft-thresholding power of co-methylation similarity of 6. This power threshold was the lowest power that approached the approximate scale-free topology criterion of 0.8, as recommended in the tutorial for this analysis (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/Consensus-NetworkConstruction-man.pdf). Similar modules were aggregated based on their

correlation (rho>0.2). For each of the resulting 23 modules, a module eigenvalue (ME) was calculated as a weighted average-methylation profile for each individual. MEs were correlated with the five phenotypic traits of interest: categorical outcome of ASD and atypical development; dimensional outcome (adaptive skills at age 3) and the two candidate endophenotypes of interest (peak look at the face at 15 months of age and Nc to face at 8 months of age). For module-trait relationships which had significant FDR adjusted p-values (FDR<0.05), correlation between gene significance and the module membership was calculated to verify that probes more associated with the trait were also the most biologically involved in the network (Langfelder & Horvath, 2008).

Subsequently, to examine early genetically driven effects on DNAm levels in the significant module, I used the mQTL database published by Gaunt and colleagues (2016, http://mqtldb.org) to extract a list of variations in nucleotide sequence, or SNPs, known to influence DNAm of the probes of interest (i.e., mQTLs) during pregnancy and at birth. I searched for all mQTLs within 1 Mb from each CpG site in the significant module (default parameter, Gaunt et al., 2016).

I then looked for biological pathways in which the mQTLs might be involved. To do this, I used eSNPO (http://bioinfo.hrbmu.edu.cn/esnpo/), which utilizes available resources to provide functional enrichment pathways for sets of SNPs (Li et al., 2016), indicating what biological function each set of SNPs play all together based on their gene expression profiles. Pathways for the two sets of SNPs previously identified (mQTLs at birth or during pregnancy) were obtained. The first choice was to look for functional enrichment of genes expressed in the brain. However, a null output was obtained when gene expressed in the brain alone were considered for this analysis, indicating that the mQTLs were expressed at the system level and not in the brain only. Therefore, all 12 tissues available in eSNPO were used to derive biological pathways for the two sets of SNPs (i.e., mQTLs at birth or during pregnancy). FDR correction was applied to control for multiple testing of all possible pathways associated with the input SNPs. The entire procedure is illustrated in **Figure 6.7**.

6.2.4.5 Analysis 4: Longitudinal analysis

Tracking the stability and change of association between DNAm and behavioural traits in the first two years of life is important to understand which probes will be potentially involved in developmental mechanisms as they show plasticity (i.e. change across development) in relation to the phenotype. Parameters estimated by the multilevel mixed-effects models used in this analysis allowed me to explore changes in the direction of the relation between DNAm and phenotype across toddlerhood, possibly reflecting epigenetic changes going along with symptom emergence.

This analysis included the subset of 33 infants with repeated measurements at the three visits. Two phenotypic measures were available at multiple visits and were therefore used as dependent variables: VABS composite score as a dimensional measure of adaptive skills (Estes et al., 2015; Robinson et al., 2016) and peak look at the face as a behavioural correlate of social visual attention engagement (Colombo & Cheatham, 2006; Hendry et al., 2018; Jones et al., 2016). The analysis was conducted on two sets of candidate probes resulting from EWASes: those associated with ASD (N=32) and those associated with atypical development (N=33) at a discovery threshold of $p<5 \times 10^{-5}$.

The relation between DNAm level at each probe and continuous phenotypic measure was modelled over time using multilevel mixed-effects linear models (Laird & Ware, 2007) to account for within- and between-child variation. A random intercept model was used to allow for individuals' differences in DNAm at the first visit, thus eliminating possible effects of ancestry differences and other between-individual sources of variability. Multilevel models handle missing values in the measurements and have been used previously in longitudinal research testing the relationship between DNAm and continuous phenotype measures (Simpkin et al., 2015).

$$meth_{ij} = (\beta_0 + u_{0i}) + \beta_1 phen_{ij} + \beta_2 T2 + \beta_3 T3 + \beta_4 phen_{ij} * T2 + \beta_5 phen_{ij} * T3 + \varepsilon_{ij}$$

$\varepsilon_{ij} \sim N(0; \sigma^2_{\varepsilon})$

$u_{0\,i} \sim N(0; \sigma 2u)$

(Equation 6.2)

In **Equation 6.2**, i indexes the individuals, j = 1; 2; 3 indexes the measurement occasion T1, T2 and T3, u_{0i} is a random intercept, which allows children to have different starting DNAm levels. β_1 gives the average change in DNAm per increase in phenotype; β_2 gives the average change in DNAm between T1 and T2; β_3 is the difference in methylation levels between T1 and T3. The estimates of interest to capture developmental role of DNAm in relation to behavioural change were β_4, which represents the change in the direction of the relationship between methylation levels and phenotype between T1 and T2 and β_5 is the change in slope between T1 and T3.
In order to control for multiple testing of many probes, p-values were adjusted using FDR method (Benjamini & Hochberg, 1995). A summary table of the number of samples included in each of the analyses described above is provided (see **Table 6.3**).

Table 6.3 *Number of samples included in all the analyses for the present study* and specification of the time point when the samples were collected (T1: 8 months, T2: 15 months, T3: 25 months).

	Sample for the present study	
	Total N	**Visit**
Analysis 1: Global methylation*	107	T1 (N=51), T2 (N=24), T3 (N=32)
Analysis 2: Epigenome-Wide Association analysis	51	T1
Analysis 3: Weighted Gene Correlation Network Analysis	51	T1
Analysis 4: Longitudinal analysis*	33 VABS/ 27 Face pop-out	T1,T2,T3

* model accounting for repeated measurements from the same individual.
N: number of participants; VABS: Vineland Adaptive Behavior Scales.

6.3 RESULTS

6.3.1 Chronological age estimation for quality control

Estimated DNAm age, obtained using the epigenetic clock algorithm (Horvath, 2013), was significantly correlated with the chronological age at the time of samples collection (rho=0.269, p=0.0051; for comparison, correlation between actual and estimated age in 1,263 blood samples in Hannon et al. (2018) was rho=0.139). This confirmed that DNA isolated from cheek-swabs generated reliable DNAm data for age-specific analyses. Horvath's algorithm (2013) also provides estimates of the tissue of origin and gender of the samples. Those were correctly estimated for the 107 samples used in the current study, providing further proof of data validity.

6.3.2 Analysis 1: Global DNA methylation levels

The multilevel mixed-effect model accounting for differences in global DNAm level in the entire cohort across time points revealed that there was no difference between children with and without ASD in terms of global level of methylation (β=0.0005, s.e.=0.0007, p=0.428). Similarly, there was no significant difference between children with typical and atypical development (β=0.0001, s.e.=0.0005, p=0.86). Global DNAm level was not associated with adaptive behaviour at three years (β=-0.0001, s.e.=0.0003, p=0.66). These results held after correcting for batch,

age (in months) at the time of testing and prenatal maternal smoke and alcohol intake (**Tables A6.2**, **A6.3**, **A6.4**).

DNAm levels increased with time ($\chi^2(6)=23.46$, $p<0.0001$), with global methylation levels being significantly higher at 25 months than at 8 months ($\beta=0.002$, s.e.=0.004, $p<0.0001$). This result was expected based on a previous study reporting general increase in DNAm in buccal cells the first two years of life (Martino et al., 2013). No difference was observed between T1 and T2 ($\beta=0.0006$, s.e.=0.0005, $p=0.212$). Developmental trajectories did not differ between children with and without ASD ($\chi^2(9)=0.588$, $p=0.745$). Similarly, there was no difference in trajectories of global methylation level change when comparing children with typical development at 3 years (LR + HR-TD, N=28) with children with atypical outcome at 3 years (HR-Aty + HR-ASD, N=23, $\chi^2(9)=0.302$, $p=0.86$).

6.3.3 Analysis 2: Epigenome-Wide Association analyses (EWAS)

EWAS results for the categorical and dimensional outcomes are summarized in **Table 6.4**. Results for the power analyses for the signals with lower p-value and highest effect size for the three EWASes are reported in **Table 6.5**. No probes reached the p-value threshold for corrected significance in any analysis, but all analyses revealed a number of probes that were significant at a discovery threshold of $p<5 \times 10^{-5}$. In all cases, the percentage of probes identified in the vicinity of genes in the SFARI database of high-confidence ASD genes was higher, though not significantly, than would be expected by chance (4.1%).

Table 6.4 *Summary of the results of the Epigenome-Wide Association Analyses* for the three phenotypes of interest: ASD, atypical development and parent-reported adaptive skills at age 3.

Phenotype	Participant numbers by outcome group at T4	Normality	λ	Discovery sign. probes	Effect Size >0.01	Max Effect Size	% in SFARI (4.1% by chance)	% foetal brain dev. (7.1% by chance)
ASD	41 no-ASD (9 LR, 19 HR-TD, 13 HR-Aty) vs. 10 HR-ASD	/	1	32	32	0.22	6.25% (SND1, CACNA2D1)	6.25% (DDR2, CFLAR)
Atyp. Dev.	28 Typ. Dev. (9 LR, 19 HR-TD) vs. 23 Atyp. Dev. (13 HR-Aty, 10 HR-ASD)	/	1.12	33	33	0.07	9% (CHD1, PLXNA3, GIGYF1)	6% (cg01257697, ECE2)
Adaptive Skills	N=49 (9 LR, 18 HR-TD, 13 HR-Aty, 9 HR-ASD)	W=0.98, p=0.4	1.01	23	22	0.04	8.97% (PGLYRP2, CDKL5)	8.97% (FOXK1, ADCY10)

Normality: normality of the distribution assessed with Shapiro-Wilk test for the three continuous phenotypes; λ: genomic inflation for each EWAS, where 1 represents no-inflation. Discovery sign. probes: number of probes which were significant at a discovery threshold of $p<5 \times 10^{-5}$; Effect Size>0.01: number of probes with effect size higher than 1%, where the effect size corresponded to the beta estimates of the effect size resulting from the multiple regression; Max Effect Size: maximum absolute effect size observed among the discovery significant probes; % in SFARI: percentage of discovery significant probes in gene enriched for ASD according to the SFARI dataset (Banerjee-Basu & Packer, 2010, last release Nov. 2018); % foetal brain dev: percentage of discovery significant probes which were also found to be implicated in foetal brain development by (Spiers et al., 2015); no-ASD: infants with no diagnosis of ASD at age 3; LR: Low-Risk infants; HR-TD: High-Risk infants with Typical Development; HR-Aty: High Risk infants with Atypical development who did not fully meet criteria for Autism Spectrum Disorder (ASD); HR-ASD: High-Risk infants who received diagnosis of ASD at age 3; Typ. Dev.: Typical Development at age 3; Atyp. Dev.: Atypical Development at age 3;

Table 6.5 *Results from the power analysis of the Epigenome-Wide Association (EWAS) analyses* with the three phenotypes of interest: ASD, atypical development and adaptive skills at age 3. Results for the most significant signal and for the signal with the highest effect size are reported. Columns indicate, for each phenotype (row): the probe's name and UCSC reference gene in parenthesis, the effect size as β estimate in the EWAS (Effect Size β), the effect of the predictor in multiple regression model (f^2), the power of the observed results at the EWAS p-value for each probe (Power observed result), the power for obtaining an epigenome-wide significant result for that signal at a p-value threshold <2.4 x 10^{-7}, recommended by Saffari et al. (2018) for 450k arrays (Power EWAS signal) and the estimated number of participants for obtaining epigenome-wide significant results with 80% power (N for EWAS signal).

	EWAS most significant signal						**EWAS signal with the highest effect size**					
	Probe name (gene)	**Effect size (β)**	**f^2**	**Power observed result**	**Power EWAS signal**	**N for EWAS signal**	**Probe name (gene)**	**Effect size (β)**	**f^2**	**Power observed result**	**Power EWAS signal**	**N for EWAS signal**
ASD	cg15976650 (TUFT1)	0.02	0.75	0.77	0.48	64	*cg23367851 (CYCS)*	0.22	0.63	0.75	0.32	73
Atyp. Dev.	cg21973914 (F10)	0.06	1	0.79	0.77	53	cg06963664 (PLXNA3)	0.07	0.56	0.74	0.23	80
Adaptive Skills	cg26862175 (STAB1)	0.02	0.88	0.89	0.58	57	cg13707005 (CUGBP2)	0.05	0.57	0.72	0.2	79

6.3.3.1 Categorical outcome

ASD diagnosis

EWAS was first performed for prediction of categorical ASD outcome comparing no-ASD (LR, HR-TD and HR-Aty) versus ASD (HR-ASD). No probe was statistically significant at the empirical epigenome-wide significant threshold after controlling for multiple testing ($p<2.4 \times 10^{-7}$, see Saffari et al., 2018). At a discovery threshold of $p<5 \times 10^{-5}$, 32 probes were significantly associated with ASD, of which 2 probes (6.25%) are in genes that have been previously linked with ASD, according to the SFARI dataset. Of note, 4% of the total number of analysed probes were associated with genes in the SFARI dataset, so the higher enrichment observed in this EWAS was not statistically significant ($\chi^2(1)=0.373$, p=0.27).

Discovery-level significant probes are reported in **Table A6.5**. **Figure 6.2** illustrates patterns of group differences for three selected probes: TUFT1, the gene associated with the probe with the most significant (i.e. lowest) p-value; CYCS, the gene associated with the probe with the largest effect size; and SND1, whose mutation leads to presynaptic myasthenic congenital syndrome 7 (GeneCards, Stelzer et al. 2016) and whose common (Holt et al., 2010) and rare (Iossifov et al., 2012) variations have been previously associated with ASD.

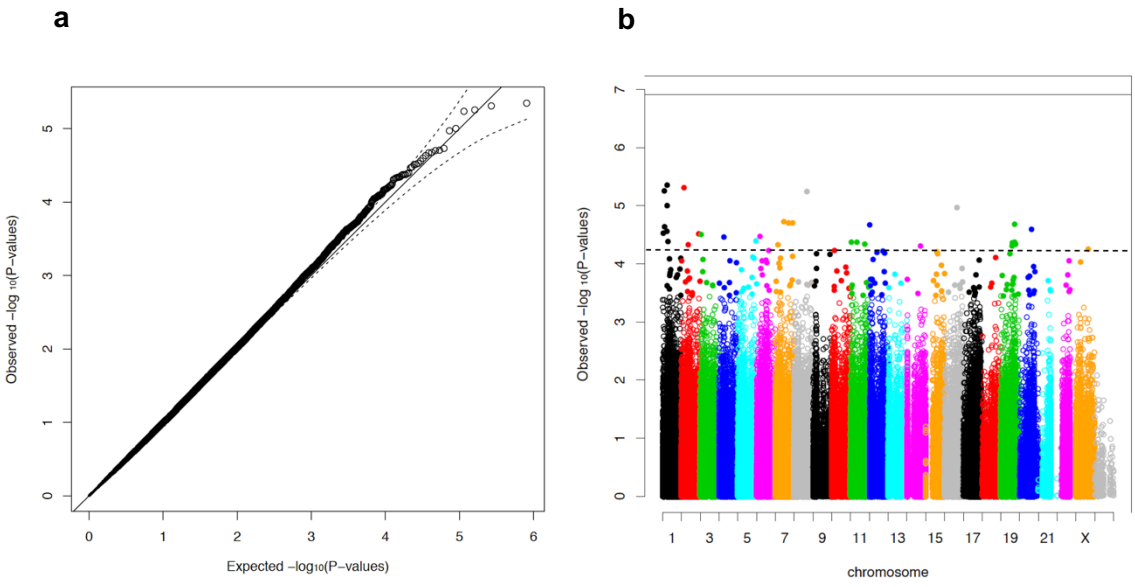

Figure 6.1 *Epigenome-wide association with ASD*. **a** Q-Q plot of the EWAS for no-ASD versus ASD, with dashed lines representing 95% CIs. **b** Manhattan plot of p-values from the ASD EWAS. The solid horizontal line indicates experiment-wide significance ($p<1.2 \times 10^{-7}$). The dashed horizontal line indicates the discovery threshold ($p<5 \times 10^{-5}$).

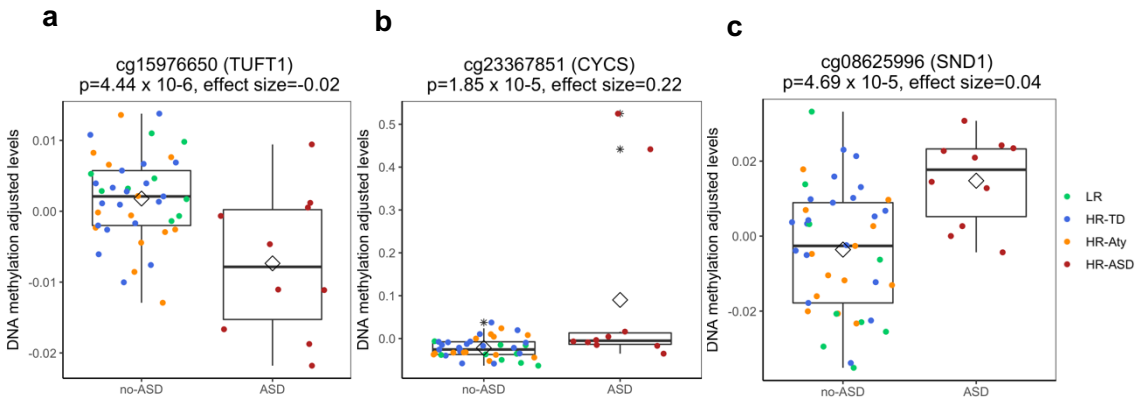

Figure 6.2 *Boxplots illustrating DNA methylation levels by ASD outcome for three of the top-ranked probes associated with ASD*. **a** the most significant signal (probe cg15976650 in the CpG island of gene TUFT1), **b** the signal with the highest effect size (probe cg23367851 in the shore of gene CYCS) and **c** a gene previously associated with ASD (cg08625996 located in gene SND1). In all figures, individual data are represented by points, colour-coded by outcome group. The lower and upper hinges of the boxplots correspond to the first and third quartiles (the 25th and 75th percentiles). The upper and lower whiskers extend from the hinges to the largest and smallest values, respectively, no further than 1.5 x inter-quartile range. Data beyond the end of the whiskers are plotted individually and represented by stars. Horizontal lines within boxplots indicate the median value, while rhombi represent mean values.

Sample size estimation to obtain 80% power for these results was based on the amount of variance in DNAm levels explained by having ASD, as in the EWAS model accounting for 10 PCs and batch effect (f^2, Faul et al., 2009). For CYCS, seventy-three individuals would be needed under the current study design, with a cases-controls ratio of 1:4, to reach 80% power of detecting a difference in DNAm with a epigenome-wide significance threshold estimated for 450k array (Saffari et al., 2018). Sixty-four individuals would be necessary to obtain epigenome-wide significant results for the most significant probe with 80% power, while 81 would allow higher confidence in the result for the probe in the ASD-related gene SND1. **Figure 6.3** shows the sample size needed to obtain higher power for the same EWAS under the current study design.

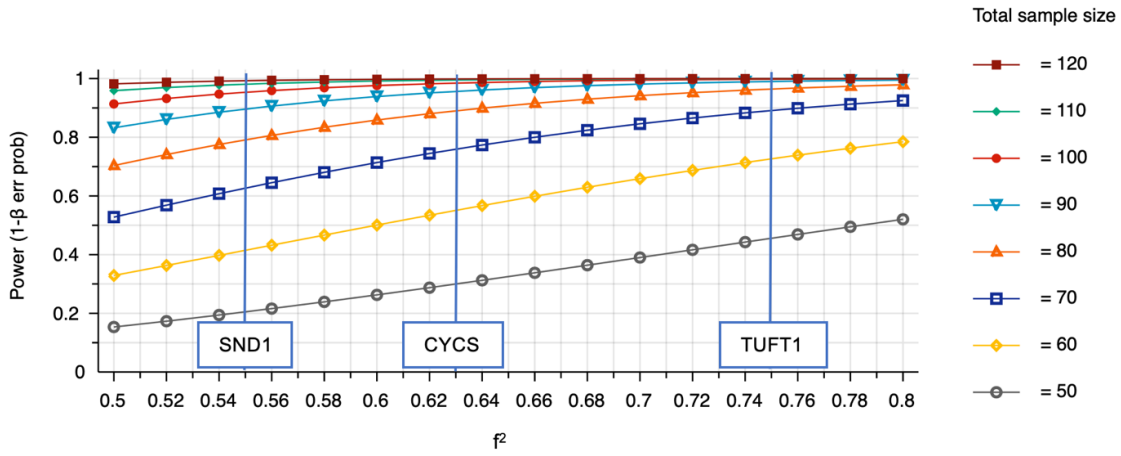

Figure 6.3 *Sample sizes needed to obtain significant results at an epigenome-wide significant threshold* of 2.4 x 10^{-7} (Saffari et al., 2018) under the current design, as a function of power and f^2 for the ASD EWAS. Cohen's f2 is reported in the x-axis as a measure of the relative amount of DNA methylation variance explained by ASD in the multiple regression models used for EWAS calculation (Faul et al., 2009). Labelled blue lines represent Cohen's f2 values resulting from the EWAS models for probe cg08625996 (SND1), cg23367851 (CYCS) and cg15976650 (TUFT1).

Atypical development at age 3

An EWAS analysis for atypical development was conducted to compare DNAm levels at 8 months between infants who later underwent typical (LR+HR-TD, N=38) versus atypical development (HR-Aty+HR-ASD, N=25). The top hit for this analysis, probe cg21973914 located in the shore of gene F10, was most significantly associated with outcome with an FDR-corrected p-value of 0.13 (p=3.34 x 10^{-7}). Gene F10's primary role is to encode the vitamin K-dependent coagulation factor X of the blood coagulation cascade, and is involved in calcium ion binding and phospholipid binding (GeneCards, Stelzer et al. 2016). The probe with the highest effect size (cg06963664), which was 7.3% more methylated in infants who showed signs of atypical development at 3 years of age, is located in gene PLXNA3, whose deleterious variants have been found in ASD probands from the Simons Simplex Collection, Autism Sequencing Consortium and Chinese population (Niklas Krumm et al. 2014; Guo et al. 2017). This signal was detected with 74% power, while 80 individuals would be needed to obtain 80% power for this analysis under the current study design.

Overall, 33 probes were associated with atypical development at a discovery p-value threshold <5 x 10^{-5} (**Figure 6.4**, **Table A6.6**). Three of the top probes (9%, slightly higher than what expected by chance: $\chi^2(1)= 1.83$, p = 0.088) have been found to be associated with genes within the SFARI dataset. One of these has been previously described as the probe with the highest effect size

(cg06963664, gene PLXNA3). Additionally, probe cg21082921 is located in the promoter region of gene CHD1. Missense and *de novo* mutation of this gene have been found in individuals with ASD (Iossifov et al., 2014; Neale et al., 2012) and other neurodevelopmental disabilities such as speech apraxia and developmental delay with and without seizures (Pilarowski et al., 2018). Last, probe cg05922723 is located in the GIGYF1 gene, whose *de novo* mutation has been recognised in ASD probands by three different studies (De Rubeis et al. 2014; Iossifov et al. 2014; Krumm et al. 2015).

Two of the discovery significant probes (6%, that is less than 7.1% expected by chance, $\chi^2(1)=0.05$, p=0.82) have been previously highlighted by Spiers et al. (2015) as possibly contributing to brain development during the foetal period: probe cg01257697 and probe cg04729574 located in gene ECE2, which is involved in processing various neuroendocrine peptides (GeneCards, Stelzer et al. 2016). Results of the power analysis for the signals identified with atypical development EWAS were qualitatively similar to those of the ASD EWAS (**Figure A6.1**, **Table 6.5**).

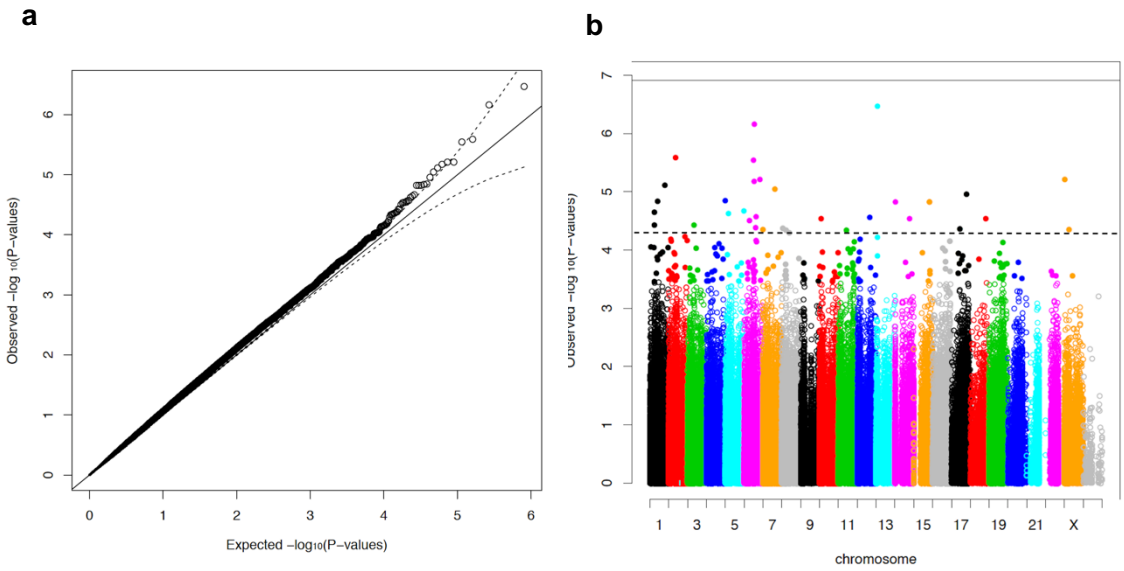

Figure 6.4 *Epigenome-wide association with categorical outcome of atypical development*. **a** Q-Q plot of the EWAS for typical versus atypical development. **b** Manhattan plot of p-values from the atypical development EWAS. The solid horizontal line indicates experiment-wide significance ($p<1.2 \times 10^{-7}$). The dashed horizontal line indicates the discovery threshold ($p<5 \times 10^{-5}$).

6.3.3.2 Dimensional outcome

Adaptive skills

The VABS Composite Score was used to measure adaptive skills at age 3. The most significant probe associated with adaptive skills was probe cg26862175 ($p=1.08 \times 10^{-5}$). This probe is located in gene STAB1, which encodes a large, transmembrane receptor protein involved in calcium ion binding and protein disulfide oxidoreductase activity. Its paralog, STAB2, has been associated with La Cross Encephalitis, which mainly occurs in children younger than 16 (GeneCards, Stelzer et al. 2016).

The probe with the highest effect size for this analysis, cg13707005, showing a 4.5% decrease in methylation in the CUGBP2 gene, was associated with one standard deviation increase in adaptive skills at 3 years. The CUGBP2 gene is related to autosomal dominant Childhood Absence Epilepsy and Neuroblastoma, which both emerge during childhood (GeneCards, Stelzer et al. 2016). Power analysis revealed that a sample of 78 male infants would be needed to obtain at least 80% power to detect a significant signal at a threshold of $p<2.4 \times 10^{-7}$ for this analysis (**Figure A6.2**).

Twenty-three probes were significantly associated with VABS Composite Score at 36 months using a discovery threshold of 5×10^{-5}, but all with FDR adjusted p-values were >0.8 (**Figure 6.5**, **Table A6.7**). Of those, 2 probes are enriched for ASD-related genes (8.7% vs. 4.1% expected by chance, $\chi^2(1)=1.086$, p=0.149). Probe cg26853265 can be found in the CpG island of gene PGLYRP2, whose evidence for implication in a functional role in ASD comes from mice studies. Arentsen et al. (2017) showed that PGLYRP2 regulates the development and formation of brain circuits by passing the blood-brain barrier. In fact, it is highly expressed in the neonate brain despite being responsible for recognition of the peptidoglycan which can be found in the gut microbiota (Arentsen et al., 2017). PGLYRP2 knock-out male juvenile mice showed higher preference for social situations compared with controls, and reduced expression of the ASD risk gene c-Met in the striatum, the brain structure devoted to integration of social information into goal-directed and rewarding behaviours (Báez-Mendoza & Schultz, 2013). The present results indicate that lower methylation levels in the CpG island of gene PGLYRP2 predicts lower higher adaptive skills at three years. Probe cg01283227 is located in a gene on chromosome X, CDKL5, which is considered syndromic for ASD. It is involved in the BDNF signalling pathway and its mutations lead to Rett's Syndrome, Early Infantile Epileptic Encephalopathy and various symptoms of neurodevelopmental disorder (Talkowski et al. 2012). It is a recognized cause of

monogenic ASD in males (Codina-Solà et al., 2015). In the present study, increased DNAm in the shore of this gene was associated with better adaptive skills at age three.

Two of the discovery significant probes (8.7%, when 7.12% that would have been expected by chance, $\chi^2(1)=0.073$, p=0.39), have been previously associated with brain development in the foetal period by Spiers et al. (2015). Cg26344392 is located in a gene, ADCY10, which has a role in mammalian spermatogenesis, while the other, cg00208274, is located in gene FOXK1, which is involved in transcriptional regulation especially of myogenic cells and plays a role in remodelling processes of muscles occurring in response to physiological stimuli (GeneCards, Stelzer et al. 2016).

Three of the discovery-significant probes resulting from this analysis (cg07152030, on gene ARF1, cg05175964 located in gene NR2E3 and cg23281307 in gene CASP8) were also highly associated with atypical development in the previous EWAS, suggesting that these two analyses were partly observing the same signal.

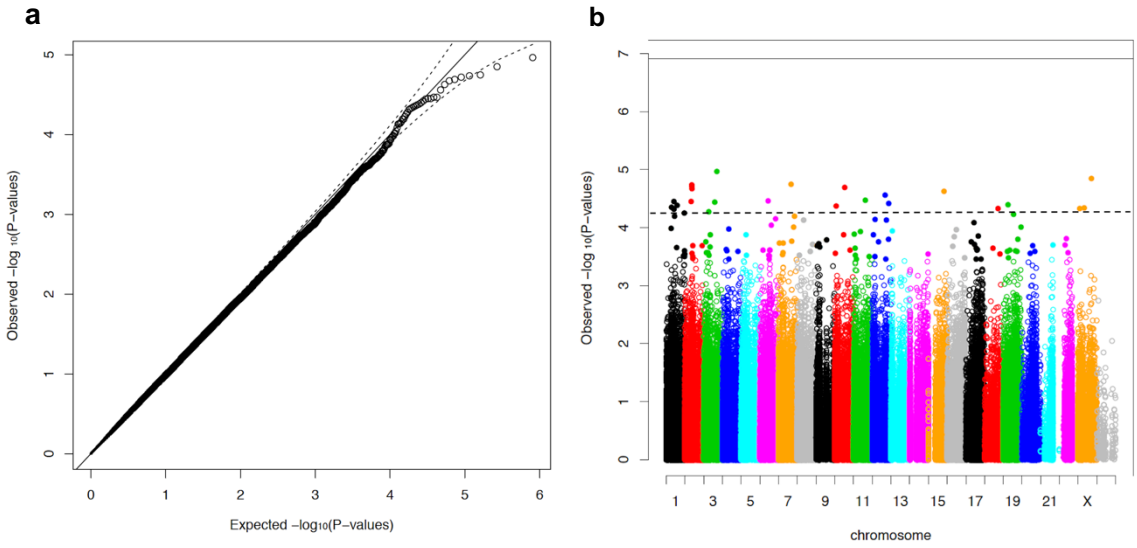

Figure 6.5 *Epigenome-wide association with dimensional outcome*. The phenotype for this analysis was a measure of parent-reported adaptive skills, by the Vineland Adaptive Behavior Scales (VABS) Composite Score at age 3. **a** Q-Q plot of the EWAS for adaptive skills. **b** Manhattan plot of p-values from the adaptive skills EWAS. The solid horizontal line indicates experiment-wide significance ($p<1.2 \times 10^{-7}$). The dashed horizontal line indicates the "discovery" threshold ($p<5 \times 10^{-5}$).

6.3.4 Analysis 3: Weighted Gene Co-methylation Network Analysis (WGCNA)

Summary results for WGNCA conducted for both categorical and dimensional outcomes and candidate endophenotypes (**Figure 6.6**) for all infants' DNAm data at 8 months revealed that one network (the *slate blue* module) was significantly associated with Nc mean amplitude with a p-value=4 x 10^{-7} (FDR<0.0001). No other association reached corrected significance. Probes in the slate blue module showed higher DNAm in relation to enhanced neural activation in response to Noise over faces with direct gaze (**Figure A6.4**). This result was driven by a case that showed extreme, though plausible (see **Figure A6.5**) values of Nc amplitude difference between conditions, with greater Nc in response to the noise stimulus. This subject also had an extreme methylation profile (ME) for the slate blue module, though he did not show overall atypical DNAm genome-wide (see **Figure A6.6**). This result must be treated with caution given that it is significant only in the presence of one influential case. However, as there was no a priori reason to exclude the subject from the analysis, I explored this finding with additional analyses aimed to understand whether it could reflect plausible biological mechanisms that, if altered due to abnormal methylation, could be related to neural atypicalities.

Significant correlations between module membership and gene significance (rho=0.69, p=0.0008) suggested that more relevant hubs in the network were also more associated with the trait, although only 20 probes were involved in this module. The list of annotated probes is reported as **Table A6.8**. None of these probes resulted significantly associated with the phenotypes of interest in the EWASes. One probe, cg18962750, is located in a gene which has been previously associated with ASD, DIXDC1 (5% of the probes in the module, not significantly larger than what expected by chance, $\chi^2(1)=0.04$, p=0.42).

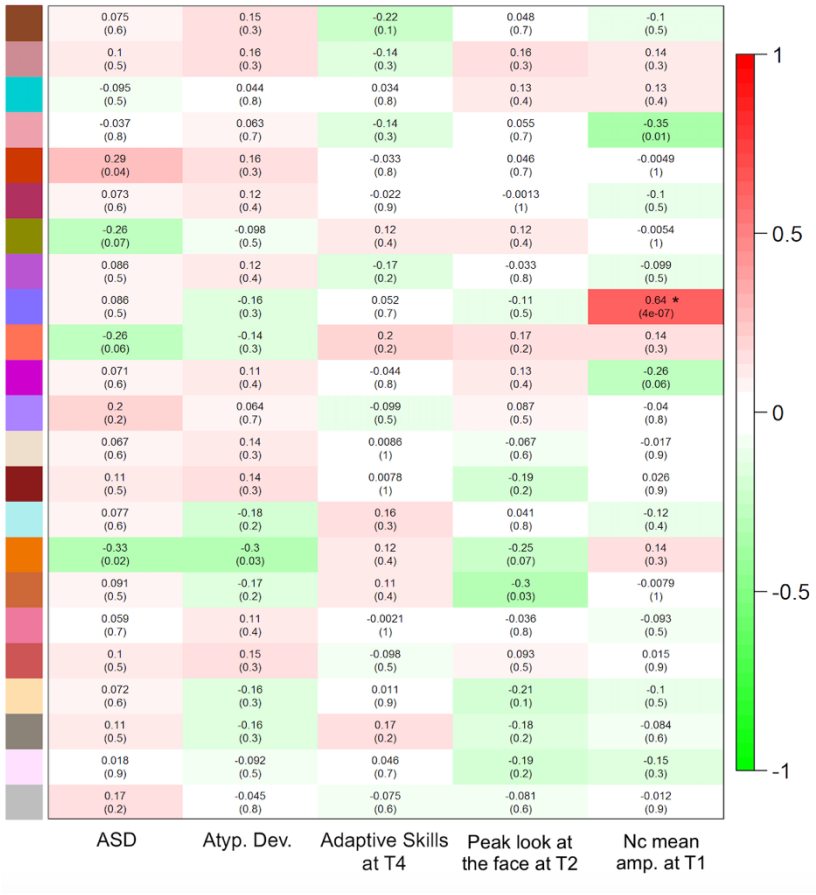

Figure 6.6 *Heatmap representing the correlation between module eigenvalues resulting from the Weighted Gene Co-methylation Network Analysis* (WGCNA) and the five phenotypes of interest: ASD at age 3, atypical development at age 3, adaptive skills at age 3, peak look at the face at 15 months, and Nc mean amplitude difference between the face with direct gaze and the Noise condition in the face/non-face EEG task. Rows represent modules, identified by a colour, and columns represents phenotypes. Strong positive correlation is indicated by red, negative correlation is indicated by green, and no correlation is indicated by white, as displayed in the colour scale bar. Cells contain the correlation and, in parentheses, p-values for each pair of module-phenotype. Star represents that the p-value is significant after controlling for multiple testing using FDR.

A method for interpreting the relevance of epigenetic signatures is to explore the profile of genes that exert a direct effect over methylation levels in those probes (Gaunt et al., 2016). To do this, I identified the quantitative trait loci that influence DNAm of probes in the significant network (mQTLs) during pregnancy and at birth, using the mQTL Database published by Gaunt et al., 2016. I found 573 mQTLs (571 in *cis* and 2 in *trans*) during pregnancy, while 385 SNPs (369 in *cis* and 16 in *trans*) are mQTLs of these probes at birth. 356 probes were shared signals, indicating that they influenced infants' DNAm at both time points.

Functional expression pathways were identified for the two sets of mQTLs (birth and pregnancy) using eSNPO (Li et al., 2016). FDR-significant functional pathways enriched in the set of genes regulating DNAm levels of the network associated with Nc mean amplitude are reported in **Tables A6.9** and **A6.10**. Highly significantly enriched GO pathways for mQTL SNPs at birth and

during pregnancy which are plausible mechanisms involved in ASD were: biological processes involving forebrain ventricular zone progenitor cell division, cerebral cortex radially oriented cell migration, negative regulation of neuron differentiation (all FDR*s*<2 x 10^{-16} for mQTLs during pregnancy and birth), synaptic transmission (FDR=3.31 x 10^{-6} and 1.38 x 10^{-7}, respectively) and cellular component of myelin sheath (FDR=5.89 x 10^{-5} and 2.40 x 10^{-5}, respectively). Other SNPs-GO pathways potentially associated with ASD were: biological processes for response to ischemia (FDR*s*<2 x 10^{-16}) which is one potential source of vulnerability for the autistic brain (Johnson 2017), sleep (FDR*s*<2 x 10^{-16}), which is atypical in people with ASD (Won, Feldman, & Huffman, 2019) and, for mQTL SNPs at birth, calcineurin complex (FDR=0.002) and calcineurin-NFAT signalling cascade (FDR=0.009) involved in activity-dependent GABAergic interneuron tuning (Bannai et al., 2009). Of interest might be also GO pathways associated with mitochondrial biological processes, given that mitochondrial dysfunctions are a proposed mechanism for ASD (Essa et al., 2013; Siddiqui, Elwell, & Johnson, 2016): pyruvate dehydrogenase (NAD+) activity at birth (FDR<2 x 10^{-16}) and mitochondrial pyruvate dehydrogenase complex in both SNPs sets (FDR*s*<2 x 10^{-16}); and cellular components: mitochondrial matrix (FDR=0.045 during pregnancy and FDR=0.015 at birth), and mitochondrion (FDR=0.049 at birth but non-significant during pregnancy).

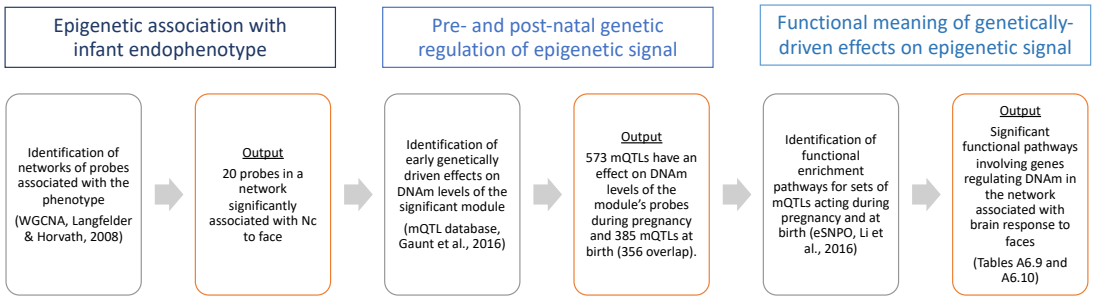

Figure 6.7 *Diagram illustrating the steps (grey boxes) and outputs (orange) of the Weighted Gene Co-methylation Network Analysis*, methylation quantitative trait loci analysis and functional enrichment pathway analysis.

WGCNA: weighted gene co-methylation network analysis, Nc: negative central event-related potential component; DNAm: DNA methylation; mQTLs: methylation quantitative trait loci.

6.3.5 Analysis 4: Longitudinal analyses

The sample for the present analysis included 33 and 27 children with multiple DNAm and adaptive skills and peak look at the face, respectively (five of those received ASD diagnosis at age 3). The number of subjects for each visit, divided by outcome group, is shown in Table 6.6.

Table 6.6 *Number of participants for each time point and total number of unique participants included in Analysis* 4 as providing valid DNA methylation data and phenotypic measures for at least two of the three visits (T1, T2 and T3). Number of participants are reported for the two phenotypic measures of interest: parent-reported adaptive skills (measured by the standard composite score of the Vineland Adaptive Behavior Scales) and peak look at the face during a face pop-out eye-tracking task.

	T1 (8 months)		T2 (15 months)		T3 (25 months)		Total N unique	
	Adaptive skills	Peak look at the face	Adaptive skills	Peak look at the face	Adaptive skills	Peak look at the face	Adaptive skills	Peak look at the face
LR	8	7	9	6	11	7	12	9
HR-TD	9	6	3	2	9	6	10	7
HR-Aty	6	4	5	4	3	4	6	6
HR-ASD	5	5	5	3	3	3	5	5
Total N	28	22	22	15	26	20	33	27

LR: Low-Risk infants; HR-TD: High-Risk infants with Typical Development; HR-Aty: High Risk infants with Atypical development who did not meet criteria for Autism Spectrum Disorder (ASD); HR-ASD: High-Risk infants who received diagnosis of ASD at age 3.

One potentially valuable feature of longitudinal analysis is the possibility to examine the probes that changed the direction of association with phenotypes at the later ages, providing insights on the timing of DNAm influence on the phenotypic trait. Longitudinal analyses focused on the probes which were identified for suggestive significance by the EWAS for ASD (N=32, **Table A6.5**) and atypical development (N=33, **Table A6.6**).

For probes associated with ASD, DNAm levels of one probe (cg21348771) showed an age-specific change of association with peak look at the face between T1 and T2 (β=-0.008, s.e.=0.003, p=0.003, FDR=0.08, **Figure 6.8**). Interestingly, this probe is in the shore of gene GFOD1, which has been associated with ADHD (GeneCards, Stelzer et al., 2016). No other signals were significant after controlling for multiple testing (all FDR*s*>0.1, **Tables A6.11** and **A6.12**).

When analysing the probes associated with atypical development, DNAm levels of one probe (cg04089240 in gene TRPM5, **Figure 6.9**) showed significant change in direction of the association between DNAm levels and adaptive skills at 15 months (β=-0.003, s.e.=0.0009, p=0.002, FDR=0.03). Of note, de novo loss of function mutation in the TRPM5 gene was found to be associated with ASD (Neale et al., 2012). No other probe showed significant association with adaptive skills (**Table A6.13**) or peak look at the face (**Table A6.14**) after correcting p-values for multiple testing.

Although very preliminary due to the small sample size, these results showing changes in the relationship between phenotype and DNAm during the second year are interesting because the time period between 8 and 15 months has been associated with emerging atypicality related to later ASD in both looking behaviour (Elsabbagh, Gliga, et al., 2012; Hendry et al., 2018) and behavioural measures including the adaptive skills measured with the VABS (Bussu et al., 2018; Estes et al., 2015) in previous work.

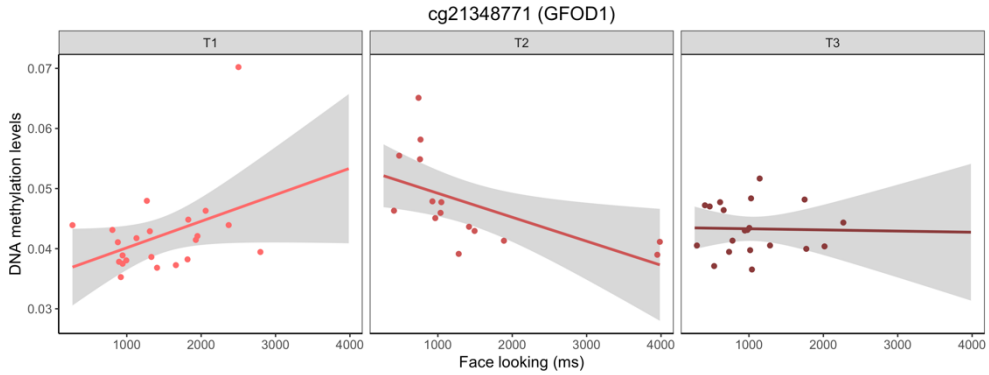

Figure 6.8 *Scatterplots representing the relationship between peak look at the face (x-axis) and DNA methylation levels of the cg21348771 probe* located in the promoter region of the GFOD1 gene (y-axis) for the three visits: T1 (8 months), T2 (15 months) and T3 (25 months). Peak look at the face was measured as average peak look duration at the face in a face pop-out eye-tracking task, in milliseconds. Regression lines are depicted, with shaded grey areas representing standard errors.

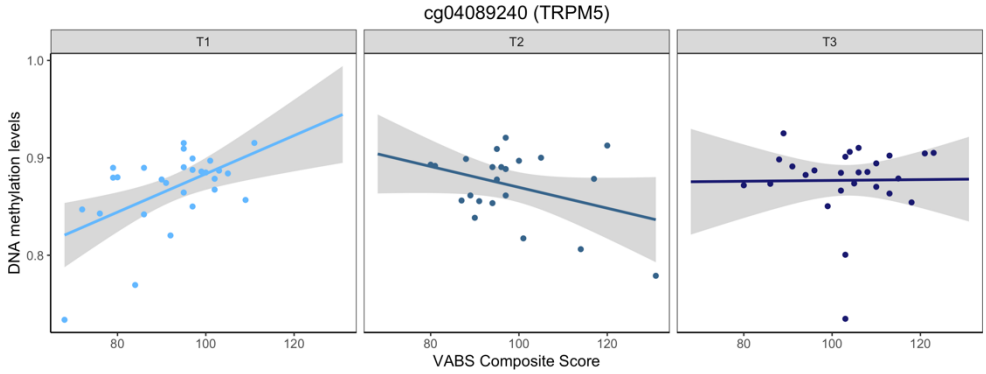

Figure 6.9 *Scatterplots representing the relationship between parent-reported adaptive skills (x-axis) and DNA methylation levels of the cg04089240 probe* located in the shore of gene TRPM5 (y-axis) for the three time-points: T1 (8 months), T2 (15 months) and T3 (25 months). Adaptive skills were measured with the composite standard score of the Vineland Adaptive Behavior Scales questionnaire. Regression lines are depicted, with shaded grey areas representing standard errors.

6.4 DISCUSSION

Incorporating epigenetic analysis into prospective longitudinal studies of infants with later ASD could help delineate the mechanisms that underpin the emergence of the disorder. First, I did not find differences in whole-genome methylation associated with ASD emergence, in line with some other reports in larger samples (Hannon et al., 2018). Second, this study showed that

although EWAS did not produce genome-wide significant hits in a sample of this size (as expected), altered DNAm profiles were identified in a number of probes that are located in close proximity of high-confidence ASD genes at a discovery-level threshold. Further, power analysis revealed that the required number of participants to obtain well-powered results under in the current study design was lower than has been previously reported, suggesting that epigenetic analysis of even modest cohorts (around 90-100 participants) is feasible and promising. Third, a network-based analysis coupled with neuroimaging phenotypes did produce significant associations after correction for multiple comparisons. Further, biological pathways identified through examination of mQTLs linked to probes in the significant network were relevant to brain development. This supports other evidence that neuroimaging metrics may be highly promising endophenotypes appropriate for analysis of genetic linkage (Geschwind & Konopka, 2009; Nikolova & Hariri, 2015; Wiers, 2012). Finally, the longitudinal analysis revealed dynamic changes in the relation between DNAm of candidate probes and ASD-relevant behaviours between the first and second years of life. Since this is the time at which behavioural markers of ASD typically emerge, such longitudinal analyses may hold promise for understanding underpinning mechanisms of change (Gliga, Jones, Bedford, Charman, & Johnson, 2014). Together, the analyses reported in this chapter indicate that epigenetic analysis may be a promising strategy for the integration of neurobiology and neurocognitive insights into ASD emergence.

6.4.1 Whole genome methylation

As expected based on previous studies of cheek-swab samples collected during in the first two years of life (Martino et al. 2013), an increase of global DNAm was observed in the genome for the entire sample between 8 and 25 months. This confirms the importance of using narrow age ranges in DNAm studies, especially when investigating mechanisms of development (Michels et al., 2013). However, I did not find significant differences in global methylation between infants with and without later ASD or with and without later atypical development. Effect sizes were <0.0005, suggesting that if any significant difference between groups existed it would have only been identified with extremely large samples. In accordance with other studies (see Dall'Aglio et al., 2018), the present study did not replicate previous claims about a general disruption of DNAm in individuals with ASD (James, Shpyleva, Melnyk, Pavliv, & Pogribny, 2013; Tsang et al., 2016). Of note, previous studies reporting differences in global DNAm in ASD have studied children over 2 (James et al., 2013; Tsang et al., 2016). It is possible that differences in global methylation between people with and without ASD emerge later in life, as environmental

influences on the epigenome become more consistent (Fraga et al., 2005). However, the present data gave no indications of different developmental pathways for children with typical and atypical development up to two years of age.

6.4.2 Single probes

No individual probes reached a strict genome-wide significance threshold in the EWAS analyses. This was expected since the sample size was relatively small (though comparable to many previous studies on DNAm and ASD; see Dall'Aglio et al. 2018 for a review). However, most effect sizes resulting from our categorical analyses are comparable with previous case-control DNAm studies, with the most highly associated probes having an effect size (β coefficients of regression) ranging from 0.005 to 0.1, indicating a difference of 0.5-10% in DNAm level between individuals with and without ASD (Dall'Aglio et al., 2018). For example, the most significant signal in this sample was a probe with 2% average difference between children with and without ASD. Previous power computations for EWAS signals have suggested that sample sizes of 110-120 cases and controls are necessary for epigenome-wide significance (Saffari et al., 2018; Tsai & Bell, 2015). Importantly, these simulation analyses have indicated that more powerful signals are found when variance in DNAm is smaller, and thus more homogenous phenotypic samples may give larger effect sizes (Tsai & Bell, 2015). Accordingly, characteristics in the infant-sibling designs might lead to higher power for EWAS analyses. For example, this study examined DNAm levels of males only, ranging between 7 and 10 months of age, all receiving accurate diagnostic assessment by the same clinical team at the same age. Results of an exploratory power analysis conducted on the observed results confirm that EWAS in infant-sibling designs may require substantially smaller sample sizes than previously estimated (see **Table 6.5**). Of note, these results should be considered with caution given that the estimates of the examined effect sizes might be unreliable as obtained in an underpowered study (Saffari et al., 2018; Tsai & Bell, 2015).

I also examined the probes that were associated to each phenotype at a discovery threshold to further probe the potential promise of this approach for the study of early neurodevelopmental trajectories. I identified probes in high confidence ASD genes according to the SFARI Gene resource (Banerjee-Basu & Packer, 2010), and probes previously related to foetal brain development (Spiers et al., 2015). In both cases, the proportion of probes in the discovery set found in the SFARI datasets was higher, although not significantly, than that expected by chance given the proportion of all the analysed probes in those datasets. With regards to probes

previously implicated in foetal brain development, the proportion of shared signals was not higher than expected by chance for all phenotypes (**Table 6.4**).

Enrichment for ASD genes was greater for the comparison between typical and atypical outcome and with dimensional variation in adaptive behaviour (around 9%) than for ASD versus no-ASD (6%). This may due to the more balanced participant numbers for the former analyses. However, the ASD case-control contrast did reveal an intriguingly strong signal for a particular probe (cg23367851). This probe was very highly methylated in two individuals (see **Figure 6.2b**), although non-parametric analysis still gave nominally significant results (Wilcoxon rank sum test: W=99, p=0.011). This result could be an artefact of the underlying genotype signal. Reassuringly, no SNP was directly associated with this probe, although one SNP (rs58704610) was annotated within 10 bp from it. Genotype information for this SNP was not available from the gBASIS study. I did not observe three groups of segregation of DNAm level similar to genotype but given the small sample this does not allow us to exclude the possibility the DNAm signal might be confounded by of an effect of genotype.

6.4.2.1 The CYCS probe

This probe is associated with the promoter region of the CYCS gene, which is a central component of the electron transport chain in mitochondria (GeneCards, Stelzer et al. 2016). Given that mitochondrial dysfunctions have been found previously in individuals with ASD (Siddiqui et al., 2016), I leveraged the availability of a range of phenotypic measures collected as part of BASIS in the first three years to conduct post-hoc exploratory verification of the plausibility of such mechanism. Examination of phenotypic data from these two individuals revealed that parents reported lower motor skills at 8 months compared to the other adaptive domains (**Figure A6.3a**). Data from standardized behavioural assessments confirmed that at 8 months they showed lower gross and fine motor skills, respectively, with respect to their peers (**Figures A6.3b** and **A6.3c**), in line with findings reporting motor delay and fatigability in individuals with mitochondrial dysfunction and ASD (Weissman et al., 2008). Longitudinal data available revealed that this effect was not persistent, suggesting that, if the obtained result reflects a true pathophysiological mechanism, this represents a transient effect (which, occurring during a critical period, could arguably have cascading consequences on development), rather than a stable pathological dysfunction. Disruptions in the electron transport chain might lead to early inefficient oxygen metabolism in the brain, having downstream effects on the hemodynamic response to social stimuli which can be observed with Near Infrared Spectroscopy (NIRS, Siddiqui et al., 2017). Observations of brain oxygenation responses measured at 5 months of age with fNIRS (Lloyd-Fox et al., 2013) seem to support the

hypothesis that increased methylation in this gene might be linked with reduced responses to social versus non-social auditory stimuli, which is a pattern observed in infants who go on to develop ASD (Lloyd-Fox et al., 2017). There was a significant correlation between fNIRS responses and CYCS methylation levels for a subset of individuals who provided NIRS data (N=12) (rho=-0.78, p=0.005, Figure A6.4). Of note, this probe is promoter-associated and non tissue-specific (**Table A6.5**), thus a systemic epigenetic effect is not implausible. Moreover, Goldenthal and colleagues compared the activity of the respiratory complex in buccal swabs between children with ASD and controls and found inefficiencies in 42% (N=39) of the children with ASD (Goldenthal et al., 2015). A deletion in the region of the genome where the CYCS gene is located was reported in one individual with a developmental delay in the Deciphering Developmental Disorders study (Fitzgerald et al., 2014), and has been found to be downregulated in a mouse model of schizophrenia (Sowers et al., 2019). Given increasing drives to stratify participants with ASD into meaningful subgroups (Jeste & Geschwind, 2014), genetics and epigenetic signals related to mitochondrial function may be a meaningful area for future investigation. Such findings can generate hypotheses for biologically possible mechanisms through which epigenetic markers could reflect or contribute to the gradual emergence of more severe phenotypes.

6.4.3 Networks

Network-based analysis is a method that takes into account the interrelation between different epigenetic probes when associating their methylation levels with particular phenotypes. The subsequently more conservative analysis may thus increase power to detect effects in small samples. Indeed, in the present study I found a significant association between one network of probes and the putative neural endophenotype of emerging ASD identified in **Chapter 2** (the difference in mean amplitude in response to social versus non-social condition of the Nc ERP, see also Jones et al. 2016). This result suggests that neuroimaging measures may be more powerful in detecting relations to epigenetics than categorical outcome. Understanding the pathophysiological pathway underlying this association and ASD is complicated, especially when observing epigenetic signal in a tissue different from the brain. **Table A6.8** reports, for the probes in the significant module, levels of mean within-individuals correlation between DNAm levels from buccal swabs and brain, extracted from the resource published by Braun et al., 2019 (IMAGE-CpG, https://han-lab.org/methylation/default/). 15% of the probes in this module showed moderate correlation (rho<0.5) between the two tissues within individuals in Braun et al. (2019). This percentage was higher, but not significantly ($\chi^2(1)=1.09$, p=0.15), than the

percentage of probes with moderate brain-buccal correlation in the reference study (8.5% of all interrogated probes).

One approach to dealing with the "tissue-issue" is to move away from the interpretation of the individual effects of significant probes and examine the functional effect of genetic variants that are known to exert upstream influences on DNAm levels of the probes in the significant network. These genetic variants are of course themselves present across tissue types. We can then examine the biological processes controlled by these genetic variants to see whether some are plausibly involved in neurodevelopment. This approach uses DNAm data as a signature readout of a combination of genetic and epigenetic differences that may impact development (Ciernia & Lasalle, 2016; Geschwind, 2008). I found that 356 genetic variants influence DNAm of the probes in the significant co-methylation network during pre- and early post-natal periods. Importantly, functional enrichment analysis revealed that genes in the significant network are significantly involved in several brain-related pathways involving neural cells migration and differentiation, synaptic transmission and composition of myelin sheath. Various pathways are also involved in mitochondrial generation function, intriguing in the light of the EWAS results for later ASD (see **Tables A6.9**, **A6.10**). This approach thus generated encouraging signals that could guide future structural genomic work in similar cohorts, and could ultimately help us to generate hypotheses for potential biomarkers that link biology to behaviour.

6.4.4 Trajectories

Although no significant change in global methylation was observed between 8 and 15 months of age, the longitudinal analysis revealed significant associations between probe-specific methylation profiles and behavioural phenotypes across times. One possibility is that altered brain development caused by genetic or environmental perturbations may affect DNAm in a system- and period-specific way, as the brain attempts to compensate for suboptimal conditions (Ciernia & Lasalle, 2016). Changes in DNAm occurring at specific loci in sensitive developmental windows are strictly regulated and highly important for neurodevelopment, as demonstrated in studies using foetal brain tissue (Numata et al. 2012). One intriguing feature of the longitudinal analysis was that significant changes in the association between DNAm and the behavioural phenotypes was observed between 8 and 15 months. Although ASD is not usually diagnosed before the third birthday, early signs of emerging atypical development can be observed from the second year of life, as reviewed in **Chapter 1** and **3** (**sections 1.3.1** and **3.1.1**). The age of 12-15 months appears to be particularly significant. For example, differences in adaptive skills between high-risk infants with and without ASD are significant at 12 but not 6 months (Estes et

al., 2015); difficulties in attention shifting emerge between 6 and 12-14 months (Elsabbagh et al., 2013; Elsabbagh, Gliga, et al., 2012); the behavioural composite symptom measured by the AOSI is elevated in infants with later ASD at 14 but not 8 months (Gammer et al., 2015); and prediction of later ASD from a range of cognitive and adaptive skills is possible with reasonable sensitivity and specificity at 14 but not 8 months (Bussu et al., 2018). The present study indicates that there may be intriguing suggestions of an increase in the degree of relation between the DNAm of particular epigenetic probes and behavioural phenotypes within this same window. Future studies with larger samples could fruitfully focus on this developmental window.

6.4.4.1 The GFOD1 probe

Longitudinal modelling revealed that, among the probes whose DNAm levels were highly associated with ASD at 3 years of age, one probe located in gene GFOD1 showed an age-specific change of association with peak look at the face between T1 and T2. Encouragingly, DNAm levels of this probe in saliva and brain have been shown to be correlated within individuals (rho=0.64, p=0.04, Braun et al., 2019). Genome-wide association analyses revealed that GFOD1 is significantly associated with ADHD at the population level (Lasky-Su et al., 2008). Moreover, this gene was found to be associated with differences in IQ in a large sample of individuals with ASD (N=1,590, Wang, Qin, Guo, Samuels, & Shugart, 2013). In the present study, decreased DNAm in this gene was associated with shorter peak look duration at 8 months but longer peak look duration at the face at 15 months. Across this period, look duration becomes increasingly under the influence of the executive attention system, which has been identified as a domain of vulnerability for a significant minority of children with ASD (Hendry et al., 2018, see also **Chapter 5**). Thus, it is possible that epigenetic variation of the GFOD1 gene contributes to this developmental mechanism. In fact, ADHD and ASD often co-occur in the same individuals and shared genetic and familial influences have been observed on ADHD and ASD, as discussed previously (**sections 5.1.2** and **5.4.2**, see also Rommelse, Franke, Geurts, Hartman, & Buitelaar, 2010). Causal modelling investigating the co-occurrence of ADHD and ASD in family members of people with a neurodevelopmental disorder revealed a strong causal link between impulsivity and social difficulties (Sokolova et al., 2017, see also **Figure 5.14** of this thesis). In agreement with **Chapter 5**, the present finding supports the idea that shared biological mechanisms might lead to the emergence of common behavioural traits at the end of the first year of life in children with risk factors for neurodevelopmental disorders (Johnson, Gliga, Jones, & Charman, 2014).

6.4.5 Limitations and future directions

To conclude, in this proof-of-principle study I explored the potential of imaging-behaviour-epigenetics studies in longitudinal cohorts of individuals at risk for ASD. Given the exploratory nature of the study, an extension, as well as replication, of these findings is warranted. Importantly, some of the significant associations identified in the current study were driven by influential cases. While it is tempting to speculate that only abnormal methylation profiles might be associated with substantial differences at a phenotypic level, it is necessary to bear in mind that only if replicated these findings could be informative of possible pathophysiological mechanisms. The study sample size, although similar to many previous studies, is too small to provide high confidence on the observed results (Sonuga-Barke & Fearon, 2018). Undoubtedly, the EWAS analyses were underpowered to provide epigenome-wide significant results (see **Table 6.5**). Larger samples can help to identify weaker signals but are counterbalanced by important limitations such as increasing heterogeneity and less precise and less informative phenotypes.

Only male infants were included in this study, in order to reduce heterogeneity, but obviously this means these results will not necessarily generalize to females. Of note, the Illumina 450K array used in the current study covers only 2% of the CpG sites; future studies should consider obtaining better genome coverage using EPIC BeadChip (which interrogates > 850,000 CpG sites) or whole-genome bisulfite sequencing (Pidsley et al., 2016), though this has cost implications. In practice there is often a trade-off between sample size and density of coverage that can be achieved. Future researchers will need to carefully evaluate means to conduct well-powered and biologically informative studies.

6.5 SUMMARY OF FINDINGS

Promising signals were observed at the single-probe level, replicating the contribution of several genes implicated in biological mechanisms for ASD. Estimation of sample sizes required to obtain significant epigenome-wide association analyses based on the observed effects suggested that homogeneity of diagnostic ascertainment and the use of dimensional neurocognitive measures might allow for the detection of meaningful signals with smaller samples than previously estimated. Mapping networks of co-methylated probes associated with neural correlates of social attention showed several candidate pathways potentially involved in brain development.

Longitudinal modelling indicated promising signals associated with cognitive development, although larger samples and complete datasets are needed to obtain generalizable results. Incorporation of epigenetic analysis into prospective longitudinal designs is a potentially fruitful approach to illuminating the neurobiological mechanisms linking social attention and ASD.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

The goal of the PhD work described in this thesis was to evaluate the hypothesis that social attention atypicalities are involved in the causal pathways linking familial risk for ASD to the emergence of behavioural symptoms of this neurodevelopmental disorder. In fact, much remains unknown about the developmental mechanisms underlying ASD and what little is understood has, admittedly, little public health significance (Iacono, Malone, & Vrieze, 2017). Moreover, despite the growing evidence showing that biological factors play a critical role in the emergence of the ASD phenotype, diagnosis as defined by the DSM depends little on biology. To push forward the current state of knowledge on the biological basis of the multifaceted ASD phenotype and overcome these limits, it is important to find and validate observable and measurable markers that link biological risk factors to clinical observations. These markers should be measurable correlates of cognitive and psychological functions that are quantitative, relevant for the clinical ASD phenotype and linked to its aetiology. Identifying these markers, or endophenotypes (see **section 1.3**), is an important step to track pathophysiological processes underlying the behavioural symptoms which guide clinical diagnosis (Gottesman & Gould, 2003). Ultimately, validated endophenotypes should also be used to guide clinicians and researchers in planning individualised prevention programs, evaluate prognosis and determine candidate targets for intervention for selected groups of individuals (Dick, 2018).

In this thesis, I evaluated the evidence for considering social attention an endophenotype of ASD. In fact, as reviewed in **Chapter 1**, theoretical and empirical work has converged in suggesting that social attention might play a role in the developmental pathway to ASD. In **section 1.3.3**, I identified the following critical questions which had not been addressed or convincingly answered by previous research:

- Which of the candidate early markers of ASD contribute to developmental trajectories towards the emergence of the ASD behavioural phenotype?
- To what extent atypicalities of social attention identified in the adult literature map onto atypicalities observed before the emergence of ASD traits?
- Are previous findings of social attention atypicalities in family members of individuals with ASD replicable?
- Do atypicalities of social attention share genetic variance with ASD traits?

In the next section, I illustrate and discuss the main findings of this thesis in relation to these research questions.

7.1 SUMMARY OF GENERAL FINDINGS

The main findings of the work described in this thesis shedding light on the role of social attention in the path to ASD are concisely reported in **Table 7.1** and summarized below.

In **Chapter 2**, I investigated whether atypical early brain responses during attention engagement to social (faces) and non-social salience-matched stimuli were observed in infants with an older sibling with ASD, considered at high familial risk. I found that reduced and shorter neural correlates of attention in response to social versus non-social stimuli were predictive of lower socialization skills at three years in high-risk infants. Thus, I observed that atypicalities in engaging attentive brain states precede the onset of ASD and dimensional social outcome.

In **Chapter 3**, I examined the contribution of neural and behavioural early signs of attention atypicalities to the developmental trajectories of cognitive and social skills which can be impaired, to various degrees, in children with ASD. I reported that early atypicalities in attentive brain states in the first year of life are associated with reduced attention engagement with the gazed-at object in a gaze-following eye-tracking task in the second year of life, which partly mediated the pathway to autistic symptoms and social traits. Another behavioural feature reflecting social attention, the duration of longest look at a static face among other non-social stimuli in an eye-tracking task, was predictive of later communication skills. These findings provide further evidence for considering social attention atypicalities as early markers involved in the path to ASD traits.

In **Chapter 4**, I examined whether atypicalities in social attention could be seen in family members of children with ASD, and to what extent this effect could be attributed to the combined effect of common additive genetic variation. In families, increased polygenic risk for ASD was associated with severity of symptoms in the restricted and repetitive behaviours domain to a greater extent than to social difficulties. Interestingly, when testing the polygenic contribution to variance in detection of eye-gaze direction, I found that polygenic risk for ASD predicted better performance in individuals from low-risk and simplex high-risk families. These findings suggested that social attention skills, although associated primarily with difficulties in the social domain of ASD, are influenced by genetic factors which have an effect on domain-general attentional functions.

Results of **Chapter 5** provided evidence that atypical looking behaviour when directing attention to faces (specifically, longer peak look duration), is associated with general risk factors for neurodevelopmental disorders, possibly including increased genetic burden for attention difficulties. Importantly, this early marker predicted increased severity of ASD traits at school age, and was also associated with lower inhibitory control in toddlers at high familial risk. This work suggests that this component of executive function might play a role in the developmental trajectory to ASD too. Importantly, the observed path was specific to responses to social stimuli.

Finally, **Chapter 6** demonstrated that the study of developmental mechanisms underpinning ASD might benefit from incorporating epigenetic analysis into prospective longitudinal studies of infants at familial risk. I found that functional pathways involved in brain development and ASD regulate prenatally DNA methylation levels in networks of probes associated with neural correlates of social attention. Moreover, significant changes in the association between DNA methylation and behavioural phenotypes, including looking behaviour during social attention tasks, were observed between 8 and 15 months of age, a critical period for the emergence of atypical developmental trajectories.

Together, these observations help to answer some of the unsolved questions highlighted in **Chapter 1** of this thesis and will direct future research aimed to uncover the causal developmental mechanisms underpinning ASD and aid the development of novel intervention strategies.

Table 7.1 *Main findings of each study relative to the role of social attention in the path to ASD*, summarized in terms of sample size, age of the participants, method, measure of social attention and key results. Italic font highlights the participants' age and methods used to measure social attention.

	Title	Sample size	Age	Method	Measure of social attention	Findings
Chapter 2	Diminished engagement of attentive brain states to faces precedes the emergence of ASD	N=131	*8 m.*, 3 y.	*EEG (ERPs and microstates),* parent-report questionnaire	Neural responses to face with direct gaze (FD) versus Noise stimuli	Infants with emerging ASD show atypical neural responses during social attention; weaker activation to faces are predictive of later socialization skills in high-risk children.
Chapter 3	Roles of attentive brain state and looking behaviour in the development of social cognition	N=247	*8 m., 14 m.*, 2 y., 3 y.	*ERPs and microstates, eye-tracking, behavioural assessment*, parent-report questionnaires	Neural responses to FD versus Noise stimuli, looking time at the gazed-at object, peak look duration at face stimuli	Neural atypicalities during social attention predict reduced engagement in joint attention situations and those two aspects both contribute to lower social skills and ASD symptoms at age 3.
Chapter 4	Familial and polygenic risk for atypical social attention	N=249 (N=189 with DNA)	*6-52 y.*	*Computerised task*, questionnaires, polygenic score	Accuracy in eye-gaze direction detection	Worse social attention is associated with more severe social difficulties but not with polygenic score for ASD in a familial sample.
Chapter 5	Signs of social attention atypicality as risk marker of neurodevelop-mental disorders	N=197	*14 m.*, 2 y., 6-10y.	*Eye-tracking*, parent-report questionnaires, polygenic score	Peak look duration at face stimuli	Atypical social attention is related to general risk factors for neurodevelopmental disorders and leads to ASD symptoms in the presence of low executive function skills.
Chapter 6	A proof-of-principle study of DNA methylation in infants at risk for ASD	N=63	*8 m., 15 m.*, 2 y., 3 y.	*EEG (ERPs), eye-tracking*, parent-report questionnaires DNA methylation	Neural responses to FD versus Noise stimuli, peak look duration at static face stimuli	DNAm patterns in probes associated with neural response during social attention are regulated prenatally by genes possibly involved in biological mechanisms to ASD. Time-specific changes in DNAm related to variation in social attention and adaptive behaviour might contribute to developmental trajectories.

N: number of participants; m.: months at the time of collection (on average); y.: years of age; EEG: electro-encephalography; ERPs: event-related potentials; FD: face with direct gaze; DNAm: DNA methylation.

7.2 SYNTHESIS AND THEORETICAL IMPLICATIONS

The goal of this thesis was to evaluate the evidence that social attention atypicalities are involved in the causal path to ASD. The approach taken was to test whether disruptions in the process of allocating attention to social stimuli might lie in the steps between (genetic, epigenetic and familial) risk factors for ASD and difficulties in socialization and adaptive behaviour, which often lead to disability in affected individuals. A marker of neurodevelopmental disorder, i.e. a psychophysiological measure that is associated with behavioural symptomatology (Iacono, 1985), is considered an endophenotype when it indexes genetic liability for relevant traits (Iacono et al., 2017). In **section 1.3.3**, I identified unsolved questions related to the hypothesis that social attention could be informative of causes and mechanisms of ASD as an endophenotype of the disorder. Those were:

- Which of the candidate early markers of ASD contribute to developmental trajectories towards the emergence of the ASD behavioural phenotype?
- To what extent atypicalities of social attention identified in the adult literature map onto atypicalities observed before the emergence of ASD traits?
- Are previous findings of social attention atypicalities in family members replicable?
- Do atypicalities of social attention share genetic variance with ASD traits?

Evidence-based responses to these questions are discussed below.

7.2.1 Candidate developmental endophenotypes

In **Chapter 1** (see **section 1.3.3** and **Table 1.1**) I summarised the evidence for considering social attention an endophenotype of ASD, according to the original requirements from Gottesman & Gould (2003). **Table 7.2** summarises whether the studies described in the chapters of this thesis did or did not provide evidence for the chosen measure of social attention to be considered an endophenotype of ASD, according to the requirements defined by Gottesman & Gould (2003). More recently, Iacono et al. (2017) revisited the concept of endophenotype in light of the recent advances in the field of molecular genetics, and proposed further criteria that should guide validation of possible endophenotypes, concerning the actual link to genetics and the clinical utility of proposed endophenotypes. I discuss the findings of the work described in this thesis in light of these updated suggestions, as they provide useful starting points for evaluating directions for future research.

Table 7.2 *Criteria for validation of endophenotypes of psychiatric disorders*, positive (√) or negative (X) outcome of the validation performed in the various chapters of this thesis.

The endophenotype…	Thesis chapter	Validation
(1) … is associated with illness in the population	Not tested	
(2) … is heritable	Not tested	
(3) … is primarily state-independent (manifests in an individual whether or not illness is active)	Chapter 2	√
	Chapter 3	√
	Chapter 5	√
(4) Within families, endophenotype and illness co-segregate	Chapter 4	√
(5) … is found in nonaffected family members at a higher rate than in the general population	Chapter 2	√
	Chapter 4	**X**
	Chapter 5	√

7.2.1.1 Shared genetic variance

Expanding the concept of heritability of an endophenotype proposed by Gottesman & Gould (2003), Iacono et al. (2017) recommended that candidate endophenotypes share genetic variance with the clinical phenotype and are associated with specific genetic variants robustly associated with the disorder. **Chapter 4** and **5** tested the former of these hypotheses using polygenic scores to evaluate the combined effect of common risk variants associated with ASD (and ADHD) on dimensional social attention traits. The results did not find evidence for shared polygenic effects between ASD and eye-gaze direction detection in a familial sample (**Chapter 4**), nor to looking behaviour at static face images recorded with eye-tracking in the second year of life (**Chapter 5**). A small proportion of the variance in the latter measure of social attention was accounted for by polygenic score for ADHD, although this result requires replication. **Chapter 6** produced suggestive evidence for a relationship between epigenetic signatures associated with genes robustly associated with ASD or brain development and measures of early social attention skills. DNA methylation has been proposed as a promising biological mechanism involved in the path to psychopathology (Barker, Walton, & Cecil, 2018), and to ASD specifically (Ciernia & Lasalle, 2016). Results described in **Chapter 6** supports the use of neurocognitive measures of social attention to identify relevant biological pathways in neurodevelopmental disorders.

7.2.1.2 Utility

Other criteria according to Iacono et al. (2017) concern the utility of endophenotypes in informing on etiological mechanisms of disorders. Those criteria are: prediction of the development of the clinical phenotype; enhancement of the theoretical understanding of brain mechanisms accounting for individual differences in the endophenotype; the possibility to inform animal models and facilitation of the identification of variants that have relatively large effects (Iacono et al., 2017).

Chapter 2, 3 and **5** presented converging evidence that early signs of reduced or atypical attention engagement to visual stimuli carrying a social content have an impact on later ASD behavioural traits at a developmental period when overt symptoms manifestations cannot be detected (requirement (3), see **Table 7.2**). My initial prediction, based on multiple theoretical accounts (Dawson, Bernier, & Ring, 2012; Klin, Shultz, & Jones, 2015; Piven, Elison, & Zylka, 2017), was that early signs of social attention atypicality were predominantly linked to later difficulties in social skills. However, **Chapter 3** and **5** revealed that they also contributed to the restricted and repetitive behaviour phenotype and language difficulties. Importantly, while accounting for some of the heterogeneity in the ASD phenotype, the early markers examined in this thesis were specifically associated with autistic traits and less so with attentional difficulties (**Chapter 5**).

Of interest, results of **Chapter 2** have been extended to individual level prediction thanks to machine learning algorithms evaluating to what extent microstate features extracted from HR 8-month-olds during the face/non-social EEG paradigm predicted categorical ASD and dimensional variation in social adaptive skills at three years. In this as yet unpublished study we found that prediction of ASD diagnosis was possible with 60% accuracy and that strength and duration of the attentive microstate in response to faces with direct gaze were predictive of social ability to a greater extent than cognitive ability at the same age. Although the application of microstate analysis to infant data is novel and replication is warranted, the result is nonetheless promising and further supports using psychophysiological measures to identify early divergence to neurodevelopmental trajectories.

Chapter 3 provided an example on how to combine information derived from EEG and eye-tracking, that allow to detect early signs of atypicalities which would not necessarily be spotted with a naked eye, to test theoretical accounts on the emergence of ASD. Integrating data collected at multiple data points during a prospective longitudinal study in structural equation models (**Chapter 3**) and mediation analysis (**Chapter 5**) offered the possibility to look at the interaction between manifestations of vulnerability and potential protective factors in a developmental perspective. This approach helps to disentangle what signs are reflecting dis-

adaptive and which adaptive mechanisms, which are indicators of disruptions and which are early compensatory strategies possibly emerging in resilient individuals (Johnson, Gliga, Jones, & Charman, 2014). It can therefore illuminate the choice on possible targets for early intervention.

Notably, the examined measures of social attention do not fully meet the utility criteria for endophenotypes as defined by Iacono et al. (2017). With respect to their informative value for animal models, research on visual social attention has been conducted in primates (Freeman & Young, 2016; Parr et al., 2016; Putnam, Roman, Zimmerman, & Gothard, 2016). However, knock-out mouse models will be hard pushed to test the effect of single genes on visual social attention using comparable paradigms, given that social behaviour is best observed from responses of other sensory systems (in particular the olfactory system) in this species (Freeman & Young, 2016). Additionally, results of this thesis did not test whether social attention endophenotypes are associated with rare(r) genetic variants of large effect. However, in line with previous studies I demonstrated in **Chapter 4** and **5** that common genetic variants in aggregate explain a small proportion on the measures of social skills (Iacono et al., 2017; Skuse et al., 2014). Epigenetic factors (**Chapter 6**) and rare variants might contribute to individual risk to a larger extent.

Taken together, converging evidence across chapters points towards two developmental endophenotypes that might be implicated in the causal path to ASD: Nc mean amplitude difference to face and non-social control stimuli measured with EEG at 6 to 11 months of age and peak look duration at the face during a face pop-out task measured with eye-tracking at 12 to 15 months of age. These two early markers of ASD are reliably measurable, close to the biological mechanisms underlying the emergence of behavioural symptoms, and likely to have cascading effects on later social and communication skills. Thus, the present findings justify further investigations of the genetic and epigenetic architecture underlying these two developmental traits in larger cohorts.

7.2.2 Insights from a relatively small sample: Risk and protective value of social attention

The use of large samples is especially advised when investigating effects of small size, as in molecular genetics research, and/or when studying a population characterised by high heterogeneity and strong environmental contributions, as in ASD research (Dick et al., 2018; Geschwind & Konopka, 2009). Particularly when undertaking exploratory, data-driven analyses,

as is current practice in genetics and epigenetics research following the replication crisis of candidate gene studies (Plomin, 2013), reliability of findings can depend on the size of the study sample (Sonuga-Barke & Fearon, 2018). However, each person's developmental trajectory is unique, and longitudinal observations at the individual level have been extremely informative of developmental processes involved in derailment from the typical path towards the emergence of ASD (Bussu et al., 2018; Thomas et al., 2009). In fact, empirically tracking patterns of developmental change at multiple time points in relation to cognitive and affective function allows us to underpin what drives individual differences in adaptive behaviour (Karmiloff-Smith, 1998, 2007). Thus, whilst large samples are warranted in face of the aetiologic and phenotypic heterogeneity of ASD, deep and longitudinal phenotyping are needed to gain insight into developmental causal chains, and that is simply not currently not feasible on a large scale.

Following-up the development of small groups of individuals with similar aetiology, as opposed to large populations with unknown aetiology, could be more effective in the attempt to track down mechanisms underlying phenotypic heterogeneity (Baker et al., 2018; Chaste et al., 2015; Distefano et al., 2016; Frohlich et al., 2016; Garg et al., 2015; Jeste et al., 2016; Kolesnik et al., 2017) and to delineate paths underlying neural and behavioural differences (Jeste & Geschwind, 2014). In this thesis, a similar approach was undertaken by looking at developmental features that differed based on risk (comparing LR versus HR groups) and/and outcome (comparing HR-TD versus HR-Aty versus HR-ASD). This allowed me to evaluate which characteristic developmental features which were specifically associated with more vulnerable starting points and/or more severe behavioural outcomes.

Features that differed between HR and LR infants, but were common to all HR groups, were hypothesised to be closer to the underlying causes of ASD, possibly appearing before the influence of individual resilience factors (Szatmari, 2018). This was the case of long peak look duration at the face in a face pop-out task, recorded at 14 months (**Chapter 5**) and also partly of Nc mean amplitude difference between face and non-social conditions recorded with EEG at 8 months (**Chapter 2**). Features that were atypical only in infants with emerging ASD are more likely to reflect early signs of a divergent developmental trajectory which leads specifically to ASD. For example, shorter latencies of the Nc in response to faces than to the non-social stimuli were found in the HR-ASD group only (**Chapter 2**). Features that are disrupted in infants with later diagnosis of ASD but atypically 'good' in individuals at risk who do not receive a diagnosis of ASD compared to LR, might reflect mechanisms of resilience. Accordingly, a potential protective value of the duration of looking time at the gazed-at object at 14 months, which was shorter in the HR-ASD group and especially long in HR-TD, compared to LR infants (**Chapter 3**),

has been detected. Similarly, accuracy in eye-gaze direction detection was enhanced in female unaffected family members of children with and without ASD, indicating the role of sex-specific protective factors on social attention (**Chapter 4**). Observing responses to these marker tasks in individuals with known syndromic cause for ASD might be crucial to map common adaptive biobehavioural responses and shared mechanisms of risk and resilience.

The availability of longitudinal data from deeply phenotyped individual cases is a precious resource to probe mechanisms, as demonstrated in **Chapter 6**. The possibility, which might be lost in large case-control studies, to triangulate multiple observations collected with different methods in single individuals might be an extremely valuable opportunity for expanding the knowledge on developmental mechanisms underlying ASD (Dick et al., 2018).

7.2.3 When is a developmental trajectory atypical?

"Atypical" literally means "different from all others of the same type" (The Cambridge Dictionary, 2019). In ASD research, this adjective is widely used to refer to characteristics which are different from what is observed in individuals without a developmental or psychiatric condition. In this thesis, "atypical" has been attributed to responses that were (statistically) different from the responses of the LR group, in line with other studies of children at familial risk for ASD (Jones, Gliga, Bedford, Charman, & Johnson, 2014). Interesting considerations on the variability of the developmental path to ASD emerged across multiple chapters when observing responses in children at high familial risk who did not receive a diagnosis of ASD at age 3, namely the HR-TD and HR-Aty children. The assumption that the LR group showed "typical" responses was crucial especially for the study described in **Chapter 2**. There, the "typical" microstate sequence during social attention was estimated on the LR group only. This choice was guided by the theoretically-informed intuition, confirmed by the ERPs results, that high-risk infants - even if they do not receive a diagnosis of ASD at age 3 - might still show signs of brain vulnerability earlier in development.

7.2.3.1 The HR-noASD children

Despite the label of "typically developing" assigned in late toddlerhood, HR-TD did not always show overlapping responses with the LR group indicating that this label does not necessarily imply that they underwent a developmental trajectory comparable to LR infants at earlier ages. In line with this argument, several pieces of research in this thesis revealed that the HR-TD group showed: reduced amplitude of the Nc in response to faces than to non-social stimuli at 8

months, differently from LR infants (**Chapter 2**); longer looking time at the gazed-at object during a gaze-following eye-tracking task, interpretable as more attention engagement (Bedford et al., 2012), than LR infants, (**Chapter 3**); similar peak look duration at face images to HR-ASD and HR-Aty children, who all show longer peak looks than the LR group (**Chapter 5**); comparable accuracy in detecting eye-gaze direction to LR children during childhood and similar levels of polygenic score for ASD (**Chapter 4**). These findings indicate that in the first years of life development of attention and looking behaviour in social contexts in individuals at risk for ASD is often not a linear path, in line with observations on cognitive and adaptive skills in at risk populations (Bussu et al., 2018; Charman, 2018; Estes et al., 2015; Kim et al., 2018).

The HR-Aty children represent a particularly interesting group and are defined as infant siblings who showed signs of developmental delay, sub-threshold symptoms of neurodevelopmental disorders or difficulties in the language domain, but did not meet criteria of ASD at age 3. A multi-site study collapsing data from the Baby Siblings Research Consortium, including 859 infant siblings at high familial risk and 473 LR controls with no family history of ASD, found that approximately 11% of all the HR infants (versus 3% of LR) had developmental delay and around 30% (versus 17% LR) showed elevated levels of autistic symptoms, measured with the ADOS (Charman et al., 2017). The HR-Aty children are assumed to have enhanced burden for neurodevelopmental disorders (Frazier, Youngstrom, Hardan, Georgiades, & Constantino, 2015), although for unknown reasons they do not develop the core symptomatology of ASD by three years of age. Notably, useful endophenotypes might not necessarily validate a DSM categorical diagnosis, but instead provide a biologically informed way to uncover etiological factors relevant to the types of dysfunction similar to those with a diagnosis experience (Beauchaine & Constantino, 2017; Iacono et al., 2017). Accordingly, collapsing the HR-ASD and HR-Aty group for one of the EWAS analyses conducted in **Chapter 6** facilitated the detection of a larger proportion of signals related to genes known to be associated with ASD. It is possible that this result was only the effect of a more balanced group design or of chance (in fact, statistical test revealed that this result was not statistically different from chance, p=0.088). However, it might also indicate that this approach might be more informative of biological (and behavioural) mechanism of derailment form a typical path of development.

7.2.3.2 A dimensional approach

Despite the utility of a final classification into diagnostic/outcome groups to identify early markers of ASD and atypical developmental outcome, a recent view is that a dimensional approach to the study of mechanisms underlying complex traits is advised as features are distributed continuously, and not bimodally, in nature (Beauchaine & Constantino, 2017). The

BASIS/STAARS protocol includes a diagnostic assessment for defining outcome groups at age 3, which is considered an age at which behavioural symptoms of ASD have emerged (Ozonoff et al., 2015). However, a recent follow-up study combining three longitudinal infant-sibling studies for a total of 484 HR and 262 LR participants revealed that 14 HR children who did not meet criteria for ASD at 3 years received a diagnosis of ASD at later ages (Ozonoff et al., 2018). Going back to previous records, the authors found that these children showed indicators of atypical trajectory from parent-report questionnaires when the first diagnostic assessment was done. Accordingly, the approach of using dimensional measures of traits, common to all chapters of this thesis, possibly offered an additional level of information on the relation between the examined markers and behavioural traits. In fact, it allowed me to track developmental changes and stability of traits over development. **Chapter 4** extended the investigation of social attention difficulties in early childhood to children who were older than 6. Additionally, **Chapter 5** provided a link between early markers and dimensional outcome obtained during mid-childhood. The demonstration of an association between social attention measures and stable clinical characteristics after age 3 is an additional value of this research and has important implications on the clinical relevance of the candidate endophenotypes (Iacono et al., 2017).

7.2.4 Social and non-social aspects of "social attention"

In this thesis, social attention has been used as an umbrella term to include all processes in which the individual allocated attentional resources to conspecifics (see **section 1.3.2**). Various experimental measures collected when subjects were paying attention to visual stimuli with a social content were assumed to reflect this construct. However, whether different endophenotypes underpin the same cognitive mechanisms needs to be established when trying to uncover potential endophenotypes in the paths from genetic loading and later traits (Beauchaine & Constantino, 2017; Flint & Munafò, 2007). In **Chapter 3**, different candidate infant markers were entered in the same SEMs to understand the relationship between those in relation to later ASD phenotypes. Disengagement reaction times and looking time at the gazed-at object were shown to share variance. Moreover, the latter measure was associated with the attentive brain state latent factor, indicating some continuity between social attention skills across early developmental periods.

Of interest, correlation between all eye-tracking measures collected at the same time point was weak (**Figure A3.1**), such that it was not appropriate to summarise them in one factor. This is in line with the theoretical account of social attention development by Salley & Colombo (2016). They proposed that in the first 18 months of life different aspects of social attention, such as

visual skills required to orient towards salient social cues, the motivation to direct attention to conspecifics supported by neural circuits for reward and the natural attitude to enact social behaviours to engage and learn from others, are not fully integrated initially. Later in development, following specialization of neural circuits and increase in the efficacy of connections between areas, a wide network in the brain is activated during social attention (see **Figure 1.4** and Johnson, 2011; Jones, Venema, Lowy, Earl, & Webb, 2015; Klein, Shepherd, & Platt, 2009). Of the six infant measures of social attention examined in this thesis, only the measure extracted from the gaze following eye-tracking task was highly correlated with accuracy in eye-gaze direction detection collected later in childhood (see **Table 7.3**). This finding is reassuring, given that both measures refer to the ability to interpret the direction of a social cue. It also indicates that precursors of later individual differences in social attention and autistic traits might be observed early in childhood (although these results are naturally preliminary given the reduced sample size for this analysis).

Importantly, the infant measure of gaze-following ability covaried with the ability to disengage fixation from a central target to shift gaze peripherally, such that longer latencies in disengagement were associated with less attention engagement when responding to joint attention (**Chapter 3**). Further, accuracy in detecting eye-gaze direction in childhood and adulthood was associated with higher polygenic risk for ASD, which in turn was predictive of severity of restricted and repetitive behaviours (**Chapter 4**) in individuals at low familial risk for ASD and of single-incidence families. These two elements point towards the possibility that an increased focused attention style interacts with risk factors in influencing visual ability when interpreting social cues.

Table 7.3 *Correlation between the infant measures of social attention and performance at the Gaze Monitoring Task* collected between 6 and 19 years of age.

	Measure of social attention	N	rho	p	FDR
T1	**Nc mean amp. FD-Noise**	26	0.06	0.765	0.847
	Nc mean lat. FD-Noise	26	0.09	0.670	0.847
	Ms mean GFP FD-Noise	25	0.27	0.185	0.370
	Ms duration FD-Noise	26	0.31	0.106	0.318
T2	**Peak look at the face**	33	0.03	0.847	0.847
	Looking time at gazed-at object	25	0.49	0.010*	0.060

T1: around 8 months of age; T2: around 14 months of age; N: number of participants included in the analysis; rho: correlation coefficient, p: p-value of the Pearson's correlation test, FDR: p-value adjusted for multiple testing using Benjamini and Hochberg False Discovery Rate method; amp. : amplitude; FD: Face with Direct Gaze; m.: months of age; lat.: latency; Ms: microstate 4.
* $p<0.05$.

Accordingly, the results discussed in **Chapter 5** supported the idea that executive function (specifically, inhibitory control) interact with early looking behaviour during social attention in infants at high risk for ASD (Hendry et al., 2018; Johnson, 2012). Beauchaine & Constantino (2017) proposed that establishing maps of expectable relations between different variables will be needed to understand the role of any single endophenotype within an individual. This approach will ultimately allow researchers to ascertain relative contributions of multiple causal factors to a multifaceted neurodevelopmental condition such as ASD (Constantino, 2009).

In line with the idea of combined cascading effects of responses to social and non-social stimuli on learning from the environment, **Chapter 2** illustrated that brain activation was atypically reduced when attending to faces but also significantly increased when attending to non-social stimuli in 8-month-old HR infants who later received a diagnosis of ASD, and in those with no developmental concerns at age 3. The combination of these two features was selected as key variable identifying increased neural vulnerability when processing visual stimuli. As mentioned in **section 7.2.1**, a further investigation was carried out on the microstate features with the contribution of G. Bussu, who used machine learning techniques to establish the predictive value of later social adaptive skills of each of the microstate features in response to the different stimuli. **Figure 7.1** illustrates the results of this analysis, revealing that both strength of the scalp field in response to the face with direct gaze and non-face control stimulus (Noise) contributed to later dimensional outcome in the HR group. Of note, there was a higher contribution of the response of the attentive microstate to the face compared to Noise.

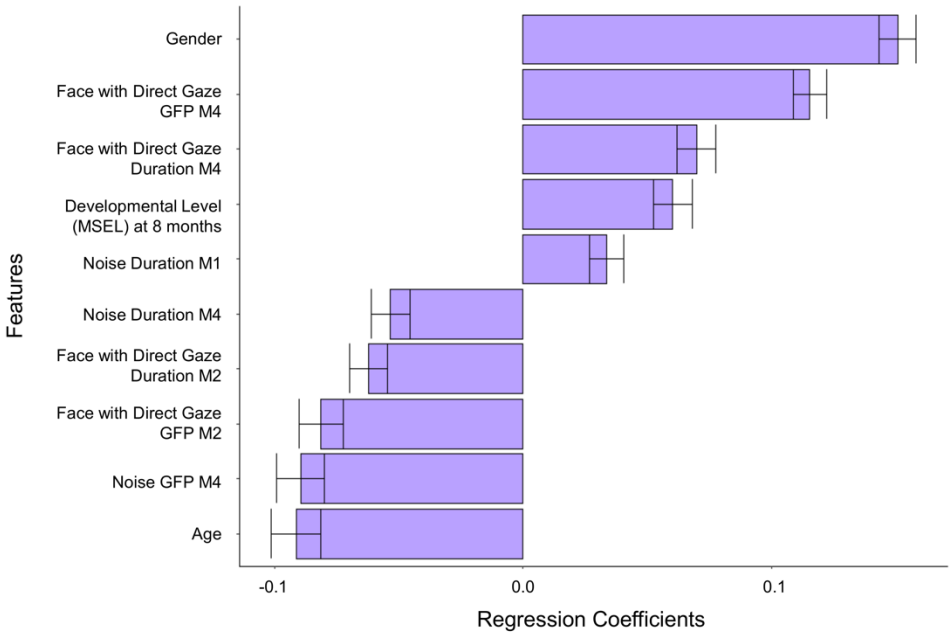

Figure 7.1 *Individual prediction of dimensional outcome by microstate features extracted in Chapter 2*. On the x-axis are displayed regression coefficients for prediction of social skills, as measured by the VABS socialization standard score at 36 months, at an individual level using demographic data (age in days, gender and developmental level as measured by the MSEL Early Learning Composite score at 8 months) and microstate features in response to faces with direct or averted gaze, and Noise. Microstate features included duration and Global Field Power (GFP) of microstates 1 (M1), 2 (M2) and 4 (M4, see **Chapter 2**). Only coefficients that were always selected by an elastic net regression model with leave-one-out cross-validation are reported. The average of regression coefficients over cross-validation folds is shown by the bars and the standard deviation by the error bars. Coefficients are in standard units *(Gui, Bussu et al., unpublished, reproduced with permission).*

In **Chapter 5**, I separately examined the same measure of attention in response to social and non-social stimuli in the pathway between risk factors and later symptoms. Differently from the social condition, peak look duration to non-face stimuli showed no association with any of the risk factors analysed nor with outcome measures. This was true also for measures related to the ADHD phenotype. Thus, atypical responses were elicited specifically in the presence of social stimuli. Wagner, Luyster, Tager-flusberg, & Nelson (2016) found that in 9-month-old HR infants without later ASD diagnosis pupil size in response to static emotional faces was related to worse social-communicative functioning at 18 months. Thus, it is possible that atypicalities of low-level responses to sensory inputs in infants at high genetic risk for ASD are enhanced specifically when attending to faces, which directly activate the brain arousal system (Senju & Johnson, 2009). In a longitudinal study of brain characteristics using fMRI, Hazlett et al. (2017) observed that, especially between 6 and 12 months of age, infants later diagnosed with ASD showed a hyper-expansion of surface area in the middle occipital cortex, associated with processing visual information in the visual areas. Of interest, brain volume overgrowth during the second year of

life was specifically associated with autistic-like social, but not non-social, deficits at age 2 (Hazlett et al., 2017; Piven et al., 2017). Taken together, these results could find plausible explanation in the hypothesis that dysfunctional modulation in the alerting, orienting and executive attention networks may influence attentional capacity especially for social information, possibly over-arousing given the complex nature of social stimuli (Keehn, Müller, & Townsend, 2013; Senju & Johnson, 2009).

7.3 LIMITATIONS

The work presented in this thesis was motivated by the ambitious plan to interrogate biological and developmental mechanisms underlying changes in psychophysiological measures associated with ASD. This was possible by leveraging a wealth of longitudinal experimental measures collected within an infant-sibling design, and recent availability of genetic and epigenetic data for a sub-set of the cohort. As discussed in **Chapter 1** (**section 1.1.4**), although this is a highly promising approach to understanding the origins of ASD, it is challenged by the high costs involved in deeply phenotyping a cohort at multiple levels, and by a high attrition rate. Both these aspects negatively impact sample size, which is critical for obtaining robust results.

7.3.1 Selection bias

The fact that psychological and biomedical research tends to oversample participants with high socio-economic status (SES) and is less representative of minority groups has been widely recognised (Gill & Redwood, 2013; Yancey, Ortega, & Kumanyika, 2005). This places limits on the generalizability of observed results to the wider population. This bias is likely to concern BASIS too. As further note on selection bias impacting the research described in this thesis relates to the reduced retention rates documented in longitudinal research for individuals from less advantageous socioeconomic backgrounds (Gustavson, Von Soest, Karevold, & Roysamb, 2012; Young, Powers, & Bell, 2006). Thus, it is possible that the attrition seen in BASIS is biased towards families with greater socioeconomic issues and more severe autism cases (Wolke et al., 2009), although no data have been analysed to verify this hypothesis. Future studies might specifically investigate whether low SES, which is often used as a proxy for 'environmental' risk factors (Amso & Lynn, 2017), has a negative impact on early social attention skills. Although Markant, Ackerman, Nussenbaum, & Amso (2016) found that selective attention in 9-month-old

infants was not affected by low levels of SES, suggesting that this function is resilient to adverse environmental conditions, these have been shown to influence early brain activity (Tomalski et al., 2013) and cognitive abilities (Bradley & Corwyn, 2002; Brito, Fifer, Myers, Elliott, & Noble, 2016) in the first years of life. This type of research could be highly informative on the (possibly protective, see Markant et al., 2016) role of social attention in moderating the negative effect of adverse environmental conditions on the development of cognitive and affective functions.

Chapter 4 contains a diagram of the original BASIS sample and the final numbers of families who agreed to participate in a new wave of behavioural and genetic data collection, named gBASIS (see **Figure 4.1**). Of note, 33% and 37% of the HR families did provide questionnaire and experimental (online task) data for gBASIS, respectively, while this percentage was lower (27%) for the LR families. For DNA collection, 49% of the HR families and 27% of the LR families were retained. This picture, although disappointing, highlights two important aspects which might be useful for planning future research. First, anecdotally, HR families were internally motivated to participate in the research by the goal to take part in the effort to improve the current knowledge on ASD, which might directly help their family members in the future. LR families were possibly less engaged in the aims of the study, having no such indirect reward for their participation; of note, no monetary compensation was offered to the gBASIS participants. Second, a higher proportion of HR families agreed to participate in genetic data collection (as compared to the behavioural data). To understand this datum, it is important to note that the majority of the HR families received a home visit during which the biological samples were collected. In contrast, questionnaire and DNA sample collection for remaining HR families and all LR families were collected by post, requiring active participation and effort on the part of the families (typically the mothers). In the future, retention rates might be maximised by maintaining contact with the participants and keeping them engaged with future recruitments, and (when possible) performing home-visits for data collection (Abshire et al., 2017).

A lower drop-out rate was observed in the earlier time points of the longitudinal study. For data collected at T1 and T2, there was a large amount of missing data dependent on the level of compliance of the young participants with the administered tasks. For example, a diagram in Chapter 2 (**Figure 2.1**) shows the original sample size for EEG data collection and the final number of children who provided valid ERP data for analyses. Reassuringly, the percentage of children with atypical outcome (45% HR-Aty and 34% HR-ASD) which were excluded from the study was comparable to the percentage of excluded LR (48%) and HR-TD (45%) participants. Moreover, the two groups were not different in terms of their cognitive and adaptive skills at the time of testing and at the time of diagnostic assessment (see **Table 2.1**). Nevertheless, the

high proportion of missing data overall reduces the utility of this early marker in screening procedures for early detection of atypical developmental trajectories and partly challenges the claim of its use as marker of efficacy for early intervention (Dawson et al., 2012). Additionally, it should be noticed that the present thesis investigated effects on infants who had an older sibling with ASD; therefore, insights on the developmental path to ASD provided by the present findings necessarily reflect processes characterising ASD in multiplex families and might not be informative of other routes to (simplex) ASD.

7.3.2 Exploration versus hypothesis testing

The sample size usually required to obtain reliable neuroimaging and behavioural research findings is notably smaller than the size required in molecular genetics research. The studies presented in this thesis observing group differences and associations with dimensional outcome in neurocognitive measures were larger than many previous research studies using the same paradigms. In particular, the choice of the neural and behavioural correlates of social attention as candidate developmental endophenotypes was based on publications including a subset of the cohort used in the current study (Bedford et al., 2012; Elsabbagh et al., 2013, 2012; Hendry et al., 2018) or other smaller independent samples (Jones et al., 2016), increasing the reliability of results. The TANOVA and microstate investigations of scalp field data described in **Chapter 2** are highly novel for infant research, therefore replication is certainly warranted. However, it is worth mentioning that the sample size for extraction of the "typical" microstate maps based on cross-validation is much larger (N=40) than the sample size used in the methodological paper illustrating the procedure for microstate extraction with RAGU in adult data (N=16). This aspect could partly compensate the fact that infant data might be noisier than adult ones. Reassuringly, results of the cross-validation procedure were comparable to those obtained in the original paper with adult data (Koenig, Stein, Grieder, & Kottlow, 2014, Habermann et al., 2014).

The most problematic aspect related to sample size of the studies presented in this thesis concerns the exploratory analyses looking the contribution of polygenic and epigenetic factors to infant measures. The expected effect sizes from previous studies using a similar approach gave rise to mixed hopes of the potential success to detect meaningful signals with the current sample size by using more precise neurocognitive measures as endophenotypes. For example, for polygenic risk score research, evidence of small effect sizes for the association with individual common genetic variants was provided for ERP and eye-tracking measures in over 4,000 individuals (Iacono et al., 2017). However, in this study the SNP-based heritability estimates were substantial (for P300 ERP component, a neural correlated of attention engagement in

adults: h^2=0.29; for the anti-saccade test, a marker task for inhibitory control, h^2=0.46). Moreover, by aggregating the effect of multiple variants, Cullen et al. (2019) found that polygenic score for psychiatric disorders significantly predicted neuroanatomic abnormality measured with fMRI in a sample of 122 infants of European ancestry (R^2=0.05). In light of these studies, the investigations carried out in the studies describes in **Chapter 4** and especially **5** could be reasonably powered.

Similarly, two large case-control studies testing DNA methyaltion differences at birth for over 1,000 individuals found no evidence for association at a single-probe level (Andrews et al., 2018; Hannon et al., 2018). However, Wong et al. (2013) examined differences in DNAm in 50 MZ twin pairs and found differences between ASD cases and controls at many loci with effect size >0.15. Moreover, strong effects of DNAm loci with a dimensional measure of ASD traits from parent-report questionnaire were observed (rho~0.28-0.40 for the reported signals, Wong et al., 2013). Further, novel methods of examining the aggregate effect of networks of probes have been shown to be extremely powerful to detect differences in early development (Spiers et al., 2015). These observations were considered when planning the series of exploratory analyses summarised in **Chapter 6**, aimed to examine the feasibility and potential for integrating DNAm measurements with parent-report behaviour, eye-tracking and electro-encephalography measures collected over the first years of life.

In 1928, the eminent statistician R. Fisher, in his book *Statistical Methods for Research Workers*, stated: "it is a useful preliminary before making a statistical estimate [...] to test if there is anything to justify estimation at all" (Fisher, 1928). A recent debate in the scientific community arose from the controversial proposal that findings should be reported based on the observed effect sizes, instead of using p-value thresholds to categorise significant and non-significant results (see Amrhein, Greenland, & McShane, 2019). However, an estimation-only approach which is not guided by hypothesis testing in the interpretation of significance of the observed results risks to provide misleading results, poor predictions and overconfident claims (Haaf, Ly, & Wagenmakers, 2019). In agreement with this argument, I acknowledge the fact that generalizability of findings of the exploratory research described in **Chapter 6** and partly in **Chapter 4** and **5** is limited by the small sample size, which reflects the challenge of obtaining complete datasets of good-quality data from richly phenotyped infant cohorts. Nevertheless, this type of work might pioneer future work on genetics, epigenetics and behavioural development. The field of human developmental neuroscience can benefit from these investigations of effect sizes, power and possible functional significance of the observed signals, and this information can be used to plan cooperative efforts for obtaining larger samples.

7.3.3 Missing information on rare genetic variants and familial environments

The limited effect of polygenic score on social attention, found in **Chapter 4** and **5**, should not be considered an exhaustive evaluation of the genetic contributions to the examined endophenotypes. Some of the methodological limitations that might explain the results have been addressed before (**sections 4.4.3** and **5.4.2**). However, a more precise picture of genetic risk would be provided by assessing whether rarer genetic variants (including inherited and de novo CNVs and SNVs) also accounting for part of the variability seen in social attention. HR infants are assumed to be at higher genetic risk of ASD and advanced paternal age is also associated with an increased risk of de novo mutations; given the established role of de novo CNVs in liability to ASD this is an avenue worth exploring in future BASIS research (Iossifov et al., 2012, Leppa et al., 2016). Having a more comprehensive genetic characterization in the infant siblings would be crucial to deeply explore the contribution of the full spectrum of genetic risk variants to social attention (Iacono et al., 2017). This would constitute a more powerful validation of social attention endophenotypes and provide more accurate information about pathophysiological processes related to the emergence of behavioural symptoms (Dick, 2018).

The target population in this thesis is composed by children at "high familial risk". Individuals within the same family share not only DNA sequence, but also the same rearing and socioeconomic environment. Moreover, as discussed in **Chapter 4** (**sections 4.4.3** and **4.4.4**), the interaction between genes and environment acts at the level of the individual as well as at the family level. For example, it has recently been demonstrated that parents' non-transmitted alleles effect their offspring's phenotype, most likely via genetic influence on environmental exposures (the 'nature of nurture' ; Kong et al., 2018). For example, polygenic scores for educational attainment in parents significantly predicts their ability to provide warm-sensitive and cognitively stimulating parenting behaviours, and this effect is independent from evocative effects of children's behaviour (Wertz et al., 2019). From a different perspective, a large adoption study (N=561 mother-child dyads) showed that the child's own genetic risk for psychopathology could negatively impact maternal reactivity since the first year of life. Importantly, the evoked effect on mothers to react negatively to infants' heritable traits was only observed in the presence of marital problems which could increase sensitivity to stress and sense of powerless (Pasco Fearon et al., 2015). This process was also observed in situations of known genetic aetiology; for example, Soukup-Ascençao, D'Souza, D'Souza, & Karmiloff-Smith (2016) found that infants with Down and Williams Syndrome, who were less attentive to their parents and show less joint activity, had parents who acted more controlling (directive) and less

sensitive behaviours during live social interaction. Importantly, parental interaction style has also a direct influence on cognitive and affective development in the first two years of age (Annette Karmiloff-Smith, 2010; Pasco Fearon et al., 2015). Measures of environmental risk for the family environment, such as SES or parental stress, were not included in this thesis. Undoubtedly, they would have added a level of explanation to the results of **Chapter 4**, **5** and **6**, which aimed to understand the degree to which parental effects, familial risk and epigenetic factors, respectively, influenced the infants' phenotype.

In summary, the findings described in this section reveal that isolating causal effects of individual risk factors on child development is complicated and requires large datasets with longitudinal measures and possibly registries of health records (Fearon, 2019). Across this thesis, the "familial risk" effects on early developmental features likely reflected the intermingled effects of gene-environment interaction for the entire family system (Asbury, Dunn, Pike, & Plomin, 2003; Oliver & Alison, 2018; Parfitt, Pike, & Ayers, 2014). A careful investigation of the environmental contribution to developmental trajectories of ASD should consider a wide range of measures, many of those closely interdependent and hard to disentangle in an infant-sibling design.

7.4 FUTURE DIRECTIONS TO FURTHER INVESTIGATE CAUSALITY

The work presented in this thesis aimed to investigate the role of social attention in the causal path to ASD. As remarked in **Chapter 1** (**section 1.3.4**), establishing causality is complicated because of the developmental timing and multiple bi-directional links between genetic factors, environmental experiences and the process of reorganization the brain undergoes during the early phases of development (Gottlieb, 2007; Johnson, 2017; Panchanathan & Frankenhuis, 2016). The research methods used in this thesis have the potential to shed light on the opportunity to pursue the investigation of the possible cascading effects of early social attention atypicalities in the emergence of the ASD phenotype. For instance, leveraging the longitudinal prospective design to assess whether social attention atypicalities preceding the emergence of the disorder are predictive of later traits (**Chapter 2**, **3** and **5**), relying on statistical models designed to test directions of the relationships between variables (**Chapter 3** and **5**), using within-family designs (**Chapter 4**), combining repeated measurements from the same subject to study the change in relationship between epigenetic signatures and behaviour and comparing effects of prenatal exposure on children methylation (**Chapter 6**) are among the strategies which

served this aim (Barker et al., 2018; Richmond, Al-amin, Smith, & Relton, 2014). However, other research methodologies have been specifically designed to test causality limiting the effect of confounders.

Testing temporal causality

To assess the causality link between biomarkers and clinical outcome, randomised controlled trials (RCTs) is a powerful method that tests whether a modification in the outcome is observed in the group receiving a treatment targeting the function of interest which does not occur in the non-treated population (Green et al., 2017). Some of the early markers used in this thesis have been used in RCTs of toddlers with ASD (Dawson et al., 2012) or infants at high familial risk for ASD (Green et al., 2015) and ADHD (Goodwin et al., 2016) devoted to improve social or attention skills. In **section 7.4.1** I propose current challenges and promising avenues to implement an intervention design targeting the measures of social attention examined in this thesis.

Testing biological causality

From a genetic perspective, powerful methods to evaluate the causal effect of genetic variants on a phenotype are Mendelian Randomization (MR) and knock-out animal models. Specifically, MR analysis is based on the idea of using single variants or polygenic scores as instruments to control for confounding variables, i.e. moderators associated with both genetics and outcome, and reverse causation, the phenomenon by which the disease influences the apparent exposure, rather than vice versa (Smith & Ebrahim, 2008). MR is increasingly used to evaluate causal effects of a third variable (i.e. an environmental exposure or treatment) on the well validated association between selected genetic variants and a phenotype of interest. In MR, individuals are grouped based on their genotype, which, at a population level, should not be associated with other genetic or environmental factors. Thus, this method grants a powerful control for reverse causation and confounding (Smith, 2010). However, MR studies require very large samples and an investment on such design associated with collection of neurocognitive data in early infancy will be conditional on identifying very robust associations between common genetic variants and ASD (Burgess, Thompson, & CRP CHD Genetics Collaboration, 2011). Before this approach is undertaken, other investigations are needed to understand whether social attention atypicalities reflect developmental responses to specific disruptions of functional genetic pathways (see **section 7.4.2**).

Mice models have illuminated the knowledge on the function of important genes and pathways on the development of ASD features. For example, OXTR polymorphisms have been often

associated with ASD therefore causal pathways involving the OXTR expression, neurodevelopment and social behaviour have been the focus of several animal studies (Feldman, Monakhov, Pratt, & Ebstein, 2016). An interesting finding that emerged from this research is that the effects of the interplay between the oxytocin and serotoninergic systems on social behaviour depend on the duration of critical windows for synaptic plasticity in early neurodevelopment (Dölen, 2015; Hung et al., 2017; Nagano, Takumi, & Suzuki, 2018; Nardou et al., 2019). This model is particularly interesting with respect to three key aspects elaborated in this thesis. First, the oxytocin and serotonin systems have been consistently associated with visual attention markers in infancy, as reviewed in **section 1.3.1**, see also (Papageorgiou & Ronald, 2013). Second, the proposed biological mechanism is in line with the described developmental models of ASD pathogenesis (**sections 1.3.4** and **3.1.2**) postulating the developmental trajectories depend on timing of adaptive responses of the brain in relation to sensitive periods (Johnson, 2017; Karmiloff-Smith, 2007; Panchanathan & Frankenhuis, 2016). Third, although mice models do not offer optimal paradigms for testing effects on visual attention, the role of oxytocin in modulating this function has been consistently validated in primates (e.g., Parr et al., 2016; Simpson et al., 2017) and humans (Clark-Elford et al., 2015; Domes, Steiner, Porges, & Heinrichs, 2013; Nishizato, Fujisawa, Kosaka, & Tomoda, 2017; Skuse et al., 2014). Thus, while animal research seems promising to provide insights on the biological underpinnings of social attention and its development, it seems unlikely that the opposite will happen, given the multiple risk and protective factors, some of which highlighted in this thesis, that have been hypothesised to be involved in the emergence of the various components of attention.

Testing transmission causality

Transmission causality implied that parents' features have an effect on their child's phenotype. This is based on the assumption that, at least in the very first period of life, parental attitude plays a major role as regulator of the environmental exposure for the child (Shultz, Klin, & Jones, 2018). One way to investigate how the exposure to a particular environment is related to behavioural outcome is through epigenetics. In fact, markers can partly reflect these processes and mediate the path between risk exposure and child psychopathology, moderated by genetic factors (Barker et al., 2018). As reviewed in **Chapter 1** (**section 1.2.3)**, disruptions in DNAm have been proposed to play a role in the emergence of ASD (Ciernia & Lasalle, 2016). To strengthen the inference on the role of DNAm as causal mediator between risk factor exposure and behavioural outcome, a triangulation of approaches are needed in order to minimise the risk of confounding reverse causation effects for causal effects. Barker et al. (2018) described some of

the designs that can be used in this sense: assisted conception or in vitro fertilization designs that make it possible to separate maternal genetics effects from the influence of prenatal exposures, natural experiments in which a population is exposed to specific events (see for example Chandak et al., 2017; Heijmans et al., 2008) and discordant twin designs (Wong et al., 2013). In an infant-sibling design, conversely, what can be done is to improve the investigation of the relation between parental measures, including genetic factors, and environmental exposures and their effects on infants' developmental trajectories. A first step would be to evaluate transmitted genetic variants, as described in **section 7.4.3**. Investigations which integrate multiple factors to model the effect of familial risk on ASD outcome could only be done in large samples (Newschaffer et al., 2012) and will require careful data analysis strategies to limit the effect of confounders (Fearon, 2019).

In the section below, I summarise possible directions to validate and extend the results obtained in this PhD work and continue the investigation of the role of social attention in the developmental path to ASD.

7.4.1 Ecological measures of social attention

Obtaining experimental measures in carefully controlled conditions is a first necessary step to identify reliable markers of psychological processes. However, testing the ecological validity of these markers is important to evaluate whether individual differences in attention as assessed using screen-based tasks might relate to individual differences in attention in naturalistic settings. Previous research has partly addressed this question. For example, Jones et al. (2015) investigated neural responses (alpha and theta power) during social and non-social attention while 6- and 12-month-old typically developing infants were presented with movies and naturalistic live interactions (woman singing versus holding a toy). They found the same pattern of results (increased theta power over the frontal regions to social stimuli) in the movies and live situations. Although the goal of the study was not to statistically compare responses obtained with the two paradigms, they did report that neural responses when attending to social live interactions were descriptively enhanced in 12-month-old infants during social attention. Thus, EEG activity seems to be similar in situations of screen-based and live presentations but naturalistic settings produce neater results (Jones et al., 2015). Of note, in the present study static images rather than videos were used, therefore a comparison between neural responses to this type of paradigm and exposure to live static human faces and

interacting humans would be important to verify ecological validity of the studied endophenotypes.

This type of comparison was performed by Wass (2014) for peak look durations recorded with eye-tracking from 11-month-old typically developing infants. Six different paradigms were used: screen-based tasks showing static simple and complex images, videos of mixed static and dynamic scenes, videos of toys spinning or short TV clips and semi-naturalistic situations during which the infant could play with one or four toys, respectively. Importantly, he found that individual differences in peak look duration were consistent across the static and dynamic screen stimuli. However, they were unrelated to individual differences in looking behaviour during free play with objects. He argued that susceptibility to high luminance contrasts and sudden changes in stimulus onset–offset may trigger individual differences on screen-based tasks, but are not relevant in more naturalistic context. In the latter, the complexity of the visual scene might have a different impact to looking behaviour compared to the limited amount of uncontrolled distractors in screen-based tasks (Wass, 2014). In light of this study, it becomes necessary to further investigate, initially in typically developing infants, whether the measures of social attention used in this thesis as candidate endophenotype of ASD are reflect responses obtained in ecologically valid contexts.

Advances in technology for recording neurophysiological measures can support this aim. In fact, sophisticated wireless technology can be now used to track EEG activity and looking behaviour during social interactions (Leong et al., 2017; Liu et al., 2018; Wass et al., 2018). Such approach should be undertaken to extend the current findings to a level of ecological validity and to evaluate the effect of every-day experiences on early brain activity. In light of the findings reported in **Chapter 2**, looking at microstates in continuous EEG data during social attention to movies could be an intermediate step to extend the current examination using approaches similar to what is used in the adult research (Rieger, Hernandez, Baenninger, & Koenig, 2016). Ultimately, a promising avenue would be to record microstates online during naturalistic social interactions, to study what elements of the environment (i.e. the interacting person) elicit attentive brain states which facilitate learning. Adults research is moving towards developing microstate-neurofeedback intervention for psychiatric patients (Hernandez, Rieger, Baenninger, Brandeis, & Koenig, 2016; Michel & Koenig, 2018). Similarly, a long-term goal would be to exploit the potential of this non-invasive technique to inform researchers about strategies that elicit typical versus adaptive responses in social contexts in infants at risk for ASD, and tailor personalised interventions with a valid application in every-day life.

7.4.2 Increasing heterogeneity to understand specificity

The study of syndromic ASD is providing important advances in characterising causal mechanisms associated with specific phenotypic features (Baker et al., 2018; Bruining et al., 2010; Distefano et al., 2016; Ousley et al., 2017). One fruitful approach is to group patients with the same syndromic cause to study mechanisms underlying phenotypic traits which emerge from etiologically homogeneous conditions (Jeste & Geschwind, 2014). However, a certain degree of variability in symptomatology has been reported within groups of individuals with the same genetic syndrome (Bruining et al., 2014). The extent to which these individuals show heterogeneous phenotypic manifestations has been shown to depend on environmental factors (Glennon, Karmiloff-smith, & Thomas, 2017) as well as different genetic background in terms of common variants (Weiner et al., 2017) which might mediate the effect of syndromic ASD risk.

More generally, the fact that individuals who carry the same genetic disruption have different development developmental trajectories fits well with the Neuroconstructivist account of development. Under this view, individual developmental paths result from cascades of experience-driven processes that occur within complex biological and ecological systems providing genetic, epigenetic and environmental constraints (Marechal et al., 2007). Timing of these internal and external influences is fundamental in determining specialization of brain circuits over the course of development (Panchanathan & Frankenhuis, 2016). The observation that similar behaviours can be driven by different underlying neural processes has been validated with computational modelling (Frankenhuis, Panchanathan, & Nettle, 2016; Karmiloff-Smith, Casey, Massand, Tomalski, & Thomas, 2014). Specifically, disruptions of the typical process of experience-driven pruning at critical time points have been argued to lead to emergent behavioural manifestations which include autistic features (Thomas, Davis, Karmiloff-Smith, Knowland, & Charman, 2016). Thus, conditions in which a sub-optimal signal processing in early life is observed at a neural level, deriving by different etiological causes, might present similar compensatory or adaptive brain processes which emerge as ASD-like phenotypes (Johnson, 2017).

A cross-syndrome approach has been undertaken to evaluate early differences and similarities in developmental trajectories of language (D'Souza, D'Souza, & Karmiloff-Smith, 2017), numeracy (Karmiloff-Smith et al., 2012), literacy (Cornish, Steele, Monteiro, Karmiloff-Smith, & Scerif, 2012; Steele, Scerif, Cornish, & Karmiloff-Smith, 2013), attention (Scerif, Longhi, Cole, Karmiloff-Smith, & Cornish, 2012) developmental and social difficulties (Hernandez et al., 2009; Jeste et al., 2016; Kolesnik et al., 2017). A potentially interesting approach could be to evaluate

whether similar signs of atypical social attention at the identified age-windows (i.e. neural atypicalities at around 8 months; atypicalities in looking behaviour at around 14 months) are observed in individuals with various syndromic conditions and "familial" idiopathic ASD, and whether these relate to similar outcomes later in development. This could be critical to inform causal mechanisms as it would become possible to identify from syndromic patients which biological mechanisms are more likely to be associated with specific trajectories of development.

A first step in this ambitious plan would be to verify whether atypicalities in these early social attention markers predict ASD in individuals with a known single-gene/region mutation. Subsequently, clustering individuals by their early social attention phenotype might help to identify pathophysiologic characteristics associated with the endophenotypes. This approach will also inform on the opportunity to plan condition-specific versus general interventions. Naturally, such research plan would require cooperation with clinical services who deal with the patients and their family members.

7.4.3 Further investigations of inherited and non-inherited genetic variants

Idiopathic ASD, which has been the focus of this thesis, is an extremely complex condition whose phenotypic characterization has been shown to derive from a multiplicity of genetic factors of relatively small and largely additive effect (see **Figure 1.2**). More than 51% of the genetic liability for ASD has been estimated to be due to inherited variants (Gaugler et al., 2014).

A powerful approach for estimating the combined influence of inherited common genetic variants in familial samples would be to conduct a polygenic transmission disequilibrium test (pTDT), which assesses the increase in polygenic risk in the offspring compared to the mid-parent value obtained averaging the PGS of the two parents. This method recently identified polygenic contribution to ASD in a sample of 6,000 trios spanning both multiplex and simplex families (Weiner et al., 2017). Interestingly, pTDT for ASD was also enhanced in non-affected siblings of individuals with ASD, suggesting that it might also capture part of the variance in BAP. It would be particularly interesting to make use of the BASIS pedigrees data to see whether pTDT is predictive of our infant social attention endophenotypes or effect developmental trajectories. Currently, the gBASIS sample includes only 83 complete trios (mother, father and infant sibling) of European ancestry and phenotypic data, but merging datasets with other infant research groups would be a good starting point to evaluate possible effects of inherited genetic variants

on early signs of atypical development. Naturally, the use of neurocognitive infant endophenotypes of social attention would probably be unfeasible, given that it is unlikely that different research teams implemented identical protocols and data-cleaning procedures (given that differences were found in this work even between different Phases of data collection from the same site). An exploratory step should initially investigate parent-report measures or scores from standardized behavioural assessments, which would be comparable across sites and less affected by missing data due to data quality issues.

This exciting research avenue could be complemented by the investigation of rates of inherited and de novo CNVs and SNVs, thus providing more information on the contribution of transmitted versus non-transmitted genetic loading in the path to ASD. To this aim, a fruitful approach would be to perform whole-exome sequencing on the families with at least four family members, including two parents, the ASD proband and a carefully phenotyped infant sibling. For example, a recent study using SNP microarray and WES data in 31 independent families identified de novo CNVs in 3 out of 26 individuals with autism (11.5%) and 1 out of 43 siblings (2.3%) (Leblond et al., 2019). Similar proportions might be expected for the present cohort (**Figure 7.2**, total N=71).

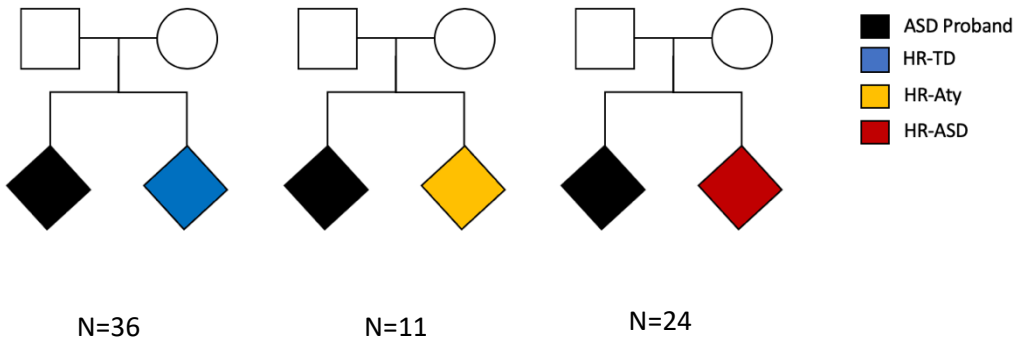

Figure 7.2 *Pedigrees of the gBASIS quartets with good quality DNA data*. In each pedigree, the top square represents the father, the top circle represents the mother, while the bottom rhombi represent the children of gender not specified (Bennett, French, Resta, & Doyle, 2008). Rhombi on the left of each pedigree indicate older children who received a community diagnosis of ASD (ASD proband); the right bottom squares represent younger siblings (BASIS participants) who were typically developing at age 3 (blue), who showed signs of atypical development but had sub-threshold autistic symptoms (orange) or who received diagnosis of ASD (red). Number of families for each pedigree are reported below.

This approach would be very informative of causal mechanisms underlying differences in developmental trajectories thanks to the fact that for all infant siblings a wide variety of measures, including measurements of brain activity and looking behaviour, are available, thanks to the longitudinal data collection carried out within BASIS. For instance, the identification of a

high penetrance and functionally deleterious de novo structural or sequence DNA variant specific to an ASD proband will shed light on the links between gene function and early behavioral measures of social attention and cognition. Of note, examining individual social attention skills per se would be of little use if values had to be considered in absolute terms. However, thanks to the analyses carried out as part of this PhD work, "disruption" or atypicality of social attention can now be evaluated relative to the entire BASIS/STAARS cohort. In the event that inherited rarer variants (previously identified in the literature as high-confidence ASD genes) are observed, the investigation could focus on performing a family-by-family characterization of the infant at-risk social attention or executive function profiles, the parental characteristics, and potential protective or moderating effects that might impact high genetic risk to influence variable phenotypic outcomes in the infants.

Examining the effects of specific disruptive genetic variation on early social attention will take research to a novel, exciting level of analysis. Multidisciplinary and translational approaches might be implemented in the future to track newly identified pathophysiological mechanisms involved in the causal pathway to ASD.

7.5 CONCLUDING REMARKS

This thesis has illuminated hitherto undocumented relations between genetic and epigenetic variation and behavioral and neural correlates of social attention in infancy preceding the emergence of ASD symptoms. It has also investigated the relationship between different markers of atypical developmental trajectories in the first stages of development and their role in the pathway from familial risk to behavioural symptoms of neurodevelopmental disorders. Moreover, it has explored the relation between polygenic risk, autistic traits and social attention skills in a familial sample, offering exciting grounds for future research on genetic and environmental effects involved in the causal path to ASD.

To conclude this thesis, I provide a summary of the results of the presented work in relation to the questions identified in **Chapter 1**:

- Which of the candidate early markers of ASD contribute to developmental trajectories towards the emergence of the ASD behavioural phenotype?

A reduced engagement of the brain in states of attention when looking at static images of faces as opposed to an enhanced and prolonged attentive state in response to non-social visual stimuli

detected as early as 6 to 10 months of life precede the development of autistic symptoms and reduced social adaptive skills. This result was obtained by applying, for the first time, microstate analysis in infant data. Importantly, this novel approach was combined with a classic ERP investigation looking at the Nc component, which critically replicated previous findings (Jones et al., 2016) in a larger, independent cohort. Moreover, reduced mean amplitude of the Nc in response to faces than to non-social stimuli was found to be associated with DNA methylation levels in a network of probes which are regulated by genes functionally involved in neuronal signalling and mitochondrial function. Longer peak look duration at a face image in a face pop-out array, recorded with eye-tracking at 14 months of age, is associated with familial and possibly polygenic risk factors for neurodevelopmental disorders. Preliminary evidence for a relation with DNA methylation changes in a gene previously involved in ADHD and intelligence emerged as result from a longitudinal analysis, though replication is warranted given the small sample size for this analysis. This early sign of atypical looking behaviour in social contexts relate to inhibitory control at 2 years and to language skills at three years. It was also shown to be predictive of ASD symptoms later in childhood (6-10 years), providing important validation of the clinical utility of this developmental endophenotype.

- To what extent atypicalities of social attention identified in the adult literature map onto atypicalities observed before the emergence of ASD traits?

Critically, I found little evidence for a relation between the abovementioned infant endophenotypes and accuracy in detecting eye-gaze direction detection recorded during a researcher-unsupervised computerised task administered over the internet to family members of children with and without ASD. However, the latter was associated with autistic traits in the familial sample and with earlier individual differences in attention engagement with the gazed-at object in an eye-tracking gaze-following task administered at 14 months of age. These intra-individual longitudinal associations were yet to be documented and constitute important information in the study of individual differences. Thus, results illustrated in this thesis suggest that performances in tasks testing eye-gaze direction detection abilities might be manifestations of the symptomatology of ASD, and therefore being observed as precursors of the disorder in infants and as part of the Broader Autism Phenotype in some family members with sub-threshold autistic traits.

The other infant measures might reflect different components of social attention. For example, as discussed in **Chapter 5**, peak look duration in the face pop-out task might depend on emerging executive attention skills. Both the Nc component and the duration of peak looks at static stimuli have been previously associated with processing load in young infants (Colombo & Cheatham,

2006; Jones et al., 2016). Overall, they might rely more on visual attention strategies directed towards the exploration of the face as a whole, differently from the measure obtained from the gaze following task. Different neural underpinnings might also characterise the two tasks. For example, Dalton, Nacewicz, Alexander, & Davidson (2007) showed that gaze processing elicited activation of the amygdala in relatives of individuals with ASD (social behaviour network in Klein et al. 2009, see **Figure 1.4**). On the contrary, the Nc component in infancy has been shown to be elicited by superior and posterior regions of the prefrontal cortex (Richards, Reynolds, & Courage, 2010) which are part of the reward and orienting networks. These observations encourage a reflection on the fact that the concept of endophenotype applies to a specific measurement and knowing how this maps onto a specific cognitive function is of particular relevance when considering its utility in clinical practice.

- Are previous findings of social attention atypicalities in family members of individuals with ASD replicable?

The analyses carried out in this thesis replicated and extended previous results in familial samples and gave rise to speculations on the potential role of protective factors contributing to social attention. Particularly, non-affected female family members of individuals with ASD showed higher accuracy compared to non-affected males and with affected females, suggesting that sex-specific protective factors might act on social attention as well as on the core symptoms of ASD. Descriptively, at 14 months of age, siblings of children with ASD who underwent a typical development at three years showed enhanced attention engagement with the gazed-at object in a gaze-following task compared to infant sibling who showed atypical or autistic behavioural features at age 3 and also to low-risk infants. This could constitute preliminary evidence for a protective role of enhanced engagement in joint attention situations for infants at high vulnerability. Differently, 14-month-old infants at high familial risk for ASD showed, independent on their clinical outcome, atypical looking behaviour during social attention in a face pop-out task. The hypothesis of a protective effect of executive function against the consolidation of ASD finds support in the observed relationship between this marker and individual differences in emerging inhibitory control.

- Do atypicalities of social attention share genetic variance with ASD traits?

The present design did not provide evidence for a significant association between accuracy in eye-gaze direction detection measured with an online computerised task and polygenic score for ASD. On the contrary, suggestive evidence was found for an association between a polygenic

score constructed using the most highly penetrant common genetic variants associated with ADHD and the duration of peak looks at the face recorded with eye-tracking at 14 months. This result should be considered with caution given the limited power of the base and target samples for this analysis. It might, however, reflect an effect of increased polygenic liability for neurodevelopmental disorders on infant endophenotypes associated with emerging disruptions in executive function and with autistic symptoms in mid childhood.

The work presented in my thesis provided preliminary evidence for considering infant neurocognitive measures of social attention as endophenotypes of ASD. One candidate endophenotypes is the pattern of neural activation from 300 ms after the stimulus onset when 6- to 11-month-old infants are attending to faces with direct gaze compared to a non-social control stimulus. Another candidate endophenotypes is peak look duration at a static face image among other non-social stimuli, measured with eye-tracking between 12 and 15 months of age. These promising markers revealed atypicalities in infants at high familial burden for neurodevelopmental disorders (**Chapter 2** and **5**) and are predictive of later social and communication skills, respectively (**Chapter 3**). Further studies are needed to corroborate findings on the possible role of genetic, familial (**Chapter 4** and **5**) and epigenetic (**Chapter 6**) factors associated with these early signs of atypical developmental trajectory, before planning early interventions for ASD targeting social attention. Nevertheless, the novel approach undertaken in this PhD represent a pioneering starting point to investigate, in larger samples, the role of social attention in the path to ASD. Integrating molecular genetic research methods in prospective longitudinal studies of infants at high familial risk for neurodevelopmental disorders offer exciting possibilities for future research on the causal mechanisms underlying individual differences in developmental trajectories.

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APPENDIX CHAPTER 2

Table A2.1 Composition characteristics of the Phase 1 and 2 sample by recruitment Phase, and mean scores of the behavioural measures collected at T1 (8 months) and T2 (14 months), T3 (2 years) and T4 (3 years).

Participants	**Phase 1**	**Phase 2**	
N current study	104	143	
Males/Females	41/62	78/65	
Low-risk/High-risk	50/54	27/116	
N HR-TD	24	64	
N HR-Aty	12	32	
N HR-ASD	17	17	
N missing outcome	1	3	
Behavioural measures	**Mean (s.d.)** **Min - Max**	**Mean (s.d.)** **Min - Max**	**p**
T1			
Age (months)^{w}	7.28 (1.19) 6 - 10	8.7 (0.83) 7 - 11	<0.001*
MSEL Composite Score^{t}	99.06 (13.17) 64 - 143	107.48 (15.29) 66 - 140	<0.001*
VABS Composite Score^{w}	96.83 (15.09) 66 - 150	94.72 (12.05) 49 - 122	0.376
T2			
Age (months)^{w}	13.78 (1.55) 11 - 20	14.82 (0.98) 13 - 18	<0.001*
MSEL Composite Score^{t}	101.49 (17.39) 56 - 154	95.79 (14.66) 49 - 141	0.008*
VABS Composite Score^{w}	96.09 (12.53) 61 - 136	97.20 (11.96) 60 - 142	0.835
T3			
Age (months) ^{t}	24.24 (1.0) 21 - 27	26.28 (1.97) 23 - 35	<0.001*
MSEL Composite Score^{w}	108.40 (18.65) 57 - 144	101.83 (20.33) 49 - 144	0.007*
VABS Composite Score^{w}	104.76 (11.77) 76 - 143	101.99 (12.99) 57 - 126	0.252
T4			
Age (months) ^{t}	37.87 (2.81) 32 - 53	39.1 (2.62) 25 - 48	<0.001*
MSEL Composite Score^{w}	110.37 (19.78) 49 - 147	105.59 (24.47) 49 - 145	0.246
VABS Composite Score^{w}	101.06 (11.97) 58 - 131	97.08 (13.40) 52 - 121	0.060
ADOS CSS^{w}	3.73 (2.50) 1- 10	2.38 (2.15) 1 - 10	<0.001*
SRS Total Score^{w}	45.54 (9.46) 35 - 92	48.60 (12.21) 35 - 89	0.251

N: number of subjects; s.d.: standard deviation; Min – Max: minimum and maximum values; p: p-value of two-tailed t-tests. MSEL: Mullen Scales of Early Learning; VABS: Vineland Adaptive Behavior Scales; ADOS CSS: Autism Diagnostic Observation Schedule Calibrated Severity Score; SRS: Social Responsiveness Scale. ^{t,w} subscripts indicate whether t-test or Wilcoxon rank sum test, respectively, was performed to compare groups. P-values refer to these statistics. Wilcoxon test was performed if non-normality of the distribution in one or both groups was found based on significant Shapiro-Wilk test.
* p<0.05.

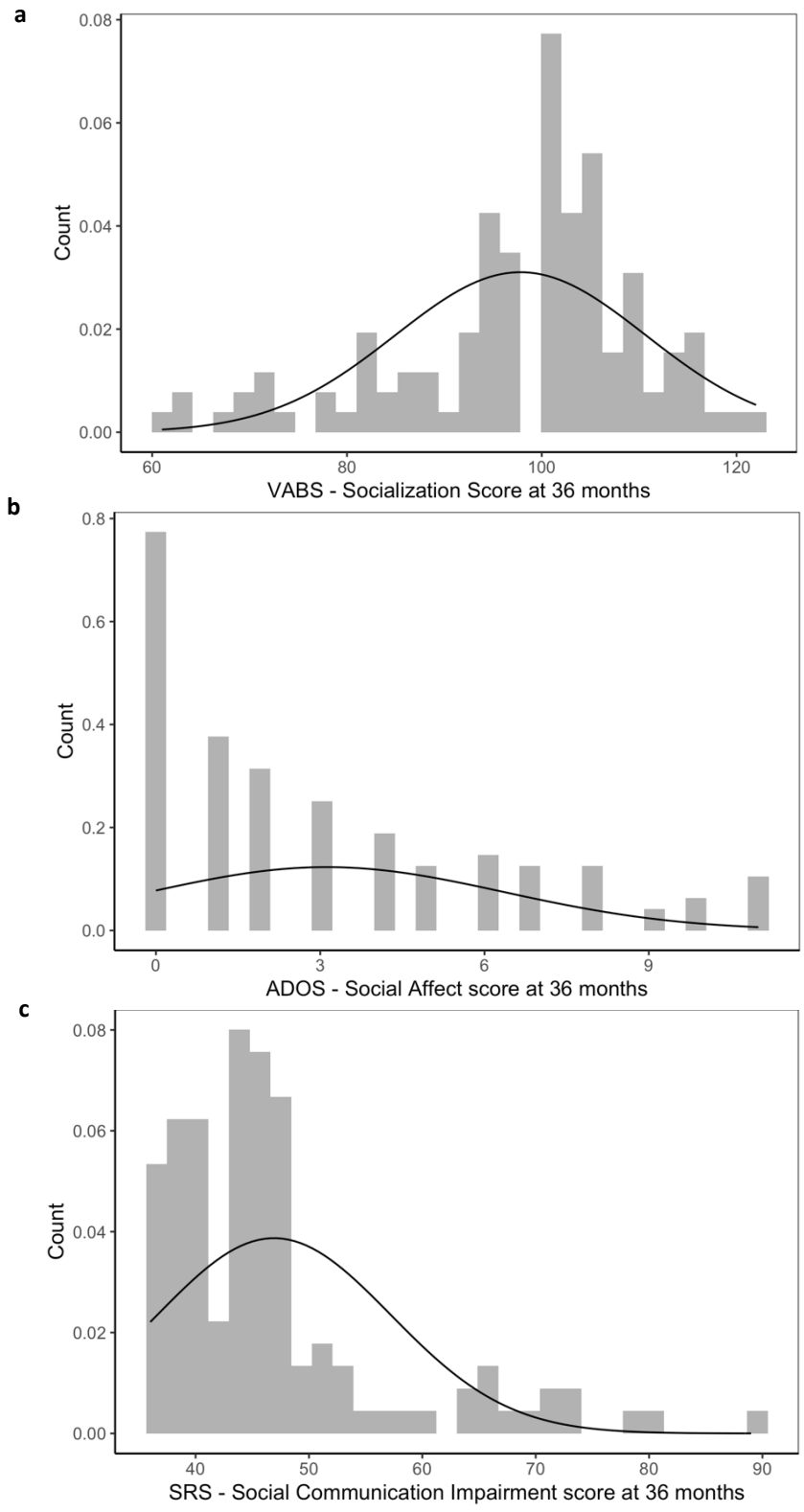


Figure A2.1 *Distribution of dimensional measures of social skills at three years for the study sample*. **a** Vineland Adaptive Behavior Scales (VABS), N= 123 – Socialization domain standard score; **b** Autism Diagnostic Observation Schedule (ADOS), N= 126 – Social Affect Calibrated Severity Score; **c** Social Responsiveness Scale (SRS) – Social Communication Impairment t-score, N= 123.

Control analyses for early and late Nc amplitude

Table A2.2 Comparison between multilevel mixed-effects models with the early Nc mean amplitude difference between Face with Direct Gaze (FD) and Noise as dependent variable. The baseline model, with subjects as random effects and region as a nested variable within subject, was compared with updated models testing the fixed effect of predictors such as proportion of attended trials in the FD and Noise condition (prop FD/Noise), sex, Phase, region, age, outcome group and interaction between these variables.

Models Early Nc mean amp. FD-N	d.f.	AIC	BIC	Log.Lik.	χ^2	p
Baseline Model	4	1723.85	1738.12	-857.9240		
Prop FD/Noise	5	1721.42	1739.26	-855.7088	4.430	0.035*
Sex	6	1722.70	1744.11	-855.3496	0.718	0.397
Phase	7	1723.64	1748.62	-854.8212	1.057	0.304
Region	8	1725.43	1753.98	-854.7174	0.208	0.649
Age	9	1724.91	1757.03	-853.4556	2.524	0.112
Age x Outcome	12	1728.18	1771.00	-852.0901	2.731	0.435
Outcome	15	1720.31	1773.83	-845.1531	13.874	0.003*
Outcome x Sex	18	1723.39	1787.62	-843.6957	2.915	0.405
Outcome x Phase	21	1725.18	1800.12	-841.5914	4.209	0.240
Outcome x Region	22	1727.18	1805.69	-841.5910	0.001	0.976
Phase x Region	25	1732.11	1821.32	-841.0566	1.069	0.785
Outcome x Phase x Region	4	1723.85	1738.12	-857.9240		

d.f.: degrees of freedom; AIC: Akaike information criterion; BIC: Bayesian information criterion; Log.Lik.: log likelihood; χ^2: chi-square statistic; p: p-value.
* $p<0.05$.

Table A2.3 Comparison between multilevel mixed-effects models with the late Nc mean amplitude difference between Face with Direct Gaze (FD) and Noise as dependent variable. The baseline model, with subjects as random effects and region as a nested variable within subject, was compared with updated models testing the fixed effect of predictors such as proportion of attended trials in the FD and Noise condition (prop FD/N), sex, Phase, region, age, outcome group and interaction between these variables.

Models Late Nc mean amp. FD-Noise	d.f.	AIC	BIC	Log.Lik.	χ^2	p
Baseline Model	4	1840.73	1855.00	-916.3648		
Prop FD/Noise	5	1839.79	1857.63	-914.8934	2.943	0.086
Sex	6	1841.78	1863.19	-914.8909	0.005	0.943
Phase	7	1841.63	1866.61	-913.8171	2.148	0.143
Region	8	1843.62	1872.16	-913.8087	0.017	0.897
Age	9	1841.44	1873.56	-911.7214	4.175	0.041*
Age x Outcome	12	1842.68	1885.50	-909.3412	4.760	0.190
Outcome	15	1837.12	1890.65	-903.5609	11.561	0.009*
Outcome x Sex	18	1841.49	1905.72	-902.7438	1.634	0.652
Outcome x Phase	21	1843.48	1918.41	-900.7389	4.010	0.260
Outcome x Region	22	1845.31	1923.81	-900.6527	0.172	0.678
Phase x Region	25	1851.28	1940.48	-900.6380	0.030	0.999
Outcome x Phase x Region	4	1840.73	1855.00	-916.3648		

d.f.: degrees of freedom; AIC: Akaike information criterion; BIC: Bayesian information criterion; Log.Lik.: log likelihood; χ^2: chi-square statistic; p: p-value.
* $p<0.05$.

Face with Direct Gaze versus Noise

Nc mean amplitude

Table A2.4 Comparison between multilevel mixed-effects models with the Nc mean amplitude difference between Face with Direct Gaze (FD) and Noise as dependent variable. The baseline model, with subjects as random effects and region as a nested variable within subject, was compared with updated models testing the fixed effect of predictors such as proportion of attended trials in the FD and Noise condition (prop FD/Noise), sex, Phase, region, age, outcome group and interaction between these variables.

Models Nc mean amp. FD-Noise	d.f.	AIC	BIC	Log.Lik.	χ^2	p
Baseline Model	4	1761.64	1775.92	-876.8219		
Prop FD/Noise	5	1759.85	1777.69	-874.9241	3.796	0.051
Sex	6	1761.67	1783.08	-874.8368	0.175	0.676
Phase	7	1761.92	1786.90	-873.9621	1.749	0.186
Region	8	1763.90	1792.45	-873.9513	0.022	0.883
Age	9	1762.24	1794.36	-872.1212	3.660	0.056
Age x Outcome	12	1764.42	1807.24	-870.2120	3.819	0.282
Outcome	15	1757.11	1810.64	-863.5566	13.311	0.004*
Outcome x Sex	18	1724.85	1789.084	-844.427	1.453	0.693
Outcome x Phase	21	1727.94	1802.87	-842.969	2.915	0.405
Outcome x Region	24	1729.38	1815.02	-840.688	4.563	0.207
Phase x Region	25	1731.38	1820.58	-840.687	0.001	0.976
Outcome x Phase x Region	28	1736.31	1836.22	-840.152	1.069	0.785

d.f.: degrees of freedom; AIC: Akaike information criterion; BIC: Bayesian information criterion; Log.Lik.: log likelihood; χ^2: chi-square statistic; p: p-value.
* $p<0.05$.

Table A2.5 Results of the higher-order model with lower AIC testing outcome differences (see Table A2.4), with Nc amplitude difference between the Face with Direct Gaze (FD) and Noise conditions as dependent variable.

Predictors Nc mean amp. FD-Noise	β	s.e.	t	p
(Intercept)	-4.533	6.102	-0.743	0.459
Prop FD/Noise	-0.179	0.098	-1.823	0.071
Sex	-0.077	1.081	-0.071	0.944
Region	-0.091	1.406	-0.065	0.949
Phase	-0.096	0.666	-0.144	0.886
Age	0.016	0.024	0.657	0.512
LR vs. HR-TD	20.469	8.332	2.457	0.015*
LR vs. HR-Aty	9.505	11.292	0.842	0.402
LR vs. HR-ASD	41.392	12.490	3.314	0.001*
Age LR vs. HR-TD	-0.076	0.033	-2.311	0.023*
Age LR vs. HR-Aty	-0.036	0.043	-0.850	0.397
Age LR vs. HR-ASD	-0.150	0.049	-3.020	0.003*

β: regression coefficient; s.e.: standard error; t: t-statistic; p: p-value. LR: Low-Risk infants, HR-TD: High-Risk infants with Typical Development, HR-Aty: High Risk infants with Atypical development but no Autism Spectrum Disorder diagnosis, HR-ASD: High-Risk infants with Autism Spectrum Disorder.
* $p<0.05$.

Table A2.6 Results of the linear regression testing the relationship between VABS Soc. at 3 years and difference between Nc mean amplitude in response to Face with Direct Gaze (FD) and Noise at 8 months (in microVolts). Amplitude differences of the Nc component measured over the left and right frontal regions were averaged together. Positive values of the predictor indicate that there was a more enhanced response to Noise than to FD, while negative values indicate a higher sensitivity to FD.

Predictors VABS Soc.	β	s.e.	t	p
(Intercept)	98.30	2.11	46.46	<0.001
Phase	-4.77	2.25	-2.12	0.036*
Sex	5.38	2.228	2.41	0.017*
Nc mean amp. FD-Noise	-0.42	0.174	-2.409	0.018*

VABS Soc.: Vineland Adaptive Behavior Scale standard scores in the Socialization domain; β: regression coefficient; s.e.: standard error; t: t-statistics; p: p-value.
* $p<0.05$.

Table A2.7 Results of the linear regression testing the relationship between VABS Mot. at 3 years and difference between Nc mean amplitude in response to Face with Direct Gaze (FD) and Noise at 8 months (in microVolts). Amplitude differences of the Nc component measured over the left and right frontal regions were averaged together. Positive values of the predictor indicate that there was a more enhanced response to Noise than to FD, while negative values indicate a higher sensitivity to FD.

Predictors VABS Mot.	β	s.e.	t	p
(Intercept)	91.91	2.001	45.91	<0.0001
Phase	-5.24	2.092	-2.50	0.014*
Sex	10.38	2.071	5.01	<0.0001*
Nc mean amp. FD-Noise	-0.15	0.155	-0.95	0.342

VABS Mot.: Vineland Adaptive Behavior Scale standard scores in the Motor Skills domain; β: regression coefficient; s.e.: standard error; t: t-statistics; p: p-value.
* p<0.05.

Nc peak latency

Table A2.8 Comparison between multilevel mixed-effects models with difference between the Nc peak latency in response to Face with Direct Gaze (FD) and to Noise as dependent variable. The baseline model, with subjects and region as random effects, was compared with updated models testing the fixed effect of predictors such as proportion of attended trials in the FD and Noise condition (prop FD/Noise), sex, Phase, region, age, outcome group and interaction between these variables.

Models Nc peak lat. FD-Noise	d.f.	AIC	BIC	Log.Lik.	χ^2	p
Baseline Model	4	3324.47	3338.74	-1658.233		
Prop FD/Noise	5	3326.37	3344.21	-1658.186	0.093	0.761
Sex	6	3327.53	3348.94	-1657.766	0.840	0.359
Phase	8	3330.85	3359.39	-1657.423	0.687	0.709
Region	7	3329.49	3354.47	-1657.747	0.649	0.421
Age	9	3332.63	3364.75	-1657.317	0.861	0.65
Age x Outcome	12	3337.38	3380.20	-1656.690	1.253	0.74
Outcome	15	3333.78	3387.30	-1651.890	9.601	0.022*
Outcome x Sex	18	3338.03	3402.26	-1651.013	1.754	0.625
Outcome x Region	21	3338.50	3413.44	-1648.251	5.523	0.137
Outcome x Phase	24	3340.62	3426.26	-1646.308	3.887	0.274
Phase x Region	25	3342.40	3431.60	-1646.198	0.221	0.638
Outcome x Phase x Region	28	3345.27	3445.17	-1644.632	3.131	0.371

d.f.: degrees of freedom; AIC: Akaike information criterion; BIC: Bayesian information criterion; Log.Lik.: log likelihood; χ^2: chi-square statistic; p: p-value.
* p<0.05.

Table A2.9 Results of the higher-order with lower AIC testing outcome differences (see Table A2.8) with Nc latency difference between the Face with Direct Gaze (FD) and Noise conditions as dependent variable.

Predictors Nc peak lat. FD-Noise	β	s.e.	t	p
(Intercept)	40.653	113.107	0.359	0.720
Prop FD/Noise	-0.676	1.813	-0.373	0.710
Sex	-31.440	20.021	-1.570	0.119
Region	-2.840	14.772	-0.192	0.848
Phase	-26.257	26.041	-1.008	0.315
Age	-0.001	0.453	-0.002	0.998
LR vs. HR-TD	47.516	154.339	0.308	0.759
LR vs. HR-Aty	123.667	209.161	0.591	0.555
LR vs. HR-ASD	-620.802	231.341	-2.683	0.008*
Age LR vs. HR-TD	-0.126	0.613	-0.205	0.838
Age LR vs. HR-Aty	-0.415	0.788	-0.526	0.600
Age LR vs. HR-ASD	2.346	0.917	2.558	0.012*

β: regression coefficient; s.e.: standard error; t: t-statistic; p: p-value. LR: Low-Risk infants, HR-TD: High-Risk infants with Typical Development, HR-Aty: High Risk infants with Atypical development but no Autism Spectrum Disorder diagnosis, HR-ASD: High-Risk infants with Autism Spectrum Disorder.
* p<0.05.

Table A2.10 Results of the linear regression testing the relationship between VABS Soc. at 3 years and difference between Nc peak latency in response to Face with Direct Gaze (FD) and Noise at 8 months (in milliseconds). Latency differences of the Nc component measured over the left and right frontal regions were averaged together.

Predictors VABS Soc.	β	s.e.	t	p
(Intercept)	96.53	2.122	45.48	<0.0001
Phase	-3.72	2.264	-1.65	0.103
Sex	5.96	2.265	2.63	0.01*
Nc peak lat. FD-Noise	0.02	0.01	1.68	0.096

VABS Soc.: Vineland Adaptive Behavior Scale standard scores in the Socialization domain; β: regression coefficient; s.e.: standard error; t: t-statistics; p: p-values.
* p<0.05.

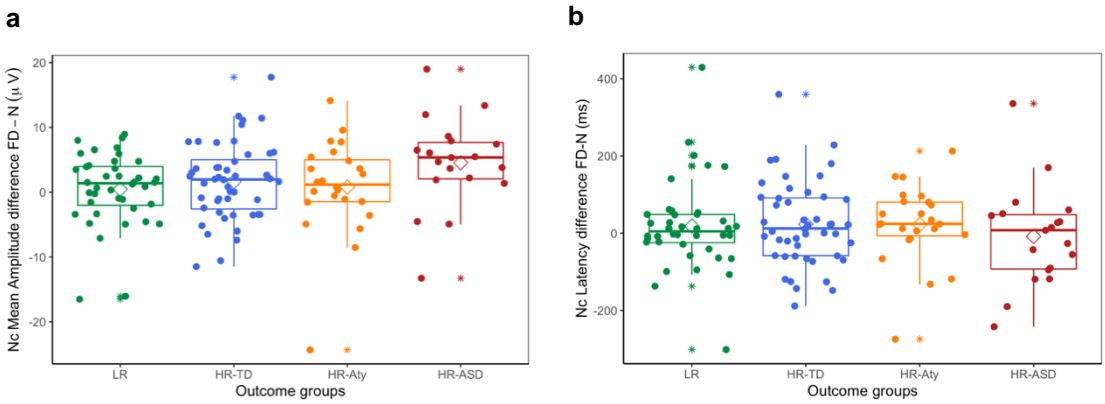


Figure A2.2 a Boxplots showing the difference in Nc mean amplitude (in microVolts) between the Face with Direct Gaze (FD) and Noise for the four outcome groups. Positive values indicate less enhanced (less negative) response to the Face than to the Noise stimulus. **b** Boxplots showing the difference in peak latency of the Nc (measured in milliseconds) between the Face with Direct Gaze (FD) and Noise for the four outcome groups. Negative values indicate shorter response to FD than to Noise. All individual data are represented by points. The lower and upper hinges of the boxplots correspond to the first and third quartiles (the 25th and 75th percentiles). The upper and lower whiskers extend from the hinges to the largest and smallest values, respectively, no further than 1.5 x inter-quartile range. Data beyond the end of the whiskers are plotted individually and represented by stars. Horizontal lines within boxplots indicate the median value, while rhombi represent mean values.

Face with Averted Gaze versus Noise

Nc Mean Amplitude

Table A2.11 Comparison between multilevel mixed-effects models with difference between the Nc mean amplitude in response to Face with Averted Gaze (FA) and to Noise as dependent variable. The baseline model, with subjects and region as random effects, was compared with updated models testing the fixed effect of predictors such as proportion of attended trials in the FA and Noise condition (Prop FA/Noise), sex, Phase, region, age, outcome and interaction between these variables.

Models Nc mean amp. FA-Noise	d.f.	AIC	BIC	Log.Lik.	χ^2	p
Baseline Model	4	1784.65	1798.93	-888.3269		
Prop FA/Noise	5	1786.42	1804.27	-888.2117	0.230	0.631
Sex	6	1787.35	1808.76	-887.6742	1.075	0.300
Phase	7	1787.34	1812.32	-886.6714	2.005	0.157
Region	8	1789.34	1817.89	-886.6708	0.001	0.971
Age	9	1790.70	1822.82	-886.3510	0.640	0.424
Age x Outcome	12	1794.62	1837.44	-885.3117	2.079	0.556
Outcome	15	1797.21	1850.74	-883.6056	3.412	0.332
Outcome x Sex	18	1800.05	1864.28	882.0229	3.165	0.367
Outcome x Region	21	1805.50	1880.43	-881.7479	0.550	0.908
Outcome x Phase	24	1809.00	1894.64	-880.5009	2.490	0.477
Phase x Region	25	1810.75	1899.96	-880.3751	0.252	0.616
Outcome x Phase x Region	28	1814.43	1914.34	-879.2148	2.321	0.509

d.f.: degrees of freedom; AIC: Akaike information criterion; BIC: Bayesian information criterion; Log.Lik.: log likelihood; χ^2: chi-square statistic; p: p-value.

Table A2.12 Results of the linear regression testing the relationship between VABS Soc. at 3 years and difference between Nc mean amplitude in response to Face with Averted Gaze (FA) and Noise at 8 months (in microVolts). Amplitude differences of the Nc component measured over the left and right frontal regions were averaged together.

Predictors VABS Soc.	β	s.e.	t	p
(Intercept)	97.55	2.118	46.06	<0.0001
Phase	-4.36	2.283	-1.91	0.058
Sex	5.9	2.276	2.59	0.011*
Nc amplitude FA-Noise	-0.27	0.21	-1.3	0.196

VABS Soc.: Vineland Adaptive Behavior Scale standard scores in the Socialization domain; β: regression coefficient; s.e.: standard error; t: t-statistic; p: p-value.
* p<0.05.

Table A2.13 Results of the comparison between multilevel mixed-effects models with difference between the Nc latency in response to Face with Averted Gaze (FA) and to Noise as dependent variable. The baseline model, with subjects and region as random effects, was compared with updated models testing the fixed effect of predictors such as proportion of attended trials in the FA and Noise condition (Prop FA/Noise), sex, Phase, region, age, outcome and interaction between these variables.

Models Nc peak lat. FA-Noise	d.f.	AIC	BIC	Log.Lik.	χ^2	p
Baseline Model	4	3323.40	3337.68	-1657.701		
Prop FA/N	5	3324.75	3342.59	-1657.374	0.653	0.419
Sex	6	3326.68	3348.09	-1657.342	0.065	0.799
Phase	7	3328.68	3353.66	-1657.341	0.001	0.978
Region	8	3330.29	3358.84	-1657.146	0.391	0.532
Age	9	3332.14	3364.25	-1657.068	0.156	0.693
Age x Outcome	12	3335.98	3378.80	-1655.992	2.153	0.541
Outcome	15	3339.06	3392.59	-1654.532	2.919	0.404
Outcome x Sex	18	3342.20	3406.43	-1653.098	2.443	0.486
Outcome x Region	21	3339.45	3414.38	-1648.724	8.748	0.033*
Outcome x Phase	24	3345.23	3430.87	-1648.615	0.123	0.989
Phase x Region	25	3346.04	3435.25	-1648.018	1.194	0.275
Outcome x Phase x Region	28	3351.10	3451.01	-1647.549	0.938	0.816

d.f.: degrees of freedom; AIC: Akaike information criterion; BIC: Bayesian information criterion; Log.Lik.: log likelihood; χ^2: chi-square statistic; p: p-value.
* p<0.05.

Table A2.14 Results of the linear regression testing the relationship between VABS Soc. at 3 years and difference between Nc latency in response to Face with Averted Gaze (FA) and Noise at 8 months (in milliseconds). Latency differences of the Nc component measured over the left and right frontal regions were averaged together.

Predictors VABS Soc.	β	s.e.	t	p
(Intercept)	96.62	1.550	62.35	<0.0001
Phase	-3.53	1.624	-2.18	0.031*
Sex	5.95	1.623	3.67	0.0003*
Nc peak lat. FA-Noise	0.00	0.006	0.53	0.594

VABS Soc.: Vineland Adaptive Behavior Scale standard scores in the Socialization domain; β: regression coefficient; s.e.: standard error; t: t-statistic; p: p-value.
* p<0.05.

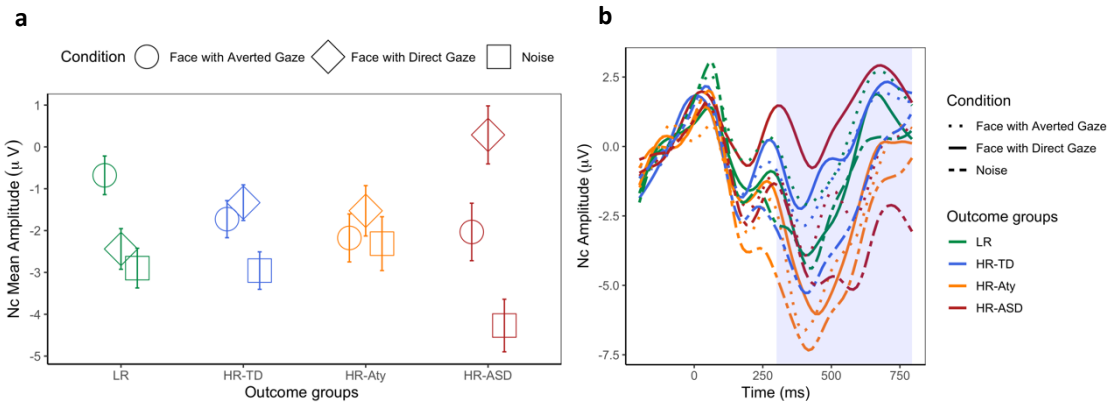


Figure A2.3 a Mean amplitude of the Nc component in response to Face with Direct Gaze (rhombus), Face with Averted Gaze (circle) and Noise (square) at 8 months in the four groups. All bars represent ± standard error. **b** Illustration of the grand average event-related potentials under the three conditions over lateral frontal electrodes at 8 months, with violet shade highlighting the Nc time window.

Microstates

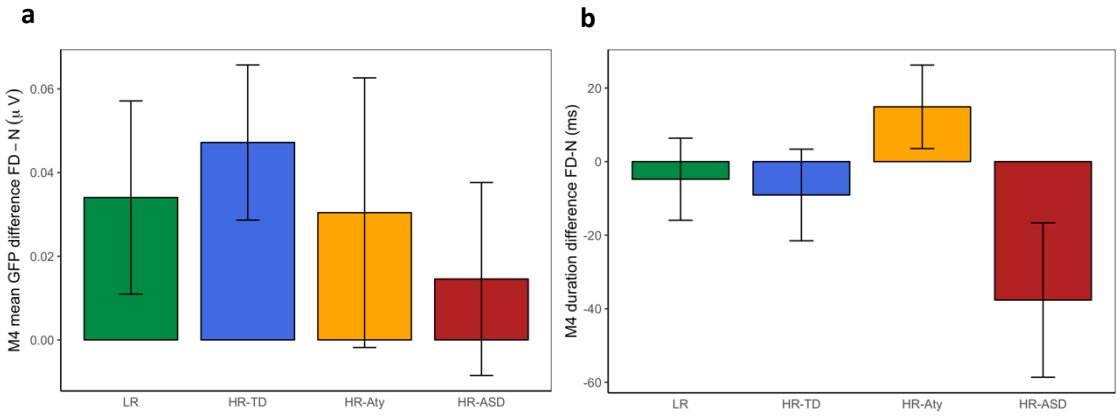


Figure A2.4 a Bar plot showing the difference in mean Global Field Power (GFP, in microVolts) between the Face with Direct Gaze (FD) and Noise for the four outcome groups. Higher values indicate more enhanced response to the Face stimulus. **b** Bar plot showing the difference in duration of M4 (measured in milliseconds) between FD and Noise for the four outcome groups. Negative values indicate shorter response to FD than to Noise. All black bars represent ± standard error.

Table A2.15 Results of the one-way ANOVA testing whether the three HR outcome groups were different in terms of difference in mean Global Field Power of Microstate 4 between the Face with Direct Gaze and the Noise condition.

	d.f.	F	p	η^2
(Intercept)	1	0.03	0.860	
Sex	1	0.02	0.891	0.0002
Phase	1	3.19	0.078	0.042
Age	1	0.38	0.538	0.005
Outcome	2	0.41	0.666	0.011
Residuals	73			

d.f.: degrees of freedom, F: F-statistic, p: p-value. η^2: partial eta-squared.

Table A2.16 Results of the one-way ANOVA testing whether the three HR outcome groups were different in terms of difference in duration of Microstate 4 between the Face with Direct Gaze and the Noise condition.

	d.f.	F	p	η^2
(Intercept)	1	0.88	0.350	
Sex	1	5.13	0.026*	0.064
Phase	1	3.85	0.054	0.049
Age	1	0.13	0.723	0.002
Outcome	2	3.40	0.039*	0.083
Residuals	75			

d.f: degrees of freedom, F: F-statistic, p: p-value. η2: partial eta-squared.
* p<0.05.

Table A2.17 Results of the robust linear regression testing the relationship between VABS Soc. at T4 and difference in Mean GFP of M4 between the Face with Direct Gaze (FD) and the Noise condition at T1 in relation to ASD diagnosis.

Predictors VABS Soc.	β	s.e.	F	p
(Intercept)	105.208	4.508	0.00	<0.001
Sex	0.701	2.322	0.09	0.763
Phase	-3.840	2.412	2.48	0.120
Mean GFP FD-Noise	20.946	9.919	4.43	0.039*
ASD	-20.778	2.932	49.42	<0.001*
Mean GFP x ASD	-42.563	29.139	2.13	0.148

VABS Soc.: Vineland Adaptive Behavior Scale standard scores in the Socialization domain; β: robust regression coefficient, s.e.: standard error, F: robust F-statistic, p: p-value; GFP: Global Field Power.
* p<0.05.

Table A2.18 Results of the robust linear regression testing the relationship between VABS Motor Skills scores at 36 months and difference in Mean GFP of M4 between the Face with Direct Gaze (FD) and the Noise condition in relation to ASD diagnosis.

Predictors VABS Mot.	β	s.e.	F	p
(Intercept)	100.736	4.308	0.00	0.000
Sex	4.861	2.227	4.77	0.032*
Phase	-6.342	2.307	7.43	0.008*
Mean GFP FD-Noise	10.845	9.448	1.34	0.250
ASD	-6.104	2.793	4.85	0.031*
Mean GFP x ASD	-23.782	27.755	0.74	0.393

VABS Mot.: Vineland Adaptive Behavior Scale standard scores in the Motor Skills domain; β: robust regression coefficient, s.e.: standard error, F: robust F-statistic, p: p-value; GFP: Global Field Power.
* p<0.05

Table A2.19 Results of the robust linear regression testing the relationship between VABS Socialization domain scores at 36 months and difference in duration of M4 between the Face with Direct Gaze (FD) and the Noise condition in relation to ASD diagnosis.

Predictors VABS Soc.	β	s.e.	F	p
(Intercept)	104.958	4.725	0.00	0.000
Sex	0.773	2.436	0.18	0.675
Phase	-3.076	2.463	41.64	<0.001*
Duration FD-Noise	-0.007	0.017	0.10	0.751
ASD	-21.082	3.220	1.53	0.220
Duration x ASD	0.034	0.035	0.91	0.343

VABS Soc.: Vineland Adaptive Behavior Scale standard scores in the Socialization domain; β: robust regression coefficient, s.e.: standard error, F: robust F-statistic (Wald test for multiple comparisons), p: p-value.
* p<0.05.

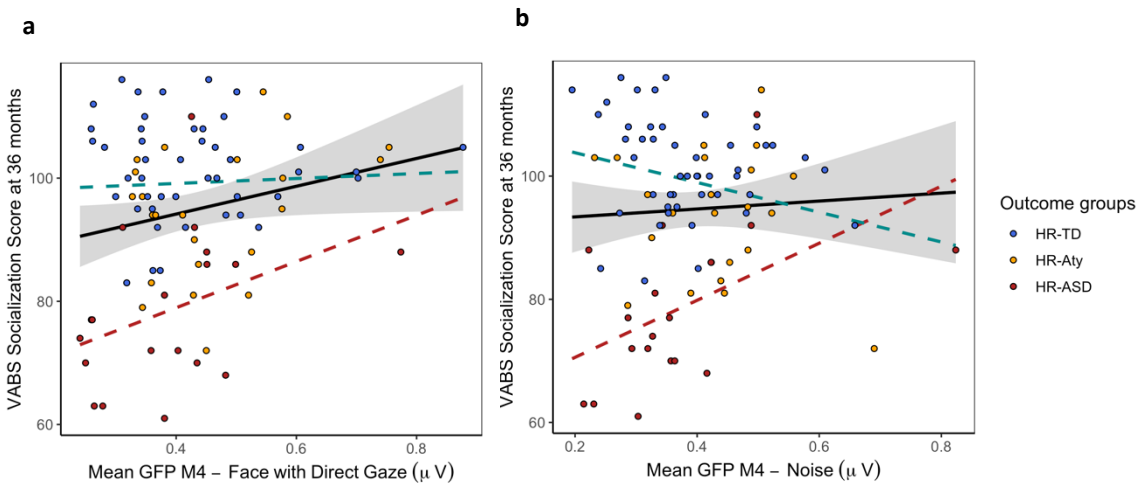


Figure A2.5 Scatterplot representing the relationship between M4 mean Global Field Power (GFP) in the Face with Direct Gaze (**a**) and Noise (**b**) conditions at 8 months (in microVolts) on the x-axis, and VABS Socialization domain standard scores at 36 months on the y-axis in the three high-risk groups. The regression line for the entire group of HR infants is displayed as a black line, with grey shadows representing standard errors. Cyan dashed lines depict the linear relationship between mean GFP and VABS socialization scores for the non-ASD groups (HR-TD and HR-Aty) while red dashed lines depict the linear relationship for the HR-ASD group.

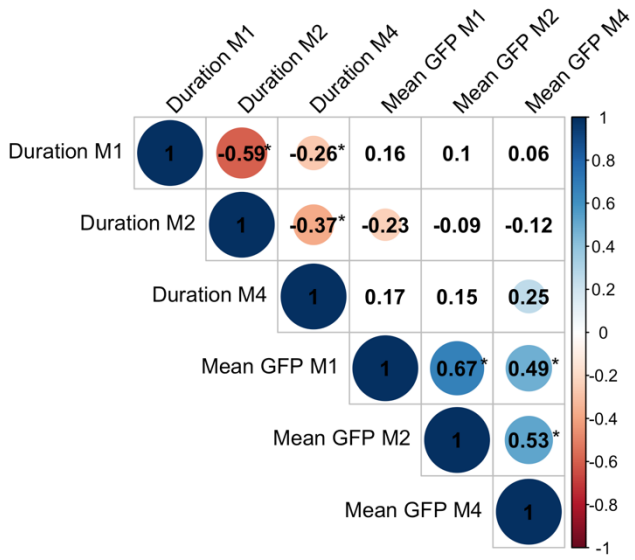


Figure A2.6 Pearson's correlation coefficients for associations between microstate features in response to Face with Direct Gaze in the entire study sample. Coloured circles highlight correlation coefficients for relationships that were statistically significant at a p-value <0.05, with red for negative correlation and blue for positive correlation. * indicates significant correlations with Bonferroni correction for multiple testing (α=0.05/15=0.003).

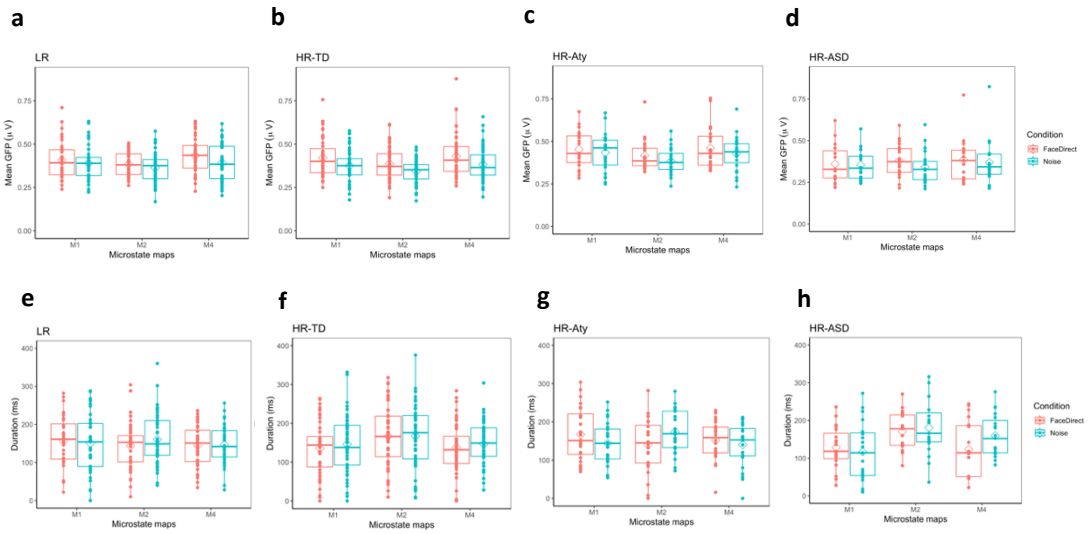


Figure A2.7 Boxplots indicating Global Field Power (GFP, in microVolts) and duration (in milliseconds) for microstates 1, 2 and 4 for the four outcome groups: **a** and **e** represent the microstates mean GFP and duration, respectively, for the LR infants, **b** and **f** for the HR-TD infants, **c** and **g** for the HR-Aty infants, and **d** and **h** for HR-ASD infants.

Table A2.20 Results of the Multivariate ANalysis Of VAriance (MANOVA) testing the main effect of condition (FD and Noise) on mean Global Field Power (GFP) of microstates 1, 2 and 4 in the LR group.

LR group: MANOVA mean GFP FD vs. Noise		**d.f.**	**F**	**p**	**η^2**
Between-subjects effects	**Intercept**	1	24.309	<0.001	0.410
	Age	1	0.080	0.778	0.002
	Phase	1	0.572	0.455	0.016
	Sex	1	0.695	0.410	0.019
	Residuals	35			
Within-subjects multivariate effects	**Condition**	1, 35	1.672	0.204	0.046
	Microstate	2, 34	4.392	0.020*	0.205

LR: low-risk infants; GFP: global field power; FD: Face with Direct Gaze; FD: Face with Direct Gaze; d.f: degrees of freedom; F: F-statistic; p: p-value; η^2: partial eta-squared.
* p<0.05.

Table A2.21 Results of the Multivariate ANalysis Of VAriance (MANOVA) testing the main effect of condition (FD and Noise) on duration of microstates 1, 2 and 4 in the LR group.

LR group: MANOVA duration FD vs. Noise		d.f.	F	p	η^2
Between-subjects effects	**Intercept**	1	127.694	<0.001	0.780
	Age	1	3.518	0.069	0.089
	Phase	1	0.569	0.456	0.016
	Sex	1	0.609	0.440	0.017
	Residuals	36			
Within-subjects multivariate effects	**Condition**	1, 36	0.249	0.621	0.007
	Microstate	2, 35	3.101	0.058	0.151

LR: Low-Risk infants; FD: Face with Direct Gaze; FD: Face with Direct Gaze; d.f: degrees of freedom; F: F-statistic; p: p-value; η^2: partial eta-squared.
* p<0.05.

Table A2.22 Results of the Multivariate ANalysis Of VAriance (MANOVA) testing the main effects and interaction between condition (FD and Noise) and the three outcome groups (HR-TD, HR-Aty, HR-ASD) on mean GFP of microstates 1, 2 and 4 in the HR group.

HR group: MANOVA mean GFP FD vs. Noise		d.f.	F	p	η^2
Between-subjects effects	**Intercept**	1	28.772	<0.001	0.265
	Outcome	2	2.374	0.100	0.056
	Age	1	0.103	0.749	0.001
	Phase	1	2.883	0.093	0.035
	Sex	1	0.830	0.365	0.010
	Residuals	80			
Within-subjects multivariate effects	**Condition**	1, 80	5.938	0.017*	0.069
	Condition x Outcome	2, 80	0.882	0.418	0.022
	Microstate	2, 79	1.700	0.189	0.041
	Microstate x Outcome	4, 158	1.755	0.141	0.043

HR: High-Risk infants; GFP: global field power; FD: Face with Direct Gaze; FD: Face with Direct Gaze; d.f: degrees of freedom; F: F-statistic; p: p-value; η^2: partial eta-squared.
* p<0.05.

Table A2.23 Results of the Multivariate ANalysis Of VAriance (MANOVA) testing the main effects and interaction between condition (FD and Noise) and the four outcome groups (LR, HR-TD, HR-Aty, HR-ASD) on duration of microstates 1, 2 and 4 in the HR group.

HR group: MANOVA duration FD vs. Noise		d.f.	F	p	η^2
Between-subjects effects	**Intercept**	1	261.095	<0.001	0.754
	Outcome	2	1.675	0.193	0.038
	Age	1	2.939	0.090	0.033
	Phase	1	0.376	0.541	0.004
	Sex	1	1.005	0.319	0.012
	Residuals	85			
Within-subjects multivariate effects	**Condition**	1, 85	5.938	0.424	0.008
	Condition x Outcome	2, 85	0.882	0.061	0.064
	Microstate	2, 84	1.700	0.001*	0.150
	Microstate x Outcome	4, 168	1.755	0.714	0.023

HR: High-Risk infants; FD: Face with Direct Gaze, d.f: degrees of freedom; F: F-statistic; p: p-value; η^2: partial eta-squared.
* p<0.05.

APPENDIX CHAPTER 3

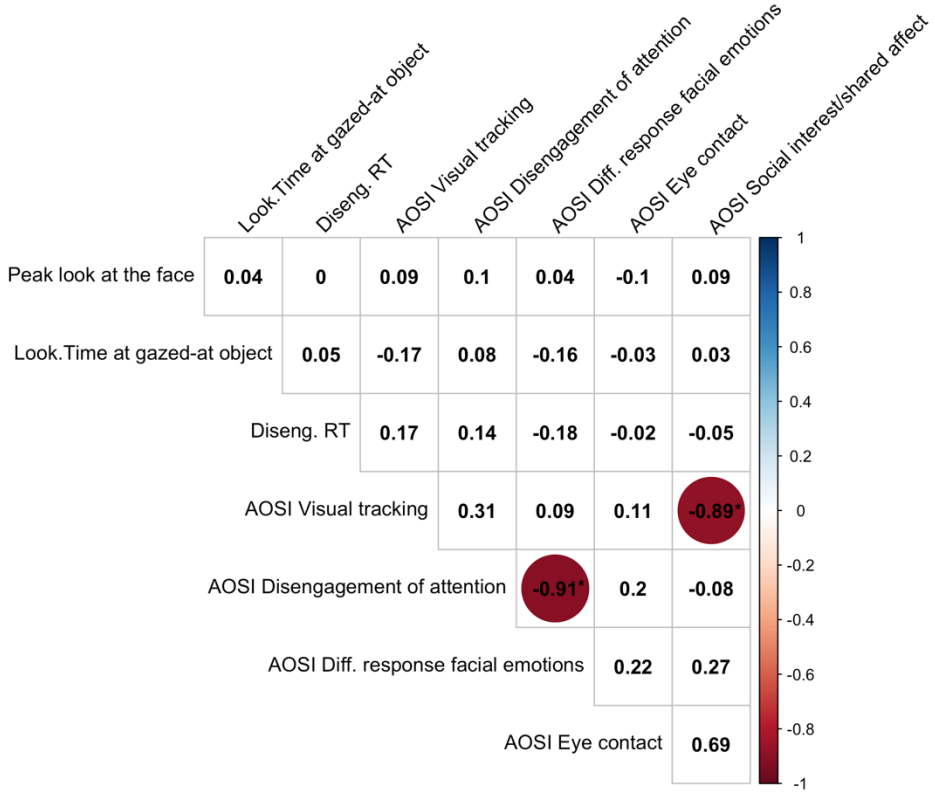


Figure A3.1 Polychoric correlation coefficients for associations between measures of experimental measures of looking behavior at 14 months (Peak look at the face: peak look duration at the face in the face pop-out task; Look. time at gazed-at object: proportion of looking time at the gazed-at object in the gaze following task, Diseng. RT: disengagement reaction times in the gap-overlap task) and observational measures of visual attention and social engagement selected from the Autism Observation Scale for Infants (AOSI Visual tracking, Disengagement of attention, Differential response to facial emotions, Eye-contact, Social interest and shared affect). Coloured circles highlight correlation coefficients for relationships that were statistically significant at a p-value<0.05, with red indicating a negative correlation. * indicates significant correlations with Bonferroni correction for multiple testing (α = 0 .05/28 = 0.001).

Table A3.1 Number of missing data and results of the Shapiro-Wilk tests assessing normality of the distribution for the observed variables used in the SEMs.

Visit	Variable	N missing	W	p
T1	Nc mean amplitude	116	0.968	0.003*
	Nc peak latency	116	0.962	0.001*
	Microstate 4 Global Field Power	118	0.98	0.052+
	Microstate 4 duration	117	0.923	<0.0001*
T2	Peak look at the face	37	0.899	<0.0001*
	Looking time at the gazed object	54	0.908	<0.0001*
	Disengagement RT	15	0.965	<0.0001*
T3	Effortful Control (ECBQ)	32	0.972	0.0003*
T4	Social adaptive skills (VABS)	14	0.953	<0.0001*
	Social Communication Impairment (SRS)	5	0.31	<0.0001*
	Restricted Repetitive Behavior (SRS)	5	0.311	<0.0001*
	Receptive Language (MSEL)	10	0.969	<0.0001*
	Expressive Language (MSEL)	10	0.977	0.0007*

N: number of participants; W: Shapiro-Wilk statistics; p: p-value; RT: reaction time; ECBQ: Early Childhood Behavior Questionnaire; VABS: Vineland Adaptive Behavior Scales; SRS: Social Responsiveness Scale; MSEL: Mullen Scales of Early Learning.
* $p<0.05$.
+ $p<0.1$.

Table A3.2 Summary of SEM results with adaptive social behaviour (VABS Socialization domain standard score) as outcome variable at T4. Unstandardised beta coefficients (β), robust standard errors (s.e.), z-values, p-values and standardised beta coefficients (St.β) are reported for each path. For latent variable and regression paths, predictors are reported in the second column.

Latent variable		**β**	**s.e.**	**z**	**p**	**St. β**
Attentive brain state	Ms duration	0.841	0.203	4.138	<0.001*	0.977
Attentive brain state	Ms mean GFP	0.127	0.104	1.227	0.22	0.151
Attentive brain state	Nc peak lat.	0.021	0.076	0.271	0.786	0.024
Attentive brain state	Nc mean amp.	-0.322	0.067	-4.777	<0.001*	-0.38
Regressions		**β**	**s.e.**	**z**	**p**	**St. β**
Adaptive social behaviour	Attentive brain state	0.186	0.071	2.602	0.009*	0.219
Adaptive social behaviour	Peak look at the face	-0.017	0.075	-0.222	0.824	-0.017
Adaptive social behaviour	Look. time at gazed-at object	0.189	0.064	2.976	0.003*	0.189
Adaptive social behaviour	Disengagement RT	-0.09	0.063	-1.435	0.151	-0.09
Adaptive social behaviour	Risk group	-0.661	0.128	-5.146	<0.001*	-0.661
Peak look at the face	Attentive brain state	0.1	0.115	0.869	0.385	0.118
Peak look at the face	Phase	0.038	0.214	0.178	0.859	0.038
Peak look at the face	Risk group	0.56	0.167	3.348	0.001*	0.56
Look. time at gazed-at object	Attentive brain state	0.145	0.076	1.917	0.055+	0.172
Look. time at gazed-at object	Phase	-1.392	0.165	-8.418	<0.001*	-1.392
Look. time at gazed-at object	Risk group	0.338	0.151	2.239	0.025*	0.338
Disengagement RT	Attentive brain state	-0.102	0.073	-1.399	0.162	-0.12
Disengagement RT	Phase	0.23	0.176	1.306	0.192	0.23
Disengagement RT	Risk group	0.008	0.165	0.051	0.959	0.008
Attentive brain state	Phase	1.215	0.264	4.61	<0.001*	1.03
Attentive brain state	Risk group	-0.953	0.25	-3.811	<0.001*	-0.808
Covariances		**β**	**s.e.**	**z**	**p**	**St. β**
Nc mean amp.	Nc peak lat.	-0.28	0.075	-3.724	<0.001*	-0.28
Look. time at gazed-at object	Disengagement RT	-0.13	0.063	-2.055	0.04*	-0.13
Peak look at the face	Look. time at gazed-at object	-0.031	0.06	-0.514	0.607	-0.031
Peak look at the face	Disengagement RT	-0.029	0.077	-0.373	0.709	-0.029

GFP: Global Field Power; lat.: latency, amp.: amplitude, Look. time: proportion of looking time, RT: Reaction Times.
* p<0.05.
+ p<0.1.

Table A3.3 Summary of SEM results with autistic traits (Social Communication Impairment, SCI, and Restricted and Repetitive Behaviours, RRB) as outcome variable at T4. Unstandardised beta coefficients (β), robust standard errors (s.e.), z-values, p-values and standardised beta coefficients (St.β) are reported for each path. For latent variable and regression paths, predictors are reported in the second column.

Latent variable		**β**	**s.e.**	**z**	**p**	**St. β**
Attentive brain state	Ms duration	0.802	0.243	3.298	0.001*	0.944
Attentive brain state	Ms mean GFP	0.134	0.096	1.4	0.161	0.161
Attentive brain state	Nc peak lat.	0.013	0.082	0.156	0.876	0.015
Attentive brain state	Nc mean amp.	-0.324	0.062	-5.232	<0.001*	-0.388
Regressions		**β**	**s.e.**	**z**	**p**	**St. β**
SCI	Attentive brain state	0.096	0.09	1.069	0.285	0.115
SCI	Peak look at the face	-0.066	0.071	-0.924	0.355	-0.066
SCI	Look. time at gazed-at object	-0.152	0.064	-2.371	0.018*	-0.152
SCI	Disengagement RT	-0.034	0.058	-0.591	0.554	-0.034
SCI	Risk group	0.258	0.142	1.818	0.069+	0.258
RRB	Attentive brain state	0.097	0.089	1.089	0.276	0.116
RRB	Peak look at the face	-0.063	0.072	-0.877	0.381	-0.063
RRB	Look. time at gazed-at object	-0.156	0.064	-2.428	0.015*	-0.156
RRB	Disengagement RT	-0.034	0.058	-0.591	0.554	-0.034
RRB	Risk group	0.257	0.142	1.808	0.071	0.257
Peak look at the face	Attentive brain state	0.126	0.123	1.025	0.305	0.15
Peak look at the face	Phase	0.006	0.229	0.027	0.979	0.006
Peak look at the face	Risk group	0.576	0.175	3.298	0.001*	0.576
Look. time at gazed-at object	Attentive brain state	0.154	0.078	1.985	0.047*	0.184
Look. time at gazed-at object	Phase	-1.415	0.175	-8.082	<0.001*	-1.415
Look. time at gazed-at object	Risk group	0.349	0.159	2.189	0.029*	0.349
Disengagement RT	Attentive brain state	-0.093	0.08	-1.165	0.244	-0.112
Disengagement RT	Phase	0.219	0.193	1.136	0.256	0.219
Disengagement RT	Risk group	0.018	0.173	0.104	0.917	0.018
Attentive brain state	Phase	1.282	0.373	3.439	0.001*	1.072
Attentive brain state	Risk group	-0.993	0.316	-3.142	0.002*	-0.83
Covariances		**β**	**s.e.**	**z**	**p**	**St. β**
Nc mean amp.	Nc peak lat.	-0.284	0.076	-3.74	<0.001*	-0.284
SCI	RRB	0.945	0.192	4.919	<0.001*	0.945
Look. time at gazed-at object	Disengagement RT	-0.129	0.064	-2.028	0.043*	-0.129
Peak look at the face	Look. time at gazed-at object	-0.036	0.061	-0.603	0.547	-0.036
Peak look at the face	Disengagement RT	-0.026	0.076	-0.339	0.735	-0.026

GFP: Global Field Power; lat.: latency, amp.: amplitude, SCI: Social Communication Impairment; RRB: Restricted and Repetitive Behaviours; Look. time: proportion of looking time, RT: Reaction Times.
* p<0.05.
+ p<0.1.

Table A3.4 Summary of SEM results with language skills (MSEL receptive and expressive language) as outcome variables at T4. Unstandardised beta coefficients (β), robust standard errors (s.e.), z-values, p-values and standardised beta coefficients (St.β) are reported for each path. For latent variable and regression paths, predictors are reported in the second column.

Latent variable		**β**	**s.e.**	**z**	**p**	**St. β**
Attentive brain state	Ms duration	0.866	0.207	4.194	<0.001*	0.993
Attentive brain state	Ms mean GFP	0.123	0.084	1.465	0.143	0.144
Attentive brain state	Nc peak lat.	0.017	0.075	0.227	0.82	0.02
Attentive brain state	Nc mean amp.	-0.321	0.068	-4.695	<0.001*	-0.376
Regressions		**β**	**s.e.**	**z**	**p**	**St. β**
Rec. language	Attentive brain state	0.218	0.096	2.271	0.023*	0.255
Rec. language	Peak look at the face	-0.184	0.067	-2.771	0.006*	-0.184
Rec. language	Look. time at gazed-at object	0.18	0.097	1.868	0.062+	0.18
Rec. language	Disengagement RT	-0.047	0.059	-0.791	0.429	-0.047
Rec. language	Risk group	-0.319	0.181	-1.758	0.079+	-0.319
Rec. language	Phase	-0.056	0.235	-0.236	0.813	-0.056
Expr. language	Attentive brain state	0.227	0.105	2.167	0.03*	0.266
Expr. language	Peak look at the face	-0.144	0.077	-1.881	0.06+	-0.144
Expr. language	Look. time at gazed-at object	0.14	0.087	1.603	0.109	0.14
Expr. language	Disengagement RT	-0.096	0.055	-1.733	0.083+	-0.096
Expr. language	Risk group	-0.298	0.184	-1.621	0.105	-0.298
Expr. language	Phase	-0.154	0.249	-0.618	0.537	-0.154
Peak look at the face	Attentive brain state	0.116	0.109	1.066	0.287	0.136
Peak look at the face	Phase	0.021	0.204	0.101	0.919	0.021
Peak look at the face	Risk group	0.572	0.164	3.487	<0.001*	0.572
Look. time at gazed-at object	Attentive brain state	0.163	0.069	2.367	0.018*	0.191
Look. time at gazed-at object	Phase	-1.404	0.171	-8.214	<0.001*	-1.404
Look. time at gazed-at object	Risk group	0.35	0.153	2.28	0.023*	0.35
Disengagement RT	Attentive brain state	-0.114	0.071	-1.597	0.11	-0.134
Disengagement RT	Phase	0.238	0.18	1.327	0.185	0.238
Disengagement RT	Risk group	0.003	0.166	0.015	0.988	0.003
Attentive brain state	Phase	1.176	0.279	4.215	<0.001*	1.005
Attentive brain state	Risk group	-0.943	0.255	-3.697	<0.001*	-0.805
Covariances		**β**	**s.e.**	**z**	**p**	**St. β**
Nc mean amp.	Nc peak lat.	-0.282	0.075	-3.755	<0.001*	-0.282
Rec. language	Expr. language	0.584	0.069	8.404	<0.001*	0.584
Look. time at gazed-at object	Disengagement RT	-0.131	0.063	-2.075	0.038*	-0.131
Peak look at the face	Look. time at gazed-at object	-0.023	0.061	-0.385	0.7	-0.023
Peak look at the face	Disengagement RT	-0.032	0.076	-0.426	0.67	-0.032

GFP: Global Field Power; lat.: latency, amp.: amplitude, Rec. language: receptive language; Expr. language: expressive language; Look. time: proportion of looking time, RT: Reaction Times.
* p<0.05.
+ p<0.1.

Table A3.5 Summary of SEM results with Effortful Control (ECBQ)as outcome variables at T3. Unstandardised beta coefficients (β), robust standard errors (s.e.), z-values, p-values and standardised beta coefficients (St.β) are reported for each path. For latent variable and regression paths, predictors are reported in the second column.

Latent variable		**β**	**s.e.**	**z**	**p**	**St. β**
Attentive brain state	Ms duration	0.867	0.275	3.153	0.002*	0.999
Attentive brain state	Ms mean GFP	0.121	0.11	1.096	0.273	0.142
Attentive brain state	Nc peak lat.	0.019	0.08	0.233	0.815	0.022
Attentive brain state	Nc mean amp.	-0.318	0.066	-4.813	<0.001*	-0.373
Regressions		**β**	**s.e.**	**z**	**p**	**St. β**
Effortful Control	Attentive brain state	0.138	0.077	1.79	0.073+	0.161
Effortful Control	Peak look at the face	-0.068	0.088	-0.777	0.437	-0.068
Effortful Control	Look. time at gazed-at object	0.121	0.077	1.567	0.117	0.121
Effortful Control	Disengagement RT	-0.021	0.067	-0.317	0.751	-0.021
Effortful Control	Risk group	-0.323	0.154	-2.096	0.036*	-0.323
Peak look at the face	Attentive brain state	0.116	0.112	1.031	0.303	0.136
Peak look at the face	Phase	0.016	0.208	0.077	0.939	0.016
Peak look at the face	Risk group	0.575	0.166	3.461	0.001*	0.575
Look. time at gazed-at object	Attentive brain state	0.147	0.071	2.063	0.039*	0.172
Look. time at gazed-at object	Phase	-1.383	0.17	-8.138	<0.001*	-1.383
Look. time at gazed-at object	Risk group	0.324	0.153	2.121	0.034	0.324
Disengagement RT	Attentive brain state	-0.08	0.069	-1.155	0.248	-0.094
Disengagement RT	Phase	0.192	0.175	1.097	0.272	0.192
Disengagement RT	Risk group	0.036	0.163	0.221	0.825	0.036
Attentive brain state	Phase	1.189	0.353	3.368	0.001*	1.014
Attentive brain state	Risk group	-0.945	0.31	-3.049	0.002*	-0.805
Covariances		**β**	**s.e.**	**z**	**p**	**St. β**
Nc mean amp.	Nc mean amp.	-0.281	0.075	-3.751	<0.001*	-0.281
Look. time at gazed-at object	Disengagement RT	-0.135	0.064	-2.124	0.034*	-0.135
Peak look at the face	Look. time at gazed-at object	-0.025	0.059	-0.426	0.67	-0.025
Peak look at the face	Disengagement RT	-0.032	0.077	-0.412	0.68	-0.032

GFP: Global Field Power; lat.: latency, amp.: amplitude; Look. time: proportion of looking time, RT: Reaction Times.
* p<0.05.
+ p<0.1.

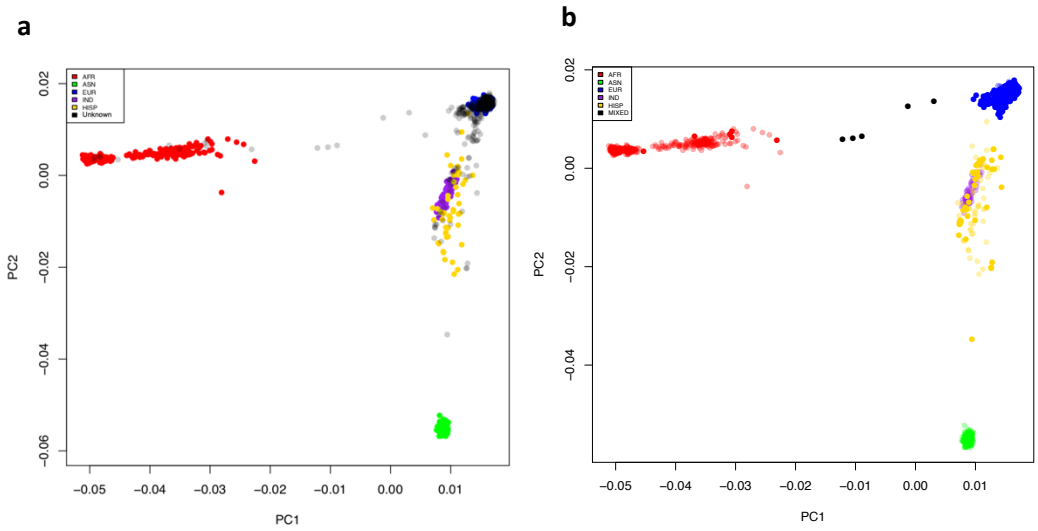


Figure A4.1 Illustration of the first two principal components extracted from a combined sample obtained by merging 717 gBASIS samples for the current study and 1,115 samples of various ancestries from the HapMap 3 reference panel (The International HapMap 3 Consortium, 2010). **a** Dots indicating HapMap 3 samples are colour-coded based on their know population, while transparent-black dots indicate the 717 samples of unknown ancestry. **b** Transparent coloured dots indicate the HapMap3 samples while solid dots indicate the previously unknown gBASIS samples colour-coded based on the new ancestry assignment. In both plots, red indicates Africans (AFR), green Asians (ASN), blue Europeans (EUR), purple Indians (IND), yellow Hispanics (HISP) and black unclassified because unknown (a) or mixed (b).

HapMap 3 population definitions: AFR includes: African ancestry in Southwest USA, Maasai in Kinyawa, Kenya, Yoruba in Ibadan, Nigeria; Luhya in Webuye, Kenya. EUR includes: Utah residents with Northern and Western European ancestry from the CEPH collection, Toscani in Italia; IND includes: Han Chinese in Beijing, China; Chinese in Metropolitan Denver, Colorado; Gujarati Indians in Houston, Texas; Japanese in Tokyo, Japan; HISP includes: Mexican ancestry in Los Angeles, California. Babysibs – NA indicates

Table A4.1 Number of gBASIS samples by ancestry after HapMap 3-based ancestry assignment. Samples were assigned to one of the following populations: Africans (AFR), Europeans (EUR), Hispanics (HISP), Mixed and non-assigned (NA). Of the NAs individuals, 2 reported "Hispanic" origins, 1 "White mixed", 1 "Arab Iraqui", 1 "British/Indian", 1 "British/Pakistani", 1 "Kurdish", 1 "Hungarian", 1 "Spanish", 6 were children of one European parent and one Hispanic parent, 4 defined themselves or their children as white "British" or "English".

Ancestry label	N
AFR	11
EUR	642
HISP	40
MIXED	5
NA	19
Total	**717**

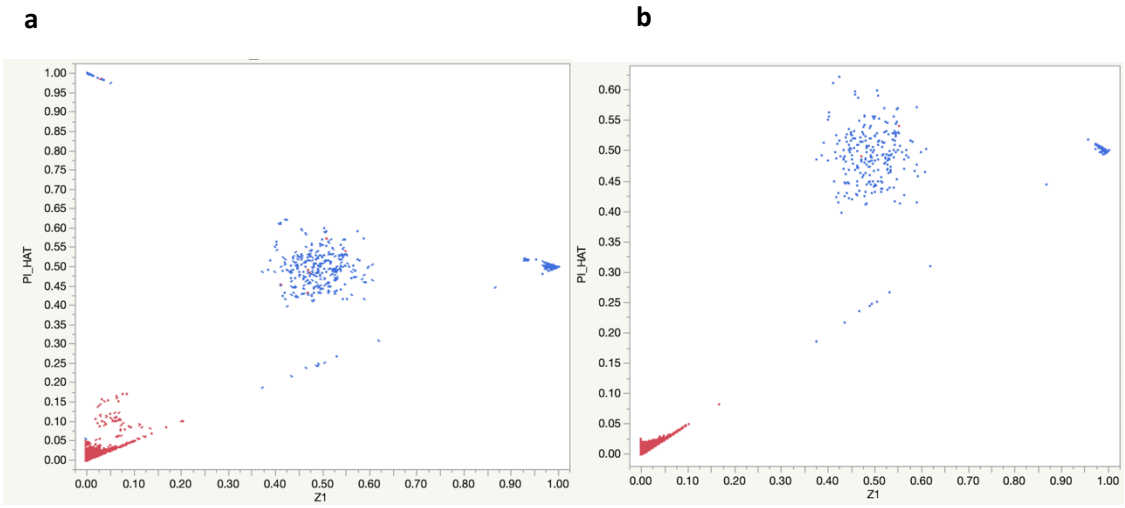


Figure A4.2 Graphical representation of pihat and z1 estimates for each pair of samples resulting from Identity-By-Descent (IBD) analysis. **a** Results of the IBD analysis performed on 717 imputed quality-checked gBASIS samples. Pairs with pihat =~ 1 and z1 =~ 0 are expected to be duplicates. Red dots represent pairs of individuals classified as unrelated from their family IDs while blue dots are expected to be related as they have the same family ID. Red dots in the cluster of pairs considered related through a full-sibling relationship (pihat =~ 0.5, z1 =~ 0.5) are likely due to mistakes in ID assignment. **b** Results of the IBD analysis performed on the 579 samples of European ancestry from which duplicates and samples with mistaken ID assignment were removed. No pairs had pihat =~ 1 and z1 =~ 0, indicating that there were no duplicates in this sample. Two red dots remained in the cluster of full siblings. Manual inspection of the data revealed that these represent a family ID mis-assignment related to the fact that one family participating in Phase 2 enrolled the youngest child in a subsequent Phase of the study (STAARS, see section 5.2.1), and this individual was assigned to a new family number. The two red dots represent misclassified relationships with the two older siblings (the proband and the Phase 2 BASIS participant), which are defined as unrelated based on the fact that they do not share the same ID.

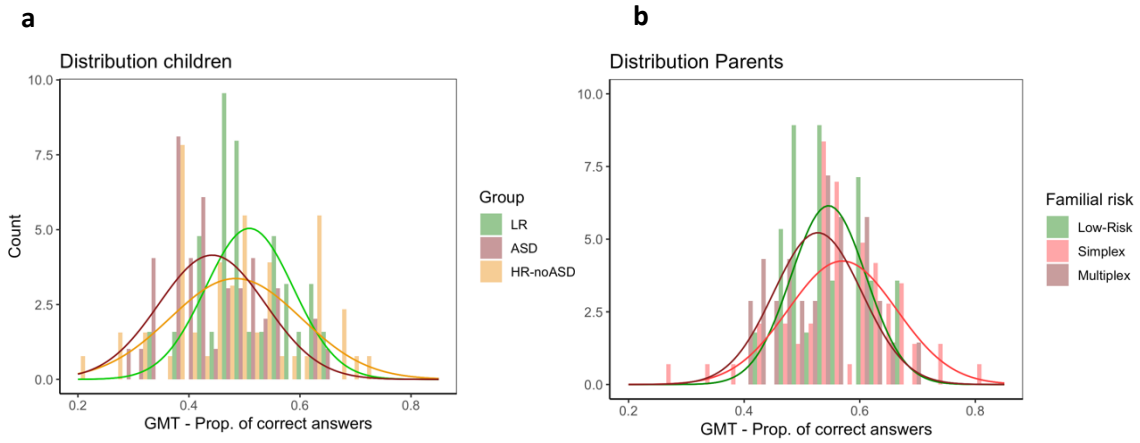


Figure A4.3 Histograms representing the distribution of the proportion of correct answers in the Gaze Monitoring Task for children (**a**) and parents (**b**).

Table A4.2 Results of the general linear model testing the relationship between performance at the Gaze Monitoring Task (GMT) measured as proportion of correct answers and Social Communication Impairment domain t-score of the Social Responsiveness Scale (SRS SCI) in children.

Predictors GMT children	β	s.e.	t	p
(Intercept)	0.477	0.05	9.40	<0.001
SRS SCI	-0.003	0.00	-3.53	0.001*
Sex	0.030	0.06	0.46	0.644
Age	0.013	0.00	3.22	0.002*
Parent Help	-0.008	0.02	-0.37	0.712
SRS SCI x Sex	0.000	0.00	-0.08	0.935

β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value.
* p<0.05.

Table A4.3 Results of the general linear model testing the relationship between performance at the Gaze Monitoring Task (GMT) measured as proportion of correct answers and Restricted and Repetitive Behaviours domain t-score of the Social Responsiveness Scale (SRS RRB) in children.

Predictors GMT children	β	s.e.	t	p
(Intercept)	0.453	0.05	8.88	<0.001
SRS RRB	-0.002	0.001	-2.933	0.004*
Sex	0.078	0.066	1.169	0.245
Age	0.012	0.004	3.021	0.003*
Parent Help	-0.008	0.023	-0.356	0.723
SRS RRB x Sex	-0.001	<0.001	-0.84	0.402

β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value.
* p<0.05.

Table A4.4 Results of the general linear model testing the relationship between performance at the Gaze Monitoring Task (GMT) measured as proportion of correct answers and the Social Communication Impairment domain t-score of the Social Responsiveness Scale (SRS SCI) in parents.

Predictors GMT parents	β	s.e.	t	p
(Intercept)	0.721	0.085	8.508	<0.001
SRS SCI	-0.003	0.002	-2.001	0.049*
Sex	-0.306	0.105	-2.906	0.005*
SRS SCI x Sex	0.006	0.002	2.965	0.004*

β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value.
* p<0.05.

Table A4.5 Results of the general linear model testing the relationship between performance at the Gaze Monitoring Task (GMT) measured as proportion of correct answers and the Restricted and Repetitive Behaviours domain t-scores of the Social Responsiveness Scale (SRS RRB) in parents.

Predictors GMT parents	β	s.e.	t	p
(Intercept)	0.709	0.078	9.031	<0.001
SRS RRB	-0.003	0.002	-2.005	0.048*
Sex	-0.309	0.108	-2.869	0.005*
SRS RRB x Sex	0.006	0.002	2.905	0.005*

β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value.
* p<0.05.

Table A4.6 Comparison between multilevel mixed-effects models with proportion of correct answers at the Gaze Monitoring Test (GMT) as dependent variable. The baseline model, with family as random effect, was compared with updated models testing the fixed effect of predictors such as age when the GMT was complete, ASD polygenic score (PGS), familial risk (multiplex, simplex, low-risk), interaction between PGS and familial risk and a binary variable indicating whether valid GMT data were obtained from all family members or for some of them (completeness fam. GMT).

Models	d.f.	AIC	BIC	Log.Lik.	χ^2	p
Prop. Correct GMT	3	-351.79	-341.78	178.90		
Age	4	-398.64	-385.29	203.32	48.844	<.0001
PGS	5	-397.54	-380.85	203.77	0.906	0.341
Familial risk	7	-400.83	-377.46	207.41	7.285	0.026*
PGS x Familial risk	9	-400.42	-370.38	209.21	3.589	0.166
Completeness fam. GMT	10	-402.90	-369.53	211.45	4.486	0.034*

d.f.: degrees of freedom; AIC: Akaike information criterion; BIC: Bayesian information criterion; Log.Lik.: log likelihood; χ^2: chi-square statistic; p: p-value.
* p<0.05.

Table A4.7 Results of the higher-order significant model (see Table A4.6) testing the relationship between proportion of correct answers at the Gaze Monitoring Test (GMT) and ASD polygenic score by familial risk.

Predictors GMT	β	s.e.	t	p
(Intercept)	0.641	0.128	4.998	<0.001*
Age	0.003	0.000	7.338	<0.001*
PGS	99.204	59.426	1.669	0.097
mHR vs. LR	0.100	0.245	0.407	0.686
mHR vs. sHR	-0.171	0.158	-1.082	0.284
Completeness fam. GMT	-0.028	0.014	-2.088	0.041*
PGS mHR vs. LR	27.450	113.040	0.243	0.809
PGS mHR vs. sHR	-97.343	73.730	-1.320	0.189

β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value; PGS: polygenic score for ASD; mHR: multiplex families; sHR: simplex families; LR: low-risk families; completeness fam. GMT: binary variable indicating whether valid SRS data were obtained from all family members or for some of them.
* p<0.05.

Table A4.8 Comparison between multilevel mixed-effects models with the Social Communication Impairment domain t-score of the Social Responsiveness Scale (SRS SCI) as dependent variable. The baseline model, with family as random effect, was compared with updated models testing the fixed effect of predictors such as ASD polygenic score (PGS), familial risk (multiplex, simplex, low-risk), interaction between PGS and familial risk and a binary variable indicating whether valid SRS data were obtained from all family members or for some of them (completeness fam. SRS).

Models	d.f.	AIC	BIC	Log.Lik.	χ^2	p
SRS SCI	3	1880.216	1890.383	-937.108		
PGS	4	1877.570	1891.126	-934.785	4.647	0.031*
Familial risk	6	1852.210	1872.545	-920.105	29.359	<.0001*
PGS x Familial risk	8	1855.359	1882.472	-919.680	0.851	0.653
Completeness fam. SRS	9	1856.778	1887.280	-919.389	0.581	0.446

d.f.: degrees of freedom; AIC: Akaike information criterion; BIC: Bayesian information criterion; Log.Lik.: log likelihood; χ^2: chi-square statistic; p: p-value.
* p<0.05.

Table A4.9 Results of the higher-order significant model (Table A4.8) testing the relationship between Social Communication Impairment domain t-score of the Social Responsiveness Scale (SRS SCI) and ASD polygenic score while controlling for familial risk.

Predictors SRS SCI	β	s.e.	t	p
(Intercept)	89.825	11.424	7.863	<0.001
PGS	6684.803	5416.005	1.234	0.219
mHR vs. LR	-24.163	2.703	-8.940	<0.001*
mHR vs. sHR	-28.767	2.561	-11.234	<0.001*

β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value; PGS: polygenic score for ASD; mHR: multiplex families; LR: low-risk families; sHR: simplex families.
* p<0.05.

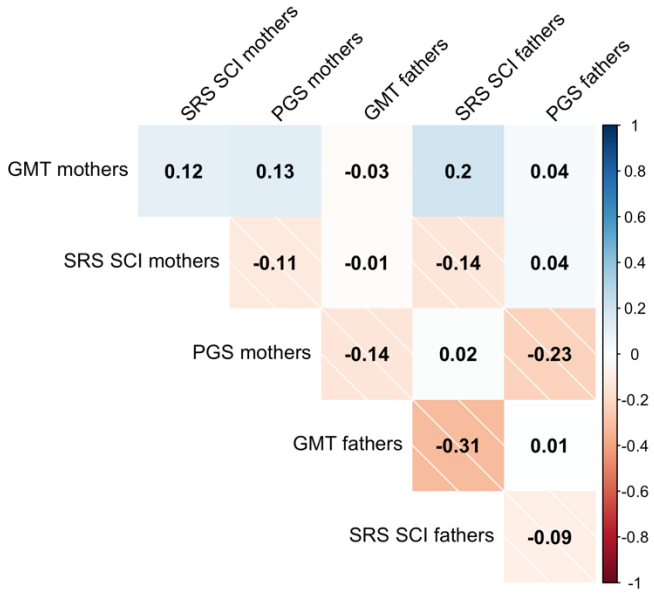


Figure A4.4 Correlation coefficients for associations between Gaze Monitoring Test (GMT), social difficulties measured as the Social Communication Impairment domain t-score of the Social Responsiveness Scale (SRS SCI) and ASD polygenic score (PGS), in 23 couples of mothers and fathers. Blue indicates a positive correlation while striped red indicates a negative correlation. No correlation reached significance at a nominal p-value threshold of $p<0.05$.

Table A4.10 Results of the three linear regressions testing the relationship between infants' Nc mean amplitude difference between Face with Direct Gaze and Noise stimulus and their parents' social attention measured as the proportion of correct answers at the Gaze Monitoring Test (GMT), social difficulties measured as the Social Communication Impairment domain t-score of the Social Responsiveness Scale (SRS SCI) and ASD polygenic score (PGS), respectively.

	Predictors Nc mean amp.	N	β	s.e.	t	p	Adj. p
Mother	**GMT**	39	-9.022	12.479	-0.723	0.474	0.949
	SRS SCI	30	0.041	0.167	0.245	0.809	0.965
	PGS	54	-5349.38	4583.89	-1.167	0.249	0.949
Father	**GMT**	29	1.940	18.703	0.104	0.918	0.965
	SRS SCI	24	0.006	0.127	0.044	0.965	0.965
	PGS	47	5243.46	5617.84	0.933	0.356	0.949

N: number of observations included in the analysis, β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value, Adj. p: p-value corrected for multiple testing (three tests for each of the two parents' measures) using False Discovery Rate method.

Table A4.11 Results of the three linear regressions testing the relationship between infants' microstate (Ms) duration difference between Face with Direct Gaze and Noise stimulus and their parents' social attention measured as the proportion of correct answers at the Gaze Monitoring Test (GMT), social difficulties measured as the Social Communication Impairment domain t-score of the Social Responsiveness Scale (SRS SCI) and ASD polygenic score (PGS), respectively.

Predictors Ms duration		N	β	s.e.	t	p	Adj. p
Mother	GMT	39	6.556	21.685	0.302	0.764	0.917
	SRS SCI	30	-0.664	0.518	-1.283	0.210	0.917
	PGS	54	-4715	12112	-0.389	0.699	0.917
Father	GMT	29	-38.601	58.142	-0.664	0.513	0.917
	SRS SCI	24	0.253	0.408	0.619	0.543	0.917
	PGS	47	679.00	15130	0.045	0.964	0.964

N: number of observations included in the analysis, β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value, Adj. p: p-value corrected for multiple testing (three tests for each of the three family members' measures) using False Discovery Rate method.

Table A4.12 Results of the three linear regressions testing the relationship between infants' peak look duration at the face in a face pop-out task and their parents' social attention measured as the proportion of correct answers at the Gaze Monitoring Test (GMT), social difficulties measured as the Social Communication Impairment domain t-score of the Social Responsiveness Scale (SRS SCI) and ASD polygenic score (PGS), respectively.

Predictors Peak look at the face		N	β	s.e.	t	p	Adj. p
Mother	GMT	52	2155.94	1166.73	1.848	0.071	0.308
	SRS SCI	41	-5.627	10.527	-0.535	0.596	0.715
	PGS	78	-435152	39,7139	-1.096	0.277	0.415
Father	GMT	40	2724.02	1639.88	1.661	0.105	0.308
	SRS SCI	34	18.923	12.950	1.461	0.154	0.308
	PGS	68	-82665	512241	-0.161	0.872	0.872

N: number of observations included in the analysis, β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value, Adj. p: p-value corrected for multiple testing (three tests for each of the three family members' measures) using False Discovery Rate method.

Table A4.13 Results of the three linear regressions testing the relationship between infants' looking time at the gazed-at object in a gaze following task and their parents' social attention measured as the proportion of correct answers at the Gaze Monitoring Test (GMT), social difficulties measured as the Social Communication Impairment domain t-score of the Social Responsiveness Scale (SRS SCI) and ASD polygenic score (PGS), respectively.

Predictors Look. time at the gazed-at object		N	β	s.e.	t	p	Adj. p
Mother	GMT	47	0.153	0.136	1.121	0.268	0.403
	SRS SCI	34	0.003	0.001	2.372	0.024*	0.144
	PGS	69	-21.264	49.712	-0.428	0.670	0.724
Father	GMT	36	-0.291	0.169	-1.725	0.094	0.250
	SRS SCI	27	0.003	0.002	1.589	0.125	0.250
	PGS	60	20.527	57.813	0.355	0.724	0.724

N: number of observations included in the analysis, β: regression coefficient, s.e.: standard error, t: t-statistic, p: p-value, Adj. p: p-value corrected for multiple testing (three tests for each of the three family members' measures) using False Discovery Rate method.
* $p<0.05$.

APPENDIX CHAPTER 5

Table A5.1 List of all phenotypic measures for the infant sibling and proband used in Chapter 5, and information on age at the time of collection and whether it was available for Phase 1, 2 or 3.

Participant	Measure	Phase 1	Phase 2	Phase 3
Infant sibling who participated in BASIS/STAARS	Collected at T2			
	Eye-tracking face-popout task	x	x	x
	Collected at T3			
	Early Childhood Behavioral Questionnaire (ECBQ)	x	x	
	Collected as part of gBASIS (6-10 years)			
	Social Responsiveness Scale – 2 pre-school (SRS)	x	x	
	Conners 3-P	x	x	
Proband (i.e. older siblings of the target children who have a community diagnosis of neurodevelopmental disorder)	Collected at T4			
	Strengths and Difficulties Questionnaire (SDQ)		x	
	Social Communication Questionnaire-Lifetime (SCQ-L)		x	

Table A5.2 Composition characteristics of the Phase 3 sample available for the present study, by risk group, and mean scores of the behavioural measures collected at 5, 10 and 14 months.

	HR(ADHD)	HR(ASD)	HR(ASD/ADHD)	LR	Total N
N current study	34	89	11	30	164
Males/Females	23/11	49/40	4/7	18/12	94/70
	Mean (s.d.) Min - Max	**Mean (s.d.) Min - Max**	**Mean (s.d.) Min - Max**	**Mean (s.d.) Min - Max**	**p**
T0					
Age	5.29 (0.47) 5 - 6	5.37 (0.64) 3 - 6	5.00 (0.00) 5 - 5	5.41 (0.50) 5 - 6	0.342[k]
MSEL Composite Score	84.71 (13.47) 59 - 122	82.98 (10.98) 63 - 108	90.40 (8.71) 75 - 95	85.63 (9.70) 66 - 102	0.427[a]
VABS Composite Score	98.96 (13.15) 72 - 118	93.58 (11.45) 68 - 118	95.33 (13.82) 70 - 112	95.67 (9.57) 79 - 113	0.276[a]
T1					
Age	10.21 (0.90) 9 - 12	10.06 (0.50) 9 - 11	9.90 (0.74) 9 - 11	10.04 (0.58) 9 - 11	0.753[k]
MSEL Composite Score	82.31 (12.84) 61 - 113	87.18 (15.11) 50 - 136	92.90 (16.88) 73 - 134	90.29 (14.06) 58 - 128	0.116[a]
VABS Composite Score	97.00 (10.80) 85 - 111	84.67 (10.53) 68 - 107	89.00 (24.27) 74 - 117	98.67 (2.31) 96 - 100	0.064[k]
T2					
Age	14.32 (0.86) 13 - 16	14.28 (0.69) 12 - 16	14.33 (0.71) 14 - 16	14.21 (0.66) 13 - 15	0.917[k]
MSEL Composite Score	77.07 (12.33) 56 - 98	77.94 (12.38) 54 - 114	71.11 (13.01) 55 - 100	78.71 (11.73) 53 - 102	0.427[a]
VABS Composite Score	96.95 (12.45) 76 - 119	92.09 (13.11) 62 - 126	95.44 (18.61) 64 - 121	97.52 (10.70) 76 - 112	0.253[a]

HR(ADHD): high-risk infants at familial risk for ADHD; HR(ASD): high-risk infants at familial risk for ASD; HR(ASD/ADHD): high-risk infants at familial risk for comorbid ASD and ADHD; LR: Low-Risk infants; N: number of subjects with available scores; s.d.: standard deviation; p: p-value of the one-way ANOVA or Kruskal-Wallis test with outcome groups as between-subjects factor. MSEL: Mullen Scales of Early Learning; VABS: Vineland Adaptive Behavior Scales; ADOS: Autism Diagnostic Observation Schedule; SRS: Social Responsiveness Scale. [a,k] subscripts indicate whether ANOVA or Kruskal-Wallis test, respectively, was performed to compare groups. P-values refer to these statistics. Kruskal-Wallis test was performed if non-normality of the distribution in at least one of the groups was found based on significant Shapiro-Wilk test.

Table A5.3 Results of the ANOVA testing the effect of risk group on peak look duration at the face at 14 months in the entire sample including Phase 1, 2 and 3 participants.

	d.f.	F	p	η^2
Risk group	1	13.716	0.0003*	0.031
Phase	2	1.517	0.221	0.009
Sex	1	0.002	0.962	<0.0001
Age (months)	1	0.936	0.334	0.003
Risk group X Phase	2	2.104	0.124	0.013
Residuals	326			

d.f.: degrees of freedom; F: F-test statistic; p: p-value; η^2 : eta-squared as a measure of the effect size.
* p<0.05.

Table A5.4 Results of the ANOVA testing the effect of risk group on peak look duration at the non-face stimuli at 14 months in the entire sample including Phase 1, 2 and 3 participants.

	d.f.	F	p	η^2
Risk group	1	0.025	0.875	0.0002
Phase	2	1.023	0.361	0.004
Sex	1	4.166	0.042*[w]	0.012
Age (months)	1	2.556	0.111	0.008
Risk group X Phase	2	0.385	0.681	0.003
Residuals	338			

d.f.: degrees of freedom; F: F-test statistic; p: p-value; η^2 : eta-squared as a measure of the effect size.
* p<0.05.
[w]Non-parametric test did not reveal the same result (W=14155, p=0.386).

Table A5.5 Results of the robust linear regression testing the relationship between peak look duration at the face recorded at around 14 months during an eye-tracking face pop-out task and familial risk for ASD and ADHD, controlling for the effect of age when the face pop-out task was administered and sex.

	β	s.e.	F	p
(Intercept)	-323.99	970.61		
ASD	259.24	132.18	3.82	0.053[+]
ADHD	19.84	159.69	0.015	0.902
Age (months)	89.81	67.99	1.725	0.192
Sex	-132.38	102.34	1.688	0.196
ASD x ADHD	-246.09	273.12	0.840	0.361

β: robust regression coefficient, s.e.: standard error, F: robust F-statistic (Wald test for multiple comparisons), p: p-value.
[+] p<0.05.

Table A5.6 Results of the robust linear regression testing the relationship between peak look duration at the non-social stimuli recorded at around 14 months during an eye-tracking face pop-out task and familial risk for ASD and ADHD, controlling for the effect of age when the face pop-out task was administered and sex.

	β	s.e.	F	p
(Intercept)	979.60	762.43	0.010	0.919
ASD	10.62	103.73	1.27	0.262
ADHD	-143.98	126.94	0.003	0.959
Age (months)	-4.52	53.37	0.007	0.933
Sex	63.51	81.32	0.616	0.434
ASD x ADHD	11.043	215.8402		

β: robust regression coefficient, s.e.: standard error, F: robust F-statistic (Wald test for multiple comparisons), p: p-value.
* p<0.05.

Figure A5.1 Results of the polygenic score (PGS) for ASD (**a**) and ADHD (**b**) predicting peak look duration at the face at various GWAS p-value thresholds, after controlling for sex. Height of bars (Y-axis) represents the model fit (R^2). X-axis represents the 9 selected p-value thresholds plus the p-value threshold selected for the high-resolution best-fit polygenic score. Numbers above bars represent p-values. Bars are coloured on a continuous scale from red (significantly higher for longer peak look durations) to light blue (lower for longer peak look durations).

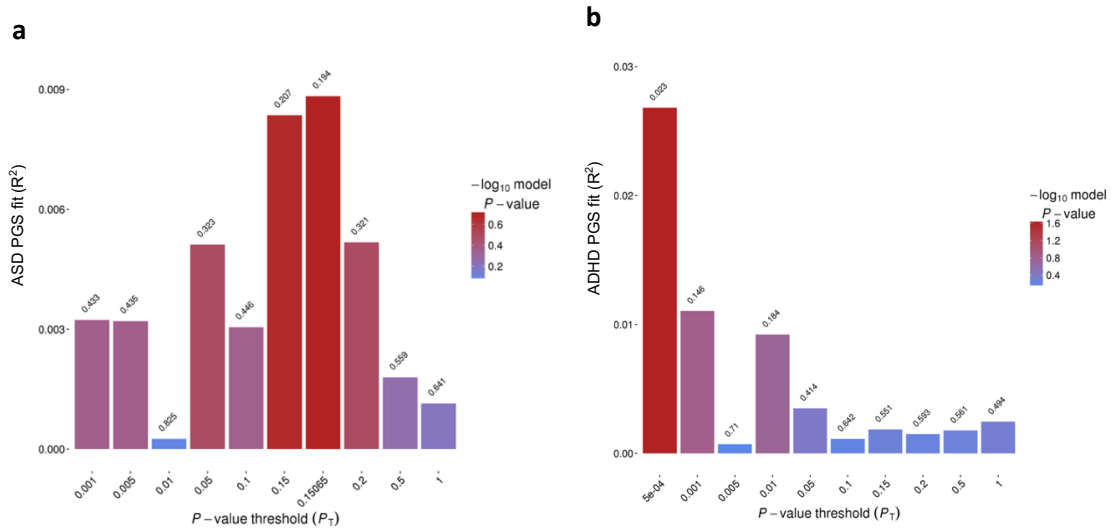


Table A5.7 Results of the ANOVA testing the effect of outcome group on peak look duration at the face at 14 months in the entire sample including Phase 1 and 2 participants.

	d.f.	F	p	η^2
Outcome	3	4.574	0.004*	0.048
Phase	1	1.770	0.185	0.012
Sex	1	0.405	0.525	0.003
Age (months)	1	0.367	0.546	0.002
Outcome X Phase	3	1.608	0.189	0.024
Residuals	197			

d.f.: degrees of freedom; F: F-test statistic; p: p-value; η^2 : eta-squared as a measure of the effect size.
* p<0.05.

Table A5.8 Results of the ANOVA testing the effect of outcome group on peak look duration at the non-face stimuli at 14 months in the entire sample including Phase 1 and 2 participants.

	d.f.	F	p	η^2
Outcome	3	0.221	0.882	0.002
Phase	1	1.016	0.315	<0.0001
Sex	1	2.269	0.133	0.010
Age (months)	1	4.605	0.033*	0.021
Outcome X Phase	3	0.546	0.651	0.008
Residuals	212			

d.f.: degrees of freedom; F: F-test statistic; p: p-value; η^2 : eta-squared as a measure of the effect size.
* p<0.05.

Table A5.9 Results of the robust linear regression testing the relationship between peak look duration at the face recorded at around 14 months during an eye-tracking face pop-out task and four ECBQ subscale scores, controlling for the effect of age when the face pop-out task was administered and sex.

	β	s.e.	F	p
(Intercept)	2760.43	744.15		
Sociability	68.95	45.03	2.37	0.125
Attention Focusing	28.99	52.23	0.29	0.589
Impulsive Behaviour	-62.76	73.77	0.71	0.400
Inhibitory Control	-137.19	46.51	8.52	0.004*
Age face pop-out (months)	-57.87	38.70	2.22	0.138
Sex	-106.93	98.79	1.17	0.281

β: robust regression coefficient, s.e.: standard error, F: robust F-statistic (Wald test for multiple comparisons), p: p-value.
* p<0.05.

Table A5.10 Results of the robust linear regression testing the relationship between peak look duration at the non-face stimuli at around 14 months during an eye-tracking face pop-out task and four ECBQ subscale scores, controlling for the effect of age when the face pop-out task was administered and sex.

	β	s.e.	F	p
(Intercept)	84.79	557.74		
Sociability	6.35	34.16	0.04	0.851
Attention Focusing	41.97	39.13	1.18	0.279
Impulsive Behaviour	17.18	56.18	0.09	0.759
Inhibitory Control	-7.65	34.28	0.050	0.822
Age face pop-out (months)	62.30	29.10	4.632	0.033*
Sex	4.95	73.67	0.004	0.947

β: robust regression coefficient, s.e.: standard error, F: robust F-statistic (Wald test for multiple comparisons), p: p-value.
* p<0.05.

Table A5.11 Summary of mediation analysis results with autistic traits (Social Communication Impairment, SCI, and Restricted and Repetitive Behaviours, RRB) as dependent variables at school age (6-10 years), peak look duration at the face at 14 months as independent variable and scores at the Inhibitory Control scale of the Early Childhood Behavior Questionnaire at 2 years as mediator.

Regressions		β	s.e.	z	p	St. β
SCI	**Inhibitory Control**	-0.475	0.166	-2.861	0.004*	-0.485
SCI	**Peak look at the face**	-0.041	0.165	-0.251	0.802	-0.042
RRB	**Inhibitory control**	-0.497	0.162	-3.068	0.002*	-0.508
RRB	**Peak look at the face**	0.014	0.187	0.073	0.942	0.014
Inhibitory Control	**Peak look at the face**	-0.354	0.153	-2.318	0.020*	-0.352
Covariance		β	s.e.	z	p	St. β
SCI	**RRB**	0.662	0.141	4.696	<0.001	0.899
Indirect effect SCI		0.168	0.096	1.753	0.080+	0.171
Indirect effect RRB		0.176	0.096	1.826	0.068+	0.179
Total effect SCI		0.127	0.194	0.651	0.515	0.129
Total effect RRB		0.190	0.231	0.820	0.412	0.193

β: unstandardized beta coefficient, s.e.: standard error, z: z-value, p: p-values; St.β: standardized beta coefficients; SCI: Social Communication Impairment; RRB: Restricted and Repetitive Behaviours.
* p<0.05.
+ p<0.1.

Figure A5.2 Histograms illustrating the distribution of total score of the Social Communication Questionnaire Lifetime (SCQ-L total, **a**) and Strengths and Difficulties Questionnaire Hyperactivity/Inattention subscale (SDQ Hyp./Inatt., **b**) for the BASIS probands (older siblings of the BASIS participants who received a community diagnosis of ASD before enrolment of their younger sibling in the longitudinal study).

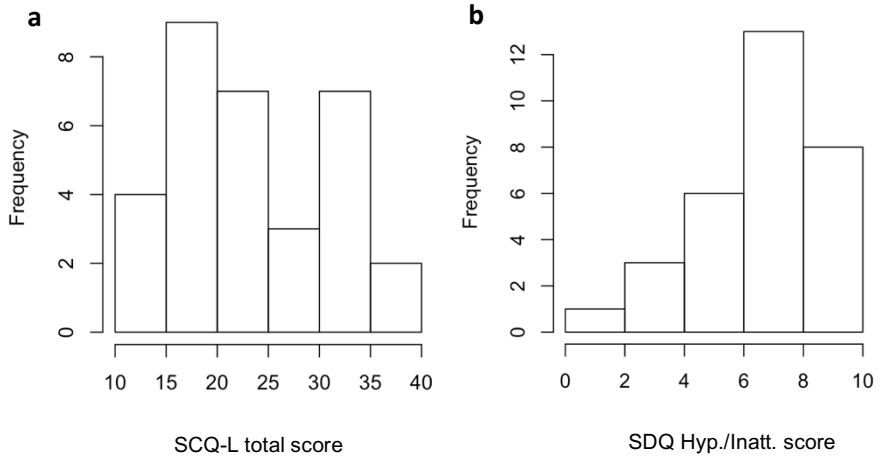
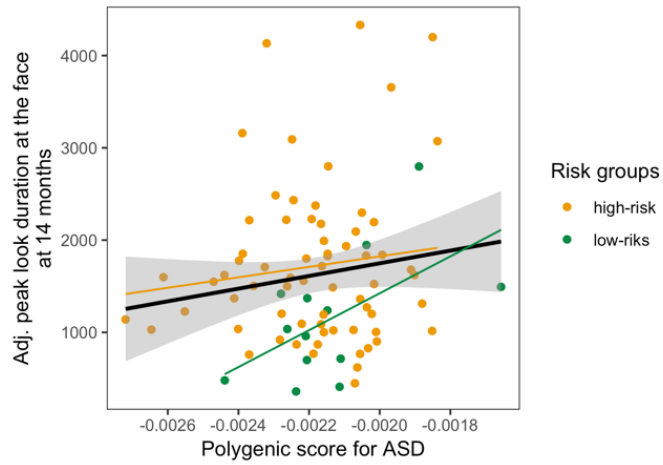


Figure A5.3 Relation between ADS polygenic score estimated on the large gBASIS sample as the best fit score for prediction of categorical ASD diagnosis (see Chapter 4), on the x-axis, and peak look duration at the face at 14 months, in milliseconds, on the y-axis. Dots represent individual data points, colour-coded in yellow for high-risk infants and green for low-risk infants. The solid black line represents regression line for the entire group, with grey shaded areas depicting standard errors. Coloured lines represent the regression lines for the high-risk (yellow) and low-risk (green) groups.



APPENDIX CHAPTER 6

Table A6.1 Total number of participants per group included in the current study, and mean (standard deviation), minimum and maximum scores of the behavioural measures collected at T1, T2, T3 and T4.

Outcome groups	**LR**	**HR-TD**	**HR-Aty**	**HR-ASD**		
N current study	14	28	14	11		
	Mean (s.d.) Min-Max	**Mean (s.d.) Min-Max**	**Mean (s.d.) Min-Max**	**Mean (s.d.) Min-Max**	**p**	η^2
T1						
MSEL Composite Score	111.43 (16.19) 78 - 140	107.50 (15.87) 66 - 133	104.43 (13.10) 82 - 130	98.91 (14.94) 73 - 126	0.22	0.07
VABS Composite Score	96.93 (10.19)$^{+}$ 80 - 115	94.88 (9.78) 76 - 111	88.00 (13.58) 68 - 114	85.27 (14.46)$^{+}$ 49 - 102	0.034*	0.14
T2						
MSEL Composite Score	100.43 (15.42)^{a} 84 - 141	98.00 (15.06)^{b} 71 - 131	90.00 (11.75) 71 - 114	82.55 (12.89)^{a,b} 65 - 106	0.008*	0.18
VABS Composite Score	99.93 (12.55) 88 - 131	96.48 (12.54) 72 - 123	94.77 (11.76) 77 - 120	89.80 (8.63) 76 - 101	0.229	0.07
T3						
MSEL Composite Score	110.00 (14.69)^{a} 80 - 132	101.46 (14.38)^{b} 79 - 143	94.27 (19.49)^{c} 76 - 140	75.73 (18.45)^{a,b,c} 49 - 113	<0.001*	0.35
VABS Composite Score	108.71 (7.97)^{a,b} 96 - 121	105.88 (10.20)^{c} 86 - 123	97.83 (9.79)^{a,d} 82 - 114	83.36 (10.79)^{b,c,d} 70 - 102	<0.001*	0.48
T4						
MSEL Composite Score	118.79 (17.75)^{a,b} 69 - 141	111.04 (17.50)^{c,d} 81 - 142	84.71 (21.57)^{a,c} 56 - 145	83.50 (27.15)^{b,d} 49 - 127	<0.001*	0.36
VABS Composite Score	104.79 (5.70)^{a,b} 96 - 114	101.26 (8.59)^{c,d} 88 - 120	87.14 (8.13)^{a,c,e} 74 - 97	73.10 (11.00)^{b,d,e} 57 - 91	<0.001*	0.66
ADOS Severity Score	2.14 (1.96)$^{+}$ 1 - 6	1.29 (0.55)^{a,b} 1 - 3	4.07 (2.50)$^{a,+}$ 1 - 8	4.90 (3.87)$^{b,+}$ 1 - 10	0.002*	0.32

LR: Low-Risk infants, HR-TD: High-Risk infants with Typical Development, HR-Aty: High Risk infants with Atypical development but not ASD, HR-ASD: High-Risk infants with Autism Spectrum Disorder. N: number of subjects with available scores; Mean; s.d.: standard deviation; p-value of the one-way ANOVA with outcome groups as between-subjects factor (with the exception of ADOS Severity score, for which Kruskal-Wallis non-parametric test was used); η^2: eta-squared as a measure of the effect size, MSEL: Mullen Scales of Early Learning; VABS: Vineland Adaptive Behavior Scales; ADOS: Autism Diagnostic Observation Schedule; ADOS-2 Calibrated Severity Scores are reported, calculated by selecting relevant items from the administered ADOS-G to obtain an Overall Total score.

*p<0.05 on one-way ANOVA testing the effect of outcome group for each behavioural measure.

^{a,b,c,d,e} Different superscript letters denote that groups are significantly different from each other based on Tukey's Honest Significant Difference post-hoc analyses with 95% family-wise confidence level for when comparing four outcome groups for all measures except ADOS Severity Score. For ADOS score, post-hoc comparison has been performed using Dunn Test. $^{+}$ indicates multiple-comparison adjusted p<0.1.

Analysis 1: Global methylation level

Table A6.2 Results of the linear model testing for differences in developmental trajectories of global methylation levels associated with ASD outcome, when accounting for the effect of possible covariates such as age in months, batch, maternal smoke and maternal alcohol intake during pregnancy.

	β	s.e.	d.f.	t	p
(Intercept)	0.468	0.002	58	267.43	<0.0001
Change T1-T2	0.001	0.001	39	1.04	0.307
Change T1-T3	0.003	0.003	39	1.25	0.217
ASD	-0.0001	0.001	58	-0.09	0.932
Age in months	-0.0001	0.0002	39	-0.43	0.666
Batch	0.001	0.001	58	1.10	0.278
Maternal smoke	0.0003	0.001	58	0.51	0.610
Maternal alcohol intake	-0.001	0.0004	58	-1.80	0.077
Change T1-T2 x ASD	-0.001	0.001	39	-0.54	0.593
Change T1-T3 x ASD	-0.0001	0.001	39	-0.06	0.954

β: regression coefficient; s.e.: standard error; d.f.: degrees of freedom; t: t-statistic; p: p-value.

Table A6.3 Results of the linear model testing for differences in developmental trajectories of global methylation levels associated with atypical development outcome (i.e. LR + HR-TD vs. HR-Aty + HR-ASD), when accounting for the effect of possible covariates such as age in months, batch, maternal smoke and maternal alcohol intake during pregnancy.

	β	s.e.	d.f.	t	p
(Intercept)	0.469	0.002	58	283.84	<0.0001
Change T1-T2	0.001	0.001	39	1.13	0.266
Change T1-T3	0.004	0.003	39	1.51	0.140
Atyp. Dev.	-0.0004	0.001	58	-0.70	0.489
Age in months	-0.0001	0.0002	39	-0.66	0.515
Batch	0.001	0.001	58	1.54	0.128
Maternal smoke	0.0003	0.001	58	0.60	0.548
Maternal alcohol intake	-0.001	0.0004	58	-2.08	0.042*
Change T1-T2 x Atyp. Dev.	-0.0001	0.001	39	-0.07	0.942
Change T1-T3 x Atyp. Dev.	-0.001	0.001	39	-0.62	0.538

β: regression coefficient; s.e.: standard error; d.f.: degrees of freedom; t: t-statistic; p: p-value.

Table A6.4 Results of the linear model testing for differences in developmental trajectories of global methylation levels associated with adaptive skills at 36 months measured by the Vineland Adaptive Behavior Scales (VABS Sparrow, Cicchetti, & Balla, 2005), when accounting for the effect of possible covariates such as age in months, batch, maternal smoke and maternal alcohol intake during pregnancy.

	β	s.e.	d.f.	t	p
(Intercept)	0.468	0.002	56	268.92	0.000
Change T1-T2	0.001	0.001	38	0.79	0.437
Change T1-T3	0.003	0.003	38	1.12	0.270
VABS at 36 months	0.0001	0.000	56	0.32	0.752
Age in months	-0.0001	0.000	38	-0.34	0.737
Batch	0.001	0.001	56	1.15	0.254
Maternal smoke	0.0004	0.001	56	0.69	0.492
Maternal alcohol intake	-0.001	0.000	56	-1.69	0.097
Change T1-T2 x VABS	0.00001	0.000	38	0.02	0.986
Change T1-T3 x VABS	0.0001	0.000	38	0.23	0.821

β: regression coefficient; s.e.: standard error; d.f.: degrees of freedom; t: t-statistic; p: p-value.

Analysis 2: Epigenome-Wide Association analyses

Table A6.5 List of the top-ranked significant probes associated with ASD at a discovery p-value threshold <5 x 10^{-5}.

Probe's name	p	FDR	Effect Size	Chr.	UCSC Ref. Gene	Relation to CpG Island	Regulatory Feature Group
cg15976650	*4.44E-06*	*0.58*	*-0.02*	*1*	*TUFT1*	*Island*	*PA*
cg05780766	4.86E-06	0.58	-0.10	2	CYP27C1		UCS
cg20963995	5.54E-06	0.58	0.02	1	PTGFRN	Island	UCS
cg18317933	5.73E-06	0.58	-0.04	8	PRKDC	Island	
cg16291048	9.92E-06	0.62	0.08	1	S100A6	Shore	PA
cg03963853	1.07E-05	0.62	0.17	16	MGRN1	Island	
cg23367851	***1.85E-05***	***0.62***	***0.22***	***7***	***CYCS***	***Shore***	***PA***
cg14896948	1.96E-05	0.62	-0.03	7	COBL		
cg10242763*	*1.97E-05*	*0.62*	*0.04*	*7*	*CACNA2D1*	*Shore*	
cg26257814	2.09E-05	0.62	0.03	19	FLJ26850	Shore	U
cg13303475	2.14E-05	0.62	0.01	12	NT5DC3	Island	
cg11469137	2.31E-05	0.62	0.04	1	PLOD1	Shore	PA
cg13525458	2.51E-05	0.62	0.03	20	ZSWIM3		PA
cg03724010	2.74E-05	0.62	-0.05	1	C1orf70	Shore	UCS
cg05398769	2.94E-05	0.62	-0.11	1	CASZ1	Shelf	
cg07153098	3.03E-05	0.62	-0.04	2	LOC388965	Island	
cg03376719	3.08E-05	0.62	0.03	3	ALCAM	Shore	
cg21348771	3.32E-05	0.62	0.01	6	C6orf114	Island	PA
cg08364334	3.40E-05	0.62	-0.09	4		Shelf	
cg19046697	3.98E-05	0.62	-0.03	5	FLJ42709	Island	UCS
cg24249925•	*4.06E-05*	*0.62*	*-0.05*	*1*	*DDR2*		
cg19320505	4.20E-05	0.62	0.03	11	ELMOD1	Shore	
cg03565750	4.21E-05	0.62	0.04	19	PNKP	Shore	PA
cg07583091	4.22E-05	0.62	0.04	11	GTF2H1	Island	U
cg14920716	4.32E-05	0.62	-0.05	19	ZNF146		
cg21929600	4.52E-05	0.62	0.02	19	ZNF552	Island	PA
cg26587228	4.57E-05	0.62	0.08	19	USP29		
cg20278936	4.58E-05	0.62	0.02	11	OSBP	Shore	PA
cg12944530•	*4.62E-05*	*0.62*	*0.04*	*2*	*CFLAR*	*Shore*	*PA*
cg08625996*	*4.69E-05*	*0.62*	*0.04*	*7*	*SND1*		
cg14005246	4.87E-05	0.62	0.02	14		Island	
cg07926644	4.92E-05	0.62	-0.05	19	PIAS4	Shelf	

p: p-value; FDR: False Discovery Rate adjusted p-value, or q-value; Chr.: chromosome; UCSC Ref. Gene: annotated gene based on the University of California Santa Cruz Genome Browser; Regulatory feature groups: PA: promoter associated, UCS: unclassified cell specific, U: unclassified.

Potentially relevant probes are indicated in italic font. Underlined is the top significant probe. In bold is the probe with the highest effect size.* Indicates that the probe is in a gene which has been previously associated with ASD, while • indicates that the probe has been found to change methylation level during foetal brain development (Spiers et al., 2015).

Table A6.6 List of the top significant probes associated with atypical development at a "discovery" p-value threshold < 5 x 10^{-5}.

Probe's name	p	FDR	Effect Size	Chr.	UCSC Ref. Gene	Relation to CpG Island	Regulatory Feature Group
cg21973914	3.34E-07	0.13	0.06	13	F10	Shore	
cg15082394	6.80E-07	0.14	-0.07	6	RGL2	Shore	
cg23281307	2.56E-06	0.29	-0.05	2	CASP8		
cg23775991	2.84E-06	0.29	-0.05	6	FLOT1	Shore	PA
cg16862641	6.07E-06	0.38	0.07	6	COL9A1	Shore	
cg13919860	6.11E-06	0.38	0.03	X	TMSB15B	Island	
cg26566415	6.65E-06	0.38	0.01	6		Shore	U
cg09061759	7.66E-06	0.39	0.01	1	JAK1	Island	
cg06425881	8.85E-06	0.40	-0.02	7	HECW1		
cg01257697•	*1.09E-05*	*0.40*	*0.05*	*17*			
cg16185996	1.43E-05	0.40	-0.07	4	FGFRL1	Shore	
cg07152030	1.44E-05	0.40	0.02	1	ARF1		
cg09234567	1.48E-05	0.40	0.01	15	GRAMD2	Island	GA
cg05175964	1.49E-05	0.40	-0.04	15	NR2E3	Shore	
cg14918743	1.49E-05	0.40	-0.05	14	ZBTB42	Shore	PACS
cg21082921*	*2.14E-05*	*0.51*	*0.03*	*5*	*CHD1*	*Shore*	*PA*
cg05927274	2.24E-05	0.51	0.03	1			
cg24093411	2.37E-05	0.51	0.03	5	TCF7	Island	
cg00275962	2.66E-05	0.51	-0.03	6	POLR1C	Island	PA
cg15727320	2.73E-05	0.51	-0.06	12	P11		
cg03566107	2.85E-05	0.51	-0.05	18	CBLN2	Shore	
cg23606751	2.86E-05	0.51	0.04	14	SNAPC1	Shore	PA
cg23661183	2.88E-05	0.51	0.03	10	ABLIM1		PA
cg16140548	3.11E-05	0.52	0.05	6	PLG		
cg04729574•	*3.66E-05*	*0.56*	*0.05*	*3*	*ECE2*	*Shore*	
cg10633176	3.67E-05	0.56	-0.02	1	ZNF687	Island	PA
cg10719970	4.07E-05	0.56	-0.07	6	FOXP4	Shore	
cg24574147	4.18E-05	0.56	0.02	8	XKR6	Shore	
cg16656864	4.29E-05	0.56	0.04	17	TAOK1	Shore	
cg06963664*	***4.39E-05***	***0.56***	***-0.07***	***X***	***PLXNA3***	***Island***	
cg05922723*	*4.47E-05*	*0.56*	*-0.06*	*7*	*GIGYF1*	*Shore*	
cg07452560	4.50E-05	0.56	-0.04	8	KIAA1688		
cg04089240	4.59E-05	0.56	-0.05	11	TRPM5	Shore	

p: p-value; FDR: False Discovery Rate adjusted p-value, or q-value; Chr.: chromosome; UCSC Ref. Gene: annotated gene based on the University of California Santa Cruz Genome Browser; Regulatory feature groups: PA: promoter associated, U: unclassified, GA: gene associated, PACS: promoter associated cell specific.

Potentially relevant probes are indicated in italic font. Underlined is the top significant probe. In bold is the probe with the highest effect size.* Indicates that the probe is in a gene which has been previously associated with ASD, while • indicates that the probe has been found to change methylation level during foetal brain development (Spiers et al., 2015).

Table A6.7 List of the top significant probes associated with dimensional outcome (adaptive skills at 3 years) at a p-value threshold < 5 x 10^{-5}.

Probe's name	p	FDR	Effect Size	Chr.	UCSC Ref. Gene	Relation to CpG Island	Regulatory Feature Group
cg26862175	1.08E-05	0.85	0.02	3	STAB1		
cg03735049	1.42E-05	0.85	0.02	X	TSR2		PA
cg00208274•	*1.78E-05*	*0.85*	*0.02*	*7*	*FOXK1*		*UCS*
cg05165940	1.82E-05	0.85	-0.01	2		Island	UCS
cg23281307	1.90E-05	0.85	0.02	2	CASP8		
cg16175077	2.03E-05	0.85	0.01	10			U
cg01044692	2.11E-05	0.85	0.00	2	FAM119A	Island	PA
cg05175964	2.35E-05	0.85	0.02	15	NR2E3	Shore	
cg10209089	2.76E-05	0.85	-0.04	12		Shelf	
cg04163147	3.38E-05	0.85	0.01	11	OR52B4		
cg16387436	3.40E-05	0.85	0.02	6	DCDC2		
cg09811127	3.54E-05	0.85	-0.03	1	MORN1		UCS
cg05781893	3.55E-05	0.85	0.03	2	PRKRA	Shelf	
cg19255722	3.58E-05	0.85	-0.01	3	SCN10A		
cg01473602	3.80E-05	0.85	-0.02	12	CSRP2		
cg26853265*	*3.99E-05*	*0.85*	*-0.03*	*19*	*PGLYRP2*	*Island*	
cg08134680	4.12E-05	0.85	0.02	1	EPHA10	Shelf	UCS
cg13707005	**4.23E-05**	**0.85**	**-0.05**	**10**	**CUGBP2**		
cg26344392•	*4.38E-05*	*0.85*	*-0.02*	*1*	*ADCY10*		
cg01283227*	*4.49E-05*	*0.85*	*0.02*	*X*	*CDKL5*	*Shore*	
cg06830348	4.60E-05	0.85	0.01	18	ST8SIA3		
cg15225044	4.70E-05	0.85	0.02	X	NKAP	Island	PA
cg07152030	4.82E-05	0.85	-0.01	1	ARF1		

p: p-value; FDR: False Discovery Rate adjusted p-value, or q-value; Chr.: chromosome; UCSC Ref. Gene: annotated gene based on the University of California Santa Cruz Genome Browser; Regulatory feature groups: PA: promoter associated, UCS: unclassified cell specific, U: unclassified.

Potentially relevant probes are indicated in italic font. Underlined is the top significant probe. In bold is the probe with the highest effect size.* Indicates that the probe is in a gene which has been previously associated with ASD, while • indicates that the probe has been found to change methylation level during foetal brain development (Spiers et al., 2015).

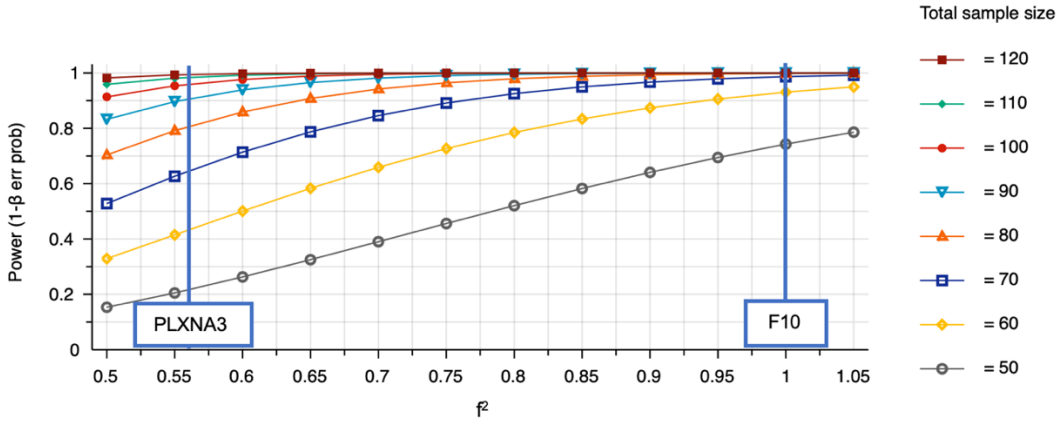


Figure A6.1 Sample sizes needed to obtain significant results at an epigenome-wide significant threshold of 2.4 x 10^{-7} (Saffari et al., 2018) under the current design, as a function of power and f^2 effect size measure for the atypical development EWAS. Cohen's f2 is reported in the x-axis as a measure of the relative amount of DNAm variance explained by ASD in the multiple regression models used for EWAS calculation (Faul, Erdfelder, Buchner, & Lang, 2009). The blue labelled line represents the Cohen's f2 value resulting from the EWAS model for probe cg06963664 (PLXNA3) and cg21973914 (F10).

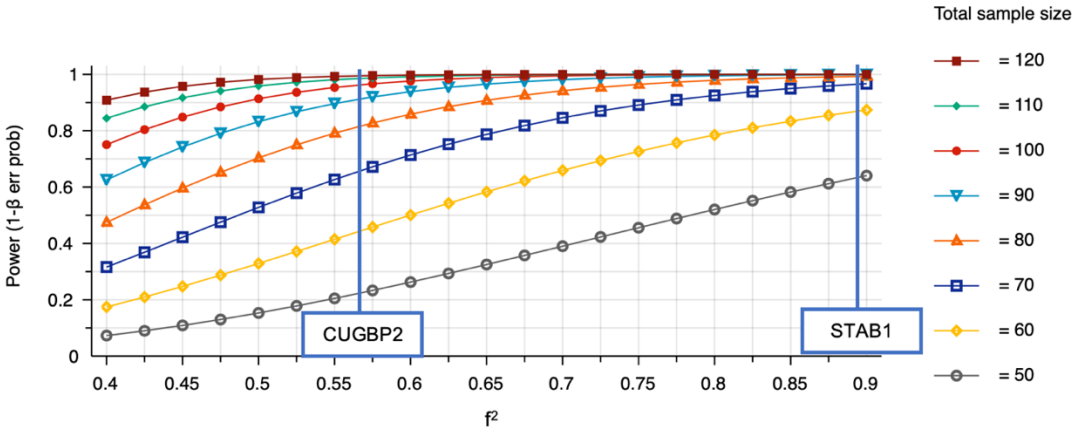
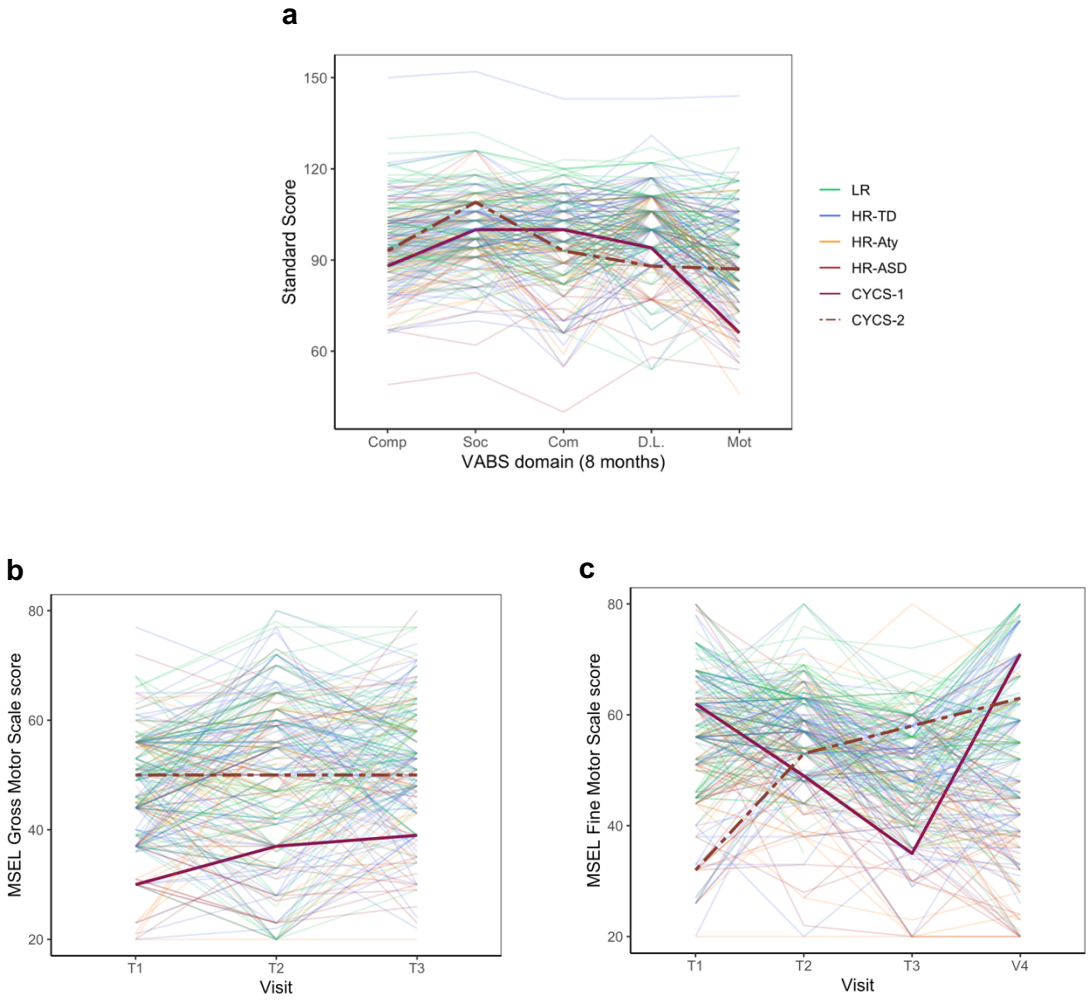


Figure A6.2 Sample sizes needed to obtain significant results at an epigenome-wide significant threshold of 2.4 x 10^{-7} (Saffari et al., 2018) under the current design, as a function of power and f^2 effect size measure for the dimensional outcome EWAS. Cohen's f2 is reported in the x-axis as a measure of the relative amount of DNAm variance explained by ASD in the multiple regression models used for EWAS calculation (Faul et al., 2009). Labelled blue lines represent Cohen's f2 values resulting from the EWAS models for probe cg13707005 (CUGBP2).

Figure A6.3 a Individual values for Vineland Adaptive Behavior Scales standard scores at 8 months for all individuals who participated in BASIS phase 1 and 2 (N=247) for each of the five domains. Comp: Composite standard score; Soc: Socialization domain standard score, Com: Communication domain standard score; D.L.: Daily living skills standard score, Mot: Motor skills standard score. **b** Gross Motor Scale score of the Mullen Scales of Early Learning (MSEL) standardized assessment, at three subsequent visits (T1: 8 months, T2: 15 months, T3: 25 months) for all individuals with available MSEL and outcome information who participated in BASIS phase 1 and 2 (N=243). **c** Fine Motor Scale score of the Mullen Scales of Early Learning (MSEL) standardized assessment, at four subsequent visits (T1: 8 months, T2: 15 months, T3: 25 months, T4: 36 months) for all individuals with available MSEL and outcome information who participated in BASIS phase 1 and 2 (N=243). In all plots, individuals with atypically high levels of DNAm in the CYCS gene, CYCS-1 and CYCS-2, are reported as solid dark magenta and dashed brick lines, respectively.



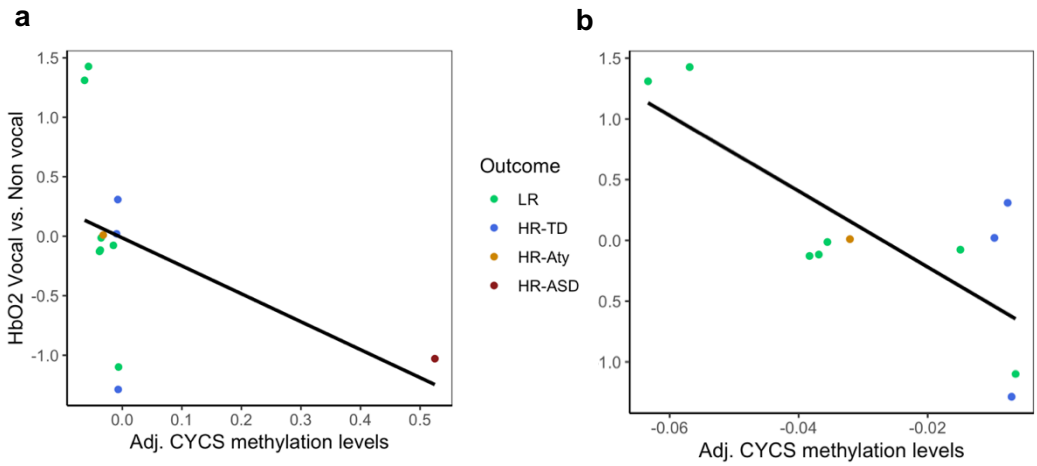


Figure A6.4 Scatter plot representing the relationship between DNA methylation of the cg23367851 probe in the CYCS gene at 8 months (x-axis) and difference in oxygenated haemoglobin concentration (HbO2) in the auditory social (vocal) vs. non-social (non-vocal) conditions at 5 months (y-axis). On the x-axis, CYCS methylation levels adjusted for the effect of principal components and batch effect are reported. **a** depicts values for all infants who provided both NIRS and DNA methylation data (N=12). **b** represents all infants excluding the individual with atypically DNA methylation levels in the CYCS gene.

The black line represents the linear relationship between the two variables. Individual data in figures a and b are represented by points. Green represents LR, i.e. low-risk controls; blue represents HR-TD, i.e. high-risk infants with typical development at 3 years; orange represents HR-Aty, i.e. high-risk infants with developmental concerns but no ASD traits; and red represents HR-ASD, i.e. high-risk infants who received clinical diagnosis of ASD at 36 months.

Analysis 3: Weighted Co-methylation Gene Network Analysis

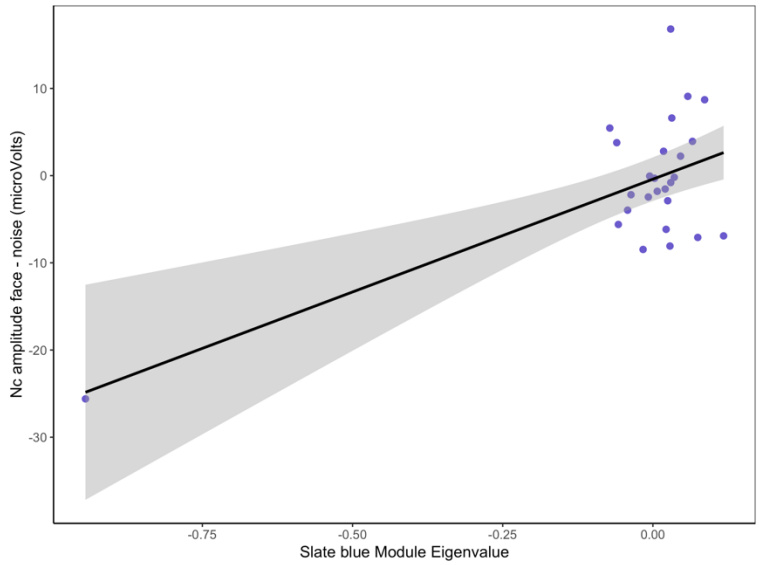


Figure A6.4 Relationship between slate blue module eigenvalue (i.e., methylation profile for the probes in the module) and Nc to face.

Figure A6.5 Distribution of the Nc to face variable by outcome group in a larger sample (N=131, in Chapter 2) including the 51 infants which were part of the present study. The influential case is circled in red. LR: low-risk infants; HR-TD: high-risk infants with typical development at T4; HR-Aty: HR infants with sub-threshold levels of ASD symptoms and/or more general developmental delays; HR-ASD: HR infants who received a diagnosis of ASD at T4.

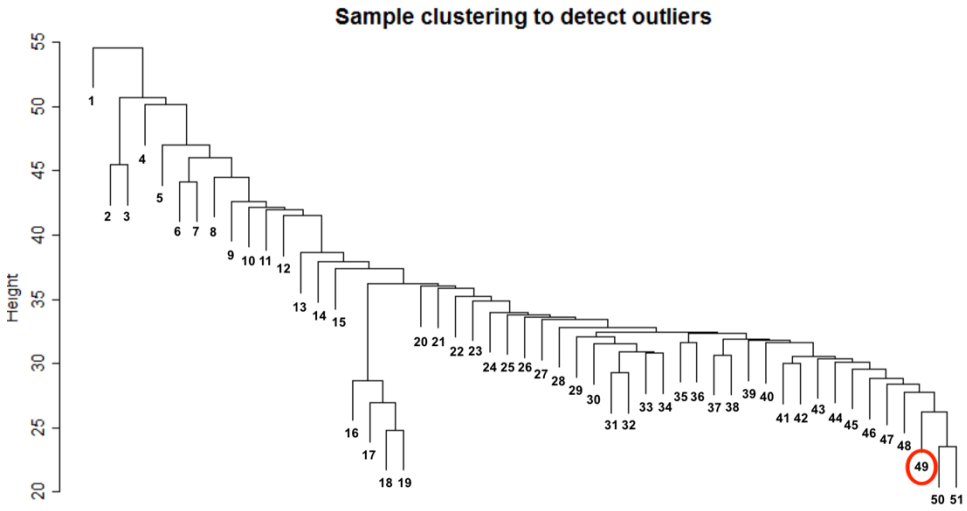


Figure A6.6 Hierarchical clustering dendrogram of 51 samples included in WGCNA based on their Euclidean distance ('hclust' function in R, as in WGCNA tutorial https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/). The influential case is circled in red.

Table A6.8 List of probes in the module associated with Nc to face at 8 months.

Probe's name	Chr.	UCSC Ref. Gene	Relation to CpG Island	Regulatory Feature Group	Rho Buccal-Brain	p Buccal-Brain
cg05413694	3				0.73	0.006*
cg21250931	15	TMC3			0.35	0.23
cg11362183	12	SRGAP1			0.20	0.50
cg09907936	19	ZNF135	Island	UCS	-0.5	0.06
cg07482220	6	AGPAT1			-0.3	0.28
cg13710937	14		Shelf		0.53	0.06
cg27491509	22	PIM3	Island	PA	0.26	0.37
cg15123087	7	ARHGEF5	Shelf		0.85	0.003*
cg26746037	19	ZFR2	Island	UCS	0.48	0.09
cg13166553	13				-0.06	0.84
cg26359712	8				-0.1	0.75
cg15022051	6				-0.1	0.76
cg17002138	1	TMEM48	Island	PA	0.41	0.15
cg17566867	22	KCNJ4	Shelf	UCS	0.25	0.40
cg23322161	1	KNCN			0.13	0.66
cg07967531	1		Shelf		0.08	0.77
cg12042737	14	KIF26A	Shore		0.15	0.61
cg05265771	6				0.13	0.65
cg21494882	6		Shelf		0.35	0.23
cg18962750*	*11*	*DIXDC1*	*Shelf*		*0.37*	*0.20*

p: p-value; FDR: False Discovery Rate adjusted p-value, or q-value; Chr.: chromosome; UCSC Ref. Gene: annotated gene based on the University of California Santa Cruz Genome Browser; Regulatory feature groups: UCS: unclassified cell specific, PA: promoter associated. Rho Buccal-Brain: Mean within-individual correlation values between DNA methylation levels in buccal and brain samples coming from 13 individuals who participated in Braun et al. (2019); p Buccal-Brain: p-values testing significant intra-individual correlation between DNA methylation from buccal and brain tissues.

Potentially relevant probes are indicated in italic font. Underlined is the top significant probe. In bold is the probe with the highest effect size.* Indicates that the probe is in a gene which has been previously associated with ASD (SFARI Gene, Banerjee-Basu & Packer, 2010).

Table A6.9 Enrichment Gene Ontology (GO) terms for mQTLs influencing probes of the significant module during pregnancy.

GO ID	Category	GO Term	count	p	FDR
GO:0021869•	*biological process*	*forebrain ventricular zone progenitor cell division*	*66*	*3.07E-212*	*1.75E-210**
GO:0070507	biological process	regulation of microtubule cytoskeleton organization	66	1.60E-163	4.56E-162*
GO:0021799•	*biological process*	*cerebral cortex radially oriented cell migration*	*66*	*5.14E-159*	*9.76E-158**
GO:0043015	molecular function	gamma-tubulin binding	66	1.62E-146	2.31E-145*
GO:0032956	biological process	regulation of actin cytoskeleton organization	66	6.48E-137	7.39E-136*
GO:0045665•	*biological process*	*negative regulation of neuron differentiation*	*66*	*3.76E-119*	*3.57E-118**
GO:0060070	biological process	canonical Wnt signalling pathway	66	8.57E-116	6.98E-115*
GO:0090263	biological process	positive regulation of canonical Wnt signalling pathway	66	2.45E-98	1.74E-97*
GO:0007049	biological process	cell cycle	66	1.88E-95	1.19E-94*
GO:0019904	molecular function	protein domain specific binding	66	5.14E-87	2.93E-86*
GO:0003779	molecular function	actin binding	66	1.28E-68	6.61E-68*
GO:0060561	biological process	apoptotic process involved in morphogenesis	24	6.82E-64	3.24E-63*
GO:0005925	cellular component	focal adhesion	66	3.20E-57	1.40E-56*
GO:2001241	biological process	positive regulation of extrinsic apoptotic signalling pathway in absence of ligand	24	1.92E-43	7.80E-43*
GO:0008601	molecular function	protein phosphatase type 2A regulator activity	24	2.48E-41	9.44E-41*
GO:0034047	biological process	regulation of protein phosphatase type 2A activity	24	2.67E-41	9.52E-41*
GO:0004742	molecular function	dihydrolipoyllysine-residue acetyltransferase activity	8	5.26E-29	1.77E-28*
GO:0005967°	*cellular component*	*mitochondrial pyruvate dehydrogenase complex*	*8*	*7.52E-27*	*2.38E-26**
GO:0022829	molecular function	wide pore channel activity	12	4.86E-25	1.46E-24*
GO:0055077	molecular function	gap junction hemi-channel activity	12	2.54E-23	7.22E-23*
GO:0006461	biological process	protein complex assembly	24	6.95E-22	1.89E-21*
GO:0034214	biological process	protein hexamerization	12	1.97E-20	5.11E-20*
GO:0005921	cellular component	gap junction	12	1.40E-18	7.82E-19*
GO:0006812†	*biological process*	*cation transport*	*12*	*2.75E-18*	*3.32E-18**
GO:0045121	cellular component	membrane raft	24	9.22E-17	6.26E-18*
GO:0006086	biological process	acetyl-CoA biosynthetic process from pyruvate	8	8.42E-15	1.35E-17*
GO:0002931†	*biological process*	*response to ischemia*	*12*	*2.03E-14*	*1.95E-16**
GO:0030431†	*biological process*	*sleep*	*8*	*5.80E-13*	*1.71E-14**
GO:0034604°	*molecular function*	*pyruvate dehydrogenase (NAD+) activity*	*8*	*1.31E-12*	*3.99E-14**

GO ID	Category	GO Term	count	p	FDR
GO:0010510	biological process	regulation of acetyl-CoA biosynthetic process from pyruvate	8	4.93E-12	2.33E-12*
GO:0005829	cellular component	cytosol	66	1.94E-10	2.33E-12*
GO:0005737	cellular component	cytoplasm	78	3.75E-10	8.51E-12*
GO:0006090	biological process	pyruvate metabolic process	8	2.94E-09	3.24E-10*
GO:0006099	biological process	tricarboxylic acid cycle	8	1.21E-08	6.10E-10*
GO:0007267	biological process	cell-cell signalling	12	2.20E-06	4.66E-09*
GO:0005886	cellular component	plasma membrane	12	6.35E-06	1.86E-08*
GO:0007268•	*biological process*	*synaptic transmission*	*12*	*1.61E-05*	*3.31E-06**
GO:0006006	biological process	glucose metabolic process	8	4.24E-05	9.28E-06*
GO:0044237	biological process	cellular metabolic process	8	2.46E-04	2.29E-05*
GO:0043209•	*cellular component*	*myelin sheath*	*8*	*2.79E-04*	*5.89E-05**
GO:0016021	cellular component	integral component of membrane	12	5.85E-04	3.34E-04*
GO:0044281	biological process	small molecule metabolic process	9	0.020	3.69E-04*
GO:0005654	cellular component	nucleoplasm	33	0.026	7.58E-04*
GO:0055085	biological process	transmembrane transport	12	0.037	0.026*
GO:0004095	molecular function	carnitine O-palmitoyltransferase activity	1	0.041	0.032*
GO:0005759°	*cellular component*	*mitochondrial matrix*	*8*	*0.085*	*0.045**
GO:0005739°	*cellular component*	*mitochondrion*	*9*	*0.098*	*0.049**
GO:0070062	cellular component	extracellular exosome	24	0.144	0.099
GO:0006853	biological process	carnitine shuttle	1	0.169	0.112
GO:0005515	molecular function	protein binding	73	0.638	0.161
GO:0005634	cellular component	nucleus	33	0.325	0.185
GO:0005741	cellular component	mitochondrial outer membrane	1	1.000	0.350
GO:0006635	biological process	fatty acid beta-oxidation	1	0.477	0.503
GO:0044255	biological process	cellular lipid metabolic process	1	0.525	0.544
GO:0005515	molecular function	protein binding	73	0.638	0.650
GO:0005741	cellular component	mitochondrial outer membrane	1	1	1

Count indicates the number of input SNPs for eSNPO with annotations in the GO term; the p value is calculated by Fisher Exact test based on the number of SNPs for the given GO term and the number of input SNPs, as indicated in Li et al. (2016) and the FDR consists in the p-value adjustment for multiple testing of several GO pathways per SNP, calculated using *qvalue* package in R (R Core Team, 2013).

Potentially relevant probes are indicated in italic font. Underlined are the GO pathways. • indicates that the pathways involved in neurodevelopment, ° indicates the pathways involved in the mitochondrial functioning, † indicates other functional pathways potentially involved in ASD. * indicates significance after correction for multiple testing using FDR.

Table A6.10 Enrichment GO terms for mQTL SNPs influencing probes of the significant module at birth.

GO ID	Category	GO Term	count	p	FDR
GO:0021869•	*biological process*	*forebrain ventricular zone progenitor cell division*	*46*	*4.68E-155*	*1.39E-152**
GO:0070507	biological process	regulation of microtubule cytoskeleton organization	46	7.68E-123	1.14E-120*
GO:0021799•	*biological process*	*cerebral cortex radially oriented cell migration*	*46*	*9.39E-120*	*9.33E-118**
GO:0043015	molecular function	gamma-tubulin binding	46	3.78E-111	2.81E-109*
GO:0032956	biological process	regulation of actin cytoskeleton organization	46	1.65E-104	9.82E-103*
GO:0045665•	*biological process*	*negative regulation of neuron differentiation*	*46*	*3.54E-92*	*1.76E-90**
GO:0060070	biological process	canonical Wnt signalling pathway	46	7.69E-90	3.27E-88*
GO:0090263	biological process	positive regulation of canonical Wnt signalling pathway	46	1.15E-77	4.27E-76*
GO:0007049	biological process	cell cycle	46	1.19E-75	3.95E-74*
GO:0019904	molecular function	protein domain specific binding	48	2.49E-74	7.41E-73*
GO:0003779	molecular function	actin binding	46	8.17E-57	2.21E-55*
GO:0005925	cellular component	focal adhesion	46	9.62E-49	2.39E-47*
GO:0004742	molecular function	dihydrolipoyllysine-residue acetyltransferase activity	8	2.61E-31	5.98E-30*
GO:0005967°	*cellular component*	*mitochondrial pyruvate dehydrogenase complex*	*8*	*3.73E-29*	*7.94E-28**
GO:0022829	molecular function	wide pore channel activity	12	1.40E-28	2.78E-27*
GO:0055077	molecular function	gap junction hemi-channel activity	12	7.37E-27	1.37E-25*
GO:0034214	biological process	protein hexamerization	12	5.87E-24	1.03E-22*
GO:0005921	cellular component	gap junction	12	4.26E-22	7.04E-21*
GO:0006812	biological process	cation transport	12	8.41E-22	1.32E-20*
GO:0005737	cellular component	cytoplasm	58	3.36E-20	5.00E-19*
GO:0002931†	*biological process*	*response to ischemia*	*12*	*6.84E-18*	*9.70E-17**
GO:0006086	biological process	acetyl-CoA biosynthetic process from pyruvate	8	4.29E-17	5.81E-16*
GO:0005829	cellular component	cytosol	48	1.37E-15	1.77E-14*
GO:0030431	*biological process*	*sleep†*	*8*	*3.02E-15*	*3.75E-14**
GO:0034604°	*molecular function*	*pyruvate dehydrogenase (NAD+) activity*	*8*	*6.84E-15*	*7.84E-14**
GO:0045254	cellular component	pyruvate dehydrogenase complex	8	6.84E-15	7.84E-14*
GO:0010510	biological process	regulation of acetyl-CoA biosynthetic process from pyruvate	8	2.60E-14	2.87E-13*
GO:0006090	biological process	pyruvate metabolic process	8	1.68E-11	1.79E-10*
GO:0006099	biological process	tricarboxylic acid cycle	8	7.10E-11	7.30E-10*
GO:0007267	biological process	cell-cell signaling	12	1.60E-09	1.59E-08*

GO ID	Category	GO Term	count	p	FDR
GO:0006006	biological process	glucose metabolic process	8	3.54E-07	3.29E-06*
GO:0044237	biological process	cellular metabolic process	8	2.39E-06	2.16E-05*
GO:0043209•	*cellular component*	*myelin sheath*	*8*	*2.74E-06*	*2.40E-05**
GO:0055085	biological process	transmembrane transport	12	1.10E-04	9.39E-04*
GO:0004723	molecular function	calcium-dependent protein serine/threonine phosphatase activity	2	2.35E-04	0.0019*
GO:0005515	molecular function	protein binding	51	2.37E-04	0.0019*
GO:0005955†	*cellular component*	*calcineurin complex*	*2*	*2.80E-04*	*0.0022**
GO:0005654	cellular component	nucleoplasm	2	3.13E-04	0.0024*
GO:0051533	biological process	positive regulation of NFAT protein import into nucleus	2	7.78E-04	0.0058*
GO:0033173†	*biological process*	*calcineurin-NFAT signaling cascade*	*2*	*0.001*	*0.009**
GO:0005759°	*cellular component*	*mitochondrial matrix*	*8*	*0.002*	*0.015**
GO:1900740	biological process	positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signalling pathway	2	0.012	0.082
GO:0004095	molecular function	carnitine O-palmitoyltransferase activity	1	0.022	0.148
GO:0097193	biological process	intrinsic apoptotic signalling pathway	2	0.062	0.411
GO:0006853	biological process	carnitine shuttle	1	0.094	0.606
GO:0042383	cellular component	sarcolemma	2	0.12	0.758
GO:0006470	biological process	protein dephosphorylation	2	0.131	0.81
GO:0005886	cellular component	plasma membrane	12	0.157	0.955
GO:0002223	biological process	stimulatory C-type lectin receptor signalling pathway	2	0.162	0.965
GO:0005509	molecular function	calcium ion binding	2	1	1
GO:0005516	molecular function	calmodulin binding	2	0.244	1
GO:0005739	cellular component	mitochondrion	9	0.703	1
GO:0005741	cellular component	mitochondrial outer membrane	1	0.603	1
GO:0006635	biological process	fatty acid beta-oxidation	1	0.291	1
GO:0006915	biological process	apoptotic process	2	0.59	1
GO:0012501	biological process	programmed cell death	2	0.331	1
GO:0016021	cellular component	integral component of membrane	12	0.55	1
GO:0038095	biological process	Fc-epsilon receptor signalling pathway	2	0.657	1
GO:0044255	biological process	cellular lipid metabolic process	1	1	1
GO:0044281	biological process	small molecule metabolic process	9	1	1
GO:0045087	biological process	innate immune response	2	0.329	1

GO ID	Category	GO Term	count	p	FDR
GO:0045944	biological process	positive regulation of transcription from RNA polymerase II promoter	2	0.438	1

Count indicates the number of input SNPs for eSNPO with annotations in the GO term; the p value is calculated by Fisher Exact test based on the number of SNPs for the given GO term and the number of input SNPs, as indicated in Li et al. (2016) and the FDR consists in the p-value adjustment for multiple testing of several GO pathways per SNP, calculated using "qvalue" package in R (R Core Team, 2013).

Potentially relevant probes are indicated in italic font. Underlined are the GO pathways. • indicates that the pathways involved in neurodevelopment, ° indicates the pathways involved in the mitochondrial functioning, † indicates other functional pathways potentially involved in ASD. * indicates significance after correction for multiple testing using FDR.

Analysis 4: Longitudinal analyses

Table A6.11 Effect of associations between EWAS-discovery significant probes associated with ASD and adaptive skills measured with the Vineland Adaptive Behavior Scales Composite Score.

ASD-EWAS probes	Effect at T1			Change T1–T2			Change T1–T3		
	β_1	p	FDR	β_4	p	FDR	β_5	p	FDR
cg15976650	0.0001	0.491	0.99	-0.0003	0.325	0.71	-0.0004	0.196	0.94
cg05780766	<1E-04	0.968	0.99	0.0013	0.365	0.71	-0.0019	0.231	0.94
cg20963995	0.0001	0.583	0.99	<1E-04	0.958	0.75	-0.0003	0.384	0.94
cg18317933	0.0001	0.752	0.99	-0.0006	0.180	0.71	-0.0001	0.880	0.96
cg16291048	0.0007	0.384	0.99	-0.0004	0.701	0.71	0.0006	0.579	0.94
cg03963853	0.0004	0.843	0.99	-0.0012	0.603	0.71	-0.0017	0.487	0.94
cg23367851	0.0001	0.813	0.99	0.0001	0.808	0.71	0.0002	0.646	0.94
cg14896948	<1E-04	0.952	0.99	-0.0009	0.267	0.71	-0.0006	0.457	0.94
cg10242763	0.0005	0.655	0.99	-0.0007	0.670	0.71	0.0020	0.209	0.94
cg26257814	-0.0004	0.208	0.99	0.0005	0.190	0.71	0.0004	0.323	0.94
cg13303475	0.0001	0.613	0.99	0.0001	0.589	0.71	-0.0001	0.565	0.94
cg11469137	-0.0001	0.861	0.99	0.0008	0.191	0.71	0.0001	0.925	0.96
cg13525458	0.0006	0.345	0.99	-0.0007	0.436	0.71	-0.0001	0.900	0.96
cg03724010	-0.0004	0.577	0.99	0.0007	0.465	0.71	-0.0010	0.352	0.94
cg05398769	0.0005	0.497	0.99	0.0004	0.672	0.71	-0.0011	0.292	0.94
cg07153098	<1E-04	0.973	0.99	-0.0002	0.807	0.71	-0.0005	0.630	0.94
cg03376719	-0.0001	0.857	0.99	0.0002	0.718	0.71	0.0007	0.280	0.94
cg21348771	0.0001	0.555	0.99	<1E-04	0.936	0.75	0.0001	0.475	0.94
cg08364334	-0.0006	0.577	0.99	-0.0008	0.627	0.71	0.0022	0.199	0.94
cg19046697	0.0001	0.884	0.99	0.0005	0.488	0.71	<1E-04	0.963	0.96
cg24249925	-0.0003	0.688	0.99	0.0003	0.774	0.71	0.0019	0.135	0.94
cg19320505	0.0004	0.362	0.99	-0.0001	0.838	0.71	0.0001	0.857	0.96
cg03565750	0.0002	0.738	0.99	0.0006	0.425	0.71	0.0004	0.637	0.94
cg07583091	0.0004	0.635	0.99	-0.0006	0.593	0.71	0.0004	0.704	0.94
cg14920716	0.0005	0.630	0.99	-0.0012	0.375	0.71	-0.0008	0.592	0.94
cg21929600	<1E-04	0.988	0.99	0.0001	0.854	0.71	0.0002	0.735	0.94
cg26587228	0.0008	0.432	0.99	-0.0005	0.746	0.71	-0.0020	0.189	0.94
cg20278936	<1E-04	0.893	0.99	-0.0001	0.850	0.71	0.0001	0.730	0.94
cg12944530	-0.0003	0.739	0.99	0.0007	0.538	0.71	0.0007	0.537	0.94
cg08625996	<1E-04	0.956	0.99	-0.0004	0.623	0.71	-0.0001	0.872	0.96
cg14005246	0.0001	0.626	0.99	-0.0003	0.458	0.71	0.0002	0.550	0.94
cg07926644	-0.0002	0.745	0.99	0.0005	0.434	0.71	-0.0001	0.893	0.96

p: p-value; FDR: p-value corrected for multiple testing of N=32 probes associated with ASD based on EWAS analysis, using false discovery rate method.

Table A6.12 Effect of associations between EWAS-discovery significant probes associated with ASD and peak look at the face.

ASD-EWAS probes	Effect at T1			Change T1–T2			Change T1–T3		
	β_1	p	FDR	β_4	p	FDR	β_5	p	FDR
cg15976650	-0.0006	0.889	0.99	-0.0001	0.990	0.99	0.0043	0.516	0.98
cg05780766	0.0072	0.749	0.99	0.0018	0.948	0.99	-0.0286	0.424	0.98
cg20963995	0.0020	0.683	0.99	-0.0006	0.920	0.99	0.0019	0.807	0.98
cg18317933	-0.0004	0.943	0.99	0.0071	0.349	0.95	0.0010	0.917	0.98
cg16291048	0.0110	0.462	0.99	0.0111	0.541	0.95	0.0013	0.955	0.98
cg03963853	0.0238	0.504	0.99	-0.0228	0.598	0.95	0.0042	0.936	0.98
cg23367851	-0.0003	0.968	0.99	0.0093	0.291	0.95	0.0021	0.853	0.98
cg14896948	-0.0083	0.302	0.99	-0.0105	0.283	0.95	0.0010	0.938	0.98
cg10242763	-0.0096	0.687	0.99	0.0089	0.760	0.99	0.0095	0.799	0.98
cg26257814	-0.0013	0.799	0.99	0.0034	0.583	0.95	-0.0026	0.743	0.98
cg13303475	0.0030	0.332	0.99	-0.0066	0.083	0.95	-0.0031	0.507	0.98
cg11469137	0.0004	0.954	0.99	-0.0025	0.787	0.99	-0.0075	0.536	0.98
cg13525458	-0.0065	0.594	0.99	0.0137	0.359	0.95	0.0154	0.424	0.98
cg03724010	0.0117	0.590	0.99	0.0027	0.918	0.99	-0.0203	0.498	0.98
cg05398769	-0.0109	0.516	0.99	0.0091	0.653	0.95	0.0203	0.413	0.98
cg07153098	-0.0090	0.196	0.99	0.0071	0.398	0.95	0.0021	0.845	0.98
cg03376719	-0.0007	0.939	0.99	0.0006	0.955	0.99	0.0094	0.500	0.98
cg21348771	*0.0043*	*0.044*	*0.71*	*-0.0083*	*0.003*	*0.08*[+]	*-0.0046*	*0.163*	*0.98*
cg08364334	0.0145	0.436	0.99	0.0157	0.489	0.95	-0.0488	0.104	0.98
cg19046697	-0.0001	0.994	0.99	0.0057	0.541	0.95	-0.0011	0.926	0.98
cg24249925	-0.0093	0.483	0.99	0.0128	0.430	0.95	0.0005	0.981	0.98
cg19320505	-0.0078	0.413	0.99	0.0062	0.595	0.95	0.0012	0.935	0.98
cg03565750	-0.0124	0.319	0.99	0.0017	0.909	0.99	0.0070	0.696	0.98
cg07583091	0.0024	0.868	0.99	-0.0153	0.388	0.95	-0.0181	0.431	0.98
cg14920716	0.0375	0.022	0.71	-0.0118	0.535	0.95	-0.0529	0.039	0.98
cg21929600	-0.0036	0.623	0.99	0.0080	0.374	0.95	0.0074	0.498	0.98
cg26587228	0.0193	0.341	0.99	-0.0166	0.497	0.95	0.0131	0.657	0.98
cg20278936	-0.0020	0.685	0.99	-0.0026	0.666	0.95	0.0033	0.655	0.98
cg12944530	-0.0079	0.343	0.99	-0.0020	0.843	0.99	0.0009	0.944	0.98
cg08625996	-0.0016	0.874	0.99	-0.0049	0.685	0.95	0.0048	0.762	0.98
cg14005246	0.0028	0.498	0.99	-0.0025	0.616	0.95	-0.0058	0.376	0.98
cg07926644	0.0024	0.801	0.99	-0.0092	0.425	0.95	0.0124	0.402	0.98

p: p-value; FDR: p-value corrected for multiple testing of N=32 probes associated with ASD based on EWAS analysis, using false discovery rate method.

[+] p<0.1.

Table A6.13 Effect of associations between EWAS-discovery significant probes associated with atypical development and adaptive skills measured with the Vineland Adaptive Behavior Scales Composite Score.

Atyp.Dev.-EWAS probes	Effect at T1			Change T1–T2			Change T1–T3		
	β_1	p	FDR	β_4	p	FDR	β_5	p	FDR
cg21973914	0.0006	0.523	0.85	-0.0015	0.218	0.45	-0.0013	0.315	0.90
cg15082394	0.0009	0.525	0.85	-0.0012	0.517	0.45	-0.0016	0.432	0.90
cg23281307	0.0013	0.178	0.81	-0.0009	0.516	0.45	-0.0020	0.161	0.90
cg23775991	0.0013	0.109	0.72	-0.0015	0.179	0.45	-0.0001	0.960	0.99
cg16862641	-0.0011	0.344	0.85	0.0018	0.262	0.45	0.0002	0.897	0.99
cg13919860	-0.0004	0.539	0.85	-0.0002	0.872	0.57	0.0005	0.647	0.93
cg26566415	-0.0003	0.088	0.72	0.0005	0.054	0.32	0.0005	0.049	0.81
cg09061759	0.0000	0.965	0.99	0.0001	0.855	0.57	0.0002	0.608	0.93
cg06425881	0.0001	0.871	0.99	0.0001	0.874	0.57	-0.0008	0.342	0.90
cg01257697	-0.0001	0.963	0.99	0.0019	0.514	0.45	0.0006	0.843	0.99
cg16185996	-0.0004	0.735	0.99	0.0000	0.979	0.62	0.0000	0.992	0.99
cg07152030	0.0000	0.910	0.99	-0.0004	0.436	0.45	-0.0003	0.549	0.93
cg09234567	-0.0004	0.024	0.26	0.0005	0.049	0.32	0.0004	0.116	0.90
cg05175964	0.0001	0.916	0.99	0.0003	0.767	0.57	-0.0007	0.515	0.93
cg14918743	0.0003	0.755	0.99	-0.0004	0.772	0.57	-0.0004	0.771	0.99
cg21082921	-0.0004	0.450	0.85	-0.0003	0.744	0.57	0.0005	0.510	0.93
cg05927274	-0.0013	0.472	0.85	0.0030	0.207	0.45	0.0032	0.212	0.90
cg24093411	-0.0012	0.008	0.12	0.0006	0.357	0.45	0.0012	0.080	0.88
cg00275962	0.0008	0.247	0.81	-0.0009	0.384	0.45	-0.0008	0.436	0.90
cg15727320	0.0014	0.239	0.81	-0.0027	0.096	0.40	-0.0003	0.877	0.99
cg03566107	0.0010	0.200	0.81	-0.0017	0.132	0.40	-0.0010	0.387	0.90
cg23606751	0.0000	0.993	0.99	0.0011	0.435	0.45	0.0001	0.943	0.99
cg23661183	-0.0004	0.528	0.85	-0.0007	0.414	0.45	0.0012	0.183	0.90
cg16140548	-0.0009	0.451	0.85	0.0031	0.061	0.32	0.0004	0.788	0.99
cg04729574	-0.0001	0.871	0.99	-0.0003	0.776	0.57	-0.0010	0.373	0.90
cg10633176	0.0001	0.777	0.99	0.0008	0.126	0.40	0.0000	0.924	0.99
cg10719970	0.0014	0.270	0.81	-0.0012	0.502	0.45	-0.0015	0.414	0.90
cg24574147	-0.0002	0.591	0.89	0.0005	0.263	0.45	0.0000	0.927	0.99
cg16656864	-0.0006	0.517	0.85	0.0013	0.328	0.45	0.0006	0.649	0.93
cg06963664	0.0019	0.252	0.81	0.0016	0.490	0.45	-0.0019	0.416	0.90
cg05922723	0.0003	0.762	0.99	-0.0010	0.490	0.45	-0.0017	0.279	0.90
cg07452560	0.0009	0.410	0.85	-0.0003	0.824	0.57	-0.0008	0.589	0.93
cg04089240	*0.0020*	*0.004*	*0.12*	*-0.0030*	*0.002*	*0.03**	*-0.0019*	*0.044*	*0.81*

p: p-value; FDR: p-value corrected for multiple testing of N=33 probes associated with ASD based on EWAS analysis, using false discovery rate method.

* p<0.1.

Table A6.14 Effect of associations between EWAS-discovery significant probes associated with atypical development and peak look at the face.

Atyp.Dev.-EWAS probes	Effect at T1			Change T1–T2			Change T1–T3		
	β_1	p	FDR	β_4	p	FDR	β_5	p	FDR
cg21973914	-0.0008	0.962	0.99	0.0238	0.226	0.32	-0.0117	0.642	0.99
cg15082394	0.0231	0.347	0.99	-0.0278	0.352	0.34	-0.0140	0.701	0.99
cg23281307	0.0115	0.538	0.99	-0.0041	0.856	0.39	-0.0687	0.023	0.76
cg23775991	0.0183	0.253	0.99	-0.0106	0.584	0.35	0.0097	0.692	0.99
cg16862641	-0.0013	0.955	0.99	-0.0378	0.198	0.32	-0.0005	0.989	0.99
cg13919860	-0.0033	0.777	0.99	0.0259	0.079	0.29	-0.0024	0.899	0.99
cg26566415	0.0013	0.689	0.99	-0.0047	0.248	0.32	-0.0021	0.674	0.99
cg09061759	0.0069	0.107	0.99	-0.0115	0.031	0.21	-0.0058	0.371	0.99
cg06425881	0.0093	0.413	0.99	-0.0079	0.564	0.35	-0.0020	0.907	0.99
cg01257697	-0.0275	0.511	0.99	0.0248	0.625	0.35	-0.0274	0.673	0.99
cg16185996	-0.0109	0.618	0.99	0.0602	0.030	0.21	0.0459	0.165	0.99
cg07152030	0.0040	0.623	0.99	0.0041	0.681	0.36	0.0010	0.937	0.99
cg09234567	-0.0005	0.875	0.99	-0.0013	0.738	0.37	0.0030	0.546	0.99
cg05175964	-0.0086	0.539	0.99	0.0216	0.211	0.32	-0.0034	0.873	0.99
cg14918743	0.0216	0.225	0.99	-0.0186	0.389	0.34	-0.0221	0.414	0.99
cg21082921	-0.0003	0.979	0.99	0.0027	0.852	0.39	0.0008	0.966	0.99
cg05927274	-0.0352	0.324	0.99	0.0465	0.287	0.32	-0.0085	0.879	0.99
cg24093411	-0.0008	0.925	0.99	0.0114	0.259	0.32	0.0056	0.661	0.99
cg00275962	-0.0052	0.692	0.99	0.0170	0.295	0.32	-0.0116	0.564	0.99
cg15727320	0.0112	0.626	0.99	0.0161	0.564	0.35	-0.0158	0.650	0.99
cg03566107	0.0025	0.875	0.99	0.0131	0.492	0.35	-0.0482	0.060	0.98
cg23606751	-0.0063	0.771	0.99	0.0056	0.831	0.39	-0.0040	0.906	0.99
cg23661183	-0.0061	0.619	0.99	0.0178	0.242	0.32	0.0078	0.688	0.99
cg16140548	0.0131	0.555	0.99	-0.0382	0.164	0.32	0.0170	0.616	0.99
cg04729574	-0.0131	0.416	0.99	-0.0201	0.307	0.32	0.0122	0.605	0.99
cg10633176	0.0006	0.931	0.99	0.0043	0.606	0.35	-0.0178	0.106	0.99
cg10719970	-0.0003	0.991	0.99	0.0277	0.397	0.34	-0.0180	0.650	0.99
cg24574147	-0.0043	0.512	0.99	0.0169	0.044	0.21	0.0074	0.472	0.99
cg16656864	-0.0149	0.404	0.99	0.0143	0.508	0.35	0.0074	0.793	0.99
cg06963664	0.0346	0.289	0.99	0.0132	0.737	0.37	-0.0254	0.619	0.99
cg05922723	0.0127	0.515	0.99	-0.0164	0.491	0.35	0.0141	0.640	0.99
cg07452560	0.0051	0.775	0.99	-0.0010	0.964	0.42	-0.0109	0.700	0.99
cg04089240	0.0036	0.707	0.99	0.0093	0.431	0.35	0.0038	0.801	0.99

p: p-value; FDR: p-value corrected for multiple testing of N=33 probes associated with ASD based on EWAS analysis, using false discovery rate method.