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Investigating the role of immunogenetic factors in autism spectrum conditions and traits

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Thesis for the Degree of Doctor of Philosophy in Neuroscience

Investigating the role of immunogenetic factors in autism spectrum conditions and traits

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Abstract

Autism spectrum disorder (ASD) is a relatively common and heterogeneous, neurodevelopmental condition that comes at high cost to both the individual and the society. ASD affects approximately 1.6% of the population; it is pervasive, chronic, and associated with increased morbidity and reduced life expectancy. Nonetheless, there are no effective pharmacological treatments for the core symptoms of ASD. One reason for this is that the underpinning mechanisms that drives these symptoms symptoms are poorly characterized.

Genetic factors are recognized to play an important role in ASD. The estimated heritability of ASD is 64-90%, and several genetic ASD risk factors have been identified. Collectively, these discoveries highlighted the role of genes that support different aspects of brain development such as synaptic formation and organization. However, the genetic – and (neuro)biological architecture of ASD is complex and currently identified 'risk' genes do not exhaustively explain it. Hence, it is crucial to investigate the genetics of ASD further. Particularly, attention should be drawn to genes that regulate the immune response. This is because immune genes are known to sustain neurodevelopment through diverse processes, including neuroprotection, neurogenesis, and synaptic formation and pruning. Although there is proof of the dysregulation of several immune functions, from inflammation to autoimmunity to ASD, the study of immune genes in ASD has been limited; and mainly restricted to the major histocompatibility complex genes, key regulators of adaptive immunity. The genetic regulators of the immune system are, however, multiple and they may exert different influences on brain development and thus ASD, which have yet to be examined.

Therefore, this thesis aims to explore the relationship between ASD and genetic factors involved in different immune mechanisms.

To address this aim, I carried out a number of studies using complementary analytic strategies.

First, I conducted a systematic literature review to gain a comprehensive overview of known immune genes that have been associated to ASD (described in **Chapter 3)**. This study laid the groundwork for my investigation since it supported a role of immune genes in ASD and their relevance to general neurodevelopment. Also, this review highlighted the need for a deeper investigation of immune genes in ASD, considering genetic factors involved in several types of immune responses.

Second, given the increasing evidence to support a genetic association between ASD and autistic-like traits in the general population, I explored the genetic underpinnings of these population-based traits. Specifically, I performed a hypothesis-free genome-wide association study meta-analysis of four autistic-like traits across multiple international cohorts (presented in **Chapter 4).** Strikingly, the top results from this hypothesis-free approach all pointed to immune-related genes and I discovered an association between immunogenetic factors and specific autistic features (i.e., rigidity and attention to details) in the general population.

Taken together, these studies provided preliminary evidence for a role of immune genes in ASD - both at a diagnostic level and at the level of population-based quantitative traits.

Therefore, in my third study I further explored the genetic relationship between ASD and both innate and adaptive forms of immune dysregulations. In brief, I investigated 1) if ASD and diverse immune-related conditions, including inflammatory, allergic, and autoimmune diseases, share genetic factors; and 2) if individual variations in genetic factors linked to these immune conditions are also associated with variation in autistic-like traits in individuals of the general populations and across sex groups. In this study (described in **Chapter 5)**, I identified significant genetic correlations between ASD and allergic and systemic autoimmunity, at both the genome-wide and local genomic level. The genetic factors underlying these correlations show increased expression in both immune and brain tissues, and an association with epigenetic changes during

neurodevelopment. Also, I demonstrated that individual variations in autoimmunity-related genetic factors are associated with rigidity in the general population.

Driven by the population-based findings presented in Chapter 5, in my fourth study I investigated if - and how - individual variations in identified immunogenetic factors are linked to different symptoms and cognitive domains in a clinical sample of ASD. To do this, I leveraged genetic and deep phenotypic data from the Longitudinal European Autism Project (LEAP). In this study, described in **Chapter 6**, I identified an association between genetic regulators linked to lymphocytic count and the worsening of repetitive and restricted behaviors over time.

In summary, my thesis supports a neurodevelopmental role of immune genes and their relevance to ASD. My findings demonstrate a role of genes that regulate autoimmune and allergic responses in ASD. Also, my work provides preliminary evidence that genetic factors involved in autoimmunity are linked specifically to the rigid aspect of the autistic phenotype, in the general population. This association was also observed in a clinical sample of ASD. Taken together my findings encourage the study of immune genes with respect to specific (i.e., rigid) features of the complex autistic phenotype. Taking a step further, this work invites to extend immunogenetic investigation to other neurodevelopmental diagnostic categories, and in relation to several neurodevelopmental windows and neural systems.

To my parents

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If you ask any student to describe their PhD journey in a word, I am confident you will get the most disparate answers.

To me, the first word that comes to mind is 'numbers'. From day one, my PhD has been filled with numbers. To start from the number of times I tested positive to COVID-19 and to continue with the number of zoom meetings I attended in my pyjamas. From the number of analyses that I ran because they were *totally* reasonable, to the number of analyses that turned out to make no sense *at all*. From the number of versions, I wrote of the same manuscript, to the number of houses I changed in between drafts. Still, none of these equal the number of people I am thankful to.

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The views expressed in this thesis are my own and not necessarily those of the Sackler family, or EU-AIMS/AIMS-2 Trials.

Declaration of contribution

The work presented in this thesis would not have been possible without the contribution of several people

JB – Dr Janita Bralten
GMA – Prof Grainne McAlonan
RT – Prof Ryad Tamouza
RM – Rugile Malatiuchive
DGM – Prof Declan G.M Murphy
GF – Giuseppe Fanelli
ML – Prof Marion Leboyer
CMP – Dr Charlotte Marie Pretzsch

Chapter 3 : MA and RM performed the study selection. MA performed the analysis and the qualitative synthesis of studies under the supervision of DGM, JB, and GMA. RM, ML provided important suggestions and helped to prepare the manuscript for submission.
Chapter 4 : MA and JB conceived and designed the study. MA performed the analyses and written the manuscript under the supervision of JB. The manuscript also received important feedback from the co-authors as listed in the relevant publication (pag. 129.)
Chapter 5 : MA conceived the study, performed the analyses under the supervision of JB, DGM and GMA. GF also contributed with insightful inputs to the study.
Chapter 6 : MA conceived the study, performed the analyses under the supervision of JB, DGM and GMA. CMP supported in the data preparation and provided valuable suggestions.

I declare that I wrote this thesis and that it was critically evaluated by DGM, JB, and GMA.

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1. Chapter 1: Introduction

This thesis addresses the immunogenetic underpinnings of autism spectrum disorders (ASD), a complex and highly heritable neurodevelopmental condition. Therefore, in this introductory chapter, I provide the theoretical and scientific rationale for studying immunogenetic factors in ASD.

In the first part of this introduction, I describe ASD; I introduce its core clinical features, and how these manifest heterogeneously and to what extent they affect the individual and the society. I highlight the need for research on potential pathophysiological mechanisms that may help the search for new treatments for autistic symptoms. In this regard, I emphasize the role of genetic factors in ASD; and how genetic research on ASD – defined clinically and dimensionally as population-based autistic-like traits – can help to identify the underlying – and potentially druggable – mechanisms. In particular, I introduce immune genes whose role in ASD needs to be explored.

In the second part of this introduction, I explain why it is important to study immune genes in ASD. Hence, I give an overview of the immune system and its complex genetic architecture; and I describe the evidence to support dysregulations across several immune mechanisms in ASD – which may (in part) be explained by variations in immune genes.

I conclude by describing my strategy to investigate the potential genetic relationship between ASD and the immune system; and I present my research questions and the analytical approaches I adopted to address each of these.

1.4. Autism spectrum disorders (ASD)

ASD is a neurodevelopmental condition characterised by core symptoms in social and non-social domains. Social symptoms include atypical social communication and interaction, whereas non-social symptoms refer to restricted and repetitive patterns of behaviours. These symptoms are usually present in early life and become fully manifested when social demand increases (e.g., during school). Also, these symptoms must cause significant impairment in personal and social functioning and must not be explained by any intellectual disability or developmental delay (American Psychiatric Association, 2013).

1.4.1. Clinical heterogeneity

The clinical presentation of ASD is heterogeneous. According to the Diagnostic and Statistical Manual of Mental disorders, fifth edition (DSM-5) (American Psychiatric Association, 2013), social deficits may present in the form of impaired social-emotional reciprocity, non-verbal communication and/or interpersonal relationship (Table 1). Restricted, repetitive behaviours may include stereotyped motor movements, insistence on sameness, fixated interests and/or atypical sensory processing. These core features can be measured dimensionally and fall along a continuum of severity. For example, social and non-social symptoms may require minimal to substantial support.

Table 1 Diagnostic definition of autism spectrum disorder according to the Diagnostic and Statistical Manual of Mental disorders, 5th edition.

Diagnostic criteria for autism spectrum disorder (ASD)

A. Persistent deficits in social communication and social interaction cross multiple contexts, as manifested by the following, currently or by history

1. Deficits in social-emotional reciprocity, ranging, for example, from abnormal social approach and failure of normal back-and-forth conversation; to reduce sharing of interests, emotions, or affect; to failure to initiate or respond to social interactions.
2. Deficits in nonverbal communicative behaviours used for social interaction, ranging, for example, from poorly integrated verbal and nonverbal communication; to abnormalities in eye contact and body language or deficits in understanding and use of gestures; to a total lack of facial expressions and nonverbal communication.
3. Deficits in developing, maintaining, and understanding relationships, ranging, for example, from difficulties adjusting behaviour to suit various social contexts; to difficulties in sharing imaginative play or in making friends; to absence of interest in peers.

Specify current severity:

Severity is based on social communication impairments and restricted, repetitive patterns of behaviour. For either criterion, severity is described in 3 levels: Level 3 – requires very substantial support, Level 2 – Requires substantial support, and Level 1 – requires support.

B. Restricted, repetitive patterns of behaviour, interests, or activities, as manifested by at least two of the following, currently or by history

1. Stereotyped or repetitive motor movements, use of objects, or speech (e.g., simple motor stereotypes, lining up toys or flipping objects, echolalia, idiosyncratic phrases).
2. Insistence on sameness, inflexible adherence to routines, or ritualized patterns of verbal or nonverbal behaviour (e.g., extreme distress at small changes, difficulties with transitions, rigid thinking patterns, greeting rituals, need to take same route or eat same food every day).
3. Highly restricted, fixated interests that are abnormal in intensity or focus (e.g., strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interests).
4. Hyper- or hypo reactivity to sensory input or unusual interest in sensory aspects of the environment (e.g., apparent indifference to pain/temperature, adverse response to specific sounds or textures,

excessive smelling or touching of objects, visual fascination with lights or movement).

Specify current severity:

Severity is based on social communication impairments and restricted, repetitive patterns of behaviour. For either criterion, severity is described in 3 levels: Level 3 – requires very substantial support, Level 2 – Requires substantial support, and Level 1 – requires support.

C. Symptoms must be present in the early developmental period (but may not become fully manifest until social demands exceed limited capacities or may be masked by learned strategies in later life).
D. Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.
E. These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay. Intellectual disability and autism spectrum disorder frequently co-occur; to make comorbid diagnoses of autism spectrum disorder and intellectual disability, social communication should be below that expected for general developmental level.

Note: Individuals with a well-established DSM-IV diagnosis of autistic disorder, Asperger's disorder, or pervasive developmental disorder not otherwise specified should be given the diagnosis of autism spectrum disorder. Individuals who have marked deficits in social communication, but whose symptoms do not otherwise meet criteria for autism spectrum disorder, should be evaluated for social (pragmatic) communication disorder.

Specify if:

With or without accompanying intellectual impairment

With or without accompanying language impairment

The idea of symptoms as dimensions of impairments also underpins the conceptualization of ASD as a spectrum of disorders in the DSM-5. The diagnostic definition of ASD in the DSM-5 replaces and integrates the previous diagnoses of autistic disorder, Asperger's disorder and pervasive developmental disorder-not otherwise specified (which were characterised by different degrees of social and cognitive impairments). For instance, Asperger's disorder was defined by lack of non-verbal communication skills, significant social difficulties, along with inflexible adherence to

routines. The diagnosis of Asperger's disorder did not encompass language and cognitive impairment, and diagnosed individuals often displayed above average intellect. Conversely, autistic disorders was defined by social impairment and additional cognitive and language impairment (Figure 1). In summary, clinical heterogeneity is intrinsic to the definition of ASD as a spectrum of conditions.

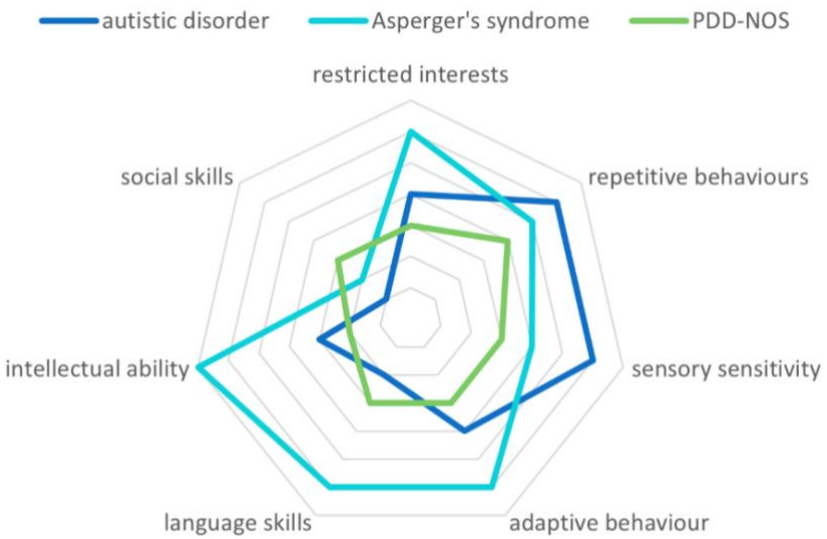

Figure 1 Illustrative representation of clinical heterogeneity of conditions across the autism spectrum. Each autistic condition is coded with a different colour. In the plot, each axis refers to a domain of impairment and the centre of the plot represents the 'zero' value for each domain. The variability across domain is captured by the shape of the polygons, which are coloured to represent the different autistic conditions. Each vertex of the coloured polygons informs about the quantity of a behavioural traits: the closer the edge of the chart a vertex is, the higher the quantity of a traits. Here, we provide an illustrative example of how domains may differ across autistic spectrum conditions, which are generally characterised by lower social skills, and variable levels of adaptive and intellectual abilities.

One further source of clinical heterogeneity refers to the presence of additional psychiatric and non-psychiatric conditions (Lai et al., 2014). Autistic individuals commonly experience: i) mental health problems such as anxiety (in 42-56% of cases) and mood disorders (12-70%), conduct

disorders (16-28%) and attention-deficit hyperactivity disorder (28-44%); and ii) physical health problems, including epilepsy (8-30%) and systemic immune dysregulations, especially allergic and autoimmune disturbances (<38%).

Notably, the adoption of the broad diagnostic definition of ASD – together with increased awareness and changes in service availability – is thought to contribute to a surge in the prevalence of ASD over the years.

1.4.2. Epidemiology

ASD is one of the most common neurodevelopmental conditions and is currently diagnosed approximately in 1.4% of the population (Chiarotti & Venerosi, 2020). This prevalence has been sharply increasing over the last decade. For instance, a 787% exponential increase in the incidence of autism diagnoses has been registered in the UK between 2008 and 2018 (Russell et al., 2022).

ASD occurs in all racial, ethnical, and socioeconomic groups; however, some differences may exist across countries. For example, it has been reported that ASD affects 0.7-1% of the European population, whereas it is diagnosed in 1.5% of the US population (Chiarotti & Venerosi, 2020). Moreover, ASD is diagnosed up to four times more often in men than women (Werling & Geschwind, 2013). Recent estimates, however, indicate an increase in the diagnoses of ASD in women (i.e., 3:1 male to female ratio), potentially due to the recognition of male biases in clinical settings (Russell et al., 2022).

The increasing prevalence of ASD is of concern, and this is because ASD has considerable impact on the individual and the society.

1.4.3. Personal and public health impact

ASD is one of the most serious neurodevelopmental conditions, with significant repercussions on personal functioning and well-being (van Heijst & Geurts, 2015). From early life, difficulties in social communication affect the formation and maintenance of interpersonal relationships with peers and caregivers. These challenges start in childhood and often continue into adulthood (Billstedt et al., 2011), and they have been associated with mood dysregulations and reduced life expectancy (Oakley et al., 2021; Smith DaWalt et al., 2019). Moreover, the cognitive difficulties experienced by individuals on the spectrum impact educational attainment and success at work. For example, the rate of employment among autistic individuals has been estimated to be only 20% (Chen et al., 2015).

ASD is also associated with a remarkable caregiver, family, and financial burden. For instance, the level of caregiver burden in ASD is comparable to that reported by persons caring for individuals with a brain injury (Cadman et al., 2012). Also, ASD incurs high financial and health care costs, that range between 1-2£ million yearly per individual in the UK and in the US respectively (Rogge & Janssen, 2019).

1.4.4. Treatment options

The high – and increasing - prevalence of diagnosed ASD, together with the impact of ASD on the individual and society, demonstrate how urgent it is to find ways to manage and ameliorate autistic symptoms – and associated challenges. Currently, the symptoms and behavioural problems experienced by autistic people are mainly targeted by behavioural and educational programs (Warren et al., 2011). Although these programs proved effective in some cases, they are often expensive and learned skills are usually difficult to 'generalise' – i.e., implement outside the

therapeutic setting (Warren et al., 2011). In addition, pharmacological therapies are often used to treat the associated clinical features of ASD (Loth et al., 2016). For example, second-generation antipsychotics, like risperidone and aripiprazole, are used to treat irritability and anxiety.

Conversely, there are currently no proven pharmacological treatments for the core symptoms of ASD (Loth et al., 2016). The reasons for this are several. First, the mechanisms underpinning the core features of ASD are poorly understood. Second, due to the clinical heterogeneity of ASD, a given pharmacological treatment may suit individuals with certain clinical profiles more than others.

Therefore, it is crucial to address these issues and 1) research pathophysiological – and potentially druggable - mechanisms for ASD; and 2) investigate these mechanisms in relation to specific clinical domains of impairments to ultimately inform personalised treatment approaches.

Since ASD has been shown to run in families, with potential genetic mechanisms involved, one way to do is through genetic investigation.

1.4.5. Genetic underpinnings

ASD is one of most heritable common neurodevelopmental disorders. Twin-based studies estimated that the heritability of ASD is 70-90% (Tick et al., 2016). The genetic architecture of ASD is complex, being polygenic and multifactorial. ASD results from the interaction of both rare and common genetic variants, along with environmental factors (Gaugler et al., 2014) (Figure 2).

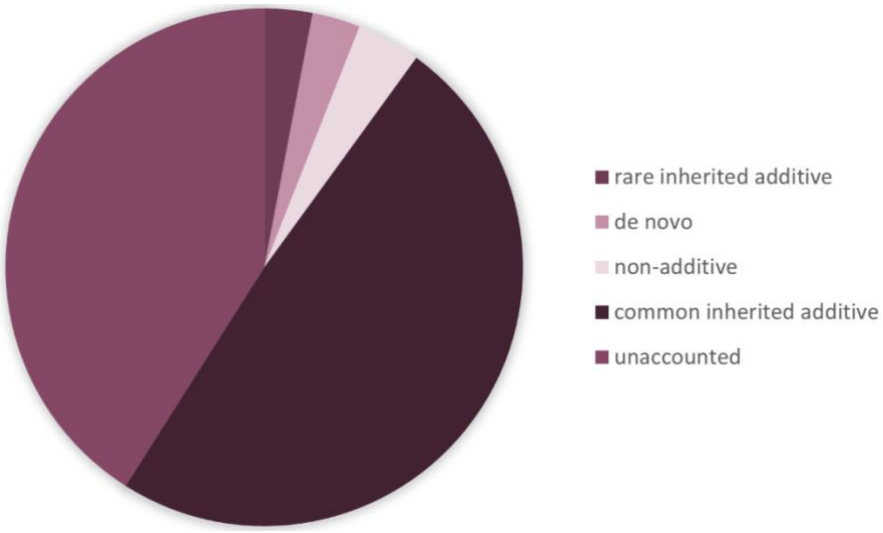

Figure 2 The genetic architecture of ASD includes both rare, de-novo and common genetic variations. Common variants play a major role, nevertheless large portion of variability remains unaccounted for. Figure adapted from Gaugler et al., 2014.

The study of rare and common genetic risk factors has provided important insights on the pathophysiology of ASD (Antaki et al., 2022; Grove et al., 2019; Rylaarsdam & Guemez-Gamboa, 2019; Satterstrom et al., 2020). Rare and common genetic research in ASD, respectively, identified several genes currently defined as ASD 'risk' genes (https://gene.sfari.org/database/human-gene/); and they collectively highlight a key role of genes regulating neurodevelopmental processes which I describe in the following paragraphs.

1.4.5.1. Rare genetic variants

Rare variants (occurring in less than 1% of the general population) are estimated to explain more than 6% of ASD heritability (Gaugler et al., 2014). Those linked to ASD include frameshift variations (e.g., nucleotide insertions or deletions), which may either alter or truncate protein translation (e.g., nonsynonymous, non-sense), hence driving a cascade of biochemical changes; or, have no translational effect (synonymous). Strong genetic risk for ASD is also conferred by copy

number variations (CNVs) consisting of deletions, duplications, translocations, and inversions of different chromosomal structures (e.g., 15q11-q13, 16p11, 22ql1-13, Xp22) (Leppa et al., 2016; Rylaarsdam & Guemez-Gamboa, 2019). These rare variants are in part inherited (3%) and therefore under strong selective pressure. However, a significant portion of rare variants in ASD occur de-novo in the germline (3%), meaning that both parents do not carry the genetic variant (Gaugler et al., 2014).

The gold standard methodologies to identify rare variants are whole genome and whole exome (protein coding regions for the genome) sequencing analyses. These studies have been carried out in samples of ~ 5,000-10,000, where the probability of detecting rare variations is increased, although larger sample size remain warranted. The largest exome sequencing study to date yielded the discovery of 53 genes carrying disruptive de-novo ASD-risk variants and demonstrated that ASD is linked to the disruption of genes regulating: i) neuronal communication, such as those controlling synaptic formation (*SHANK3, synGAP, SYNs*) and signalling, including sodium-calcium- potassium- voltage-gated channel genes (*SCN2A, CACNAs, KCNQ3*), and glutamate and GABAergic receptor genes (*GRIN2, GABRBs*); ii) neuronal and glial organization (cytoskeleton) (*DPYSL2, DYRK1A, GFAP, MAP1A)*; and iii) transcriptional processes including chromatin remodelling (*CHD8, CHD2*) and histone methylation (*KDM5B* (Satterstrom et al., 2020)). These genes have neurodevelopmental relevance and functional analyses of these demonstrated their predominant expression in early neurodevelopment (and especially in new-born excitatory, and inhibitory neurons) (Grove et al., 2019; Lee et al., 2019). Hence, there is recognition of the influence of rare variants on ASD – however these genetic factors explain only a small fraction of heritability when compared to the involvement of common genetic variants (Gaugler et al., 2014).

1.4.5.2. Common genetic variants

Common genetic variants, i.e., single nucleotide polymorphisms (SNPs) with minor allele frequency > 1% in the general population, play a major role in ASD. SNPs have been estimated to explain additively up to 49% of ASD heritability (Gaugler et al., 2014) This, therefore, encouraged numerous prior studies to investigate the associations of common genetic variants to ASD.

The 'gold standard' approach to identify common variants for ASD is generally accepted to be the case-control genome-wide association study (GWAS) (Tam et al., 2019). In these studies, the distribution of millions of SNPs between cases and controls is tested simultaneously and therefore large sample sizes are required to detect true genetic risk factors, while minimising the rate of false discoveries. To date, the largest case-control GWASs of ASD (N =46,350-55,420) led to the identification of several common genetic risk factors for ASD, of which five "top" SNPs survived stringent multiple comparison corrections ($p < 5x10\text{-}8$) (Grove et al., 2019; Matoba et al., 2020). The functional analyses of the "top" SNPs highlighted a role of genes regulating key neurodevelopmental processes in ASD, in concordance with the rare variant findings discussed above. For example, the "top" SNPs map to genes that regulate neuronal outgrowth (*NEGR1*), transcription (*KMT2E*) and splicing during neuronal formation and differentiation (*PTBP2*), synaptic transmission (*CADPS, KCNN2*) and kinase activity (*MACROD2*). A role of neurodevelopmental genes has been further supported by the analysis of SNPs associated with ASD at different levels of significance ($p > 5x10\text{-}8$). For instance, these SNPs were overrepresented in genes that: i) are expressed in progenitor cells; ii) regulate transcription in the developing brain; and iii) influence neuronal communication, like genes controlling calcium signalling at synapses (Grove et al., 2019).

These prior GWAS studies were important first steps to gain insights on polygenic and multifactorial nature of ASD. However, they also highlighted challenges to the identification of

genetic risk factors for ASD. One key challenge is genetic heterogeneity among autistic individuals, which likely accounts for heterogeneity in their clinical profiles. For example, Grove et al., indicated that individuals belonging to different clinical subgroups (e.g., childhood autism, Asperger's disorder, atypical autism) vary in their load of common genetic risk variants for ASD, captured by a so-called 'polygenic score' (Grove et al., 2019). These clinical subtypes also differ in their genetic liability to other psychiatric conditions or cognitive impairment. In addition, recent work from other groups demonstrated that genetic heterogeneity also exists between sexes in ASD (Antaki et al., 2022). For example, they reported that autistic women have a higher polygenic score for ASD - suggesting that they have a higher genetic threshold for liability than autistic men. These studies provided evidence for quantitative differences in the genetic liability across subgroups (defined by clinical symptoms and/or sex) in ASD. However, it is also possible that these groups vary in the *type* of genetic variations they carry. Hence, it is crucial to address the issue of heterogeneity when studying the genetics of ASD, and this can be done, for example, by investigating the genetics of distinct autistic symptoms or traits.

1.4.5.3. Common variants in the general population

One way to investigate the genetics of ASD, which takes into account its heterogeneity, is to investigate specific autistic symptoms or domains separately. As shown in Table 1, the DSM-5 defines ASD by impairments across different clinical dimensions. It is now recognised that variations in these "autistic dimensions" follow a continuous distribution; and that they are present to some degree – also sub clinically - in all of us (Lundström, 2012). Moreover, there is increasing evidence that common genetic variants that increase liability for ASD are also important to determine variability along the continuum of so-called "autistic-like traits" in the general population (Figure 3) (Bralten et al., 2018). To prove this, variations across autistic traits in individuals within the general population have been associated with their polygenic risk score for

ASD. These findings are of importance because they indicate that population-based genetics can tell us something about the genetics of ASD, which means that we can leverage population-based samples, being those far larger and easier to access than clinical cohorts; hence, with these large cohorts, we can increase the probability of identifying novel common genetic risk factors. These findings also imply that we are able to investigate specific autistic trait separately to identify trait-specific genetic factors, whose effects would otherwise potentially be diluted when ASD is investigated categorically.

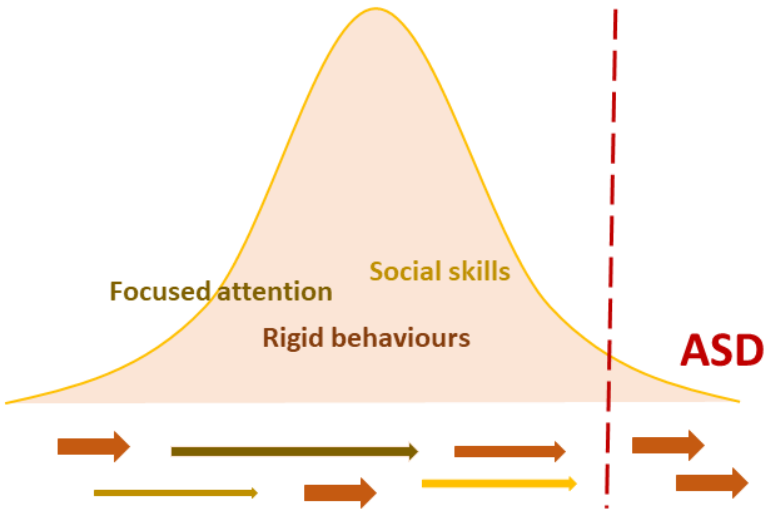

Figure 3 Continuous distribution of autistic traits in the general population. The level of autistic traits is represented on the x-axis, whereas the y-axis refers to the density. The arrows (bottom) of varying colour and size represent multiple diverse genetic factors of different effect size (large vs small) that influence the level of autistic traits in the population. The dotted line represents the diagnostic threshold, and beyond this, individuals are likely to receive a diagnosis of ASD.

In conclusion, genetic research in ASD can be key to disentangling its pathophysiology and delineating potential therapeutic targets. Research on common genetic variants plays a critical part because these genetic factors i) are major contributors to ASD, and ii) can be measured in both

the general population and in relation to specific autistic features. Nevertheless, the common genetic variations that have been identified to date only explain 11.8% of ASD heritability (Grove et al., 2019), and this highlights the need for further work. To do so, we must build on recent progress and explore in depth further genetic regulators of brain development. In the following section, I introduce immune genes as potentially suggestive study candidate and whose role in neurodevelopment and hence ASD awaits clarification.

1.4.6. Immune genes as potential risk factors

Although genes that support brain development are important for ASD, the influence of some of these genes in ASD has not been fully characterised. This is especially the case with immune genes. It is known that immune processes modulate brain development and impact the attainment of major neurodevelopment milestones (Cowan & Petri, 2018; Elmer & McAllister, 2012; Tamouza et al., 2021). This knowledge is mainly based on animal studies which demonstrate that microglia – the immune cells resident in the brain – and astrocytes regulate the vascularization of the brain and protect it from external threats at the blood brain barrier (da Fonseca et al., 2014). Moreover, in animals, microglia cells control neuronal differentiation and survival by phagocytosis and help develop neural communication, by controlling axonal projection and the formation of functional synapses, while inducing pruning of obsolete synapses (Cowan & Petri, 2018).

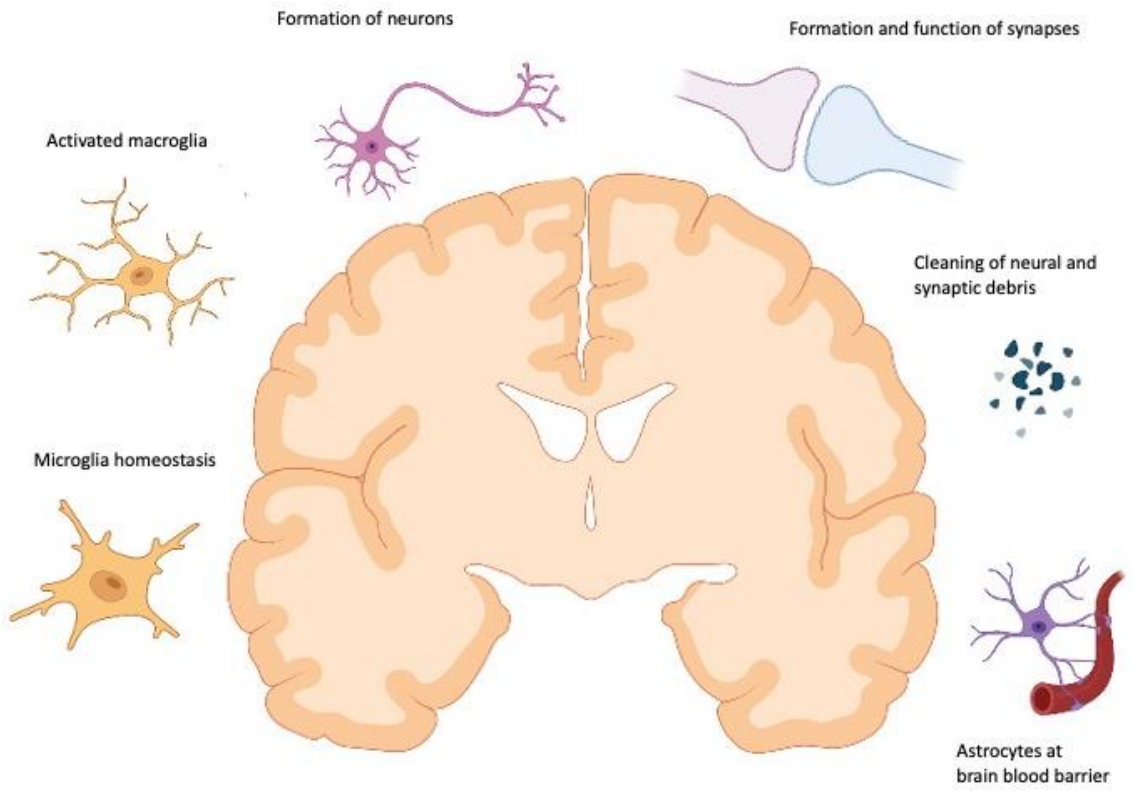

Figure 4 Illustration of immune processes regulating neuronal and synaptic formation and signalling, removal of resulting debris and shielding the brain from external threats.

The role of these immune processes in human brain development is less clear. Nevertheless, it is recognised that these immune-mediated mechanisms, from microglial activation to immune defence, are tightly regulated by immunogenetic factors (Cai et al., 2022). For example, key immune genes are major histocompatibility (MHC) genes and complement family genes (e.g., C4) which control antigen presentation during infections, apoptosis, and immune tolerance (Tamouza et al., 2021). Also, these MHC genes are widely expressed at both pre and post-synaptic interfaces and have been thus implicated in the regulation of synaptic organisation, including activity-dependent and homeostatic plasticity at the synapses; they also control the pruning of obsolete synapses through their interaction with microglial cells. Notably, variations in these MHC genes have been reported in studies of both common and rare genetic variants in ASD. Recent work identified *de-*

novo variants in MHC genes in a Chinese cohort of autistic children (Cai et al., 2022). Additionally, the latest ASD GWAS indicated that common risk variants for ASD are enriched in MCH genes and are significantly expressed in immune cells (Grove et al., 2019). There is also increasing evidence of dysregulation in the expression of these immune genes in ASD (Gandal et al., 2018; Lombardo et al., 2017). For instance, there are reports of increased expression of genes regulating microglia activation and inflammation in the peripheral blood of autistic children and in the post-mortem brain of autistic individuals.

These findings were important first steps as they demonstrate that research on immune genes may help elucidating the pathophysiology of ASD. However, to better clarify the role played by the immune system/genes, we need to investigate a wider range of immune processes.

Hence, in the second part of this introduction, I will 1) give an overview of the immune system and its constitutive mechanisms; 2) introduce the genetic factors regulating these immune mechanisms; and 3) describe evidence that links specific immune mechanisms - and related genetics - to ASD.

1.5. The immune system

The immune system is a system of cells, tissues and molecules that respond to potentially harmful microorganisms (e.g., viruses, bacteria) encountered by an individual through life (Chaplin, 2010a) (Figure 5). It is organised into two main branches, one innate and one adaptive immune component, and which are both in turn divided into humoral and cellular immunity. The innate immune system initiates a fast response that is unspecific to the encountered pathogens, and it is not memorised by the immune effector cells/molecules. Mediators of innate immunity include humoral molecules (like pattern-recognition receptors, cytokines and complement proteins) and effector cells (like phagocytes and natural killer cells). In contrast, the adaptive immune system is necessary for building a targeted immune response that is highly specific to a given pathogen. This adaptive immune response requires time to form but it is preserved as immunological memory for several months (e.g., five to nine months). The components of adaptive immunity include humoral factors, specifically antibodies (immunoglobulins) and cytokines, and cells, like B and T lymphocytes, as well as CD4 T helper and CD8 cytotoxic cells.

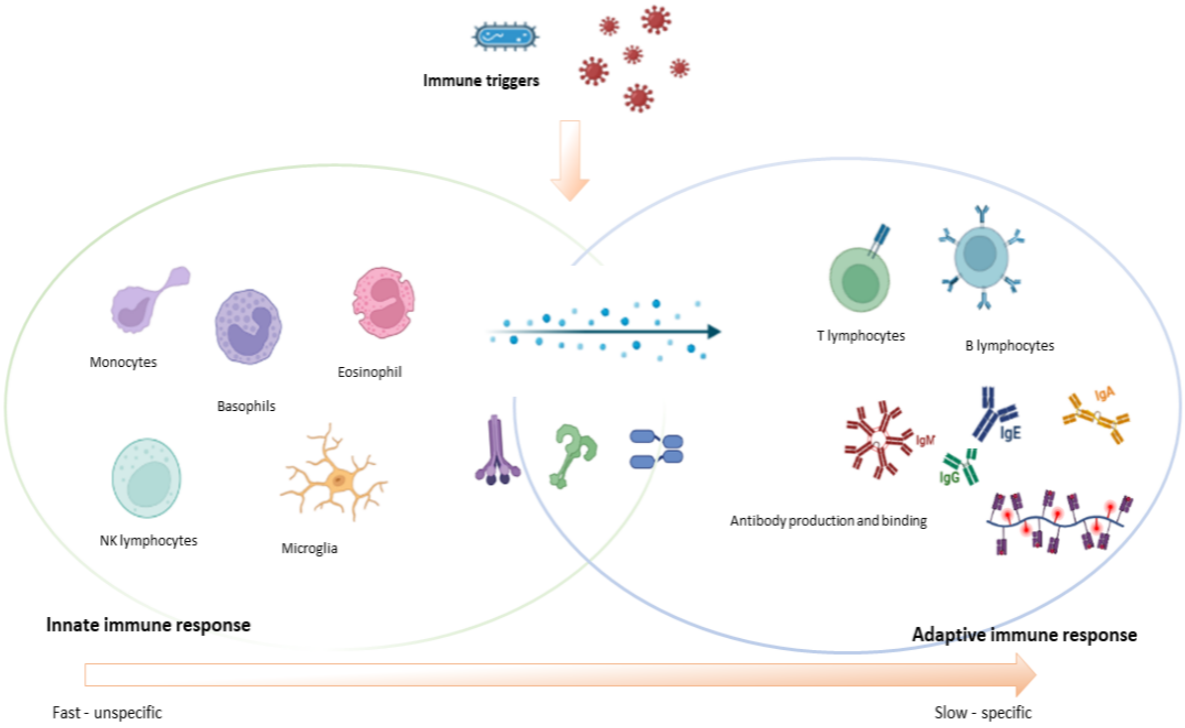

Figure 5 Illustrative organization of the immune system. From the left, some of the cellular and molecular regulators of the innate, unspecific immune response; to some of the cellular and molecular regulators of the adaptive, specific immune response on the right.

1.5.1. Genetics of immunity

Both the innate and adaptive immune response are tightly regulated by genetic factors (Knight, 2013) (Figure 6). For instance, an innate immune response is initiated when pattern-recognition receptors (PRR) (e.g., toll-like receptors) on innate effector cells recognise pathogen-associated molecular patterns on the pathogen cells. This molecular matching leads to the activation of several genetic mechanisms and transcription factors which are necessary to stimulate cytokine production and to induce an inflammatory response. The genes that support the innate immune response

include toll-like receptor genes, NF-KB pathway genes, MAPK pathway genes, oxidative response genes (*RAS, NOS*), interleukin signalling genes, complement system genes, and transcription factor genes (like *FOXP3, GATA3, STAT4, IRF*s). On the other hand, the adaptive immune response begins with the presentation of antigens and their recognition by antibodies and receptors on B and T lymphocytes. This, in turn, triggers antibody-mediated and cell-mediated immune responses, which result in complement activation, cytokine response and pathogen neutralisation. All these steps in adaptive immunity are regulated by specific immune genes. To start, antigens are presented by molecules encoded by major histocompatibility complex (MHC) genes on chromosome 6 (Shiina et al., 2009). The MHC genes comprise more than 100 genes (and pseudogenes) grouped into three functional classes: class I, class II, and class III. Class I and class II MHC genes support antigen presentation to (respectively) cytotoxic T-cells (CD8) and helper T cells (CD4), which then activate B cells. The function of class III MHC genes is less defined but thought to be mainly immunomodulatory. The MHC genes also have the highest number of alleles (> 3,000 different alleles), which is necessary for fine-tuning the adaptive response and producing antigens that are specific to a wide range of pathogens. Antigen presentation and binding is also regulated by killer-cell immunoglobulin-like receptor (KIR) genes and modulated by complement system genes (C4, C5). Other genetic regulators of adaptive immunity include chemokine and cytokine pathways genes, mTOR pathway genes, tyrosine kinase genes (Figure 6).

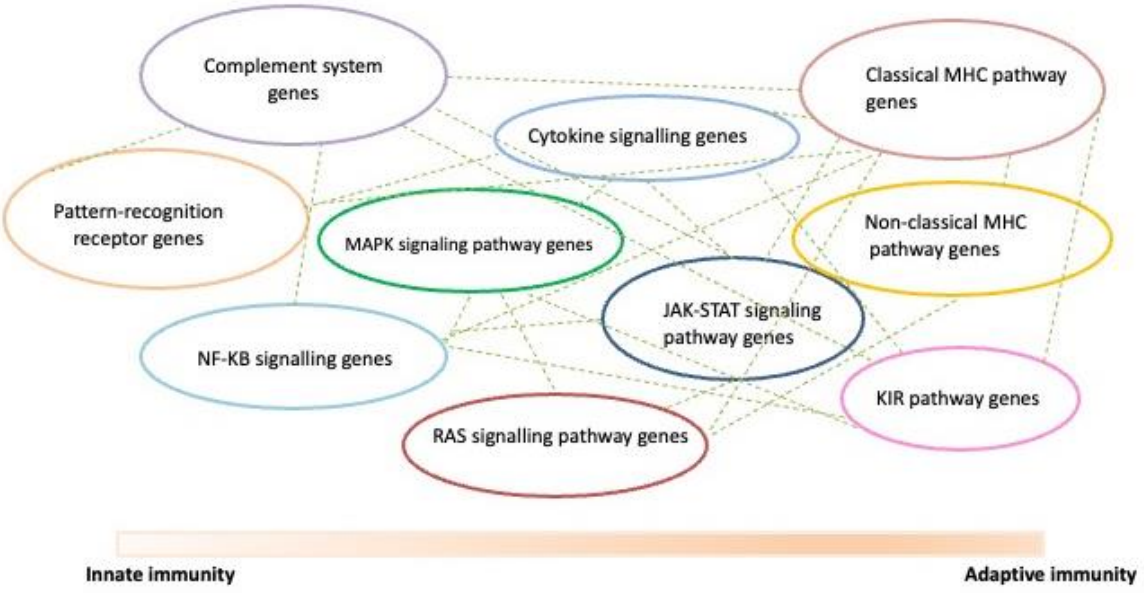

Figure 6 Illustrative representation of some of the networks of genes that regulate the innate and adaptive immune response, based on literature (Knight, 2013).

Depending on the class of immune genes considered, a range of genetic variations may compromise immune functioning and predispose the individual to immune diseases. Several genetic risk factors for immune pathologies have been identified in the entire genome by the study of rare immunodeficiency syndromes and GWASs of immune-related conditions (Hu & Daly, 2012; Knight, 2013; Zhu et al., 2018) (Figure 7). For instance, variations in genes regulating macrophage response or complement system genes may trigger an aberrant innate response and lead to immunodeficiency syndromes (Knight, 2013). In addition, variations in MHC genes have been implicated in both innate and adaptive immunity, and specifically to a failure of self-tolerance, thus promoting autoimmune diseases. Autoimmunity has been also linked to variations in genetic factors regulating B-cell responses and the functioning of T cells including T helper 1 and T helper 17 responses (Alvaro-Benito et al., 2016; Chaplin, 2010a; Hu & Daly, 2012; Wahren-Herlenius & Dörner, 2013). Moreover, genes regulating innate immunity (e.g., eosinophil activity), antigen

responses (immunoglobulin E) and the T helper 2 cell responses have been implicated in allergic diseases (Ashley et al., 2017; Chaplin, 2010b; Zhu et al., 2018).

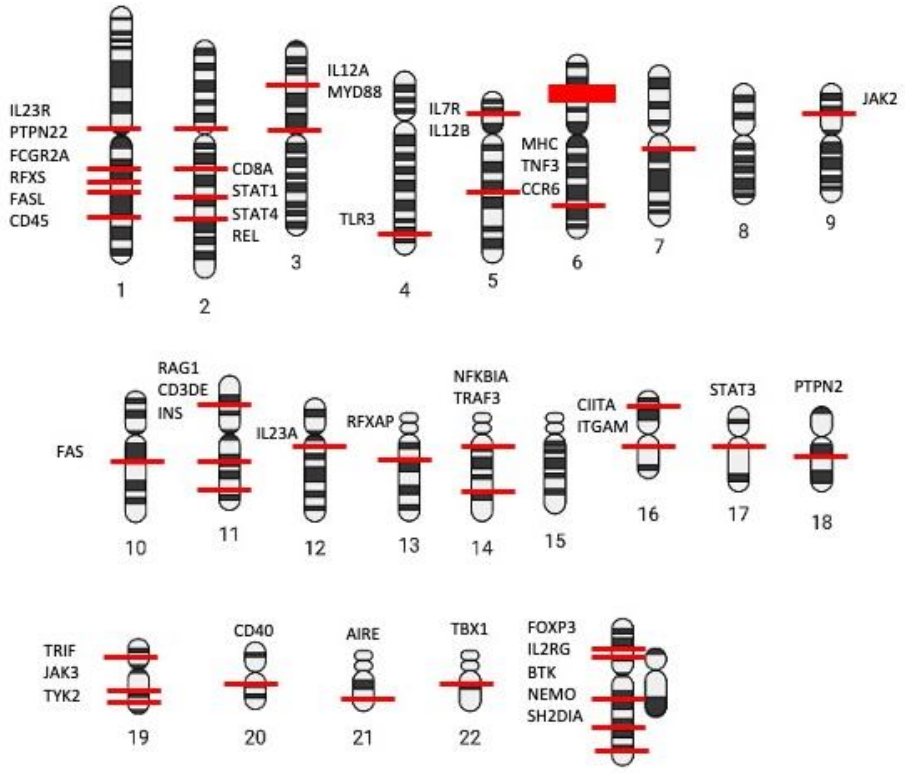

Figure 7 Genetic factors implicated in the risk for rare immunodeficiency syndromes and common autoimmune diseases. Figure adapted by Knight, 2013

In summary, the genetic landscape of immunity is broad, complex, and heterogeneous. It is unclear if – and/or which – immunogenetic mechanisms are relevant to ASD. Nonetheless, there is evidence from epidemiological studies that link ASD with dysfunctions in multiple immune processes, including autoimmunity, which hint to the role of immune genes controlling these processes (Atladóttir et al., 2009)(Vinet et al., 2015).

1.5.2. Immune dysregulations in autistic individuals and their families

Dysregulations of both innate and adaptive immunity are common in ASD (Hughes et al., 2018). Dysregulations of innate immunity that have been reported include alterations in a range of innate immune cells, such as natural killer cells, monocytes, neutrophils, and eosinophils; and in the blood levels of complement proteins and in the amount of nitric oxide (Gładysz et al., 2018; Sweeten et al., 2004). Whereas dysregulations of adaptive immunity that have been reported in ASD include variations in the number of T lymphocytes and in the cytokines that regulate the function of these cells (Hughes et al., 2018).

In addition to these differences in immune-marker levels, it has been reported that up to 20% of autistic individuals are affected by immune dysregulations (Zerbo et al., 2015). For instance, 1% of autistic individuals report autoimmune conditions, including psoriasis, type 1 diabetes, and vitiligo, which suggest a misdirected antigen and T-cell-mediated responses (Zerbo et al., 2015). In addition, autistic individuals are prone to allergic reactions (e.g., allergies to food or rhinitis, atopic dermatitis), which may reflect altered histamine signalling, eosinophil and/or certain antibody (immunoglobulin E) response (Lyall et al., 2015). Also, transcriptomic studies demonstrate an increased expression of inflammatory genes in the blood and post-mortem brain of autistic individuals (Gandal et al., 2018: Voigeneau et al., 2012).

Notably, immune dysregulations are not unique to autistic individuals but also observed in their relatives (Atladóttir et al., 2009, Croen et al., 2018) (Vinet et al., 2015). For example, autoimmune conditions in the parents have been associated with an increased likelihood of autism in children (odds ratio ~ 1.6) (Keil et al., 2010) . In particular, prior studies indicate a significant, albeit weak, association between ASD risk and the parental diagnosis of type 1 diabetes, psoriasis, rheumatic fever, systemic lupus; in particular, these associations were stronger for maternal immune diagnosis than paternal diagnosis (Keil et al., 2010) (Croen et al., 2018). Also, there is evidence of an

association between ASD and maternal asthma, and allergies – with potential differences across the types of allergic responses considered (Croen et al, 2018).

Taken together, the available evidence suggests that immune dysregulations in ASD are – in part – accounted for by genetic variations in immune processes. This suggestion is further supported by the evidence of a familial history of immune disturbances in ASD. Moreover, immunogenetic liability may also contribute to the reported association between ASD risk and exposure to immune stress, which I describe in the following paragraph.

1.5.3. Environmental immune challenges and ASD risk

ASD is multifactorial, and it results from the interaction between genetic factors and environmental risk factors. This is demonstrated by the evidence that monozygotic concordance in ASD is never complete (<100%) (Tick et al., 2016), and that shared environments exert considerable influence (Bölte et al., 2019).

Notably, some of the best documented environmental risk factors for ASD include those that interfere with immune homeostasis. For example, there is evidence linking ASD and immune overactivation in early life (Estes & McAllister, 2016b, 2016a; McAllister, 2017). This evidence, mainly based on experiments in animals, demonstrated that maternal immune activation (MIA) during gestation impairs typical neurodevelopment. Namely, these studies indicate that maternal exposure to pathogens – like active viruses or bacteria – trigger an inflammatory response, including extreme cytokine activation, *in utero*. The resulting pro-inflammatory molecules (e.g., cytokines, chemokines) can cross the placenta and interfere with immune homeostasis in the developing brain. For instance, pro-inflammatory markers may induce aberrant microglia

activation which, in turn, may affect neurogenesis, synaptic formation and pruning, and lead to abnormalities in brain structure and connectivity (Coiro et al., 2015; Pendyala et al., 2017).

In humans, there is epidemiological evidence to suggest an association between MIA and ASD risk (Jiang et al., 2016). For example, ASD has been associated with exposure to gestational infections and autoimmune flares, that are known to induce MIA(Boksa, 2010) . In addition, epidemiological studies suggest that other environmental risk factors for ASD include airborne allergens which may trigger innate and adaptive immune reactions, involving eosinophils, histamine signalling and antibodies (Immunoglobulin E) (Karimi et al., 2017). Taking into account the evidence for both genetic and environmental risk factors in the pathophysiology of ASD, it is likely that part of the harmful effect of environmental immune stressors is due to their interaction with pre-existing genetic liability.

In conclusion, immune regulation and immune genes likely play a key role in neurodevelopment and variation in those may intervene in the pathophysiology of ASD. However, the genetic architecture of the immune system is complex and heterogeneous and modulates a range of immune functions. It is, therefore, crucial to understand which specific immunogenetic mechanisms are important to ASD.

1.6. Aim and hypotheses of this thesis

In the light of the presented evidence, the overarching aim of this thesis was to characterise if, and which, immune genes may play a role in ASD. Specifically – considering the phenotypic and genetic complexity of both ASD and the immune system – in this thesis, I tested three, complementary, research hypotheses:

My **first hypothesis** was that immune genetic factors contribute to the genetic landscape of ASD and may be developmentally important.

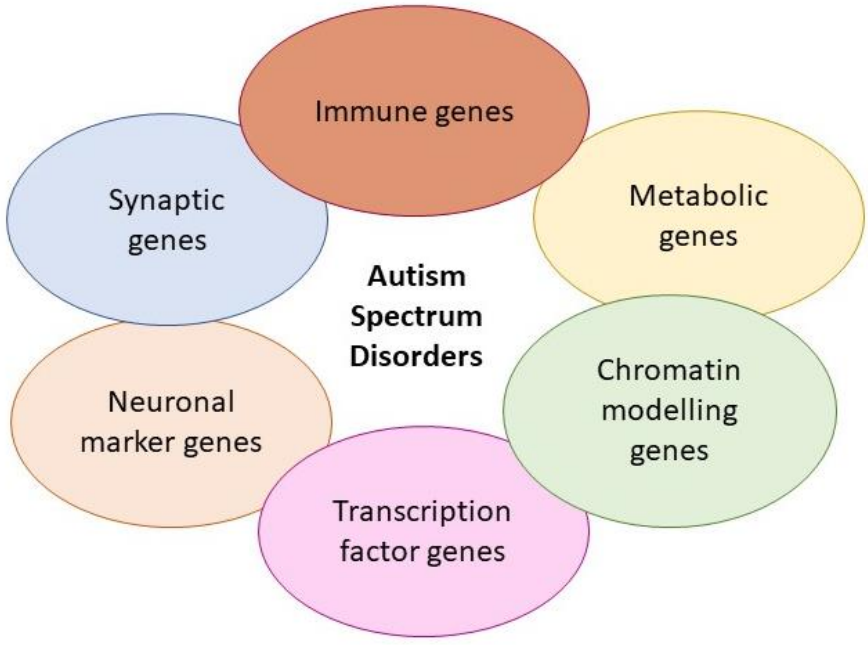

Figure 8 Research hypothesis I. Immune genes account for a portion of the genetic architecture of ASD, along with other neurodevelopmental genes.

My **second hypothesis** was that ASD share genetic factors related to autoimmune diseases, allergic diseases, asthma, and immune dysregulations reported in autistic individuals.

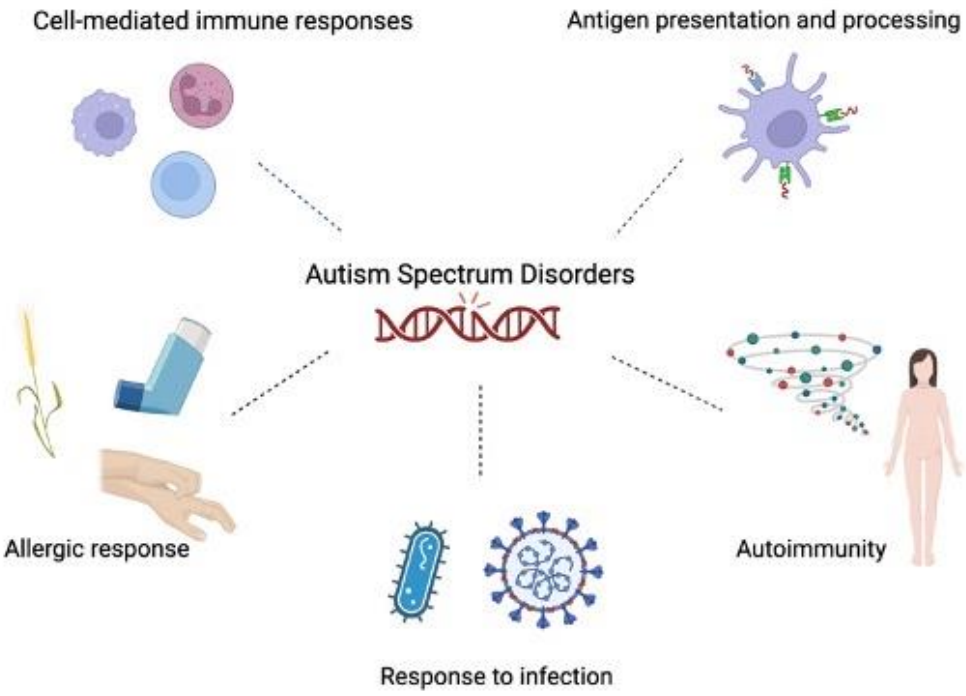

Figure 9 Research hypothesis II. Illustrative representation of the relationship between ASD genetics and genetic mechanisms regulating some specific conditions and processes of the immune system.

My **third hypothesis** was that specific immunogenetic factors – such as autoimmune-related, allergy-related and immune-cell related genetic factors - influence specific dimension of the autistic phenotype, especially rigid behaviours.

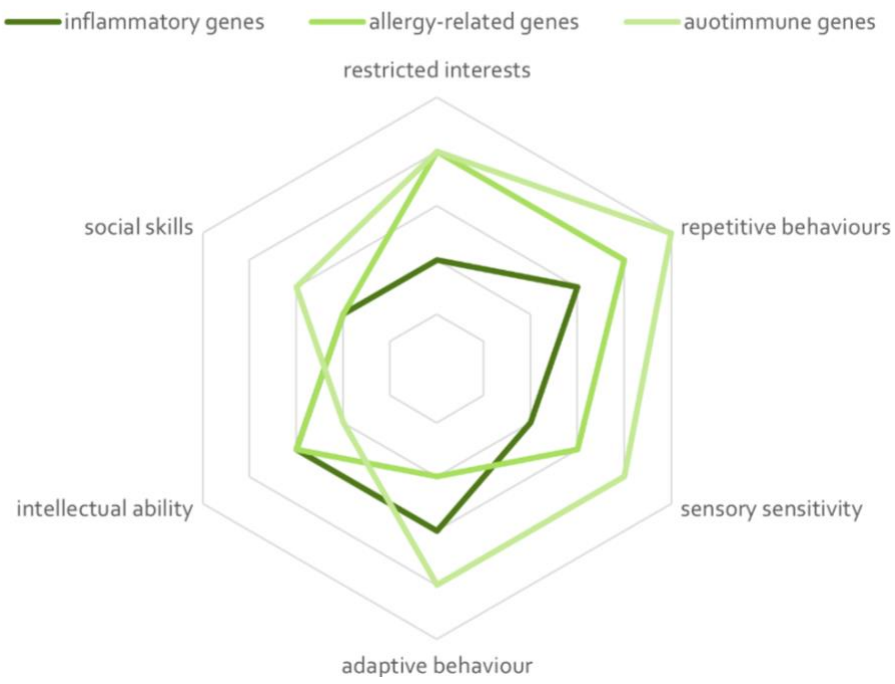

Figure 10 Research hypothesis III. Illustrative representation of the potential distribution of immunogenetic factors across specific aspects of the autistic phenotype.

To test these hypotheses, I investigated the role of common genetic factors, influencing a range of immune conditions, in ASD and across a set of population-based autistic traits. An overview of the materials and the methods that I have adopted for this investigation is provided in Chapter 2. The chapters to follow refer to the individual studies addressing each of the described research hypotheses.

To test the first hypothesis of an association between immune genes and ASD:

I conducted a systematic literature review of studies that linked immune genes and clinically defined ASD. This review is presented in Chapter 3 and supports the role of immune genes in ASD. However, this review also highlighted a literature bias towards specific immune genes (e.g., MHC genes), and, therefore, the need to investigate 1) a wider range of immunogenetic factors in ASD; and 2) their relationship to specific aspects of the autistic phenotype.

To gain insight on the genetic underpinnings of diverse autistic dimensions, I further explored common genetic variants associated with several autistic traits in the general population. To do so, I performed a meta-analysis of GWASs of five autistic traits across four international population-based cohorts. This work is described in Chapter 4 and published in the journal *Autism* 2022 Feb;26(2):361-372. Importantly, the result of this work provided preliminary evidence for a role of immune genes in population-based autistic traits, and with that in ASD.

These two initial studies laid the ground for my subsequent studies.

On the basis of prior results, I explored the second hypothesis of an association between ASD and immune genes involved in specific immune processes and diseases. Specifically, I investigated the genetic relationships between ASD and immune conditions known to be highly prevalent in autistic individuals and their relatives. Furthermore, I assessed the association between individual polygenic liability to these immune conditions and the described population-based autistic traits. This work is illustrated in Chapter 5 and demonstrated an association between ASD and genetic factors implicated in autoimmune and allergic diseases in ASD. In addition, this study indicated that immunogenetic factors are differentially associated across autistic domains.

Therefore, guided by the population-based findings, I investigated my third hypothesis of an association between immunogenetic factors and specific clinical features of ASD. To do so, I leveraged a unique clinical sample of ASD, including genetic and deep phenotyping data measured

across two time points: the Longitudinal European Autism Study (LEAP) cohort. By using these data, I tested the association between individual variations in identified immunogenetic factors and clinical features of ASD, cognition, and adaptive behaviour, also longitudinally. This work is presented in Chapter 6 and revealed a preliminary association between lymphocyte-related genetic factors and repetitive and restricted behaviours in individuals with ASD.

Last, I integrated the findings of these different studies and collectively discuss them in Chapter 7. In this chapter, I discuss my work in the context of previous studies and I elaborate on the clinical implications of my findings, and existing methodological limitations. Also, I introduce potential future research directions and preliminary findings to support these.

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2. Chapter 2: General methods

In this chapter I will describe the methods that I used in my thesis. I will give a general introduction about the type of genetic variants that I studied, and I will illustrate the genomic methods I adopted i) to identify common genetic variants linked to ASD and autistic traits and ii) to explore the genetic relationship between ASD and immunity.

2.1. Single nucleotide polymorphisms (SNPs)

The most common type of genetic variations are single nucleotide polymorphisms (SNPs), defined as variants that are observed in at least 1% of the population. A SNP refers to a change in a single nucleotide, or base (i.e., adenine, cytosine, guanine, thymine) within the DNA sequence. SNPs occur at a frequency of about one in 1,000 base pairs and it has been estimated that every individual carries up to 5million SNPs. (Shastry, Methods Mol Bio, 2009)

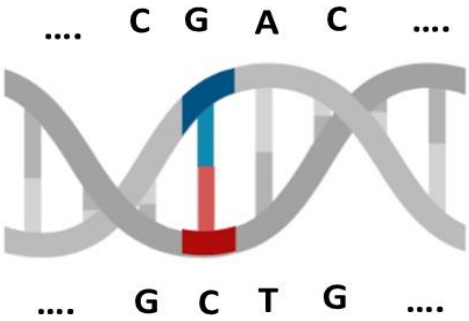

Figure 1 Single nucleotide polymorphisms are changes in individual nucleotides throughout the entire DNA sequence compared to a reference sequence.

SNPs are present throughout the genome, and they may fall within the coding sequence of genes or in non-coding regions such as the regions between genes. According to their genomic position, SNPs may have different effects. For instance, SNPs in coding regions may change the amino acid sequence and thus affect the encoding of a given protein, although it is also possible that a different SNP still leads to the same protein (known as synonymous SNPs)

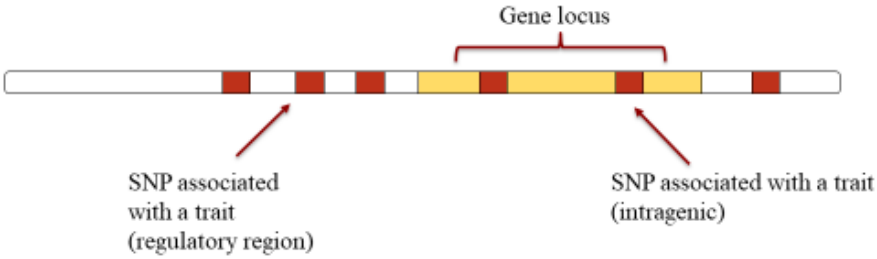

Figure 2 Single nucleotide polymorphisms can occur within the protein-coding region of a gene or in its regulatory regions.

SNPs in non-coding regions may have no effect on gene transcription in some cases, however in others they might affect gene transcription, and thus modulate the expression of a given gene, in which case we call them "expression quantitative trait loci" (eQTL).

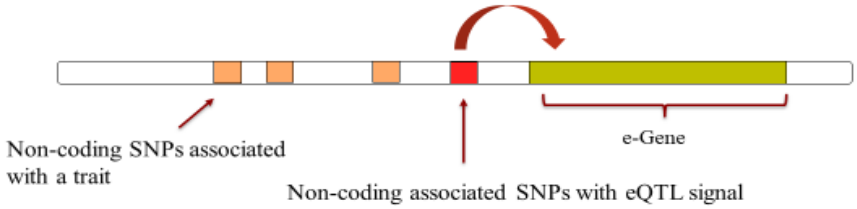

Figure 3 Single nucleotide polymorphisms, falling within non-coding regions, can have no effect on gene transcription or they can act as expression quantitative trait loci that influence the expression of genes in the proximity and/or at longer distances.

SNPs have been implicated in a range of common and complex heritable diseases or traits, including psychiatric conditions (Consortium*, 2013; Lee et al., 2019; Sullivan, 2010; Wray et al., 2014). In the context of these complex phenotypes, SNPs may help us to better understand the biological mechanisms that characterise these traits, or conditions, and that could represent potential therapeutic targets. Therefore, different approaches have been developed over the years to identify SNPs linked to complex traits. The current so-called 'gold standard' approach to identify SNPs that are associated to a disease or trait of interest is a genome-wide association study.

2.2. Genomic methodologies

2.2.1. Genome-wide association study

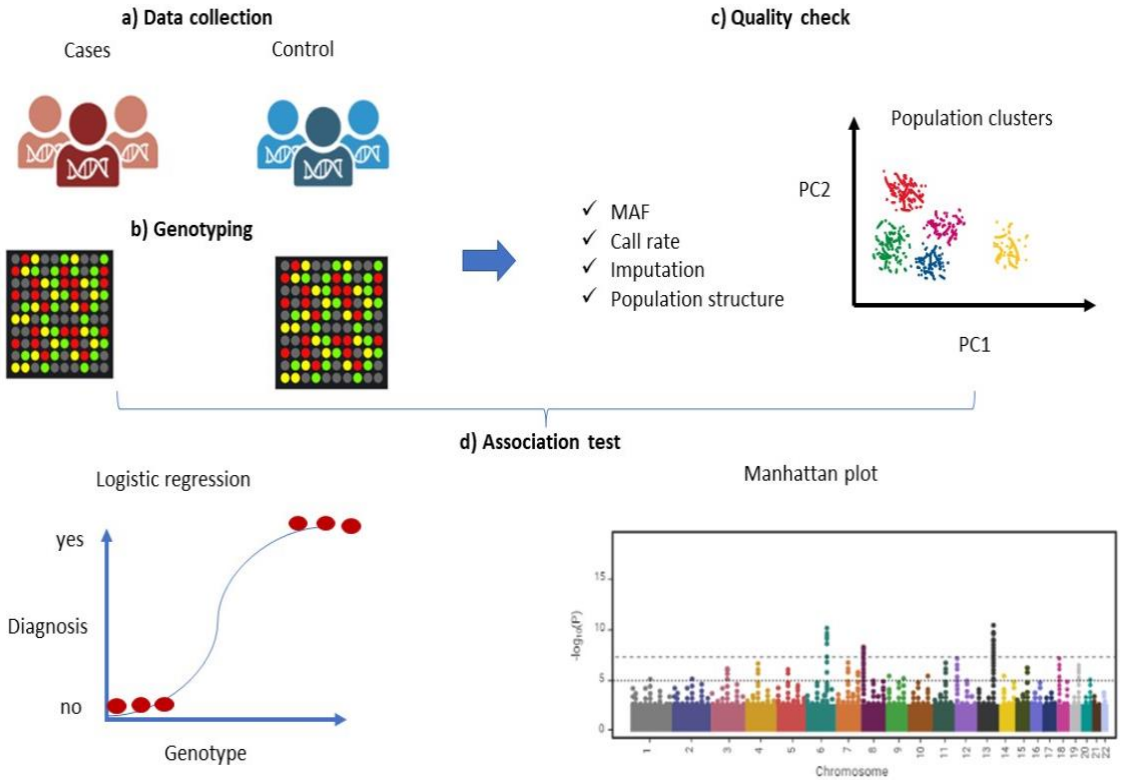

Figure 4 Schematic representation of the constitutive steps of a GWAS. Here, I show a case-control GWAS which include a) data collection from cases and controls; b) genotyping using

SNP microarrays; c) quality control steps including filtering out SNPs on frequency and imputation quality and including the assessment of population-structure; d) a logistic regression to test the association of individual SNPs with the likelihood of having a diagnosis. The results of a GWAS can be visualised in a Manhattan plot (on the right), where on the x-axis the genomic position is represented, and each dot represents single SNPs across all the chromosomes; the y-axis displays the negative logarithm of the p-values for the association between each SNPs and the disease considered.

A genome-wide association study (GWAS) is a hypothesis-free approach to identify SNPs, across the entire genome, that are associated with a trait of interest by comparing individuals that have a certain trait or condition to individuals that do not. A GWAS consists of multiple steps (Tam et al., 2019; Uffelmann et al., 2021) (Figure 4). To start, genetic data are collected from individuals that have the trait or disease/disorder of interest and individuals that do not (Figure 4a). These samples are genotyped using microarrays that capture millions of SNPs throughout the genome (Figure 4b). Subsequently genotype data undergo quality control (Figure 4c) which include filtering according to minor allele frequency, call rate, and Hardy-Weinberg equilibrium (Thomas Winkler, 2014). In addition, because microarrays are limited in the number of SNPs they capture, missing SNPs are statistically imputed (Kabisch et al., 2017). The imputation of missing SNPs relies on the linkage disequilibrium (LD) that exists between SNPs, whereby proximal SNPs tend to be co-inherited, and given patterns of co-inheritance are population specific (Slatkin, 2008). Genotype imputation, therefore, allows researchers to increase SNP coverage by identifying additional SNPs that are in the same LD block of genotyped SNPs based on a population-specific reference genomic panel (Li et al., 2009). To ensure good quality data it is essential to exclude SNPs of poor imputation quality. To reduce potential confounding factors, it is also important to explore population structure within your sample. Confounding factors known as population stratification can occur when allele frequency differences between cases and controls are biased by differences

in the composition of different ethnicities within your samples, leading to associations that are unrelated to the trait of interest. Common approaches to quantify population stratification are dimensionality reduction strategies, like principal component analyses or multidimensional scaling (Liu et al., 2013). These methods help to define clusters or components that capture the variability in genetics due to ethnicity.

After quality control, an association test (Figure 4d) is performed. Hence, good quality, genome-wide SNPs are tested for their association with the phenotype. Genetic association can be tested for using either categorical or continuous phenotypes (Tam et al., 2019). In the case of a categorical phenotype, genome-wide genotyping is carried out in individuals with a condition and those without. This approach, known as case-control GWAS, consists of a logistic regression where each SNPs is regressed against the likelihood of having the condition. This provides summary statistics for the association of all the SNPs throughout the genome with the categorical phenotype. The standard way to illustrate such an output is via a Manhattan plot (Figure 4d). In this plot, the x-axis represents genomic locations on individual chromosomes, and each dot refers to individual SNPs, whereas the y-axis represents the logarithm of the association p-value for each SNP with the phenotype.

A GWAS can also be carried for continuous phenotypes (Tam et al., 2019). This approach, referred as continuous-trait GWAS, tests if each SNP increases or decreases the level of a trait, hence fitting a linear regression model. As a result, SNPs that are associated with variations in a certain trait are identified. To avoid potential biases, both logistic and linear GWAS regression models can include covariates of relevance, like age, sex, and the principal components of population structure (the 5-10 first components) (Liu et al., 2013). There are multiple software packages available to perform quality control and conduct GWASs, including plink (Li et al., 2009, 2010; Purcell et al., 2007) .In chapter four, I performed continuous-trait GWASs of five autistic traits to identify SNPs associated with autistic traits in the general population.

It is important to note that in GWASs substantial numbers (i.e., multiple) comparisons are performed and therefore there is a substantial multiple-testing burden (Uffelmann et al., 2021). This is because, in a GWAS, millions of SNPs are tested at the same time for their association with the phenotype, and the number of independent signals within the human genome has been estimated to be around 1 000 000. Therefore, to reduce the rate of type I error (i.e., false positives), association results are considered genome-wide significant only if falling below the p-value threshold of 5x10-8 (i.e. 0,05 divided by 1 000 000) (Uffelmann et al., 2021). Another important cautionary note is that GWAS findings can be indirect due to LD (Slatkin, 2008), whereby a SNP may be associated with a phenotype not because of the SNP itself is casual but merely due to being in LD with a causal genetic risk variant. In addition, in many instances the individual SNPs effect size in complex phenotypes is small, making them more difficult to find.

Hence, in order to both achieve adequate statistical power and increase the chance of identifying significant associations, GWAS requires a large sample size (Zeggini & Ioannidis, 2009). One way to increase sample size is through meta-analysis.

2.2.2. Meta-analysis of GWAS

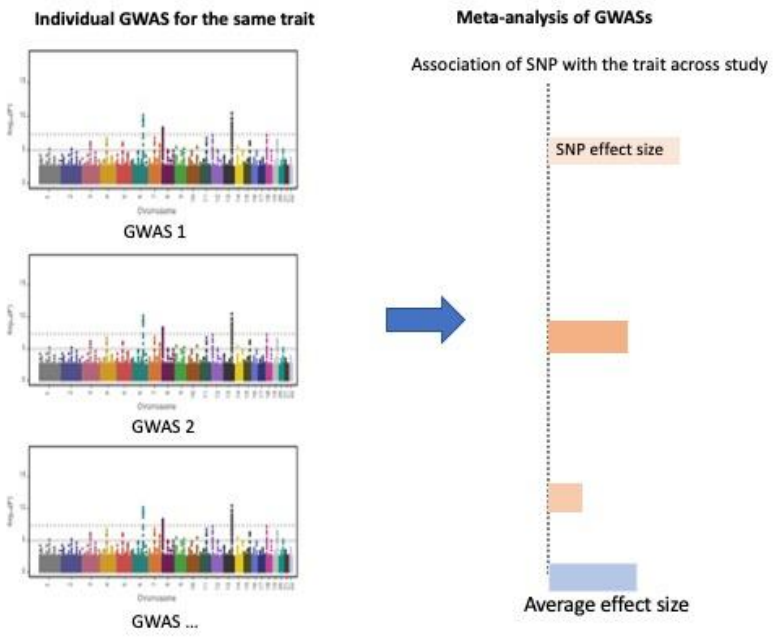

Figure 5 Representation of a GWAS meta-analysis. First, Individual GWAS investigating the same trait are gathered (on the left), and then these are meta-analysed (right). Hence, in this step, the average effect size of each SNP across individual GWAS is estimated.

A meta-analysis is a method which can be used to combine the results of individual GWASs for the same trait of interest; and calculate the sample-size weighted average effect for each of the SNPs on the phenotype across studies (Zeggini & Ioannidis, 2009) (figure 5). This, therefore, allows researchers to obtain a total sample size that is larger than individual studies and increases the likelihood of finding significant SNPs. The meta-analysis of multiple GWAS is usually carried out in the context of large consortia, e.g., the Psychiatric Genomic Consortium (PGC) (https://pgc.unc.edu/), where data from multiple cohorts are integrated using software packages like METAL (Willer et al., 2010).

A crucial aspect of the meta-analysis of multiple GWASs is harmonization between studies (Winkler et al., 2014; Zeggini & Ioannidis, 2009). Namely, it is important that individual GWASs follow the same analytical plan, use harmonised phenotypes, and standardise their results to allow cross-study comparisons. The meta-analysis can be performed by weighting the results from each

individual GWAS by its sample size or using inverse variance-weighted average method (Winkler et al., 2014). In psychiatry, especially owing to the international consortia like the psychiatric genomic consortium (PGC) multiple GWAS meta-analyses have been performed for a range of psychiatric disorders. These studies led to the identification of SNPs that are associated, for example, with autism spectrum disorders, schizophrenia, major depressive disorder, and attention deficit hyperactivity disorder (Consortium*, 2013; Grove et al., 2019; Lee et al., 2019; Sullivan, 2010).

In chapter four, I meta-analysed the results of GWASs of autistic traits conducted in four international cohorts. This analysis allowed me to obtain optimal statistical power to identify genome-wide significant SNPs for autistic traits.

Hence, taken together, SNPs associated with a disease or trait of interest can be detected through the meta-analysis of independent GWASs. However, to understand the biological function of these SNPs, it is necessary to perform subsequent studies (e.g., gene-based analyses), to prioritise genes and to identify biological pathways of importance.

2.2.3. Gene-based analysis

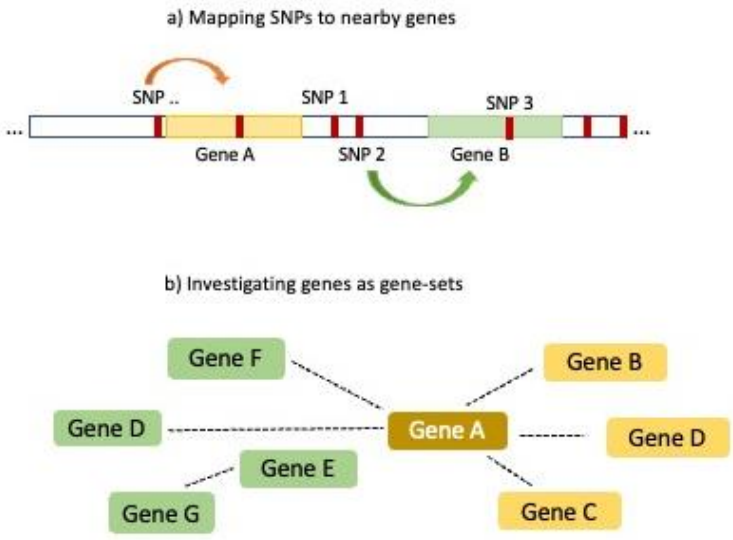

Figure 6 Starting from individual SNPs it is possible to a) prioritize genes that are associated with the investigated trait; and b) explore these genes as a set supporting specific biological processes.

Gene-based analysis allows us to determine the association of a given gene with the trait of interest starting from the association values of SNPs that are mapped to such gene(s). (Figure 6a). This is achieved via a linear regression model where the effects of single SNPs are aggregated to obtain gene-level statistics (de Leeuw et al., 2015). For each gene, SNPs that fall within the spatial coordinates of a given gene, or (optionally) in the areas flanking the gene location, are considered. Also, the resulting gene-level statistics are corrected for biases that may arise due to the LD present between gene-related SNPs. In conclusion, gene-wide analysis allows us to map associated SNP to genes and provide information on genes that have the strongest association with the trait.

Because this analysis considers ~ 20,000 genes annotated in the human genome, gene-level association p-values should be multiple comparison corrected to reduce type I errors. A widely adopted tool to perform such analysis is the Multimarker Analysis of Genomic Annotation (MAGMA) software package (de Leeuw et al., 2015) *(*https://ctg.cncr.nl/software/magma *)*.

In chapter four, I adopted this method to identify genes associated with autistic traits.

2.2.4. Gene-set association analysis

Gene-set association analysis is an extension of gene-based analyses, which test if genes – that are involved in specific biological processes – are jointly associated with the trait of interest (Figure 6b) (de Leeuw et al., 2015). A first step for this analysis is to define the sets of genes that belong to the biological pathways or process the researcher wants to investigate. The investigated gene sets can therefore be defined by the researcher based on prior literature or can be selected from publicly available gene ontology annotations of human genes (The Gene Ontology Consortium, 2019) http://geneontology.org/). Once the gene-sets are defined, the association of any given gene-set with the trait is assessed using, for example, the MAGMA software package (de Leeuw et al., 2015) (https://ctg.cncr.nl/software/magma). The test consists of a linear regression model, which includes a vector containing all the genes in the set as independent variable. To ensure that associations are not biased towards genes spanning large genomic regions with high numbers of SNPs, gene size and density are included as covariates in the regression model. The outcome of this analysis is a gene-set statistics per gene-set which provides information on the strength of the association of given gene-set with the trait; and which is deemed as significant if falling below a Bonferroni-corrected p-value threshold. Overall, this analysis provides a biological context to the SNPs and genes that have been linked with a trait.

In chapter four, I adopted this method to explore potential biological roles of the SNPs I identified as being associated with autistic traits.

2.2.5. Gene-based expression analysis

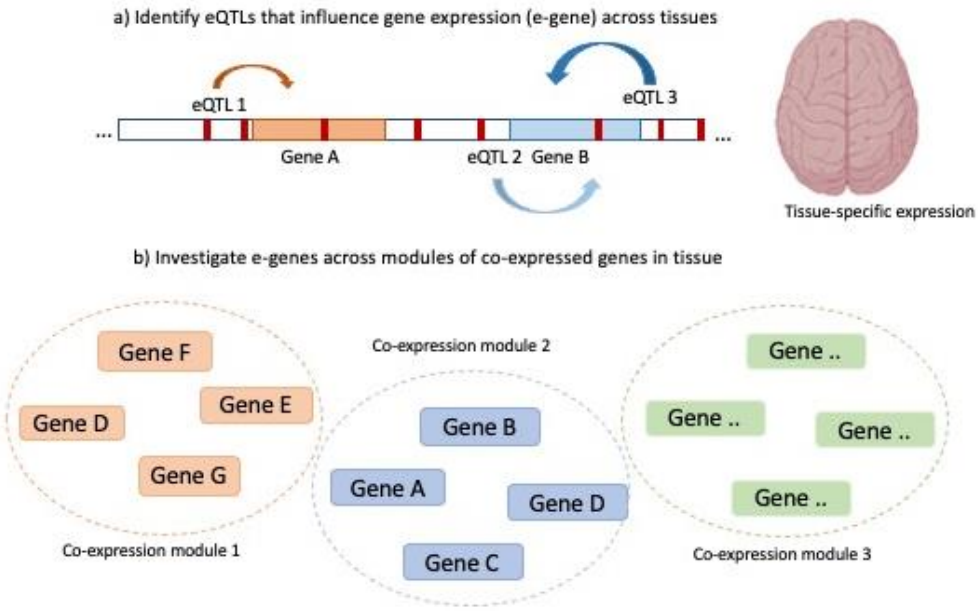

Figure 7 SNPs may act as eQTLs and influence gene expression across specific tissues. It is thus possible to a) identify genes expressed in a given tissue (e.g., brain) and that are associated with a trait; and b) explore how the representation of these genes is in large networks or modules of co-expressed genes, that may serve diverse biological functions.

As mentioned previously, SNPs may fall within non-protein coding regions of the genome. In this case, the functional role of these SNPs is unclear and may, for example, involve the regulation of the expression of genes located nearby (cis) but also at longer distances (trans) (Uffelmann et al., 2021) and these are called expression quantitative trait loci (eQTLs). Importantly, eQTLs influence gene expression in a tissue-dependent manner, whereby the same eQTL may influence the expression of two different genes in two different tissues respectively (GTEx Consortium, 2017). Prior efforts delineated pairs of cis-eQTL and expressed genes (e-gene), these referred to a specific range of tissues, and are currently available at the GTex portal (GTEx Consortium, 2017) (https://gtexportal.org/). By leveraging this information, it is then possible to map SNPs/eQTLs from GWAS to gene expression across tissues (Figure 7a). This can be done using an extension of MAGMA: e-MAGMA (Gerring et al., 2021). Specifically, e-MAGMA aggregates the effect of

single eQTLs into a gene-level statistics. This, therefore, provides information about the association of e-genes, expressed in a given tissue, with the trait.

In chapter four, I adopted this method to identify genes expressed in the brain that are associated with autistic traits. In chapter five, I used this method to define genes expressed in the brain and in the immune system and that are shared between ASD and immune system traits.

2.2.6. Gene co-expression network analysis

Gene co-expression network analysis is a method to complement the identification of e-genes associated with a trait (Gerring et al., 2019). Specifically, this step allows researchers to explore whether sets of e-genes - that are known to be expressed together in a certain tissue – are jointly associated with a trait (Figure 7b). These sets of e-genes are so-called 'gene co-expression modules' and are constructed by exploring the correlations existing between the pool of expressed genes per tissue (Langfelder & Horvath, 2008). These modules can be obtained by using, for example, the weighted gene co-expression network analysis (WGCNA) R-package (Langfelder & Horvath, 2008). Using these modules, gene co-expression network analysis can be performed using the e-MAGMA tool (Gerring et al., 2019). The outcome of this analysis is a gene-set/module-based statistic which provides information about the association between each co-expression network of genes in a tissue and the phenotype of interest. Moreover, to gain further biological insights, it is possible to examine the enrichment of genes within associated co-expression networks across a range of biological functions (Maleki et al., 2020). This enrichment analysis uses a Fisher's exact test to examine the non-random distribution of genes within a biological pathway (van Belle et al., 2004). There are several tools to help test enrichment and these include the R-packages g:Profiler and GeneOverlap (Kull et al., 2007; Shen, 2014). To summarise, from the association of individual tissue-specific e-genes, it is possible to estimate networks of co-expressed genes associated with

the trait. These co-expression networks differ across tissues, and, for each tissue, they may highlight biological processes relevant to the trait investigated.

In chapter four, I adopted this method to explore the potential biological functions of gene expressed in the brain that are associated with autistic traits.

2.2.7. Shared genetic aetiology analysis

Because complex traits are highly polygenic, and heterogeneous and multiple traits occur together, it is possible that SNPs and genes associated with one trait are also relevant for another trait (Bulik-Sullivan, Finucane, et al., 2015; van Rheenen et al., 2019). If this happens at multiple locations of the genome, genetic overlap might exist between multiple phenotypes. This genetic overlap may, therefore, provide insights on the mechanisms underpinning complex traits and which may, for example, explain instances when complex traits co-occur in the population (Bulik-Sullivan, Finucane, et al., 2015). There are different methods that can be used to explore the genetic overlap between traits. These include methods testing the genetic correlation between two traits, (Bulik-Sullivan, Finucane, et al., 2015; Werme et al., 2022), or polygenic score-based methods that allow the exploration of how genetic variants associated with a given traits are also related to individual variability in another trait (Choi et al., 2019). In the following paragraphs I will describe these methods.

2.2.7.1. Global correlation analyses

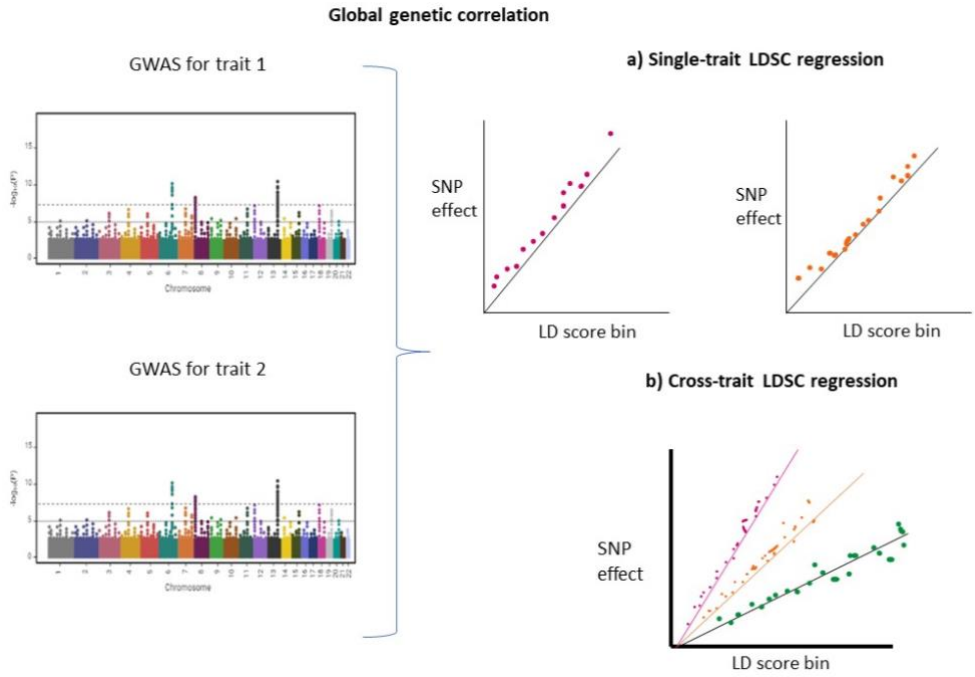

Figure 8 Representation of the LD-score based regression method. On the left, summary statistics from the GWAS of two traits of interested are gathered. Subsequently, for each trait, single trait LD-score based regression is performed to estimate its heritability and exclude potential biases due to LD (top right panel); after this, the cross-trait LD-score based regression is performed to estimate the global genetic correlation between the two traits (bottom right panel).

Genetic correlation analysis is a method that can be used to estimate the correlation in the effects of SNPs across a pair of traits (Bulik-Sullivan, Finucane, et al., 2015). This is made possible by leveraging SNP-based effects listed in the summary statistics of GWASs for the traits of interest. The standard approach to estimate genetic correlation is via cross-trait LD Score (LDSC) regression (Figure 8) (Bulik-Sullivan, Finucane, et al., 2015; Bulik-Sullivan, Loh, et al., 2015) (https://github.com/bulik/ldsc). This method consists of two steps. A first step is to perform a single-trait LDSC regression where the effects of each SNP on a trait are regressed against the level of LD between different SNPs, captured by population-specific LD scores (Figure 8a) (Bulik-Sullivan, Loh, et al., 2015). This analysis, therefore, returns the proportion of variance in a trait

explained by SNPs, so-called SNP-based heritability. This step is carried out for both the traits considered in any pair-wise genetic correlation. If both traits demonstrate reliable SNP-based h2 (i.e., non-infinite, positive estimates) and no genomic inflation, the second step is then to perform cross-trait LDSC regression (Figure 8b). This step jointly considers the effects of each SNP on trait 1 and trait 2 and regress those against the LD scores (Bulik-Sullivan, Finucane, et al., 2015). The output of this analysis is a measure of the covariance between the SNP-based effects across the two traits – while controlling for LD. The estimated covariance is then divided by the sample size, thus providing a measure of correlation between SNP-based effects across traits. This correlation estimate represents the average cross-trait genetic correlation, which considers the whole of set of SNPs analysed (van Rheenen et al., 2019). The analysis is restricted to SNPs imputed to the HapMap 3 reference panel and that respect quality criteria, such as having a good imputation score (>.7) and minor allele frequency > 1%. Also, it is important to consider LD scores that are estimated in the same population as the one used to estimate SNP effects in the GWAS; and it is common practice to exclude scores for the MHC region due to the high LD in this region (Bulik-Sullivan, Finucane, et al., 2015).

In chapter five, I used this method to estimate the global genetic correlation between ASD and a range of immune-related diseases and traits.

2.2.7.2. Local genetic correlation analyses

Local genetic correlation analysis is a method to estimate correlation in the effects of SNPs confined to specific genomic loci across a pair of traits (Werme et al., 2022). This method overcomes limitations that are intrinsic to the analysis of genetic correlation on a global scale. Because global genetic correlation analyses estimate the average of genetic correlation between two traits and across all SNPs, it may miss genetic relationship that are more localized in the genome (van Rheenen et al., 2019). For example, it may fail to detect genetic correlations that are confined to specific genetic locations, or that have opposite directions at different loci.The R-package local analysis of covariant association (LAVA) is a tool to estimate local genetic correlation (Werme et al., 2022) (https://ctg.cncr.nl/software/lava). This tool implements cross-trait LDSC regression analyses between the pair of traits of interest; and repeat those across ~2,500 loci created by partitioning the genome into blocks of circa 1 megabase, while minimising LD between them. The LAVA analysis is divided in two steps. First, a univariate test is performed to understand if SNPs within each loci explain significant portion of heritability (h2) in each trait using single trait LDSC-based regression (Bulik-Sullivan, Loh, et al., 2015). Subsequent, genetic correlation between traits is performed only for the loci where SNPs have significant effect on both traits using cross-trait LDSC-based regression(Bulik-Sullivan, Finucane, et al., 2015). In conclusion, the output of this analysis is an estimate of genetic correlation for each of the loci tested for the investigated traits. This allows to prioritize regions of the genome that may be particularly relevant for the pair of traits investigated.

In chapter five, I used this method to complement global genetic correlation analyses between ASD and immunity and thus to identify local genetic correlations.

2.2.7.3. Polygenic scores

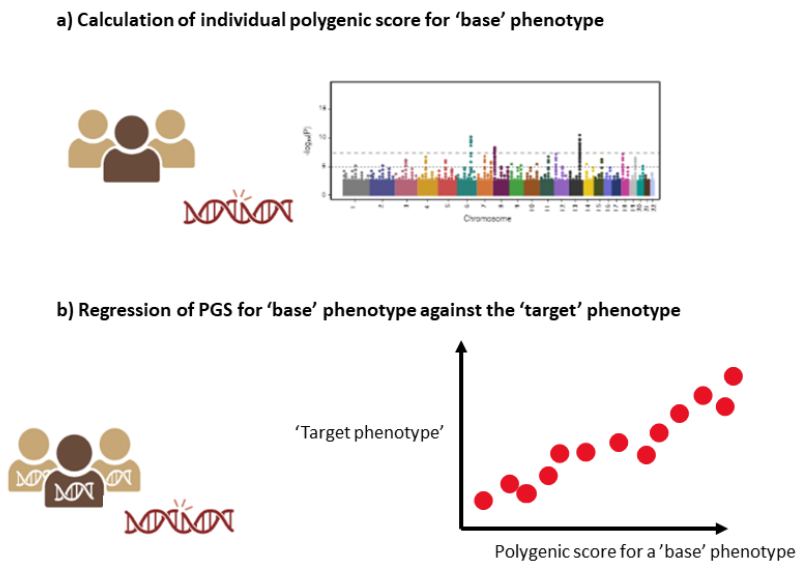

Figure 9 Polygenic score calculation. First, a) an individual polygenic score for a 'base' phenotype is calculated; then b) the obtained score is tested for association with variability in a trait of interest, so called 'target' phenotype.

Polygenic score analysis is a method that aggregates genetic variants and their effects on a known phenotype in individuals to provide an estimate of their genetic risk to a certain phenotype, which subsequently can be associated to the phenotype in those individuals (Choi et al., 2020; Choi & O'Reilly, 2019). In detail, a polygenic risk score is an individual score calculated by computing the sum of SNPs associated with a trait of interest (Figure 9a). The SNPs included in the sum are weighted by (the logarithm of) their effect size in the GWAS available for the tested trait (the so called 'base' phenotype) (Choi et al., 2020). It is important to consider as 'base', GWASs that are well-powered, and that rely on large sample size and have significant SNP-based heritability (Choi et al., 2020). Generally, a polygenic score (PGS) encompasses genome-wide SNPs that are associated with a given phenotype. One procedure to estimate PGS is to perform clumping and thresholding of SNPs, and this can be done using software packages like PRSice2 (Choi & O'Reilly, 2019). In this procedure, clumping is used to account for LD between SNPs. Specifically clumping

is a statistical approach that denotes an 'index' SNP (i.e., most associated SNP) for each LD block; this approach then forms 'clumps' of all other SNPs that are within a distance from this 'index' SNP chosen by the researcher (e.g., ±500 kilobases) (Choi et al., 2020). PGS calculation is then restricted to only index SNPs per each LD block. Subsequently, thresholding is used to filter on SNPs that report the highest association with the trait of interest. Thresholding can consider different p-value thresholds of association, such as the seven broad p-value thresholds included in the PRSice software package or the best-fit threshold, which includes only the associated SNPs that could explain most of the variability in the target phenotype.

Once the PGS for a trait is calculated for each individual, it is possible to explore how given PGS is associated with the phenotype of the individual, either for the same trait or a different trait, which is then defined as 'target' phenotype (Figure 9b) (Choi et al., 2020). This is tested via a regression model. In this model, the target phenotype is referred as the dependent variable, and the polygenic score for the 'base' phenotype represents the independent variables. Covariates are included and these are generally age, sex and the principal components accounting for population structure. The regression model can be either logistic or linear according to the nature of the target phenotype, i.e., categorical, or continuous. The output of this regression model will inform about the proportion of variability in the 'target' phenotype that is captured by the PGS (R2 for linear regression, or Niagelkerke's R2 for logistic regression) (Choi et al., 2020). To exclude potential confounders, an adjusted R2 is considered which is a modified version of R2 corrected by the number of predictors included in the regression models. Additionally, it is possible to include in the regression model individual PGSs that include SNPs associated with the 'base' phenotype at different levels of significance (or p-value thresholds (Pts)). For example, PGS including only the SNPs that have the strongest association with a trait or testing SNPs associated with the trait at different p-value thresholds (Pts) and defined a-priori (e.g., Pt =0.0001; 0.001; 0.01; 0.05) (Choi et al., 2020).

In chapter four, I performed the polygenic score analyses to explore the association between PGS for ASD and autistic traits in the general population. In chapter five, I adopted this method to examine if PGSs for different immune diseases are associated with autistic traits. In chapter six, I also adopted this method to link PGS for immune diseases and symptoms and cognitive profiles in a clinical sample of ASD.

In conclusion, I employed the genomic methods described above to study the genetic underpinnings of autism and its related population-based traits. This allowed me to better understand the biology of ASD and autistic traits, and, to estimate the genetic overlap between these phenotypes/traits and diseases of the immune system. In this chapter, I illustrated the general features of these methods, I explained which information they provide, and what are they used for. Further details about how I used these methods across my studies are provided in the relevant experimental chapter.

2.3. Study populations

To address my research questions and adopt the methods described above, in my studies I made use of multiple datasets which include genetic and phenotypic data, and summary statistics of GWAS. Table 1 provides an overview of the type of datasets used in each study, and further details are provided in the relevant chapter.

Table 2 Overview of the datasets utilised to conduct empirical studies described in the chapters of this thesis

Phenotype	**Data type**	**Study Design**	**Thesis chapter**
ASD			
i-PSYCH-PGC meta-analysis (Grove et al., 2019)	GWAS Summary statistics	Case-control	Chapter 4
i-PSYCH-PGC-SPARK meta-analysis (Matoba et al., 2020)	GWAS summary statistics	Case-control	Chapter 5
Autistic-like Trait			
NBS (Galesloot et al., 2017) BIG (Franke et al., 2010) GeneofCog (Pinar et al., 2018) Raine (Jones, 2015)	Genotype data autistic trait measures	Population-based	Chapter 4, 5
Autistic symptoms			
LEAP (Loth et al., 2017)	Genotype data	Case-control	Chapter 6

	clinical measures at time 1 clinical measures at time 2			
Immune Phenotypes				
Allergic diseases, Asthma Autoimmune diseases (SLE, RA, AID) (Bentham et al., 2015; Forgetta et al., 2020; Y. Han et al., 2020; Okada et al., 2014; Saevarsdottir et al., 2020; Zhu et al., 2018)	GWAS summary statistics		Case-control	Chapter 5
white blood cell count (eosinophil, lymphocyte, monocyte, neutrophil), C-RP levels (X. Han et al., 2020; Vuckovic et al., 2020)	GWAS summary statistics		Population-based	Chapter 5

Abbreviations: GWAS = genome-wide association study; NBS = Njimegen Biomedical Study; BIG = Brain Imaging Genetic study; GenofCog = Genetics of Cognition; PGC = Psychiatric Genomic Consortium; iPSYCH = Lundbeck foundation for Integrative Psychiatry; SPARK = Simon Foundation Powering Autism Research and Knowledge; SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; AID = autoimmune thyroid diseases; LEAP = Longitudinal European Autism Project;

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3. Chapter 3: Immunogenetics of autism spectrum disorder: a systematic literature review

3.1. Introduction

Autism spectrum disorder (ASD) is one of the most common neurodevelopmental conditions, affecting approximately 1 in 59 individuals worldwide (Chiarotti & Venerosi, 2020). ASD is characterised by difficulties in social communication and interaction, and repetitive patterns of behaviors and interests (American Psychiatric Association, 2013). In addition, hyper and/or hypo reactivity to sensory stimulation may be present (Hazen et al., 2014). These so-called core symptoms limit everyday functioning and personal well-being (Billstedt et al., 2011; van Heijst & Geurts, 2015). They also incur high societal costs, with a pro-capita estimate of circa $1.4-2.4 million in the US and £1.2-2 million in the UK (Rogge & Janssen, 2019). Nevertheless, at the moment we lack effective pharmacological intervention options for the 'core' symptoms of ASD (Loth et al., 2016), in part as the pathophysiological mechanisms driving them remain still unclear.

ASD refers to a heterogeneous group of disorders with different etiologies, phenotypes and trajectories. Twin studies demonstrated that genetic factors play an important role in ASD, which has an estimated heritability of 70-90% (Tick et al., 2016). Some of the genes that have been so far linked to ASD include genetic regulators of synaptic formation and signaling (Bourgeron, 2015; Rodriguez-Gomez et al., 2021). However, these putative risk loci do not fully capture the complex biological landscape of ASD (Wegener Sleeswijk et al., 2019) and this highlights the necessity to explore the role played by additional gene families and their related pathways in ASD.

One potentially relevant (genetic) mechanism involves genes controling the immune system. A role of the immune system in ASD has been long hypothesized; and this has been corroborated by recent findings in both animal models and humans (Masi et al., 2017). For example, studies in mice support an association between maternal immune activation (MIA) during pregnancy and the

onset of autistic-like behaviors in newborns (Choi et al., 2016; Reed et al., 2020; Rudolph et al., 2018). Notably, the effects of MIA on the offspring's behaviour have been showed to be modulated by the interleukin (IL) -6 pathway (Choi et al., 2016; Reed et al., 2020; Rudolph et al., 2018). Studies in humans also link ASD to immune dysregulations, including inflammation, oversensitivity to allergens, auto-antibodies production, and deregulated anti-infectious processes (Ashwood et al., 2011; Croen et al., 2019; Zerbo et al., 2015). Albeit environmental stressors of the immune system are likely to contribute to these associations, prior work supports the influence of immunoregulatory genes on ASD(Leboyer et al., 2016; Torres et al., 2012). For instance, candidate gene analyses have reported an association between ASD and specific human leukocytes antigens (HLA) haplotypes (Bennabi et al., 2018; Torres et al., 2012). Moreover, genome-wide assocation studies of ASD and population-based studies of autistic-like traits have identified assocations with common variants, or single nucleotide polymphorisms (SNPs), in immune system genes (Arenella et al., 2021; Grove et al., 2019). Of interest, a meta-analysis of GWAS performed in several psychiatric disorders, including autism, identified the MHC (Major Histocompatibility Complex) region wich hosts the human leucocyte antigen (HLA) cluster, as a strong signal of risk (Lee et al., 2019).Transcriptomic analyses of post-mortem brain tissues of autistic individuals have also described dysregulations of several immune gene pathways (Gandal et al., 2018). Furthermore, indirect support of a role of immune-related genetic factors comes from epidemiological studies, which demonstrated an association between family history of autoimmune and inflammatory conditions and ASD (Atladóttir et al., 2009; Vinet et al., 2015).

Although prior findings suggest that immune genes contribute to ASD, it is important to acknowledge the complexity of immune system genetics and the multitude of functionally diverse immune gene pathways (Parkin & Cohen, 2001). Currently, it is unclear if - and which – specific immunogenetic mechanisms are relevant to ASD. To address this question, it is crucial to gather prior findings of association between immune genes and ASD, and examine the immunological function of associated immune genes.

Hence, we conducted the first – to the best of our knowledge - systematic review of studies exploring the assocation between immune genes and ASD. Our work aggregates findings from both (immune-related) genetic and transcriptomic studies of ASD spanning the period from 2010 to 2022. Synthesized findings provide an overview of bona fide ASD-related immune genes, along with information about their specific immune function. Additionally, we explore the representation of immune genes among established ASD risk genes and evaluated their potential neurodevelopmental role (Figure 1). This effort is key to better define the potential immunogenetic underpinnings of ASD.

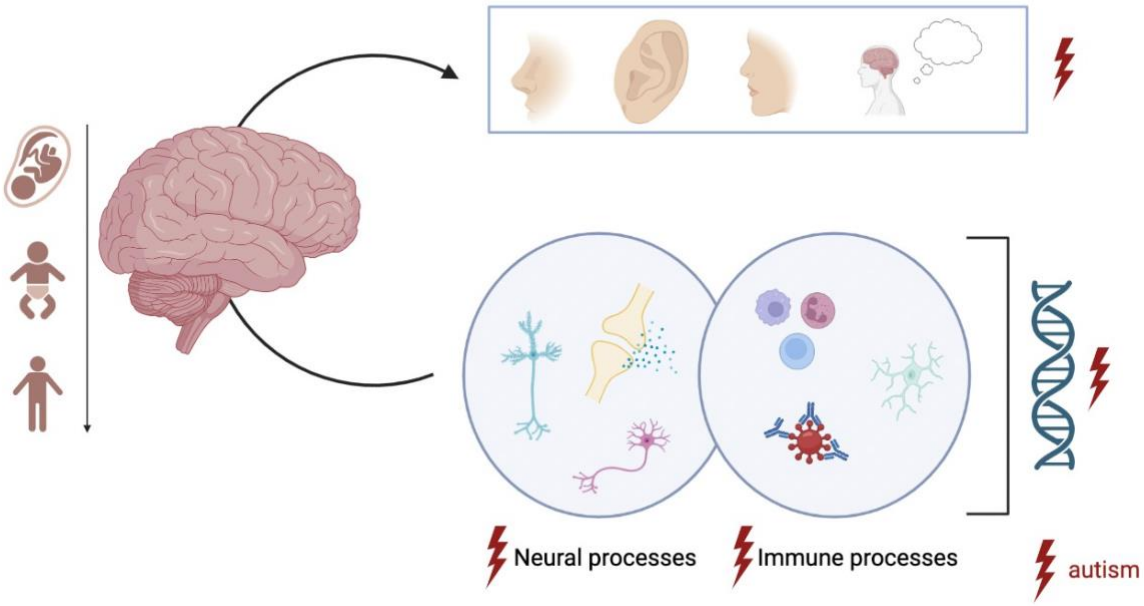

Figure 1 Representation of the neuro-immune crosstalk influencing brain development through pre, peri- and post-natal life. Genetics (on the left) influence the relationship between brain and immunity, and perturbances may increase the liability towards autism spectrum disorders.

3.2. Methods

Following the PRISMA guidelines (Moher et al., 2009), we conducted a systematic literature review of immunogenetic research in ASD. The systematic review was registered on PROSPERO with number CRD42021222673. Our literature search covered the period from January 1, 2010, through August 1, 2022. We excluded articles before 2010 so as to only include studies using state-of-the-art genomic approaches and analytical guidelines (e.g., updated genome build and reference genomic panel), and the most updated ASD diagnostic criteria (e.g., based on the Diagnostic and Statistical Manual of Mental Disorder (DSM) – VI or 5 or the International Classification of Disease (ICD) -10). Our literature search was conducted using the scientific literature databases PubMed and Web of Science. For both databases, a search query was created including terms for ASD and immune-related genetics: [(ASD OR autism OR autistic OR autistic disorder OR autism spectrum disorder OR Asperger's syndrome OR Kanner's syndrome OR pervasive neurodevelopmental disorder) AND (immune gene OR immune genetics OR immune genetic polymorphism OR inflammatory gene OR inflammation genetics OR immune RNA))].

We filtered reports to extract original research articles, written in English, and confined to human populations. The literature assessment followed four steps consisting of 1) identification 2) screening 3) eligibility 4) interpretation (see Figure 1). Two authors (MA and RM) independently performed the search and assessed the resulting articles.

First, results from the initial queries in PubMed and Web of Science were combined and recorded. Articles classified as duplicates were removed.

Second, articles were screened based on title and abstract for their relevance to the purpose of the review. We excluded review articles, as well as papers that did not primarily investigate ASD (e.g., articles focusing on other neurodevelopmental conditions).

Third, studies were assessed for eligibility. At this stage, full-text articles were retrieved and evaluated using the Newcastle-Ottawa Scale (NOS) for case-control studies (http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp) (Wells et al., 2000). In brief, the NOS allowed us to assess studies based on: a) selection, that refers to the definition/ ascertainment of cases - here based on the consensus diagnostic measures for ASD, as defined by the British Association of Psychopharmacology (Howes et al., 2018) (e.g., DSM, ADOS/ADI-R) - and the selection of control groups (i.e., individuals without clinical records and unrelated to the case groups); b) comparability, that refers to the use of strategies to control for potential confounding factors, such as sex, medication use, genetic syndromes, comorbidities and c) exposure, that refers to the adoption of the same ascertainment and analytical approach between cases and controls. Studies were classified as 'eligible' if receiving a NOS-based quality score above 4 at least, scoring at least 1 point in each of the a), b), c) categories. We also filtered studies based on statistical power. Because of this we considered studies with sample size large enough to guarantee statistical power according to previous power analyses and dependending on the genetic methodology used (Meurs, 2016; Owzar et al., 2012)(Spencer et al., 2009) . On average, we included studies that counted on sample sizes : N> 30-40 for brain and blood expression studies; N> 100 for SNP genotyping; N > 4000 for genome-wide association studies).

Eligible studies were included in a qualitative synthesis. For these studies, the reported genes were cross-checked for their relevance to the immune system in the ImmGen and innatedb portals, offering curated, comprehensive list of human genes involved in immune functioning (https://www.immgen.org/; https://innatedb.com/).

To estimate whether immune genes were also found among genes implicated in ASD, we examined the most recent list of 1,075 ASD genes (July 2022) from the Simons Foundation Autism Research Initiative (SFARI) (https://gene.sfari.org/). Within these genes, we examined the overlap with genes that are known to be functionally involved in the immune responses and which are annotated

in two different immune databases: innatedb (N = 3,714 genes; https://innatedb.com/) and ImmGen (N= 2,483 genes; https://www.immgen.org/), using a Fisher's exact test. Moreover, we used the "GENE2FUNC" function of the web-based platform Functional Mapping and Annotation of Genome-wide association studies (FUMA) (Watanabe et al., 2017) to explore the functional role of the overlapping genes by mapping genes to biological and molecular pathways (i.e., KEGG pathways). By leveraging BrainSpan data and Gtex data, FUMA allowed us to estimate gene expression in the brain throughout the life span and overall gene expression across bulk tissues. These enrichment analyses were performed using all the genes annotated throughout the genome as background to define the function of the overlapping ASD-immune genes as compared to the rest of the genome.

3.3. Results

Our search strategy led to 106 scientific reports of which 28 original research articles were deemed eligible for review after the 4-step selection process described above (Figure 2).To those, we manually added the most recent GWAS meta-analyses of ASD (Grove et al., 2019). This study reported an association between ASD and immune genes only in the supplementary materials, and therefore it would have been filtered out based on abstract and title screening. Overall, our review supports the role of immune genetic factors in ASD - as confirmed by both genomic and transcriptomic analyses in autistic individuals. 11 of the 29 studies consisted of genotype analyses of specific immune gene polymorphisms, and bioinformatic analyses on genome-wide SNP-based association of ASD (e.g., enrichment tests). Included genotype-based studies are presented in Table 1. In addition, 18 of the 29 studies were based on expression analyses of candidate immune-related genes or immune gene pathways in the blood and post-mortem brain tissues of autistic individuals (Table 2).

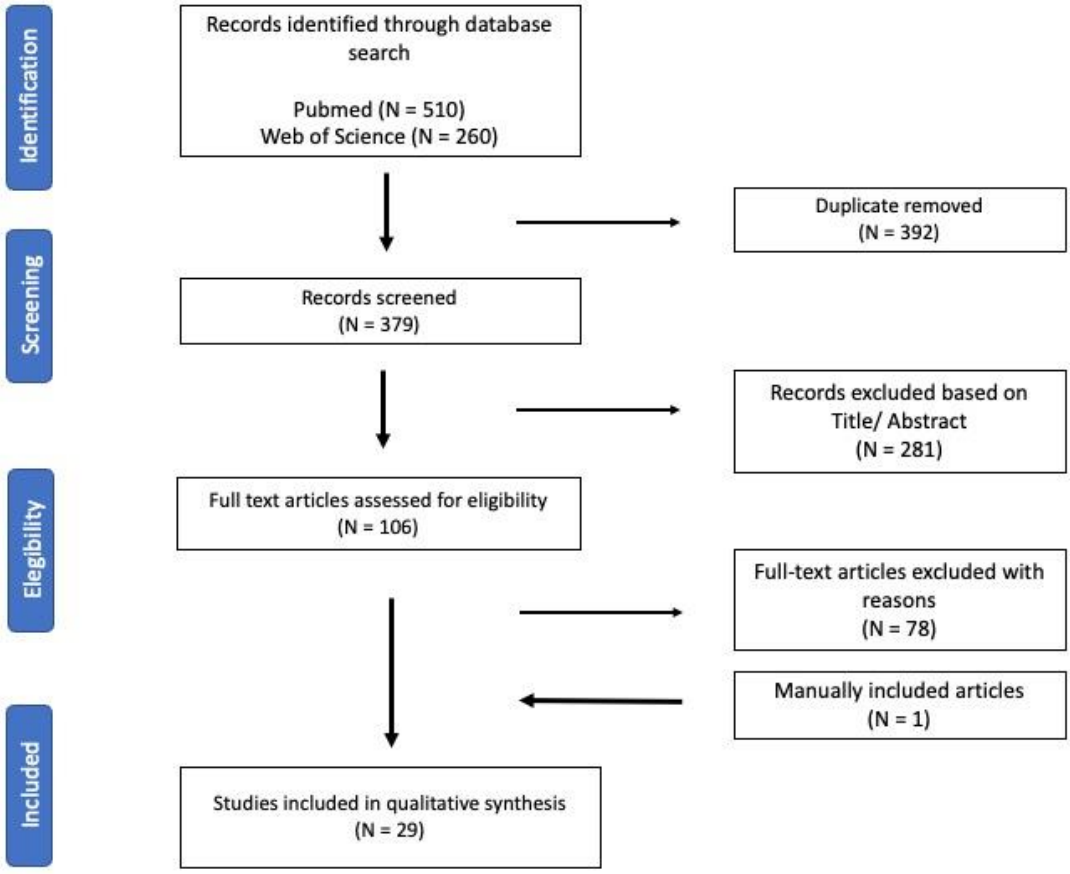

Figure 2 Flow chart of the study selection based on the 4 steps: identification, screening, eligibility and inclusion to qualitative synthesis. Reasons for exclusion of full-text articles included lower sample size and statistical power, irrelevant focus, inappropriate NOS scoring.

Table 3 List of genotype-based studies exploring the association of immune-related genes and ASD and included in the qualitative synthesis. Description about the study sample and identified immune genes are provided together with the review assessment scores.

Study reference						
Sayad et al (2018) *Neuropsychiatric Diseases and Treatment*	Guerini et al (2018) *Brain Behaviour and Immunity*	Bennabi et al (2018) *Scientific Reports*	Safari et al (2017) *Gene*	Mo et al (2017) *Nordic Journal of Psychiatry*	Bennabi et al (2015) *PLOS One*	
Study sample						
103 ASD; 180 TD;	111 ASD; 260 TD;	483 ASD; 352 TD;	532 ASD; 472 TD;	201 ASD; 200 TD;	478 ASD; 351 TD;	
Immune gene						
HLA-A *HLA-B,* *HLA-DRB*	*HLA-G*	*HLA-DRB1* *HLA-DQB1*	*FOXP3*	*CD157* *AIM2* *JARID2*	*CLEC7A*	
Ethnicity						
Middle Eastern	European	European	Middle Eastern	Asian	European	
NOS score						
6	6	6	6	6	6	
Effect Size						
OR = 1.9 (p = .04)	OR 1 = 0.5 (p = .0001) OR 2 = 7.3 (p= .002);	OR 1 = 1.75 (p= .001) OR 2 = 0.75 (p = .002)	OR = 1.35 (p = .03)	OR = 0.5-1.5 (p > .05)	OR = 1.36 (p = .01)	
doi						
https://doi.org/10.2147/NDT.S186673	https://doi.org/10.1016/j.bbi.2017.09.007	https://doi.org/10.1038/s41598-018-25974-9	https://doi.org/10.1016/j.gene.2016.10.019	https://doi.org/10.1080/08039488.2017.1410570	https://doi.org/10.1371/journal.pone.0137339	

Abbreviations : ASD : autism spectrum disorder; TD : typically developing; NOS : Newcastle Ottawa Scale; OR : odd ratio; β : beta; p : p-value;	Chen et al (2022) *Neuropsychopharmacology Biological Psychiatry*	Pekkoc Uyanik et al (2021) *Immunological Investigations*	Saad et al (2020) *Progress in Neuro-Psychopharmacology and Biological Psychiatry*	Guerini et al (2020) *Autism Research*	Grove et al (2019) *Nature*
	18; 381 ASD; 27,969 TD;	95 ASD; 84 TD;	80 ASD; 60 TD;	100 ASD; 187 TD;	18,381 ASD; 27,969 TD;
	IP-10	*IL-1β* *IL6*	*IL-1β* *IL-1RA*	*VDR*	MHC gene-set
	RANTES gene-set				
	European	Middle Eastern	African	European	European
	5	6	6	5	6
	OR 1 = 0.7 (p = 0.02)	OR = 2.3 (p = 0.02)	OR 1 = 1.8 (p = .001)	OR = 1.63 (p = .01)	β = 0.4 (p = .01)
	OR 2 = 1.07 (p = 0.03)		OR 2 = 1.33 (p = .01)		
	https://doi.org/10.1016/j.pnpbp.2022.110534	https://doi.org/10.1080/08820139.2020.1870489	https://doi.org/10.1016/j.pnpbp.2020.109999	https://doi.org/10.1002/aur.2279	https://10.1038/s41588-019-0344-8

Table 4 List of transcription (RNA-sequencing)-based studies investigating the expression of immune genes in both blood and post-mortem brain of autistic individuals, included in the qualitative synthesis. Description about the study design and identified immune genes are provided together with the review assessment scores.

Ahmad et al (2017) *Molecular Neurobiology*	Patel et al. (2016) *Molecular Neurobiology*	Pramparo et al (2015) *JAMA Psychiatry*	Gupta et al (2014) *Nature Communications**	Voineagu et al (2011) *Nature**	**Study reference**
40 ASD; 32 TD;	13 ASD; 13 TD;	91 ASD; 56 TD;	40 ASD; 32 TD;	19 ASD; 17 TD;	**Sample**
FOXP3 *RORyt* *STAT3* *T-bet* *GATA-3*	*IFN-y,* *IL1*β	Type II interferon pathway	Microglia activation Type I interferon pathway	Astrocyte response Microglia activation	**Immune gene and pathway**
Whole blood	Frontal cortex	Leukocytes	Occipital cortex Frontal cortex	Frontal cortex Temporal cortex Cerebellum	**Tissue**
Middle Eastern	European, African American	European, African American	European, African American	European, African American	**Ethnicity**
5	4	5	4	5	**NOS**
https://doi.org/10.1007/s12035-016-9977-0	https://doi.org/10.1007/s12035-015-9178-2	https://10.1001/jamapsychiatry.2014.3008	https://doi.org/10.1038/ncomms6748	https://doi.org/10.1038/nature10110	**doi**

Balestrieri (2019) *Frontiers in Immunology*	Ahmad et al (2019) *Psychopharmacology*	Ahmad et al (2019) *Molecular Immunology*	Gandal et al (2018) *Science**	Balta (2018) *Molecular Biology Reports*	Wright et al (2017) *Translational Psychiatry**	Lombardo et al (2017) *Molecular Autism**
31 ASD; 14 TD;	53 ASD; 28 TD;	10 ASD; 40 TD;	1,695 ASD; 1,000 TD;	30 ASD; 30 TD;	13 ASD; 39 TD;	76 ASD; 109 TD;
HERV-H *HERV-K* *HERV-W* *HEMO* *TNF-a* *IFNy* *IL-10* *IL-8*	*IL16*	*TIM-3* *CD11a* *CD11b* *CD14* *IL-B1* *IFNy*	Astrocyte response Interferon signaling pathway	*VDR*	Histamine signaling pathway *HNMT* *HRH1* *HRH2* *HRH3*	Interferon signaling pathway Complement system
Whole blood	Whole blood	Whole blood	Frontal cortex Temporal cortex	Whole blood	Frontal cortex	Frontal cortex Occipital cortex Temporal cortex
European	Middle Eastern	Middle Eastern	European, African American	European	European, African American	European, African American
5	5	5	5	5	5	4
https://doi.org/10.3389/fimmu.2019.02244	https://doi.org/10.1007/s00213-018-5120-4	https://doi.org/10.1016/j.molimm.2018.12.020	https://10.1126/science.aat8127	https://doi.org/10.1007/s11033-018-4191-y	https://doi.org/10.1038/tp.2017.87	https://doi.org/10.1186/s13229-017-0147-7

Hughes et al (2022), *Translational Psychiatry*	Li et al (2022), *Frontiers in Neuroinformatics*	Sabaie et al (2021) *Frontiers in Molecular Bioscience*	Lombardo et al (2020) *Brain Sciences**	Fallah et al (2020) *Metabolic Brain Diseases*	Guan et al (2019) *Translational Psychiatry**
17 ASD; 22 TD;	18 ASD; 11 TD;	30 ASD; 30 TD;	84 ASD; 109 TD;	50 ASD; 50 TD;	40 ASD; 32 TD;
E. coli response pathway	*JUN* *PDGFRA* T helper 17/1/2 cell pathway	JAK-STAT signaling pathway Insulin signaling pathway	Infection-related pathways Prion diseases	*IFNy, IFNy-AS1*	Nitric oxide synthase biosynthesis
LPS-stimulated monocyte	Cerebellum	Whole blood	Frontal cortex Temporal cortex Occipital cortex Corpus callosum Cerebellum Leukocyte whole blood	Whole blood	Frontal cortex Occipital cortex
European, African American	European, African American		European, African American	Middle Eastern	European, African American
4	5	5	4	5	4
https://10.1038/s41398-021-01766-0	https://doi.org/10.3389/fninf.2022.800079	https://doi.org/10.3389/fmolb.2021.754296	https://doi.org/10.3390/brainsci10040200	https://doi.org/10.1007/s11011-019-00510-4	https://doi.org/10.1038/s41398-019-0488-4

*Abbreviations: ASD: autism spectrum disorder; TD: typically developing; NOS: Newcastle Ottawa Scale; LPS: lipopolysaccharides; *overlapping populations;*

The following sections illustrate the findings from, respectively, immune genotype association studies and gene expression studies. Also, the findings from our cross-comparison of ASD-risk genes and immune-related genes are presented, together with their functional annotation.

3.3.1. Immune genes associated with ASD

Genotype-based studies indicated associations between ASD and three main classes of immune-related genes involved in 1) the processing and presentation of antigens; 2) immune regulation (i.e., transcription factors); and 3) cytokine signalling. Table 3 provides an overview of the immune genes associated with ASD, with information about their immune function and the signaling pathway to which they have been annotated.

Table 3. List of immune genes associated with ASD in genotype analyses with description of their immunological and neurodevelopmental function.

Gene ID	Gene name	Immune function and gene pathway	Brain function
CLEC7A	C-type Lectin Domain 7A	Innate immune response: TLR signaling Dectin-1 signaling	Neuroprotection Microglia response

IP-10	*IL-6*	*IL-1RA*	*IL-1β*	*HLA-G*	*HLA-DRB1*	*HLA-DQB1*	*FOXP3*
Interferon gamma inducible protein 10	Interleukin 6	Interleukin 1 receptor antagonist	Interleukin 1 β	Human Leukocyte Antigen G, MHC non-classical I	Human Leukocyte Antigen DR B1, MHC class II	Human Leukocyte Antigen DQ B1, MHC class II	Fox-head Box P3
Innate and Adaptive immune response: Chemokine signaling Viral response T cell regulation	Innate and Adaptive immune response: Maternal-fetal tolerance Apoptosis MIF signaling Cytokine signaling	Adaptive immune response: NF-KB signaling cytokine signaling Bacterial response	Immune and Adaptive immune response: NF-KB signaling MIF signaling Cytokine signaling TLR signaling	Adaptive immune response: Antigen processing and presentation to CD8+ T-cell, B-cell, NK-cell	Adaptive immune response: Antigen processing and presentation to CD4+ T-cell	Adaptive immune response: Antigen processing and presentation to CD4+ T-cell	Immune regulation: NF-KB signaling IL-2 signaling
Neuroprotection	MAPK/ERK signaling JAK-STAT signaling	Neuroprotection	MAPK/ERK signaling JAK-STAT signaling	Neurodevelopment Neuronal / synaptic plasticity	Neuronal/ synaptic plasticity	Neuronal/synaptic plasticity	WNT/ Notch signaling

Abbreviations: NF-KB: nuclear factor kappa-light-chain enhancer of activated B cells; TLR: toll-like receptor; MIF : macrophage migration inhibitory factor; Ca2+ : calcium; IL : interleukin;	*VDR*	Vitamin D receptor	*RANTES*
			Regulated on Activation, Normal T cell Expressed and Secreted
	Immune regulation:		Innate and Adaptive immune responses:
	Vitamin D/Ca2 + signaling Transcription factor		Chemokine signaling T cell (CD8+) signaling Viral response
	Neurite outgrowth Synaptic signaling (Ca2+ dependent)		Neuroprotection Blood-brain-barrier preservation

3.3.2. Expression of immune genes in brain and blood in ASD

Expression changes have been measured either in the peripheral blood or in the post-mortem brains of autistic individuals, and variations in the expression of different immune genes and immune gene pathways were consistenly reported across studies. Tables 4-5 list the immune genes and gene pathways with altered expression in the blood and in the brain of autistic individuals, respectively, with details about direction of effect, immune and potential neural functions.

Table 4. List of immune genes pathways with altered expression in the blood of autistic individuals. Table includes information about the immune and neural functions of identified genes. Arrows indicate the direction of regulation of given pathways as observed in ASD (↑= increased; ↓= decreased).

Vitamin D signalling	T-cell receptor	T-reg cell signalling	Immune transcription factor	Endogenous retrovirus signalling	Cytokine signalling	Antigen processing and presentation	**Gene Pathway**	**Table 4. Immune gene and gene pathways with altered expression in the peripheral blood of autistic individuals**
VDR	*TIM-3*	*FOXP3*	*STAT3*	*HEMO*	*IFNY*	*HLA-DR*	**Genes**	
			GATA3	*HERV/K/W*	*IL1B*			
			T-Rɒt		*II-8*			
↑	↑	↓	↑	↑	↑	↑	**Direction**	
Immune regulation	Immune activation, autoimmunity	Immune regulation	Th1, Th2, Th17 cell transcription	Infection response	Th1/Th2/Th17 response	T-reg cell signalling	**Immune function**	
Synaptic transmission and plasticity	Microglia activation	WNT/Notch signalling	Neurodevelopment Neuronal progenitor state	Neuroinflammation	Microglia activation	Embryonic neurodevelopment Neuroimmune homeostasis	**Brain function**	

Abbreviations: T-reg: T regulatory; Th: T-helper;

Table 5. List of immune genes pathways with altered expression in the post-mortem brain of autistic individuals. Table includes information about the immune and neural functions of identified genes. arrows indicate the direction of regulation of given pathways as observed in ASD (↑= increased; ↓= decreased).

Table 5. Immune gene and gene pathways with altered expression in post-mortem brain of autistic individuals									
Gene pathway				**Brain region**			**Direction**		**Immune function**
Histamine signalling	Complement system	Astrocyte signalling	Antigen processing and presentation	Frontal cortex	Temporal cortex	Occipital cortex			
Frontal cortex	Frontal cortex	Frontal cortex	Frontal cortex	↑	↑	↑			
	Temporal cortex	Temporal cortex	Temporal cortex						
	Occipital cortex		Occipital cortex						
↑	↑	↑	↑						
Immune activation (inflammation)	Inflammatory response	Immune activation	Adaptive immune response						
Neurodevelopment, Cortico-striatal signalling	**Neurodevelopment, Neuronal /Synaptic plasticity**	**Synaptic plasticity, communication, demyelination**	**Neurodevelopment, Neuronal/synaptic plasticity**						**Brain function**

Th17 cytokine signalling	T and B cell receptor signalling	JAK/STAT signalling	NK cell functioning	NF-KB signalling	mTOR signalling	Microglia response	MAPK signalling	Leukocyte activation	Interferon signalling	Infection and prion disease
Frontal cortex Temporal cortex Occipital cortex	Frontal cortex Temporal cortex Occipital cortex	Frontal cortex Temporal cortex Occipital cortex	Frontal cortex Temporal cortex Occipital cortex	Frontal cortex Temporal cortex	Frontal cortex Temporal cortex Occipital cortex	Frontal cortex Temporal cortex	Frontal cortex Temporal cortex Occipital cortex	Frontal cortex Occipital cortex	Frontal cortex Temporal cortex Occipital cortex	Frontal cortex Temporal cortex Occipital cortex
↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
Adaptive immune response	Cell-mediated immune response	Immune cell and cytokine transcription	Innate immunity	Immune activation (B-cell)	Cell cycle regulation and apoptosis	Immune activation	Cell cycle regulation and apoptosis	Immune activation	Immune activation	Immune activation
Neuroinflammation	Neuroinflammation	Neuronal /Synaptic plasticity	Neuroinflammation demyelination	Neural/Synaptic plasticity	Neuronal /Synaptic plasticity	Neural/Synaptic plasticity	Neuronal /Synaptic plasticity	Neuronal /Synaptic plasticity	Apoptosis, Demyelination	Neuroinflammation

Viral immune response (innate)	Frontal cortex Temporal cortex Occipital cortex ↑	Immune activation	Neuroinflammation	
Abbreviations: NF-KB: nuclear factor kappa-light-chain enhancer of activated B cells*; NK: natural killer cells; Th: T-helper;*				

3.3.3. Immune genes among ASD-related genes

The cross-comparison of annotated immune genes and ASD-related genes (SFARI) indicated an overlap of 98 genes., which did not reach statistical significance ($\chi^2 = 2.4$; $p = 0.12$). The 98 overlapping immune-ASD genes are presented in Table S 1 and a description of their immune function and SFARI ASD liability score is provided. Moreover, FUMA-based analyses provided further details on the biological function of these overlapping genes. For example, these genes were reported to be significantly eriched for immunological pathways involved in mTOR signaling, NK and T cell signaling, and allograft rejection (Figure S 1). Notably, these genes also displayed an enrichment in neurodevelopmental pathways, regulating axon guidance and neurotrophic signaling, and in the estrogen signaling cascade. Temporal expression analyses of these genes in the brain indicated an increased expression in the late prenatal period and late infancy. Further, cross-tissue expression analyses indicated a significant up-regulation of these genes in the adipose tissue and a down-regulation in testis as compared to genome-wide genes, in line with the key role of immune genes on broad endocrine signalling (e.g., metabolism, and hormons)(Figure S 2).

3.4. Discussion

There is increasing evidence supporting the contribution of the immune system to the pathophysiology of ASD (Masi et al., 2017). However, the mechanisms through which the immune system may influence the individual liability to ASD are unclear. One possibility is that immune genetic factors play a role. This hypothesis is reinforced by recent findings suggesting immune genes as one of the mechanisms contributing to the complex genetic architecture of ASD (Arenella et al., 2022; Grove et al., 2019; Tamouza et al., 2020).

To explore this hypothesis, we reviewed the literature on immune genetic studies in ASD available to date. Collectively, prior studies support an association between ASD and 1) inherited variations in genes controlling both innate and adaptive immune responses (Balta et al., 2018; Bennabi et al., 2018; Fallah et al., 2020; Guerini, Bolognesi, Chiappedi, Ripamonti, et al., 2018a; Mo et al., 2018; Pekkoc Uyanik et al., 2021; Saad et al., 2020; Safari et al., 2017, Tamouza et al, 2020, 2021); and 2) altered expression of these immune genes along their pathways in both the brain and at the systemic level (Gandal et al., 2018; Gupta et al., 2014; S. D. Lombardo et al., 2020; M. v. Lombardo et al., 2017; Patel et al., 2016; Pramparo et al., 2015; Sabaie et al., 2021; Voineagu et al., 2011; Wright et al., 2017). By specifically investigating the overlap between genes regulating the immune response and genes that have been linked to ASD risk (https://gene.sfari.org/), we were also able to gain insights on the potential neurodevelopmental functions of immune genes that may be relevant to ASD. The immune genes linked to increased ASD risk, for example, are known to support neuronal migration and synaptogenesis and are expressed in key stages of brain development, such as pregnancy and early childhood. Taken together, our findings suggest that immune genes play a key modulatory role in ASD by affecting early brain development and ontogeny.

3.4.1. Inherited immunogenetic polymorphisms

Overall, SNP-based association studies link ASD to diverse immune system genes. Most of the studies reviewed focused on the major histocompatibility complex (MHC) and its hosted human leukocyte antigen (HLA) genes including those belonging to the class II cluster, namely HLA-DRB1 and HLA-DQB1 (Bennabi et al., 2018; Guerini, Bolognesi, Chiappedi, Ghezzo, et al., 2018; Guerini, Bolognesi, Chiappedi, Ripamonti, et al., 2018a; Guerini et al., 2015; Tamouza et al., 2020). These genes encode molecules that control antigen presentation to CD4+ T helper cell with

consequent induction of the humoral immune response and production of antigen-specific antibodies by the B lymphocyte compartment. In addition, there is also evidence of a role for the non-classical HLA class I gene, *HLA-G*, a poweful immunodulator of immune responses which help to achieve allogenic tolerance necessary to proper fetal development during pregnancy. Dysfunctions at the level at these interfaces may induce inflammatory molecules passage through the placental with auto-immune consequences (Ferreira et al., 2017).

The findings of variations in HLA genes in ASD reconcile with reports of immune dysregulations in autistic individuals and in their family members. For example, HLA class I and class II genes are central to autoimmune pathologies (e.g., systemic lupus erythematoseous, rheumatoid arthritis) which have been frequently reported in the mothers of autistic children (Atladóttir et al., 2009; Cho & Feldman, 2015; Vinet et al., 2015). Also, due to their effects on the maternal-fetal interface, variations in HLA class I genes may contribute to maternal immune (over)activation that has been linked to a range of neurodevelopment conditions including ASD (Estes & McAllister, 2016; Scola & Duong, 2017) .

Other ASD-related immune genes include genes controlling the response of T-regulatory cells (Safari et al., 2017), and genes coding for cytokines and their receptors, which are key mediators of immune system homeostasis and activation (Fallah et al., 2020; Pekkoc Uyanik et al., 2021); and also, the *CLEC7A* gene, which encode for molecules that constitute major censors of the antifungal immune responses and thus may explain part of the dysbiosis observed in ASD (Bennabi et al., 2015).

However, we also found that genotype-based studies – and especially those investigating HLA genes – recorded a complex association between immune gene polymorphisms and ASD. For example, certain HLA polymorphisms have been associated with increased liability to ASD, and they have been particularly linked to regressive forms of the condition (Tamouza et al., 2020). On the contrary, other HLA alleles have been suggested to have a protective effect against ASD as

their frequency in autisitc individuals is significantly lower than non-autistic controls (Bennabi et al., 2018; Guerini, Bolognesi, Chiappedi, Ripamonti, et al., 2018a; Guerini et al., 2015). Some example of potentially protective HLA haplotyples include the HLA DRB-3 and DQB02 which belong to the Ancestral Haplotype HLA 8.1 (Bennabi et al., 2018). These haplotypes, and others, have been regarded as beneficial to human evolution, given their pro-inflammatory properties and their capacity to mount a reaction against external/harmful antigens, thus supporting brain homeostasis (Debnath et al., 2018). Notably, these haplotypes have been reported in association with high cognitive performance, as indexed by IQ levels, and they have been observed in high functioning autistic subgroups, such as individuals diagnosed with Asperger's disorder (Bennabi et al., 2015, 2018; Debnath et al., 2018).

3.4.2. Increased transcription of immune genes

The majority of the reviewed studies pointed to immune gene dysregulation, predominantly increased expression of immune genes in ASD (Gandal et al., 2018; Gao et al., 2021; Gupta et al., 2014; M. v Lombardo, 2018; M. v. Lombardo et al., 2017; Nazeen et al., 2016; Voineagu et al., 2011). This immune gene up-regulation suggests a state of immune activation and immune over-sensitization. Functional assessment of the immune genetic factors observed to be upregulated in ASD points to a role of genetic processes controlling general inflammatory response. These genes included mediators of the innate immune response, such as genes regulating the NK cell signaling pathway, and mediators of the adaptive immune response, such as genes controlling the T and B cell-mediated responses. However, the drivers of the expression changes reported in these studies are unclear. Potential drivers include external triggers such as viruses and bacteria. For example, the observed immunogenetic dysregulations are similar to expression changes observed in animals following viral (synthetic viral RNA polyinosinic:polycytidylic acid) and/or bacterial

(lipopolysaccharide) stimulation *in utero* (M. v. Lombardo et al., 2018). Given the findings of inherited variations in transcription factor genes in ASD (Balta et al., 2018; Safari et al., 2017), potential environmental triggers may act on the top of a pre-existing immunogenetic vulnerability in some cases of ASD.

Notably, the upregulation of immune genes appears persistent across the lifetime and it is reported in autistic children, autistic adults, and in the post-mortem brains of autistic individuals (Fallah et al., 2020; Gupta et al., 2014; Pramparo et al., 2015; Wright et al., 2017). It is possible that these expression changes are initiated at a very early stage of life. Increasing evidence suggests that gestation may be a period of increased immune sensitivity, and fine-tuning of immune gene regulation (Hsu & Nanan, 2014; Mandal et al., 2013; Morelli et al., 2015). During gestation, the fetal immune system is shaped and it is endowed with a range of immune responses to adopt later in life. Factors that interfere with the immune milieu at this stage of life, including genetic background and environmental triggers such as infectious agents, can program the immune system towards a pro-inflammatory state. This phenomenon is so-called 'fetal programming' and it impacts health outcome and susceptibility to disease throughout the entire life span (Mandal et al., 2013). For example, fetal exposure to immune stimulation has been linked to a life-long pro-inflammatory state in mice (Mandal et al., 2013). Some autistic individuals also exhibit signs of inflammation – indexed by elevated levels of pro-inflammatory cytokines – which may originate from gestational immune activation (Edmiston et al., 2018; Xu et al., 2015).

Moreover, our study synthesis demonstrates that immune activation is likely to be pervasive, and occur both peripherally and in the central nervous system (S. D. Lombardo et al., 2020). This, therefore, corroborates the hypothesis that ASD should be regarded as a systemic disorder. However, in the light the neurodevelopmental nature of ASD, most studies focused on the expression of immune genes in brain (Gupta et al., 2014; M. v. Lombardo et al., 2017; Voineagu et al., 2011; Wright et al., 2017). In particular, they investigated gene expression in brain regions

that are functionally (Kennedy & Courchesne, 2008) and structurally (Stanfield et al., 2008; van Rooij et al., 2018) relevant to ASD. These studies, for example, reported an increased expression of cytokine and leukocyte activation genes in the temporal lobe and in the frontal cortex (Gupta et al., 2014; M. v. Lombardo et al., 2017; Voineagu et al., 2011; Wright et al., 2017) , which have been implicated in social and cognitive features of autism(Mundy, 2018). Notably, these brain regions are also those that exhibit immune gene upregulation in mice exposed to immune challenges (for a review see (Woods et al., 2021)), suggesting that these brain structures may be particularly sensitive to the effect of immune stimulation. Moreover, recent findings indicate that immune/inflammatory genes are significantly enriched in brain areas where deviate from a so-called typical neuroanatomical range (Ecker et al., 2022). It does, however, remain unclear if up-regulation of immune genes is a cause or a consequence of neural anomalies.

3.4.3. The neurodevelopmental function of immune genes

To further understand the importance of immune genes in ASD, we explored the most recent list of genes associated with ASD risk and curated by the SFARI initiative (Banerjee-Basu & Packer, 2010). Hence, among these ASD-risk genes, we identified a set of immune genes and explored their neurobiological and specific immunological functions. Specifically, we demonstrated that these immune genes, also linked to ASD risk support multiple immune pathways, and include for example regulator of the innate immune response, NK-cell response, and also the adaptive, T-cell-mediated response. Notably, the identified immune, ASD-risk genes, showed also to be enriched for key neurodevelopmental processes, which include neuronal signalling, axon guidance, and the mTOR signaling cascade. We also examined the pattern of expression of these genes across a wide range of time windows. This analysis revealed that the overlapping ASD-immune genes are specifically expressed in the late prenatal period, and early childhood. Both these periods are critical

for brain development. The late prenatal period brings formation and organization of synapses between neurons (Goddings & Giedd, 2014; Tau & Peterson, 2010) and the start of axonal myelination (Tau & Peterson, 2010). Late infancy (up to the beginning of adolescence), in contrast, coincides with the refinement of neural circuits and synaptic pruning that underpins sensory processing and learning (Goddings & Giedd, 2014). Immune activation – and up-regulation of immune genes - at these life stages may have a cascading effect on the formation of neurons and the connectivity among them. For example, the the activation of microglia - and the consequent circulation of pro-inflammatory cytokines - is known to influence the normal process of engulfment and pruning of disused synapses (Boulanger, 2009; Garay & McAllister, 2010). Moreover, the (over) activation of innate immune cells (e.g., NK cells) – one of the molecular pathway upregulated in ASD - has been linked to damage to the myelin sheath and altered neuronal transmission in experimental models of multiple sclerosis (Shi et al., 2000).

The findings of a partial, albeit not statistically significant, overlap between immune and neurodevelopmental genes, and their upregulation in critical neurodevelopmental stages demonstrates that immune genes are important factors in brain development. Previous work exploring the neurodevelopmental function of key immune genes confirms this (Debnath et al., 2018; Miller et al., 2013). Studies in mice indicate that *HLA* genes aid neuronal migration and are central to the formation of synapses (Elmer & McAllister, 2012; Yirmiya & Goshen, 2011). Evidence also supports the role of cytokine genes in the formation of neuronal and glial cells, and in the establishment of neural connectivity (reviewed by (Deverman & Patterson, 2009)). Furthermore, the analyses of main immunogenetic pathways (e.g., TNF and IL-6 signaling) reveal that these comprise a pool of neurotrophic factors, such as STAT3 and AKT, in their signaling cascade (Yang et al., 2018). The activation of these pathways may consequently have downstream effects on neuronal formation and organization (Zegeye et al., 2018). To further support the interaction between immune genes and neurodevelopmental genes, prior transcriptomic findings indicate that immune gene upregulation in autism is coupled with downregulation of

neurodevelopmental genes (M. v. Lombardo et al., 2017). The opposite directions of these effects suggest that the over-activation of immune genes may have inhibitory effects on the normal execution of neurodevelopmental processes.

3.4.4. Sources of inter-individual immune gene variability

Although most associations between immune genes and autism are replicated across studies, there are some inconsistencies. For example, variations in some immune genes (*HLA, CD157, AIM2, JARID2*) are observed in autistic individuals of European ancestries but not replicated in other ethnical groups (Kara et al., 2018; Mo et al., 2018; Ramos et al., 2012). *HLA* genes, in particular, are known to be both highly polymorphic and highly variable across ethnical groups (Ramos et al., 2015; Shiina et al., 2009). These discordant genetic effects may be accounted by fluctuations in allele distribition across populations, which may occur randomly (in case of genetic drift) and/or non-randomly (due to in/out-breeding), and they are also affected by population's geographical relocation and exposure to different environmental challenges (Charlesworth & Charlesworth, 2017). A potential approach that could overcome, even partly, such biases is the study of HLA ancestral haplotypes that are conserved across population due to their immune properties (Price et al, 1999; Dorak et al, 2006; Bennabi et al, 2018).

Some studies indicate that the effects of immunogenetic variations are male-specific (Safari et al., 2017). The male-specific effects are in line with findings in rodents, which indicate a perturbed antigen response following MIA in male but not in female mice (Carlezon et al., 2019). However, multiple factors may account for these sex differences. First, there may be a statistical power issue. Study populations of ASD are highly male-skewed due to an increased incidence of ASD in males and/or underdiagnosis of autism in females (Halladay et al., 2015). These populations may be, therefore, underpowered to detect significant effects in females. Second, there may be an influence

of other sex-related factors. It is, for example, recognized that sex hormones modulate the immune response (Roved et al., 2017). Androgens – and particularly testosterone – display immuno-suppressive properties, while estrogens act as enhancers and regulators of the immune response (Roved et al., 2017). Given their immunoregulatory role, estrogens may counteract the effect of immunogenetic variations in autistic females. Conversely, testosterone may exacerbate the effects of immune gene variations. One major hypothesis of ASD links intra-uterine testosterone to the onset of autistic behaviors (James, 2014). In this context, our analysis suggests that immunogenetic factors may intervene in the potential relationship between testosterone, or sex hormones in general, and ASD. Last, it is important to note that the X chromosome hosts the largest number of immune genes, which therefore makes males more sensitive to the effect of variations affecting any of these X-linked immune genes (Schurz et al., 2019).

Previous studies also indicate that immunogenetic variations may be associated with specific clinical subgroups, such as regressive autism (Tamouza et al., 2020). In this particular subset of patients with ASD, the GI tract was demonstrated to be central (Bennabi et al., 2015; Hughes et al., 2018) . By studying the distribution of HLA haplotypes in patients with and without regression, we identified a risk HLA class II sub haplotype namely, the HLA DPRB1*17-DPQ1*02 (Bennabi et al., 2018). Of interest, this haplotype has also been reported to be protective against intestinal auto-immune disorders like pediatric autoimmune celiac disease (Dubois et al., 2010). Conversely, we indetified a protective HLA haplotype, the HLA-DRB1 *11-DQB1*07, which is less frequent in autistic individuals. Although further research is needed to clarify these effects – and their direction, one possibility is that immunogenetic variability in ASD is confined to specific clinical groups and that, in these subgroups, these genetic variation translate into immune problems, such as dysbiosis. The hypothesis of immune clinical subtypes have been reviewed previously (Jyonouchi et al., 2014), and it is reinforced by the evidence that immune dysregulations are observed in only a portion of autistic individuals(Zerbo et al., 2015). It is crucial to explore this possibility as it may help to define individuals that could potentially benefit the most from

interventions aimed at minimizing the likelihood of immune (over-)activation and targeting ongoing immune dysregulations.

3.4.5. Current limitations and potential guidelines for the future

Our review exposed a number of the limitations of previous immunogenetic research in ASD. First, prior studies investigated few candidate genes and relied mainly of a hypothesis-driven approach. Althouhg these genes refer to key genetic factors (e.g., HLA alleles) of the immune system (Bennabi et al., 2018; Guerini, Bolognesi, Chiappedi, Ripamonti, et al., 2018b; Guerini et al., 2015; Sayad et al., 2018), they did not cover the entire range of immune mechanisms that may influence the autistic phenotype. For a fuller picture, it would be useful to extend immunogenetic investigation in ASD to other, functionally diverse, immune genetic factors. For example, this could be achieved by performing hypothesis-free genome-wide association analyses that would explore the entire genome without any selection bias in contrast with hypothesis-driven candidate gene studies. Second, previous research was mostly based on the samples of European descendants and male individuals. As discussed, it is important to include a wide range of ethnical groups (Ramos et al., 2015; Roved et al., 2017), and potentially investigate immunogenetic factors in autistic men and women separately. Furthermore, previous studies did not systematically assess the presence and/or family history of immune conditions in the examined population. Given the heterogeneity of both ASD and the immune response, detailed immune phenotyping of autistic individuals may help to refine the relationship between different classes of immune genes and autistic symptoms. For example, a recent deep analysis of both phenotype and functions of NK cells in adult with high functioning ASD allow to identify phenotype specificities along and functional alteration highly suggesting the involvement of a yet to identified trigger (Bennabi et al, 2019). We are also aware that, although we followed standard review guidelines, we screened articles based on abstract and therefore we could have missed immunogenetic findings that may

have been not listed in articles' abstract. For example, we added manually the Grove et al.'s study which report immunogenetic associations only in the supplement, but we cannot exclude the possibility of other additional relevant studies. Another limitation was the relatively poor assessment of the effects of age. Most studies include individuals of different age groups, spanning from early childhood to adulthood. Although this allows us to understand the life-long impact of immunogenetic variability, it is likely that, through life, other biological mechanisms intervene to counteract the effect of immune genetic variations. This may be especially the case for transcriptional changes that are known to be dynamic and tuned to environmental signals (Li et al., 2018). Future research should, therefore, investigate the effects of immunogenetic variations and immune gene transcription in clinical groups of different ages, for example adopting cross-sectional designs or even longitudinal approches if possible. This is extremely important, especially in light of the neurodevelopmental role of immune genes in early life. Finally, given the neurodevelopmental involvement of immune genes, it is crucial to investigate the influence of immunogenetic factors on the brain structure and functions in autistic individuals. This may help to answer the question of whether neural (dys)functions bridge between immunogenetic factors and behavioral alterations.

3.4.6. Conclusions and clinical implications

Genetic factors are likely to be one of the mediators in the relationship between the immune system and ASD. Immune genes appear to influence the autistic phenotype via inherited variations and/or changes in the expression levels of genomic products. These immune genes participate in key neurodevelopmental processes and show upregulation during key stages of neurodevelopment, such as during gestation. These findings have valuable clinical implications. First, they may support strategies to optimize outcomes. The findings of increased immunogenetic tuning/sensitvity in the prenatal and perinatal period highlight the importance of ensuring a (immune-)protected maternal

and fetal environment during these life stages. Also, they highlight the importance of integrating medical history and clinical assessment of mothers, so as to identify women carrying a higher load of immunogenetic variations and who may therefore be more susceptible to exaggerated immune activation during pregnancy (e.g.viral exposure). These women, for instance, may benefit from preventive strategies, such as protection to common allergens or inactivated vaccine (e.g., inactivated seasonal flu vaccine). Additionally, our findings highlight the importance of integrating clinical observations in childhood with the systematic recording of familial history and episodes of immune disturbances. This information may help to define subgroups of children with a higher chance of immune dysregulation, and to further explore whether – genetic predisposition to – immune dysregulations in these children may be a precursor to behavioral and cognitive alterations typical of ASD. Last, our findings may inform novel intervention approaches. The identification of specific immunogenetic pathways associated with ASD may guide future clinical trials to test the efficacy of these pathways as putative intervention targets. In this context, transcriptomic signature may be considered as valuable biomarkers to i) screen for autistic individuals with higher immunogenetic variability and for them to iii) monitor the effects of targeted immunomodulatory therapies.

In sum, we emphasize the need for a more systematic investigation of immune genes in ASD. Previous studies have invested attention on well-characterized immune genes and genetic pathways. However, to accommodate the heterogeneity of the immune system and ASD, future research should extend to additional immune mechanisms and investigate these across different clinical profiles, sexes, and age groups.

3.5. Supplementary Materials

Table S 1 List of immune genes included in the SFARI list of ASD-risk genes with information about immune function and SFARI-based category and association score with ASD.

Gene-symbol	Gene name	SFARI category	SFARI score	Immune annotation
ACE	angiotensin I converting enzyme	Rare Single Gene Mutation, Genetic Association	3	innate immunity
ACHE	Acetylcholinesterase (Yt blood group)	Rare Single Gene Mutation	2	jnk signalling
ADRB2	adrenergic, beta-2-, receptor, surface	Genetic Association	3	adrenorecptor / cytokine receptor
AGTR2	angiotensin II receptor, type 2	Rare Single Gene Mutation	3	cytokine receptor
AR	androgen receptor	Genetic Association	3	androgen / cytokine receptor

AVPR1A	arginine vasopressin receptor 1A	Rare Single Gene Mutation, Genetic Association	2	cytokine receptor
AVPR1B	arginine vasopressin receptor 1B	Genetic Association, Functional	3	cytokine receptor
AZGP1	alpha-2-glycoprotein 1, zinc-binding	Rare Single Gene Mutation	3	antigen processing
BRAF	v-raf murine sarcoma viral oncogene homolog B	Rare Single Gene Mutation, Syndromic	1	natural killer cell toxicity
C4B	complement component 4B	Rare Single Gene Mutation, Genetic Association, Functional	3	complement system
CAMK2A	calcium/calmodulin dependent protein kinase II alpha	Rare Single Gene Mutation, Syndromic, Genetic Association, Functional	3	trl signalling and ifn-1 signalling
CARD11	caspase recruitment domain family member 11	Rare Single Gene Mutation	3	caspase signalling bcr signal
CTNNB1	catenin beta 1	Rare Single Gene Mutation, Syndromic	1	ifn signalling / nfkb signalling crp regulation
CX3CR1	Chemokine (C-X3-C motif) receptor 1	Rare Single Gene Mutation, Functional	3	cytokine receptor
DHCR7	7-dehydrocholesterol reductase	Rare Single Gene Mutation, Syndromic	1	ifn signalling macrophage
EIF4E	eukaryotic translation initiation factor 4E	Rare Single Gene Mutation, Genetic Association	3	nfkbia1 - nkfb signalling
ESR2	estrogen receptor 2 (ER beta)	Rare Single Gene Mutation, Syndromic, Genetic Association	3	estrogen / cytokine receptor
ESRRB	estrogen-related receptor beta	Rare Single Gene Mutation, Genetic Association	3	estrogen / cytokine receptor
FABP5	fatty acid binding protein 5 (psoriasis-associated)	Rare Single Gene Mutation, Functional	3	antimicrobial
FGF14	fibroblast growth factor 14	Rare Single Gene Mutation	3	cytokine
FGFR1	fibroblast growth factor receptor 1	Genetic Association	2	cytokine receptor
GFAP	glial fibrillary acidic protein	Rare Single Gene Mutation	1	antimicrobial

HLA-A	major histocompatibility complex, class I, A	Genetic Association	3	antigen processing
HLA-B	Major histocompatibility complex, class I, B	Genetic Association	3	antigen processing
HLA-DPB1	major histocompatibility complex, class II, DP beta 1	Rare Single Gene Mutation, Genetic Association	3	antigen processing
HLA-DRB1	major histocompatibility complex, class II, DR beta 1	Genetic Association	3	antigen processing
HLA-G	major histocompatibility complex, class I, G	Genetic Association	3	natural killer cell toxicity
HMGN1	high mobility group nucleosome binding domain 1	Genetic Association	2	nf-kb signalling
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	Rare Single Gene Mutation, Syndromic, Genetic Association	1	natural killer cell toxicity
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A	Rare Single Gene Mutation, Genetic Association, Functional	3	cytokine receptor
HTR3C	5-hydroxytryptamine (serotonin) receptor 3, family member C	Rare Single Gene Mutation, Genetic Association	3	cytokine receptor
IGF1	insulin like growth factor 1	Rare Single Gene Mutation, Functional	3	cytokine
IL1R2	interleukin 1 receptor, type II	Rare Single Gene Mutation	3	interleukine receptor
IL1RAPL1	interleukin 1 receptor accessory protein-like 1	Rare Single Gene Mutation	3	interleukine receptor
IL1RAPL2	interleukin 1 receptor accessory protein-like 2	Rare Single Gene Mutation, Genetic Association	3	interleukine receptor
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	Rare Single Gene Mutation, Genetic Association	2	innate immunity
ITPR1	inositol 1,4,5-trisphosphate receptor type 1	Rare Single Gene Mutation, Genetic Association	3	fcgr mediated phagocytosis
LEP	Leptin	Rare Single Gene Mutation	3	trl2 signalling in monocytes
LILRB2	leukocyte immunoglobulin like receptor B2	Rare Single Gene Mutation	3	macrophage function

LRP1	LDL receptor related protein 1	Rare Single Gene Mutation	2	antimicrobial
MAPK3	mitogen-activated protein kinase 3	Rare Single Gene Mutation, Functional	3	natural killer cell toxicity
MET	met proto-oncogene (hepatocyte growth factor receptor)	Rare Single Gene Mutation, Genetic Association, Functional	2	cytokine receptor
MSR1	macrophage scavenger receptor 1	Rare Single Gene Mutation	3	trl4 siagnal macrophage inhibition
MTOR	Mechanistic target of rapamycin (serine/threonine kinase)	Rare Single Gene Mutation, Syndromic, Functional	2	trl2 trl4 mediated neutrophil activation
MUC4	mucin 4, cell surface associated	Rare Single Gene Mutation	3	antimicrobial
NEO1	Neogenin 1		3	antimicrobial
NOTCH2NL	notch 2 N-terminal like	Functional	3	innate mcroglia response
NR1D1	nuclear receptor subfamily 1 group D member 1	Rare Single Gene Mutation	3	cytokine receptor
NR2F1	nuclear receptor subfamily 2 group F member 1	Rare Single Gene Mutation, Syndromic, Functional	3	cytokine receptor
NR3C2	Nuclear receptor subfamily 3, group C, member 2	Rare Single Gene Mutation, Syndromic	1	cytokine receptor
NR4A2	nuclear receptor subfamily 4 group A member 2	Rare Single Gene Mutation, Syndromic	1	cytokine receptor
NRP2	neuropilin 2	Rare Single Gene Mutation, Genetic Association	3	cytokine receptor
OXT	oxytocin/neurophysin I prepropeptide	Rare Single Gene Mutation, Genetic Association	3	cytokine
OXTR	oxytocin receptor	Rare Single Gene Mutation, Genetic Association, Functional	2	cytokine receptor
PAK1	p21 (RAC1) activated kinase 1	Rare Single Gene Mutation, Syndromic		natural killer cell toxicity
PAK2	p21 (RAC1) activated kinase 2	Rare Single Gene Mutation	2	tcr signalling
PDCD1	programmed cell death 1	Rare Single Gene Mutation	3	tcr signalling
PIK3CG	phosphoinositide-3-kinase, catalytic, gamma polypeptide	Genetic Association	3	akt signalling trl-signalling

PIK3R2	phosphoinositide-3-kinase regulatory subunit 2	Rare Single Gene Mutation, Syndromic		natural killer cell toxicity
PLAUR	Plasminogen activator, urokinase receptor	Rare Single Gene Mutation, Genetic Association	3	trl3 mediated neutrophil expression
PLXNA3	plexin A3	Rare Single Gene Mutation	3	cytokine receptor
PLXNA4	Plexin A4	Rare Single Gene Mutation, Functional	2	trl-signalling mediating cytokine expression
PLXNB1	plexin B1	Rare Single Gene Mutation	2	cytokine receptor
PRKCA	protein kinase C alpha	Rare Single Gene Mutation	3	myd88 depdent cytokine expression
PRKCB	protein kinase C beta	Rare Single Gene Mutation, Genetic Association	2	myd88 depdent cytokine expression
PRKDC	protein kinase, DNA-activated, catalytic polypeptide	Rare Single Gene Mutation, Syndromic, Functional	3	pdrkc xxcc6/5
PSMD12	proteasome 26S subunit, non-ATPase 12	Syndromic	1	antigen processing
PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	Rare Single Gene Mutation, Syndromic, Functional	1	
PTGS2	prostaglandin-endoperoxide synthase 2	Rare Single Gene Mutation, Genetic Association, Functional	3	nf-kb signal
PTPN11	protein tyrosine phosphatase, non-receptor type 11	Rare Single Gene Mutation, Syndromic	1	natural killer cell toxicity
PTPRC	protein tyrosine phosphatase, receptor type, C	Rare Single Gene Mutation, Genetic Association	3	jak, cytokine signalling via Il-3 signal mediated
RAC1	Rac family small GTPase 1	Syndromic, Functional		trl signall mtor-pik3
RHEB	Ras homolog, mTORC1 binding	Syndromic		mtor my-dependent signalling, dentritic cell

				developme nt
RNF135	Ring finger protein 135	Rare Single Gene Mutation, Syndromic, Genetic Association	3	rig-I signalling
ROBO2	roundabout guidance receptor 2	Rare Single Gene Mutation, Genetic Association, Functional	2	cytokine receptor
RORA	RAR-related orphan receptor A	Rare Single Gene Mutation, Syndromic, Genetic Association, Functional		innate type 2 immunte - nf-kb
RORB	RAR related orphan receptor B	Rare Single Gene Mutation, Syndromic, Functional	1	cytokine receptor
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	Rare Single Gene Mutation, Genetic Association, Functional	2	cytokine
SERPINE1	serpin family E member 1	Genetic Association	3	host defense response
SLC22A15	Solute carrier family 22, member 15	Genetic Association	3	nf-kb signal mapk resolute infalmmatio n
SMAD4	SMAD family member 4	Rare Single Gene Mutation	2	smad signal regulated tgf signal
SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	Rare Single Gene Mutation, Syndromic, Genetic Association		swi/snf family important for cell differentiati on
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	Rare Single Gene Mutation, Syndromic	2	swi/snf family
SOD1	superoxide dismutase 1	Genetic Association, Functional	3	imune protector, inf signal regulation
SYP	synaptophysin	Rare Single Gene Mutation	3	il6st jak2 il-11 signal

TCF4	Transcription factor 4	Rare Single Gene Mutation, Syndromic, Genetic Association, Functional	1	dendritic cell developme nt (plasmoyd)
TEK	TEKreceptortyrosine kinase	Rare Single Gene Mutation	1	cytokine receptor
TET2	Tet methylcytosine dioxygenase 2	Rare Single Gene Mutation	2	repression of il6 transcriptio n to resolute inflammatio n
THBS1	Thrombospondin 1	Rare Single Gene Mutation, Genetic Association	3	decreased apoptosis
THRA	thyroid hormone receptor alpha	Rare Single Gene Mutation, Functional	3	NF-kb signalling
TRAF7	TNF receptor associated factor 7	Rare Single Gene Mutation, Syndromic	1	trl-mediated nf-kb signalling
TRIM32	tripartite motif containing 32	Rare Single Gene Mutation, Functional	3	TMEM173, INGB signalling
TSC1	tuberous sclerosis 1	Rare Single Gene Mutation, Syndromic	1	TRL response inhibitor, through MTOR JNK signal
USP7	Ubiquitin specific peptidase 7 (herpes virus-associated)	Rare Single Gene Mutation, Syndromic	2	TRL-mediated nfkb signal JAK
VDR	vitamin D receptor	Genetic Association, Functional	3	nf-kb signalling
YY1	YY1transcription factor	Rare Single Gene Mutation, Syndromic, Functional		TLR3-signalling
ZBTB16	Zinc finger and BTB domain containing 16	Genetic Association	3	cxcr4 regulation
ZMYND11	Zinc finger, MYND-type containing 11	Rare Single Gene Mutation, Syndromic	2	nf-kb activation

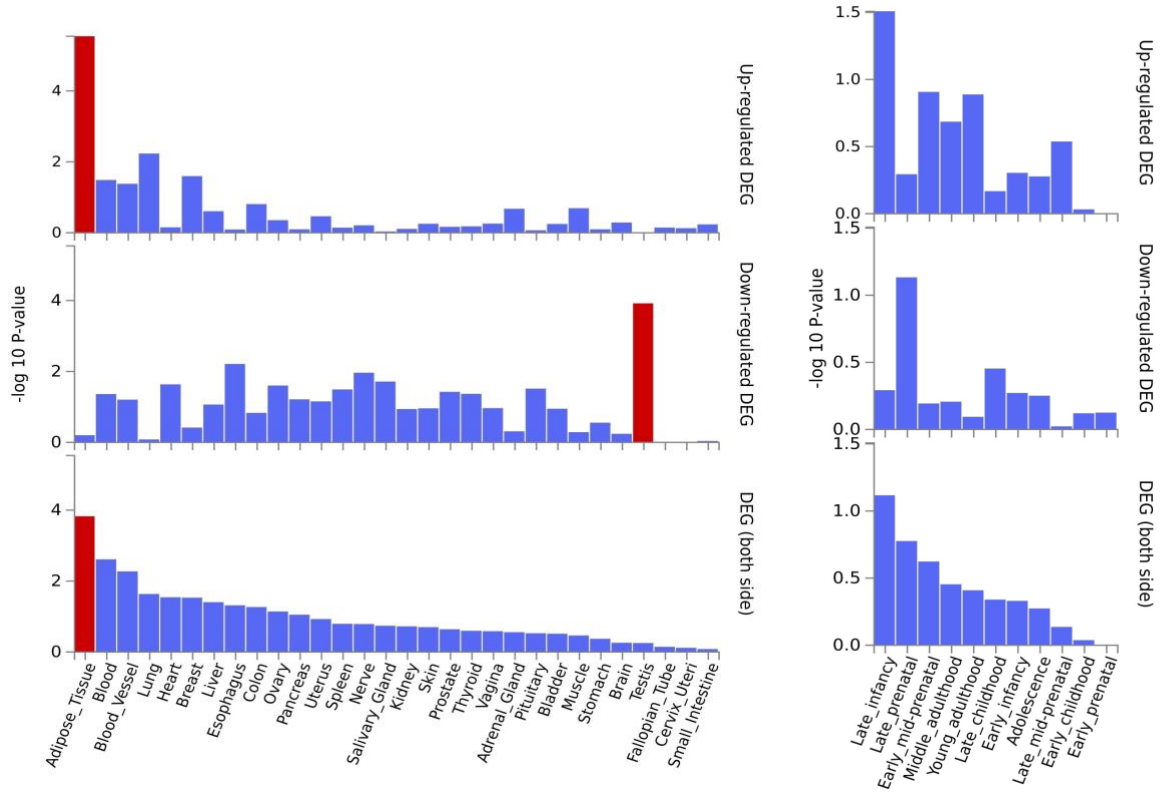

Figure S 1 On the left, FUMA-based results of the tissue expression of identified immune, ASD-risk genes in human tissues from Gtex. On the right, FUMA-based results of the temporal expression of identified immune, ASD-risk genes across neurodevelopmental time epochs from Brain Span. Each graph represents a bar plot showing the degree of enrichment of these genes across tissues, reported on the x-axis. The degree of the enrichment is quantified by the negative logarithm of enrichment p-values on the y-axis. The graphs show genes that are upregulated (top graphs), genes that are downregulated (mid graphs) and overall genes that present expression changes in both directions. Red bars represent significant gene expression results, hence expression changes that occur above the chance level.

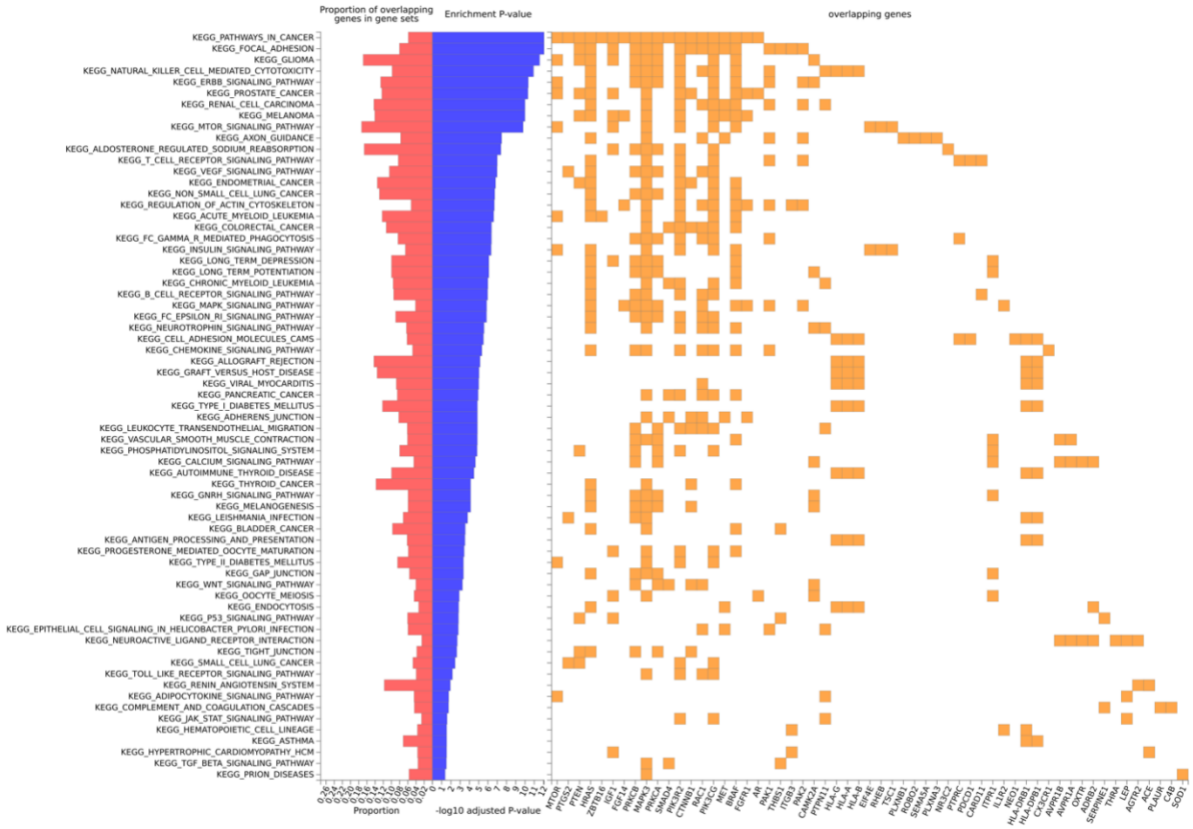

Figure S 2 FUMA-based results of biological enrichment of immune, ASD-risk genes. The top enriched gene-pathways are represented on the y-axis. On the x-axis, the proportion of immune, ASD-risk genes contained in each pathway is reported together with the resulting enrichment p-values. Moreover, an overview of which specific genes are included in each pathway is available on the right-hand side of the graph.

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4. Chapter 4. Genetics of population-based autistic-like traits

In the present chapter, I investigated the genetic underpinnings of four autistic-like traits, together with a total autistic score, that have been measured in the general population. The rationale of this study was that autistic symptoms may manifest at different degrees of severity across individuals. These symptoms are in essence quantitative, they occur to some extent in the general population, and they lead to a diagnosis of ASD when exceeding a clinical 'liability' threshold. This phenotypic continuity between subclinical autistic-like behaviours and clinical ASD has been purported to be underpinned by shared genetic influences. In this chapter, I explore this possibility. First, I performed a meta-analysis of genome-wide association studies of five autistic-like traits carried out in four independent cohorts. This allowed me to define genetic factors associated with distinct autistic-like traits, or dimensions, and their (neuro)biological function. Hence, I explored the genetic relationship between these autistic-like behaviours in the general population and a diagnosis of ASD in a clinical international sample. In brief, this work provides novel suggestions about the influence of immunogenetic factors on specific autistic traits, like rigidity and attention to detail. More generally, I demonstrated that genetic study of autistic-like traits may be useful to gain insights on the complex genetics of ASD, whose definition is challenged by the considerable heterogeneity of the condition.

Potential role of immune-related genes in autism spectrum disorders: Evidence from genome-wide association meta-analysis of autistic traits

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Potential role for immune-related genes in autism spectrum disorders: Evidence from genome-wide association meta-analysis of autistic traits

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Abstract

The clinical heterogeneity of autism spectrum disorders majorly challenges their genetic study. Autism spectrum disorders symptoms occur in milder forms in the general population, as autistic-like traits, and share genetic factors with autism spectrum disorders. Here, we investigate the genetics of individual autistic-like traits to improve our understanding of autism spectrum disorders. We meta-analysed four population-based genome-wide association studies investigating four autistic-like traits – 'attention-to-detail', 'imagination', 'rigidity' and 'social-skills' ($n = 4600$). Using autism spectrum disorder summary statistics from the Psychiatric Genomic Consortium ($N = 46{,}350$), we applied polygenic risk score analyses to understand the genetic relationship between autism spectrum disorders and autistic-like traits. Using MAGMA, we performed gene-based and gene co-expression network analyses to delineate involved genes and pathways. We identified two novel genome-wide significant loci – rs6125844 and rs3731197 – associated with 'attention-to-detail'. We demonstrated shared genetic aetiology between autism spectrum disorders and 'rigidity'. Analysing top variants and genes, we demonstrated a role of the immune-related genes *RNF114, CDKN2A, KAZN, SPATA2* and *ZNF816A* in autistic-like traits. Brain-based genetic expression analyses further linked autistic-like traits to genes involved in immune functioning, and neuronal and synaptic signalling. Overall, our findings highlight the potential of the autistic-like trait–based approach to address the challenges of genetic research in autism spectrum disorders. We provide novel insights showing a potential role of the immune system in specific autism spectrum disorder dimensions.

Lay abstract

Autism spectrum disorders are complex, with a strong genetic basis. Genetic research in autism spectrum disorders is limited by the fact that these disorders are largely heterogeneous so that patients are variable in their clinical presentations. To address this limitation, we investigated the genetics of individual dimensions of the autism spectrum disorder phenotypes, or autistic-like traits. These autistic-like traits are continuous variations in autistic behaviours that occur in the general population. Therefore, we meta-analysed data from four different population cohorts in which autistic-like traits were measured. We performed a set of genetic analyses to identify common variants for autistic-like traits, understand how these variants related to autism spectrum disorders, and how they contribute to neurobiological

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processes. Our results showed genetic associations with specific autistic-like traits and a link to the immune system. We offer an example of the potential to use a dimensional approach when dealing with heterogeneous, complex disorder like autism spectrum disorder. Decomposing the complex autism spectrum disorder phenotype in its core features can inform on the specific biology of these features which is likely to account to clinical variability in patients.

Keywords
autism spectrum disorders, genetics, immune system, molecular and cellular biology

Introduction

Autism spectrum disorders (ASDs) refer to a class of common and pervasive conditions with an early life onset (Lai et al., 2014). Core ASD characteristics are impaired social communication and interaction, and repetitive, restrictive interests and behaviours, along with sensory abnormalities (Hazen et al., 2014). These symptoms impact on patients' quality of life and on individual caretakers and society (Billstedt et al., 2011). Considering the increasing prevalence of ASDs (~2%) and the lack of effective treatments, there is an imperative to understand ASD aetiology (Fombonne, 2018; Kim et al., 2011).

Twin studies indicate that ASDs are highly heritable (h^2~70%–90%), demonstrating the importance of genetic research on these conditions (Tick et al., 2016). However, ASDs are genetically complex and multifactorial, with rare and common variants involved (de la Torre-Ubieta et al., 2016; Grove et al., 2019). Genome-wide association studies (GWASs), comparing cases to controls, represent the gold standard for identifying common genetic risk variants for multifactorial disorders like ASDs. To date, GWASs have been limited as extremely large samples are needed to find robustly associated risk variants. The most recent ASD-GWAS meta-analysis included 18,381 cases and 27,969 controls and detected five independent genome-wide significant loci (Grove et al., 2019). Two additional genome-wide significant loci were identified after meta-analysing these data with genetic data from the European cohort of the Simons Foundation Powering Autism Research Knowledge (SPARK) project, leading to a total sample size of 55,420 (Matoba et al., 2020). Functional analysis of the ASD-GWAS top findings, together with rare genetic variant and animal studies, revealed a broad molecular landscape for ASD, involving steroidogenesis, and neurobiological processes, like neurite outgrowth and synaptic function (Poelmans et al., 2013). The neurobiological nature of ASDs is confirmed by neuroimaging studies reporting neuroanatomical alterations in ASDs, although results differ across individuals (Chen et al., 2019; Van Rooij et al., 2018). Nevertheless, the largest to-date meta-analysis showed robust differences in the frontal and striatal regions in ASD cases compared to controls (Van Rooij et al., 2018).

Overall, phenotypic heterogeneity constitutes a major obstacle to the study of ASD aetiology, and, therefore, it is important to address this issue. It has been demonstrated that complex disorders like ASDs represent the extreme manifestation of quantitative traits occurring in the general population along a continuum (Constantino & Todd, 2003). Autistic-like traits (ALTs) refer to those continuous variations in social skills and repetitive behaviours that in their most severe forms define ASDs (Constantino & Todd, 2003). Each ALT captures a distinct ASD feature, parsing the complex autistic phenotype. The continuous ALT distribution is determined by the cumulative effect of many common, small-effect genetic variants that collectively increase ASD risk (Weiner, 2017). Hence, the genetic study of ALTs may constitute a route to disentangle the complex ASD genetics. Previous studies highlighted the potential of ALT-based research to investigate ASD aetiology (Colvert et al., 2015; Constantino & Todd, 2003; Jones, 2015; Lundström et al., 2012; Robinson et al., 2013, 2016; Taylor et al., 2019). These studies, in fact, demonstrated genetic correlations between ASDs and social and communication skills in the general population. In addition, Bralten et al. (2018) defined five core ALTs – attention-to-detail, childhood behaviour, imagination, rigidity and social skills – through factor analyses of ASD measurements, and indicated a shared genetic aetiology between specific traits and clinical ASDs.

The GWAS approach has gained popularity in studying population-based, quantitative traits (McCarthy et al., 2008). For ALTs, GWASs revealed suggestive associated genomic loci (Jones, 2015). However, previous ALT-GWASs in unrelated individuals from the general population relied on limited samples (N=~2000), lacking power to detect robust genome-wide associations (Bralten et al., 2018; Robinson et al., 2016). Future research on larger study populations is therefore needed to detect ALT-associated variants. Moreover, ALT-GWASs thus far investigated social skills, whereas rigidity and attention have been under-represented (Jones, 2015). Considering the ASD phenotypic diversity, research encompassing a wide range of ALTs is needed. Well-powered, comprehensive ALT research may lead to novel genetic findings and reveal biological pathways involved in the ALT-ASD continuum.

This study investigates the genetics and biology of four ALTs: 'attention-to-detail', 'imagination', 'rigidity' and 'social-skills'. First, we aimed to identify common genetic risk variants associated with ALTs. Therefore, we

Table 1. Descriptive statistics of the four population cohorts included in the meta-analysis.

Cohort	*N*	Mean age (SD)	Gender (% male)	Genotyping platform	Mean ALT scores (SD)			
					Attention	Imagination	Rigidity	Social skills
NBS	2847	28.4 (2.7)	46%	Illumina Human OmniExpress BeadChip	6.4 (1.2)	5.4 (1.7)	8.4 (2.6)	6.3 (2.1)
BIG	372	25.6 (4.5)	43%	Affymetrix GeneChip Array 6.0	6.3 (1.4)	4.3 (1.4)	8.3 (2.1)	6.11 (1.8)
Genetics of Cognition	416	24.4 (4.7)	39%	Illumina Infinium PsychArray-24 BeadChip	5.8 (2.1)	4.6 (1.78)	7.6 (1.6)	5.6 (2.2)
Raine	965	19.7 (0.7)	49%	Illumina Human 660W Quad array	5.4 (1.5)	4.0 (1.4)	9.7 (1.7)	7.6 (1.4)

SD: standard deviation; ALT: autistic-like trait; NBS: Nijmegen Biomedical Study; BIG: Brain Imaging Genetics.
Information about sample size, age, gender and genotyping platform for the four cohorts included (Franke et al., 2010; Galesloot et al., 2017; Jones et al., 2013; Pinar et al., 2018).

meta-analysed GWAS data for the ALTs from four cohorts of in total 4600 individuals and we assessed the shared genetic aetiology between ALTs and ASDs. Subsequently, we identified ALT-associated genes through gene-wide analyses that we combined with gene-expression network analyses to identify biological pathways associated with ALTs across established ASD-related brain regions.

Methods

Study cohorts and consortia

This study is an international collaboration including raw genotyping data and GWAS summary statistics from four study cohorts (Table 1) Community members were not included in this study.

Nijmegen Biomedical Study. The Nijmegen Biomedical Study (NBS; http://www.nijmegenbiomedischestudie.nl/) is a population-based study set up by the Department of Health Evidence and the Department of Laboratory Medicine of the Radboud University Medical Center (Radboudumc) in Nijmegen, The Netherlands. The NBS investigates the role of genetic and environmental factors on individual well-being (Galesloot et al., 2017). The study was approved by the Institutional Review Board of Radboudumc (CMO 2001/055). All participants completed written informed consent. This study included imputed genotyping data and ALT scores for a total of 2847 Dutch individuals participating in the NBS.

Brain Imaging Genetics. The Brain Imaging Genetics (BIG; http://www.cognomics.nl/big.html) project is an initiative promoted by the Human Genetics Department of the Radboudumc, the Donders Centre for Cognitive Neuroimaging of the Radboud University, and the Max Planck Institute for Psycholinguistics in Nijmegen, The Netherlands. The BIG project investigates genetic variation linked to behaviour, cognition, brain structure and function in the general population (Franke et al., 2010). The BIG study was approved by the regional medical ethics committee (CMO Regio Arnhem/Nijmegen). All participants provided written informed consent. This study included imputed genotyping data and ALT scores for a total of 372 individuals of European ancestry participating in the BIG project.

The Raine Study. The Raine Study (https://rainestudy.org.au/) is a large prospective cohort study of pregnancy, childhood, adolescence and adulthood based in Western Australia. The study investigates the role of genetic and environmental factors on individual well-being using a longitudinal approach (Jones et al., 2013). The Raine Study was approved by the Human Research Ethics Committee at the King Edward Memorial Hospital and University of Western Australia. The Raine Study Gen2 participants and their family provided written informed consent. This study used GWAS summary statistics of four ALTs for a total of 945 European individuals participating in the Raine Gen2-20 year follow-up study.

Genetics of Cognition. Genetics of Cognition (GenofCog) is a general population study set up by the Turner Institute of Brain and Mental Health and the Monash University in Melbourne, Australia. The study investigates genetic variations and neural correlates related to cognition and psychopathology (Pinar et al., 2018). The study was approved by the Monash University Ethics Committee. All participants provided written informed consent. This study used imputed genotyping data and ALTs score for 436 European individuals participating in the GenofCog study.

Assessment of autistic traits

The Autism Spectrum Quotient (AQ) (Baron-Cohen et al., 2001) and a customised ALT questionnaire developed by Bralten et al. (2018) were used to measure the four ALTs across our cohorts. The AQ, a self-report 50-item questionnaire, was adopted to assess ALTs in the Raine Study and in the GenofCog cohorts. The customised ALT questionnaire is a self-report, shorter measure consisting of 18 items of which 12 are AQ-derived and six refer to ASD criteria described in the Diagnostic and Statistical Manual

of Mental Disorder (Bralten et al., 2018). The questionnaire demonstrated construct validity, as shown by a moderately high internal consistency (Cronbach's $\alpha = 0.70$) of the total score to the 18 items (Bralten et al., 2018). Bralten et al. (2018) showed that the 12 AQ-derived items of this questionnaire converge on four factors – 'attention-to-detail', 'imagination', 'rigidity' and 'social-skills' – while the six Diagnostic and Statistical Manual of Mental Disorders (DSM)-based items cluster into a fifth factor: 'childhood-behaviour'(Bralten et al., 2018). These items explain 50.7% of the variance in the total autistic score. This questionnaire was adopted to measure ALTs in the NBS and BIG samples. To ensure homogeneity across studies, we exclusively considered the 12 items shared between the two questionnaires and therefore investigated the ALTs 'attention-to-detail', 'imagination', 'rigidity' and 'social-skills'. A list of the 12 items considered for this study can be found in Supplementary Table 2. Individual ALT scores were calculated, and outliers (2 standard deviations (2SD) from the mean) removed (see Table 1 for mean ALT values). Scores were then corrected for age and sex and residuals were used in following analyses after being checked for independence and log-transformed to ensure normality using SPSS21.

Genome-wide association analyses and meta-analyses

Genotyping was performed on study-specific platforms (Table 1). Initial single nucleotide polymorphism (SNP) filtering was applied on call rate (>95%), Hardy–Weinberg equilibrium (HWE $<1^{-6}$) and minor allele frequency (MAF >0.01). In each cohort, genotyped data for the autosomal chromosomes were imputed to increase genotype density and achieve fine genome-wide mapping. For all data sets, imputation followed the ENIGMA protocol (http://enigma.ini.usc.edu/wp-content/uploads/2012/07/ENIGMA2_1KGP_cookbook_v3.pdf), that adopts the 1000 Human Genome Project reference panel and the software MACH (Li et al., 2010). SNPs with imputation quality <0.7 were excluded. Multidimensional scaling (MDS) was performed to assess population structure. Next, independent ALT-GWASs were performed in each cohort implementing a linear regression model in Mach2qtl, fitting the quantitative nature of ALTs. The model included sex, age and the four MDS components as covariates. Cohort-specific results were quality-controlled using the *clean* function of the *EASYQC* package in R (Winkler et al., 2014) and data were combined in four ALT-specific inverse-variance weighted meta-analyses in METAL (Willer et al., 2010). The final analysis included a total of 8,284,544 autosomal SNPs. We applied the significant p-threshold of $p < 1.25^{-9}$, referring to the canonical GWAS p-threshold ($p < 5^{-8}$) divided by the ALTs tested. In order to estimate the statistical power we had to find genome-wide significantly associated SNPs in our meta-analysis of 4600 individuals, we performed a power calculation in QUANTO (Gauderman, 2002). The power analysis showed that GWAS of quantitative traits, under the assumption of normal trait distribution, required more than 4000 individuals to identify SNPs with frequency >1% at 80% power.

Shared genetic aetiology analysis

Polygenic risk score (PRS)–based analyses were applied to estimate the extent of shared, common variant, genetic aetiology between clinical ASDs and ALTs using PRSice (v1.25) (Euesden et al., 2015). To set the 'base ASD phenotype' we used publicly available ASD-GWAS summary statistics by an independent cohort, not-overlapping with the ALT cohorts from the Psychiatric Genomic Consortium (PGC) and the Lundbeck Foundation Initiative for Integrative Psychiatric Research (iPSYCH) counting 18,381 cases and 27,969 controls (The Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium, 2017). Summary statistics for ALTs (obtained from the meta-analysis of our cohort-specific GWAS data) were used to define the 'target' phenotypes. Four separate PRS-based analyses have been conducted between ASDs and each ALT at a time, using the summary–summary statistics-based approach (Euesden et al., 2015). Clumping, using PLINK, preceded the actual PRS calculation to ensure that only index SNPs for each linkage disequilibrium (LD) block ($r^2 < 0.25$, 500 kb) throughout the genome were considered. Next, PRSs were calculated on the clumped ASD summary statistics and included only SNPs exceeding seven broad p-value thresholds (i.e. $P_T < 0.001$, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5). For each threshold, ASD-PRS was extracted and used to estimate the extent of shared genetic aetiology with ALTs. p-values were corrected using the False Discovery Rate (FDR) method in R.

Gene-wide analyses

Gene-wide analyses on ALT-GWAS results were performed to identify ALT-associated genes using the Multi-marker Analysis of GenoMic Annotation (MAGMA) software (de Leeuw et al., 2015). First, ALT-SNPs were annotated to genes using 100 kb downstream and upstream flanking regions to include regulatory regions. Next, a gene-specific Z-statistic was obtained considering the p-values of gene-related SNPs, while correcting for LD. We applied the significance p-value threshold $p = 2.8^{-6}$, accounting for the total number of genes tested.

Gene co-expression network analyses

To explore the functional role of ALT-associated SNPs, we performed gene co-expression network analysis using the

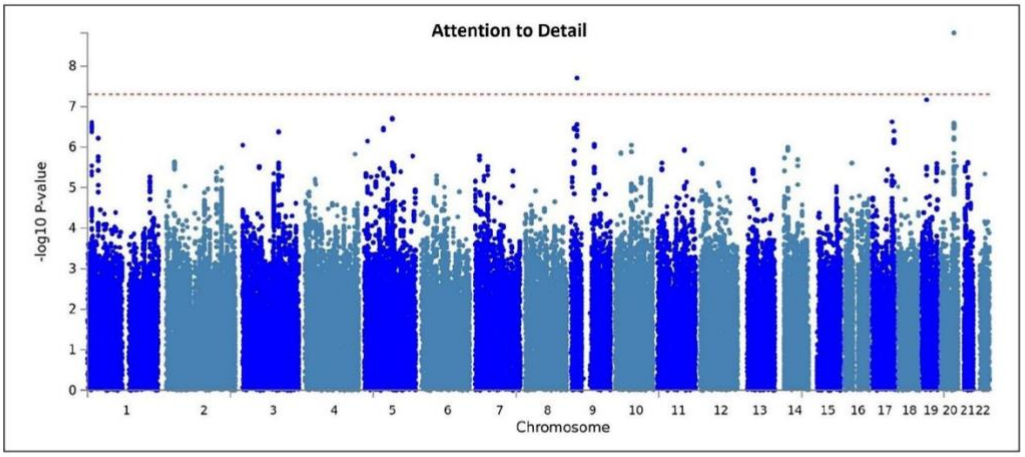

Figure 1. Manhattan plot of the GWAS meta-analysis for 'attention to detail'.
Each dot represents the result of the linear regression analysis for each single variant taking the attention to detail mean score as dependent variable and correcting for age, sex, gender and four MDS components. The *x*-axis shows the chromosomes and the *y*-axis shows the −log (two-sided) *p*-value of the association. The red dotted line indicates the threshold for genome-wide significance.

eQTL-MAGMA (e-MAGMA) software package (Gerring et al., 2019). Analyses followed the procedure described in https://github.com/eskederks/eMAGMA-tutorial. Accordingly, we mapped SNPs to genes based on available annotation files, that were tissue-specific and referred to significant (FDR <0.05) SNP-gene associations from GTEx (https://www.gtexportal.org/home/). Next, we performed gene-wide analyses to link ALT-GWAS SNPs to eQTL-associated gene (eGenes) using annotation files for seven ASD-associated brain regions (i.e. total cortex, frontal cortex, anterior cingulate cortex, putamen, caudate, nucleus accumbens and amygdala) (Van Rooij et al., 2018). Gene-wide analyses adopted the MAGMA approach and provided an eGene-specific *Z*-statistic reflecting association with ALTs. Subsequently, we performed gene-set expression analyses using gene-set annotations referring to region-specific co-expression gene-networks. These region-specific co-expression networks are divided into sets or modules (indexed by colour) of correlated genes. Using the MAGMA gene-set approach, we performed a competitive test testing the association of each module with the ALTs. Results were then Bonferroni-corrected (i.e. accounting for the total of gene-modules tested). Finally, we performed post hoc analyses on the significant gene-set associations to define the biological functions of the identified gene-expression modules. We used the g:GOst tool from the g:Profiler webserver (https://biit.cs.ut.ee/gprofiler/gost) (Kull et al., 2007), which performed gene-set enrichment analyses on input gene lists using a Fisher's one-tailed test, based on Gene Ontology (GO) annotations (http://geneontology.org/). The g:SCS option was chosen to correct for multiple testing while controlling for the inter-correlation between GO terms.

We integrated brain-specific gene expression analyses for ALTs with gene expression analyses across a wider range of human tissues. To do so, we exploited the tool for functional mapping and annotation (FUMA) of GWAS that refers to tissue-specific expression patterns based on GTEx v6 RNA-seq data (Watanabe et al., 2017). We used the summary statistics for each ALT as input.

Results

Meta-analysis of genome-wide association with autistic traits

ALT-based meta-analyses revealed genome-wide significant associations of 'attention-to-detail' with two SNPs, *rs6125844* ($p = 1.52 \times 10^{-9}$) and *rs3731197* ($p = 1.9 \times 10^{-8}$) (Figure 1). The *rs6125844*-association survived our stringent correction ($p < 1.25 \times 10^{-9}$), whereas the *rs3731197*-association exceeded the genome-wide significance threshold ($p < 5 \times 10^{-8}$). No SNP-association reached significance for the other ALTs (*see Supplementary Information*).

Shared genetic aetiology between ASDs and autistic traits

Using the PRS-based approach, we found a shared genetic aetiology between clinical ASDs and 'rigidity' (Figure 2). The most predictive thresholds were $P_T = 0.05$, $P_T = 0.1$ and $P_T = 0.5$ (FDR-corrected $p < 0.01$). Considering the common genetic variants captured by our analyses, we did not find a statistically significant genetic sharing between ASDs and other ALTs (see *Supplementary Information*).

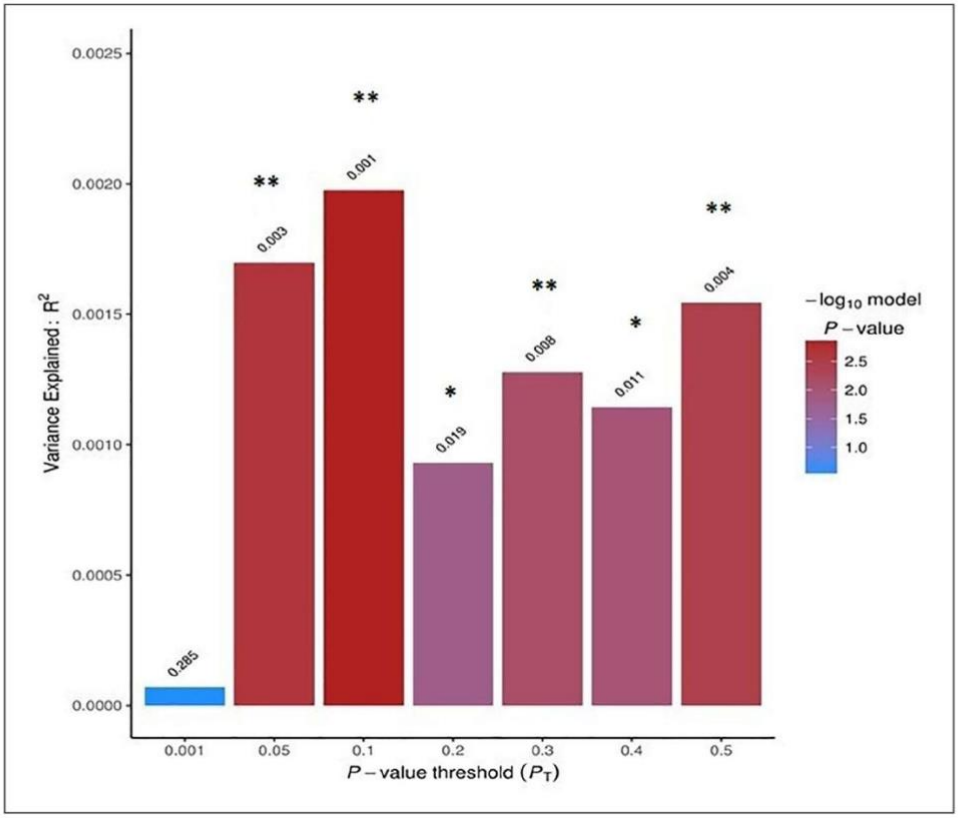

Figure 2. Polygenic risk-based results of ASDs and 'rigidity'.
Polygenic risk score-based results showing the degree of shared genetic aetiology between ASDs ('base' phenotype) and 'rigidity' ('target' phenotype) at seven broad *p*-value thresholds (P_T). The bar plot was created with PRSice1. The *x*-axis displays the seven *p*-value thresholds tested and the *y*-axis displays the variance explained by the genetics of the 'base' phenotype in the 'target' phenotype. The colours of the bars indicated the −log10 *p*-value of the association.
*p-values < 0.05 after FDR-correction; **p-values < 0.01 after FDR-correction.

Gene-wide analyses

SNPs included in the ALT meta-analyses mapped onto a total of 17,867 autosomal genes. Gene-wide analyses showed that four genes – *RNF114, CDKN2A, SPATA2, KAZN* – were significantly associated with 'attention-to-detail'. In addition, *ZNF816* was significantly associated with 'social skills' (Table 2). Literature-based analyses of these genes indicated an involvement in immune regulation and inflammatory phenotypes, like psoriasis. No gene-association survived the Bonferroni-correction for 'imagination' and 'rigidity'.

Gene co-expression network analyses

Gene co-expression network analyses revealed ALT-specific associations with gene-expression modules across ASD-related brain regions. Namely, we observed that attention-to-detail eGenes were statistically associated with the expression-module for total cortex ($p=0.001$), while nominally associated with expression-module for putamen ($p=0.01$). Imagination-related eGenes were associated the expression-module for nucleus accumbens ($p=0.003$) and amygdala ($p=0.001$). Rigidity-related eGenes were nominally associated with modules for anterior cingulate cortex ($p=0.01$) and nucleus accumbens ($p=0.01$). Finally, social skill–related eGenes were associated with an expression-module for anterior cingulate cortex ($p=0.001$) and putamen ($p=0.002$); functional enrichment analyses revealed a expression-module enrichment for biological processes, including synaptic signalling, neurogenesis. Among the enriched pathways we also identified immune-related processes, such as cytokine signalling, adding support to the results of our gene-wide analyses of ALTs. Results of the e-MAGMA and enrichment ALT-analyses are presented in the *Supplementary*

Table 2. MAGMA-based significant results of gene-wide analyses.

ALT	Associated genes	*p*-value
Attention to detail	*RNF114*	2.05e−7
	CDNK2	4.52e−7
	SPATA2	2.30e−6
	KAZN	4.67e−7
Imagination	–	–
Rigidity	–	–
Social skills	*ZNF816A*	6.4e−7

MAGMA: Multi-marker Analysis of GenoMic Annotation; ALT: autistic-like trait.
Association results from MAGMA-based gene-wide analyses. Indicated top genes exceeded the Bonferroni-corrected threshold of $p = 2.8^{-6}$ to account for the number of genes tested ($N = 17{,}867$).

Information (Supplementary Table 1), as well as results from FUMA-based analyses of gene expression across multiple human tissues beyond brain *(Supplementary Figures 8–11)*. Additional data about GWASs results, PRS-based analyses and gene co-expression network analyses can be found in the *Supplementary Information* materials.

Discussion

In this study, we investigated the genetics of four ALTs – 'attention-to-detail', 'imagination', 'rigidity' and 'social-skills' – by meta-analysing GWAS data of 4600 individuals from the general population. We found two common genetic variants (*rs6125844* and *rs3731197*) that were significantly associated with 'attention-to-detail'. Our PRS-based analysis was significant for the comparison between ASDs and 'rigidity'. Next, we showed significant associations between 'attention-to-detail' and *RNF114, CDKN2A, SPATA2* and *KAZN*, and between 'social-skills' and *ZNF816A*. Finally, we demonstrated that ALT-eQTLs are associated with gene-networks in ASD-related brain regions. Biological characterisation of these gene-networks showed enrichment in synaptic signalling, neurogenesis and the immune response.

By meta-analysing the largest available population-based data sets for 'attention-to-detail', 'imagination', 'rigidity' and 'social-skills', we were able to identify two SNPs, r*s6125844* and *rs3731197*, significantly associated with 'attention-to-detail'. The SNP *rs6125844* is mapped to a *cis*-regulatory region for *RNF114*, potentially influencing its genetic expression. *RNF114* is an E3-ubiquitin ligase that has been implicated in immune reactivity through direct regulation of the NF-kB pathway and relation with innate immunity mediators, suggesting a role of immunity-related genetics in 'attention-to-detail' (Bijlmakers et al., 2011). Furthermore, *RNF114* promotes the ubiquitination and degradation of cyclin-dependent kinase inhibitors (CKIs), that contribute to neuronal functions, like axon guidance and synaptic signalling which have been linked to ASD pathophysiology (Kawauchi et al., 2013; Poelmans et al., 2013). These CKIs have also been proposed as putative targets for the resolution of ongoing inflammation (Laphanuwat & Jirawatnotai, 2019). In agreement with that, our second top-associated SNP, *rs3731197*, is located in an intron of *CDKN2A* and in a *cis*-regulatory region for *CDKN2B*, both belonging to the CKI complex. Both *CDKN2A* and *CDKN2B* encode key proteins important for neurodevelopment and show immune-regulatory properties (Kawauchi et al., 2013). In brief, our top SNP-associations suggest a link between 'attention-to-detail' and immune regulators, that deserves consideration in future ALT-based research.

Our findings confirm our hypothesis and previous results of a degree of genetic association between ALTs and ASDs (Robinson et al., 2016). The results of our PRS-based analyses, linking common variants for ASDs and 'rigidity', are in line with previous analyses on a subset of our data set that showed genetic sharing between ASDs and this ALT (Bralten et al., 2018). In general, these findings indicate an existing ALT-to-ASD genetic continuity and validate the idea of using ALT-data to address the complex genetics of ASDs. The quantitative-trait approach also fits with the research domain criteria (RDoC) paradigm promoted by the National Institute of Mental Health (NIMH) that aims to dismiss categorial classification of mental disorders in favour of dimensional definitions (Insel, 2014). By looking at specific functional domains, that cut across psychiatric categories, a quantitative-trait approach may circumvent the heterogeneity and comorbidity associated with DSM-based diagnoses. For instance, 'rigidity' is a trait observed in ASDs, obsessive-compulsive disorder (OCD) and anxiety disorders (Morris & Mansell, 2018). Research on rigidity may therefore offer insights into molecular mechanism(s) underlying all these conditions and ideally defining common target(s) of intervention.

Following GWAS meta-analyses, gene-wide analyses revealed that four genes – *RNF114, CDKN2A, SPATA2* and *KAZN* – were significantly associated with 'attention-to-detail'. As mentioned, *RNF114* and *CDKN2A* encode proteins directly involved in immune regulation through their action on the NF-kB signalling pathway (Bijlmakers et al., 2011; Pramanik et al., 2018). Moreover, *SPATA2* is also shown to regulate TNF-induced NF-kB signalling and appears specifically expressed in testis Sartori cells, an immune-privileged site and has been implicated in inflammation (Schlicher et al., 2017). Given the strong male prevalence in ASDs, *SPATA2* expression in the testis may reveal a sex-specific effect of this gene in immune regulation. This hypothesis should be addressed in future research by adopting a sex-stratified approach to ALTs. Besides, we also observed a significant association between 'social-skills' and *ZNF816*. Like *RNF114, ZNF816* encodes for a zinc-finger protein involved in immune processes, like

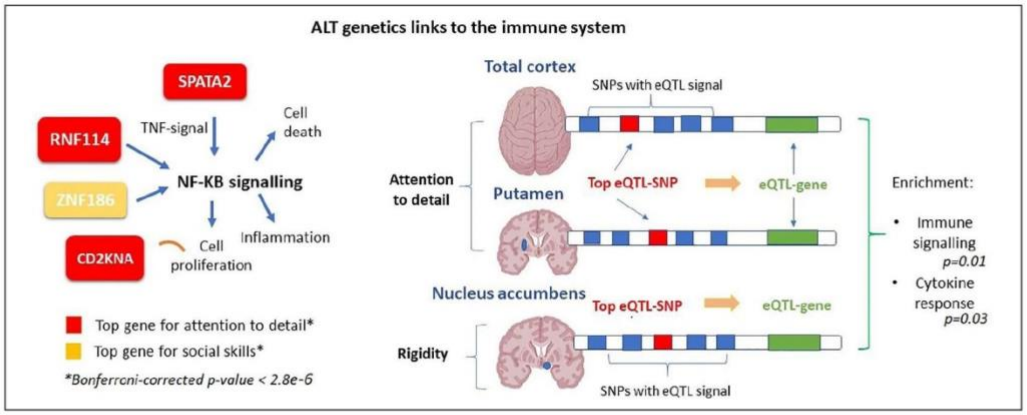

Figure 3. Summary figure of immune-related findings in ALT genetics.
Summary figure to illustrate our findings pointing to a relationship between ALTs and the immune system. On the right, results of gene-wide analyses highlight significant ALTs associations with genes that are involved in immune functioning (NF-KB signalling). On the left, results of gene co-expression network analyses show that eQTL-genes, linked to ALTs, for total cortex, putamen and nucleus accumbens are enriched in immune processes (association *p*-values for the immune pathways are reported).
*Genes exceeded the Bonferroni-corrected *p*-value threshold of 2.8e−6.

NF-KB signalling, and implicated in autoimmune diseases as shown in previous studies (Kallionpää et al., 2014; Stuart et al., 2015). These results, therefore, further suggest a role of immunity-related genetics in ALTs. Given the genetic correspondence between ASDs and ALTs, these results stress the importance to further investigate the relationship between immunogenetics and ASDs. Immune dysregulation, either in the form of ongoing inflammation or autoimmunity, is prevalent in ASDs (McAllister, 2017). ASDs are, in fact, associated with family history of autoimmune diseases, like celiac disease and rheumatoid arthritis (Atladóttir et al., 2009; Ludvigsson et al., 2013), and either increased or decreased levels of inflammatory markers have been found in the blood of ASD individuals (Goines & Ashwood, 2013). However, immune dysregulation varies among patients and it is purported to be confined to a specific ASD-subgroup (Careaga, 2017). This, together with our finding of a trait-specific immune link, suggests that immunity might be associated specifically with certain autistic features. ALT-based research could, then, clarify the complex relationship between immunity and ASDs, by revealing ALT-related immunobiological mechanisms and pathways for dimension-specific pharmacotherapy. This research line is of particular relevance for ASDs given the high heterogeneity of these disorders, that suggests the improbability of a one-fit-all treatment, but the need for an intervention that is tailored on the patient's characteristics. To this regard, PRS-based analyses could help to identify potential clinical subtypes, that would benefit from specific treatment(s).

We followed our results with gene co-expression network analyses that assess the expression of genes derived by genetic variation in particular tissues, in our case established ASD-related brain regions. These analyses revealed an enrichment of neuronal and synaptic signalling in the cortex for 'imagination'. These results are in line with previous literature showing that ASD-related genes are over-represented in neuronal processes and (glutamatergic) synaptic signalling (Poelmans et al., 2013). However, the fact that we observed trait-specific associations demonstrate biological variability between ALTs. This is supported by evidence from Warrier et al. (2019) of dissociable genetics between the ASD-like empathising and systematising behaviours. Importantly, synaptic dysregulation also occur in OCD (Ting & Feng, 2008), that often co-exists with ASDs and with these, shares behavioural rigidity (Meiran et al., 2011). Synaptic functioning may therefore constitute a common mechanism, potentially influencing the cross-disorder phenotype rigidity. In addition, enrichment analyses showed that ALT-related gene-networks across brain regions are involved in the immune response. Namely, we found that attention-to-detail and rigidity-related genes for total cortex, putamen and nucleus accumbens were enriched in immunological processes. These results corroborate SNP and gene-level findings results of an immune link to ALTs. Figure 3 provides an overview of our findings pointing to a link between the immune system and ALT genetics. In general, the observed enrichment of ALT-genes in neuro-immune processes demonstrates that both the immune and nervous system contribute to ALTs. This fits the evidence of a neuro-immune cross-talk during neurodevelopment and the findings of immune-related molecules driving neuronal growth and communication (Debnath et al., 2018; Nutma et al., 2019). Since ASDs are linked to aberrant neurodevelopment, it is important to understand

the role of immune-related molecules along the ALT-to-ASD continuum.

This study should be evaluated in the light of some strengths and limitations. First, trait-oriented research exploits population-based cohorts for which large data are accessible at little cost. Relying on population-based cohorts, we combined data from multiple sources worldwide to obtain a sample large enough to perform a well-powered genetic ALT-investigation. The resulting sample size was, indeed, increased with respect to previous GWASs of ALTs that counted ~2000 unrelated individuals. Second, the analysis of our top findings offered new insights into the biology of autistic-like behaviours, hinting to the immune response. This does not only indicate potential areas for future investigation, but it helps to clarify the molecular profile underlying diverse autistic dimensions. The aggregation of multiple cohorts, however, increased the variability of this study population. Differences in gender distribution exist in the cohorts used (see Table 1). Although gender differences are documented in population-based surveys (Nutma et al., 2019), this might influence the representativeness of study cohorts and, therefore, more gender-balanced samples should be investigated. Also, our population included individuals exposed to different cultural and geographical backgrounds. Such differences may have biased, for example, the individual interpretation of ALTs and ultimately, the self-reporting of these traits. We indeed referred to self-report measurement that is intrinsically limited by the respondents' interpretation of the items. The increase of heterogeneity, resulting from the aggregation of multiple datasets, could, in fact, explain our failure to replicate previous findings of a significant association between 'rigidity' and the *MET* gene as observed in only the NBS cohort. Also, although the levels of ALTs in our cohort have been checked for normality, we could not exclude the possibility that any individual received a formal ASD diagnosis. To this regard, we believe that future ALT-based research, adopting an ASD diagnosis as exclusion criteria and potentially even larger sample sizes, could help to validate our conclusions. Moreover, this study explored genetic variants associated with factors extracted from a validated ALT questionnaire. Our findings of genetic diversity between ALTs should therefore encourage and seek for replication in future ALT research relying on questionnaires developed for each of these ALTs.

Our PRS-based analyses indicate a significant genetic association between polygenic risk scores for ASDs and 'rigidity'; however, ASD-related variants could explain at most 0.20% of the genetic variance for 'rigidity'. This result differs from previous a report that demonstrated a genetic correlation between population-based autistic traits and autistic formal diagnoses of ~0.50–0.60 (Colvert et al., 2015). The polygenic nature of ALTs, the phenotypic variability in our cohorts and the difference between base and target sample sizes could potentially account for the low variance explained in this study. However, our population-based genetic analyses investigate specifically common genetic variants, while previous twin studies also included rare variants and gene × environment interactions that might contribute to the observed difference in findings. However, our results seems consistent with previous reports applying the PRS-based method to neuropsychiatric phenotypes. Namely, PRS for OCD explained only 0.2% of the variance in obsessive-compulsive symptoms in the general population (den Braber et al., 2016). Also, although we observed significant association between PRS for ASDs and rigidity at different thresholds (P_T=0.05, 0.1 and 0.5), we could not find significant association when considering SNPs at the most conservative threshold (P_T=0.001). This result may indicate a combined effect of a wide range of small-effect ASD-related SNPs in predicting ALT variability. However, further PRS-based analyses, involving larger samples, are needed to clarify the genetic relationship between ASDs and ALTs. To further clarify the ALT-ASD genetic relationship, it would be valuable to assess the extent of symptoms variance explained by each ALT's genetics specifically within individuals diagnosed with ASD. Large patient-based studies would be needed to address this point in future studies.

Finally, this study specifically investigated common genetic variations associated with population-based autistic traits. In light of the confirmed role of both common and rare variants in ASDs (De Rubeis et al., 2015; Grove et al., 2019; Iossifov et al., 2014), we believe that our understanding of the genetic architecture of ALTs could benefit from analysis of rare genetic variations, which should be considered as object of study in the future.

Conclusion

Our analyses revealed genetic loci linked to ALTs in the general population which may be of relevance for ASDs. Our data demonstrate genetic concordance between ALTs and clinical ASDs demonstrating the potential to use population-based ALTs to address the complex ASD genetics. ALT-associated SNPs and genes seem involved in the immune response and eQTL signals for different ALTs are enriched for immune-related processes in the brain. These findings suggest an immune-ALT link that should inform further investigation. Overall, research on disorder-related traits has the potential to parse the heterogeneity of disorders and highlight dimension-specific biological pathway also important for pharmacotherapy.

Author contributions

M.A. has contributed to the design of the work, analysis and interpretation of the data and writing of the manuscript. J.B. has contributed to the design of the work, analysis and interpretation of the data. She also contributed to drafting the work and revising

it critically for important intellectual content. G.C. has contributed to the acquisition and analysis of the data, revising and final approval of the manuscript version to be published; W.D.W. has contributed to the analysis of the data; R.M.J. has contributed to the acquisition and analysis of the data, revising and final approval of the manuscript version to be published; A.J.O.W. has contributed to the acquisition and analysis of the data, revising and final approval of the manuscript version to be published; E.K.M. has contributed to the acquisition and analysis of the data, revising and final approval of the manuscript version to be published; A.F. has contributed to the acquisition of the data, revising and final approval of the manuscript version to be published; M.A.B. has contributed to the acquisition of the data, revising and final approval of the manuscript version to be published; J.K.B. has contributed to revising the manuscript critically for important intellectual content and final approval of the version to be published; L.A.K. has contributed to revising the manuscript critically for important intellectual content and final approval of the version to be published; G.P. has contributed to revising the manuscript critically for important intellectual content and final approval of the version to be published.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship and/or publication of this article: In the past 3 years, J.K.B. has been a consultant to, member of advisory board of and speaker for Takeda/Shire, Roche, Medice, Novartis, Angelini and Servier. He is not an employee of any of these companies, and a stock shareholder of any of these companies. He has no other financial or material support, including expert testimony, patients and royalties. G.P. is the director of Drug Target ID, Ltd. The other authors declare no conflict of interest.

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Supplemental material

Supplemental material for this article is available online.

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4.1. Supplementary Materials

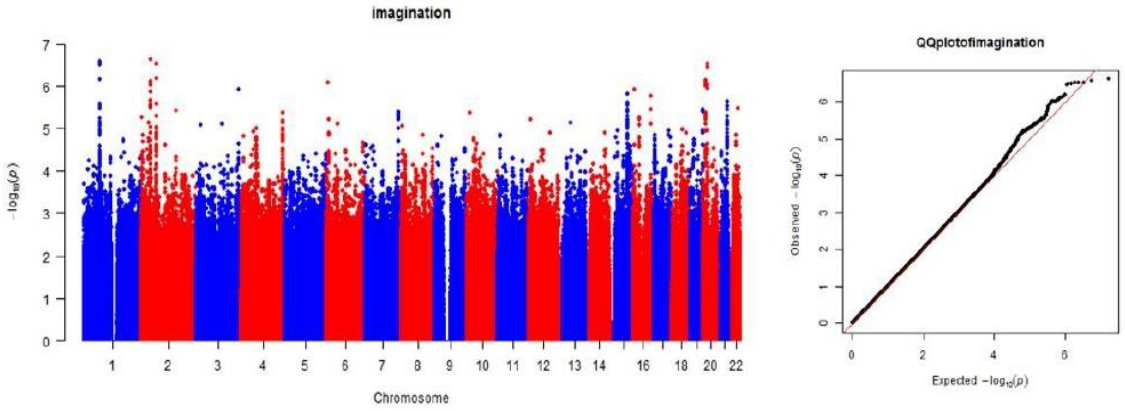

Figure 1. Manhattan and Q-Q plot of the GWAS meta-analysis for 'imagination'

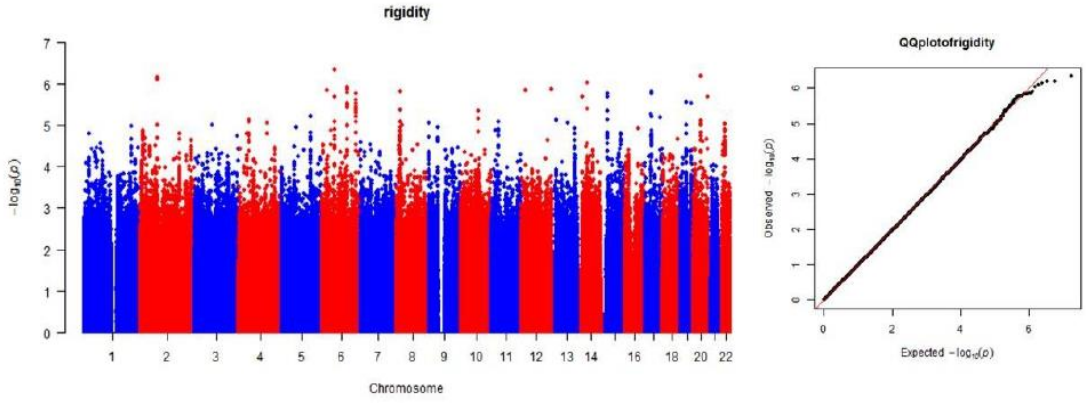

Figure 2. Manhattan and Q-Q plot of the GWAS meta-analysis for ' rigidity'

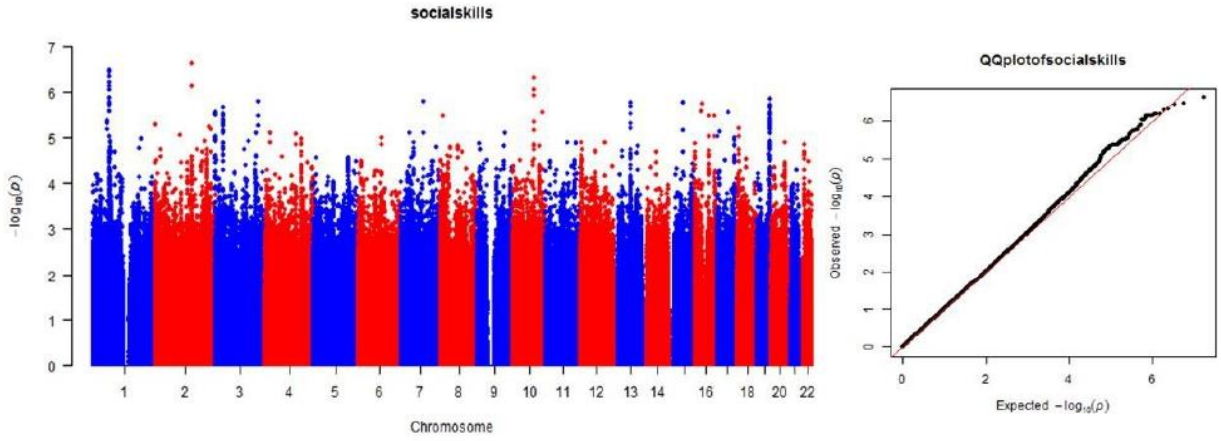

Figure 3 Manhattan and Q-Q plot of the GWAS meta-analysis for 'social skills'

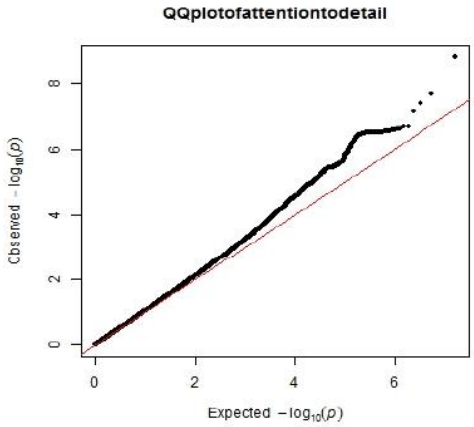

Figure 4 Q-Q plot for 'attention to detail'

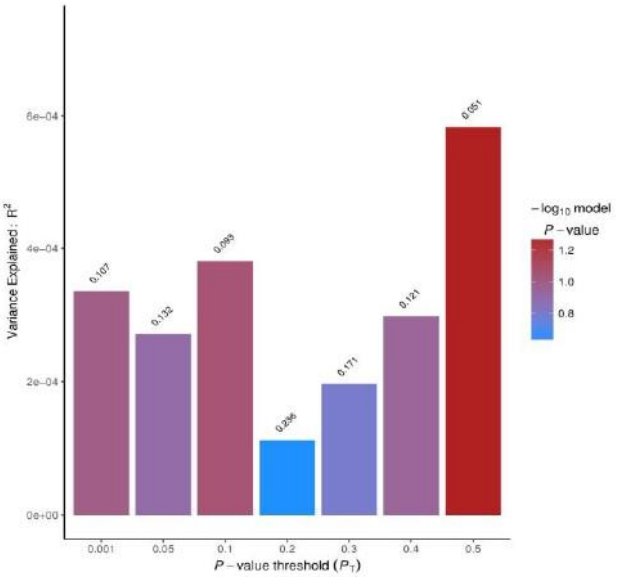

Figure 5 PRS-based results for ASD and 'attention to detail'

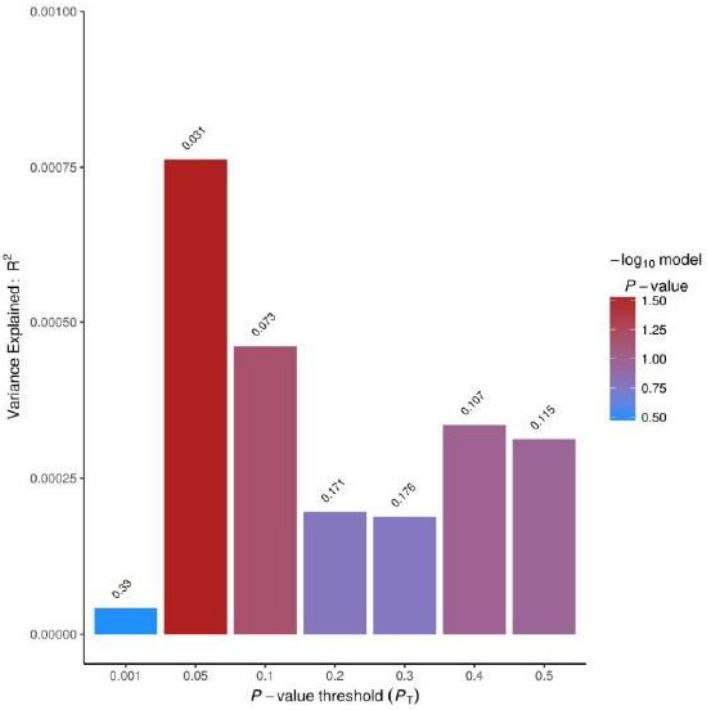

Figure 6 PRS-based results for ASD and 'imagination'

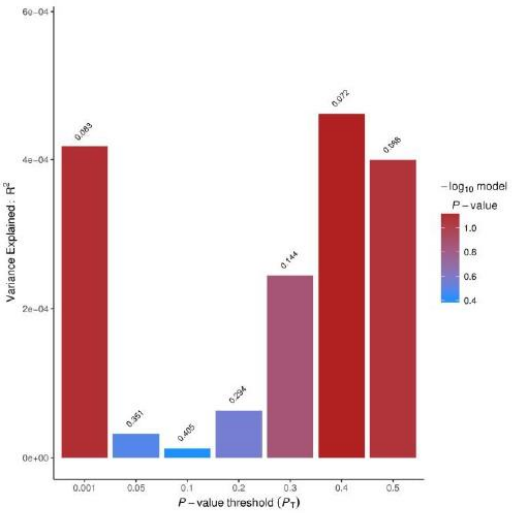

Figure 7 PRS-based results for ASD and ' social skills'

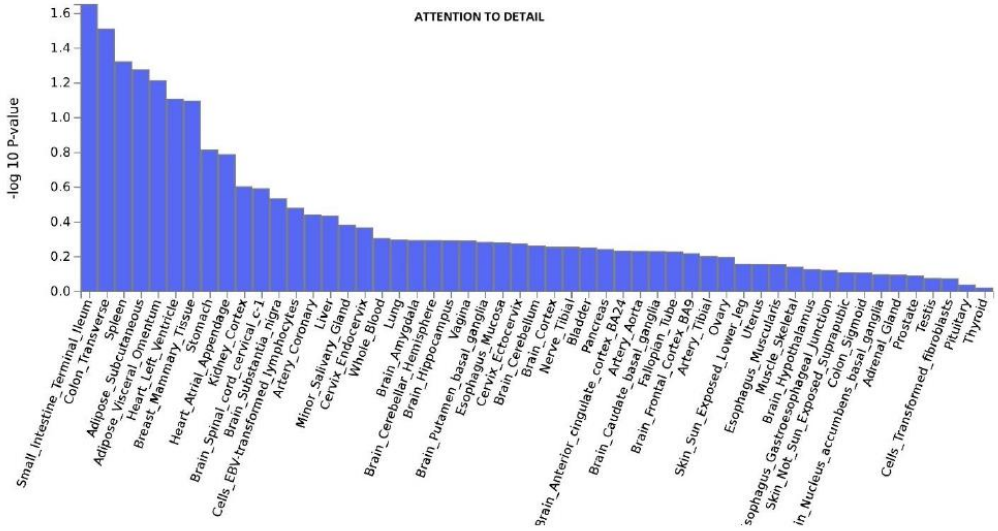

Figure 8 Gene expression linked to attention-to-detail across 53 GTEX-derived tissue types (FUMA-based analyses)

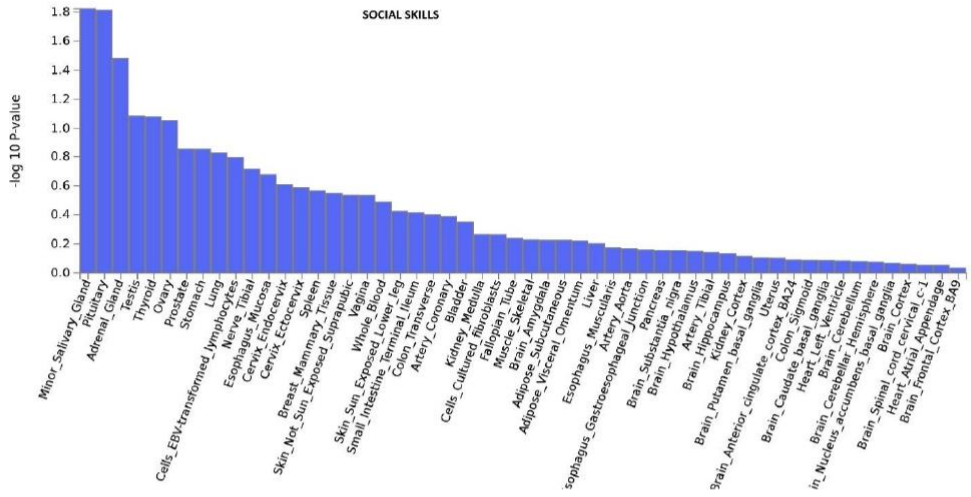

Figure 11 Gene expression linked to social skills across 53 GTEX-derived tissue types (FUMA-based analyses)

Supplementary Tables

Supplementary Table 1. Results of e-MAGMA-based gene co-expression network analyses for the 4 ALTs

Trait	Brain regions	Gene-set association p-value	Gene modules	Top ranked GO:BP
Attention	Putamen	0.01	salmon	Immune response
	Total Cortex	0.03	cyan	Response to cytokine
Imagination	Nucleus accumbens	0.003**	Dark red	Synaptic signalling
	Total Cortex	0.001**	black	neurogenesis

	amygdala	0.001**	Skyblue3	CNS development
Rigidity	Nucleus accumbens	0.01	red	Immune signalling
	Anterior cingulate cortex	0.01	lightcyan	Axon ensheatment
Social	Putamen	0.002**	midnightblue	Mitochondrial translation
	Anterior cingulate cortex	0.001**	magenta	RNA processing
	Frontal cortex	0.01	brown	Cellular metabolic process
	Amygdala	0.01	white	RNA metabolic process

Results of e-MAGMA gene co-expression network analyses. Reported are the significant ALT-specific association with gene modules across brain regions. Modules represent gene expression networks indexed by colour and referring to GO-based biological processes. CNS= central nervous system; GO= gene onthology; BP= biological processes. ** = significant results after Bonferroni-corrected *p = 0.007* (p-value divided by the number of region tested).

5. Chapter 5. Genetic relationship between the immune system and autism spectrum disorder and autistic-like traits

Building on the findings of chapter 3, this chapter investigates the genetic relationship between ASD and a broader range of immune system dysregulations. The rationale for this study was to extend prior studies that focused on specific immune genes and to identify specific immune phenotypes that share genetic underpinnings with ASD. Also, driven by the findings of chapter 4, the present chapter also explored if there is a specific genetic relationship between aspects of immunity and dimensions of ASD, conceptualised as autistic-like traits in the general population. This study allowed to refine the genetic association between ASD and immunity, and suggest that genetic factors linked to autoimmunity and allergy may especially relate to rigid behaviour typical of ASD, in the general population.

This work is currently under revision and considered for peer-review. The complete article and supplementary material are enclosed in the paragraphs to follow.

5.1. Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental condition, with a strong genetic component and estimated heritability of 70-90% (Tick et al., 2016). ASD is common and it is diagnosed in approximately 1.6% of the population, with a 4:1 male-to-female ratio (Chiarotti & Venerosi, 2020). Clinically, ASD is characterised by different symptoms, that include impaired social communication and interaction abilities, repetitive patterns of behaviours and interests, and often atypical sensory processing (American Psychiatric Association, 2013). These symptoms generally persist throughout life and influence several aspects of personal and interpersonal functioning (van Heijst & Geurts, 2015). ASD symptoms also incur high social costs (medical and

non) (Rogge & Janssen, 2019). Nevertheless, there is limited understanding of their underlying pathophysiological mechanisms.

However, increasing evidence, from both animal and human studies, suggests that the immune system (and especially immune over-activation) may play a key role in ASD. For example, findings in rodents link maternal immune activation (MIA) during pregnancy to the onset of ASD-like behaviours in their offspring (Boulanger-Bertolus et al., 2018; Estes & McAllister, 2016; K. Liu et al., 2023). In humans, prior studies support the presence of inflammation and autoimmunity in autistic individuals, as indexed by increased blood levels of pro-inflammatory cytokines and anti-neuronal antibodies respectively (Edmiston et al., 2018; Mostafa et al., 2013). Also, there are reports of immune pathologies, like allergies, and autoimmune diseases, in (a portion of) autistic individuals (Zerbo et al., 2015).

Notably, research in animals and in humans both suggest that the genetic factors may intervene in the relationship between ASD and immunity. For instance, experiments in animal models of MIA demonstrated that genetic regulators of immunity, such as interleukin-17 pathway genes, may mediate the effects of MIA on the offspring behaviours. (G. B. Choi et al., 2016; Lombardo et al., 2018; Smith et al., 2007; Traglia et al., 2018). In humans, the contribution of immune genes to ASD is supported by i) epidemiological research which demonstrates an association between ASD and family history of autoimmune and inflammatory conditions (Atladóttir et al., 2009; Zerbo et al., 2015), and by ii) prior genetic studies. Namely, candidate gene analyses, reported an association between genes belonging the human leukocyte antigen (HLA) region, such as *HLA-G, HLA-DRB1* and *HLA-DQB1* genes, and ASD (Bennabi et al., 2018; Torres et al., 2016). These genetic associations have been confirmed by hypotheses-free genetic approaches- such as genome-wide association studies (GWAS) that linked ASD and common genetic variants enriched in pathways controlling antigen presentation, and leukocyte and cytokine activation (Grove et al., 2019). Additionally, our group reported that common genetic variations in genes involved in

inflammatory processes are related to specific autistic-like traits – such as rigidity and attention to detail – in the general population (Arenella et al., 2021). Transcriptomic studies further demonstrated that ASD is linked to dysregulated expression of immune genes. Specifically, mRNA analyses of post-mortem brain tissues in ASD demonstrated up-regulation of several immunoregulatory and inflammatory gene pathways (Gandal et al., 2018); and recent in vivo studies using magnetic resonance imaging 'virtual histology' approaches revealed that immune gene dysregulations characterise cortical regions where autistic individuals have anatomical variations from the neurotypical range (Ecker et al., 2022).

Taken together, these findings support a role of the immune system in the pathophysiology of ASD; and, in particular, suggest that immunogenetic factors are important. However, prior studies have implicated a wide range of immune mechanisms, from autoimmunity to inflammation (Ashwood & van de Water, 2004; McAllister, 2017), and it is unclear which particular immune phenotypes link to ASD through genetics. To address this challenge, we estimated the genetic correlation between ASD and diverse classes of immune conditions and markers. In addition, due to the heterogeneous phenotype of ASD we investigated if the immune-related genetic factors link to particular autistic-like traits in the population (Figure 1).

First, we tested the existence of genome-wide genetic correlations between different types of immune diseases or general markers of inflammation and clinically diagnosed ASD. As genetic correlation may not be constant throughout the genome, we subsequently explored local genetic correlations between these immune-related phenotypes and ASD. For the loci that were found to be significantly related we explored the role of loci-specific variants in immune regulation and brain development. Last, we investigated whether the aggregated genetic risk for immune diseases, as captured by polygenic scores, are associated with the severity of autistic-like traits in the general population. Additionally, given the sex differences in the prevalence of both ASD (Halladay et al.,

2015) and immune conditions (Angum et al., 2020), we stratified the polygenic score analyses by sex.

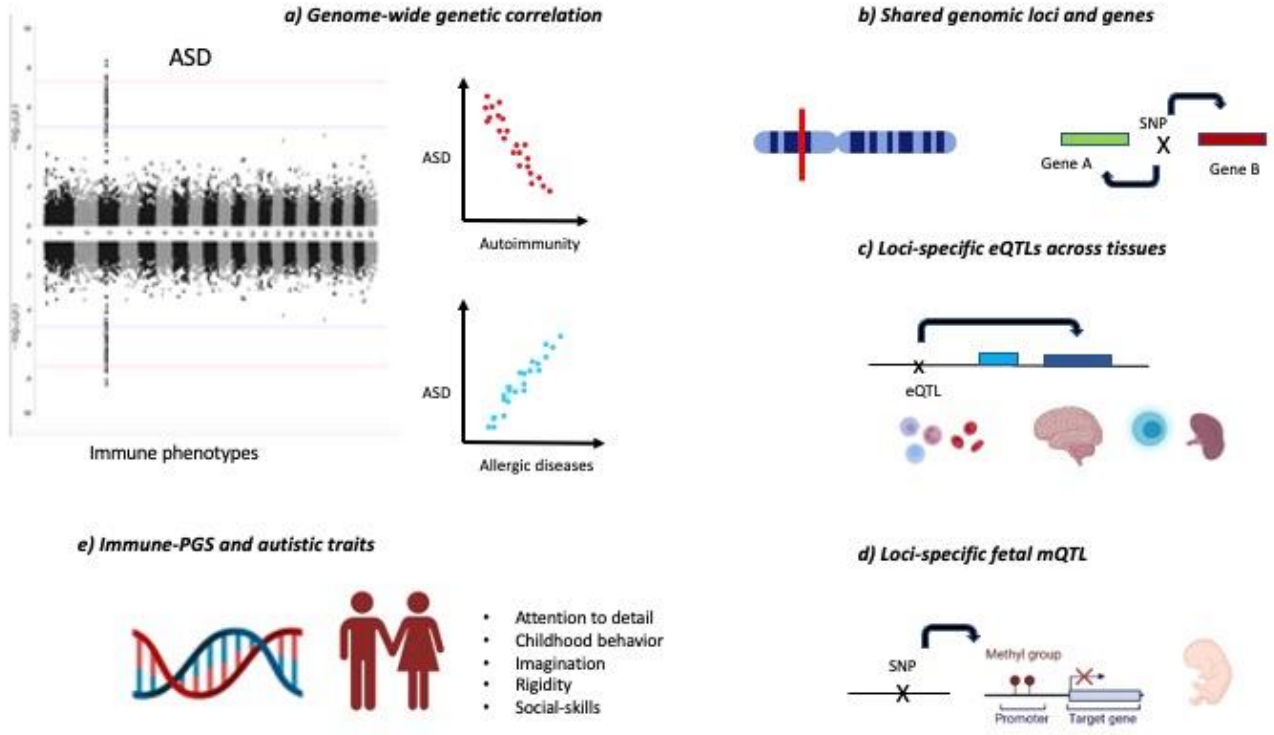

Figure 1 Illustrative graph of the analytical flow of the study. a) Genome-wide genetic correlation between ASD and immunity was estimated, using GWAS summary statistics for ASD and immune phenotypes. Both positive and negative genetic correlation were identified. b) Local genetic correlation analyses identified shared loci. Within the loci, SNPs were mapped to genes based on position. c) SNPs acting as eQTL were identified in each locus and mapped to genes expressed in brain and immune tissues. d) SNPs acting as mQTL in the fetal brain were identified in each locus and mapped to genes. e) In the general population, polygenic scores for immune phenotypes were associated with five autistic-like traits, and across sexes.

5.2. Materials and Methods

5.2.1. Genome-wide association studies summary statistics

To explore the genetic relationships between ASD and immune-related phenotypes, we leveraged publicly available summary statistics of the largest genome-wide association studies (GWASs) on ASD and immune phenotypes (Table 1). Inclusion criteria for GWAS data were: European ancestry, annotation to the Genome Reference Consortium Human (GRCh) 37/hg19 build, and a sample size (N effective) > 5,000.

Autism spectrum disorders

We used the summary statistics of the meta-analysis of the GWAS of ASD including seven cohorts, from the iPSYCH, Psychiatric Genomic Consortium samples, and the Simon Foundation Powering Autism Research for Knowledge (SPARK) sample (N = 55,420) (Matoba et al., 2020).

Immune phenotypes

We used GWAS summary statistics for:

a) a set of immune-related diseases that have been reported in autistic individuals and their families (Atladóttir et al., 2009; Zerbo et al., 2015), including autoimmune thyroid diseases (AID) (Saevarsdottir et al., 2020), celiac disease (CD)(Dubois et al., 2010), rheumatoid arthritis (RA) (Okada et al., 2014), systemic lupus erythematosus (SLE) (Bentham et al., 2015), and type 1

diabetes mellitus (T1DM) (Forgetta et al., 2020), and conditions associated with hypersensitivity to allergens (i.e., allergic diseases (ALG) (Zhu et al., 2018) and asthma (Y. Han et al., 2020));

b) blood levels of C-reactive protein (CRP) (X. Han et al., 2020), a peripheral biomarker of inflammation; and

c) the total counts (and/or relative percentage) of white blood cells (Vuckovic et al., 2020) involved in the fast response to infection (neutrophils), T and B cell-mediated response (lymphocytes), allergic reaction (eosinophils), and phagocytosis (monocytes).

Table 5. Characteristics of the samples used as input for the genetic correlation and polygenic score (PGS) analyses.

Phenotype	***N total***	***N cases***	***N controls***	***Reference***
Systemic lupus erythematosus (SLE)	*14,256*	*5,201*	*9,066*	*(Bentham et al., 2015)*
Rheumatoid arthritis (RA)	*58,284*	*14,361*	*43,923*	*(Okada et al., 2014)*
Autoimmune thyroid disease (AIT)	*755,406*	*30,234*	*725,172*	*(Saevarsdottir et al., 2020)*
Type 1 diabetes mellitus (T1DM)	*24,840*	*9,358*	*15,705*	*(Forgetta et al., 2020)*

Asthma	*303,859*	*64,538*	*239,321*	*(Y. Han et al., 2020)*
Allergic disease (ALG)	*102,453*	*25,685*	*76,768*	*(Zhu et al., 2018)*
Celiac disease (CD)	*15,283*	*4,533*	*10,750*	*(Dubois et al., 2010)*
Autism spectrum disorder (ASD)	*55,420*	*22,458*	*29,386*	*(Matoba et al., 2020)*
Lymphocyte count (LYMPH)	*408,112*	-	-	*(Vuckovic et al., 2020)*
Lymphocyte percentage (LYMP%)	*408,112*	-	-	*(Vuckovic et al., 2020)*
Neutrophil count (NEU)	*408,112*	-	-	*(Vuckovic et al., 2020)*
Monocyte count (MON)	*408,112*	-	-	*(Vuckovic et al., 2020)*
Eosinophil count (EOS)	*408,112*	-	-	*(Vuckovic et al., 2020)*

C-reactive protein (CRP)	*401,696*	-	-	*(X. Han et al., 2020)*

5.2.2. Genotype data for autistic-like traits

To examine whether the immune-related phenotypes that are genetically correlated with ASD, are linked to specific autistic dimensions in the general population, we explored population-based genotype data and measures of autistic-like traits in the Nijmegen Biomedical Study.

The Nijmegen Biomedical Study (NBS)

We used genotype and behavioural data from a Dutch population-based cohort of 2,847 individuals who participated in the Nijmegen Biomedical Study (NBS) (mean age 28.4; 54% females). The NBS is a project managed by the Department of Health Evidence and the Department of Laboratory Medicine of the Radboud University Medical Center. The study was approved by the Institutional Review Board and aimed to investigate genetic factors, lifestyle, and environmental exposures underlying a range of traits and diseases (for further information, see (Galesloot et al., 2017)). In this cohort, genotyping was performed using the Illumina Human OmniExpress Beadchip platform. Initial single nucleotide polymorphism (SNP) filtering was applied on call rate (>95%), Hardy-Weinberg equilibrium (HWE<$1x10^{-6}$), minor allele frequency (MAF > 0.01), and imputation quality (> 0.7). Autosomal SNPs were imputed to the 1000 Genome Reference Panel (1KGRP) phase 3 release, using Minimach. To assess population structure, multidimensional scaling (MDS) was performed in PLINK (Purcell et al., 2007) and the first four MDS components were retained as covariates in subsequent analyses. In addition, participants were asked to complete a self-report questionnaire on autistic-like traits, developed by qualified clinicians at Radboudumc and previously validated in the Dutch population (Arenella et al., 2022; Bralten et al., 2018). The questionnaire consists of 18-items, rated on a 4-point Likert scale, based on the autism quotient (AQ) questionnaire and the ASD criteria listed in the Diagnostic and Statistical Manual of Mental disorder – 5^{th} edition. The items cover the three main

dimensions of ASD (social communication, social interaction, and repetitive behaviours). Moreover, some items enquire about the level of autistic behaviours (based on the DSM) present in childhood (i.e., childhood behavior). Factor analysis of these items identified five autistic-like traits: attention-to-detail, imagination, rigidity, social skills, and childhood behaviour (see Table S1, and (Arenella et al., 2022; Bralten et al., 2018) further details). These traits, along with a total autistic-like traits score, were normalised and adopted as target phenotypes for polygenic risk score (PGS) analyses described below.

5.2.3. Shared genetic etiology between ASD and immune phenotypes

Global genetic correlations analyses

Global genetic correlation was estimated between ASD and immune-related diseases (i.e., AIT, ALG, Asthma, CD, RA, SLE, T1DM), and population-based variations in immune-inflammatory response as indexed by CRP blood levels, the blood count of eosinophils, lymphocytes, monocytes, and neutrophils (Table 1). Pair-wise global genetic correlation between ASD and each immune-related phenotype was estimated via Linkage Disequilibrium SCore (LDSC) regression as implemented in the LDSC v1.0.1 tool (https://github.com/bulik/ldsc) (Bulik-Sullivan et al., 2015). Analyses used pre-computed linkage disequilibrium (LD) scores based on the 1kGRP reference, which are suitable for European-centred GWASs. LDSC analyses consisted of two steps: 1) converting summary statistics data to the standard LDSC format (i.e., exclusion of HLA region and merging to the HapMap3 reference panel); 2) estimating genetic correlation. A block jack-knife procedure was used to estimate standard errors and calculate corresponding *p*-values. P-values of genetic correlation estimates (*rg*) were false discovery rate (FDR)-corrected, given the medium-high genetic intercorrelations and co-heritability of the immune phenotypes themselves (Fig 1; Table S2). Global genetic correlation analyses were restricted to GWAS summary statistics

with sample size > 5,000 individuals, SNP-based heritability (h^2_{SNP}) > 0.05 and mean chi square > 1.02 as recommended in (Zheng et al., 2017).

Local genetic correlation analyses

Local genetic correlation analyses complemented global genetic correlation between ASD and the immune-related phenotypes. This step allowed us to identify scenarios in which ASD-immune genetic correlations are restricted to specific genomic regions, and to determine shared genetic factors located in the genomic regions. Local genetic correlations analyses were performed using the R-package 'Local Analysis of [co]Variant Association (LAVA)' (Werme et al., 2022). Local genetic correlation was estimated across 2,495 loci defined by partitioning the genome into blocks of ~1 Mb while minimising LD between them. The analyses consisted of two steps: 1) univariate association analyses, to detect the local h^2_{SNP} signal of each phenotype within each genomic locus; and 2) bivariate association analyses, to estimate the pair-wise genetic correlation between two phenotypes of interest at the chosen locus. Bivariate association analyses were restricted to those genomic loci showing a significant h^2_{SNP} signal for both phenotypes ($p<1x10^{-4}$). The p-values of local *rg* were Bonferroni-corrected, considering the number of loci tested in the bivariate association analyses. Subsequently, we identified SNPs included in each locus based on GRCh 37 positions and mapped these SNPs to genes using the *g:snpense* function of the R-package 'g:profiler2' (Kull et al., 2007). In addition, we queried to PubMed to explored if identified genes have been implicated in immunity. Since some SNPs (i.e., *cis*-eQTLs) may influence the transcription of proximal genes with differences across tissues, we tested if SNPs within each locus were linked to gene expression across tissues. For this, we used 'e-MAGMA' (https://github.com/eskederks/eMAGMA-tutorial) (Gerring et al., 2021), a tool which converts GWAS summary statistics for a phenotype of interest into a e-gene-level statistics that refers to a gene expressed in a given tissue (e-gene). The conversion takes into account LD between SNPs

and is based on a reference list of eQTL-to-gene association (FDR p-value<0.05) across different tissues from GTEx v8 (https://www.gtexportal.org/). The considered tissues were those relevant to neurodevelopment (brain cortex) and immune regulation and activation (spleen, lymphocytes, and whole blood).

To further investigate the impact of SNPs mapped within each genetically correlated locus on gene regulation, we examined their influence on *cis*-methylation of CpG sites in the developing brain (i.e., if they act as fetal mQTLs). To explore this, we adopted a frequentist approach and tested the enrichment of fetal mQTLs among loci-specific SNPs (Fisher's exact test) (van Belle et al., 2004). This analysis was based on a publicly available compendium of ~16,000 Bonferroni significant mQTLs in the developing brain (see (Hannon et al., 2016); https://epigenetics.essex.ac.uk/mQTL/).

Polygenic score analyses

Polygenic score (PGS) analyses were conducted to explore if the additive effect of common genetic variants to the immune-related phenotypes was associated with autistic-like traits in a population-based sample. Linear regression models were used to test the association of the genetic liability to immune-related phenotypes with five autistic-like traits (i.e., rigidity, attention-to-detail, social skills, imagination, childhood behaviour) and the total autistic score in the target NBS cohort.

The GWAS summary statistics for the immune-related phenotypes showing genome-wide genetic correlation with ASD were individually used as base datasets for PGS calculation. The summary statistics underwent a preliminary clumping step using PLINK (Purcell et al., 2007) to ensure that only the most significant independent SNP for each LD block (r2>0.25, clumping window=±500kb) was considered. PGSs were then calculated on the target NBS individual-level genotype data using PRSice2 (S. W. Choi & O'Reilly, 2019). For each base immune phenotype

showing genome-wide genetic correlation with ASD, PGSs were computed including SNPs exceeding seven *a priori* defined GWAS *p*-value thresholds (Pt) (i.e., Pt=0.0001, 0.001, 0.01, 0.05, 0.1, 0.2, 0.3). Multiple linear regressions were performed, considering PGSs for the immune-related phenotypes as independent variables and autistic-like traits as dependent variables. Age, sex, body mass index (BMI), and population structure (MDS) components were included as covariates. The variance explained by the PGS of each immune-related phenotype (PRS-R2 = full model R2 – null model R2) for each of the five autistic-like traits and the total score was calculated separately. The p-values of each association test were adjusted using FDR correction, considering the number of autistic-like traits and immune-related phenotypes tested. Results were considered statistically significant when $p_{FDR} < 0.05$.

Sex-stratified PGS analyses

To investigate if associations between immune-based PGS and autistic-like traits were sex-specific, PGS analyses were performed after stratifying the target NBS sample according to sex. Hence, we tested multiple general linear models for both sex group which included age, BMI, and MDS components as covariates. To reduce the burden of multiple between-sexes comparisons, and also minimise variations in effect size, we considered for each immune disease, the PGS that best explain variability in each autistic trait, so-called 'best-fit' PGS. We considered results statistically significant only if $p_{FDR} < 0.05$.

5.3. Results

5.3.1. Global genetic correlations between ASD and immune phenotypes

We identified significant positive global genetic correlations between ASD and asthma (*rg*=0.08, se=0.006, p_{FDR}= 0.02) and between ASD and allergic diseases (*rg*=0.14, se=0.1, p_{FDR}=0.01). Additionally, ASD showed significant negative genetic correlations with autoimmune disorders (RA and SLE) and lymphocyte count/percentage (rg = -0.06-0.17; se = 0.02-0.06; p_{FDR}=0.01) (Figure 2, Table S3).

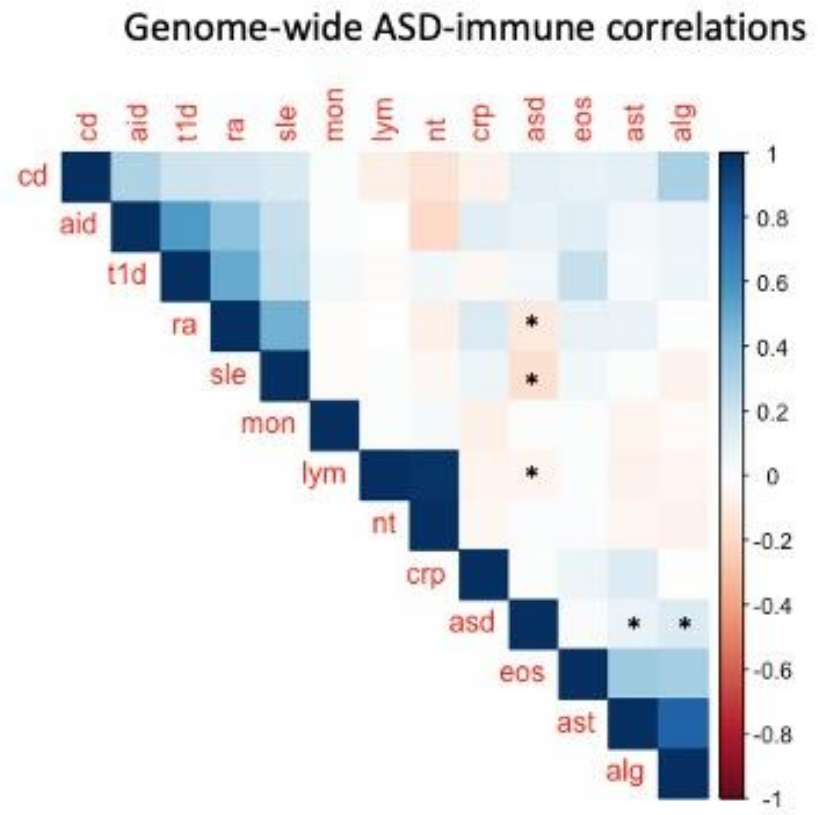

Figure 2. Genetic correlation plot summarising the results of the global genetic correlation analyses between ASD and immune-related phenotypes. Colour bar indicates variation in the strength and direction of genetic correlation estimates (rg) with positive rg in blue and negative rg in red. The FDR-corrected significant correlations are marked with an asterisk (*).

5.3.2. Local genetic correlations between ASD and immune phenotypes

For each ASD-immune pairwise comparison, we identified multiple loci with significant h^2_{SNP} for both ASD and the immune phenotype considered ($p < 1x10\text{-}4$) (see Table S3). Of these loci, we registered significant genetic correlation – i.e., surviving multiple comparison correction – at 11 unique loci shared between ASD and AIT, RA, CRP, EOS, LYMP, MON, NEU. Among those, two loci - the chr11:95-96Mb locus and the chr17: 43-44Mb locus – showed genetic correlation between ASD and multiple immune phenotypes (AIT, EOS, and Lymph). We also observed local genetic correlation between ASD and CRP at the chr6:29-30Mb locus containing the HLA region, a key immune-related region that is not covered by the LDSC analyses. Table 2 illustrates the significant genetic correlation loci and the genes belonging to these genetic regions.

Table 6. Loci with a Bonferroni-significant genetic correlation signal between ASD and immune-related phenotypes.

Immune phenotype	Chr	Start (bp)	End (bp)	rg	95% CI	p-value	Mapped genes
EOS	1	200134006	201067952	-0.66	-1 - -0.26	0.0020	*KIF14* ***DDX59*** *CACNA1S* *KIF21B* *CAMSAP2* ***NR5A2*** ***MROH3P*** ***INAVA***

Lymph	5	126992837	128067414	-0.90	-1 - -0.61	5.1x10-6	*FBN2* *CCDC192* *SCL27A6* *SCL12A2*
NEU	5	87943483	89584466	0.41	0.23-0.63	2.1x10-5	***MEF2C***
NEU	5	74245355	75239302	0.45	0.25-0.66	2.3x10-5	*GCNT4* *ANKRD31* ***HMGCR*** ***CERT1*** *POLK* ***ANKDD1B*** *POC5* *SLC25A5P9* *BIN2P2*
CRP	6	29529756	29833843	0.74	0.39-1	0.00048	*GABBR1* ***HLA-F*** *MOG* ***HLA-G*** *OR2H2* ***HLA-P*** ***MICE*** ***ZFP57*** ***HLA-V*** ***IFITM4P***

RA	10	38566461	42392742	-0.84	-1 - -0.50	0.00021	***PLD5P1*** *HSD1787P2* ***SEPTIN7P9*** *ABCD1P2* *SCL9B1P3* *ACTR3BP5* *CHEK2P5*
NEU	10	129134739	129831969	-0.39	-0.66--0.19	0.00020	***DOCK1*** *NPS* ***FOXI2*** *CLRN3* ***PTPRE***
AIT	11	95327211	96150134	-0.44	-0.88 - -0.12	0.0078	***FAM76B*** *CEP57* ***MTMR2***
RA	11	95327211	96150134	-0.74	-1 - -0.35	0.00050	***MAML2*** *CCDC82* *JRKL*
MONO	12	68839662	70097805	0.46	0.24 – 0.73	5.2x10-5	*BEST3* ***CPSF6*** *CCT2* *FRS2* *CPM* ***RAP1B***

							YEAST4
							NUP107
							MDM2
							SLC35E3
							LYZ
CRP	13	66382287	67718879	0.93	0.54	3.5x10-	***PCDH9***
					- 1	5	
AID	17	43460501	44865832	0.55	0.15-	0.0079	*WNT3*
					0.92		*NSF*
EOS	17	43460501	44865832	-	-0.80	0.0022	***AL17A***
				0.50	- -		*NSFP1*
					0.25		***ARL17B***
Lymph	17	43460501	44865832	-	-0.91	4.1x10-	***CHRH1***
				0.62	- -	5	***MAPT***
					0.37		*KANSL1*
							MAPK8IP1P1
							MAPK8IP1P2
							PLEKMH1
							LRRC37A4P
							DND1P1
							ARHGAP17

Abbreviations: rg = genetic correlation; Chr = chromosome; CI = confidence intervals; EOS = eosinophil count; Lymph = lymphocyte count; NEU = neutrophil count; CRP = c-reactive protein; AID = autoimmune thyroid disease; RA = rheumatoid arthritis; MONO = monocyte count; italics = negative local genetic correlation. ***bold*** *= genes implicated in immunity based on literature.*

5.3.3. Brain and immune-related e-QTLs in shared loci

For four out of the 11 shared genomic loci between ASD and immune-related phenotypes (chr1:200-201Mb, chr6:29-30Mb, chr12:68-70Mb, chr17:43-44Mb), we identified e-genes, expressed in the brain and in immune tissues (Table S4). Specifically, at the chr1:200-201Mb locus, *DDX9* expression in the cortex and immune cells was significantly associated with ASD and eosinophil count (p = 0.01-0.0009); at the locus chr6:29-30Mb, the expression of *RNF39* in the cortex and the expression of *HLA-F* and *ZFP57* immune tissues were significantly associated with ASD and CRP (p = 0.03-2.9x10-5); at the locus chr12:68-70Mb, the expression of *YEATS4* in the cortex and immune cells, and the expression of *LYZ* and *MDM2* was significantly associated with ASD and monocyte count (p= 0.01-3.1x10-8); and last, at the locus chr17:43-44Mb, the expression of *KANSL1, ARL17A, LRRC37A2, LRRC37A* in the brain and in immune tissues, and the expression of *WNT3* and *MAP3* in immune tissues was significantly associated with ASD and lymphocyte and neutrophil count (p= 9.67x10-7-1.22x10-14). We did not identify genes expressed in the brain and immune tissues significantly associated with ASD and immune phenotypes at the other shared loci.

5.3.4. Enrichment of fetal mQTLs in shared loci

We registered a complete overlap (100%) fetal mQTLs with ASD-related SNPs specifically falling with the locus (chr17:43-44Mb), where we registered a correlation between ASD and (respectively) AID, eosinophil, and lymphocyte count (figure S1). At this locus, fetal brain mQTLs were associated with (cis) methylation at CpG islands at eight unique DNA locations corresponding to the *LRRC37A* and *MAPT* genes (Table S5). We did not identify any significant overlap between ASD-related SNPs and fetal mQTLs at the other shared loci (p > 0.05).

5.3.5. Immune-based polygenic scores association with the autistic-like traits

PGS analyses indicated specific associations between genetic liability to immune-related phenotypes and autistic-like traits in a population-based sample. The strongest association existed between rigidity and PGS for SLE (best Pt=0.006; p_{FDR} = 0.03) (Figure 3; Table S6). Rigidity was also associated with PGSs for RA (Pt = 0.12; p_{FDR} =0.03) and ALG (Pt=0.052; p_{FDR} =0.03). In addition, we detected associations between the total autistic score and PGSs for ALG (Pt= 0.2; p_{FDR} =0.03), Lymph (Pt = 0.01; p_{FDR} = 0.03) and SLE (Pt= 0.0001; q=0.03) (Figure S2). Last, there was an association between childhood behaviour and PGS for LYMPH (Pt=0.0004; p_{FDR} =0.03; Figure 4). The association between immune-based PGS and the other tested autistic-like traits did not survive FDR-correction (p_{FDR} > 0.05: Table S6: Figure S3-S5). Also, sex-stratified associations between immune-based PGSs and autistic-like traits were not significant after FDR-correction (p_{FDR} > 0.05) (see Table S7).

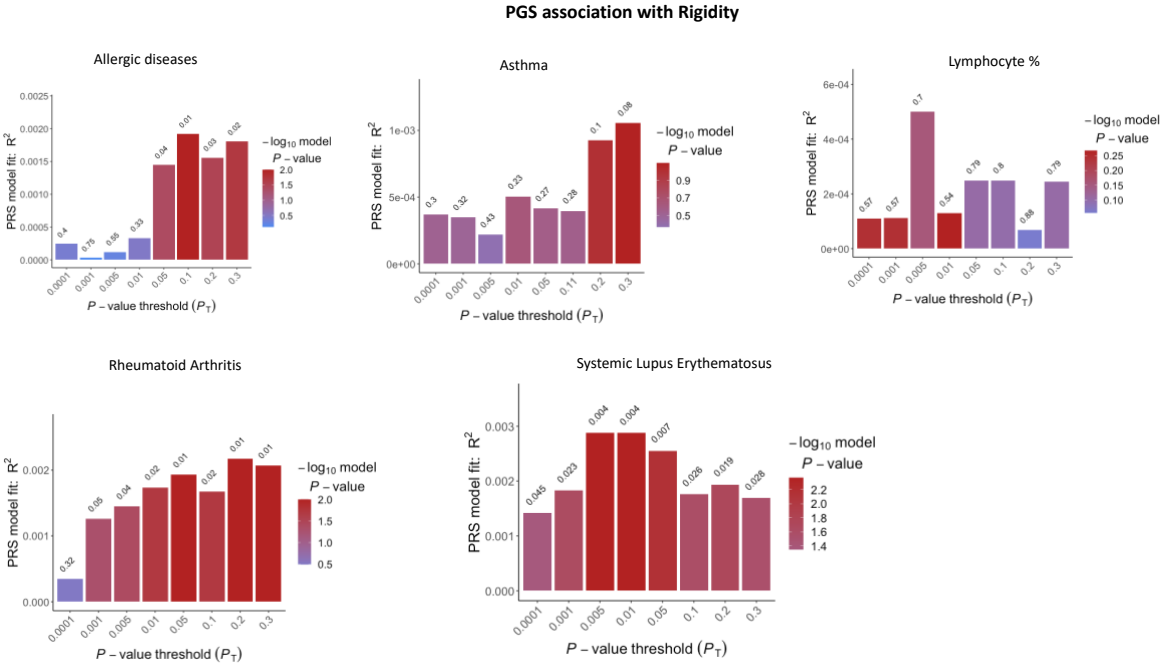

Figure 3 Bar plots for the association between polygenic scores for different immune-related phenotypes and 'rigidity'. Each bar corresponds to the PGS calculated at the GWAS p-value threshold (Pt) listed on the x-axis. The height of the bar (y-axis) represents the degree of variance explained by each PGS in rigidity. The bar colour indicates the significance of the association (according to the -log10(p-value)). The p-value of association for each PGS is reported on the top of each bar,

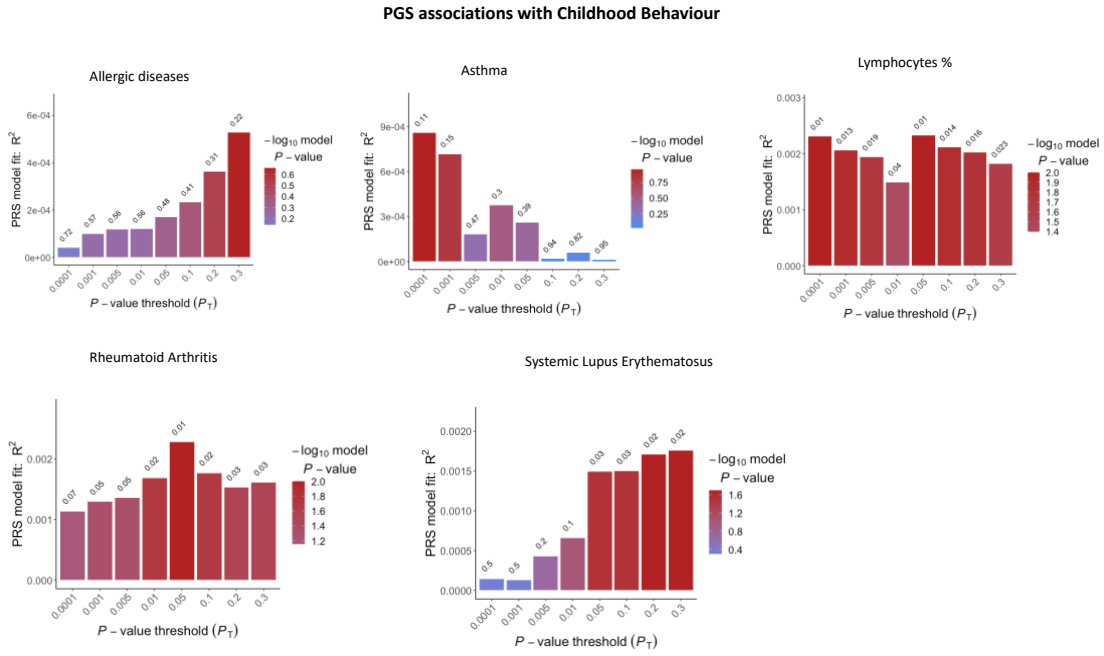

Figure 4 Bar plot results indicating the variance that polygenic scores for immune phenotypes associated with ASD explain in 'childhood behaviour'.

5.4. Discussion

In this study, we demonstrated that several autoimmune and atopic diseases share genetic liability with ASD. The genetic relationship between these immune phenotypes and ASD is complex, and its direction varies according to the specific immune phenotype considered. To further explore this genetic relationship, we investigated local genetic correlation and identified specific shared genomic loci. Some of these loci demonstrated an enrichment for common variants regulating gene expression in both immune tissues and brain; and which participate in methylation during neurodevelopment. Furthermore, our results indicate that immunogenetic associations exist between specific autistic-like dimensions in general population.

Considering ASD as a clinical category, we reported a positive global genetic correlation between ASD and diseases associated with increased sensitivity to common allergens (i.e., allergies and asthma). These genetic correlations are in line with reports on the high prevalence of various allergic conditions in ASD (Miyazaki et al., 2015) and are consistent with prior findings of dysregulated expression of histamine signalling genes – key modulators of allergic reaction – in post-mortem ASD brains (Wright et al., 2017). In addition, we detected a negative global genetic correlation between ASD and lymphocyte count, which suggests that genetic factors associated with higher likelihood for ASD are also link to lower levels of peripheral lymphocytes, and *vice versa*. These findings, therefore, suggest the possibility of faulty adaptive, lymphocyte-mediated immune response. Notably, dysregulations in lymphocyte levels, and especially T cells, have been documented in the peripheral blood of autistic individuals (Ashwood et al., 2011). We also detected a negative genetic correlation between autoimmune conditions (RA and SLE) and ASD, suggesting that variants associated with and increased likelihood of having ASD may be associated with resilience towards autoimmune diseases, and *vice versa*. These findings differ from epidemiological reports of a high rate of autoimmune conditions in the relatives of autistic individuals (Atladóttir et al., 2009). The evidence of both positive and negative genetic association suggest that ASD is linked to dysregulation in very refined immunogenetic mechanisms. To understand which of these mechanisms may be important, it is crucial to consider the aetiology of the immune phenotypes considered here. For example, prior studies suggest that allergic responses and autoimmunity may be ascribed both an imbalance between different classes of T helper (Th) lymphocytes, like Th1/Th17 and Th2 cells (Ashley et al., 2017; Bolon, 2012; Chaplin, 2010; Wahren-Herlenius & Dörner, 2013). However, while allergic responses have been associated with an increased Th2 cell activity as compared to Th1 cells, autoimmunity has been linked to a predominant Th1 response (Ashley et al., 2017; Bolon, 2012; Chaplin, 2010; Ramos et al., 2015). In this context, our findings suggest that deeper interrogation of whether ASD associate with genetic factors regulating the Th1/Th2 homeostasis are warranted.

The negative genetic correlation findings reported here should be interpreted in the light of some methodological challenges. For example, the global LDSC based genetic correlation analyses exclude common HLA polymorphisms – due to the complex LD structure of the HLA region. This region is, however, central to the aetiology of most autoimmune conditions (B. Liu et al., 2021; Wahren-Herlenius & Dörner, 2013) and may further contribute to the co-occurrence of those conditions in ASD. Moreover, global genetic correlations analyses fail to detect scenarios in which the genetic correlation between two phenotypes varies (or has opposite directions) across different genomic regions, being masked when summed on a global scale (Werme et al., 2022). To overcome these limitations, we also assessed genetic correlation at the level of specific loci – including loci within the HLA region. The results of these analyses supported a role of HLA-specific SNPs encompassing the chr6:29Mb locus in the relationship between ASD and CRP. Genetic variants at this locus map to, and regulate, the expression of the *HLA-G* gene, which is known to intervene in the maternal-fetal interface and has been implicated in a range of neurodevelopmental conditions, including ASD (Guerini et al., 2019). When we examined HLA-loci in the relationship between ASD and autoimmunity, local genetic correlations at these loci did not survive multiple comparison correction, suggesting that other factors may drive the association between ASD and autoimmune diseases. A useful next step would be further studies that, for example, rely on *ad-hoc* imputation of HLA loci, to elucidate the influence of HLA-related SNPs on ASD.

Notably, the local genetic correlation approach also led to the identification of loci that are shared (pleiotropic) between ASD and multiple immune phenotypes. One of the loci with higher pleiotropy spans the chr17q21.31 region. This region on chromosome 17 includes an inversion polymorphism, which is common in the European population and that has been previously implicated in ASD and brain morphology (Adams et al., 2016; Ikram et al., 2012; Pain et al., 2019). Our analyses also indicated that variants at this locus influence expression in the brain and tissues of the immune system, suggesting a role of this genomic region in potential neuro-immune

alterations. Last, we demonstrated that ASD-related variants in these regions act as mQTLs in the fetal brain, suggesting that these genetic factors may be important in the prenatal period and potentially interact with prenatal environmental challenges, including MIA and its cascade effects on brain development.

Another explanation for the complex pattern of correlations observed between ASD and immune phenotypes may be ascribed to the phenotypic heterogeneity of ASD. ASD is defined by different combinations of cognitive and behavioural symptoms (Georgiades et al., 2013). Prior work also suggested that these symptoms may be genetically distinct (Arenella et al., 2022; Warrier et al., 2019). Therefore, immunogenetic mechanisms may influence specific symptom domains, and these specific genetic effects may be diluted or transformed when adopting categorical definitions of ASD (Warrier et al., 2019). To address this point, we investigated if immunogenetic factors were associated with specific autistic dimensions or traits in the general population. We adopted a PGS approach, which considers the additive effect of common genetic variants across the genome, including the HLA region (S. W. Choi & O'Reilly, 2019). Our results demonstrate an association between immune-related genetic variations and rigidity and childhood behaviour. This is consistent with our prior work demonstrating an enrichment of SNPs associated with autistic-like traits, including rigidity and attention to detail, in eQTLs influencing the expression of immunogenetic pathways in human brain cortex (Arenella et al., 2022). Notably, we also demonstrated that autoimmune-related genetic factors show a positive association with rigidity whereas a negative association with social skills. These, therefore, findings support the thesis that immunogenetic factors may relate to particular autistic features (rigidity) and not others; and – more generally - they prove that trait-specific analyses may clarify complex associations that exists when considering heterogenous phenotype like ASD.

Last, potential confounder in the genetic relationship between ASD and autoimmune diseases is sex. This is because sex hormones differentially modulate the immune responses (Roved et al.,

2017). Namely, testosterone is known to act as an immunosuppressant, whereas oestrogens have immunoregulatory properties (leading to autoimmunity in extreme cases) (Roved et al., 2017). As a result of these modulatory effects, immune diseases differentially affect the two sexes and are more prevalent in women (with an approximate female to male ratio of 10:1 (Wahren-Herlenius & Dörner, 2013). This sex-specific regulation suggests that immunogenetic factors may also have different effects on autistic phenotypes across sexes. One obstacle to the evaluation of the possibility of any inter-sex variability is that women are under-represented in the ASD clinical population due to higher prevalence of diagnosed ASD in men as compared to women (Halladay et al., 2015), but also the 'masking' of ASD symptoms in females (Lockwood Estrin et al., 2020). However, the low female sample size in sex stratified GWAS of ASD did not allow us to test the relationship between ASD and immune phenotypes across sexes (Martin et al., 2021). To address this issue, we examined the association between immune-related genetic factors and autistic-like traits separately in women and in men from a general population sample, which had a balanced representation of both sexes. However, the lower sample size did not allow to perform reliable between-sexes comparison analyses. Our results from PGS analyses did not show significant sex-stratified associations. Larger (female) samples sizes in both general population and clinical ASD populations are required to investigate this question further, including the tests of between-sexes differences.

Our work has both strengths and limitations. We explored the genetic relationship between ASD and the immune system, by leveraging the largest GWAS summary statistics for immune phenotypes being linked to different immunopathology (i.e., autoimmunity, atopy, and inflammation). In this regard, we used both categorical and dimensional approaches, as well as sex-stratified analyses, to disentangle the complex relationship between the immune system and autistic phenotypes. Another strength is the use of state-of-the-art genomic techniques to estimate global, and local genetic sharing between immune and autistic phenotypes. In contrast, one limitation of our study is the correlational/observational nature of our approach. Therefore, we cannot infer

any causal role of immunogenetic factors in ASD. Moreover, we limited our analyses to immune phenotypes for which we could exploit well-powered GWAS data (i.e., based on sample size and h^2_{SNP}), and therefore we could not investigate other likely relevant immune phenotypes, like cytokine markers (including both Th1 and Th2- related cytokines) that may have provided further insights on ASD-linked immune mechanisms (Nath et al., 2019). In addition, the self-report questionnaire to measure ALTs in the general population suffer some limitations. For example, the childhood behaviour factors include a wide range of items, whereas other factors like attention to details refer to fewer items and largely involved in social processes. In light of this, replication analyses leveraging other instruments to assess autistic traits are warranted. It is also important to note that our PGS-based findings – albeit reaching significance -demonstrated that immune-based PGSs only account for a small proportion of phenotypic variance in the autistic-like traits, in line with other studies adopting the same methods (Den Braber et al., 2016). Furthermore, our study was restricted to European populations and therefore our findings cannot be generalised to other ethnicities.

5.5. Conclusions

Our study demonstrates that genetic factors involved in autoimmunity and allergic responses may be important to ASD. However, while allergy-related genetic factors are associated with increased likelihood of having ASD, autoimmunity-related genetic factors link to reduced ASD likelihood. By leveraging different methods, we gain insights on i) genomic loci – and the genes within those - that register an association between ASD and immunity, and ii) specific autistic features, that – in the general population – associate with these immunogenetic factors. Overall, we demonstrated that immunogenetic factors, linked to ASD, may have a regulatory function in both the mature and the developing brain; and that these immunogenetic factors are specifically linked to autistic-like traits 'rigidity' and 'childhood behaviour'.

5.6. Supplementary Materials

Table S 2 Questions used in the Nijmegen Biomedical Study to measure autistic-like traits

Questions used to measure four autistic-like traits
Attention to detail
By looking at someone face, I find easy to work out what is he or she is thinking or feeling
I can quickly workout whether someone is fascinated by what I say
I tend to notice details that others do not [a]
Imagination
I find making stories up easy
As a child, I enjoyed playing games involving pretending with other children
Rigidity
People tell me that I keep going on and on about the same thing [a]
I often get so absorbed that I lose sight of other things [a]
It upsets me if my daily routine is disturbed [a]
I prefer to do things the same way over and over again [a]
Social skills

I find it hard to make new friends [a]

I enjoy social occasions as birthdays, receptions, etc.

I don't know how to keep a conversation going [a]

Childhood behaviour

As a child, I was a late talker, or I had other speech-related problems [a]

As a child, I often retreated to my own world, or I rarely played with other children [a]

As a child, I moved in a rigid way, or I tended to repeat certain movements [a]

As a child, I often repeated the same words, or I made up new words [a]

As a child, I often took statements and jokes [a]

As a child, I frequently moved became upset by sudden and unexpected changes [a]

Table S 3 Matrix showing the genetic inter-correlation between the different immune phenotypes

	ast	**alg**	**aid**	**cd**	**crp**	**eos**	**lymph**	**monoc**	**neutr**	**ra**	**sle**	**t1d**
ast	1,00	0,80	0,05	0,11	0,15	0,35	-0,07	-0,06	-0,05	0,08	0,01	0,03
alg	0,80	1,00	0,07	0,32	0,01	0,33	-0,05	-0,03	-0,07	-0,01	-0,07	0,06
aid	0,05	0,07	1,00	0,30	0,12	0,12	-0,01	0,01	-0,20	0,40	0,22	0,57
cd	0,11	0,32	0,30	1,00	-0,07	0,09	-0,09	0,01	-0,15	0,18	0,16	0,20
crp	0,15	0,01	0,12	-0,07	1,00	0,07	-0,06	-0,08	-0,04	0,15	0,07	-0,04
eos	0,35	0,33	0,12	0,09	0,07	1,00	0,01	0,01	0,02	0,09	0,05	0,22
lymph	-0,07	-0,05	-0,01	-0,09	-0,06	0,01	1,00	0,01	0,97	-0,01	0,01	-0,03
monoc	-0,06	-0,03	0,01	0,01	-0,08	0,01	0,01	1,00	0,03	-0,02	-0,02	0,04
neutr	-0,05	-0,07	0,20	-0,15	-0,04	0,02	0,97	0,03	1,00	-0,08	-0,04	0,05
ra	0,08	-0,01	0,40	0,18	0,15	0,09	-0,01	-0,02	-0,08	1,00	0,47	0,50
sle	0,01	-0,07	0,22	0,16	0,07	0,05	0,01	-0,02	-0,04	0,47	1,00	0,24

t1d	0,03	0,06	0,57	0,20	-0,04	0,22	-0,03	0,04	0,05	0,50	0,24	1,00

Abbreviations: ast = asthma; alg = allergic disease; aid = autoimmune thyroid diseases; cd = celiac disease; crp = c-reactive protein; eos = eosinophil count; lymph = lymphocyte count; monoc = monocyte count; neutr = neutrophil count; ra = rheumatoid arthritis; sle = systemic lupus erythematosus; t1d = autoimmune type 1 diabetes.

Table S 4 Global genetic correlation results between immune phenotypes and ASD and number of shared loci

Immune phenotypes	**rg**	**se**	**p**	**q**	**N loci with significant h^2_{SNP}**
Allergy	0.14	0.04	0.006	0.01	21
Asthma	0.08	0.03	0.01	0.02	17
AID	0.09	0.04	0.03	0.06	9
CRP	0.0008	0.02	0.97	0.97	37
CD	0.11	0.07	0.11	0.18	110
Eos	0.02	0.03	0.40	0.57	34
Lymph	-0.06	0.02	0.005	0.01	21
Monoc	0.002	0.02	0.93	0.97	20
Neutr	0.02	0.03	0.55	0.66	78
RA	-0.12	0.04	0.005	0.01	17
SLE	-0.17	0.06	0.004	0.01	34
T1D	0.058	0.074	0.43	0.57	27

Abbreviations : AID = autoimmune thyroid diseases; HLA= human leukocyte antigen; CD = celiac disease; CRP = c-reactive protein; Eos = eosinophil count; Lymph = lymphocyte count; Monoc = monocyte count; Neutr = neutrophil count; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; *T1D = autoimmune type 1 diabetes.; rg = genetic correlation; se = standard error; p = p-value; q = q-value;* h^2_{SNP} = *SNP-based heritability*

Table S 5 Loci-specific genes expressed in the brain and immune system significantly associated with ASD and immune phenotypes

Locus	e-Gene	Tissues	Trait-pair (p1-p2)	Z_p1	P_p1	Z_p2	P_p2
Locus chr 1	*DDX9*	Transformed lymphocytes	ASD-Eos	3.07	0.001	1.71	0.04
	-	spleen		3.04	0.001	1.99	0.02
	-	Blood		3.09	0.0009	2.12	0.01
Locus chr 6	*RNF39*	Brain cortex	ASD-CRP	1.9	0.02	1.79	0.03
	HLA-F	Blood		2.37	0.008	3.28	0.0005
	HLA-F	Transformed lymphocytes		2.01	0.02	3.28	0.0005
	HLA-A						
	ZFP57	Transformed lymphocytes		1.75	0.03	e4.01	2.9x10-5
Locus chr 12	*YEATS4*	Brain cortex	ASD-Mon	2.05	0.01	3.63	0.0001
		Blood		3.17	0.0007	4.16	1.5x10-5
		Transformed lymphocytes		2.47	0.006	3.59	0.0001
	LYZ	blood		3.00	0.001	5.4	3.1x10-8
		spleen		2.33	0.006	3.6	0.0001
	MDM2	blood		1.17	0.01	3.78	7.8x10-5
Locus chr 17	*KANSL1*	blood	ASD-lymph/ntr	4.86	5.7x10-7	7.8	2.8x10-15
	ARL17A	blood		4.81	7.2x10-7	7.75	4.59x10-15
		spleen		4.7	8.9x10-7	7.6	1.03x10-14
		Brain cortex		4.83	6.73x10-7	7.85	2.05x10-15
	LRRC37A	blood		4.86	5.7x10-7	7.89	1.4x10-15
		Spleen		4.9	4.26x10-7	8.6	3.08x10-18
		Brain cortex		4.8	6.2x10-7	7.9	1.21x10-15
	LRRC37A2	blood		4.9	2.9x10-7	8.0	2.76x10-15
	-	Transformed lymphocytes		4.76	9.67x10-7	7.73	5.4x10-14
		Spleen		4.79	7.9x10-7	7.78	3.4x10-15

-	Brain cortex	4.81	7.4x10-7	7.97	7.836x10-16
WNT3	Transformed lymphocytes	4.56	2.5x10-7	7.6	1.22x10-14
	Spleen	2.5	0.003	7.2	2.5x10-13
MAPT	Spleen	4.5	2.9x10-6	7.4	4.8x10-14

Abbreviations: chr = chromosome; Z = standardised effect size; p = pvalue; p1 = phenotype 1; p2 = phenotype 2; ASD = autism spectrum disorder; Eos = eosinophil count; Mon = monocyte count; CRP = c-reactive protein; Lymph = lymphocyte count; Neutr = neutrophil count;

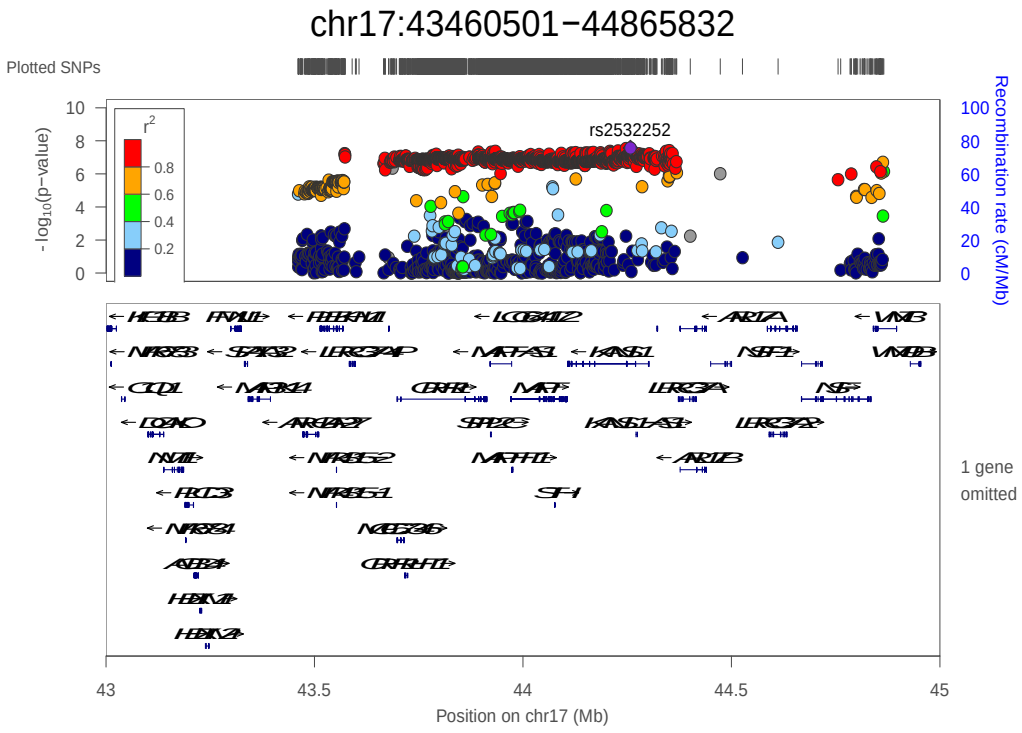

Figure S 1. Locus with rg signal between ASD and Autoimmune Thryoid diseases, Eosinophil count, Lymphocyte count. This locus registered a complete overlap between associated SNPs and significant fetal mQTLs.

Table S 6 Genomic locations of epigenetic modifications in the fetal brain associated overlapping with ASD-related SNPs at the locus chr17:43-44Mb.

Site of DNA modification (chr:bp)	***Gene***	***Gene location***
17:43662623	*LRRC37A*	TSS
17:43662625	*LRRC37A*	TSS
17:43663208	*LRRC37A*	TSS
17:43663579	*LRRC37A*	TSS
17:43971911	*MAPT*	TSS promoter
17:43971919	*MAPT*	TSS promoter
17:43972573	*MAPT*	Enhancer
17:43973522	*MAPT*	Enhancer Low activity region
Abbreviations: chr = chromosome; bp = basepair; TSS = transcription starting site;		

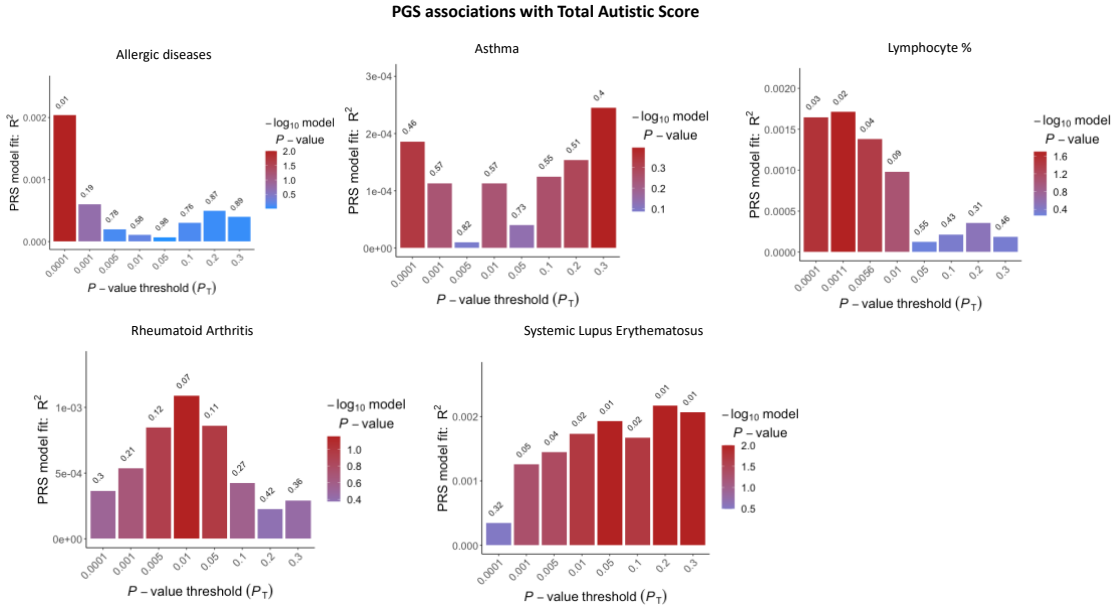

Figure S 2 Bar plot results indicating the variance that Polygenic scores for immune phenotypes associated with ASD explain in 'total autistic score'.

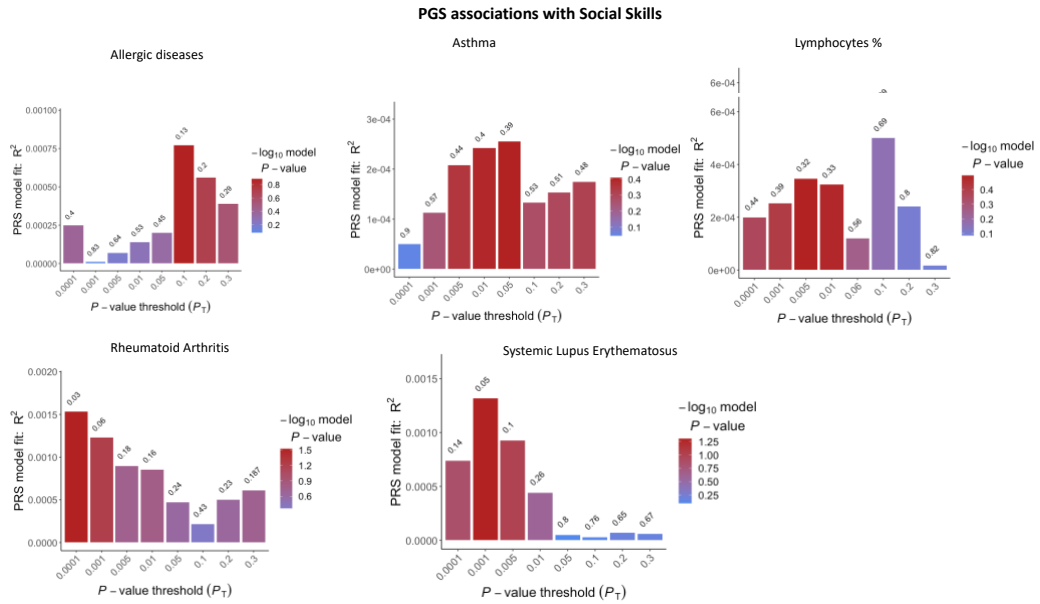

Figure S 3. Bar plot results indicating the variance that Polygenic scores for immune phenotypes associated with ASD explain in 'social skills'.

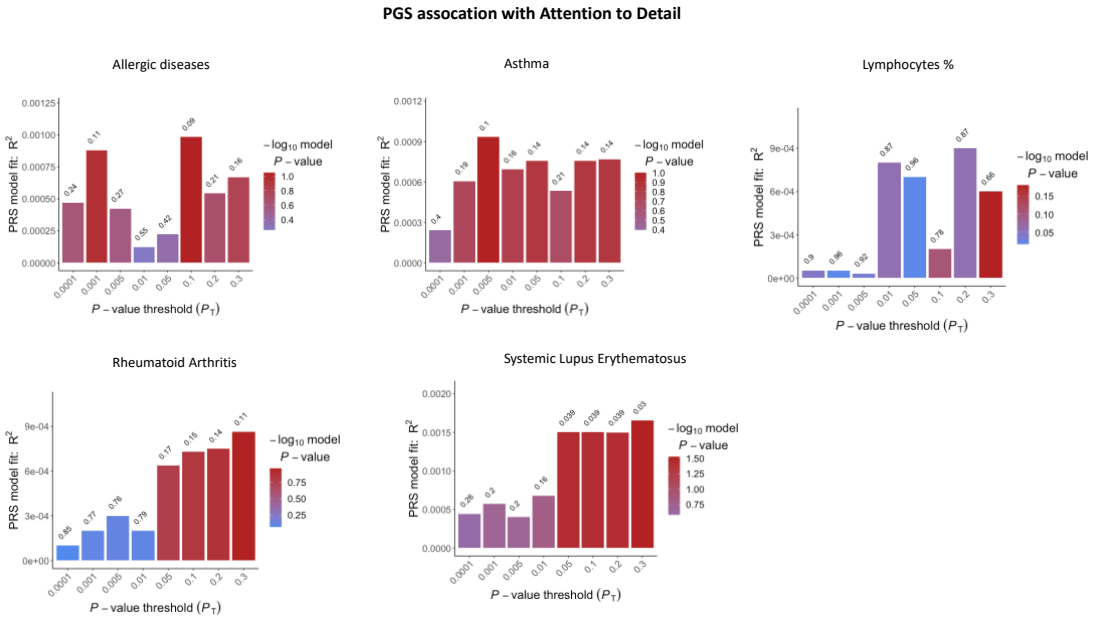

Figure S 4. Bar plot results indicating the variance that Polygenic scores for immune phenotypes associated with ASD explain in 'attention to detail'.

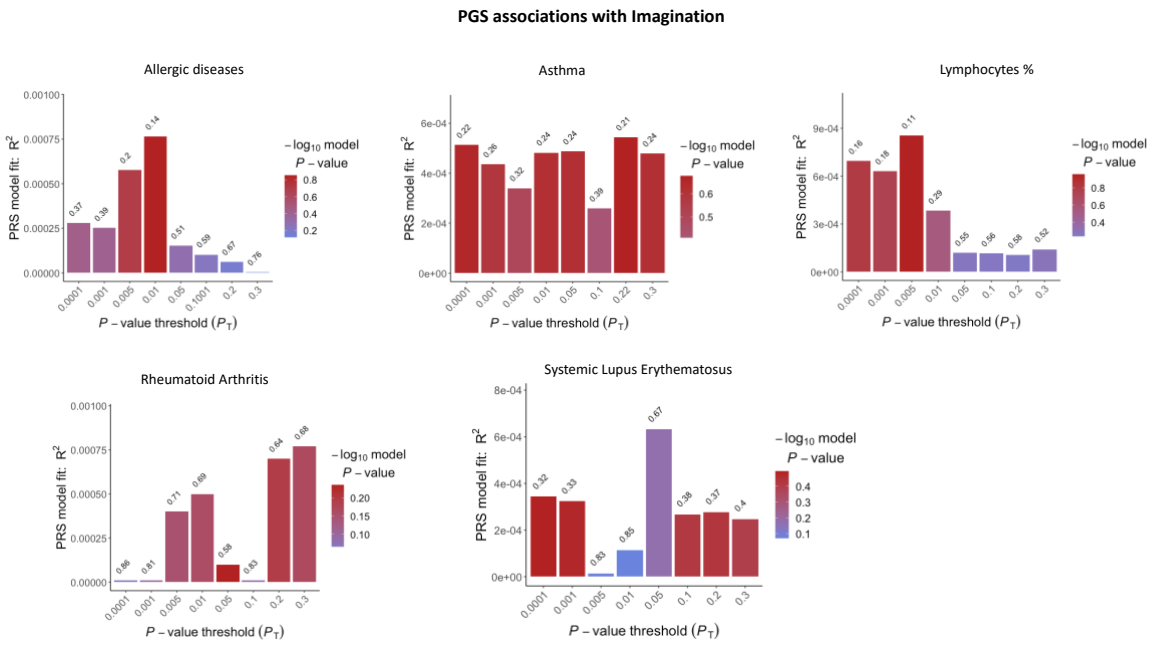

Figure S 5 Bar plot results indicating the variance that Polygenic scores for immune phenotypes associated with ASD explain in 'imagination'.

Table S 7 Results of regression analyses of polygenic score for immune phenotypes on each autistic-like trait.

Childhood behavior	Base GWAS	threshold	Full R2	beta (se)	p-value	FDR-p value
	ALG	0.0001	0.0024	-5.12(0.29)	0.07	0.13
	AST	1	0.0006	3.31(0.24)	0.18	0.24
	Lymph	**0.0004**	**0.004**	**8.0(0.028)**	**0.005**	**0.03**
	RA	0.009	0.0015	3.9(0.03)	0.37	0.39
	SLE	**1**	**0.0034**	**1.23(0.06)**	**0.01**	**0.03**
Rigidity						
	ALG	**0.13**	**0.0045**	**1.59(0.059)**	**0.007**	**0.03**
	AST	1	0.0030	4.16(0.23)	0.07	0.13
	Lymph	0.003	0.0023	5.2(0.49)	0.28	0.32
	RA	**0.052**	**0.0043**	**2.5(0.09)**	**0.009**	**0.03**
	SLE	**0.006**	**0.0051**	**1.1(0.03)**	**0.002**	**0.03**
Social skills						
	ALG					
	AST	0.04	0.0026	7.56(0.71)	0.2	0.26
	Lymph	0.01	0.0026	1.3(0.11)	0.21	0.27
	RA	0.005	0.0037	-2.5(0.15)	0.11	0.16
	SLE	0.001	0.0035	-3.5(0.17)	0.04	0.11
Attention						
	ALG	0.09	0.0012	5.93(0.37)	0.07	0.13
	AST	0.008	0.0013	2.9(0.16)	0.07	0.13
	Lymph	0.01	0.0003	3.4(0.61)	0.57	0.58
	RA	0.24	0.0010	-2.5(0.15)	0.11	0.16
	SLE	1	0.0020	-7.2(0.31)	0.02	0.06
Imagination						
	ALG	0.01	0.0009	4.29(0.26)	0.10	0.16
	AST	0.0006	0.0013	1.4(0.10)	0.17	0.24
	Lymph	0.005	0.0017	1.1(0.07)	0.11	0.16
	RA	0.05	0.00010	-6.5(0.51)	0.58	0.58
	SLE	0.17	0.0013	-3.0(0.26)	0.23	0.27
Total						
	ALG	**0.00010**	**0.0035**	**-3.4(0.14)**	**0.01**	**0.03**
	AST	1	0.0019	1.43(0.13)	0.30	0.33
	Lymph	**0.0004**	**0.0035**	**3.8(0.16)**	**0.01**	**0.03**
	RA	0.008	0.0029	3.6(0.18)	0.05	0.13
	SLE	**0.21**	**0.0039**	**3.5(0.013)**	**0.009**	**0.03**

Abbreviations: ALG = allergic disease; AST = asthma ; Lymph = lymphocyte count ; RA = rheumatoid arthritis ; SLE = systemic lupus erythematosus ; R2 = variance explained

by the model (model fit) ; se = standard error; FDR = false discovery rate; bold = signficant association after FDR-correction.

Table S 8 Results of regression analyses of polygenic score analyses on autistic-like traits stratified by sex

Autistic-like traits	**Immune phenotype**	**Males**			**Females**		
		Beta (se)	**p**	**q**	**Beta (se)**	**p**	**FDR-p**
Childhood Behavior	Allergy	-0.024 (0.027)	0.36	0.64	-0.03.5(0.025)	0.15780	0.32
	Asthma	0.036 (0.027)	0.18	0.42	0.018 (0.025)	0.46761	0.58
	Lymph	0.018 (0.027)	0.50	0.72	0.071 (0.025)	0.00469	0.09
	RA	0.022 (0.027)	0.40	0.64	0.0168(0.025)	0.50792	0.60
	SLE	0.036 (0.027)	0.18	0.42	0.050 (0.025)	0.04561	0.19
Rigidity	Allergy	0.028 (0.026)	0.28	0.56	0.007 (0.025)	0.7507	0.77
	Asthma	0.051 (0.026)	0.04	0.37	0.039 (0.025)	0.1156	0.26
	Lymph	0.012 (0.026)	0.64	0.77	-0.008 (0.025)	0.7481	0.77
	RA	0.039 (0.026)	0.13	0.40	0.045 (0.025)	0.0719	0.26
	SLE	0.04 (0.026)	0.06	0.37	0.040 (0.025)	0.1107	0.26
Social skills	Allergy	-0.05 (0.027).	0.04	0.37	-0.026 (0.025)	0.294	0.40
	Asthma	-0.036 (0.027)	0.18	0.42	0.030 (0.0252)	0.226	0.36
	Lymph	0.028 (0.027)	0.91	0.94	0.064 (0.025)	0.0101	0.09
	RA	-0.056 (0.027)	0.03	0.37	0.013 (0.025)	0.602	0.69
	SLE	-0.011 (0.027)	0.68	0.78	0.058 (0.025)	0.0194	0.11
Attention to detail	Allergy	0.022 (0.027)	0.40	0.64	0.033 (0.025)	0.183	0.34
	Asthma	0.044 (0.027)	0.10	0.40	0.041 (0.024)	0.103	0.26
	Lymph	0.033 (0.027)	0.19	0.42	-0.020 (0.025)	0.935	0.93
	RA	-0.012 (0.027)	0.64	0.77	0.027 (0.025)	0.274	0.39

	SLE	-0.040 (0.027)	0.13	0.40	0.035 (0.025)	0.160	0.32
Imagination	Allergy	0.018 (0.026)	0.49	0.72	0.020 (0.025)	0.4117	0.53
	Asthma	-0.015 (0.027)	0.57	0.75	0.039 (0.025)	0.1147	0.26
	Lymph	0.002 (0.027)	0.94	0.94	0.029 (0.025)	0.2464	0.36
	RA	0.0040 (0.026)	0.88	0.94	-0.030 (0.025)	0.2256	0.36
	SLE	-0.041 (0.026)	0.12	0.40	-0.010(0.025)	0.6643	0.73
Total	Allergy	-0.051 (0.026)	0.05	0.37	-0.029 (0.024)	0.240	0.36
	Asthma	0.015 (0.026)	0.56	0.75	0.053 (0.024)	0.0323	0.16
	Lymph	0.0089 (0.023)	0.73	0.81	0.06.2 (0.02)	0.0123	0.09
	RA	0.044 (0.026)	0.09	0.40	0.043 (0.024)	0.0785	0.26
	SLE	0.022 (0.026)	0.38	0.64	0.064 (0.024)	0.00973	0.09

Abbreviations: ALG = allergic disease; AST = asthma ; Lymph = lymphocyte count ; RA = rheumatoid arthritis ; SLE = systemic lupus erythematosus ; se = standard error; p = p-values; FDR = false discovery rate.

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6. Chapter 6. Immunogenetic underpinnings of clinical symptoms in ASD

6.1. Introduction

In the previous chapters, I demonstrated that a) there is a link between immune genes and ASD (Chapters 3-5) and that b) genetic factors linked to lymphocytic response, and to autoimmune and allergic conditions, are relevant to ASD and to certain autistic dimensions (rigidity) in the general population (Chapters 4-6). Based on these latter findings, I hypothesized that given immunogenetic factors are also related to rigid and stereotypic behaviors, a 'core' symptom domain in individuals diagnosed with ASD.

A connection between immune factors and particular ASD symptoms has been established by prior research in both animals and humans; but the genetic basis for this is poorly understood. For instance, animal studies using mice models of maternal immune activation (MIA) demonstrated an association between MIA and the occurrence of repetitive and rigid behaviors, such as grooming and marble burying in the offspring (Boulanger-Bertolus et al., 2018; Estes & McAllister, 2016). Also, an increased rate of stereotypies has been reported in rhesus monkey when exposed to maternal antibodies (immunoglobulin G) during gestation (Martin et al., 2008). In addition, in autistic children, there is evidence of a correlation between the level of repetitive behaviors and dysregulations in immune cells, including dendritic cells and T-lymphocytes, and their cytokine products and antibodies (Breece et al., 2013; Gladysz et al., 2018; Hollander et al., 1999; Onore et al., 2012; Robinson-Agramonte et al., 2022). Notably, prior studies also suggest that immune dysregulations may characterize clinical subgroups of ASD defined by more severe behavioral impairments. These studies, specifically, indicate that clinical severity in ASD is associated with increased levels of several proinflammatory cytokines (e.g., interleukins, interferons, chemokines) and increased T-lymphocyte activation (Careaga et al., 2017; Robinson-Agramonte et al., 2022).

Of note, these dysregulated immune markers point to multiple and heterogeneous immune processes. It has therefore, been suggested that ASD is underpinned by multiple immune mechanisms, and that these may map to diverse immune sub-phenotypes within ASD (Careaga et al., 2017; Robinson-Agramonte et al., 2022).

Nonetheless, to the best of my knowledge, there are neither reports on the association of immune genes with specific clinical features, and especially rigidity; and nor in relation to clinical outcome/severity. I now have the unique opportunity to address this issue and study the association between immune genetics and 'core' symptom domains in autistic individuals by accessing a rich sample of autistic individuals from the EU-AIMS Longitudinal European Autism Project (LEAP), that has been deeply phenotyped and longitudinally followed, (Charman et al., 2017; Loth et al., 2017).

In brief, the LEAP sample collected genetic and imaging data, along with a comprehensive range of clinical measures that assessed symptom domains of ASD (Charman et al., 2017) (Figure 1). This has previously allowed us to link genetic mechanisms, including genes associated with inflammatory responses (e.g., microglia activation), to variations in cortical thickness throughout the brain in autistic individuals (Ecker et al., 2022). Of note, in the LEAP sample, behavioral measures have been collected at two time points separated by ~12-24 months. I was, thus, also able to explore the impact of specific genetic mechanisms on how behavioral variability *change* over time.

Moreover, the LEAP study includes (longitudinal) measures of adaptive behavior. Adaptive behavior refers to the set of abilities required for the attainment of personal independence and social sufficiency and is thus considered to be a clinically meaningful measure of social behaviour (Pretzsch et al., 2022; Tillmann et al., 2019). Recent work from our group demonstrated - for the first time - that change in adaptive behavior in ASD is associated with variations in cortical thickness and surface area of specific brain regions, including 'social brain' regions, and genetic

pathways regulating neurogenesis and autophagy (Pretzsch et al., 2022). Therefore, by using these data, I further had the, unprecedented, opportunity to i) identify autistic individuals who improve or worsen over time (i.e., 'good' vs 'poor' clinical outcome), and ii) explore if immunogenetic factors may underpin individual differences in the direction and magnitude of clinical change.

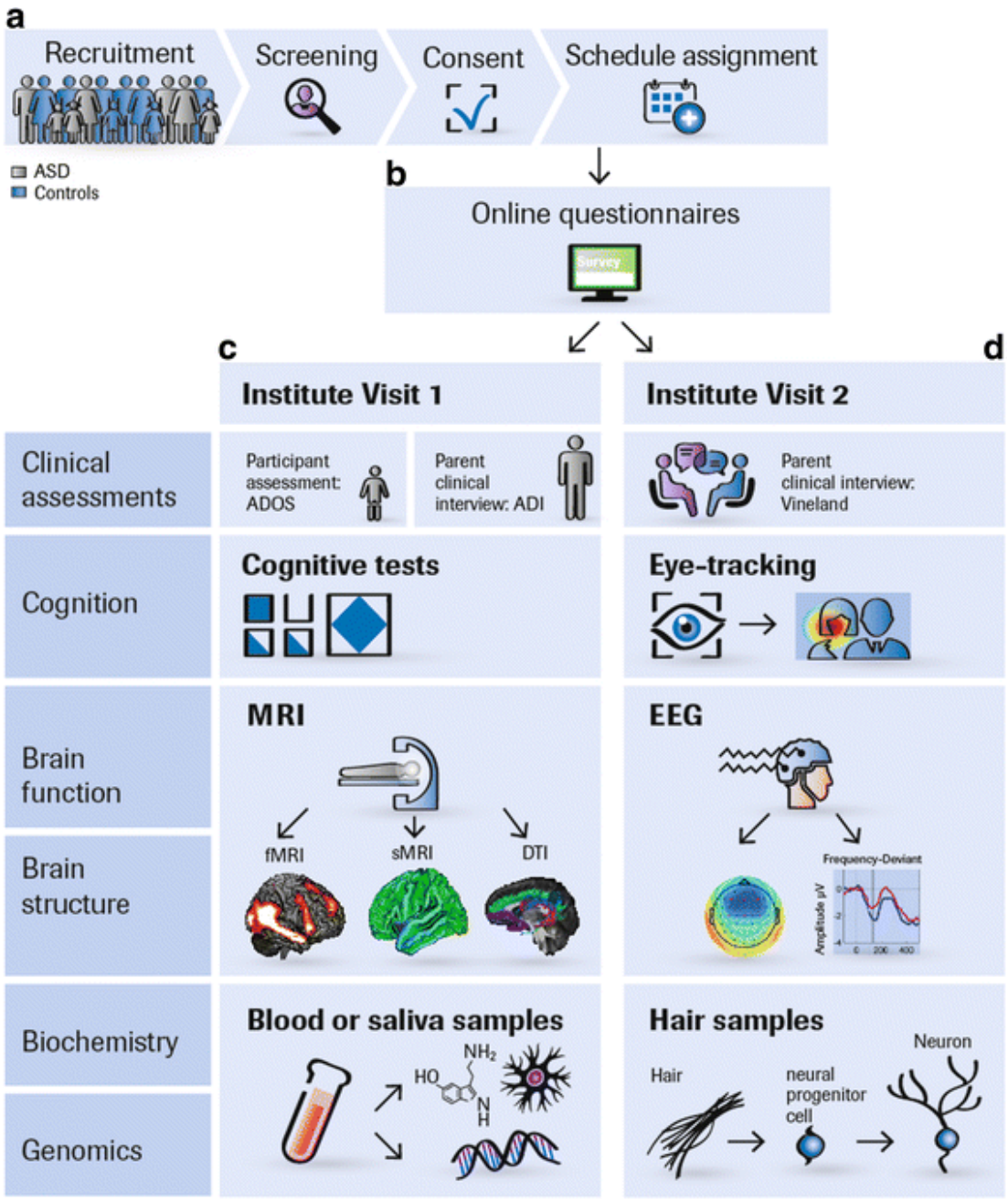

Figure 11 Figure from (Loth et al., 2017)*Illustration of the study design of the Longitudinal European Autism Project (LEAP). A) Participants are recruited and assessed across six international centres in Europe and B) online questionnaire are sent to participants and/or their parents and consent forms are obtained. C-D) The participants and guarantor (parent) visit the centre twice, and four weeks apart. During the visits, the participants undergo cognitive tests, magnetic resonance imaging scans, eye tracking and electroencephalography assessment. Bio samples (blood, saliva, and hair) are taken for biomarker and genomic analyses. After 12-24 months, the participants visit the centre again and undergo all the assessments in order to monitor any longitudinal changes.*

In summary, building on my prior findings (Chapter 5-6) and taking advantage of the rich data resource that the LEAP study is, I conducted the present study.

Here, I specifically addressed two aims. My first aim was to extend prior findings in the general population to a sample of autistic individuals. Specifically, I explored the relationship between immunogenetic factors (i.e., genetic variants associated with lymphocytic levels, autoimmunity, and allergic responses) and the clinical manifestation of rigidity, such as stereotypic behaviours in the autistic participants of LEAP. Additionally, in these participants, I investigated the relationship between immunogenetic factors and clinical outcome, as indexed by adaptive behavior.

My second aim was to explore whether the relationship between immunogenetic factors and both repetitive behaviours and adaptive behavior varied over time in ASD. To address this aim, I leveraged the longitudinal design of LEAP, and evaluated the association between immune-PGS and behavioral change from time point 1 to time point 2.

In addition, I followed these findings with further, pilot post-hoc analyses. I preliminarily explored the possibility of using immunogenetic factors (immune-PGSs) to group autistic individuals into more homogenous immune(genetic) subtypes or clusters. The rationale for these pilot analyses was two-fold. First, it is recognized that the immune phenotypes I considered in this work share genetic mechanisms (Ashley et al., 2017; Han et al., 2020; Ramos et al., 2015; Shirakawa et al., 2000; Zhu et al., 2018). For example, genetic regulators of the antigen response influence both the autoimmune diseases (SLE, RA) and the lymphocyte levels (Ramos et al., 2015). Therefore, it may be simplistic to only investigate one-to-one relationships between the genetics of each phenotype (e.g., immune-PGS) and behaviour. Second, there is suggestive evidence for multiple and diverse immune sub phenotypes within ASD (Careaga et al., 2017; Robinson-Agramonte et al., 2022), although there is poor knowledge on potential immunogenetic variability across those subtypes. Moreover, in this exploratory work, I also assessed whether these immunogenetic-based clusters of autistic individuals vary in terms of behavior.

6.2. Materials and methods

6.2.1. The Longitudinal European Autism Project (LEAP)

The overall goal of the LEAP study is to identify biomarkers for ASD which may improve the diagnostic process and favor the identification of effective – and personalized – treatment approaches ((Charman et al., 2017; Loth et al., 2017)). LEAP is a multi-site project including six European specialist ASD centers: the Institute of Psychiatry, Psychology and Neuroscience, King's College London (KCL), United Kingdom Autism Research Centre at the University of Cambridge (UMCU), United Kingdom, Radboud University Nijmegen Medical Centre (RUMC), University Medical Centre Utrecht (UMCU), the Netherlands, Central Institute of Mental Health (CIMH), Mannheim, Germany, and the University Campus Bio-Medico (UCBM), Rome, Italy. Participants, consisting of both autistic individuals and neurotypicals, were recruited from existing research databases/cohorts, clinical referrals, special needs and mainstream schools, and local communities.

Autistic participants were included if they had an existing clinical diagnosis of ASD in accordance with Diagnostic and Statistical Manual of Mental Disorder (DSM)-IV/ICD-10 or DSM-5 criteria (American Psychiatric Association, 2013). The considered age range was 6-30 years. As up to 70% of autistic individuals present with one or more co-occurring psychiatric conditions (Simonoff et al., 2008), all psychiatric comorbidities (except for psychosis and bipolar disorders) were allowed. We also included participants on stable medication since medication for side symptoms, including aggression and hyperactivity are regularly prescribed to autistic individuals (30-50% in Europe (Wong et al., 2014) and 70% in the US (Frazier et al., 2011)). The study was approved by national and local ethics review boards at each study site. This included the London-Central and Queen Square Health Research Authority Research Ethics Committee (UCAM, KCL; ID 13/LO/1156), the UMM University Medical Mannheim Medical Ethics Commission II (Mannheim University;

ID 2014-540N-MA), the RUMC Institute Ensuring Quality and Safety Committee on Research Involving Human Subjects Arnhem-Nijmegen (RUMC; UMCU; ID 2013/455), and the UCBM Committee De Roma (Rome University; ID 18/14 PAR ComET CBM). All the participants and/or their legal guardian (if appropriate) gave written informed consent. This study was carried out to Good Clinical Practice (ICH GCP) standards.

6.2.2. Clinical measures of interest

In this study, I first focused on clinical measures of rigidity such as restricted and repetitive behaviors in autistic participants to the LEAP study. Repetitive and restricted behaviours have been evaluated using the repetitive behavior scale revised (RBS-R) (Charman et al., 2017). The RBS-R is a questionnaire that captures the breadth of repetitive and restricted behaviors in ASD, ranging from stereotypic behaviors, ritualistic/sameness behaviors, and restricted interests (Wolff et al., 2016). For this study, I considered parent-reports of participant's total scores on the repetitive behaviors scale (RBS). Leveraging the longitudinal design of LEAP, I was also able to measure change in participants' RBS scores (Δ_{RBS}) between visits (RBS-t2 – RBS-t1).

Additionally, I also considered measures of adaptive behavior, defined as the individuals' ability to adopt independently skills needed for everyday functions (e.g., social, practical, and conceptual skills) (Tillmann et al., 2019). To study adaptive behavior, I used scores on the parent interview on the Vineland Adaptive Behavior Scale (VABS-II), which is the primary instrument to capture adaptive behavior (Sparrow & Cicchetti, 1989). For this study, I considered the parent-reports of Vineland's adaptive behavior composite summary or standard scores (VABSC) of the autistic participants. Additionally, I considered measures of change in adaptive behaviors between visits (i.e., Δ_{VABSC} = VABSC-t2 – VABSC-t1). The included measures (RBS, VABSC, Δ_{RBS}, Δ_{VABSC}) were

checked for normality, and the extracted standardized values have been then adopted to perform statistical analyses.

6.2.3. Genotype data

DNA isolated from blood or saliva was genotyped at the 'Centre National de Recherche en Genomique Humaine' (CNRGH) in Paris using the Infinium OmniExpress-24v1 BeadChip (>700K markers) from Illumina. Participants with a sample call rate below 95%, heterozygosity above or below 3 standard deviations from the mean, or a mismatch in reported and genetic sex were excluded. Also, filtering was applied on SNPs that deviated from Hardy-Weinberg Equilibrium (p>1x10-6) and had a genotype call rate below 95% using PLINK v1.9 (Purcell et al., 2007). Imputation of additional SNPs was performed using the 700k genotyped SNPs on the Michigan Imputation Server and based on the Human Reference Consortium r1.1 (2016) reference panel. A Principal Component Analysis (PCA) of a variance standardized relationship matrix was used to evaluate the ancestry of the participants and to provide components for covariate adjustments in the subsequent analyses. Specifically, the first four genetic components (PC1 to PC4) encompassing population variability have been retained and utilized in further analyses. To cluster individuals based on ancestry, the dimensionality was further reduced with uniform manifold approximation and projection (UMAP), reducing the first 8 PCA components to 2 components for better visualization and easier interpretation. Finally, to derive subpopulation clusters, density-based clustering was performed on these clusters (HDBSCAN), and only individuals of European genetic ancestries were selected.

6.2.4. Polygenic score calculation

For each individual, I computed polygenic scores (PGS) referred to their genetic liability to immune conditions that I previously associated with ASD (see chapter 5). The PGS scores considered additively the effects of common genetic variants associated with allergy, asthma, lymphocytresponse levels, systemic lupus erythematosus, and rheumatoid arthritis. To calculate each immune-related PGS of interest, I used the GWAS summary statistics for the respective immune condition or marker (mentioned above and described in chapter 5 – Table 1) as 'base' dataset(s). The summary statistics underwent a preliminary clumping step using PLINK to ensure that only the most significant independent SNP for each linkage disequilibrium (LD) block ($r2>0.25$, clumping window=±500kb) was considered. PGSs were then calculated on the target individual-level genotype data from the LEAP participants using PRSice2 (Choi & O'Reilly, 2019). For each base immune phenotype showing genome-wide genetic correlation with ASD, PGSs were computed including SNPs at a fixed p-value threshold of 0.3 which was chosen to reduce the number of testing necessary when considering multiple p-value thresholds. The choice of this p-value threshold was dictated by the low sample size and selected in accordance with prior reports that showed a good signal-to-noise ratio of this inclusive threshold (Choi et al., 2020). Then, individual immune-based PGSs were standardised and adopted in subsequent analyses described below.

6.2.5. Immune-PGSs and clinical measures of interest

To extend prior findings in the general population (Chapter 5), first I tested the association between individual immune-based PGSs and repetitive behaviors in autistic individuals. Additionally, I tested the association between immune-PGSs and the degree of relative change in

RRBs from time 1 to time 2 in these participants (i.e., considering individual baseline RRB levels). Subsequently, I investigated the association between immune-based PGSs and the participants' level of adaptive behavior at time 1, along with the degree of relative change from time 1 to time 2. To test these associations, I performed multiple regression models correcting for age, sex, site, IQ and 4 population PCs. Due to the high number of comparisons tested, I adopted a 'False-Discovery Rate' (FDR) correction for the resulting p-values and considered significant only FDR-p-value < 0.01. The analyses have been performed in R.

6.2.6. Clustering on immune-PGSs

Subsequently, I performed a preliminary analysis to explore the possibility to use immune-based-PGSs to cluster autistic individuals. This analysis consisted of two steps.

First, I used all the 5 immune-PGS together to identify subgroups of autistic individuals that present more similar/homogenous pattern of variability across immune-PGSs (i.e., immune-PGS based clusters). This was done by adopting the '*k-mean* clustering' algorithm (Likas et al., 2003), which is an unsupervised classification technique that allows to group individuals based on input variables – and, in our case, the immune-PGSs. The number of optimal clusters is represented by *k*. To choose the number of separate clusters that could best represent our data, we adopted the 'elbow method' (Likas et al., 2003). This method explores how the variance explained by the clusters changes as a function of number of clusters considered. Hence, I plotted the total variance (i.e., within-clusters sum of squares) against the number of clusters, and I used the 'elbow' in the plotted curve as a criterion to select the optimal number of clusters to consider.

Second, once established the number of clusters and grouped autistic individuals accordingly, I explored if specific immune-PGS-based clusters differed in terms of repetitive behaviors and adaptive outcome. Between clusters differences were tested using one-way Analysis of variance

(ANOVA), or its non-parametric equivalent (Kruskal-Wallis's test) in case of non-normally distributed data (Sheskin, 2003). Post-hoc analyses were performed to determine which groups showed statistical differences, if any. Due to the multiple pair-wise comparisons being performed, I adopted a 'Bonferroni' correction for the test p-values. The analyses have been performed in R.

6.3. Results

6.3.1. Descriptive characteristics of autistic individuals

For this study, I included a total of 168 autistic individuals from the LEAP study. The sample had a mean age of 15.5 years (standard deviation (SD) = 5.7). Females represented the 29% of the participants, consistent with reported male-to-female ratios in ASD (Charman et al., 2017). Participants had a mean IQ of 103.7 (SD = 15.7). Genotype data were available for all the individuals. Total RBS measures were available for 168 participants; and of these, 96 individuals also had measures of change in RBS. VABSC scores were available for a total of 146 autistic individuals and the measure of change in VABSC was available in 116 individuals. Table S1 provides an overview of mean values, and dispersion indexes, for each of the phenotypic measures. Normality checks indicates a non-normal distribution of phenotypic measures and SLE-PGS and lymph-PGS (Shapiro-Wilk test p-value < 0.05). Figures S1-4 show the distribution of measures and PGSs and related data outliers.

6.3.2. Immune-PGS and behaviour

Given the presence of statistical outliers in the data, I performed multiple non-parametric Spearman's partial correlations between the immune-PGS and clinical measures. The results

indicate no statistically significant associations between RBS and any of the individual immune-PGS (p-value > 0.05) in this sample. I observed no significant association between immune-PGS and VABSC scores (p > 0.05; Table 1).

Table 1. Association between immune-PGS and clinical measures of interest in ASD with rho (p-value).

Phenotype	*Allergy PGS*	*Asthma PGS*	*Lymph PGS*	*RA PGS*	*SLE PGS*
RBS total	-0.02 (0.7)	-0.06 (0.4)	0.05 (0.4)	-0.08 (0.3)	-0.09 (0.2)
VABSC total (N = 146)	0.04 (0.6)	0.08 (0.3)	-0.12 (0.1)	-0.04 (0.5)	-0.04 (0.5)
Δ_{RBS} (N=96)	-0.14 (0.15)	-0.08 (0.42)	**0.28 (0.005)**	-0.11 (0.27)	-0.04 (0.64)
Δ_{VABSC} (N=116)	-0.01 (0.8)	-0.17 (0.06)	-0.05 (0.53)	0.01 (0.8)	0.04 (0.6)

Abbreviations: rho = Spearman correlation coefficient; PGS = polygenic scores; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; RBS = repetitive behaviour scale; VABSC = Vineland adaptive behaviour scale composite score; Δ_{RBS} = change in repetitive behavior scores; Δ_{VABSC} = change in adaptive behavior scores. ***Bold*** *= significant correlation value;*

6.3.3. Immune-PGS and change in behaviour

I identified a significant association between PGS for lymphocyte counts and a positive Δ_{RBS} (i.e., worsening of rigid/repetitive behaviors over time) (rho =0.28; p= 0.005). In contrast, there were no significant correlations between immune-PGS and Δ_{VABSC} in autistic individuals (P > 0.05; Table 1).

6.3.4. Immune-PGS-based clusters

Subsequently, I used the immune-PGSs to divide autistic individuals into genetically similar subgroups. The 'elbow method' demonstrated that the optimal number of clusters (*k*) was 4 (Figure 2).

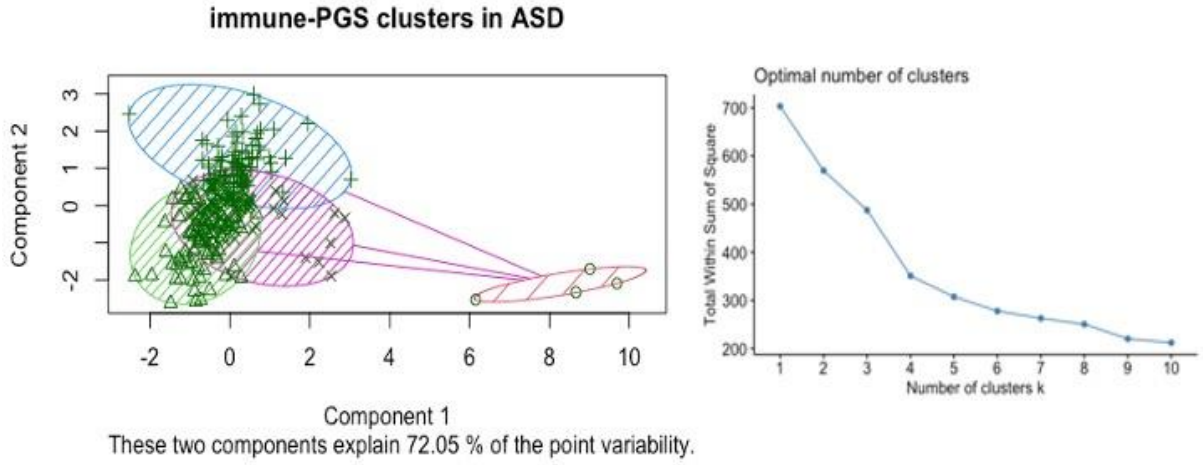

Figure 12 On the left, Identification of k clusters of individuals based on their variability along the 5 immune-related polygenic scores. The first polygenic scores are plotted respectively on the x and y-axis; On the right, Representation of variance within clusters. The 'elbow' in the curve was used to identify the number of clusters (k) – on the x-axis- that optimally represented variability in the data.

These 4 clusters varied in the degree of immune-PGSs (Figure 3, S2). For example, the 1st cluster was characterized by higher PGSs for allergy and asthma, whereas the 4th cluster presented the highest PGSs for lymphocyte count and SLE. However, the 4th cluster only included 3 autistic individuals and therefore this group was not considered for subsequent statistical comparisons (Table S2).

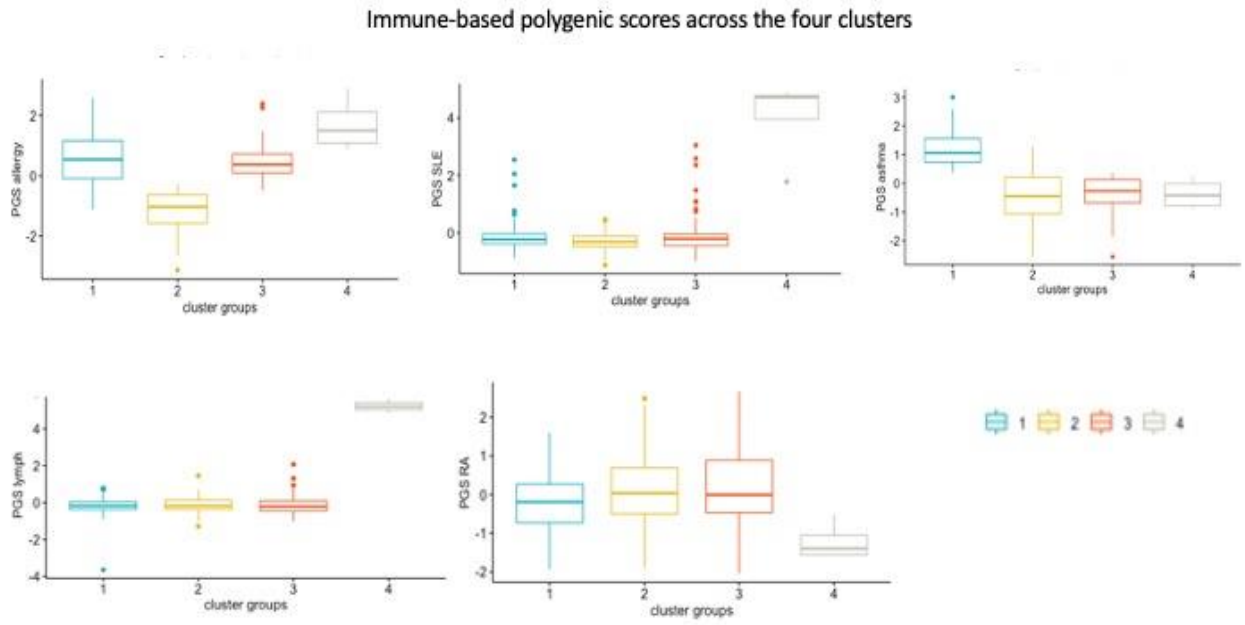

Figure 3 Visualisation of the distribution of immune-based polygenic scores across the different clusters in the autistic participants. The x-axis displays the four genetically based clusters; the y-axis displays the degree of individuals' PGS for each immune phenotype. Each graph indicates the distribution of each immune-PGS investigated.

6.3.5. Behavioral variability across immune-PGS clusters

In the autistic participants, I identified a statistically significant difference in the level of RBS across immune-PGS clusters (KW χ^2 = 14.53, p-value = 0.002: Figure 4- Table S3). Post-hoc analyses, using Dunn's test', indicated that the 2nd group had significantly higher levels of RBS as compared to the 1st cluster (2-1; Z = -2.4, adjusted-p-value = 0.04). Moreover, we reported a significant

difference in the level of VABS scores across immune-PGS clusters (KW χ^2=13.4; p=0.003; Figure 4; Table S3). Specifically, VABSC scores were statistically different between the 2nd cluster group and respectively the 1st cluster group (1-2; Z = 2.6; adjusted-p=0.02) and 3rd cluster groups (2-3; Z = -2.7; adjusted-p= 0.03). We also examined if degree of behavioral change (Δ_{RBS}, Δ_{VABSC}) varied across immune-PGS clusters in ASD. The immune-PGS clusters did not report significant differences in behavioral change across time points (p > 0.05; Table S3).

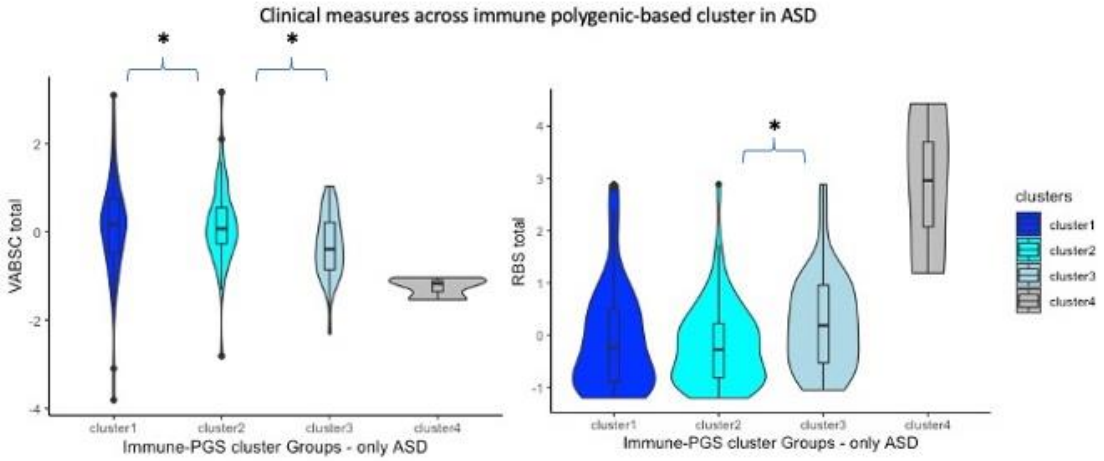

Figure 4 Distribution of repetitive, restricted behaviours and adaptive behaviours across immune-PGS-based clusters of autistic participants. Statistically significant differences (p < .05) are marked by an asterisk.

6.4. Discussion

This study aimed to understand if immunogenetic liability, captured by immune-based PGSs, relates to the level of stereotypic and adaptive behaviors in autistic individuals. A further aim was to explore this relationship across time points. My results demonstrate that immune-based PGS was not associated with repetitive/restricted behaviors or adaptive behavior in autistic individuals at the time of recruitment to the LEAP. However, polygenic variability in lymphocyte levels was correlated with *clinical change* (an increase in repetitive/restricted behaviors over time) in these individuals.

Additionally, here, I also preliminarily explored the possibility of using immune-based PGS to cluster autistic individuals into more similar immune(genetic) subgroups. Although this work was only pilot and limited by sample size, my results suggest that the identified immunogenetic subgroups differ in terms of repetitive behaviors and adaptive behaviors.

Here, I sought to expand my previous findings of an association between immunogenetic factors and rigidity from the general population to an autistic sample. This was made possible by using a longitudinal, and most comprehensively phenotyped, sample of autistic individuals: the LEAP study (Charman et al., 2017; Loth et al., 2017). Namely, in this sample, I explored the association between immunogenetic factors – linked to lymphocytic leveles, autoimmune diseases and allergies – and clinical manifestations resembling rigidity (i.e., repetitive and restricted behaviors) in autistic participants. Due to the longitudinal nature of the sample, I was able to investigate – for the first time - if the relationship between immunogenetic factors and rigid behaviors varied across time. Collectively my findings support an association between immunogenetic factors and clinical rigidity. However, it is important to note that immunogenetic factors, and specifically genetic

factors linked to lymphocytic levels, were associated with *change* in repetitive behaviors and not with these measures at baseline. However, it is important to note that change scores may be subjected to 'regression to the mean' for which individuals with more extreme baseline values may display the highest the degree of change. Here, we centered our measures at both time 1 and time 2 to control for potential baseline outliers, but other approaches are also popular including covarying for baseline values or testing differences between subgroups defined by baseline value cut-offs. Nonetheless, my findings are in line with prior evidence of altered levels of peripheral lymphocytes in autistic participants (Breece et al., 2013; Gładysz et al., 2018; Hollander et al., 1999; Onore et al., 2012; Robinson-Agramonte et al., 2022); and support a role of genetic factors in these dysregulations. Moreover, the finding of an association between immunogenetic factors and worsening of repetitive behaviors over time is in line with prior evidence that immune variability may characterize autistic individuals with more severe behavioral impairments (Careaga et al., 2017; Robinson-Agramonte et al., 2022), and hence more likely to regress over time. Notably, a high incidence of repetitive behaviors, in the form of compulsions, is observed in pediatric autoimmune neuropsychiatry conditions triggered by streptococcal infections (PANDAS) in early life (Frick et al., 2016).

Moreover, in the LEAP sample, I also had the unique opportunity to investigate the relationship between immunogenetic factors and adaptive behavior in ASD. This was especially relevant because it allowed me to understand if immunogenetic factors relate to individual's everyday functioning, and if these factors are associated with more favorable prognosis, or the opposite. However, in contrast with repetitive behaviors, I reported no association between immunogenetic factors and either adaptive behavior at baseline or change in this behavior over time. Taken together, these findings stress the importance of investigating the relationship between immunogenetic factors and clinical dimensions further and in larger samples. In particular, further studies are needed to clarify if this relationship is specific to rigid or restricted behaviors in autistic participants or also relate to other clinical features.

Of note, prior studies demonstrated the presence of diverse forms of immune dysregulations in autistic participants, including alterations in both adaptive and innate immunity. This, therefore, led to the hypothesis of several immune sub phenotypes within ASD. Also, in my analyses I considered multiple immune phenotypes underpinned by different and likely pleiotropic genetic mechanisms. (Ashley et al., 2017; Han et al., 2020; Ramos et al., 2015; Shirakawa et al., 2000; Zhu et al., 2018). Considering the possibility of multiple immune phenotypes, I then explored if immunogenetic information (i.e., immune-based PGS) may help to identify groups of individuals with more homogenous immune(genetic) profiles. Namely, I performed a pilot cluster analysis whereby I clustered individuals based on their variability along all the immune-PGS considered. The results of this exploratory analysis suggest that both repetitive behaviors and adaptive behavior varied between immunogenetic-based ASD subgroups (or clusters). In particular, autistic individuals with higher genetic variability in lymphocytic levels and autoimmunity registered poorer behavioral performance. Nonetheless, these findings should be regarded as preliminary, and limited by the low sample size and thus statistical power. To prove this, one of the identified immunogenetic-based subcluster (4^{th}) only included 3 individuals and was then discarded from the statistical analyses while ensure a fairly even distribution of participants across clusters. Because of this, replication of cluster analyses in larger samples is warranted and necessary. If successful, these future studies may provide proof of concept for using genetic information to cluster individuals into subgroups with more homogenous genetic underpinning, and which may map to specific clinical features/profiles. Taking a step further, immunogenetic-based subtyping may pave the way towards better, personalized immune-based therapies for autistic individuals/subtypes (Simmons & Quinn, 2014).

This study has several limitations. First, the study included a relatively small sample size. This is especially limiting considering the low predictive power of the polygenic score-based regression methods which are generally able to explain only a small fraction of phenotypic variability (Murray et al., 2021). Moreover, the analyses demonstrated the presence of participants that were outliers

in terms of immune-based polygenic liability, and also behavior. This was evident in the cluster analyses, whereby I identified few autistic individuals (4^{th}) with extremely skewed levels of both lymphocyte-related PGS and SLE and repetitive behaviors. In this regard, it is crucial to assess larger clinical cohorts and understand if additional autistic individuals with such genetic and clinical profiles may be identified or not.

One further limitation is that this study only explored the role of genetic factors linked to the general liability for immune conditions, like autoimmunity and allergic disease; and genetic factors linked to lymphocytic response. I did not investigate more specific immunogenetic mechanisms that may contribute to these immune conditions or responses. In particular, based on the association between behavior and PGS for lymphocyte, it is important to narrow these associations down to defined types of lymphocytes, such as B or T (and CD4 helpter or CD8-cytotoxic) lymphocytes (Chaplin, 2010). This is crucial, because it may help to better refine the mechanistic underpinnings linking ASD and immunity: and potentially help identify viable and modifiable therapeutic targets. Finally, this study was underpowered to explore any potential between sexes differences in the relationship between immunogenetic scores and behaviors due to the small sample size. Between-sexes comparisons are largely encouraged and should be explored in larger study populations; also, one potential approach would be to explore the impact of sex-specific immune-related genetic variants on behavior if sex-stratified GWAS summary statistics become available in the future.

In conclusion, my findings support an association between immunogenetic factors and increased repetitive behaviors over time in autism. This also provides further support to prior findings of a specific role of immune genes in behavioral rigidity. Also, my work provides preliminary evidence to support further work on the use of genetic information to identify subgroups of autistic individuals that are more homogenous in terms of biology and may better respond to specific treatment options.

6.5. Supplementary Materials

Table S 7. Distribution of clinical measures of interest in autistic participants from LEAP

Phenotype	N	Mean	SD
RBS total	168	15.5	12.2
VABSC total	146	73.4	14.03
Δ_{RBS}	96	-0.89	8.1
Δ_{VABSC}	116	0.4	9.7

Abbreviations: RBS = repetitive behaviour scale; VABSC = Vineland adaptive behaviour scale composite score; Δ_{RBS} = change in repetitive behavior scores; Δ_{VABSC} = change in adaptive behavior scores. SD = standard deviation;

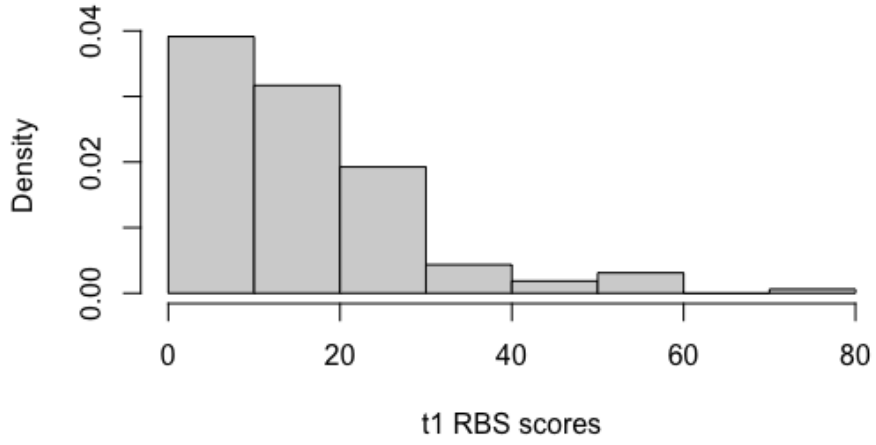

Figure S 1 Distribution of Repetitive and Restricted Behaviours levels in the study LEAP sample.

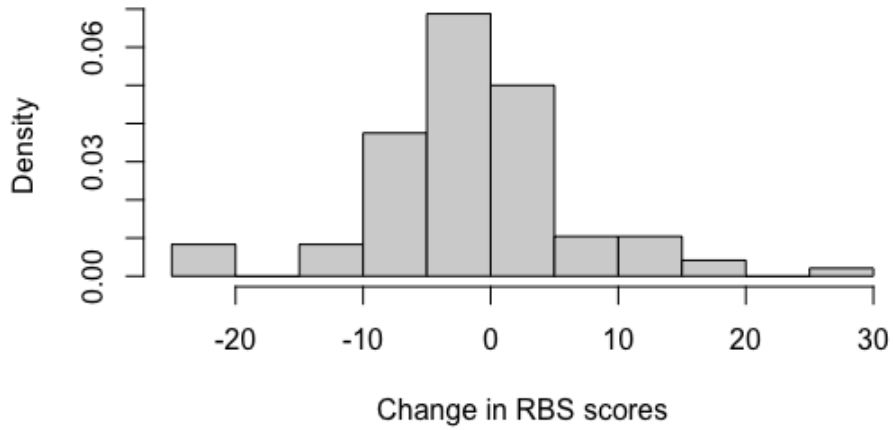

Figure S 2 Distribution of the change in Repetitive and Restricted Behaviours levels through time in the study LEAP sample.

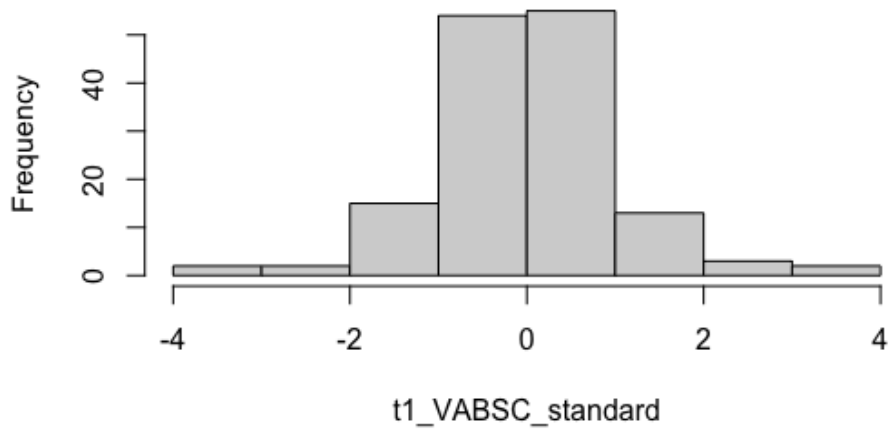

Figure S 3 Distribution of Vineland Adaptive Behaviour Scores in the study LEAP sample.

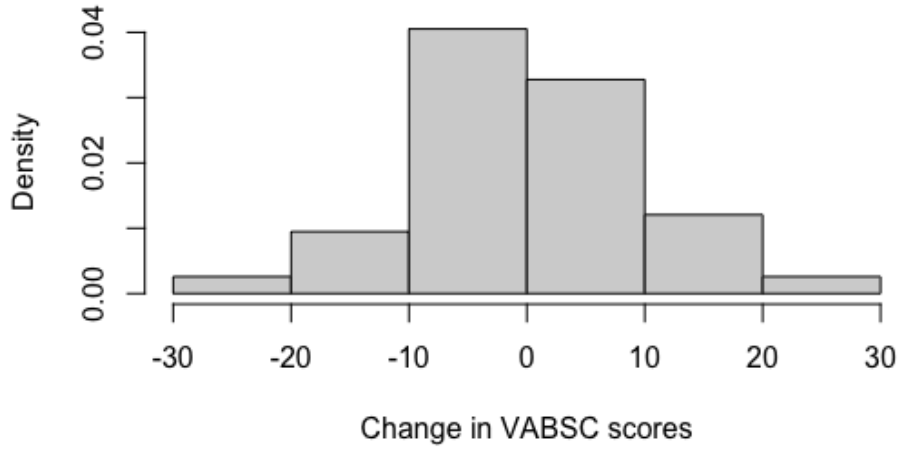

Figure S 4 Distribution of Vineland Adaptive Behaviour Scores through time in the study LEAP sample.

Table S 2 Distribution of immune-based PGS across cluster groups.

Immune-based PGS	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**
(Mean (SD))	(N = 66)	(N = 63)	(N = 36)	(N = 3)
Allergy PGS	-0.24 (0.33)	0.87 (0.52)	-1.57 (0.52)	1.64 (0.90)

Asthma PGS	0.6(0.1)	0.16(0.88)	-0.17 (0.9)	-0.57 (0.41)
Lymphocyte PGS	-0.29 (0.55)	-0.10 (0.40)	-0.11 (0.47)	5.2 (0.38)
RA PGS	-0.18 (0.78)	0.09 (0.85)	0.11 (0.76)	-1.4 (0.19)
SLE PGS	-0.20 (0.61)	-0.26 (0.29)	-0.11 (0.62)	3.7 (1.3)

Abbreviations: PGS = polygenic scores; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; SD = standard deviation;

Table S 3 Differences in clinical measures across immune PGS-based clusters

Phenotype	***KW χ^2***	***p-value***
RBS total	**14.5**	**(0.002)**
VABSC total	**13.4**	**(0.003)**
Δ_{RBS} (N=96)	1.4	0.68
Δ_{VABSC} (N=116)	4.3	0.2

Abbreviations: KW χ^2 = Kruskal Wallis' Chi-square; RBS: repetitive behavior scale; VABSC: Vineland adaptive behavior scale composite score; Δ_{RBS} = change in repetitive behavior scores; Δ_{VABSC} = change in adaptive behavior scores. ***Bold*** *= significant differences;*

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7. Chapter 7: General discussion

In this PhD thesis, I investigated the relationship between immune genes and ASD. My study was driven by three main hypotheses. First, I hypothesised that variations in immune genes are associated with individual liability to ASD. Second, I hypothesised that this relationship is determined by specific aspects of immune system genetics. Third, I hypothesised that variations in immune system genetics is related to specific features of the autistic phenotype.

To test these hypotheses, I adopted different analytical approaches which led to the following findings.

I started by conducting a systematic review of the available literature on immune genes in ASD (Chapter 3). This was an essential first step to inform my subsequent experimental studies. Overall, this review confirmed the importance of immune genetic factors not only with respect to ASD, but also to neurodevelopment in general; further, it highlighted the need to clarify which specific genetic aspects of immunity may be neurodevelopmentally most important.

I followed up these review findings with several genomic analyses (Chapter 4-6). First, based on the notion that phenotypic manifestations typical of ASD also occur as a continuum in the general population (Plomin et al., 2009), I explored the genetics of four distinct autistic-like population-based traits (Chapter 4). Specifically, I performed a meta-analysis of genome-wide association studies which demonstrated that a) immune genes contribute to autistic-like variability in the general population and that b) these genes may particularly relate to specific aspects of the autistic phenotype, especially autistic-like attention and rigidity.

Subsequently, I explored the genetic relationship between distinct aspects of immunity and ASD, defined both categorically and as population-based traits (Chapter 5). By applying different statistical methods, I demonstrated that genetic factors regulating the adaptive immune response, autoimmunity, and allergic responses may be important to ASD; and that these immunogenetic factors are also linked to neurodevelopment.

I also reported a specific association between the identified immunogenetic processes and autistic-like rigidity, in the general population. Therefore, to understand if these immunogenetic factors also relate to specific clinical features of ASD (e.g., rigid behaviours), I leveraged data from a large scale, deep phenotyped, clinical ASD cohort and tested immunogenetic factors in relation to clinical manifestation of rigidity and adaptive behaviour in these participants (Chapter 6). Here, I demonstrated that genetic factors involved in the lymphocytic response are associated with an increase in repetitive and rigid behaviours in autistic individuals over time. Also, I provided preliminary evidence that subgroups of individuals with higher genetic susceptibility towards autoimmunity and lymphocytic activity may be characterised by increased levels of rigid behaviours along with lower adaptive skills.

In conclusion, this thesis supports a role of immune genetic variants linked to aberrant immune activation in ASD; and perhaps especially in relation to the 'rigidity' aspect of this complex

phenotype. More generally, this work suggests the crucial role of the immune system – and its genetic regulators – to both typical and atypical neurodevelopment, here exemplified by ASD.

In the following paragraphs, I will discuss the key findings of this thesis and the implications of these for clinical practice (where relevant). Also, I will describe limitations to my work, and illustrate potential novel research directions that may in future clarify the influence of immune genes on neurodevelopment.

7.1. Immune overactivation in ASD

Collectively, my findings demonstrate that ASD links to genetic factors predisposing towards aberrant/overactive immune responses.

Namely, I reported an association between ASD and genetic factors involved in allergic conditions, which reflect hypersensitivity to common allergens, such as airborne substances or food (Miyazaki et al., 2015). I further demonstrated a genetic association between ASD and autoimmune pathologies, which represents an exaggerated immune reactivity towards one's own tissues and cells (Wahren-Herlenius & Dörner, 2013). In addition, I discovered evidence suggesting a state of immune gene up-regulation in both the blood and the brain of autistic individuals (Gandal et al., 2018; Gupta et al., 2014; Voineagu et al., 2011; Wright et al., 2017).

These genetic association findings are consistent with previous biomarker analyses in autistic populations (Ashwood et al., 2011; Balestrieri et al., 2019; Masi, Glozier, et al., 2017; Pecorelli et al., 2016; Wright et al., 2017; Xie et al., 2017; N. Xu et al., 2015). For instance, there are prior

reports in ASD of increased blood levels of cells/molecules involved in allergic responses, such as eosinophils and mast cells, immunoglobulins (IgE) and histamine (Miyazaki et al., 2015; Wright et al., 2017). Further analyses demonstrated that autistic individuals present increased levels of pro-inflammatory cytokines, along with a heightened antigen response, indexed by the level of specific MHC molecules in the blood, and anti-neuronal antibodies in the brain (Balestrieri et al., 2019; Edmiston et al., 2018; Hughes et al., 2018; Masi, Glozier, et al., 2017; Pecorelli et al., 2016; N. Xu et al., 2015). Epidemiological studies also reinforce my genetic findings. Specifically, they identified increased rate of allergic diseases, including asthma and allergic rhinitis, and infections among autistic people (Lyall et al., 2015; Miyazaki et al., 2015; Zerbo et al., 2015). Moreover, there is evidence of autoimmune pathologies in both autistic individuals, and their family members (Atladóttir et al., 2009; Edmiston et al., 2018; Vinet et al., 2015).

While these prior studies indicated a state of enhanced immune activity in ASD, the work of this thesis offers novel insights on the role of genetic factors in the reported immune overactivity. In this regard my findings suggest that inherited variations in immune genes, and/ or their altered expression, may affect immune homeostasis in ASD and thus increase individuals' sensitivity to external threats (from viruses to allergens) and even towards the self (autoimmunity). Nonetheless, further research is needed to clarify, for example, the direction of the association between ASD and autoimmunity-related genes, which have previously been reported as either protective or risk factors (Bennabi et al., 2018). Also, future studies should elucidate the mechanisms through which immune activation – and its many and specific genetic correlates – are related variations in the manifestation of autistic symptoms.

7.2. Immune activation and brain development

To define the relationship between immune-related genetic variants and ASD, it is crucial to also understand the role of the immune system in brain biology.

The immune system interacts with the central nervous system, and diverse immune cells reside in the brain, such as astrocytes and microglia (Cowan & Petri, 2018; Dantzer, 2018; Garay, 2010; Morimoto & Nakajima, 2019). These immune cells and their molecular product (cytokines) are essential to neuroprotection - as they manage immune surveillance and facilitate tissue repair after damage (Becher et al., 2017; Filiano et al., 2017). Moreover, immune cells support key neurobiological processes at various stages, and especially during neurodevelopment (Faust et al., 2021; Garay, 2010). For instance, immune cells and their messengers have been implicated in the formation of neurons, and the synapses between those, which are essential for learning and for communication within the brain (Cowan & Petri, 2018; Deverman & Patterson, 2009; Faust et al., 2021; Garay, 2010). Specifically, cellular, and molecular immune markers modifythe transmission of electrical signals across synapses (e.g., by insulating axons), and ensure that obsolete synapses are removed efficiently without clogging the brain environment (Faust et al., 2021; Garay, 2010). These immune markers are tightly regulated in their function by (immune genetic) mechanisms (Benacerraf, 1981; Chaplin, 2010; Knight, 2013; Orru, 2013). Hence, any perturbation of the immune response – at the genetic and molecular level - may have dramatic consequences for brain function and development.

In this context, the association I observed between variations in immune genes and ASD may potentially be explained by the effect of immune gene alterations on brain development, and so in turn lead to phenotypic manifestations typical of ASD. This interpretation is supported by diverse types of evidence both from my work and that of others (Debnath et al., 2018; Pendyala et al., 2017; Tamouza et al., 2021). For instance, in my review (chapter 3), I gathered evidence that linked specific immune genes (e.g., MHC genes) to synaptic development and homeostasis (Debnath et al., 2018; Tamouza et al., 2021). I also demonstrated how many immunogenetic constituents act

as neural transcription factors and modulate neurobiological processes that support neuronal formation, migration, and signalling (e.g., mTOR signalling cascade, JAK-STAT signalling pathway). To confirm the importance of immunity – and immune genes – in neurodevelopment, I also demonstrated that immune genes are highly expressed in early human neurodevelopmental stages (chapter 3). Specifically, these genes are upregulated in the pre-natal and peri-natal periods, which are key time points for the formation of neurons, their migration and differentiation, and the constitution and myelination of synapses.

Moreover, dysregulations in immune genes may exacerbate the effects of immune triggers present in the environment (M. v. Lombardo et al., 2018; Vuillermot et al., 2012; X. Xu et al., 2020). For example, a genetic predisposition towards immune over activation, or a state of immune gene up-regulation, as those I reported here, may make the individual more susceptible to the effect of viral or bacterial infections – and perhaps especially during key stages of neurodevelopment, such as pregnancy (X. Xu et al., 2020). Given this, it is possible that immunogenetic liability may mediate the well-known association between neurodevelopmental disorders and gestational infections, and the maternal immune activation resulting from it (Boulanger-Bertolus et al., 2018; Estes & McAllister, 2016; Morelli et al., 2015; X. Xu et al., 2020). To further support my suggestion of an interaction between immune genes and environment stressors, I also demonstrated that immunogenetic factors linked to ASD are – in part – sensitive to epigenetic modifications observed in early development (Hannon et al., 2016).

In summary, to elucidate the role of immune genetic variations in ASD, it is crucial to explore these genes taking a neurodevelopmental perspective. Because of the role of immunity in brain development and homeostasis (Cowan & Petri, 2018; Estes & McAllister, 2016; Yirmiya & Goshen, 2011), it is likely that perturbations of immune genes impact on the trajectory of brain development and, thus, lead to behavioural and cognitive aberrations manifested by autistic children.

7.3. Phenotypic specificity

This work also highlights the importance of examining the relationship between immune genetic variants and specific symptom domains of ASD.

It is widely acknowledged that i) ASD is an overly complex and heterogeneous condition (Masi, DeMayo, et al., 2017a); and that ii) clinical heterogeneity likely reflects the contribution of multiple and varied underlying genetic and biological mechanisms (Jeste & Geschwind, 2014). In this context, my findings suggest that variations in immune genes – and particularly genetic factors involved in autoimmune and adaptive immunity – may particularly contribute to symptoms of rigidity in ASD.

A relationship between immunity and rigid, and repetitive, behaviours has been previously reported by other studies assessing either animal models of ASD or in autistic children. For instance, studies in mice indicated that maternal immune activation, induced through bacterial product lipopolysaccharide (LPS) or viral RNA polyinosinic polycytidylic acid (Poly I:C) exposures, was linked to increased levels of repetitive behaviours in the offspring, such as repetitive grooming and marble burying behaviours (Liu et al., 2023). Similarly, studies in autistic children reported an association between experimentally induced immune activation and the worsening of restrictive and repetitive behaviours (Gładysz et al., 2018; Hughes et al., 2022; Onore et al., 2012). Also, there is prior evidence of a link between immune aberrations and motor rigidity in autistic individuals (Onore et al., 2012). My work both supports and extends this previous work, by demonstrating that genetic factors may intervene in the relationship between rigid behaviours and/or movements and the immune response(s) in ASD. Relatedly, an association between motor/behavioural rigidity and immune alterations has been also described by non-psychiatric

research and precisely in clinical populations suffered from inflammatory and autoimmune pathologies (BARRY et al., 2011; Whiteley et al., 2021).

Nonetheless it is unlikely that inflammatory process *only* impact on repetitive and rigid behaviours, as immunity and immune genes have also been associated with other clinical symptoms in ASD. For instance, prior work reported an association between immune dysregulations, like autoimmunity and neuroinflammation, and autistic regression (i.e. the loss of previously acquired speech and social skills and that can occur in some at around the age of 2 (Prosperi et al., 2019; Whiteley et al., 2021). Autistic regression has also been linked to variations in HLA genes by prior genetic studies, as shown in my review (Tamouza et al., 2020). Notably, studies on autistic regression indicate that this phenomenon is accompanied by both immune dysregulations and motor abnormalities such as rigidity (Prosperi et al., 2019; Whiteley et al., 2021), and thus they further reinforce a relationship between immunity and rigid behaviours in ASD (albeit potentially non-specific).

Notably, my study on well-characterised cohort of autistic individuals (Chapter 6) also supported a link between immunogenetic factors and increase of repetitive behaviours over time, specifically. Not only, in this cohort – extending my prior population-based findings – I also demonstrated that immunogenetic factors may especially relate to reduced adaptive outcome in autistic individuals. However, it is important to acknowledge that this work was affected by important statistical power issues; these being a small sample size used and the low predictive power of polygenic score methods (Murray et al., 2021). Therefore, studies in larger clinical cohorts are warranted.

Taken together, my findings support the hypothesis that immune genes relate to specific aspects of ASD, and perhaps especially rigidity. Although further work is required, this suggestion highlights the importance of addressing factors underpinning clinical – and the underlying biological - heterogeneity when considering complex phenotypes as ASD. One strategy to achieve

this is to adopt a domain, or a 'trait-oriented' approach, such as the one illustrated in this thesis. This is because a trait-oriented investigation allows investigators to reduce clinical heterogeneity, and thus help to clarify the influence of specific biological mechanisms, including immunogenetic processes, on behaviour (Bralten et al., 2018).

7.4. Clinical implication

The evidence I report showing immunogenetic variability in ASD carries several potential clinical implications.

To begin with, the influence of the immune system – and its genetic regulators – on neurodevelopment demonstrates how crucial it is to maintain immune homeostasis during key developmental stages such as pregnancy. This could, for instance, be achieved through vaccinations, diet, or nutrient supplementations, and ensuring an environment shielded from relevant toxic or allergenic substances during pregnancy.

Further, my findings hold promises for what is currently regarded as 'precision medicine', that is a medical paradigm encouraging the use of individual genetic and biological information to aid the prevention, diagnosis and ultimately treatment of diseases (Insel, 2014). In this perspective, my research supports the use of genetic information and/or medical records to identify familial contexts with higher susceptibility to immune dysregulations; and where preventive measures aimed at maintaining immune health and limiting the chances of immune stress, and especially in fetal/early life, may result especially suitable.

Besides prevention, immunogenetic studies in ASD may open new frontiers in terms of screening. Namely, research on specific immunogenetic factors may help to define precise immune mechanisms/pathways - and related markers - that are more likely to influence ASD. Although

more work is needed to acquire this knowledge, the potential of it is tremendous. This is because immune markers have the advantage of being measurable in blood at little cost, and thus they may represent valuable tools to aid early diagnosis. For instance, the use of inflammatory markers in blood has been already considered in the diagnosis and management of depression across different studies (Osimo et al., 2020).

In addition, the identification of specific immune pathways in ASD potentially reveals novel treatment opportunities (Gładysz et al., 2018). Namely, these immune pathways may help to better define targets for pharmacological new therapies in ASD - for which we still lack effective pharmacological interventions for so-called core symptoms (e.g., repetitive, and restricted behaviors) (Loth et al., 2016; Masi, DeMayo, et al., 2017b).

Notably, my work stresses the importance of embracing clinical and biological heterogeneity that typically occur in clinical settings. Here, I describe an association between immunogenetic liability and autistic-like rigidity. Put in context, this observation suggests that immune profiling and immune-based therapies may be particularly suited for autistic individuals with a certain clinical profile and especially those individuals with higher levels of repetitive and restricted behaviours.

However, more research is needed to fully exploit the potential of immunogenetic information in clinical settings. Also, my work has important limitations that should be addressed to gain a deeper understanding of immunogenetic mechanisms in ASD and make findings translatable to the clinic.

7.5. Limitation & research considerations

First, in this work I explored the genetic relationship between ASD and multiple immune phenotypes, like autoimmune diseases and inflammatory markers. Although these findings hint at a role of particular immune states (i.e., immune overactivity), the immune phenotypes I studied

are complex and reflect disruptions across multiple immunogenetic mechanisms (Ashley et al., 2017; Bolon, 2012; Chaplin, 2010; Wahren-Herlenius & Dörner, 2013; Zhu et al., 2018). Hence, my findings do not allow me to draw conclusions regarding which of these immunogenetic mechanisms has a primary influence on ASD. Future research should, therefore, systematically investigate the role of the many immune-related mechanisms implicated in autistic individuals.

Second, my work suffered from statistical power limitations. Although I had the unique opportunity to use deeply phenotyped *and* longitudinal data from autistic participants, the sample size available for my analyses was relatively small in genetic terms. Here, we reported a small, but significant association between immunogenetic factors and increase in repetitive and rigid behaviour over time. However, these findings may have been likely driven by outliers in the data. Because of this, Because of this, future studies should be conducted using larger samples or considering meta-analysis to clarify how immune genes related to autistic features in clinical groups. Large study cohorts may also help to assess the predictive value of genetic information, as in the form of polygenic scores, on symptoms through robust simulation models (Choi et al., 2020). Also, by leveraging larger sample sizes, we may be able to test if immunogenetic information can be used to cluster autistic individuals into more biologically homogenous subgroups and potentially identify clinical subtypes with higher immunogenetic liability.

Third, I could not exclude that additional factors may intervene in the association between immune genes and ASD. Particularly, I am aware of the interaction that exists between the immune system and endocrine signalling (Stelzer & Arck, 2016). For example, immune mechanisms are intertwined with metabolic processes, and it is likely that metabolic dysregulations – and relevant genes - intervene in the relationship between ASD and immunity (Alwarawrah et al., 2018). In addition, the immune response is tightly modulated by sex hormones (Roved et al., 2017). Namely, oestrogens induce immune tolerance and associated with higher autoimmune susceptibility, whereas testosterone has been linked to a weakened immune response (Roved et al., 2017). In the

light of the sex difference in the prevalence of ASD (Werling & Geschwind, 2013), it is informative to understand if the relationship between immune genes and ASD is modulated by sex. It is recognised that sex hormones exert opposite effects on the immune response, with oestrogens showing immune-enhancing properties and testosterone acting as immunosuppressant. This, therefore, makes women more likely to suffer from autoimmunity (i.e., an increased but self-directed immune response), and men more susceptible to infectious/ inflammatory pathologies. In my work, I reported negative associations between autoimmune genetics and ASD, suggesting that genetic risk factors for autoimmunity are associated with reduced risk for ASD. However, these opposite directions of effects are likely biased by the differences in the proportion of men and women in autistic population (male-skewed) and in cohorts affected by autoimmune diseases (female-skewed). Hence, future studies should be conducted in samples with balanced representations of both sexes, or separately in males and females and/or including records on any relevant metabolic issues. This may help to identify any (endocrine) factors that may modulate the relationship between immune genes and ASD, and potentially define clinical groups more likely to benefit from immune-based profiling and therapies.

Fourth, I focused my research on common genetic variations in immune processes and how they influence ASD. It is recognised that rare variants – and especially de-novo variations – play a considerable role in ASD (Ashitha & Ramachandra, 2020; Krumm et al., 2015; Leblond et al., 2019; Satterstrom et al., 2020); and that there can be interactions between the effects of rare variants and common genetic factors on ASD (Antaki et al., 2022). Moreover, prior studies suggest that some of the genetic loci hosting common polymorphisms are also likely to harbour de-novo variations or rare genetic variations, of potential larger effect size (Arenella et al., 2023; Leblond et al., 2019; Satterstrom et al., 2020). It is very possible that ASD is also influenced by rare variants affecting immune genes (Cai et al., 2022). Future studies should explore the occurrence of rare immunogenetic variants in autistic children since this may help to further delineate immune pathways carrying higher genetic susceptibility.

7.6. Future directions

In the previous paragraph, I outlined some limitations of my work and here I propose research strategies that may help to overcome these limitations in the future. In addition, my findings also highlight other areas of study that are worth of future consideration. Therefore, in the following paragraphs I will describe the steps that I will undertake in my future career, and these include early life analyses, transdiagnostic analyses and imaging genetic analyses.

7.6.1. Immune genes across the neurodevelopmental spectrum

One potential direction is to study the relevance of immunogenetic factors across the entire neurodevelopment spectrum (Figure 1). It is recognised that neurodevelopmental disorders tend to co-occur (Lai et al., 2019), and they share core symptoms and traits (Kushki et al., 2019). Notably, prior work indicates that these disorders are genetically inter-correlated, and that genetic effects are overlapping across diagnoses (Demontis et al., 2019; Grove et al., 2019; Kushki et al., 2019; Lee et al., 2019). This is likely the case also for immunogenetic factors. This suggestion is supported by both genetic and transcriptomic studies that described variations in immune genes (HLA genes, C4 alleles) in individuals with schizophrenia, and attention-deficit/ hyperactivity disorder (ADHD) (Debnath et al., 2018; Mokhtari & Lachman, 2015; Radwan et al., 2020). Moreover, epidemiological studies indicate the presence of immune dysregulations in a range of neurodevelopmental conditions, including ADHD and obsessive-compulsive disorders, and which may be accounted for in some degree by immunogenetic liability (Estes & McAllister, 2016;

Murphy et al., 2010; Rodriguez et al., 2019; Şimşek et al., 2016; Tistarelli et al., 2020; Tsetsos et al., 2020). This evidence, therefore, suggests the need for future studies to take a transdiagnostic approach to study the role of immune genes in relation to neurodevelopment. This approach may shed light on pathophysiological mechanisms underpinning neurodevelopmental processes that cut across current diagnostic definitions.

Additionally, the possibility of transdiagnostic effects may provide a theoretical basis for future studies on immunogenetic mechanisms – and others – in relation to cross-cutting, neurobehavioral domains; and thus help the move away from diagnostic categories that are highly affected by their intrinsic heterogeneity (Insel, 2014). For example, my work – in line with others – suggests that immune genes relate to rigidity, which is a behavioural domain or trait that characterises multiple conditions such as schizophrenia or obsessive-compulsive disorder, besides ASD (Meiran et al., 2011; Morris & Mansell, 2018).

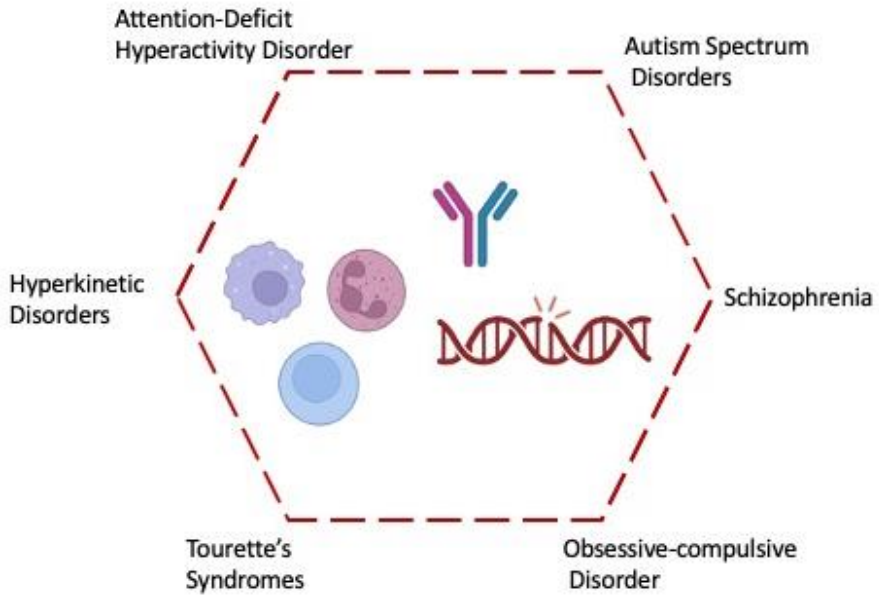

Figure 1 Investigate genetic regulators of immunity across multiple neurodevelopmental diagnostic categories

7.6.2. Immune genes across the life span

A further future direction is to explore the influence of immune genes across the life span (Figure 2). As illustrated above, immune processes and genetic factors regulate key aspects of neurodevelopment. However, how the influence of immune genetic mechanisms on neurodevelopmental results in variable outcomes remains unclear (e.g., 'risk vs resilience' to developing specific conditions/symptoms). To gain insight on these dynamics, it may be useful to track the relationship between immune genes and behaviour over time. Here, I attempted to explore this by leveraging the longitudinal design of the LEAP study (Loth et al., 2017), which indeed demonstrated an association between immunogenetic factors and increase in repetitive and rigid behaviors over time. However, although I had the opportunity to evaluate the influence of immunogenetic factors on behavioural change across time points, these analyses included mainly adolescents and young adults. Because of the neurodevelopmental function of immune genes, it is important to also include additional age groups and especially very young populations such as neonates. For this purpose, pregnancy cohorts and studies in infants or pre-school children would be especially suitable. These studies may help to identify early behavioural markers associated with immunogenetic liability. Also, if integrated with studies in younger children and adults, they may allow us to directly observe how immunogenetic liability impacts the neurodevelopmental trajectory. Importantly, a life span analysis makes it possible to investigate the effect of other potential modulatory factors - such as environmental exposures or the endocrine variations mentioned before - that likely impact the influence of immune genes on neurodevelopmental outcome.

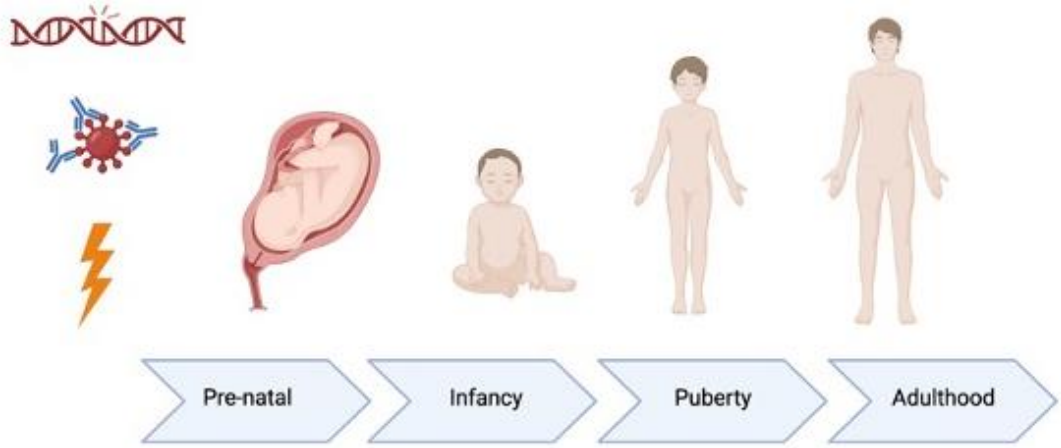

Figure 13 Investigate the influence of immune-related genetic factors, and exposures, across the life span, starting from the pre-natal period until adulthood

7.6.3. Immune genes and neuroanatomy

It is crucial to investigate the impact of immune genes on brain. I illustrated initial evidence that links immune genes to neural processes at the microscopic level, such as neuronal migration and synaptogenesis (Coiro et al., 2015; Deverman & Patterson, 2009; Pendyala et al., 2017). These findings are, however, based on studies in vitro or experiments in animal models (Coiro et al., 2015; Deverman & Patterson, 2009; Pendyala et al., 2017). The impact of immune genes on the human brain is less established. For instance, there is evidence for immune gene dysregulations in the brain of autistic individuals, but these findings are based on the analyses of post-mortem human brain tissues (Gupta et al., 2014; M. v. Lombardo et al., 2017). Also, these prior studies did not explore specific immunogenetic mechanisms and only investigated few brain systems (S. D. Lombardo et al., 2020; M. v. Lombardo et al., 2017; Voineagu et al., 2011). However, it is now possible to explore the relationship between immune genes and human brain by leveraging brain

imaging data that measures various aspects of brain anatomy and function (Ecker et al., 2022; Thompson et al., 2014; van Rooij et al., 2018).

To explore this possibility, I conducted some preliminary analyses linking immunogenetic and brain imaging data in ASD. Specifically, building on the findings of this thesis, I explored a) the expression of immunogenetic factors highlighted in this thesis throughout the entire brain; and I then tested b) how individual variations in these immunogenetic factors influence individual neuroanatomical variation in brain regions with higher expression of given immune genetic factors.

To address point a), I used the e-MAGMA method (Gerring et al., 2021) (see chapter 2 and chapter 4) and virtually assessed the expression of genetic variations involved in autoimmune diseases – being systemic lupus erythematosus and rheumatoid arthritis - in brain tissues from cortical and subcortical regions (i.e., basal ganglia, amygdala, hippocampus). These analyses revealed a significant expression of autoimmune-related genes the caudate and in the nucleus accumbens ($p=0.001$), while not in the cortical regions ($p > 0.05$). Therefore, I focused my subsequent analyses on subcortical areas.

Next, I addressed point b) and tested if autoimmune-based polygenic scores (based on chapter 5) were associated with volumetric variation across subcortical brain regions in autistic individuals and neurotypicals from the LEAP cohort (see chapter 6; (Loth et al., 2017)). To do this, I used raw genotype data and brain imaging data (for further details on imaging measures see Ecker et al., 2022) extracted from the LEAP participants. In this pilot analysis, I included both autistic people ($N=242$) and neurotypicals ($N=225$); and I tested a series of regression models including immune-based polygenic scores as independent variables and subcortical volume variability as dependent variables. The models included age, sex, IQ, site, total intracranial volume, and the population structure components as covariates. These analyses demonstrated a significant association between increased polygenic scores for autoimmune systemic lupus erythematosus and reductions in

subcortical volumes (beta=-.17, p=0.003). This association was specific to the autistic group, and not observed in neurotypicals (p>0.05: figure 3).

Additionally, as post-hoc analysis, I explored if autistic individuals grouped by the level of SLE-polygenic variance exhibited differences in subcortical anatomy. My results indicated statistically significant differences in the volume of caudate and pallidum in autistic individuals divided into groups with low vs high SLE-polygenic scores (T_caudate = 7.9, p = 0.004; T_pallidum = 4.6, p = 0.02).

Although these analyses were only preliminary, they suggest that immunogenetic factors contribute to variations in the development/anatomy of specific brain systems, which may in turn influence behaviour. These preliminary findings hint at an important link between immune genes and subcortical structures (Figure 3), which are known to be implicated in motor control and flexibility (van Rooij et al., 2018). This, therefore, aligns with my previously observed association between immune genes and behavioural/ motor rigidity, and suggests that their impact on subcortical development may play a key role. However, these analyses should be considered as initial steps and future studies should explore in depth the relationship of specific immunogenetic pathways and brain phenotypes, including structural but also and importantly both functional and anatomical connectivity.

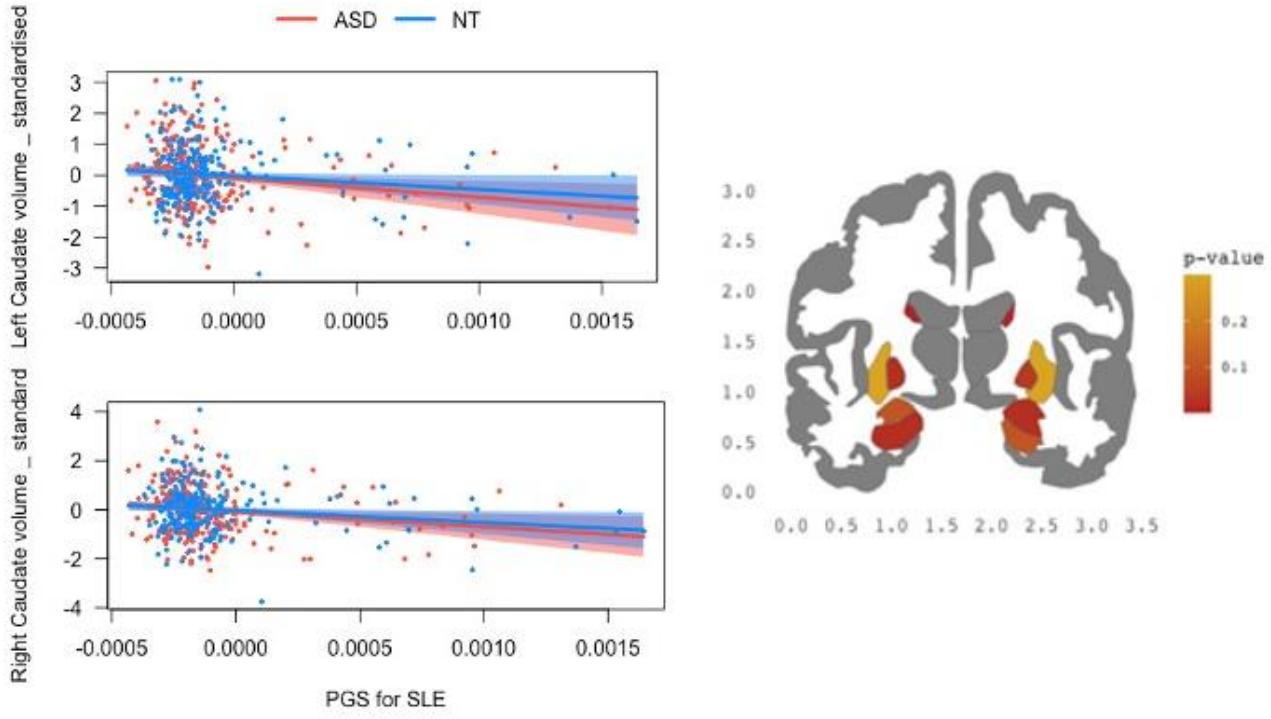

Figure 3 Preliminary results of the association between immune-based polygenic scores and subcortical volumes in the LEAP study. On the left, the correlation between PGS for systemic lupus erythematosus (SLE) (x-axis) and bilateral caudate volumes (y-axis) is plotted by diagnostic group. Blue dots represent neurotypicals, while red dots represent autistic individuals. On the right, representation of the subcortical structure reporting differences across autistic individuals divided in subgroups with high vs low autoimmune polygenic scores.

7.7. Conclusions

In this thesis, I investigated the relationship between immune genes and ASD. Taking into account the heterogenous nature of both ASD and immunity, my aims were 1) to identify specific

immunogenetic factors of relevance to ASD; and 2) to explore if these immunogenetic factors are linked to specific features of ASD.

This work highlights the relevance of immune genetic factors in ASD. Specifically, I demonstrated that ASD is linked to genetic factors predisposing towards excessive immune responses, from allergies to autoimmunity. Moreover, I provide evidence for an association between these immunogenetic factors and autistic-like rigidity in the general population. Future research is, however, needed to clarify this association in clinical settings.

My findings may have important implications for clinical practice and potentially help to identify clinical subgroups that may especially benefit from immune-based prevention and/or interventions. Moreover, this work emphasizes the importance of future work in exploring immune genes in a broader neurodevelopmental perspective. This could be achieved by studying the transdiagnostic influence of immune genes, and throughout the life span. Importantly, future research should explore the impact of immune genes on brain, so to identify potential neurobiological systems (e.g., subcortical circuits) that may underpin the association between immune genes and behaviour.

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