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IDENTIFICATION OF BIOBEHAVIORAL MARKERS OF NEURODEVELOPMENTAL DISORDERS IN TWINS

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Cover: Six years old twin sisters, both diagnosed with ASD. Photographer: Martin Schoeller.
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Identification of Biobehavioral Markers of Neurodevelopmental Disorders in Twins

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Autism Spectrum Disorder (ASD) and Attention-Deficit/Hyperactivity Disorder (ADHD) are complex neurodevelopmental disorders (NDD), heterogeneous in phenotypes and etiology. The exact mechanisms driving the phenotypes are still largely unknown. The overall aim of this thesis is to find new leads for ASD and ADHD etiology, focusing on environmental factors through the examination of discordant twins. For this purpose, two ASD enriched and well characterized twin cohorts were investigated; the Roots of Autism and ADHD Twin Study of Sweden (RATSS) and the Californian Autism Twin Study (CATS). In study I, we are discussing the background the aims and the rationale for the RATSS program.

In study II, the association between ASD and IQ is examined. There is a robust and negative phenotypic correlation between IQ and ASD, categorical as well as dimensional, underpinned by genetic and non-shared environmental (NSE) factors. The role of non-shared environment in ASD, focusing on early medical events, is explored in Study III. There is an association between the total load of early medical events and ASD, both categorically as well as dimensionally defined. The association is particularly driven by behavioral dysregulation in infants (feeding and sleeping problems, excessive crying and worriedness) as a function of NSE factors. In study IV, we test the hypothesis of a link between ASD and pre- and postnatal dysregulation of metals. ASD cases demonstrate higher lead levels during a period of 20 weeks before and 30 weeks after birth, lower manganese levels 17 weeks prenatally to 30 weeks postnatally, and a reduction in zinc 10 weeks prenatally to five weeks postnatally.

In study V we study executive functioning as a behavioral marker of ADHD. There is a link between ADHD on one hand, and foresighted planning and inhibitory control on the other hand, mediated by NSE factors.

In summary, the findings support ASD to be continuously distributed in the population with clinical phenotypes being the extremes of these continuums. They point to a cumulative multifactor threshold model, including both genetic and NSE components in the etiology of ASD. More specifically, the results support that systemic pre- and postnatal elemental dysregulation increase ASD risk, and an association between early medical events and ASD risk. The findings also indicate low IQ to be a behavioral marker for ASD, and poor executive functioning to be a behavioral marker for ADHD. Both these association are underpinned by NSE factors.

SCIENTIFIC PAPERS IN THE THESIS

I. Bölte, S, **Willfors, C,** Bergren, S, Norberg, J, Poltrago, L, Mevel, K, Coco, C, Fransson, P, Borg, J, Sitnikov, R, Toro, Tammimies, K, Anderlid, B-M, Nordgren, A Falk, A, Meyer, U, Kere, J, Landén, M, Dalman, K, Ronald, A, Anckarsäter, H and Lichtenstein, P. The Roots of Autism and ADHD Twin Study in Sweden (RATSS). *Twin Research and Human Genetics.* **17**(3),164-76 (2014).

II. **Willfors, C**, O'Hara, R, Taylor, M J, Kuja-Halkola, R, Cauvet, E, Tammimies, K, Lichtenstein, P, Bölte, S, Hallmayer, J. Nature and Nurture of the Autism-IQ Link. In manuscript.

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IV. Arora, M, Reichenberg, A, **Willfors, C**, Austin, C., Gennings, C, Berggren, S, Lichtenstein, P, Anckarsäter, H, Tammimies, K, and Bölte, S. Fetal and Postnatal Metal Dysregulation in Autism. In manuscript.

V. **Willfors, C**, Poltrago, L, Berggren, S, Coco, C, Anckarsäter, H, Lichtenstein, P, Ronald, A, and Bölte, S. On the Role of Non-shared Environment for Executive Functioning in ADHD: A Twin-difference Design Study. *International Journal of Developmental Disabilities*. **60**(3), 163-173 (2014).

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II. Zander, E, **Willfors, C,** Bergren, S, Choque-Olsson, N, Coco, C, Elmlund, A, Hedfors Moretti, Å, Holm, A, Jifält, I, Kosiedarzki, R, Linder, J, Nordin, V, Olafsdottir, K, Poltrago, L, and Bölte, S. The objectivity of the Autism Diagnostic Observation Schedule (ADOS) in naturalistic clinical settings. *European Child & Adolescent Psychiatry.* **25**(7), 769-780 (2015).

CONTENTS

LIST OF ABBREVIATIONS

ABA	Applied Behavioral Analysis
ABCL	Adult Behavior Check-List
ACE	Additive genetics, Common environment, unique Environment
ADHD	Attention Deficits-Hyperactivity Disorder
ADI-R	Autism Diagnostic Interview – Revised
ADOS	Autism Diagnostic Observation Schedule
AP	Attention Problem scale
ASD	Autism Spectrum Disorder
ASR	Adult Self-Report
BAP	Broader Autism Phenotype
CATSS	Child and Adolescents Twin Study in Sweden
CBCL	Child Behavior Check-List
CI	Confidence Interval
CNV	Copy Number Variation
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DDS	Department of Developmental Services
D-KEFS	Delis-Kaplan Executive Functioning System
DIVA	Diagnostic Interview for ADHD in Adults
DLM	Distributed Lag Model
DNA	Deoxyribonucleic Acid

DSM	Diagnostic and Statistical Manual of Mental Disorders
DTI	Diffusion Tensor Imaging
DZ	Dizygotic
GABA	Gamma-aminobutryc Acid
GxE	Gene-Environment interaction
GWAS	Genome-Wide Association Studies
GEE	Generalized Estimating Equations
ICC	Intra-Class Correlation Coefficient
ICD	International Classification of Diseases
ID	Intellectual Disability
iPS	Induced Pluripotent Stem
IQ	Intelligence Quotient
K-SADS	Schedule for Affective Disorders and Schizophrenia School Age Children
LA-ICP-MS	Laser Ablation Inductively Coupled Plasma Mass Spectrometry
MRI	Magnetic Resonance Imaging
MZ	Monozygotic
NDD	Neurodevelopmental Disorder
NIH	National Institute of Health
NIST	National Institute of Standards and Technology
NSE	Non-Shared Environment
NWR	New Wave Research
OR	Odds Ratio

PPVT-3	Peabody Picture Vocabulary Test 3rd Edition
RATSS	Roots of Autism and ADHD Twin Study in Sweden
RRB	Restricted and Repetitive Behaviors
SCID	Structural Clinical Interview for DSM-IV-TR
SE	Standard Error
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
SPECT	Single Photon Emission Tomography
SRM	Standard Reference Materials
SRS	Social Responsiveness Scale
SB-5	Stanford-Binet 5th Edition
STR	Swedish Twin Registry
TEDS	Twins Early Development Study
TD	Typically Developing
TTTS	Twin-to-Twin Transfusion Syndrome
WCST	Wisconsin Card Sorting Test
WAIS-IV	Wechsler Adult Intelligence Scale 4th Edition
WISC-IV	Wechsler Intelligence Scale for Children 4th Edition
YSR	Youth Self-Report

1 INTRODUCTION

1.1 NEURODEVELOPMENTAL DISORDERS

1.1.1 Symptomatology, Prevalence and Comorbidity

1.1.1.1 Neurodevelopmental Disorders

Neurodevelopmental disorders (NDDs) are defined by early and persistent developmental behavior difficulties leading to functional impairments in everyday life.[1,2] As indicated by the terminology, neurological as well developmental disturbances are assumed as their causes, although the exact mechanisms and pathways driving the phenotypes are still unknown. The literature to date confirms that NDD phenotypes are related to disturbances in brain structure and functioning, affecting the development of motor-, sensory-, language-, social- and cognitive skills.[3,4] In the current and 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), an umbrella concept of NDD is introduced for the first time,[5] emphasizing the significance of abnormal neurodevelopment in the etiology of Autism Spectrum Disorder (ASD) and Attention-Deficit/Hyperactivity Disorder (ADHD). The NDD category in DSM-5 includes, besides ASD and ADHD, intellectual disability (ID), communication-, specific learning- and motor disorders.[6]

1.1.1.2 Autism Spectrum Disorder

ASD is defined by impairments in the areas of verbal and non-verbal communication, social reciprocity and interaction, and by stereotyped and repetitive behaviors and interests. Autistic phenotypes were described already in the 1940s by Leo Kanner and Hans Asperger.[7,8] In Kanner's article, based on a detailed description of eleven children with autistic features, he makes the conclusion that the fundament of their impairments is "the children's *inability to relate themselves* in the ordinary way to people and situations from the beginning of life".[7, p 242] Further he notes "…these children have come into the world with annate inability to form the usual, biologically provided affective contact with people, just as other children come into the world with annate physical or intellectual handicaps".[7, p 250] Autism has over time been one of the most stable psychiatric diagnoses in terms of core symptoms, although the criteria for the disorder have become more and more inclusive. In contrary to the behavioral criteria, the view on the causes of autism has been shifting. Autism was already with the first descriptions of the phenotype suggested to be familial.[7,8] Later, for decades, it was viewed as environmentally caused, mainly through callous and emotionally distant mothers. This view was questioned by results from the first valid twin studies in the 1970s,

reporting substantially higher concordance in monozygotic (MZ) compared with dizygotic (DZ) twin pairs, and indicating a high heritability.[9] Numerous twin studies have thereafter confirmed a strong genetic component in the etiology of autism, for categorical diagnoses as well as autistic traits, reporting heritability estimates as high as 80-90%.[10] However, lately the high heritability in ASD has been questioned, and a more equal distribution between genes and environment is suggested,[11,12] with the environmental components comprising pre-, peri- and early postnatal factors.[13]

Autism is heterogeneous in phenotypes as well as functional deficits. Severity ranges from high-functioning expressions (e.g., many previously diagnosed with Asperger's syndrome), to low-functioning individuals with no or little language and typically, comorbid intellectual disabilities. Nevertheless, an ASD diagnosis always implies impairments in one or several areas of daily life.[1] The literature supports autistic phenotypes to be continuously distributed in the population, with clinical diagnoses representing extremes on this continuum.[14-16] In the DSM-5, the previous separate diagnoses within the spectrum (i.e., Autistic Disorder, Pervasive Developmental Disorder Not Otherwise Specified, Asperger's Syndrome, and Childhood Disintegrative Disorder), are merged into one ASD diagnose. Of note, the Rett's Syndrome which is characterized by a known molecular etiology, is defined as a separate diagnose. Severity levels are specified based on the degree of required support in two areas of symptoms; i) social communication and ii) restricted, repetitive behaviors. The accumulated genetic and environmental evidence in ASD is acknowledged, by adding a specifier of known genetic or environmental conditions to the diagnostic criteria, alongside specifiers for intellectual disability and language impairment.[5]

1.1.1.3 Attention-Deficit Hyperactivity Disorder

ADHD, also known as hyperkinetic disorder, is characterized by inattention and/or hyperactivity and impulsivity problems. One of the first descriptions of the phenotype was made in 1775 by a German physician, Melchior Adam Weikard.[17] Since then, the knowledge and evidence of the diagnosis and treatment has been growing. In DSM-5, three presentations of ADHD symptomatology are described: predominantly inattentive, predominantly hyperactive-impulsive, and the combination of the two areas, with additional unspecified presentations. The severity of impairments is ranked from mild to severe, with a specification if in partial remission.[5] Although not part of the diagnostic criteria, central areas of deficits are also executive dysfunction and impaired emotion regulation. The cognitive profiles within

ADHD vary widely. Most individuals have impairments in some behavioral domains and only a minority are impaired in all the above mentioned areas.

In addition to the DSM-5 subgroups (i.e., based on the summed number of criteria in each symptom domain), attempts have been made to identify phenotypical subgroups based on biological markers and related traits. Six alternative subgroups, based on temperament dimension and with different biological underpinnings, have been identified. These were calculated from community detection and based on graph theory combined with functional magnetic resonance imaging (fMRI) and physiological measures. It is suggested that these subgroups would better represent differences in etiology, than the current diagnostic categories.[18,19] As for ASD, the literature supports ADHD phenotypes to be continuously distributed in the normal population, with clinical diagnoses as the extremes.[20]

1.1.1.4 Prevalence

There are no good worldwide prevalence estimates available for NDDs in total, but data from the United States indicates a frequency of 12% in the general population.[21] In the past decade, an increasing prevalence has been reported for both ASD and ADHD.[22,23] Current estimates of ASD are 1–2%, and the figures are stable over the life span.[24,25] The prevalence of ADHD is around 5% in school-children,[26] and diminishes up in adult age. Hence, many adults express a number of ADHD symptoms on a subclinical level, but are not longer filling criteria for diagnosis.[27]

The origins of the increased prevalence of NDDs are debated. Improved knowledge and increased awareness, access to better services, and reduced stigmatization might be important factors.[28] One longitudinal Swedish twin study showed no real increase in ASD prevalence numbers over a 10-year period. They collected two types of ASD measures over the 10-year period; a parental telephone interview and clinical diagnostic data from national registers. A comparison between the measures showed an increase in registered clinical diagnoses, but stable figures of ASD as measured the telephone interview. The findings suggest the increasing prevalence numbers to be explained by changes in clinical procedures of diagnosing, rather than reflecting a real increase of ASD in the population.[29]

The increase in ADHD prevalence is at least partly due to alterations of the diagnostic criteria for ADHD with the 5th edition of DSM (i.e., change of the age for onset from seven to 12 years).[3] If there is also a true increase in prevalence, due to for example environmental factors such as exposure to environmental toxins or toxic metals, it is still to be confirmed.[30,31]

1.1.1.5 Sex Differences

There is a skewed sex distribution within ASD (all ages) and ADHD (in children) in which males are affected 2–4 times more often than females.[3,32]

Differences in ASD phenotypes between males and females have been reported, such as more prominent repetitive and stereotyped behaviors in males.[33] In contrast, diagnosed females often present with more severe variants of ASD, and comorbidity with intellectual disability (ID) is more frequent.[34] Consequently, the sex ratio of ASD has been reported to be almost equal in the low IQ range, while being highly skewed towards males in the upper range of intellectual abilities.[35] It has been suggested that the diagnostic criteria favors male phenotypes, resulting in only the most severely impaired females reaching cut-off for diagnoses.[36]

One of the most cited theories on gender and ASD is Simon Baron-Cohens' of an extreme male brain.[37] It is supported by studies showing individuals with ASD to present with increased systemizing (male feature), combined with decreased empathizing (female feature). The theory hypothesize a masculinization of the brain to occur during the fetal brain development by elevated levels of prenatal androgens and potential involvement of the X- and Y-chromosomes in the etiology.[37,38] However, the evidence for this extreme male brain theory of autism, is inconsistent.[32,39]

While the skewed sex ratio is stable over the life span in ASD, the distribution becomes closer to equal between males and females in adult age in ADHD.[3] Like ASD, there are phenotypical differences in ADHD with girls displaying greater intellectual impairments combined with less symptoms of hyperactivity and other externalizing problems.[40] A functional brain imaging study showed sex-related differences in neural activity linked to ADHD. Brain connectivity was measured during a working memory task with findings of altered patterns of neural activity in males with ADHD, but not in females with ADHD.[41]

Genetic studies favor a polygenetic threshold model for both disorders that varies with sex. The model hypothesize the female sex to be a protective factor for the disorders, and girls to have a higher threshold for the genetic liability to manifest ASD and ADHD.[42-44] This model is supported by females presenting with more severe phenotypes, as well as findings of affected females having a higher degree of affected relatives in comparison to affected males (assuming a familiar transmission).[32,45,46]

1.1.1.6 Comorbidity

Comorbidity is high in NDDs.[3,4] The overlap between ASD and ADHD is in the range of 28-44%. In ASD, comorbidity with intellectual disability is common (~45%), as well as tic disorders (14-38%), anxiety (42-56%), and depression (12-70%).[4] In addition, individuals with ASD often suffer from somatic conditions such as epilepsy (8-30%), gastrointestinal problems (9-70%), and sleeping disorders (50-80%). A recent epidemiological study showed a markedly increased mortality in ASD in comparison to the general population with and odds ratio (OR) of 2.56, and 95% confidence interval (CI) between 2.38 and 2.76. The authors concluded the increase to be caused by a range of co-occurring somatic conditions.[47]

A meta-analysis of ADHD yielded a 10.7-fold risk for conduct disorder, 5.5-fold risk for depression and 3-fold risk for anxiety, in comparison to the general population.[48] ADHD symptoms are further associated with an increased risk of stressful life events (OR=1.8, 95% CI 1.3-2.4).[49] An increased mortality is assumed, owing to high levels of impulsivity, accidents, smoking and drug use, although more epidemiological studies confirming these results are needed.[50]

The high overlap between ASD and ADHD have been modelled in different ways: i) as three independent disorders (i.e. ADHD, ASD, and ASD-ADHD combined); ii) as two disorders with alternating manifestations of the same underlying factors; iii) as a few correlated risk factors for the disorders.[51] Support for all three models can be found in the literature to date, leaving it to future research to determine which model best describes the true etiological relationship.[52]

1.1.1.7 Intelligence Quotient (IQ)

Even though co-occurrence with ID is common, ASD appears on the full range of intelligence.[53] High IQ is one of the strongest predictors of functional outcome in ASD in terms of employment, higher education, social relationships and independent living.[54] ASD, in combination with low IQ (<70), is almost exclusively accompanied by poorer outcomes, such as relaying on family and social services for every-day living, lack of permanent employment, few friends, and poor communication-, reading- and spelling abilities.[55]

Despite the overlap between ID and ASD, few studies have investigated the etiology of the overlap, and mixed findings are reported. A high genetic correlation of 0.95 (95% CI .60-1.00) for the phenotypical overlap was found in a small Japanese twin sample (N=45 pairs). A considerable lower genetic correlation have been reported from two studies based on the

Twin Early Development Study (TEDS) cohort ranging from -0.04 to -0.27.[56,57] The latter studies suggest autistic traits in the extremes and the normal range are substantially genetically independent of IQ.[56] Common genetics have also been indicated for ASD and IQ in molecular genetic studies, with many genes predisposing for both conditions.[58] Also environmental factors such as perinatal risk factors (i.e. gestational age, birth weight and 5-min Apgar score) have been associated with both ID and ASD.[59] Common epigenetic causes are suggested. There are findings of factors such as prenatal exposure to valproate (primarily used to treat epilepsy, bipolar disorders, and migraine) causing epigenetic dysregulation that potentially are linked to both ID and ASD risk.[60]

A weak link has been shown between ADHD and IQ, in the magnitude of two to eight IQ points lower in individuals with ADHD than controls.[61] A meta-analysis including 18 studies on the topic showed a significant, but not clinically relevant, effect of slightly lower IQ in ADHD cases compared to controls. The results suggested only a subset of the ADHD group, also presenting with comorbid disorders, to have lower IQ than the general population.[62]

1.1.2 Diagnostic Assessments

Both ASD and ADHD are behaviorally defined disorders. For diagnostic evaluations, multidisciplinary assessments are recommended, including anamnestic information and assessments of symptom severity, neurocognitive and intellectual abilities, and every-day functioning.

1.1.2.1 Diagnosis of ASD

Symptoms of ASD are measurable from the age of 12 months.[63] Still, most cases are diagnosed considerably later,[24] as early behavioral measures are limited in sensitivity and specificity.[64] As early identification ensures access to intervention with increased chances for improved outcomes, establishing reliable early detection of ASD remains a clinical research priority.[65] The first choice diagnostic instruments for ASD assessments are the Autism Diagnostic Interview – Revised (ADI-R),[66] in combination with the Autism Diagnostic Observation Scale (ADOS).[67,68] Both instruments have shown good psychometric properties, in particular when combined.[69-71] The ADI-R is a semi-structured anamnestic interview with the caregiver as respondent, focusing on early and present symptoms in the areas of social reciprocity, communication, repetitive behaviors and interests, and early developmental delays. The ADOS is a semi-structured observation, that samples and quantifies an

individual's behavior in a naturalistic setting. Both ADI-R and ADOS provide diagnostic classifications as well as dimensional scores.

1.1.2.2 Diagnosis of ADHD

Early symptoms are typically unclear and interpreted as insufficient maturity in ADHD, and early screening is less emphasized than in ASD. Still, a six year long study indicated that screening with parent-rated questionnaires promote earlier detection of impairments.[72] ADHD assessments include information from anamnestic and diagnostic interviews, such as Schedule for Affective Disorders and Schizophrenia School Age Children (K-SADS)[73] and Diagnostic Interview for ADHD in adults (DIVA).[74] These assessments might be combined with caregiver-, teacher- or self-rated symptom scales, neuropsychological testing, and assessment of adaptive functioning.

Since both ASD and ADHD phenotypes are continuously distributed, categorical (for assessment of diagnosis) as well as continuous (for assessments of traits) measures are needed to capture the entire phenotypes. Dimensional traits are typically measured by continuous caregiver- or self-rated scales such as the Social Responsiveness Scale (SRS) for autistic traits,[75] and the Conners for ADHD traits.[76]

Comorbidity is common in both disorders, hence assessments of other psychiatric and somatic symptoms are essential in the diagnostic evaluation. Assessment of comorbidity might include interviews and/or rating scales for psychiatric disorders, medical examinations, medical anamnestic information, and when applicable, magnetic resonance imaging (MRI) of the brain, and genetic testing, for exclusion of other neurological and genetic disorders.

1.1.3 Treatment

There are various behavioral interventions for ASD symptoms, including applied behavioral analysis (ABA) based treatments, structured teaching, skilled- and behavior-based interventions, interventions addressing anxiety and aggression, and early parent-mediated interventions. The effects of these interventions on core ASD symptomatology are low to moderate.[4,77,78] There are no pharmacological treatment available for the core ASD symptomatology, although related symptoms such as anxiety and sleeping problems can be treated with a positive effect on the individual's general functioning, and indirectly on ASD symptoms.[79]

In contrast, pharmacological interventions are the most efficient treatment alternatives in ADHD. Meta-analysis support both stimulants and non-stimulants to effectively reduce ADHD symptoms in children as well as in adults.[80] There are two types of stimulants used, methylphenidate and amphetamine products, both blocking dopamine transporters (see below for a more details on the role of dopamine in ADHD). Amphetamine products also promote the release and reverse transport of dopamine. For non-stimulants, the most effective medication is a selective noradrenaline reuptake inhibitor. A review of 351 studies on long-term treatment effects in ADHD reported significantly improved outcomes with treatment, in comparison to untreated individuals with ADHD, although not reaching the level of non-affected individuals.[81] However, one meta-analysis of methylphenidate treatment in ADHD, also report uncertainty regarding the magnitude of treatment effects (due to low quality of underpinning evidence), and associations between methylphenidate and adverse events such as sleep problems and decreased appetite.[82] Non-pharmacological treatments for ADHD include dietary and behavioral treatments.[83,84] The results from clinical trials of non-pharmacological treatments in ADHD show substantially smaller effect-sizes than for pharmacological interventions, on the other hand, they induce minimal risk of reverse events.[80]

1.1.4 Neurobiology

Neuroimaging, in combination with molecular genetics, points at atypical neural connectivity in ASD. The literature reports on various and heterogeneous brain functional connectivity alterations, among which the reduced long range versus increased short range (frontal) connectivity is the most popular.[85,86] Some findings suggest reduced functional connectivity to be associated with multiple cognitive impairments such as language, working memory, problem solving, social cognition, and social perception.[87] Diffusion tensor imaging (DTI) has demonstrated alterations mostly in associative white matter fiber bundles connecting different brain regions, which subtend high-level cognitive functions (e.g., social cognition, language) in ASD.[87]

In addition, there are anatomical findings that are frequently reported in ASD. The most popular is an early brain overgrowth, observed between six and 24 months of age. A meta-analysis of head circumference and brain size in ASD indeed showed that both head circumference and total brain volume were significantly larger in ASD cases compared to controls. The effect sizes were higher in low-functioning groups in comparison to high-functioning, and largest in early childhood.[88] However, a study on the Californian Autism Twin Study (CATS) (see below for a more detailed description of the CATS sample), showed

no association between ASD and head circumference.[89] Further structural alterations have been reported, including changes in white matter volumes and abnormal growth in the frontal lobe, temporal lobes, and limbic structures, such as the amygdala.[90]

Findings from neurochemical studies with animal models and pharmacological studies are inconclusive in ASD. Genetic differences in serotonin transportation seem to have the most empirical support for a role in ASD etiology.[90] A gamma-aminobutyric acid (GABA)-glutamate (i.e. an inhibitory/excitatory) imbalance is also suggested to be associated with ASD, although studies investigating this hypothesis have reported contradictory results.[91]

Several brain regions and functional pathways have been implicated in ADHD. Meta-analysis on structural MRI studies in ADHD have reported on smaller total brain volume (3-5%), due to reduction of grey matter. In particular smaller volumes in the right globus pallidus, right putamen, caudate nucleus and cerebellum are linked to ADHD. Interestingly, data supports pharmacological treatments to reduce the structural brain alterations in ADHD patients.[92] Functional MRI studies have examined brain activity during tasks calling attention, inhibitory control, and working memory in ADHD subjects. These studies have reported on an under-activation in the frontoparietal network, involved in goal-directed executive processes, in the frontostratial network, mediating reorientation of attention, and the ventralstratial network, which is related to reward-processing. In addition, a hyper-activation in somatomotor and visual systems has been reported.[3]

Some of the most robust neurobiological findings in ADHD relate to alterations in dopamine receptors. Dopamine has a central role in the regulation of motor activity, motivation, inhibitory control, timing and attention functions. The striatal dopamine transporters are the target for the two most commonly used drugs in ADHD: methylphenidate and amphetamine.[93] A meta-analysis including studies using positron emission tomography (PET) and single photon emission tomography (SPECT) to study dopamine in ADHD, reported the striatal dopamine density to be 14% higher in ADHD cases compared to controls. In addition, the dopamine density was higher in ADHD patients with previous medication in comparison to drug-naïve patients.[94] Genetic studies support this hypothesis. Although together with a large number of other candidate genes, dopamine transporter and receptor genes have been reported to be among the most important genes in ADHD etiology.[95]

1.1.5 Neuropsychology

There are several psychological and neurocognitive theories modelling the impairments in ASD and ADHD. Below is a non-exhaustive list of some of the more popular ones.

1.1.5.1 Social Cognition/Theory of Mind

The concept that autistic individuals lack a "theory of mind" was first introduced in 1985.[96] The theory of mind refers to an ability of meta-representation and to attribute beliefs in others, predicting their thoughts and actions. It implies that individuals are unable to differentiate between physical states (objects) and mental states (persons), and an inability to ascribe mental states in self as well as in others, resulting in multiple difficulties related to social interaction.[97] A crucial step in development of these abilities is the understanding of false believes. The Sally and Anne task is a widely used test for false belifes.[98] In the test the child is shown two dolls, Sally and Anne, whom have a basket, a box and a toy. Sally puts the toy in the basket and leaves the room. When Sally is out, Anne moves the toy to the box. The child is then asked where Sally will look for the toy when she returns. By answering the basket, the child shows an understanding of the mental representation of Sally, which is different from the child's own (who knows that the toy is moved to the box), and make a prediction of the behavior of Sally based on this understanding. Children with ASD have larger difficulties to pass this test in comparison to e.g. typically developed children or children with Down's syndrome.[96] In support of this, fMRI studies show altered activity in prefrontal cortex and in the right tempoparietal junction during theory of mind tasks in ASD cases, in comparisons to controls,[99] and eye-tracking studies have shown altered tracking in ASD cases during false belief tasks, in comparison to controls.[100]

1.1.5.2 Weak Central Coherence

Although the Theory of Mind hypothesis well describes the social challenges in ASD, it does not clearly address communicational problems or the repetitive and stereotyped behaviors. Weak central coherence refers to a cognitive style with enhanced detail-focus and failure to extract global form and meaning. It was introduced as an attempt to also capture the non-social impairments in ASD.[101] A review of over 50 studies on the topic give robust evidence for local bias in ASD, but mixed results regarding weak global processing. The authors suggest weak central coherence to be a biased cognitive style, rather than a deficit, and to explain the non-social features in ASD.[102]

1.1.5.3 Dual and Triple Pathways-models of ADHD

In addition to executive dysfunction, non-executive deficits are present in ADHD such as altered reward and aversion signaling leading to impulsivity. The delay aversion construct is defined as an emotional and motivational response style described as a "negative emotional reaction to the imposition of delay".[103, p 103] Delay aversion has been tested in a range of studies, showing individuals with ADHD to have a tendency of choosing smaller and sooner, over larger and later rewards.[104] ADHD phenotypes are heterogeneous and it is common to demonstrate deficits in one of the two areas of symptoms exclusively, suggesting a dual pathway. The dual pathway proposes executive versus non-executive phenotypes to be caused by different etiological pathways: the first being a dorsal fronto-striatal dysregulation mediated by inhibitory executive dysfunction. The second being underpinned by ventral fronto-striatal circuits and linked to the delayed reward/aversion processing.[105] In addition, Sonuga-Barke and colleagues have proposed a triple pathway model; i) inhibition, ii) timing and iii) delay. They have showed subgroups within ADHD to be impaired in only one of these three areas, hence suggesting separate etiologies are suggested for the three domains. The results point at genetic underpinnings for inhibition and timing, and a more environmentally driven etiology for impaired delay functions.[106]

1.1.5.4 Executive Functions in ASD and ADHD

There are overlapping deficits in ASD and ADHD, and most prominent is executive dysfunction. Executive functioning directs goal-directed cognitions including planning, working memory, problem solving, mental flexibility, and decision making. These abilities enable independent and purposeful every day functioning, affecting all areas of life including social relationships, academic- and work achievements.[107]

Executive dysfunction is frequently reported in individuals with ASD. This holds especially true for cognitive inflexibility, reported as a life-long complication of ASD.[108,109] Areas of working memory, initiation and organizational functions typically become more problematic when individuals with ASD become older and due to the rising demands of independence.[109]

Behavioral inhibition and working memory are proposed as core underlying difficulties in ADHD,[110] with neurobiological correlates including alterations in the frontal lobes, caudate nucleus, and cerebellar vermis.[111]

1.2 ETIOLOGY

1.2.1 Behavior Genetics

Behavior genetics is the study of the relative distribution of genetic and environmental contributions to behavioral phenotypes. It is based on the underlying notion that if a trait or a disorder is heritable, individuals that are more genetically similar should also be more similar regarding that trait or disorder status. By studying individuals with different degrees of relatedness and/or environmental exposure (e.g. twin designs, adoption designs), estimations can be made of the relative effect of genes (heritability) and environment on behavioral traits.

1.2.1.1 Heritability, Shared and Non-shared Variation

Twin modelling is taking advantage of the fact that monozygotic (MZ) twins share 100% of their genetic variation on deoxyribonucleic acid (DNA) sequence level, while dizygotic (DZ) twins share on average 50% of their genetic variation. By comparing MZ and DZ pairs, the relative distribution of Additive genetics, Common environment, and unique Environment can be modelled (ACE).[112]

Two categories of environmental factors are explored in behavior genetics: environmental factors shared by family members, making them similar (e.g. prenatal factors, sex, age, socioeconomic status) and environmental factors not shared between family members (NSE), making them dissimilar (e.g. medical events, dietary, traumatic life events). In addition to NSE, there are stochastic *de novo* mutations potentially adding to intra-pair differences. However, these mutations are rare and usually not taken into account in twin modelling.

1.2.1.2 The Limitations of Twin Designs

Twin designs are powerful in etiological research, however, there are some limitations that need to be addressed.

Two assumptions are made in twin-modelling: i) the equal environment assumption, assuming no difference in environmental effects between MZ and DZ pairs; and ii) the non-appearance of assortative mating, assuming that parents do not choose partners being phenotypically more similar to themselves. If the assumption of equal environment is in any way violated, the heritability estimates would be affected, most likely leading to an overestimation of the heritability. However, this assumption has been tested and proven to be reasonable for most traits.[e.g. 113] Possible exceptions are prenatal factors. MZ twins are more often affected by prenatal factors than DZ twins, particularly if the twins are sharing the same

chorion. These pairs will in most cases experience a higher intra-pair prenatal competition, presumably resulting in greater environmental effects than DZ pairs (e.g., for birth weight).[114]

If the assumption of no assortative mating is violated, the DZ pairs would share more than 50% of their genes due to their parents being more genetically similar, while the genetic similarity for MZ twin pairs would be unaltered. A few studies have investigated the degree of assortative mating in ASD with mixed findings.[e.g. 15] Ronald and Hoekstra[10] argue that since the intra-pair correlations are higher in studies using spousal report,[e.g. 115] compared with studies using self-report,[e.g. 57] the similarity between partners are more likely to be due to shared values and common beliefs, rather than shared genetic effects. However, more studies with broader family designs are needed to further investigate the occurrence of assortative mating in ASD/ADHD.

In within twin-pair comparisons, there might be a risk of a sibling interaction effect.[116] This can either be cooperative or competitive, leading either to increased or decreased similarity between the twins. The effect can be reflective of a true relationship, due to tendencies in the twins to either differentiate or to influence each other. It can also be due to parental rating patterns, and parents to either emphasize the differences or the similarities between their children. If the effect is cooperative, it will increase the variance in both MZ and DZ pairs, but more in MZ, and if the effect is competitive, it will decrease the variance in both groups, but again with a larger effect in the MZ pairs.[116] This has particularly been demonstrated in twin difference designs focusing on discordancy using parent reports.[e.g. 39]

An often discussed limitation of twin designs is the generalizability of results from twin samples to non-twin populations. For instance, it has been suggested that twinning itself could be a risk factor for ASD. However, when tested in two UK population-based samples, the opposite was found for dimensional as well as categorical ASD.[117] The same has been shown for related abilities such as IQ and academic performance, reporting no differences between twins and singletons, when adjusting for birth weight.[118]

1.2.2 Nature vs Nurture

An increasing number of twin studies in the past decades have contributed substantially to the understanding of the etiology of ASD and ADHD, demonstrating both genetic and environmental contributions.[10,20]

Heritability estimates for ASD range from 38-55%,[11,12] and up to 95%.[14] Although one twin study claims shared environment to play a major role in ASD etiology,[12] most literature

supports NSE to be the predominant environmental factor.[10,11] These results have been replicated in the normative range as well as in extreme phenotypes and for different age groups.[10,119] The literature support two or three symptom domains to constitute ASD, with partly different etiologies.[120] A population-based Swedish twin study suggest a common pathway for the triad of ASD symptom domains, but also genetic influences that are separate for each domain.[121]

The heritability in ADHD is estimated somewhere between 67% and 88%, in both children and adults.[122] However, one longitudinal twin study including >600 twin pairs reported on an increasing heritability with age, from 33% (age three years) to 67% (age 13 years).[123] A recent population based study including >59 000 Swedish twins estimated heritability to 88% in the total population and slightly less, 72%, in adults only. While previous twin studies have reported higher heritability estimates in females,[46,124] no sex related difference for the etiological components were found in the Swedish cohort. The environmental effect is due to NSE factors, and no or limited effects of shared environment are reported.[125] The two core domains of symptoms, inattention and impulsivity/hyperactivity, are both strongly influenced by genetic factors (71% and 73%, respectively). The type of genetic effect differ though, and dominant genetic effect is more prominent for inattentive symptoms, and additive genetic effect for impulsivity/hyperactivity.[126]

There is a genetic overlap between ASD and ADHD, and 50-72% of the phenotypic variance is estimated to be due to additive genetics shared between the disorders.[127] A Swedish twin study of >17 000 twins investigated the overlap between different symptoms domains in ASD/ADHD. They found the largest overlaps, phenotypic as well as genetic, to be between repetitive and restricted symptoms and hyperactive/impulsive as well as inattentive symptoms, with genetic correlations of 0.61-0.64.[128] However, exploration of the shared etiology of the disorders has been limited by a restriction in the DSM-IV, not allowing concurrent ADHD and ASD diagnoses. This is now changed in the present DSM-5, and will hopefully lead to more research and a better understanding of shared causal mechanisms as well as clues for enhanced treatment options for patients with both disorders.

1.2.2.1 Genetic Factors

Although considerable progress in genetic research in ASD during the past decade, and a rapid methodological advancement in the field, the genetic causes of ASD are still largely unknown.[129] A minority of individuals with ASD (~15%) have an identifiable single-gene disorder, such as fragile X, tuberous sclerosis or neurofibromatosis, which may account for

the phenotype. In addition, recent advances in genome-wide methods including detection of copy number variations (CNVs) and single nucleotide polymorphisms (SNPs) from sequencing studies, have revealed multiple genes and variants linked to ASD risk.[130] The majority of findings have emerged from *de novo* CNVs and single nucleotide variations (SNVs) affecting genes involved in synaptic functioning, and chromatin modification. Estimates suggest the existence of at least several hundred ASD risk genes.[131]

Genome-wide association studies (GWAS) report around 40% of the heritability in ADHD is due to common genetic variants, and the remaining are due to CNVs and rare genomic insertion and deletions (<1%).[3] Identified ADHD relevant genes are involved in dopaminergic, noradrenergic, serotonergic, cholinergic, and the central nervous system (CNS) development pathways.[95]

The literature shows various genetic effects to influence both ASD and ADHD phenotypes. Some of the risk alleles seem to have a pleiotropic effect, meaning they can result in either ASD or ADHD, while others are disorder specific.[52]

1.2.2.2 Environmental Factors

In addition to a strong genetic component in the etiology of ASD and ADHD, twin studies consistently point at a NSE factor in their etiology. In line with an early developmental pathogenesis, the literature suggests pre- and perinatal factors to be the dominant environmental risk factors for NDDs.

Multiple single environmental factors have been implicated in ASD, including parental age, birth injury and trauma, gestational diabetes, neonatal anemia, maternal valproate intake, and exposure to toxic chemicals, especially during the third trimester.[13,132,133] A British study including >13 600 twins found only a weak correlations between total neonatal problem load and autism traits.[134] Maternal intake of periconceptional folic acid supplements is reported to have a protective effect.[60] Exposure to metal toxicants, is potentially increasing ASD risk.[31] For example, one study investigating hair metal concentrations of ASD cases, showed reduced zinc and magnesium levels and elevated levels of toxicants, such as lead, associated with ASD.[135] The lead hypothesis is supported by findings of enhanced levels in blood.[136]

Animal studies suggest the gut to influence various behaviour linked to ASD phenotypes[137] and gastrointestinal problems are common in ASD patients.[138] Although most of the mechanisms by which the microbiome can affect the brain and human behaviour are still to

be established, the possibility of microbe based treatments for ASD has attracted a lot of attention.[138]

In ADHD, a strong genetic effect is suggested for the onset of the disorder, and environmental factors to affect the degree of the behavioral compensatory strategies defining the persistence. Mainly pre- and perinatal factors have been associated with ADHD, such as low birth weight, prematurity, in-utero exposure to maternal stress, maternal smoking, and maternal alcohol and prescribed drug intake. However, evidence of causal relationship has only been shown for low birth weight, while the others might be due to unmeasured confounding factors.[3,139] Associations between environmental toxins, such as early childhood exposure to lead and pesticides, have been linked to ADHD risk. Dietary and psychosocial factors have been suggested to increase ADHD risk, however the findings are inconclusive.[139]

There are overlapping risk factors for both ASD and ADHD such as low birth weight, prematurity and maternal exposure to environmental toxins.[3,4,46,140] Exposure to air pollution has been suggested to increase NDD risk, and to add to the increasing prevalence.[141] Yet, a recent population-based Swedish twin study including >3000 twins found no associations between pre- and postnatal exposure to air pollution and neither ADHD nor ASD risk.[30]

1.2.2.3 Epigenetic Mechanisms

The literature show environmental factors in ASD and ADHD to mainly act via gene-environment interaction (GxE), such as epigenetic dysregulation.[142,143] Epigenetic mechanisms are modifications of gene expression, controlled by something other than the DNA sequence, that are potentially reversible. These factors are influenced by the maturational stage, tissue type, and environment. There is evidence that dysregulation of epigenetic markers or mechanisms, such as DNA methylation, play a significant role in ASD and ADHD etiology, integrating genetic and environmental factors to dysregulate neurodevelopmental processes.[60,144,145] One factor that might cause epigenetic dysregulation is prenatal programming, caused by a stressful in-utero environment. The programming is dependent of the type and timing of exposure, the sex of the fetus, and is depending on cognitive inputs from the environment.[146] Literature, primarily based on rodent studies, have proposed a three-hit concept of vulnerability and resilience to maternal stress. The first hit is the effect of genetic disposition, the second hit is the effect of the prenatal environment, and the third hit is an altered genetic expression leading to phenotypes with differing vulnerability to later exposure.[147] A population-based Swedish study including almost three million individuals, examined the associations between maternal stress and ASD/ADHD diagnoses.

They found maternal bereavement during the third trimester to increase the risk of both disorders, with an adjusted hazard ratios of 1.58 for ASD and 1.31 for ADHD.[148]

There are methodological challenges in the field. Nevertheless a rapid development of techniques using induced pluripotent stem (iPS) cells and facilitating sophisticated *in vivo* modelling of epigenetic mechanisms, might lead to novel discoveries in the future.[149]

1.2.2.4 A Cumulative Multifactor Threshold Model

The literature favors a multifactorial causal model for both ASD and ADHD, with a variety of possible etiological pathways leading to the phenotypes. Evidence points at an interplay between genetic and environmental factors, effecting development of the CNS and immune system.[150,151] In ASD, the timing of events is considered decisive with certain critical time windows associated with increased or decreased susceptibility. The cumulative degree of risk factors has been correlated to the severity of traits, suggesting future research to focus on the combined risk factor profiles, rather than trying to identify specific causal agents.[152]

1.2.3 Twin Difference Designs

A powerful design for exploration of NSE is the assessment of twins discordant for the phenotype of interest, also called twin difference or co-twin control design.[153] Particularly powerful is the investigation of MZ twin pairs discordant for disorders or traits that are assumed to have a high heritability. Since MZ pairs are identical on a DNA sequence level, the design implicitly controls for genetic factors in addition to all other factors being shared within twin pairs. Therefore, differences within the pairs must be due to NSE (including epigenetic mechanisms), rare *de novo* genetic mutations, or measurement errors. In highly familial disorders, such as ASD and ADHD, MZ discordant pairs are rare, making recruitment of participants challenging. To date only a moderate number of studies have particularly addressed MZ pairs discordant for these disorders, and they are in general of small sample size and limited generalizability.

1.2.3.1 Discordant MZ Twin Pair Design Studies in ASD

A handful of studies have examined neurological differences in ASD discordant pairs. Among those, Kate and colleagues published four studies on structural brain imaging in partly overlapping samples including from one up to 15 MZ discordant pairs, and various numbers of controls.[154-157] The findings show reduced caudate, amygdala, hippocampus and cerebellar volumes, and differences in cortical folding in the partial lobes in ASD twins.

Two studies of MZ pairs discordant for ASD have examined gene expression.[158,159] The findings show the most differentially expressed and methylated genes in discordant pairs are genes related to development, function and structure of the CNS. Many of the affected genes also map closely to chromosomal regions containing previously reported autism candidate genes. Quantitative discordance (i.e., intra-pair differences on continuous measures for ASD traits) has been assessed in the British Twins Early Development Study (TEDS) cohort.[144] In this study, the authors listed the top 50 differentially-methylated probes associated with ASD, indicating a large heterogeneity of epigenetic factors potentially involved in ASD pathogenesis. One case study of an ASD discordant pair (one twin with ASD, and one twin with developmental delay and hyperactivity) showed a genetic discordance, reporting a 2p25.3 deletion in the non-ASD twin. A mitotic non-allelic recombination is suggested to have occured during blastomeric divisions of the zygote, resulting in a chromosome imbalance causing the phenotypical discordance.[160]

1.2.3.2 Discordant MZ Twin Pair Designs in ADHD

Also for ADHD, there is a limited number of studies based on discordant MZ twin pairs. The studies have proved early environmental factors such as birth weight, delayed physical growth, motor development and being second born in the pair, to be associated with ADHD risk.[140,161-163] Two discordant twin pair studies have explored brain alterations in ADHD, the first linking structural differences (i.e., reduced caudate nucleus and prefrontal lobes volumes) to ADHD.[164] The second explored brain activation while performing executive functioning tasks, and showed both decreased activity (in dorsolateral prefrontal, parietal and temporal brain regions) and increased activity (in premotor cortex and regions associated with visual selective attention processing) to be associated with ADHD.[165]

One study investigated the effect of *de novo* and inherited CNVs on ADHD risk. They found one pre- and one post-twinning CNV to be associated with ADHD, and an association between overall larger CNVs and increased ADHD risk.[166]

1.3 SUMMARY

ASD and ADHD are complex neurodevelopmental disorders with heterogeneous phenotypes as well as etiology. No reliable biomarkers are available for neither of the disorders, hence both ASD and ADHD are exclusively behaviorally defined. Most patients are diagnosed much later than the appearance of first symptoms, decreasing the possibility of successful behavioral treatment interventions, particularly in ASD. There are effective pharmacological interventions available for ADHD symptoms, but no biological based treatments for the core

symptoms of ASD. Comorbidities are common, both with other NDDs and with psychiatric as well as somatic conditions.

Strong genetic influences have been demonstrated for both disorders, with environmental factors potentially acting as triggers (ASD), or directing the course of the disorder over the life span (ADHD). Both phenotypes are continuously distributed in the population, with clinical phenotypes being the extremes. Twin studies have showed partly distinguishable genetic causes for different symptom domains, but also common pathways for different areas of symptoms within the disorders. The overlap between ASD and ADHD is substantial, phenotypically as well as etiologically, with partly shared genetic as well as environmental risk factors.

The literature favors multifactor causal models, inclusive of different etiological pathways leading to the phenotypes. Evidence points to an interplay between genetic and environmental factors affecting development of the CNS and the immune system.[150,151] The timing of events is considered decisive with certain critical time windows associated with increased or decreased susceptibility.[3,150] It is suggested that the cumulative degree of risk factors is correlated to the severity of traits.[152]

In ASD, the literature show a neuronal-cortical organization that impacts the developmental trajectories of social cognition (theory of mind),[167] executive functioning,[168] and top-down vs bottom-up processing (attention to detail/central coherence).[169] In ADHD, the more consistent findings from brain imaging studies point at neuronal alterations affecting the frontal-subcortical-cerebellar pathways that control attention, inhibition, salience to rewards and motor control.[111] Neurochemical findings in ADHD involve hypo functioning of catecholaminergic circuits (involved in dopamine release), particularly those projecting to the prefrontal cortex.[170]

The study of discordant twin pairs (dimensionally and categorically) for ASD and ADHD gives high control of genetic and shared environmental factors and is therefore powerful in identifying NSE-driven behavioral and biological markers. The results from these designs in determining the risk or causes of ASD and ADHD are promising, however studies to date are limited in sample size, scope and generalizability.

2 AIMS AND RATIONALE

The overall aim of this thesis is to identify new leads for ASD and ADHD etiology by the examination of discordant twin pairs. More specifically, the studies focus on identifying NSE-driven behavioral markers and environmental risk factors, through twin-difference designs.

The objective of **study I** is to give an introduction and overview of the Roots of Autism and ADHD Twin Study in Sweden (RATSS), which is the framework for the other studies. In this protocol paper we describe the background, motivation, rationale and future perspectives of the RATSS.

In **study II**, the objective is to investigate the phenotypical association between IQ and autism, both categorically and dimensionally, and the effect of genes and environment on the associations. For this purpose, a combined cohort of Swedish and Californian twins is examined, stratified on zygosity.

In **study III**, the aim is to explore the associations between early medical history events and autism (categorical as well as dimensional), and to test the hypothesis of a cumulative effect of NSE factors on ASD risk. In this study, an explorative approach is taken examining a smaller sample of qualitatively discordant pairs for ASD. Next, the hypotheses generated from the first step of analysis are tested in a larger sample of quantitatively discordant ASD pairs, stratified on zygosity.

In **study IV**, the aim is to investigate the association between a prenatal and early life excess or deficiency of metals, autistic traits and ASD. For this, a novel method is applied using tooth-matrix biomarkers to establish fetal and postnatal profiles in deciduous teeth. ASD qualitatively discordant and control pairs are included.

In **study V**, the aims are to examine executive functioning as a behavioral marker in dimensionally-defined ADHD, and the effects of NSE. For this purpose, a sample of MZ twin pairs discordant for ADHD traits is examined.

3 METHODS

3.1 DESIGN

All studies included in this thesis are based on discordant twin pair designs (see above for a more detailed description of the rationale) and within-pair comparisons. The pairs are either qualitatively or quantitatively discordant. Qualitative discordancy is defined as a diagnosis of ASD or ADHD in the proband (i.e. the twin serving as starting point), and either no diagnosis or a different psychiatric diagnosis in the co-twin. Quantitative discordancy is defined by at least one-point intra-pair difference on dimensional ASD (i.e. SRS score or ADOS Comparison score) or ADHD scales (i.e. Child or Adult Behavior Checklist List; CBCL/ABCL).

As a second step in study II and study III, the samples are stratified by zygosity. By comparing the within-pair regression coefficients between MZ and DZ pairs, an indication of the genetic and NSE contributions on the associations are given.[171] In comparison to the ACE-modelling, the comparison of the magnitude of the associations in MZ and DZ pairs is crude. It gives an indication of genetic and NSE influences, but no estimation of the impact of the factors, nor any estimation of the shared environmental effects.[172] See figure 1.

In study IV, MZ and DZ pairs are not separated, and between-group comparisons are made between pairs discordant and concordant for ASD. In study V, the study sample is limited to MZ pairs.

3.1.1 The Roots of Autism and ADHD Twin Study in Sweden

The studies presented here are all based on a Swedish twin sample collected within the Roots of Autism and ADHD Twin Study in Sweden (RATSS) program, with the addition of a Californian sample in study II (see below for more details). The RATSS data collection started in August 2011 at the Center of Neurodevelopmental Disorders at Karolinska Institutet (KIND). Exclusion criteria for RATSS are indication of profound intellectual disability (IQ < 35), serious psychiatric (e.g., paranoid schizophrenia) or neurological (e.g. intractable epilepsy) conditions, or any well-defined genetic syndrome (e.g., fragile X). The subsamples are selected on qualitative and quantitative discordance for ASD or ADHD, zygosity, available samples (teeth) and time point for analysis, see table 1.

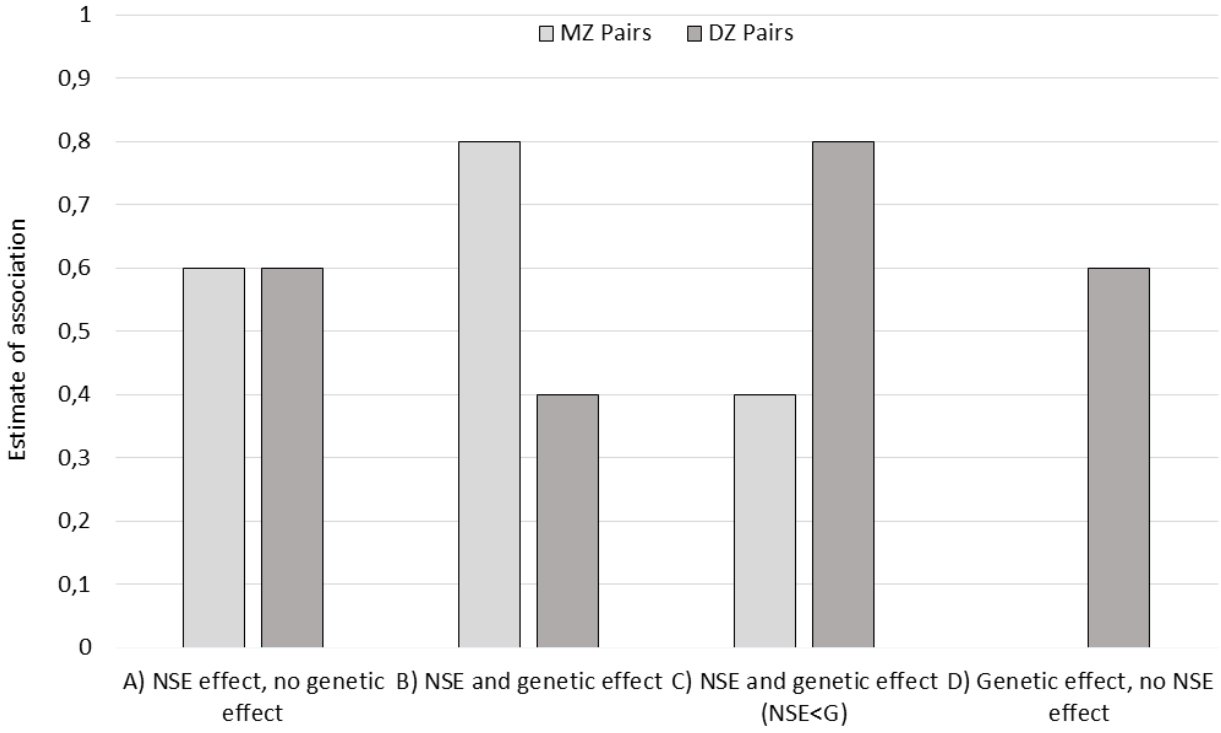

Figure 1. The figure shows different hypothetical intra-pair associations between exposure and outcome in MZ and DZ pairs. Since the MZ pairs share 100% of their genetic variation, the within-pair associations found in MZ pairs are adjusted for genetics and shared environmental factor, and the associations found must be driven by NSE factors. Since the environmental factors are assumed to be equal between MZ and DZ pairs, all differences between the zygosity groups must be due to genetic factors. Hence, (A) an equal intra-pair correlation in MZ and DZ pairs indicates an effect of NSE and no genetic effect on the association. (B) A stronger correlation in MZ than in DZ pairs, indicates both a NSE and a genetic effect. The genetic effect is in this case masking the NSE in the DZ pairs, resulting in a stronger association in MZ pairs when adjusting for genetics confounding. (C) An association in both groups, that is stronger in DZ pairs, indicates both a genetic and NSE effect, and the genetic effect being more dominant. (D) An association in the DZ pairs and no association in MZ pairs, indicates a genetic effect and no NSE effect.

3.1.1.1 Procedure in RATSS

The twins were identified and recruited via several sources. The main source was the Swedish Twin Registry (STR), either via studies based on the STR, such as i) the Child and Adolescent Twin Study in Sweden (CATSS) targeting all twins born in Sweden since 1994; with a response rate of approximately 80% and >25,000 twins born between 1994 and 2004,[173] or by combining STR with other registries, such as ii) the patient registry of the Swedish Board of Health and Welfare; iii) the clinical registries of the Division of Child and Adolescent Psychiatry, and iv) with the Habilitation and Health centers and pediatric units in Stockholm County. In addition, families were recruited via summons in the journals of the

National Society for neurodevelopmental disorders (Riksförbundet Attention), the National Society for Autism and Asperger syndrome (Autism och Aspergerföreningen), and the national twin organization (Tvillingklubben), exhibitions at their (bi-) annual meetings, and other forms of public media (e.g., reports on the project in newspapers and on television).

The families were typically first contacted via mail and then via telephone. The majority of twins and their parents were either assessed at the Astrid Lindgren's Children Hospital or at the KIND research clinic in Stockholm. A minority were assessed in their homes. Information, consents, and questionnaires were sent to the families for completion ahead of their visit. For families not living in the Stockholm area, travel and accommodation were arranged by the study nurse. The twins and the parents were all examined, tested and interviewed separately. Each family was assessed by three psychologists, or psychology students under supervision, trained on the different instruments. The order of tests was switched within and between the pairs. Total assessment duration was seven to eight hours including a longer lunch break and several shorter intervals. Best estimate psychiatric clinical consensus diagnoses based on all gathered information were made by the team of psychologists.

Blood and saliva samples were collected at the clinic by a research nurse during the study visit. The teeth were naturally shed deciduous teeth that had been stored in the participants' home. Teeth were brought to the study team in person by twins or parents and thereafter stored at room temperature at the clinic.

3.1.2 The Californian Autism Twin Study

The Californian twin sample was collected within the Californian Autism Twin Study (CATS) at Stanford University School of Medicine. The twins were born between year 1987 and 2004, and the data was collected during 2005-2009. Inclusion and exclusion criteria were: "i) At least 1 child in the pair had a qualifying diagnosis (see later) in the Department of Developmental Services (DDS) electronic file or in client records; each twin in a pair who met this criterion was considered a proband.; ii) maternal residence in California at the time of delivery; iii) both co-twins were alive and residing in California at the time of enrollment; iv) no history among proband of neurogenetic conditions that might account for ASD (i.e., fragile X syndrome, Down syndrome, tuberous sclerosis, and neurofibromatosis); v) proband had a mental age more than 18 months; and vi) at least one parent with sufficient verbal and reading ability in English or Spanish was able to respond to interviews and checklists".[12, p 1096]

3.1.2.1 Procedure in CATS

Records from the DDS were used for identification of the Californian twin pairs. The DDS consists of 21 regional centers, coordinating services for persons with ASD, intellectual and other developmental disabilities. Patients are referred to the DDS centers from primary care providers, educators, public health clinics, other service agencies, and parents throughout California. For identification of twin births and to obtain demographic data, the electronic DDS client files were linked to Californian birth records. The parents were first contacted via a letter from the local DDS center, explaining the study and asking their consent to send identifying information to study staff at Stanford University. Next, they research group at Stanford University would the contact them to further describe the study and obtain written consent. Families in northern California were assessed by a research team at Stanford University and families in southern California by a research team at the Autism Genetic Resource Exchange.[12]

3.1.3 Sub-samples

See table 1 for an overview of the samples included in the different studies. Study II consisted of a merged sample of 341 pairs, 132 pairs from the RATSS cohort and 209 pairs from the CATS cohort. The zygosity ratio was 127 MZ and 204 DZ pairs, ranging from ages 4 to 28 years, with a mean age of 11.8 years. Thirty-six percent were females. Study III includes a total of 126 pairs. A two-step analysis was done. First, a subsample of 13 MZ pairs qualitatively discordant for ASD and 13 MZ typically developed (TD) control pairs was included for an in-depth exploration of medical records. Second, the findings from the first medical records review were confirmed in the remaining sample of 100 pairs, splitting the sample by zygosity groups (MZ pairs N=54; DZ pairs N=46). Study IV is based on tooth samples collected in 32 complete pairs (17 MZ and 15 DZ) and 12 individuals from twin pairs whose co-twin did not donate teeth. In this study, seven were complete pairs discordant for ASD diagnosis, six pairs concordant for ASD, and 19 pairs concordant non-ASD. In study V, a subsample of 27 MZ pairs quantitatively discordant for ADHD traits was selected.

Table 1. Description of samples and variables included in study II-V.

	Study II	Study III, Step 1	Study III, Step 2	Study IV	Study V
Research questions	The association between ASD and IQ	Explorative identification of medical factors contributing to ASD risk	The effect of a cumulative load and specific medical factors on ASD risk	The effect of metal regulation during pre- and postnatal development on ASD risk	The role of NSE on executive functioning in ADHD
Total N	341 pairs	26 pairs	100 pairs	32 pairs + 12 singletons	27 pairs
Sex (M:F)	218:123	32:20	109:91	46:30	40:14
Zygosity	137 MZ pairs 204 DZ pairs	26 MZ pairs	54 MZ pairs 46 DZ pairs	17 MZ pairs 15 DZ pairs	27 MZ pairs
Concordance (N pairs)	70 conc. ASD, 173 disc. ASD, 90 conc. non-ASD	13 disc. ASD, 13 conc. TD	All quantitative disc. ASD traits	7 disc. ASD, 6 conc. ASD, 19 conc. non-ASD	All quantitative disc. ADHD traits
Exposure measures / Independent variables	• IQ	• Intra-pair differences in medical records data of early medical events	• Total load of medical events • Dysregulation • Birth weight	• Exposure of ten different metals during pre- and postnatal development	• CBCL/ABCL score • ADHD diagnosis
Outcome measures / Dependent variables	• ASD diagnoses • SRS scores	• ASD diagnosis	• SRS score	• ASD diagnosis, • SRS score • ADOS Comparison score	• The Tower test score • WCST score
Covariates	• Sex • Age • Site	The groups were matched on: • Sex • Age	• Sex • IQ • ADHD diagnoses	• Zygosity • Sex • Gestational age • Birth weight	• Age • IQ • Sex

3.2 MEASURES

3.2.1 Zygosity

In the RATSS cohort, zygosity was determined by genotyping of saliva or whole-blood derived DNA using Infinium Human-CoreExome chip (Illumina). The estimating identity by descent is analyzed using the PLINK software[174] after quality control and removal of SNPs with a minor allele frequency less than 0.05 within the samples. All pairs of DNA samples showing $\hat{\pi} \geq 0.99$ were considered as MZ pairs. For a minority of twins without DNA samples, an algorithm based on five questions on twin similarity derived from 571 pairs of twins with known zygosity from the CATSS sample was used. Only twins with more than 95% probability of being correctly classified were assigned a zygosity by this method.[173] In the CATS cohort, "zygosity of sex-concordant twin pairs was determined in batches of 10-20 pairs concomitantly. Nine short tandem repeat loci and the X/Y amelogenin locus were amplified. Twin pairs discordant for at least one marker were considered DZ. Twin pairs concordant on all markers were considered MZ".[12, p 1096]

3.2.2 Behavioral Measures

All twins from the RATSS sample were assessed with an extensive behavioral protocol, including observations, neuropsychological tests, and self- and parent reports supported by data from medical records. Clinical consensus diagnosis (or lack of diagnosis) were supported by results from medical history, the Autism Diagnostic Interview – Revised (ADI-R),[66] the Autism Diagnostic Observation Schedule Second Edition (ADOS-2),[175] the Kiddie Schedule for Affective Disorders and Schizophrenia (K-SADS)[73] or the Structural Clinical Interview for DSM-IV-TR Axis I (SCID)[176] combined with the Diagnostic Interview for ADHD in adults (DIVA).[74] Either the Wechsler Intelligence Scales for Children or Adults Fourth Editions (WISC-IV/WAIS-IV), the Leiter-revised (Leiter-r) scales in combination with the Peabody Picture Vocabulary Test Third Edition (PPVT-3) (in cases of low verbal abilities), and the parent rated Adaptive Behavior Assessment Scale 2nd Edition (ABAS-II)[177] were collected for assessment of intellectual abilities and adaptive functions.

Autistic traits were measured by parent reported Social Responsiveness Scale (SRS).[75] The SRS has good to excellent psychometric properties for test-retest reliability (0.80-0.97), as well as interrater reliability (0.75-0.95), and satisfactory convergent validity (0.35-0.58).[178,179] ADHD traits were measured with the Attention problems (AP) scale from the parent-reported Child or (CBCL/6-18) or the Adult Behavior Check-List (ABCL/18-59), and by the self-rated AP scale from Youth Self Report (YSR/11-18) or Adult Self Report (ASR/18-59).[180,181]

Executive functioning was measured by two neuropsychological tests, a computerized version of the Wisconsin Card Sorting Test (WCST)[182] and the Tower test from the broader Delis-Kaplan Executive Function System (D-KEFS).[183] Both tests primarily measure planning skills. In addition the WCST measures cognitive flexibility and set shifting, while the Tower test also requires rule learning and inhibition skills.

The CATS protocol included the same ASD measures: the ADOS, the ADI-R, and the SRS. The 5th edition of the Stanford-Binet 5th (SB-5) scale was collected for assessment of intellectual abilities.[184] A study on the convergent validity between SB-5 and the WISC-IV in an ASD sample found a high correlation between corresponding intelligence scores (r=0.78-0.88), though some differences were found, indicating a tendency for individuals to score higher on SB-5 than on WISC-IV.[185]

3.2.3 Medical History

For the medical history, an index of medical risk factors was created by an in-depth review of all factors loading on ASD cases in MZ pairs qualitatively discordant for ASD. Detailed information on medical and developmental history, with a focus on the first five years of life, was collected from questionnaires and medical records data. Intra-pair differences for the appearance, frequency and age of onset for developmental alterations, medical complications and life events were registered. All medical history events identified as differentiating within ASD discordant pairs (summing up to 31 factors), were added up to generate a total load for each individual. Adjustment for missing data was done according the formula below.

$$\text{Cumulative load} = \frac{\Sigma \text{ medical history events}}{31 - \text{NA}}$$

The index was evaluated by calculation of intraclass correlations coefficients (ICC) between the index data and the medical records/questionnaire data. The agreement ranged from moderate for cumulative load of early medical events (ICC=.55, 95% CI:.22-.75), substantial for dysregulation (ICC=.76, 95% CI:.58-.86), and almost perfect for birth weight (ICC=.93, 95% CI:.88-.96).

3.2.4 Tooth-matrix Methods

In study IV, a novel tooth-matrix biomarker is used to measure multiple elements at a fine temporal resolution during early development (from 20 weeks before birth to 30 weeks after birth). Correlations between ASD measures and 10 different metals (i.e. Barium, Chromium,

Copper, Lithium, Magnesium, Manganese, Lead, Tin, Strontium, and Zinc) were tested for different time points during pre- and postnatal development.

The teeth were analyzed using laser ablation-inductively coupled plasma mass spectrometry (LA-ICP-MS) and assigning developmental times.[186,187] Herein, teeth are sectioned and the neonatal line (a line in enamel and dentine formed at the time of birth), as well as stepwise markings, were used to assign chronological information to sampling points. The laser ablation unit used was a New Wave Research (NWR) 193 system (ESI, USA) equipped with an ArF excimer laser. Helium was used as the carrier gas and mixed with argon after the ablation cell via a Y-piece. To increase sensitivity, the system was tuned daily using National Institute of Standards and Technology (NIST) standard reference materials (SRM) 612.

3.3 STATISTICS

3.3.1 Paired-data Analysis

The first step of the analyses in study III, and the main analysis in Study V, are based on tests for paired-data. Due to the small sample sizes, non-parametric Wilcoxon signed rank test for continuous variables and McNemar's test for dichotomous variables were used. In study III, an exploratory approach was taken, using two-tailed tests, while in study V, a direction of the relationship was hypothesized, and one-tailed tests performed.

3.3.2 Generalized Estimating Equation Analysis

In study II and III, conditional linear regression models, based on generalized estimating equations (GEE) analysis, were fitted. Each twin pair was entered as a separate stratum in the model. Since the conditional model quantify the association between the variables on within twin pair level, it adjust for all factors that are shared within the pairs (e.g., all genetic and shared environmental factors in MZ pairs), in turn allowing for adjustments of covariates not shared between the twins. In comparison to standard methods for repeated measures based on ordinary least squares, GEE modelling allows dependence between the variables and are here used for continuous, ordinal, and dichotomous variables.[188,189]

3.3.3 Distributed Lag Non-linear Models

Distributed lag models (DLMs) are used to model exposure-response relationships with delayed time effects. A non-linear DLM is a framework that can model non-linear exposure-response dependencies and time-delayed effects simultaneously. It is based on a methodology that is using a bi-dimensional space of functions, describing the shape of a relationship based

on the space of the predictor as well as the lag dimension of its occurrence.[190] In the tooth analysis in study IV, a DLM was fitted to detailed chronological data generated by tooth-matrix biomarkers to detect critical developmental windows for the selected metals. This method allowed estimation of the effect of exposure at a specific time window, while adjusting for exposures at other times.

3.3.4 Difference-in-difference Analysis

In study IV, three steps of analyses were performed. The primary analysis determined developmental periods when there were disparities in elemental concentrations associated with ASD in twin pairs discordant for ASD and control twin pairs. The parameter estimate in this analysis was the smoothed mean differences in log concentrations of discordant pairs minus mean differences in control pairs:

$$(X_{case} - X_{control}) - (X_{control\text{-}1} - X_{control\text{-}2})$$

Thus, for example, when ASD cases from the discordant pairs have higher concentrations than their non-ASD co-twins, and these differences exceed the differences observed in control twin pairs, the association is positive. In a sensitivity analysis, we similarly compared ASD-discordant pairs with ASD-concordant pairs. Finally, correlations between element concentrations and ASD scores were calculated, using data from all participants.

3.4 ETHICAL CONSIDERATIONS

Collection of the RATSS cohort was approved by the Swedish national ethical board (diary no Ö 32-2010). The collection of the CATS cohort was approved by the California Health and Welfare Agency Committee for the Protection of Human Subjects and by the institutional review board at Stanford University (protocol id 13820).

There are several ethical issues to consider in clinical studies like these. Most of the participants were children and adolescents (aged <18 years) and the majority had neurodevelopmental impairments, typically paired with low intellectual abilities, making it challenging for some participants to fully understand all possible risks and benefits implicated with their participation. The research teams made great efforts to explain all parts of the study in an adequate way adapted to the intellectual level of each individual (e.g., via images and sound recordings). No assessments or examinations were done if there were any signs of hesitation from the participant to take part, still, the motivation for young and low-functioning participants could be questioned. On the other hand, the scientific and clinical

value of these group (low-functioning children with NDD) is particularly high as they are typically excluded from clinical studies.

Participating in a study including multilevel assessments (cognitive assessments, genetic testing, biosampling, etc.), as in the RATSS project, could potentially raise awareness and worries about abnormalities that would not have been raised otherwise, affecting the well-being of the twins and parents. Then again, study participation included a thorough neuropsychiatric, neurological and genetic assessment. In several cases, the participation in the study led to the notion of impairments not previously addressed. In these cases, the families were informed and referred for further examinations and interventions. In several cases, these were assessments that the families for long had asked for, but had not been provided with in the regular health care services. From the author's clinical impression, from meeting and discussing with most of the participating families, the psychological and practical benefits reported by the families far exceeded the draw-backs they might have experienced related to participation.

4 RESULTS

4.1 STUDY 1

This paper supports cross-disciplinary multilevel twin studies as a powerful and unique tool to explore NDD etiology. The overall objective with the RATSS project is to generate a launch pad for novel surveys to understand the complex interplay between genes, environment and phenotypes. The project is mainly targeting MZ twin pairs discordant for categorical and dimensional ASD and ADHD. To increase the specificity of the results, control groups of DZ pairs, ASD and ADHD concordant pairs, and TD pairs are included. The multilevel data collection includes comprehensive behavioral phenotyping with assessments of psychopathology, neurocognitive profiles, heritability, medical history and anamnestic information. Within the study, the twins are physically and dysmorphologically assessed, and structural, functional and molecular brain imaging data is collected. Specimens are collected for induced pluripotent stem (iPS) cells, (epi)genetic, gut bacteria, protein-/monoamine, and metal analyses. Experts from several research groups, within and outside Sweden, are involved in the project. To the author's best knowledge, the RATSS is the largest multilevel clinical study of ASD and ADHD using MZ twin pairs.

4.2 STUDY 2

4.2.1 Preliminary Analyses (not published)

Initially, we planned to do ACE-modelling on the combined Californian and Swedish cohort. In preparation for this, we first explored the two main variables of interest (IQ and autism), and the two samples separately. First, probandwise concordance rates were calculated for the categorical variables; ASD diagnoses and ID (here defined as IQ<70), stratified on zygosity (see table 2). Next, we explored the continuous variables and the relationship between them, by calculations of cross-pair correlations for SRS and IQ scores (ICC within pairs) and cross-trait correlations for SRS and IQ scores (ICC between SRS score in twin 1 and IQ score in twin 2). The results revealed substantial differences between the samples, in particular for ID diagnoses and SRS scores, indicating that the combined sample was not appropriate for ACE modelling. The differences between the samples might be due to differences in recruitment, age, and severity. In particular the RATSS sample is not randomly selected, but rather selected on discordancy, which would bias the results in ACE-modelling. Therefore, we decided on an alternative statistical model that would not be biased by differences between

the cohorts, nor a non-randomized selection: conditional regression models. By fitting a GEE model, we were controlling for all factors shared within pairs, including differences between the cohorts (both twins in a pair were always from the same cohort).

Table 2. Initial analyses of familiarity for ASD (categorical ASD and continuous SRS total score) and IQ (categorical ID and continuous IQ full scale score), stratified on zygosity and site.

Calculations	Californian sample	Swedish sample
Probandwise concordance for ASD	MZ: 63.7% DZ: 21.9%	MZ: 48.0% DZ: 12.5%
Probandwise concordance for ID	MZ: 60.4% DZ: 58.8%	MZ: 87.5% DZ: 57.1%
Cross-pair ICC for SRS	MZ: 0.13 DZ: -0.32	MZ: 0.62 DZ: 0.27
Cross-pair ICC for IQ	MZ: 0.37 DZ: -0.03	MZ: 0.78 DZ: 0.47
Cross-trait ICC for SRS and IQ	MZ: -0.44 DZ: 0.27	MZ: -0.47 DZ: -0.13

4.2.2 Final Results

The descriptive analyses showed that the ASD twins in discordant pairs scored similarly high for autistic traits (SRS total score; z=-1.34, $p>.05$) and low IQ scores (full scale IQ; z=-0.86, $p>.05$), as the ASD twins in concordant pairs. Correspondingly, the non-ASD twins in discordant pairs showed similarly high IQ scores (z=-0.91, $p>.05$), as the non-ASD concordant twin. Although both non-ASD groups scored low for autistic traits, the non-ASD twins in discordant pairs presented significantly lower on autistic traits than the concordant non-ASD twins (z=-5.18, $p<.001$).

Across all individuals, there was a significant negative association between IQ and ASD diagnoses (OR=0.92, 95% CI: 0.91-0.94) as for IQ and autistic traits (β=-0.55, 95% CI:-0.60,-0.47, corresponding to a correlation of r=-0.57). Adjustment for sex, site, and age did not alter the significances of neither association.

We then proceeded to examine if the associations remained at within-twin level, and thereby adjusting for all variables shared within pairs. There was a negative association between IQ and categorical ASD (OR=0.87, 95% CI: 0.83-0.91) also on within pair level, as for IQ and

autistic traits (β=-0.83, 95% CI: -0.91,-0.75). Next we tested the effect of zygosity, sex, site, and age on the association between IQ and autistic traits. The association was significant for both DZ (β=-0.87, 95% CI:-0.96,-0.78) and MZ twin pairs (β=-0.65, 95% CI:-0.81,-0.48), but significantly stronger in the DZ pairs (χ^2 =5.47, p=.019). The IQ-autistic trait association was significant for both female (β=-0.93, 95% CI:-1.13,-0.73) and male (β=-0.71, 95% CI:-0.83,-0.56) same-sex pairs, with no significant difference between the groups (p>0.05). The association was true in both the Swedish (β=-0.59, 95% CI:-0.83,-0.35) and the Californian cohorts (β=-0.87, 95% CI:-0.95,-0.78), but significantly stronger in the Californian (χ^2 =4.65, p=.031). The association was significant in all age groups (age 4-11; β=-0.93, 95% CI:-1.04,-0.81, age 11-17; β=-0.75, 95% CI:-0.86,-0.63, age 18-28; β=-0.57, 95% CI:-0.96,-0.19), but stronger in younger pairs (χ^2 =6.85, p=.033) (see Table 3).

Table 3. Regression estimates for associations between IQ and autistic traits at within-pair level, and comparisons of estimates between groups.

Samples	**Association between IQ and SRS score**			**Between-groups comparisons**	
	β	**95% CI**	***p***	***χ***	***p***
Total (N=306)	-0.83	-0.91, -0.75	<.001	-	-
Male SS (N =154)	-0.71	-0.83, -0.56	<.001	3.4	.062
Female SS (N =74)	-0.93	-1.13, -0.73	<.001		
MZ (N=122)	-0.65	-0.81, -0.48	<.001	5.47	.019
DZ (N=184)	-0.87	-0.96, -0.78	<.001		
Californian cohort (N=176)	-0.87	-0.95, -0.78	<.001	4.65	.031
Swedish cohort (N=130)	-0.59	-0.83, -0.35	<.001		
Age 4-10y (N=128)	-0.93	-1.04, -0.81	<.001	6.853	.033
Age 11-17y (N=146)	-0.75	-0.86, -0.63	<.001		
Age 18-28y (N=32)	-0.57	-0.96, -0.19	.003		

Last, we corroborated the association by testing the triad ASD symptom domains and verbal and non-verbal IQ, separately. There was a significant association between autist traits and both verbal IQ (β=-0.80, 95% CI:-0.87,-0.72) and non-verbal IQ (β=-0.82, 95% CI:-0.91,-

0.74)). The association with IQ was significant for all ASD symptom domains: social reciprocity (β=-0.42, 95% CI:-0.46,-0.39) communication (β=-0.03, 95% CI:-0.04,-0.03), and repetitive behavior and interests (β=-0.12, 95% CI:-0.13,-0.11).

4.3 STUDY 3

In study III, there was an association between ASD and the cumulative load of early medical events when controlling for IQ and ADHD comorbidity. This association was true for both clinical ASD (Z=-2.85, p=.004) and dimensional traits (β=78.18, p=.002), in MZ pairs. A single, early medical factor correlated with the extent of autistic traits was dysregulation (i.e., feeding, sleeping abnormalities, excessive crying and worriedness) during the first year of life (β=31.75, p=.03). The association for birth weight and ASD traits was not significant, but showed a similar trend (β=-.01, p=.05). The associations were only true in MZ pairs, when adjusting for genetic factors, and not in DZ pairs with a larger genetic variance. See table 4.

Table 4. Conditional linear regression between SRS total score and cumulative load of medical events, dysregulation problems and birth weight, including IQ and ADHD diagnosis as covariates

Variables	MZ (n=54 pairs)			DZ (n=46 pairs)		
	β	**SE**	***p***	***β***	**SE**	***p***
Cumulative load	78.18	26.59	.01	-8.71	46.03	.43
IQ	-0.19	0.25	.22	-1.20	0.29	<.001
ADHD	3.30	5.64	.26	39.18	8.09	<.001
Dysregulation	31.75	16.2	.03	11.85	20.60	.28
IQ	-0.33	0.24	.08	-1.14	0.30	<.001
ADHD	2.78	5.87	.32	40.63	8.22	<.001
Birth weight	-0.01	0.01	.05	-0.01	0.01	.30
IQ	-0.39	0.24	.05	-1.19	0.28	<.001
ADHD	0.71	5.05	.44	40.10	7.90	<.001

4.4 STUDY 4

In study IV, we found ASD cases to have higher lead levels over the third trimester and first three months postnatally, in comparison to non-ASD twins. Zinc levels were reduced in ASD cases prenatally, and manganese levels were consistently lower in ASD cases both pre- and postnatally. The manganese deficiency was highest seven months after birth.

By fitting DLMs, different developmental periods when elemental levels varied within the discordant pairs were identified. The greatest difference of lead levels was observed eight weeks postnatally, when ASD cases had 1.7 times higher lead than their non-ASD co-twins. Cases showed manganese deficiency 17 weeks before birth to 30 weeks postnatally, reaching seven-fold lower levels six months postnatally. Cases had reduced zinc levels, being 28% lower than their co-twins eight weeks prenatally. Analyses of clinical ASD severity (ADOS-2 comparison score) and autistic traits (SRS-2 total scores) showed that eight of ten metals examined were significantly associated with ASD. Manganese was inversely associated with autistic traits as defined by SRS-2 (strongest association at 30 weeks, r=-0.37) and ASD severity on ADOS-2 (strongest association at 2 weeks, r=-0.30). Higher lead levels between eight weeks before birth and up to approximately week 25 postnatally were significantly positively associated with these indices, but the association was weaker than that observed for manganese (maximum r with SRS-2=0.10 at 13 weeks postnatally, and maximum r with ADOS-2=0.18 at seven weeks postnatally). A weak inverse association of zinc with the SRS-2 was also observed in the third trimester (maximum r at 12 weeks before birth=-0.03) and a positive association with ADOS-2 before birth and postnatally (maximum r=0.07 at 30 weeks postnatally, 95% CI:0.04-0.1).

4.5 STUDY 5

In study V, results showed a positive correlation between the intra-pair variation in ADHD traits and sub-scores of the Tower test; Total rule violation (r_s=.41, p=.017) and Rule-violation-per-item-ratio (r_s=.38, p=.03), both primarily measuring inhibition. There was no association between ADHD and the Tower test total score, nor between ADHD and WCST scores (p>.05). Adjusting for age, IQ, and sex did not alter the significance of the results. Finally, the analyses were executed excluding pairs exposed to twin-to-twin-transfusion syndrome and pairs in which at least one twin was currently medicated for ADHD. Neither exclusion altered the significance of the results.

5 DISCUSSION

5.1 STUDY 1

One major challenge in etiological research is to link different levels of data, to not only identify single risk factors, but to also reach a broader understanding of the interplay between factors and to identify causal pathways. Ideally, multilevel data collection of large samples of well characterized cases and controls, would facilitate such investigations. To focus on the most informative and exceptionally well matched case-control pairs, such as MZ twin pairs discordant for the phenotype of interest, is a powerful design for this purpose. The RATSS study design has several strengths, in particular, the access to nation-wide registry data enabling the recruitment of an exclusive sample of discordant twins, which is combined with a comprehensive phenotyping and multilevel data collection. There are several techniques applied by collaborators in RATSS that have not previously been used in ASD/ADHD discordant twin designs. These include tooth-matrix for exploration of pre- and postnatal exposure of metals, reprogramming of iPS cells, mass spectrometry-based proteomic techniques for CSF analysis, and multimodal brain connectome approaches. Taken together the cross-disciplinary expertise in the study team, the identification and recruitment of twin pairs via nation-wide registers, and the comprehensive and multi-level data collection, the RATSS program has potential of finding novel leads for pathogenic pathways and informative biomarkers in ASD and ADHD.

5.2 STUDY 2

This study examined the link between IQ and ASD, for categorical ASD diagnoses and autistic traits, in a sample of carefully phenotyped twins across two countries. Genetic and environmental underpinnings were explored. Our findings show that lower IQ, clinical diagnoses as well as autistic traits, is associated with an increase of the presence of clinical ASD and increased autistic traits. The IQ-autistic trait link for MZ twins was significant, indicating NSE effects; whereas the stronger associations in DZ compared to MZ pairs suggest an additional genetic effect on the association. The phenotypic correlation between IQ and autistic traits is robust and it was significant for both sexes, for different age groups, across both the Californian and Swedish cohorts, for verbal and non-verbal IQ, as well as for all domains of the ASD symptom triad.

Our results for ASD diagnoses and the phenotypical overlap with IQ, are in line with the previous literature showing a high overlap between the ASD and ID.[4] However, the results for IQ and autistic traits are partly novel. Previous literature indicates no or modest correlations between IQ and autistic traits,[56,57,178,179,191] whereas our findings show a robust association between autistic traits and full scale IQ. In comparison to previous studies, our results are based on in-person IQ testing with first choice clinical IQ measures, while aforementioned studies have relied on either clinical records data, or IQ estimates based on online, parent- or telephone administrated assessments.[56,57,178,179,191]

The literature on the heritability of the genetic overlap between IQ and ASD is inconclusive. Twin studies based on the TEDS cohort are reporting modest genetic correlations,[56,57] whereas one small Japanese twin study is reporting a high genetic correlation.[192] Our results add to the previous knowledge by showing a NSE component in the etiology of the IQ-ASD link. The literature show several NSE risk factors common for both ASD and ID, such as gestational age, birth weight and 5-min Apgar score.[59,60] If the IQ-ASD association is underpinned by changeable or reversible NSE factors, these might be areas with large potential for proactive interventions. Hence, our findings emphasize the importance of further research on the NSE factors in relation to the IQ-ASD overlap.

5.3 STUDY 3

The results from study III show early dysregulation, and foremost, the cumulative load of early medical events to index children at risk of ASD. While the findings of a cumulative effect of somatic symptoms on ASD risk is novel and contradictory to previous results,[134] early dysregulation as precursor of behavioral problems is in line with the literature.[193,194] A large population-based study including over 4000 infants reported that early regulatory problems predict behavioral problems (i.e., external, internal and attentional problems) later in life.[193] Similarly, a Swedish clinical study found that regulatory issues were frequent in children later diagnosed with ASD.[194] Our findings show the associations to be significant in MZ pairs (adjusting for all genetic effect), but not in DZ pairs (only partly adjusting for the genetics). This indicates genetic effects due to the differences between the groups (i.e. only the degree of relatedness is assumed to differ between MZ and DZ pairs) as well as NSE effects, since the association is significant in MZ where genetic and shared environmental factors are adjusted for. Hence, while earlier studies have been unable to differentiate genetic and environmental effects on these phenomena, our results advance the results as they demonstrate an environmental effect on the association between early regulation difficulties and ASD.

From a clinical perspective, these results highlight early dysregulation problems and low birth weight, and foremost, a combination of several early medical events, as red flags for children at risk for developing ASD. Although symptoms of ASD emerge as early as at 12 months of age,[63] and ASD can quite reliably be diagnosed between 24 and 36 months of age, many children are not diagnosed until around the age of eight years.[24] This is unfortunate as early detection is a prerequisite for early intervention,[195] which is associated with better outcomes.[196] The screening tools available for ASD before the age of 24 months are limited in sensitivity and specificity, and do not include early medical features.[196] Including the total load of early medical events in screening might for subgroups of children facilitate earlier ASD diagnosis.

5.4 STUDY 4

In study IV, the findings indicate associations between ASD and pre- and postnatal dysregulation of lead, zinc and manganese. This supports the hypothesis that prenatal and early childhood disruption (excess or deficiency) of multiple metals during critical developmental windows is associated with ASD, suggesting a systemic elemental dysregulation pathway for ASD etiology. At least one previous study used teeth to analyse metal toxicants in relation to ASD, reporting no significant differences between ASD cases and controls for lead, zinc or manganese,[77] making our findings novel. In this previous study grounded teeth were analysed, not facilitating any investigation of the timing of the exposure, which might explain the contradictory results. Our study supports the evidence of joint interaction of environmental exposures with genetic variations in the etiology of ASD. The results show that the brain's capacity to regulate metals varies with developmental age, suggesting *in utero* fluctuations in expression of metal transporter genes. Overall, it is likely that alterations in metal regulation in ASD cases involve multiple disruptions within the complex networks that regulate elemental uptake and distribution.

5.5 STUDY 5

In study V, the findings indicate an association between ADHD and inhibitory control, when adjusting for genetic and shared environmental factors. Since the within-pair design automatically control for genetic and shared environmental factors, the results indicate NSE components in the etiology of the association. Further, the findings support inhibition to be a core deficit in ADHD,[197] and confirm ADHD to be an extreme on a continuum. The results suggest that planning and inhibitory control in ADHD, might be areas with larger potential

for change in comparison to other areas of executive functioning with higher degree of genetic influence. Hence, systematically studies of environmental effects on different aspects of executive functions in ADHD might lead to improved development of treatment and habilitation programs in ADHD.

5.6 LIMITATIONS

There are some limitations that need to be addressed in relation to the studies included here.

In study II, the study sample is a combined sample from two different cohorts, and partly different measurements and procedures. Although the same standardized instruments for assessment of ASD were used at both sites (i.e., ADOS, ADI-R, SRS), a risk of discrepancies in testing procedure cannot be out ruled and no inter-rater reliability measures across sites are available. Four different IQ measures were used (i.e. SB-5, Wechsler scales, Leiter-r and PPVT-3). Though, with few exceptions, both twins in a pair were tested with the same test, limiting the risk for potential measurement bias. The same applies to all other factors shared between twins, and that automatically were adjusted in the within-pair analyses. Also, since the results were constant over two independent samples, with partly different measures, it confirms the robustness of the results.

In study III the primary exposure variables (the cumulative load of early medical events, dysregulation and birth weight) are not primary risk factors for ASD, but secondary (or tertiary), although they precede an ASD diagnosis. Hence, in this study, we are unable to draw firm conclusions about the possible primary factors. Further, this study is unable to weight the different factors summing up to the cumulative load, making the estimations crude.

For study III-IV, one might consider the sample sizes to be small with a noteworthy risk of type II errors, due to low statistical power. However, the significances reported still indicate strong and robust findings, although the non-findings have to be interpreted with caution. Further, MZ pairs discordant for highly heritable conditions such ASD and ADHD are rare, and our ASD and ADHD discordant sample actually represents a rather large minority of those discordant pairs in Sweden.[198]

In study V, analyses of categorical clinical diagnoses were lacking power due to the small sample size, hence it would be of interest to confirm the results in a larger sample of pairs qualitatively discordant for ADHD. The outcome measure (executive functioning) is based on two neuropsychological tests primarily measuring planning skills. However, since inhibition

turned out to be the outcome variable significantly associated with ADHD, further studies with a twin design and more comprehensive measures of ADHD, inhibitory control and other executive functioning measures, would be valuable for confirmation of the results.

In these studies we are stressing the significance of NSE as a function of differences in MZ pairs. Even if rare, it has to be noted that the differences between the MZ twins could also be due to post-twining *de novo* mutations and measurement errors.

6 CONCLUSION AND FUTURE DIRECTIONS

This thesis is exploring different aspects of NDD etiology, in particular focusing on NSE factors in ASD. The studies are based on discordant twin pair designs and within-twin pair analyses, allowing for adjustment of genetic and shared environmental factors, with the aim of identifying NSE driven associations and markers.[114]

Our ASD results are in favour of a multiple pathway model for ASD etiology. This implies ASD to be underpinned by a genetic predisposition with triggering pre- and postnatal NSE factors such as systemic elemental dysregulation (e.g., elevated lead-, and diminished zinc and manganese levels), and a total load of early medical events and/or manifestations (e.g., decreased intra-uterine growth, behavioral dysregulation). These factors might affect the developing brain if occurring during critical developmental time windows, resulting in ASD deficits and related phenotypes such as low IQ. See figure 2.

In agreement with heritability studies suggesting NSE and genetic components in the etiology of ASD and ADHD,[10] our results support NSE factors to underpin behavioral as well as biological markers in both ASD and ADHD. We show an association between early medical events and ASD, driven by NSE factors. In addition to genetic underpinnings, we show a NSE influence on the IQ-ASD link as well as on the link between executive functions and ADHD. If the identified phenotypical correlations are owing to changeable or reversible NSE factors, these might be areas with larger potential for change in comparison to other areas of behavioral functioning with higher degree of genetic influence. Hence, our results emphasize the importance of further exploration of environmental factors or areas of factors, involved in NDD etiology.

The ASD findings (study II-IV) support the notion that ASD is continuously distributed in the population, with overlapping etiology between traits in the normal population and clinical phenotypes,[10,14] as well as partly different etiology between different symptom domains.[121] The latter is demonstrated in study II. The different symptom domains, as defined by ADI-R, are all negatively correlated with IQ, but with significant differences in the strengths of the associations. In line with the Research Domain Criteria (RDoC), proposed by the American National Institutes of Health (NIH),[199] our results emphasize the relevance of focusing on phenotypical and neurobiological correlates rather than in diagnostic constructs in NDD research. Also in ASD there might be subgroups with related behavioral characteristics (e.g., level of intellectual abilities) and neurobiological correlates, similar to on-going ADHD research.[18,19]

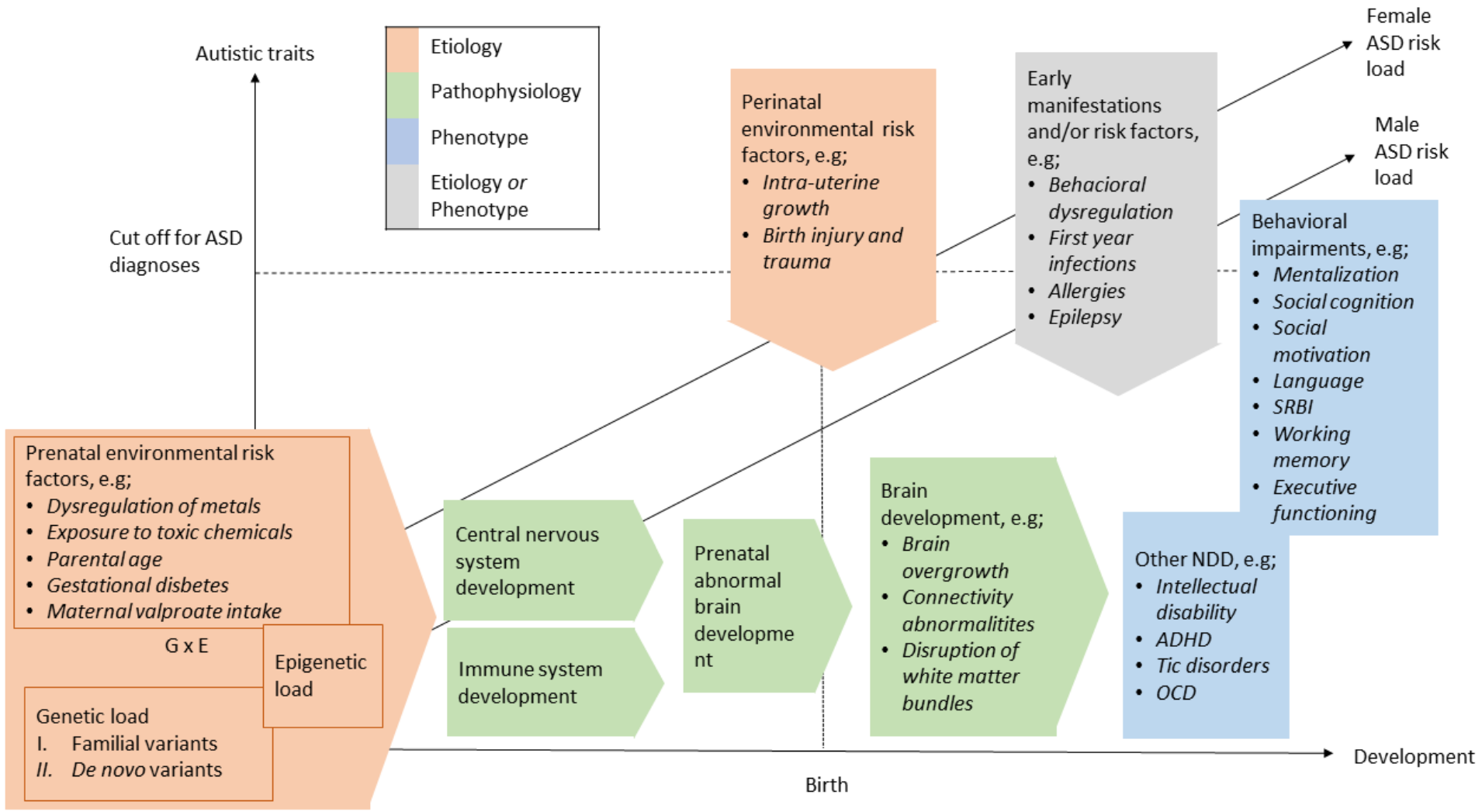
Autistic traits
Etiology
Pathophysiology
Phenotype
Etiology *or* Phenotype
Cut off for ASD diagnoses
Perinatal environmental risk factors, e.g;
• *Intra-uterine growth*
• *Birth injury and trauma*
Early manifestations and/or risk factors, e.g;
• *Behacioral dysregulation*
• *First year infections*
• *Allergies*
• *Epilepsy*
Female ASD risk load
Male ASD risk load
Behavioral impairments, e.g;
• *Mentalization*
• *Social cognition*
• *Social motivation*
• *Language*
• *SRBI*
• *Working memory*
• *Executive functioning*
Prenatal environmental risk factors, e.g;
• *Dysregulation of metals*
• *Exposure to toxic chemicals*
• *Parental age*
• *Gestational disbetes*
• *Maternal valproate intake*
G x E
Epigenetic load
Genetic load
I. Familial variants
II. De novo variants
Central nervous system development
Immune system development
Prenatal abnormal brain developme nt
Brain development, e.g;
• *Brain overgrowth*
• *Connectivity abnormalitites*
• *Disruption of white matter bundles*
Other NDD, e.g;
• *Intellectual disability*
• *ADHD*
• *Tic disorders*
• *OCD*
Development
Birth

Figure 2 (previous page). The figure demonstrates a multifactor threshold model for ASD etiology. It shows autistic traits as a continuum on the y-axis, and time on the x-axis, from early prenatal to peri- and postnatal development. The marked female and male ASD risk load demonstrates a hypothesized line of the total sum of risk factors having a cumulative effect on autistic phenotypes. The two different lines for female and male risk load, demonstrate that the threshold for females to present a clinical phenotype is higher than for males. The total load includes a gene and environment interaction of genetic, epigenetic and prenatal environmental risk factors, as well as peri- and potentially postnatal risk factors (etiology). These factors effect the development of the immune- and the central nervous system, leading to an abnormal prenatal development of the brain, and resulting in postnatal brain abnormalities (pathophysiology), resulting in autistic and other neurodevelopmental phenotypes, that might be expressed as early manifestations such as behavioral dysregulation.

ASD and ADHD diagnosis without comorbid conditions are rare.[200] Still, many clinical studies are aiming at specific results, either excluding cases with known comorbidity or overlooking the assessment of comorbidity. In the studies presented here, we aim at an exploration of the most common comorbid conditions, such as ADHD and intellectual disability in ASD.

As we show in study II, there is robust association between IQ and ASD, both for categorical and dimensional measures. Being the most crucial factors for prediction of ASD outcomes, we conclude that IQ is a key factor to consider in behavioral assessments of ASD. Although important for clinical outcomes, the inclusion of IQ in etiological studies of ASD might be debated. Whether it is appropriate to adjust for IQ, depends on the nature of the effect (confounding or mediating).[61,201] If there is a confounding effect, IQ is related to both the risk factor and to the phenotype and falsely obscure or accentuate the relationship between them (as it is hypothesized for medical risk factors and ASD). The adjustment of IQ is then providing an undistorted estimate of the association between for example medical risk factors and ASD. If instead IQ would have had a mediating effect, either direct or indirect, adjustment might falsely obscure a true association. Hence, our results emphasize the importance of including IQ assessments in ASD assessments, but also that adjustment of IQ should to be interpreted with caution.

Identification of causal environmental risk factors or markers, are essential for the appropriate targeting and development of environmental interventions. In the studies included here, we show a prenatal metal toxicant uptake of zinc, lead and manganese, during specific prenatal developmental time windows, to be associated with ASD risk. The results support the hypothesis of an *in utero* systemic elemental dysregulation in ASD. Prenatal elemental dysregulation in ASD might act via epigenetic modification of genes coding for metal transporters. In particular zinc plays an important role in protein synthesis, cell replication, tissue growth and repair.[135,202] In addition, the literature suggest zinc to have a role as a regulator of multiple other metal homeostasis pathways, from early in the transcriptional

process to the optimal functioning of metal transporters.[31] Hence, factors such as placental insufficiency and epigenetic alterations of metal transporters may disrupt zinc homeostasis and result in an elemental dysregulation of several metals potentially increasing ASD risk.

Furthermore, we show a cumulative effect of peri- and postnatal medical events on ASD risk, suggesting factors to act both in utero and postnatally. Via an explorative approach, we replicated previous findings of postnatal dysregulation (excessive crying, feeding and sleeping difficulties) during the first years of life to be associated with ASD risk.[194] If these are early manifestations, such as feeding problems being early symptom of an abnormal gut microbiota,[138,203] and sleeping and excessive crying are early symptoms of difficulties with transition and increased stress in infants with ASD,[194] is still to be explored.

There are two main strengths with the studies included here. A multifactor model for NDD implies many causal factors with small effects. Identification of risk factors with a minor effect, might either be done by the inclusion of large samples or by holding interfering factors constant. The discordant MZ twin pair design is a demonstration of the latter, making it possible to explore less salient NSE factors. If there are strong genetic components in the etiology, as for ASD and ADHD, the genetic variation in ordinary case-control studies might supress small environmental effects.[201,204] Secondly, our studies are based on an extensive and in-depth behavioural phenotyping. This facilitates exploration of different subgroups and cognitive profiles within the diagnoses (e.g., medical records data, IQ, executive functioning, first choice autism assessments, full psychiatric screening), while adjusting for several co-variates.

Based on the literature and findings presented here, I find it motivated to follow up with the following lines of research:

i) Collection of larger samples of NDD discordant twin pairs for multilevel analyses. Extensive phenotyping in combination with novel techniques in a highly informative sample, such as the RATSS cohort, will facilitate causal modelling of GxE interactions,[205] multi-modal brain imaging (combining anatomical, diffusion, and functional neuroimaging methods),[86] and *in vivo* modelling of neurons by stem cell methods using iPS cells.[149] This might lead to identificaion of strong hypothesis for causal pathways and reliable biomarkers, potentially facilitating the development of effective behavioral and pharmacological interventions, and earlier and more valid diagnostics.

ii) Further exploration of the effects of genes and environement on the phenotypic correlations (i.e., inhibitory control in ADHD, IQ in ASD). For this, large samples of randomly selected twins would be needed for bivariate twin modelling for exploration of the etiology of the phenotypic overlaps.

iii) Combining extensive phenotyping and machine learning techniques for identification of subgroups based on behavioral measures, neurocognitive profiles and neurobiological correlates, to identify informative subgroups and further corroborate the underlying causes of the heterogentity of the disorders.[206] For this, large population-based samples, would be needed for accurate training of algorithms.

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9 APPENDIX