From the Department of Women's and Children's Health
Karolinska Institutet, Stockholm, Sweden

ENVIRONMENTAL ETIOLOGIES OF AUTISM AND OTHER NEURODEVELOPMENTAL CONDITIONS: TWIN STUDIES OF THE CUMULATIVE EFFECT OF EARLY MEDICAL EVENTS

Torkel Carlsson

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Environmental Etiologies of Autism and Other Neurodevelopmental Conditions: Twin Studies of the Cumulative Effect of Early Medical Events
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Torkel Carlsson

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Principal Supervisor:
Professor Sven Bölte, PhD
Karolinska Institutet
Department of Women's and Children's Health
Division of Neuropsychiatry, Center of Neurodevelopmental Disorders (KIND)

Co-supervisor(s):
MaiBritt Giacobini, MD, PhD
Karolinska Institutet
Department of Molecular Medicine and Surgery
Division of Clinical Genetics

Associate Professor Ulf Jonsson, PhD
Karolinska Institutet
Department of Women's and Children's Health
Division of Neuropsychiatry, Center of Neurodevelopmental Disorders (KIND)

Associate Professor Kristiina Tammimies, PhD
Department of Women's and Children's Health
Division of Neuropsychiatry, Center of Neurodevelopmental Disorders (KIND)

Associate Professor Mark Taylor, PhD
Karolinska Institutet
Department of Medical Epidemiology and Biostatistics

Opponent:
Professor Søren Dalsgaard, MD, PhD
Aarhus University
Department of Economics and Business Economics
Centre for Integrated Register-based Research (CIRRAU)

Examination Board:
Professor David Mataix-Cols, PhD
Karolinska Institutet
Department of Clinical Neuroscience
Centre for Psychiatry Research

Associate Professor Renee Gardner, PhD
Karolinska Institutet
Department of Global Public Health

Professor Karin Källén, PhD
Lund University
Department of Laboratory Medicine
Division of Occupational and Environmental Medicine

To Katrina and Eira Elise

POPULAR SCIENCE SUMMARY OF THE THESIS

Neurodevelopmental conditions (NDC), such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), are characterized by alterations in the functioning, structure, and maturation of the brain. These changes cause cognitive challenges and impairments in social, educational, occupational, and other important areas of life. NDC are common, and although highly heritable, the environment also contributes to their etiology. When studying potential causal environmental factors in humans, there is always a risk of bias. One type of bias is called *familial confounding*. Familial confounders are factors shared within family members making them similar. Since many potential environmental factors are in themselves heritable, it may be that reported associations are driven by genetic links between the studied environment and the studied outcome, rather than the environment itself. By comparing exposure across relatives with and without the outcome, twin and sibling studies hold the potential to separate the true effects of the studied environment from confounding measured or unmeasured, genetic and environmental factors. Beyond single factors, the cumulative stress hypothesis proposes that vulnerability for given conditions, such as NDC, is enhanced if adversities accumulate during early life.

The overarching aim of this thesis was:

- to explore associations between environmental factors and ASD and other NDC;
- to identify early medical events associated with ASD and other NDC, and;
- to test the hypothesis of a cumulative environmental effect.

A comprehensive systematic review of previously conducted twin or sibling studies was performed to map all early environmental factors of NDC beyond familial confounding. In total, 140 studies were included. Advanced paternal age, low birth weight, congenital malformations, and perinatal respiratory stress were found to be associated with ASD, and low birth weight, low gestational age and low family income were associated with ADHD. Several previously suspected factors, including pregnancy-related ones, were deemed due to familial confounding.

Among a rare monozygotic (MZ) twin sample of ASD discordant twins – that is one twin in the pair having an ASD diagnosis and the other one not – all medical records were scrutinized for early medical events not shared with the other twin. A list of 31 non-shared early medical events were found within the discordant MZ sample and a cumulative effect on autistic traits was confirmed in a larger sample of twins.

Then, in a large population-based twin cohort, the cumulative effect of the early medical events identified in the systematic review (that is low birth weight, congenital malformations, and perinatal respiratory stress) were tested against ASD and ASD symptoms. Being exposed to all three medical events, compared with no exposure for the co-twin, doubled the odds of an ASD diagnosis, but the result was not statistically significant. Having a higher load of

early exposure was consistently associated with more autistic symptoms for the affected twin than their co-twin.

The final study suggests that this cumulative environmental effect of early medical events acts through a common latent NDC factor, that in turn affects neurodevelopment. Thereby affecting ASD as well as ADHD, tics and learning difficulties.

There is a critical need for more genetically informed studies of good quality in the quest for the environmental etiologies of NDC.

POPULÄRVETENSKAPLIG SAMMANFATTNING AV AVHANDLING

Utvecklingsrelaterade neuropsykiatriska tillstånd eller funktionsnedsättningar (NPF), såsom autismspektrumtillstånd (AST) och uppmärksamhetsstörning/hyperaktivitet (ADHD), kännetecknas av förändringar i hjärnans uppbyggnad, funktion och mognad. Detta orsakar kognitiva utmaningar som påverkar sociala, pedagogiska och yrkesmässiga delar av livet. NPF är vanliga, och även om de till hög grad är ärftliga, så bidrar också miljöfaktorer till deras uppkomst. När man studerar potentiella kausala, orsaksmässiga, miljöfaktorer hos människor finns det alltid en risk för systematiska fel. En typ av systematiskt fel kallas för *familial confounding* på engelska. Detta uppstår av faktorer som delas av familjemedlemmar och som gör dem lika. Eftersom många potentiella miljöfaktorer i sig är ärftliga kan det inte uteslutas att en rapporterad association drivs av genetiska kopplingar mellan den studerade miljön och det studerade tillståndet, snarare än miljön i sig. Med tvilling- eller syskonstudier går det att skilja de verkliga effekterna av den studerade miljön från systematiska fel uppkomna av familial confounding genom att jämföra exponering mellan familjemedlemmar där den ena har tillståndet och den andra inte – eller omvänt, jämföra familjemedlemmar som utsatts för exponering i olika grad. Utöver enstaka faktorer, så menar *the cumulative stress hypothesis* att sårbarheten för ett givet tillstånd, såsom NPF, ökar om miljöfaktorer ansamlas under de första levnadsåren.

De övergripande målen med denna avhandling var:

- att undersöka sambandet mellan miljöfaktorer och AST och andra NPF;
- att identifiera tidiga medicinska händelser associerade med AST och andra NPF, och;
- att testa the cumulative stress hypothesis.

En omfattande systematisk översikt av tidigare utförda tvilling- eller syskonstudier genomfördes för att kartlägga alla tidiga miljöfaktorer bakom NPF, med hänsyn tagen till familial confounding. Totalt ingick 140 studier. Hög ålder hos fäder, låg födelsevikt, medfödda missbildningar och respiratorisk stress runt födseln var associerade med AST, och låg födelsevikt, för tidig födsel och låg familjeinkomst eller inkomstbortfall var associerade med ADHD. Flera tidigare misstänkta faktorer, inklusive graviditetsrelaterade sådana, befanns bero på familial confounding.

Hos 13 par av enäggstvillingar diskordanta för AST – det vill säga där en tvilling i paret har AST och den andra inte – granskades hela deras medicinska journaler för att hitta tidiga medicinska händelser som inte delades med den andra tvillingen. En lista med 31 icke-delade tidiga medicinska händelser hittades. I en större grupp av tvillingar sågs sedan en koppling mellan skillnad i antal medicinska händelser och skillnad i mängd autistiska drag.

I en stor tvillingkohort baserad på den svenska befolkningen studerades sedan sambandet mellan ASD och ASD-symtom å ena sidan och å andra sidan effekten av ansamling av de tidiga medicinska händelser som identifierats i den systematiska översikten (det vill säga låg

födelsevikt, medfödda missbildningar och respiratorisk stress runt födseln). De som exponerats för alla de tre medicinska händelserna jämfört med ingen exponering hade jämfört med sin tvilling ett fördubblat odds för en ASD-diagnos, men resultatet var inte statistiskt säkerställt. Att ha en högre förekomst av tidiga medicinska händelser var konsekvent förknippat med fler autistiska symtom, vid jämförelse inom tvillingparen.

Den sista studien antyder att denna kumulativa miljöeffekt av tidiga medicinska händelser verkar genom en gemensam bakomliggande NPF-faktor, som i sin tur påverkar utvecklingen av NPF, ASD inkluderat, tillsammans med ADHD, tics och inlärningssvårigheter.

Det finns ett behov av fler studier av god kvalitet som tar hänsyn till familial confounding, i sökandet efter miljöfaktorer bakom NPF.

ABSTRACT

Background

Neurodevelopmental conditions (NDC), such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), are characterized by alterations in the architecture, functioning, and maturation of the brain causing cognitive challenges and impairments in social, educational, occupational, and other important areas of life. NDC are common, with a prevalence of 10 to 15%. Heritability estimates leave space for environmental etiological contributions, but the exact etiology remains poorly understood. Observational studies of the etiology of NDC often suffer from familial confounding.

Objectives

The overarching aim of this thesis was:

- to explore associations between environmental factors and ASD and other NDC;
- to identify early medical events associated with ASD and other NDC, and;
- to test the hypothesis of a cumulative environmental effect.

Methods

A comprehensive systematic review of twin or sibling studies was performed to map all early environmental factors of NDC beyond familial confounding. Within a rare monozygotic (MZ) twin sample of ASD discordant twins, medical records were scrutinized for non-shared early medical events, and a co-twin control design was used to test the cumulative effect of early medical events in a larger twin sample discordant for autistic traits. In a large population-based twin cohort, the association of ASD and ASD symptoms, and the cumulative effect of early medical events identified in the systematic review (low birth weight, congenital malformations, and perinatal respiratory stress) were studied. Finally, confirmatory factor analysis was performed to model a common latent NDC factor to test if this cumulative effect acted through a common NDC pathway, ASD included.

Results

In total, 140 studies were included in the systematic review. Beyond familial confounding, advanced paternal age, low birth weight, congenital malformations, and perinatal respiratory stress were associated with ASD, and low birth weight, gestational age and low family income were associated with ADHD. The systematic review deemed several previously suspected factors, including pregnancy-related ones, due to familial confounding. A list of 31 non-shared early medical events were found within the discordant MZ sample and a cumulative effect on autistic traits was confirmed. In the large population-based twin cohort the within pair odds ratio (OR) for an ASD diagnosis when having exposure of three early medical events were 2.39, but not statistically significant (95%CI;0.62–9.24). Having a higher load of early exposure was consistently associated with autistic symptoms after adjusting for familial confounding and sex with OR 3.45 (1.66–7.15) for one exposure to OR

7.36 (1.99–27.18) for three exposures. Cumulative exposure to early medical events was also associated with a non-linear increase in the common latent NDC factor, from ß=0.12 (95%CI, 0.07–0.17) for one exposure to ß=0.62 (0.34–0.90) for three exposures. In a monozygotic twin difference analysis, with familial confounding being fully accounted for, the whole exposure effect was captured by the common latent factor, with residual associations fully attenuated for the respective symptoms of ASD, ADHD, tics and learning difficulties, at all levels of cumulative exposure.

Conclusions

This thesis advances our understanding of ASD and NDC in mainly four areas:

1. It comprehensively maps our present knowledge from twin and sibling studies on environmental etiologies of NDC.
2. Owing to environmental contributions, it places early medical events into the dimensional model of autism and the liability threshold model, associating them with symptoms of ASD continuously distributed in the general population.
3. It confirms the cumulative stress hypothesis of ASD in a large human sample, beyond familial confounding.
4. It suggests that this cumulative environmental effect of early medical events acts through a common latent NDC factor, that in turn affects neurodevelopment, ASD included.

There is a critical need for more genetically informed studies of good quality in the quest of the environmental etiologies of NDC.

LIST OF SCIENTIFIC PAPERS

I. **Carlsson, T.**, Molander, F., Taylor, M. J., Jonsson, U., & Bölte, S. (2021, Oct). Early environmental risk factors for neurodevelopmental disorders - a systematic review of twin and sibling studies. *Development and Psychopathology, 33*(4), 1448-1495. https://doi.org/10.1017/S0954579420000620

II. Willfors, C., **Carlsson, T.**, Anderlid, B. M., Nordgren, A., Kostrzewa, E., Berggren, S., Ronald, A., Kuja-Halkola, R., Tammimies, K., & Bölte, S. (2017, Jan 31). Medical history of discordant twins and environmental etiologies of autism. *Translational Psychiatry, 7*(1), e1014. https://doi.org/10.1038/tp.2016.269

III. **Carlsson, T.**, Rosenqvist, M., Butwicka, A., Larsson, H., Lundström, S., Pan, P-Y. Lundin Remnelius, K., Taylor, M. J. & Bölte, S. (2022, Feb) Association of cumulative early medical factors with autism and autistic symptoms in a population-based twin sample. *Translational Psychiatry, 12*(1), 73. https://doi.org/10.1038/s41398-022-01833-0

IV. **Carlsson, T.**, Larsson, H., Lundström, S., Bölte, S., & Taylor, M. J. Association of cumulative early medical events and major neurodevelopmental disorders through a common latent factor – a population-based twin study. *(Submitted).*

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Myers, L., Ho, M. L., Cauvet, E., Lundin, K., **Carlsson, T.**, Kuja-Halkola, R., Tammimies, K., & Bölte, S. (2020, Dec 29). Actionable and incidental neuroradiological findings in twins with neurodevelopmental disorders. *Scientific Reports, 10*(1), 22417. https://doi.org/10.1038/s41598-020-79959-8

CONTENTS

LIST OF ABBREVIATIONS

AMEND	Anterior Modifiers in the Emergence of Neurodevelopmental Disorders
AUC	Area Under the Curve
ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism Spectrum Disorder
A-TAC	Autism-Tics, ADHD and other Comorbidities inventory
CD	Communication Disorder
CI	Confidence Interval
CFA	Confirmatory Factor Analysis
PECOS	Defined Population, Exposure, Controls, Outcome and Study Design for Systematic Reviews
DCD	Developmental Coordination Disorder
DAG	Directed Acyclic Graphs
DZ	Dizygotic
GEE	Generalized Estimation Equations
hiPSCs	Human-induced Pluripotent Stem Cells
HDP	Hypertensive Disorders of Pregnancy
ID	Intellectual Disability
IQ	Intelligence Quotient
ICC	Intraclass Correlation Coefficient
MZ	Monozygotic
NDC	Neurodevelopmental Conditions
NOS	Newcastle-Ottawa Scale
OR	Odds Ratio
PCB	Polychlorinated Biphenyls
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RCT	Radomized Control Trial
RMSEA	Root Mean Square Error of Approximation
SSRI	Selective Serotonin Reuptake Inhibitors
SLD	Specific Learning Disorder

SE	Standard Error
DSM-5	The 5th Edition of the Diagnostic and Statistical Manual of Mental Disorders
CATSS	The Child and Adolescent Twin Study in Sweden
ICD	The International Classification of Diseases
NPR	The National Patient Register
RATSS	The Roots of Autism and ADHD Twin Study Sweden
SRS-2	The Social Responsiveness Scale, 2nd edition

1 INTRODUCTION

1.1 NEURODEVELOPMENTAL CONDITIONS

Neurodevelopmental conditions (NDC) are characterized by alterations in the structure and development of the brain causing challenges in cognitive functioning and impairments in important areas of life, such as education, occupation, and social life (1). NDC are common, with a prevalence of 10-15% in the general population (2). According to DSM-5, NDC include intellectual disability (ID), autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), communication disorder (CD), specific learning disorders (SLD), developmental coordination disorder (DCD), and tic disorders (TD) (3). NDC are increasingly diagnosed worldwide (4). The most commonly diagnosed NDC are ASD and ADHD, with prevalence estimates ranging from 0.70-2.64% for ASD (4, 5) and 5-10% for ADHD (2, 6, 7). Males present with NDC more often than females, but NDC in females might be underdiagnosed (8, 9). NDC phenotypes are heterogeneous, with complexity further expanded by high comorbidity with other conditions, such as psychiatric, neurological, and immunological disorders, congenital anomalies, and gastrointestinal disturbances (10-12).

The causes of NDC are multiple (13, 14) but the exact etiologies driving atypical neurodevelopment remain poorly understood. Twin and family studies have shown that NDC are highly heritable (15-17), with both common and rare genetic variants being contributory to the phenotypes (2). Research focus has mostly been on genetic causes (18-21), although heritability estimates leave space for significant environmental contributions as well (22-26), with estimates ranging from 93-98% for ID, 64%-95% for ASD, 77%-92% for ADHD, and 70-85% for TD (15-17, 27-31). With knowledge about the substantial individual burden on subjects and their families, and the societal costs these conditions bring on health care and educational and long-term support systems, knowledge of factors involved in the etiology of NDC are of great importance (32-35).

1.2 THE DIMENSIONAL MODEL OF NEURODEVELOPMENTAL CONDITIONS

For several NDC, and repeatedly shown regarding ASD (13, 36), there is a continuum of traits ranging from broader phenotypes in the general population to clinical phenotypes, with overlapping etiologies (13). Therefore, it is important to examine outcomes of NDC not only categorically as diagnoses, but also dimensionally as traits and symptoms. There are at least two reasons for this. First, dimensional definitions may be more sensitive to subtle sub-clinical effects, and with a continuous measurement of the outcome, a detailed exposure-response profile may be studied. This, in turn, may enable future testing of complex functional relationships including, but not limited to, brain structure and behavior (37). Second, due to the etiological overlap between clinical phenotypes, broader phenotypes, and traits, studying larger general population samples of people with traits or symptoms might generate novel hypotheses that later can be tested in clinical samples. On the semantic topic of symptoms and traits, throughout this thesis, the term symptom will be used to describe measures derived from diagnostic symptom criteria for a specific condition (i.e., the A-TAC

questionnaire for symptoms of ASD, ADHD, tics and learning difficulties), and traits will be used for measures of behavioral characteristics generally associated with a certain condition, but not derived from diagnostic symptom criteria (i.e., the SRS-2 questionnaire for autistic traits).

1.3 THE LIABILITY THRESHOLD MODEL

The liability threshold model relates to the dimensional model. It assumes that the liability to a dichotomous condition – having or not having a diagnosis – is normally distributed in the population, but that the condition occurs only when a certain threshold of liability is exceeded (Figure 1). The model has been vastly used in twin studies seeking to discern the heritability of a condition, but less so with regards to specific environmental contributions (38).

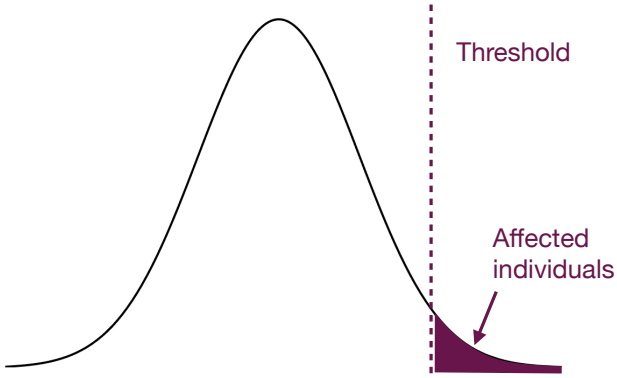

Figure 1. The Liability-Threshold Model

1.4 ENVIRONMENTAL CONTRIBUTIONS TO NDC

Prior research including animal, human cell, and epidemiological studies has suggested a wide range of environmental factors impacting neurodevelopment. One of many factors that has been associated with several NDC (i.e., ID, ASD, and ADHD) is prenatal maternal anemia (39). Also, low birth weight, low gestational age and several exposures during pregnancy have in earlier systematic and non-systematic reviews been suggested as environmental factors common to many NDC, as shown in the following list.

Regarding ID suggested environmental factors are:

- advanced maternal age, pregnancy related factors of maternal alcohol and tobacco use, hypertension, diabetes, epilepsy, and asthma, together with preterm birth and low birth weight (40).

For ASD, suggested environmental factors are:

- advanced parental age, and the pregnancy related factors of altered zinc-copper cycles, immune activation, and steroidogenic activity, and maternal diabetes,

valproate intake, toxic chemical exposure, and treatment with selective serotonin reuptake inhibitors (41).

Regarding ADHD suggested environmental factors are:

- alcohol and cigarette exposure during pregnancy, food additives and diets, lead contamination, and low birth weight (42).

For TD and tic severity, suggested environmental factors are:

- prenatal psychosocial stress, pregnancy nausea, low birth weight and maternal smoking (43).

Regarding reading disabilities, suggested environmental factors are:

- low birth weight and low gestational age.

While inconclusive findings have been found for:

- maternal smoking, risk of miscarriage, and family history of medical and psychiatric diseases (44).

For motor difficulties in childhood, suggested environmental factors are:

- the pregnancy related factors of diabetes, antidepressant medication, alcohol consumption, iron or iodine deficiency, and fish consumption, as well as neonatal complications, low birth weight, and postnatal depression (45).

1.5 THE CUMULATIVE STRESS HYPOTHESIS AND THE THREE-HIT CONCEPT

As noted, several models of underlying genetic and environmental etiologies may be relevant to NDC. Regarding the environment, apart from single environmental factors outlined above, the cumulative stress hypothesis suggests that liability for a given condition, such as NDC (46), is enhanced if adversities accumulate during early life (47). The cumulative stress hypothesis is in turn incorporated as a second hit within the etiological model of the three-hit concept (48). The three-hit concept also includes a first hit of genetic predisposition and a third hit of later-life environment. Evidence for the three-hit concept with regards to ASD has so far only been found in animal studies (49-51). Merging the dimensional model and the liability threshold model, these underlying genetic and environmental factors are assumed to form a continuous distribution of liability to a categorical outcome (52). The cumulative environmental effect on NDC has not been studied in a human sample before.

1.6 THE CAUSALITY DEBATE AND OBSERVATIONAL STUDIES

Since randomized control trials – our gold standard for causal inference – are either not feasible or unethical to perform to elucidate the etiological role of suspected environmental factors of NDC, we are left with observational studies. Observational studies are more prone

to bias. The classic Hill criteria for causal inference from 1965 include the strength of the association, consistency over several studies, specificity of the association, temporality, a dose response gradient, plausibility, coherence to prior knowledge, experiment findings, and analogy (53). Although being a basis of modern medicine and public health, the Hill criteria are debated (54). Most importantly, a strict criterion-based approach will lack the utility and the validity that is necessary in complex multicomponent causal systems – as in the case of NDC (55).

1.7 THE SIGNIFICANCE OF FAMILIAL CONFOUNDING

One major potential bias in the literature disentangling environmental factors in the causal web of NDC is familial confounding. Familial confounders are factors shared within families making family members similar. This includes both measured and unmeasured genetic and shared environmental factors. Since many suggested environmental factors are in themselves heritable, it may be that reported environmental associations are driven by genetic links between the studied exposure and the studied outcome, rather than the environment itself. One way to keep the genetic and shared environmental factors constant, while studying a suspected exposure, is to use twin or sibling studies, either by comparing exposure across relatives discordant for the outcome, or conversely, by comparing the likelihood of a given outcome in relatives differentially exposed to a given factor. Since monozygotic (MZ) twins share 100% and dizygotic (DZ) twins and siblings share 50% of their genome, while also sharing many environmental factors that are difficult to measure, twin and sibling studies hold the potential to separate the true effect of the studied environment from confounding, measured or unmeasured, genetic and environmental factors (56, 57). To exemplify the importance of familial confounding, a meta-analysis estimated the odds ratio (OR) for ASD to be 1.52 (95% CI, 1.09-2.12) following SSRI exposure during pregnancy, with none of the included studies using a genetically informed sample (58). A later epidemiological study suggested an association beyond familial confounding, but the association attenuated significantly in a sibling comparison due to confounding familial factors in the first estimate (59). Another example of this concept is the strong association between ADHD and cigarette smoking during pregnancy. Other factors such as parental intellectual abilities, socioeconomic status, and parental psychiatric problems also predict offspring ADHD, and smoking during pregnancy is influenced by genetic factors in itself (56). Therefore, we may well have a genetic link, or a shared environmental link, explaining the association between smoking during pregnancy and offspring ADHD. This potential bias needs to be accounted for. If we falsely assume that there are no concurrencies of the associations among a pertinent environmental factor, the confounding variables, and the outcome of interest, we potentially induce a bias. The same holds true for many other environmental factors, making control for familial confounding crucially important for causal inference.

When comparing sibling and twin studies, the within pair comparisons among twins, especially those in MZ twin pairs, are generally best suited for adjustment for familial confounding when studying environmental factors. There are, however, situations when

sibling studies are preferred over twin studies. First, siblings are more common than twins. Therefore, it is easier and less costly to gather a large enough cohort of siblings, than that of twins. Second, the twin design makes use of the within pair difference in exposure and outcome, but it is almost impossible to measure prenatal exposure differences in twins sharing the same prenatal environment, and sometimes, as in the case of gestational age, there is no within pair difference to measure. Therefore, regarding prenatal exposures, we are left with studying siblings from different pregnancies, or we could also control for familial confounding using adoptions or in vitro fertilization designs (60). Compared to traditional twin or sibling designs, these designs may examine the environment of family interaction and child development, as well as control for passive gene-environment interaction (i.e., confounding genetic influences on family environmental variables that arise postnatally) (61). It is, therefore, possible to estimate how familial confounding differentially applies to prenatal versus postnatal environmental factors (62). Compared to family designs, adoptions or in vitro fertilization-designs are accompanied by more practical hurdles when gathering a large enough sample, making them more cost demanding and less feasible.

2 RESEARCH AIMS

The overarching aim of this thesis was:

- to explore associations between environmental factors and ASD and other NDC;
- to identify early medical events associated with ASD and other NDC, and;
- to test the hypothesis of a cumulative environmental effect.

2.1 STUDY I

The aim of Study I was:

- to summarize the evidence from twin and sibling studies about the role of environmental factors for NDC, defined both dimensionally and categorically, controlling for familial confounding.

2.2 STUDY II

The aim of Study II was:

- to explore the associations between potential environmental factors, ASD and autistic traits by identifying early medical events, and;
- to test the hypothesis of their cumulative effect on autistic traits, while controlling for familial confounding.

2.3 STUDY III

The aim of Study III was:

- to test the hypothesis of a cumulative effect of environmental factors on ASD and ASD symptoms using a large population-based twin cohort, while controlling for familial confounding.

2.4 STUDY IV

The aim of Study IV was:

- to explore if the association between the cumulative effect of early medical events, beyond familial confounding, was specific for ASD, and;
- to test the hypothesis that the cumulative effect is not specific for ASD, but rather associated with a common latent NDC factor that in turn affects symptoms of NDC, ASD included.

3 MATERIALS AND METHODS

3.1 DESIGN

3.1.1 Systematic Review (Study I)

A systematic review attempts to combine all existing evidence that meets pre-defined eligibility criteria to answer a specific, pre-stated research question. The review aims to minimize bias with a clearly outlined systematic approach that is well documented beforehand (63). As depicted in the hierarchy of evidence pyramid (Figure 2), systematic reviews are generally regarded as a reliable source of evidence, able to assist decision making in clinical practice and guide future research. On top of the pyramid lies meta-analyses, a set of increasingly used statistical techniques where statistical power is gained through the pooling of data from the primary studies included in the systematic review. Synthesis of randomized controlled trials (RCTs) is generally considered as the highest level of clinical evidence. In contrast to RCTs, observational studies (i.e., cohort or case control studies) are more prone to bias and often present greater heterogeneity between studies. Hence, meta-analyses of observational studies may result in a seemingly precise, but incorrect, point estimate (64).

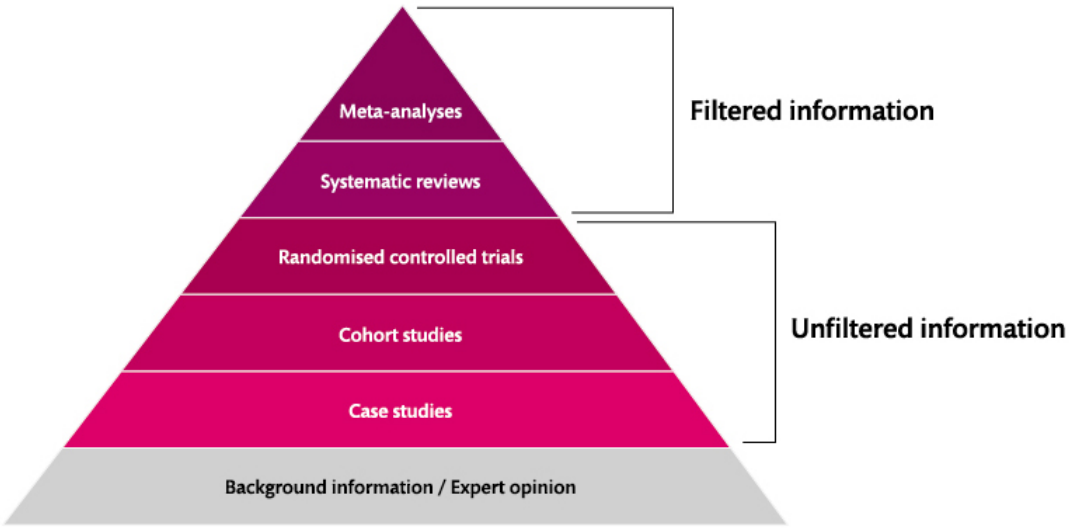

Figure 2. The hierarchy of evidence pyramid

Study I was conducted and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement (65). The protocol was registered in advance with PROSPERO (CRD42018079513) to provide methodological transparency. Details of the method are given in **Study I**.

3.1.1.1 Search strategy

A systematic literature search was performed by two librarians at Karolinska Institutet in October 2017 in the following databases: Medline (Ovid), PsychInfo (Ovid), Embase, Web of Science Core Collection, and Cochrane Library. The search was updated in March 2019 for recently published articles.

3.1.1.2 Eligibility criteria – PECOS: Population, Exposure, Comparator, Outcome, Study design

Peer-reviewed case-control and cohort studies, including twin or sibling comparison, published in English were eligible for inclusion. Case-control studies included twins or siblings discordant for one or more NDC according to the DSM-5(3), with the unaffected or less affected twin or sibling as the comparator. Cohort studies included twins or siblings discordant for exposure and with one or more NDC as the outcome. Studies with a specified environmental factor with exposure time up to the age of 5 years were included. Eligible studies included one or more of the NDC as defined in DSM-5 as outcomes (ASD, ADHD, ID, CD, SLD, DCD and TD). The outcomes could either be reported as diagnoses or symptom or trait severity. Eligible studies reported the within pair association of the exposure with one or more NDC, or with symptom or traits severity.

3.1.1.3 Study selection and data extraction

The titles and abstracts of all references were screened independently by two reviewers. Publications found to be of potential relevance by at least one of the reviewers were obtained in full text and assessed for eligibility independently by two reviewers. Main study characteristics and results were extracted independently based on the Cochrane EPOC Data Collection Checklist (63). Extracted information was the following: author; publication year; country; study design; study cohort; sample size; sex; age; sibling or twin control; condition(s) studied; environmental factor(s) studied; study methodology; recruitment method; completion rates; missing data; outcome(s) and type of measure(s); and the main results.

3.1.1.4 Risk of bias assessment

The overall risk of bias of each study was rated according to the Newcastle-Ottawa Scale (NOS) for longitudinal case control and cohort studies (66). The NOS were chosen over other risk of bias instruments as a consistent tool easy to adapt to both case-control and cohort studies.

3.1.1.5 Synthesis

Identified environmental factors were sorted according to chronology (prenatal; perinatal/neonatal; and infancy/childhood) and grouped by category for readability. For studies with categorical NDC outcomes, the relevant estimated association(s) were extracted. Since studies with dimensional measures routinely reported several estimated associations, an

evaluation of these studies was conducted to determine if the overall findings provided a signal of an association or not (yes; possibly; or no).

A narrative synthesis of the eligible studies for each NDC was performed. When appropriate, meta-analyses of the results on specific environmental factors and conditions were planned, unless prevented by heterogeneity of the included studies' exposures, study characteristics, or data presentation (63).

3.1.2 Co-Twin Control Design (Study II-IV)

The co-twin design is a powerful tool to elucidate the effect of a putative environmental factor, while controlling for familial confounding. This effect can be demonstrated either in a cohort study by comparing the likelihood of a given outcome in twins differentially exposed to a given factor, or in a case-control study by comparing exposure(s) between twins discordant for the outcome.

One way of depicting this is to use the Directed Acyclic Graphs (DAG) developed by Pearl (67). DAGs enable a graphical depiction of the underlying theory, and the following reasonable assumptions. In a DAG, prior knowledge about variables of interest and their inter-correlations are laid out as boxes and paths (or absence of a path if the assumption is that no correlation between two variables exist). Figure 3A shows in a schematic way all variables and paths of interest in a co-twin design. In a case where we do not control for shared factors within a family, the direct causal path (blue) and the indirect causal path (yellow) are left open, along with a biasing path through familial confounding (68). In contrast, when applying adjustment for within pair confounding, we can distill an eventual correlation to only catch the direct causal path between the exposure and outcome, thus making possible for us to draw conclusions about a potential environmental origin for some of the outcome-variability in the population (Figure 3B).

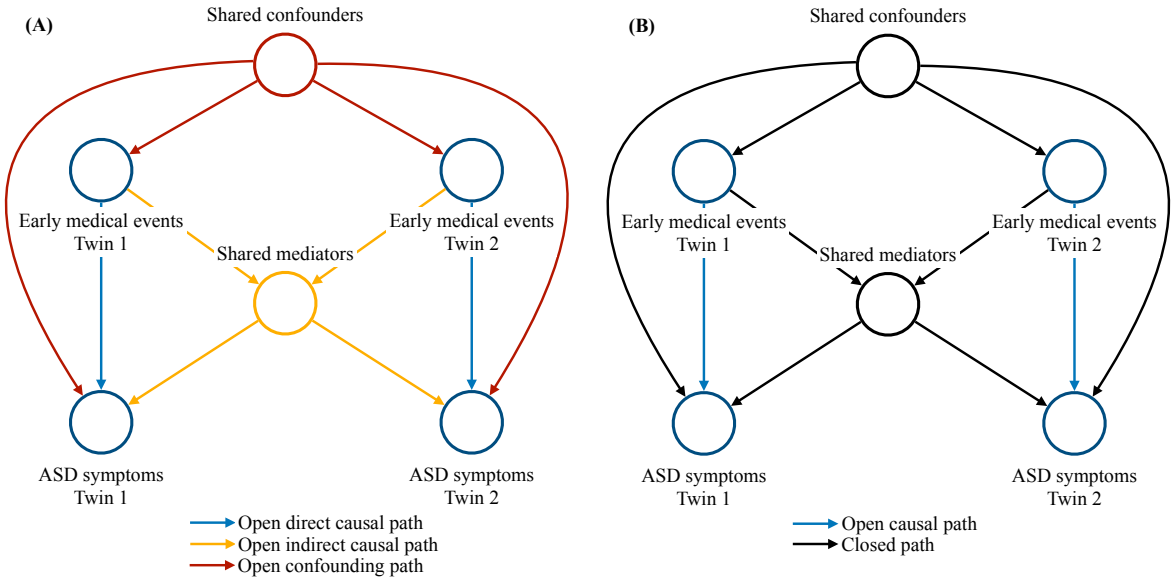

Figure 3. (A) Directed Acyclic Graph (DAG) illustrating causal (blue and yellow) and non-causal (red) influences on an observed correlation between early medical events (exposure) and ASD symptoms (outcome), when the model is unadjusted for the familial confounding. A potential correlation will be the sum of the true causal path (blue), the path from the exposure via shared mediators (yellow), and the open path due to unadjusted shared confounders (red). **(B)** DAG illustrating how a model that adjusts for the twin relationship leaves only the direct causal path (blue) open between the exposure and the outcome, while closing (black) the contribution from known and unknown shared confounders and mediators.

3.2 SUBJECTS

3.2.1 The Roots of Autism and ADHD Twin Study Sweden (RATSS)

Twins in the Roots of Autism and ADHD Twin Study Sweden (RATSS) (69) are recruited from four sources. 1) The primary resource is the Child and Adolescent Twin Study in Sweden (CATSS) (70), with 45.5% of the sample's origin. 2) The patient registry of the Swedish Board of Health and Welfare. 3) The clinical registries of the Division of Child and Adolescent Psychiatry, the Habilitation and Health centers, and pediatric units in Stockholm County. 4) Advertisement to autism societies and twin organizations, and in media.

The first step for **Study II** was to use an exclusive ASD discordant MZ twin sample of 13 MZ twin pairs discordant for clinical ASD and 13 MZ typically developing control pairs (n = 52 individuals) matched for sex (16 males and 10 females in each group). In a second hypothesis-testing step, 100 twin pairs quantitatively discordant for autistic traits were included (54 MZ pairs and 46 DZ pairs). See **Study II** for details.

3.2.2 The Child and Adolescent Twin Study in Sweden (CATSS)

Twins for **Study III and IV** were recruited from the longitudinal, population-based CATSS-study, which was initiated in 2004 (70). In CATSS, all parents of twins aged 9 years (earlier cohorts included 12-year-olds) born in Sweden are invited to report on the twins'

neurodevelopmental symptoms using a validated structured interview. Study III included a cohort of 15,701 MZ and DZ twin pairs, and **Study IV** included 10,254 MZ and same-sex DZ twin pairs, with data collected from individuals born in every year between 1992 and 2008 (see **Study III and IV** for details). CATSS has a participation rate of 75% since 2004, and selected sample characteristics have been shown to be representative for the general population in Sweden (71).

3.3 MEASUREMENTS IN STUDIES

3.3.1 Measurements in RATSS (Study II)

3.3.1.1 Medical history

For the exploratory first step of analyses comparing ASD discordant MZ twins to typically developing MZ twin controls, detailed information on medical and developmental history with a focus on the first 5 years of life was collected from parent reported questionnaires and the twins' complete medical records. Intraclass Correlation Coefficient (ICC) analysis showed good agreement between the questionnaire and the medical record information. The complete medical records comprised of prenatal records, birth records, pediatric clinic records, and medical and psychiatric care unit documentation.

Medical history in the total sample was assessed from the parent reported questionnaire. The 114 items of the questionnaire covered medical history factors such as current diagnosis and medications, family situation at birth, family medical history, pre-, peri- and postnatal factors, child disease history, and diagnostic tests.

Intra-pair differences for the frequency and age of onset for developmental alterations, medical complications, and life factors were noted. Registered medical history factors were coded binary ('1' for present, '0' for not present in each individual). In addition, the medical history factors were categorized according to the type of factors and summarized into an ordinal cumulative load. All medical history factors were identified as differing within ASD discordant pairs (that is, present in only one twin in a pair) by all four researchers, were added up to generate a cumulative load of early medical factors for each participant.

3.3.1.2 Diagnostic assessment

The participants were diagnosed by three experienced clinicians according to DSM-5 criteria using clinical consensus supported by results from a neuropsychiatric evaluation based on ADI-R and ADOS-2 for ASD criteria, the Kiddie-SADS, or the DIVA for ADHD criteria, the WISC or WAIS, 4th Editions, or the Leiter-revised scales in combination with the Peabody Picture Vocabulary Test, 3rd Edition and the parent-rated ABAS-2, to assess general cognitive and verbal abilities and adaptive functional level. Autistic traits were measured by the parent report version of the Social Responsiveness Scale- 2 (SRS-2) (see **Study II** for details).

3.3.2 Measurements in CATSS (Study III and IV)

3.3.2.1 Early medical events

The early medical events of low birth weight, congenital malformations, and perinatal respiratory stress were examined. These early, adverse environmental events were chosen as they yielded associations with ASD beyond familial confounding in the systematic review of **Study I**. Since paternal age does not differ within twin pairs, this factor was not included. We linked CATSS to the Swedish Medical Birth Register (MBR), which covers more than 90% of all deliveries in Sweden (72), and the National Patient Register (NPR), which records inpatient diagnoses (with nationwide coverage from 1987) and outpatient diagnoses from 2001 (73), with follow-up to November 30, 2018. From there we obtained detailed obstetric and neonatal information, as well as all diagnosis codes of interest for all participants throughout their lives. A binary variable was created to indicate whether each factor was present or not for each participant by identifying diagnostic codes for each medical factor according to the International Classification of Diseases, Ninth Revision (ICD-9; 1987–1996) and Tenth Revision (ICD-10;1997–2013), and from relevant obstetric information from the MBR and CATSS parental interview using SAS version 15.1. To create an ordinal cumulative exposure load variable of early medical events, the presence of binary factors was summed up for each participant. See **Study III and IV** for details.

3.3.2.2 Diagnostic assessment

3.3.2.2.1 Diagnosis of ASD (Study III)

All diagnosis codes for pervasive developmental disorders under ICD-10 code F84 were extracted from the NPR and coded binary for each participant, excluding Rett Syndrome (F84.2), other childhood disintegrative disorders (F84.3), and overactive disorder associated with intellectual disability and stereotyped movements (F84.4). The validity of the registry-based diagnosis is high (73, 74).

3.3.2.2.2 Symptoms of NDC (Study III and IV)

All participants were evaluated for ASD (**Study III**) and NDC (**Study IV**) symptoms at the age of 9 using the Autism-Tics, ADHD and other Comorbidities inventory (A-TAC) (75). Its validity is well established through clinical and population-based samples, with excellent predictive properties for ASD (area under the curve (AUC=0.98), and ADHD (AUC=0.93), and good for tics (AUC=0.86) and learning difficulties (AUC=0.87) (76, 77). Items can be answered yes (scored as 1), yes, to a certain degree (0.5), or no (0). Seventeen items address ASD. The sample distribution of the score is skewed, ranging from 0 to 17. Nineteen items address ADHD with a similarly skewed sample distribution, three items address tics, and three items address learning difficulties. For **Study III**, a series of binary outcomes was created for each 5th percentile of ASD symptom level, from the 55th to the 95th percentile with a "1" designated for individual scoring above each percentile cut off, and a "0" if scoring below.

3.4 STATISTICAL ANALYSIS

3.4.1 Co-Twin Control Design – the MZ ASD diagnosis discordant subsample (Study II)

In the ASD discordant MZ subsample of **Study II**, owing to the sample size, nonparametric Wilcoxon signed-rank test for continuous and ordinal data and McNemar's test for binary data were used to assess the within-pair effect for the identified medical events. All the tests were two-tailed.

3.4.2 Co-Twin Control Design – Generalized Estimation Equations (GEE) (Study II-IV)

In the second, hypothesis testing step of **Study II**, and in **Study III and IV**, conditional regressions were performed using generalized estimation equations (GEE), with doubly robust sandwich estimators (*R* package drgee) (78, 79). GEE accounts for related individuals in the sample. In **Study II,** a conditional linear regression model was fitted estimating the within-pair effect, adjusting for full-scale IQ, ADHD diagnoses (binary), and sex. In addition, the sample was split into zygosity groups comparing the within-pair effects in the MZ and DZ pairs. This adjusts for all factors shared within twins, which in MZ twins includes all genetic and shared environmental influences, and in DZ twins, it includes approximately 50% of genetic influences and all shared environmental influences. As such, these models adjust for familial confounding. In **Study III and IV,** regressions were performed first between all individuals in the sample and then within twin pairs. In **Study III,** a series of logistic regressions were performed for every autistic symptom percentile, and for **Study IV,** linear regressions were used.

Of interest in a within twin analysis is the within-cluster mean difference in the outcome, comparing exposed and unexposed twins. In within-cluster mean difference analysis, the only informative twin pairs in a within twin analysis are the outcome discordant pairs that are simultaneously exposure discordant. The simultaneous outcome and exposure discordancy is either in the direction that the twin with more symptoms has more exposures than their co-twin, or reversed, that the twin with more symptoms has less exposures. When adjusting for covariates, twins that are discordant on covariates and outcome will also be informative since the regression model treats exposures and covariates equally.

When using linear regressions, as **in Study II and IV**, the degree that individuals within clusters are outcome discordant matters. In **Study III**, however, even though looking at different outcome levels, the approach made use of logistic regression where the outcome discordancy is binary.

A within twin analysis is a special case for the doubly robust GEE estimator, discussed as follows by its creators, regarding unadjusted analysis (79):

"We observe that clusters with little variation in the exposure will contribute less to the [DRGEE] estimator than clusters with high variation in the exposure. In particular, if $n_i = 2$ for all i, then the [DRGEE] estimator further reduces to

$$\frac{\sum_{i=1}^{m}(X_{i1}-X_{i2})(Y_{i1}-Y_{i2})}{\sum_{i=1}^{m}(X_{i1}-X_{i2})^2},$$

so that only the exposure-discordant pairs (i.e., those pairs for which $X_{i1} \neq X_{i2}$) contribute to the estimator."

The "$n_i = 2$ for all i " is true in our samples, since all clusters consists of two twins in a pair. Thus, for the within pair analysis in a conditional regression, the informative twin pairs are only those that are simultaneously discordant for exposure/covariates ($X_{i1} \neq X_{i2}$) and outcome ($Y_{i1} \neq Y_{i2}$). However, to make a sound interpretation – and perhaps a causal interpretation– of a within-twin analysis, it is important to first perform a between all analysis and establish that an association exists. Thus, we cannot overall do this analysis without the concordant pairs statistically or scientifically.

3.4.3 Confirmatory Factor Analysis (CFA) (Study IV)

Early in the last century, Spearman (80, 81) constructed the first factor model that allowed for testing of a latent unobserved factor for level of human intelligence by collecting other testable data. This was in 1969 further developed by Jöreskog (82) into confirmatory factor analysis (CFA). CFA is based on theory and/or prior research, where the objective is to test whether the data fit a hypothesized pre-defined measurement model. **Study IV** made use of CFA with the hypothesis that the environmental cumulative effect of early medical events associated with ASD beyond familial confounding (**Study III**) is not specific for ASD, but rather, that a cumulative effect is associated with a common latent NDC factor that in turn affects symptoms of NDC, ASD included (Figure 4).

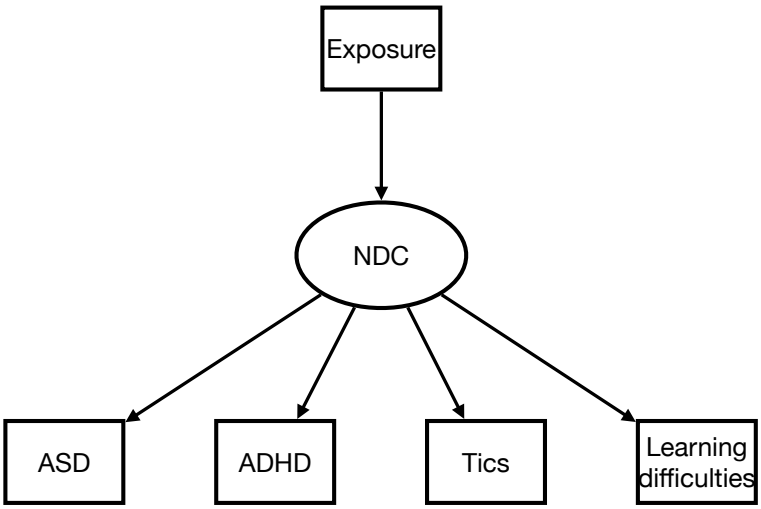

Figure 4. A common latent NDC factor model for confirmatory factor analysis in Study IV. The latent (unobserved) factor is depicted as a round block and observed factors as square blocks.

CFA was performed using *R* version 4.1.1 and the lavaan package version 0.6-9 (83), to model a common latent NDC factor that captures variance common to ASD, ADHD, tics, and learning difficulties A-TAC subscales. Standardized factor loadings for each A-TAC subscale and fit statistics were calculated using maximum likelihood estimation with robust standard errors and a Satorra-Bentler scaled test statistic. Scaled and robust root mean square error of approximation (RMSEA) were used to evaluate model fit, with RMSEA at 0.05 considered good.

3.4.3.1 Between all

First, the effect of the standardized common latent NDC factor was regressed out from each A-TAC subscale, respectively, using linear regressions, creating residual outcome variance scores for each individual. Second, a linear regression of the common latent NDC factor on level of cumulative exposure was performed, both crude and adjusted for sex and birth year. Third, linear regressions of the respective outcome residuals on level of cumulative exposure, were performed to test the degree to which cumulative exposure was associated with each NDC after adjusting for the common latent NDC factor.

3.4.3.2 Within twin

Using the twin difference design (84) to control for genetic and shared environmental influences, while modelling a common latent NDC factor, we calculated within twin pair difference scores for each twin pair, both for the cumulative exposure and for each A-TAC subscale. A linear regression of the standardized common latent NDC factor on level of cumulative exposure twin difference was performed. To test the degree to which within twin pair cumulative exposure difference was associated with each A-TAC subscale difference

after adjusting for the common latent NDC factor, linear regressions were performed on the respective residual outcome difference variances on level of cumulative exposure difference. Finally, to fully account for familial confounding, the same approach was used for a within twin analysis grouped by zygosity, with the above steps reperformed on MZ and DZ twin pairs separately.

3.5 ETHICAL CONSIDERATIONS

This project raises several highly relevant ethical issues related to research in humans and their privacy. It includes children, with comprehensive assessments of behavior and medical history. All parts of the project have been approved in full by the national Swedish, responsible regional ethical review board (RATSS: Dnr 2016/1452-31; CATSS: Dnr 2016/2135-31, Dnr 2018/2013-32, Dnr 2020-04248).

The experience from the RATSS study is that children, adolescents, and their parents usually find it stimulating and interesting to be interviewed and to carry out various tasks in connection with psychological testing. At the same time, it can also be strenuous, concerning, and time consuming to undergo a large battery of tests, interviews, and observations. Therefore, it is important to adapt the procedures to each subject's pace and need for breaks with food and drink and rest. It is also important to be responsive to the subjects' questions both during and after the examination. In some cases, a neuropsychiatric diagnosis will be made for the first time and then the research team must assist with counseling and referral to appropriate activities where necessary measures and efforts can be offered.

For most of the subjects, there is no direct benefit from being part of the project. However, unmet clinical needs and new relevant psychological and medical information about the participants can be identified throughout the study. In such cases, a referral may be made to the appropriate institution within the health care or other institution and thereby improve the participant's life situation.

Participation in a study, and especially in a longitudinal study, can make a subject feel stigmatized. Participation can also arouse both unjustified hope for cure and concern about the personal situation. On the other hand, participation can give a sense of security because you are under the observation of experts, have access to special information as well as contribute to increased self-esteem by participating in a study that could prove significant.

The framing of NDC as a less optimal outcome raises critical ethical questions, not least because of the dimensional model of autism where various degrees of autistic traits may in certain circumstances come with benefits (85). Both RATSS and CATSS encourages constructive collaborations between people with NDC, their parents, and researchers to serve the community's interests and accommodate the varied experiences and preferences of people with NDC and their families.

4 RESULTS

4.1 STUDY I

In the comprehensive systematic review of **Study I**, a total of 140 studies were identified for inclusion. The search provided 7,315 unique citations. After reviewing the abstracts, 254 citations were examined in full text, and of these, 114 did not meet the eligibility criteria and were excluded. The included texts were 58 studies (22 cohort and 36 case control studies) on ASD, 69 studies (53 cohort and 16 case control studies) on ADHD, 26 studies (21 cohort and five case control studies) on ID or a dimensional measure of IQ, 13 studies (12 cohort and one case control study) for DCD, eight studies (seven cohort and one case control study) for CD, two studies for TD, and no relevant studies for SLD.

In summary, and beyond familial confounding, low birth weight, congenital malformations, advanced paternal age, and perinatal respiratory stress are consistently associated with a diagnosis of ASD, and low birth weight, low gestational age, and low family income or income decline during childhood is associated with ADHD, both categorically and dimensionally. On the contrary, the result points in the direction of evidence of *no* association beyond familial confounding regarding ASD and the pregnancy and delivery related factors of maternal uterine bleeding, preeclampsia, gestational diabetes, pre-pregnancy body mass index, and elective and emergency cesarean section; nor regarding a diagnosis of ADHD with the pregnancy related factors of antidepressive medication, maternal infection, maternal body weight, and maternal smoking during pregnancy.

Studies with conflicting findings beyond familial confounding were found regarding the associations of antidepressive medication during pregnancy, advanced maternal age, preterm birth, labor induction, and neonatal jaundice with ASD, and alcohol use during pregnancy, and parental age with ADHD, both categorically and dimensionally. Of all 58 studies on ASD, only two studies used a dimensional measure of ASD symptoms.

Single studies, not yet replicated, suggest potential associations beyond familial confounding for ASD diagnosis with measles or mumps infections during pregnancy, metal uptake in uterus (lead and manganese), low serum level of vitamin D at birth, a parity greater than two, neonatal incubation and neonatal respiratory infection, recurrent infections in childhood, dysregulation during first year of life, and medical events the first 5 years of childhood; for ADHD diagnosis with head circumference at birth, orofacial clefts, composite score of pre-, peri-, and neonatal complications, parental divorce and maternal depression during early childhood, and; for different ADHD-symptoms with paracetamol exposure, history of miscarriage, neonatal heart surgery, hypothyroidism, neuroblastoma, and higher levels of phenylalanine exposure. Overall, there is a lack of geographic distribution, with most studies being conducted in Scandinavia and North America.

4.2 STUDY II

From the in-depth investigation of medical records, a list of 31 early medical events was found to differ within the 13 ASD discordant MZ twins. Statistically significant differences within the pairs were dysregulation during the first year of life (i.e., feeding and sleeping problems, excessive crying and worrying; Z = -2.56, P=0.011), birth weight (Z=-2.20, P=0.028), and the cumulative load of early medical events (Z=-2.85, P=0.004). None of these intra-pair differences were observed in the 13 typically developing MZ pairs.

Tested in the whole RATSS sample, autistic traits were associated with dysregulation during the first year of life (β=31.75, SE=16.2), and the cumulative load of the 32 early medical events (β=78.18, SE=26.59). An effect indicating an intra-pair difference of three points for autistic traits on the SRS-2 scale for every single medical event's difference. No significant gender effect was found.

4.3 STUDY III

Between all participants, a higher level of early cumulative exposure to medical events was associated with a diagnosis of ASD, with a sex and birth year adjusted OR 1.17 (95% CI, 0.94–1.45) for one exposure, OR 1.88 (1.42–2.48) for two exposures, and OR 3.33 (1.79–6.20) for three exposures. Furthermore, a higher level of early cumulative exposure was consistently associated with having more autistic symptoms, ranging from OR 1.20 (1.13–1.27) at the 55th autistic symptom percentile to OR 1.45 (1.28–1.65) at 95th percentile for one exposure, from OR 1.39 (1.26 –1.53) to OR 1.68 (1.40–2.02) for two exposures, and from OR 2.12 (1.57–2.86) to OR 3.39 (2.2–5.24) for three exposures (Figure 5).

The association to a diagnosis of ASD seen in the unconditional, between all, logistic regressions attenuated for exposure levels one and two, when adjusted for familial confounding and sex, with OR 0.92 (0.60–1.42) for one exposure, and OR 0.91 (0.39–2.16) for two exposures. For the level of three exposures, however, the odds ratio remained similar to that of the unconditional, between all, association, with OR 2.39 (0.62–9.24), although not statistically significant at the p=0.05 level. Higher loads of early cumulative exposure in one twin was consistently associated with having more autistic symptoms than their co-twin at every symptom level cut-off, after adjusting for familial confounding and sex, with increasing ORs with each increasing symptom level ranging from 1.35 (1.17–1.55) at the 55th symptom percentile to OR 1.52 (1.14–2.03) at 95th percentile for one exposure, from OR 1.50 (1.11–2.02) to OR 2.03 (1.16–3.58) for two exposures, and from OR 3.45 (1.66–7.15) to OR 7.36 (1.99–27.18) for three exposures (Figure 5).

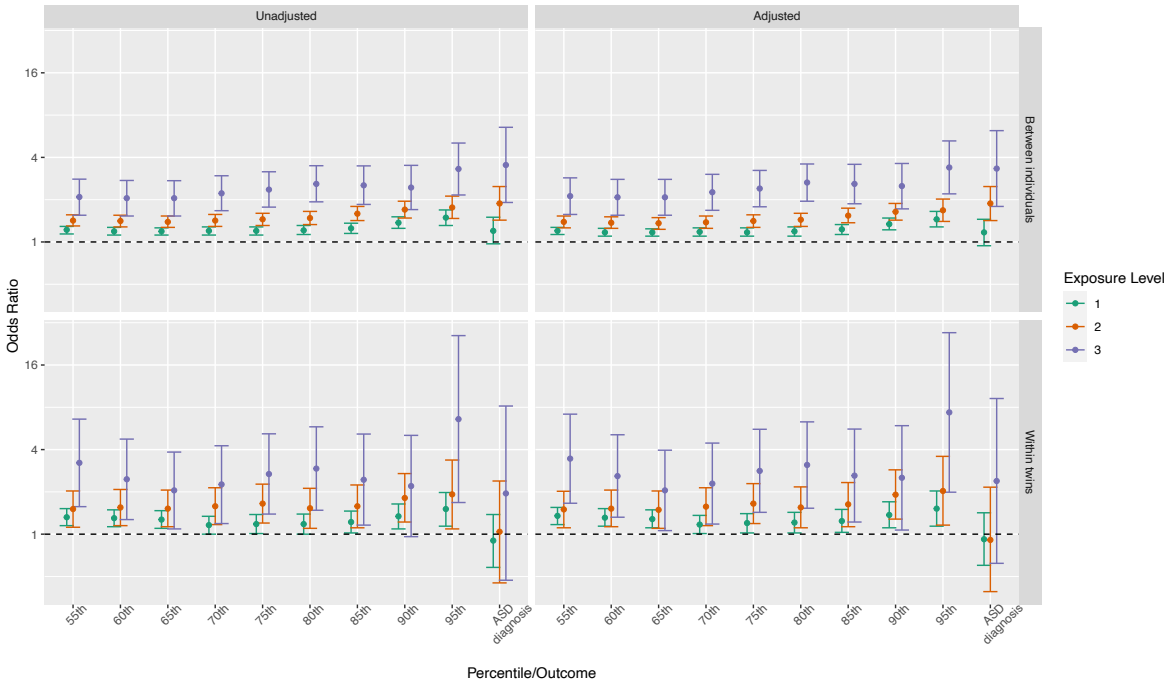

Figure 5. Between individual (upper panels) and within twin-pair (lower panels) associations between the cumulative exposure level of early medical events and a diagnosis of ASD, and being above each percentile cut-off of ASD symptoms, respectively. Forest plots illustrating odds ratios (ORs, dots) and 95% confidence interval (CI, bars) for unadjusted associations to each exposure level (left panels), and sex and birth year adjusted between individual (upper right panel) and familial confounding and sex adjusted (lower right panel) within twin pair associations (**Study III**).

4.4 STUDY IV

CFA were used to fit the three correlated factor models to the four A-TAC subscale outcomes and all models fit the data well (see **Study IV** for details).

4.4.1 The Common Latent NDC Factor Model – Between All Participants

There was a non-linear increase in the standardized common latent NDC factor by the level of exposure, with ß=0.12 (95%CI, 0.07–0.17) for exposure level 1, ß=0.25 (95%CI, 0.17–0.33) for exposure level 2, and ß=0.62 (95%CI, 0.34–0.90) for exposure level 3. Residual exposure effects on the respective outcomes not captured by the common latent NDC factor were all small or negative at all exposure levels, with slightly larger and the only statistically significant effects found for residual learning difficulties and the exposure levels 2 (ß=0.10 (95%CI, 0.07–0.13)) and 3 (ß=0.26 (95%CI, 0.13–0.38)) (Table 1).

4.4.2 The Common Latent NDC Factor Model – Within Twin Pairs

When accounting for familial confounding, the associations of the standardized common latent NDC factor on the level of cumulative exposure difference in the whole sample existed beyond familial confounding, with ß=0.10 (95%CI, 0.05–0.16) for exposure difference level 1, and ß=0.25 (95%CI, 0.05–0.45) for exposure difference level ≥2. Residual exposure effects

not captured by the common latent NDC factor on the respective residual outcome variances were all small or negative at all exposure difference levels (Table 1).

4.4.3 The Common Latent NDC Factor Model – Within Twin Pairs Split by Zygosity

The associations of the standardized common latent NDC factor on the level of cumulative exposure difference were statistically significant for the MZ subsample with ß=0.08 (95%CI, (0.03–0.13)) for exposure level difference 1, and ß=0.25 (95%CI, 0.10–0.41) for exposure level difference ≥2, and for the DZ subsample with ß=0.11 (95%CI, 0.02–0.21) for exposure level difference 1, but not for exposure level difference ≥2 (ß=0.20 (95%CI, -0.22–0.63)), although with a similar effect size. Residual exposure effects on the respective outcomes not captured by the common latent NDC factor were similar, but in some instances, slightly larger in the DZ subsample compared to the whole sample. When familial confounding was fully accounted for, as in the MZ subsample, the residual associations for all outcomes attenuated completely at all levels of cumulative exposure difference (Table 1).

Table 1. Linear regressions of common latent NDD factor and residual outcome variances on level of cumulative exposure

Mode/Sample		Regression	Exposure level 1 ß	Exposure level 1 (95% CI)	Exposure level 1 SE	Exposure level 1 *p*	Exposure level 2 ß	Exposure level 2 (95% CI)	Exposure level 2 SE	Exposure level 2 *p*	Exposure level 3 ß	Exposure level 3 (95% CI)	Exposure level 3 SE	Exposure level 3 *p*
Between all	Crude	Exp~NDD	**0.14**	**(0.09–0.19)**	**0.03**	**<0.001**	**0.28**	**(0.20–0.36)**	**0.04**	**<0.001**	**0.63**	**(0.35–0.91)**	**0.14**	**<0.001**
		Exp~resid. ASD	*0.00*	*(-0.03–0.02)*	*0.01*	*0.876*	***-0.06***	***(-0.10–-0.01)***	*0.02*	*0.008*	*-0.09*	*(-0.24–0.05)*	*0.08*	*0.216*
		Exp~resid. ADHD	*0.00*	*(-0.06–0.05)*	*0.03*	*0.927*	*0.01*	*(-0.08–0.10)*	*0.05*	*0.798*	*-0.17*	*(-0.51–0.16)*	*0.17*	*0.302*
		Exp~resid. Tics	*-0.02*	*(-0.03–0.00)*	*0.01*	*0.040*	***-0.03***	***(-0.06–-0.01)***	*0.01*	*0.010*	*-0.06*	*(-0.15–0.02)*	*0.04*	*0.160*
		Exp~resid. LD	*0.02*	*(0.00–0.04)*	*0.01*	*0.068*	***0.09***	***(0.06–0.12)***	*0.02*	*<0.001*	***0.27***	***(0.14–0.39)***	*0.06*	*<0.001*
	Adjusted	Exp~NDD[1]	**0.12**	**(0.07–0.17)**	**0.03**	**<0.001**	**0.25**	**(0.17–0.33)**	**0.04**	**<0.001**	**0.62**	**(0.34–0.90)**	**0.14**	**<0.001**
		Exp~resid. ASD	*0.00*	*(-0.02–0.02)*	*0.01*	*0.982*	***-0.06***	***(-0.10–-0.01)***	*0.02*	*0.010*	*-0.09*	*(-0.24–0.06)*	*0.08*	*0.228*
		Exp~resid. ADHD	*-0.01*	*(-0.06–0.05)*	*0.03*	*0.810*	*0.00*	*(-0.09–0.09)*	*0.05*	*0.996*	*-0.16*	*(-0.49–0.17)*	*0.17*	*0.330*
		Exp~resid. Tics	*-0.02*	*(-0.03–0.00)*	*0.01*	*0.024*	***-0.03***	***(-0.06–-0.01)***	*0.01*	*0.007*	*-0.06*	*(-0.15–0.02)*	*0.04*	*0.152*
		Exp~resid. LD	*0.02*	*(0.00–0.04)*	*0.01*	*0.046*	***0.10***	***(0.07–0.13)***	*0.02*	*<0.001*	***0.26***	***(0.13–0.38)***	*0.06*	*<0.001*

			Exposure difference level = 1 ß	Exposure difference level = 1 (95% CI)	Exposure difference level = 1 SE	Exposure difference level = 1 *p*	Exposure difference level ≥ 2 ß	Exposure difference level ≥ 2 (95% CI)	Exposure difference level ≥ 2 SE	Exposure difference level ≥ 2 *p*
Within twins	Whole sample	Exp~NDD	**0.10**	**(0.05–0.16)**	**0.03**	**<0.001**	**0.25**	**(0.05–0.45)**	**0.10**	**0.016**
		Exp~resid. ASD	*0.00*	*(-0.04–0.04)*	*0.02*	*0.950*	*0.03*	*(-0.04–0.04))*	*0.07*	*0.652*
		Exp~resid. ADHD	*0.03*	*(-0.07–0.14)*	*0.05*	*0.519*	*-0.15*	*(-0.53–0.23)*	*0.20*	*0.443*
		Exp~resid. Tics	*0.00*	*(-0.03–0.02)*	*0.01*	*0.932*	***-0.12***	***(-0.21–-0.03)***	*0.05*	*0.013*
		Exp~resid. LD	*-0.01*	*(-0.04–0.02)*	*0.01*	*0.466*	*0.10*	*(0.00–0.21)*	*0.05*	*0.043*
	MZ	Exp~NDD	**0.08**	**(0.03–0.13)**	**0.03**	**0.003**	**0.25**	**(0.10–0.41)**	**0.08**	**0.001**
		Exp~resid. ASD	*0.04*	*(-0.02–0.09)*	*0.03*	*0.191*	*0.00*	*(-0.16–0.16)*	*0.08*	*0.987*
		Exp~resid. ADHD	*-0.08*	*(-0.19–0.02)*	*0.05*	*0.109*	*0.01*	*(-0.30–0.32)*	*0.16*	*0.941*
		Exp~resid. Tics	*0.02*	*(-0.01–0.06)*	*0.02*	*0.191*	*-0.04*	*(-0.15–0.06)*	*0.05*	*0.411*
		Exp~resid. LD	*0.00*	*(-0.03–0.03)*	*0.01*	*0.829*	*0.01*	*(-0.07–0.10)*	*0.05*	*0.744*
	DZ	Exp~NDD	**0.11**	**(0.02–0.21)**	**0.05**	**0.020**	0.20	(-0.22–0.63)	**0.22**	**0.344**
		Exp~resid. ASD	*-0.02*	*(-0.08–0.04)*	*0.03*	*0.549*	*0.14*	*(-0.12–0.40)*	*0.13*	*0.298*
		Exp~resid. ADHD	*0.14*	*(-0.03–0.32)*	*0.09*	*0.111*	*-0.63*	*(-1.41–0.15)*	*0.40*	*0.115*
		Exp~resid. Tics	*-0.02*	*(-0.06–0.02)*	*0.02*	*0.346*	***-0.23***	***(-0.41–-0.06)***	*0.09*	*0.008*
		Exp~resid. LD	*-0.01*	*(-0.06–0.03)*	*0.02*	*0.575*	***0.26***	***(0.06–0.47)***	*0.10*	*0.011*

[1] Adjusted for sex (ß=0.73, SE=0.03, p=<0.001) and birthyear (ß=1.02, SE=0.00, p=<0.001)

Exp~resid. Regression of exposure level and residual variance, *NDD* Neurodevelopmental disorder latent factor, *ASD* Autism spectrum disorder symptoms, *ADHD* Attention deficit hyperactivity disorder symptoms, *LD* Learning difficulties, *MZ* Monozygotic, *DZ* Dizygotic

5 DISCUSSION

5.1 PRESENT KNOWLEDGE FROM TWIN AND SIBLING STUDIES ON ENVIRONMENTAL ETIOLOGIES OF NDC (STUDY I)

Study I is important as a first comprehensive systematic review including 140 articles in the growing field of research trying to rule out familial confounding in the search for causal environmental factors for NDC. Lines of discussion, otherwise hard to be made, are possible thanks to the systematic review's broad approach on all NDC, rather than a single diagnosis only, its inclusion of studies on both dimensional and categorical outcomes, and its diversity of the studied exposures ranging from pregnancy to early childhood.

Regarding the comparison of categorical and dimensional outcomes, three discussions follow. First, fetal growth showed an association to the level of IQ, but not a diagnosis of ID. It could, therefore, be a different environmentally driven mechanism behind a clinical diagnosis of ID compared to differing IQ levels. This is in line with previous research suggesting that severe ID is separate from milder ID, with differing genetic and environmental underpinnings (86). Second, it is notable that for smoking during pregnancy, despite no evidence of it being associated to a diagnosis of ADHD beyond familial confounding, three of the four studies looking at dimensional outcomes noted a link to symptoms of hyperactivity and impulsivity, but not to inattention. Therefore, these symptoms might have different underlying environmental etiologies. More so, previous research has implicated these dimensions differentially in neuropsychological impairment (87) and suggested they may have distinguishable underlying pathways (88-91). Third, **Study I** cannot answer whether this holds true also for different environmental factors and symptom dimensions of ASD. First, only two of the included studies on ASD used a dimensional measure; and second, those two only used a combined measure of total ASD symptom severity, not separated on social and restrictive/repetitive symptoms, respectively. It has previously been shown that social and restrictive/repetitive symptoms of ASD are genetically dissociable (92). However, it remains unclear if symptom dimensions of ASD are environmentally dissociable. Although the value of a dimensional approach in NDC research is clear, dimensional data do not necessarily have clinical significance. There could be a shift in mechanisms along the symptomatic continuum.

Study I invites a discussion regarding mechanisms linked to the fathers' age at conception. Paternal age has been shown to correlate to the number of de novo mutations in offspring (93), which in turn is linked to ASD (94-96), making it a possible explanation. Low family income, or income decline during the first years of life being associated with ADHD beyond familial confounding, is an important finding in times of global economic hardship in the wake of the COVID-19 pandemic (97). As for possible mechanisms, prenatal stress in rodent models, induced by exposure to bright light or sleep deprivation, produce offspring with higher levels of pro-inflammatory markers and disturbed functional brain connectivity, synaptic pruning, and neurogenesis, together with ADHD-like behaviors (98). Importantly, without the result showing an association beyond familial confounding, such link to animal model mechanistic findings would be too speculative. The risk of bias due to familial

confounding is clear since evidence exists of a strong association between low socio-economic status and the prefrontal working memory system (99) – described as a neuropsychological ADHD endophenotype (100). Similarly, regarding the increased likelihood of ASD due to smoking during pregnancy – where a discussion on the toxicity of cigarette smoke would naturally follow – an odds ratio of 1.4 (95% CI 1.1-1.8) has been reported (101), but a recent study using sibling comparisons, better explains this link by familial confounding (102). In fact, as noted previously, the exposure of maternal smoking is associated with many socio-economic related factors, and there is a possibility that genes affect both exposure and outcome. Again, this highlights that suspected environmental factors might not be strictly environmental (103), emphasizing the importance of genetically informed studies of potential environmental factors.

The negative finding that several obstetrical factors are not associated with ASD beyond familial confounding has recently been confirmed by a systematic review and meta-analysis of existing studies on single obstetric factors (104). It concluded that shared familial liability explained associations between obstetric complications and ASD rather than them being causal. The authors were able to perform a meta-analysis since more studies have emerged since March 2019 – the end date for inclusion in **Study I** – and by limiting the analysis to only include twin cohort studies, thereby reaching acceptable homogeneity among included studies. As previously noted, in contrast to RCTs, observational studies are more prone to bias and often present a greater challenge of heterogeneity among studies, risking a seemingly precise, but incorrect, point estimate (64). Consequently, no meta-analysis could be performed within **Study I**.

Based on the results of **Study I,** two important gaps in research should be noted. First, there is little to no research on NDC other than ASD and ADHD, and to some extent lower IQ and ID. This is remarkable since other NDC are common in the general population (36, 105). Second, there is a lack of geographic distribution with most twin and sibling studies being conducted in Scandinavia and North America. These are highly developed areas of the world both with regards of environmental regulations and health care. It is therefore difficult to generalize our finding of many obstetrical complications not being associated with NDC beyond familial confounding to areas of the world with less developed obstetrical and neonatal care. Furthermore, additional factors, not yet identified, could potentially be of relevance for NDC in other parts of the world. The limited geographical dispersion points to a global research divide for NDC. Only 1.13% of the worldwide psychiatric research productivity originates from low and lower-middle income countries (106). The results of **Study I** reflect that.

5.2 IN-DEPTH INVESTIGATION OF EARLY MEDICAL EVENTS (STUDY II)

The important finding of **Study II** is not the exact list of early medical events, but the notion of a cumulative effect in the context of an environmental ASD etiology. This is in line with a previous population-based twin study (107). But in comparison to that study, **Study II** showed a stronger association between cumulative early medical events and autistic traits,

which may be due to the exposure measure being based on in-depth examinations of medical records. In line with **Study II**'s finding of a cumulative environmental effect on ASD etiology, a recent study has found that cumulative exposure to phthalate during pregnancy is associated with elevated autistic traits (108).

The second finding of **Study II** – the importance of early dysregulation as a precursor of behavioral problems and autistic traits – has been shown previously. For instance, a large population-based study reported that early regulatory problems predicted external, internal, and attentional problems later in life (109), and that regulatory issues were more frequent in children later diagnosed with ASD (110). Importantly, neither of these studies were genetically informed, which means that their findings may have been affected by familial confounding.

5.3 THE CUMULATIVE STRESS HYPOTHESIS AND EARLY MEDICAL EVENTS (STUDY III)

As a population-based registry-linked twin study, **Study III** further supports the cumulative stress hypothesis of ASD beyond familial confounding. Furthermore, it demonstrates that there might be a continuum of environmental influences across ASD symptom severity supporting the dimensional model of autism.

Based on findings from **Study I and II**, the premise for the hypothesis-driven approach of **Study III** was to include any known environmental factor associated to ASD, beyond familial confounding. Since the only shown environmental factors associated to ASD beyond familial confounding were medical events (low birth weight, congenital malformations, and perinatal respiratory stress), we were able to measure these events by linking national medical registries to our sample. However convenient, this might be an example of the streetlight effect (111) – the exposures we study stems from prior research investigating only what has been possible to investigate. This is named the streetlight effect since it is like searching for your lost keys not necessarily where you lost them, but where the light is. Importantly, this weakness of the study may also be its strength – even though the study is selective and may not have included many other potential environmental exposures, it still shows the importance of the accumulation of environmental factors. If we were able to include more of the factors outside of the streetlight, there is reason to believe that we could detect even more of a cumulative effect.

As for underlying mechanisms, the cumulative stress hypothesis points to several possibilities involving early neurodevelopment, like developmental neurotoxicity due to oxidative stress, toxic chemicals, maternal nutrient depletion during pregnancy, or psychosocial stress. Oxidative stress can alter many key processes in brain development, including neurogenesis, neuronal differentiation, synaptogenesis, and establishment of functional connectivity network (112) – alterations associated with NDC (113-116). Next, it has recently been stressed the importance to systematically review the relationship between ASD and toxic chemicals such as chlorpyrifos, lead, and polychlorinated biphenyls (PCBs) (50), and animal

studies show that PCBs may modulate signaling pathways connected to ASD (117). Last, studies have shown an association between short intervals between pregnancies and neurodevelopment, with a proposed mechanism of insufficient recovery time from pregnancy and the subsequent period of lactation leading to nutrient depletion (118).

The potential mechanisms above provide examples of environmental etiologies fitting into the liability threshold model (Figure 1). It is interesting to note that for the cumulative effect of three exposures in **Study II** (OR 2.39 (0.62–9.24)), the point estimate of the odds ratio remained similar beyond familial confounding; however, it was not statistically significant. Cautiously, this represents the possibility of a threshold being reached in the extreme end of the distribution and for a minority of the sample, with an underlying environmental contribution to liability, affecting the whole sample.

5.4 EARLY MEDICAL EVENTS AND A COMMON LATENT NDC FACTOR (STUDY IV)

Study IV extends the finding from **Study III** demonstrating that the cumulative effect of early medical events associated with ASD symptoms beyond familial confounding is similarly associated with symptoms of ADHD, tics, and learning difficulties through a common latent NDC factor. The existence of an association beyond familial confounding supports a causal association between cumulative exposure of early medical events and NDC more generally, and not only in ASD, as seen in **Study III**. The result is further strengthened as familial confounding was fully accounted for in this large population-based twin sample, with full attenuation of residual outcome associations in the MZ-subsample.

The finding of ADHD and ASD being similarly impacted is particularly intriguing. With regards to a general factor for NDC (119), earlier findings have highlighted the role of genetics, with environmental contributions being less clear. It has been shown that restricted fetal growth is associated with a moderate increase in a latent NDC factor (120), also connecting to the cross-disorder finding from **Study I**. But inconsistencies on the level of symptom dimensions of NDC also exist. For example, different symptoms of ASD have been linked differentially to different symptoms of ADHD, with nonshared environmental correlations being lower than genetic correlations (92). Research has indicated that pre- and perinatal risk factors might play a role in diverging developmental pathways leading up to either disorder (121). The difference in results between these studies and ours might be explained by our cumulative approach to the exposures studied.

5.5 LIMITATIONS

Several limitations need to be addressed with regards to **Study I.** First, while useful, the inclusion of early studies dating back decades adds studies of potential lesser quality. With updated study designs and statistical methods, previously suggested environmental factors for ASD such as rubella infection during pregnancy and labor induction have been found to be confounded by familial factors, compared to results from earlier studies. Incorrectly applied family designs may identify factors as being free from familial confounding, when in fact, the

full information that twins and siblings provide is not properly utilized. Second, as already stated, there are other ways to control for familial confounding than twin and sibling studies, such as multi-generational population-based cohorts, which not only utilize siblings, but also half-siblings and cousins. Also, adoption or in vitro fertilization designs (60) are advantageous, compared to family designs, since they allow for examination of associations between patterns of family interaction and child development, with control for passive gene-environment interaction (61). Furthermore, with adoption studies, the effects of both the prenatal and the postnatal environment can be estimated, investigating familial confounding differentially for prenatal versus postnatal environmental factors (62). Third, there is modest control of comorbidity in the included studies, a limitation impossible to address, owing to a lack of reporting on any comorbidity beside the studied outcome. This is unfortunate since comorbidity influences NDC phenotypes (10-12). Fourth, there are discrepancies in the age of diagnosis. For ASD, most included cohort studies lack information regarding age in the sibling subsamples. However, the overall assessment of the included studies' methodologies concludes that a misclassification bias is improbable. On the contrary for dimensional measures of ADHD, some results rely on symptom measures at a young age, thereby introducing a risk of misclassification bias. Finally, it is important to caution against the general conclusion that absence of evidence of association equals evidence of absence.

For **Study II** the list of factors found, as well as their cumulative load, might not be directly linked environmental factors for ASD. However, the important finding of **Study II** is not the exact list of early medical events, but the notion of a cumulative effect in the context of an environmental etiology. The risk for both selection and confirmation bias together with the possibility of a reverse causation further restrain conclusions. However, medical records are a reliable source of information, making a recall bias less likely. Last, a minority of cases with ASD discordance could be due to rare post-twining de novo mutations, and if so, affect the control for shared genetics.

Four limitations are common for **Study II, III and IV.** The first regards generalizability from twins to singletons. Suspiciously, in **Study III and IV** there was a somewhat higher percentage of MZ pairs in the higher exposure load groups. However, there are three objections to be raised. 1) The role of zygosity for perinatal outcomes is unclear. Zygosity has been linked in one previous retrospective study to lower birth weight and prematurity (122), while in a later study, the effect of zygosity was less clear (123). 2) The results did not differ when twin pairs in **Study III** with twin-to-twin transfusion syndrome were excluded. 3) Being a twin is not associated with ASD (124), nor autistic traits (125). The second common limitation is how the cumulative score was created by summing exposures based on them being present or absent. With this crude approach, we were not able to account for the possibility of different effect sizes for each exposure. The third limitation regards the risk of residual bias in observational studies prohibiting far reaching causal interpretations. Specifically for **Study III**, by comparing twins, we rule out the effect of parental genetics, but we cannot completely rule out confounding by child specific genetic effects since we did not have the power to look at MZ and DZ twins separately. The only way to rule out

confounding from child genetics is to compare the within pair difference of MZ twins, as in **Study II and IV**. As a fourth common limitation, a potential residual bias is measurement error at the within-pair level. However, the presence of a measurement error or misclassification at the within-pair level leads to an attenuation of both the between individuals and the within pair association, compared to the true association (126).

As previously mentioned, the informative twin pairs are only those that are simultaneously discordant for exposure and outcome. Even with the large sample sizes of Study **III and IV**, the number of exposure and outcome discordant pairs were quite low, especially with regards to a diagnosis of ASD as the outcome, and furthermore when considering the number of pairs with higher exposure for the twin without ASD. This limitation needs to be acknowledged.

For the common latent NDC factor models created in **Study IV**, model fitting was probably complicated by the skewed distribution of the data. Despite the large sample, this led to higher RMSEAs. However, one should not rely solely on a fixed value, but fit indices are to be interpreted holistically (127). Unfortunately, **Study IV** had only power to study NDC outcomes dimensionally as symptoms, and not categorically as a diagnosis of ASD, ADHD, and TD, respectively. Therefore, a connection to the liability threshold model could not be drawn.

Finally, even though this thesis contributes new knowledge regarding environmental etiologies to NDC, it is important to acknowledge that it does not provide evidence for causal interpretations at an individual level. Even though the relative risk of the cumulative effect of early medical events is statistically significant beyond familial confounding, the signal is weak, only explaining a small share of the ASD and NDC symptom variability in the samples. The increased risk is low in absolute terms – which is more important when interpreting the findings at an individual level. Suspected environmental factors that were shown to have association to NDC beyond familial confounding in **Study I** are the only results that are somewhat interpretable on an individual level. However, it is important to caution against the general conclusion that absence of evidence of association equals evidence of absence.

6 CONCLUSIONS

This thesis advances our understanding of ASD and NDC in mainly four areas:

1. It comprehensively maps our present knowledge from twin and sibling studies on environmental etiologies of NDC.
2. Owing to environmental contributions, it places early medical events into the dimensional model of autism and the liability threshold model, associating them with symptoms of ASD continuously distributed in the general population.
3. It confirms the cumulative stress hypothesis of ASD in a large human sample, beyond familial confounding.
4. It suggests that this cumulative environmental effect acts through a common latent NDC factor, that in turn affects neurodevelopment, ASD included.

In this thesis, **Study I** points to a need for more genetically informed studies of good quality regarding the environmental causes of NDC, especially regarding NDC other than ASD and ADHD. It shows that after adjustment for familial confounding, advanced paternal age, low birth weight, congenital malformations, and perinatal respiratory stress are associated with ASD, and that low birth weight, gestational age and low family income are associated with ADHD. Study I also identifies previously suspected factors, including pregnancy-related ones, as due to familial confounding. It also lists potential environmental factors where replication studies are needed.

Study II suggests that the load of early medical events and early dysregulation is associated with autistic traits and ASD, and that these events are likely driven by environment and not shared within the twin pairs. **Study III** supports the cumulative stress hypothesis of ASD beyond familial confounding. It demonstrates that there might be a continuum of environmental influences across ASD symptom severity giving support to the dimensional model of autism and the liability threshold model involving environmental etiologies.

Study IV connects the exposure of cumulative early medical events to symptoms of ASD, ADHD, tics, and learning difficulties through a common latent NDC factor, beyond familial confounding, suggesting a causal pathway. It confirms the hypothesis that the environmental effect of cumulative early medical events previously associated with ASD beyond familial confounding is not specific for ASD, but rather, associated with a common latent NDC factor that in turn affects symptoms of NDC, ASD included.

7 POINTS OF PERSPECTIVE

Study I points to the need of future studies on NDC other than ASD and ADHD. Furthermore, several single studies suggest potential environmental factors beyond familial confounding, where future replication studies are warranted. It is also important to apply the most recent and sophisticated methods for twin and family data. Therefore, it would be valuable to reexamine suggested environmental factors from the past decades with a modern statistical and methodological approach. Additionally, **Study I** points to a lack of control for comorbidity. Future studies should be more thorough and complete in mapping psychiatric and somatic comorbidity, which are frequent in NDC (11, 128) since they may have a significant impact on developmental mechanisms.

The finding in **Study II** of early dysregulation during the first year of life being connected to later autistic traits is worth continued investigation. There exist many longitudinal study cohorts investigating early development. This thesis adds support to the proposed Anterior Modifiers in the Emergence of Neurodevelopmental Disorders (AMEND) framework, which proposes future research to elucidate the etiology of NDC using prospective longitudinal designs that separates genetic and environmental factors associated with the cumulative impact of early-stage events (129).

Future research should also focus on gene-environment interaction. As previously noted, evidence for the three-hit concept in ASD etiology has previously only been found in animal studies (49-51). Studies on humans are often forced to rely on observational data which is more prone to bias. Although population-based twin studies can be used to overcome much of this bias, it is difficult to obtain enough statistical power for observational gene environment interaction studies (130). One exciting future possibility for an experimental approach could be to use human-induced pluripotent stem cells (hiPSCs) and brain organoids to further expand the result of a cumulative effect to a wider range of environmental factors. These are potential tools to explore the cumulative effects of environmental factors since experimental studies on humans would be unethical or unpractical to conduct (131). By reprogramming a persons' somatic cells into hiPSCs, and differentiating them into neurons, a brain organoid can be created, resembling brain tissue. Then, environmental exposures can be experimentally tested comparing brain organoids from subjects with NDC to brain organoids derived from control subjects without the condition. No study has yet been conducted on environmental impacts on brain organoids derived from subjects with NDC. However, a few studies have recently suggested the use of hiPSCs to study environmental factors' impact on neurodevelopment (132-134). Further development is needed to create reliable NDC brain organoids before the effect of environmental factors would be possible to test experimentally. If so, this thesis emphasizes the importance of studying the cumulative effects of the environmental factors.

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