UNIVERSITY OF COPENHAGEN
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Prenatal risk factors for neurodevelopmental disorders

PhD Thesis

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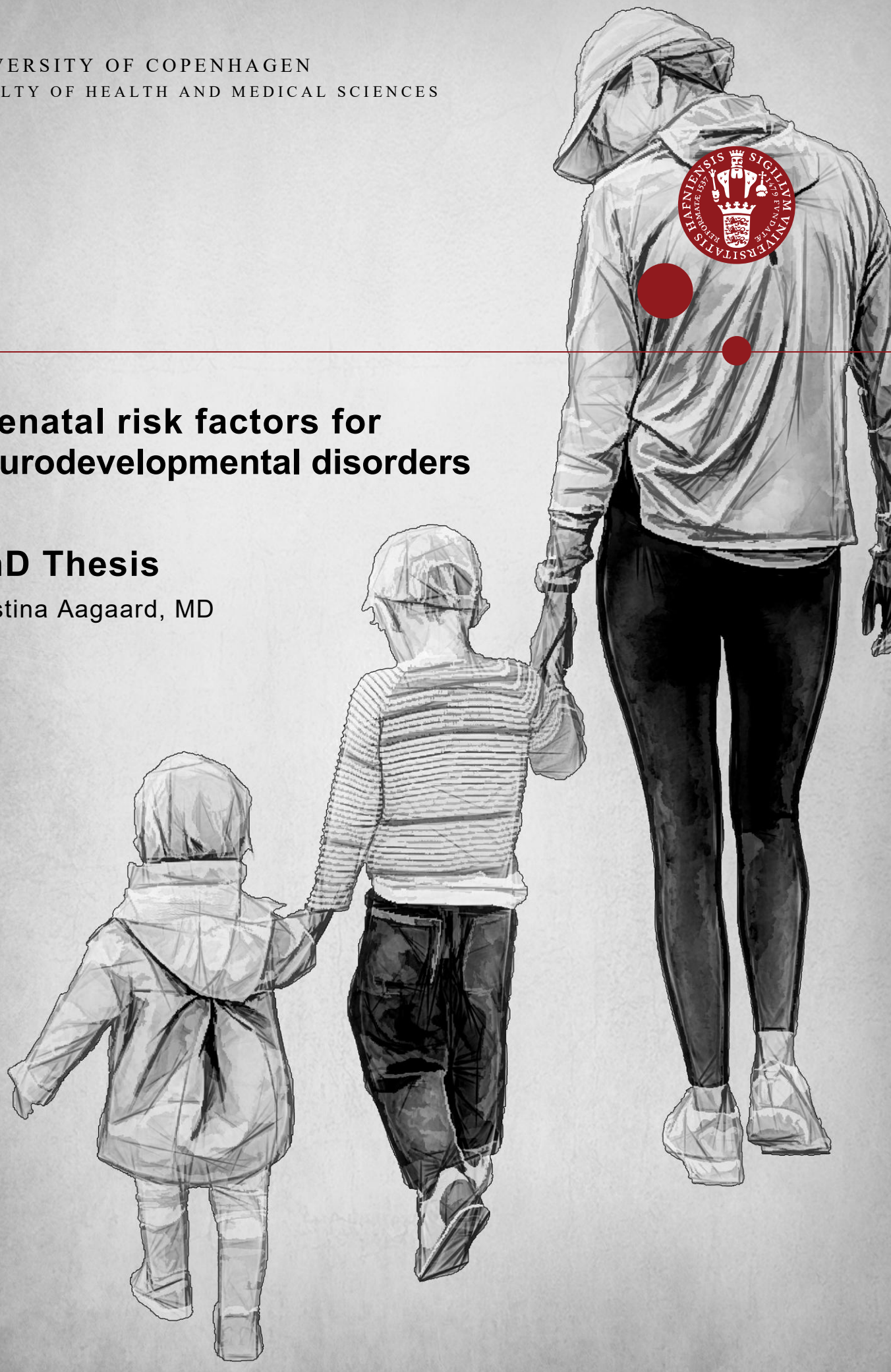

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Submitted:	27th of October 2025

PAPERS

This thesis is written as a synopsis and is based on the scientific papers listed below referred to by their roman numerals. Full scientific papers and the corresponding supplementary materials are included in the Appendix.

I. **High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10 - A randomized clinical trial**

Kristina Aagaard, Jens Richardt Møllegaard Jepsen, Astrid Sevelsted, David Horner, Rebecca Vinding, Julie Bøjstrup Rosenberg, Nicklas Brustad, Anders Eliasen, Parisa Mohammadzadeh, Nilofar Følsgaard, María Hernández-Lorca, Birgitte Fagerlund, Birte Y Glenthøj, Morten Arendt Rasmussen, Niels Bilenberg, Jakob Stokholm, Klaus Bønnelykke*, Bjørn H. Ebdrup* and Bo Chawes* (*Authors contributed equally)

Published in The American Journal of Clinical Nutrition, February 2024[1]

II. **High-dose vitamin D3 supplementation during pregnancy and test-based cognitive performance at age 10 - a post-hoc analysis of a randomized clinical trial**

Olivia Frigast Frederiksen, Jens Richardt Møllegaard Jepsen, Nicklas Brustad, Rebecca Vinding, Julie Bøjstrup Rosenberg, Parisa Mohammadzadeh, María Hernández-Lorca, Ann-Marie Malby Schoos, Nilo Vahman, Birte Y. Glenthøj, Birgitte Fagerlund, Niels Bilenberg, Klaus Bønnelykke, Bjørn H. Ebdrup*, Kristina Aagaard* and Bo Chawes* *Under review*

III. **Genetic investigation of the association between maternal dietary patterns and offspring ADHD**

Kristina Aagaard, Casper-Emil T. Pedersen, David Horner, Anders Eliasen, Nicklas Brustad, Rebecca Vinding, Mohammad Talaei, Seif O. Shaheen, Julie B. Rosenberg, Jakob Stokholm, Bo Chawes, Morten Arendt Rasmussen, Jens Richardt M Jepsen, Bjørn H. Ebdrup, Alexandra Havdahl, Laurie J. Hannigan* and Klaus Bønnelykke* *Under review*

IV. **Inflammation in pregnancy and child neurodevelopment: A trio polygenic score and Mendelian randomization study in the Norwegian Mother, Father and Child Cohort**

Kristina Aagaard, Anders Eliasen, Jens Richardt M Jepsen, Casper-Emil T. Pedersen, Rebecca Vinding, Julie B. Rosenberg, Tingting Wang, Nicklas Brustad, Susanne Brix, Bo Chawes, Morten Arendt Rasmussen, Bjørn H. Ebdrup, Ida Henriette Caspersen, Robyn E. Wootton, Helga Ask, Alexandra Havdahl, Klaus Bønnelykke* and Laurie J. Hannigan* *Submitted*

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ACKNOWLEDGEMENTS

"It ain't what you don't know that gets you into trouble.
It's what you know for sure that just ain't so"

Mark Twain

Birth cohort studies, such as those presented in this PhD thesis, are only possible thanks to the remarkable commitment of the participating families, who generously consent to take part and contribute their time and energy. I am deeply grateful for the opportunity to work with such valuable data and have done my utmost to make the best possible use of it - striving to repay this trust with thoroughness in my research.

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Kristina Aagaard

Copenhagen, October 2025

ABBREVIATIONS

25(OH)D = 25-hydroxyvitamin D

ADHD = Attention-Deficit/Hyperactivity Disorder

ADHD-RS = ADHD-Rating Scale

ALSPAC = Avon Longitudinal Study of Parents and Children

ASD = Autism Spectrum Disorder

ASQ = Ages and Stages Questionnaire

BIS = Barwon Infant Study

BMI = Body Mass Index

BRIEF = Behaviour Rating Inventory of Executive Function

CANTAB = Cambridge Neuropsychological Test Automated Battery

CBCL = Child Behaviour Checklist

CDI = Child Development Inventory

CI = Confidence Interval

CNSR = Centre for Neuropsychiatric Schizophrenia Research

COPSAC = Copenhagen Prospective Studies on Asthma in Childhood

COPSYCH = Copenhagen Prospective Study on Neuro-PSYCHiatric Development

CRP = C-reactive protein

DAG = Directed Acyclic Graph

DAWBA = Development and Well-Being Assessment

DNBC = Danish National Birth Cohort

DOHaD = The Developmental Origins of Health and Disease

DSM-V = Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

FDR = False Discovery Rate

FFQ = Food Frequency Questionnaire

GlycA = Glycoprotein Acetyls

GWAS = Genome-Wide Association Study

Hs-CRP = High sensitivity C-reactive Protein

ICD-10 = International Classification of Diseases, 10th Revision

IL-6 = Interleukin-6

IU = International Units

K-SADS-PL = Kiddie Schedule for Affective Disorders and Schizophrenia – Present and Lifetime Version

LCPUFA = Long-Chain Polyunsaturated Fatty Acids

MIA = Maternal Immune Activation

MoBa = Norwegian Mother, Father, and Child Cohort Study

MR = Mendelian Randomization

NPR = Norwegian Patient Registry

OR = Odds Ratio

OSF = Open Science Framework

PC = Principal Component

PGS = Polygenic Score

RCT = Randomized Clinical Trial

RS-DBD = Rating Scale for Disruptive Behaviour Disorders

SCQ = Social Communication Questionnaire

SD = Standard Deviation

SE = Standard Error

SNP = Single Nucleotide Polymorphism

SRS-2 = Social Responsiveness Scale, Second Edition

TOMAL-2 = Test of Memory and Learning, Second Edition

VDAART = Vitamin D Antenatal Asthma Reduction Trial

US = United States

WISC-IV = Wechsler Intelligence Scale for Children, Fourth Edition

SUMMARY

This thesis investigates prenatal risk factors for neurodevelopmental disorders, with a particular focus on Attention-Deficit/Hyperactivity Disorder (ADHD) and autism. The identification of early life exposures associated with later presentation of these disorders may provide valuable insights for future prevention strategies. ADHD and autism are known to be highly heritable, with genetic predisposition representing the strongest risk factor. This strong genetic component to neurodevelopmental disorders may introduce bias in observational studies investigating early environmental influences. In the attempt to address this, this thesis investigates the causality of proposed early life risk factors using genetically informed study designs that have the ability to account for potential confounding due to genetics.

The studies included in this thesis focused on the effect of a high-dose vitamin D3 supplementation throughout third trimester of pregnancy on risk of ADHD and autism as well as impaired cognitive functioning evaluated at the age of 10 at the Copenhagen Prospective Study on Neuro-PSYCHiatric Development (COPSYCH) visit. The COPSYCH 10-year visit consisted of an extensive psychopathological and neurocognitive evaluation of the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) 2010 cohort constituting 700 mother-child pairs followed prospectively since week 24 of pregnancy. The thesis further investigated the potential role of genetic confounding of the observed influence of a maternal unhealthy dietary pattern in pregnancy and maternal inflammation in pregnancy on neurodevelopment utilizing both the COPSAC cohort and two larger external mother-child cohorts: the Avon longitudinal study of parents and children (ALSPAC) including 1,199 trios with information on maternal, paternal and child genetics (trios) and the Norwegian mother, father, and child cohort study (MoBa) including 41,580 trios.

As a post hoc analysis of the high-dose vitamin D3 randomized clinical trial (RCT) conducted in the COPSAC2010 cohort throughout third trimester of pregnancy, we did not show any effect of this intervention on offspring risk of ADHD or autism at the age of 10 in **Paper I**. The paper further included observational analyses of the association between maternal pre-intervention blood levels of vitamin D and offspring risk of neurodevelopmental disorders. Adjusted for potential confounders, higher serum vitamin D in pregnancy was associated with a decreased risk of autism, fewer autistic traits, and a decreased risk of ADHD.

As existing observational studies also have pointed to an association between pregnancy vitamin D and offspring cognition, we in **Paper II** conducted an additional post hoc analysis of the COPSAC2010 vitamin D3 intervention investigating the effect hereof on 11 cognitive functions likewise tested at the COPSYCH visit at age 10. The results were suggestive of a positive effect of the high-dose supplementation on verbal memory, visual memory and flexibility/set shift. However, these effects did not pass multiple test correction. In observational analyses, we only showed a positive association between higher serum vitamin D in pregnancy and offspring improved flexibility/set shift.

In **Paper III** we conducted a genetic trio analysis of the suggested association between an unhealthy maternal dietary pattern in pregnancy and offspring risk of ADHD. These analyses were performed using genetic risk scores for a healthy/unhealthy diet. By including maternal, paternal and child genetic risk scores in the analyses, we were able to investigate potential causal effects of maternal diet in pregnancy on ADHD adjusting out potential bias from overlapping genetic predisposition to both eating pattern and ADHD risk. The analyses in the COPSAC2010 cohort suggested a causal negative effect of an unhealthy diet in pregnancy on severity of ADHD traits, however this finding was not replicated in the larger ALSPAC and MoBa cohorts.

Inflammation in pregnancy has been associated with risk of offspring neurodevelopmental disorders. Therefore, we in **Paper IV** investigated potential causal effects of pregnancy inflammation on neurodevelopmental traits and diagnoses using genetic risk scores for C-reactive protein (CRP), interleukin-6 (IL-6) and glycoprotein acetyls (GlycA) in pregnancy. The analyses were conducted in the MoBa cohort using the same genetic trio approach as described for Paper III. We included two additional methods to investigate the association: intergenerational mendelian randomization (MR) to test for causal effects of inflammation and classic two-sample MR to investigate potential confounding of observational associations. Neither the trio analyses nor the intergenerational MR analyses suggested causal effects. Two-sample MR suggested risk of genetic confounding of previously reported observational associations.

In conclusion, this thesis provides a thorough investigation of the influence of vitamin D in pregnancy on offspring neurodevelopment at the age of 10. A high dose vitamin D3 intervention in third trimester of pregnancy did not affect the risk of neurodevelopmental disorders at age 10. The supplementation however had a suggestive positive effect on verbal memory, visual memory

and flexibility/set shift; however, these results did not pass multiple test correction. Finally, using genetically informed study designs, we investigated the causal influences of maternal dietary pattern and maternal inflammation in pregnancy on offspring risk of neurodevelopmental disorders. Our findings were not supportive of causal effects of either diet or inflammation in pregnancy on offspring neurodevelopment, but suggested risk of genetic confounding of observational associations.

DANSK RESUME

Formålet for denne afhandling er at undersøge potentielle prænatale risikofaktorer for ADHD og autisme. Kendskab til sepcifikke eksponeringer i det tidlige liv som kausalt øger risikoen for disse tilstande, kan være et vigtigt skridt på vejen mod fremtidige præventive tiltag som kan enten mindske sygeligheden forbundet med tilstandene eller helt forhindre disse i at opstå. Udviklingen af ADHD og autisme er forbundet med høj arvelighed, og genetisk disposition er den stærkeste kendte risikofaktor. Den betydningsfulde genetiske komponent for udviklingen af disse tilstande kan medføre confounding (årsagsforveksling) i observationelle studier, som undersøger konsekvenserne af miljøpåvirkninger i det tidlige liv for fremtidig risiko for ADHD og autisme. I denne afhandling genundersøger vi derfor tidligere foreslåede risikofaktorer for udviklingsforstyrrelser i genetiske analyser, som har potentialet til at detektere kausale effekter under hensyntagen til potentiel confounding grundet genetisk disposition.

To af studierne i denne afhandling undersøger effekten af høj-dosis D-vitamin tilskud i tredje trimester af graviditeten på senere risiko for udviklingsforstyrrelserne ADHD og autisme samt kognitiv funktion vurdereret ved 10-årsalderen som en del af Copenhagen Prospective Study on Neuro-PSYCHiatric Development (COPSYCH) projektet. COPSYCH-projektet bestod af en ekstensiv psykopatologisk og neurokognitiv undersøgelse af Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) 2010 kohorten som følger 700 mor-barn par prospektivt siden graviditetsuge 24. Ydermere undersøger de sidste to studier i denne afhandling den potentielle indflydense af genetisk confounding på de tidligere rapportede associationer imellem både et usundt kostmønster samt betændelsestilstande i graviditeten og senere udviklingsforstyrrelser. Til dette formål benyttede vi både data fra COPSAC2010 kohorten samt to eksterne, større mor-barn kohorter: the Avon longitudinal study of parents and children (ALSPAC) og the Norwegian mother, father, and child cohort study (MoBa).

I en post hoc analyse af et høj-dosis D-vitamin randomiseret klinisk forsøg udført i COPSAC2010 kohorten, fandt vi i **Artikel I** ingen effekt af høj-dosis D-vitamin i tredje trimester på risikoen for ADHD og autisme vurderet ved COPSYCH 10-årsbesøget. Vi undersøgte endvidere associationen mellem mødrenes præ-interventions D-vitamin niveau i blodet og risikoen for senere udviklingsforstyrrelser i observationelle analyser. I analyser justeret for potentielle confoundere, fandt vi en association mellem højere præ-interventionsniveauer af D-vitamin og lavere risiko for autisme, sværhedsgrad af autistiske træk, og lavere risiko for ADHD.

Idet resultater fra tidligere studier har peget mod en sammenhæng mellem D-vitamin niveauer i graviditeten og barnets senere kognitive funktion, udførte vi i **Artikel II** endnu en post hoc analyse på COPSAC2010 høj-dosis D-vitamin interventionen, hvor vi undersøgte effekten heraf på 11 kognitive funktioner hos barnet ligeledes målt ved 10-årsalderen i COPSYCH-studiet. Resultaterne fra dette studie indikerede en positiv effekt af høj-dosis D-vitamin tilskud i graviditen på barnets verbale hukommelse, visuelle hukommelse og mentale fleksibilitet. Dog var disse fund ikke længere signifkante efter justering for multipel testning. I observationelle analyser fandt vi alene en positiv association mellem højere præ-interventions D-vitamin niveau og forbedret mental fleksibilitet.

I **Artikel III** foretog vi en genetisk trioanalyse af den foreslåede association mellem et usundt maternelt kostmønster i graviditeten og barnets senere risiko for ADHD. Disse analyser var baseret på genetiske risikoscores for et sundt/usundt kostmønster. Ved at inkludere mor, far og barns genetiske risikoscores i analysemodellerne, var vi i stand til at undersøge potentielle kausale effekter af morens kostmønster i gravidieten på barnets risiko for ADHD under hensyntagen til potentiel confounding grundet overlappende genetisk disposition til både kostmønster og ADHD. Analyser baseret på COPSAC2010 data antydede en kausal negativ effekt af et usundt kostmønster i graviditeten på barnets sværhedsgrad af træk fra ADHD, dog var dette fund ikke replikerbart i de større mor-barn kohorter ALSPAC og MoBa.

Mors inflammationsniveau i graviditeten er blevet koblet til risikoen for udviklingsforstyrrelser hos barnet. Derfor foretog vi i **Artikel IV** en genetisk undersøgelse af de potentielle kausale effekter af mors inflammationsniveau i graviditeten på barnets risiko for senere udviklingsforstyrrelser og træk herpå baseret på genetiske risikoscores for niveau af CRP, IL-6 og GlycA i graviditeten. Disse anlayser blev foretaget i MoBa kohorten med brug af samme trio analysetilgang, som beskrevet for Artikel III. Vi inkluderede to yderligere metoder til at undersøge associationen: intergenerationel Mendelian randomization til at teste for kausale effekter af inflammation og klassisk to-stikprøver (two-sample) Mendelian randomization til at undersøge for potentiel confounding af observationelle associationer. Hverken trioanalyser eller intergenerationel Mendelian randomization kunne påvise kausale effekter. To-stikprøver (two-sample) Mendelian randomization viste risiko for genetisk confounding i observationelle studiedesigns.

Overordnet bidrager denne afhandling først med en grundig undersøgelse af D-vitamins betydning i graviditeten for barnets senere mentale helbred. Højdosis D-vitamin tilskud i tredje trimester af graviditeten påvirkede ikke risikoen for udvikling af ADHD og autisme hos barnet. D-vitamin tilskud havde en potentiel positiv effekt på verbal hukommelse, visuel hukommelse og mental fleksibilitet hos barnet; dog var disse fund ikke længere signifikante efter justering for multipel testning. Herudover bidrager denne afhandling med genetiske analyser med potentialet til at undersøge kausale effekter af kostmønster og inflammationsniveau i graviditeten for barnets senere risiko for udviklingsforstyrrelser. Vi fandt ingen overordnet evidens for kausale effekter af hverken kostmønster eller inflammationsniveau, herimod fandt vi indikation på at observationelle studier er under risiko for confounding fra genetisk disposition.

PREFACE

During my PhD at the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC), I have devoted approximately half of my working hours to clinical responsibilities. My primary clinical task, performed together with the remaining COPSAC clinical team, has been conducting the $COPSAC_{2010}$ 13-year visit, a comprehensive 6-hour program of clinical tests and examinations with a particular focus on metabolic health. These visits also included follow-up assessments of longitudinally registered outcomes in the COPSAC2010 cohort, covering asthma, allergy, atopic dermatitis, and psychopathology. In addition, I performed outpatient visits of the $COPSAC_{2000}$ and $COPSAC_{2010}$ cohort with the aim of diagnosing and treating atopic diseases. As part of the COPSAC clinic, I have also had the important task of teaching new colleagues how to perform examinations and clinical interviews in a uniform and stringent manner to secure the highest standard of COPSAC research data.

The main outcomes of this thesis were obtained at the 10-year visit of the COPSAC2010 cohort which included a comprehensive clinical evaluation of psychopathology and neurocognition as part of the Copenhagen Prospective Study on Neuro-PSYCHiatric Development (COPSYCH) project. The final COPSYCH examinations were conducted at the beginning of my PhD and I had the privilege of directly observing the dedication and meticulous work of the trained personnel conducting the interviews and tests, as well as the remarkable commitment of the COPSAC participants during these extensive sessions lasting approximately 5-6 hours. Throughout my PhD, I have been actively engaged in the COPSYCH research collaboration, participating in regular research meetings and presenting at the COPSYCH research symposium in the fall 2023. Moreover, I contributed to a publication investigating the dimensionality of clinician-rated ADHD symptoms within COPSYCH, assessed using the Kiddie-Schedule for Affective Disorders and Schizophrenia - Present and Lifetime Version (K-SADS-PL) - a potential novel method for dimensional psychopathological assessment.[2]

In addition to the research activities related to the COPSAC birth cohorts, COPSAC has, during my PhD, recruited participants and conducted clinical visits for two additional research projects in which I have been actively involved: the randomized clinical trial “Azithromycin and high-dose vitamin D for treatment and prevention of asthma-like episodes in hospitalised preschool children” and the $COPSAC_{Severe}$ study. I have been actively involved in recruitment and data collection for the azithromycin/vitamin D RCT at several hospitals across Zealand. My primary

contribution regarding management has been to the $COPSAC_{Severe}$ study, a nationwide investigation of severe and exacerbation-prone asthma. For this project, I have been involved in the daily management of study activities and collaborated closely with the $COPSAC_{Severe}$ team to establish two new study sites in Denmark, as well as to arrange laboratory and data exchange contracts.

During my PhD, I have had the opportunity to gain insights into several external mother–child cohorts. Papers III and IV were based on collaboration with researchers from the external cohorts ALSPAC and MoBa. I have been particularly involved in establishing the collaboration with the MoBa cohort, working closely with my main supervisor, Klaus Bønnelykke, to draft analysis plans for the joint projects and secure agreements for data sharing. I have visited the MoBa cohort two times to plan and perform analyses for our shared projects, receiving outstanding support from the MoBa research team - particularly senior researcher Laurie Hannigan, whose expertise has been invaluable for all aspects of the collaboration, including developing analysis plans, conducting analyses, and teaching me genetic approaches to investigating causal effects of prenatal risk factors. For my research exchange, I had the privilege of visiting the Barwon Infant Study (BIS), led by Peter Vuillermin and Anne-Louise Ponsonby, located in Melbourne and Geelong as part of the University of Melbourne and Deakin University, respectively. BIS and COPSAC share many similarities, particularly their focus on the impact of early-life exposures on child neurodevelopment, including dietary and inflammatory factors. During my stay, I presented my research several times and received constructive feedback and valuable suggestions. I also participated in weekly research meetings and was actively engaged in the research environment, which proved to be a great source of inspiration.

Finally, I have had the privilege of closely supervising medical master's student Olivia Frigast Frederiksen during my PhD. Olivia has drafted the second paper of this study which focuses on vitamin D in pregnancy and its effects on childhood cognition at the age of ten. Prior to Olivia's involvement, I worked in close collaboration with Jens Richardt Møllegaard Jepsen, whose expertise in childhood psychopathology and cognition has been invaluable to this project, to select the cognitive domains assessed in the COPSYCH neurocognitive test battery, which serve as the main outcomes of this study. I also conducted the analytical backbone for this project (data cleaning, deciding on statistical methods, performing main analyses), providing a foundation for Olivia to build upon during her research stay. Supervising her has been an invaluable learning experience for me and an important step in my development as a researcher. It has been

particularly rewarding to witness Olivia's growth as a young scientist where she has demonstrated both remarkable dedication and talent.

For this thesis, artificial intelligence (AI) has only been used to a very limited extent (ChatGPT 5). AI suggested rephrasing or basic R code has been closely reviewed, edited and verified before possible inclusion. Conclusively, no content in this thesis is a direct product of AI.

INTRODUCTION

This thesis examines prenatal risk factors for child neurodevelopmental disorders, with a specific focus on the long-term effects of maternal vitamin D levels during pregnancy on childhood neurodevelopmental disorders, traits, and cognitive function. In addition, it investigates whether unhealthy dietary patterns and pregnancy-related inflammation contribute causally to the development of neurodevelopmental disorders in children, using genetically informed study designs. The Introduction outlines key neurodevelopmental disorders and cognitive functions, summarizes current knowledge on the selected prenatal risk factors, and presents the rationale for using multiple study designs to investigate causal relationships.

NEURODEVELOPMENTAL DISORDERS

The umbrella term "Neurodevelopmental disorders" refers to a group of chronic disorders presenting in the developmental period which originate from early disruptions in brain development.[3,4] The development of the human brain is a tightly coordinated and intricate process with windows of higher or lesser susceptibility to external exposures. A variety of potential perturbations may occur through this developmental process resulting in distinct clinical presentations.[4,5] The category of "Neurodevelopmental disorders" was introduced in the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-V) which includes the following disorders: attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder (ASD), intellectual disorder, specific learning disorder, motor disorders, communication disorders and other neurodevelopmental disorders.[3,6,7] Even though these disorders vary significantly in clinical presentation, response to treatments, and underlying causes, the validity of the grouping is strengthened by the significant overlap between the different disorders and their constituent symptom dimensions – with the overlap between for example ADHD and ASD reported between 40-70% (percentage of children with ASD presenting with ADHD).[6,8,9]

ASD and ADHD present the primary outcomes of focus of this thesis. They will therefore be described in detail in the following paragraphs.

ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

The prevalence of ADHD in children and adolescents is high and has in a large global umbrella review including 3,277,590 participants been estimated to 8.0% (95%CI 6.0-10%).[10] However, prevalence estimates have varied across studies and have been reported in a range from 5.9-14% according to the World Federation of ADHD International Consensus Statement which published 208 empirically supported statements for ADHD. This consensus statement aimed at presenting the large amount of scientific evidence for the validity of the ADHD diagnosis and correcting misconceptions about the mental disorder.[11] Through the past years the extent to which ADHD is under- or over-diagnosed has been subject of an ongoing debate.[12] The ADHD Consensus Statement suggests that ADHD has not become more prevalent throughout the last three decades (1990-2020) however the disorder is more likely to be recognized by clinicians. Yet, research using high-quality data (e.g. data including information on ADHD referrals, private assessments, exact dates for diagnosis) is still warranted in order to draw firmer conclusions on trends for the prevalence and incidence of ADHD.[11,12]

ADHD is a diagnosis based on thorough clinical evaluations of symptoms (both current and past) and coinciding functional impairment. The disorder is characterized by inattention, motor hyperactivity, and impulsivity which present in more than one of the child's environmental settings.[13] The condition is diagnosed as ADHD in the DSM-V (DSM-V was utilized for the Copenhagen Prospective Study on Neuro-PSYCHiatric Development (COPSYCH) evaluations in the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) 2010 cohort[14]), whereas in the WHO's International Classification of Diseases 10th Revision (ICD-10) classification system, the equivalent diagnosis is referred to as hyperkinetic disorder. For this thesis we utilize ICD-10 diagnoses as this is the predominantly used diagnostic classification system in Europe, however retain DSM-based terminology to describe ADHD.[15]

Research in potential preventive strategies for the development of ADHD is important as a diagnosis of ADHD is associated with several adverse outcomes, including lower quality of life, poorer occupational functioning, and economic disadvantage.[16–18] Further, ADHD often co-occurs with other psychopathological conditions thereby increasing the disease burden for the individual.[19]

AUTISM SPECTRUM DISORDER

ASD is a common disorder with a global prevalence of ~1 %.[20] Higher prevalences have been reported for example in the United States (US) where the prevalence in 2020 was estimated to 2.3%.[21] For ASD; the prevalence has increased in the US the past decade which has been ascribed factors including an increased awareness and enhanced ascertainment.[22] ASD is characterized by a large heterogeneity in both phenotype - disease manifestation - and genotype.[23] Despite many differences between individuals with ASD, all present with core ASD symptoms which are present within two domains: 1) social communication and 2) restricted, repetitive sensory-motor behaviours.[24]

Comorbidity is very common in ASD. A wide range of diseases including other neurodevelopmental disorders, sleeping problems, overweight, gastrointestinal disorders, and epilepsy have been associated with the disorder.[24] It has been suggested that this overrepresentation of comorbid disorders is linked to a higher proportion of perinatal exposures among children with ASD and common genetic risk factors.[25] Early diagnosis and identification of such comorbid disorders are important to improve the effectiveness of treatment interventions.[24]

DIMENSIONAL AND CATEGORICAL PERSPECTIVE

Neurodevelopmental disorders can be considered in both a categorical - diagnosis yes/no - and a dimensional perspective - severity of traits - which allows for evaluating severity of symptoms/problems also within individuals not meeting the threshold for a diagnosis. The validity of the dimensional perspective for neurodevelopmental disorders is supported by studies showing that diagnosis and traits can be predicted by the same genetic and environmental risk factors.[6,14] The dimensionality of ADHD is further supported by a large twin study which showed strong genetic links between ADHD research diagnoses and the presentation of ADHD symptoms below the diagnostic threshold - suggesting shared etiological factors.[26]

COGNITIVE FUNCTIONING

Cognitive functions are fundamental, as they enable us to think, learn, and solve problems. These abilities form and evolve throughout the course of brain development and maturation and are dependent on a stable and caring early life environment.[27] Genetic disposition is an important determinant of cognitive function.[28] Additionally, exposures prenatally are also known to be important, and may have long lasting effects on cognitive performance.[29] However, cognitive

development does not end at birth - neuronal connections and pruning continue to occur in response to life experiences and cognitive development can be viewed as an ongoing process throughout life.[27] This developmental trajectory is crucial, as successful cognitive development positively influences life quality through multiple factors including physical health and socioeconomic success.[30]

For ADHD, a large review from 2017 of 34 meta-analyses reported consistently lower cognitive performance, including executive functioning, among individuals with ADHD compared to healthy controls with differences increasing with age. Highest differences between ADHD and healthy controls were found for intelligence and reaction time variability.[31] The authors conclude that, given the broad range of the reported effect estimates, clinically meaningful neurocognitive deficits are not consistently found within individuals with behavioral symptoms of ADHD.[31]

ASD is associated with cognitive deficits, where children have generally been described as having an uneven cognitive profile.[32] Deficits in executive functioning is a characteristic of ASD and may be even worse among children with comorbid Intellectual disability.[33] Further, ASD is associated with alterations of social cognition as implied by its core symptoms.[34] In a review from 2023 authors concluded based on reports on Wechsler Intelligence test that autistic groups score lower in working memory and processing speed, however presented with normal verbal and non-verbal reasoning.[32]

EARLY LIFE RISK FACTORS FOR NEURODEVELOPMENTAL DISORDERS

Interest in how an adverse fetal environment may increase risk of future disease began with The Developmental Origins of Health and Disease (DOHaD) theory proposed by Barker and colleagues.[35] This theory was based on epidemiological studies associating fetal growth measures - as proxies of fetal undernutrition - to adult mortality and disease.[36–38] The theory expands to neurodevelopment, where several environmental exposures, including dietary factors during the intrauterine phase, have been linked to an increased risk of neurodevelopmental disorders.[39]

For both ADHD and ASD, genetic predisposition is the most important risk factor with heritability estimates recorded to 74% for ADHD (mean heritability estimate from 37 studies on twins)[40] and 83% for ASD[41] (in meta-analysis reported within range of 64-91%).[42] Only a small percentage of the disorders can be assigned specific genetic alterations – and the disorders are

considered polygenic i.e. arising from the effects of multiple risk genes.[40,43,44] Gene-environment interactions may be encompassed in the high heritability estimates, increasing the influence of environmental risk factors for the development of these disorders.[13] Overall, neurodevelopmental disorders are multifactorial and proposedly arise from a complex combination of genetic and environmental risk factors.[45] Environmental influences may also occur through maternal genotype, and a recent study found such genetic environmental effects to play a significant role for the development of ADHD.[46]

Several prenatal risk factors have been associated with ADHD and ASD in observational studies. These risk factors include, among others, low birth weight[47,48], prematurity[49], maternal body mass index (BMI)[50], lower prenatal vitamin D[51], pregnancy inflammation[52], and unhealthy dietary patterns.[53] This thesis focuses on pregnancy vitamin D, pregnancy inflammation and an unhealthy dietary pattern in pregnancy as prenatal risk factors for neurodevelopmental disorders and cognitive functioning. The current knowledge and scientific background for these proposed risk factors are therefore described in depth below.

PREGNANCY VITAMIN D AND CHILD NEURODEVELOPMENT AND COGNITION

Vitamin D deficiency is highly prevalent in pregnancy. Utilizing a cut-off of 50 nmol/L, vitamin D deficiency has been estimated to occur in above 50% of pregnancies.[54] The fetus relies on maternal vitamin D passing through the placenta - and neonatal and maternal vitamin D levels have been shown to be highly correlated.[55] In animal studies, vitamin D has been demonstrated to be important for fetal brain development where deficiency has been linked to morphological alterations and changes in gene expressions of importance for neurodevelopment[56], and vitamin D supplementation has been shown to reverse negative effects of inflammation on behaviours analogous to those seen in neurodevelopmental disorders.[57]

In observational studies, low vitamin D status in pregnancy has been linked to an increased risk of ADHD[58,59] and more severe ADHD traits[60,61] as well as ASD[62,63] and more severe autistic traits[64,65]. For cognitive functions, lower pregnancy vitamin D has been associated with poorer early mental and psychomotor development[66], intelligence[67,68], executive functioning[69], working memory[69], and overall cognition[70,71]. Existing studies have also reported no association between pregnancy vitamin D and neurodevelopmental disorders[72–74] or measures of cognitive performance[75–79]. A meta-analysis of the effects of prenatal vitamin D on offspring cognition and

neurodevelopment found higher levels of vitamin D to overall improve cognitive development and to be protective of ADHD and traits of autism.[51]

For both neurodevelopmental disorders and cognitive measures as outcomes, observational studies may be influenced by residual confounding from the many lifestyle factors related to especially vitamin D status in pregnancy[80] and the investigated outcomes. Randomized supplementation studies overcome this limitation by their design and can therefore be an important step for assessing causality of vitamin D in pregnancy as a risk factor for offspring neurodevelopmental disorders and cognitive functioning. To our knowledge there are no available studies on vitamin D supplementation on risk of neurodevelopmental disorders in other cohorts than COPSAC2010. One small study involving mothers who had previously given birth to a child with autism, showed they could reduce the recurrence rate of autism supplementing with high dose vitamin D3 in pregnancy (7000 IU/day), during breastfeeding (1000 IU/day), and in case of no breastfeeding the child received 1000 IU/day. The 19 children were followed-up at age 3, and the authors showed the recurrence rate of autism was reduced to 5% instead of the expected 20%.[81] In the COPSAC2010 cohort, on which the vitamin D analyses within this thesis are based, the effect of high-dose pregnancy vitamin D3 supplementation was tested on measures of offspring cognitive development using the Bayley Scales of Infant and Toddler Development at age 2.5 and neurodevelopmental measures up to age 6 including information on motor milestones, language development, general neurodevelopment (Ages and Stages Questionnaire) and emotional and behavioural problems (Strengths and Difficulties Questionnaire). The study did not show that the vitamin D3 intervention improved neurodevelopment.[82] For cognition as outcome, one preexisting supplementation study showed higher Brigance language scores in offspring of mothers receiving 2000 IU/day of vitamin D compared to standard dose (400IU per day) from the second trimester of pregnancy. No effect was observed with a supplementation of 4000 IU/day compared to standard dose.[83]

PREGNANCY DIETARY PATTERNS AND CHILD NEURODEVELOPMENT

There is an increasing interest in the effects of maternal diet in pregnancy on offspring mental health with existing research linking an insufficient intake of specific micronutrients and macronutrients during pregnancy to increased child risk of abnormal neurodevelopment.[84] Research has also investigated the impact of pregnancy dietary patterns for childhood neurodevelopmental disorders - which in contrast to studies focusing on specific nutrients may

capture a broader aspect of diet including relative intakes of different food groups as well as combined effects hereof.[85]

Unhealthy dietary patterns in pregnancy derived from maternal self-reported dietary intake in food frequency questionnaires (FFQs) have been associated to an increased risk of childhood neurodevelopmental disorders in multiple studies from separate mother-child cohorts. In the large Norwegian Mother, Father and Child Cohort study (MoBa) higher pregnancy maternal diet quality was associated with lower risk of ADHD diagnosis and less severe ADHD traits at age 8.[86] Combining data from MoBa and the Avon Longitudinal Study of Parents and Children (ALSPAC) further showed lower risk of autism diagnosis and fewer social communication difficulties among pregnant women with a more healthy dietary pattern.[87] The French EDEN cohort also found that a more westernized diet in pregnancy was associated with increased number of inattention/hyperactivity symptoms in the offspring.[88]

Based on data from the COPSAC2010 cohort, David Horner et al.[53] showed a significant association between a higher principal component (PC) derived Western dietary pattern score in pregnancy and increased risk of offspring ADHD and autism at the age of 10. A Western dietary pattern was also associated with increased severity of traits of both disorders. The associations were robust to extensive confounder control including maternal ADHD polygenic score (PGS). The findings were corroborated by replication in 3 independent mother-child cohorts including a total of >60,000 individuals. The replication cohorts included 59,725 mother-child pairs from the Danish National Birth Cohort (DNBC), 656 mother child-pairs from the Vitamin D Antenatal Asthma Reduction Trial cohort (VDAART), and 328 children from the COPSAC2010 cohort.[53]

To our knowledge no randomized clinical trial (RCT) has tested the causality of the observational findings between pregnancy diet and offspring neurodevelopmental disorders. However, one RCT did find a positive effect of a Mediterranean dietary intervention in pregnancy on early neurodevelopmental measures at age 2.[89]

INFLAMMATION IN PREGNANCY AND CHILD NEURODEVELOPMENT

Maternal immune activation (MIA) in pregnancy, induced by various environmental exposures, has been associated with offspring neurodevelopment.[90] MIA in the form of low-grade systemic chronic inflammation in pregnancy may be triggered by factors including obesity, smoking, and pregnancy complications in form of pre-eclampsia and diabetes, while acute high-grade

inflammation is caused by infections.[52] Of these factors both infections[91,92] and chronic inflammatory states for example induced by hypertensive disorders of pregnancy[93] and obesity in pregnancy[94] have been associated with adverse neurodevelopmental outcomes.

The degree of inflammation in pregnancy reflected by pregnancy levels of inflammatory markers has also been associated with risk of neurodevelopmental disorders. This thesis investigates causal effects of three inflammatory markers previously associated with adverse neurodevelopment and cognitive performance: C-reactive protein (CRP), interleukin-6 (IL-6) and glycoprotein acetyls (GlycA). In the COPSAC2010 cohort we found an association between maternal inflammatory activity, measured by CRP in pregnancy week 24, and increased offspring risk of ADHD and increased ADHD trait severity. Results were robust to confounder adjustment including maternal ADHD PGS. Risk of ADHD increased with higher levels of CRP showing a dose-response relationship which is supportive of causal effects.[95] Higher pregnancy CRP has also previously been associated with poorer early life neurodevelopmental scores.[96] Higher pregnancy IL-6 has in the COPSAC2010 cohort been associated with impaired everyday but not test-based executive functioning at child age 10[97] - an impairment related to neurodevelopmental disorders.[11,33] Further, proteomic data on pregnancy inflammation from the COPSAC2010 cohort suggested IL-6 as an important protein associated with neurodevelopmental disorders.[98] GlycA is a newer metabolomic marker reflecting sustained systemic inflammation.[99] Inflammation measured by GlycA has been shown to mediate the negative effect of an unhealthy pregnancy dietary pattern on offspring early life cognitive scores.[100]

GENETIC CONFOUNDING

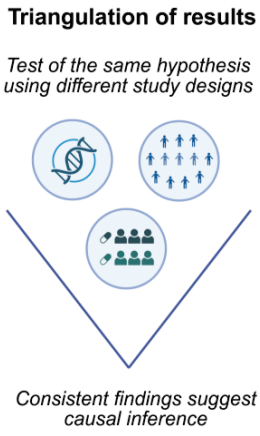
Observational associations between prenatal exposures and offspring neurodevelopment may be confounded by genetic predisposition both increasing the risk of the observed exposure and the measured outcome. Genetic confounding is especially important to consider in the case of disorders with high heritability such as ADHD and autism. Newer research has sought to investigate the potential influence of genetic confounding using genetic risk scores for ADHD. Utilizing the comprehensive genetic data from the Norwegian Mother, Father and Child Cohort Study (MoBa), maternal ADHD PGS has been shown to associate with pregnancy-related factors previously linked to an increased risk of ADHD including pregnancy smoking, pre-pregnancy BMI and intake of supplements which suggests potential risk of genetic confounding in observational analyses of these factors.[101] E.g. the observed association between pregnancy

smoking and offspring risk of ADHD underlines the importance of taking genetic confounding into account. The increased risk of offspring ADHD after in-utero exposure to maternal smoking has been replicated across observational studies, however when utilizing study designs taking shared family factors such as genetics into account the association attenuates/dissapears.[11,102]

TRIANGULATION

To investigate causality for observational associations, which are at risk of residual confounding and reverse causality, results can be sought triangulated across separate study designs which are affected by different sources of bias. With similar findings across different study designs the observed association is more likely to be causal (See Figure 1). Confounding can be accounted for statistically or by the specific study design. With the aim of triangulating results, design-based approaches are important and include for example randomized clinical trials (RCTs), sibling-comparison designs or genetic studies as trio PGS or Mendelian randomization analyses which are utilized in this thesis.[103]

Figure 1. showing the concept of triangulation. Made with inspiration from Munafó et al.: "Triangulating Evidence through the Inclusion of Genetically Informed Designs".[103] *Created in BioRender. Bønnelykke, K. (2025) https://BioRender.com/d3zi1lo*

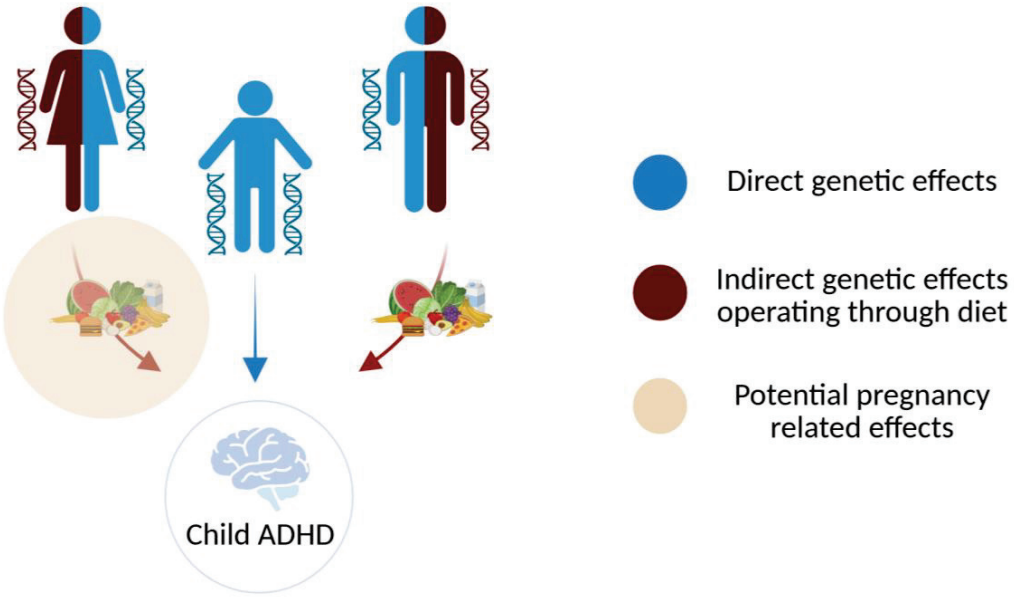


In this thesis we present post-hoc analyses of a high-dose vitamin D3 supplementation RCT to investigate potential positive effects of vitamin D3 in pregnancy on child neurodevelopment and child cognitive performance. As there, to our knowledge, are no existing randomized clinical trials investigating the effect of vitamin D3 supplementation in pregnancy and offspring neurodevelopmental disorders and broad cognitive performance in late childhood, this is an

important addition to the existing observational studies and animal studies investigating the association between pregnancy vitamin D and these outcomes.

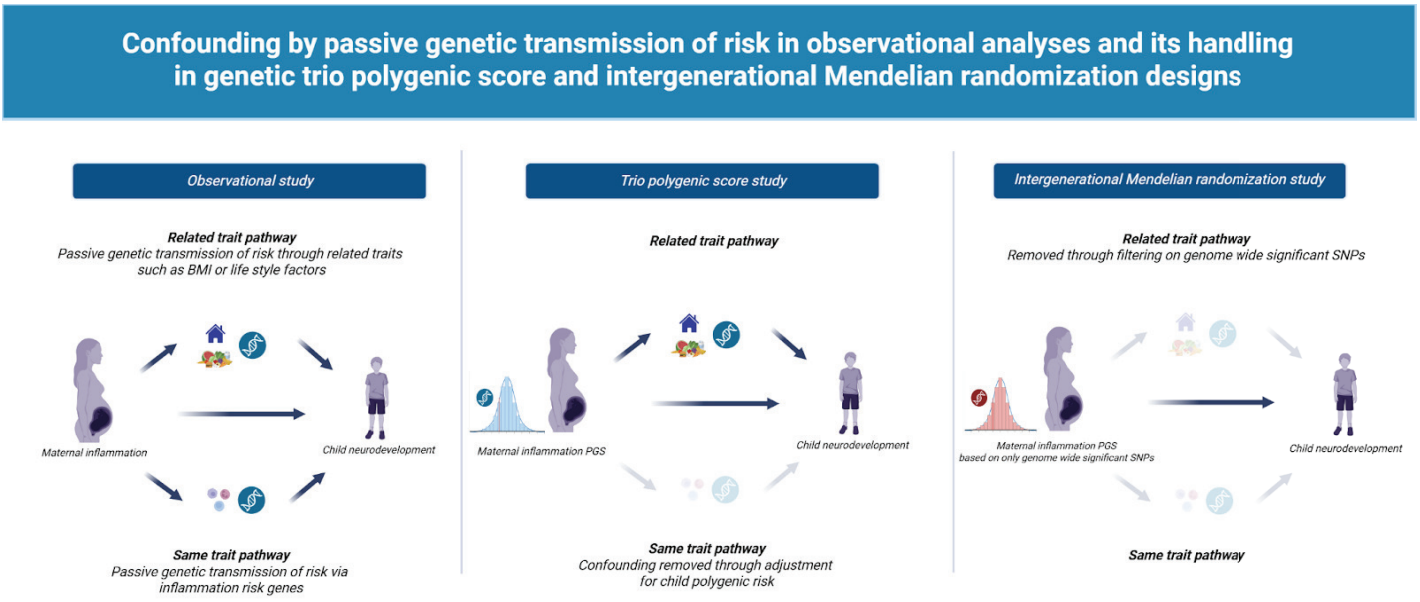
Further, this thesis uses genetically informed study designs to investigate the causality of suggested prenatal risk factors, unhealthy diet and inflammation, for neurodevelopmental disorders. To investigate the impact of pregnancy dietary patterns on offspring risk of ADHD we utilize trio polygenic score (PGS) analyses including mother, father and child PGSs for dietary patterns. Trio PGS analyses can detect maternal indirect genetic effects - genetic effects operating through the environment - accounting for potential genetic confounding.[104] The detection of indirect genetic effects of dietary patterns would support the validity of the reported observational association. See Figure 2 and Appendix, Paper III, Figure 1 for more detail on the trio model.

Figure 2. Graphical presentation of trio PGS analyses with the aim of detecting indirect genetic effects of pregnancy diet indicative of causal effects. This figure builds on concepts previously illustrated in a directed acyclic graph of the trio model by Pingault et al.[104] (See Appendix, Paper III, Figure 1) *Created in BioRender. Bønnelykke, K. (2026) https://BioRender.com/ghuyslo*



The causality of the observational association between pregnancy inflammation and childhood neurodevelopmental disorders is investigated in this thesis using two genetically informed study designs: trio PGS analyses and intergenerational Mendelian randomization (MR) analyses. Where intergenerational MR possesses the ability to test actual causal effects of pregnancy inflammation on the investigated outcomes. Figure 3 shows how genetic confounding was handled in Paper IV by testing for maternal effects of inflammation in pregnancy on offspring neurodevelopment utilizing both trio PGS and intergenerational MR analyses. The intergenerational MR analyses supplement the trio PGS analyses by seeking to further reduce genetic confounding through related trait pathways such as lifestyle factors and BMI. See Appendix, Paper IV, Figure 1 for more detail.

Figure 3. Confounding by passive genetic transmission in observational studies, and how it is addressed in trio PGS and intergenerational MR analyses of pregnancy inflammation as a potential risk factor for child neurodevelopmental disorders. (See Appendix, Paper IV, Figure 1) *Created in BioRender. Bønnelykke, K. (2026) https://BioRender.com/znhs3q2*



AIM AND OBJECTIVES

This thesis investigates dietary and inflammatory prenatal risk factors for childhood neurodevelopmental disorders. Papers I and II examine the role of maternal vitamin D3 supplementation and circulating vitamin D in pregnancy in relation to subsequent child neurodevelopment and cognitive function. Papers III and IV assess the potential causal effects of two proposed risk factors - an unhealthy pregnancy dietary pattern and inflammation during pregnancy - on childhood neurodevelopment, using genetically informed study designs.

PAPER I

Paper I is a post-hoc analysis of the COPSAC2010 high-dose vitamin D3 trial in pregnancy. The study investigates the potential protective effect of vitamin D3 supplementation on child neurodevelopmental disorders ADHD and autism as well as traits hereof. Additionally, the study estimates the association between circulating levels of pregnancy vitamin D and neurodevelopmental outcomes.

The main study question was: Is high-dose vitamin D3 supplementation in pregnancy associated with a decreased risk of neurodevelopmental disorders and traits hereof at age 10?

PAPER II

Paper II is an additional post-hoc analysis of the COPSAC2010 high-dose vitamin D3 trial in pregnancy. The study investigates the potential protective effect of vitamin D3 supplementation on child cognitive functions at age 10. As for paper I, this study also includes observational analyses on circulating levels of vitamin D in pregnancy and cognitive functions.

The main study question was: Is high-dose vitamin D3 supplementation in pregnancy associated with improved cognitive function at age 10?

PAPER III

Paper III uses genetic data from mother, father and child trios from the COPSAC2010 cohort to investigate the potential causal effect of an unhealthy dietary pattern in pregnancy on offspring risk of ADHD and severity of ADHD traits. In this study replication was sought in two external mother-child cohorts: the Norwegian Mother, Father and Child Cohort Study (MoBa) and the Avon Longitudinal Study of Parents and Children (ALSPAC).

The main study question was: Is an unhealthy dietary pattern in pregnancy causally associated with offspring risk of ADHD and severity of ADHD traits?

PAPER IV

Paper IV utilizes data from the large MoBa cohort to investigate the potential causal effects of pregnancy inflammation on offspring neurodevelopment. Analyses were based on polygenic scores for C-reactive protein (CRP), interleukin-6 (IL-6) and glycoprotein acetyls (GlycA) which were sought validated in the COPSAC2010 cohort.

The main study question was: Are higher levels of inflammation in pregnancy causally associated with offspring risk of neurodevelopmental disorders?

METHODS

The methods described in the following section cover all 4 papers included in this thesis. At the end of the section, the statistical approach for each paper is described separately within the subsection “statistical approach”.

MOTHER-CHILD COHORTS

For this thesis we utilized data from three separate mother-child cohorts. Paper I and Paper II investigating the influence of pregnancy vitamin D on offspring neurodevelopment and cognition were solely based on the COPSAC2010 cohort. Paper III and Paper IV were genetically informed study designs investigating the causality of the proposed risk factors for impaired childhood neurodevelopment: an unhealthy dietary pattern in pregnancy (Paper III) and pregnancy inflammation (Paper IV). Paper III was conducted in the COPSAC2010 cohort and findings were externally validated in the MoBa and ALSPAC mother-child cohorts. Paper IV was conducted in the MoBa cohort with use of the COPSAC2010 cohort for validation of genetic instruments.

COPSAC2010

The Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) 2010 cohort is an unselected mother-child cohort. Using information on mandatory antenatal pregnancy visits at the general practitioner, pregnant women were invited to participate. The period of recruitment was from 2008 until 2010. The following exclusion criteria were applied: daily intake of vitamin D supplement exceeding 600IU, medical conditions related to the endocrine system, heart or kidneys. Enrolled women were invited to a first visit at the COPSAC research unit at pregnancy week 22-26. In total, COPSAC2010 included 700 mother-child pairs, and the included children were followed closely and deeply phenotyped with regular visits at the COPSAC research unit. From birth until age 10, where main outcomes included in this thesis were assessed, the children attended 14 visits.[105]

MOBA

Data from the large Norwegian Mother, Father and Child Cohort Study (MoBa) was used for replication in study III and for main analyses in paper IV. MoBa is a population-based pregnancy cohort study which has obtained information on multiple pregnancy and childhood exposures including genetic exposures which can be linked to numerous health and developmental

outcomes in the children obtained through both questionnaire data and linkage to Norwegian National health registries. Pregnant women were invited to participate at routine ultrasound examinations. Of the invited women, 41% agreed to participate. The included children were born between 1999-2008. Approximately 114,500 children, 95,200 mothers and 75,200 fathers are included in the cohort, hereof approximately 16.400 women participated with more than one pregnancy.[106] The MoBa cohort provides a unique possibility to perform genetically informed study design as the cohort constitutes the, to our knowledge, largest available sample of genotyped mother, father and child trios (N~40,000). Genotyping and quality control have been described previously.[107]

ALSPAC

Data from The Avon Longitudinal Study of Parents and Children (ALSPAC) was used for replication in Paper III. ALSPAC is a prospective mother-child cohort which was designed to investigate the influence of a variety of exposures including environmental, genetic and psychological exposures on multiple health and developmental outcome measures.[108] Pregnant women with an expected delivery date between April 1991 and December 1992 were recruited from the Southwest of England, Avon. 72% of invited pregnant women consented to participate. The final sample size for data collected after the age of 7 was 15,447 pregnancies wherefrom 14,901 children were alive a age 1.[108,109] Also partners were invited to participate, which resulted in a total of 12,113 partners providing data to the study.[110]

VITAMIN D EXPOSURES IN COPSAC2010

VITAMIN D3 RANDOMIZED CLINICAL TRIAL

Pregnant women enrolled in the COSPAC2010 cohort were randomized 1:1 to a high-dose vitamin D3 supplementation or matching placebo from week 24 of pregnancy until 1 week postpartum. The high-dose intervention consisted of a daily 2400 IU cholecalciferol D3 supplementation. All pregnant women within the cohort were instructed to continue the recommended daily dose by the Danish National Board of health of 400IU vitamin D3 which resulted in a total dose of 2800IU vitamin D3 in the intervention group and 400IU in the placebo group. Thus, the high-dose supplementation corresponded totally to a vitamin D3 intake that was seven times the daily recommended dose. To assess compliance, women were instructed to return unopened capsules.[105] In total, 74% of the women adhered to the intervention with adherence defined as intake of a minimum of 80% of the prescribed capsules.[111] With the exception of medical emergency, the study was blinded until the age of three. The cohort was

powered according to the main outcome which was recurrent wheeze within the first three years of life. Analyses on neurodevelopmental and cognitive outcomes reported in this thesis are all post hoc follow-up analyses of the vitamin D3 trial.

Throughout the same time span as for the vitamin D3 intervention, the women were included in a fish oil trial in a factorial 2×2 design. The fish oil intervention consisted of a daily dose of 2400 mg/day of n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) or placebo capsule containing olive oil.[105]

MATERNAL SERUM 25(OH)D

Serum 25-hydroxyvitamin D (25(OH)D) levels were analysed by isotope dilution liquid chromatography-tandem mass spectrometry at pregnancy week 24 and 1 week postpartum - corresponding to before and after the vitamin D3 intervention period.[112] Venous blood samples for the analyses were centrifuged at 4300 rpm with a duration of 10 minutes. The separated serum was hereafter frozen and stored at -80°C until analysis. Analyses were performed at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark.

CHILD SERUM 25(OH)D

Child serum concentrations of 25(OH)D were determined at age 6 months and 6 years. These analyses were performed at the Channing Division of Network Medicine Boston, USA. Quantitative determination of 25(OH)D was performed by a chemiluminescence immunoassay (CLIA) with use of the DiaSorin LIAISON 25(OH)D Vitamin D Total Assay. US National Institute of Standards and Technology (NIST) level 1 protocol was used by the laboratory.[113,114]

GENETIC INSTRUMENTS

In Paper III and Paper IV the causality of dietary pattern in pregnancy and pregnancy inflammation as risk factors for child impaired neurodevelopment is investigated in trio PGS and intergenerational Mendelian randomization (MR) analyses conducted using genetic proxies for the investigated exposures. These genetic instruments are described below.

POLYGENIC SCORES FOR DIETARY PATTERN

Polygenic scores for dietary pattern were constructed for mothers, fathers and children in the COPSAC, MoBa and ALSPAC mother-child cohorts and used for trio PGS analyses. The dietary pattern PGSs were based on a GWAS by Cole et al. which utilized data from 449,210 European adults with available information from 24-hour recall food frequency questionnaires (FFQs).

Based on the FFQ data, Cole et al. derived dietary patterns by principal component analyses. The PGSs were calculated based on the Cole et al. GWAS of the highest heritable principal component derived dietary pattern for which the heritability estimate was reported to 0.136. The dietary pattern was characterized by higher values associated with a healthy dietary pattern (higher intake of wholegrain bread, fruit/vegetables, oily fish) and lower values with an unhealthy/Western dietary pattern (higher intake of white bread, butter and oil spread, processed meat, high-fat milk).[115]

As dietary pattern PGSs were constructed according to dietary information from an adult non-pregnant population, the validation of the maternal dietary pattern PGSs against pregnancy dietary information was crucial. For this purpose, we utilized pregnancy dietary information which was available for alle 3 cohorts included in Paper III: COPSAC2010, MoBa and ALSPAC. Pregnancy diet was reported for 43 food groups at pregnancy week 24 in the COPSAC2010 cohort[116], for 98 food groups at pregnancy week 22 in the MoBa cohort[117] and for 43 food groups in the ALSPAC cohort at pregnancy week 32.[118]

POLYGENIC SCORES FOR PREGNANCY INFLAMMATION

PGSs for pregnancy inflammation were calculated based on publicly available summary statistics from GWASs on circulating levels of CRP (N = 675,531)[119,120], IL-6 (N=21,758)[121] and GlycA (N=115,078)[122] in European individuals. For intergenerational MR analyses, PGSs were calculated based on exclusively genome wide significant single nucleotide polymorphisms (SNPs) to reduce horizontal pleiotropy.

Inflammation PGSs and intergenerational MR instruments were sought validated against circulating levels of maternal inflammatory markers in pregnancy. The validity of the CRP, IL-6 and GlycA PGSs inflammation were all tested in the COPSAC2010 which contained available information on pregnancy measures of all three inflammation markers. For CRP we performed additional validation of the PGS utilizing data from a subsample from the MoBa study including 2,976 pregnancy women with available data on circulating levels of high-sensitivity C-reactive protein (hs-CRP).[123]

NEURODEVELOPMENTAL OUTCOMES

COPSAC 2010

The COPSAC2010 cohort underwent an extensive clinical evaluation of psychopathology and neurocognition at the age of ten at the COPSYCH visit (Copenhagen Prospective Study on Neuro-PSYCHiatric Development). COPSYCH is a collaborative research project between COPSAC and Centre for Neuropsychiatric Schizophrenia Research (CNSR) with the aim of investigating early life risk factors for neurodevelopmental disorders utilizing the two RCTs imbedded within the COPSAC cohort and the extensive longitudinal information on environmental exposures. Examinations were performed between January 2019 and December 2021.[14]

Categorical psychopathology was assessed by the semi-structured clinical diagnostic interview Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version.[124] Interviews were videorecorded to allow for supervision by senior researcher and psychologist and specialist in child and adolescent psychiatry Jens Richardt Møllegaard Jepsen. External validation of diagnoses was performed by clinical professor in child and adolescent psychiatry Niels Bilenberg. Research diagnoses were based on all available clinical information and were assigned according to the ICD-10. In the COPSYCH study, DF84.0, DF84.5, and DF84.8 diagnostic codes were assigned for autism, and DF90.0, DF90.8 and DF98.8 for ADHD. ADHD diagnoses were grouped following the DSM-V terminology where ADHD Combined Presentation included DF90.0 and DF90.8, and ADHD predominantly Inattentive Presentation included DF98.8C.

Dimensional psychopathology was assessed by clinically evaluated symptom load of psychopathology calculated based on number of endorsed symptoms for autism and ADHD using the K-SADS-PL. From the K-SADS-PL, we derived symptoms component scores for both autism and ADHD by applying multiple correspondence analyses from which we extracted the first component as the primary score. Parent-rated trait scores of ADHD and autism were assessed by the well-validated questionnaires ADHD rating scale (ADHD-RS)[125] and the Social Responsiveness Scale 2 (SRS-2).[126,127]

MOBA

From MoBa, diagnoses of ADHD and autism were obtained through the Norwegian Patient Registry (NPR).[128] We utilized lifetime diagnoses and required a minimum of two registrations

of ICD-10 diagnostic codes for either ADHD (categories F90 and F98.8) or autism (category F84). Information on neurodevelopmental traits were obtained from questionnaires filled out by the mothers between child age three to eight years. We included information on language and motor development as well as ADHD and autistic traits assessed from the questionnaires described below. Language development was evaluated at age 3 and 5 by the Ages and Stages Questionnaire (ASQ).[129] Motor development was evaluated at age 3 using the ASQ motor items and at age 5 years by the Child Development Inventory (CDI).[130] Traits of ADHD were evaluated at age 3 and 5 years by the Child Behaviour Checklist (CBCL)[131] and again at age 8 by the Rating Scale for Disruptive Behavior Disorders (RS-DBD).[132] Traits of autism were evaluated by the Social Communication Questionnaire (SCQ) at ages 3 and 8 years.[133] For all calculated measures of neurodevelopmental traits based on this data, higher scores were indicative of fewer skills/more problems.

ALSPAC

From the ALSPAC cohort we utilized mother-reported ADHD traits at child age 7 which were evaluated by the structured diagnostic parent interview Development and Wellbeing Assessment (DAWBA).[134]

COGNITIVE OUTCOMES COPSAC2010

At the COPSYCH 10-year visit, neurocognition was assessed by an extensive test battery. To reduce the number of tested outcomes, we refined the cognitive domains which were outlined in the COPSYCH protocol prioritizing automated cognitive tests and the well-validated Wechsler Intelligence Scale for Children - fourth edition (WISC-IV).[14] See Table 2.1 for specification of cognitive domains and functions included as cognitive outcomes in Paper II. For more detail see Appendix, Paper II, Supplementary material.

Table 2.1. Overview of cognitive domains and functions including the specific tests utilized to assess each domain. (See Appendix, Paper II, Supplemental Material, Table 1)
CANTAB = Cambridge Neuropsychological Test Automated Battery, WISC-IV = Wechsler Intelligence Scale - Fourth edition, TOMAL-2 = Test of Memory and Learning - Second edition

Domain	Function	Test	Outcome metric
Intelligence	Estimated intelligence	Vocabulary (WISC-IV)	Total number correct
		Matrices (WICV-IV)	Total number correct
Processing speed	Speed of processing	Coding (WISC-IV)	Total number correct
		Symbol search (WISC-IV)	Sum of total number correct. errors subtracted
Reaction time	Reaction time	Reaction time (CANTAB)	Simple- and five-choice reaction time
Attention	Sustained attention	Rapid visual information processing (CANTAB)	A-prime (unitless sensitivity score)
Motor function	Motor speed	Reaction time (CANTAB)	Simple- and five-choice movement time
Memory	Verbal memory	Word selective reminding - immediate recall (TOMAL 2)	Total number words recalled over six learning trials
		Object recall (TOMAL 2)	Total number object recalled over five learning trials
	Visual memory	Paired associates learning (CANTAB)	Total errors (adjusted)
Working memory	Verbal working memory	Digit span (WISC-IV)	Total number correct forward and backward sequencing
		Letter-number sequencing (WISC-IV)	Total number correct sequences
Executive function	Flexibility/Set shift	Intra-extra dimensional set shift (CANTAB)	Extra-dimensional stage errors
	Spatial Working Memory	Spatial working memory (CANTAB)	Total errors
	Planning	Stockings of Cambridge (CANTAB)	Problems solved in minimum moves

COVARIATES

PAPER I and PAPER II

In analyses on vitamin D3 supplementation, we included the following covariates: child sex, season of birth, pre-intervention maternal 25(OH)D levels and n-3 LCPUFA supplementation.

Observational analyses on the association between maternal pre-intervention circulating 25(OH)D and neurodevelopmental outcomes were adjusted for: child sex, birth weight, gestational age, season of week 24 25(OH)D measurement, social circumstances (PC based on maternal age, household income and maternal education), maternal smoking in third trimester of pregnancy, maternal pre-pregnancy weight, and fathers´ age. For the cognitive measures, covariates included in observational analyses of maternal preintervention 25(OH)D were selected based on a directed acyclic graph (DAG) which depicted the association of interest and potential confounding factors (See Appendix, Paper II, Supplemental Figure 1). For these analyses, additional covariates were added based on existing knowledge on risk factors for impaired cognitive performance, including COPSAC research findings[97,135], indicating associations between inflammation and poorer cognitive function: gestational diabetes mellitus, preeclampsia, alcohol during pregnancy, maternal pregnancy inflammation and maternal pregnancy diet. Finally, all analyses on cognitive outcomes were adjusted for age at the COPSYCH visit as cognitive measures were included raw i.e. unadjusted for child age and sex.

PAPER III and PAPER IV

In genetic analyses using COPSAC and ALSPAC data, PGS analyses were adjusted for child sex. Due to the larger size and the longer inclusion time of the MoBa cohort these analyses were additionally adjusted for birth year, and the genetic instruments were adjusted for genotyping and imputation batch and population structure (first 20 PCs).

ETHICS

All three research cohorts (COPSAC, MoBa and ALSPAC) were approved by the relevant regional or national research ethics committees and conducted in accordance with internationally recognized ethical standards, including the principles of the Declaration of Helsinki.

The COPSAC2010 study was approved by the Local Ethics Committee (H-B-2008-093, and the Danish Data Protection Agency (2015-41-3696). Both parents gave written informed consent before enrolment.

MoBa was established based on a license from the Norwegian Data Protection Agency and was approved by the Regional Committees for Medical and Health Research Ethics. Currently, the MoBa cohort is under regulation by the Norwegian Health Registry Act. Written informed consent was obtained from both parents.[136]

Ethical approval for the ALSPAC study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Informed consent was obtained from participants according to recommendations from the ALSPAC Ethics and Law Committee.[110] See full information on consent procedures here: https://www.bristol.ac.uk/alspac/researchers/research-ethics/.

STATISTICAL APPROACH

PAPER I

We estimated the effect of high-dose vs. standard dose vitamin D3 supplementation on risk of neurodevelopmental disorders (autism and ADHD) by logistic regression models. For continuous outcomes, autism and ADHD symptoms component scores which reflect symptom load, we used linear regression models. The association between maternal pre-intervention circulating 25(OH)D and neurodevelopmental disorders and symptom load were likewise estimated by logistic and linear regression models respectively. We visualized the association between maternal 25(OH)D measured pre-intervention and autism symptom load in a partial residual plot adjusted for covariates. For both the vitamin D3 supplementation and circulating 25(OH)D we performed analyses crude and adjusted for potential confounders. Effect estimates for linear regression models were per 10 nmol/L increase on 25(OH)D.

To test if the effect of the vitamin D3 supplementation differed between mothers with either high or low 25(OH)D prior to the intervention, we tested for interaction between the circulating pre-interventional 25(OH)D and the vitamin D3 supplementation on both categorical and dimensional measures of autism and ADHD. This was done by adding cross products to the models. Hereafter, we performed analyses on the effect of the vitamin D3 supplementation across measures of pre-interventional 25(OH)D. Further, interaction with sex was tested for all analyses.

We performed sensitivity analyses with additional adjustment for maternal polygenic risk for neurodevelopmental disorders to account for potential genetic confounding of observational analyses. Lastly, to support findings on clinician rated outcomes, we included analyses on parent-rated traits of autism and ADHD reported in the well-validated SRS-2 and ADHD-RS questionnaires.

PAPER II

The effect of the high-dose vitamin D3 supplementation and maternal pre-intervention 25(OH)D was tested on 11 cognitive functions (organized within 8 cognitive domains) by linear regression models. Analyses were performed both crude and adjusted for potential confounders. Effect estimates for linear regression models were per 10 nmol/L increase in 25(OH)D.

As for Paper I, we tested for interaction between the vitamin D3 supplementation and maternal

pre-intervention 25(OH)D. Likewise as for Paper I, we tested for interaction with child sex. To evaluate if long-term effects of the vitamin D3 supplementation could be improved by high childhood circulating 25(OH)D, we tested the interaction between the vitamin D3 supplementation and child 25(OH)D at both age 6 months and 6 years.

Since ADHD is associated with cognitive impairments, we explored if the effects were driven by the presence of neurodevelopmental disorders by performing analyses excluding individuals with ADHD.

Due to the number of outcomes and the limited correlation between these (See Appendix: Paper II, Supplementary material, Figure 2) we FDR adjusted p-values (adjustment performed separately for effects of the RCT and for pre-intervention 25(OH)D analyses).

PAPER III

The predictive value of the maternal dietary pattern PGS on pregnancy diet was tested by linear regression models for the COPSAC2010 and the MoBa cohort (intake of each food group measured as number of grams per day). For validation using ALSPAC data, we used quasi-Poisson models (intake of each food group was estimated as weekly consumption frequency). The overlap between the maternal dietary pattern PGS and principal component-derived pregnancy Western dietary pattern (previously associated to offspring neurodevelopmental disorders[53]) was tested by Pearson correlation.

We tested the associations between maternal, child, and paternal dietary pattern PGS and ADHD outcomes using logistic regression models for ADHD diagnosis and linear regression models for ADHD total trait scores adjusted for sex of the child. We included sensitivity-analyses on ADHD presentations and ADHD trait subscales. We conducted trio models based on a single regression model which included maternal, paternal, and child dietary pattern PGSs as predictors of offspring ADHD, with each PGS mutually adjusted for the others.[104]

We used MoBa and ALSPAC data to replicate COPSAC2010 findings using a similar statistical approach.[136,137,108] In sensitivity analyses, we repeated MoBa analyses defining ADHD diagnosis by a minimum of two registered ICD-10 diagnostic codes. Further, due to the inclusion of siblings in MoBa, we performed sensitivity analyses with robust standard errors clustered on maternal ID.

PAPER IV

Firstly, genetic instruments for CRP, IL-6 and GlycA utilized in this study were validated against measured pregnancy inflammatory markers in the COPSAC2010 cohort and a subsample of the MoBa cohort using linear regression models.

Unadjusted PGS analyses, trio PGS analyses, and intergenerational MR analyses were all conducted using linear regression models for continuous measures of neurodevelopment and logistic regression models for diagnostic outcomes. Trio PGS analyses based on inflammation PGSs and intergenerational MR analyses were conducted in a similar manner including genetic instruments for all three inflammation genetic instruments for each family member i.e. mother, father and child.

We conducted two-sample MR based on publicly available summary statistics to investigate potential confounding of the observed association between pregnancy inflammation and child neurodevelopment - i.e. confounding arising from maternal genetic disposition to neurodevelopmental disorders increasing both pregnancy inflammation and offspring risk of the disorders. We used the Generalized Summary-data-based Mendelian Randomization (GSMR) method implemented in the GCTA software toolkit (version v1.94.1)[138] to investigate the potential causal relationship between inflammatory markers[119,121,122] and ADHD[43,44] and autism in the individual.

Missing data was imputed using the mice package from R and analyses were performed using robust standard errors clustered on maternal ID.

Paper IV was pre-registered on the Open Science Framework (OSF) (URL: https://osf.io/xrhs3).

Statistical analyses for all papers were performed using R statistical software versions 4.2.1, 4.3.1, 4.4.1 (the R Foundation, Vienna). Statistical significance was set as <0.05, 2-sided.

RESULTS

PAPER I

Paper I estimated the effect of high-dose vs standard dose vitamin D3 supplementation on offspring neurodevelopmental outcomes. Further, Paper I investigated the association between maternal pregnancy 25(OH)D (measured pre-intervention) and offspring neurodevelopmental outcomes in observational analyses.

BASELINE CHARACTERISTICS

The COPSAC2010 cohort included a total of 700 mother-child pairs. Hereof, 591 participated in the COPSYCH 10-year visit including the K-SADS-PL interview from which diagnoses and symptom loads of autism and ADHD were obtained (numbers reported after exclusion of individuals with birth weight <1500 g and gestational age < 28 weeks).

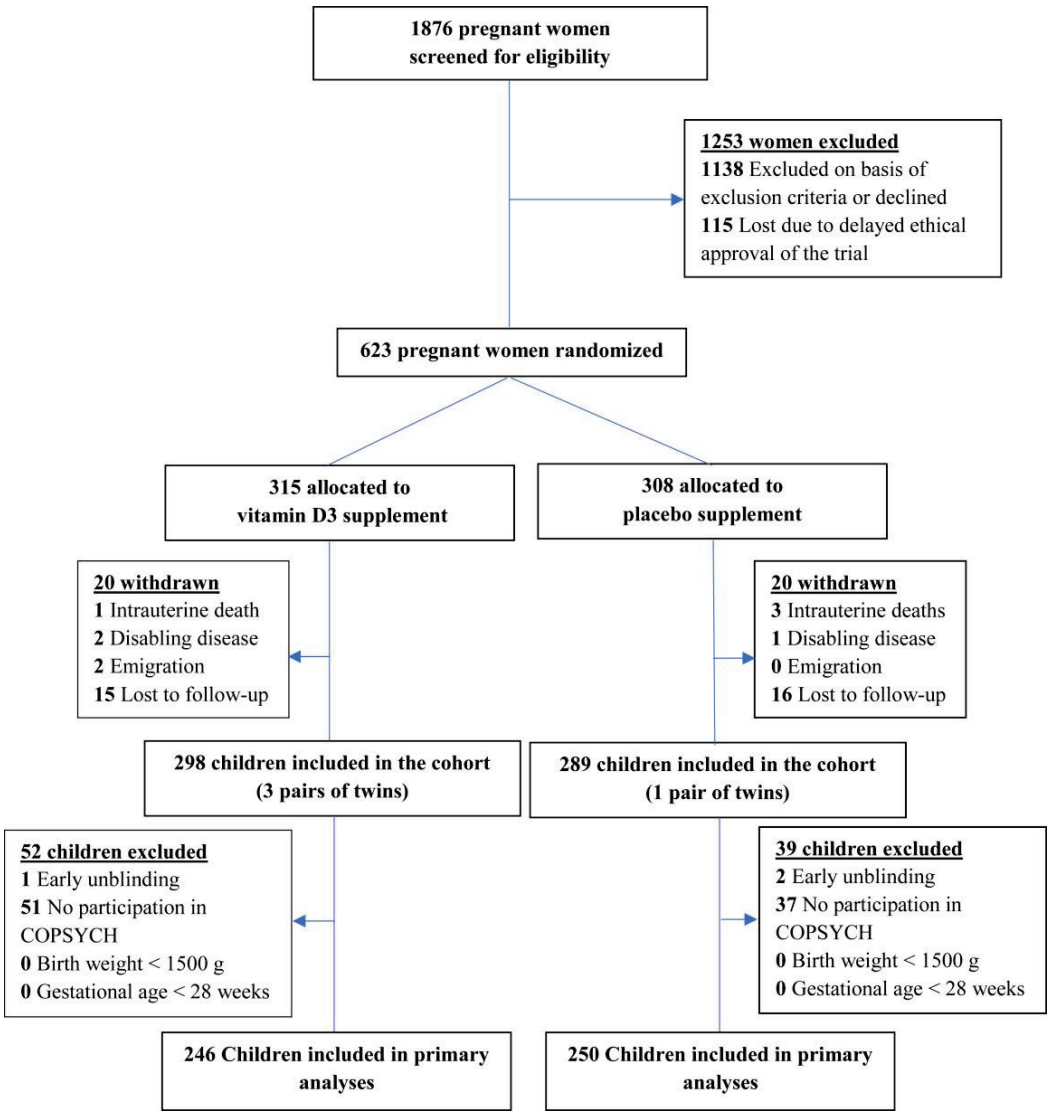
Due to a delay in ethical approval, analyses on the vitamin D3 supplementation were restricted to a total of 496 children wherefrom 246 received the high-dose supplementation and 250 standard dose of vitamin D3, See **Paper I, Figure 1 and Appendix, Paper I, Figure 1**. Baseline characteristics of mother-child pairs included in the vitamin D3 supplementation trial are shown in **Paper I, Table 1 and Appendix, Paper I, Table 1**. No major differences were observed between the high-dose and standard-dose groups. The high-dose vitamin D3 supplementation increased maternal post-intervention serum 25(OH)D significantly. Mean pre-intervention 25(OH)D was 76.05 (SD 25.92) nmol/L and 25(OH)D increased post-intervention (measured 1 week post-partum) to 108.33 (SD 35.31) nmol/L. Mean difference in 25(OH)D was 32.24 (95% CI = 27.13-37.35, $p<0.001$. The presentation of autism and ADHD among individuals with participation in the vitamin D3 trial has been presented in **Paper I, Table 1**. In total, 2.4% were diagnosed with autism and 11.7% with ADHD. Of notice, only a total of 12 cases (5 within the high-dose group, and 7 within the standard dose group) of autism were present in the sample.

For analyses on maternal pre-intervention serum 25(OH)D we were able to include all 591 individuals who participated in the COPSYCH K-SADS-PL evaluations. Baseline characteristics for the entire COPSYCH cohort can be found in the **Appendix, Paper I, Supplementary Material, Table 1**. Among the total 591 children eligible for analyses on maternal pre-intervention 25(OH)D, 2.7% were diagnosed with autism (N = 16) and 11.0% were diagnosed

with ADHD (N = 65). K-SADS-PL clinically rated symptoms were present for autism in 8.3% of the children and for ADHD in 28.8%.

Paper I, Figure 1. CONSORT participant flow diagram.

The figure is reproduced from Aagaard K. et al.: High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10: A randomized clinical trial. Am J Clin Nutr. 2024;119(2):362-370. No changes were made. This figure was made available under the CC BY 4.0 license: https://creativecommons.org/licenses/by/4.0/.



Paper I, Table 1. Baseline characterization of participants with participation in both the vitamin D3 RCT and the COPSYCH evaluation

The table is reproduced from Aagaard K. et al.: High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10: A randomized clinical trial. Am J Clin Nutr. 2024;119(2):362-370. No changes were made. This table was made available under the CC BY 4.0 license: https://creativecommons.org/licenses/by/4.0/.

Baseline characterization of participants of the vitamin D3 RCT included in the COPSYCH project

Stratified by participation in the vitamin D3 RCT	All	Placebo	Vitamin D3
	n = 496	*n* = 250	*n* = 246
Diagnosis of autism, *n* (%)	12 (2.4)	7 (2.8)	5 (2.0)
Individuals presenting clinically rated symptoms of autism, *n* (%)	40 (8.1)	25 (10.0)	15 (6.1)
Diagnosis of ADHD, *n* (%)	58 (11.7)	31 (12.4)	27 (11.0)
Individuals presenting clinically rated symptoms of ADHD, *n* (%)	143 (28.8)	76 (30.4)	67 (27.2)
Long-chain n-3 PUFA supplementation, *n* (%)	249 (50.2)	122 (48.8)	127 (51.6)
Maternal preintervention 25(OH)D, nmol/L, [mean (SD)]	75.81 (25.66)	75.57 (25.44)	76.05 (25.92)
Maternal preintervention 25(OH)D, ≥75 nmol/L, *n* (%)	253 (51.4)	125 (50.4)	128 (52.5)
Maternal post-intervention 25(OH)D, nmol/L, [mean (SD)]	89.99 (38.09)	71.86 (31.54)	108.33 (35.31)
Maternal post-intervention 25(OH)D,≥75 nmol/L, *n* (%)	313 (64.0)	108 (43.9)	205 (84.4)
Maternal age at childbirth, y [mean (SD)]	32.33 (4.28)	31.98 (4.24)	32.69 (4.30)
Maternal pre-pregnancy weight, kg, [mean (SD)]	69.17 (13.57)	69.15 (13.18)	69.19 (13.99)
Parity			
1, *n* (%)	219 (44.2)	123 (49.2)	96 (39.0)
2, *n* (%)	198 (39.9)	91 (36.4)	107 (43.5)
≥3, *n* (%)	79 (15.9)	36 (14.4)	43 (17.5)
Alcohol intake in pregnancy, *n* (%)	81 (16.4)	39 (15.6)	42 (17.1)
Smoking third trimester, *n* (%)	17 (3.4)	11 (4.4)	6 (2.4)
Exclusive lactation, weeks (median [IQR])	17.43 [8.57, 21.57]	17.57 [8.93, 21.68]	17.43 [8.29, 21.39]
Maternal educational level (%)			
Low (Elementary school or college graduate)	41 (8.3)	24 (9.6)	17 (6.9)
Medium (Tradesman certification or bachelor's degree)	314 (63.3)	162 (64.8)	152 (61.8)
High (Master's degree or higher)	141 (28.4)	64 (25.6)	77 (31.3)
Household income (%)			
Low (< 100.000DKK[1])	44 (8.9)	23 (9.2)	21 (8.5)
Medium (100.000-200.000 DKK)	257 (51.8)	134 (53.6)	123 (50.0)
High (> 200.000 DKK)	195 (39.3)	93 (37.2)	102 (41.5)
Fathers age, y, [mean (SD)]	34.63 (5.19)	34.29 (5.20)	34.98 (5.17)
Gestational age, d [mean (SD)]	279.43 (10.86)	279.32 (10.25)	279.54 (11.46)
Season of birth			
Winter, *n* (%)	179 (36.1)	87 (34.8)	92 (37.4)
Spring, *n* (%)	97 (19.6)	50 (20.0)	47 (19.1)
Summer, *n* (%)	100 (20.2)	50 (20.0)	50 (20.3)
Fall, *n* (%)	120 (24.2)	63 (25.2)	57 (23.2)
Sex, male, *n* (%)	256 (51.6)	123 (49.2)	133 (54.1)
Race, White, *n* (%)	475 (95.8)	240 (96.0)	235 (95.5)

Abbreviations: ADHD, attention deficit hyperactivity disorder; RCT, randomized controlled trial; SD, standard deviation; IQR, interquartile range; N = number.
SI Conversion: vitamin D can be converted to ng/mL by dividing by 2.496.
Alcohol intake in pregnancy describes any intake of alcohol during pregnancy, yes/no.
Smoking in third trimester describes any smoking in third trimester of pregnancy, yes/no.
Information on race was obtained through parental interviews and was defined as either white or non-white.
[1] *DKK = 0.14 USD.*

VITAMIN D3 SUPPLEMENTATION

There were no effects of the high-dose vitamin D3 supplementation as compared to standard dose vitamin D3 on offspring risk of either diagnoses or symptom load of autism and ADHD, **Paper I, Table 2 and Appendix, Paper I, Table 2**. There were no interactions with sex.

Paper I, Table 2: The effect of high-dose vitamin D3 supplementation in pregnancy on autism and ADHD
The table is reproduced from Aagaard K. et al.: High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10: A randomized clinical trial. Am J Clin Nutr. 2024;119(2):362-370. No changes were made. This Table was made available under the CC BY 4.0 license: https://creativecommons.org/licenses/by/4.0/.

Vitamin D3 supplementation and K-SADS-PL evaluation of autism and ADHD

K-SADS-PL measure	*N* (N cases)	Odds ratio estimate (CI)	*N* (N cases)	Odds ratio estimate (CI) adjusted[1]
Autism	496 (12)	0.72 (0.21,2.29)	492 (12)	0.72 (0.21,2.32)
ADHD	496 (58)	0.87 (0.50,1.51)	492 (57)	0.87 (0.49,1.53)
K-SADS-PL measure	*N*	Beta estimate (CI)	*N*	Beta estimate (CI) adjusted[1]
Autistic symptoms component score	496	−0.08 (−0.19,0.03)	492	−0.08 (−0.19,0.03)
ADHD symptoms component score	496	−0.06 (−0.18,0.07)	492	−0.07 (−0.19,0.05)

ADHD, attention deficit hyperactivity disorder; CI, confidence interval; K-SADS-PL, Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version; *N*, number.
ICD-10 diagnostic codes of Autism assigned at the COPSYCH visit included the DF84.0, DF84.5 and DF84.8 diagnostic codes, and of ADHD DF90.0, DF90.8 and DF98.8.
[1] Adjusted for week 24 vitamin D levels, season of birth, child sex, and the n-3 long-chain PUFA intervention.

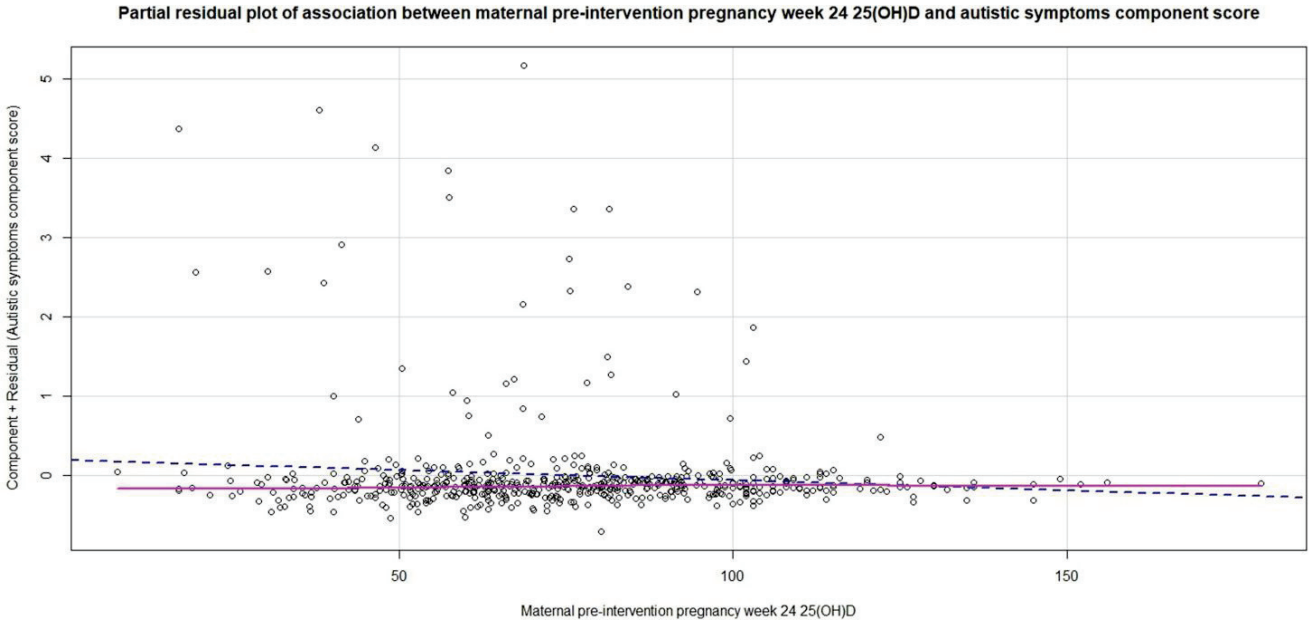
MATERNAL PRE-INTERVENTION SERUM 25(OH)D

In covariate adjusted analyses on maternal pre-intervention 25(OH)D levels, higher maternal 25(OH)D was associated with a decrease in risk of autism diagnosis (odds ratio (OR) = 0.76, 95%CI = 0.59-0.97, p = 0.034) and a decrease in autistic symptom load (beta = -0.03, 95%CI = -0.05-0.00, p = 0.024). For ADHD, higher maternal 25(OH)D associated with a decrease in risk of diagnosis (OR = 0.88, 95%CI = 0.78-0.99, p = 0.033), but there was no association with symptom load (beta = -0.02, 95%CI = -0.04-0.00, p = 0.122). All estimates are per 10 nmol/L increase in 25(OH)D. See **Appendix, Paper I, Supplementary material Table 6**. There were no interactions with sex. The association between maternal pre-intervention 25(OH)D and autism symptom load (N = 569) was presented in **Paper I, Figure 2 and Appendix, Paper 1, Figure 2** with adjustment for potential confounding variables.

In sensitivity analyses, the observational associations were additionally adjusted for autism[43] and ADHD[139] PGS to account for genetic confounding due to maternal disposition to neurodevelopmental disorders. In these analyses, the association between maternal pre-intervention 25(OH)D and ADHD attenuated. See **Appendix, Paper I, Supplementary**

Material, Table 15. Additionally, the associations reported between maternal 25(OH)D and K-SADS-PL symptoms loads could not be reproduced using parent-rated SRS-2 and ADHD-RS traits of autism and ADHD. See **Appendix, Paper I, Supplementary Material, Table 13**.

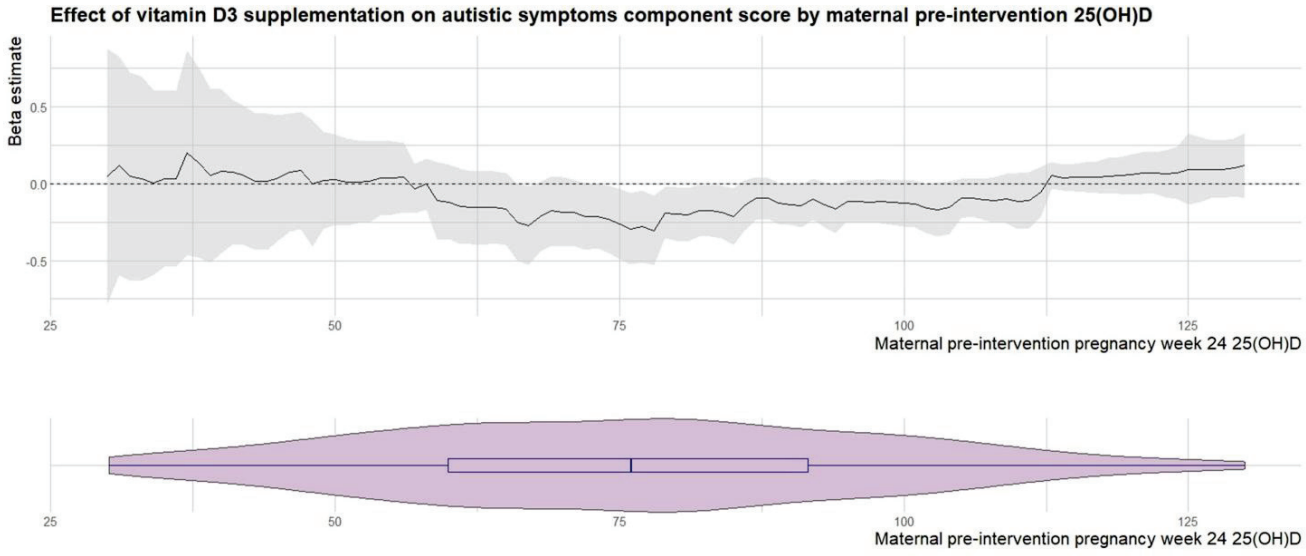
Paper I, Figure 2. *"Partial residual plot of the covariate adjusted linear association between maternal preintervention pregnancy week 24 25(OH)D and autistic symptom load measured by Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children - Present and Lifetime Version among 569 individuals. The linear fit is represented by the broken blue line and a smooth (loess) of the partial residuals by a solid magenta line (R package: crPlots). Adjusted for child sex, birth weight, gestational age, season of week 24 25(OH)D measurement, social circumstances, maternal smoking in third trimester of pregnancy, maternal pre-pregnancy weight, and fathers' age. The study population included all individuals included in the COpenhagen Prospective Study on Neuro-PSYCHiatric Development 2010 cohort with available measurements of 25(OH)D in pregnancy week 24 and with offspring participating in the COpenhagen Prospective Study on Neuro-PSYCHiatric Development visit at age 10 regardless of participation in the vitamin D3 trial. 25(OH)D, 25-hydroxy-vitamin D."* The Figure and legend are reproduced from Aagaard K. et al.: High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10: A randomized clinical trial. Am J Clin Nutr. 2024;119(2):362-370. No changes were made. This Figure was made available under the CC BY 4.0 license: https://creativecommons.org/licenses/by/4.0/.



THRESHOLD ANALYSIS

We detected a significant interaction between pre-intervention 25(OH)D and vitamin D3 supplementation on autism diagnosis (crude P-interaction 0.03) but not on ADHD diagnosis or symptoms loads of either autism or ADHD. Analyses on the effect of the vitamin D3 supplementation on autism risk by strata of maternal pre-intervention 25(OH)D was limited by the few autism cases in the sample (See **Appendix, Paper I, Supplementary Material, Figure 2 & 3**). We performed a threshold analysis to visualize any differences in the effect of the high-dose vitamin D3 supplementation on autistic symptom across different levels of pre-intervention 25(OH)D. The analysis suggested protective effects among mothers with normal to high serum 25(OH)D prior to the vitamin D3 supplementation, **Paper I, Figure 3**.

Paper I, Figure 3. *"Threshold analysis of the effect of high-dose vitamin D3 supplementation on autistic symptom load measured by Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children - Present and Lifetime Version according to maternal preintervention serum 25(OH)D measured at pregnancy week 24. Overall sample size was 492 individuals. The violin plot below shows the distribution of the measured maternal preintervention serum 25(OH)D. Linear regression was used to estimate the effect of the intervention according to maternal preintervention 25(OH)D within a moving window of ± 20 nmol/L. Black line marks the β estimate and gray area the corresponding 95% confidence interval. Estimates are unadjusted. 25(OH)D, 25-hydroxy-vitamin D."* The Figure and legend have been reproduced from Aagaard K. et al.: Aagaard K, Møllegaard Jepsen JR, Sevelsted A, et al. High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10: A randomized clinical trial. Am J Clin Nutr. 2024;119(2):362-370. No changes were made. This Figure was made available under the CC BY 4.0 license: https://creativecommons.org/licenses/by/4.0/.



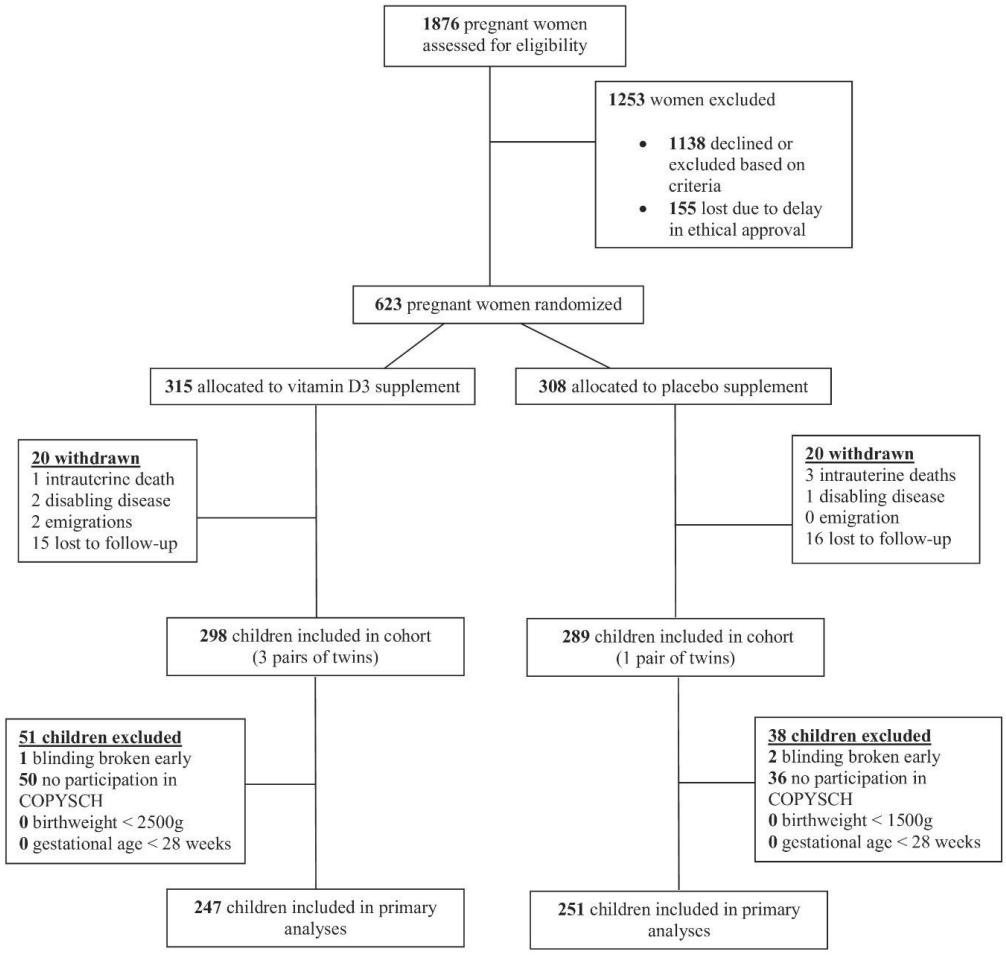
PAPER II

Paper II aimed to estimate the effect of high-dose vs standard dose vitamin D3 supplementation in pregnancy on offspring cognitive performance at age 10. Further, Paper II investigates the association between maternal pregnancy 25(OH)D (measured pre-intervention) and offspring cognitive performance in observational analyses. The main manuscript was drafted by Olivia Frigast Frederiksen who has been closely supervised by Kristina Aagaard (with Professor Bo Chawes as official main supervisor) during her research stay at COPSAC where she wrote her Masters' thesis.

BASELINE CHARACTERISTICS

A total of 498 children from the original COPSAC2010 cohort participated in both the vitamin D3 trial and the COPSYCH cognitive evaluation. Of the 498 children, 247 were in the high-dose pregnancy vitamin D3 supplementation group and 251 were in the standard dose group. See CONOSRT flow diagram, **Paper II, Figure 1 and Appendix Paper II, Figure 2**. Baseline characteristics were comparable to those presented for Paper 1. See **Appendix, Paper II, Supplementary Material, Table 2**. Mean estimated intelligence at the age of 10 was 107.6 (SD 15) for the high-dose group and 107.8 (SD 13.2) for the standard dose group. Mean scores for the remaining tests of the cognitive test battery can be viewed in **Appendix, Paper II, Supplementary Material, Table 3**.

Paper II, Figure 1. CONSORT participant flow diagram



VITAMIN D3 SUPPLEMENTATION

A high-dose as compared to standard-dose vitamin D3 supplementation in third trimester of pregnancy was associated with an improvement in 3 cognitive functions: verbal memory, visual memory and flexibility/set shift in covariate adjusted models. However, none of these findings were significant after FDR correction. See **Paper II, Table 1 and Appendix, Paper II, Table 1**. Interaction analyses did not suggest any modification by maternal pre-intervention serum 25(OH)D, sex, or childhood serum 25(OH)D of the effect of the vitamin D3 supplementation on verbal memory, visual, memory, and flexibility/set shift. **See Appendix, Paper II, Supplementary Material, Table 4**

Paper II, Table 1. Effect of vitamin D3 supplementation on cognitive functions

Analyses have been conducted in collaboration with medical student Olivia Frigast Frederiksen who has presented these in her Masters' Thesis titled: High-dose vitamin D3 supplementation during pregnancy and test-based cognitive performance at the age of 10 – a post-hoc analysis of a randomized clinical trial. Please see Co-author Statements and Preface section for details on the individual contributions made by Kristina Aagaard and Olivia Frigast Frederiksen.

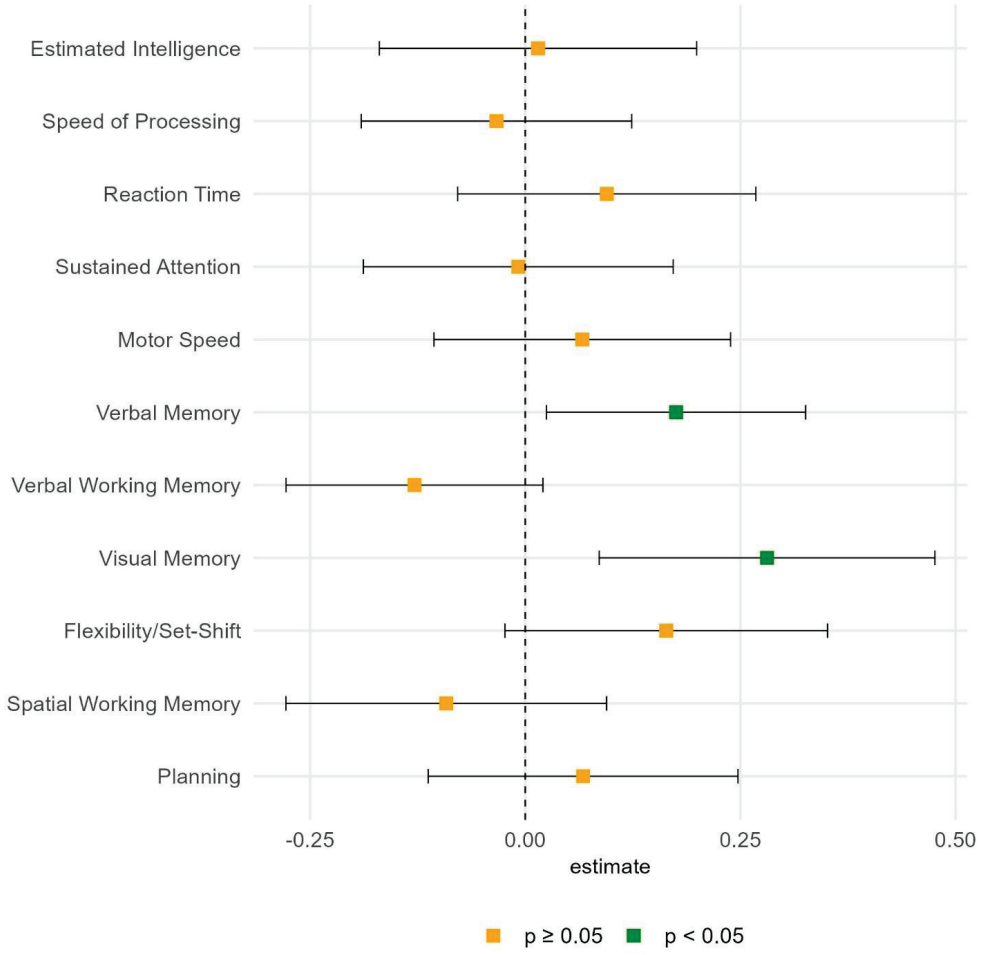
Function	N	Estimate [CI]	p-value	N	Estimate [CI], adjusted[1]	p-value	FDR p-value[2]
Estimated Intelligence	495	-0.01 [-0.19;0.16]	0.897	491	-0.01 [-0.19;0.16]	0.892	0.980
Speed of Processing	498	-0.04 [-0.19;0.12]	0.654	494	-0.02 [-0.17;0.13]	0.807	0.980
Reaction Time	496	0.12 [-0.05;0.28]	0.166	492	0.11 [-0.05;0.28]	0.183	0.335
Sustained Attention	495	-0.03 [-0.21;0.15]	0.766	491	-0.03 [-0.21;0.16]	0.785	0.980
Motor Speed	496	0.10 [-0.07;0.27]	0.245	492	0.09 [-0.08;0.25]	0.312	0.490
Verbal Memory	497	0.14 [-0.00;0.29]	0.058	493	0.17 [0.03;0.32]	**0.021**	0.116
Verbal Working Memory	498	-0.11 [-0.25;0.04]	0.159	494	-0.01 [-0.25;0.04]	0.173	0.335
Visual Memory	497	0.25 [0.07;0.44]	**0.007**	493	0.24 [0.06;0.42]	**0.011**	0.116
Flexibility/Set Shift	495	0.20 [0.02;0.38]	**0.027**	491	0.19 [0.01;0.37]	**0.035**	0.128
Spatial Working Memory	498	-0.11 [-0.29;0.06]	0.201	494	-0.12 [-0.30;0.06]	0.180	0.335
Planning	497	0 [-0.18;0.17]	0.959	493	0 [-0.17;0.17]	0.980	0.980

[1]Adjusted for sex, age at testing, n-3 LCPUFA intervention, season of birth and pre-interventional 25(OH)D level
[2]Benjamini-Hochberg false discovery rate (5%) applied across 11 tests; $p<0.05$ considered significant

To investigate if findings were driven by the most prevalent disorder in the COPSAC2010 cohort, ADHD, we performed analyses stratified according to the status hereof. In these analyses the observed effect on verbal memory and visual memory persisted among individuals without an ADHD diagnosis (**Paper II, Figure 2 and Appendix, Paper II, Figure 3**). However, all FDR p-values were above 0.05 (See **Appendix, Paper II, Supplementary Material, Table 5)**.

Paper II, Figure 2. Effect of vitamin D3 supplementation on cognitive functions among individuals without ADHD.

Analyses have been conducted in collaboration with medical student Olivia Frigast Frederiksen who has presented these in her Masters' Thesis titled: High-dose vitamin D3 supplementation during pregnancy and test-based cognitive performance at the age of 10 – a post-hoc analysis of a randomized clinical trial. Please see Co-author Statements and Preface section for details on the individual contributions made by Kristina Aagaard and Olivia Frigast Frederiksen.



Results are adjusted for sex, age at COPYSCH visit, n-3 LCPUFA intervention, season of birth and 25(OH)D level at week 24 of pregnancy. Statistical significance reported according to nominal p-values. FDR p-values all above threshold of 0.05.

MATERNAL PRE-INTERVENTION SERUM 25(OH)D

Lastly, we tested the effect of maternal pre-intervention serum 25(OH)D on cognitive outcomes which reflect early pregnancy levels of 25(OH)D. In adjusted analyses, higher serum 25(OH)D was positively associated with improved flexibility/set shift of all 11 tested functions. Again, these findings were not significant after FDR correction. **See Paper II, Table 2 and Appendix, Paper II, Table 2**.

Paper II, Table 2. Association between maternal pregnancy 25(OH)D and offspring cognitive functions

Analyses have been conducted in collaboration with medical student Olivia Frigast Frederiksen who has presented these in her Masters' Thesis titled: High-dose vitamin D3 supplementation during pregnancy and test-based cognitive performance at the age of 10 – a post-hoc analysis of a randomized clinical trial. Please see Co-author Statements and Preface section for details on the individual contributions made by Kristina Aagaard and Olivia Frigast Frederiksen.

Function	N	Estimate [CI]	p-value	N	Estimate [CI], adjusted[1]	p-value	FDR p-value[2]
Estimated Intelligence	584	0 [-0.03;0.03]	0.925	483	-0.02 [-0.06;0.02]	0.355	0.987
Speed of Processing	587	0 [-0.03;0.03]	0.833	487	0 [-0.03;0.04]	0.650	0.987
Reaction Time	585	-0.01 [-0.04;0.02]	0.537	484	0.01 [-0.03;0.04]	0.924	0.987
Sustained Attention	582	-0.01 [-0.04;0.03]	0.644	481	0 [-0.04;0.04]	0.855	0.987
Motor Speed	585	0.01 [-0.02;0.04]	0.548	484	0.03 [-0.01;0.07]	0.330	0.987
Verbal Memory	587	0.01 [-0.02;0.04]	0.522	486	-0.01 [-0.04;0.03]	0.936	0.987
Verbal Working Memory	588	0.02 [-0.01;0.05]	0.164	487	0 [-0.03;0.04]	0.702	0.987
Visual Memory	586	0.01 [-0.02;0.04]	0.517	485	0 [-0.04;0.04]	0.674	0.987
Flexibility/Set Shift	584	0.03 [-0.01;0.06]	0.110	483	0.05 [0.01;0.09]	**0.026**	0.286
Spatial Working Memory	587	0 [-0.03;0.03]	0.958	486	0 [-0.04;0.04]	0.987	0.987
Planning	586	0 [-0.03;0.04]	0.788	485	-0.01 [-0.05;0.03]	0.828	0.987

[1]Adjusted for sex, birthweight, gestational age, maternal pre-pregnancy BMI, season of week 24 measurement, gestational diabetes, preeclampsia, smoking during pregnancy, alcohol during pregnancy, maternal pregnancy IL6 and CRP, maternal education, household income, diet, maternal age, paternal age and child age at COPSYCH visit

[2]FDR adjustment applied across 11 tests with threshold of $p<0.05$.

PAPER III

The main aim of Paper III was to test the potential causal effects of pregnancy dietary pattern on offspring risk of ADHD using a genetically informed study design.

BASELINE CHARACTERISTICS

Without performing any exclusions, a total of 593 individuals from the COPSAC2010 cohort were clinically evaluated at the COPSYCH 10-year visit for any neurodevelopmental disorders. In addition, 11 individuals had information on questionnaire-based parent-rated traits of neurodevelopmental disorders. Hereof, 437 trios (mother, father and child) had complete information on dietary pattern PGS and were eligible for trio PGS analyses (See flow chart of study participants in **Appendix, Paper III, Supplementary Material, Figure 1**). Baseline characteristics of complete trios are presented in **Paper III, Table 1 and Appendix, Paper III, Table 1** according to ADHD status. Overall, the prevalence of ADHD was 12% and median total ADHD-RS trait score 7 (IQR 7-15) among complete trios.

Results were sought replicated in the independent mother-child cohorts MoBa and ALSPAC. MoBa had complete information on a total of 41,580 trios for ADHD diagnoses (ADHD prevalence 7.5%) and 18,629 trios for ADHD symptoms (median RS-DBD total score 7 (IQR 4-11)). ALSPAC contained information on 1,199 complete trios for ADHD traits (median total DAWBA score 2 (IQR 0-7)).

VALIDATION OF PREGNANCY DIETARY PATTERN PGS

We sought validation of the maternal dietary pattern PGS in both COPSAC and replication cohorts. The dietary pattern PGS predicted pregnancy diet in COPSAC and MoBa in a similar pattern as originally described in the GWAS by Cole et al. from which the PGS was derived (**Paper III, Figure 1; Appendix, Paper III, Figure 2 and Supplementary Figure 5**). The validation analysis using ALSPAC FFQ data was less conclusive. However, consistent with Cole et al., there was a positive association with intake of whole bread and a negative association with white bread (**Appendix, Paper III, Supplementary material, Figure 6**).

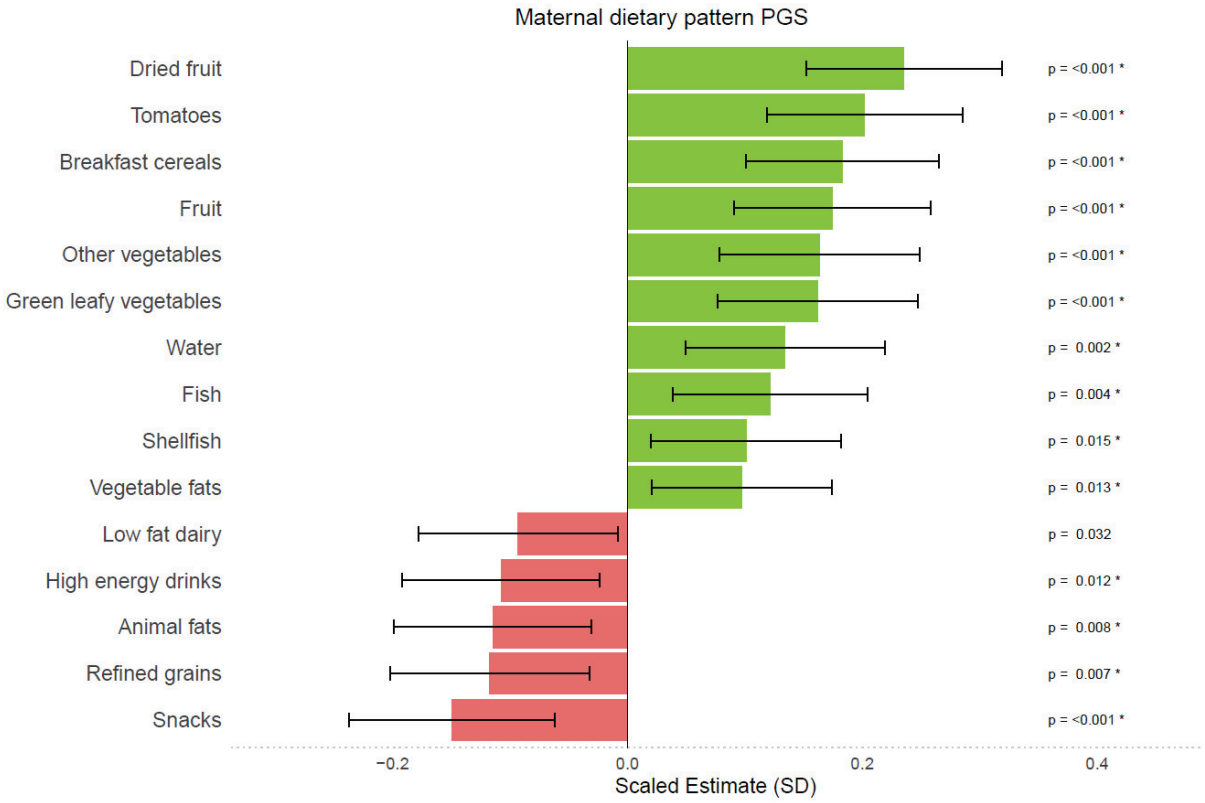
Important for testing causality of previous reported observational COPSAC findings on unhealthy pregnancy diet[53], the maternal dietary PGS significantly correlated significantly with the COPSAC2010 maternal Western dietary pattern PC (Pearson's r = -0.28; 95%CI= -0.36-0.20, $p < 0.001$).

Paper III, Table 1: Baseline characteristics of $COPSAC_{2010}$ families with complete trio information, stratified according to diagnosis of ADHD

	No ADHD	ADHD	P value
	n = 384	n = 53	
Child dietary pattern PGS, mean (SD)	0.12 (1.00)	-0.43 (1.11)	<0.001
Maternal dietary pattern PGS, mean (SD)	-0.04 (1.05)	-0.36 (0.91)	0.037
Paternal dietary pattern PGS, mean (SD)	0.06 (0.99)	-0.18 (0.92)	0.090
Sex, male, n (%)	180 (46.9)	41 (77.4)	<0.001
Gestational age, d, mean (SD)	279.17 (11.16)	280.49 (8.66)	0.285
Birthweight, kg, mean (SD)	3.54 (0.53)	3.64 (0.48)	0.122
Maternal educational level, n (%)			0.005
Elementary school	10 (2.6)	4 (7.5)	
College graduate	20 (5.2)	4 (7.5)	
Tradesman certification	67 (17.4)	18 (34.0)	
Bachelors' degree	173 (45.1)	19 (35.8)	
Masters' degree or higher	114 (29.7)	8 (15.1)	
Paternal educational level, n (%)			0.093
Elementary school	14 (3.7)	3 (5.7)	
College graduate	26 (6.8)	0 (0.0)	
Tradesman certification	124 (32.4)	25 (47.2)	
Bachelors' degree	111 (29.0)	12 (22.6)	
Masters' degree or higher	108 (28.2)	13 (24.5)	
Household income, n (%)			0.286
<100.000 DKK	30 (7.8)	3 (5.7)	
100.000-150.000 DKK	90 (23.4)	17 (32.1)	
150.000-200.000 DKK	116 (30.2)	16 (30.2)	
200.000-250.000 DKK	87 (22.7)	13 (24.5)	
>250.000 DKK	61 (15.9)	4 (7.5)	
Alcohol intake in pregnancy, yes, n (%)	60 (15.6)	6 (11.5)	0.397
Smoking in pregnancy, yes, n (%)	24 (6.2)	6 (11.3)	0.195
Parity, n (%)			0.269
1	171 (44.5)	20 (37.7)	
2	159 (41.4)	22 (41.5)	
≥3	54 (14.1)	11 (20.8)	
Maternal pre-pregnancy BMI, mean (SD)	24.53 (4.28)	26.43 (5.66)	0.004
Maternal Western dietary pattern PC, mean (SD)	-0.02 (0.93)	0.51 (1.07)	0.001
Maternal age, y, mean (SD)	32.05 (4.13)	32.03 (4.61)	0.671
Paternal age, y, mean (SD)	34.52 (5.14)	34.54 (5.10)	0.681
Vitamin D intervention, yes, n (%)	152 (47.8)	23 (50.0)	0.627
Fish oil intervention, yes, n (%)	209 (54.6)	24 (45.3)	0.181

ADHD = attention deficit hyperactivity disorder, PGS = polygenic score, BMI = Body Mass Index

Paper III, Figure 1. Association between the maternal dietary pattern polygenic score and maternal pregnancy diet. Nominal significant associations are depicted and FDR significant p-values marked with an asterisk (*).



UNADJUSTED PGS ANALYSES

Firstly, we performed unadjusted trio PGS analyses without including family members' dietary pattern PGSs. In these analyses, both higher maternal and child PGSs, corresponding to a healthier dietary pattern, were associated with lower risk of ADHD diagnosis and fewer ADHD traits (See **Paper III, Figure 2 and Appendix, Paper III, Figure 3**).

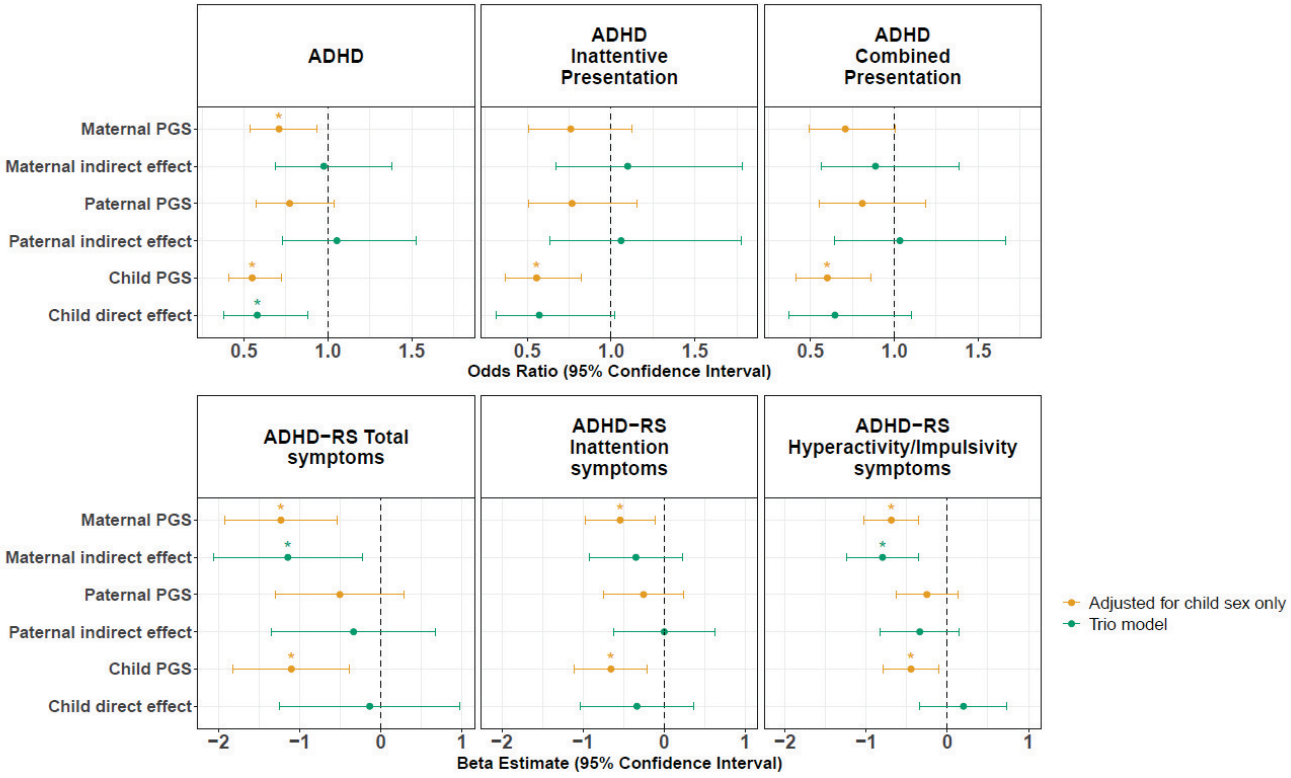
TRIO PGS ANALYSES

In trio PGS analyses including dietary pattern PGSs for all family members we removed genetic confounding from passive genetic transmission of risk through dietary pattern genetics.

In trio analyses, only child PGS remained significantly associated with risk of ADHD diagnosis indicating direct genetic effects supportive of potential genetic confounding of observational associations between pregnancy diet and ADHD.

Oppositely, the maternal dietary pattern PGS remained associated with the ADHD trait score while the child PGS association disappeared in trio analyses. This maternal indirect effect on ADHD trait score is supportive of potential causal effects of pregnancy dietary pattern on offspring ADHD traits (**Paper III, Figure 2 and Appendix, Paper III, Figure 3**).

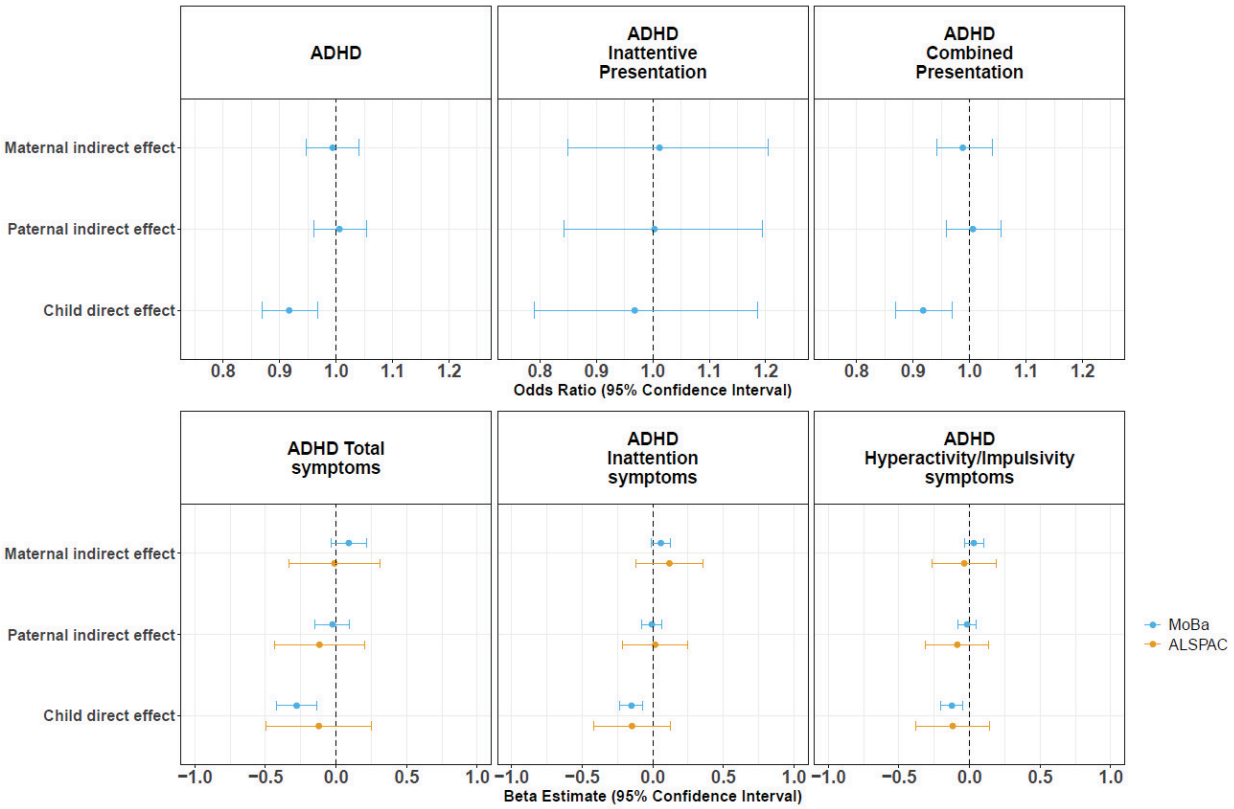
Paper III, Figure 2. Unadjusted and trio models of the association between maternal, paternal and child dietary pattern PGS and offspring ADHD diagnosis as well as ADHD trait score in the $COPSAC_{2010}$ cohort.



REPLICATION IN MOBA AND ALSPAC MOTHER-CHILD COHORTS

We did not replicate COPSAC maternal indirect genetic effects using data from larger independent mother-child cohorts MoBa and ALSPAC. Trio analyses in MoBa only showed evidence of direct genetic transmission of risk with significant findings in the child trio model for both ADHD diagnosis and ADHD traits. Results from the ALSPAC cohort were of similar directionality and size however estimated with lower precision due to the smaller sample size compared to MoBa (**Paper III, Figure 3 and Appendix, Paper III, Figure 4**). Results from the MoBa cohort were unchanged when requiring a minimum of two registered diagnoses for the same individual to classify as having ADHD and when performing analyses with robust standard errors.

Paper III, Figure 3. Maternal, paternal and child trio models of the association between dietary pattern PGS and ADHD diagnosis as well as ADHD trait score for MoBa and ALSPAC. Regression models were adjusted for sex of the child and MoBa analyses were further adjusted for birth year due to the longer period of inclusion.



PAPER IV

The main aim of Paper IV was to test the potential causal effects of pregnancy inflammation on offspring risk of neurodevelopmental disorders using genetically informed study designs.

BASELINE CHARACTERISTICS

As missing outcome data was imputed in **Paper IV**, we were able to include all 41,531 complete genotyped trios from the MoBa cohort (children with missing information on sex and one twin from each pair were excluded). In a drop out analysis, we tested differences between complete genotyped trios and the remaining MoBa cohort which showed consistently lower scores (indicative of more skills achieved/fewer problems) and lower proportion of ADHD and autism diagnoses among complete genotyped trios. Mean maternal CRP PGS was also slightly lower within the complete trios. See **Appendix, Paper IV, Supplementary Material, Table 2**. Only minor deviations were made from the preregistration, these are listed in **Appendix, Paper IV, Supplementary Material, Table 1**.

VALIDATION OF GENETIC INSTRUMENTS

We were able to sufficiently validate the CRP PGS (F-statistics 132.8) and the CRP MR instrument (F-statistics 60.6) using data from 2,323 pregnant mothers from a MoBa subsample with information on mid-pregnancy hs-CRP and genotype. Validation analyses for all inflammatory makers were performed using data from the smaller COPSAC cohort (N = 675). These analyses suggested some predictive value of both CRP and GlycA genetic instruments (For more detail see **Appendix, Paper IV, Supplementary Material**). Results presented below focus on estimates based on the sufficiently validated CRP PGS.

PGS AND INTERGENERATIONAL MENDELIAN RANDOMIZATION ANALYSES

Unadjusted PGS analyses showed associations between higher maternal CRP PGS and poorer language scores at age 3 and 5 as well as a higher degree of autistic traits at age 8 (repetitive behaviours) and ADHD traits at age 5 and 8 years. However, none of these associations survived FDR correction. See **Paper IV, Figure 1 and Appendix, Paper IV, Figure 2**. For diagnostic outcomes higher maternal CRP PGS increased risk of ADHD (OR = 1.06, 95%CI = 1.03–1.09, p = 0.002). There was some evidence of an association to autism diagnosis however not significant after FDR-correction (OR = 1.07, 95%CI = 1.02–1.12, p = 0.063).

We performed trio PGS analyses adjusted for inflammatory PGSs of family members accounting for genetic confounding through same trait pathways (See **Introduction, Figure 3 and**

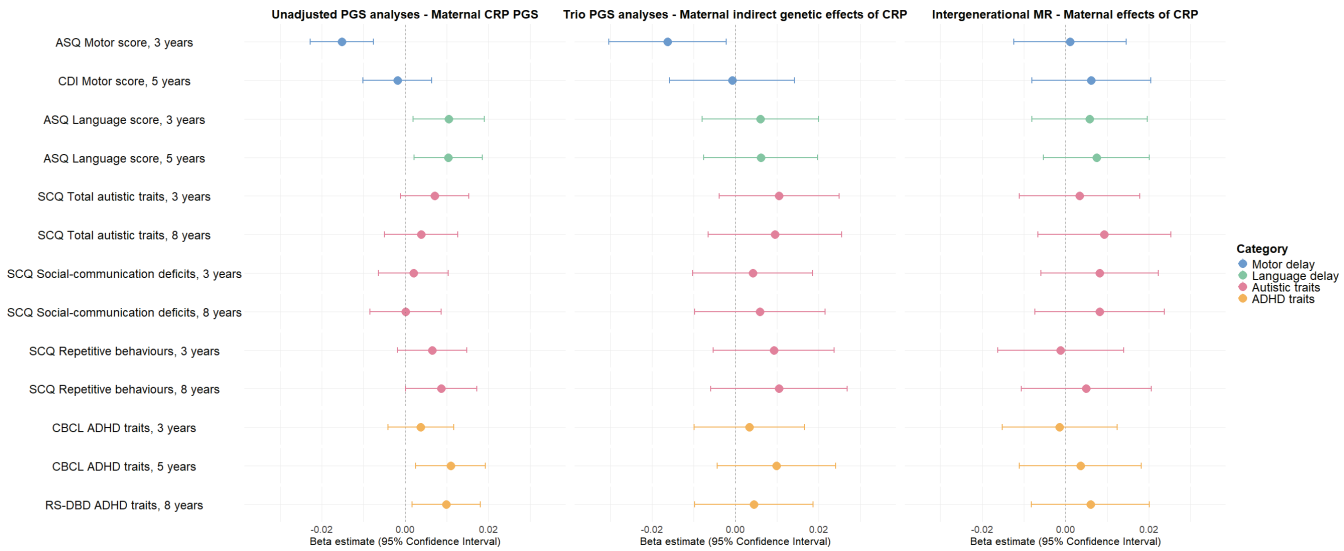
Appendix, Paper IV, Figure 1). In these analyses we did not detect any maternal indirect genetic effects which could support the causality of CRP as a risk factor for neurodevelopmental disorders (**Paper IV, Figure 1 and Appendix, Paper IV, Figure 2**). Estimated maternal indirect genetic effects of CRP on ADHD diagnosis was: OR = 1.04, 95%CI = 0.99–1.09, p = 0.867. Likewise, there were no indirect genetic effects of CRP on autism diagnosis: OR = 1.04, 95%CI = 0.95–1.13, p = 0.941.

Lastly, intergenerational MR analyses which further corrected for potential genetic confounding through related trait pathways (See **Introduction, Figure 3 and Appendix, Paper IV, Figure 1**) did not show causal effects of inflammation as measured by CRP (**Paper IV, Figure 1 and Appendix, Paper IV, Figure 2**). Estimated effect of CRP on ADHD diagnosis was: OR = 1.00, 95%CI = 0.95–1.05, p = 0.979. The effect on autism diagnosis was: OR = 1.05, 95%CI = 0.97–1.15, p = 0.900.

Results for non-validated genetic instruments for IL-6 and GlycA were null in PGS and intergenerational MR analyses. These are presented in **Appendix, Paper IV, Supplementary Material, Figures 1-2 and Table 3**.

Conducting analyses only including genetic instruments for CRP in the statistical models did not reveal any significant findings (**Appendix: Paper IV, Supplementary Material, Tables 4-5**). Further, results from complete case analyses were comparable to those reported using imputed datasets (**Appendix: Paper IV, Supplementary Material, Tables 6-7**).

Paper IV, Figure 1. Results for CRP from unadjusted PGS analyses, trio PGS analyses and intergenerational Mendelian randomization analyses. Analyses conducted using 30 imputed datasets including 41.531 complete trios. All FDR p-values > 0.05.



FOLLOW-UP TWO SAMPLE MR

As opposed to the intergenerational effects of inflammation tested with intergenerational MR, the two-sample MR based on publicly available GWAS summary statistics tested the effects of inflammation on ADHD and autism within the individual. The two-sample MR showed within-person causal effects of ADHD on CRP, IL-6 and GlycA. The only inflammatory marker causally increasing ADHD risk within the individual was CRP (**Paper IV, Table 1 and Appendix, Paper IV, Table 1**). Hence, results from the two-sample MR were consistent with potential confounding of observational analyses investigating the effects of pregnancy inflammation on neurodevelopmental disorders as maternal genetic liability to ADHD may act as a classic confounder both increasing inflammatory activity in the mother and child risk of ADHD.

Paper IV, Table 1. Two-sample Mendelian randomization analyses

Exposure	Outcome	Beta	SE	p-value	nSNPs	p-value, FDR
CRP	ADHD	0.07	0.015	<0.001	783	<0.001
CRP	AUTISM	-0.01	0.020	0.622	995	0.749
IL6	ADHD	-0.056	0.041	0.169	5	0.253
IL6	AUTISM	0.026	0.060	0.665	5	0.749
GLYCA	ADHD	-0.047	0.015	0.011	188	0.020
GLYCA	AUTISM	-0.066	0.020	0.038	232	0.018
ADHD	CRP	0.056	0.006	<0.001	32	<0.001
AUTISM	CRP	-	-	-	-	-
ADHD	IL6	0.006	0.036	0.874	36	0.002
AUTISM	IL6	-	-	-	-	-
ADHD	GLYCA	0.040	0.012	<0.001	36	0.012
AUTISM	GLYCA	-	-	-	-	-

SE: Standard Error, SNP = Single-nucleotide polymorphism, FDR = False discovery rate

Discussion

MAIN FINDINGS

First, this thesis investigated the role of pregnancy vitamin D3 supplementation for offspring future neurodevelopment and cognitive functioning. These analyses were post-hoc analyses of the vitamin D3 RCT conducted throughout third trimester of pregnancy in the COPSAC2010 mother-child cohort. In **Paper I**, we investigated the effect of high-dose vitamin D3 supplementation in pregnancy on offspring risk of neurodevelopmental disorders autism and ADHD as well as clinically evaluated symptom load and parent-rated traits of both disorders. There were no positive effects of a high-dose vitamin D3 supplementation of 2800 IU from pregnancy week 24 until 1 week postpartum on risk of diagnoses or severity of symptom load/traits when compared to standard dose i.e. 400IU. Higher maternal serum 25(OH)D measured prior to the intervention was associated with lower risk of autism, ADHD and less severe clinically evaluated autistic symptom load. In a threshold analysis, we saw indications of a positive effect of the vitamin D3 intervention on autistic symptom load within mothers with normal to high pre-intervention levels of 25(OH)D. In **Paper II,** we investigated the effect of high-dose vs. standard dose vitamin D3 supplementation on offspring cognitive functioning. We found that a high-dose supplementation in pregnancy improved verbal memory, visual memory and flexibility/set shift in the children at age 10. However, these associations did not remain significant after FDR correction. We investigated if these effects were driven by underlying psychopathology in the children by restricting the analyses to children without ADHD. In these analyses, the positive effects on verbal and visual memory were still present. Finally, we estimated the association between pre-intervention serum 25(OH)D and offspring cognition. Here we only found an association to flexibility/set shift, however again this finding did not pass multiple test correction.

Second, this thesis sought to investigate the causality of suggested prenatal risk factors for childhood neurodevelopmental disorders. In **Paper III**, we used genetic risk scores for an unhealthy pregnancy dietary pattern in trio analyses to investigate potential causal effects of an unhealthy pregnancy dietary pattern on offspring ADHD. Analyses on COPSAC2010 data indicated potential causal effects of pregnancy diet on offspring ADHD trait severity but not on ADHD diagnosis. Utilizing data from the larger MoBa and ALSPAC cohorts we were unable to provide any evidence of a causal effect of diet in pregnancy on either ADHD or severity of

ADHD traits. Results indicated that previously reported observational findings likely are influenced by genetic confounding. In **Paper IV**, we sought to validate pregnancy inflammation as a causal risk factor for neurodevelopmental disorders. We used genetic proxies for pregnancy CRP, IL6 and GlycA in trio PGS analyses and intergenerational MR analyses. None of these methods were able to support a causal role of pregnancy inflammation for impaired neurodevelopment. Important to the interpretation of the results, only the genetic instruments for CRP could be sufficiently validated using information on mid-pregnancy circulating hs-CRP from a subsample of the MoBa cohort. Finally, **Paper IV** included two-sample MR analyses investigating the effect of ADHD on inflammation within the individual. These analyses showed causal effects of ADHD on increased CRP which is indicative of potential genetic confounding of the intergenerational link between pregnancy CRP and offspring ADHD.

STRENGHTS AND LIMITATIONS

STRENGTHS

A major strength for **Papers I-III** of this thesis is the high quality of the COPSYCH evaluations resulting in an expectedly low risk of misclassification of neurodevelopmental disorders.[14] Further, video recordings of the clinical examinations allowed us to estimate inter-rater reliability which was high. Inter-rater reliability was tested based on clinical evaluations of 10 participants where ratings of Jens Richardt Møllegaard Jepsen were determined as the gold standard. Overall agreement on present symptoms was 88.48 %, 95%CI (82.60-92.92).[140] For cognitive outcomes, the large COPSYCH test battery enabled us to prioritize the automated CANTAB tests over paper-and-pencil tests which may provide more precise estimates of cognitive functioning with higher precision regarding timing of the tests and smaller risk of human administration variability influencing the results. Further, we were able to adjust for important potential confounders utilizing the extensive amount of data collected longitudinally for the COPSAC2010 cohort including factors such as prenatal inflammation and pregnancy diet which have previously been associated with cognitive performance.[97,135] For **Papers III & IV** which were genetically informed study designs based on PGSs, which still show a generally low predictive ability for the outcomes of interest, a major strength was the possibility to perform analyses based on the lager MoBa cohort. MoBa contains the to our knowledge highest number of genotyped trios (more than 40,000 complete trios).[141] Important for **Paper III & IV** we were able to sufficiently validate both genetic instruments for dietary pattern and CRP using pregnancy data on food intake and serum inflammatory markers.

LIMITATIONS

Papers I & II present post hoc analyses of the COPSAC2010 vitamin D3 RCT for which persistent wheeze was the main outcome. As these analyses were conducted post-hoc the risk of the findings being data-driven is increased, and interpretation of reported effects should be done with caution. However, since no randomized trials exist regarding the effect of high-dose vitamin D3 supplementation on neurodevelopmental disorders and cognition in middle childhood, **Papers I & II** provide important information for potential future clinical trials. Further arguing for the relevance of these studies are the difficulties associated with conducting clinical trials due to long follow-up and relatively high numbers required to investigate effects on less common childhood disorders such as autism. See post hoc power analyses in **Appendix, Paper I, Supplementary Material**. As indicated by these post-hoc power analyses, **Paper I** was limited by the low number of especially autism cases within the COPSAC2010 cohort. In contrast to clinical trials, preregistration is not a general requirement for observational studies. However, there is a trend towards an increased registration of observational studies as this reduces risk of bias from selective reporting of outcomes or selective publication dependent on study results i.e. publication bias. [142] We therefore pre-registered **Paper IV** using The Open Science Framework.

A major limitation to the observational analyses on maternal pre-intervention 25(OH)D in **Paper I & II** is the lack of information on parental psychopathology in the COPSAC2010 cohort which may serve as a confounder. To account for this, we performed sensitivity analyses in **Paper I** adjusted for maternal autism or ADHD PGS which removed the association between maternal pre-intervention 25(OH)D and severity of ADHD clinically evaluated symptom load. Finally, **Paper I & II** were limited by the relatively high baseline 25(OH)D levels in the COPSAC2010 cohort which limited our ability to draw conclusions on the effects of the vitamin D3 intervention among pregnant women with insufficient levels of 25(OH)D.

For **Paper IV**, pregnancy IL-6 and GlycA were only measured in the COPSAC2010 cohort why validation analyses of genetic instruments for these inflammatory markers were limited by low numbers. To our knowledge, no GWASs have been conducted using pregnancy inflammatory markers however this could potentially have increased the predictive ability of these genetic instruments.

INTERPRETATION

Paper I did not find any effects of high-dose vitamin D3 supplementation on risk of offspring autism or ADHD. For cognition, findings from **Paper II** suggested a positive effect of vitamin D3 supplementation on offspring verbal memory, visual memory and flexibility/set shift - however these potential effects did not hold for FDR correction. To the best of our knowledge, no studies besides from the COPSAC2010 cohort have investigated the effect of a high-dose vitamin D3 supplementation in pregnancy on offspring neurodevelopment and only one study investigated the effect on offspring early life cognition. In support of the positive effect of vitamin D3 on childhood cognitive function found in **Paper II**, a post-hoc analysis of a pregnancy high-dose vitamin D3 intervention found a positive effect of 2000IU of vitamin D3 in pregnancy on language scores assessed at ages 3-5 in 156 children.[83] With regards to previous results from the COPSAC2010 cohort, the findings from **Paper I** align with earlier reports showing no effect of vitamin D3 supplementation on early neurodevelopment and cognition.[82] The findings we report in **Paper II** are thus potential positive effects of the vitamin D3 supplementation which have presented and become testable later in childhood. This may be a plausible explanation to the differences in results as cognitive functions continue to mature throughout childhood and become increasingly differentiated allowing for a more precise estimation.[143] However, it cannot be definitely ruled out that the effects reported in **Paper II** are chance findings especially as these findings do not hold for multiple test correction.

In **Paper I** we found associations between pre-intervention pregnancy 25(OH)D and autism, ADHD and severity of clinically evaluated autistic symptom load. Since **Paper I** has been published, a large Danish study has utilized neonatal dried blood spot measurements of 25(OH)D from a population-based, case-cohort sample to investigate associations with seven mental disorders: anorexia nervosa, major depressive disorder, bipolar disorder, autism, ADHD and schizophrenia. The study found that higher neonatal 25(OH)D levels were linearly associated with a significantly lower risk of autism, ADHD, and schizophrenia. In addition to higher study numbers, another major difference compared to **Paper I** was a mean neonatal 25(OH)D of 23.6 nmol/L which is significantly lower than mean pregnancy levels in the COPSAC2010 cohort (~75 nmol/L).[144] The findings reported in this paper were adjusted for parental history of psychopathology, thus overcoming one of the most important limitations from **Paper I** and to some extent **Paper II**. Still, the reported results may be affected by residual confounding. In **Paper II** we only found an association between maternal pre-intervention 25(OH)D and offspring flexibility/set shift, which did not pass multiple test correction. One previous study

reported an association between lower maternal pregnancy 25(OH)D in third trimester and poorer offspring executive functioning estimated by the Behaviour Rating Inventory of Executive Function (BRIEF) questionnaire, however no association was observed to cognitive flexibility assessed by BRIEF. Comparable to results from **Paper II**, this study did not report any association between pregnancy 25(OH)D and offspring full-scale intelligence quotient. A major strength of this study was the extensive confounder control and the ability to adjust analyses for, when relevant, approximate mean intelligence quotient of parents and emotional symptomatology of mothers to reduce the influence of genetic confounding.[69]

The difference - in both **Paper I & Paper II** - between results regarding the effects of the vitamin D3 supplementation and observational associations for pre-intervention serum 25(OH)D may reflect differences in timing of the measured exposure. Since there was no effect of the intervention but an association between early pregnancy 25(OH)D levels and neurodevelopment, findings from **Paper I** indicate that the vitamin D3 intervention potentially was introduced too late in pregnancy to exert a positive effect on neurodevelopment. On the contrary, findings from **Paper II** suggest that 25(OH)D concentrations later in pregnancy were of greater importance for cognitive development. Human brain development is a prolonged process, and existing research has not yet conclusively identified which developmental phases are affected in common childhood neurological disorders.[145] These discrepancies may also be explained by other mechanisms besides from differences in exposure windows such as residual confounding of observational associations or high baseline levels of 25(OH)D. Finally, the discrepancy between the findings may argue against causal effects of vitamin D in pregnancy on neurodevelopment and cognitive function.

A MoBa study has investigated the causality of pregnancy vitamin D on offspring neurodevelopment using a genetically informed study design: intergenerational MR (trio MR).[146] This study did not report any evidence of causal maternal effects of vitamin D on offspring neurodevelopment. Using classical two-sample MR based on summary statistics, the study showed a causal effect of autism on lower vitamin D levels within the individual which argues for potential genetic confounding of previous reported associations.[146] Similar studies using intergenerational MR have to the best of our knowledge not been conducted for the effect of maternal vitamin D on offspring cognitive outcomes (investigated in **Paper II**), however the aforementioned study did not find evidence of causal effects on early language scores or motor

development scores which have been shown to correlate with measures of cognitive performance later in childhood.[147,148]

In **Paper III & Paper IV**, we did not find evidence of causal effects of an unhealthy dietary pattern in pregnancy on offspring ADHD or inflammation in pregnancy on neurodevelopment. A potential explanation to these null findings relates to the genetic instruments and their ability to measure the actual causal exposure. For **Paper III**, the lack of significant findings may indicate that there are no causal effects of an unhealthy dietary pattern in pregnancy on offspring ADHD. Alternatively, the causal exposure which explains the reported observational associations between pregnancy dietary pattern and ADHD[53,86] is not related to the overall dietary pattern but instead specific dietary components. Pregnancy intake of a range of nutrients has been associated with offspring neurodevelopment. These nutrients include vitamins, polyunsaturated fatty acids, folic acid, choline, iron, iodine - and the dietary pattern PGS might not have captured potential effects hereof.[84,149] The CRP PGS utilized in **Paper IV** showed a higher predictability of inflammation in pregnancy among pregnant women with low grade inflammation (defined as CRP < 10). Further, the GWAS was conducted based on UK Biobank data where the CRP measurements were generally within the low range (range:1-80 mg/L, median of 1.33 mg/L).[120] Therefore, the CRP PGS likely reflects predisposition to low grade inflammation more accurately than acute inflammation related to infections. Given that a potential true causal association between pregnancy inflammation and offspring neurodevelopment is driven by infections as opposed to low grade systemic inflammation, this might explain the lack of evidence of causal relationships in **Paper IV**. This potential explanation is supported by pre-existing studies, including studies from MoBa and the Danish National Birth Cohort, associating pregnancy infections and fever to offspring ADHD.[150–152] Evidence also exists for increase in risk of offspring autism following pregnancy infections.[153,154]

To our knowledge no previous studies have investigated the effect of neither inflammation in pregnancy nor diet in pregnancy on offspring neurodevelopmental disorders using trio PGS or intergenerational MR analyses. However, existing studies have incorporated alternative design-based methods to handle potential genetic confounding. E.g. the conclusion drawn in **Paper IV** regarding the importance of accounting for genetic confounding when estimating the association between pregnancy inflammation and ADHD is supported by a large register study which showed attenuation of the association between pregnancy infections and offspring neurodevelopment in sibling comparison analyses which removes confounding from time-stable

family-related factors including genetic predisposition.[91] Another study likewise based on registry data, found evidence that the observed association between maternal infection in pregnancy and offspring autism is likely explained by unmeasured confounding. The study both reported an association between pre-pregnancy infections and autism risk (pre-pregnancy infection was included as a negative control outcome) and no association between pregnancy infection and autism risk in siblings comparison analyses.[155] For diet in pregnancy as a risk factor for offspring ADHD, there - to our knowledge - are no existing studies with a matching degree of control for genetic confounding. A MoBa study reported an association between poorer prenatal diet quality (diet quality was calculated based on percentage of recommended intake achieved for specific food items) and offspring ADHD and ADHD traits. The study found similar associations when performing analyses stratified according to maternal ADHD symptoms.[86]

Paper I-IV included in this thesis all investigate the influence of potential prenatal risk factors for either childhood neurodevelopmental disorders or impaired cognitive function. All 4 papers seek to investigate causality by their designs which include analyses within an RCT-setting and genetic analyses with the potential of detecting environmental effects without the risk of genetic confounding. Despite study designs with the potential of detecting causal effects, we are limited in our ability to draw firm conclusions on the causality of these proposed risk factors, as each study alongside their strengths have significant limitations which have been presented and discussed previously. The most important limitations are for **Paper I & II** the fact that these studies were designed for another main outcome - recurrent wheeze within the first 3 years of life, why the presented analyses are performed post-hoc and the positive results are therefore more likely to be data driven. Additionally, this limitation also resulted in an assumingly too small sample size when investigating the protective effects of vitamin D3 supplementation for the less prevalent neurodevelopmental disorders. For **Paper III & IV**, limitations are primarily related to the strength of the genetic instruments, which may not have been compensated for by the large MoBa sample size, and the extent to which the genetic instruments accurately measure the intended exposure. Collectively, these papers add to the existing evidence by utilizing - for the given risk factor and outcome - study designs which have not to our knowledge been utilized previously. We thereby add to the purpose of triangulating evidence for these proposed risk factors, which is important, as firm conclusions as a rule should not be drawn based on single studies, but instead on the cumulative published evidence. However, a such triangulation should

ideally be performed prospectively with pre-specification of different data sources and planned study designs including pre-registration hereof.[103]

CONCLUSIONS AND PERSPECTIVES

In conclusion, we did not find evidence of an effect of high-dose vitamin D3 supplementation in pregnancy on offspring risk of neurodevelopmental disorders evaluated at the age of 10. We did detect potential positive effects of pregnancy high-dose vitamin D3 supplementation on offspring verbal memory, visual memory and flexibility/set shift evaluated at age 10, however these findings did not pass multiple test correction. With the risk of residual confounding influencing these observational results, we found associations between lower pregnancy serum 25(OH)D and ADHD, autism, severity of autistic symptom load and poorer flexibility/set shift. Finally, we were unable to provide evidence of causal effects of an unhealthy dietary pattern and pregnancy inflammation on childhood neurodevelopment utilizing genetically informed study designs with the ability to account for genetic confounding. Instead, our results indicate that genetic confounding is likely to influence observational associations between these prenatal exposures and childhood neurodevelopmental outcomes. Overall, it is important to state that firm conclusions should not be drawn based on single studies, and future research is still warranted to clarify the impact of the potential prenatal risk factors for child neurodevelopmental disorders investigated in this thesis.

FUTURE RESEARCH

To more securely conclude on the potential beneficial effects of high-dose vitamin D3 on offspring neurodevelopment, this would require larger sample sizes. COPSAC has initiated two RCTs which aim to investigate the effects of fish oil (2,4 g/day) and high-dose vitamin D3 (3600 IU/day) vs placebo or vitamin D3 standard dose (400 IU), respectively. Supplementation will be initiated between pregnancy week 22-26 and continue until 1 week after childbirth. The primary outcome is as for the COPSAC2010 vitamin D3 trial, persistent wheeze or asthma until the age of 3.The study plans to include a total of 2000 women in each RCT.[156] Secondary outcomes from both trials include a behavioural screening by the Strengths and Difficulties Questionnaire and BRIEF as well as psychopathological screening (Child Behaviour Checklist, ADHD-RS, SRS-2) at the age of 6. Thus, data from this larger vitamin D3 trial which is currently recruiting will enable us to test the effects of pregnancy vitamin D3 supplementation in an RCT setting with a significantly larger sample sizes than we had available from the COPSAC2010 cohort in **Paper I & Paper II**. Further the RCT setting, given the randomization is successful, should expectedly remove potential genetic confounding. Larger sample sizes would further enable us to perform analyses stratified according to pre-intervention serum levels of vitamin D - analyses which were

especially restricted by the low number of autism cases for **Paper I**. However, results from **Paper I** did suggest that the effect of the vitamin D3 supplementation on autism risk was dependent on early pregnancy serum levels of vitamin D.

To explore the potential effects of vitamin D3 supplementation in early pregnancy, it would further be interesting to conduct supplementation studies on women planning a pregnancy to secure sufficient vitamin D levels during first trimester. Fetal development evaluated by growth measures during the early stage of pregnancy have been linked to later mental health[157], and higher vitamin D serum levels throughout the first trimester of pregnancy have previously been associated with improved neurodevelopment.[67,69,158]

The results from **Paper III & Paper IV** stress the importance of handling potential genetic confounding in future studies investigating the impact of pregnancy dietary patterns and inflammation on risk of neurodevelopmental disorders. For the COPSAC2010 cohort we plan to obtain information on parental psychopathology at the next follow-up of the cohort, and therefore future COPSAC studies will have the potential to test the influence of genetic predisposition for these associations. For diet in pregnancy, future RCTs testing the potential positive effects of a healthy dietary pattern in pregnancy on neurodevelopment are possible, however conducting such will be both costly and require a relatively long period of follow-up. Furthermore, the results we present in this thesis suggest that dietary interventions would prove ineffective to protect against offspring ADHD. Therefore, it could be of relevance to further test the effects of dietary patterns in pregnancy on offspring ADHD utilizing similar genetically-informed study designs[103] as for **Paper III & Paper IV** based on alternative genetic instruments explaining more of the variance in the exposure data. Such genetic instruments could e.g. be made available by performing GWAS analyses on pregnancy food intake and inflammation data from large pregnancy cohorts such as MoBa.[117] To further investigate potential causal effects of diet and inflammation which we did not capture in **Paper III & IV**, future genetic trio analyses should use genetic instruments for specific nutrients and alternative inflammatory proteins in genetic trio analyses. For diet, this could be e.g. genetic instruments for macronutrients such as the intake of fatty acids where e.g. the intake of long-chain polyunsaturated fatty acids in pregnancy has been link to improved neurodevelopment.[159,160] Analyses on the pregnancy proteome could inform future trio PGS studies on inflammation. In the COPSAC2010 cohort the level of the following inflammatory proteins during pregnancy have been shown to be important for the risk of neurodevelopmental disorders: vascular endothelial growth factor A, C-C motif

chemokine ligand 3, CD5, interleukin 12B, fibroblast growth factor-23, and monocyte chemoattractant protein-1.[98] Given sufficient heritability, genetic instruments for these alternative proteins could therefore be utilized in future genetic approaches to test the influence of inflammation on offspring neurodevelopment. Finally, a genetic instrument for infections in pregnancy could prove useful, and such have previously shown a modest genetic overlap with mental disorders.[161]

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Appendix, Paper I, II, III, IV

I. High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10 - A randomized clinical trial

Kristina Aagaard, Jens Richardt Møllegaard Jepsen, Astrid Sevelsted, David Horner, Rebecca Vinding, Julie Bøjstrup Rosenberg, Nicklas Brustad, Anders Eliasen, Parisa Mohammadzadeh, Nilofar Følsgaard, María Hernández-Lorca, Birgitte Fagerlund, Birte Y Glenthøj, Morten Arendt Rasmussen, Niels Bilenberg, Jakob Stokholm, Klaus Bønnelykke*, Bjørn H. Ebdrup* and Bo Chawes* (*Authors contributed equally)

Published in The American Journal of Clinical Nutrition, February 2024[1]

II. High-dose vitamin D3 supplementation during pregnancy and test-based cognitive performance at age 10 - a post-hoc analysis of a randomized clinical trial

Olivia Frigast Frederiksen, Jens Richardt Møllegaard Jepsen, Nicklas Brustad, Rebecca Vinding, Julie Bøjstrup Rosenberg, Parisa Mohammadzadeh, María Hernández-Lorca, Ann-Marie Malby Schoos, Nilo Vahman, Birte Y. Glenthøj, Birgitte Fagerlund, Niels Bilenberg, Klaus Bønnelykke, Bjørn H. Ebdrup*, Kristina Aagaard* and Bo Chawes* *Under review*

III. Genetic investigation of the association between maternal dietary patterns and offspring ADHD

Kristina Aagaard, Casper-Emil T. Pedersen, David Horner, Anders Eliasen, Nicklas Brustad, Rebecca Vinding, Mohammad Talaei, Seif O. Shaheen, Julie B. Rosenberg, Jakob Stokholm, Bo Chawes, Morten Arendt Rasmussen, Jens Richardt M Jepsen, Bjørn H. Ebdrup, Alexandra Havdahl, Laurie J. Hannigan* and Klaus Bønnelykke* *Under review*

IV. Inflammation in pregnancy and child neurodevelopment: A trio polygenic score and Mendelian randomization study in the Norwegian Mother, Father and Child Cohort

Kristina Aagaard, Anders Eliasen, Jens Richardt M Jepsen, Casper-Emil T. Pedersen, Rebecca Vinding, Julie B. Rosenberg, Tingting Wang, Nicklas Brustad, Susanne Brix, Bo Chawes, Morten Arendt Rasmussen, Bjørn H. Ebdrup, Ida Henriette Caspersen, Robyn E. Wootton, Helga Ask, Alexandra Havdahl, Klaus Bønnelykke* and Laurie J. Hannigan* *Submitted*

PAPER I

High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10 - A randomized clinical trial

Kristina Aagaard, Jens Richardt Møllegaard Jepsen, Astrid Sevelsted, David Horner, Rebecca Vinding, Julie Bøjstrup Rosenberg, Nicklas Brustad, Anders Eliasen, Parisa Mohammadzadeh, Nilofar Følsgaard, María Hernández-Lorca, Birgitte Fagerlund, Birte Y Glenthøj, Morten Arendt Rasmussen, Niels Bilenberg, Jakob Stokholm, Klaus Bønnelykke*, Bjørn H. Ebdrup* and Bo Chawes*

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Original Research Article

High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10: A randomized clinical trial

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A B S T R A C T

Background: Vitamin D deficiency in pregnancy may increase the risk of autism and attention deficit hyperactivity disorder (ADHD).
Objective: The objective of this study was to estimate the effect of vitamin D3 supplementation in pregnancy on risk of autism and ADHD.
Design: This randomized clinical trial was part of the COpenhagen Prospective Study on Neuro-PSYCHiatric Development (COPYCH) project nested within the Copenhagen Prospective Studies on Asthma in Childhood 2010 (COPSAC2010) cohort comprising a population-based sample of 700 healthy mother-child pairs enrolled at week 24 of pregnancy. Maternal 25-hydroxy-vitamin D (25(OH)D) was measured at inclusion and 623 mothers were randomized 1:1 to either high-dose (2800 IU/d) or standard dose (400 IU/d) vitamin D3 until 1 wk postpartum (315 received high-dose, 308 standard dose). At age 10, diagnoses and symptom load of autism and ADHD, respectively, were established using the Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version.
Results: The psychopathologic evaluation was completed by 591 children aged 10 y, and 16 children (2.7%) were diagnosed with autism and 65 (11.0%) with ADHD. Hereof, 496 children participated in the vitamin D3 trial (246 received high-dose, 250 standard dose). Of these, 12 children (2.4%) were diagnosed with autism and 58 (11.7%) with ADHD. *Higher maternal preintervention 25(OH)D levels were associated with a decreased risk of autism [odd ratio (OR) per 10 nmol/L: 0.76 (0.59,0.97); P = 0.034], lower autistic symptom load [β per 10 nmol/L: −0.03 (−0.05,0.00); P = 0.024), and decreased risk of ADHD diagnosis (OR per 10 nmol/L: 0.88 (0.78,0.99); P = 0.033].* High-dose vitamin D3 supplementation was not associated with risk of autism or ADHD.
Conclusions: *Higher maternal preintervention 25(OH)D was associated with a decreased risk of autism, lower autistic symptom load, and decreased risk of ADHD diagnosis, but* high-dose vitamin D3 supplementation in pregnancy had no effect on risk of autism and ADHD.
This trial was registered at clinicaltrials.gov as *NCT00856947*.

Keywords: ADHD, Autism, neurodevelopment, supplementation, vitamin D

Abbreviations: ADHD, *attention deficit hyperactivity disorder*; *ADHD-RS*, *ADHD-Rating Scale*; *COPSAC*, Copenhagen Prospective Studies on Asthma in Childhood; COPSAC2010, Copenhagen Prospective Studies on Asthma in Childhood 2010; COPSYCH, COpenhagen Prospective Study on Neuro-PSYCHiatric Development; ICD-10, the International Classification of Disorders 10th Revision; K-SADS-PL, Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children - Present and Lifetime Version; n-3 long-chain PUFA, omega-3 long-chain polyunsaturated acids; RCT, randomized clinical trial; *SRS-2*, *Social Responsiveness Scale 2*; 25(OH)D, 25-hydroxy-vitamin D.

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Introduction

During fetal development, the brain undergoes rapid growth and development. Early environmental exposures during this vulnerable phase may have long-term consequences including affected risk of common neurodevelopmental disorders, such as autism and attention deficit hyperactivity disorder (ADHD) [1,2].

The prevalence of vitamin D deficiency in pregnancy has globally been estimated to be present in >50% of pregnant women [3]. Animal models have shown that vitamin D is crucial for the developing brain, as it contributes to functions including modulation of neurotransmission and neuroprotection [4,5]. Because the fetus relies on vitamin D passing from the mother through the placenta, maternal vitamin D deficiency may potentially affect fetal brain development [6].

Previous observational studies have reported that maternal vitamin D deficiency in pregnancy is associated with the risk of autism and ADHD in the offspring, however, results are ambiguous, and potentially confounded by diet, lifestyle, and season [7–15]. Further, lower gestational vitamin D levels have been shown to increase the severity of traits and symptoms of autism and ADHD in childhood in some studies [7,8,16–19], but not in others [20–23]. However, no randomized clinical trials (RCTs) of vitamin D supplementation in pregnancy have investigated the effect on neurodevelopmental disorders.

Based on a hypothesized protective effect of higher serum vitamin D in pregnancy, we investigated the effect of high-dose compared with standard dose of vitamin D3 supplementation in an RCT during the third trimester of pregnancy on the risk of autism and ADHD and corresponding symptom load evaluated clinically at age 10 as part of the *COpenhagen Prospective Study on Neuro-PSYCHiatric Development (COPSYCH) project* [24].

Methods

Study design

The *COPSYCH project is nested within the* Copenhagen Prospective Studies on Asthma in Childhood 2010 (*COPSAC2010) cohort comprising 700 mother-child pairs enrolled at wk 24 of pregnancy. Pregnant women living in Zealand (Latitude 55° N), Denmark, were recruited by a written invitation sent out after their first pregnancy visit at the general physician. Women not fluent in Danish, with an intake of >600 IU vitamin D3 per day, and/or with any kidney, heart, or endocrine disorder were excluded. From the COPSAC2010 cohort, 623 pregnant women were included in the vitamin D3 trial. The offspring were followed prospectively and deeply phenotyped at the COPSAC unit through 14 visits until age 10* [25]. *At age 10, the children underwent an extensive neuropsychiatric evaluation. Children with a birth weight < 1500 g or a gestational age < 28 wk were excluded from the analyses* [1,2]. *For further details see* Supplementary Material.

The study was conducted according to the guiding principles of the Declaration of Helsinki and was approved by the Local Ethics Committee (H-B-2009-014, COPSAC2010: H-B-2008-093), and the Danish Data Protection Agency (COPSAC2010: 2015-41-3696). All study participants provided informed consent.

Vitamin D intervention

The pregnant women were randomly assigned (1:1) to a daily vitamin D3 supplementation of 2400 IU or placebo starting from the first visit at the COPSAC research unit at week 24 of pregnancy until 1 wk postpartum. The vitamin D3 intervention was performed between 4 March, 2009, and 17 November, 2010. An external investigator with no additional involvement in the RCT performed the randomization by a computer-generated list of random numbers. All included women were instructed to continue consuming a daily vitamin D3 supplementation of 400 IU throughout pregnancy as recommended by the Danish National Board of Health. Total supplementation was therefore 2800 IU/d vitamin D3 in the intervention (high-dose) group and 400 IU/d in the control (standard dose) group. Mothers were asked to return capsules after the intervention period to estimate the adherence. The study was double-blinded until the youngest child had reached the age of 3 y, with the exception of medical emergency (3 cases of early unblinding). From this age information on treatment group was available to all parents. In a factorial 2×2 design, pregnant women were simultaneously randomly assigned to a daily fish oil (n-3 long-chain PUFA) supplement of 2400 mg or olive oil capsules (ClinicalTrials.gov: NCT00798226) [25].

Serum measures of 25-hydroxy-vitamin D

Maternal serum 25-hydroxy-vitamin D (25(OH)D) levels were measured before and after intervention at wk 24 of pregnancy and 1 wk postpartum, respectively. Child serum 25(OH)D concentrations were measured at 6 mo and 6 y [26].

The COPSYCH 10-y visit

The COPSYCH 10-y clinical visit was a post hoc follow-up of the vitamin D3 RCT (*clinicaltrials.gov identifier: NCT00856947*). The visit was carried out over 2 d and included an extensive evaluation of psychopathology, neurocognition, and brain structure and function using magnetic resonance imaging [24]. Examinations were performed between January 2019 and December 2021. Categorical psychopathology was established by the use of semi-structured clinical diagnostic interview Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL) [27]. All K-SADS-PL interviews were administered by trained medical doctors, nurses, and psychologists and were video recorded to enable supervision by a psychologist (JRJM) and for external validation diagnostic conferences with a clinical professor of child and adolescent psychiatry (NB) both specialists in child and adolescent psychiatry to reach consensus on diagnostics [24]. Clinician verified symptom load of psychopathology (*symptoms component scores), were established based on number of symptoms endorsed for each disorder assessed by the K-SADS-PL. Autistic and ADHD symptoms component scores were defined as the first component (describing 41% and 46%, respectively, of the variation in data) from a multiple correspondence analysis of the registered autistic and ADHD symptoms (R package: FactoMineR)* [28]. *Further, parent-rated severity of ADHD symptoms and autistic traits were obtained with the ADHD-Rating Scale (ADHD-RS) and Social Responsiveness Scale 2 (SRS-2), respectively* [29–31]. Research diagnoses based on all sources of clinical information were assigned according to the International Classification of Disorders 10th Revision (ICD-10) [32]. ICD-10 diagnostic codes of autism assigned at the COPSYCH visit included the DF84.0, DF84.5, and DF84.8 diagnostic codes, and of ADHD DF90.0, DF90.8, and DF98.8.

Statistical Analysis

We estimated the effect of maternal preintervention and postintervention serum 25(OH)D as well as child serum 25(OH)D at age 6 mo and 6 y on diagnosis of autism and ADHD and continuous autistic and ADHD symptoms component scores reflecting symptom load by

logistic and linear regression analyses. The covariate adjusted association between maternal preintervention 25(OH)D and autistic symptom load was visualized in a partial residual plot. Covariates were included based on known risk factors for autism, ADHD, and known influencers of serum 25(OH)D (See Supplementary Methods). In analyses regarding maternal and child serum 25(OH)D and risk of autism and ADHD, we included all individuals attending the COPSYCH 10-y visit regardless of participation in the vitamin D3 trial.

Among COPSYCH participants included in the vitamin D3 trial, we estimated the effect of high-dose compared with standard dose of vitamin D3 on the prevalence of autism and ADHD diagnoses and symptom load by logistic and linear regression analyses. *We tested for* interactions between preintervention serum 25(OH)D levels and the vitamin D3 intervention on psychopathologic outcomes by adding cross products to the models and performed analyses stratified according to maternal preintervention serum 25(OH)D at wk 24 of pregnancy to assess the intervention effect according to early pregnancy 25(OH)D levels. Analyses were performed both crude and adjusted for preintervention pregnancy wk 24 25(OH)D levels, season of birth, child sex, and n-3 long-chain PUFA intervention. *In all analyses, we investigated for interaction with sex due to pre-existing studies suggesting sex differences in the effect of vitamin D on risk of psychopathology* [18,20,33].

To corroborate identified associations to K-SADS-PL diagnostic outcomes, we investigated the effect of maternal as well as child circulating 25(OH)D and the effect of the vitamin D3 intervention on the severity of parent-reported autistic traits and ADHD symptoms.

In all analyses, statistical significance was set as <0.05, 2-sided. Owing to the relatively few individuals with missing information, missing data were not imputed. We have not controlled for multiple testing because analyses were performed based on a strong hypothesis generated from existing research. Statistical analyses were performed using R statistical software version R4.2.1 (the R Foundation, Vienna). For complete overview of included exposure and outcome measures see Supplementary Figure 1.

Results

Baseline characteristics

From the total COPSAC cohort including 700 mother-child pairs, 591 children were included in the COPSYCH 10-y visit and eligible for analyses on maternal and child serum 25(OH)D levels. For baseline characteristics of the COPSYCH 10-y visit see Supplementary Table 1.

From the total COPSAC cohort, a subgroup of 496 children participated both in the vitamin D3 trial and in the COPSYCH 10-y visit and were eligible for primary analyses on the intervention effect: 246 received high dose and 250 standard dose of vitamin D3 (Figure 1).

There were no significant differences in serum 25(OH)D at baseline or in season of birth between the intervention and the placebo group. Before the intervention, 51.4 % of mothers had levels of serum 25(OH)D $\geq$75nmol/L. Demographics are provided in Table 1. See the Supplementary Material for additional descriptive tables stratified according to outcome measures (Supplementary Tables 2–5).

In total, 74% of the mothers adhered to the intervention, which was defined as an intake of $\geq$80% of the prescribed capsules [34,35]. The safety profile of the RCT has been reported previously [35].

Serum 25(OH)D and risk of autism and ADHD

Of the total 591 individuals included in the COPSYCH visit, 16 children (2.7%) were diagnosed with autism and 65 (11.0%) with ADHD. Clinically rated symptoms of autism were present in 49 individuals (8.3%) and of ADHD in 170 (28.8%). *Adjusted analyses showed that higher maternal preintervention 25(OH)D level was associated with a decreased risk of autism [odds ratio (OR) per 10 nmol/L 0.76 (0.59,0.97); P = 0.034)], lower autistic symptom load [β per 10 nmol/L −0.03 (−0.05,0.00); P = 0.024], and lower risk of ADHD diagnosis [OR per 10 nmol/L 0.88 (0.78,0.99); P = 0.033], but not ADHD symptom load [β per 10 nmol/L −0.02 (−0.04,0.00), P = 0.122]* (see Supplementary Table 6). *See Figure 2 for visualization of the effect of maternal preintervention 25(OH)D on autistic symptom load. Beta coefficients for all variables in adjusted models are provided in the* Supplementary Tables 7–10. *We did not observe sex differences (P-interactions > 0.05).*

Maternal post-intervention serum 25(OH)D level or child level age 6 mo or 6 y were not associated with autism or ADHD (Supplementary Table 11).

High-dose vitamin D3 supplementation and risk of autism and ADHD

In the high-dose vitamin D3 supplementation group, 5 (2.0%) children were compared with 7 (2.8%) children in the standard dose group diagnosed with autism at age 10. Clinically rated symptoms of autism were present in 15 (6.1%) in the high-dose group compared with 25 (10%) in the standard dose group, ADHD diagnosis in 27 (11%) compared with 31 (12.4%), and clinically rated symptoms of ADHD in 67 (27.2%) compared with 76 (30.4%).

Vitamin D3 treatment group was not significantly associated with risk of autism (crude OR: 0.72; 95% CI: 0.21,2.29; P = 0.580), symptom load of autism (crude β: −0.08; 95% CI: −0.19, 0.03; P = 0.142), ADHD diagnosis (crude OR: 0.87; 95% CI: 0.50,1.51; P = 0.622), or symptom load of ADHD (crude β: −0.06; 95% CI: −0.18,0.07; P = 0.375) and there was no interaction with sex (Table 2). Results were unchanged after adjustments.

Within the high-dose vitamin D3 supplementation group, no children of mothers with preintervention 25(OH)D levels ≥75 nmol/L at wk 24 (n = 128) were diagnosed with autism when compared to 5 children of mothers with 25(OH)D <75 nmol/L (n = 116), and there was a significant interaction between preintervention 25(OH)D concentrations and the vitamin D3 intervention on autism risk (interaction term coefficient = 0.97, crude P-interaction = 0.030). Barnard's exact test inferred a significant protective effect of the vitamin D3 intervention on risk of autism within mothers with preintervention 25(OH)D levels ≥75 nmol/L; OR = 0, P = 0.044. There was no significant interaction between preintervention 25(OH)D levels and the vitamin D3 intervention on autistic symptom load (crude P-interaction = 0.261), ADHD diagnosis (crude P-interaction = 0.687), or ADHD symptom load (crude P-interaction = 0.703). (See Supplementary Methods, Supplementary Figure 2, and Supplementary Figure 3)

A threshold analysis (moving average) suggested a U-shape effect of the high-dose vitamin D3 intervention with a protective effect on autistic symptom load in cases of maternal preintervention 25(OH)D concentrations within the normal to high range of serum 25(OH)D (approximately 55–110 nmol/L) (Figure 3).

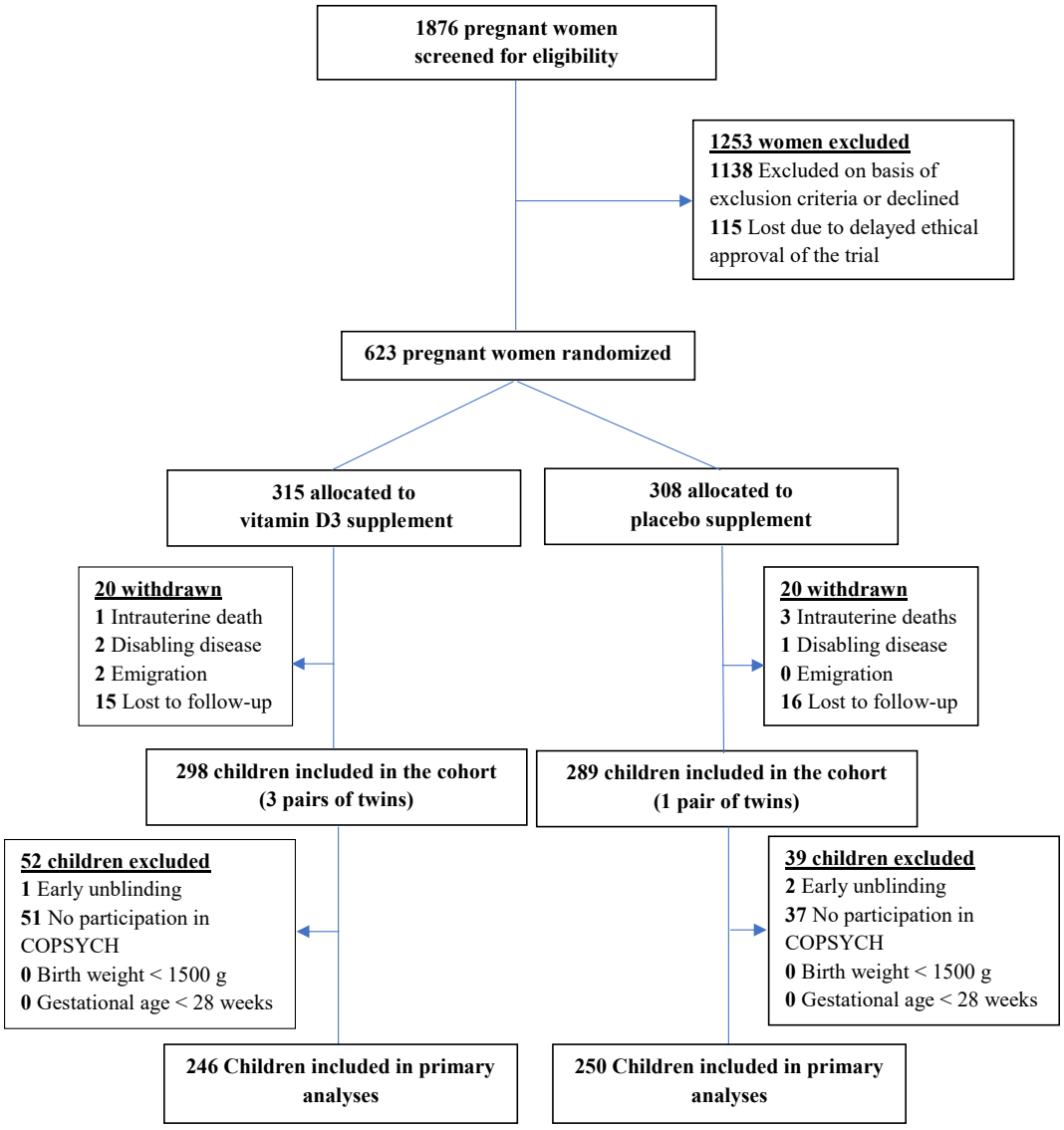

Figure 1. CONSORT participant flow diagram.

Vitamin D and parent-rated autistic traits severity and ADHD symptoms

No significant associations were found between the vitamin D3 supplementation, maternal pre- or post-intervention or child serum 25(OH)D and parent-rated severity of autistic traits measured by SRS-2 or ADHD symptoms measured by ADHD-RS [Supplementary Table 12 (vitamin D3 intervention), Table 13 (maternal 25(OH)D), and Table 14 (child 25(OH)D)]. However, estimates suggested lower SRS-total scores with increasing maternal preintervention 25(OH)D and child 6-mo 25(OH)D levels.

Sensitivity analyses

The association between maternal preintervention *25(OH)D and risk of ADHD disappeared after* additional adjustment for maternal ADHD PRS (OR, 0.94 (0.83,1.07); $P = 0.366$). *The association between higher maternal preintervention 25(OH)D and decreased autistic symptom load did replicate using alternative statistical models, however, results were nonsignificant. (see* Supplementary Methods *and* Supplementary Tables 15–16*).*

Discussion

In this RCT, investigating the effect of high-dose (2800 IU/d) compared with standard dose (400IU/d) of vitamin D3 from pregnancy wk 24 until 1 wk after birth, we found no overall protective effect on risk of autism, ADHD, or symptom loads. *Higher maternal pre-intervention 25(OH)D was associated with a decreased risk of autism, lower autistic symptom load, and decreased risk of ADHD.*

The present study overcomes important limitations of existing observational studies investigating the association between gestational serum 25(OH)D and risk of autism and ADHD in the offspring. First, the RCT design allows for an unbiased investigation of the effect of vitamin D3 supplementation in late pregnancy, a period characterized by rapid brain growth and neuronal development. This is of high importance due to the many known lifestyle factors influencing serum levels of vitamin D [36]. Second, this study was based on thorough clinical evaluations performed by uniformly trained nurses and medical doctors at the COPSAC research unit as opposed to parent-reported or registry-based outcomes more prone to bias [37,38]. Lastly,

Table 1
Baseline characterization of participants of the vitamin D3 RCT included in the COPSYCH project

Stratified by participation in the vitamin D3 RCT	All	Placebo	Vitamin D3
	n = 496	*n* = 250	*n* = 246
Diagnosis of autism, *n* (%)	12 (2.4)	7 (2.8)	5 (2.0)
Individuals presenting clinically rated symptoms of autism, *n* (%)	40 (8.1)	25 (10.0)	15 (6.1)
Diagnosis of ADHD, *n* (%)	58 (11.7)	31 (12.4)	27 (11.0)
Individuals presenting clinically rated symptoms of ADHD, *n* (%)	143 (28.8)	76 (30.4)	67 (27.2)
Long-chain n-3 PUFA supplementation, *n* (%)	249 (50.2)	122 (48.8)	127 (51.6)
Maternal preintervention 25(OH)D, nmol/L, [mean (SD)]	75.81 (25.66)	75.57 (25.44)	76.05 (25.92)
Maternal preintervention 25(OH)D, ≥75 nmol/L, *n* (%)	253 (51.4)	125 (50.4)	128 (52.5)
Maternal post-intervention 25(OH)D, nmol/L, [mean (SD)]	89.99 (38.09)	71.86 (31.54)	108.33 (35.31)
Maternal post-intervention 25(OH)D,≥75 nmol/L, *n* (%)	313 (64.0)	108 (43.9)	205 (84.4)
Maternal age at childbirth, y [mean (SD)]	32.33 (4.28)	31.98 (4.24)	32.69 (4.30)
Maternal pre-pregnancy weight, kg, [mean (SD)]	69.17 (13.57)	69.15 (13.18)	69.19 (13.99)
Parity			
1, *n* (%)	219 (44.2)	123 (49.2)	96 (39.0)
2, *n* (%)	198 (39.9)	91 (36.4)	107 (43.5)
≥3, *n* (%)	79 (15.9)	36 (14.4)	43 (17.5)
Alcohol intake in pregnancy, *n* (%)	81 (16.4)	39 (15.6)	42 (17.1)
Smoking third trimester, *n* (%)	17 (3.4)	11 (4.4)	6 (2.4)
Exclusive lactation, weeks (median [IQR])	17.43 [8.57, 21.57]	17.57 [8.93, 21.68]	17.43 [8.29, 21.39]
Maternal educational level (%)			
Low (Elementary school or college graduate)	41 (8.3)	24 (9.6)	17 (6.9)
Medium (Tradesman certification or bachelor's degree)	314 (63.3)	162 (64.8)	152 (61.8)
High (Master's degree or higher)	141 (28.4)	64 (25.6)	77 (31.3)
Household income (%)			
Low (< 100.000DKK[1])	44 (8.9)	23 (9.2)	21 (8.5)
Medium (100.000-200.000 DKK)	257 (51.8)	134 (53.6)	123 (50.0)
High (> 200.000 DKK)	195 (39.3)	93 (37.2)	102 (41.5)
Fathers age, y, [mean (SD)]	34.63 (5.19)	34.29 (5.20)	34.98 (5.17)
Gestational age, d [mean (SD)]	279.43 (10.86)	279.32 (10.25)	279.54 (11.46)
Season of birth			
Winter, *n* (%)	179 (36.1)	87 (34.8)	92 (37.4)
Spring, *n* (%)	97 (19.6)	50 (20.0)	47 (19.1)
Summer, *n* (%)	100 (20.2)	50 (20.0)	50 (20.3)
Fall, *n* (%)	120 (24.2)	63 (25.2)	57 (23.2)
Sex, male, *n* (%)	256 (51.6)	123 (49.2)	133 (54.1)
Race, White, *n* (%)	475 (95.8)	240 (96.0)	235 (95.5)

Abbreviations: ADHD, attention deficit hyperactivity disorder; RCT, randomized controlled trial; SD, standard deviation; IQR, interquartile range; N = number.
SI Conversion: vitamin D can be converted to ng/mL by dividing by 2.496.
Alcohol intake in pregnancy describes any intake of alcohol during pregnancy, yes/no.
Smoking in third trimester describes any smoking in third trimester of pregnancy, yes/no.
Information on race was obtained through parental interviews and was defined as either white or non-white.
[1] *DKK = 0.14 USD.*

consecutive follow-up and longitudinal deep phenotyping of the COPSAC2010 cohort allowed us to investigate the effects of serum 25(OH)D from the prenatal period until childhood controlling for potential confounders.

The study was limited by the low number of clinically evaluated cases of autism [39], which could explain why, we found no overall intervention effect. *A post hoc power calculation showed that the present study was underpowered (See* Supplementary Results*).* Additionally, we were unable to determine whether a higher dose initiated earlier in pregnancy or even pre-pregnancy would have caused an effect. Further, the observational result of the study was limited by missing information on parental mental health status—an important potential confounder considering both autism and ADHD are highly heritable (heritability estimate for autism 74–93% and for ADHD 70–80%) [1,2]. Analyses on the effect of maternal preintervention serum 25(OH)D adjusted for the mother's genetic risk of autism or ADHD removed the effect on ADHD (Supplementary Table 15). Lastly, the external validity of the present study is limited by the relatively high levels of maternal serum 25(OH)D when compared to global reports hereof [3].

Regardless of the intervention, but with the risk of lifestyle confounding, we observed that higher maternal preintervention 25(OH)D was associated with a reduced risk of autism and ADHD and lower clinically evaluated symptom load of autism but not ADHD. However, this association with autistic symptoms was not significant using parent-reported autistic traits. Higher maternal serum 25(OH)D in pregnancy has previously been associated with low risk of both autism and ADHD diagnosis [9,11,13,14]. The Generation R study reported an association between higher maternal gestational 25(OH)D and lower parent-rated autistic trait severity among offspring measured by SRS-2 in a large cohort comprising 2866 mother-child pairs [16]. In a smaller case-control study, higher gestational 25(OH)D was also associated with fewer autistic symptoms measured by childhood autism rating scale completed by health care professionals [7,40]. Finally, a large birth cohort study reported no association between gestational 25(OH)D and later parent-reported symptoms of ADHD [21].

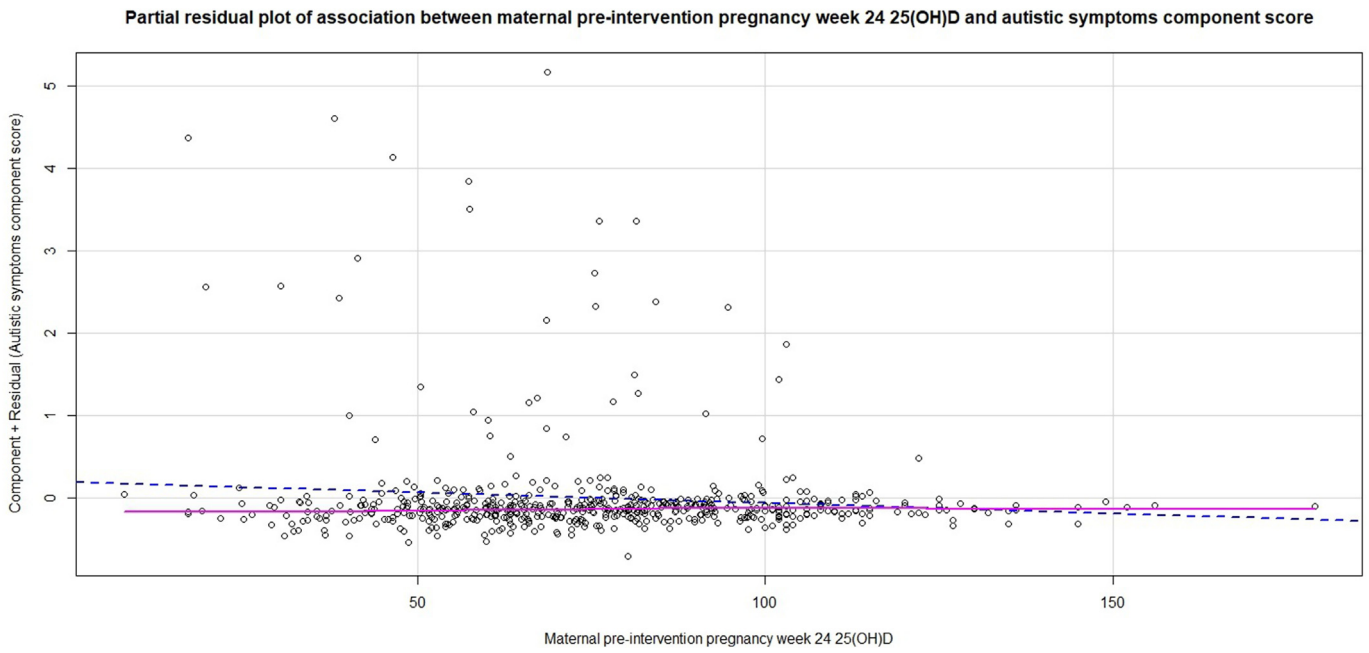

Figure 2. Partial residual plot of the covariate adjusted linear association between maternal preintervention pregnancy week 24 25(OH)D and autistic symptom load measured by Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children - Present and Lifetime Version among 569 individuals. The linear fit is represented by the broken blue line and a smooth (loess) of the partial residuals by a solid magenta line (R package: crPlots). Adjusted for child sex, birth weight, gestational age, season of week 24 25(OH)D measurement, social circumstances, maternal smoking in third trimester of pregnancy, maternal pre-pregnancy weight, and fathers´ age. The study population included all individuals included in the COpenhagen Prospective Study on Neuro-PSYCHiatric Development 2010 cohort with available measurements of 25(OH)D in pregnancy week 24 and with offspring participating in the COpenhagen Prospective Study on Neuro-PSYCHiatric Development visit at age 10 regardless of participation in the vitamin D3 trial. 25(OH)D, 25-hydroxy-vitamin D.

Table 2
Vitamin D3 supplementation and K-SADS-PL evaluation of autism and ADHD

K-SADS-PL measure	*N* (N cases)	Odds ratio estimate (CI)	*N* (N cases)	Odds ratio estimate (CI) adjusted[1]
Autism	496 (12)	0.72 (0.21,2.29)	492 (12)	0.72 (0.21,2.32)
ADHD	496 (58)	0.87 (0.50,1.51)	492 (57)	0.87 (0.49,1.53)
K-SADS-PL measure	*N*	Beta estimate (CI)	*N*	Beta estimate (CI) adjusted[1]
Autistic symptoms component score	496	−0.08 (−0.19,0.03)	492	−0.08 (−0.19,0.03)
ADHD symptoms component score	496	−0.06 (−0.18,0.07)	492	−0.07 (−0.19,0.05)

ADHD, attention deficit hyperactivity disorder; CI, confidence interval; K-SADS-PL, Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version; *N*, number.
ICD-10 diagnostic codes of Autism assigned at the COPSYCH visit included the DF84.0, DF84.5 and DF84.8 diagnostic codes, and of ADHD DF90.0, DF90.8 and DF98.8.
[1] Adjusted for week 24 vitamin D levels, season of birth, child sex, and the n-3 long-chain PUFA intervention.

The high-dose vitamin D3 supplementation during pregnancy did not show overall protection against child autism and ADHD diagnosis or symptom load. To our knowledge, no RCT has previously investigated the effect of vitamin D supplementation in pregnancy on risk of autism or ADHD. In a prospective study from 2016, vitamin D supplementation in pregnancy among mothers of children with autism decreased the recurrence rate of autism in newborn siblings from previously reported 20% to 5% [41]. These mothers were prescribed 5000 IU vitamin D3 per day during pregnancy as opposed to 2800 IU/day in the present study. In the study, newborns were also supplemented with 1000 IU/day vitamin D until the age of 3. A lower intervention dose of vitamin D3 in our RCT may therefore to some extent explain the discrepancy with our results.

Among mothers with early pregnancy serum 25(OH)D $\geq$*75 nmol/L*, we found that the high-dose vitamin D3 intervention may lower the risk of autism diagnosis. This may suggest that high 25(OH)D in early pregnancy is of particular importance for typical brain development. This is supported by a large registry-based study showing an association between lower maternal serum 25(OH)D in first and early second trimester of pregnancy and increased risk of autism diagnosis [11]. Furthermore, early pregnancy may represent a vulnerable phase because basics of the neural system are established already during the embryonic stage [42]. Hence, it is plausible that the vitamin D3 intervention in our study was introduced too late in pregnancy or that the intervention dose was too low to achieve an effect among mothers with vitamin D deficiency at randomization.

We performed a threshold analysis of the effect of the vitamin D3 supplementation on autistic symptom load according to maternal pre-intervention serum 25(OH)D. The analysis revealed a U-shaped association indicative of a protective effect of vitamin D3 intervention

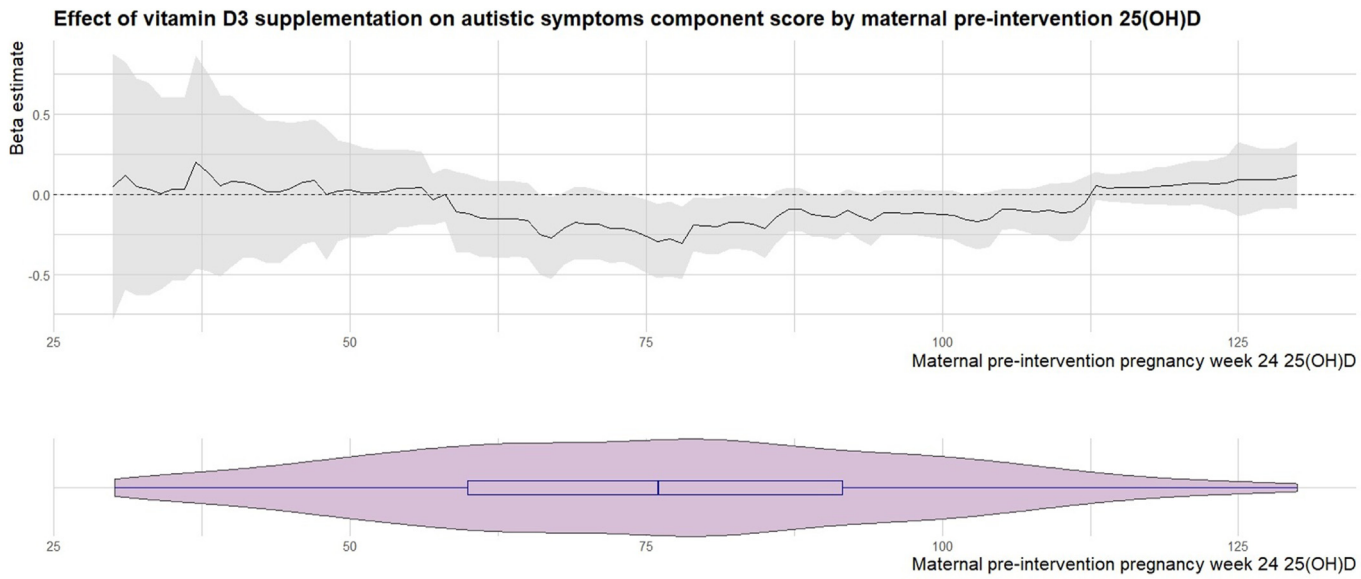

Figure 3. Threshold analysis of the effect of high-dose vitamin D3 supplementation on autistic symptom load measured by Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children - Present and Lifetime Version according to maternal preintervention serum 25(OH)D measured at pregnancy week 24.
Overall sample size was 492 individuals. The violin plot below shows the distribution of the measured maternal preintervention serum 25(OH)D. Linear regression was used to estimate the effect of the intervention according to maternal preintervention 25(OH)D within a moving window of ± 20 nmol/L. Black line marks the β estimate and gray area the corresponding 95% confidence interval. Estimates are unadjusted. 25(OH)D, 25-hydroxy-vitamin D.

among mothers with preintervention levels between ~55 and 110 nmol/L. U-shaped associations between vitamin D and bone health as well as aeroallergen sensitization have been described previously [43,44]. Further, a case-control study has also reported higher risk of schizophrenia among children with either low or high 25(OH)D measured in neonatal dried blood samples [45]. Future studies on vitamin D and autism should be aware of potential non-linear associations.

Two previous studies have reported sex differences in the relationship between early life vitamin D status and autism, where one study reported a protective association of higher neonatal vitamin D only among girls [20], whereas another study reported a protective association of higher gestational vitamin D among boys and an opposite association among girls [33]. In our study, there was no interaction between sex and the intervention for neither autism nor ADHD.

Neither the suggested protective effect on autism diagnosis of the vitamin D3 intervention within mothers with early pregnancy levels of 25(OH)D ≥75nmol/L nor the protective association of higher gestational serum 25(OH)D on autistic symptom load could be replicated using the parent-reported SRS-2. However, SRS-2 scores are influenced by factors not specific to autism such as developmental difficulties and behavioral problems. Thus, SRS scores may to some extent reflect parent-evaluated general impairment of the child instead of the severity of core autistic traits [38]. Therefore, compared to a clinician rating of autistic symptoms, the SRS-2 may be a more unspecific measure of autistic trait severity, which may have prevented us from replicating our findings.

Finally, we found no associations of maternal post-intervention serum 25(OH)D or child serum 25(OH)D at either age 6 mo or 6 y on risk of autism or ADHD, which contrasts a recent meta-analysis showing evidence of lower serum 25(OH)D in children and adolescents with autism [46]. However, all included studies were case-control and may be prone to bias from lifestyle factors such as picky eating patterns, less time spent outdoors, and medication influencing 25(OH)D levels [46], factors which would probably not be as influential in childhood measurements. A meta-analysis from 2018 also suggested an association between low childhood 25(OH)D status and risk of ADHD [15], but a Mendelian randomization study did not find evidence of a causal relationship [47].

In conclusion, *higher maternal preintervention 25(OH)D was associated with a decreased risk of autism, lower autistic symptom load, and decreased risk of ADHD diagnosis.* High-dose vitamin D3 supplementation from pregnancy wk 24 until 1 wk postpartum did not reduce the overall risk of autism and ADHD diagnosis or symptom load in the offspring at age 10 when compared to standard dose vitamin D3.

Acknowledgments

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Authors contributions

The authors' responsibilities were as follows –; KA: drafted the manuscript. All co-authors (JRMJ, AS, DH, RV, JBR, NB, AE, PM, NF, MH, BF, BYG, MAR, NB, JS, KB, BHE, BC): have provided important intellectual input and contributed considerably to the analyses and interpretation of the data. All authors: guarantee that the

accuracy and integrity of any part of the work have been appropriately investigated and resolved and all have approved the final version of the manuscript. The corresponding author had full access to the data and had final responsibility for the decision to submit for publication. No honorarium, grant, or other form of payment was given to any of the authors to produce this manuscript, and all authors: read and approved the final manuscript.

Conflict of interest

BHE is part of the Advisory Board of Eli Lilly Denmark A/S, Janssen-Cilag, Lundbeck Pharma A/S, and Takeda Pharmaceutical Company Ltd; and has received lecture fees from Bristol-Myers Squibb, Boehringer Ingelheim, Otsuka Pharma Scandinavia AB, Eli Lilly Company, and Lundbeck Pharma A/S. BG has been the leader of a Lundbeck Foundation Centre of Excellence for Clinical Intervention and Neuropsychiatric Schizophrenia Research (CINS) (January 2009 – December 2021), which was partially financed by an independent grant from the Lundbeck Foundation based on international review and partially financed by the Mental Health Services in the Capital Region of Denmark, the University of Copenhagen, and other foundations. All grants are the property of the Mental Health Services in the Capital Region of Denmark and administrated by them. She has no other conflicts to disclose. All other authors report no conflicts of interest. *The funding agencies did not have any role in design and conduct of the study; collection, management, and interpretation of the data; or preparation, review, or approval of the manuscript. No pharmaceutical company was involved in the study.*

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Governance

We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) Good Clinical Practice guidelines and the Helsinki Declaration. Privacy is important to us which is why we follow national and international legislation on General Data Protection Regulation (GDPR), the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

Data availability

Data described in the manuscript, code book, and analytic code will be made available upon request pending approval by author Klaus Bønnelykke (kb@copsac.com) and a signed data access agreement.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2023.12.002.

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High-dose vitamin D3 supplementation in pregnancy and risk of neurodevelopmental disorders in the children at age 10 - A randomized clinical trial

Supplementary Material

Supplementary Methods

Study Design

The COPSYCH project is nested within the ongoing COPSAC2010 cohort comprising 700 mother-child pairs. From the COPSAC2010 cohort, 623 pregnant women were included in the vitamin D3 trial, inclusion in the vitamin D3 trial was influenced by a delay in ethical approval. The offspring were followed prospectively and deeply phenotyped by trained nurses and medical doctors at the COPSAC unit.(1)

Serum measures of 25(OH)D

One serum 25(OH)D measure of 0.0 was corrected to half of the lowest observed value of 25(OH)D.

The COPSYCH 10-year visit

Research diagnoses were based on all clinical information at the 10-year visit and assigned according to both the International Classification of Disorders 10th Revision (ICD-10) of Mental and Behavioural Disorders: Clinical descriptions and diagnostic guidelines and Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5).(2,3) All diagnoses and every 20th mentally healthy child were re-evaluated at monthly consensus meetings with a professor of child and adolescent psychiatry (NB).(4) Autism was included by the ICD-10 diagnostic codes DF84.0 (Childhood autism), DF84.5 (Asperger's syndrome), and DF84.8 (Other pervasive developmental disorders). ADHD was included by the ICD-10 diagnostic codes DF90.0 (Disturbance of activity and attention), DF90.8 (Other hyperkinetic disorders), and DF98.8 (Other specified behavioral and emotional disorders with onset usually occurring in childhood and adolescence).

An estimation of interrater reliability on symptom-level was performed based on video-recordings of 10 participants with JRJM as gold standard. Overall agreement (across symptoms currently present and symptoms currently not present): 99.48%, 95%CI (99.25-99.66). Agreement on present symptoms: 88.48 %, 95%CI (82.60-92.92).

Information on covariates

Child sex was obtained through the Central Person Register (CPR) number and defined as either male or female. Information on birth weight and gestational age was obtained through medical records, the Danish Medical Birth Registry (MFR, Fødselsregisteret), and the Dansh Database in Fetal Medicine. Maternal pre-pregnancy weight was obtained from medical records (Vandrejournal) and the Danish database in fetal medicine (FØTOdatabasen). Season of week 24 25OHD measurement was calculated based on date of blood sample. The social circumstances variable was derived from a principal component analysis (PCA) including information on maternal age, household income, and mothers highest completed education at child age 2. A higher PCA indicated higher income, educational level, and maternal age. Maternal smoking in third trimester of pregnancy was self-reported by included mothers and was defined as any smoking in third trimester of pregnancy. Fathers´ age was obtained through the CPR number.

Statistical analysis

Main analyses

The difference in maternal pre- and post-intervention serum 25(OH)D in the high-dose vitamin D3 intervention group was tested using a paired t-test. Further, we estimated the Spearman correlation between both autistic and ADHD symptom loads and the number of K-SADS-PL symptoms.

Secondary analyses

Analyses regarding maternal serum 25(OH)D were adjusted for child sex, birth weight, gestational age, season of week 24 25(OH)D measurement/season of birth, social circumstances, maternal smoking in third trimester of pregnancy, maternal pre-pregnancy weight, and fathers´ age. Analyses regarding child serum 25(OH)D were adjusted for child sex, birth weight, child BMI z-score at age of 25(OH)D measurement, child age at 25(OH)D measurement, season of 25(OH)D measurement, social circumstances, and maternal smoking in third trimester of pregnancy. Covariates included were based on known risk factors for autism, ADHD, and 25(OH)D status.(5–9)

In adjusted analyses child sex, season of week 24 25OHD measurement, and maternal smoking in third trimester of pregnancy were added as factor variables. Birth weight, gestational age, social circumstances, maternal pre-pregnancy weight, fathers´ age, child BMI z-score and child age as continuous variables.

We tested the association between outcomes and covariates in univariate analyses using linear regression and logistic regression.

Sensitivity analyses

We tested the intervention effect among mothers with pre-intervention 25(OH)D <50 nmol/L and ≥50 nmol/L as this is the Danish cutoff to define sufficiency.(10)

To rule out residual confounding from unregistered parental autism or ADHD, we performed additional analyses adjusted for maternal autism or ADHD polygenic risk score (PRS).(11,12)

K-SADS-PL measure of symptom load, ADHD symptoms (ADHD-RS), and autistic traits (SRS-2) were left-skewed and were not transformed to preserve clinical leverage points. To ensure the correctness of the selected statistical model, we performed sensitivity analyses where associations between maternal pre-intervention serum 25(OH)D and K-SADS-PL symptom loads were sought replicated using alternative statistical approaches including analyses restricted to individuals with a minimum of one symptom of autism (in order to increase the linearity of the data) and analyses with the autistic symptoms component score as a categorical variable (any symptom compared with no symptoms).

Supplementary Results

Baseline characteristics

Across intervention and placebo group, mean maternal pre-intervention 25(OH)D was 75.81 nmol/L. The intervention significantly increased maternal serum 25(OH)D at 1 week postpartum (mean [SD], 76.05 [25.92] nmol/L compared with. 108.33 [35.31] nmol/L; mean difference 32.24 (95% CI, 27.13,37.35), p<0.001).

The autistic and ADHD symptom loads were highly correlated with the number of K-SADS-PL symptoms of autism and ADHD (Spearman, r = 0.82 and r = 0.98, respectively).

Serum 25(OH)D and risk of autism and ADHD

After exclusion of children with birth weight < 1500g and gestational age < 28 weeks (n<5), a total of 591 children from the COPSAC-2010 cohort attended the K-SADS-PL psychopathological evaluation at the COPSYCH 10-year visit. Of the 591 children, 16 (2.7%) were diagnosed with autism and 65 (11.0%) were diagnosed with ADHD. Clinically rated symptoms of autism were present in 49 (8.3%) and of ADHD in 170 (28.8%). Measures of serum 25(OH)D were normally distributed and hence not transformed in the analyses.

We performed univariate analyses showing significant associations between autism and maternal pre-pregnancy weight; ADHD and sex, social circumstances, and smoking in third trimester of pregnancy; autistic symptom load and social circumstances; and ADHD symptom load and sex, social circumstances, and pre-pregnancy weight.

Sensitivity analyses

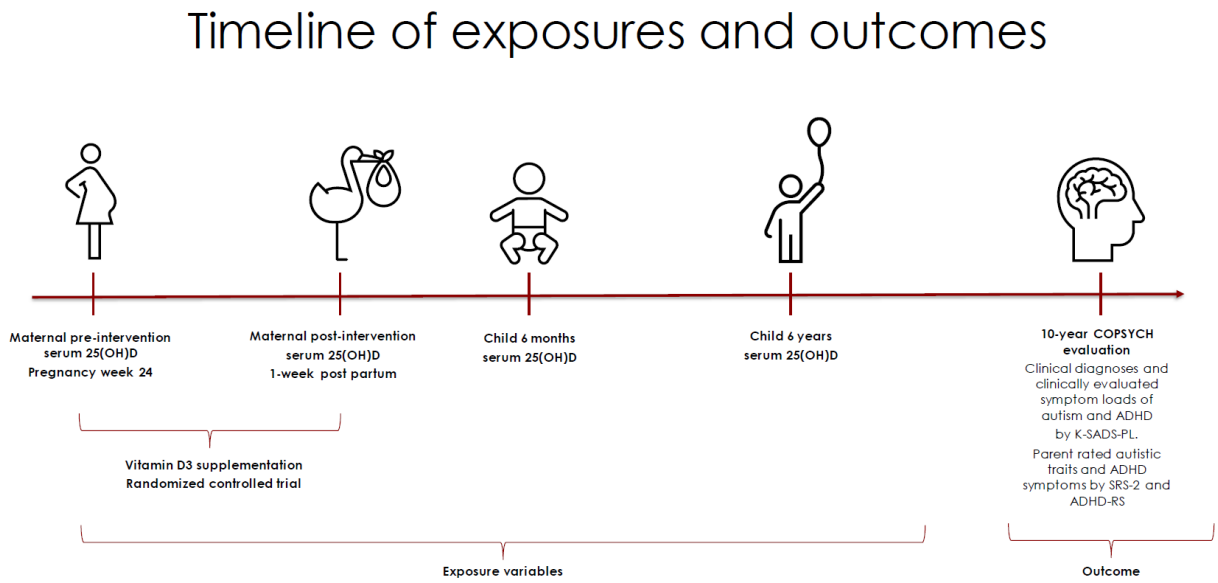
The effect of the vitamin D3 intervention by maternal pre-intervention 25(OH)D on risk of autism diagnosis and severity of autistic symptom load was comparable when using 50 nmol/L as the cutoff. However, the effect on autism diagnosis was only marginally significant (Supplementary Figure 3).

This RCT was powered according to the primary outcome of persistent wheeze or asthma. We therefore performed a post hoc power analysis to determine the needed population size to detect similar but significant effect estimates of the vitamin D3 supplementation on autism diagnosis and ADHD diagnosis. Regarding the vitamin D3 supplementation effect on autism diagnosis, a study population of 1873 individuals in each intervention group was

needed to detect a significant 50% reduction in risk of autism with a corresponding autism prevalence of 1.2% in the high-dose group as compared to 2.4% in the standard-dose group accepting a significance level of 5% and a power of 80%. Regarding the vitamin D3 supplementation effect on ADHD diagnosis, a study population of 358 individuals in each intervention group was needed to detect a significant 50% reduction in risk of ADHD with a corresponding ADHD prevalence of 5.85% int the high-dose group as compared to the 11.7% in the standard-dose group accepting a significance level of 5% and a power of 80%. (R package: pwr)

Supplementary tables and figures

Supplementary Figure 1: Timeline of exposures and outcomes



Supplementary Table 1. Characterization of the COPSAC-2010 cohort and participants hereof included in the COPSYCH K-SADS psychopathological evaluation after exclusion of individuals with low birth weight and/or low gestational age.

Stratified by COPSYCH participation	Complete COPSAC-2010 Cohort n = 698	Participation in COPSYCH K-SADS-PL evaluation n = 591	No participation in COPSYCH K-SADS-PL evaluation n = 107
Vitamin D3 supplementation, n (%)	297 (50.9)	246 (49.6)	51 (58.0)
Long chain n-3 LPUFA supplementation, n (%)	346 (49.7)	303 (51.4)	43 (40.6)
Maternal pre-intervention vitamin D, nmol/L, (mean (SD))	75.66 (25.21)	75.14 (25.46)	78.49 (23.67)
Maternal pre-intervention vitamin D, ≥75 nmol/L, n (%)	345 (49.9)	288 (49.1)	57 (53.8)
Maternal post-intervention vitamin D, nmol/L, (mean (SD))	87.88 (36.54)	88.10 (37.02)	86.66 (33.96)
Maternal post-intervention vitamin D, ≥75 nmol/L, n (%)	421 (61.5)	358 (61.8)	63 (59.4)
Maternal age at childbirth, years (mean (SD))	32.28 (4.37)	32.35 (4.33)	31.88 (4.54)
Maternal pre-pregnancy weight, kg, (mean (SD))	68.93 (13.12)	69.31 (13.21)	66.79 (12.46)
Parity			
1, n (%)	321 (46.0)	270 (45.7)	51 (47.7)
2, n (%)	267 (38.3)	230 (38.9)	37 (34.6)
≥3, n (%)	110 (15.8)	91 (15.4)	19 (17.8)
Alcohol intake in pregnancy, n (%)	100 (14.4)	89 (15.1)	11 (10.4)
Smoking third trimester, n (%)	25 (3.6)	19 (3.2)	6 (5.6)
Exclusive lactation, days (median [IQR])	17.43 [6.79, 21.43]	17.43 [8.07, 21.50]	16.71 [3.50, 21.11]
Maternal educational level (%)			
Low (Elementary school or college graduate)	55 (7.9)	50 (8.5)	5 (4.7)
Medium (tradesman certification or bachelor's degree)	451 (64.6)	374 (63.3)	77 (72.0)
High (Master's degree)	192 (27.5)	167 (28.3)	25 (23.4)
Household income (%)			
low (below 100.000DKK)	67 (9.6)	55 (9.3)	12 (11.2)
medium (100.000-200.000 DKK)	370 (53.1)	310 (52.5)	60 (56.1)
high (above 200.000 DKK)	260 (37.3)	225 (38.1)	35 (32.7)
Fathers age, years, (mean (SD))	34.50 (5.27)	34.61 (5.21)	33.83 (5.56)
Gestational age, days (mean (SD))	279.19 (11.14)	279.38 (11.14)	278.10 (11.10)
Season of birth			
Winter, n (%)	214 (30.7)	181 (30.6)	33 (30.8)
Spring, n (%)	185 (26.5)	162 (27.4)	23 (21.5)
Summer, n (%)	149 (21.3)	125 (21.2)	24 (22.4)
Fall, n (%)	150 (21.5)	123 (20.8)	27 (25.2)
Sex, male, n (%)	359 (51.4)	304 (51.4)	55 (51.4)
Race, White, n (%)	668 (95.7)	565 (95.6)	103 (96.3)

Child 6 months 25(OH)D, nmol/L (mean (SD))	84.86 (23.88)	84.61 (24.15)	86.50 (22.10)
Child 6 years 25(OH)D, nmol/L (mean (SD))	64.07 (19.77)	64.39 (19.95)	59.97 (16.88)

Abbreviations: IQR = interquartile range. N = number. RCT = Randomized controlled trial. SD = standard deviation.
SI Conversion: vitamin D can be converted to ng/mL by dividing by 2.496. 1 DKK = 0.14 USD.
Alcohol intake in pregnancy describes any intake of alcohol during pregnancy, yes/no.
Smoking in third trimester describes any smoking in third trimester of pregnancy, yes/no.
Information on race was obtained through parental interviews and was defined as either white or non-white.

Supplementary Table 2. Descriptive table of covariates included in analyses on vitamin D3 supplementation stratified according to diagnosis of autism

Descriptive table of covariates included in analyses on vitamin D3 supplementation stratified according to diagnosis of autism

	No autism	Autism
	N = 484	N = 12
Maternal pre-intervention vitamin D, nmol/L, (mean (SD))	76.19 (25.58)	60.38 (24.76)
Season of birth		
Winter, n (%)	177 (36.6)	2 (16.7)
Spring, n (%)	95 (19.6)	2 (16.7)
Summer, n (%)	96 (19.8)	4 (33.3)
Fall, n (%)	116 (24.0)	4 (33.3)
Sex, male, n (%)	247 (51.0)	9 (75.0)
Long chain n-3 LPUFA supplementation, n (%)	244 (50.4)	5 (41.7)

Abbreviations: N = number. n-3 LCPUFA = omega-3 long-chain polyunsaturated fatty acids, SD = standard deviation.
SI Conversion: vitamin D can be converted to ng/mL by dividing by 2.496.

Supplementary Table 3. Descriptive table of covariates included in analyses on vitamin D3 supplementation stratified according to diagnosis of ADHD

Descriptive table of covariates included in analyses on vitamin D3 supplementation stratified according to diagnosis of ADHD

	No ADHD	ADHD
	N = 438	N = 58
Maternal pre-intervention vitamin D, nmol/L, (mean (SD))	76.51 (24.67)	70.44 (31.94)
Season of birth		
Winter, n (%)	160 (36.5)	19 (32.8)
Spring, n (%)	85 (19.4)	12 (20.7)
Summer, n (%)	87 (19.9)	13 (22.4)
Fall, n (%)	106 (24.2)	14 (24.1)
Sex, male, n (%)	211 (48.2)	45 (77.6)
Long chain n-3 LPUFA supplementation, n (%)	222 (50.7)	27 (46.6)

Abbreviations: N = number. n-3 LCPUFA = omega-3 long-chain polyunsaturated fatty acids, SD = standard deviation.
SI Conversion: vitamin D can be converted to ng/mL by dividing by 2.496.

Supplementary Table 4. Descriptive table of covariates included in analyses on maternal pre-intervention 25(OH)D stratified according to diagnosis of autism

Descriptive table of covariates included in analyses on maternal pre-intervention 25(OH)D stratified according to diagnosis of autism

	No autism	Autism
	N = 575	N = 16
Sex, male, n (%)	294 (51.1)	10 (62.5)
Birth weight (mean (SD))	3.55 (0.53)	3.59 (0.58)
Gestational age, days (mean (SD))	279.33 (11.22)	281.44 (7.87)
Season of week 24 25(OH)D measurement		
Winter, n (%)	157 (27.5)	7 (43.8)
Spring, n (%)	111 (19.5)	3 (18.8)
Summer, n (%)	124 (21.8)	3 (18.8)
Fall, n (%)	178 (31.2)	3 (18.8)
Social circumstances principal component (mean (SD))	0.02 (0.99)	-0.08 (1.16)
Smoking third trimester, n (%)	19 (3.3)	0 (0.0)
Maternal prepregnancy weight, kg, (mean (SD))	69.02 (12.75)	79.69 (22.89)
Fathers age, years, (mean (SD))	34.59 (5.18)	35.43 (6.32)

Abbreviations: SD = standard deviation. N = number, n-3 LCPUFA = omega-3 long-chain polyunsaturated fatty acids.
SI Conversion: vitamin D can be converted to ng/mL by dividing by 2.496.
Alcohol intake in pregnancy describes any intake of alcohol during pregnancy, yes/no.
Smoking in third trimester describes any smoking in third trimester of pregnancy, yes/no.

Supplementary Table 5. Descriptive table of covariates included in analyses on maternal pre-intervention 25(OH)D stratified according to diagnosis of ADHD

Descriptive table of covariates included in analyses on maternal pre-intervention 25(OH)D stratified according to diagnosis of ADHD

	No ADHD	ADHD
	N = 526	N = 65
Sex, male, n (%)	255 (48.5)	49 (75.4)
Birth weight (mean (SD))	3.54 (0.54)	3.61 (0.49)
Gestational age, days (mean (SD))	279.26 (11.42)	280.40 (8.58)
Season of week 24 25(OH)D measurement		
Winter, n (%)	151 (28.9)	13 (20.3)
Spring, n (%)	99 (19.0)	15 (23.4)
Summer, n (%)	113 (21.6)	14 (21.9)
Fall, n (%)	159 (30.5)	22 (34.4)
Social circumstances principal component (mean (SD))	0.05 (0.98)	-0.28 (1.04)
Smoking third trimester, n (%)	14 (2.7)	5 (7.7)
Maternal prepregnancy weight, kg, (mean (SD))	68.97 (12.74)	72.05 (16.44)
Fathers age, years, (mean (SD))	34.59 (5.15)	34.83 (5.67)

Abbreviations: SD = standard deviation. N = number, n-3 LCPUFA = omega-3 long-chain polyunsaturated fatty acids.
SI Conversion: vitamin D can be converted to ng/mL by dividing by 2.496.
Alcohol intake in pregnancy describes any intake of alcohol during pregnancy, yes/no.
Smoking in third trimester describes any smoking in third trimester of pregnancy, yes/no.

Supplementary table 6: Maternal pre-intervention serum 25(OH)D at week 24 of pregnancy and K-SADS-PL evaluation of autism and ADHD

K-SADS-PL measure	N (N cases)	Odds Ratio Estimate (CI)	N (N cases)	Odds Ratio Estimate (CI) Adjusted[1]
Autism	586 (16)	0.73 (0.58,0.91)	569 (16)	0.76 (0.59,0.97)
ADHD	586 (64)	0.89 (0.80,0.99)	569 (63)	0.88 (0.78,0.99)
K-SADS-PL measure	N	Beta Estimate (CI)	N	Beta Estimate (CI) Adjusted[1]
Autistic symptoms component score	586	-0.03 (-0.05,-0.01)	569	-0.03 (-0.05,0.00)
ADHD symptoms component score	586	-0.02 (-0.04,0.00)	569	-0.02 (-0.04,0.00)

[1]Adjusted for child sex, birth weight, gestational age, season of week 24 25(OH)D measurement, social circumstances, maternal smoking in third trimester of pregnancy, maternal pre-pregnancy weight, and fathers´ age. Beta estimates per 10 nmol/L 25(OH)D
ADHD = attention deficit hyperactivity disorder. CI = confidence interval. K-SADS-PL = Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version. N = number.
ICD-10 diagnostic codes of Autism assigned at the COPSYCH visit included the DF84.0, DF84.5 and DF84.8 diagnostic codes, and of ADHD DF90.0, DF90.8 and DF98.8.
The study population included all individuals included in the COPSAC2010 cohort with available measurements of 25(OH)D in pregnancy week 24 and with offspring participating in the COPSYCH visit at age 10 regardless of participation in the vitamin D3 trial.
Beta coefficients for all variables in adjusted models are provided in the Supplementary Tables 6-9.

Supplementary Table 7. Beta estimates for the effect of maternal pre-intervention 25(OH)D levels on risk of autism diagnosis

	Autism diagnosis		
Predictors	Odds Ratios	95% Confidence Interval	p-value
(Intercept)	0.00	0.00 – 160.45	0.228
Pre intervention 25OHD	0.76	0.59 – 0.97	0.034
Child sex [Male]	1.78	0.62 – 5.65	0.298
Birth weight	0.64	0.18 – 2.21	0.483
Gestational age	1.02	0.96 – 1.09	0.513
Season of week 24 25OHD measurement [spring]	0.62	0.13 – 2.40	0.508
Season of week 24 25OHD measurement [summer]	1.10	0.21 – 4.70	0.905
Season of week 24 25OHD measurement [fall]	0.54	0.11 – 2.11	0.396
Social circumstances	0.98	0.54 – 1.80	0.938
Smoking in third trimester of pregnancy [1]	0.00	-	0.992
Maternal prepregnancy weight	1.04	1.01 – 1.07	0.012
Fathers age	1.02	0.91 – 1.13	0.652
Observations	569		

Supplementary Table 8. Beta estimates for the effect of maternal pre-intervention 25(OH)D levels on risk of ADHD diagnosis

	ADHD diagnosis		
Predictors	Odds Ratios	95% Confidence Interval	p-value
(Intercept)	0.00	0.00 – 2.37	0.089
Pre intervention 25OHD	0.88	0.78 – 0.99	0.033
Child sex [Male]	3.76	2.06 – 7.22	<0.001
Birth weight	1.14	0.57 – 2.24	0.714
Gestational age	1.01	0.98 – 1.04	0.674
Season of week 24 25OHD measurement [spring]	1.67	0.74 – 3.82	0.216
Season of week 24 25OHD measurement [summer]	1.88	0.78 – 4.57	0.157
Season of week 24 25OHD measurement [fall]	2.05	0.97 – 4.49	0.064
Social circumstances	0.67	0.49 – 0.92	0.014
Smoking in third trimester of pregnancy [1]	4.06	1.17 – 12.54	0.018
Maternal prepregnancy weight	1.01	0.99 – 1.03	0.364
Fathers age	1.05	0.99 – 1.11	0.090
Observations	569		

Supplementary Table 9. Beta estimates for the effect of maternal pre-intervention 25(OH)D levels on autistic symptom load

	Autistic symptom load		
Predictors	Estimates	95% Confidence Interval	p-value
(Intercept)	-1.44	-3.01 – 0.12	0.071
Pre intervention 25OHD	-0.03	-0.05 – -0.00	0.024
Child sex [Male]	0.04	-0.07 – 0.15	0.440
Birth weight	-0.14	-0.27 – -0.00	0.044
Gestational age	0.01	-0.00 – 0.01	0.077
Season of week 24 25OHD measurement [spring]	-0.14	-0.30 – 0.01	0.076
Season of week 24 25OHD measurement [summer]	-0.04	-0.20 – 0.12	0.629
Season of week 24 25OHD measurement [fall]	-0.03	-0.17 – 0.11	0.709
Social circumstances	-0.05	-0.12 – 0.01	0.084
Smoking in third trimester of pregnancy [1]	-0.15	-0.46 – 0.15	0.327
Maternal prepregnancy weight	0.01	0.00 – 0.01	0.001
Fathers age	0.00	-0.01 – 0.01	0.627
Observations	569		

Supplementary Table 10. Beta estimates for the effect of maternal pre-intervention 25(OH)D levels on ADHD symptom load

	ADHD symptom load		
Predictors	Estimates	95% Confidence Interval	p
(Intercept)	-1.72	-3.28 – -0.16	0.031
Pre intervention 25OHD	-0.02	-0.04 – 0.00	0.122
Child sex [Male]	0.39	0.29 – 0.50	<0.001
Birth weight	-0.04	-0.17 – 0.09	0.529
Gestational age	0.00	-0.00 – 0.01	0.264
Season of week 24 25OHD measurement [spring]	0.10	-0.05 – 0.26	0.191
Season of week 24 25OHD measurement [summer]	0.10	-0.06 – 0.25	0.231
Season of week 24 25OHD measurement [fall]	0.12	-0.02 – 0.26	0.088
Social circumstances	-0.09	-0.15 – -0.03	0.004
Smoking in third trimester of pregnancy [1]	0.25	-0.06 – 0.56	0.112
Maternal prepregnancy weight	0.01	0.00 – 0.01	0.014
Fathers age	0.01	-0.00 – 0.02	0.073
Observations	569		

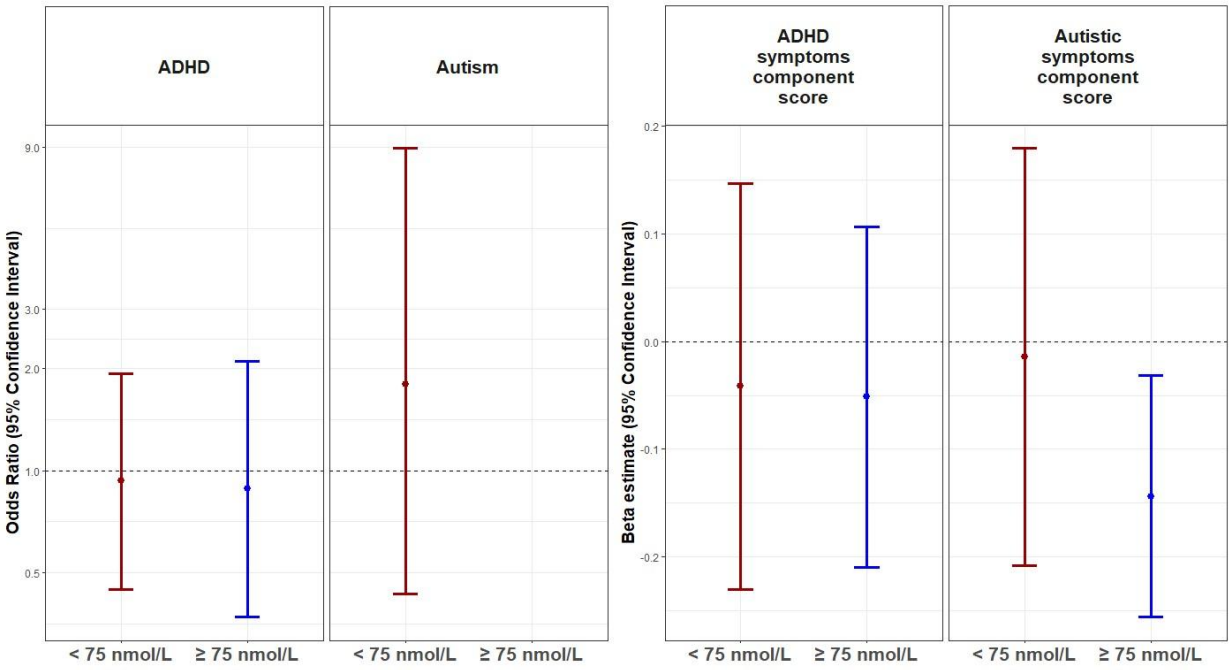
Supplementary Table 11: Serum 25(OH)D and K-SADS-PL evaluation of autism and ADHD

	Maternal post-intervention 1-week post-partum serum 25(OH)D			Child 6 months serum 25(OH)D			Child 6 years serum 25(OH)D		
K-SADS-PL measure	N	Beta Estimate (CI) Adjusted[1]	P value	N	Beta Estimate (CI) Adjusted[2]	P value	N	Beta Estimate (CI) Adjusted[3]	P value
Autism	563	1 (0.85,1.16)	0.956	549	0.89 (0.69,1.13)	0.348	467	0.93 (0.64,1.31)	0.695
ADHD	563	1.02 (0.94,1.09)	0.625	549	1.01 (0.90,1.14)	0.823	467	1.07 (0.90,1.27)	0.440
K-SADS-PL measure	N	Beta Estimate (CI) Adjusted[1]	P value	N	Beta Estimate (CI) Adjusted[2]	P value	N	Beta Estimate (CI) Adjusted[3]	P value
Autistic symptoms component score	563	0.00 (-0.01,0.01)	0.907	549	-0.01 (-0.04,0.01)	0.224	467	-0.02 (-0.05,0.01)	0.270
ADHD symptoms component score	563	0.00 (-0.01,0.02)	0.770	549	0.00 (-0.03,0.02)	0.934	467	0.00 (-0.03,0.04)	0.829

[1]Adjusted for child sex, birth weight, gestational age, season of birth, social circumstances, maternal smoking in third trimester of pregnancy, maternal pre-pregnancy weight, and fathers´ age. Beta estimates per 10 nmol/L 25(OH)D.
[2]Adjusted for child sex, child age at 6 months *25(OH)D* measurement, season of 6 months *25(OH)D* measurement, child 6 months BMI z-score, social circumstances, child birth weight, maternal smoking in third trimester, and child gestational age.
[3]Adjusted for child sex, child age at 6 months *25(OH)D* measurement, season of 6 months *25(OH)D* measurement, child 6 months BMI z-score, social circumstances, child birth weight, maternal smoking in third trimester, and child gestational age.
ADHD = attention deficit hyperactivity disorder. BMI = body mass index. CI = confidence interval. K-SADS-PL = Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version. N = number.
Beta estimates per 10 nmol/L 25(OH)D.

Supplementary Figure 2. Vitamin D3 supplementation and K-SADS-PL evaluation of autism and ADHD according to maternal 25(OH)D ≥75 nmol/L or <75 nmol/L at pregnancy week 24

239 individuals were included in analyses regarding 25(OH)D<75 nmol/L and 253 in analyses regarding 25(OH)D ≥75 nmol/L. Analyses were performed using logistic regression for binary diagnoses and linear regression for continuous symptom loads. Barnard's exact test inferred a significant protective effect of the vitamin D3 intervention on risk of autism, OR=0, p=0.044. 95%CI not computable and results are therefore not shown in figure due to zero cases of autism among mothers with 25(OH)D ≥ 75 nmol/L receiving the high-dose intervention. Estimates are unadjusted.



Supplementary Table 12: Vitamin D3 intervention and parent-rated autistic traits and ADHD symptoms severity by SRS-2 and ADHD-RS

	N	Beta Estimate (CI) Crude	P value	N	Beta Estimate (CI)[1] Adjusted	P value
ADHD-RS total score, question 1-18	500	0.03 (-1.54,1.60)	0.968	496	-0.08 (-1.63,1.47)	0.919
ADHD-RS inattention score, question 1-9	502	0.33 (-0.63,1.29)	0.502	498	0.30 (-0.65,1.25)	0.535
ADHD-RS impulsivity/hyperactivity score, question 10-18	500	-0.30 (-1.05,0.46)	0.441	496	-0.38 (-1.13,0.36)	0.314
SRS-2 total scale	501	0.01 (-3.09,3.11)	0.997	497	-0.05 (-3.17,3.07)	0.975
SRS-2 Restricted, Repetitive Behaviors and Interests scale	501	0.22 (-0.46,0.89)	0.529	497	0.17 (-0.51,0.85)	0.617
SRS-2 Social Communication and Interaction scale	501	-0.21 (-2.76,2.34)	0.871	497	-0.22 (-2.79,2.34)	0.865

[1]Adjusted for week 24 vitamin D levels, season of birth, child sex, and n-3 LCPUFA intervention.
ADHD = attention deficit hyperactivity disorder. ADHD-RS: ADHD-Rating Scale. CI: confidence interval. N = number. SRS-2: Social Responsiveness Scale 2.

Supplementary Table 13: Maternal serum 25(OH)D and parent-rated autistic traits and ADHD symptoms severity by SRS-2 and ADHD-RS

	Maternal pre-intervention pregnancy week 24 serum 25(OH)D			Maternal post-intervention 1-week post-partum serum 25(OH)D		
	N	Beta Estimate (CI)[1] Adjusted	P value	N	Beta Estimate (CI)[2] Adjusted	P value
ADHD-RS total score, question 1-18	570	-0.18 (-0.47,0.11)	0.222	565	-0.02 (-0.21,0.17)	0.838
ADHD-RS inattention score, question 1-9	574	-0.10 (-0.28,0.08)	0.285	568	-0.03 (-0.15,0.09)	0.591
ADHD-RS impulsivity/hyperactivity score, question 10-18	570	-0.08 (-0.22,0.06)	0.268	565	0.01 (-0.08,0.10)	0.812
SRS-2 total scale	574	-0.44 (-1.03,0.16)	0.148	568	-0.02 (-0.41,0.36)	0.901
SRS-2 Restricted, Repetitive Behaviors and Interests scale	574	-0.09 (-0.22,0.04)	0.188	568	0.00 (-0.08,0.09)	0.950
SRS-2 Social Communication and Interaction scale	574	-0.35 (-0.84,0.14)	0.161	568	-0.03 (-0.35,0.29)	0.867

[1]Adjusted for child sex, birth weight, gestational age, season of week 24 25(OH)D measurement, social circumstances, maternal smoking in third trimester of pregnancy, maternal pre-pregnancy weight, and fathers´ age. Beta estimates per 10 nmol/L 25(OH)D.
[2]Adjusted for child sex, birth weight, gestational age, season of birth, social circumstances, maternal smoking in third trimester of pregnancy, maternal pre-pregnancy weight, and fathers´ age. Beta estimates per 10 nmol/L 25(OH)D.
ADHD = attention deficit hyperactivity disorder. ADHD-RS: ADHD-Rating Scale. CI: confidence interval. N = number. SRS-2: Social Responsiveness Scale 2.

Supplementary Table 14: Child serum 25(OH)D and parent-rated autistic traits and ADHD symptoms severity by SRS-2 and ADHD-RS

	Child 6 months serum 25(OH)D			Child 6 years serum 25(OH)D		
	N	Beta Estimate (CI)[1] Adjusted	P value	N	Beta Estimate (CI)[2] Adjusted	P value
ADHD-RS total score, question 1-18	550	0.00 (-0.30,0.30)	0.984	463	0.11 (-0.32,0.53)	0.620
ADHD-RS inattention score, question 1-9	553	0.02 (-0.16,0.20)	0.836	466	0.16 (-0.11,0.42)	0.238
ADHD-RS impulsivity/hyperactivity score, question 10-18	550	-0.02 (-0.17,0.13)	0.782	463	-0.05 (-0.26,0.15)	0.606
SRS-2 total scale	553	-0.41 (-1.02,0.19)	0.180	466	-0.27 (-1.14,0.60)	0.548
SRS-2 Restricted, Repetitive Behaviors and Interests scale	553	-0.08 (-0.21,0.05)	0.217	466	-0.03 (-0.22,0.16)	0.784
SRS-2 Social Communication and Interaction scale	553	-0.33 (-0.84,0.17)	0.195	466	-0.24 (-0.96,0.48)	0.514

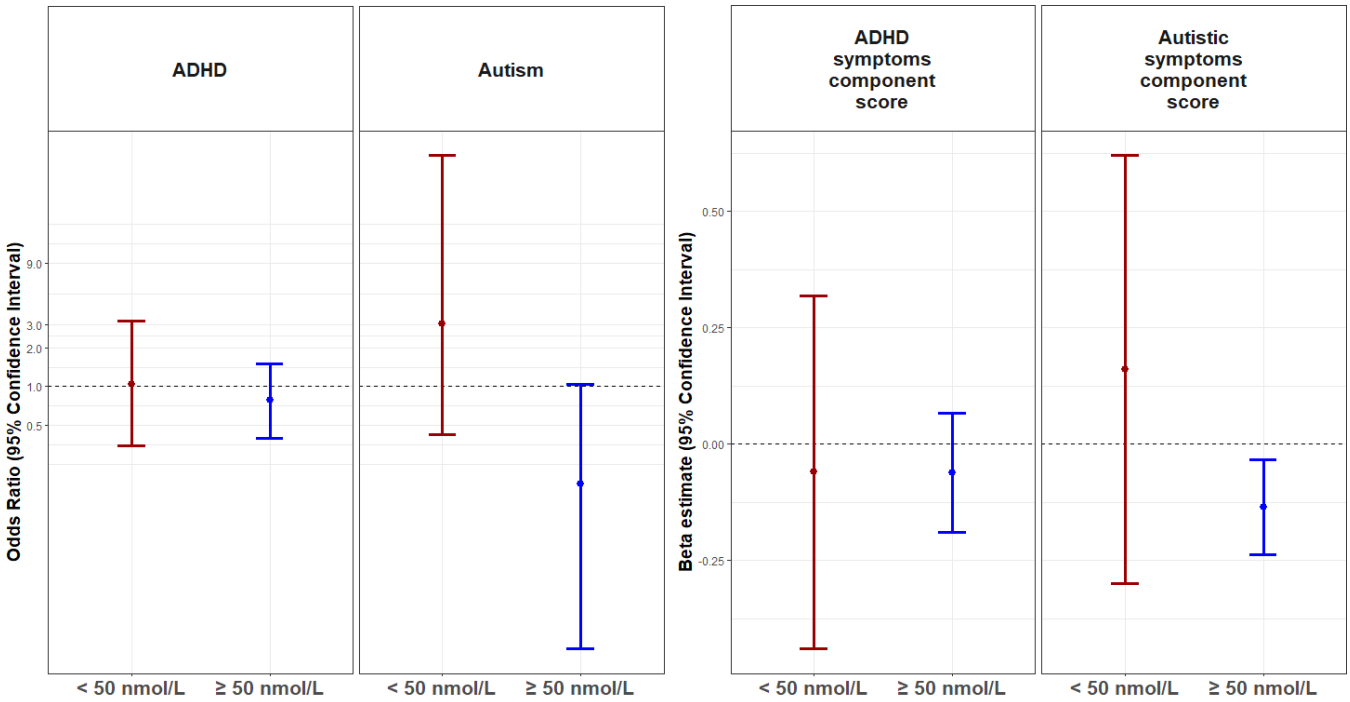
[1]Adjusted for child sex, child age at 6 months *25(OH)D* measurement, season of 6 months *25(OH)D* measurement, child 6 months BMI z-score, social circumstances, child birth weight, maternal smoking in third trimester, and child gestational age. Beta estimates per 10 nmol/L 25(OH)D.

[2]Adjusted for child sex, child age at 6 years *25(OH)D* measurement, season of 6 years *25(OH)D* measurement, child 6 years BMI z-score, social circumstances, child birth weight, maternal smoking in third trimester, and child gestational age.

ADHD = attention deficit hyperactivity disorder. ADHD-RS: ADHD-Rating Scale. BMI = body mass index. CI: confidence interval. N = number. SRS-2: Social Responsiveness Scale 2.

Supplementary Figure 3: Vitamin D3 supplementation and K-SADS-PL evaluation of autism and ADHD according to maternal 25(OH)D ≥50 nmol/L or <50 nmol/L at pregnancy week 24

74 individuals were included in analyses regarding 25(OH)D<50 nmol/L and 418 in analyses regarding 25(OH)D ≥50 nmol/L. Analyses were performed using logistic regression for binary diagnoses and linear regression for continuous symptom loads. Estimates are unadjusted.



Supplementary Table 15: Maternal pre-intervention serum 25(OH)D at week 24 of pregnancy and K-SADS-PL evaluation of autism - Additional adjustment for autism and ADHD polygenic risk score.			
K-SADS-PL measure	N	Estimate (95%CI) Adjusted[1]	P-value
Autism	477	OR 0.75 (0.57,0.97)	0.034
Autistic symptoms component score	477	β -0.03 (-0.05,-0.00)	0.028
K-SADS-PL measure	N	Estimate (95%CI) Adjusted[2]	P-value
ADHD	477	OR 0.94 (0.83,1.07)	0.366
ADHD symptoms component score	477	β -0.01 (-0.03,0.02)	0.613

[1]Adjusted for child sex, season of week 24 vitamin D measurement, social circumstances, birth weight, gestational age, smoking in third trimester of pregnancy, maternal pre-pregnancy weight, fathers age, and maternal autism polygenic risk score.

[2]Adjusted for child sex, season of week 24 vitamin D measurement, social circumstances, birth weight, gestational age, smoking in third trimester of pregnancy, maternal pre-pregnancy weight, fathers age, and maternal ADHD polygenic risk score.

ADHD = attention deficit hyperactivity disorder. CI = confidence interval. K-SADS-PL = Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version. N= number. OR = odds ratio.

Supplementary Table 16: Maternal pre-intervention serum 25(OH)D at week 24 of pregnancy and K-SADS-PL evaluation of autism and ADHD symptom load using alternative statistical approaches

K-SADS-PL measure (Statistical model)	N	Estimate (95%CI) Adjusted[1]	P value
Autistic symptoms component score (Linear regression)	569	β -0.03 (-0.05,-0.00)	0.024
Autistic symptoms component Analysis limited to individuals with minimum 1 registered K-SADS-PL symptom of autism (Linear regression)	48	β -0.14 (-0.36,0.09)	0.231
Number of K-SADS symptoms of autism generated as categorical variable (0 vs. >0) (Logistic regression)	569	OR 0.91 (0.80,1.04)	0.172
ADHD symptoms component score (Linear regression)	569	β -0.02 (-0.04,0.00)	0.122
ADHD symptoms component score Analysis limited to individuals with minimum 1 registered K-SADS-PL symptom of ADHD (Linear regression)	163	β 0.00 (-0.05,0.05)	0.898
Number of K-SADS symptoms of ADHD generated as categorical variable (0 vs. >0) (Logistic regression)	569	OR 0.94 (0.86,1.01)	0.103

[1]Adjusted for child sex, birth weight, gestational age, season of week 24 25(OH)D measurement, social circumstances, maternal smoking in third trimester of pregnancy, maternal pre-pregnancy weight, and fathers´ age. Beta estimates per 10 nmol/L 25(OH)D
ADHD = attention deficit hyperactivity disorder. CI = confidence interval. IRR = incidence rate ratio. K-SADS-PL = Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version. N= number. OR = odds ratio.

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PAPER II

High-dose vitamin D3 supplementation during pregnancy and test-based cognitive performance at age 10 - a post-hoc analysis of a randomized clinical trial

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Under review

High-dose vitamin D3 supplementation during pregnancy and test-based cognitive performance at age 10 - a post-hoc analysis of a randomized clinical trial

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Abbreviations

ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder; CANTAB, Cambridge Neuropsychological Test Automated Battery; $COPSAC_{2010}$, Copenhagen Prospective Studies on Asthma in Childhood 2010; COPSYCH, COpenhagen Prospective Study on Neuro-PSYCHiatric Development; n-3-LCPUFA, omega-3 long-chain polyunsaturated acids; RCT, randomized clinical trial; WISC-IV, Wechsler Intelligence Scale for Children – Fourth Edition; 25(OH)D, 25-hydroxy-vitamin D

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KEY FINDINGS

Question: Does high-dose vitamin D3 supplementation during pregnancy have a positive effect on cognition in offspring?

Findings: In this post hoc analysis of a randomized clinical trial including 498 children, high-dose vitamin D3 supplementation had a positive effect on verbal memory, visual memory, and flexibility/set shift at age 10 when compared to standard-dose, although these effects did not remain significant after FDR-correction.

Meaning: High-dose vitamin D3 supplementation during pregnancy may improve cognitive functioning at age 10.

ABSTRACT

Importance: Observational studies have reported associations between pregnancy vitamin D levels and offspring cognition, but no randomized clinical trial (RCT) has investigated the lasting impact of high-dose vitamin D3 supplementation in pregnancy on cognition into middle childhood.

Objective: To determine whether high-dose vitamin D3 supplementation during pregnancy improves offspring cognition at age 10.

Design: Post hoc analysis of a blinded, placebo-controlled RCT conducted from 2008-2010. Participants were unblinded at 3 years of age, while investigators remained unaware of group assignments. The 10-year visits were conducted from January 2019 - December 2021. Analyses were conducted February-June 2025.

Setting: Participants were from the Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$) cohort.

Participants: The cohort included 700 mother-child pairs from Denmark, of whom 623 were randomized. Exclusion criteria were vitamin D intake >600 IU/day, endocrine, kidney or heart disease,

and insufficient Danish language proficiency. Cognitive assessments were conducted in the offspring at age 10 years excluding those born <28 weeks gestation and birth weight <1500g.

Intervention: High-dose (2800 IU/day) or standard-dose (400 IU/day) vitamin D3 from pregnancy week 24 to one week postpartum.

Main outcome and measures: The primary outcome was cognitive functioning across eleven functions assessed at age 10 using a comprehensive neuropsychological test battery as part of the COpenhagen Prospective Study on Neuro-PSYCHiatric Development (COPSYCH).

Results: The vitamin D3 RCT and the COPSYCH visit was completed by 498 children (240 [48%] female) (age 10.3 years [SD 0.4]), including 247 prenatally exposed to high-dose and 251 to standard-dose vitamin D3. Covariate adjusted analyses of standardized scores showed positive effects on verbal memory (β=0.17 SD, 95% CI:0.03-0.32, p=.021), visual memory (β=0.24 SD, 0.06-0.42, p=.011) and flexibility/set shift (β=0.19 SD, 0.01-0.37, p=.035), which, however, did not pass multiple test corrections.

Conclusion: In this post hoc analysis of an RCT, there was indication that high-dose vitamin D3 supplementation in pregnancy can have a positive effect on visual memory, verbal memory, and flexibility/set shift in the offspring measured at age 10. These findings strengthen the evidence on the impact of prenatal vitamin D exposure on childhood cognition.

Trial registration: clinicaltrials.gov as NCT00856947.

INTRODUCTION

Childhood cognition is a predictor of socio-economic status, occupational achievement, and cognitive abilities later in life[1–4]. Although heritability estimates for cognition are reported as high as 80%[5], meta-analyses show that several prenatal exposures can be linked to worse cognitive outcomes[6,7], illustrating how early environmental factors may shape cognitive development.

Globally, vitamin D deficiency is a widespread problem among pregnant women[8]. Vitamin D contributes to brain development during pregnancy[9], with in vitro and rodent models highlighting its involvement in essential neurodevelopmental processes, including neuronal differentiation, neurotransmitter synthesis, intracellular calcium signaling, and antioxidant activity[10,11]. The distribution of vitamin D receptors and 1-a-hydroxylase in the human brain further supports its important role in neurodevelopment[12]. Moreover, experimental rat models demonstrate associations between vitamin D deficiency and cognitive functions, including learning and memory impairments[13,14].

Prenatal vitamin D deficiency has been linked to neuropsychiatric disorders such as autism spectrum disorder (ASD)[15,16], attention-deficit/hyperactivity disorder (ADHD)[17,18], and schizophrenia[19]. These disorders are associated with impairments in several cognitive domains including attention and executive functioning[20–22].

In two previous studies we have examined the impact of prenatal vitamin D supplementation in the $COPSAC_{2010}$ cohort; Sass et al.[23] found no effect on offspring neurodevelopment from birth to 6 years of age, and Aagaard et al.[24] reported no effect on neurodevelopmental disorders at age 10, but hitherto we have not investigated a potential effect on cognitive function at this age.

Studies examining gestational vitamin D levels and cognitive functioning in the offspring have reported

inconsistent findings. Existing observational studies differ in exposure and outcome measurements. Exposure measurements have varied between first[25–30], second[25,30–35], third trimester[25,29–31,36–40] and cord blood[25,30–32,34,36,41–44] levels of 25(OH)D. Outcome measurements varied in age at testing, cognitive function assessed, and types of tests used. Positive associations have been reported for language skills[25,33,40,45], gross motor skills[25,27,44,45], executive functioning[28] and intelligence[31,46]. Two meta-analyses of observational studies found positive associations between maternal vitamin D during pregnancy and offspring cognitive abilities as well as fewer ADHD and autistic traits[47,48]. To date, only one other RCT has examined the role of vitamin D supplementation during pregnancy on offspring cognition. The study reported a positive effect of 2000 IU/day supplementation from 12-16 weeks until delivery on receptive and expressive language at age 3-5 years, though it was limited by a relatively small sample size (n=156)[49]. To our knowledge, no RCT has investigated the effect of prenatal vitamin D supplementation on offspring cognition in middle childhood.

In this post-hoc analysis of an RCT we aimed to investigate the hypothesis that high-dose compared to standard-dose vitamin D3 supplementation during pregnancy has a positive effect on test-based cognitive performance evaluated at age 10 as part of the COpenhagen Prospective Study on Neuro-PSYCHiatric Development (COPSYCH) project[50].

METHODS

The COPSYCH study consists of cognitive and psychopathological assessments of the $COPSAC_{2010}$ cohort, comprising 700 mother-child pairs. The cohort was powered according to the original primary outcome of recurrent wheeze[51]. Pregnant women from Zealand, Denmark, were enrolled at 22-26 weeks gestation from 2008-2010. Exclusion criteria were daily intake of vitamin D >600 IU, chronic heart, kidney or endocrine disease and insufficient Danish language proficiency. Children were followed from

birth to age 10 with a minimum of 14 visits at the COPSAC clinic. Additional visits were conducted if the child experienced respiratory, allergy, or skin symptoms[51] (*supplement 1*).

Study intervention

In total, 623 pregnant women were randomized 1:1 from pregnancy week 24 to 1 week postpartum to receive either high-dose vitamin D3 supplementation of 2400 IU daily in addition to the recommended 400 IU, or standard-dose vitamin D3 of 400 IU daily as advised by the Danish National Board of Health. The participants were unblinded when all children had reached three years of age. Adherence was determined by counting returned capsules. The intervention had a 2x2 factorial trial design. In addition to vitamin D3 the participants were randomized to receive a daily fish oil (n-3-LCPUFA) supplement or olive oil capsules from week 24 to 1 week postpartum[51].

The study compiled with the Declaration of Helsinki and was approved by the Local Ethics Committee (H-B-2008-093), and the Danish Data Protection Agency (2015-41-3696). Both parents gave written informed consent before enrolment.

Vitamin D measurement

Maternal 25(OH)D levels were measured at week 24 of pregnancy (pre-intervention) and 1 week postpartum (post-intervention). Child levels were measured at 6 months and 6 years of age[24].

Outcome measures

The outcome was assessed with neuropsychological tests administered at the COPYSCH 10-year visit which was a post-hoc follow-up. The visit was carried out between January 2019 and December 2021 and extended over two days. Day one consisted of a neurocognitive and neuropsychiatric assessment, and day two of a brain MRI scan[50].

The neurocognitive test battery included subtests from several cognitive tests. Completion of the test battery took approximately two hours, with breaks provided when needed[50]. The test battery was administered by trained professionals. Description of all included tests can be found in *online methods*.

The cognitive domains are described in the COPSYCH protocol[50]. We included eight distinct cognitive domains encompassing eleven functions (*Figure 1).*

Seven subtests from CANTAB[52] were included. Rapid Visual Information Processing[53] tested sustained attention. Reaction Time[53] tested reaction time and motor speed. Paired Associates Learning[53] tested visual memory. Intra-Extra Dimensional Set Shift[53] tested flexibility/set shift, Stockings of Cambridge[53] tested planning and Spatial Working Memory[53]. Two subtests from Test of Memory and Learning 2nd Edition, Word Selective Reminding[54], and Object Recall[54], both tested verbal memory. Six subtests from WISC-IV were included. Coding[55] and Symbol Search[55] both tested speed of processing. Digit Span[55] and Letter-Number Sequencing[55] tested verbal working memory. Vocabulary[55] and Matrices[55] were used to estimate level of intelligence[56] (*Table S1*).

All raw scores were standardized into z-scores and, when necessary, direction was reversed to ensure that higher scores indicate better performance. Normal distribution was observed in most cognitive functions, except for visual memory (left-skewed), flexibility/set shift (bimodal) and spatial working memory (left-skewed).

Statistical methods

In the main analyses we estimated the effect of high-dose vs standard-dose vitamin D3 on the eleven cognitive functions using linear regression. Analyses were performed crude and adjusted for child sex and age at the COPSYCH visit, season of birth, pre-intervention 25(OH)D levels and the n-3-LCPUFA intervention.

As a secondary analysis we estimated the association between pre-intervention 25(OH)D levels and cognitive functions using crude and adjusted linear regression. 25(OH)D concentrations were divided by a factor of 10, so estimates reflected the change per 10 nmol/L increase of 25(OH)D. A directed acyclic graph guided covariate selection, based on known influencers of 25(OH)D levels and cognition (*Figure S1)*. In adjusted analyses we added sex, season of measurement, gestational diabetes mellitus, preeclampsia, smoking during pregnancy, alcohol during pregnancy, maternal education, and household income as factor variables. Birthweight, gestational age, maternal pre-pregnancy BMI, maternal pregnancy inflammation (IL6, CRP), maternal pregnancy diet[57], maternal age, paternal age, and age at COPSYCH visit were added as continuous variables.

As sensitivity analyses, we tested interaction between the vitamin D3 intervention and pre-intervention 25(OH)D levels by adding cross-product terms to the linear regression models. Likewise, we tested interaction with sex based on known sex differences in neurodevelopment[58], and interaction with child 6 month and 6 year 25(OH)D levels based on the hypothesis that higher childhood vitamin D status may enhance the effect of prenatal supplementation. Second, to explore whether potential supplementation effects were driven by underlying psychopathology, we repeated the analyses separately among children with and without an ADHD diagnosis. Finally, we tested each individual subtest, to identify those driving the observed effects on the cognitive functions.

Statistical significance was set as <0.05, two-sided. Missing data was not imputed. Analyses were performed using R 4.3.1. The primary results of the RCT were adjusted using FDR at 5% *(supplement 2)*.

RESULTS

Baseline characteristics

Of the 700 mother-child pairs enrolled in the $COPSAC_{2010}$ cohort, 623 were randomized to receive high-dose or standard-dose vitamin D3. In total, 498 children participated in both the COPSYCH visit and the

prenatal vitamin D3 trial. Of these, 251 had been prenatally exposed to placebo and 247 to high-dose vitamin D3 (*Figure 1*).

Baseline characteristics of the included mother-child pairs are outlined in *Table S2* and descriptive statistics of cognitive test scores in *Table S3*. There were no significant differences in pre-intervention 25(OH)D levels or season of birth between the supplementation and placebo group. Overall mean pre-intervention levels were 75.8 nmol/L [25.6] with 14.9% having levels under 50 nmol/L. Post-intervention 25(OH)D levels between the two groups were *placebo: 71.7 nmol/L [31.6] vs supplementation: 108.2 [35.4]*. The safety profile of the intervention has been published previously[59].

In the secondary analyses of the week 24 levels, we also included mothers from the cohort who did not participate in the vitamin D3 trial (N=90). In this combined group mean 25(OH)D levels at pregnancy week 24 were 75.7 nmol/L [25.2].

High-dose vitamin D3 supplementation and cognitive outcomes

Mean (SD) estimated intelligence score for offspring at 10 years was 107.6 (15) and 107.8 (13.2) for the vitamin D and placebo group, respectively.

Of the eleven cognitive functions, vitamin D3 supplementation was positively associated with verbal memory (*β=0.17 SD)*, visual memory (*β=0.24 SD)* and flexibility/set-shift (*β=0.19 SD)* after covariate adjustment but did not pass multiple test correction. No significant differences were found between the vitamin D3 supplementation and placebo group for the remaining eight cognitive functions *(Table 1*).

Pre-intervention 25(OH)D levels and cognitive outcomes

The crude analyses showed no association between pre-intervention 25(OH)D levels and cognitive functions. After adjustment for a priori chosen potential confounders, only flexibility/set-shift showed a positive association *(β per 10 nmol/L = 0.05),* however this result did not remain significant after FDR correction (*Table 2*).

Sensitivity analyses

Interaction analyses assessing whether sex, pre-intervention 25(OH)D or child levels at 6 months and 6 years modified the effect of the vitamin D3 supplementation revealed no significant interactions on verbal memory, visual memory or flexibility/set shift (*Table S4)*.

Based on the a priori hypothesis that the effect of vitamin D3 supplementation on cognitive function would differ by ADHD, we examined the effects stratified by ADHD diagnosis (*Figure 3*). Significant associations were found among children without ADHD in both verbal memory *(β=0.18 SD, 95% CI: 0.03-0.33, p=0.023)* and visual memory *(β=0.28 SD, 0.09-0.48, p=0.005),* but the interaction analysis was not significant (*Table S5*). Removing children with autism in these analyses did not alter results.

Of the 17 individual cognitive tests which made up the cognitive functions/domains, the vitamin D3 supplementation had a positive association with Paired Associates Learning (total errors) *(β=0.25 SD, 0.07-0.44, p=0.01)* and Intra Extra Dimensional Set Shift (ED stage errors) *(β=0.2 SD, 0.02-0.38, p=0.03)* in the crude analyses. These remained significant in the adjusted analyses along with Word Selective Reminding (total number recalled) (*β=0.2 SD, 0.03-0.37, p=0.02*). The remaining 14 tests were not significantly associated with the vitamin D supplementation (*Table S6)*. However, neither of the associations were significant after FDR correction.

DISCUSSION

In this post-hoc analysis of a RCT, we explored the long-term impact of high-dose vs standard-dose vitamin D3 supplementation during pregnancy on cognitive functions in children at 10 years of age. We found indication that high-dose supplementation had a positive effect on 3 of 11 functions assessed, which were verbal memory, visual memory, and flexibility/set shift. However, these results were not significant after correction for multiple testing. Observationally, serum 25(OH)D in pregnancy was only

associated with a marginally better flexibility/set shift. Together, these findings support the hypothesis that prenatal vitamin D3 exposure may have an effect on a subset of cognitive functions in childhood.

Interpretation

Although a previous analysis of this RCT reported no significant effect of prenatal vitamin D3 supplementation on cognition at 2.5 years using the Bayley Scales[23], our current findings at 10 years could indicate that the effects of vitamin D3 supplementation on a subset of cognitive functions may become measurable later in childhood. This is supported by literature on cognitive development in children which highlights how cognition and particularly executive functions, become increasingly fine-tuned and differentiated throughout childhood[60,61].

The only other published vitamin D prenatal RCT[49] reported a positive effect of 2000 IU/day vitamin D3 supplementation from pregnancy week 12-16 until delivery, on the language component of the Brigance Screen at 3-5 years in the offspring, supporting the hypothesis that prenatal vitamin D3 has a positive effect on cognitive functioning. However, our results did not pass multiple test corrections, however, this may also be an overcorrection as the cognitive functions are intercorrelated.

Our results indicate a potential effect of prenatal vitamin D3 supplementation on the memory domain. To our knowledge, no previous human studies have specifically examined the association between gestational vitamin D status and memory in offspring. This finding is supported by one existing rat study[62] reporting a positive association between maternal vitamin D deficiency and impaired memory. A cross-sectional study in adolescents found that higher serum levels of 25(OH)D at age 9-13 were associated with better performance on visual, but not verbal, memory[63].

To investigate the influence of neurodevelopmental disorders, we tested the supplementation effect in children with and without ADHD. The signal on visual and verbal memory persisted when restricting the

analyses to children without the diagnosis, indicating that potential effects are not driven by children with ADHD. However, no significant interaction between the vitamin D3 supplementation and ADHD status was observed. A recent large Danish study reported inverse associations between neonatal 25(OH)D levels and risk of ADHD, ASD, and schizophrenia[64]. However, our previous study of the vitamin D RCT in the $COPSAC_{2010}$ cohort found no effect of the prenatal supplementation on ADHD diagnosis, though power may have been reduced by the limited number of children with ADHD in the cohort[24]. Interaction analyses between vitamin D3 supplementation and child 6 month and 6 year 25(OH)D levels were non-significant, implying that the observed effect of the supplementation on cognitive functioning is not affected by 25(OH)D levels in early childhood. This suggests that prenatal exposure may represent a critical window during which vitamin D influences cognitive development, supporting the idea of a primarily prenatal programming effect. However, postnatal levels may be influenced by numerous confounding factors and may not accurately reflect long-term vitamin D status, meaning that small postnatal effects cannot be ruled out[65].

A previous Danish prospective cohort study showed a positive association between early pregnancy and cord blood levels of 25(OH)D and intelligence in boys at age 7, measured using WISC-V. Notably, in that cohort, the median cord blood level was below 50 nmol/L[31]. In contrast, we observed no effect on intelligence in our cohort where mean pre-intervention levels were >75 nmol/L, suggesting that associations with intelligence may be more apparent at lower baseline 25(OH)D levels. Several prospective studies have similarly reported a positive association between vitamin D levels during pregnancy and various cognitive functions in childhood, including intelligence, attention and executive functions[25,28,46]. In contrast, other observational studies report no such association with some focusing specifically on IQ[32,35] and others assessing specific cognitive functions[38,41,43].

Analyses of observational data revealed only one modest association between maternal pre-intervention serum 25(OH)D levels and flexibility/set-shift. Several factors may explain the discrepancy between findings from the supplementation analyses and those based on pre-intervention levels. Residual confounding may remain despite adjustment for multiple variables. The relatively high mean pre-intervention 25(OH)D levels in our cohort may have limited our ability to detect associations if effects are more evident at deficient levels. Pre-intervention levels primarily reflect vitamin D status during the first and second trimesters, whereas supplementation from week 24 primarily influences levels during the third trimester. The discrepancy may partly be explained by differences in timing of exposure, as brain regions follow distinct developmental trajectories. The latter half of pregnancy is a period of rapid cortical maturation which may be particularly sensitive to vitamin D[66,67].

Strengths and limitations

Few RCTs have examined the effect of vitamin D3 supplementation during pregnancy on offspring cognitive functions[23,49]. To our knowledge, this is the first RCT to assess cognitive functions in children beyond 5 years of age using performance-based outcomes. Strengths of this study include the large sample size and the deep phenotyping of children enabled by their participation in the $COPSAC_{2010}$ cohort. Furthermore, the cognitive test battery consisted solely of performance-based subtests covering a broad range of cognitive functions. ADHD diagnoses were based on clinical interviews[50].

This study also has several limitations. It is a post-hoc analysis of an RCT that was not pre-specified, thus increasing the risk of spurious findings. The high pre-interventional mean 25(OH)D level limits our ability to assess potential benefits in participants with low vitamin D status. The small number of children with ADHD reduces the power to detect subgroup-specific effects. Moreover, due to the timing of the supplementation, we only have the opportunity to examine the potential effect of high-dose supplementation in late pregnancy. In the observational analyses residual confounding cannot be ruled

out. We lack data on parental intelligence and psychopathology, both of which are heritable[68–70]. However, we do have data on income and educational level which are known to be associated with intelligence[71] and adjusting for these factors did not substantially modify the results. Finally, the generalizability of the study is limited by the predominance of white participants with a high mean 25(OH)D.

CONCLUSION

This post-hoc analysis of an RCT indicates that high dose vitamin D3 supplementation from week 24 of pregnancy to 1 week postpartum has a positive effect on visual memory, verbal memory and flexibility/set shift in the offspring at age 10 when compared to standard-dose. Importantly, none of these effects were significant after FDR-correction, and research is still warranted to clarify the potential positive effect of vitamin D3 supplementation on cognitive performance.

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ChatGPT 5 has been used in the creation of figures by providing help with R code.

AUTHOR CONTRIBUTIONS

Author responsibilities were as follows; OFF drafted the manuscript. All co-authors (JRMJ, NB, RV, JBR, PM, MHL, AMS, NV, BYG, BF, NB, KB, BHE, KA, BC) have provided important intellectual input and contributed considerably to the analyses and interpretation of the data. All authors guarantee that the accuracy and integrity of any part of the work have been appropriately investigated and resolved,

and all have approved the final version of the manuscript. The corresponding author had full access to the data and had final responsibility for the decision to submit for publication. No honorarium, grant, or other form of payment was given to any of the authors to produce this manuscript.

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CONFLICT OF INTEREST

BE is part of the Advisory Board of Boehringer Ingelheim, Lundbeck Pharma A/S, and Orion Pharma A/S; and has received lecture fees from Boehringer Ingelheim, Otsuka Pharma Scandinavia AB, and Lundbeck Pharma A/S. ORCID: 0000-0002-2590-5055

All other authors report no conflicts of interest.

GOVERNANCE

We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. Privacy is important to us which is why we follow

national and international legislation on General Data Protection Regulation (GDPR), the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

DATA SHARING STATEMENT

Individual-level personally identifiable clinical data from the children participating in the cohort cannot be made freely available, to protect the privacy of the participants and their families, in accordance with the Danish Data Protection Act and European Regulation 2016/679 of the European Parliament and of the Council (GDPR) that prohibit distribution even in pseudo-anonymized form. However, research collaborations are welcome, and data can be made available under a joint research collaboration by contacting COPSAC. Requests will be answered within two weeks. Data use is restricted to purposes within childhood health and disease.

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Table 1. Effect of high-dose vs standard-dose vitamin D3 supplementation during pregnancy on the cognitive functions at age 10

Function	N	Estimate [CI]	p-value	N	Estimate [CI], adjusted[1]	p-value	FDR p-value[2]
Estimated Intelligence	495	-0.01 [-0.19;0.16]	0.897	491	-0.01 [-0.19;0.16]	0.892	0.980
Speed of Processing	498	-0.04 [-0.19;0.12]	0.654	494	-0.02 [-0.17;0.13]	0.807	0.980
Reaction Time	496	0.12 [-0.05;0.28]	0.166	492	0.11 [-0.05;0.28]	0.183	0.335
Sustained Attention	495	-0.03 [-0.21;0.15]	0.766	491	-0.03 [-0.21;0.16]	0.785	0.980
Motor Speed	496	0.10 [-0.07;0.27]	0.245	492	0.09 [-0.08;0.25]	0.312	0.490
Verbal Memory	497	0.14 [-0.00;0.29]	0.058	493	0.17 [0.03;0.32]	**0.021**	0.116
Verbal Working Memory	498	-0.11 [-0.25;0.04]	0.159	494	-0.01 [-0.25;0.04]	0.173	0.335
Visual Memory	497	0.25 [0.07;0.44]	**0.007**	493	0.24 [0.06;0.42]	**0.011**	0.116
Flexibility/Set Shift	495	0.20 [0.02;0.38]	**0.027**	491	0.19 [0.01;0.37]	**0.035**	0.128
Spatial Working Memory	498	-0.11 [-0.29;0.06]	0.201	494	-0.12 [-0.30;0.06]	0.180	0.335
Planning	497	0 [-0.18;0.17]	0.959	493	0 [-0.17;0.17]	0.980	0.980

[1]Adjusted for sex, age at testing, n-3 LCPUFA intervention, season of birth and pre-interventional 25(OH)D level
[2]Benjamini-Hochberg false discovery rate (5%) applied across 11 tests; $p<0.05$ considered significant

Table 2. Associations of maternal 25(OH)D levels at week 24 and offspring cognitive functions at age 10

Function	N	Estimate [CI]	p-value	N	Estimate [CI], adjusted[1]	p-value	FDR p-value[2]
Estimated Intelligence	584	0 [-0.03;0.03]	0.925	483	-0.02 [-0.06;0.02]	0.355	0.987
Speed of Processing	587	0 [-0.03;0.03]	0.833	487	0 [-0.03;0.04]	0.650	0.987
Reaction Time	585	-0.01 [-0.04;0.02]	0.537	484	0.01 [-0.03;0.04]	0.924	0.987
Sustained Attention	582	-0.01 [-0.04;0.03]	0.644	481	0 [-0.04;0.04]	0.855	0.987
Motor Speed	585	0.01 [-0.02;0.04]	0.548	484	0.03 [-0.01;0.07]	0.330	0.987
Verbal Memory	587	0.01 [-0.02;0.04]	0.522	486	-0.01 [-0.04;0.03]	0.936	0.987
Verbal Working Memory	588	0.02 [-0.01;0.05]	0.164	487	0 [-0.03;0.04]	0.702	0.987
Visual Memory	586	0.01 [-0.02;0.04]	0.517	485	0 [-0.04;0.04]	0.674	0.987
Flexibility/Set Shift	584	0.03 [-0.01;0.06]	0.110	483	0.05 [0.01;0.09]	**0.026**	0.286
Spatial Working Memory	587	0 [-0.03;0.03]	0.958	486	0 [-0.04;0.04]	0.987	0.987
Planning	586	0 [-0.03;0.04]	0.788	485	-0.01 [-0.05;0.03]	0.828	0.987

[1]Adjusted for sex, birthweight, gestational age, maternal pre-pregnancy BMI, season of week 24 measurement, gestational diabetes, preeclampsia, smoking during pregnancy, alcohol during pregnancy, maternal pregnancy IL6 and CRP, maternal education, household income, diet, maternal age, paternal age and child age at COPSYCH visit

[2]Benjamini-Hochberg false discovery rate (5%) applied across 11 tests; $p<0.05$ considered significant

Figure 1: $COPSAC_{2010}$ and COPSYCH measures[72]

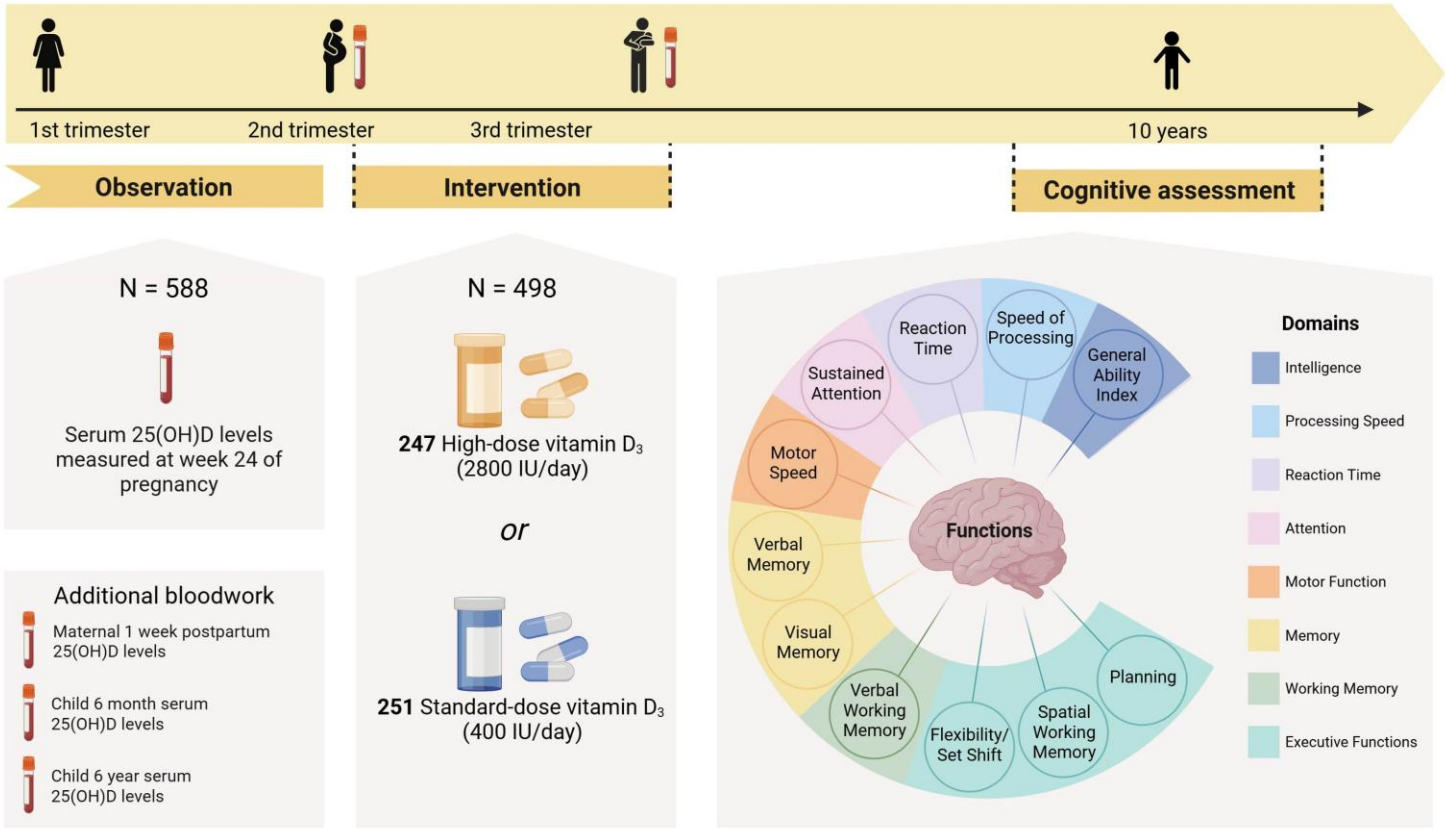


Figure 2. CONSORT flow diagram

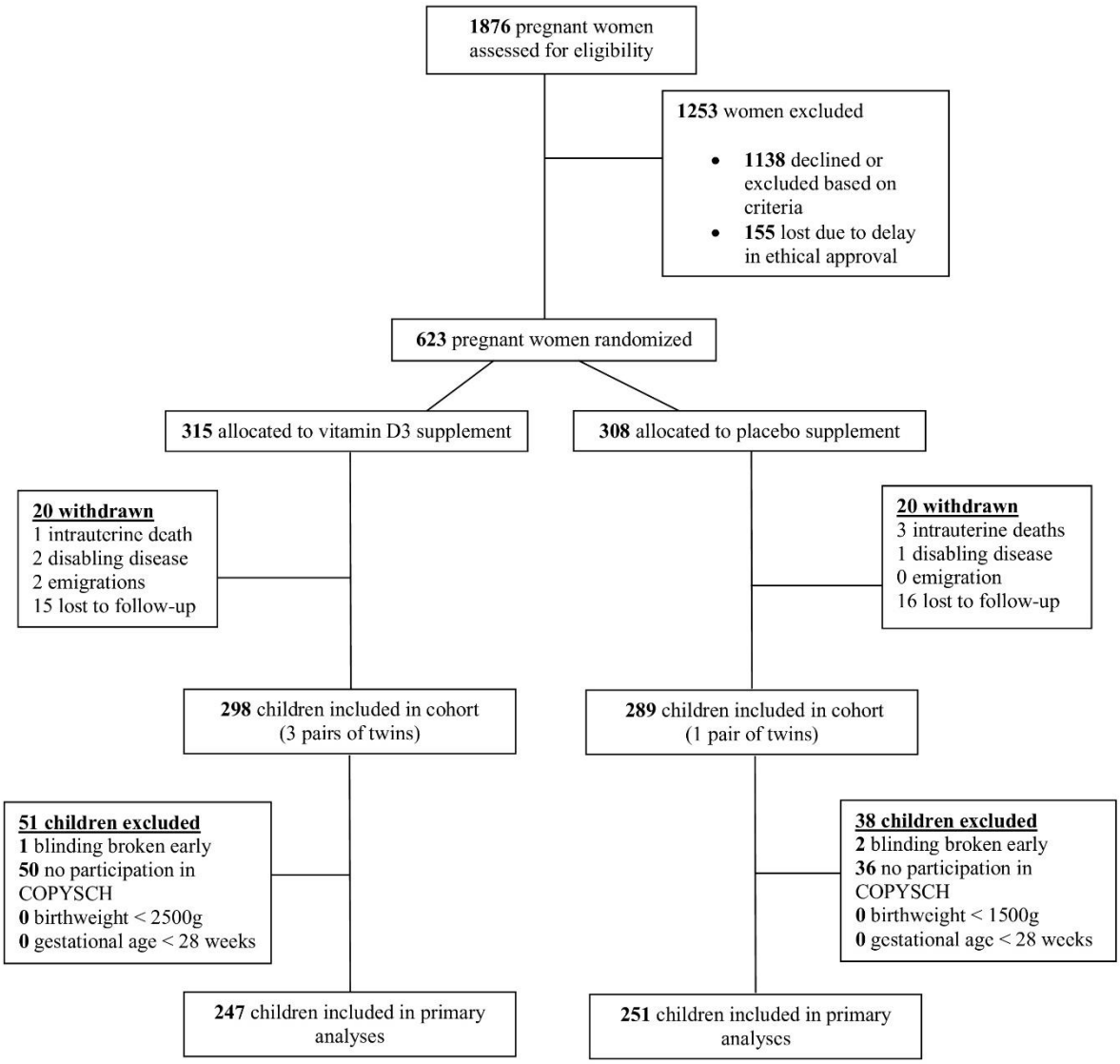
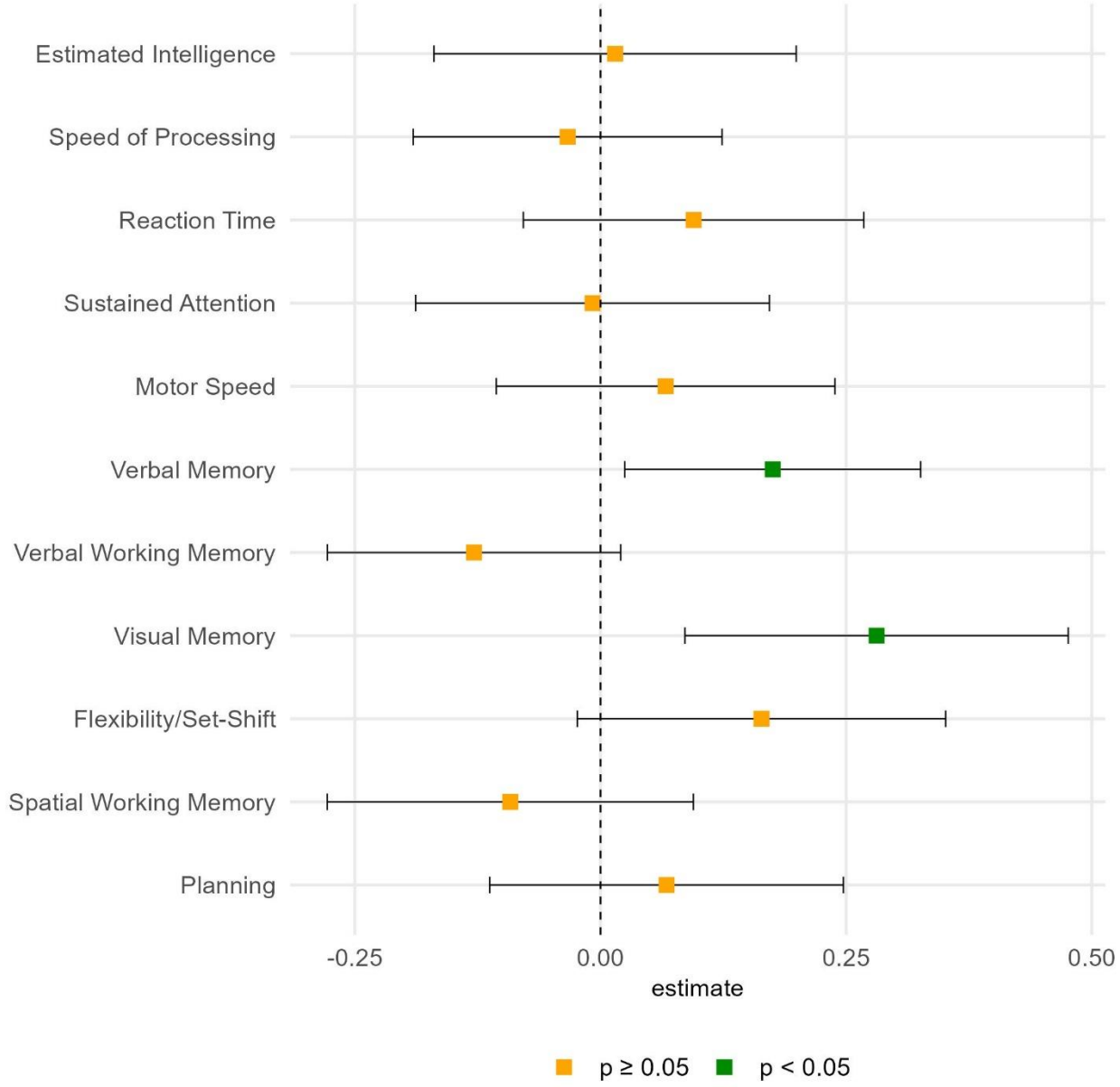


Figure 3. Effect of high-dose vs standard-dose vitamin D3 supplementation during pregnancy on the cognitive functions at age 10 in children without an ADHD diagnosis



Adjusted for sex, age at COPYSCH visit, n-3 LCPUFA intervention, season of birth and 25(OH)D level at week 24 of pregnancy.
Statistical significance is reported according to nominal p-values, all FDR p-values above threshold of 0.05.

Supplemental Online Content

eAppendix: Supplemental Methods

Study intervention
The randomization was performed at the Pharmacy of Glostrup by a computer-generated list of random numbers. The randomization was performed by an external investigator with no additional involvement in the RCT.

Serum measures of 25(OH)D
One serum 25(OH)D measure of 0.0 was corrected to half of the lowest observed value of 25(OH)D.

The obtainment of serum vitamin D measures has been described previously in Sass et al[1]: *"The venous blood samples obtained before and after the intervention were centrifuged for 10min at 4300rpm to separate serum, and thereafter frozen at -80oC until analysis. The serum samples were transported on dry ice for duplicate analyses for 25-hydroxyvitamin D2(25(OH)-Vitamin D2) and 25(OH)-Vitamin D3 at the Dept. of Clinical Biochemistry, Aarhus University Hospital, Denmark. Serum 25-hydroxyvitamin D levels were analysed by isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS)[2,3]. Calibrators traceable to NIST SRM 972 (Chromsystems, DE) were used."*

Outcome measures
Details on inter-rater reliability of agreement is published here: https://doi.org/10.1016/j.psychres.2023.115481

The cognitive domains described in the COPSYCH protocol were refined post hoc to reduce the number of outcomes. When possible, we chose to prioritize the automated Cambridge Neuropsychological Test Battery (CANTAB) tests over paper-and-pencil tests in cases where both tests assessed the same cognitive function. We also prioritized Wechsler Intelligence Scale for Children - fourth edition (WISC-IV) measures as these are well-validated and widely used in previous literature[4–6].

Pearson correlation analyses were performed for all functions that were indexed by more than one test outcome to determine whether test scores could be aggregated into a single composite score (e*Figure 2)*. An acceptable correlation threshold was set to ± 0.4. When two tests did not meet the threshold, one was excluded. This applied to only the spatial working memory function that was indexed by Spatial Working Memory task (SWM) and Spatial Span task (SS). We included SWM rather than SS as literature has shown it is a more effective measure of executive function[7].
The estimated General Ability Index (GAI) from the WISC-IV estimated overall intelligence based on verbal comprehension (Vocabulary) and perceptual reasoning (Matrices) indices. Each subtest outcome score was age-corrected and multiplied by three, acting as a proxy for the absent tests in its respective category, and their sum was converted to GAI score using the General Ability Index Conversion Table with Danish Norms[8].

In total, seven computer-based subtests were included from CANTAB[9]. Rapid Visual Information Processing[10] assessed sustained attention with a working memory component. The child had to watch a stream of moving numbers and respond whenever a specific three-number sequence appeared. Reaction Time[10] consisted of a screen with one (simple mode) or five circles (five-choice mode). The child had to select a circle when it

changed color, movement time was a measure of motor speed, while time to react (i.e. time to lift finger from screen) assessed reaction time. Paired Associates Learning[10] assessed visual memory. Boxes on the screen were automatically opened and one or more contained a pattern, after closing the child chose the box where a specific pattern was located. The outcome was adjusted to include scores for all potential rounds, allowing comparison regardless of how many rounds the child completed.

The following CANTAB tests assessed executive functioning; Intra-Extra Dimensional Set Shift[10] measured the ability to shift attention by requiring the child to choose between patterns, learning which is correct through feedback. In the process rule changes shift to a new pattern (extra-dimensional), where the outcome was extra-dimensional stage errors. Spatial Working Memory[10] required the child to find hidden tokens in boxes, while avoiding previously searched boxes, as each box held only one token. Stockings of Cambridge[10] measured planning by having the child recreate a pattern of balls by moving one at a time and using as few moves as possible. For detailed information on all the included tests from CANTAB see official website: https://cambridgecognition.com/

The Word Selective Reminding and Object Recall subtests from Test of Memory and Learning (TOMAL-2) both assessed verbal memory. For Word Selective Reminding[11] the examiner read 12 words aloud which the child was asked to recall. If they forgot one or more words, the examiner provided reminders. The child completed six trials. In Object Recall[11], pictures of objects were shown and there were no reminders. The child completed five trials.

Six subtests from WISC-IV were included. Coding[12] and Symbol Search[12] both measured speed of processing. Digit Span[12] and Letter-Number Sequencing[12] assessed verbal working memory. Vocabulary[12] and Matrices[12] estimated intelligence.

Statistical methods

Statistical variables were expressed as mean and standard deviation (SD) for normally distributed variables or median and interquartile range (IQR) for skewed data. Categorical variables were reported as numbers and percentages.

The main analyses were adjusted for sex and age at COPSYCH visit as the scores from the cognitive test battery are raw and unadjusted.

For the secondary analyses we constructed a directed acyclic graph to determine appropriate covariates a priori, based on known influencers of 25(OH)D levels and cognition[13–20] (*figure S2)*.

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eTable 1: Overview of domains, functions, tests and outcome metrics in the neurocognitive test battery

Domain	Function	Test	Outcome metric
Intelligence	Estimated intelligence	Vocabulary (WISC-IV)	Total number correct
		Matrices (WICV-IV)	Total number correct
Processing speed	Speed of processing	Coding (WISC-IV)	Total number correct
		Symbol search (WISC-IV)	Sum of total number correct. errors subtracted
Reaction time	Reaction time	Reaction time (CANTAB)	Simple- and five-choice reaction time
Attention	Sustained attention	Rapid visual information processing (CANTAB)	A-prime (unitless sensitivity score)
Motor function	Motor speed	Reaction time (CANTAB)	Simple- and five-choice movement time
Memory	Verbal memory	Word selective reminding - immediate recall (TOMAL 2)	Total number words recalled over six learning trials
		Object recall (TOMAL 2)	Total number object recalled over five learning trials
	Visual memory	Paired associates learning (CANTAB)	Total errors (adjusted)
Working memory	Verbal working memory	Digit span (WISC-IV)	Total number correct forward and backward sequencing
		Letter-number sequencing (WISC-IV)	Total number correct sequences
Executive function	Flexibility/Set shift	Intra-extra dimensional set shift (CANTAB)	Extra-dimensional stage errors
	Spatial Working Memory	Spatial working memory (CANTAB)	Total errors
	Planning	Stockings of cambridge (CANTAB)	Problems solved in minimum moves

CANTAB; Cambridge Neuropsychological Test Automated Battery
WISC-IV; Wechsler Intelligence Scale for Children - fourth edition
TOMAL 2; Test of Memory and Learning - second edition

eTable 2: Baseline characteristics of participants included in the RCT and the COPSYCH project

	Overall N = 498	**Vitamin D** N = 247	**Placebo** N = 251
Maternal BMI, mean (SD)	24.7 (4.6)	24.5 (4.6)	24.9 (4.5)
Maternal age, y, mean (SD)	32.4 (4.3)	32.7 (4.4)	32.0 (4.2)
Pre-interventional 25(OH)D level, nmol, mean (SD)	75.8 (25.6)	76.0 (25.9)	75.5 (25.4)
Pre-interventional 25(OH)D level, >75 nmol/L, N (%)	253 (51.2)	128 (52.2)	125 (50.2)
Post-interventional 25(OH)D level, nmol, mean (SD)	89.8 (38.1)	108.2 (35.4)	71.7 (31.6)
Post-interventional 25(OH)D level, >75 nmol/L, N (%)	313 (63.7)	205 (84.0)	108 (43.7)
Unhealthy diet PC, mean (SD)[1]	0.04 (1.01)	-0.04 (0.94)	0.1 (1.1)
Maternal IL6, median [IQR]	0.3 [0.2, 0.4]	0.3 [0.2, 0.4]	0.3 [0.2, 0.4]
Maternal CRP, median [IQR]	5.1 [2.5, 10.2]	5.2 [2.6, 10.0]	5.1 [2.5, 10.3]
Maternal education			
Elementary	18 (3.6)	7 (2.8)	11 (4.4)
High School	23 (4.6)	10 (4.0)	13 (5.2)
Tradesman	100 (20.1)	42 (17.0)	58 (23.1)
Bachelor's degree	215 (43.2)	110 (44.5)	105 (41.8)
Master's degree	142 (28.5)	78 (31.6)	64 (25.5)
Household income, N (%)[2]			
<100.000 DKK	44 (8.8)	21 (8.5)	23 (9.2)
100.000-150.000 DKK	105 (21.1)	48 (19.4)	57 (22.7)
150.000-200.000 DKK	154 (30.9)	76 (30.8)	78 (31.1)
200.000-250.000 DKK	121 (24.3)	61 (24.7)	60 (23.9)
>250.000 DKK	74 (14.9)	41 (16.6)	33 (13.1)
Alcohol use during pregnancy, yes, N (%)[3]	81 (16.3)	42 (17.1)	39 (15.5)
Smoking during pregnancy, yes, N (%)[3]	37 (7.4)	16 (6.5)	21 (8.4)
Parity, N (%)			
1	221 (44.4)	97 (39.3)	124 (49.4)
2	198 (39.8)	107 (43.3)	91 (36.3)
>2	79 (15.9)	43 (17.4)	36 (14.3)
n3-LCPUFA intervention, yes, N(%)	249 (50.0)	127 (51.4)	122 (48.6)
Preeclampsia, yes, N (%)	25 (5.0)	13 (5.3)	12 (4.8)
Antibiotics during pregnancy, yes, N (%)	171 (34.4)	82 (33.3)	89 (35.5)
Gestational diabetes mellitus, yes, N (%)	7 (1.4)	2 (0.8)	5 (2.0)
Paternal age, y, mean (SD)	34.6 (5.2)	35.0 (5.2)	34.3 (5.2)
Paternal education, N (%)			
Elementary	22 (4.5)	7 (2.9)	15 (6.1)
High School	25 (5.2)	13 (5.4)	12 (4.9)
Tradesman	150 (30.9)	69 (28.9)	81 (32.9)
Bachelor's degree	146 (30.1)	76 (31.8)	70 (28.5)
Master's degree	142 (29.3)	74 (31.0)	68 (27.6)
Birthweight, kg, mean (SD)	3.55 (0.53)	3.56 (0.55)	3.53 (0.50)
Gestational age at birth, days, mean (SD)	279.4 (10.9)	279.5 (11.5)	279.4 (10.3)
Sex, female, N (%)	240 (48)	113 (46)	127 (51)
Season of birth, N (%)			

Winter	180 (36.1)	93 (37.7)	87 (34.7)
Spring	98 (19.7)	47 (19.0)	51 (20.3)
Summer	100 (20.1)	50 (20.2)	50 (19.9)
Fall	120 (24.1)	57 (23.1)	63 (25.1)
Race, white, N (%)	476 (96)	236 (96)	240 (96)
Solely breastfed, days, median [IQR]	122 [60, 151]	122 [56.5, 149.5]	123.00 [64, 151.5]
ADHD, yes, N (%)	58 (11.7)	27 (11.0)	31 (12.4)
ASD, yes, N (%)	12 (2.4)	5 (2.0)	7 (2.8)

Abbreviations: SD, Standard deviation; IQR, interquartile range; N, number; ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder
[1]Unhealthy diet represents a Western dietary pattern identified via PCA in [Horner D, Nature Metabolism, 2025
[2]Income is self-reported combined household income 3 months before birth]
[3]Alcohol and smoking during pregnancy reflects use at any time during pregnancy

eTable 3: Mean values of all tests included in the cognitive test battery from the COPSYCH visit at age 10

	Vitamin D		Placebo		
Test	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**Outcome metric**
Estimated Intelligence	245	107.6 (15)	250	107.8 (13.2)	Index score
Matrices	246	23.5 (3.9)	251	23.4 (3.8)	Total number correct
Vocabulary	246	29.5 (6.3)	250	29.6 (5.6)	Total number correct
Symbol Search	247	23.6 (4.7)	250	23.8 (4.5)	Sum of total number minus errors
Coding	247	37.7 (7.8)	251	37.8 (8)	Total number correct
Reaction Time, simple	246	364.2 (50.3)	249	369.5 (45.9)	milliseconds
Reaction Time, five-choice	247	412.8 (57.6)	249	419.7 (57.2)	milliseconds
Rapid Visual Information Processing	247	0.8 (0.1)	248	0.8 (0.1)	A'
Movement Time, simple	246	219.1 (62.7)	249	223.9 (73)	milliseconds
Movement Time, five-choice	247	240.5 (52.9)	249	246.3 (54.9)	milliseconds
Word Selective Reminding	245	56.9 (7)	250	55.7 (7.4)	Total number recalled
Object Recall	246	51.7 (8.9)	250	50.6 (8.7)	Total number recalled
Paired Associates Learning	247	11.1 (9.8)	250	13.9 (13.1)	Total errors
Letter-Number Sequencing	247	16.5 (2.8)	251	16.8 (2.8)	Total number correct
Digit Span	247	14 (2.5)	251	14.3 (2.4)	Total number correct
Intra Extra Dimensional Set Shift	244	15.4 (10.6)	251	17.5 (10)	Extra-dimensional errors
Spatial Working Memory	247	11.4 (7.9)	251	10.5 (7.5)	Total errors
Stockings of Cambridge	247	7.3 (2)	250	7.3 (1.8)	Problems solved in minimum moves

eTable 4: Interaction analyses of vitamin D3 supplementation with sex, maternal week 24 25(OH)D level and offspring 6 months and 6 year 25(OH)D levels

Function	Week 24[1] p-value	6 month[2] p-value	6 year[3] p-value	Sex p-value
Estimated Intelligence	0.24	0.72	0.34	0.26
Speed of processing	0.79	0.06	0.73	0.20
Reaction time	0.42	0.21	0.43	0.44
Sustained attention	0.55	0.39	0.76	0.92
Motor speed	**0.04**	0.48	0.84	0.98
Verbal memory	0.12	0.51	0.74	0.76
Verbal working memory	0.62	0.66	0.58	1.00
Visual memory	0.92	0.75	0.73	0.72
Flexibility/Set shift	0.30	0.06	0.95	0.57
Spatial Working Memory	**0.02**	0.26	0.10	0.34
Planning	0.18	**0.04**	0.44	0.37

[1]maternal pre-interventional 25(OH)D level
[2]25(OH)D level in offspring at 6 months of age
[3]25(OH)D level in offspring at 6 years of age

eTable 5: Effect of high-dose vs standard-dose vitamin D3 supplementation during pregnancy on the cognitive functions at age 10, split by children with and without an ADHD diagnosis, including p-interaction

	Without ADHD				With ADHD				
Function	**N**	**Estimate [CI]**	**p-value**	**FDR p-value[1]**	**N**	**Estimate [CI]**	**p-value**	**FDR p-value[1]**	**p-interaction[2]**
Estimated Intelligence	433	0.01 [-0.17;0.20]	0.874	0.930	56	-0.22 [-0.77;0.33]	0.428	0.785	0.328
Speed of Processing	435	-0.03 [-0.19;0.12]	0.676	0.826	57	-0.06 [-0.59;0.47]	0.824	0.824	0.913
Reaction Time	435	0.09 [-0.08;0.27]	0.283	0.612	55	0.31 [-0.22;0.83]	0.242	0.665	0.678
Sustained Attention	435	-0.01 [-0.19;0.17]	0.930	0.930	54	-0.11 [-0.82;0.60]	0.764	0.824	0.476
Motor Speed	435	0.07 [-0.11;0.24]	0.450	0.637	55	0.31 [-0.37;0.98]	0.367	0.785	0.580
Verbal Memory	435	0.18 [0.03;0.33]	**0.023**	0.126	56	0.08 [-0.40;0.57]	0.731	0.824	0.809
Verbal Working Memory	435	-0.13 [-0.28;0.02]	0.091	0.250	57	0.08 [-0.45;0.60]	0.772	0.824	0.473
Visual Memory	435	0.28 [0.09;0.48]	**0.005**	0.055	56	-0.07 [-0.70;0.56]	0.819	0.824	0.237
Flexibility/Set shift	434	0.16 [-0.02;0.35]	0.086	0.250	55	0.50 [-0.09;1.10]	0.095	0.522	0.300
Spatial Working Memory	435	-0.09 [-0.28;0.10]	0.334	0.612	57	-0.48 [-1.04;0.07]	0.088	0.522	0.478
Planning	435	0.07 [-0.11;0.25]	0.463	0.637	56	-0.37 [-0.93;0.19]	0.189	0.665	0.073

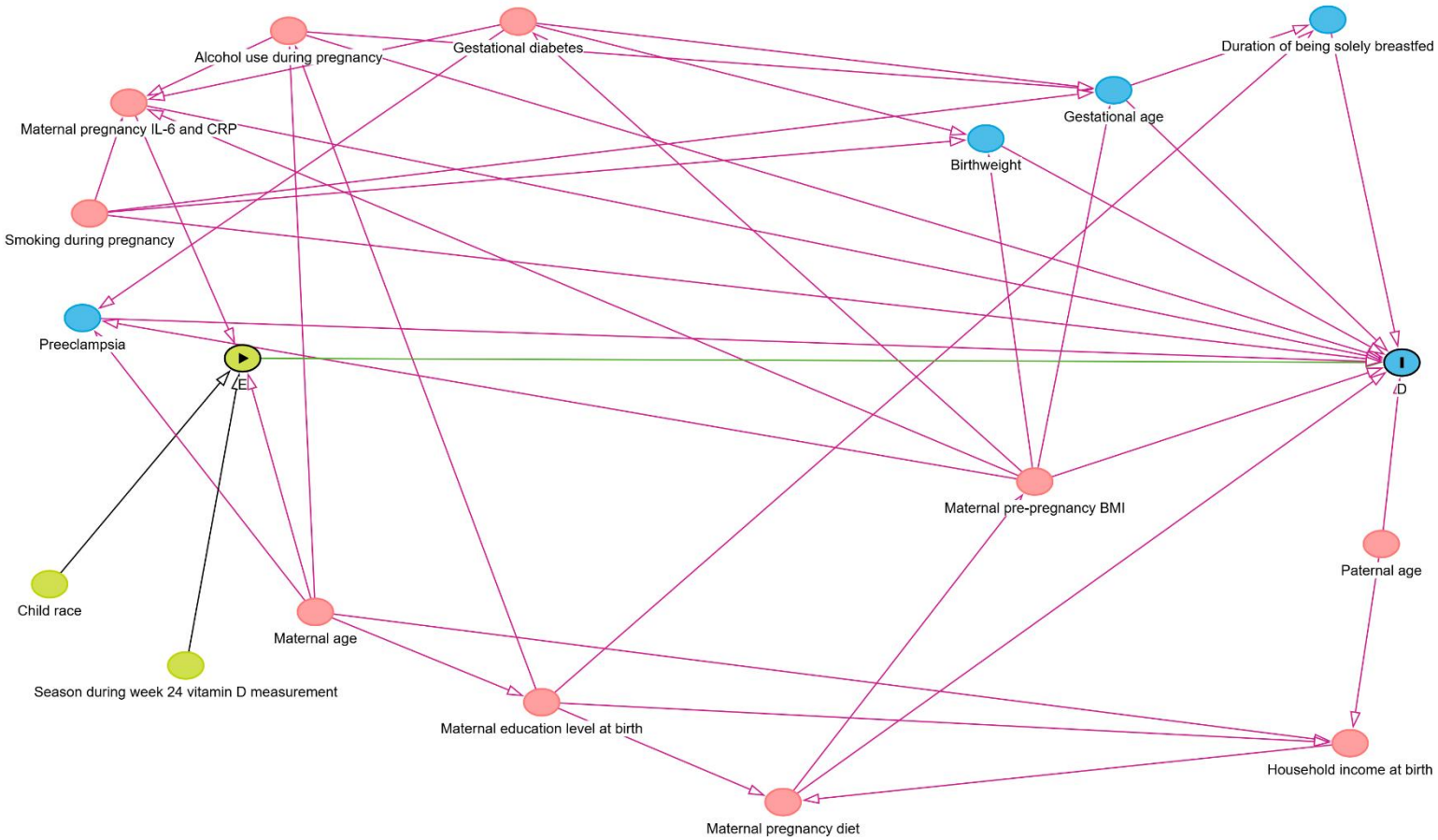
ADHD = attention-deficit/hyperactivity disorder
Results adjusted for sex. age at testing. PUFA intervention. season of birth and pre-interventional 25(OH)D level
[1]Benjamini-Hochberg false discovery rate (5%) applied across 11 tests; $p < 0.05$ considered significant.
[2]interaction analyses from linear regression models of vitamin D3 intervention x ADHD diagnosis

eTable 6: Effect of high-dose vs standard-dose vitamin D3 supplementation on all the tests included in the neurocognitive test battery

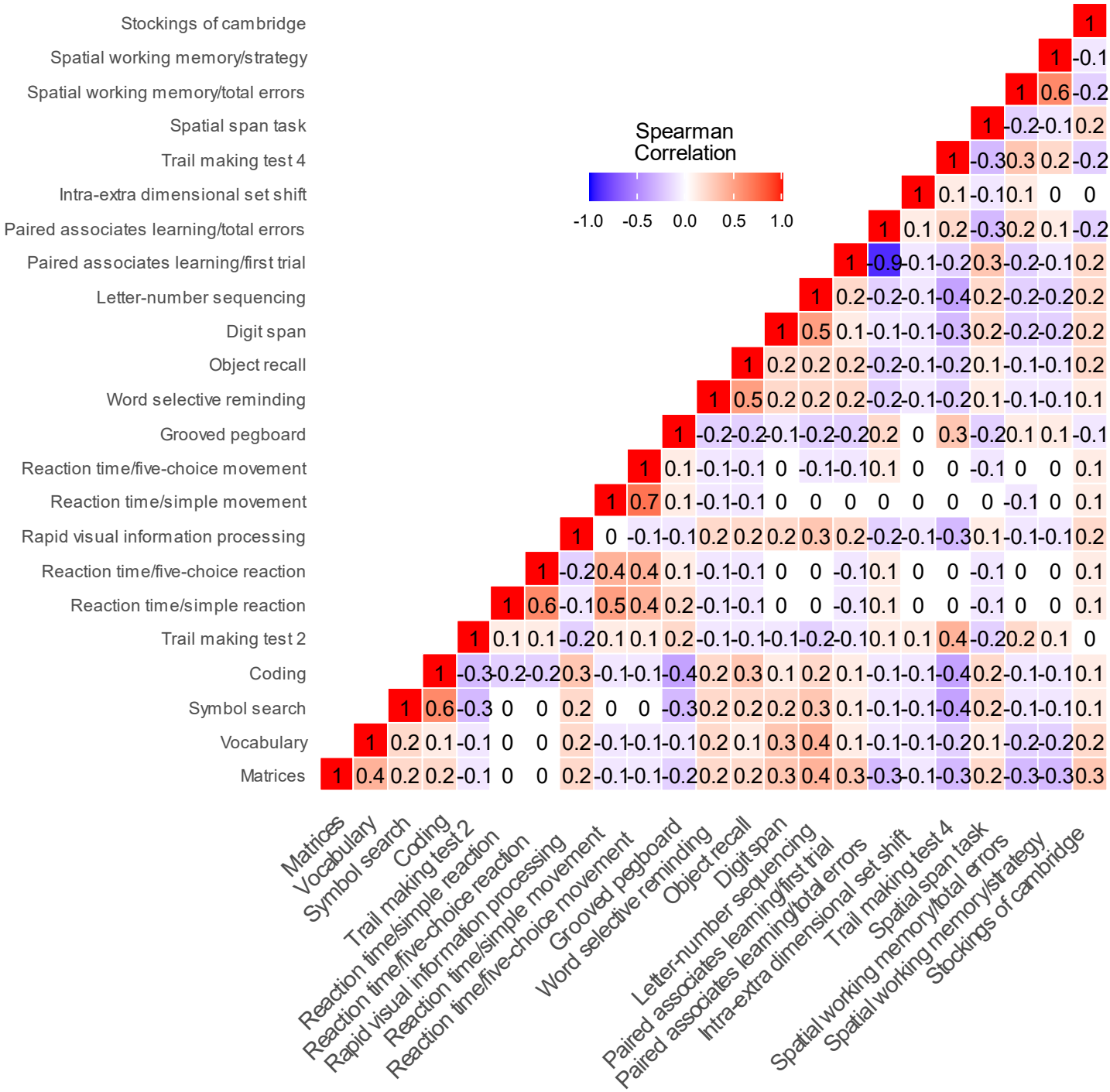
	Crude			Adjusted[1]			
Test	**N**	**Estimate [CI]**	**p-value**	**N**	**Estimate [CI]**	**p-value**	**FDR p-value[2]**
Matrices	497	0.03 [-0.14;0.21]	0.71	493	0.04 [-0.13;0.21]	0.65	0.85
Vocabulary	496	-0.02 [-0.2;0.15]	0.80	492	0.03 [-0.2;0.14]	0.75	0.90
Coding	498	-0.01 [-0.19;0.16]	0.87	494	0.01 [-0.16;0.18]	0.94	0.98
Symbol search	497	-0.05 [-0.23;0.12]	0.55	493	0.04 [-0.22;0.13]	0.64	0.85
Reaction time, simple	495	0.11 [-0.07;0.29]	0.22	491	0.11 [-0.07;0.3]	0.22	0.47
Reaction time, five-choice	496	0.12 [-0.06;0.3]	0.18	492	0.11 [-0.07;0.29]	0.21	0.47
Rapid visual information processing	495	-0.03 [-0.21;0.15]	0.77	491	-0.03 [-0.21;0.16]	0.79	0.90
Movement time, simple	495	0.08 [-0.11;0.26]	0.42	491	0.07 [-0.12;0.25]	0.49	0.76
Movement time, five-choice	496	0.11 [-0.07;0.29]	0.23	492	0.09 [-0.08;0.26]	0.31	0.53
Word selective reminding	495	0.17 [0;0.34]	0.06	491	0.2 [0.03;0.37]	**0.02**	0.17
Object recall	496	0.13 [-0.05;0.3]	0.15	492	0.15 [-0.02;0.31]	0.09	0.38
Paired associates, total errors	497	0.25 [0.07;0.44]	**0.01**	493	0.24 [0.06;0.42]	**0.01**	0.17
Digit span	498	-0.12 [-0.29;0.05]	0.17	494	-0.12 [-0.29;0.06]	0.19	0.47
Letter number sequencing	498	-0.09 [-0.26;0.08]	0.30	494	-0.09 [-0.26;0.08]	0.31	0.53
Intra extra dimensional set shift	495	0.02 [0.02;0.38]	**0.03**	491	0.19 [0.01;0.37]	**0.03**	0.17
Spatial working memory	498	-0.11 [-0.29;0.06]	0.20	494	-0.12 [-0.3;0.06]	0.18	0.47
Stockings of Cambridge	497	0 [-0.18;0.17]	0.96	493	0 [-0.17;0.17]	0.98	0.98

All estimates are based on standardized z-scores; higher values indicate better performance.
[1]Adjusted for sex. age at testing. PUFA intervention. season of birth and pre-interventional 25(OH)D level
[2]Benjamini-Hochberg false discovery rate (5%) applied across 17 tests; $p < 0.05$ considered significant.

eFigure 1: Directed acyclic graph: pre-interventional 25(OH)D levels and childhood cognition
Created using: https://www.dagitty.net/dags.html#



eFigure 2: Heatmap: Spearman correlation between all cognitive tests from the original COPSYCH protocol



PAPER III

Genetic investigation of the association between maternal dietary patterns and offspring ADHD

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Governance: We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. Privacy is important to us which is why we follow national and international legislation on General Data Protection Regulation (GDPR), the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

Abbreviations:

ADHD = attention deficit hyperactivity disorder

ADHD-RS = ADHD Rating Scale

ALSPAC = Avon Longitudinal Study of Parents and Children

$COPSAC_{2010}$ = COpenhagen Prospective Studies on Asthma in Childhood2010

COPSYCH = COpenhagen Prospective Study on Neuro-PSYCHiatric Development

CRP = C-reactive protein

DAWBA = Development and Wellbeing Assessment

FFQ = food frequency questionnaire

GWAS = genome wide association study

ICD-10 = International Classification of Disorders 10th revision

K-SADS-PL = Kiddie Schedule for Affective Disorders and Schizophrenia Present and Lifetime Version

MoBa = Norwegian, Mother, Father and Child Cohort Study

NPR = Norwegian Patient Registry

PC = principal component

PGS = polygenic score

RS-DBD = Parent Rating Scale for Disruptive Behaviour Disorders

SNP = single nucleotide polymorphism

UK = United Kingdom

ABSTRACT

In observational studies, an unhealthy dietary pattern during pregnancy is associated with an increased likelihood of offspring ADHD. We investigated whether dietary pattern in pregnancy is a causal predisposing factor for offspring ADHD using a genetically informed study design. Polygenic scores (PGSs) for a healthy dietary pattern were calculated for mother, father, and child trios in the $COPSAC_{2010}$ cohort. Diagnoses and trait scores of ADHD were assessed in the children at age 10. Using trio models, we tested whether the association between maternal diet and offspring ADHD reflects genetic confounding or a causal effect. In $COPSAC_{2010}$ trio models (N-trio=437), a maternal healthy dietary pattern PGS was associated with reduced ADHD trait score after adjustment for child and paternal dietary pattern PGS, suggesting indirect genetic effects consistent with causal effects from maternal diet. However, this was not replicated in MoBa (N-trio=41 580) or ALSPAC (N-trio=1 199). Conclusively, trio models in $COPSAC_{2010}$ were consistent with causal effects of diet during pregnancy on offspring ADHD trait score, but not on diagnoses, but this was not replicated in larger cohorts, where direct genetic effect estimates implied an important role for genetic confounding in observed associations between maternal diet and offspring ADHD. Collectively, these genetic results do not provide support for the hypothesis that maternal dietary pattern in pregnancy represents a substantial causal predisposing factor for offspring ADHD and indicate that genetic confounding is likely to inflate observed associations between the two.

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a common neurodevelopmental disorder reported to affect 5.9-7.1% of children and adolescents.(1) It is well established that most of the variability in liability to ADHD is driven by a combination of genetic predisposition and early environmental exposures, where especially the intrauterine environment is believed to play a pivotal role.(2) Questions concerning the causality of these pregnancy-related environmental associations with ADHD have recently emerged, as their links with ADHD may be confounded by shared familial factors such as maternal genetic liability.(3–5) For example, smoking in pregnancy has been associated with a twofold incidence of ADHD in large population-based studies.(2,6,7) However, using genetically informed study designs including sibling designs, this association has been consistently shown to be driven by familial confounding, meaning that smoking in pregnancy is not likely a causal predisposing factor for offspring ADHD, rather a genetic predisposition to ADHD increases the likelihood of being exposed to smoking in utero.(2,3,8,9)

There is emerging evidence from observational studies(10–12) and pre-clinical animal models(10,13) that an unhealthy dietary pattern during pregnancy may increase the likelihood of child neurodevelopmental conditions, including ADHD and higher load of ADHD traits. Inferring causality from observational associations between maternal diet and offspring ADHD is challenging due to the strong genetic component of ADHD, with heritability estimates as high as 80%.(3,14) Maternal genetic predisposition to ADHD likely confounds these associations by influencing both dietary intake in pregnancy and an increased likelihood of ADHD in the child. In the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) mother-child cohorts, we previously identified an association between a Western dietary pattern during pregnancy and an increased risk of offspring ADHD. This finding was replicated across three independent mother-child cohorts, strengthening the validity of the association. Importantly, the relationship persisted after rigorous confounder adjustment, including maternal polygenic scores for

ADHD to partially account for genetic predisposition.(15) Family-based study designs as the genetic trio model offer an alternative and more robust methodological approach to disentangle genetic and environmental effects, which may be a step towards identifying true causal relationships and inform the design of randomized controlled trials (RCTs) and potential intervention strategies.(16) With growing knowledge about the genetic underpinnings of dietary patterns(17), it is now possible to calculate PGSs that reflect individual predisposition to specific dietary behaviours. A large-scale genome-wide association study (GWAS) based on Food Frequency Questionnaires (FFQs) found a considerable heritable component to dietary intakes, where common genetic variants explained 13.6% of the variance for a FFQ-derived dietary pattern.(17) This suggests that the observed association between maternal diet and offspring ADHD may be partially due to underlying genetics conferring a tendency to have an unhealthy diet and also increased likelihood of ADHD, which is then passed on to the child, so-called "genetic confounding".

Genetic trio models, incorporating both parents' and child's genetic liability to a given phenotype, can be used to decompose associations between parental exposures and child outcomes, and overcome some limitations of studies using sibling-pairs or adopted children.(18) Trio models leverage the fact that parents can transmit genetic disposition for a disorder to the child, both directly through inherited alleles and indirectly through non-transmitted alleles that influence the environment surrounding the child (See Figure 1).(19,20) Evidence of direct genetic effects implies that an observational association between the same trait is subject to "genetic confounding", whereas indirect genetic effects including mechanisms operating through environmental pathways, as such, are consistent with causality underpinning observed associations. Trio models have previously been used to examine suggested predisposing factors for ADHD, including psychopathological conditions, smoking, alcohol use, and educational attainment. Using genetic trio models for these factors, direct genetic effects were predominant, and indirect genetic effects were demonstrated only for maternal neuroticism.(18)

Here, we use genetic trio models to investigate dietary patterns in pregnancy as a predisposing factor for offspring ADHD. The goal of the study is to estimate the likely role of direct genetic transmission in explaining observed links between maternal diet in pregnancy and offspring ADHD. To the extent that trio models reveal direct effects of children's genetic liability to particular dietary patterns on their ADHD symptoms and likelihood of receiving ADHD diagnoses, these links will be subject to genetic confounding. In contrast, maternal indirect genetic effect estimates from the trio models - where mothers' genetic liability to specific dietary patterns independently predicts offspring ADHD outcomes - are consistent with the possibility of a causal mechanism underpinning the observed links between the two. To strengthen the validity and generalizability of our findings, we sought external replication in two large independent family cohorts; the Norwegian, Mother, Father and Child Cohort Study (MoBa) and the Avon Longitudinal Study of Parents and Children (ALSPAC).

METHODS

Study population

This project was set in the $COPSAC_{2010}$ cohort, which includes a population-based sample of 700 mother-child pairs. Pregnant women were recruited to the cohort between November 2008 and November 2010. Mothers were followed at the COPSAC research unit from week 24 of pregnancy, and the offspring with 14 clinical visits from birth until the age of 10, where the COpenhagen Prospective Study on Neuro-PSYCHiatric Development (COPSYCH) neurodevelopmental evaluation was conducted.(21,22) In the current study, we excluded one individual from each of the 10 total set of twins in the $COPSAC_{2010}$ cohort and removed genetically determined non-biological parents. The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and was approved by the Local Ethics Committee (H-B-2008-093, and the Danish Data Protection Agency (2015-41-3696). Both parents gave written informed consent before enrolment. (See Supplementary Methods)

We sought replication of findings from the $COPSAC_{2010}$ cohort using data from the MoBa and ALSPAC cohorts.

The Norwegian MoBa study is a population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health. Participants were recruited from all over Norway from 1999-2008. The women consented to participation in 41% of the pregnancies.(23) The cohort includes approximately 114,500 children, 95,200 mothers, and 75,200 fathers. MoBa is regulated by the Norwegian Health Registry Act. Blood samples were obtained from both parents during pregnancy and from mothers and children (umbilical cord) at birth.(24) The current project uses information on child sex and birth year from the Medical Birth Registry (MBRN) which is a national health registry containing information about all births in Norway.

ALSPAC recruited pregnant women from the Southwest of England, Avon, with an expected delivery between April 1991 and December 1992. Of all eligible pregnancies, 14,541 pregnant women, corresponding to 72%, consented to participate (13,988 alive at age one). An additional enrollment of pregnancies resulted in a sample size of 15,447 pregnancies for data collected after the age of seven. Of these, 14,901 children were alive at 1 year of age.(25,26) G0 partners (partners of the original cohort) were invited to complete questionnaires by the mothers at the start of the study and they were not formally enrolled at that time. 12,113 G0 partners have been in contact with the study by providing data and/or formally enrolling when this started in 2010. 3,807 G0 partners are currently enrolled.(27) Please note that the study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool.(28) Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees.

In ALSPAC we removed all twins, and in the larger MoBa one twin from each set was kept (See Supplementary Methods).

Polygenic scores for dietary pattern

The polygenic score (PGS) for dietary pattern was based on a GWAS by Cole et al. on 449,210 individuals of European ancestry with dietary information from FFQs.(17) The GWAS analyzed principal component analysis (PCA) derived dietary patterns, where the dietary pattern PGS in the present study was calculated according to the first principal component (PC1) which was the highest heritable dietary pattern (heritability estimate = 0.136) and had the most genome-wide significant loci (significant GWAS loci = 140). PC1 explained 8.63% of the phenotypic variance in curated measures of single food intake and was defined by foods that have been described in the literature as Western and prudent dietary factors.(17) Significant positive loadings for PC1 included increased intake of wholegrain bread, fruits and vegetables, oily fish, and water (a “healthy” dietary pattern). Significant negative loadings included

intake of white bread, butter and oil spread, processed meat, and high-fat milk (an "unhealthy" dietary pattern).(17,29)

Polygenic scores were constructed using PRS-CS for the $COPSAC_{2010}$ and ALSPAC cohorts (See Supplementary Methods). For MoBa, the PGS was constructed using the comparable LDpred2 method and was adjusted for genotyping and imputation batch and population structure (first 20 PCs).(30,31) Details on the genotyping of MoBa and ALSPAC trios are available elsewhere.(32,33) Reported associations are for 1 SD increase in the PGS corresponding to genetic predisposition to eating a healthier dietary pattern.

Validation of dietary pattern polygenic scores

We tested whether the dietary pattern PGS predicted reported consumption of similar food items in pregnant women from the $COPSAC_{2010}$ cohort as the PC1 described by Cole et al.(17) This was done by plotting the maternal PGS against the intake of 43 food items reported in a validated semi-quantitative FFQ by mothers in week 24 of pregnancy.(34) The predictive value of the dietary pattern PGS was further tested by its correlation to a maternal pregnancy Western dietary pattern derived from a principal component analysis based on energy, macronutrient, and micronutrient intake, also calculated from FFQs, which was previously associated with offspring neurodevelopmental disorders in $COPSAC_{2010}$.(15) We also examined the effect of the dietary pattern PGS on the 20 food groups most strongly associated with the Western dietary pattern, and conversely, the effect of the Western pattern on those most strongly linked to the PGS. Lastly, we calculated mean Western dietary pattern PC for 2 SD intervals of the maternal dietary pattern PGS.

Dietary pattern PGSs were likewise validated in the replication cohorts using FFQ data. In MoBa, a 255-item validated FFQ was administered at gestational week 22, covering maternal dietary habits since she became pregnant.(35–38) FFQ items were sorted into intake of 98 food groups measured by grams per

day.(39) In ALSPAC, the FFQ was administered at week 32 of pregnancy and covered the current intake of the 43 most frequently consumed foods in the UK. More detailed questions were asked for an additional 8 foods usually consumed daily, which enabled us to include the estimated weekly intake of wholemeal and white bread. A healthy pregnancy dietary pattern PC based on the FFQ data (explaining 10.6% of variance) with high loadings of, among others, salad, fruit, oat and bran-based cereals, fish, and non-white bread, was likewise used for validation of the dietary pattern PGS in the ALSPAC cohort.(40) (See Supplementary Methods)

Neurodevelopmental outcomes

At the age of 10 years the $COPSAC_{2010}$ cohort underwent an in-depth clinical psychopathological evaluation where the study's main outcomes, diagnoses of ADHD and ADHD trait scores, were assessed.

Child and parent(s) were interviewed separately with the semi-structured clinical diagnostic interview Kiddie Schedule for Affective Disorders and Schizophrenia Present and Lifetime Version (K-SADS-PL) by trained interviewers, including medical doctors, nurses, and psychologists.(41) Research diagnoses were based on a collective evaluation of the K-SADS-PL and all other available clinical information and assigned according to the International Classification of Disorders 10^{th} revision (ICD-10).(42) ADHD was defined by the diagnostic codes DF90.0, DF90.8, and DF98.8C. We grouped diagnoses according to DSM-5 terminology with ADHD Combined Presentation including DF90.0 and DF90.8, and ADHD Inattentive Presentation including DF98.8C. (See Supplementary Methods)

ADHD trait scores from all included cohorts referred to the 18 DSM-IV items for ADHD, with 9 items for inattentive and 9 items for hyperactivity/impulsivity symptoms. For all scales, responses were made on a 4-point Likert scale. Scores were not transformed in analyses.

In $COPSAC_{2010}$, ADHD trait score was assessed by the ADHD Rating Scale (ADHD-RS) filled out by parents.(43)

Neurodevelopmental outcomes in MoBa included child ADHD diagnoses obtained from the Norwegian Patient Registry (NPR). ADHD was defined by at least one registered ICD-10 diagnostic code of hyperkinetic disorder including DF90.0, DF90.1, DF90.8, DF90.9, and DF98.8 regardless of age at registration. Combined Presentation included DF90.0, DF90.1, DF90.8, DF90.9 and Inattentive Presentation included DF98.8. The prevalence of ADHD diagnoses from the NPR after the exclusion of one twin from each pair was 7,5% (N ADHD = 8,305). Mother reported traits of ADHD were assessed at the age of 8 by the Parent Rating Scale for Disruptive Behaviour Disorders (RS-DBD).(44)

In ALSPAC, mother-reported ADHD traits were evaluated using the structured diagnostic interview Development and Wellbeing Assessment (DAWBA) at the age of 7.(45,46) (See Supplementary Methods)

Statistical analysis

We used linear regression and quasi-Poisson models to test the ability of the dietary pattern PGS to predict food intake measured by FFQ. The Pearson correlation was tested between dietary pattern PGS and principal component-derived pregnancy Western dietary pattern and maternal ADHD PGS.(47) Additionally, we tested the genetic correlation using linkage disequilibrium score regression (LDSC) between maternal dietary pattern genetics and ADHD genetics.(48)

In the main analyses, the associations between maternal, child, and paternal dietary pattern PGS and ADHD outcomes were tested using logistic regression for ADHD diagnosis and linear regression for ADHD total trait scores. Sensitivity analyses were performed with ADHD presentations and ADHD trait subscales as outcomes. All trio models were based on a single regression model including maternal, paternal, and child dietary pattern PGSs as predictors of offspring ADHD, with each PGS mutually adjusted for the others.(18) In follow-up analyses, we evaluated the effect of the dietary pattern PGS in the general population by performing trio models on ADHD trait scores within individuals without an

ADHD diagnosis. Further, we performed trio models on the dietary pattern PGS adjusted for the matching ADHD-PGSs to separate the effects of dietary genetics from ADHD genetics.(47) All analyses were adjusted for the sex of the child. Trio models indicating indirect genetic effects were rerun with adjustment for social circumstances variables and putative prenatal predisposing factors for ADHD to adjust for measured confounding. We sought replication using a similar statistical approach in the MoBa and ALSPAC cohorts. Trio models on MoBa data were additionally adjusted for birth year to account for the longer inclusion time. Additional sensitivity analyses were performed for MoBa; trio models on ADHD diagnosis were performed, requiring a minimum of two registered ADHD diagnoses in NPR, and further trio models were performed using clustered robust standard errors based on maternal ID to account for the inclusion of siblings.

All statistical tests were performed with an alpha of 5%. Data was analyzed using the statistical software R, version 4.4.1.

RESULTS

Baseline Characteristics

In $COPSAC_{2010}$, a total of 593 individuals were clinically evaluated for neurodevelopmental and other psychiatric disorders at age 10 years, and an additional 11 individuals had questionnaire-based information on symptom loads. 530 had data on diet PGS for at least one family member and 437 had complete trio information (Supplementary Figure 1). Cohort demographics are described according to diagnosis of ADHD in Table 1. Among complete trios 12.1% were classified with ADHD as compared to 11.0% among the total 593 evaluated children. A drop-out analysis comparing complete trios to the remaining cohort showed a higher degree of participation in the fish oil intervention and a marginally higher mean Western dietary pattern PC among complete trios (Supplementary Table 1).

Validation of the dietary pattern polygenic score

The maternal dietary pattern PGS showed an association to maternal diet in pregnancy in COPSAC2010 comparable to findings of Cole et al.(17) where the diet PGS was generated (Figure 2) and showed a significant correlation with the PC derived Western dietary pattern in pregnancy previously associated with neurodevelopmental disorders in $COPSAC_{2010}$ (Pearson's r = -0.28; 95%CI= -0.36-0.20, $p <$ 0.001).(15) Top food groups associated with the maternal dietary PGS were associated in a similar pattern to the Western dietary pattern PC. When depicting top food groups for the Western dietary pattern PC, the maternal dietary pattern PGS also showed similar directionality of associations (See Supplementary Figures 2-3). Mean maternal dietary pattern PC decreased with higher values of the maternal dietary pattern PGS (See Supplementary Figure 4). The maternal dietary PGS was associated with maternal smoking in pregnancy, maternal and paternal education, but not with other strong ADHD correlates such as maternal pregnancy C-reactive protein (CRP)(49) and maternal pre-pregnancy body mass index (BMI) (See Supplementary Table 2).(50) Maternal and paternal dietary pattern PGSs were not correlated (r =

0.04; 95%CI= -0.05-0.12, $p = 0.406$). For validation cohorts, the validity of the maternal dietary pattern PGS was tested on pregnancy FFQ data. In MoBa, 83 of the included 98 food groups were significantly associated with the maternal dietary pattern PGS after FDR correction. Consistent with the pattern described by Cole et al., a higher PGS positively associated with intake of olive oil, vegetables, water, wholegrain cereals, and brown bread, and negatively associated with intake of sugar sweetened soft drinks, processed meat, white bread, chips, and animal fats (See Supplementary Figure 5). In ALSPAC, maternal PGS was positively associated with the intake of pudding, wholegrain cereals, juice, whole meal bread, and negatively associated with the intake of white bread and roast potatoes (See Supplementary Figure 6), and further positively correlated with maternal healthy diet PC (Pearson's r = 0.04 p-value 0.001).

Dietary and ADHD genetic overlap

Maternal dietary pattern PGS showed a weak correlation to maternal ADHD PGS (Pearson's r = -0.10; 95%CI = -0.18-0.20, $p = 0.015$) in the $COPSAC_{2010}$ cohort, and there was a modest genetic correlation between dietary pattern genetics and ADHD genetics based on summary statistics from published GWAS (r = -0.24; SE 0.04, $p < 0.001$).

Dietary pattern PGS and ADHD outcomes in the $COPSAC_{2010}$ cohort

Without adjustment for any other family members dietary pattern PGSs, a one standard deviation increase in maternal dietary pattern PGS, corresponding to a higher genetic predisposition to a healthy diet, was associated with a 29% lower likelihood of ADHD diagnosis (OR = 0.71; 95%CI = 0.53–0.93, $p = 0.015$). Paternal dietary pattern PGS showed a similar effect size (OR = 0.77; 95%CI= 0.57–1.03, $p = 0.085$). Child dietary pattern PGS had the strongest association with ADHD diagnosis (OR = 0.55; 95%CI = 0.41–0.73), $p < 0.001$). Higher maternal and child dietary pattern PGS were also associated with lower offspring ADHD-RS total score (mother beta = -1.23; 95%CI = -1.93–-0.53, $p < 0.001$; child beta = -1.10;

95%CI = -1.82–-0.38, p = 0.003). No association was observed between paternal dietary pattern PGS and ADHD-RS total score (beta = -0.50; 95%CI = -1.30-0.29, p = 0.212). (Supplementary Table 3)

Trio analyses in the $COPSAC_{2010}$ cohort

The association between maternal and paternal dietary pattern PGS and offspring ADHD diagnoses attenuated after adjustment for direct genetic transmission in the trio models (mother OR = 0.98; 95%CI = 0.69–1.38, p = 0.893; father OR = 1.05; 95%CI = 0.73–1.53, p = 0.780), while the child dietary pattern PGS association remained consistent (child OR = 0.58; 95%CI = 0.38–0.88, p = 0.011). This indicates that direct genetic effects may contribute to observational associations between diet in pregnancy and ADHD diagnosis.

In contrast, there was evidence of maternal indirect genetic effects on ADHD trait score (mother beta = -1.15; 95%CI = -2.06 –-0.23, p = 0.015) and no evidence of direct genetic effects, with the child dietary pattern PGS estimate attenuating in trio models for this outcome (child beta = -0.14; 95%CI = -1.25-0.98, p = 0.810). No paternal indirect genetic effects on ADHD trait scores were observed, suggesting that the observed effects are exclusively maternal (father beta = -0.34; 95%CI = -1.35–0.68, p = 0.514).(51) In analyses on ADHD-RS subscales, there was evidence of indirect genetic effects also for Impulsivity and Hyperactivity traits, but not for Inattentive traits. (Figure 3 & Supplementary Table 4)

Exclusion of ADHD cases and adjustment for ADHD PGS in trio models did not alter the results (Supplementary Figure 7 & 8). The maternal indirect genetic effect on ADHD trait score was attenuated after adjustment for social circumstances variables and putative predisposing factors for ADHD including maternal pregnancy week 24 CRP, maternal pre-pregnancy BMI, maternal age, paternal age, household income, maternal educational level, sex, birth weight, gestational age, and fish oil supplementation in third trimester: adjusted beta = -0.84; 95%CI = -1.73–-0.05, p = 0.051.

Replication in the MoBa and ALSPAC cohorts

Analyses using data from the MoBa cohort included 41,580 complete trios in analyses on ADHD diagnoses (ADHD prevalence 7,0 %) and 18,629 complete trios for ADHD symptoms (median RS-DBD total score 7 (IQR 4-11). Child, maternal, and paternal dietary pattern PGSs for ADHD were all associated with ADHD diagnosis and ADHD total trait score in unadjusted models. In trio analyses, there was no evidence of indirect genetic effects (maternal OR for ADHD = 0.99; 95%CI = 0.95–1.04, p = 0.784, maternal beta for ADHD traits = 0.09; 95%CI = -0.03–0.21, p = 0.156) , and only child dietary pattern PGS remained associated with ADHD outcomes indicating direct genetic transmission of risk only (child OR for ADHD = 0.92; 95%CI = 0.87–0.96, p = 0.002, child beta for ADHD traits =-0.28; 95%CI = -0.42–0.14, $p < 0.001$). Results from 1,199 complete trios (median total DAWBA score 2 (IQR 0-7)) from the ALSPAC cohort were consistent in direction and magnitude, though less precisely estimated, meaning that no effects were different from zero (See Supplementary Results, Supplementary Figures 9-10, and Supplementary Tables 5-8).

DISCUSSION

We used genetic trio models to investigate potential mechanisms underpinning observed associations between maternal dietary pattern in pregnancy and ADHD in the offspring. Overall, trio models applied across three cohorts indicated a likely role for genetic confounding in observational associations between maternal dietary pattern and offspring ADHD diagnosis, with consistent evidence for direct effects from transmitted genetic liabilities to specific dietary patterns on the likelihood of receiving the diagnosis among offspring. Our results did not provide support for a causal link between maternal dietary pattern and offspring ADHD diagnoses, with a lack of evidence of effects of maternal dietary pattern PGS after control for genetic liability directly transmitted to offspring. In relation to ADHD trait outcome, trio models in the $COPSAC_{2010}$ cohort showed evidence for indirect genetic effects that would be consistent with a causal relationship. However, this effect on ADHD trait score was not reproducible in the larger external replication cohorts MoBa and ALSPAC. Taken together, these findings are not consistent with the hypothesis that maternal dietary pattern in pregnancy is a substantial or universal causal predisposing factor for ADHD traits in the offspring.

To our knowledge, this is the first study to explore dietary patterns as a causal predisposing factor for ADHD using a genetic trio model approach.(11) By using a healthy dietary pattern PGS, we showed evidence of direct genetic transmission, suggesting that previously reported observational findings may be impacted by genetic confounding. Genetic confounding describes a scenario where an observational association is wholly or partly attributable to the fact that the same genetic factors influence both the exposure and outcome. Existing studies have to some extent accounted for this. In the $COPSAC_{2010}$ cohort, the substantial increase in likelihood of ADHD following a Western prenatal dietary pattern was robust to adjustment for maternal ADHD PGS and was replicated across several mother-child cohorts.(15) The study further found that ADHD genetics moderated the effect of the Western dietary

pattern supporting the role of genetics for such associations.(15) An observational study from the MoBa cohort reported small, though robust, associations between quality of pregnancy diet and offspring likelihood of ADHD diagnoses and ADHD traits across strata of registered maternal ADHD traits in an attempt to account for maternal ADHD genetic disposition.(12) Using the largest available number of genotyped trios, we did not find evidence in our genetic-based models that would be consistent with effects of diet in pregnancy, as defined by a healthy dietary pattern PGS, on either ADHD diagnosis nor number of ADHD traits, including individuals not reaching the diagnostic threshold for ADHD. Our findings suggest that, if any causal effects of diet in pregnancy on offspring ADHD exist, these are likely to either be of small magnitude or somewhat context specific.

We found some evidence consistent with a causal effect of diet on ADHD trait score in the $COPSAC_{2010}$ cohort, which was not replicated in MoBa or ALSPAC. The UK Biobank GWAS from which the dietary pattern PGS was derived reported a significant genetic correlation with factors not related to diet, including physical activity, educational attainment, socioeconomic status, and smoking status. For BMI, the study reported that there may be a shared genetic etiology but that the underlying genetic architectures are predominantly distinct.(17) In $COPSAC_{2010}$, we confirmed that the maternal dietary pattern PGS was associated with parental educational attainment, maternal smoking in pregnancy, and that there was a genetic correlation with ADHD. The indirect genetic effect in $COPSAC_{2010}$ trio analyses may be a product of confounding from such lifestyle factors related to both ADHD and dietary pattern genetics, and the attenuation of the effect to $p=0.051$ after adjustment for lifestyle factors indicated that this could be the case. Alternatively, the indirect genetic effects detected in $COPSAC_{2010}$ for ADHD trait score and not for ADHD diagnosis, reflect a higher sensitivity of a dimensional approach than of a binary. The indirect genetic effects may potentially not reflect causal mechanisms, or the inability to replicate may be due to population differences between the cohorts or variances in the assessment of traits.

The trio design is a major strength of this study. To investigate the observational associations between diet in pregnancy and ADHD further, results should be sought triangulated across different study designs with different sources of bias. For this purpose, genetically informed studies such as ours are an important contribution, due to the design-based handling of confounding which is believed to be a strong complementary approach to traditional statistical adjustment.(16) The reliability of our findings is significantly substantiated by the ability to validate the dietary pattern PGS derived from summary statistics based on a non-pregnant population in all three included cohorts using FFQ data. Though the $COPSAC_{2010}$ cohort includes the smallest number of trios compared to MoBa and ALSPAC, the major strength of including analyses within this cohort is the high quality of the phenotypic outcomes based on the thorough COPSYCH clinical examinations, thus avoiding misclassification of a clinician evaluated ADHD diagnosis.(22)

Assortative mating and population structure may confound associations from a GWAS and consequently the reported effects in our study. Supportive of a potential bias from assortative mating, we found that parental PGSs were weakly correlated in MoBa and ALSPAC. Bias from assortative mating or population stratification would be expected to inflate indirect genetic effect estimates, but leave estimates of direct genetic effects unchanged.(18,52) Assortative mating is therefore unlikely to explain the conclusions drawn in this paper. Even though the study included a high number of trios, we cannot rule out the possibility that we may have been underpowered to detect small indirect genetic effects, due to the low specificity of PGS's in general, which only explain a small part of the phenotypic variance.(52) Further, the PGS was generated based on the intake of a specific dietary pattern primarily driven by type of bread consumed and therefore may not capture effects from specific nutrients.(17) The Cole et al GWAS from where the dietary pattern PGS was derived, was based on a 24-hour recall FFQ obtained from an adult population. Data from a GWAS on pregnancy diet from a larger time span was not available, however, such data could have increased the predictive value of the PGS. Additionally the FFQ data may have been

subject to bias due participation dependent on health status. Finally, the study was restricted to individuals of European ancestry, which limits the generalizability of our findings.

Conclusion

In conclusion, using a genetically informative design applied across multiple independent cohorts, we found consistent evidence that genetic liabilities to specific dietary patterns transmitted from parents to children are associated with their ADHD diagnoses and traits – indicating a likely role for genetic confounding in observational links between maternal diet and offspring ADHD. We did not find strong evidence, in the form of indirect genetic effects in genetic trio models, that would be consistent with a causal mechanism underpinning these links. Taken together, our results suggest that influences of a healthy diet in pregnancy on ADHD outcomes are likely either of small magnitude or highly context specific.

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Disclaimer:

Data from the Norwegian Patient Registry has been used in this publication. The interpretation and reporting of these data are the sole responsibility of the authors, and no endorsement by the Norwegian Patient Registry is intended nor should be inferred.

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Tables

Table 1: Baseline characteristics of COPSAC$_{2010}$ families with complete trio information, stratified according to diagnosis of ADHD

	No ADHD	ADHD	P value
	n = 384	n = 53	
Child dietary pattern PGS, mean (SD)	0.12 (1.00)	-0.43 (1.11)	<0.001
Maternal dietary pattern PGS, mean (SD)	-0.04 (1.05)	-0.36 (0.91)	0.037
Paternal dietary pattern PGS, mean (SD)	0.06 (0.99)	-0.18 (0.92)	0.090
Sex, male, n (%)	180 (46.9)	41 (77.4)	<0.001
Gestational age, d, mean (SD)	279.17 (11.16)	280.49 (8.66)	0.285
Birthweight, kg, mean (SD)	3.54 (0.53)	3.64 (0.48)	0.122
Maternal educational level, n (%)			0.005
Elementary school	10 (2.6)	4 (7.5)	
College graduate	20 (5.2)	4 (7.5)	
Tradesman certification	67 (17.4)	18 (34.0)	
Bachelors' degree	173 (45.1)	19 (35.8)	
Masters' degree or higher	114 (29.7)	8 (15.1)	
Paternal educational level, n (%)			0.093
Elementary school	14 (3.7)	3 (5.7)	
College graduate	26 (6.8)	0 (0.0)	
Tradesman certification	124 (32.4)	25 (47.2)	
Bachelors' degree	111 (29.0)	12 (22.6)	
Masters' degree or higher	108 (28.2)	13 (24.5)	
Household income, n (%)			0.286
<100.000 DKK	30 (7.8)	3 (5.7)	
100.000-150.000 DKK	90 (23.4)	17 (32.1)	
150.000-200.000 DKK	116 (30.2)	16 (30.2)	
200.000-250.000 DKK	87 (22.7)	13 (24.5)	
>250.000 DKK	61 (15.9)	4 (7.5)	
Alcohol intake in pregnancy, yes, n (%)	60 (15.6)	6 (11.5)	0.397
Smoking in pregnancy, yes, n (%)	24 (6.2)	6 (11.3)	0.195
Parity, n (%)			0.269
1	171 (44.5)	20 (37.7)	
2	159 (41.4)	22 (41.5)	
≥3	54 (14.1)	11 (20.8)	
Maternal pre-pregnancy BMI, mean (SD)	24.53 (4.28)	26.43 (5.66)	0.004
Maternal Western dietary pattern PC, mean (SD)	-0.02 (0.93)	0.51 (1.07)	0.001
Maternal age, y, mean (SD)	32.05 (4.13)	32.03 (4.61)	0.671
Paternal age, y, mean (SD)	34.52 (5.14)	34.54 (5.10)	0.681
Vitamin D intervention, yes, n (%)	152 (47.8)	23 (50.0)	0.627
Fish oil intervention, yes, n (%)	209 (54.6)	24 (45.3)	0.181

ADHD = attention deficit hyperactivity disorder, PGS = polygenic score, BMI = Body Mass Index

Figures

Figure 1. Graphical presentation of the trio polygenic score model to estimate direct and indirect genetic effects of diet in pregnancy on offspring likelihood of attention deficit hyperactivity disorder. The figure was drawn based on a directed acyclic graph of the trio model by Pingault et al.(18) *Created in BioRender. Bønnelykke, K. (2026) https://BioRender.com/ghuyslo*

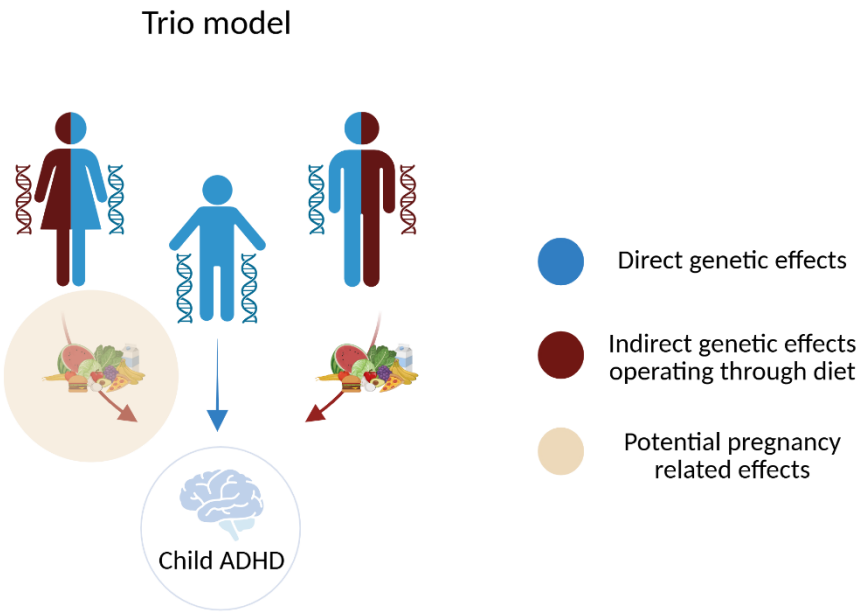


Figure 2. Maternal dietary pattern polygenic score plotted against maternal diet as reported by food frequency questionnaires in week 24 of pregnancy. Only nominal significant associations are shown. FDR significant p-values are marked with an asterisk (*).

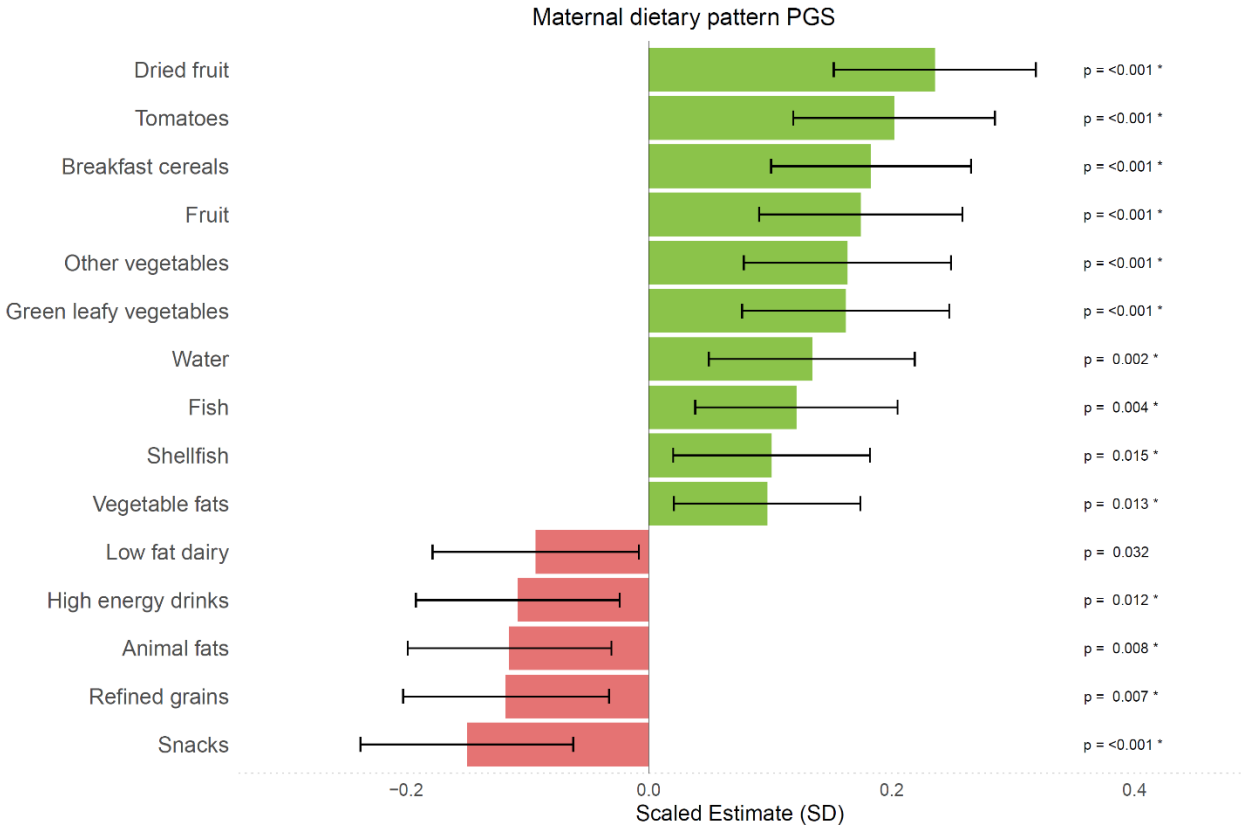


Figure 3. Unadjusted and trio models of the association between dietary pattern PGS and ADHD K-SADS-PL diagnosis as well as ADHD-RS trait score in the $COPSAC_{2010}$ cohort.

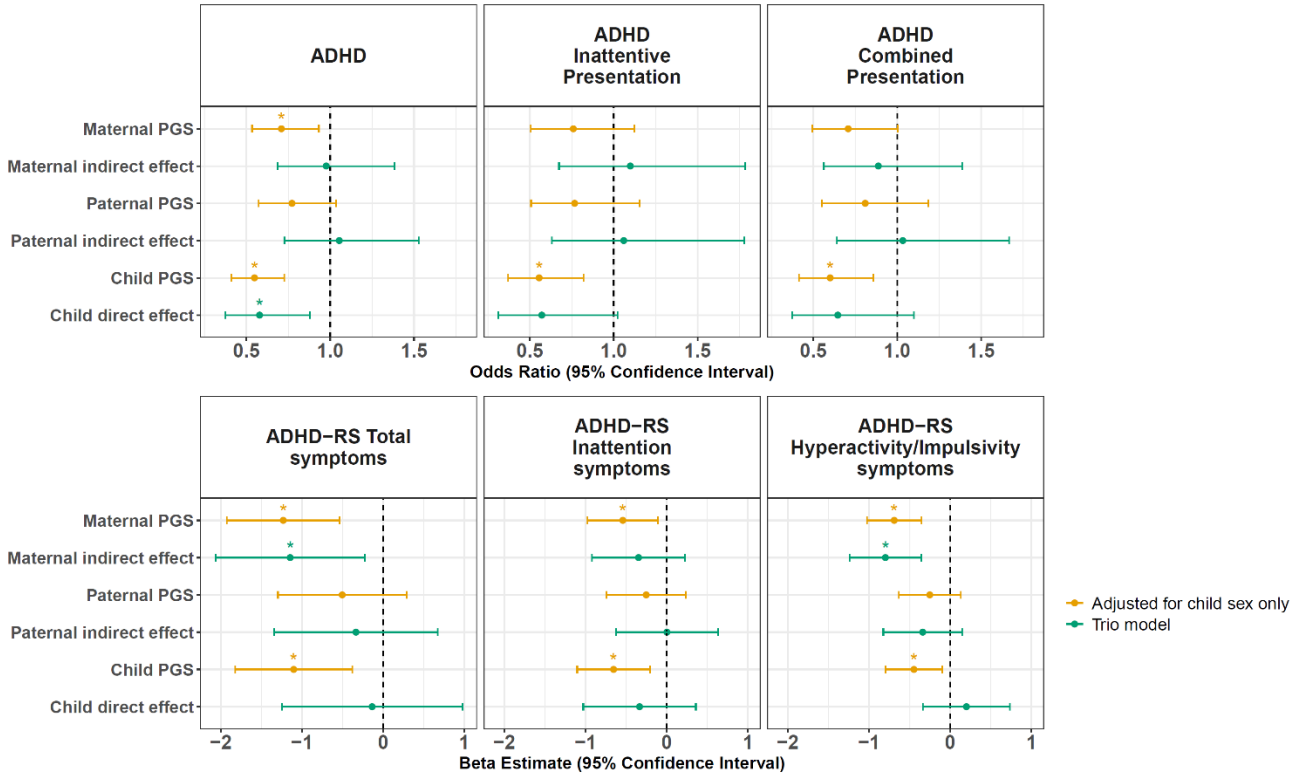
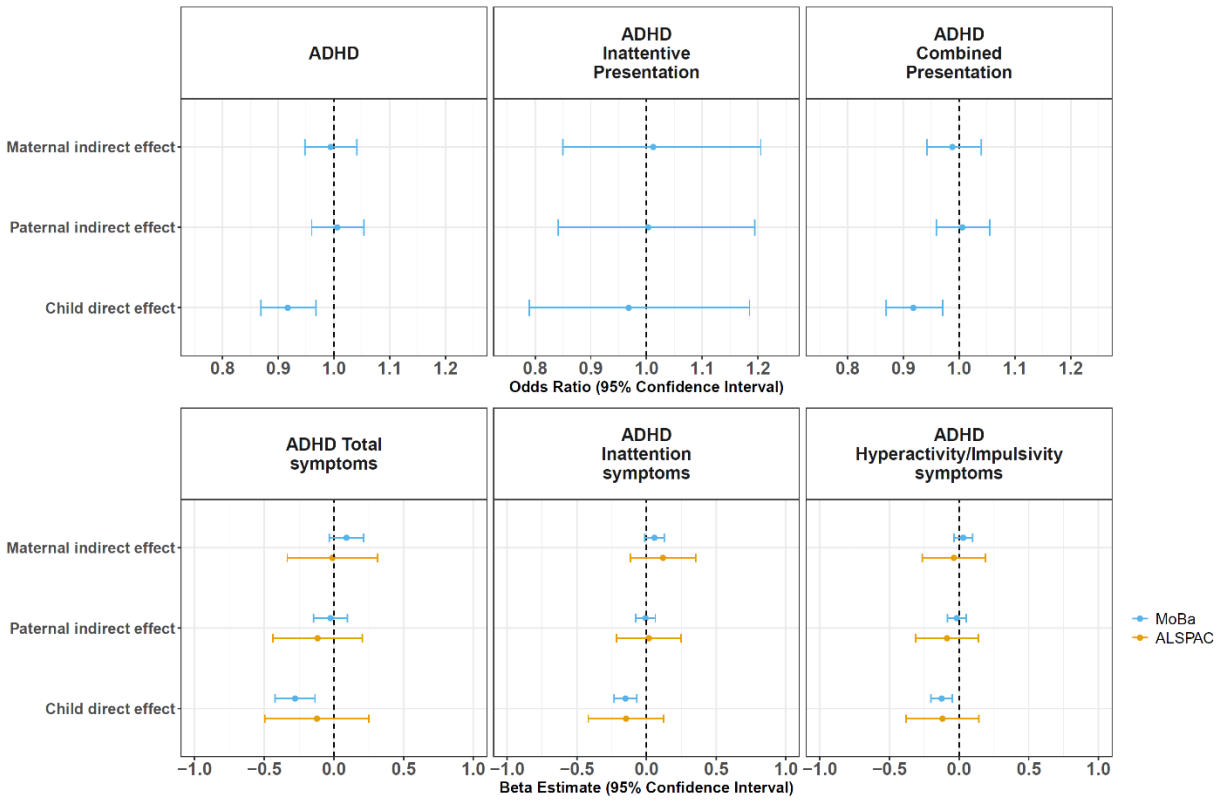


Figure 4. Trio models of the association between dietary pattern PGS and ADHD diagnosis as well as ADHD trait score for MoBa and ALSPAC. ALSPAC analyses were adjusted for sex of the child and MoBa analyses included additional adjustment for birth year due to the longer period of inclusion.



Supplementary Information

Supplementary methods

$COPSAC_{2010}$ Study population

To be included, women were required to speak Danish, consume no more than 600 IU vitamin D during pregnancy; and not suffer from any endocrine, cardiovascular, or nephrological disorders. Two randomized controlled trials were conducted within the cohort, where mothers in their third trimester were randomized to receive supplementation with fish oil and high-dose vitamin D3 in a factorial 2x2 design.

$COPSAC_{2010}$ FFQ data

The FFQ contained 360 items covering their dietary intake with a one-month recall period. Intake of foods was reported either in natural units or frequency of consumption as appropriate for the given item. Number of grams per day for 43 food groups was calculated based on an assumption of standard portion sizes. Implausible values of energy intake corresponding to below 4,200 kJ/day or above 16,700 kJ/day were excluded.

$COPSAC_{2010}$ neurodevelopmental outcomes

COPSYCH examinations took place between January 2019 and December 2021. All research diagnoses were assigned in consensus with senior researcher JRJM. An external validation of research diagnoses was performed together with a clinical professor in child and adolescent psychiatry. Video recordings of the clinical examinations allowed for both supervision and testing of interrater reliability which was high. Overall agreement across symptoms currently present and symptoms currently not present was 99.5% CI (99.3-99.7). Agreement of present symptoms was 88.5% CI (82.6-93.0).

Dietary pattern polygenic scores in $COPSAC_{2010}$

In the $COPSAC_{2010}$ cohort, high throughput genome-wide single-nucleotide polymorphism (SNP) genotyping of the parents and children was performed using the IlluminaInfinium™ II HumanHap 550K SNP BeadChip technology (Illumina, San Diego) and further by the Illumina Infinium HumanOmniExpressExome BeadChip in the children. We used the PRS-continuous shrinkage (CS) method to construct the score using the 1KG reference panel.[1] The final score consisted of 1,071,676 SNPs available in both the GWAS summary statistics, reference panel, and the COPSAC cohort. Given the sample size of the GWAS, the phi parameter, controlling the global shrinkage, was estimated based on the data. Each individual SNP effect on diet was updated for each SNP and combined to an aggregated score using the software PLINK(28) and scaled to mean 0 with 1 standard deviation (SD) for each dataset.

The MoBa study

The current study is based on Version 12 of the quality-assured data files released for research in January 2019. The establishment of MoBa and initial data collection was based on a license from the Norwegian

Data Protection Agency and approval from The Regional Committees for Medical and Health Research Ethics. The MoBa cohort is currently regulated by the Norwegian Health Registry Act.

MoBa FFQ data
Implausible values of calorie intake corresponding to an intake of ≥ 4780 (20,000 kJ) or ≤ 1076 kcal (4,502 kJ) were removed. Number of grams per day were calculated for each food group.

The ALSPAC study
Consent for biological samples has been collected in accordance with the Human Tissue Act (2004). Informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time.

ALSPAC FFQ data
As previously reported, we converted FFQ data registered with five different frequency options to weekly frequency of intake.[2] Implausible values of energy intake were excluded based on the MoBa cutoffs.

ALSPAC neurodevelopmental outcomes
The ALSPAC DAWBA ADHD trait scores were based on the following variables (See ALSPAC data documentation for further detail): Kr460a indicating total number of inattention/activity symptoms. Variable 459b and 447b covering prorated scores for attention and activity traits.

Statistical analysis
Demographics of the $COPSAC_{2010}$ cohort were reported according to the presence of ADHD, and differences between groups were tested by either t-test or chi-squared test. Associations between maternal dietary pattern PGS and selected social circumstance and prenatal exposure variables were calculated in univariate linear regression models. To test for assortative mating, a potential source of bias in trio models, we calculated the correlation between maternal and paternal dietary pattern PGSs.

Supplementary results

Assortative mating in the MoBa and ALSPAC cohorts
Maternal and paternal dietary pattern PGS were weakly correlated in both the MoBa (Pearson's r =-0.03, p-value 4.30×10^{-16}) and ALSPAC cohort (Pearson's r =-0.06, p-value 0.044).

MoBa neurodevelopmental outcomes
After the exclusion of one twin from each set, median number of ADHD RS-DBD traits of ADHD was 7 (IQR 4 -11), and prevalence of ADHD diagnoses from the NPR was 7,5% for all ADHD diagnoses, 7.2% for ADHD combined presentation, and 0.5% for ADHD inattentive presentation.

ALSPAC neurodevelopmental outcomes
After the exclusion of twins, the median number of total parent-rated ADHD DAWBA rated traits was 2 (IQR 0-7).

MoBa sensitivity analyses

In the larger MoBa cohort main trio analyses were performed using robust standard errors clustered on maternal ID. This did not alter the results. Changing the diagnostic grouping of ADHD diagnoses from requiring only one to two registered diagnoses in the Norwegian Patient Registry did not alter the results either.

Supplementary References

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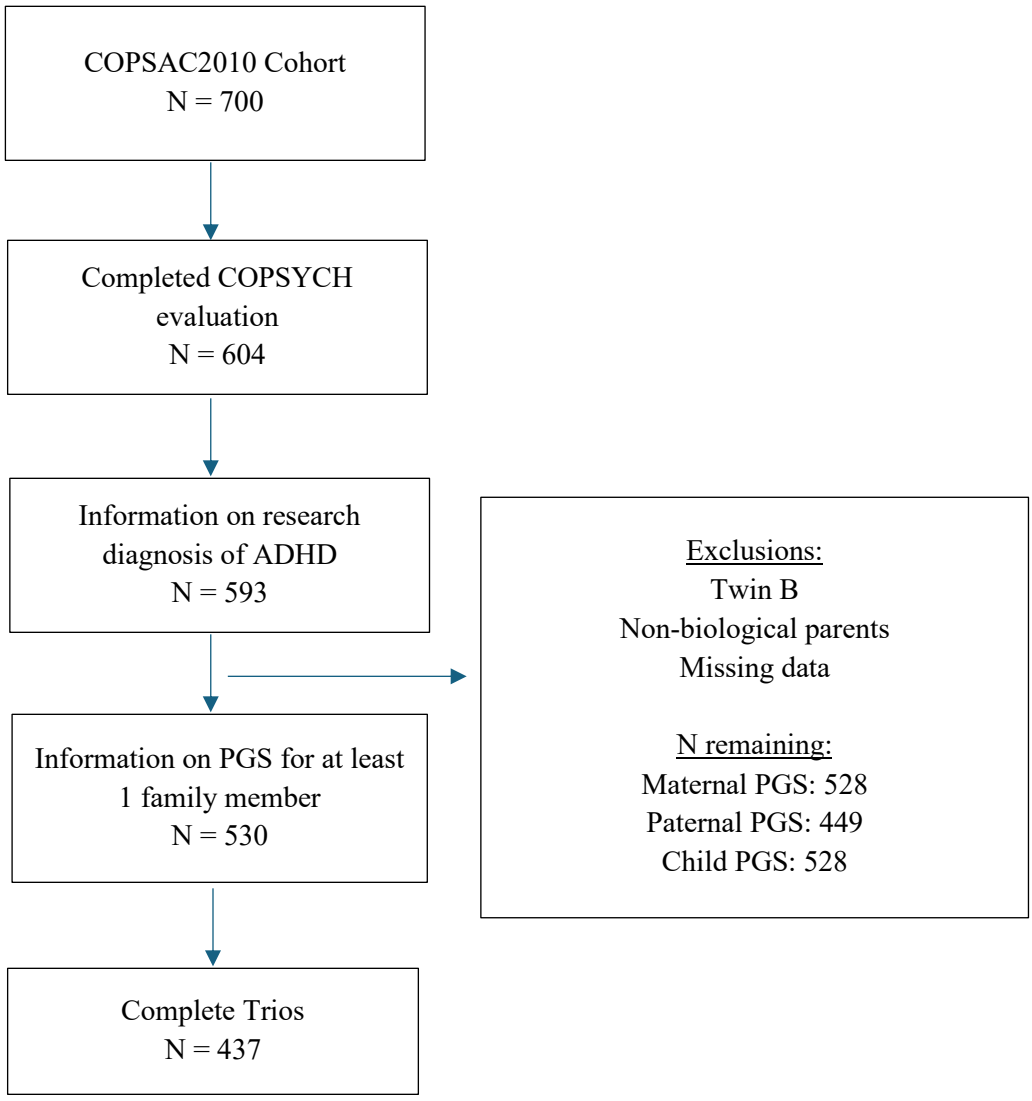
Supplementary tables and figures

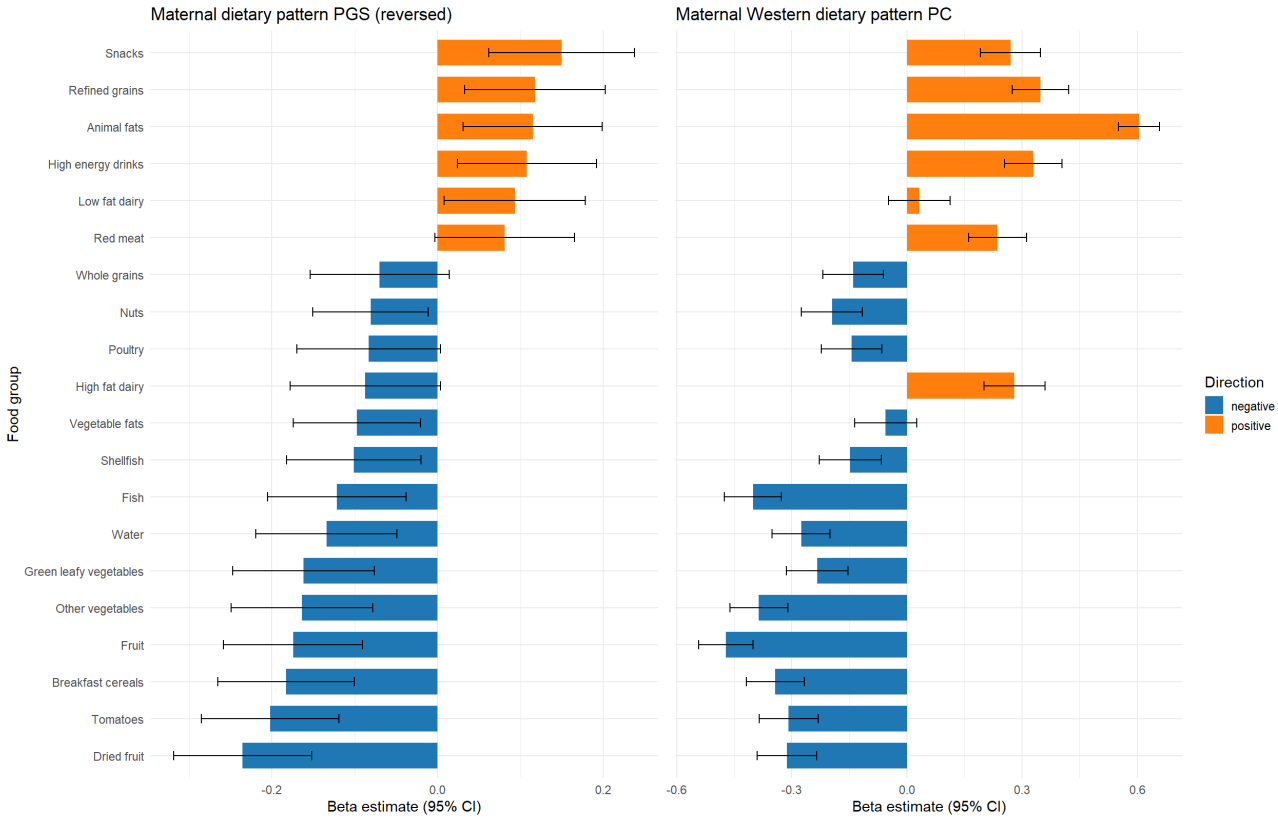
Supplementary Figure 1. Flow chart of the $COPSAC_{2010}$ study cohort



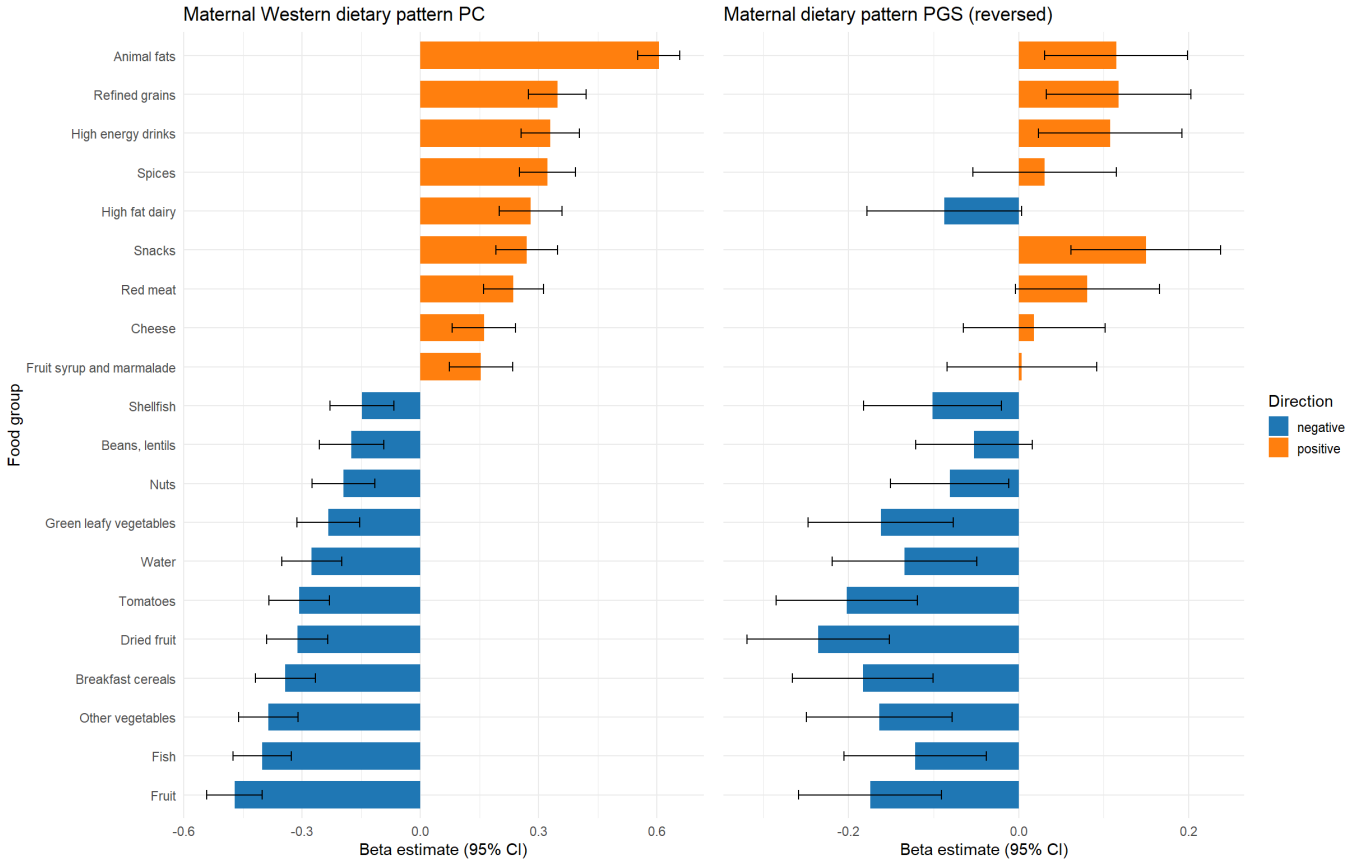
Supplementary Table 1: Drop out analysis

	Total cohort	Incomplete Trios	Complete trios	P-value
	n =700	n =263	n = 437	
Child dietary pattern PGS, mean (SD)	0.04 (1.00)	0.02 (0.95)	0.05 (1.03)	0.531
Maternal dietary pattern PGS, mean (SD)	-0.04 (1.03)	0.08 (1.01)	-0.08 (1.03)	0.078
Paternal dietary pattern PGS, mean (SD)	0.05 (0.98)	0.12 (0.97)	0.03 (0.99)	0.299
Sex, male, n (%)	360 (51.4)	139 (52.9)	221 (50.6)	0.426
Gestational age, d, mean (SD)	279.00 (11.65)	278.46 (12.81)	279.33 (10.89)	0.233
Birthweight, kg, mean (SD)	3.54 (0.55)	3.51 (0.59)	3.55 (0.52)	0.271
Maternal educational level, n (%)				0.449
Elementary school	20 (2.9)	6 (2.3)	14 (3.2)	
College graduate	35 (5.0)	11 (4.2)	24 (5.5)	
Tradesman certification	130 (18.6)	45 (17.1)	85 (19.5)	
Bachelor's' degree	321 (45.9)	129 (49.0)	192 (43.9)	
Masters' degree or higher	194 (27.7)	72 (27.4)	122 (27.9)	
Paternal educational level, n (%)				0.128
Elementary school	31 (4.6)	14 (5.7)	17 (3.9)	
College graduate	39 (5.7)	13 (5.3)	26 (6.0)	
Tradesman certification	213 (31.3)	64 (26.2)	149 (34.2)	
Bachelor's' degree	206 (30.3)	83 (34.0)	123 (28.2)	
Masters' degree or higher	191 (28.1)	70 (28.7)	121 (27.8)	
Household income, n (%)				0.129
<100.000 DKK	67 (9.6)	34 (13.0)	33 (7.6)	
100.000-150.000 DKK	170 (24.3)	63 (24.0)	107 (24.5)	
150.000-200.000 DKK	200 (28.6)	68 (26.0)	132 (30.2)	
200.000-250.000 DKK	157 (22.5)	57 (21.8)	100 (22.9)	
>250.000 DKK	105 (15.0)	40 (15.3)	65 (14.9)	
Alcohol intake in pregnancy, yes, n (%)	101 (14.5)	35 (13.4)	66 (15.1)	0.411
Smoking in pregnancy, yes, n (%)	54 (7.7)	24 (9.1)	30 (6.9)	0.242
Parity, n (%)				0.071
1	323 (46.1)	132 (50.2)	191 (43.7)	
2	267 (38.1)	86 (32.7)	181 (41.4)	
≥3	110 (15.7)	45 (17.1)	65 (14.9)	
Maternal pre-pregnancy BMI, mean (SD)	24.55 (4.39)	24.19 (4.18)	24.76 (4.50)	0.070
Maternal Western dietary pattern PC, mean (SD)	-0.1 (0.99)	-0.12 (1.05)	0.05 (0.96)	0.046
Maternal age, y, mean (SD)	32.28 (4.36)	32.67 (4.62)	32.05 (4.18)	0.069
Paternal age, y, mean (SD)	34.51 (5.27)	34.48 (5.52)	34.53 (5.13)	0.629
Vitamin D intervention, yes, n (%)	297 (50.9)	122 (55.5)	175 (48.1)	0.069
Fish oil intervention, yes, n (%)	347 (49.7)	114 (43.5)	233 (53.4)	0.014

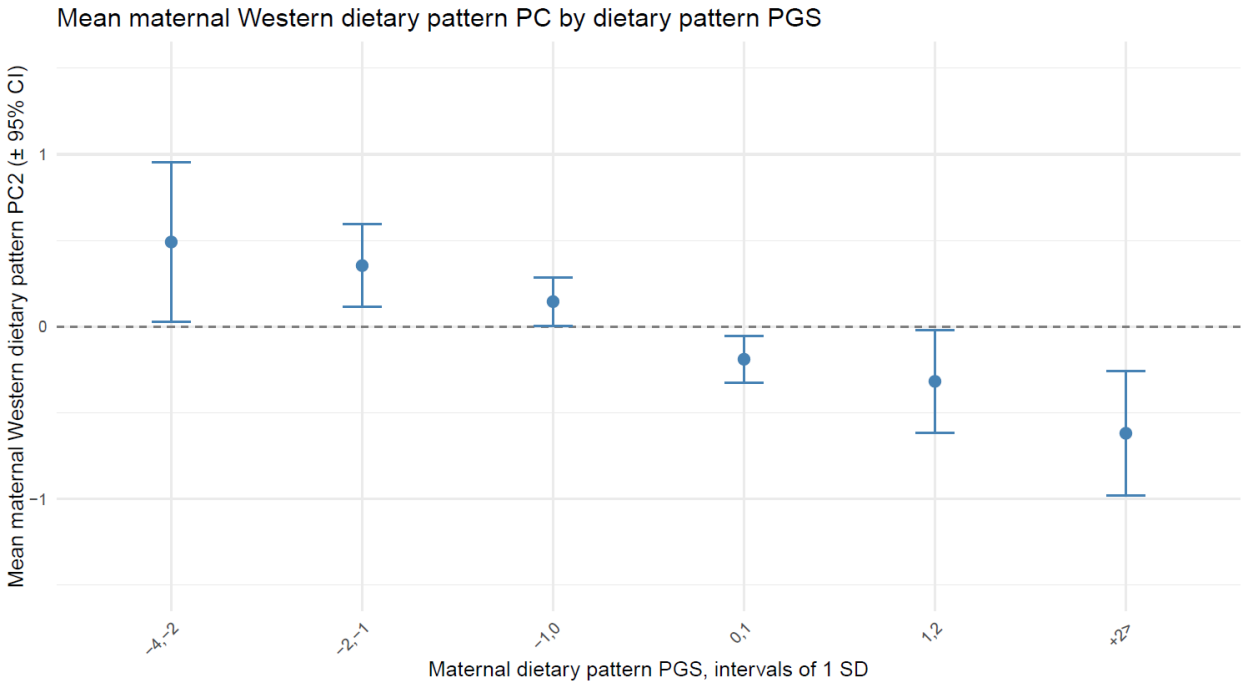
Supplementary Figure 2. Top 20 food groups associated with the maternal dietary pattern PGS (reversed so that higher PGS associates with an unhealthier diet) and their association with COPSAC maternal Western dietary pattern PC. The maternal Western dietary pattern PC has been presented previously by Horner et al and associated to neurodevelopmental conditions.[3]



Supplementary Figure 3. Top 20 food groups associated with the COPSAC maternal Western dietary pattern PC and their association with maternal dietary pattern PGS (reversed so that higher PGS associates with an unhealthier diet). The maternal Western dietary pattern PC has been presented previously by Horner et al and associated to neurodevelopmental conditions.[3]



Supplementary Figure 4. Mean maternal Western dietary pattern PC according to intervals of maternal dietary pattern PGS.

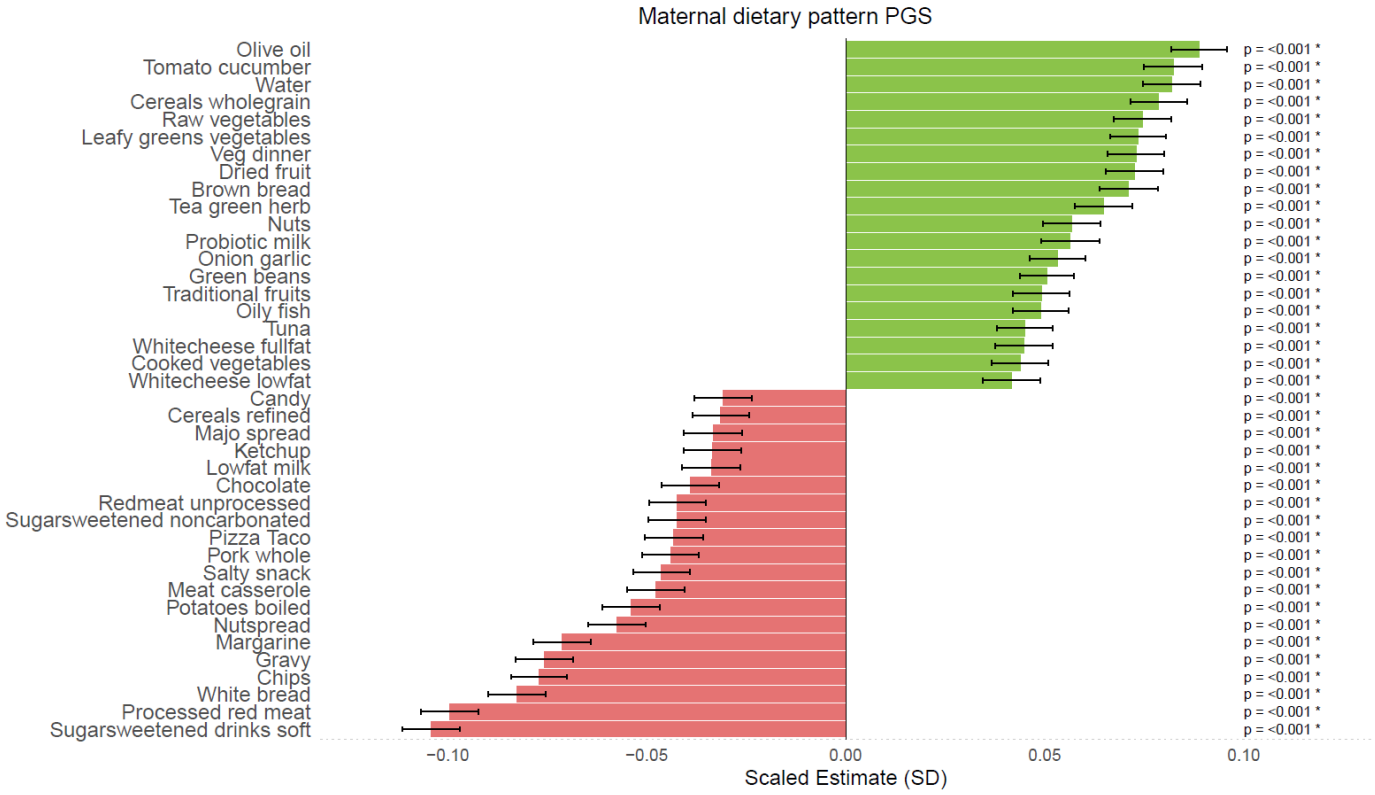


Supplementary Table 2: Association between selected social circumstance and prenatal exposure variables and maternal dietary pattern PGS

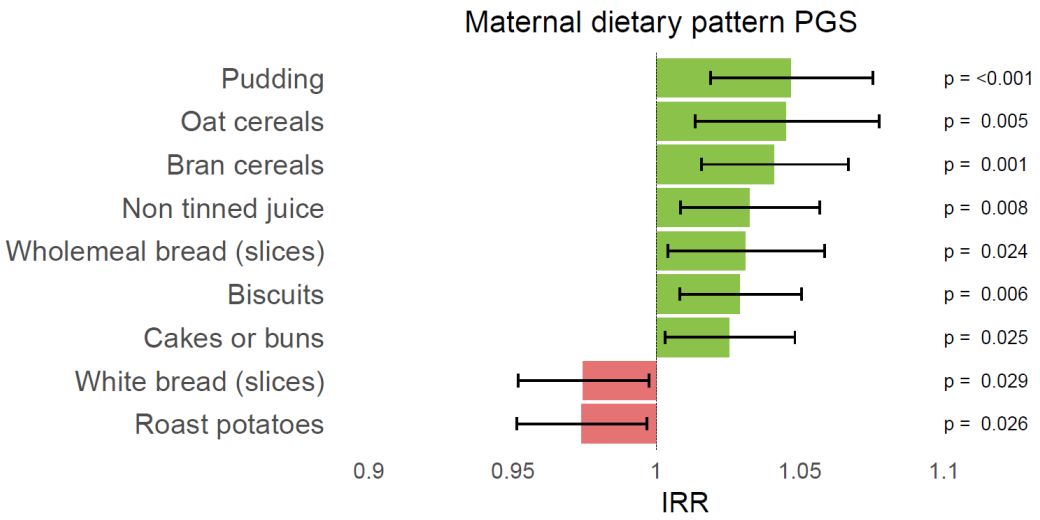
	n	β [95%CI]	p-value
Sex, male	597	-0.04 [-0.20;0.13]	0.657
Child birth weight, kg	597	0.04 [-0.11;0.20]	0.610
Child gestational age, days	597	0.00 [-0.01;0.00]	0.382
Maternal smoking in pregnancy	597	-0.41 [-0.73;-0.10]	0.010
Maternal alcohol intake in pregnancy	595	0.00 [-0.23;0.24]	0.982
Maternal parity, n (1, 2, ≥3)	597	0.03 [-0.09;0.14]	0.608
Maternal use of antibiotics in pregnancy	596	0.06 [-0.11;0.23]	0.481
Maternal pre-eclampsia in pregnancy	596	0.01 [-0.39;0.41]	0.960
Paternal age at birth, years	583	0.02 [-0.00;0.03]	0.064
Maternal CRP at pregnancy week 24, log2 transformed	594	-0.03 [-0.09;0.02]	0,228
Maternal glycA at pregnancy week 24	595	-0.61 [-1.43;0.20]	0,137
Maternal pre-pregnancy BMI	594	0.00 [-0.02;0.02]	0.999
Maternal age at child birth, years	597	0.01 [-0.01;0.03]	0.220
Maternal education at child birth	597		0.002*
Elementary		Ref	
College		0.18 [-0.41;0.77]	
Tradesman		0.08 [-0.42;0.57]	
Medium		0.25 [-0.22;0.73]	
University		0.54 [0.06;1.02]	
Paternal education at child birth	582		<0.001*
Elementary		Ref	
College		0.81 [0.29;1.33]	
Tradesman		0.36 [-0.07;0.78]	
Medium		0.53 [0.11;0.96]	
University		0.69 [0.27;1.12]	
Household income at child birth	596		0.312*
<100.000		-0.08 [-0.41;0.25]	
100.000-150.000		Ref	
150.000-200.000		0.11 [-0.11;0.34]	
200.000-250.000		0.13 [-0.11;0.37]	
>250.000		0.25 [-0.03;0.52]	

Linear regression model used to test association between continuous covariates and dietary pattern polygenic score.
BMI = Body Mass Index. CRP = C-reactive protein. *P-value from likehood ratio test

Supplementary Figure 5. Maternal dietary pattern polygenic score (PGS) plotted against maternal diet as reported by food frequency questionnaires in the MoBa cohort. The plot depicts the 20 most positively and the 20 most negatively associated food groups. FDR significant p-values are marked with an asterisk (*).



Supplementary Figure 6. Maternal dietary pattern polygenic score (PGS) plotted against maternal diet as reported by food frequency questionnaires in the ALSPAC cohort. Only nominal significant associations were included in the figure. None of the associations were significant after FDR-correction.



Supplementary Table 3: Association between maternal, paternal, and child dietary pattern PGS and offspring ADHD diagnosis and ADHD-RS traits in the COPSAC$_{2010}$ cohort

	Maternal PGS n = 511		**Paternal PGS** n = 449		**Child PGS** n = 528	
	OR [95%CI]	**p-value**	**OR [95%CI]**	**p-value**	**OR [95%CI]**	**p-value**
ADHD	0.71 [0.53;0.93]	0.015	0.77 [0.57;1.03]	0.085	0.55 [0.41;0.73]	<0.001
ADHD Inattentive presentation	0.76 [0.51;1.12]	0.174	0.77 [0.51;1.16]	0.206	0.56 [0.37;0.82]	0.004
ADHD Combined presentation	0.71 [0.49;1.00]	0.055	0.81 [0.55;1.19]	0.279	0.60 [0.41;0.86]	0.006
	Maternal PGS n = 513		**Paternal PGS** n = 450		**Child PGS** n = 530	
	β [95%CI]	**p-value**	**β [95%CI]**	**p-value**	**β [95%CI]**	**p-value**
ADHD-RS Total traits	-1.23 [-1.93;-0.53]	<0.001	-0.50 [-1.30;0.29]	0.212	-1.10 [-1.82;-0.38]	0.003
ADHD-RS Inattentive traits	-0.54 [-0.98;-0.11]	0.015	-0.25 [-0.74;0.24]	0.313	-0.65 [-1.10;-0.21]	0.004
ADHD-RS Impulsivity/Hyperactivity traits	-0.69 [-1.03;-0.35]	<0.001	-0.25 [-0.64;0.13]	0.196	-0.45 [-0.80;-0.10]	0.012

All analyses were adjusted for child sex

ADHD: Attention deficit hyperactivity disorder. ADHD-RS: ADHD Rating Scale. CI: Confidence Interval. OR: Odds ratio. PGS: polygenic score.

Supplementary Table 4: Trio models of the association between maternal, paternal, and child dietary pattern PGS and offspring ADHD diagnosis and ADHD-RS traits in the COPSAC$_{2010}$ cohort

	Maternal PGS Trio model* n = 437		**Paternal PGS Trio model**** n = 437		**Child PGS Trio model***** n = 437	
	OR [95%CI]	**p-value**	**OR [95%CI]**	**p-value**	**OR [95%CI]**	**p-value**
ADHD	0.98 [0.69;1.38]	0.893	1.05 [0.73;1.53]	0.780	0.58 [0.38;0.88]	0.011
ADHD Inattentive presentation	1.1 [0.67;1.78]	0.702	1.06 [0.63;1.78]	0.825	0.57 [0.31;1.02]	0.063
ADHD Combined presentation	0.89 [0.56;1.39]	0.603	1.03 [0.64;1.67]	0.893	0.65 [0.37;1.10]	0.111
	Maternal PGS Trio model* n = 438		**Paternal PGS Trio model**** n = 438		**Child PGS Trio model***** n = 438	
	β [95%CI]	**p-value**	**β [95%CI]**	**p-value**	**β [95%CI]**	**p-value**
ADHD-RS Total traits	-1.15 [-2.06;-0.23]	0.015	-0.34 [-1.35;0.68]	0.514	-0.14 [-1.25;0.98]	0.810
ADHD Inattentive traits	-0.35 [-0.92;0.23]	0.235	0.00 [-0.63;0.63]	0.995	-0.33 [-1.03;0.36]	0.345
ADHD Impulsivity/Hyperactivity traits	-0.80 [-1.24;-0.36]	<0.001	-0.34 [-0.83;0.15]	0.171	0.20 [-0.34;0.74]	0.467

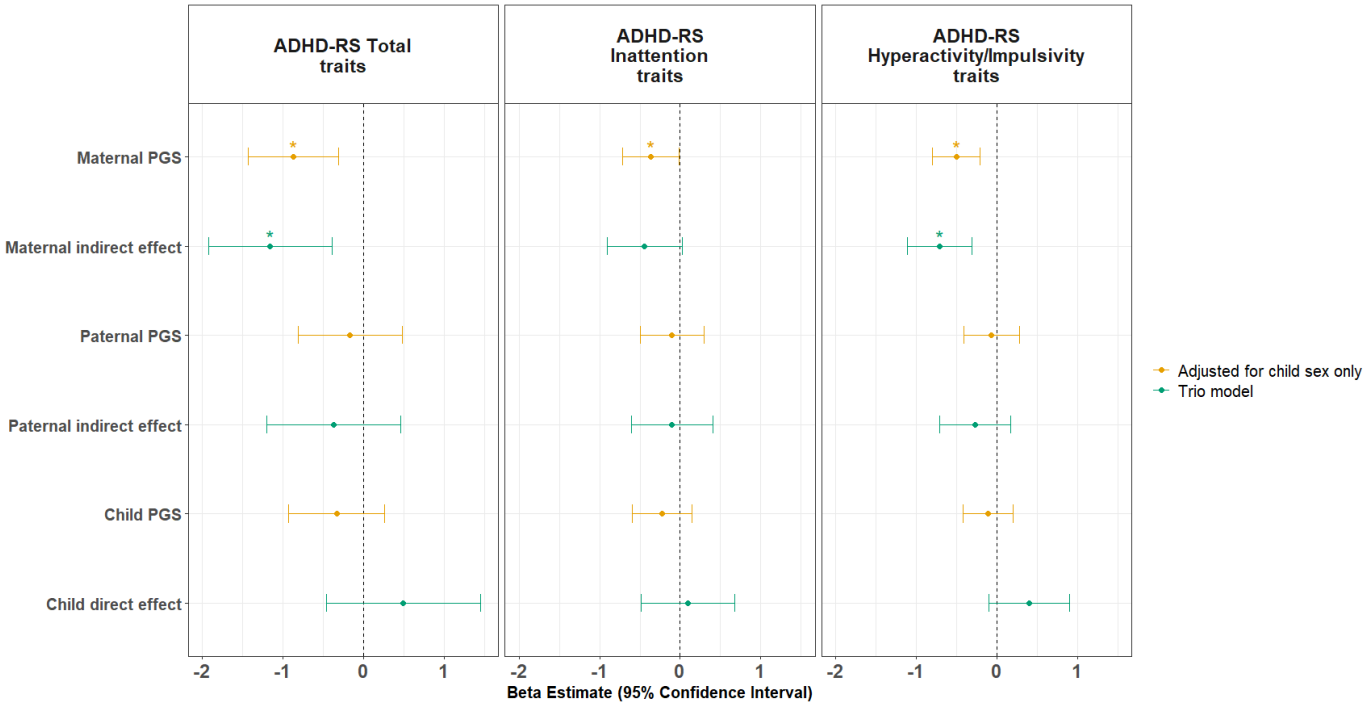
*Adjusted for child sex and child PGS

**Adjusted for sex, maternal PGS and child PGS

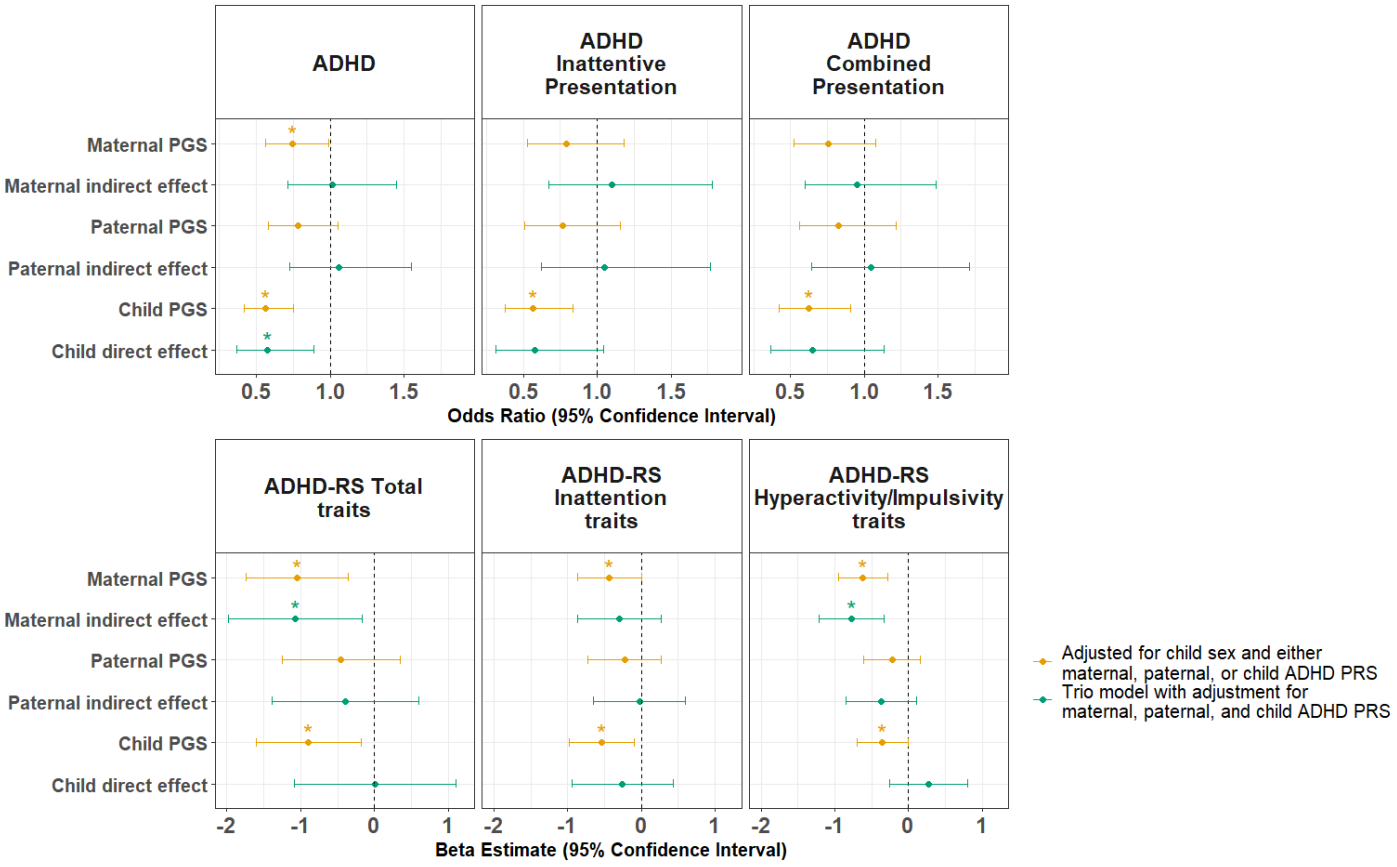
***Adjusted for sex, maternal PGS and paternal PGS

ADHD: Attention deficit hyperactivity disorder. ADHD-RS: ADHD Rating Scale. CI: Confidence Interval. OR: Odds ratio. PGS: polygenic score.

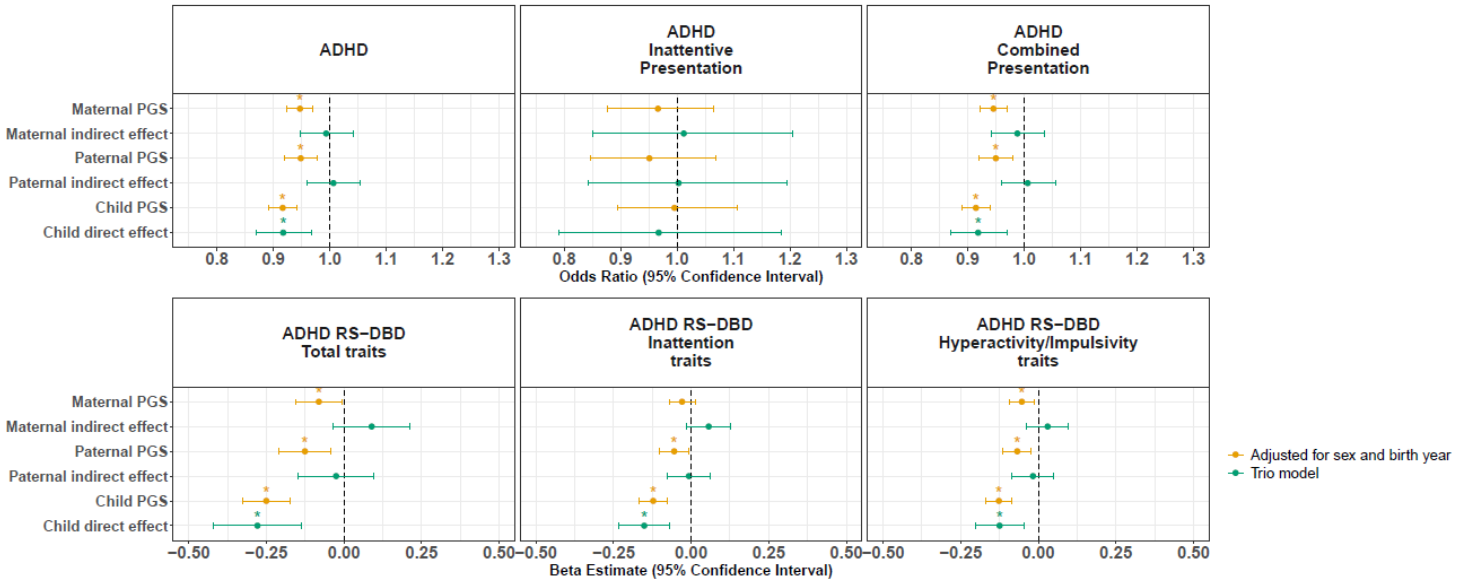
Supplementary Figure 7. Unadjusted and trio models of the dietary pattern PGS association to ADHD-RS traits after exclusion of individuals with a diagnosis of ADHD in the $COPSAC_{2010}$ cohort



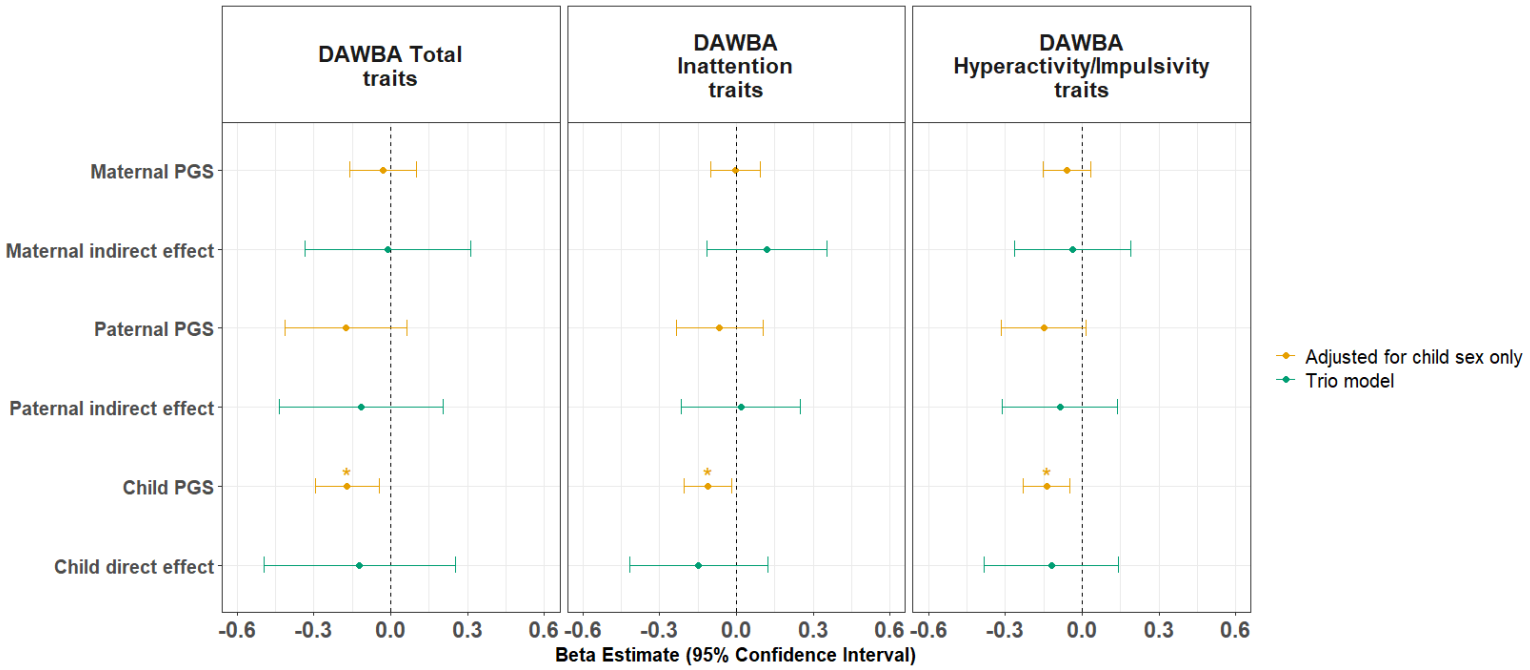
Supplementary Figure 8. Unadjusted and trio models of the dietary pattern PGS association to ADHD diagnosis and ADHD-RS traits in the $COPSAC_{2010}$ cohort including additional adjustment for child, maternal, and paternal ADHD PGS



Supplementary Figure 9. Unadjusted and trio models of the association between dietary pattern PGS and NPR ADHD diagnosis as well as ADHD RS-DBD traits in the MoBa cohort



Supplementary Figure 10. Unadjusted and trio models of the association between dietary pattern PGS and DAWBA ADHD traits in the ALSPAC cohort



Supplementary Table 5: Association between maternal, paternal, and child dietary pattern PGS and offspring ADHD NPR diagnosis and RS-DBD ADHD traits in the MoBa cohort

	Maternal PGS n = 91,931		Paternal PGS n = 62,353		Child PGS n = 75,342	
	OR [95%CI]	**p-value**	**OR [95%CI]**	**p-value**	**OR [95%CI]**	**p-value**
ADHD	0.95 [0.92;0.97]	<0.001	0.95 [0.92;0.98]	<0.001	0.92 [0.89;0.94]	<0.001
ADHD Inattentive presentation	0.97 [0.88;1.06]	0.485	0.95 [0.85;1.07]	0.401	1.00 [0.90;1.11]	0.933
ADHD Combined presentation	0.94 [0.92;0.97]	<0.001	0.95 [0.92;0.98]	0.001	0.91 [0.89;0.94]	<0.001
	Maternal PGS n = 36,131		**Paternal PGS** n = 26,619		**Child PGS** n = 31,191	
	β [95%CI]	**p-value**	**β [95%CI]**	**p-value**	**β [95%CI]**	**p-value**
RS-DBD Total traits	-0.08 [-0.15;-0.01]	0.031	-0.13 [-0.21;-0.04]	0.003	-0.25 [-0.33;-0.17]	<0.001
RS-DBD Inattentive traits	-0.03 [-0.98;-0.11]	0.173	-0.05 [-0.10;-0.01]	0.026	-0.12 [-0.17;-0.08]	<0.001
RS-DBD Impulsivity/Hyperactivity traits	-0.05 [-0.09;-0.01]	0.009	-0.07 [-0.11;-0.02]	0.003	-0.13 [-0.17;-0.08]	<0.001

All analyses were adjusted for child sex and birth year

ADHD: Attention deficit hyperactivity disorder. CI: Confidence Interval. NPR: Norwegian patient registry. OR: Odds ratio. PGS: polygenic score. RS-DBD: Disruptive Behavior Disorders Rating Scale.

Supplementary Table 6: Trio models of the association between maternal, paternal, and child dietary pattern PGS and offspring ADHD NPR diagnosis and RS-DBD ADHD traits in the MoBa cohort

	Maternal PGS Trio model* n = 41,580		**Paternal PGS Trio model**** n = 41,580		**Child PGS Trio model***** n = 41,580	
	OR [95%CI]	**p-value**	**OR [95%CI]**	**p-value**	**OR [95%CI]**	**p-value**
ADHD	0.99 [0.95;1.04]	0.784	1.01 [0.96;1.05]	0.790	0.92 [0.87;0.96]	0.002
ADHD Inattentive presentation	1.01 [0.85;1.20]	0.894	1.00 [0.84;1.194]	0.975	0.97 [0.79;1.18]	0.749
ADHD Combined presentation	0.99 [0.94;1.04]	0.613	1.01 [0.96;1.06]	0.802	0.92 [0.87;0.97]	0.002
	Maternal PGS Trio model* n = 18,629		**Paternal PGS Trio model**** n = 18,629		**Child PGS Trio model***** n = 18,629	
	β [95%CI]	**p-value**	**β [95%CI]**	**p-value**	**β [95%CI]**	**p-value**
RS-DBD Total traits	0.09 [-0.03;0.21]	0.156	-0.03 [-0.15;0.10]	0.686	-0.28 [-0.42;-0.14]	<0.001
RS-DBD Inattentive traits	0.06 [-0.01;0.13]	0.117	-0.01 [-0.08;0.06]	0.843	-0.15 [-0.20;-0.05]	0.002
RS-DBD Impulsivity/Hyperactivity traits	0.03 [-0.04;-0.10]	0.382	-0.02 [-0.08;0.05]	0.613	-0.12 [-0.80;-0.10]	0.012

*Adjusted for sex, birth year, and child PGS
**Adjusted for sex, birth year, maternal PGS, and child PGS
***Adjusted for sex, birth year, maternal PGS, and paternal PGS

ADHD: Attention deficit hyperactivity disorder. CI: Confidence Interval. NPR: Norwegian patient registry. OR: Odds ratio. PGS: polygenic score. RS-DBD: Disruptive Behavior Disorders Rating Scale.

Supplementary Table 7: Association between maternal, paternal, and child dietary pattern PGS and offspring DAWBA ADHD traits in the ALSPAC cohort

	Maternal PGS n = 5,529		**Paternal PGS** n = 5,967		**Child PGS** n = 1,498	
	β [95%CI]	**p-value**	**β [95%CI]**	**p-value**	**β [95%CI]**	**p-value**
DAWBA Total traits	-0.03 [-0.16;0.10]	0.650	-0.18 [-0.41;0.06]	0.146	-0.17 [-0.30;-0.05]	0.008
DAWBA Inattentive traits	-0.00 [-0.10;0.09]	0.954	-0.07 [-0.24;0.10]	0.447	-0.11 [-0.20;-0.02]	0.020
DAWBA Impulsivity/Hyperactivity traits	-0.06 [-0.15;0.03]	0.206	-0.15 [-0.31;0.02]	0.078	-0.14 [-0.23;-0.05]	0.003

All analyses were adjusted for child sex

ADHD: Attention deficit hyperactivity disorder. CI: Confidence Interval. DAWBA: Development and Well-Being Assessment. PGS: polygenic score.

Supplementary Table 8: Trio models of the association between maternal, paternal, and child dietary pattern PGS and offspring DAWBA ADHD traits in the ALSPAC cohort

	Maternal PGS Trio model* n = 1,199		**Paternal PGS Trio model**** n = 1,199		**Child PGS Trio model***** n = 1,199	
	β [95%CI]	**p-value**	**β [95%CI]**	**p-value**	**β [95%CI]**	**p-value**
DAWBA Total traits	-0.01 [-0.34;-0.31]	0.946	-0.12 [-0.44;0.20]	0.473	-0.12 [-0.50;-0.25]	0.522
DAWBA Inattentive traits	0.12 [-0.12;0.35]	0.324	0.02 [-0.22;0.25]	0.886	-0.15 [-0.42;0.12]	0.283
DAWBA Impulsivity/Hyperactivity traits	-0.04 [-0.27;0.19]	0.747	-0.09 [-0.31;0.14]	0.450	-0.12 [-0.38;0.14]	0.450

*Adjusted for child sex and child PGS
**Adjusted for sex, maternal PGS and child PGS
***Adjusted for sex, maternal PGS and paternal PGS

ADHD: Attention deficit hyperactivity disorder. CI: Confidence Interval. DAWBA: Development and Well-Being Assessment. PGS: polygenic score.

PAPER IV

Inflammation in pregnancy and child neurodevelopment: A trio polygenic score and Mendelian randomization study in the Norwegian Mother, Father and Child Cohort

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Inflammation in pregnancy and child neurodevelopment: A trio polygenic score and Mendelian randomization study in the Norwegian Mother, Father and Child Cohort

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collection, management, and interpretation of the data; or preparation, review, or approval of the manuscript. No pharmaceutical company was involved in the study. BE is part of the Advisory Board of Boehringer Ingelheim, Lundbeck Pharma, and Orion Pharma; and has received lecture fees from Boehringer Ingelheim, Otsuka Pharma Scandinavia AB, and Lundbeck Pharma (ORCID: 0000-0002-2590-5055).

Governance: We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. Privacy is important to us which is why we follow national and international legislation on General Data Protection Regulation (GDPR), the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

Abbreviations:

ADHD = attention-deficit/hyperactivity disorder

ASQ = Ages and Stages Questionnaire

BMI = body mass index

CBCL = Child Behavior Checklist

CDI = Child Development Inventory

CRP = C-reactive protein

COPSAC = Copenhagen Prospective Studies on Asthma in Childhood

FDR = false discovery rate

GlycA = glycoprotein acetyls

GSMR = Generalized Summary-data-based Mendelian Randomization

GWAS = genome wide association study

HEIDI = HEterogeneity In Dependent Instrument

ICD-10 = International Classification of Diseases, 10th Revision

IL-6 = interleukin-6

IVW = inverse variance weighted

LD = linkage disequilibrium

MIA = maternal immune activation

MoBa = Norwegian Mother, Father and Child Cohort Study

NEB = Norwegian environmental biobank

NMR = Nuclear Magnetic Resonance

NPR = Norwegian patient registry

OR = odds ratio

OSF = Open Science Framework

PGS = polygenic score

PC = principal component

RS-DBD = Rating Scale for Disruptive Behavior Disorders

SCQ = Social Communication Questionnaire

SNP = single nucleotide polymorphism

SSB = Statistics Norway

ABSTRACT

Background: Inflammation in pregnancy induced by chronic disease, infection, or environmental exposures has been associated with neurodevelopmental conditions in observational study designs.

Objective: To investigate causal effects of pregnancy inflammation on offspring neurodevelopment in the Norwegian Mother, Father and Child Cohort Study (MoBa) using a genetically informed design.

Methods: In pre-registered analyses, we tested potential causal effects of increased C-reactive protein (CRP), interleukin-6 (IL-6), and glycoprotein acetyls (GlycA) in pregnancy on neurodevelopmental outcomes using trio polygenic score (PGS) and intergenerational Mendelian randomization (MR) analyses. We first sought to validate PGSs and MR genetic instruments against measured pregnancy inflammatory markers in the $COPSAC_{2010}$ cohort and in a MoBa subsample. PGS and MR genetic instruments were used to predict neurodevelopmental outcomes in the MoBa cohort, which included mother-reported neurodevelopmental traits registered from age 3 to 8 and lifetime diagnoses of ADHD and autism. In both the trio PGS and intergenerational MR analyses, simultaneous inclusion of mothers', fathers', and children's genotypes allowed for effects consistent with the hypothesized causal pathway (maternal inflammation) to be estimated independent of familial confounding factors.

Results: Validation analyses of genetic instruments only showed strong predictive value for CRP. Trio PGS in 41,531 complete trios did not reveal maternal indirect effects of genetic liability to inflammation on offspring neurodevelopmental outcomes. The results of the trio MR also failed to support a causal link between maternal inflammation during pregnancy and offspring neurodevelopment.

Conclusion: This study provided no evidence consistent with causal effects of maternal inflammation in pregnancy on offspring neurodevelopment.

Keywords, MeSH: pregnancy, inflammation, Mendelian randomization, MoBa, MBRN

INTRODUCTION

Neurodevelopmental conditions are thought to originate from early alterations of brain development driven by both environmental exposures and underlying genetic predispositions.[1] These conditions present early in life and affect functioning in several neuropsychological domains to varying degrees and with wide variability in support needs.[2,3] Neurodevelopment can be understood through a dimensional perspective, where individuals reaching the threshold for a diagnosis present with a significantly high degree of traits.[1] Genetic and environmental predisposing factors have been shown to influence both the development of traits and the likelihood of a diagnosis of the common neurodevelopmental conditions attention-deficit/hyperactivity disorder (ADHD) and autism.[1] The substantial genetic influence challenges the causality of early life predisposing factors reported in observational studies, as genetic predisposition may serve as a confounder affecting both the measured exposure and the neurodevelopmental outcome.[4]

The maternal immune activation (MIA) hypothesis, supported by evidence from animal models and epidemiological studies of pregnant women, proposes that maternal inflammatory dysregulation in pregnancy disrupts fetal neurodevelopment.[5] Various factors may stimulate maternal immune response during pregnancy and increase the likelihood of neurodevelopmental conditions in offspring. These factors include chronic inflammatory states such as autoimmune disorders, gestational diabetes, obesity, smoking and stress as well as acute inflammatory factors i.e. infections.[6] Results from animal models suggest that the effect is independent of the risk factors that drive inflammation.[5]

Inflammation reflected by the level of circulating inflammatory markers (e.g. C-reactive protein (CRP) and interleukin 6 (IL-6)) in pregnancy has been linked to an increased likelihood of offspring neurodevelopmental conditions. Large register and cohort studies have investigated the association between CRP and neurodevelopmental conditions including ADHD and autism with inconsistent results.[7–10] In the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC), there was a

robust association between CRP measured in week 24 of pregnancy and both ADHD diagnosis and trait scores with a clear dose-response relationship suggesting causal effects.[11] IL-6 levels in pregnancy and neonatally have likewise been associated with neurodevelopmental conditions, ADHD traits and autism likelihood.[12–14] Pregnancy IL-6 has further been associated with impaired executive functioning - a cognitive domain frequently impaired in neurodevelopmental conditions.[15,16] Glycoprotein acetyls (GlycA) is a newer metabolomic marker that to a higher extent reflects chronic inflammation.[17,18] Measures of GlycA in pregnancy have been associated with neurodevelopmental delay and lower cognition scores in the offspring.[8,19]

Maternal inflammation in pregnancy is associated with non-infectious inflammatory risk factors influenced by lifestyle, anthropometrics and genetic disposition. Given that genetic dispositions are directly transmitted from mothers to their children, this represents a potential source of confounding in reported associations between maternal inflammation in pregnancy and offspring neurodevelopmental conditions in observational studies. The high heritability of neurodevelopmental conditions, and the demonstrably broad associations of the common genetic variants contributing to this heritability increase the likelihood of a genetic confounding pathway playing some role in these associations.[4,20,21] One way to assess the role of genetic confounding in intergenerational associations is to use polygenic scores (PGS) in trio analyses. In such models, the direct effect of children's own genetic liabilities for prenatal exposures - i.e., the confounding pathway - can be estimated; while any causal effects of the exposure are estimated as part of maternal indirect genetic effects.[22] The heritability of CRP and IL-6 have previously been reported to respectively 21% and 45% in a twin-family design suggesting that genetic studies are a useful tool to study these traits.[23] Causal effects can be further investigated in intergenerational MR analyses, which extend trio PGS models with stricter assumptions to attempt to isolate causal mechanisms.[24] These approaches have previously been applied to investigate predisposing factors for ADHD as well as causal effects of intrauterine growth and pregnancy levels of vitamin D and fish oil on later neurodevelopmental traits.[22,25,26]

We set out to investigate potential causal effects of pregnancy inflammation on offspring neurodevelopment. We used trio PGS analyses and intergenerational MR analyses in the Norwegian Mother Father and Child Cohort study (MoBa), accounting for various sources of confounding to robustly assess the genetic evidence for a causal mechanism linking these traits.

METHODS

Study population

The Norwegian Mother, Father and Child Cohort Study (MoBa) is a population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health.[27–29] Participants were recruited from all over Norway from 1999-2008. The women consented to participation in 41% of the pregnancies. The cohort includes approximately 114,500 children, 95,200 mothers and 75,200 fathers. MoBa is regulated by the Norwegian Health Registry Act. Written informed consent was obtained from participating mothers and fathers at enrollment. The current study was approved by The Regional Committees for Medical and Health Research Ethics (2016/1702). The COPSAC2010 cohort was used for validation of inflammatory PGSs utilized in this project. The COPSAC2010 cohort is an ongoing cohort study of 700 mother-child pairs followed since week 24 of pregnancy [30] conducted in accordance with the guiding principles of the Declaration of Helsinki and approved by the Local Ethics Committee (H-B-2008-093), and the Danish Data Protection Agency (2015-41-3696). Written informed consent was obtained from both parents prior to enrollment.

Inflammation polygenic scores

We used genetic data from the MoBa cohort derived from blood samples drawn during pregnancy from both parents and from the child's umbilical cord at birth. Genotyping and QC procedures for the MoBa cohort which contains interrelated families have been described previously.[31] Polygenic scores for CRP, IL-6, and GlycA were generated using LDpred2 restricting to only well-imputed SNPs (INFO score >0.95)[32–34], based on publicly available GWAS summary statistics comprising 575,531 individuals of European ancestry for CRP [32,35], 21,758 for IL-6 [33], and 115,078 for GlycA.[34] PGSs for the MoBa cohort were corrected for genotyping and imputation batch and population structure (first 20 PCs).[36] In

COPSAC, PGSs for validation against measured inflammatory markers in pregnancy were constructed from the same summary statistics using the comparable method PRS-CS.[37]

Instrumental variables for intergenerational Mendelian randomization

The same GWAS summary statistics were used as the basis of the instrumental variables for intergenerational Mendelian randomization (MR) analyses. This time, we created PGSs for each exposure using only independent (kb 250, r_2 0.10) SNPs that were genome-wide significant ($p < 5 \times 10^{-8}$) in original discovery efforts. The main assumptions of MR analyses are as follows: 1) the instrumental variable must be robustly associated with the exposure, 2) there is no confounding of the instrumental variable and the outcome, and 3) the instrumental variable only affects the outcome through the exposure.[25,38] To test the first assumption, we validated the exposures in pregnancy using inflammatory data available in MoBa and COPSAC2010. Neither of the remaining MR assumptions are directly testable. Correcting the MR PGSs for population structure (first 20 PCs) and performing analyses in a within-family design reduces potential bias from ancestry/population stratification and assortative mating and goes some way towards satisfying the second assumption, while the restriction to a small number of robustly associated variants is designed to reduce the risk of horizontal pleiotropy (Figure 1) and ensure that the third assumption is also plausibly met. All steps were performed in PRSice2.[39]

Inflammation markers in MoBa and the COPSAC cohort

We validated inflammation PGSs and MR instrumental variables in the COPSAC2010 cohort using information on pregnancy inflammatory markers of CRP, IL-6 and GlycA. Maternal blood samples were obtained at gestational week 24 and stored at -80 °C after centrifugation. High sensitivity-CRP (hs-CRP) and IL-6 were measured in plasma using a high-sensitive electrochemiluminescence-based immunoassay from Meso Scale Discovery. The lower level of detection was 0.007 ng/mL for hs-CRP and 0.178 pg/mL for IL-6.[11,15] GlycA was derived by Nuclear Magnetic Resonance (NMR) Spectroscopy from EDTA

plasma samples performed by Nightingale Health. The measured NMR GlycA signal reflects the level of several acute-phase proteins and has been shown to have a higher long-term stability than CRP and is a newly introduced biomarker reflecting systemic inflammation.[40] For validation of CRP genetic instruments, we further utilized data from a MoBa cohort subsample with available measurements of plasma hs-CRP from mid-pregnancy collected around gestational week 18 at a routine ultrasound visit (See Supplementary Methods). This data was collected as a part of the Norwegian Environmental Biobank (NEB) project. The NEB is a sub-study within the MoBa cohort established with the aim of biomonitoring nutrients and environmental contaminants in MoBa participants. The study included 2,999 pregnant women with available genetic data who had donated blood and urine samples and had responded to pregnancy questionnaires and child questionnaires until age 3 (questionnaire 1-6) in MoBa.[27,41]

Neurodevelopmental measures in the MoBa cohort

Mother reported neurodevelopmental traits

Developmental evaluations of language and motor skills were obtained using the Ages and Stages Questionnaire (ASQ) and the Child Development Inventory (CDI). From the ASQ, we included language-related items at ages 3 (6 items) and 5 years (7 items), as well as motor-related items at age 3 years (4 items).[42] From the CDI, we included 12 motor-related items assessed at age 5.[43] ADHD-related traits were assessed using the ADHD scale from the Child Behaviour Checklist (CBCL) at ages 3 and 5, each including 6 items.[44] At age 8, ADHD-related traits were obtained from 18 items from the Rating Scale for Disruptive Behavior Disorders (RS-DBD).[45] Autism-related traits were measured using the Social Communication Questionnaire (SCQ) at ages 3 and 8, each consisting of 40 items.[46] The SCQ was further divided into subscales reflecting repetitive behaviours (11 items) and social-communication deficits (22 items). For all scores, higher values represent more problems/fewer skills achieved.

Scale scores were calculated by multiplying the mean of available items by the total number of items in the scale, provided that ≥50% of item responses were non-missing.

Diagnoses of autism and ADHD

From the Norwegian Patient Registry (NPR), we included lifetime diagnoses of ADHD and autism (registered between 2008-2023). ADHD was defined as having at least two registered ICD-10 diagnostic codes within categories F90 or F98.8. Autism was defined as having at least two registered ICD-10 codes within category F84.[47]

Statistical analyses

Validation of PGS and MR instruments

Inflammation PGSs and MR instruments for CRP, IL-6, and GlycA were validated against measurement of the corresponding inflammatory marker by linear regression models (without additional covariate adjustment) from which we evaluated both regression estimates and F-statistics. No transformation of maternal inflammation markers was performed in these models.

In the main models described below, we included all inflammation PGS/MR instruments (CRP, IL-6, and GlycA) as predictors in each model – regardless of whether we were able to directly validate them. *Post hoc* sensitivity analyses were restricted to demonstrably valid genetic instruments.

Trio-PGS analyses

After validation, we first performed unadjusted PGS analyses (i.e., regressing outcomes on maternal PGS without accounting for child and paternal PGSs). Multiple linear regression was used to model associations between maternal PGSs and continuous (scale) outcomes, while multiple logistic regression was applied for binary diagnostic outcomes. Next, we expanded the models to additionally include child

and paternal PGS as predictors, allowing us to isolate maternal indirect genetic effects consistent with a causal role for inflammation during pregnancy on offspring neurodevelopmental traits and diagnoses in the MoBa cohort. All models included adjustment for year of birth and child's sex.

Intergenerational MR analyses

As a final step in our investigation of possible causality in links between maternal inflammation during pregnancy and offspring neurodevelopmental outcomes, we ran intergenerational MR analyses. These are structurally equivalent to the trio PGS analyses described above, but were conducted using maternal, paternal and child MR instruments for CRP, IL-6 and GlycA. Because these instruments are restricted to only those genetic variants most strongly linked to inflammation, maternal indirect genetic effect estimates from these models are less vulnerable to confounding via horizontal pleiotropy (i.e., effects of genetic variants on outcomes that do not operate through inflammation).

Figure 1 provides an overview of the confounding factors affecting observational studies of maternal pregnancy effects on offspring neurodevelopment (left-most panel) and illustrates how they are progressively controlled for in our analytic pathway.

Two sample MR

To supplement the main analytical pipeline, we conducted two sample MR to investigate a specific potential source of genetic confounding in the observed intergenerational link between inflammation and neurodevelopment - namely, the upstream causal influence of maternal neurodevelopmental conditions on their own inflammation. Bidirectional Mendelian Randomization (MR) analyses were conducted using the Generalized Summary-data-based Mendelian Randomization (GSMR) method implemented in the GCTA software toolkit (version v1.94.1)[48], corresponding to the standard inverse variance weighted (IVW) MR.[49] The approach was used to investigate the potential causal relationship – within individuals – between inflammation markers (IL6[33], GlycA[34], and CRP[32,35]) and ADHD[50] and autism[51] in both

directions (See Supplementary Methods). We included three MR sensitivity methods: MR Egger, weighted median, and weighted mode (R package: TwoSampleMR).

Multiple imputation

Missing information on neurodevelopmental traits was imputed using the mice package from R.[52] Information on covariates (sex and birth year) was complete. A total of 30 datasets were imputed with 10 iterations. Additional variables were added from the Medical Birth Registry Norway (MBRN) (length at birth, weight at birth, maternal age, paternal age) and Statistics Norway (SSB) (household income and parental years of education) to act as auxiliary predictors. The quality of the imputation model was evaluated by visualizing convergence across iterations and the distribution of the final imputed variables. Sensitivity analyses were performed using only complete cases.

Maternal clustering

Trio PGS and intergenerational MR analyses were performed with robust standard errors clustered on maternal ID in order to account for the inclusion of siblings in MoBa. In cases of identical or fraternal twins, only one twin per pair was included in the analyses.

Multiple testing

To account for multiple comparisons, we applied false discovery rate (FDR) correction using the Benjamini-Hochberg method. We applied FDR correction separately to all estimates for maternal genetic effects in trio PGS analyses and the intergenerational MR analyses, based on 45 tests across 15 outcomes. For the two-sample MR, FDR correction was applied based on the number of IVW estimates. All reported p-values are FDR adjusted, and results were considered statistically significant if below the 0.05 threshold after FDR adjustment.

Statistical analyses were performed using R version 4.4.1. Analytic code for the project is publicly available at https://github.com/psychgen/preg-inflammation-neurodev. This project was pre-registered on the Open Science Framework (OSF) (URL: https://osf.io/xrhs3).[53] A deviations Table is provided in the supplementary material (Supplementary Table 1).[54]

RESULTS

A total of 41,531 trios had complete information on genotyping in the MoBa cohort after removing one twin from each pair (n excluded = 2,423, including individuals with missing information on plurality) and children without available information on sex (n = 209). We observed small differences between both exposure and outcome measures between complete trios and the remaining MoBa cohort with consistently lower scores (indicating fewer problems) among complete genotyped trios (See Supplementary Table 2). The prevalence of neurodevelopmental condition diagnoses among complete trios was 2.1% (N = 853) for autism and 6.5% (N = 2,697) for ADHD, compared with 2.1% and 7.3%, respectively, in the remaining cohort. A significant proportion of individuals included in the MoBa cohort did not respond to the follow-up assessments utilized in the present study. The highest proportion of missing data for the questionnaire-based outcomes in the total cohort was 64% for the ASQ at 5 years, and the lowest proportion of missing 48% for the ASQ at age 3.

Validation of genetic instruments for inflammation during pregnancy

The CRP PGS and the CRP MR instrument were validated in a subsample of 2,323 women in MoBa with information on pregnancy CRP (measurements of GlycA and IL-6 were not available). F-statistics (132.8 for the CRP PGS and 60.6 for the CRP MR instrument) were conclusively above 10, a heuristic threshold for weak instrument bias in MR.[55] We additionally sought validation for all inflammation PGSs and MR instrumental variables among 675 individuals in the COPSAC2010 cohort. The results indicated that the CRP (r squared 0.02, F-statistics 14.9) and to some extent also the GlycA MR instruments (r squared 0.02, F-statistics 12.1) derived from non-pregnant populations had a weak predictive value for pregnancy inflammation, though none of the PGS predicted inflammation in this sample. Full information on the validation in both cohorts is available in the Supplementary Information.

Unadjusted maternal PGS analyses

Unadjusted PGS analyses showed associations between maternal CRP PGS and ASQ language scores from 3 and 5 years, SCQ repetitive behaviours at age 8, and ADHD traits measured at 5 and 8 years (p-values > 0.05). Unadjusted PGS analyses on diagnostic outcomes showed an association between maternal CRP PGS and ADHD (OR = 1.06 , 95%CI = 1.03–1.09, p = 0.002,) as well as autism diagnosis (OR = 1.07 , 95%CI = 1.02–1.12, p = 0.063) (Figure 2). There were no associations between non-validated genetic instruments for IL-6 and GlycA and neurodevelopmental outcomes (See Supplementary Table 3 and Supplementary Figures 1-2).

Trio-PGS and intergenerational Mendelian randomization analyses

We did not find evidence of maternal indirect genetic effects of CRP on neurodevelopmental traits (Figure 2) or diagnoses of ADHD (OR = 1.04 , 95%CI = 0.99–1.09, p = 0.867) and autism (OR = 1.04 , 95%CI = 0.95–1.13, p = 0.941) in trio PGS analyses. Consistent with this, intergenerational MR analyses did not provide any evidence for substantive causal effects of inflammation on neurodevelopmental traits (Figure 2) or ADHD diagnoses (OR = 1.00 , 95%CI = 0.95–1.05, p = 0.979) and autism (OR = 1.05 , 95%CI = 0.97–1.15, p = 0.900). Analyses using non-validated genetic instruments, IL-6 and GlycA, also did not provide evidence for causal effects (See Supplementary Table 3 and Supplementary Figures 1-2).

As *post hoc* analyses, we reran both trio PGS and intergenerational MR analyses including only the validated CRP PGS and MR instrument, i.e., without mutually adjusting for IL-6 and GlycA genetic liabilities (Supplementary Table 4-5). The pattern of results did not change. In complete case analyses, there were indications of maternal indirect genetic effects of CRP and IL-6 on child autistic traits, and intergenerational MR showed an effect of higher pregnancy GlycA on autistic trait severity. However, these reported results from complete case analyses were not statistically significant after FDR correction (Supplementary Table 6-7).

Follow-up two sample MR

In two sample MR analyses, we found evidence consistent with a within-person causal effect of ADHD on CRP (IVW: 0.06, SE 0.01, FDR $p < 0.001$), IL-6 (IVW: 0.01, SE 0.04, FDR $p = 0.002$) and GlycA (IVW: 0.04, SE 0.01, FDR $p = 0.02$). Due to fewer than three independent genome-wide significant SNPs, we were unable to test the effect of autism on all three inflammatory markers. Only genetic disposition to higher CRP causally increased likelihood of ADHD (IVW 0.07, Standard error (SE) 0.02, FDR $p < 0.0001$). Unexpectedly, GlycA decreased liability to both ADHD (IVW: -0.05, SE 0.02, FDR $p = 0.020$) and autism (IVW: -0.07, SE 0.02, FDR $p = 0.018$) in a manner consistent with causal effects. There were no causal effects detected regarding the effect of CRP or IL-6 on autism likelihood (Table 1). Using sensitivity methods, the effect of ADHD on increased CRP was confirmed (See Supplementary Table 8).

DISCUSSION

This study aimed to investigate potential causal mechanisms underpinning the widely reported relationship between maternal inflammation during pregnancy and offspring neurodevelopment. We tested the effect of genetic proxies for inflammatory markers CRP, IL-6, and GlycA on neurodevelopment utilizing trio PGS and intergenerational MR analyses. Results from analyses using a strong instrument for CRP were not consistent with causal effects of maternal inflammation on neurodevelopment. Results for IL-6 and GlycA were also null, but these instruments could not be conclusively validated as predictive during pregnancy and so results must be considered in relation to possible weak instrument bias. Utilizing classical two-sample MR there was evidence of genetic predisposition to ADHD causally increasing liability to inflammation within individuals, which indicates a potential mechanism for genetic confounding in associations between pregnancy inflammation and offspring neurodevelopmental conditions. Collectively, the results presented in this paper did not provide evidence for a casual effect of pregnancy inflammation measured by level of CRP on offspring neurodevelopment.

Interpretation

Existing studies on pregnancy inflammation and neurodevelopment have primarily been observational in design. Some observational studies have included methods to evaluate the potential confounding from maternal genetic liability to neurodevelopmental conditions. In the $COPSAC_{2010}$ cohort, the reported association between pregnancy week 24 CRP and ADHD was robust to adjustment for maternal ADHD PGS (estimated to explain a maximum of 5% of variance in ADHD status in the iPSYCH cohort)[56] to account for potential genetic confounding since information on maternal ADHD status was unavailable.[11] A case-control study nested within Stockholm Youth cohort, investigated the association between several acute phase reactants including CRP in pregnancy on offspring autism. The study reported an association between higher CRP and autism likelihood; however, this association was attenuated using a sibling-

comparison design. The authors suggest the association may be influenced by shared genetics influencing immune signaling and autism likelihood. Further, they observed a significant interaction with maternal psychiatric history, and suggest CRP may be a more important risk factor among individuals with low genetic predisposition to autism.[57] Conversely, a recent paper reported a gene-environment interaction where perinatal inflammation potentiated the influence of genetic predisposition to ADHD.[58] Based on existing knowledge, genetic predisposition – while potentially moderating the role of inflammation as a predisposing factor – likely also confounds the association between inflammation and neurodevelopment. Our findings align with this picture, since we show that maternal genetic liabilities for inflammation are linked to offspring neurodevelopmental traits and conditions, but that such links are fully mediated by offspring genetic liabilities.

Two-sample MR based on publicly available GWAS summary statistics were in this study conducted to further investigate potential confounding of the observed association between maternal inflammation during pregnancy and childhood neurodevelopmental conditions. The two-sample MR showed causal effects of ADHD on inflammation measured by CRP, IL-6 and GlycA within the individual. These findings substantiate the risk of genetic confounding of the intergenerational association between pregnancy inflammation and child neurodevelopmental conditions. This is because maternal genetic predisposition to ADHD *both* increases inflammatory activity in the mother *and* is transmitted to the child at conception, increasing their likelihood of developing ADHD. Two-sample MR results have previously been reported for ADHD and CRP utilizing the same summary statistics with similar results suggesting a bidirectional relationship.[59] Previous, two-sample MR studies including genetic proxies of CRP and IL-6 have failed to show a causal link to autism.[60,61]

To our knowledge, this is the first study to use trio PGS and intergenerational MR to investigate the effect of pregnancy inflammation on neurodevelopment. One existing case-control study included a GWAS on pregnancy CRP to test whether genetic disposition to CRP influenced the association between higher CRP and autism liability. In analyses including 500 children with autism and 580 controls, two significant

SNPs for CRP did not account for the observed association, and the SNPs were not significantly associated with autism likelihood. The authors did not have the power to conduct MR to establish causality.[62]

Strengths

Our study used the largest available sample of genotyped trios. This is a major strength, as the large sample size may have been able to partially compensate for the small predictive power of inflammation PGSs in trio PGS and intergenerational MR analyses. Further, the genetic instruments for CRP were validated in a subsample from the MoBa cohort with F-statistics above 10, implying that the risk of weak instrument bias for CRP is low.[55] We were able to include the total number of genotyped individuals from the MoBa cohort regardless of participation in the follow-up questionnaires, by performing multiple imputation including potential explanatory variables from the MBRN. This is important as non-participation is likely to be non-random and may introduce selection bias.[52] Moreover, incorporating diagnostic outcomes from a linked health registry secured an almost complete follow-up of the cohort for these outcomes and bolstered the imputation of other outcomes.

Limitations

The biggest limitation of the present study was the inability to conclusively validate PGSs for IL-6 and GlycA for use in pregnancy, as these were constructed from GWASs conducted within non-pregnant adults. However, as IL-6 and GlycA measurements in pregnancy were not available in the MoBa subsample, the validation analyses were limited by the smaller sample size available in the COPSAC2010 cohort. This trade-off was behind our decision to proceed, in the main analyses, with all instruments regardless of whether we were able to directly demonstrate their validity in the target population. In general, power for studies using genetic liabilities as proxies is limited by the predictive capacity of these genetic instruments. That is, the proportion of a true causal exposure effect detectable in such studies depends on a) the heritability of the exposure (SNP-based heritability for CRP reported to 13%)[35] and b)

the proportion of that heritability explained by currently available PGS (r squared for CRP PGS in MoBa 0.05). For this study, the predictive ability of the inflammation genetic instruments may have been improved if summary statistics from GWAS on pregnancy inflammation levels had been available, as the measured levels of all 3 inflammation markers included in this paper have been shown to increase throughout pregnancy.[8,63,64] Another limitation may be the nature of the inflammation tagged by PGS in our analytic sample. Causal effects are thought to be driven by infections characterized by high CRP levels.[65] However, in MoBa, the PGS for CRP showed the highest predictiveness among individuals with CRP <10 (r squared 0.15) – suggesting that it may primarily proxy low-grade chronic inflammation, rather than inflammation due to infections. Next, residual bias in the genetic instruments used for trio PGS and intergenerational MR from horizontal pleiotropy is possible. This means that the instruments may include variants acting via maternal anthropometrics or smoking, rather than via inflammation *per se.* However, given that such traits would likely associate with inflammation and neurodevelopmental outcomes in a similar direction, the effect of their confounding in these analyses would be an inflation of effects – and, as such is not likely to explain the lack of evidence consistent with causal effects in this study. Lastly, we included multiple neurodevelopmental outcomes and did not predefine a primary endpoint, which gave us a substantial multiple testing burden. Applying appropriate corrections for this multiple testing reduced our power and could have limited our ability to detect true associations of small magnitude. Nevertheless, only a few findings reached nominal significance, as reflected by the reported uncorrected confidence intervals.

Conclusion

Conclusions regarding the causal role of inflammation should not be based on results from a single design in isolation. Instead, triangulation should be sought across different study designs to infer causality. The present genetic investigation can serve as part of the evidence base for this triangulation, as it employed a previously unused approach for investigating causal effects of pregnancy inflammation on offspring

neurodevelopment, finding no evidence consistent with causal effects of maternal CRP, IL-6 or GlycA on offspring neurodevelopment. Future studies should aim at approaching the association using alternatives to the reported observational findings, with a special focus on studies providing design-based methods for handling confounding.[66]

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Tables

Table 1. Two-sample Mendelian randomization analyses based on publicly available GWAS summary statistics

Exposure	Outcome	Beta	SE	p-value	nSNPs	p-value, FDR
CRP	ADHD	0.07	0.015	<0.001	783	<0.001
CRP	Autism	-0.01	0.020	0.622	995	0.749
IL-6	ADHD	-0.056	0.041	0.169	5	0.253
IL-6	Autism	0.026	0.060	0.665	5	0.749
GlycA	ADHD	-0.047	0.015	0.011	188	0.020
GlycA	Autism	-0.066	0.020	0.038	232	0.018
ADHD	CRP	0.056	0.006	<0.001	32	<0.001
Autism	CRP	-	-	-	-	-
ADHD	IL-6	0.006	0.036	0.874	36	0.002
Autism	IL-6	-	-	-	-	-
ADHD	GlycA	0.040	0.012	<0.001	36	0.012
Autism	GlycA	-	-	-	-	-

SE: Standard Error, SNP = Single-nucleotide polymorphism, FDR = False discovery rate

Figures

Figure 1. Confounding by passive genetic transmission of risk. *Created in BioRender. Bønnelykke, K. (2026) https://BioRender.com/znhs3q2*

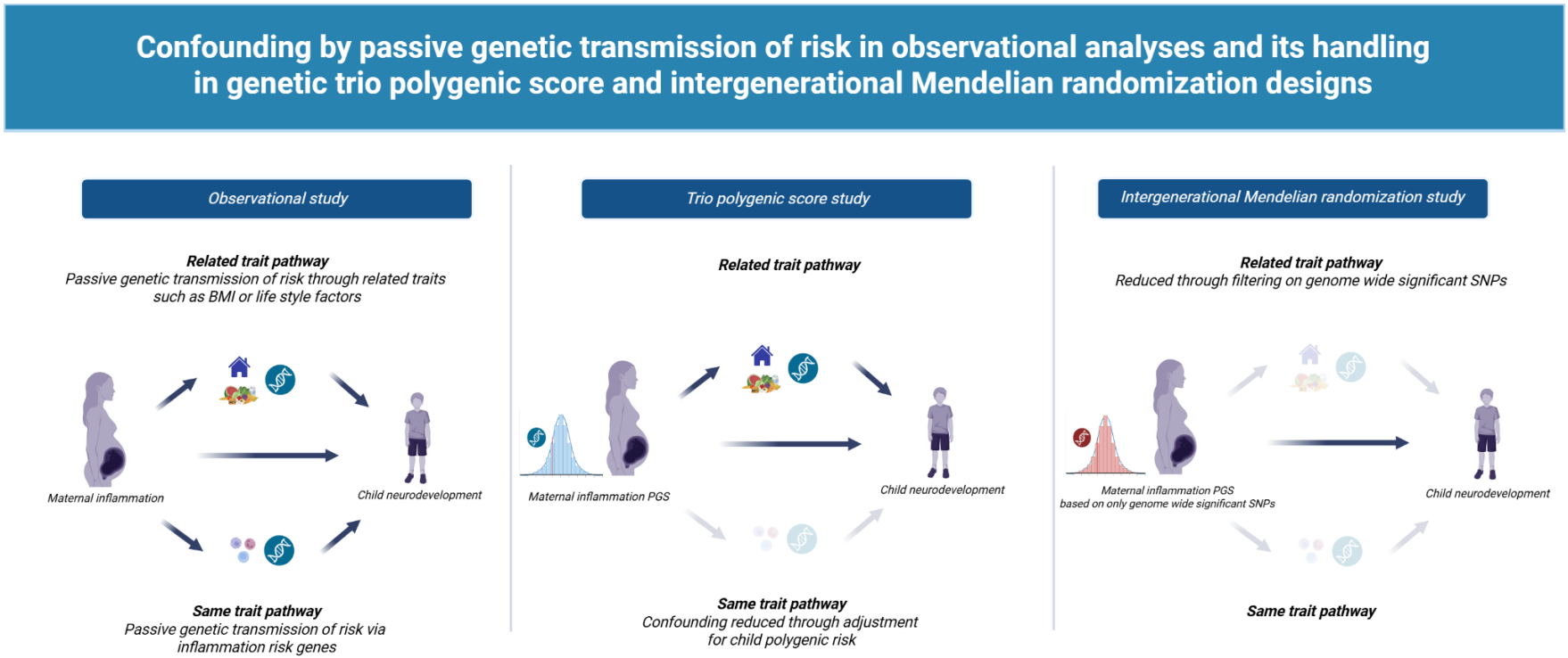
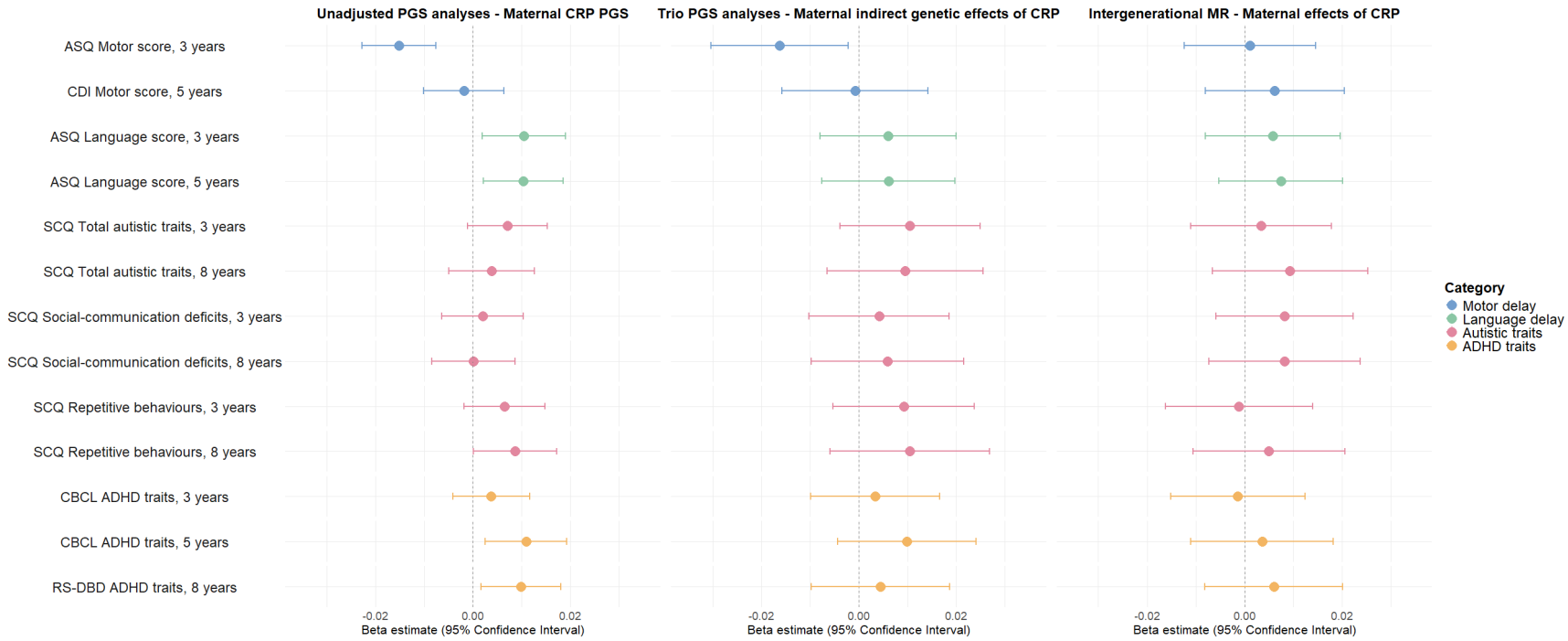


Figure 2. Unadjusted PGS analyses, maternal indirect effects from trio PGS analyses and intergenerational Mendelian randomization analyses for C-reactive protein. Results are based on multiple imputed datasets including a total of 41,531 trios. All FDR p-values > 0.05.



Supplementary Information

Supplementary Methods

Two sample Mendelian randomization

Genome-wide association (GWAS) summary statistics for each trait were used as input for the exposure and outcome, respectively. For each trait, we formatted the summary statistics to match the format required for the GSMR analysis. GWAS summary statistics were for GlycA[1] downloaded from the OpenGWAS database (https://opengwas.io/datasets/met-d-GlycA), for CRP[2] and IL-6[3] from the NHGRI-EBI GWAS Catalog (CRP: https://www.ebi.ac.uk/gwas/publications/36376304, IL-6: https://www.ebi.ac.uk/gwas/publications/33067605) and for autism[4] and ADHD[5] from the Psychiatric Genomics Consortium (https://pgc.unc.edu/).

The following criteria were used to select genetic instruments: We only considered genome-wide significant variants ($p < 5e-08$) as exposures. Variants were clumped in a similar manner as for the MR instrumental variables - within 250kb windows with an r^2 threshold of 0.1 to generate a list of independent variants. A minimum of 3 independent genome-wide significant exposures were required for each trait to perform the MR analysis. We used the default frequency filter of 0.2, where variants with allele frequency differences between the GWAS summary statistics and the LD reference sample were removed.

To account for horizontal pleiotropy, we applied the HEterogeneity In Dependent Instrument (HEIDI) outlier test implemented in GSMR using a HEIDI-outlier p-value threshold of 0.01 to identify and exclude SNPs with evidence of horizontal pleiotropy.[6]

CRP measurement MoBa

CRP in MoBa was measured with a Multigent CRP Vario (CRPVa) assay at the Department of Government Services, Finnish Institute for Health and Welfare (THL) in Helsinki, Finland.

Supplementary Results

Validation of the CRP, IL-6, and GlycA PGS in the COPSAC cohort

The inflammation PGSs and MR instrumental variables were validated in the COPSAC2010 cohort among 675 individuals (no exclusions performed to obtain the highest number of individuals) with available information on both inflammation PGS and pregnancy blood inflammatory markers. CRP was measured in mg/L, IL-6 in pg/mL and GlycA in mmol/L. In linear regression models for each inflammation PGS, r squared was -0.00 for CRP, -0.00 for IL-6, and 0.00 for GlycA, beta estimate was 0.55 for CRP, 0.03 for IL-6, and 0.01 for GlycA. F-statistics was 0.6 for CRP, 0.7 for IL-6, and 3.1 for GlycA. All p-values above 0.05. In linear regression models including MR instrumental variables, r squared was 0.02 for CRP, 0.01 for IL-6, and 0.02 for glycA, beta estimate was 2.7 for CRP, 0.03 for IL-6, and 0.01 for GlycA. F-statistics was 14.9 for CRP, 1.0 for IL-6, and 12.1 for GlycA. P-values from linear regression models were significant for CRP and glycA and below <0.001.

Validation of the CRP PGS and MR instrument in the MoBa cohort

The CRP PGS and the CRP MR instrument were validated in a subsample of 2,976 women with information on pregnancy CRP (no exclusions performed to obtain the highest number of individuals). Hereof, 2,323 individuals had available information on both PGS and blood measurement of CRP. Mean CRP was slightly higher among those with information on PGS (mean 6.49 mg/L, SD 7.80) as compared to those without (mean 6.28 mg/L, SD 7.17). Linear regression models for the CRP PGS and the CRP MR instrument showed beta estimates of 1.82 and 1.28, r-squared of 0.05 and 0.03, and p-values <0.001 respectively. F-statistics were 132.8 for the CRP PGS and 60.6 for the CRP MR instrument. To test the ability of the PGSs to predict low-grade inflammation we performed a *post hoc* validation analysis restricted to 1,944 individuals with CRP measurements ≤ 10. Within this subsample, linear regression models for the CRP PGS and the CRP MR instrument showed beta estimates of 0.94 and 0.68, r-squared

of 0.15 and 0.08, and p-values <0.001. F-statistics were 348.6 for the CRP PGS and 164.7 for the CRP MR instrument.

Supplementary References

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Supplementary Tables and Figures

Supplementary Table 1. Deviations from preregistration[7]

Deviations					
#	Details		Original Wording	Deviation Description	Reader Impact
1	Type	Variables	*Polygenic scores will be created using ldpred2 using a version of the pipeline outlined here (https://privefl.github.io/bigsnpr/articles/LDpred2.html), based on SNPs with high imputation quality (INFO > 0.95).*	*PRS-CS method applied for generation of PGS in COPSAC.* *Reason: Main PGS pipeline in COPSAC used.*	*Comparable to planned method. No impact.*
	Reason	Other (Please Explain)			
	Timing	After data access			
2	Type	Analysis	*Two sample MR analysis will be performed to test potentially causal associations between maternal neurodevelopmental conditions and levels of inflammation (H3). These analyses will be run using summary statistics via the TwoSampleMR R package.*	*GSMR from the GCTA software toolkit used for analyses.* *Reason: Main COPSAC MR pipeline used.*	*Comparable to planned method. No impact.*
	Reason	Other (Please Explain)			
	Timing	After data access			
3	Type	Analysis	*Appropriate MR sensitivity analyses to assess potential biases will be run according to recommendations – see https://pmc.ncbi.nlm.nih.gov/articles/PMC5386135/*	*Sensitivity analyses were conducted as planned for two sample MR analyses. Only sensitivity analysis of intergenerational MR was the validation of genetic instruments as there was no evidence of causal effects.*	*No impact as residual bias of genetic instruments expectedly would inflate estimates.*
	Reason	New knowledge			
	Timing	After results known			
Unregistered Steps					
#	Details		Original Wording	Unregistered Step Description	Reader Impact
1	Type	Analysis		*Unadjusted PGS analyses*	*No impact, since these analyses do not test the main hypotheses.*
	Timing	After results known			

2	Type	Analysis		*Analyses restricted to validated genetic instruments only.*	*Important as these analyses strengthen the reliability of the results.*
	Timing	After results known			

Supplementary Table 2. Drop-out analysis, investigating differences between complete trios and the remaining MoBa cohort.				
Exposure/outcome	**Overall**	**Complete trios**	**Remaining cohort**	**p-value**
	n = 111,069	n = 41,531	n = 69,538	
CRP PGS, maternal (mean (SD))	0.00 (1.00)	-0.01 (1.00)	0.01 (1.00)	0.018
CRP PGS, paternal (mean (SD))	0.00 (0.99)	0.00 (0.99)	0.00 (1.00)	0.933
CRP PGS, child (mean (SD))	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.433
IL-6 PGS, maternal (mean (SD))	0.00 (1.00)	-0.01 (1.00)	0.00 (1.00)	0.341
IL-6 PGS, paternal (mean (SD))	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.833
IL-6 PGS, child (mean (SD))	0.00 (1.00)	-0.01 (1.00)	0.01 (1.00)	0.020
GlycA PGS, maternal (mean (SD))	0.00 (1.00)	0.00 (1.00)	0.00 (0.99)	0.556
GlycA PGS, paternal (mean (SD))	0.00 (1.00)	-0.01 (1.00)	0.01 (1.00)	0.039
GlycA PGS, child (mean (SD))	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.598
ASQ motor score, 3 years (mean (SD))	1.17 (1.35)	1.17 (1.34)	1.17 (1.35)	0.544
CDI motor score, 5 years (mean (SD))	0.87 (1.49)	0.84 (1.43)	0.89 (1.54)	<0.001
ASQ language score, 3 years (mean (SD))	0.67 (1.19)	0.62 (1.11)	0.70 (1.25)	<0.001
ASQ language score, 5 years (mean (SD))	0.76 (1.35)	0.72 (1.24)	0.80 (1.43)	<0.001
SCQ total autistic traits, 3 years (mean (SD))	6.26 (3.41)	6.11 (3.31)	6.38 (3.48)	<0.001
SCQ total autistic traits, 8 years (mean (SD))	3.41 (2.97)	3.31 (2.88)	3.49 (3.03)	<0.001
SCQ social-communication deficits, 3 years (mean (SD))	2.30 (1.84)	2.24 (1.76)	2.35 (1.90)	<0.001
SCQ social-communication deficits, 8 years (mean (SD))	2.62 (2.46)	2.58 (2.43)	2.65 (2.48)	0.001
SCQ repetitive behaviors, 3 years (mean (SD))	3.85 (2.55)	2.77 (2.49)	3.91 (2.59)	<0.001
SCQ repetitive behaviors, 8 years (mean (SD))	0.67(1.19)	0.62 (1.12)	0.71 (1.24)	<0.001

CBCL ADHD traits, 3 years (mean (SD))	3.45 (2.24)	3.42 (2.22)	3.48 (2.26)	0.002
CBCL ADHD traits, 5 years (mean (SD))	2.55 (2.19)	2.49 (2.15)	2.59 (2.22)	<0.001
RS-DBD ADHD traits, 8 years (mean (SD))	8.53 (7.22)	8.37 (7.06)	8.66 (7.33)	0.001
ADHD (yes) %	7,762 (7.0)	2,697 (6.5)	5,065 (7.3)	<0.001
Autism (yes) %	2,274 (2.1)	853 (2.1)	1,421 (2.1)	1.000

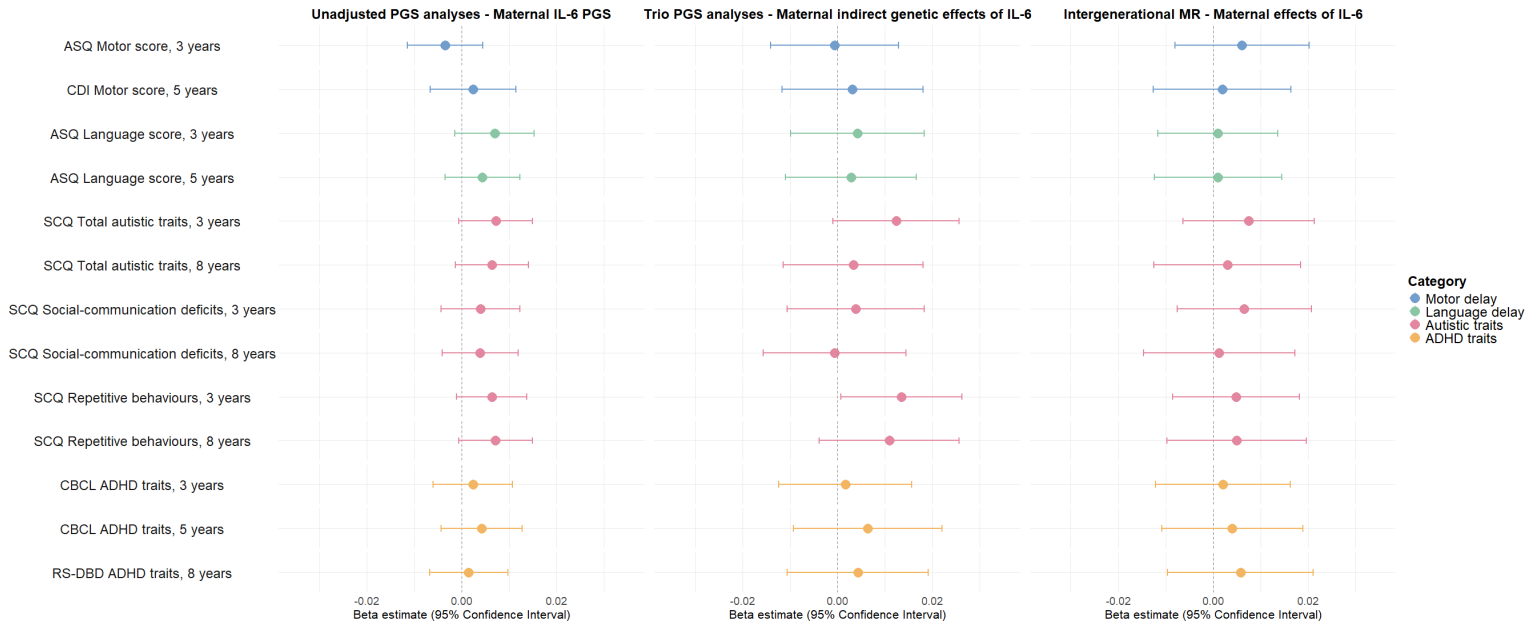
ADHD = Attention Deficit Hyperactivity Disorder, ASQ = Ages and Stages Questionnaire, CBCL = Child Behavior Checklist, CDI = Child Development Inventory, RS-DBD = Rating Scale for Disruptive Behavior Disorders, SCQ = Social Communication Questionnaire
Differences between groups tested with t-test (for continuous variables) and chi-square test (for categorical variables).

Supplementary Table 3. Unadjusted PGS, trio PGS and intergenerational MR analyses for non-validated genetic instruments and the risk of offspring ADHD or autism (N trio PGS and intergenerational MR = 41,531; N unadjusted PGS analyses = 92,155), maternal effects

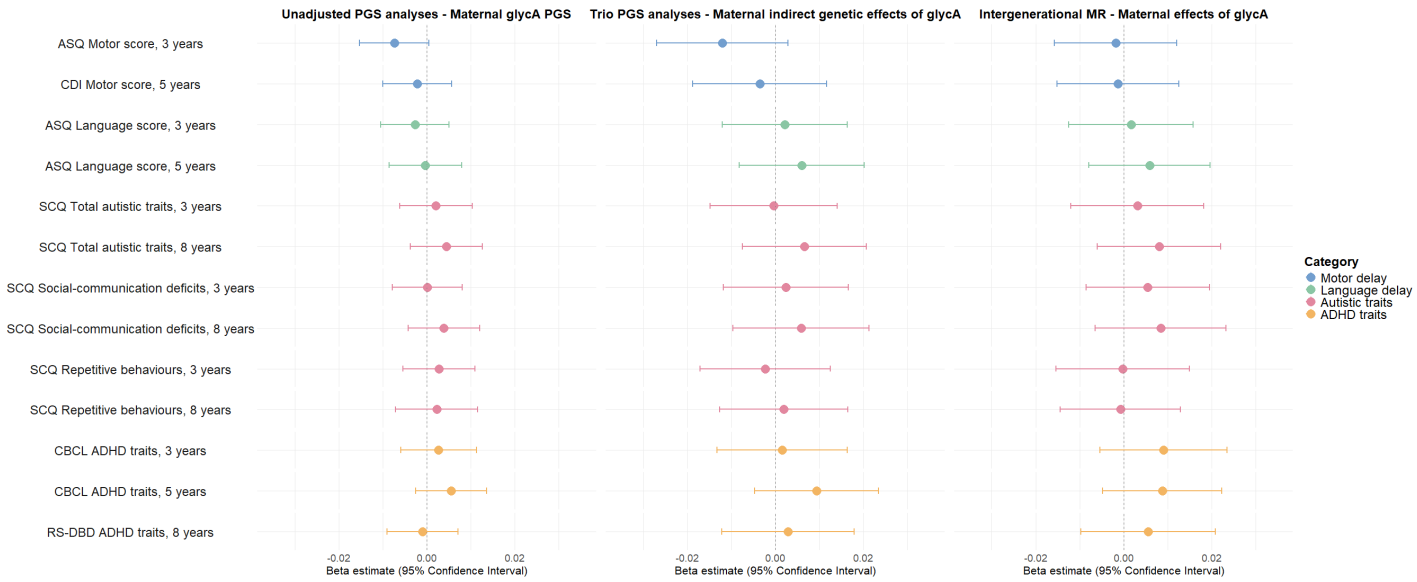
Exposure	Outcome	Odds ratio	95% CI, lower limit	95%CI, upper limit	FDR p-value
Unadjusted PGS analyses					
IL-6	ADHD	1.02	0.99	1.05	0.373
IL-6	Autism	1.02	0.98	1.07	0.606
GlycA	ADHD	1.00	0.98	1.03	0.815
GlycA	Autism	1.01	0.96	1.06	0.776
Trio PGS analyses					
IL-6	ADHD	1.02	0.97	1.07	0.941
IL-6	Autism	1.00	0.92	1.09	0.962
GlycA	ADHD	1.00	0.95	1.05	0.962
GlycA	Autism	1.04	0.95	1.13	0.941
Intergenerational MR analyses					
IL-6	ADHD	1.03	0.98	1.08	0.900
IL-6	Autism	1.03	0.95	1.12	0.900
GlycA	ADHD	1.03	0.98	1.08	0.900
GlycA	Autism	1.08	0.99	1.18	0.900

ADHD = Attention Deficit Hyperactivity Disorder, CI = confidence interval, FDR = False discovery rate, MR = Mendelian Randomization, PGS = polygenic score
P-values shown are FDR adjusted.

Supplementary Figure 1. Unadjusted PGS analyses, maternal indirect effects from trio PGS analyses and intergenerational Mendelian randomization analyses for interleukin-6. Results are based on multiple imputed datasets including a total of 41,531 trios. All FDR p-values > 0.05.



Supplementary Figure 2. Unadjusted PGS analyses, maternal indirect effects from trio PGS analyses and intergenerational Mendelian randomization analyses for GlycA. Results are based on multiple imputed datasets including a total of 41,531 trios. All FDR p-values > 0.05.



Supplementary Table 4. Trio PGS analyses restricted to CRP PGS (N = 41,531), maternal effects

Exposure	Outcome	Beta Estimate	95% CI, lower limit	95%CI, upper limit	p-value
CRP	ASQ motor score, 3 years	-0.02	-0.03	-0.01	0.006
CRP	CDI motor score, 5 years	0.00	-0.02	0.01	0.813
CRP	ASQ language score, 3 years	0.01	-0.01	0.02	0.381
CRP	ASQ language score, 5 years	0.01	-0.01	0.02	0.283
CRP	SCQ total autistic traits, 3 years	0.01	0.00	0.02	0.203
CRP	SCQ total autistic traits, 8 years	0.01	-0.01	0.03	0.188
CRP	SCQ social-communication deficits, 3 years	0.00	-0.01	0.02	0.554
CRP	SCQ social-communication deficits, 8 years	0.01	-0.01	0.02	0.364
CRP	SCQ repetitive behaviors, 3 years	0.01	-0.01	0.02	0.307
CRP	SCQ repetitive behaviors, 8 years	0.01	-0.01	0.03	0.229
CRP	CBCL ADHD traits, 3 years	0.01	0.00	0.03	0.110
CRP	CBCL ADHD traits, 5 years	0.00	-0.01	0.02	0.591
CRP	RS-DBD ADHD traits, 8 years	0.00	-0.01	0.02	0.504
Exposure	**Outcome**	**Odds ratio**	**95% CI, lower limit**	**95%CI, upper limit**	**p-value**
CRP	ADHD	1.04	0.99	1.09	0.162
CRP	Autism	1.04	0.96	1.14	0.314

ADHD = Attention Deficit Hyperactivity Disorder, ASQ = Ages and Stages Questionnaire, CBCL = Child Behavior Checklist, CDI = Child Development Inventory, CI = confidence interval, FDR = False discovery rate, PGS = polygenic score, RS-DBD = Rating Scale for Disruptive Behavior Disorders, SCQ = Social Communication Questionnaire
P-values shown are not FDR adjusted. All FDR adjusted p-values above 0.05.

Supplementary Table 5. Intergenerational Mendelian randomization analyses restricted to CRP PGS (N = 41,531), maternal effects

Exposure	Outcome	Beta Estimate	95% CI, lower limit	95%CI, upper limit	p-value
CRP	ASQ motor score, 3 years	0.00	-0.01	0.01	0.998
CRP	CDI motor score, 5 years	0.01	-0.01	0.02	0.415
CRP	ASQ language score, 3 years	0.01	-0.01	0.02	0.421
CRP	ASQ language score, 5 years	0.01	0.00	0.02	0.236
CRP	SCQ total autistic traits, 3 years	0.00	-0.01	0.02	0.747
CRP	SCQ total autistic traits, 8 years	0.01	-0.01	0.03	0.247
CRP	SCQ social-communication deficits, 3 years	0.01	-0.01	0.02	0.309
CRP	SCQ social-communication deficits, 8 years	0.01	-0.01	0.02	0.286
CRP	SCQ repetitive behaviors, 3 years	0.00	-0.02	0.01	0.805
CRP	SCQ repetitive behaviors, 8 years	0.00	-0.01	0.02	0.587
CRP	CBCL ADHD traits, 3 years	0.00	-0.02	0.01	0.869
CRP	CBCL ADHD traits, 5 years	0.00	-0.01	0.02	0.646
CRP	RS-DBD ADHD traits, 8 years	0.01	-0.01	0.02	0.440
Exposure	**Outcome**	**Odds ratio**	**95% CI, lower limit**	**95%CI, upper limit**	**p-value**
CRP	ADHD	1.00	0.95	1.05	0.965
CRP	Autism	1.05	0.97	1.14	0.253

ADHD = Attention Deficit Hyperactivity Disorder, ASQ = Ages and Stages Questionnaire, CBCL = Child Behavior Checklist, CDI = Child Development Inventory, CI = confidence interval, FDR = False discovery rate, PGS = polygenic score, RS-DBD = Rating Scale for Disruptive Behavior Disorders, SCQ = Social Communication Questionnaire
P-values shown are not FDR adjusted. All FDR adjusted p-values above 0.05.

Supplementary Table 6. Complete case analyses, maternal indirect genetic effects from trio PGS analyses

Exposure PGS	Outcome	N	Beta Estimate	95% CI, lower limit	95%CI, upper limit	p-value
CRP	ASQ motor score, 3 years	24,527	-0.02	-0.04	-0.01	0.004
IL-6	ASQ motor score, 3 years	24,527	0.00	-0.02	0.01	0.845
GlycA	ASQ motor score, 3 years	24,527	-0.02	-0.03	0.00	0.033
CRP	CDI motor score, 5 years	18,136	0.00	-0.01	0.02	0.749
IL-6	CDI motor score, 5 years	18,136	0.01	-0.01	0.02	0.388
GlycA	CDI motor score, 5 years	18,136	-0.01	-0.03	0.00	0.108
CRP	ASQ language score, 3 years	24,600	0.01	-0.01	0.02	0.326
IL-6	ASQ language score, 3 years	24,600	0.00	-0.01	0.02	0.500
GlycA	ASQ language score, 3 years	24,600	0.00	-0.01	0.02	0.826
CRP	ASQ language score, 5 years	18,088	0.01	-0.01	0.02	0.460
IL-6	ASQ language score, 5 years	18,088	0.01	-0.01	0.02	0.444
GlycA	ASQ language score, 5 years	18,088	0.00	-0.01	0.02	0.640
CRP	SCQ total autistic traits, 3 years	24,584	0.02	0.00	0.03	0.044
IL-6	SCQ total autistic traits, 3 years	24,584	0.02	0.00	0.03	0.019
GlycA	SCQ total autistic traits, 3 years	24,584	0.00	-0.02	0.01	0.890
CRP	SCQ total autistic traits, 8 years	18,525	0.02	0.00	0.04	0.055
IL-6	SCQ total autistic traits, 8 years	18,525	0.01	-0.01	0.02	0.501
GlycA	SCQ total autistic traits, 8 years	18,525	0.01	0.00	0.03	0.123
CRP	SCQ social-communication deficits, 3 years	24,582	0.00	-0.01	0.02	0.546
IL-6	SCQ social-communication deficits, 3 years	24,582	0.00	-0.01	0.02	0.624
GlycA	SCQ social-communication deficits, 3 years	24,582	0.01	-0.01	0.02	0.455
CRP	SCQ social-communication deficits, 8 years	18,515	0.01	-0.01	0.03	0.230
IL-6	SCQ social-communication deficits, 8 years	18,515	0.00	-0.02	0.02	0.917
GlycA	SCQ social-communication deficits, 8 years	18,515	0.01	-0.01	0.03	0.165
CRP	SCQ repetitive behaviors, 3 years	24,539	0.02	0.00	0.03	0.045

IL-6	SCQ repetitive behaviors, 3 years	24,539	0.02	0.01	0.04	0.005
GlycA	SCQ repetitive behaviors, 3 years	24,539	-0.01	-0.02	0.01	0.476
CRP	SCQ repetitive behaviors, 8 years	18,623	0.02	0.00	0.04	0.024
IL-6	SCQ repetitive behaviors, 8 years	18,623	0.02	0.01	0.04	0.010
GlycA	SCQ repetitive behaviors, 8 years	18,623	0.00	-0.02	0.02	0.901
CRP	CBCL ADHD traits, 3 years	24,543	0.00	-0.01	0.02	0.817
IL-6	CBCL ADHD traits, 3 years	24,543	0.00	-0.01	0.02	0.739
GlycA	CBCL ADHD traits, 3 years	24,543	0.00	-0.01	0.02	0.847
CRP	CBCL ADHD traits, 5 years	18,137	0.01	0.00	0.03	0.132
IL-6	CBCL ADHD traits, 5 years	18,137	0.01	-0.01	0.03	0.318
GlycA	CBCL ADHD traits, 5 years	18,137	0.01	0.00	0.03	0.141
CRP	RS-DBD ADHD traits, 8 years	18,610	0.01	-0.01	0.03	0.276
IL-6	RS-DBD ADHD traits, 8 years	18,610	0.01	-0.01	0.02	0.518
GlycA	RS-DBD ADHD traits, 8 years	18,610	0.00	-0.01	0.02	0.719
Exposure PGS	**Outcome**	**N**	**Odds Ratio**	**95% CI, lower limit**	**95%CI, upper limit**	**p-value**
CRP	ADHD	41,511	1.04	0.99	1.09	0.153
IL-6	ADHD	41,511	1.02	0.97	1.07	0.361
GlycA	ADHD	41,511	1.00	0.95	1.05	0.977
CRP	Autism	41,511	1.04	0.95	1.13	0.418
IL-6	Autism	41,511	1.00	0.92	1.09	0.918
GlycA	Autism	41,511	1.04	0.95	1.13	0.444

ADHD = Attention Deficit Hyperactivity Disorder, ASQ = Ages and Stages Questionnaire, CBCL = Child Behavior Checklist, CDI = Child Development Inventory, CI = confidence interval, FDR = False discovery rate, PGS = polygenic score, RS-DBD = Rating Scale for Disruptive Behavior Disorders, SCQ = Social Communication Questionnaire
P-values shown are not FDR adjusted. All FDR adjusted p-values above 0.05.

Supplementary Table 7. Complete case analyses, maternal effects from intergenerational MR analyses

Exposure	Outcome	N	Beta Estimate	95% CI, lower limit	95%CI, upper limit	p-value
CRP	ASQ motor score, 3 years	24,527	0.00	-0.01	0.02	0.843
IL-6	ASQ motor score, 3 years	24,527	0.01	-0.01	0.03	0.203
GlycA	ASQ motor score, 3 years	24,527	0.00	-0.02	0.01	0.648
CRP	CDI motor score, 5 years	18,136	0.01	-0.01	0.03	0.200
IL-6	CDI motor score, 5 years	18,136	0.00	-0.01	0.02	0.610
GlycA	CDI motor score, 5 years	18,136	-0.01	-0.02	0.01	0.370
CRP	ASQ language score, 3 years	24,600	0.01	-0.01	0.02	0.329
IL-6	ASQ language score, 3 years	24,600	0.00	-0.01	0.02	0.813
GlycA	ASQ language score, 3 years	24,600	0.00	-0.01	0.02	0.787
CRP	ASQ language score, 5 years	18,088	0.01	-0.01	0.03	0.186
IL-6	ASQ language score, 5 years	18,088	0.00	-0.01	0.02	0.600
GlycA	ASQ language score, 5 years	18,088	0.00	-0.01	0.02	0.596
CRP	SCQ total autistic traits, 3 years	24,584	0.00	-0.01	0.02	0.559
IL-6	SCQ total autistic traits, 3 years	24,584	0.01	0.00	0.03	0.174
GlycA	SCQ total autistic traits, 3 years	24,584	0.00	-0.01	0.02	0.550
CRP	SCQ total autistic traits, 8 years	18,525	0.02	0.00	0.04	0.045
IL-6	SCQ total autistic traits, 8 years	18,525	0.01	-0.01	0.02	0.565
GlycA	SCQ total autistic traits, 8 years	18,525	0.02	0.00	0.04	0.039
CRP	SCQ social-communication deficits, 3 years	24,582	0.01	0.00	0.03	0.112
IL-6	SCQ social-communication deficits, 3 years	24,582	0.01	-0.01	0.02	0.242
GlycA	SCQ social-communication deficits, 3 years	24,582	0.01	-0.01	0.02	0.217
CRP	SCQ social-communication deficits, 8 years	18,515	0.02	0.00	0.04	0.060
IL-6	SCQ social-communication deficits, 8 years	18,515	0.00	-0.02	0.02	0.835
GlycA	SCQ social-communication deficits, 8 years	18,515	0.02	0.00	0.04	0.031
CRP	SCQ repetitive behaviors, 3 years	24,539	0.00	-0.02	0.01	0.851

IL-6	SCQ repetitive behaviors, 3 years	24,539	0.01	-0.01	0.02	0.334
GlycA	SCQ repetitive behaviors, 3 years	24,539	0.00	-0.02	0.01	0.849
CRP	SCQ repetitive behaviors, 8 years	18,623	0.01	-0.01	0.02	0.389
IL-6	SCQ repetitive behaviors, 8 years	18,623	0.01	-0.01	0.03	0.217
GlycA	SCQ repetitive behaviors, 8 years	18,623	0.00	-0.02	0.02	0.919
CRP	CBCL ADHD traits, 3 years	24,543	0.00	-0.02	0.01	0.589
IL-6	CBCL ADHD traits, 3 years	24,543	0.00	-0.01	0.02	0.581
GlycA	CBCL ADHD traits, 3 years	24,543	0.01	0.00	0.03	0.062
CRP	CBCL ADHD traits, 5 years	18,137	0.01	-0.01	0.03	0.297
IL-6	CBCL ADHD traits, 5 years	18,137	0.00	-0.02	0.02	0.773
GlycA	CBCL ADHD traits, 5 years	18,137	0.02	0.00	0.04	0.051
CRP	RS-DBD ADHD traits, 8 years	18,610	0.01	-0.01	0.03	0.166
IL-6	RS-DBD ADHD traits, 8 years	18,610	0.01	-0.01	0.03	0.336
GlycA	RS-DBD ADHD traits, 8 years	18,610	0.01	-0.01	0.03	0.342
Exposure PGS	**Outcome**	**N**	**Odds Ratio**	**95% CI, lower limit**	**95%CI, upper limit**	**p-value**
CRP	ADHD	41,511	1.00	0.95	1.05	0.955
IL-6	ADHD	41,511	1.03	0.98	1.08	0.280
GlycA	ADHD	41,511	1.03	0.98	1.08	0.184
CRP	Autism	41,511	1.05	0.97	1.15	0.235
IL-6	Autism	41,511	1.03	0.95	1.12	0.485
GlycA	Autism	41,511	1.08	1.00	1.18	0.065

ADHD = Attention Deficit Hyperactivity Disorder, ASQ = Ages and Stages Questionnaire, CBCL = Child Behavior Checklist, CDI = Child Development Inventory, CI = confidence interval, FDR = False discovery rate, PGS = polygenic score, RS-DBD = Rating Scale for Disruptive Behavior Disorders, SCQ = Social Communication Questionnaire
P-values shown are not FDR adjusted. All FDR adjusted p-values above 0.05.

Supplementary Table 8. Two-sample Mendelian randomization analyses based on publicly available GWAS summary statistics, results from sensitivity analyses.

Exposure	Outcome	Beta	SE	p-value	nSNPs	Method
CRP	ADHD	-0.10	0.03	0.003	824	MR Egger
CRP	ADHD	0.02	0.03	0.564	824	Weighted Median
CRP	ADHD	-0.02	0.03	0.443	824	Weighted mode
CRP	Autism	-0.03	0.04	0.456	1019	MR Egger
CRP	Autism	-0.02	0.04	0.667	1019	Weighted Median
CRP	Autism	0.01	0.040	0.783	1019	Weighted mode
IL-6	ADHD	0.01	0.13	0.945	5	MR Egger
IL-6	ADHD	-0.05	0.05	0.345	5	Weighted Median
IL-6	ADHD	-0.04	0.06	0.505	5	Weighted mode
IL-6	AUTISM	0.05	0.32	0.883	5	MR Egger
IL-6	AUTISM	0.04	0.07	0.557	5	Weighted Median
IL-6	AUTISM	0.06	0.08	0.508	5	Weighted mode
GlycA	ADHD	-0.03	0.04	0.479	192	MR Egger
GlycA	ADHD	-0.03	0.03	0.343	192	Weighted Median
GlycA	ADHD	-0.02	0.04	0.613	192	Weighted mode
GlycA	AUTISM	-0.32	0.06	<0.001	243	MR Egger
GlycA	AUTISM	-0.08	0.04	0.070	243	Weighted Median
GlycA	AUTISM	-0.06	0.09	0.497	243	Weighted mode
ADHD	CRP	-0.05	0.05	0.353	34	MR Egger
ADHD	CRP	0.04	0.01	<0.001	34	Weighted Median
ADHD	CRP	0.05	0.01	0.010	34	Weighted mode
Autism	CRP	-	-	-	-	MR Egger
Autism	CRP	-	-	-	-	Weighted Median
Autism	CRP	-	-	-	-	Weighted mode
ADHD	IL-6	-0.36	0.16	0.027	34	MR Egger
ADHD	IL-6	0.00	0.05	0.934	34	Weighted Median
ADHD	IL-6	0.01	0.10	0.902	34	Weighted mode
Autism	IL-6	-	-	-	-	MR Egger

Autism	IL-6	-	-	-	-	Weighted Median
Autism	IL-6	-	-	-	-	Weighted mode
ADHD	GlycA	-0.05	0.05	0.304	34	MR Egger
ADHD	GlycA	0.03	0.02	0.049	34	Weighted Median
ADHD	GlycA	0.03	0.03	0.325	34	Weighted mode
Autism	GlycA	-	-	-	-	MR Egger
Autism	GlycA	-	-	-	-	Weighted Median
Autism	GlycA	-	-	-	-	Weighted mode

GWAS = Genome-Wide Association Study, SE: Standard Error, SNP = Single-nucleotide polymorphism.
P-values not FDR corrected.