Emilie Willoch Olstad

Prenatal Medication Exposure, DNA Methylation and Child Neurodevelopment

Thesis submitted for the degree of *Philosophiae Doctor*

Pharmacoepidemiology and Drug Safety Research Group
PharmaTox Strategic Research Initiative

Department of Pharmacy,
Faculty of Mathematics and Natural Sciences

2023

© Emilie Willoch Olstad, 2023

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 2613

ISSN 1501-7710

All rights reserved. No part of this publication may be
reproduced or transmitted, in any form or by any means, without permission.

Cover: UiO.
Print production: Graphic Center, University of Oslo.

Til Morfar og Farfar

PREFACE

The present thesis is submitted in partial fulfillment of the requirements for the degree of *Philosophiae Doctor* at the University of Oslo. The research presented was conducted under the supervision of Prof. Hedvig Nordeng and Dr. Kristina Gervin.

The thesis is a collection of four papers resulting from research conducted at the Department of Pharmacy, University of Oslo, between October 2019 and January 2023. The overarching theme of the thesis is to explore the relationship between prenatal medication exposure, DNAm and neurodevelopment. All studies are joint works, conducted together with Prof. Nordeng, Dr. Gervin and other collaborators.

The thesis synopsis is written to highlight the common thread of the thesis, as well as to elucidate the relations between and the interdependence of the papers. The synopsis introduces the fields of prenatal pharmacoepidemiology, epigenetics and the combination of the two, and subsequently presents important methodological considerations and results of the research. It concludes with a discussion of the findings in relation to the current literature and outlines future directions of the research. The thesis synopsis should equip the reader with sufficient knowledge to understand and interpret the findings of the present thesis.

ACKNOWLEDGEMENTS

First, thanks a lot to my supervisors Prof. Hedvig Nordeng and Dr. Kristina Gervin. I will keep this short and sweet, credits to Kristina. I am really grateful to have had such engaged, knowledgeable and caring supervisors. Despite some challenges along the way, you have always had my back, believed in me and provided valuable advice, both academically and personally. Thank you!

Thanks to all collaborators contributing to the doctoral project and a special thanks to Prof. Geir Kjetil Sandve. I have really enjoyed our essay-long e-mail discussions. I much appreciate you taking your time: I have learnt a lot and it has added a new perspective to my research. I hope for and look forward to future discussions!

Thanks to all other parties making this thesis possible, including the participants of the Norwegian Mother, Father and Child Cohort Study (MoBa), and funding from the PharmaTox Strategic Research Initiative. Several research schools have enabled me to broaden my network and have hosted interesting courses and conferences, including the Norwegian PhD School of Pharmacy, the Norwegian Research School in Neuroscience, the National Research School in Population-Based Epidemiology, the Digital Life Norway Research School, and the National Research School in Bioinformatics, Biostatistics and Systems Biology.

To all my fellow PhD students (former and present!): thank you for creating such an open and warm-hearted working environment. A special thanks to Lene and Elin, being in the infamous boat with me. At times it felt like we were all paddling for our lives, but with laughs and cries and pep-talks, we made it through. Elin, thanks for all your advice and help, you really paved the way for me to "just" hand in my thesis. Lene, thanks for checking in on me and for being the best office mate (Sarah, you were too!).

I am very fortunate to have a lovely, supportive family. Mamma and Pappa, thank you for being there. Always. Thank you, Mamma, for the warm hugs, fresh pancakes for lunch when I was buried in papers, and all the cheering and encouragement along the way. Pappa, thanks a lot for your ever-lasting curiosity and for critically appraising my work, even if what caught your

interest the most was the brief mentioning of DNA methylation landscapes ("Finally something geological!"). Thanks to my siblings Martin and Annika, and to their better halves Tiril and Daniel, for showing up for coffee breaks and lunch dates. I always leave these meet-ups happier than when I arrived.

Thanks to my wonderful grandparents, Farmor and Mormor, and the late Morfar and Farfar. Farmor, thanks for always being available for a chat or a cooking class, for taking an interest in what I do and for calling me to let me know there is an article in the newspaper about medication use during pregnancy. Mormor, I love your warmth and caring nature, as well as our frequent dates where we always happen to find *the best* spot for a coffee and cake. Morfar, thanks for sparking my scientific interest and encouraging my curiosity by taking me to *Teknisk Museum*, and for gifting me the microscope I *so much wished for* when I was eight (and thanks for the blood sample for my very important research, Mamma). Farfar, thanks for always asking me how I was doing and for making me laugh, like the time I told you I was contemplating doing a PhD and you replied, without hesitance: "Well, surely it's better to just take as many degrees as you possibly can?!".

Christiana and Vilde, thanks for our years together in Trondheim, studying for our master's in neuroscience, and for our current long-distance relationship. You always cheer me up and your support means a lot to me. Christiana, thanks for all the meaningful conversations we have had (and will have), for your belief in me, and for being such an inspiration! Vilde, thanks for all our late-night FaceTime calls, where I stare at the laboratory ceiling while you put your mice under the knife. Thanks for asking the tough questions and staying for the answers. You are both such wonderful, clever, caring, bright and funny women, and I am so grateful to have you in my life.

Finally, Oda. Unfortunately, however much I try and twist and turn academic tradition, you do not qualify as a co-author. But for the record, even if only a meager consolation, I know that I could never have written any part of this thesis without you. Thanks for being here!

Emilie Willoch Olstad,

Oslo, 03.02.2023

ABSTRACT

Background: The Developmental Origin of Health and Disease (DOHaD) hypothesis proposes that intrauterine exposures can impact the development of diseases later in life. To this end, prenatal exposure to several medications including analgesics and psychotropics, has been associated with abnormal neurodevelopment, including attention-deficit/hyperactivity disorder (ADHD). The underlying link between prenatal medication exposure and neurodevelopment is not clear, but epigenetic modifications have been suggested as one possible mechanism. Several medications are known to interact with the epigenetic machinery and pioneering studies have revealed significant associations between prenatal medication exposure and newborn DNA methylation (DNAm). Yet, whether DNAm is underlying the associations of prenatal medications with neurodevelopment remains elusive. To this end, the aim of this thesis was to explore the relationship between prenatal medication exposure, DNAm and neurodevelopment.

Materials and methods: The thesis consists of one systematic literature review of prenatal pharmacoepigenetic studies and three original research papers. In the original research, we analyzed DNAm in cord blood from the Norwegian Mother, Father and Child Cohort Study (MoBa) biobank. MoBa contains questionnaire data on maternal medication use during pregnancy and information on child neurodevelopmental outcomes assessed by internationally recognized psychometric tests. Two epigenome-wide association studies (EWASs) were conducted to examine the association of prenatal paracetamol, (es)citalopram or folic acid exposure with cord blood DNAm and child neurodevelopment. Additionally, a comparative study of DNAm measurements from the Infinium microarray platforms 450k and EPIC was performed, using duplicate samples assessed by both platforms.

Results: In the systematic literature review, 18 studies were identified. Overall, there were few overlapping findings across studies. However, a fair comparison of the studies was challenging, partly due to substantial heterogeneity in methodology, genome coverage, processing of data and statistical modelling. To this end, 10 recommendations for future studies within prenatal pharmacoepigenetics were proposed. These recommendations

were also the fundament for the three original research papers of this thesis. In the first EWAS on paracetamol, folic acid, DNAm and ADHD, we found no significant association of prenatal paracetamol exposure with differential DNAm in children with ADHD. Further, folic acid did not influence these results substantially. In the EWAS on (es)citalopram, DNAm and neurodevelopment, no sites were significantly differentially methylated between the (es)citalopram, depression and control groups. This was also true when assessing the interaction effect of (es)citalopram and DNAm on neurodevelopment. However, multiple sites were associated with the developmental trajectories of communication and psychomotor skills. Several of these sites annotated to genes relevant to neurodevelopmental processes and have previously been associated with ADHD. In the comparative study of microarray platforms, we identified a considerable number of probes exhibiting poor cross-platform reliabilities. The probe reliabilities were also influenced by the preprocessing of DNAm data.

Discussion: We found that an overall challenge within prenatal pharmacoepigenetics is the non-overlapping findings between studies. Along the same lines, our two EWASs neither found any of the previously identified associations of prenatal paracetamol or (es)citalopram exposure with DNAm. The non-replication of our previous study on paracetamol and DNAm could not be explained by the low reliability of probes across microarray platforms. Yet, low probe reliabilities may in some cases explain non-replication in other studies. Low reliability-probes also exhibit decreased power, and therefore, may have implications for the estimation of appropriate sample sizes in EWASs. Due to the paucity of overlapping findings across studies, we proposed 10 recommendations to promote the quality, interpretability and comparability of prenatal pharmacoepigenetic studies.

Conclusion: The findings of this thesis elucidate multiple challenges of prenatal pharmacoepigenetic studies. There is a paucity of replication of findings across studies, which may in part be explained by heterogeneity in methodologies, genome coverage, processing of DNAm data and statistical modelling. With improvements in technologies and methodologies, it should be feasible to overcome current challenges, which may provide more robust results of clinical value in the near future.

SAMMENDRAG

Bakgrunn: DOHaD-hypotesen postulerer at ulike faktorer i fosterlivet kan påvirke sykdomsutvikling senere i livet. Prenatal eksponering for flere ulike medisiner, slik som analgetika og psykofarmaka, er assosiert med utviklingsforstyrrelser i hjernen, blant annet hyperkinetisk forstyrrelse (ADHD). Den underliggende sammenhengen mellom medisineksponering i fosterlivet og hjerneutvikling er ikke kjent, men epigenetiske modifikasjoner er foreslått som én mulig mekanisme. Det er kjent at en rekke medisiner påvirker epigenetiske prosesser og tidlige studier har sett en sammenheng mellom medisineksponering i fosterlivet og DNA-metylering (DNAm) hos nyfødte. Det er likevel uklart hvorvidt DNAm bidrar inn i forholdet mellom prenatal medisineksponering og hjerneutvikling. Målet med denne avhandlingen var derfor å undersøke forholdet mellom medisineksponering i fosterlivet, DNAm og utviklingsforstyrrelser i hjernen.

Materiale og metode: Denne avhandlingen består av én systematisk litteraturgjennomgang av studier innen prenatal farmakoepigenetikk og tre originale forskningsartikler. I de tre originale arbeidene, analyserte vi DNAm i navlestrengsblod fra biobanken i Den norske mor, far og barn undersøkelsen (MoBa). MoBa har spørreskjemadata om mors medisinbruk under svangerskapet, samt informasjon om barnets utvikling målt med flere internasjonalt anerkjente psykometriske tester. Vi gjennomførte to epigenom-vide assosiasjonsstudier for å undersøke sammenhengen mellom paracetamol-, (es)citalopram- eller folsyreeksponering i fosterlivet med DNAm i navlestrengsblod og utviklingsforstyrrelser med opphav i hjernen. Vi utførte også en studie der vi sammenliknet prøver med målinger av DNAm fra to Infinium mikromatriseplattformer (450k og EPIC).

Resultater: I den systematiske litteraturgjennomgangen fant vi 18 relevante studier. Generelt var det lite overlapp mellom funnene i hver studie. Det var imidlertid vanskelig å sammenlikne de forskjellige studiene, blant annet grunnet ulikhet i metode, genomdekning, normalisering av DNAm-data og statistisk modellering. Derfor foreslo vi 10 anbefalinger for framtidige studier innen prenatal farmakoepigenetikk. Disse anbefalingene la også grunnlaget for de tre originale forskningsartiklene i avhandlingen. I den første epigenom-

vide assosiasjonsstudien på paracetamol, folsyre, DNAm og ADHD fant vi ingen signifikant sammenheng mellom paracetamoleksponering i fosterlivet og endret DNAm i barn med ADHD. Folsyre forandret ikke dette resultatet nevneverdig. I den epigenom-vide assosiasjonsstudien på (es)citalopram, DNAm og hjerneutviklingsforstyrrelser var det heller ingen seter som hadde en signifikant annerledes DNAm da (es)citalopram-, depresjons- og kontrollgruppene ble sammenliknet. Vi fant heller ingen interaksjonseffekt mellom (es)citalopram og DNAm på hjerneutviklingsutfall. Det var derimot flere seter der DNAm var assosiert med ulike utviklingsforløp for kommunikasjonsferdigheter og psykomotorikk. Flere av disse setene lå i eller nær gener som er relevante for utviklingen av hjernen og som tidligere er blitt assosiert med ADHD. I sammenlikningsstudien av mikromatriseplattformene, fant vi en stor andel prober hvis målte DNAm stemte dårlig overens på tvers av plattformer. Graden av overensstemmelse var i tillegg avhengig av hvordan DNAm-dataene var blitt normalisert før sammenlikningen.

Diskusjon: Vi fant at en utfordring innen prenatal farmakoepigenetikk generelt er ikke-overlappende funn på tvers av slike studier. Dette så vi også i de to epigenom-vide studiene som heller ikke fant endret DNAm på seter som tidligere har blitt assosiert med paracetamol eller (es)citalopram. Det at vi ikke klarte å gjenskape funnene fra den tidligere studien vår på paracetamol og DNAm, kunne ikke forklares med den lave overensstemmelsen mellom prober på tvers av mikromatriseplattformene. Dette utelukker imidlertid ikke muligheten for at probene i noen tilfeller kan påvirke replikasjon i andre studier. Ettersom prober med lavt samsvar på tvers av plattformer også har redusert statistisk styrke, kan disse probene i tillegg påvirke beregningen av passende utvalgsstørrelser i epigenom-vide studier. Grunnet mangelen på overlappende funn mellom studier, foreslo vi 10 anbefalinger for å fremme kvaliteten på, tolkbarheten av og sammenlikningen på tvers av prenatale farmakoepigenetiske studier.

Konklusjon: Funnene i denne avhandlingen belyser flere utfordringer ved studier innen prenatal farmakoepigenetikk. Det er mangelfull replikasjon av funn på tvers av studier, som blant annet, men ikke utelukkende, kan forklares ved ulikhet i metode, genomdekning, normalisering av DNAm-data og statistisk modellering. Framtidige forbedringer av teknologi og metoder kan gjøre det mulig å overkomme de nåværende utfordringene, og dermed gi mer robuste resultater av klinisk verdi i nær framtid.

TABLE OF CONTENTS

Preface ... **III**

Acknowledgements ... **V**

Abstract ... **VII**

Sammendrag ... **IX**

Table of Contents ... **XI**

List of Papers ... **XV**

List of Figures ... **XVII**

Abbreviations ... **XIX**

1 Background ... **1**

1.1 Prenatal Pharmacoepidemiology ... *1*
1.1.1 Why are Prenatal Pharmacoepidemiological Studies Needed? ... 2
1.1.2 Analgesic and Psychotropic Use During Pregnancy ... 3
1.1.3 Prenatal Medication Exposure and Neurodevelopment ... 5

1.2 Epigenetics ... *7*
1.2.1 What is Epigenetics? ... 7
1.2.2 Mediators of DNA Methylation and Demethylation ... 8
1.2.3 DNAm Landscape ... 10
1.2.4 The Influence of DNAm on Gene Transcription ... 10
1.2.5 DNAm Variability ... 11
1.2.6 DNAm in Embryonic and Fetal Development ... 12
1.2.7 Epigenetic Epidemiology ... 16

1.3 Prenatal Pharmacoepigenetics ... *16*

2 Thesis Aims ... **19**

3 Materials and Methods ... **21**

3.1 Research Methodologies ... *21*
3.1.1 Systematic Literature Review ... 21

3.1.2 Epigenome-Wide Association Studies 22
3.1.3 Comparative Study of Microarray Platforms 22

3.2 Data Sources 23
3.2.1 The Mother, Father and Child Cohort Study 23
3.2.2 The Medical Birth Registry of Norway 24
3.2.3 The Norwegian Patient Registry 24

3.3 Comparison Groups and Selection Criteria 25

3.4 Measures 26
3.4.1 Prenatal Medication and Supplement Exposure 26
3.4.2 Maternal Mental Health 27
3.4.3 Neurodevelopmental Outcomes 27

3.5 Measuring DNAm – the Microarray Technology 28

3.6 Statistical Considerations 31
3.6.1 Analysis Pipelines and Normalization Methods 31
3.6.2 Selection of Covariates 32
3.6.3 Cell Type Composition 32
3.6.4 Regression Models 33

3.7 Ethics and Data Protection 33

4 Results 35

4.1 Objective 1: Review of Prenatal Pharmacoepigenetic Studies 35

4.2 Objective 2: Prenatal Medications, DNAm and Neurodevelopment 38

4.3 Objective 3: DNAm Variation Between Microarrays 39

5 Discussion 41

5.1 Replication of Findings 42

5.2 Study Power 43
5.2.1 Sample Sizes 44
5.2.2 Infinium Microarray Probe Reliability 45

5.3 Cell Type and Tissue-Specific DNA Methylation 46

5.4 Interpreting DNA Methylation Differences 48

5.5 Neurodevelopmental Outcomes 49

5.6 Genetic Considerations 50

5.7 Causal Inference: Mechanisms and Biomarkers 50

6 Conclusion **53**

7 Future Perspectives **55**

References **59**

Appendix A **83**

Appendix B – ***Errata*** **85**

Paper I **87**

Paper II **111**

Paper III **135**

Paper IV **149**

LIST OF PAPERS

PAPER I

Olstad EW, Nordeng HME, Gervin K. Prenatal medication exposure and epigenetic outcomes: a systematic literature review and recommendations for prenatal pharmacoepigenetic studies. *Epigenetics*. 2022 Apr;17(4):357-380. doi: 10.1080/15592294.2021.1903376. Epub 2021 Apr 29.

PAPER II

Olstad EW, Nordeng HME, Lyle R, Gervin K. No impact of prenatal paracetamol and folic acid exposure on cord blood DNA methylation in children with attention-deficit/hyperactivity disorder. Submitted to *Epigenetics*.

PAPER III

Olstad EW, Nordeng HME, Sandve GK, Lyle R, Gervin K. Low reliability of DNA methylation across Illumina Infinium platforms in cord blood: implications for replication studies and meta-analyses of prenatal exposures. *Clinical Epigenetics*. 2022 Jun 28;14(1):80. doi: 10.1186/s13148-022-01299-3.

PAPER IV

Olstad EW, Nordeng HME, Sandve GK, Lyle R, Gervin K. Effect of prenatal exposure to (es)citalopram and maternal depression during pregnancy on DNA methylation and child neurodevelopment. Submitted to *Molecular Psychiatry*.

Please also see a relevant associated paper not included in the thesis in Appendix A.

LIST OF FIGURES

Figure 1. Overview of two main types of epigenetic modifications. 9
Figure 2. Schematic of DNA methylation in cell differentiation. 15
Figure 3. Fetal development, teratogens and DNA methylation..................18
Figure 4. Overview of the objectives and papers of the present thesis. 20
Figure 5. Overview of the Infinium microarray DNAm assay. 30
Figure 6. Infographic summarizing the main findings of Paper III. 40
Figure 7. The 10 recommendations of the systematic review......................42
Figure 8. Project description of the RealArt Convergence Environment. ...57

ABBREVIATIONS

ADHD	Attention-deficit/hyperactivity disorder
ASD	Autism spectrum disorder
ASQ	The Ages and Stages Quesionnaire
bp	Base pairs
CBCL	The Child Behavior Checklist
CGI	CpG island
CpG	Cytosine-phosphate-guanine site
DNA	Deoxyribonucleic acid
DNAm	DNA methylation
DOHaD	Developmental Origin of Health and Disease
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
EMA	European Medicines Agency
ENCODE	Encyclopedia of DNA Elements
ESC	Embryonic stem cell
(Es)citalopram	Citalopram and escitalopram
EWAS	Epigenome-wide association study
FACS	Fluorescence-activated cell sorting
FDA	United States' Food and Drug Administration
GWAS	Genome-wide association study
ICD-10	International Classification of Diseases and Other Health Problems, 10th Revision
IQ	Intelligence quotient
LCGA	Latent class growth analysis
MBP	Methyl-CpG-binding protein

MBRN	The Medical Birth Registry of Norway
MoBa	The Norwegian Mother, Father and Child Cohort Study
mQTL	Methylation quantitative trait locus
mRNA	Messenger RNA
NIPH	The Norwegian Institute of Public Health
NK	Natural killer
NorPD	The Norwegian Prescription Registry
NPR	The Norwegian Patient Registry
NSAID	Non-steroidal anti-inflammatory drug
nRBC	Nucleated red blood cell
PC	Principal component
PCA	Principal component analysis
PGC	Primordial germ cell
PR^2	Partial R^2
RNA	Ribonucleic acid
SCL	Hopkins Symptom Checklist
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor

1 BACKGROUND

The Developmental Origin of Health and Disease (DOHaD) hypothesis proposes that prenatal exposures can impact later development of diseases in childhood, adolescence and adulthood [1–4]. The mechanisms underlying such relations are not clear, but epigenetic modifications have been proposed as one possible link [5, 6]. For instance, prenatal paracetamol exposure has been associated with both epigenetic patterns [7] and child neurodevelopmental outcomes [8].

To further explore the epigenetic safety of prenatal medication exposure, unifying knowledge and methodology from prenatal pharmacoepidemiology and epigenetics is essential. In this chapter, I first provide a brief overview of prenatal pharmacoepidemiology, with a particular focus on analgesics, psychotropics and child neurodevelopment. Second, the field of epigenetics is presented, focusing on the aspects relevant to prenatal pharmacoepidemiology. Finally, I briefly describe prenatal pharmacoepigenetics, uniting prenatal pharmacoepidemiology and epigenetics.

1.1 PRENATAL PHARMACOEPIDEMIOLOGY

Pregnant women world-wide are facing difficult decisions with regards to medication use, concerning both their own and the fetus' health. About 4 in 5 women report using at least one medication during pregnancy [9, 10]. Still, for medications intended for long-term use, such as antidepressants, pregnancy is one of the main predictors of medication discontinuation [11,

12]. Understanding the risks and benefits of medication for both the mother and the fetus is pivotal to ensure the reproductive safety of medications used during pregnancy.

In this section, I first provide an overview of prenatal pharmacoepidemiological studies, including the motivation for such research. Then, I focus on prenatal exposure to analgesics and psychotropics, and the association of prenatal medication exposure with child neurodevelopmental outcomes.

1.1.1 WHY ARE PRENATAL PHARMACOEPIDEMIOLOGICAL STUDIES NEEDED?

Historically, women, and pregnant women in particular, have been excluded from clinical studies on medication use and safety, being referred to as a "vulnerable" population [13, 14]. The limited knowledge about medication safety during pregnancy was devastatingly exposed by the thalidomide tragedy in the 1950s. Thalidomide was advertised as a completely safe medication to treat morning sickness and insomnia in early pregnancy, but turned out to cause severe birth defects in more than 10,000 children, and likely even more miscarriages [15]. Since then, the importance of including pregnant women in studies on medication safety has received increased attention [13]. Nonetheless, of the 290 pharmaceutical substances submitted for approval to the United States' Food and Drug Administration (FDA) between 2010 and 2019, 89.3% did not have data on use during pregnancy from human studies [16]. Hence, knowledge on the safety of medications during pregnancy is still limited.

Today, the focus on pregnant women as "vulnerable" has shifted to women being "scientifically complex", referring to multiple ethical, juridical and biological considerations [14]. For instance, exposing women to potentially fetotoxic medications during pregnancy is ethically challenging, if not impossible. Further, while women can provide an informed consent to participate, the legal protection of the fetus is challenged as it does not have the capacity to consent. Finally, maternal physiological changes during pregnancy may impact medication metabolism and availability [17, 18], and many medications may cross the blood-placenta barrier and influence the fetus [19, 20]. Thus, understanding medication effects and mechanisms in

pregnant women is crucial, but limited by ethical and juridical restrictions in clinical trials.

The ethical and juridical challenges of including pregnant women in clinical trials render prenatal pharmacoepidemiological studies based on observational data pivotal to ensure reproductive safety of medications for both mother and child. Prenatal pharmacoepidemiological studies using observational data on medication use, e.g., self-reports or prescription records, minimize the ethical and juridical challenges of studying pregnant women. Pharmacoepidemiological data are used to describe utilization patterns among pregnant women and to investigate an association of prenatal medication exposure with an outcome of interest, such as malformations, birth weight, preterm births and psychomotor development.

In summary, the thalidomide tragedy motivated an increased focus on safe medication use during pregnancy. Pregnant women are physiologically different from non-pregnant women, but inclusion in clinical trials is oftentimes infeasible for ethical and juridical reasons. Therefore, prenatal pharmacoepidemiological studies based on observational data are pivotal to ensure reproductive safety of medications.

1.1.2 Analgesic and Psychotropic Use During Pregnancy

Medications are frequently used by pregnant women, with about 4 in 5 women using at least one medication during pregnancy [9, 10]. Overall, the most common medications during pregnancy include gastrointestinal and antiemetic agents, antibiotics and analgesics [9, 10], of which the two latter are usually used for shorter periods of time to treat short-term/acute conditions. While most pregnant women contract short-term illnesses, 1 in 5 women also have long-term and/or chronic diseases during pregnancy [10].

Several of the short-term conditions during pregnancy are oftentimes treated with paracetamol. Paracetamol is recommended by both the European Medicines Agency (EMA) [21] and the FDA [22] to treat pain and fever during pregnancy. Reflecting these recommendations, 50–65% of pregnant women are reported to use paracetamol [8, 10, 23–25]. By contrast, other painkillers like opioid analgesics and non-steroidal anti-inflammatory drugs (NSAIDs; contraindicated during pregnancy) are used in approximately 3–8%

of pregnancies each [9, 10]. Paracetamol's mechanism of action is still debated, but it has been suggested to selectively inhibit the enzyme COX-3 in the central nervous system [26], and thereby relieve pain and reduce fever. The most frequent conditions treated with paracetamol during pregnancy are headache, migraine, pain and various infections, while fever is less commonly paracetamol-treated [24, 27]. Notably, a systematic review of prenatal paracetamol exposure and child neurodevelopment, found no data supporting indication bias due to the maternal condition for paracetamol use [27].

One of the most frequent long-term conditions during pregnancy is depression, with more than 1 in 10 pregnant women suffering from this disorder [28]. Lasting depressive symptoms during pregnancy may contribute to both adverse maternal and child outcomes [29, 30]. Pregnant women are increasingly prescribed antidepressants to treat moderate to severe depression [31–33], with 1–7% of pregnant women using selective serotonin reuptake inhibitors (SSRIs) [9, 10, 31, 32, 34]. In turn, the most frequently used SSRIs are the structurally similar citalopram and escitalopram (hereafter, (es)citalopram), sertraline and fluoxetine [31, 32, 34]. Like other SSRIs, (es)citalopram acts by inhibiting serotonin reuptake in the brain and thereby increases the extracellular serotonin levels, including in the synaptic cleft between neurons [35]. Serotonin is a neurotransmitter implicated in both mood, sleep and memory. At early developmental stages serotonin serves multiple other functions as well [36], including a regulatory role of cell migration and differentiation [37]. It is believed that the increased serotonergic neurotransmission, resulting from the heightened serotonin levels in the synapses, lessens the depressive symptoms [35, 38].

Notably, pregnancy is one of the main predictors of antidepressant medication discontinuation [11]. In general, women are more reluctant to use any medications during pregnancy [39, 40] and overestimate the risks associated with prenatal medication exposure [40, 41]. Yet, some medications are perceived more harmful than others: pregnant women consider antibiotics and paracetamol low-risk, high-benefit medications, while antidepressants and anxiolytics are assumed to exhibit the highest risk [40]. Indeed, in one study assessing the perceived risk of malformations upon use of various medications, prenatal exposure to antidepressants was regarded just as high-risk as prenatal thalidomide exposure [41]. In line with this, women with depression show low adherence to their medication treatment [12]. The low

adherence was associated with the women's negative beliefs about psychotropic use during pregnancy [12]. These findings underscore the importance of studying the safety of medication use during pregnancy, to support pregnant women and health care personnel in weighing the risks against the benefits of medication use.

1.1.3 PRENATAL MEDICATION EXPOSURE AND NEURODEVELOPMENT

Pregnant women's perception of risks associated with medication exposure mainly regards concerns about harming the fetus [12, 39–42]. Indeed, some medications are known teratogens, i.e., the exposure to which may lead to malformations or abnormal development. Examples of such medications include thalidomide [43] and the antiepileptic valproic acid [44]. Importantly, not all medications are teratogenic and the timing of exposure is important [45, 46].

The brain develops throughout pregnancy and into childhood (see Section 1.2.6) [47]. Abnormal development of the brain may result in neurodevelopmental disorders. In the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5), neurodevelopmental disorders are defined as abnormal development of the nervous system, which may impact both cognitive and behavioral abilities, such as language, executive functioning, memory, emotions, and social and psychomotor skills [48]. Examples of neurodevelopmental disorders include attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder (ASD), learning and intellectual disabilities, Tourette's disorder and language disorder [48].

As covered in Section 1.1.2, paracetamol is generally considered safe to use during pregnancy [21, 22]. However, a recent review by Bauer *et al.* (2021) prompted discussion about the toxicity of even therapeutic dosages of paracetamol during pregnancy, with respect to certain reproductive, urogenital and neurodevelopmental conditions [8]. Indeed, several studies find a modest, but consistent, association of prenatal paracetamol exposure with abnormal neurodevelopment in the children [8, 23, 27, 49, 50]. The most frequently studied neurodevelopmental outcomes are ADHD and ASD, and related symptoms such as language and communication skills, attention and executive functioning [8]. Most of the recent systematic reviews also suggest

that the duration of paracetamol exposure is important, with longer duration increasing the risks of abnormal neurodevelopmental outcomes [8, 23, 27, 50]. Nevertheless, the reviews find considerable heterogeneity in results between studies, and conclude that the current evidence for an association of prenatal paracetamol exposure with neurodevelopmental outcomes suffers from methodological shortcomings, such as misclassification bias [8, 23, 27, 50].

The effects of prenatal antidepressant exposure on child neurodevelopment have been extensively studied, albeit showing conflicting results [51–64]. Overall, recent systematic reviews of the literature find that many studies report significant associations between prenatal antidepressant exposure and multiple different neurodevelopmental outcomes [51–64]. The outcomes include ADHD, ASD, pervasive developmental disorder, scholastic skills, intelligence quotient (IQ), mental retardation and developmental delays in various domains such as language, psychomotor, cognitive and socio-emotional skills [51–64].

Meta-analyses of antidepressants and ADHD [54, 56, 57, 60], ASD [53, 55, 57, 59, 60] or mental retardation [60] have also been performed, although such analyses were discouraged in other reviews, due to the heterogeneity in methodologies across studies [61, 63, 64]. Overall, the meta-analyses show some evidence of an association between antidepressants and abnormal neurodevelopment, but all conclude that this association can be partially or entirely explained by confounding by indication [53–57, 59, 60]. This was also found in an umbrella review reporting convincing evidence of a prenatal antidepressant-ASD association which was attenuated when accounting for maternal depression [65].

In line with the meta-analyses, the systematic reviews conclude that more rigorous investigations are needed, with methodologies to reduce biased results, including misclassification bias of medication exposure and neurodevelopmental outcomes [52, 61, 63, 66], and confounding by indication [32, 51, 52, 58, 61, 62, 64]. Some reviews also highlight the utility of the discordant-sibling design to address confounding by indication, by controlling for the shared genetics and environment between siblings [51, 57–59, 61–63]. Accordingly, the meta-analyses restricted to sibling studies all show no association between antidepressant exposure and either ADHD [54, 56] or

ASD [53, 59]. To summarize, while many studies find an association between prenatal antidepressant exposure and neurodevelopmental outcomes, most associations attenuate when controlling for maternal psychiatric illness.

Indeed, in many instances, using the medication may be more beneficial than any potential negative sequelae of using it. For instance, untreated maternal fever may be associated with adverse child outcomes, both acutely (e.g., neural tube and congenital heart defects) and on the long-term (e.g., ASD and ADHD) [67–69]. Depression has also been associated with adverse offspring outcomes, both on the short-term, such as preterm birth and low birth weight [29], and on the long-term, such as increased risk of ASD [70] and poorer child language, psychomotor, cognitive and socio-emotional development [30]. Therefore, even though maternal medications may increase the risk of certain neurodevelopmental outcomes, leaving the condition untreated may prove more harmful to the child.

1.2 EPIGENETICS

The mechanism linking prenatal medication exposure and child neurodevelopmental outcomes remains unclear. In accordance with the DOHaD hypothesis, epigenetics may be a possible link. In this section, I elaborate on what epigenetics is and its importance to embryonic and fetal development, focusing on aspects relevant to prenatal pharmacoepidemiology.

1.2.1 WHAT IS EPIGENETICS?

The answer to the question of what epigenetics is has changed multiple times during the past eight decades [71]. The term “epigenetics” was first coined by the developmental biologist Conrad Waddington in 1942 [72]. Waddington defined the “epigenotype” as the developmental processes occurring *between* the genotype and the phenotype, i.e., the mechanisms underlying any association of the genotype with the phenotype [72]. This definition also reads from the term itself: the prefix “epi-” means “on top of” or “in addition to” in Ancient Greek, and thus, epigenetics literally means “in addition to genetics”. According to a common modern-day definition, epigenetics is mechanisms that regulate gene transcription and genome stability without changing the DNA sequence. These modifications can be maintained through cell divisions

and are reversible. The entire collection of all epigenetic marks in the human is called the human epigenome.

There are two main categories of epigenetic modifications: histone modifications and DNA methylation (DNAm; Figure 1). Other important factors also regulating gene expression are microRNAs, nucleosome positioning and many protein complexes [73]. DNA is wound about 1.8 times around octamers of histone proteins, together called a nucleosome. Multiple nucleosomes wrap into chromatin making up the condensed chromosomes. The histones extend so-called histone tails, which can bind different molecular groups, including methyl, acetyl, phosphate and ubiquitin groups [74]. Histone modifications influence the chromatin structure directly and indirectly, thereby impacting gene transcription [74].

DNAm is the most commonly studied epigenetic modification, probably due to the relative ease of assaying compared to histone modifications [75]. DNAm refers to the attachment of a methyl group ($–CH_3$) to the fifth carbon of a cytosine nucleotide (Figure 1, bottom panel) [76]. Methylation usually occurs on cytosine nucleotides preceding guanine nucleotides (5’-cytosine-phosphate-guanine-3’ sites [CpGs]) [76]. Of note, methylation of cytosines in non-CpG contexts has also been found, particularly in the developing and adult human brain [77–79]. Yet, most research to date focuses on DNAm in the CpG context.

1.2.2 MEDIATORS OF DNA METHYLATION AND DEMETHYLATION

Methylation of the DNA is facilitated by DNA methyltransferases. These enzymes catalyze the transfer of one methyl group from S-adenyl methionine to the cytosine nucleotide. Different transferases act depending on the context of methylation. Dnmt3a and -3b are *de novo* DNA methyltransferases, meaning they can establish new DNAm patterns [76], such as during the major remodeling events of the epigenome early in development (detailed in Section 1.2.6). In contrast, Dnmt1 establishes DNAm patterns during replication, using the complimentary methylated DNA strand as a template [76].

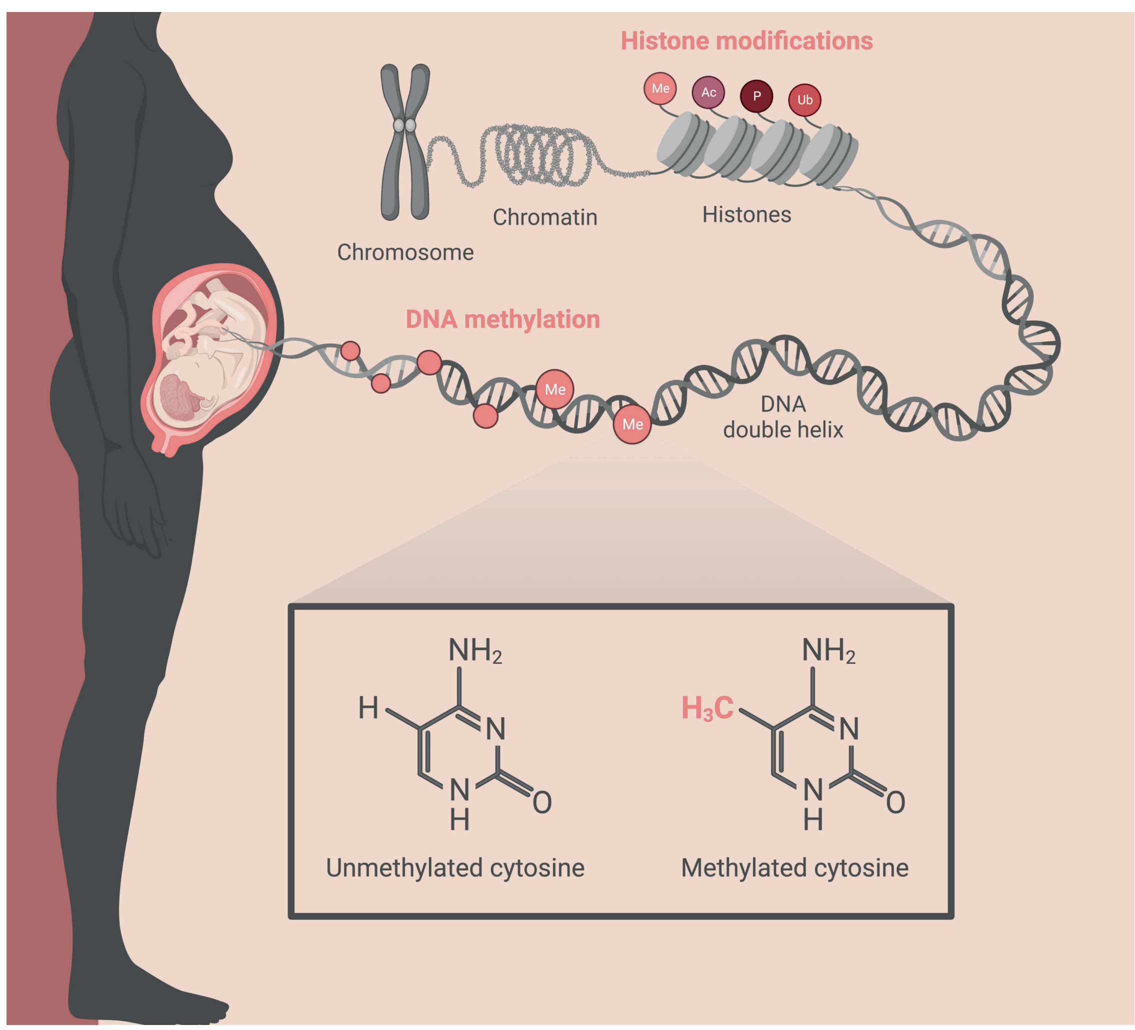


Figure 1. *Overview of two main types of epigenetic modifications.*

The DNA double helix is wound around proteins called histones, forming a complex called chromatin. Condensed chromatin is called chromosomes. Histones extend histone tails upon which different molecular groups, such as methyl (Me), acetyl (Ac), phosphate (P) and ubiquitin (Ub), can be attached to influence chromatin structure. There are also epigenetic modifications attached directly to the cytosine nucleotides of DNA, called DNA methylation. Created with BioRender.com.

DNA demethylation occurs both passively and actively. Passive demethylation may happen during cell division, if Dnmt1 is inhibited or dysfunctional. Active demethylation occurs via several mechanisms, all including a series of chemical reactions. One group of enzymes known to actively mediate demethylation is the ten-eleven translocation enzymes, Tet1, -2 and -3, which catalyze the addition of a hydroxyl group (–OH) to the methyl group to create hydroxymethyl cytosine [76, 80]. The Tet enzymes subsequently oxidize hydroxymethyl-cytosine to formyl-cytosine and then carboxy-cytosine. Finally, the carboxy group is removed to create

unmethylated cytosine [76, 80]. Interestingly, in neurons, hydroxymethylation may account for up to 20% of the total cytosine methylation and hydroxymethylation [77]. This finding prompted the question of whether hydroxymethylation is not merely a step in the demethylation process, but itself serves as a gene regulator [76, 77, 80].

1.2.3 DNAm Landscape

The total DNAm pattern across all CpGs in the genome is called the methylome. While DNAm occurs throughout the genome, some genomic regions are more CpG-dense than others. CpG islands (CGIs) refer to regions of the genome which are rich in CpGs, usually defined as longer than 500 base pairs (bp) and with more than 55–60% CpGs [81, 82]. Regions of 2,000 bp flanking the CGIs upstream ("north") and downstream ("south") are called shores, while the shelves are located 2,000 bp upstream and downstream of the CpG shores [82]. CpGs outside of the shelves are often referred to as open sea CpGs.

Notably, CpG density correlates with DNAm status. Overall, the majority of CpGs in CGIs tend to be unmethylated [83]. However, such hypomethylation is the exception, as 70–80% of all CpGs in mammalian genomes are methylated [83]. Thus, most CpGs in low-density regions are methylated [83]. Importantly, there are deviations from these overall DNAm patterns. For instance, 16% of CGIs in the adult brain are methylated [84], and 10% of the CpGs in low-density regions are hypomethylated [83].

1.2.4 The Influence of DNAm on Gene Transcription

The transcription of genes is the first step toward translating the DNA to proteins. During transcription, an RNA copy of a gene is generated (messenger RNA [mRNA]) through a complex interplay between proteins, including transcription factors and RNA polymerase. The original hypothesis of the DNAm-transcription relation was proposed in the 1970s, suggesting that DNAm directly silences gene expression by inhibiting transcription [85, 86]. However, the link between DNAm and transcription has proven more complicated [87]. It is true that highly methylated promoters and enhancers are typically associated with gene silencing [87–89]. Promoters are genomic regions to which proteins bind to initiate or repress transcription, located nearby the transcription start site, and enhancers are short genomic regions binding proteins which may activate gene transcription not necessarily close

to the transcription start site. Silencing via DNAm in these regions occurs either directly, by obstructing the binding of the transcription machinery, or indirectly, by methyl-CpG-binding proteins (MBPs) [90]. MBPs act by recruiting histone modifiers and chromatin remodelers which are associated with transcriptional repression [90].

Approximately 60–70% of all gene promoters overlap with a CGI and these CGIs commonly appear close to transcription start sites [91–93]. As promoter CGIs are usually unmethylated, these genes are not repressed by methylation [84]. Indeed, CGI-overlapping promoters primarily correspond to genes expressed in most cells, such as housekeeping genes [91–93]. In contrast to the largely unmethylated promoter CGIs, 20–30% of intragenic (gene body) CGIs are methylated [84]. In fact, DNAm in gene bodies is widespread even outside of CGIs, and such DNAm is believed not to preclude gene transcription [84, 94]. Rather, high intragenic methylation may maintain transcription efficiency, for instance, by silencing alternative promoters [84, 87].

In summary, the effect of DNAm on gene transcription is highly context-dependent. The relation depends on both the genomic context and available transcription factors. There are also additional important considerations not detailed here, including the complex interactions between DNAm and other epigenetic factors like histone modifications [83, 95], and the role of DNAm in the splicing of mRNA, giving rise to multiple different transcripts from the same gene [96–98]. Consequently, the relationship between DNAm and gene transcription is still being unraveled, and direct inferences about gene transcription from DNAm status alone is challenging.

1.2.5 DNAm Variability

The epigenome is highly variable and changes over time, according to both internal and external conditions. There are three main factors contributing to site-level DNAm variation between individuals: genetic, environmental and stochastic factors. Approximately 20% of inter-individual DNAm variation is explained by *genetic variation* [99–101]. To this end, researchers have identified several 100,000s loci in the DNA which impact DNAm status at specific CpGs, called methylation quantitative trait loci (mQTLs) [99, 100, 102]. *Environmental influences* include a variety of non-genetic exposures which influence CpG DNAm status. The unique environment of an individual

is estimated to account for around 80% of DNAm variability [101]. In humans, a multitude of environmental exposures have been reported to influence DNAm [103]. For instance, several conventional medications are known to directly interact with enzymes in the epigenetic machinery (e.g., valproic acid and opioids) [104, 105], and up to 5% of available non-epigenetic medications are predicted to interact with epigenetic enzymes [106]. Finally, DNAm changes may also occur *stochastically*, e.g., by random errors during DNA replication [107]. Stochastic DNAm changes accumulate throughout life [107].

DNAm patterns also vary within one individual. In particular, cell types and tissues exhibit distinct DNAm patterns [79, 83, 89, 108]. The origin of these differences is described in the following section.

1.2.6 DNAm in Embryonic and Fetal Development

In accordance with the DOHaD hypothesis, fetal life is a critical period which may influence an individual's health throughout the lifetime. Indeed, this is also true with regards to epigenetics: embryonic and fetal development is a critical time for the establishment of epigenetic patterns. During this time, the epigenome is particularly plastic, making it even more susceptible to environmental influences. The epigenetic plasticity accommodates the complex process of cellular differentiation, whereby the fertilized egg cell becomes the more than 200 specialized cell types of the adult human body.

Fertilization of the egg cell with a sperm cell creates a zygote, in which cell division is initiated to generate cells called blastomeres [109]. The blastomeres are totipotent stem cells, that is, they have the potential to become any cell type in the body. At the eight-cell stage, the blastomeres start to differentiate into two types of cells. The *embryonic stem cells* (*ESCs*) of the inner cell mass, which will develop to the embryo, and the *trophoblasts*, which will form parts of the placenta (Figure 2A) [109]. Thus, the ESCs can differentiate to most cell types except extraembryonic structures, and therefore, are pluripotent. Further cell divisions create a morula (16–32 cells) and subsequently a blastocyst. The blastocyst is eventually implanted into the uterine wall to continue development [109].

During early developmental stages, large-scale epigenetic changes also take place. Two major waves of global DNAm re-programming, occur in ESCs

and primordial germ cells (PGCs; Figure 3). First, just after fertilization, the global DNAm levels decrease substantially, in a wave of global DNAm re-programming (Figure 2A) [110, 111]. In this process, most DNAm of the paternal genome is removed, while demethylation of the maternal genome is less prominent [110, 111]. Spared for this global erasure are imprinting control regions and some transposable elements [110, 111]. The partial erasure and subsequent re-establishment of new DNAm patterns are necessary to ensure the pluripotent features of the ESCs [110]. Only when the blastocyst is implanted into the uterine wall do the global DNAm levels start increasing [80, 112], likely due to the differentiation of ESCs into more specialized cell lineages [112]. The second wave of global demethylation and subsequent re-methylation occurs in the PGCs (Figure 3), which later give rise to egg and sperm cells. Notably, only 6–8% of the global DNAm remains at gestational weeks 10 and 11, in female and male PGCs, respectively [113]. Female PGC re-methylation initiates at around 11 gestational weeks [113], considerably earlier than male PGCs, which may initiate re-methylation around gestational week 19 [113, 114]. Notably, these two waves of global changes in DNAm are coordinated with extensive genome-wide changes also in histone modifications. Hence, these events are not contributing to locus-specific regulation of transcription, but rather a global reprogramming of the epigenome as a whole [80].

In addition to the two major waves of DNAm re-programming during early development, smaller-scale changes in DNAm in a cell type- and tissue-specific manner also occur. After the blastocyst implants into the uterine lining, the pluripotent ESCs develop into different multipotent stem cells, which are progenitors to various highly specialized cell types. For instance, neural stem cells are multipotent stem cells that can become most of the cell types in the brain, such as neurons, astrocytes and oligodendrocytes. This cell differentiation depends on a timely and sequential expression of genes. Indeed, while a neuron and a muscle cell have the same DNA, they exhibit strikingly different features due to their differential expression of genes (Figure 2B).

The regulation of gene silencing and activation throughout differentiation is partly contributed by dynamic DNAm patterns during embryogenesis (Figure 2B) [80, 115]. The dynamic patterns are cell type- and tissue-specific, with 15–21% of CpGs' DNAm status varying between cell types and tissues [116,

117]. These variable CpGs are primarily found in enhancers [77, 115, 116]. The sequential methylation and demethylation of variable CpGs is hypothesized to be mediated by the local binding of transcription factors which causes demethylation [80]. However, how the transcription factors contribute to demethylation is not clear [80]. The dynamic methylation and demethylation during fetal development are the main contributors to the diversity in DNAm found in differentiated cells and tissues later in life [79, 80, 83, 118].

Besides cell differentiation, fetal development also involves other cellular processes, wherein DNAm plays an important role. For instance, neurodevelopment involves complex processes like neuronal migration, synaptic transmission and myelination of neurons [119]. DNAm supports a precise transcriptional regulation of these processes to ensure normal brain development and function [120]. Studies on neurodevelopment and DNAm indicate that considerable DNAm remodeling takes place throughout pregnancy and even postnatally [120, 121]. Further, schizophrenia-associated differential DNAm is enriched for sites that are dynamically altered specifically during fetal brain development, suggesting a neurodevelopmental component of the disorder [121, 122]. Thus, these studies suggest that disorders with some neurodevelopmental component, may be contributed by early epigenetic mechanisms.

To summarize, major changes to the methylome occur during fetal life, including waves of de- and re-methylation, as well as cell differentiation requiring tight transcriptional control. The considerable epigenetic plasticity renders the developing fetus' epigenome particularly susceptible to influence from environmental factors. As dynamic changes in brain DNAm extend into early childhood and occur in genes essential to neurodevelopment, environmental factors such as prenatal medication exposure could in principle influence neurodevelopment.

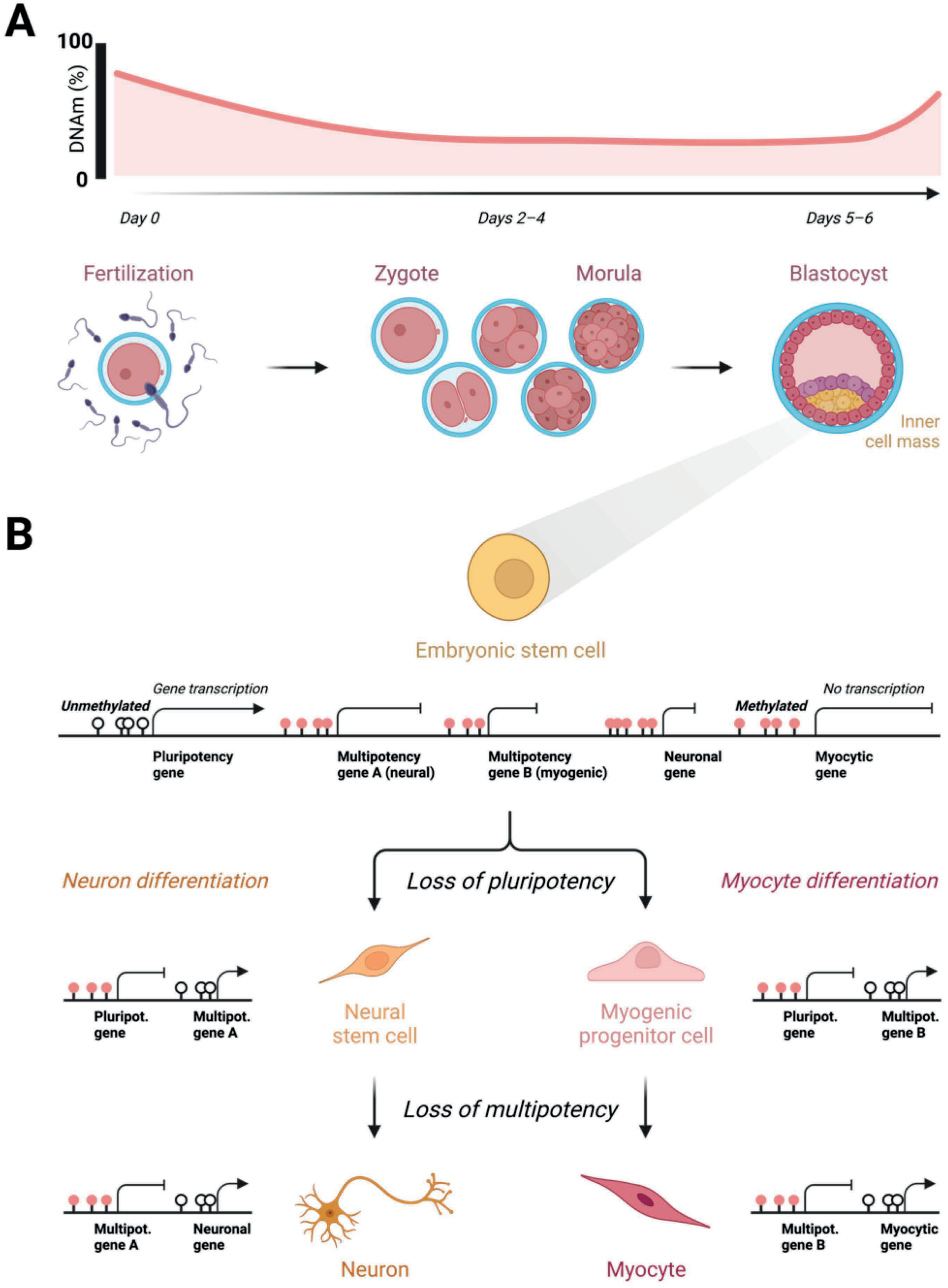


***Figure 2.** Schematic of DNA methylation in cell differentiation.*

Overview of *(**A**) the early stages of human development and (**B**) a very simplified cell differentiation path. (**A**) Just after fertilization, most paternal and maternal DNAm is erased. During this time, the dividing cell exhibits totipotent potential and eventually forms a blastocyst with an inner cell mass of pluripotent embryonic stem cells.*

Figure 2 continued. ***(B)*** *The embryonic stem cells express genes important to maintain pluripotency, while cell type-specific genes are silenced which may partly be due to hypomethylation of regulatory regions, e.g., promoters and enhancers. During cellular differentiation, pluripotency genes are silenced, while genes important for establishing and maintaining a multipotent cell identity are turned on. Neural stem cells will express Gene A but not Gene B, and myogenic progenitor cells will express Gene B but not Gene A, contributing to define particular cell lineages. Finally, cell type-specific genes are differentially expressed based partly on local DNAm patterns. Created with BioRender.com.*

1.2.7 Epigenetic Epidemiology

The ability of DNAm to change dependent on environmental influences is pivotal in epigenetic epidemiology. Early pioneering research, such as the Heijmans *et al.* (2008) [123] study on prenatal famine exposure and *IGF2* DNAm, inspired a wave of research within epigenetic epidemiology [124]. Studies in this field apply epidemiological methods to epigenetic studies [125], to understand the plausible epigenetic etiology of diseases [126].

Early studies within epigenetic epidemiology focused on candidate genes, i.e., gene(s) of interest were selected *a priori* and DNAm was measured at CpGs in the selected gene(s) [75]. In the late 2000s, high-throughput DNAm assays like the Illumina Infinium HumanMethylation microarrays became available [127]. These microarrays enabled increased coverage of the epigenome, while still remaining relatively time- and cost-efficient, facilitating epigenome-wide association studies (EWASs). In EWASs, the DNAm across 100,000s of CpGs are measured and the association of DNAm with an exposure or phenotype of interest is tested [75]. As such, current epigenetic epidemiology studies more elaborately map the epigenetic landscape than traditional candidate gene studies [75]. Indeed, continuous technological, statistical and methodological developments improve studies within epigenetic epidemiology, and the number of such studies is increasing every year.

1.3 Prenatal Pharmacoepigenetics

Pharmacoepigenetics broadly covers three different concepts: development of drugs interacting with the epigenetic machinery, how epigenetic patterns alter the response to medications and conversely, how medications may influence epigenetic patterns. For the purposes of this thesis, prenatal

pharmacoepigenetics refers to how prenatal exposure to medications may influence DNAm.

According to the DOHaD hypothesis, early-life exposures may impact later disease development, possibly mediated through epigenetic modifications. The embryonic and fetal periods are developmental stages particularly susceptible to teratogens (Figure 3). The brain develops throughout pregnancy and into childhood [47], and this process depends on a tightly orchestrated epigenetic program [120, 121]. Prenatal pharmacoepidemiological studies have identified associations of paracetamol and antidepressants with child neurodevelopmental outcomes [8, 23, 27, 49–64]. Further, many medications interact with the epigenetic machinery [104–106]. Yet, whether DNAm is underlying the relations of prenatal medications with neurodevelopment remains elusive.

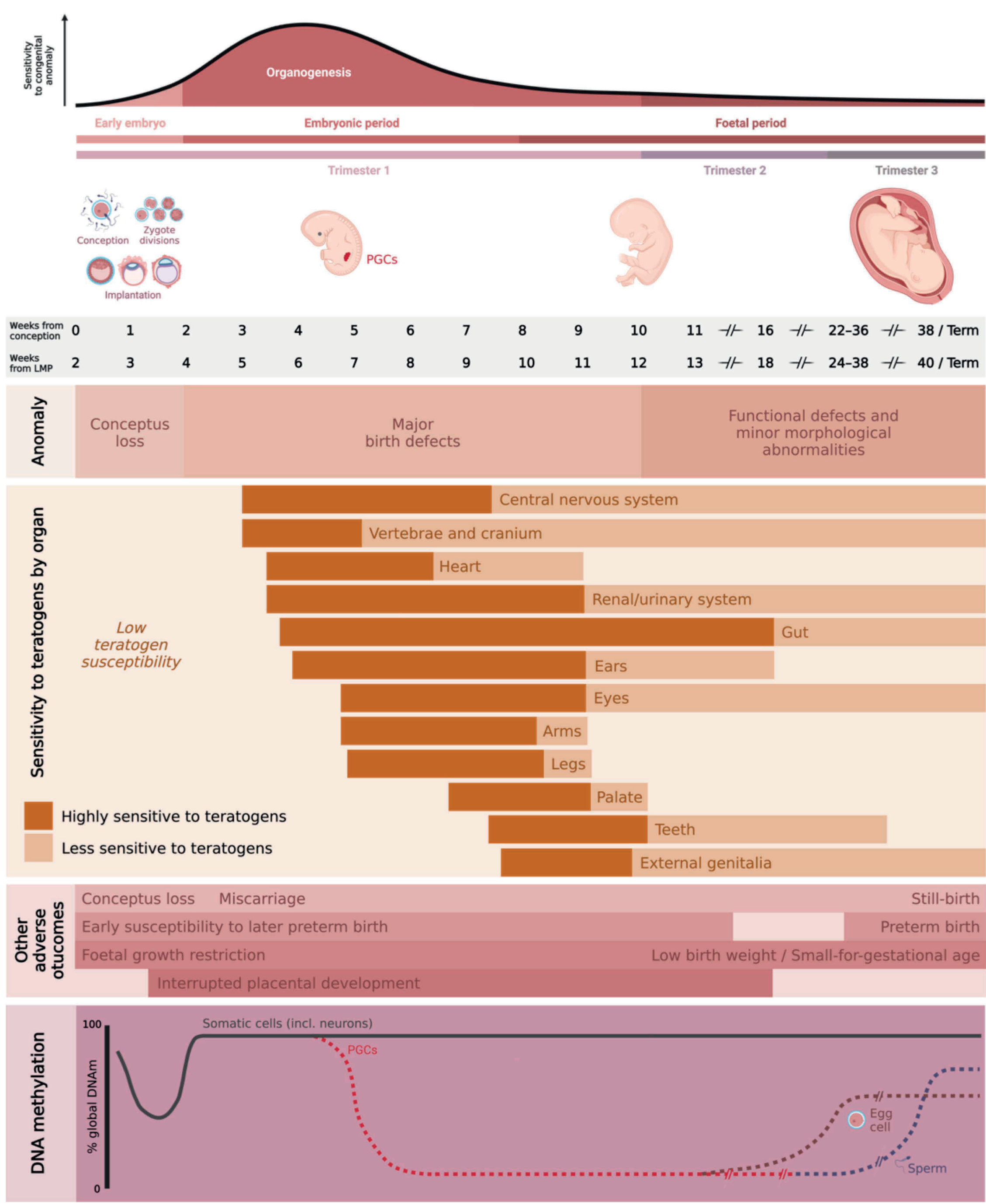


Figure 3. *Fetal development, teratogens and DNA methylation.*

Overview of fetal development, windows of susceptibility to teratogens, adverse outcomes and global DNAm levels. Note that the timing and extent of global re-methylation in the primordial germ cells (PGCs) are not certain [113, 114]. *Modified from Moore (1988)* [128], *Ciernia* et al. *(2016)* [112] *and Gkountela* et al. *(2015)* [114]. *Created with* BioRender.com.

2 THESIS AIMS

The aim of this doctoral work was to explore the relationship between prenatal medication exposure, DNAm and neurodevelopment. The hypothesis was that prenatal exposure to paracetamol or (es)citalopram is associated with DNAm and child neurodevelopment.

To explore the main aim and hypothesis, the specific objectives were to:

(1) Provide an overview of the current literature on prenatal pharmacoepigenetics, focusing on psychotropics and analgesics, and propose recommendations for future studies in the field.

(2) Examine the relationship of prenatal medication exposure, DNAm and neurodevelopment in a Norwegian birth cohort by:

  (a) Conducting EWASs to investigate whether prenatal exposure to paracetamol or (es)citalopram is associated with differences in cord blood DNAm.

  (b) Assessing the association of DNAm with neurodevelopment, and investigating the relation between medications, DNAm and neurodevelopment using interaction terms.

(3) Explore technical variation and reliability of measured DNAm levels in cord blood between Infinium microarray platforms.

The relation of each of these objectives to the papers of the thesis is presented in Figure 4.

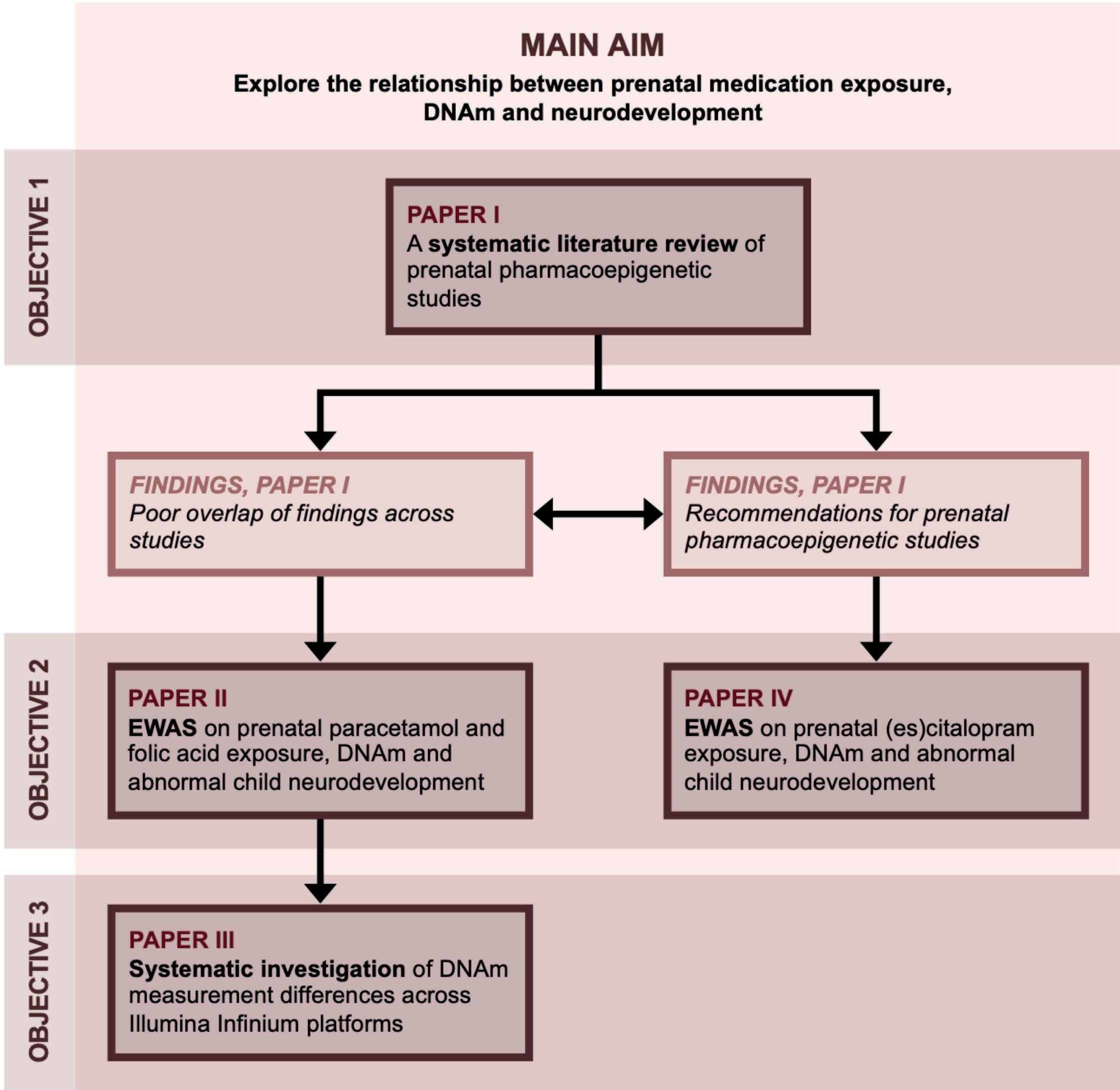


Figure 4. *Overview of the objectives and papers of the present thesis.*

Overview of the main aim, objectives and papers of the present thesis, including the four papers (dark red) and the main findings of the first paper (pink). The findings and recommendations of Paper I provided a foundation for the Papers II–IV, as indicated by the arrows. Which objective the respective papers relate to is indicated in the left margin (see main text for details).

3 MATERIALS AND METHODS

This thesis comprises papers with various research approaches, including a systematic literature review, two EWASs and a comparative study of DNAm measurements from different Infinium microarray platforms. Therefore, in the first section, I briefly overview the distinct study methodologies and approaches. In subsequent sections, I overview topics mainly related to the three original research papers (**Papers II–IV**), including data sources, sample selection, measures, microarray technology and statistical considerations.

3.1 RESEARCH METHODOLOGIES

In this section, I briefly summarize the different research methodologies of the papers. **Paper I** provides an overview of the current literature of prenatal pharmacoepigenetics, while **Papers II–IV** are original research based on data from the Norwegian Mother, Father and Child Birth Cohort Study (MoBa).

3.1.1 SYSTEMATIC LITERATURE REVIEW

In **Paper I**, a systematic literature review was conducted. Systematic literature reviews should compile all the literature relevant to the research question of the review [129]. This requires a systematic and transparent approach, wherein all papers are assessed in the same way. To limit bias by the reviewer, at least two reviewers should preferably be included in the assessment of all articles from the database searches and in the extraction of data from the selected articles [129]. To support researchers in conducting systematic literature reviews, the *Preferred Reporting Items for Systematic review and*

Meta-Analysis protocols (PRISMA) guidelines have been developed, which provides a list of items to be reported [130].

We took multiple measures to make the systematic literature review transparent and the results reliable. First, the search strategy and inclusion criteria were determined *a priori*, and were discussed by all authors and two experienced librarians to ensure an unambiguous protocol. Second, the protocol was registered in PROSPERO, which is a prospective register of systematic reviews [131, 132]. The register is open to everyone and thus ensures methodological transparency. Third, we searched multiple databases, including MEDLINE, EMBASE, PsycINFO, Scopus and Web of Science. Additionally, the reference lists of all included papers and 35 relevant reviews were screened to ensure full coverage of the literature. Fourth, all relevant papers identified in the database searches were screened independently by two reviewers. The reviewers were blinded to each other's assessment until all articles were screened using the Rayyan application [133]. Any disagreement between reviewers were resolved by consulting a third reviewer. Finally, the extraction of data was performed by adhering to the PRISMA guidelines [130], when applicable.

3.1.2 Epigenome-Wide Association Studies

Epigenome-wide association studies (EWASs) aim to examine the association of epigenetic modifications across the genome (primarily, DNAm) with an environmental exposure or phenotype of interest [75, 134]. The two EWASs conducted in **Papers II and IV** are based on data from MoBa, a large prospective birth cohort study (detailed in Section 3.2.1) [135, 136]. Prospective cohort studies are longitudinal, usually conducted over several years, and follow subjects from prior to the occurrence of the phenotype of interest. During the study period, a multitude of variables may be recorded, such as physical and mental health, lifestyle and socioeconomic status.

3.1.3 Comparative Study of Microarray Platforms

In **Paper III**, we systematically investigated the concordance of DNAm measurements. We relied on a subset of data from **Paper II** and the original paper by Gervin *et al.* (2017) [7]. The 17 overlapping samples between the two studies were used to systematically investigate differences in DNAm measurements from two microarray-based platforms.

3.2 DATA SOURCES

Below, I present the data sources used for **Paper II, III and IV**.

3.2.1 THE MOTHER, FATHER AND CHILD COHORT STUDY

The Norwegian Mother, Father and Child Cohort Study (MoBa) is a population-based prospective birth cohort study conducted by the Norwegian Institute of Public Health (NIPH) [135, 136]. All pregnant women in Norway between 1999 and 2008 were invited to participate, and 40.6% of women consented to participation, including linkage of their responses to relevant health registries [135, 136]. MoBa includes approximately 114,500 children, 95,200 mothers and 75,200 fathers [135, 136].

In MoBa, questionnaires regarding e.g., maternal, paternal and child health, lifestyle and school performances, have been distributed multiple times during pregnancy (pregnancy weeks 15, 22 and 30), childhood (0.5, 1.5, 3, 5, 7, 8, 11 years) and adolescence (13, 14 and 16–17 years). The study is still ongoing. Depending on child age, parents, children and/or (pre-)school teachers were asked to reply. Importantly, MoBa also comprises a biobank, with (whole and umbilical cord) blood, urine and milk teeth [137]. The biobank contains more than 90,000 blood samples from parents and children. Whole blood samples from both parents were collected at the routine ultrasound appointment at 17–18 pregnancy weeks. At birth, umbilical cord blood was collected, and maternal whole blood was collected within two days post-partum [137].

A major advantage of MoBa is its size, being one of the world's largest birth cohorts. Further, the cohort contains detailed information on pregnancy exposures, including use of over-the-counter medications and child behavior and cognition [136, 138]. Finally, the multiple different biospecimens [137] offer possibilities to explore the molecular underpinnings of epidemiological findings. An important limitation of MoBa is that the MoBa population does not necessarily reflect the Norwegian population of women giving birth between 1999 and 2008 as a whole. In particular, young women, women smoking or living alone, and mothers with more than two previous pregnancies, previous/current still births or neonatal deaths, were markedly under-represented in MoBa as compared to all women giving birth in Norway in the same time period (22–45% less frequent in MoBa) [139]. By contrast, women taking folic acid or multivitamins were clearly overrepresented in

MoBa (31–43% more frequent in MoBa) [139]. The children still enrolled in MoBa at 3 years old were more frequently the firstborn child and the mother exhibited higher education than other mothers giving birth in Norway during the same time period [140]. Importantly, only the prevalence estimates appeared biased due to self-selection (selection bias) in MoBa, while tested exposure-outcome associations were not biased [139].

The doctoral project is based on questionnaires (Q-) at gestational weeks 15 (Q1) and 30 (Q3), and at 0.5 years (Q4), 1.5 years (Q5), 3 years (Q6) and 5 years (Q5y). The relatively small EWAS sample sizes and the increasing loss to follow up with child age in MoBa [141] render questionnaires beyond 5 years futile to include in our studies. DNAm was measured in cord blood samples from the MoBa biobank.

3.2.2 THE MEDICAL BIRTH REGISTRY OF NORWAY

The Medical Birth Registry of Norway (MBRN) is a national health registry with information on all births in Norway from 1967 and onwards. The registry is administered by NIPH. All Norwegian maternity units are obliged to notify births to the MBRN, this includes all pregnancies ending later than pregnancy week 12 and even pregnancy terminations after this week. This notification should include information on maternal health before and during pregnancy (e.g., medication use, and upon maternal consent, information on maternal occupation, smoking, alcohol use, and whether pregnancy resulted from assisted conception), as well as complications during pregnancy and/or birth (e.g., labor interventions, live or still births, congenital abnormalities and apparent child diagnoses).

3.2.3 THE NORWEGIAN PATIENT REGISTRY

The Norwegian Patient Registry (NPR) contains health information on patients in contact with the Specialist health care services in Norway, provided by specialists in outpatient clinics and governmental hospitals. The registry was established in 2008 and is administered by the Norwegian Directorate of Health. Diagnoses in the NPR are classified based on the International Classification of Diseases and Other Health Problems, 10th Revision (ICD-10).

3.3 COMPARISON GROUPS AND SELECTION CRITERIA

In this section I briefly outline the selection criteria of the various EWAS comparison groups. In both EWASs, only pregnancies which were singleton and term births (≥37 weeks) were included.

In **Paper II**, cases and controls were selected based on both prenatal paracetamol exposure and ADHD diagnosis. ADHD was included as a selection criterion to enrich for ADHD in the study, as ADHD is a relatively rare condition among children in MoBa (~3.0%). Cases were defined as children having received an ADHD diagnosis by a health professional *and* being exposed to paracetamol ≥20 days. All samples having these features were included, as only 61 samples in the entire MoBa exhibited these characteristics. The study included two additional comparison groups: one group of children with an ADHD diagnosis who were not exposed to paracetamol and one group of children neither exposed to paracetamol nor having an ADHD diagnosis. As both of these groups consisted of >100 subjects, 100 individuals for each group were randomly sampled to be included in the study.

In **Paper IV**, any pregnancies where the fetus had been exposed to psycholeptics or antiepileptics were excluded. The cases were defined as being prenatally exposed to either citalopram or escitalopram (collectively referred to as (es)citalopram). To assess confounding by indication, i.e., that the underlying depression and not the antidepressants is associated with DNAm, we also included a depression group of women with unmedicated depression or anxiety during pregnancy. All women in MoBa fulfilling the inclusion criteria of either the (es)citalopram or the depression groups were included, due to relatively low numbers (n = 305 and n = 309, respectively). The control group included women that neither used (es)citalopram nor experienced depression during pregnancy. As this group greatly outnumbered the other comparison groups (n = 17,228), propensity scores were used to match the controls to the (es)citalopram group, resulting in a control group of n = 347 samples. The propensity scores were the probability of the subject taking (es)citalopram provided a list of relevant pre-treatment characteristics [142, 143]. Specifically, propensity scores were generated by using a logistic regression model with (es)citalopram (yes/no) as the dependent variable and various characteristics as independent variables, including measures of

parental lifestyle and socioeconomic status. From this list, covariates with a *p* value <0.1 were selected for inclusion in the final model matching the (es)citalopram subjects to controls.

3.4 MEASURES

3.4.1 PRENATAL MEDICATION AND SUPPLEMENT EXPOSURE

Medication use in both **Papers II and IV** were retrieved from self-reports in MoBa. Provided a list of conditions, pregnant women were asked to report any medication use on each of the indications. Use was reported for every 4-week interval throughout pregnancy. Q1 and Q3 covered gestational weeks 0–4, 5–8 and 9–12, and 13–16, 17–20, 21–24, 25–28 and ≥29, respectively. Q4 covered the last weeks of pregnancy, from gestational week 30 until delivery. Women also reported the total number of days the medication was used for each indication. Medications are denoted by their Anatomical Therapeutic Chemical (ATC) code. Vitamin, mineral and dietary supplement use in MoBa is registered similar to medications. Provided a list of various supplements, women are asked to report any use per 4-week interval throughout pregnancy. In both **Papers II and IV**, medication and supplement use were coded as binary variables ("yes"/"no"), where "yes" indicated use at any time during pregnancy.

In **Paper II**, we defined prenatal paracetamol exposure as maternal use of paracetamol (ATC code: N02BE01) on any indication listed in the MoBa questionnaires for ≥20 days. Maternal folic acid use during pregnancy was also examined. Self-reports of folic acid use in MoBa correspond well with maternal folic acid serum concentrations in gestational week 18 [144, 145]. Maternal folic acid use is also recorded in MBRN upon interview during the first antenatal consultation (gestational weeks 6–12). To be defined as using folic acid during pregnancy, we required recorded use in *both* MoBa and MBRN.

In **Paper IV**, maternal use of citalopram (ATC code: N06AB04) or escitalopram (ATC code: N06AB10) were assessed on indications relating to mental health problems except eating disorders. Any reported use of (es)citalopram, irrespective of the total number of days, were defined as prenatal (es)citalopram exposure, as antidepressants are commonly taken as

one dose per day over a prolonged period of time. Antidepressant self-reports in MoBa and redeemed prescriptions registered in the Norwegian Prescription Database (NorPD) are shown to exhibit good agreement, where 87.0% of reports of antidepressant use in MoBa also have a corresponding filled prescription for the antidepressant in NorPD [146].

3.4.2 MATERNAL MENTAL HEALTH

To examine the impact of the underlying maternal disease in **Paper IV**, we compared mothers using (es)citalopram during pregnancy to a depression group. Maternal depression was ascertained using two measures. First, self-reported depression recorded as answering "Yes" to having depression (Q1, Q3), anxiety (Q1), other psychological problems (Q3) or mental health problems (Q4) during pregnancy. Second, depression was assessed using items from the Hopkins Symptom Checklist (SCL) relating to symptoms of depression and anxiety (SCL-5 items in Q1 and SCL-8 items in Q3) [147–150]. A mean SCL-5 score ≥2.0 is indicative of depression [150, 151]. The SCL is an internationally recognized instrument to measure symptoms of mental disorders [147, 148]. The SCL-5 and -8 short-version SCLs included in MoBa have been shown to correlate well with the full-item instrument SCL-90 (0.90 and 0.92 respectively) [150]. The SCL-5 has a sensitivity of 82.0% and a specificity of 96.0% [151].

3.4.3 NEURODEVELOPMENTAL OUTCOMES

Different domains of neurodevelopment were assessed in **Papers II and IV**. In both papers, ADHD diagnoses recorded in the NPR were utilized. In **Paper IV**, additional psychometric test scores were used to examine features that may be, but are not necessarily, related to ADHD [152, 153].

ADHD DIAGNOSIS IN NPR

Diagnoses in the NPR are registered by specialists in outpatient clinics and governmental hospitals in accordance with the ICD-10 coding system. ADHD was defined as an ICD-10 diagnosis of hyperkinetic disorder (F90) [154]. The ICD-10 F90 diagnostic criteria are relatively similar to the ADHD diagnostic criteria in the Diagnostic and Statistical Manual, 5th edition (DSM-5) [48, 154], albeit the DSM-5 criteria are less conservative than the ICD-10 criteria [155].

THE CHILD BEHAVIOR CHECKLIST, DSM-ADHD SUBSCALE

The Child Behavior Checklist DSM-oriented (CBCL-DSM) ADHD subscale measures ADHD symptoms [156, 157]. In MoBa, the CBCL-DSM is included in questionnaires distributed at 1.5 (Q5), 3 (Q6) and 5 (Q5y) years old. When compared to ADHD diagnoses from semi-structured clinical interviews, the CBCL-DSM ADHD subscale exhibits a moderate agreement ($\kappa = 0.51$), a sensitivity of 81% and a specificity of 70% [158].

THE AGES AND STAGES QUESTIONNAIRE

The Ages and Stages Questionnaire (ASQ) communication, fine motor and gross motor subscales [159] were also included in **Paper IV**. Only a subset of the original ASQ items were included in MoBa and the items included in MoBa span different age ranges of the ASQ questionnaires, to introduce more variation in scores across individuals. Overall, the ASQ exhibits good agreement (84%) compared to standardized assessments, having a sensitivity of 72% and a specificity of 86% [159]. The Norwegian version of the ASQ has also been validated [160].

3.5 MEASURING DNAM – THE MICROARRAY TECHNOLOGY

DNAm can be measured in different ways, broadly categorized based on the experimental approach: *enzyme digestion*-, *affinity enrichment*- and *bisulfite conversion*-based methods [161]. Additionally, third generation methods have also proven useful in assaying DNAm, allowing for direct detection of methylation from the DNA molecule, e.g., the NanoPore technology [162]. Selecting an appropriate assay depends on multiple factors, including budget, time, DNA quantity, throughput, resolution and genome coverage [161]. The Illumina Infinium microarray offers a good balance between throughput and genome coverage versus the costs and time [161], and therefore, these microarrays are the most common method for assaying DNAm to date [134].

To this end, the Infinium microarrays were also used for assaying DNAm in this doctoral project. The different steps of Infinium microarray analysis, are delineated in Figure 5. First, DNA is treated with bisulfite, which deaminate cytosine bases to uracil. Methylated cytosines are not converted to the same degree. Second, the bisulfite-treated DNA is amplified and enzymatically

fragmented. Third, the amplified DNA is hybridized to the microarray. Fourth, the microarray is scanned to reveal probe intensities.

The microarray consists of 100,000s of oligonucleotides called probes, which are target-specific, i.e., each probe should recognize an individual CpG [82, 127]. In the Infinium methylation assays, there are two different probe designs, named type I and II [82]. The type I design has two distinct beads per CpG: one recognizing the methylated CpG and the other recognizing the unmethylated CpG. The bead matching the methylated CpG has a probe which will be extended by one base only if the target sequence has retained the cytosine after bisulfite treatment. Conversely, the bead matching the unmethylated CpG has a probe which will be extended by one base only when the cytosine has been converted to a thymine. DNA polymerase, the enzyme that synthesizes DNA, can only extend the probe by one nucleotide when the preceding base pair matches (i.e., when cytosine binds guanine or adenine binds thymine). Since the base preceding the CpG in the 5' to 3' direction of the DNA will be the same regardless of CpG methylation status, the two beads will both be detected in the same color channel. By contrast, the type II design has only one bead type with one probe extending by a single base. The fluorophore-labeled base reveals whether the cytosine has been converted to thymine based on the color detected in the scanner. After scanning, the intensities measured are "translated" into the methylation level of the CpG (the β value) defined as the proportion of methylated intensity to the total intensity (see enlarged box in Figure 5).

The first Infinium methylation assay covered approximately 27,000 CpGs [127] and used only type I probes. Since type II probes require half the number of beads per CpG, it was utilized when the methylation assay was expanded to include ~480,000 CpGs, named the 450k platform [82]. This was the main microarray used in EWASs throughout the past decade [134], until it was replaced by the Infinium EPIC v1.0 platform covering ~850,000 CpGs. Currently, Illumina offers two different microarray assays to assess human DNAm: the EPIC platform v2.0 (poor-performing probes from v1.0 has been removed and replaced by an additional 186,000 CpGs) and the Infinium custom methylation kit, allowing researchers to customize the microarray with probes of interest (up to 100,000 CpGs) [163]. In summary, the Infinium microarrays are affordable high-throughput assays widely used in EWASs today and are continuously evolving.

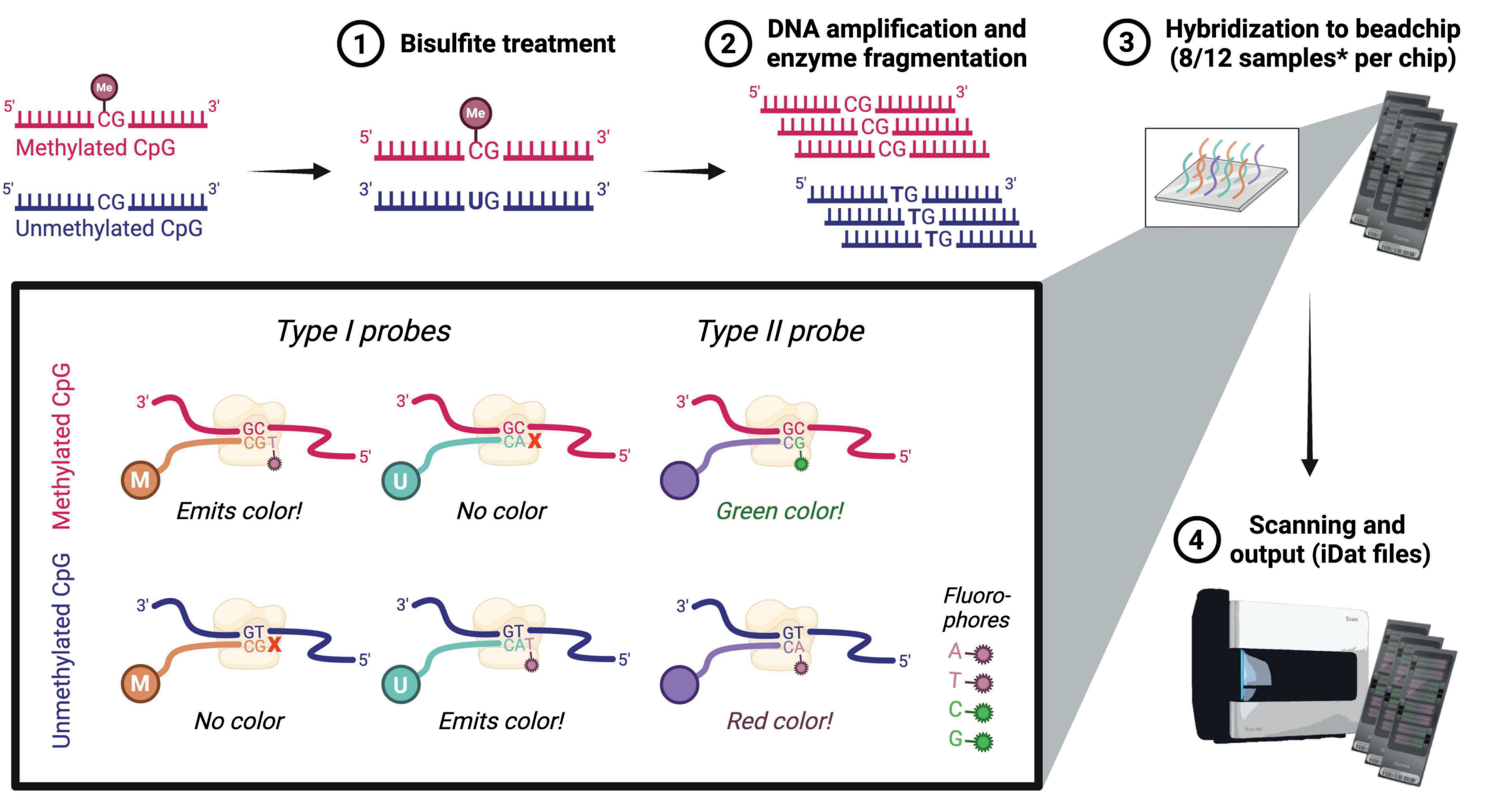


Figure 5. *Overview of the Infinium microarray DNAm assay.*

DNA is treated with bisulfite converting unmethylated cytosine (C) to thymine (T; via uracil [U]). The DNA is amplified and fragmented, before hybridization to a beadchip with two different probe designs (see main text). Microarray scanning outputs red and green color intensities translating to DNAm levels. Created with BioRender.com.

** Depending on microarray platform: 12 samples on the 450k beadchip and 8 samples on the EPIC beadchip.*

3.6 STATISTICAL CONSIDERATIONS

In the below paragraphs, I delineate some statistical considerations that are important in EWASs. Please note that the list is not exhaustive, but focuses on selected topics specifically relevant to this thesis.

3.6.1 ANALYSIS PIPELINES AND NORMALIZATION METHODS

Multiple analysis pipelines have been developed to aid in the processing and interpretation of DNAm data. Most pipelines are embedded in R packages, such as *minfi* [164], *ChAMP* [165, 166], *ENmix* [167], *RnBeads* [168, 169] and *wateRmelon* [170]. The packages feature similar preprocessing steps, including normalization and filtering of the raw data. However, the default selection of normalization method(s) differs across packages. Broadly, normalization of DNAm data may be divided into three categories: *background*, *probe-type* and *dye-bias normalization*. These normalizations adjust for background noise, different probe type chemistries, and bias in dye intensities (red versus green color channel), respectively. There is currently no consensus with respect to which normalization methods are better, and this may also vary depending on the study. For instance, functional normalization is better for studies when large differences between comparison groups are expected (such as in studies on cancer) [171], while *ssNoob* (single-sample normal-exponential using out-of-band probes normalization) has been suggested for the conjunct analysis of 450k and EPIC data [172].

In **Paper III**, the per-CpG reliability of DNAm across platforms for different common analysis pipelines in R was assessed. Based on the findings from these investigations, the default *ENmix* pipeline seemed most appropriate to best conserve concordance of measurements across platforms. Therefore, in **Papers II and III**, the default *ENmix* pipeline was used, including *ENmix.oob* (exponential-normal mixture out-of-band) background normalization [173], *RELIC* (REgression on Logarithm of Internal Control probes) dye-bias correction [174] and the *RCP* (regression on correlated probes) probe-type normalization [175]. In **Paper IV**, background noise was corrected for using *ENmix.oob* [173] and *BMIQ* (beta-mixture quantile normalization) was used for probe-type normalization [176]. The *ENmix.oob* and *BMIQ* methods are both frequently used in EWASs [134], and have been reported to perform better than other normalization methods in conserving concordance across duplicate samples analyzed on the same microarray platform [177, 178].

3.6.2 SELECTION OF COVARIATES

Systematic variation in DNAm data may contribute to false positives. In EWASs, such variation can occur if technical or biological covariates differentially impact DNAm, e.g., due to batch or sex, respectively. Yet, including overly many covariates in a regression model may overburden it and as such reduce the statistical power [134].

In line with this, we found considerable variation in which covariates were included in prenatal pharmacoepigenetic studies in our systematic literature review (**Paper I**). Thus, we implemented a systematic approach in the EWASs of **Papers II and IV**, to determine appropriate covariates to include in the models. First, we summarized the DNAm variation using principal component analysis (PCA), to have a measure of the overall, genome-wide variation in DNAm. The principal components (PCs) accounting for the most DNAm variation were then tested for association with a list of putative relevant covariates. These covariates were identified from prenatal pharmacoepigenetic studies in **Paper I**. Second, the covariates significantly associated with the selected PCs were included in PC-PR^2, a method for assessing the contribution of each covariate to the between-sample DNAm variability [179, 180]. Finally, the covariates contributing the most to DNAm variability between samples were assessed for significant differences in mean or proportion between the comparison groups. If the covariate both contributed considerably to DNAm variability and was significantly different between the comparison groups, we included the covariate in the regression model. By using this systematic approach in selecting covariates, we aimed to include relevant but not excessively many covariates in our models.

3.6.3 CELL TYPE COMPOSITION

As DNAm vary across cell and tissue types, the estimated cell type composition is important to assess in EWASs. Like most current EWASs, we used one of several established deconvolution methods. Such methods may be reference-based or reference-free, based on whether the method uses a reference DNAm database of cell types in the tissue of interest or not [181]. Generally, when a reference database of DNAm is available for the tissue type of interest, a reference-based approach is recommended [182]. Since a validated reference database for umbilical cord blood exists [183], we used this in **Papers II and IV**.

3.6.4 *Regression Models*

In EWASs, linear regression models are most often used to investigate the strength of association between DNAm at individual CpGs and an exposure or phenotype of interest. DNAm data violate the assumptions of linear regression, including that of homoscedasticity [184]. To limit heteroscedasticity, M values ($\log_2(\beta / (1-\beta))$) are suggested for statistical analyses in EWASs rather than β values [184].

Accordingly, we used linear regression models and M values when assessing the association between DNAm and prenatal medication exposure in **Papers II and IV**. We also assessed interaction where two variables interact to influence the response variable. In **Paper II** we asked whether two prenatal exposures (paracetamol and folic acid) could interact to influence DNAm and in **Paper IV**, we asked whether DNAm and (es)citalopram exposure interacted to impact neurodevelopmental outcomes.

In **Paper IV** we also used the unsupervised clustering method latent class growth analysis (LCGA; also called group-based trajectory modelling) to find trajectories of neurodevelopmental outcomes across multiple timepoints [185]. This enabled the consideration of development of such outcomes over time, which may be more informative than symptoms at one particular timepoint.

3.7 Ethics and Data Protection

The linking of data across MoBa, MBRN and NPR was performed using the personal, 11-digit identification number, unique to every permanent resident of Norway. All data are de-identified, and the linkage between MoBa and the different health registries was handled by NIPH and the relevant registries.

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 (reference: S-97045 and S-95113). The MoBa cohort is currently regulated by the Norwegian Health Registry Act. The MoBa biobank is registered as the research biobank number 169 in the Norwegian Biobank Registry. The original studies of this thesis were approved by the Regional Committees for Medical and Health Research Ethics (reference: 23136, 2014/163).

MBRN and NPR are both governed by the *Personal Health Data Registries Act §11* [Norwegian]. Additionally, both MBRN and NPR each have specific regulations for data handling (*Regulation on collection and processing of health information in the Medical Birth Registry of Norway* and *Regulation on collection and processing of health information in the Norwegian Patient Registry* [both in Norwegian]).

4 RESULTS

In the following paragraphs, the main results of the four papers of the thesis are presented, in the order of the thesis objectives (Figure 4).

4.1 OBJECTIVE 1: REVIEW OF PRENATAL PHARMACOEPIGENETIC STUDIES

In **Paper I**, we performed a systematic literature review of studies on prenatal exposure to analgesics or psychotropics with epigenetic modifications as the outcome. We also proposed 10 recommendations for future studies. The results of the systematic literature review provided a foundation for the three remaining papers of the thesis.

We identified 18 eligible studies published before September 1, 2020. The most frequently studied medication group was antidepressants, examined in 12 different studies. The remaining six studies investigated antiepileptic drugs (n = 2), paracetamol (n = 2), acetylsalicylic acid (n = 1) and methadone (n = 1). All studies examined the association between medication exposure and DNAm, either by performing EWASs (n = 7), conducting candidate gene studies (n = 9) or using both approaches (n = 2). DNAm was primarily assessed in cord blood (n = 13), but some studies investigated placentae (n = 5) or buccal cells (n = 2). In addition to DNAm, eight studies also examined child phenotypic outcomes such as ADHD, neonatal abstinence syndrome and stress reactivity.

Notably, among the EWASs investigating the same medication or medication group (paracetamol, antidepressants and antiepileptic drugs), none of the significant CpGs overlapped between studies. Further, for both paracetamol and antiepileptic drugs, one study reported several hundreds of significant CpGs, while the other study found no or only a few significant CpGs. All but one candidate gene study investigated prenatal antidepressant exposure. There were four genes which were investigated in more than one study. For one gene the findings were consistent across studies: there was no difference in DNAm between antidepressant exposed and unexposed groups in the glucocorticoid receptor gene (*NR3C1*).

The systematic review revealed inconsistencies in the prenatal pharmacoepigenetic literature with regards to methodology, materials, genome coverage and statistical modelling. These differences made the interpretation of findings challenging, in particular with regards to comparison across studies. Therefore, we proposed 10 recommendations to improve the quality, interpretability and comparability of future prenatal pharmacoepigenetic studies. The recommendations were focused on how established epidemiological practices can be utilized in epigenetic studies, as well as particular considerations important in epigenetic studies (Box 1).

Box 1. Recommendations for future prenatal pharmacoepigenetic studies. Reproduced from Olstad *et al.* (2021) [187].

(1) HYPOTHESIS: candidate gene studies should use a plausible hypothesis to guide the study design

Hypotheses should be defined prior to designing a candidate gene study, and be guided by principles of teratology, knowledge of pharmacological mechanisms, and epidemiological and biological observations. Hypothesis-free EWASs are also important as the field of prenatal pharmacoepigenetic studies is still emerging.

(2) MEDICATION SELECTION: investigate individual medications rather than medication classes

Unless the pharmacological and epigenetic mechanisms of action of medications are expected to be similar across the medication class, medications should be analysed on an individual substance level.

(3) STATISTICAL POWER: ensure sufficient sample sizes to detect relevant DNAm differences

To detect biologically relevant DNAm associations and to ensure valid interpretation of the results, tools developed for power assessments in epigenetic studies should be used when planning such studies.

Box 1. ***Continued.***

(4) STUDY DESIGN: include a disease comparison group to disentangle medication from indication

Studies should include a disease comparison group to better differentiate the effects of exposure to medication from the underlying maternal disease. This may reduce the impact of confounding by indication.

(5) SYSTEMATIC ERROR: assess selection bias, information bias, and confounding

Selection bias should be assessed by comparing characteristics of study samples to the target population. The validity of medication exposure, neonatal phenotype, and other covariates should be reported, and information bias and misclassification addressed. Measured confounders of the exposure–outcome association(s) are to be adjusted for and residual confounding investigated. Importantly, cell type heterogeneity should be considered a confounding factor in epigenetic studies.

(6) TISSUE SELECTION: biomarkers and extrapolation of DNAm patterns across tissues

If the research aim is not only to report a tissue-independent biomarker, but to extrapolate results to other target tissues, the limitations of such translation should be recognized, and reduced using software applications or data sets on cross-tissue correlations of modifications.

(7) LONGITUDINAL PERSPECTIVE: assess persistence of DNAm patterns throughout childhood

The follow-up of epigenetic patterns later in childhood is essential to assess the relevance of these changes over time, as they may suggest a long-term impact on the phenotypic outcome.

(8) DATA INTEGRATION: integrate epigenetic data with complementary omics data

Integration of complementary omics data, such as genomic and transcriptomic data, can strengthen functional and causal inferences of the findings.

(9) CAUSAL INFERENCE: provides a framework for interpreting exposure-outcome associations

Causal inference methods, such as two-step Mendelian randomization, may support the inference of causation from exposure–outcome associations, including how medication may impact phenotypic outcome via DNAm changes. Importantly, the underlying assumptions of causal methods are often untestable and, therefore, such methods should be used carefully.

(10) REPLICATION: replicate findings using different methods and independent cohorts

Replication both across methods and in independent cohorts is essential to increase the validity of the findings and the generalizability of the results to enhance clinical relevance.

4.2 OBJECTIVE 2: PRENATAL MEDICATIONS, DNAM AND NEURODEVELOPMENT

In **Papers II and IV**, we examined associations between medication exposure, DNAm and child neurodevelopment.

In **Paper II**, we aimed to replicate and expand on a study conducted by our research group on prenatal paracetamol exposure, DNAm and ADHD [7]. In contrast to the original study, we found no significant CpGs associated with paracetamol and ADHD. We also examined whether folic acid could interact with paracetamol in the effect on DNAm, as folic acid is an important methyl donor for DNAm [186]. We did not find any significant interaction effects of paracetamol and folic acid on cord blood DNAm. In summary, this study could not replicate previous findings in MoBa, and did not identify any interaction effects of paracetamol and folic acid on DNAm in children with ADHD.

In **Paper IV**, we aimed to investigate the relationship between prenatal (es)citalopram, cord blood DNAm and later neurodevelopmental outcomes. We found no significant differentially methylated CpGs between the three comparison groups: the (es)citalopram, the depression and the control groups. In line with some previous pharmacoepidemiological studies, we identified a significantly higher proportion of ADHD in the (es)citalopram and depression groups compared to controls. To examine any molecular underpinning of these differences, we investigated whether there was an interaction effect of (es)citalopram exposure and DNAm on neurodevelopment. These analyses did not identify any significant interaction effects of (es)citalopram exposure and DNAm on the neurodevelopmental outcomes. Children with neurodevelopmental conditions often present heterogeneity in the developmental course. We used LCGA to identify different developmental trajectories for ADHD symptoms, and communication and psychomotor skills. We found that DNAm measured at birth before symptom onset was significantly associated with later developmental trajectories of communication (126 significant CpGs) and psychomotor skills (32 significant CpGs). Interestingly, several of the identified CpGs annotated to genes previously associated with ADHD (e.g., *PEX10*, *KCNJ5* and *SHANK2*) and neurodevelopmental processes (e.g., *BEGAIN* and *HOXC4*). Some of the significant CpGs also showed good blood-brain correlation.

To summarize, the two EWASs complement and build on the systematic literature review, by implementing several of the recommendations to improve study quality and interpretability. Neither of the EWASs identified significant associations between prenatal medication exposure and DNAm.

4.3 OBJECTIVE 3: DNAM VARIATION BETWEEN MICROARRAYS

In **Paper III**, we conducted a systematic investigation of the concordance of DNAm measurements between the Infinium microarray platforms 450k and EPIC (Figure 6). This study was prompted by our non-replication in **Paper II**.

We assessed 17 samples with DNAm measured on the 450k and EPIC platforms. Comparing six popular analysis pipelines for DNAm data (*ChAMP*, *ENmix*, *minfi*, *RnBeads*, customized *RnBeads* [*ENmix.oob* and *BMIQ*] and *wateRmelon*), we found considerable differences in cross-platform CpG reliability when preprocessing with the different pipelines. The *ENmix* pipeline exhibited the best cross-platform reliabilities. We found relatively small differences in the mean absolute DNAm differences between platforms, with 1.6% of the CpGs exhibiting a mean absolute difference in DNAm >0.1 across platforms. However, the per-CpG correlation was generally low, with a mean correlation of 0.237. This was also reflected in the cross-platform reliabilities, as only about 26.7% of probes exhibited a moderate or better reliability (intra-class correlation coefficient ≥0.5). Finally, we examined the reliability of the significant CpGs of our previous paracetamol-DNAm study [7], and found that most of these CpGs exhibited low reliabilities, but this could not explain our non-replication of findings in **Paper II**.

In summary, we found relatively poor concordance of DNAm measurements across microarray platforms. While these findings cannot explain the non-replication reported in our **Paper II**, other replication studies may be affected by the poor cross-platform concordance.

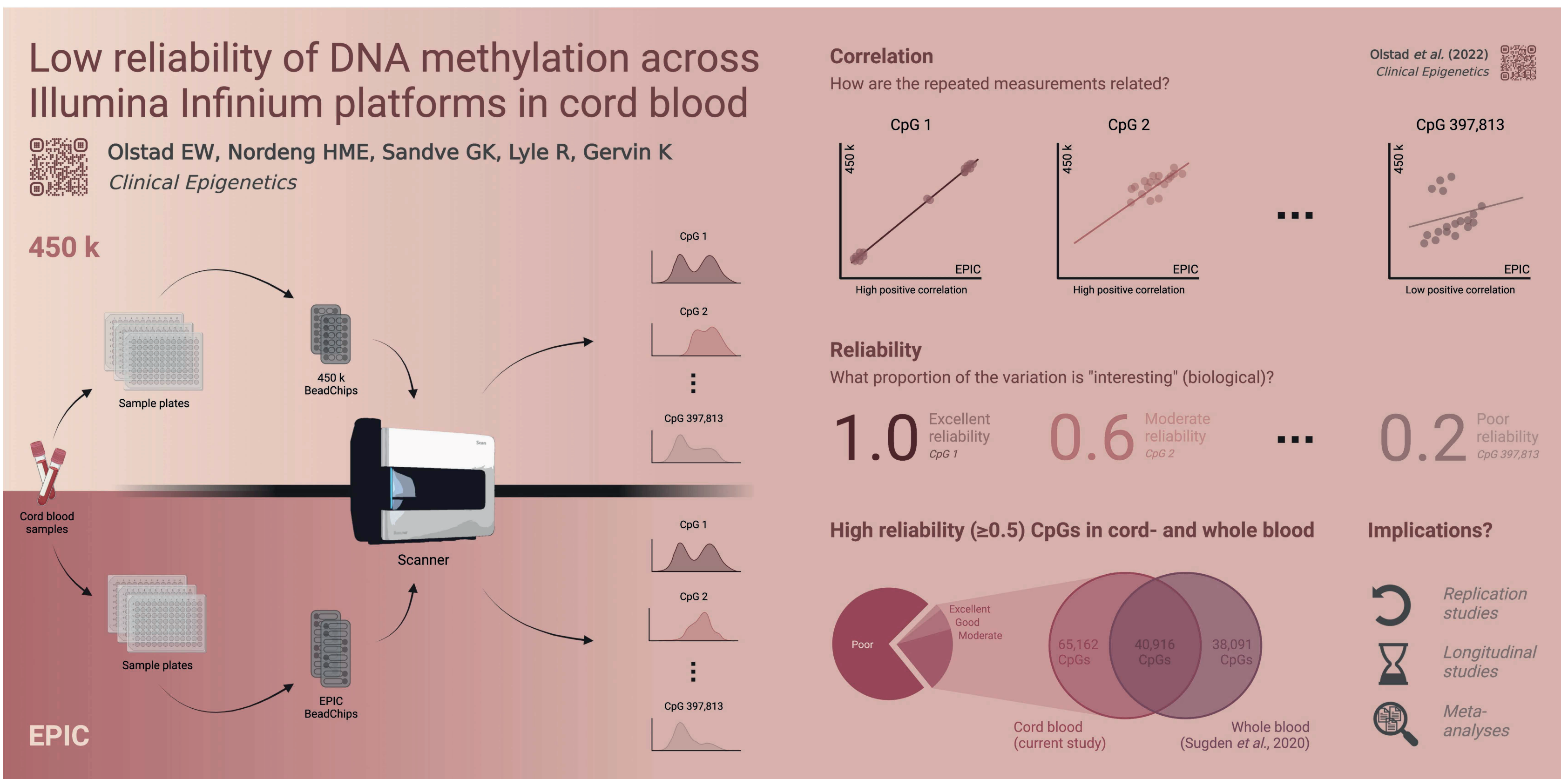


Figure 6. *Infographic summarizing the main findings of Paper III.*

The paper included duplicate samples assayed on either the 450k or EPIC microarray platforms. CpGs with high correlation and accuracy show good reliability (CpG 1), while a decrease in accuracy (CpG 2) or correlation or both (CpG 397,813) reduces the reliability. Among the high-reliability CpGs identified in Paper III, approximately 38.6% overlapped with the high-reliability CpGs of Sugden et al. *(2020)* [205]. *Our findings may have important implications for both replication studies, longitudinal studies and meta-analyses. Created with BioRender.com.*

5 DISCUSSION

To explore the current state in the field of prenatal pharmacoepigenetics, we conducted a systematic literature review. This review identified 18 studies with few overlapping findings across same-medication studies. Yet, a fair comparison of the studies was challenging, partly due to substantial heterogeneity in methodology, genome coverage, processing of data and statistical modelling. To this end, we proposed 10 recommendations to improve the interpretability, comparability and quality of prenatal pharmacoepigenetic studies (Figure 7). The systematic literature review provided an inspiration and foundation for the three remaining papers. In the first EWAS, we found no significant association of prenatal paracetamol exposure with differential DNAm in children with ADHD. Further, folic acid did not interact with paracetamol to influence DNAm. This non-replication of our previous study on paracetamol and DNAm could not be explained by the low reliability of probes across microarray platforms. Nevertheless, the finding of poor concordance of measured DNAm levels across platforms can in principle contribute to non-replication in other instances. Finally, in the study on (es)citalopram, DNAm and neurodevelopment, no CpGs were significantly differentially methylated between the (es)citalopram, depression and control groups. This was also true when assessing the interaction effects of (es)citalopram and DNAm on neurodevelopment. However, we identified multiple CpGs associated with the developmental trajectories of communication and psychomotor skills. Several of these CpGs annotated to genes relevant to neurodevelopmental processes and genes previously associated with ADHD.

Figure 7. *The 10 recommendations of the systematic review.*
Visualization of the 10 recommendations proposed in the systematic literature review. *See Box 1, p. 36–37 for details.*

This thesis highlights several challenges of the current prenatal pharmacoepigenetic studies (Figure 7). Here, I discuss several of these challenges in relation to the other findings of this thesis, and briefly mention the implications for future prenatal pharmacoepigenetic studies.

5.1 Replication of Findings

Replication is essential to ensure that the study findings are robust and valid. In our systematic literature review, we found no overlapping findings across studies on the same medication or medication group. Since we concluded the systematic literature review in September 2020, several new studies in prenatal pharmacoepigenetics have been published. In particular, five new EWASs (two on opioids [188, 189], two on antidepressants [190, 191] and one on paracetamol [192]) and one candidate gene study on opioids [193]. Importantly, none of the new studies change the main finding of mostly non-overlapping results in the systematic literature review.

Along the same lines, we could not replicate the findings of our previous EWAS on paracetamol and ADHD [7]. Further, our study on (es)citalopram did not find any CpGs associated with DNAm, contrasting all but one previous EWAS on antidepressants and cord blood DNAm [190, 194–197].

Regrettably, there is currently a paucity of robust and replicable findings within prenatal pharmacoepigenetics. This challenge is also reported in related fields, such as prenatal exposure to poor maternal wellbeing [198], suggesting that the challenge of replicating findings is not particular to prenatal pharmacoepigenetics, but a broader issue within epigenetic epidemiology.

To this end, we suggested 10 recommendations to improve interpretability, comparability and replicability of prenatal pharmacoepigenetic studies, which were followed up in our (es)citalopram study. First, we investigated only a single antidepressant, instead of all antidepressants (Figure 7 and Box 1, pt. 2 “Medication selection”). Further, we included a disease comparison group of subjects prenatally exposed to unmedicated maternal depression (Figure 7 and Box 1, pt. 4 “Study design”). We also reduced the impact of variables other than the exposure by using propensity score matching to select our controls and systematically assessed potential covariates (Figure 7 and Box 1, pt. 5 “Systematic error”). The validity of all measures of medication exposure and neurodevelopmental outcomes were reported (Figure 7 and Box 1, pt. 5 “Systematic error”). To strengthen the relevance of the findings to brain phenotypes, we also assessed the correlation of DNAm at significant CpGs in cord blood with the same CpGs in different brain areas (Figure 7 and Box 1, pt. 6 “Tissue selection”). Finally, we included a substantially larger number of samples in the exposure group than any of the previous epigenetic studies on prenatal antidepressant exposure. While we believe such measures are important to implement also in future studies, it is still uncertain whether the 10-fold increase in sample size is sufficient to ensure an appropriately powered study.

5.2 Study Power

The power of a study is the probability of rejecting the null hypothesis when the alternative hypothesis is true. I.e., a high power indicates a larger likelihood of identifying a true difference between the comparison groups. Conversely, lower power results in more false negatives and consequently, a reduced likelihood that the identified positives are true positives. Therefore, having sufficient power is essential for the study findings to be robust, interpretable and replicable.

5.2.1 SAMPLE SIZES

Study power is inherently related to sample sizes. However, estimating sufficient sample sizes to achieve an acceptable power in EWASs is challenging [75, 199, 200]. Such calculations depend not only on the significance threshold, the effect sizes and variability in DNAm, but also the cell and/or tissue types and the proportion of differentially methylated CpGs [75, 199].

The median total sample size of the EWASs we reviewed was 19. By comparison, current genome-wide association studies (GWASs), examining the association between single nucleotide polymorphisms (SNPs) and a phenotype, includes 10,000s to 100,000s of samples. Arguably, a pertinent question is why GWASs would require more than a 1,000-fold more samples than EWASs. Indeed, early studies in GWASs also included a few hundred samples. Then, in a powerful review in 2005 [201], Wang *et al.* argued for increasing sample sizes from the order of 100s to 1,000s. Only with these numbers of samples, Wang *et al.* stated, would GWASs be able to detect the small effect sizes of infrequent SNPs [201]. In 2010, Rakyan *et al.* stated that the appropriate sample size of EWASs should not be expected to be any smaller than what is appropriate in GWASs [75]. In particular, additional complexities of epigenetic data include cell- and tissue-specific DNAm patterns, that the DNAm level can take any value between 0 and 1, and inherent measurement errors [75].

Currently, there are two sample size calculators specifically developed for EWASs available, by Graw *et al.* (2019) [199] and Mansell *et al.* (2018) [200]. In the antidepressant EWASs included in the systematic literature review, the median number of exposed samples was 14. According to the Mansell *et al.* calculator [200], with a group size of 14, only 13.4% of CpGs will have a power >80%[1]. Notably, in the (es)citalopram EWAS of this thesis, 98.7% of CpGs have a power >80%[a].

These estimations suggest that most of the sample sizes currently used in prenatal pharmacoepigenetic EWASs are too low for studies to exhibit an appropriate power. While these considerations cannot rule out that some of the significant findings reported in prenatal pharmacoepigenetic studies are

[1] **Parameters:** sample size = 28/650; $\alpha = 9.42*10^{-8}$; diff. DNAm = 0.05; bins = 500.

true positive findings, they suggest that current results should be interpreted with caution. Likely, the rapid development of technology to measure DNAm will enable even more cost-effective DNAm assays in the years to come, which will support increasing sample sizes in EWASs.

5.2.2 *INFINIUM MICROARRAY PROBE RELIABILITY*

In our comparative study of measured DNAm levels across Infinium microarray platforms, we uncovered relatively poor per-CpG reliability between platforms. I.e., the DNAm level measured for a CpG with the 450k platform usually did not correspond well with the DNAm level measured for the same CpG on the EPIC platform. These findings are in line with previous reports in cord blood [202] and multiple other tissues [202–207].

Some studies report that such differences are not impacting EWAS results [202, 203], while others find that the CpGs with higher reliability across platforms also have the highest probability of being replicated [205]. Similarly, poor reliability of probes has been associated with a reduced power of individual CpGs, increasing the number of false negative findings [205, 208, 209]. Consequently, if our non-replication of paracetamol-DNAm associations was entirely explained by low-reliability probes, we would expect no or only a few significant CpGs also in the previous study, which was not the case [7]. Importantly, this does not exclude the possibility of low-reliability probes influencing replication of other studies using different microarray platforms. For CpGs that are truly different between two comparison groups but have low cross-platform probe reliabilities, we are less likely to identify these CpGs across studies using different Infinium platforms, i.e., the CpGs are harder to replicate.

Low probe reliabilities have also been identified for same-platform comparisons of duplicate samples processed conjunctly [173, 178, 203, 205, 209–214]. Yet, to my knowledge, current EWAS power calculators are not explicitly incorporating the low-reliability aspect. Considering the impact of reliability on power and the considerable proportion of low-reliability probes both across and within platforms [173, 178, 211–215, 202–207, 209, 210], it is plausible that low-reliability probes would require much larger sample sizes to attain the same power as high-reliability probes. A further investigation of this hypothesis could provide useful insights into the power of EWASs. It may

also elucidate whether sample sizes of 300–400 are truly sufficient for an appropriate study power, as suggested by current EWAS power calculators.

We also found that the cross-platform probe reliability depended on the preprocessing of the data. Hence, whether DNAm levels are preserved across platforms, also depends on the preprocessing pipeline. Notably, recent studies have reported that changes in analysis pipeline may impact the p values of CpGs upon testing differential DNAm [216], as well as the consistency of DNAm predictors and their association with phenotypes [217].

To summarize, the various microarray platforms and the preprocessing of DNAm data may influence the replicability of prenatal pharmacoepigenetic findings. Therefore, a corollary of welcoming technological improvements and novel bioinformatic tools is the challenges associated with microarray platforms and analysis pipelines. However, a more extensive exploration of the plausible impact of probe reliabilities on power will be important to ensure that current studies are not underpowered to detect true group differences.

5.3 Cell Type and Tissue-Specific DNA Methylation

The cell type- and tissue-specific DNAm patterns confer considerations particular for epigenetic studies. First, changes in cell type proportions rather than actual DNAm changes may be driving differential DNAm between groups. Second, extrapolation between tissues is not straight-forward, and oftentimes peripheral tissues are used due to inaccessibility of the tissue of interest. Third, DNAm measurements from cell-type heterogenous tissues are an average over all the cell types of the tissue.

In the two EWASs of this thesis, we investigated DNAm in umbilical cord blood. Cord blood consists of hematopoietic stem cells, nucleated red blood cells (nRBCs) and various leukocytes, including monocytes, granulocytes and lymphocytes. The constituents of the latter are natural killer (NK) cells, B cells, and $CD4^+$ and $CD8^+$ T cells. These different cell types show distinct DNAm patterns [183, 218]. Consequently, if medication exposure alters the cell type composition of cord blood, differences between the exposed and unexposed groups may not reflect a change in the actual DNAm patterns, but rather in the relative proportions of cell types. Thus, the importance of

accounting for cell type composition in EWASs has been highlighted many times [181, 219, 220].

To this end, potential covariates, including cell type composition, were thoroughly examined in the EWASs of this thesis. As expected, we found that cell types contributed the most variability to DNAm by far, explaining 5.0–13.7% of the between-sample DNAm variability. Importantly, we did not find considerable differences between groups with regards to cell type composition. These investigations suggested that in our cases, cell type composition was likely not a driving force of DNAm differences between comparison groups.

The second challenge with DNAm differences between tissues is whether the DNAm of a peripheral surrogate tissue is relevant to the tissue of interest. For instance, we investigated cord blood DNAm, but were interested in whether DNAm was associated with neurodevelopmental outcomes. Ideally, we would investigate DNAm in fetal brain tissue. However, very few such specimens are available [221, 222]. Even if *post mortem* fetal brain samples were more readily available, such tissue would likely in large part stem from abortions, for which the generalizability to live birthed individuals is questionable. Therefore, a frequently used approach is to examine more available peripheral tissues as proxies for brain tissue. The frequent use of such surrogate tissues in EWASs have led to the investigation of surrogate tissue-brain DNAm correlation and the establishment of databases of such correlations [223–225]. Utilizing these resources may help inferring the biological relevance of significant DNAm differences between groups also in the tissue of interest.

To this end, in the (es)citalopram study, we assessed the CpGs associated with communication and psychomotor developmental trajectories using the BECon online tool [224]. This tool has data from the 450k platform for blood and brain. Notably, current blood-brain correlation resources only exhibit whole blood versus brain correlations [223, 224]. Since DNAm patterns changes as the immune system matures in early childhood, cord blood and whole blood exhibit differential DNAm patterns [218], and extensive changes also occur in the brain methylome throughout development. Therefore, the biological interpretation of the CpGs in the EWAS on (es)citalopram is limited by unavailable tissue correlation resources.

Another limitation of using bulk tissue, whether it is cord blood, whole blood or brain tissue, is the fact that DNAm measurements from cell type heterogenous tissues are an average over all cell types of the tissue [75]. Thus, if there is a considerable change in DNAm in a subset of cell types, this may be concealed by the non-changing DNAm of the other cells. One way to combat this challenge is by analyzing single cell types. To enable sorting of cell types, e.g., by the fluorescence-activated cell sorting (FACS) technique, fresh tissue is needed. However, biobanks of large cohort studies typically contain frozen samples, and thus, disable cell sorting.

5.4 INTERPRETING DNA METHYLATION DIFFERENCES

The effect sizes in the epigenetic epidemiology of complex diseases are expected to be small, on the order of 0.05 and upwards [126, 226]. These small differences are likely a result of the multitude of different factors believed to influence complex disease development and the aforementioned limitations of cell type heterogeneity in tissues.

Inference of the functional implications of differential DNAm will depend on genomic position and how DNAm "translates" to gene transcription. As outlined in Section 1.2.4, a direct inference from DNAm to gene transcription is challenging. Therefore, the functional meaning of changes in DNAm will require integration of other -omics data, including data on the genome, transcriptome and on histone modifications [181]. There are also online resources available to support making functional inferences about DNAm findings. An example of a multi-omics initiative is the Encyclopedia of DNA Elements (ENCODE) project, aiming to elucidate all functional elements of the human genome by mapping a host of different data including transcripts, epigenetics, transcription factor binding sites and genotypes [227–229].

None of the EWASs in the systematic literature review integrated other -omics data. This is also a limitation of the EWASs of this thesis. While single-omics studies may be valuable in identifying potential biomarkers, the mechanistic implications of the findings are difficult to infer from DNAm alone. Increasing utilization of available online resources and focus on multi-omics approaches, may promote the functional interpretation of prenatal pharmacoepigenetic studies.

5.5 NEURODEVELOPMENTAL OUTCOMES

The term neurodevelopmental outcomes covers a variety of different conditions and disorders, whose common denominator is that they origin during the development of the brain. Therefore, neurodevelopmental outcomes may be measured in many different ways, e.g., diagnoses asserted by specialists, parental or self-reported psychometric tests, and school results [66]. The manifestations and developmental courses of neurodevelopmental conditions are heterogenous, and there is considerable symptom overlap between such disorders [230]. To this end, researchers within developmental sciences advocate for a transdiagnostic approach to developmental disorders whereby trajectories of the many domains implicated in such disorders are modeled [231–233].

In both EWASs of this thesis, ADHD was examined. ADHD is characterized by inattention and/or hyperactivity with varying severity [152, 153, 234]. As such, ADHD is inherently a heterogenous disease. Further, the disorder follow different developmental courses [153, 234]. Consequently, a diagnosis may be reductionistic and overly simplistic. Indeed, this is an important limitation of our EWAS on paracetamol wherein we used ADHD diagnoses only.

While diagnoses neatly classify the subjects into two categories, a single-minded focus on neurodevelopmental diagnoses when investigating medication safety and long-term neurodevelopmental outcomes has been questioned [235]. Importantly, longitudinally assessing trajectories of ADHD symptom development, may better reflect subgroups of individuals with the disorder, with differing symptom severity and developmental courses [152, 153, 234]. This approach also enables identification of subclinical individuals, which are not captured with the dichotomous diagnostic label [152, 153, 234]. Indeed, two recent EWASs have used repeated measurements of ADHD symptoms to identify symptom trajectories [236] or to increase the precision of the ADHD measure [237].

In our (es)citalopram study, we included both ADHD diagnosis and symptoms to more robustly assess ADHD. We did not identify a significant association of neither ADHD diagnoses nor ADHD symptom trajectories with differential DNAm. However, delayed communication and psychomotor development were associated with differential DNAm in genes previously associated with

ADHD in childhood [236, 238]. Following up on these findings and covering a broader spectrum of neurodevelopmental conditions, may reveal novel insights into the disease etiology of such disorders.

5.6 Genetic Considerations

An important consideration with regards to DNAm and ADHD, and other neurodevelopmental disorders, is genetics. The EWASs of this thesis could not account for the genetic component of ADHD. In the (es)citalopram study, we observed significantly more children with ADHD and other neurodevelopmental symptoms both among (es)citalopram-treated and depressed mothers. Future studies including genetic data may help to elucidate the impact of maternal genetic susceptibility to child ADHD and other neurodevelopmental outcomes. Further, understanding the correlation of child polygenic risk scores with neurodevelopmental outcomes will also be important.

Genetics contributes about 20% of inter-individual DNAm variation [99–101]. To this end, researchers have identified several 100,000s loci in the DNA which impact DNAm status at specific CpGs, called methylation quantitative trait loci (mQTLs) [99, 100, 102]. Consequently, genetic data may also be used to explore the contribution of genetics to DNAm variation.

Finally, genetic data can be utilized to strengthen the causal interpretation of associations. In particular, mQTLs can also be used for strengthening the causal interpretation of findings. First, by assessing the overlap of mQTLs with EWAS hits. If overlapping, this may suggest that the DNAm mediates an increased disease risk at the specific loci. For instance, studies have demonstrated mQTLs which substantially overlap with genetic variants associated with schizophrenia [239–241] and autism [242]. Second, mQTLs may be used in two-step Mendelian randomization [243, 244], as briefly described in the following section.

5.7 Causal Inference: Mechanisms and Biomarkers

One important question is whether the significant CpGs associated with a phenotype are causal. In the DOHaD framework, DNAm is hypothesized as a

mediator between prenatal exposures and later disease development [5, 6]. In this perspective, DNAm is part of a causal path from exposure to outcome. While understanding disease etiology is important, non-causal associations may also be valuable, for instance as prognostic or diagnostic biomarkers [245].

The mechanism underlying the associations between prenatal medication exposure and neurodevelopment is not known. Understanding whether DNAm is part of this mechanism may guide the use of medications with good safety profiles in pregnancy. Moreover, such investigations can give rise to new therapeutic agents interacting with the epigenetic machinery. For instance, several approved cancer medications are DNA methyltransferase inhibitors [245], as early studies on the epigenetics of cancer identified extensive changes to the methylome [246].

However, even if DNAm plays no mediating role in the medication-neurodevelopmental outcome relationship, it may still be a valuable biomarker. For instance, DNAm biomarkers can be useful to infer exposure status or risk for neurodevelopmental disorders [245, 247, 248]. Further, DNAm biomarkers are already used for diagnostic and prognostic purposes, as well as to predict response to therapy [245]. Notably, biomarkers can be both causal and non-causal.

Most EWASs to date focus on associations only and cannot explore causal relations directly. For instance, our EWASs used linear regression models to assess the association of prenatal medication exposure with DNAm. This analysis alone cannot be used to infer causal relationships.

One approach to explore the role of DNAm as a causal mediator in EWASs, is two-step Mendelian randomization [243, 244]. While this method has been used to assess the role of DNAm in mediating the association of prenatal exposure with child phenotypic outcomes [249, 250], no study within prenatal pharmacoepigenetics has utilized this approach to date. Causal inference methods should be used with caution, however, if the aim of a study is to explore causal pathways, such approaches are pivotal.

6 CONCLUSION

The aim of this thesis was to explore the relationship between prenatal medication exposure, DNAm and neurodevelopment. To this end, a systematic literature review was conducted to summarize the literature on prenatal pharmacoepigenetics and propose recommendations for future studies. Several of the recommendations were followed up on in two EWASs.

We hypothesized that prenatal exposure to paracetamol or (es)citalopram influences DNAm, and may be associated with child neurodevelopmental outcomes. However, in our EWASs, we could not replicate findings from previous studies on the association of prenatal paracetamol or (es)citalopram exposure with DNAm. We did, however, find significant associations between child neurodevelopmental trajectories and DNAm. These findings should be replicated in other independent cohorts.

The finding of non-overlapping results seemed to be a general challenge within prenatal pharmacoepigenetics. As we identified considerable variation in measured DNAm levels across microarray platforms, we suggest that this variation may contribute to the observed paucity of replication of findings. Importantly, other factors likely also contribute to the non-replication, several of which were outlined in this thesis.

In conclusion, the findings of this thesis elucidate multiple challenges of prenatal pharmacoepigenetic studies. The apparent lack of robust findings renders the current clinical relevance of such studies uncertain. With improvements in technology and methodology, it should be possible to overcome the present challenges. This may provide more robust results of greater clinical value in the near future.

7 FUTURE PERSPECTIVES

One important aim of prenatal pharmacoepigenetic studies is the translation of findings into clinic. However, the current state of the field limits such translation. This thesis points out several challenges and possibilities within prenatal pharmacoepigenetics. Below, I outline prospects for future research.

We discovered poor probe reliabilities across platforms and propose that this may influence the power calculations of EWASs (Section 5.2.2). To my knowledge, current EWAS power calculators do not directly include probe reliabilities when estimating study power. Investigating the effect of including probe reliabilities in such calculators may contribute to ensure adequately powered prenatal pharmacoepigenetic studies.

Cell type heterogeneity may conceal DNAm changes in specific cell types (Section 5.3). Currently, obtaining single-cell samples is expensive and infrequently available from large cohort studies. As technology progresses and new cohorts and clinical studies store samples which can be used for cell sorting (e.g., by isolating peripheral mononuclear cells from blood before freezing), future studies may be able to investigate DNAm changes in individual cell types. Such studies may find novel signals currently concealed in cell type heterogenous samples.

Neurons have been discovered to exhibit considerable hydroxymethylation, as well as methylation at non-CpGs [77–79]. These epigenetic modifications are likely also important in gene regulation [80]. Therefore, it would be interesting to assess such modifications. As persistent hydroxymethylation has primarily been found in human brain tissue, ideally, this modification should be investigated in that tissue. However, as pointed out, brain tissues

from live humans are infrequently available and *post mortem* tissues are mostly used. It is also possible to investigate hydroxymethylation in other model systems of differentiating neurons. Notably, as is always important when using model systems, caution is needed when drawing inferences to humans. An examination of hydroxymethylation and non-CpG methylation may be pivotal to better elucidate associations between prenatal medication exposure, (hydroxy)methylation of DNA and child neurodevelopment.

With regards to ADHD and other neurodevelopmental conditions, it would be interesting to assess parental-reported ADHD symptoms and estimate developmental trajectories also in the paracetamol data set (Section 5.5). This could allow for detection of ADHD subpopulations or subclinical individuals, not accounted for when using only the diagnostic label. Furthermore, this thesis mainly focused on ADHD, however, other neurodevelopmental outcomes should also be investigated, e.g., ASD and learning disabilities.

A limitation of the EWASs in this thesis was that we could not assess how maternal genetic susceptibility influences neurodevelopmental outcomes in the child or the correlation of child polygenic risk scores with neurodevelopmental outcomes. Genetic data in MoBa is now available and therefore, an investigation of genetics is feasible. This will allow for an examination of child polygenic risk scores for ADHD and other neurodevelopmental outcomes, as well as an examination of the genetic contribution to DNAm variation. Additionally, mQTLs can be used to strengthen the causal interpretation of findings, as described in Section 5.6.

When integrating genetic and epigenetic data to improve causal inference, the complexity of the data set increases, being both static and dynamic in nature. Whether the simple linear integrative -omics models capture this complexity is not clear. Using artificial data where the ground truth is known may prove a valuable approach to explore such questions. Creating simulated “synthetic” data sets by mimicking statistical patterns in real-world data, enables testing of how varying one parameter influences the estimates of other parameters. As such, simulated data allow for testing of models and assumptions, and can be used to uncover complex interactions and non-linearities in the data. In this regard, newly established initiatives such as the RealArt convergence environment at the University of Oslo hold great promise for improving our knowledge on prenatal pharmacoepigenetics (Figure 8).

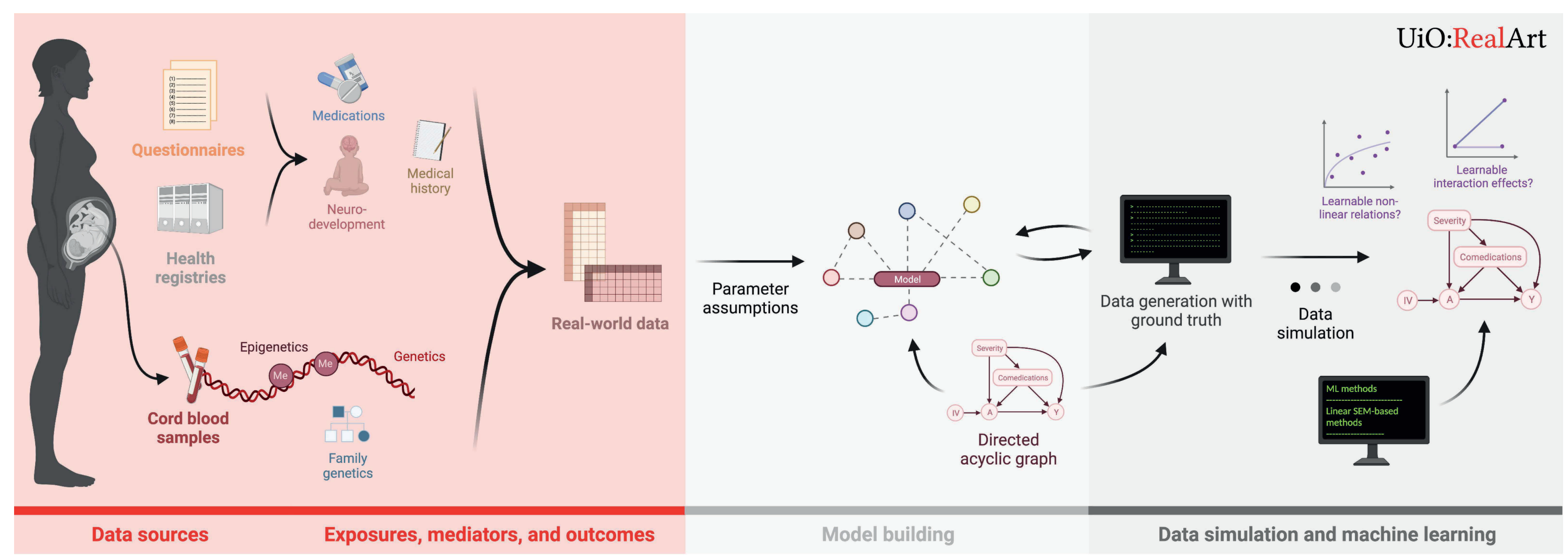


Figure 8. *Project description of the RealArt Convergence Environment.*

Data simulation will be used to generate synthetic data sets mimicking real-world epidemiological, genetic and epigenetic data. This will allow for investigations of complex interactions and non-linearities in the data. Created with BioRender.com.

REFERENCES

1. Barker DJP, Osmond C, Winter PD, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. Lancet. 1989;334:577–80.
2. Barker DJP. The developmental origins of adult disease. J Am Coll Nutr. 2004;23:588S-595S.
3. Gluckman PD, Hanson MA, Pinal C. The developmental origins of adult disease. Matern Child Nutr. 2005;1:130–41.
4. O'Donnell KJ, Meaney MJ. Fetal origins of mental health: The developmental origins of health and disease hypothesis. Am J Psychiatry. 2017;174:319–28.
5. Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD, Hanson MA. Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease. Pediatr Res. 2007;61:5–10.
6. Bianco-Miotto T, Craig JM, Gasser YP, van Dijk SJ, Ozanne SE. Epigenetics and DOHaD: from basics to birth and beyond. J Dev Orig Health Dis. 2017;8:513–9.
7. Gervin K, Nordeng H, Ystrom E, Reichborn-kjennerud T, Lyle R. Long-term prenatal exposure to paracetamol is associated with DNA methylation differences in children diagnosed with ADHD. Clin Epigenetics. 2017;9.
8. Bauer AZ, Swan SH, Kriebel D, Liew Z, Taylor HS, Bornehag CG, et al. Paracetamol use during pregnancy — a call for precautionary action. Nat Rev Endocrinol. 2021;17:757–66.
9. Haas DM, Marsh DJ, Dang DT, Parker CB, Wing DA, Simhan HN, et al. Prescription and Other Medication Use in Pregnancy. Obstet Gynecol. 2018;131:789.
10. Lupattelli A, Spigset O, Twigg MJ, Zagorodnikova K, Mårdby AC, Moretti ME, et al. Medication use in pregnancy: A cross-sectional, multinational web-based study. BMJ Open. 2014;4:4365.
11. Petersen I, Gilbert RE, Evans SJW, Man SL, Nazareth I. Pregnancy as a major determinant for discontinuation of

antidepressants: An analysis of data from The Health Improvement Network. J Clin Psychiatry. 2011;72:979–85.

12. Lupattelli A, Spigset O, Björnsdõttir I, Hämeen-Anttila K, Mårdby AC, Panchaud A, et al. Patterns and factors associated with low adherence to psychotropic medications during pregnancy - A cross-sectional, multinational web-based study. Depress Anxiety. 2015;32:426–36.

13. Committee Opinion No. 646. Obstet Gynecol. 2015;126:e100–7.

14. Blehar MC, Spong C, Grady C, Goldkind SF, Sahin L, Clayton JA. Enrolling pregnant women: issues in clinical research. Womens Health Issues. 2013;23.

15. Vargesson N. Thalidomide-induced teratogenesis: History and mechanisms. Birth Defects Res. 2015;105:140.

16. Byrne JJ, Saucedo AM, Spong CY. Evaluation of Drug Labels Following the 2015 Pregnancy and Lactation Labeling Rule. JAMA Netw open. 2020;3:e2015094.

17. Pariente G, Leibson T, Carls A, Adams-Webber T, Ito S, Koren G. Pregnancy-Associated Changes in Pharmacokinetics: A Systematic Review. PLOS Med. 2016;13:e1002160.

18. Westin AA, Reimers A, Spigset O. Should pregnant women receive lower or higher medication doses? Tidsskr Den Nor legeforening. 2018;138.

19. Goasdoué K, Miller SM, Colditz PB, Björkman ST. Review: The blood-brain barrier; protecting the developing fetal brain. Placenta. 2017;54:111–6.

20. Tetro N, Moushaev S, Rubinchik-Stern M, Eyal S. The Placental Barrier: the Gate and the Fate in Drug Distribution. Pharm Res. 2018;35:1–16.

21. European Medicines Agency. PRAC recommendations on signals: Adopted at the 12-15 March 2019 PRAC meeting [Internet]. 2019 [cited: 2023 Jan 9]. Available from: https://www.ema.europa.eu/en/documents/prac-recommendation/prac-recommendations-signals-adopted-12-15-march-2019-prac-meeting_en.pdf.

22. The U.S. Food and Drug Administration. FDA Drug Safety Communication: FDA has reviewed possible risks of pain

medicine use during pregnancy [Internet]. 2015 [cited: 2023 Jan 9]. Available from: https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-fda-has-reviewed-possible-risks-pain-medicine-use-during-pregnancy.

23. Masarwa R, Levine H, Gorelik E, Reif S, Perlman A, Matok I. Prenatal Exposure to Acetaminophen and Risk for Attention Deficit Hyperactivity Disorder and Autistic Spectrum Disorder: A Systematic Review, Meta-Analysis, and Meta-Regression Analysis of Cohort Studies. Am J Epidemiol. 2018;187:1817–27.

24. Bandoli G, Palmsten K, Chambers C. Acetaminophen use in pregnancy: Examining prevalence, timing, and indication of use in a prospective birth cohort. Paediatr Perinat Epidemiol. 2020;34:237–46.

25. Werler MM, Mitchell AA, Hernandez-Diaz S, Honein MA. Use of over-the-counter medications during pregnancy. Am J Obstet Gynecol. 2005;193:771–7.

26. Chandrasekharan N V., Dai H, Roos KLT, Evanson NK, Tomsik J, Elton TS, et al. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: Cloning, structure, and expression. Proc Natl Acad Sci. 2002;99:13926–31.

27. Bauer AZ, Kriebel D, Herbert MR, Bornehag CG, Swan SH. Prenatal paracetamol exposure and child neurodevelopment: A review. Horm Behav. 2018;101:125–47.

28. Woody CA, Ferrari AJ, Siskind DJ, Whiteford HA, Harris MG. A systematic review and meta-regression of the prevalence and incidence of perinatal depression. J Affect Disord. 2017;219:86–92.

29. Jarde A, Morais M, Kingston D, Giallo R, MacQueen GM, Giglia L, et al. Neonatal outcomes in women with untreated antenatal depression compared with women without depression: A systematic review and meta-analysis. JAMA Psychiatry. 2016;73:826–37.

30. Rogers A, Obst S, Teague SJ, Rossen L, Spry EA, MacDonald JA, et al. Association between Maternal Perinatal Depression and Anxiety and Child and Adolescent Development: A Meta-analysis. JAMA Pediatr. 2020;174:1082–92.

31. Charlton R, Jordan S, Pierini A, Garne E, Neville A, Hansen A, et al. Selective serotonin reuptake inhibitor prescribing before, during and after pregnancy: a population-based study in six European regions. BJOG. 2015;122:1010–20.

32. Molenaar NM, Bais B, Lambregtse-van den Berg MP, Mulder CL, Howell EA, Fox NS, et al. The international prevalence of antidepressant use before, during, and after pregnancy: A systematic review and meta-analysis of timing, type of prescriptions and geographical variability. J Affect Disord. 2020;264:82–9.

33. Mitchell AA, Gilboa SM, Werler MM, Kelley KE, Louik C, Hernández-Díaz S. Medication use during pregnancy, with particular focus on prescription drugs: 1976-2008. Am J Obstet Gynecol. 2011;205:51.e1-51.e8.

34. Zoega H, Kieler H, Norgaard M, Furu K, Valdimarsdottir U, Brandt L, et al. Use of SSRI and SNRI antidepressants during pregnancy: A population-based study from Denmark, Iceland, Norway and Sweden. PLoS One. 2015;10.

35. Maul A. Citalopram. In: Enna SJ, Bylund DB, editors. xPharm: The Comprehensive Pharmacology Reference. Version 2. Elsevier; 2008. p. 1–5.

36. Thompson BL, Levitt P, Stanwood GD. Prenatal exposure to drugs: effects on brain development and implications for policy and education. Nat Rev Neurosci. 2009;10:303–12.

37. Vitalis T, Ansorge MS, Dayer AG. Serotonin homeostasis and serotonin receptors as actors of cortical construction: special attention to the 5-HT3A and 5-HT6 receptor subtypes. Front Cell Neurosci. 2013;7.

38. Waugh J, Goa KL. Escitalopram: A review of its use in the management of major depressive and anxiety disorders. CNS Drugs. 2003;17:343–62.

39. Nordeng H, Koren G, Einarson A. Pregnant women’s beliefs about medications - A study among 866 Norwegian women. Ann Pharmacother. 2010;44:1478–84.

40. Ceulemans M, Lupattelli A, Nordeng H, Odalovic M, Twigg M, Foulon V. Women’s Beliefs About Medicines and Adherence to Pharmacotherapy in Pregnancy: Opportunities for Community Pharmacists. Curr Pharm Des. 2019;25:469–82.

41. Nordeng H, Ystrøm E, Einarson A. Perception of risk regarding the use of medications and other exposures during pregnancy. Eur J Clin Pharmacol. 2010;66:207–14.

42. Bjørndal LD, Tauqeer F, Heiervang KS, Clausen HK, Heitmann K, Lupattelli A. Original research: Perceived risk of neurodevelopmental outcomes in offspring related to psychotropic and mental illness exposures in pregnancy and breastfeeding: a cross-sectional survey of women with past or current mental illness. BMJ Open. 2022;12:e061159.

43. Friedman JM, Weber-Schöndorfer C. 2.13: Antineoplastic drugs. In: Schaefer C, Peters P, Miller RK, editors. Drugs During Pregnancy and Lactation: Treatment Options and Risk Assessment. 3rd ed. Academic Press; 2014. p. 373–99.

44. Chambers C, Schaefer C. 2.10: Epilepsy and antiepileptic drugs. In: Schaefer C, Peters P, Miller RK, editors. Drugs During Pregnancy and Lactation: Treatment Options and Risk Assessment. 3rd ed. Academic Press; 2014. p. 251–91.

45. Wood ME, Andrade SE, Toh S. Safe expectations: current state and future directions for medication safety in pregnancy research. Clin Ther. 2019;41:2467.

46. Peters P, Miller RK, Schaefer C. 1: General commentary on drug therapy and drug risks in pregnancy. In: Schaefer C, Peters P, Miller RK, editors. Drugs During Pregnancy and Lactation: Treatment Options and Risk Assessment. 3rd ed. Academic Press; 2014. p. 1–23.

47. Stiles J, Jernigan TL. The Basics of Brain Development. Neuropsychol Rev. 2010;20:327–48.

48. American Psychiatric Association. Neurodevelopmental Disorders. In: Diagnostic and Statistical Manual of Mental Disorders. 5th ed. American Psychiatric Publishing, Inc.; 2013. p. 31–86.

49. Alemany S, Avella-García C, Liew Z, García-Esteban R, Inoue K, Cadman T, et al. Prenatal and postnatal exposure to acetaminophen in relation to autism spectrum and attention-deficit and hyperactivity symptoms in childhood: Meta-analysis in six European population-based cohorts. Eur J Epidemiol. 2021;36:993–1004.

50. Gou X, Wang Y, Tang Y, Qu Y, Tang J, Shi J, et al. Association

of maternal prenatal acetaminophen use with the risk of attention deficit/hyperactivity disorder in offspring: A meta-analysis. Aust N Z J Psychiatry. 2019;53:195–206.

51. Besag FMC, Vasey MJ. Should Antidepressants be Avoided in Pregnancy? Drug Saf. 2023;46:1.

52. Lebin LG, Novick AM. Selective Serotonin Reuptake Inhibitors (SSRIs) in Pregnancy: An Updated Review on Risks to Mother, Fetus, and Child. Curr Psychiatry Rep. 2022;24:687–95.

53. Zhou XH, Li YJ, Ou JJ, Li YM. Association between maternal antidepressant use during pregnancy and autism spectrum disorder: An updated meta-analysis. Mol Autism. 2018;9:1–7.

54. Man KKC, Chan EW, Ip P, Coghill D, Simonoff E, Chan PKL, et al. Prenatal antidepressant exposure and the risk of attention-deficit hyperactivity disorder in children: A systematic review and meta-analysis. Neurosci Biobehav Rev. 2018;86:1–11.

55. Mezzacappa A, Lasica PA, Gianfagna F, Cazas O, Hardy P, Falissard B, et al. Risk for Autism Spectrum Disorders According to Period of Prenatal Antidepressant Exposure: A Systematic Review and Meta-analysis. JAMA Pediatr. 2017;171:555–63.

56. Jiang HY, Peng CT, Zhang X, Ruan B. Antidepressant use during pregnancy and the risk of attention-deficit/hyperactivity disorder in the children: a meta-analysis of cohort studies. BJOG. 2018;125:1077–84.

57. Leshem R, Bar-Oz B, Diav-Citrin O, Gbaly S, Soliman J, Renoux C, et al. Selective Serotonin Reuptake Inhibitors (SSRIs) and Serotonin Norepinephrine Reuptake Inhibitors (SNRIs) During Pregnancy and the Risk for Autism spectrum disorder (ASD) and Attention deficit hyperactivity disorder (ADHD) in the Offspring: A True Effect or a Bias? A Systematic Review & Meta-Analysis. Curr Neuropharmacol. 2021;19:896.

58. Rommel AS, Bergink V, Liu X, Munk-Olsen T, Molenaar NM. Long-Term Effects of Intrauterine Exposure to Antidepressants on Physical, Neurodevelopmental, and Psychiatric Outcomes: A Systematic Review. J Clin Psychiatry. 2020;81:10661.

59. Vega ML, Newport GC, Bozhdaraj D, Saltz SB, Nemeroff CB, Newport DJ. Implementation of advanced methods for reproductive pharmacovigilance in autism: A meta-analysis of

the effects of prenatal antidepressant exposure. Am J Psychiatry. 2020;177:506–17.

60. Halvorsen A, Hesel B, Østergaard SD, Danielsen AA. In utero exposure to selective serotonin reuptake inhibitors and development of mental disorders: a systematic review and meta-analysis. Acta Psychiatr Scand. 2019;139:493–507.

61. Sujan AC, Öberg AS, Quinn PD, D'Onofrio BM. Annual Research Review: Maternal antidepressant use during pregnancy and offspring neurodevelopmental problems - a critical review and recommendations for future research. J Child Psychol Psychiatry. 2019;60:356–76.

62. Fitton CA, Steiner MFC, Aucott L, Pell JP, Mackay DF, Fleming M, et al. In utero exposure to antidepressant medication and neonatal and child outcomes: a systematic review. Acta Psychiatr Scand. 2020;141:21–33.

63. Morales DR, Slattery J, Evans S, Kurz X. Antidepressant use during pregnancy and risk of autism spectrum disorder and attention deficit hyperactivity disorder: Systematic review of observational studies and methodological considerations. BMC Med. 2018;16:1–14.

64. Prady SL, Hanlon I, Fraser LK, Mikocka-Walus A. A systematic review of maternal antidepressant use in pregnancy and short- and long-term offspring's outcomes. Arch Womens Ment Health. 2018;21:127.

65. Dragioti E, Solmi M, Favaro A, Fusar-Poli P, Dazzan P, Thompson T, et al. Association of Antidepressant Use With Adverse Health Outcomes: A Systematic Umbrella Review. JAMA Psychiatry. 2019;76:1241–55.

66. Hjorth S, Bromley R, Ystrom E, Lupattelli A, Spigset O, Nordeng H. Use and validity of child neurodevelopment outcome measures in studies on prenatal exposure to psychotropic and analgesic medications - A systematic review. PLoS One. 2019;14:e0219778.

67. Dreier JW, Andersen AMN, Berg-Beckhoff G. Systematic review and meta-analyses: Fever in pregnancy and health impacts in the offspring. Pediatrics. 2014;133:e674–88.

68. Croen LA, Qian Y, Ashwood P, Zerbo O, Schendel D, Pinto-Martin J, et al. Infection and Fever in Pregnancy and Autism

Spectrum Disorders: Findings from the Study to Explore Early Development. Autism Res. 2019;12:1551.

69. Gustavson K, Ask H, Ystrom E, Stoltenberg C, Lipkin WI, Surén P, et al. Maternal fever during pregnancy and offspring attention deficit hyperactivity disorder. Sci Rep. 2019;9.

70. Gentile S, Fusco ML. Placental and fetal effects of antenatal exposure to antidepressants or untreated maternal depression. J Matern Neonatal Med. 30:1189–99.

71. Bird A. Perceptions of epigenetics. Nat. 2007;447:396–8.

72. Waddington CH. The epigenotype. Endeavour. 1942;18–20.

73. Cavalli G, Heard E. Advances in epigenetics link genetics to the environment and disease. Nat. 2019;571:489–99.

74. Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. Cell Res. 2011;21:381–95.

75. Rakyan VK, Down TA, Balding DJ, Beck S. Epigenome-wide association studies for common human diseases. Nat Rev Genet. 2011;12:529–41.

76. Moore LD, Le T, Fan G. DNA Methylation and Its Basic Function. Neuropsychopharmacology. 2013;38:23–38.

77. Lister R, Mukamel EA, Nery JR, Urich M, Puddifoot CA, Johnson ND, et al. Global epigenomic reconfiguration during mammalian brain development. Science. 2013;341.

78. Kinde B, Gabel HW, Gilbert CS, Griffith EC, Greenberg ME. Reading the unique DNA methylation landscape of the brain: Non-CpG methylation, hydroxymethylation, and MeCP2. Proc Natl Acad Sci U S A. 2015;112:6800–6.

79. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. Nat. 2009;462:315–22.

80. Luo C, Hajkova P, Ecker JR. Dynamic DNA methylation: In the right place at the right time. Science. 2018;361:1336–40.

81. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A. 2002;99:3740–5.

82. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, et al. High density DNA methylation array with single CpG site resolution. Genomics. 2011;98:288–95.

83. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, et al. Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nat. 2008;454:766–70.

84. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, et al. Conserved role of intragenic DNA methylation in regulating alternative promoters. Nat. 2010;466:253–7.

85. Riggs AD. X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet. 1975;14:9–25.

86. Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. Science. 1975;187:226–32.

87. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet. 2012;13:484–92.

88. Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes Dev. 2011;25:1010.

89. Varley KE, Gertz J, Bowling KM, Parker SL, Reddy TE, Pauli-Behn F, et al. Dynamic DNA methylation across diverse human cell lines and tissues. Genome Res. 2013;23:555.

90. Du Q, Luu PL, Stirzaker C, Clark SJ. Methyl-CpG-binding domain proteins: Readers of the epigenome. Epigenomics. 2015;7:1051–73.

91. Larsen F, Gundersen G, Lopez R, Prydz H. CpG islands as gene markers in the human genome. Genomics. 1992;13:1095–107.

92. Saxonov S, Berg P, Brutlag DL. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc Natl Acad Sci U S A. 2006;103:1412–7.

93. Weber M, Hellmann I, Stadler MB, Ramos L, Pääbo S, Rebhan M, et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet. 2007;39:457–66.

94. Ball MP, Li JB, Gao Y, Lee JH, Leproust EM, Park IH, et al. Targeted and genome-scale strategies reveal gene-body

methylation signatures in human cells. Nat Biotechnol. 2009;27:361–8.

95. Roadmap Epigenomics Consortium, Kundaje A, Meuleman W, Ernst J, Bilenky M, Yen A, et al. Integrative analysis of 111 reference human epigenomes. Nat. 2015;518:317–29.

96. Gelfman S, Cohen N, Yearim A, Ast G. DNA-methylation effect on cotranscriptional splicing is dependent on GC architecture of the exon-intron structure. Genome Res. 2013;23:789–99.

97. Lev Maor G, Yearim A, Ast G. The alternative role of DNA methylation in splicing regulation. Trends Genet. 2015;31:274–80.

98. Shayevitch R, Askayo D, Keydar I, Ast G. The importance of DNA methylation of exons on alternative splicing. RNA. 2018;24:1351–62.

99. Gaunt TR, Shihab HA, Hemani G, Min JL, Woodward G, Lyttleton O, et al. Systematic identification of genetic influences on methylation across the human life course. Genome Biol. 2016;17:61.

100. Min JL, Hemani G, Hannon E, Dekkers KF, Castillo-Fernandez J, Luijk R, et al. Genomic and phenotypic insights from an atlas of genetic effects on DNA methylation. Nat Genet. 2021;53:1311–21.

101. Van Dongen J, Nivard MG, Willemsen G, Hottenga JJ, Helmer Q, Dolan C V., et al. Genetic and environmental influences interact with age and sex in shaping the human methylome. Nat Commun. 2016;7:1–13.

102. Hannon E, Spiers H, Viana J, Pidsley R, Burrage J, Murphy TM, et al. Methylation QTLs in the developing brain and their enrichment in schizophrenia risk loci. Nat Neurosci. 2015;19:48–54.

103. Martin EM, Fry RC. Environmental Influences on the Epigenome: Exposure- Associated DNA Methylation in Human Populations. Annu Rev Public Health. 2018;39:309–33.

104. Kringel D, Malkusch S, Lötsch J. Drugs and Epigenetic Molecular Functions. A Pharmacological Data Scientometric Analysis. Int J Mol Sci. 2021;22:7250.

105. Csoka AB, Szyf M. Epigenetic side-effects of common

pharmaceuticals: a potential new field in medicine and pharmacology. Med Hypotheses. 2009;73:770–80.

106. Lotsch J, Schneider G, Reker D, Parnham MJ, Schneider P, Geisslinger G, et al. Common non-epigenetic drugs as epigenetic modulators. Trends Mol Med. 2013;19:742–53.

107. Vershinina O, Bacalini MG, Zaikin A, Franceschi C, Ivanchenko M. Disentangling age-dependent DNA methylation: deterministic, stochastic, and nonlinear. Sci Rep. 2021;11:9201.

108. Loyfer N, Magenheim J, Peretz A, Cann G, Bredno J, Klochendler A, et al. A DNA methylation atlas of normal human cell types. Nat. 2023;613:1–10.

109. Schoenwolf G, Bleyl S, Brauer P, Francis-West P. 1: Gametogenesis, Fertilization, and First Week. Larsen's Human Embryology. 6th ed. Elsevier; 2020. eBook.

110. Smith ZD, Chan MM, Humm KC, Karnik R, Mekhoubad S, Regev A, et al. DNA methylation dynamics of the human preimplantation embryo. Nat. 2014;511:611–5.

111. Guo H, Zhu P, Yan L, Li R, Hu B, Lian Y, et al. The DNA methylation landscape of human early embryos. Nat. 2014;511:606–10.

112. Ciernia AV, LaSalle J. The landscape of DNA methylation amid a perfect storm of autism aetiologies. Nat Rev Neurosci. 2016;17:411–23.

113. Guo F, Yan L, Guo H, Li L, Hu B, Zhao Y, et al. The Transcriptome and DNA Methylome Landscapes of Human Primordial Germ Cells. Cell. 2015;161:1437–52.

114. Gkountela S, Zhang KX, Shafiq TA, Liao WW, Hargan-Calvopiña J, Chen PY, et al. DNA Demethylation Dynamics in the Human Prenatal Germline. Cell. 2015;161:1425–36.

115. Slieker RC, Roost MS, van Iperen L, Suchiman HED, Tobi EW, Carlotti F, et al. DNA Methylation Landscapes of Human Fetal Development. PLOS Genet. 2015;11:e1005583.

116. Ziller MJ, Gu H, Müller F, Donaghey J, Tsai LTY, Kohlbacher O, et al. Charting a dynamic DNA methylation landscape of the human genome. Nat. 2013;500:477–81.

117. Schultz MD, He Y, Whitaker JW, Hariharan M, Mukamel EA,

Leung D, et al. Human body epigenome maps reveal noncanonical DNA methylation variation. Nat. 2015;523:212–6.

118. Smith ZD, Chan MM, Mikkelsen TS, Gu H, Gnirke A, Regev A, et al. A unique regulatory phase of DNA methylation in the early mammalian embryo. Nat. 2012;484:339–44.

119. Lister R, Mukamel EA. Turning over DNA methylation in the mind. Front Neurosci. 2015;9:252.

120. Spiers H, Hannon E, Schalkwyk LC, Smith R, Wong CCY, O'Donovan MC, et al. Methylomic trajectories across human fetal brain development. Genome Res. 2015;25:338–52.

121. Jaffe AE, Gao Y, Deep-Soboslay A, Tao R, Hyde TM, Weinberger DR, et al. Mapping DNA methylation across development, genotype and schizophrenia in the human frontal cortex. Nat Neurosci. 2015;19:40–7.

122. Pidsley R, Viana J, Hannon E, Spiers H, Troakes C, Al-saraj S, et al. Methylomic profiling of human brain tissue supports a neurodevelopmental origin for schizophrenia. Genome Biol. 2014;15:1–11.

123. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A. 2008;105:17046–9.

124. Bakulski KM, Fallin MD. Epigenetic Epidemiology: Promises for Public Health Research. Environ Mol Mutagen. 2014;55:171.

125. Relton CL, smith DG. Is epidemiology ready for epigenetics? Int J Epidemiol. 2012;41:5–9.

126. Mill J, Heijmans BT. From promises to practical strategies in epigenetic epidemiology. Nat Rev Genet. 2013;14:585–94.

127. Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, Shen R, et al. Genome-wide DNA methylation profiling using Infinium® assay. Epigenomics. 2009;1:177–200.

128. Moore KL. The Developing Human: Clinically Oriented Embryology. 4th ed. Moore KL, editor. Saunders; 1988.

129. Denison HJ, Dodds RM, Ntani G, Cooper R, Cooper C, Sayer AA, et al. How to get started with a systematic review in epidemiology: an introductory guide for early career researchers.

Arch Public Heal. 2013;71:21.

130. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med. 2009;6:e1000097.

131. Page MJ, Shamseer L, Tricco AC. Registration of systematic reviews in PROSPERO: 30,000 records and counting. Syst Rev. 2018;7:32.

132. Centre for Reviews and Dissemination University of York; National Institute for Health Research. PROSPERO [Internet]. Available from: https://www.crd.york.ac.uk/prospero/.

133. Ouzzani M, Hammady H, Fedorowicz Z, Elmagarmid A. Rayyan-a web and mobile app for systematic reviews. Syst Rev. 2016;5.

134. Campagna MP, Xavier A, Lechner-Scott J, Maltby V, Scott RJ, Butzkueven H, et al. Epigenome-wide association studies: current knowledge, strategies and recommendations. Clin Epigenetics. 2021;13(1):214.

135. Magnus P, Irgens LM, Haug K, Nystad W, Skjærven R, Stoltenberg C, et al. Cohort profile: The Norwegian Mother and Child Cohort Study (MoBa). Int J Epidemiol. 2006;35:1146–50.

136. Magnus P, Birke C, Vejrup K, Haugan A, Alsaker E, Daltveit AK, et al. Cohort Profile Update: The Norwegian Mother and Child Cohort Study (MoBa). Int J Epidemiol. 2016;45:382–8.

137. Paltiel L, Haugan A, Skjerden T, Harbak K, Bækken S, Stensrud NK, et al. The biobank of the Norwegian Mother and Child Cohort Study – present status. Nor Epidemiol. 2014;24:29–35.

138. Rønningen KS, Paltiel L, Meltzer HM, Nordhagen R, Lie KK, Hovengen R, et al. The biobank of the Norwegian Mother and Child Cohort Study: a resource for the next 100 years. Eur J Epidemiol. 2006;21:619–25.

139. Nilsen RM, Vollset SE, Gjessing HK, Skjærven R, Melve KK, Schreuder P, et al. Self-selection and bias in a large prospective pregnancy cohort in Norway. Paediatr Perinat Epidemiol. 2009;23:597–608.

140. Biele G, Gustavson K, Czajkowski NO, Nilsen RM, Reichborn-Kjennerud T, Magnus PM, et al. Bias from self selection and loss to follow-up in prospective cohort studies. Eur J Epidemiol.

2019;34:927–38.

141. Vejrup K, Magnus P, Magnus M. Lost to follow-up in the Norwegian mother, father and child cohort study. Paediatr Perinat Epidemiol. 2022;36:300–9.

142. Austin PC. Optimal caliper widths for propensity-score matching when estimating differences in means and differences in proportions in observational studies. Pharm Stat. 2011;10:150–61.

143. Wood ME, Lapane KL, van Gelder MMHJ, Rai D, Nordeng HME. Making fair comparisons in pregnancy medication safety studies: An overview of advanced methods for confounding control. Pharmacoepidemiol Drug Saf. 2018;27:140–7.

144. Roth C, Bjørke-Monsen AL, Reichborn-Kjennerud T, Nilsen RM, Smith GD, Stoltenberg C, et al. Use of folic acid supplements in early pregnancy in relation to maternal plasma levels in week 18 of pregnancy. Mol Nutr Food Res. 2013;57:653–60.

145. Bjørke-Monsen AL, Roth C, Magnus P, Midttun Ø, Nilsen RM, Reichborn-Kjennerud T, et al. Maternal B vitamin status in pregnancy week 18 according to reported use of folic acid supplements. Mol Nutr Food Res. 2013;57:645–52.

146. Skurtveit S, Selmer R, Odsbu I, Handal M. Self-reported data on medicine use in the Norwegian Mother and Child cohort study compared to data from the Norwegian Prescription Database. Nor Epidemiol. 2014;24:209–16.

147. Derogatis LR, Lipman RS, Covi L. The SCL-90: an outpatient psychiatric rating scale. Psychopharmacol Bull. 1973;9:13–28.

148. Derogatis LR, Lipman RS, Rickels K, Uhlenhuth EH, Covi L. The Hopkins Symptom Checklist (HSCL): a self-report symptom inventory. Behav Sci. 1974;19:1–15.

149. Hesbacher PT, Rickels K, Morris RJ, Newman H, Rosenfeld H. Psychiatric illness in family practice. J Clin Psychiatry. 1980;41:6–10.

150. Tambs K, Røysamb E. Selection of questions to short-form versions of original psychometric instruments in MoBa. Nor Epidemiol. 2014;24:195–201.

151. Strand BH, Dalgard OS, Tambs K, Rognerud M. Measuring the

mental health status of the Norwegian population: a comparison of the instruments SCL-25, SCL-10, SCL-5 and MHI-5 (SF-36). Nord J Psychiatry. 2003;57:113–8.

152. Cecil CAM, Nigg JT. Epigenetics and ADHD: Reflections on Current Knowledge, Research Priorities and Translational Potential. Mol Diagnosis Ther. 2022;26:581–606.

153. Franke B, Michelini G, Asherson P, Banaschewski T, Bilbow A, Buitelaar JK, et al. Live fast, die young? A review on the developmental trajectories of ADHD across the lifespan. Eur Neuropsychopharmacol. 2018;28:1059–88.

154. World Health Organization. The ICD-10 classification of mental and behavioural disorders: clinical descriptions and diagnostic guidelines. 10th ed. 1992.

155. Thomas R, Mitchell GK, Batstra L. Attention-deficit/hyperactivity disorder: are we helping or harming? BMJ. 2013;347.

156. Achenbach TM, Ruffle TM. The Child Behavior Checklist and related forms for assessing behavioral/emotional problems and competencies. Pediatr Rev. 2000;21:265–71.

157. Nøvik TS. Validity of the child behaviour checklist in a Norwegian sample. Eur Child Adolesc Psychiatry. 1999;8:247–54.

158. Skarphedinsson G, Jarbin H, Andersson M, Ivarsson T. Diagnostic efficiency and validity of the DSM-oriented Child Behavior Checklist and Youth Self-Report scales in a clinical sample of Swedish youth. PLoS One. 2021;16.

159. Squires J, Potter J, Bricker D. The ASQ User's Guide. 2nd ed. Paul H. Brookes Publishing Co.; 1999.

160. Richter J, Janson H. A validation study of the Norwegian version of the Ages and Stages Questionnaires. Acta Paediatr. 2007;96:748–52.

161. Yong WS, Hsu FM, Chen PY. Profiling genome-wide DNA methylation. Epigenetics Chromatin. 2016;9:1–16.

162. Wescoe ZL, Schreiber J, Akeson M. Nanopores discriminate among five C5-cytosine variants in DNA. J Am Chem Soc. 2014;136:16582–7.

163. Microarray Kits | Illumina array kits for genotyping & epigenetics [Internet]. Available from: https://www.illumina.com/products/by-type/microarray-kits.html.

164. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2014;30:1363–9.

165. Morris TJ, Butcher LM, Feber A, Teschendorff AE, Chakravarthy AR, Wojdacz TK, et al. ChAMP: 450k Chip Analysis Methylation Pipeline. Bioinformatics. 2014;30:428–30.

166. Tian Y, Morris TJ, Webster AP, Yang Z, Beck S, Feber A, et al. ChAMP: updated methylation analysis pipeline for Illumina BeadChips. Bioinformatics. 2017;33:3982–4.

167. Xu Z, Niu L, Li L, Taylor JA. ENmix: a novel background correction method for Illumina HumanMethylation450 BeadChip. Nucleic Acids Res. 2016;44:e20.

168. Assenov Y, Müller F, Lutsik P, Walter J, Lengauer T, Bock C. Comprehensive analysis of DNA methylation data with RnBeads. Nat Methods. 2014;11:1138–40.

169. Müller F, Scherer M, Assenov Y, Lutsik P, Walter J, Lengauer T, et al. RnBeads 2.0: Comprehensive analysis of DNA methylation data. Genome Biol. 2019;20:55.

170. Pidsley R, Y Wong CC, Volta M, Lunnon K, Mill J, Schalkwyk LC. A data-driven approach to preprocessing Illumina 450K methylation array data. BMC Genomics. 2013;14:1–10.

171. Fortin JP, Labbe A, Lemire M, Zanke BW, Hudson TJ, Fertig EJ, et al. Functional normalization of 450k methylation array data improves replication in large cancer studies. Genome Biol. 2014;15:1–17.

172. Fortin JP, Triche TJ, Hansen KD. Preprocessing, normalization and integration of the Illumina HumanMethylationEPIC array with minfi. Bioinformatics. 2017;33:558.

173. Xu Z, Niu L, Li L, Taylor JA. ENmix: A novel background correction method for Illumina HumanMethylation450 BeadChip. Nucleic Acids Res. 2016;44:e20.

174. Xu Z, Langie SAS, De Boever P, Taylor JA, Niu L. RELIC: a novel dye-bias correction method for Illumina Methylation BeadChip. BMC Genomics. 2017;18.

175. Niu L, Xu Z, Taylor JA. RCP: a novel probe design bias correction method for Illumina Methylation BeadChip. Bioinformatics. 2016;32:2659–63.

176. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, et al. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics. 2013;29:189–96.

177. Marabita F, Almgren M, Lindholm ME, Ruhrmann S, Fagerström-Billai F, Jagodic M, et al. An evaluation of analysis pipelines for DNA methylation profiling using the Illumina HumanMethylation450 BeadChip platform. Epigenetics. 2013;8:333.

178. Xu Z, Niu L, Taylor JA. The ENmix DNA methylation analysis pipeline for Illumina BeadChip and comparisons with seven other preprocessing pipelines. Clin Epigenetics. 2021;13:1–8.

179. Fages A, Ferrari P, Monni S, Dossus L, Floegel A, Mode N, et al. Investigating sources of variability in metabolomic data in the EPIC study: the Principal Component Partial R-square (PC-PR2) method. Metabolomics. 2014;10:1074–83.

180. Perrier F, Novoloaca A, Ambatipudi S, Baglietto L, Ghantous A, Perduca V, et al. Identifying and correcting epigenetics measurements for systematic sources of variation. Clin Epigenetics. 2018;10.

181. Teschendorff AE, Relton CL. Statistical and integrative system-level analysis of DNA methylation data. Nat Rev Genet. 2017;19:129–47.

182. Teschendorff AE, Breeze CE, Zheng SC, Beck S. A comparison of reference-based algorithms for correcting cell-type heterogeneity in Epigenome-Wide Association Studies. BMC Bioinformatics. 2017;18:1–14.

183. Gervin K, Salas LA, Bakulski KM, Van Zelm MC, Koestler DC, Wiencke JK, et al. Systematic evaluation and validation of reference and library selection methods for deconvolution of cord blood DNA methylation data. Clin Epigenetics. 2019;11.

184. Du P, Zhang X, Huang C-C, Jafari N, Kibbe WA, Hou L, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics. 2010;11:587.

185. Wood ME, Lupattelli A, Palmsten K, Bandoli G, Hurault-Delarue C, Damase-Michel C, et al. Longitudinal Methods for Modeling Exposures in Pharmacoepidemiologic Studies in Pregnancy. Epidemiol Rev. 2021;43:130.

186. Li M, Francis E, Hinkle SN, Ajjarapu AS, Zhang C. Preconception and Prenatal Nutrition and Neurodevelopmental Disorders: A Systematic Review and Meta-Analysis. Nutr. 2019;11:1628.

187. Olstad EW, Nordeng HME, Gervin K. Prenatal medication exposure and epigenetic outcomes: a systematic literature review and recommendations for prenatal pharmacoepigenetic studies. Epigenetics. 2021.

188. Radhakrishna U, Vishweswaraiah S, Uppala L V., Szymanska M, Macknis J, Kumar S, et al. Placental DNA methylation profiles in opioid-exposed pregnancies and associations with the neonatal opioid withdrawal syndrome. Genomics. 2021;113:1127–35.

189. Borrelli KN, Wachman EM, Beierle JA, Taglauer ES, Jain M, Bryant CD, et al. Effect of Prenatal Opioid Exposure on the Human Placental Methylome. Biomedicines. 2022;10:1150.

190. Kallak TK, Bränn E, Fransson E, Johansson Å, Lager S, Comasco E, et al. DNA methylation in cord blood in association with prenatal depressive symptoms. Clin Epigenetics. 2021;13:1–14.

191. Inkster AM, Konwar C, Peñaherrera MS, Brain U, Khan A, Price EM, et al. Profiling placental DNA methylation associated with maternal SSRI treatment during pregnancy. Sci Reports. 2022;12:1–14.

192. Eslamimehr S, Jones AD, Anthony TM, Arshad SH, Holloway JW, Ewart S, et al. Association of prenatal acetaminophen use and acetaminophen metabolites with DNA methylation of newborns: analysis of two consecutive generations of the Isle of Wight birth cohort. Environ Epigenetics. 2022;8:1–10.

193. Wachman EM, Wang A, Isley BC, Boateng J, Beierle JA,

Hansbury A, et al. Placental OPRM1 DNA methylation and associations with neonatal opioid withdrawal syndrome, a pilot study. Explor Med. 2020;1:124–35.

194. Schroeder JW, Smith AK, Brennan PA, Conneely KN, Kilaru V, Knight BT, et al. DNA methylation in neonates born to women receiving psychiatric care. Epigenetics. 2012;7:409–14.

195. Non AL, Binder AM, Kubzansky LD, Michels KB. Genome-wide DNA methylation in neonates exposed to maternal depression, anxiety, or SSRI medication during pregnancy. Epigenetics. 2014;9:964–72.

196. Gurnot C, Martin-Subero I, Mah SM, Weikum W, Goodman SJ, Brain U, et al. Prenatal antidepressant exposure associated with CYP2E1 DNA methylation change in neonates. Epigenetics. 2015;10:361–72.

197. Cardenas A, Faleschini S, Cortes Hidalgo A, Rifas-Shiman SL, Baccarelli AA, Demeo DL, et al. Prenatal maternal antidepressants, anxiety, and depression and offspring DNA methylation: Epigenome-wide associations at birth and persistence into early childhood. Clin Epigenetics. 2019;11.

198. Ryan J, Mansell T, Fransquet P, Saffery R. Does maternal mental well-being in pregnancy impact the early human epigenome? Epigenomics. 2017;9:313–32.

199. Graw S, Henn R, Thompson JA, Koestler DC. PwrEWAS: A user-friendly tool for comprehensive power estimation for epigenome wide association studies (EWAS). BMC Bioinformatics. 2019;20.

200. Mansell G, Gorrie-Stone TJ, Bao Y, Kumari M, Schalkwyk LS, Mill J, et al. Guidance for DNA methylation studies: Statistical insights from the Illumina EPIC array. BMC Genomics. 2019;20:1–15.

201. Wang WYS, Barratt BJ, Clayton DG, Todd JA. Genome-wide association studies: theoretical and practical concerns. Nat Rev Genet. 2005;6:109–18.

202. Solomon O, MacIsaac J, Quach H, Tindula G, Kobor MS, Huen K, et al. Comparison of DNA methylation measured by Illumina 450K and EPIC BeadChips in blood of newborns and 14-year-old children. Epigenetics. 2018;13:655–64.

203. Logue MW, Smith AK, Wolf EJ, Maniates H, Stone A, Schichman SA, et al. The correlation of methylation levels measured using Illumina 450K and EPIC BeadChips in blood samples. Epigenomics. 2017;9:1363–71.

204. Pidsley R, Zotenko E, Peters TJ, Lawrence MG, Risbridger GP, Molloy P, et al. Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. Genome Biol. 2016;17.

205. Sugden K, Hannon EJ, Arseneault L, Belsky DW, Corcoran DL, Fisher HL, et al. Patterns of Reliability: Assessing the Reproducibility and Integrity of DNA Methylation Measurement. Patterns. 2020;1:100014.

206. Fernandez-Jimenez N, Allard C, Bouchard L, Perron P, Bustamante M, Bilbao JR, et al. Comparison of Illumina 450K and EPIC arrays in placental DNA methylation. Epigenetics. 2019;14:1177–82.

207. Cheung K, Burgers MJ, Young DA, Cockell S, Reynard LN. Correlation of Infinium HumanMethylation450K and MethylationEPIC BeadChip arrays in cartilage. Epigenetics. 2020;15:594–603.

208. Dugué PA, English DR, MacInnis RJ, Joo JE, Jung CH, Milne RL. The repeatability of DNA methylation measures may also affect the power of epigenome-wide association studies. Int J Epidemiol. 2015;44:1460–1.

209. Dugue PA, English DR, MacInnis RJ, Jung CH, Bassett JK, Fitzgerald LM, et al. Reliability of DNA methylation measures from dried blood spots and mononuclear cells using the HumanMethylation450k BeadArray. Sci Rep. 2016;6.

210. Bose M, Wu C, Pankow JS, Demerath EW, Bressler J, Fornage M, et al. Evaluation of microarray-based DNA methylation measurement using technical replicates: The atherosclerosis risk in communities (ARIC) study. BMC Bioinformatics. 2014;15:1–10.

211. Shvetsov YB, Song MA, Cai Q, Tiirikainen M, Xiang YB, Shu XO, et al. Intra-individual variation and short-term temporal trend in DNA methylation of human blood. Cancer Epidemiol Biomarkers Prev. 2015;24:490.

212. Forest M, O'Donnell KJ, Voisin G, Gaudreau H, MacIsaac JL,

McEwen LM, et al. Agreement in DNA methylation levels from the Illumina 450K array across batches, tissues, and time. Epigenetics. 2018;13:19.

213. Xu Z, Taylor JA. Reliability of DNA methylation measures using Illumina methylation BeadChip. Epigenetics. 2021;16:495–502.

214. Zaimi I, Pei D, Koestler DC, Marsit CJ, De Vivo I, Tworoger SS, et al. Variation in DNA methylation of human blood over a 1-year period using the Illumina MethylationEPIC array. Epigenetics. 2018;13:1056–71.

215. Moran S, Arribas C, Esteller M. Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. Epigenomics. 2016;8:389–99.

216. Lussier AA, Zhu Y, Smith BJ, Simpkin AJ, Smith ADAC, Suderman MJ, et al. Updates to data versions and analytic methods influence the reproducibility of results from epigenome-wide association studies. Epigenetics. 2022;17:1373–88.

217. Ori APS, Lu AT, Horvath S, Ophoff RA. Significant variation in the performance of DNA methylation predictors across data preprocessing and normalization strategies. Genome Biol. 2022;23:1–21.

218. Jones MJ, Dinh L, Razzaghian HR, de Goede O, MacIsaac JL, Morin AM, et al. Differences in DNA methylation of white blood cell types at birth and in adulthood reflect postnatal immune maturation and influence accuracy of cell type prediction. bioRxiv. 2018;399279.

219. Jaffe AE, Irizarry RA. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. Genome Biol. 2014;15:1–9.

220. Houseman EA, Kim S, Kelsey KT, Wiencke JK. DNA Methylation in Whole Blood: Uses and Challenges. Curr Environ Heal reports. 2015;2:145–54.

221. A priceless resource. Nat. 2011;478:427–427.

222. Jourdon A, Scuderi S, Capauto D, Abyzov A, Vaccarino FM. PsychENCODE and beyond: transcriptomics and epigenomics

of brain development and organoids. Neuropsychopharmacol. 2020;46:70–85.

223. Hannon E, Lunnon K, Schalkwyk L, Mill J. Interindividual methylomic variation across blood, cortex, and cerebellum: implications for epigenetic studies of neurological and neuropsychiatric phenotypes. Epigenetics. 2015;10:1024.

224. Edgar RD, Jones MJ, Meaney MJ, Turecki G, Kobor MS. BECon: a tool for interpreting DNA methylation findings from blood in the context of brain. Transl Psychiatry. 2017;7:e1187–e1187.

225. Sommerer Y, Ohlei O, Dobricic V, Oakley DH, Wesse T, Sedghpour Sabet S, et al. A correlation map of genome-wide DNA methylation patterns between paired human brain and buccal samples. Clin Epigenetics. 2022;14:1–10.

226. Heijmans BT, Mill J. Commentary: The seven plagues of epigenetic epidemiology. Int J Epidemiol. 2012;41:74–8.

227. Feingold EA, Good PJ, Guyer MS, Kamholz S, Liefer L, Wetterstrand K, et al. The ENCODE (ENCyclopedia of DNA Elements) Project. Science. 2004;306:636–40.

228. Myers RM, Stamatoyannopoulos J, Snyder M, Dunham I, Hardison RC, Bernstein BE, et al. A User's Guide to the Encyclopedia of DNA Elements (ENCODE). PLOS Biol. 2011;9:e1001046.

229. Dunham I, Kundaje A, Aldred SF, Collins PJ, Davis CA, Doyle F, et al. An integrated encyclopedia of DNA elements in the human genome. Nat. 2012;489:57–74.

230. Morris-Rosendahl DJ, Crocq MA. Neurodevelopmental disorders—the history and future of a diagnostic concept. Dialogues Clin Neurosci. 2020;22:65–72.

231. Astle DE, Fletcher-Watson S. Beyond the Core-Deficit Hypothesis in Developmental Disorders. Curr Dir Psychol Sci. 2020;29:431.

232. Astle DE, Holmes J, Kievit R, Gathercole SE. Annual Research Review: The transdiagnostic revolution in neurodevelopmental disorders. J Child Psychol Psychiatry. 2022;63:397–417.

233. Fletcher-Watson S. Transdiagnostic research and the neurodiversity paradigm: commentary on the transdiagnostic

revolution in neurodevelopmental disorders by Astle et al. J Child Psychol Psychiatry Allied Discip. 2022;63:418–20.

234. Nigg JT, Karalunas SL, Feczko E, Fair DA. Toward a Revised Nosology for Attention-Deficit/Hyperactivity Disorder Heterogeneity. Biol Psychiatry Cogn Neurosci Neuroimaging. 2020;5:726–37.

235. Nordeng H, Lupattelli A, Wood M. Prenatal exposure to antidepressants and increased risk of psychiatric disorders. BMJ. 2017;358.

236. Walton E, Pingault JB, Cecil CAM, Gaunt TR, Relton CL, Mill J, et al. Epigenetic profiling of ADHD symptoms trajectories: a prospective, methylome-wide study. Mol Psychiatry. 2017;22:250–6.

237. Neumann A, Walton E, Alemany S, Cecil C, González JR, Jima DD, et al. Association between DNA methylation and ADHD symptoms from birth to school age: a prospective meta-analysis. Transl Psychiatry. 2020;10:1–11.

238. Wilmot B, Fry R, Smeester L, Musser ED, Mill J, Nigg JT. Methylomic analysis of salivary DNA in childhood ADHD identifies altered DNA methylation in VIPR2. J Child Psychol Psychiatry. 2016;57:152–60.

239. Hannon E, Spiers H, Viana J, Pidsley R, Burrage J, Murphy TM, et al. Methylation QTLs in the developing brain and their enrichment in schizophrenia risk loci. Nat Neurosci. 2015;19:48–54.

240. Hannon E, Dempster E, Viana J, Burrage J, Smith AR, Macdonald R, et al. An integrated genetic-epigenetic analysis of schizophrenia: Evidence for co-localization of genetic associations and differential DNA methylation. Genome Biol. 2016;17:176.

241. Lin D, Chen J, Perrone-Bizzozero N, Bustillo JR, Du Y, Calhoun VD, et al. Characterization of cross-tissue genetic-epigenetic effects and their patterns in schizophrenia. Genome Med. 2018;10:13.

242. Hannon E, Schendel D, Ladd-Acosta C, Grove J, Hansen CS, Andrews S V., et al. Elevated polygenic burden for autism is associated with differential DNA methylation at birth. Genome Med. 2018;10:19.

243. Teschendorff AE, Relton CL. Statistical and integrative system-level analysis of DNA methylation data. Nat Rev Genet. 2018;19:129–47.

244. Relton CL, Davey Smith G. Two-step epigenetic mendelian randomization: A strategy for establishing the causal role of epigenetic processes in pathways to disease. Int J Epidemiol. 2012;41:161–76.

245. Berdasco M, Esteller M. Clinical epigenetics: seizing opportunities for translation. Nat Rev Genet. 2018;20:109–27.

246. Nishiyama A, Nakanishi M. Navigating the DNA methylation landscape of cancer. Trends Genet. 2021;37:1012–27.

247. Yousefi PD, Suderman M, Langdon R, Whitehurst O, Davey Smith G, Relton CL. DNA methylation-based predictors of health: applications and statistical considerations. Nat Rev Genet. 2022;23:369–83.

248. Bakulski KM, Halladay A, Hu VW, Mill J, Fallin MD. Epigenetic Research in Neuropsychiatric Disorders: the “Tissue Issue.”. Curr Behav Neurosci Rep. 2016;3:264.

249. Caramaschi D, Sharp GC, Nohr EA, Berryman K, Lewis SJ, Davey Smith G, et al. Exploring a causal role of DNA methylation in the relationship between maternal vitamin B12 during pregnancy and child’s IQ at age 8, cognitive performance and educational attainment: a two-step Mendelian randomization study. Hum Mol Genet. 2017;26:3001–13.

250. Allard C, Desgagné V, Patenaude J, Lacroix M, Guillemette L, Battista MC, et al. Mendelian randomization supports causality between maternal hyperglycemia and epigenetic regulation of leptin gene in newborns. Epigenetics. 2015;10:342–51.

APPENDIX A

A relevant associated paper not part of the thesis.

Gomes JDA, **Olstad EW**, Kowalski TW, Gervin K, Vianna FSL, Schüler-Faccini L, Nordeng HME. Genetic Susceptibility to Drug Teratogenicity: A Systematic Literature Review. Front Genet. 2021 Apr 27;12:645555. doi: 10.3389/fgene.2021.645555.

APPENDIX B – *ERRATA*

After submission of the thesis, some typographical and grammatical mistakes were identified by the evaluation committee and the doctoral candidate. The printed thesis has been updated to correct these mistakes with permission from the committee. Further, parts of the reference list have been reformatted in accordance with the opponents' suggestions. Lastly, the final figures and tables of the Paper IV manuscript were added. Importantly, none of these corrections changed the scientific content and meaning of the thesis. A list of the corrections made after submission is available upon request from the author of the thesis, Emilie Willoch Olstad (e.w.olstad@farmasi.uio.no).

I

Paper I

Prenatal medication exposure and epigenetic outcomes: a systematic literature review and recommendations for prenatal pharmacoepigenetic studies

Olstad EW, Nordeng HME, Gervin K

Epigenetics. 2022 Apr;17(4):357-380. Epub 2021 Apr 29.
doi: 10.1080/15592294.2021.1903376.

EPIGENETICS
2022, VOL. 17, NO. 4, 357–380
https://doi.org/10.1080/15592294.2021.1903376

REVIEW

OPEN ACCESS

Prenatal medication exposure and epigenetic outcomes: a systematic literature review and recommendations for prenatal pharmacoepigenetic studies

Emilie Willoch Olstad [a,b], Hedvig Marie Egeland Nordeng [a,b,c], and Kristina Gervin [a,b,d]

[a]Pharmacoepidemiology and Drug Safety Research Group, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway; [b]PharmaTox Strategic Research Initiative, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway; [c]Department of Child Health and Development, Norwegian Institute of Public Health, Oslo, Norway; [d]Department of Research, Innovation and Education, Division of Clinical Neuroscience, Oslo University Hospital, Oslo, Norway

ABSTRACT

When used during pregnancy, analgesics and psychotropics pass the placenta to enter the foetal circulation and may induce epigenetic modifications. Where such modifications occur and whether they disrupt normal foetal developme nt, are currently unanswered questions. This field of prenatal pharmacoepigenetics has received increasing attention, with several studies reporting associations between *in utero* medication exposure and offspring epigenetic outcomes. Nevertheless, no recent systematic review of the literature is available. Therefore, the objectives of this review were to (i) provide an overview of the literature on the association of prenatal exposure to psychotropics a nd analgesics with epigenetic outcomes, and (ii) suggest recommendations for future studies within prenatal pharmacoepigenetics. We performed systematic literature searches in five databases. The eligible studies assessed human prenatal exposure to psychotropics or analgesics, with epigenetic analyses of offspring tissue as an outcome. We identified 18 eligible studies including 4,419 neonates exposed to either antidepressants, antiepileptic drugs, paracetamol, acetylsalicylic acid, or methadone. The epigenetic outcome in all studies was DNA methylation in cord blood, placental tissue or buccal cells. Although most studies found significant differences in DNA methylation upon medication exposure, almost no differences were persistent across studies for similar medications and sequencing methods. The reviewed studies were challenging to compare due to poor transparency in reporting, and heterogeneous methodology, design, genome coverage, and statistical modelling. We propose 10 recommendations for future prenatal pharmacoepigenetic studies considering both epidemiological and epigenetic perspectives. These recommendations may improve the quality, comparability, and clinical relevance of such studies. PROSPERO registration ID: CRD42020166675.

ARTICLE HISTORY
Received 7 November 2020
Revised 3 March 2021
Accepted 9 March 2021

KEYWORDS
Epigenetics; DNA methylation; prenatal exposure; psychotropics; analgesics; literature review; EWAS; epigenetic epidemiology; epidemiology; recommendations

Background

Every day, pregnant women use medications for which the scientific evidence on foetal safety is limited or inconclusive. As most medications pass both the placental and blood-brain barriers during gestation [1–4], common medications such as analgesics and psychotropics may exhibit pharmaceutical effects in the foetus and potentially disrupt normal foetal development. This reasoning is based on the Developmental Origins of Health and Disease (DOHaD) hypothesis, which is a conceptual framework linking prenatal environmental exposures to health and disease in later life [5–9]. Indeed, many studies have reported a variety of adverse developmental outcomes as sociated with *in utero* medication exposure, including developmental delays and abnormalities (comprehensively reviewed in [10–18] and the textbook by Schaefer *et al.* [19]).

T he mechanisms by which prenatal exposure to medications impacts foetal development remain largely unknown. One suggested mechanism is the direct or indirect influence of epigenetic modifications in the developing foetus [9,20]. Epigenetics encompasses regulatory mechanisms that can impact genome stability and gene transcription, such as histone modifications and DNA methylation (DNAm) of cytosine-phosphate-

CONTACT Emilie Willoch Olstad e.w.olstad@farmasi.uio.no Pharmacoepidemiology and Drug Safety Research Group, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway; PharmaTox Strategic Research Initiative, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway

Supplemental data for this article can be accessed here.

© 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

guanine sites (CpGs). Such modifications are reversible and can be influenced by both genetics and environmental factors, such as medications [21], making epigenetic changes plausible mediators of the prenatal environmental impact on developmental outcomes [9,20].

The research on epigenetic modifications in neonates exposed to medications *in utero*, hereafter referred to as prenatal pharmacoepigenetics, has gained increasing attention in recent years. Although the literature on prenatal pharmacoepigenetics is growing, only one systematic review summarizing the findings on medications that potentially interfere with foetal development is available [22]. However, this review only included studies on antidepressants. Therefore, the primary aim of the current review is to provide an overview of the literature on the association of prenatal exposure to psychotropics and analgesics with epigenetic outcomes. In addition, by evaluating the eligible studies from both epidemiological and epigenetic perspectives, this review also aims to provide recommendations for future prenatal pharmacoepigenetic research to improve the overall quality, comparability, and clinical relevance of prenatal pharmacoepigenetic association studies.

Methods

Search strategy

Literature searches were performed in the MEDLINE, EMBASE, PsycINFO, Scopus, and Web of Science databases. The searches were first completed on 19 January 2020, and any new studies meeting the eligibility criteria, published before 1 September 2020, were included in the final review. In addition, the reference lists of the eligible articles and references of 35 relevant reviews were screened to ensure complete coverage of the literature. Prior to performing the literature searches, a detailed search strategy and vocabulary were developed with support from experienced librarians in medicine, pharmacy, and psychology. We included studies investigating (i) prenatal exposure to (ii) psychotropics and analgesics with (iii) an epigenetic outcome. The search terms for these three criteria are listed in Supplementary Table S1. Supplementary Table S2 provides an example of a search in EMBASE. The review is reported in adherence to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [23], and the protocol and search strategy are available in the PROSPERO database (registration ID: CRD42020166675) [24,25].

Inclusion criteria

The studies included in this review were selected based on the participants, intervention/exposure, comparison group, outcome, and study design (PICOS) criteria [26]. *Participants* were defined as children (<18 y old) prenatally exposed to psychotropics or analgesics for which epigenetic data were available. Anatomical Therapeutic Chemical (ATC) codes were used to identify medication groups in accordance with the World Health Organization ATC index [27]. The *exposure* was defined as use of antidepressants (ATC code: N06A), psycholeptics (N05), antiepileptic drugs (AEDs; N03), analgesics (N02), or non-steroidal anti-inflammatory drugs (NSAIDs; M01A) during pregnancy. We specifically selected analgesics and psychotropics, based on the expertise of our research group, biological plausibility, and the emerging number of pharmacoepigenetic studies on analgesics and psychotropics. The *comparison group* included children of mothers who did not use the medication of interest during pregnancy. The *outcome* was epigenetic measurements in tissue samples from exposed and unexposed children (<18 y old). If the study also included data on immediate or long-term developmental outcomes in the children, we reported these as well. Studies investigating the same data sets were all eligible if they reported on different exposures and/or outcomes. Only original articles with the *study designs* case-control, cohort, or randomized controlled trial were included. No limitations were applied regarding the time of publication, but only articles in English or Scandinavian languages were eligible.

Data extraction

After searching and retrieving the results from the databases, any duplicates were removed in EndNote X8.2 and the remaining records

uploaded to the online systematic review data management platform Rayyan [28]. Two reviewers (KG and EWO) independently screened the titles and abstracts, excluding studies that did not meet the inclusion criteria. If the eligibility of a paper was unclear based on the title and abstract, it was included for the next round of screening. In the second screening, the full-text versions of all papers were read and the final exclusion of papers performed. Any disagreement between the two reviewers was resolved by a third reviewer (HMEN).

Results

Outcomes of the screening and selection process

The initial searches yielded a total of 2,159 records: 488 records in MEDLINE, 880 records in EMBASE, 88 records in PsycINFO, 194 records in Scopus, and 509 records in Web of Science (Figure 1). A total of 871 duplicated records were removed, leaving 1,288 unique articles to screen the titles and abstracts in Rayyan [28]. After the first screening, 1,262 papers were excluded due to being non-original studies (n = 605) or failing to meet the defined PICOS criteria (n = 657). After reading the complete texts of the 26 records remaining from the first round, we excluded 11 records due to wrong exposure according to our criteria (could not differentiate medication exposure across groups; n = 5), wrong population according to our criteria (participants were too old upon exposure or sampling; n = 3), or wrong comparison groups according to our criteria (did not include a non-medicated group; n = 3). By screening the reference lists of the 15 remaining records and 35 relevant reviews, we identified 1 additional article. Two additional studies meeting the

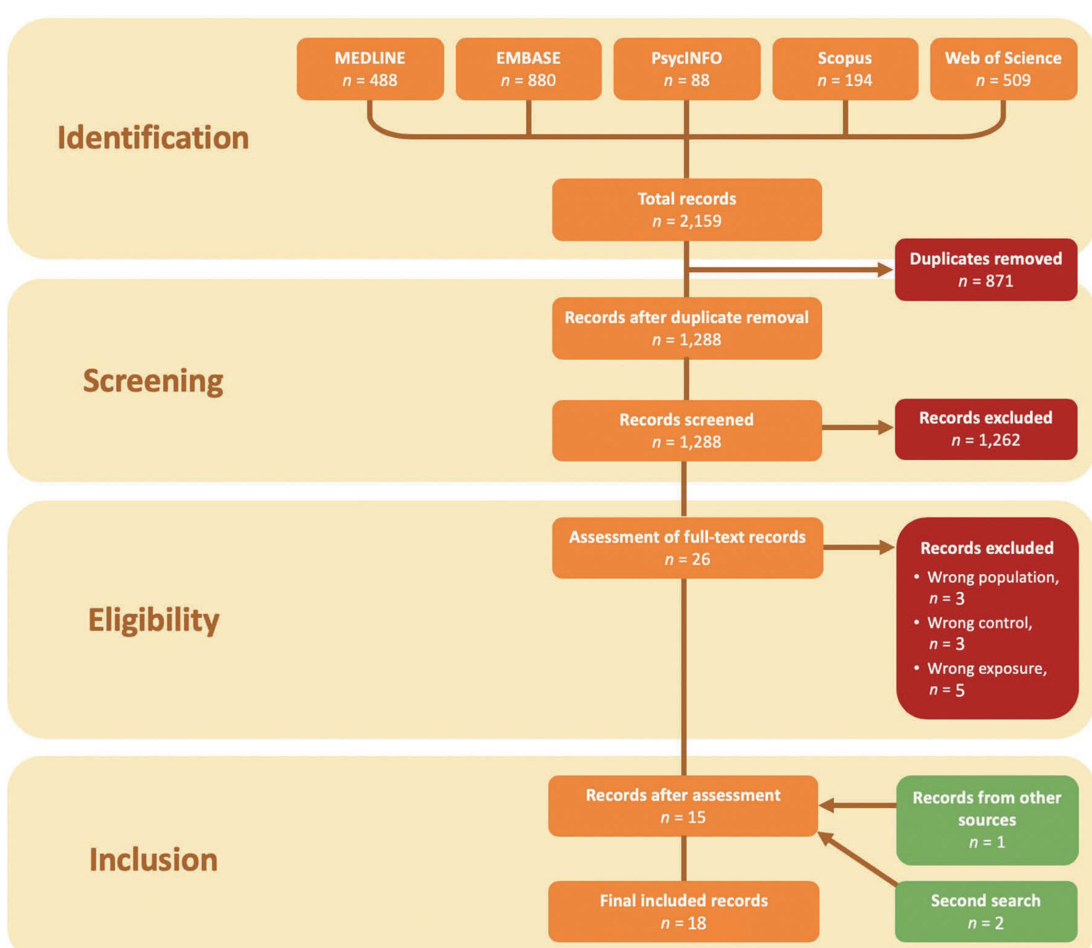


Figure 1. Flow chart of article screening and selection based on the template from PRISMA [23]. 'Second search' refers to eligible studies published during the manuscript revision process.

eligibility criteria were published during the revision process (before 1 September 2020) and were also included. Consequently, a total of 18 records were included in the final review.

Overview of the eligible studies

All of the eligible articles were based on data from single birth cohorts, except for one study validating results in an independent cohort [29], and one study being a randomized controlled trial [30]. Of the eligible articles, nine were epigenome-wide association studies (EWASs; median sample size 241 neonates [interquartile range; IQR: 284]), and eleven were candidate gene studies (median sample size 115 neonates [IQR: 168]). Hence, two studies, including 46 and 58 neonates, combined epigenome-wide and candidate gene approaches [31,32]. The medications included were the psychotropics antidepressants (12 studies; median sample size 201 neonates [IQR: 354]), and AEDs (2 studies; 18 and 201 neonates), and the analgesics paracetamol (2 studies; 281 and 384 neonates), acetylsalicylic acid (1 study; 358 neonates), and methadone (1 study; 53 neonates). The epigenetic outcome investigated in all papers was DNAm, in cord blood (13 studies; median sample size 201 neonates [IQR: 341]), placental tissue (5 studies; median sample size 236 neonates [IQR: 38]), and/or buccal cells (2 studies; 236 and 53 neonates). The neonatal tissues were sampled within 72 h after birth in all studies, except by Cardenas *et al.* (2019), who also collected blood from children aged 3–5 y and 7–11 y [29]. All studies adjusted for potential covariates and/or confounders in their statistical analyses or by design, but the number of variables under consideration differed greatly (Supplementary Table S3). The covariates most frequently accounted for were maternal age (n = 16), smoking during pregnancy (n = 13), infant sex (n = 12), gestational age (n = 10), and folate use in pregnancy (n = 10).

In addition to the epigenetic outcomes, several studies reported phenotypic outcomes in children, specifically poor foetal growth (n = 1) [33], birth weight (n = 2) [31,34], severity of neonatal abstinence syndrome (n = 1) [34], ADHD (n = 1) [35], stress reactivity (n = 2) [36,37], and soothability (n = 1) [38] (Supplementary Table S4). One study performed a mediation analysis of medication exposure, epigenetic modification, and neonatal phenotypic outcome [37]. This study assessed whether the DNAm of a CpG in the placental NR3C2 gene acted as a mediator of the effect of maternal depressive symptoms on cortisol reactivity in 12-month-old infants [37]. The effects of maternal depression on cortisol levels were decomposed into direct effects and DNAm-mediated indirect effects, finding that, although the indirect effect of DNAm was positive, it did not overcome the larger negative direct effect of depressive symptoms on infant cortisol levels [37]. However, the analysis demonstrated an increased DNAm at the *NR3C2* CpG upon *in utero* antidepressant exposure, suggesting that maternal antidepressant use during pregnancy enhances the indirect effect of *NR3C2* DNAm on the infant stress response [37].

All EWASs used the Illumina platform [39] to assess DNAm with the MethylationEPIC (n = 2), HumanMethylation 450 (n = 3), or HumanMethylation 27 (n = 4) bead chips. To assess the association between CpG DNAm and medication exposure, the majority of the EWASs used linear regression models (n = 6). In most of the EWASs, a result was considered significant if the false discovery rate (FDR) adjusted p-value was <0.05 (n = 8), except for one study that used an FDR adjusted $p < 0.1$ [32].

In the candidate gene studies, several methods were used to investigate DNAm: the Illumina platform (n = 1) [33], the SEQUENOM MassARRAY EpiTYPER platform (n = 3), and the PyroMark system (n = 7). The studies reported the methylation percentages (n = 6), mean methylation percentages of triplicates (n = 4) or the β value from the Illumina microarray (n = 1) [33]. Various statistical tests were applied to assess differential DNAm. For these tests, three studies used FDR adjusted p-values ($p < 0.25$ in one study; $p < 0.05$ in two studies), two studies used the Bonferroni-corrected p-value, and the last six studies applied an unadjusted $p < 0.05$. Further details on the studies are available in Tables 1 and 2. For excellent discussions of statistical approaches in epigenetic studies, we

recommend the recently published reviews by Teschendorff and Relton [40], van Rooij *et al.* [41], and Mansell *et al.* [42].

Results of prenatal medication exposure and neonatal DNA methylation

The most examined medication group in prenatal pharmacoepigenetics was antidepressants, investigated in 12 studies of 3,320 neonates (2 EWASs, 8 candidate gene studies, and 2 studies combining an epigenome-wide and a candidate gene approach). In the most recent EWAS, Cardenas *et al.* [29] discovered 130 differentially methylated CpGs in cord blood samples collected from neonates exposed to antidepressants *in utero*. One of these sites that mapped to *ZNF575* was replicated in an independent cohort [29]. Schroeder *et al.* [43] found that the exposed neonates had two differentially methylated CpGs in *TNFRSF21* and *CHRNA4*. However, the authors disregarded these findings as false positives considering the small effect sizes (DNAm changes of 1–3%) [43]. In the EWAS conducted by Gurnot *et al.* [31], three CpGs were differentially methylated in neonates prenatally exposed to serotonin reuptake inhibitors (SRIs; *CYP2E1, EVA1*, and *SLMAP*). However, in the EWAS by Non *et al.* [32], no CpGs were significantly different in neonates exposed to selective serotonin reuptake inhibitors (SSRIs) *in utero*.

The candidate gene studies investigated CpGs in a total of 32 different genes (Supplementary Table S6). Most of the included genes were chosen based on their suggested association with psychiatric disorders (e.g., the serotonin transporter gene *SLC6A4*) [32,38,44], stress reactivity (e.g., the glucocorticoid and mineralocorticoid receptor genes *NR3C1* and *NR3C2*) [32,36,37,45], or adverse early life events (e.g., the brain-derived neurotrophic factor gene *BDNF*) [32,44]. In the studies combining epigenome-wide and candidate gene approaches [31,32], the candidate gene investigation was used to verify the epigenome-wide results. However, except for the verification of *CYP2E1* DNAm by Gurnot *et al.* [31], neither of the significant genes in either of the candidate gene studies were also significant in the EWASs of antidepressants.

Four genes involved in neurotransmitter receptor or transporter activity (*NR3C1, SLC6A4*, and *FKBP5*) or neuronal differentiation (*BDNF*) were investigated across several studies (Table 3). The DNAm of neither *NR3C1* nor *BDNF* was associated with prenatal exposure to antidepressants in any of the studies investigating these genes [32,36,37,45]. For *SLC6A4*, the results were contradictory. Although Gartstein *et al.* [38] found an increase in DNAm at six CpGs in cord blood upon prenatal SSRI exposure, Non *et al.* [32] reported a decrease in DNAm at one CpG in cord blood upon prenatal SSRI exposure when examined by pyrosequencing but not in the epigenome-wide approach. Finally, Devlin *et al.* [44] found no association between *in utero* exposure to SSRIs or serotonin and noradrenaline reuptake inhibitors (SNRIs), and DNAm of *SLC6A4* in cord blood. A CpG in *FKBP5*, which encodes a co-regulator of the glucocorticoid receptor, was negatively associated with *in utero* SSRI exposure in cord blood [32], but not in the placenta [33]. In summary, the results of studies on prenatal antidepressant exposure and DNAm are largely inconsistent.

Prenatal AED exposure was investigated in two EWASs [46,47], which reported discrepant results. Emes *et al.* [46] found no global DNAm differences in the cord blood of neonates exposed to AEDs *in utero*, whereas Smith *et al.* [47] observed decreased global DNAm in the cord blood of neonates prenatally exposed to AEDs and no global DNAm differences in placental tissue. Furthermore, Emes *et al.* [46] reported differential DNAm at 662 CpGs when comparing cord blood from neonates that were exposed and not exposed to AEDs *in utero*, whereas Smith *et al.* [47] found 14 CpGs with significantly reduced DNAm in the same tissue, including three CpGs that were also significant in placentae.

Two EWASs examined the association between *in utero* paracetamol exposure and DNAm in placentae [48] or cord blood [35]. Addo *et al.* [48] reported 24 differentially methylated CpGs in placental tissue when comparing exposed and

Table 1. Overview of the studies included in the literature review.

	Reference	Country; setting (study period)	Sample size*n*(groups)	Research aim(s)	Exposure(s) (data source)	Pharmacoepigenetic outcome(s)
Epigenome-wide association studies	Yeung *et al.* (2020) [30]	USA; the EAGeR (Effects of Aspirin in Gestation and Reproduction) randomized trial (2006–2012)	n = 358 (acetylsalicylic acid, n = 185; no acetylsalicylic acid, n = 173)	Investigate the impact of maternal use of low-dose acetylsalicylic acid prior to and during pregnancy on cord blood DNAm	*Acetylsalicylic acid* (randomly assigned prior to conception; 81 mg/d until gestational week 36 [49])	• Differential DNAm at 1 CpG associated with prenatal acetylsalicylic acid exposure (cg2002882; 3,500 bp upstream of the *POU4F1* promoter)
	Addo *et al.* (2019) [48,100]	USA; Extremely Low Gestational Age Newborns (2002–2004)	n = 281 (paracetamol, n = 165; no paracetamol, n = 116)	Examine DNAm of CpGs in placentae of paracetamol-exposed and non-exposed neonates	*Paracetamol* (≥ 1 during pregnancy; self-reported Tylenol use)	• Differential DNAm at 24 CpGs associated with prenatal paracetamol exposure
	Cardenas *et al*. (2019) [29]	USA; Project Viva (1999–2002) The Netherlands; Generation R Study (2002–2006)	n_{ProV} = 479 (antidepressant[a], n = 14; depression, n = 33; anxiety, n = 40) n_{GenR} = 999 (antidepressant, n = 14; depression, n = 31; anxiety, n = 56)	Identify DNAm differences in neonates associated with exposure to maternal anxiety, depression, or antidepressant use in pregnancy	*Antidepressants* (≥ 1 prescription in pregnancy; medical record in Project Viva, self-reported and prescription-validated in Generation R) *Maternal depression* (EPDS at mid-pregnancy in Project Viva; BSI at 20 weeks in Generation R) *Maternal anxiety* (PRAS at mid-pregnancy in Project Viva; BSI at 20 weeks in Generation R)	• Differential DNAm at 130 CpGs in Project Viva in neonates prenatally exposed to antidepressants compared to non-exposed neonates, 5 confirmed in Generation R (1 under Bonferroni significance; reduced DNAm on cg22159528 *ZNF575* of exposed children) • No DMRs associated with prenatal antidepressant exposure
	Gervin *et al.* (2017) [35]	Norway; the Norwegian Mother, Father and Child Cohort Study (1999–2008)	n = 384 (no paracetamol & no ADHD, n = 96; paracetamol & no ADHD, n = 96; no paracetamol & ADHD, n = 96; short-term paracetamol & ADHD, n = 77; long-term paracetamol & ADHD, n = 19)	Investigate if differential DNAm is associated with prenatal paracetamol exposure and ADHD development	*Paracetamol* (long-term [≥ 20 d] and short-term [6–19 d] exposure; self-reported in questionnaires during pregnancy) *ADHD* (diagnosis in the Norwegian Patient Registry)	• In children with ADHD, prenatal long-term exposure to paracetamol was associated with differential DNAm compared to children without ADHD not exposed to paracetamol (6211 CpGs), children with ADHD and not exposed to paracetamol (193 CpGs), and short-term paracetamol-exposed children with ADHD (2089 CpGs)
	Emes *et al*. (2013) [46]	UK; EFFECT-M study	n = 18 (epilepsy & AEDs[b], n = 9; no epilepsy & no AEDs, n = 9)	Examine association between prenatal AED exposure and DNAm, and if AEDs affect the foetal DNAm by lowering the maternal folate level	*AEDs* (self-reported and validated by medical record)	• DNAm difference in 662 CpGs (652 different genes) in AED-exposed compared to non-exposed neonates • No difference in global DNAm levels between AED-exposed and non-exposed children
	Smith *et al.* (2012) [47]	USA; the Emory Women's Mental Health Program	n = 201 (AEDs[c] & epilepsy/psychiatric disorder[d], n = 53; no AEDs or epilepsy/psychiatric disorder, n = 148)	Examine the impact of prenatal AED exposure on DNAm patterns in neonates	*AEDs* (self-reported every 4–6 weeks in pregnancy and validated by concentrations of AEDs in maternal blood) *Psychiatric disorder and/or epilepsy* (questionnaire on medical and psychiatric history and SCID at intake, SCID and seizure history at 4–6-week intervals)	• Prenatal AED exposure associated with decreased global DNAm in cord blood, not in placenta • Longer prenatal AED exposure associated with decreased DNAm in 14 cord blood CpGs • In placental tissue, 3 of the 14 cord blood CpGs also exhibited decreased DNAm (*PGC*, *ZNF384*, and *C15orf2*) • DNAm patterns neither specific to AED type nor more extreme differences for polydrug treatment
	Schroeder *et al.* (2012) [43]	USA; the Emory Women's Mental Health Program	n = 201 (Several different comparisons[†]: • Current MDD, n = 118; no current MDD, n = 83 • Antidepressants[e], n = 151; no antidepressants, n = 50)	Investigate the association of maternal psychiatric disorder, symptoms and severity of depression, and medication treatment in pregnancy with neonatal DNAm patterns	*Psychotropics* (medication evaluation upon visits every 4–6 weeks during pregnancy) *Maternal mood disorder diagnosis* (life-time history and MDEs; SCID every 4–6 weeks during pregnancy, SCID Mood Module assessed MDEs) *Depressive symptoms* (depression severity and clinically significant depressive symptoms; HRSD17 and BDI)	• Prenatal antidepressant exposure associated with DNAm in 2 CpGs (1.9% decrease in *TNFRSF21* and 3% increase in *CHRNA4*), independent of antidepressant type and duration • No association between prenatal hypnotic, antiemetic, benzodiazepine, or atypical antipsychotic exposure and differential DNAm, independent of duration of exposure • Prenatal exposure to atypical antipsychotics associated with DNAm at 1 CpG

(*Continued*)

Table 1. (Continued).

	Reference	Country; setting (study period)	Sample size *n* (groups)	Research aim(s)	Exposure(s) (data source)	Pharmacoepigenetic outcome(s)
Combined epigenome-wide and candidate gene studies	Gurnot *et al.* (2015) [31]	Canada; University of British Columbia	$n_{epigenome\text{-}wide} = 23$ (depressed & SRIs[f], $n = 11$; depressed & no SRIs, $n = 12$) $n_{candidate\ gene} = 44$ (depressed & SRIs[g], $n = 19$; depressed & no SRIs, $n = 25$)	Examine if prenatal SRI exposure and/or maternal mood is associated with DNAm across the genome and in *CYP2E1*; investigate if DNAm is also associated with birth outcomes	*SRIs* (cord blood/maternal blood drug ratios at birth; maternal whole blood and neonatal cord blood concentrations of SRIs, drawn directly after birth) *Maternal mood* (HAM-D at 26 and 36 weeks of pregnancy, mean score)	• Prenatal SRI exposure associated with DNAm in 3 CpGs (*CYP2E1, EVA1*, and *SLMAP*; EWAS) • In *CYP2E1*, DNAm highly negatively correlated with prenatal maternal depressive mood only if prenatally exposed to SRIs concurrently • Pyrosequencing of *CYP2E1* yielded DNAm values that correlated with the microarray findings
	Non *et al.* (2014) [32]	USA; Harvard Epigenetic Birth Cohort (2007–2009)	$n = 58$ (SSRIs[h], $n = 22$; depression/anxiety & no SSRIs, $n = 13$; no depression/anxiety or SSRIs, $n = 23$)	Examine differences in DNAm patterns across the genome in neonates prenatally exposed or non-exposed to SSRIs and/or maternal depression/anxiety	*SSRIs* (medical charts) *Maternal depression/anxiety* (explicitly noted by obstetrician in labour and delivery forms)	• No association between prenatal SSRI exposure and differential DNAm (EWAS) • No regional clusters of CpGs (1 kb) associated with prenatal SSRI exposure (EWAS) • Pyrosequencing: 6 CpGs in *Col7a1* exhibited lower DNAm at all 6 CpGs and a lower mean DNAm in neonates prenatally exposed to SSRIs • Pyrosequencing: prenatal SSRI exposure associated with 1 CpG in *NFKB2*, 1 CpG in *SLC6A4*, 1 CpG in *FKBP5*, and 1 CpG in *DNMT3a*
Candidate gene studies	Galbally *et al.* (2020) [37]	Australia; Mercy Pregnancy and Emotional Wellbeing Study (2012–2015)	$n = 236$ (antidepressants, $n = 43$; non-medicated & depression/dysthymia, $n = 24$; non-medicated & no depression/dysthymia, $n = 169$)	Investigate associations between maternal depression during pregnancy and the DNAm of placental and buccal *NR3C1* and *NR3C2*, which may mediate an indirect effect of maternal depression on 12-month infant cortisol reactivity	*Antidepressants* (self-reported and hospital records, validated by concentration in cord and maternal whole blood, converted to SEDs) *Maternal mental health* (SCID at ≤ 20 weeks, EPDS/STAI in week 20, 3rd trimester, and 6 and 12 months after birth) *Maternal stress* (PRAMS at week 20 and in 3rd trimester)	• 1 differentially methylated CpG in placental *NR3C2* when comparing medicated and non-medicated depression • No differential methylation of *NR3C1* or *NR3C2* in buccal cells
	Galbally *et al.* (2018) [51]	Australia; Mercy Pregnancy and Emotional Wellbeing Study (2012–2015)	$n = 239$ (untreated current MDD, $n = 24$; antidepressant-treated current MDD, $n = 28$; antidepressant-treated not meeting MDD diagnostic criteria, $n = 15$; no current or past MDD, $n = 172$)	Explore DNAm of *OXTR* in the placentae of women depressed during pregnancy and in women using antidepressant(s) during pregnancy	*Antidepressants* (self-reported at recruitment and in 3rd trimester, hospital records in 3rd trimester [converted to SEDs], and measurement of whole blood and cord blood concentrations at birth) *Maternal depression* (SCID at recruitment and EPDS in 3rd trimester)	• Decreased DNAm in *OXTR* CpG 8 upon foetal exposure to antidepressants (self-reported) • Increased DNAm of *OXTR* CpG 8 upon higher cord blood concentrations of antidepressants
	McLaughlin *et al.* (2017) [34]	UK; Princess Royal Maternity Hospital	$n = 53$ (methadone-maintained opioid-dependent mothers, $n = 21$; smoking, "deprived" & opioid-naive mothers, $n = 17$; non-smoking, "affluent" & opioid-naive mothers, $n = 15$)	Explore if prenatal opioid exposure is associated with a differential DNAm in opioid-related genes (*ABCB1, CYP2D6*, and *OPRM1*)	*Methadone* (venous blood concentration 24–72 hours after birth to methadone-maintained opioid-dependent mothers) *Smoking* (case records) *Poverty* (DepCat score calculated from postal codes provided in case records)	• Increased DNAm in *ABCB1, CYP2D6*, and *OPRM1* in neonates of methadone-maintained opioid-dependent mothers compared to neonates of opioid-naive mothers
	Mansell *et al.* (2016) [45]	Australia; The Barwon Infant Study (2010–2013)	$n = 481$ (Various group comparisons: • Depressive symptoms, $n = 88$; • No depressive symptoms, $n = 357$ • Anxiety, $n = 77$; no anxiety, $n = 368$ • Stress scores, $n = 481$)	Investigate the association between maternal mental well-being and the DNAm of cord blood *NR3C1*	*Antidepressants* (self-reported at 28 weeks) *Depression* (EPDS ≥ 10 at 28 weeks) *Anxiety* (EPDS anxiety subscale ≥ 5 at 28 weeks) *Stress* (PSS score week 28)	• Increased DNAm of *NR3C1* CpG 35 associated with prenatal antidepressant exposure, but the association diminished when included as a covariate in the multivariate model of maternal pregnancy well-being and neonatal DNAm of *NR3C1*

(*Continued*)

Table 1. (Continued).

Reference	Country; setting (study period)	Sample size*n*(groups)	Research aim(s)	Exposure(s) (data source)	Pharmacoepigenetic outcome(s)
Gartstein *et al.* (2016) [38]	Canada; University of British Columbia	$n = 115$ (SSRI exposure, $n = 46$; no SSRI exposure, $n = 69$)	Examine the association between prenatal SSRI exposure and neonatal *SLC6A4* DNAm, and the influence on soothability	*SSRIs* (self-reported prescription retrieval and number of days used) *Maternal internalizing symptoms* (sum of EPDS, HAM-D, and HAM-A scores in 33–36 weeks of pregnancy)	• Prenatal SSRI exposure positively associated with neonate *SLC6A4* CpG DNAm of CpGs 3, 5, 7, and 9 and mean DNAm status of CpGs 9 and 10
Ciesielski *et al.* (2015) [33]	USA; Women and Infants Hospital in Providence Rhode Island (2008–2010)	$n = 184$ (psychiatric diagnosis[i] & antidepressants[j], $n = 12$; psychiatric diagnosis & no antidepressants, $n = 13$; no psychiatric diagnosis & no antidepressants, $n = 159$)	Investigate how DNAm in placentae is related to growth restriction observed in mothers with psychiatric illnesses (some of which are treated with antidepressants)	*Antidepressants* (medical records) *Maternal psychiatric disease* (depression, anxiety, and/or OCD/panic disorder prior to and/or during pregnancy; medical records)	• No difference in likelihood of unequal median DNAm between the antidepressant-exposed group, and the group with no psychiatric diagnosis and no antidepressants
Soubry *et al.* (2011) [52]	USA; the Newborn Epigenetics Study (2005–2008)	$n_{IGF2} = 356$ (antidepressants[k], $n = 35$; depressed, $n = 56$; non-medicated non-depressed, $n = 265$) $n_{H19} = 411$ (antidepressants, $n = 43$; depressed, $n = 65$; non-medicated, non-depressed, $n = 303$)	Examine the association between prenatal antidepressant and/or maternal depression exposure and DNAm of two DMRs in *IGF2* (*H19*)	*Antidepressants* (any use; medical charts) *Depression during pregnancy* (self-reported and interviewer-based questionnaires, diagnosis validated by clinical charts)	• Prenatal antidepressant exposure not associated with DNAm in neonatal *IGF2* or *H19* DMRs • Higher DNAm in *H19* DMR associated with prenatal antidepressant exposure in African-Americans but not in Caucasians
Devlin *et al.* (2010) [44]	Canada; cohort part of a study on how psychotropic medication exposure impact neonatal health	$n = 82$ (SRIs, $n = 33$; no SRIs, $n = 49$)	Examine the impact of maternal *MTHFR* C677T genotype on maternal mood, and the association with DNAm in maternal and neonatal *SLC6A4* and *BDNF*	*SRIs* (data source not stated) *Depressed mood* (EPDS and HAM-D in the 2nd [week 26] and 3rd [week 33] trimesters)	• SRI exposure not associated with neonatal DNAm levels in *SLC6A4* or *BDNF*
Oberlander *et al.* (2008) [36]	Canada; cohort part of a study on how psychotropic medication exposure impact neonatal health	$n = 82$ [l] (depression & SRIs[m], $n = 36$; depression & no SRIs, $n = 13$; no depression & no SRIs, $n = 33$)	Investigate any association between maternal depressed or anxious mood during pregnancy and DNAm of *NR3C1* in neonates; examine the association between *NR3C1* DNAm and stress reactivity at 3 months	*SSRIs* (data source not stated) *Maternal mood* (EPDS, HAM-A and HAM-D in week 26 and 33; EPDS, HAM-A, HAM-D, PSI-SF 3 months after birth)	• Prenatal SRI exposure not associated with neonatal *NR3C1* DNAm status • Global neonatal DNAm did not differ between SRI exposed and non-exposed infants

[†]Complete list of comparisons is available in Supplementary Table S4.
[a]1 amitriptyline; 1 bupropion; 2 citalopram hydrobromide; 2 desipramine; 3 fluoxetine; 2 paroxetine; 5 sertraline (12/14 women used SSRIs only).
[b]4 carbamazepine; 3 lamotrigine; 2 polytherapy (1 carbamazepine/valproic acid; 1 lamotrigine/valproate).
[c]36 lamotrigine; 3 valproate; 3 levetiracetam; 2 carbamazepine; 1 topiramate; 1 phenytoin; 1 gabapentin; 6 polytherapy (not specified).
[d]26 epilepsy; 27 psychiatric disorder (20 bipolar disorder; 5 MDD; 2 anxiety).
[e]Define two classes: class 1 of SRIs (SSRIs; SNRIs; TCAs), and class 2 of bupropion.
[f]2 paroxetine; 2 fluoxetine; 2 sertraline; 1 citalopram; 4 venlafaxine.
[g]2 paroxetine; 3 fluoxetine; 2 sertraline; 2 citalopram; 10 venlafaxine.
[h]11 sertraline; 6 fluoxetine; 4 citalopram; 2 paroxetine.
[i]Depression, anxiety, obsessive-compulsive disorder, or panic disorder.
[j]18 SSRIs (13 sertraline); 5 atypical antidepressants.
[k]SSRIs (72%); SNRIs; TCAs; SARIs; bupropion.
[l]Numbers not fixed; some mothers in untreated, non-depressed group became depressed during the study, whereas others started receiving pharmacological treatment.
[m]18 paroxetine; 6 fluoxetine; 5 sertraline; 2 venlafaxine; 5 citalopram.
ADHD: attention-deficit/hyperactivity disorder; AED: antiepileptic drug; BDI: Beck Depressive Inventory; BSI: Brief Symptom Inventory; DepCat: measure of socioeconomic status in Scotland (affluent–deprived); DMR: differentially methylated region; DNAm: DNA methylation; EPDS: Edinburgh Postnatal Depression Scale; GO: Gene Ontology; HAM-A: Hamilton Rating Scale for Anxiety; HAM-D: Hamilton Rating Scale for Depression; HRSD17: 17-item Hamilton Rating Scale for Depression; IBQ: Infant Behaviour Questionnaire; MDD: major depressive disorder; MDE: major depressive episode; OCD: obsessive-compulsive disorder; PRAS: Pregnancy-related Anxiety Scale; PSI-SF: Parenting Stress Index – Short Form; PSS: Perceived Stress Scale; SARI: serotonin antagonist and reuptake inhibitor; SCID: Structured Clinical Interview for DSM-IV; SED: sertraline-equivalent dosage; SNRI: serotonin and noradrenaline reuptake inhibitor; (S)SRI: (selective) serotonin reuptake inhibitor; STAI: State-trait Anxiety Inventory; TCA: tricyclic antidepressant.

Table 2. Overview of the methodology and statistical analysis.

	Reference	Sample tissue	Individual sites, regional or global level	Method	Epigenetic outcome	Statistical tests	Statistical thresholds	Model covariates†	Cell count correction	Authors' inference about exposure–outcome associations*
Epigenome-wide association studies	Yeung *et al.* (2020) [30]	Cord blood	Site-by-site	Infinium MethylationEPIC BeadChip (Illumina)	Subset quantile normalized β values	Linear mixed-effects models	FDR cut-off < 0.05	6	Reference data for cord blood [101]	Association
	Addo *et al.* (2019) [48,100]	Placenta	Site-by-site	Infinium MethylationEPIC BeadChip (Illumina)	*M* values for statistical procedures, β values for data presentation	Robust linear regression models	FDR cut-off < 0.05 (2 CpGs reached Bonferroni significance at $p < 6.3 \times 10^{-8}$)	14 (and PCA)	Houseman [102]	Association
	Cardenas *et al.* (2019) [29]	Cord blood	Site-by-site and regional	Infinium Human-Methylation450 BeadChip (Illumina)	*M* values for statistical procedures, β values for data presentation	Robust linear regression models	FDR cut-off < 0.05 (16 CpGs reached Bonferroni significance at $P < 1.34 \times 10^{-7}$ in Project Viva, 1 site of which was confirmed in the replication cohort)	10 (and PCA)	Houseman [102]	Association
	Gervin *et al.* (2017) [35]	Cord blood	Site-by-site and regional	Infinium Human-Methylation450 BeadChip (Illumina)	*M* values for statistical procedures, β values for data presentation	Linear regression models	FDR cut-off < 0.05	6 (and SVA)	Houseman [102] and reference data for cord blood [103]	Association
	Emes *et al.* (2013) [46]	Cord blood	Site-by-site and global (LINE-1)	Infinium Human-Methylation27 BeadChip (Illumina)	β values (log_2-transformed upon *t* testing)	Hierarchical clustering of the β values of all sites, then *t*-tests of the mean scores across the identified clusters to infer differentially methylated sites	FDR cut-off < 0.05	0	No	Association

(*Continued*)

Table 2. (Continued).

	Reference	Sample tissue	Individual sites, regional or global level	Method	Epigenetic outcome	Statistical tests	Statistical thresholds	Model covariates[†]	Cell count correction	Authors' inference about exposure–outcome associations[*]
	Smith *et al.* (2012) [47]	Cord blood and placenta	Site-by-site and global (average measure across all investigated CpGs)	Infinium Human-Methylation27 BeadChip (Illumina)	*M* values for statistical procedures, β values for data presentation	Linear mixed effects models	FDR cut-off < 0.05 Follow-up in placental tissue: one-sided *p*-values	4	No	Association
	Schroeder *et al.* (2012) [43]	Cord blood	Site-by-site	Infinium Human-Methylation27 BeadChip (Illumina)	*M* values for statistical procedures, β values for data presentation	Linear mixed effects models	FDR cut-off < 0.05	4	No	Association
Combined epigenome-wide and candidate gene studies	Gurnot *et al.* (2015) [31]	Cord blood	***Genome-wide*** Site-by-site ***Gene-specific*** *CYP2E1* both individual sites (16 CpGs) and global methylation	***Genome-wide*** Infinium Human-Methylation27 BeadChip array (Illumina) ***Gene-specific*** PyroMark Q96 ID pyrosequencer (Qiagen)	***Genome-wide*** β values ***Gene-specific*** Methylation percentage	*Genome-wide* Non-parametric Wilcoxon tests and linear regression models *Gene-specific* Linear regression models	FDR cut-off < 0.05	0	No	The authors propose an epigenetic mediation mechanism based on three observations, suggesting that the DNAm of *CYP2E1* is an epigenetic mechanism to protect the unborn child from the adverse effects of SRIs
	Non *et al.* (2014) [32]	Cord blood	***Genome-wide*** Site-by-site and regional ***Gene-specific*** Site-by-site; 10 candidate genes	***Genome-wide*** Infinium Human-Methylation450 BeadChip (Illumina) ***Gene-specific*** Pyromark Q24 Pyrosequencer (Qiagen)	***Genome-wide*** β values ***Gene-specific*** Methylation percentage	Multivariate robust standard error regression models	*Genome-wide* FDR cut-off < 0.1 *Gene-specific* Bonferroni correction accounting for the number of probes tested in each gene	4	No; the authors do not expect shifts in cell populations to influence much, as neither of the identified genes are important in inflammation or immune system functioning	Association

(*Continued*)

Table 2. (Continued).

	Reference	Sample tissue	Individual sites, regional or global level	Method	Epigenetic outcome	Statistical tests	Statistical thresholds	Model covariates[†]	Cell count correction	Authors' inference about exposure–outcome associations[*]
Candidate gene studies	Galbally *et al*. (2020) [37]	Placenta and buccal cells (24–72 hours after birth)	Site-by-site; *NR3C1* and *NR3C2* (13 placental CpGs and 11 buccal CpGs for both genes)	SEQUENOM MassARRAY EpiTYPER platform	Mean methylation percentage of triplicate samples	Univariate ANOVAs	FDR cut-off < 0.25	0	No, but state this as a limitation	Hypothesize that the DNAm of placental *NR3C2* CpG 24 mediates the indirect effect of maternal depression on cortisol reactivity at 12 months. In this model, antidepressants may modify the effect of depression on DNAm. To strengthen the hypothesis, the authors performed a mediation analysis based on Hayes *et al*. [104]. From this analysis, the authors concluded that CpG 24 methylation in the placental *NR3C2* reduces the association between maternal depression and infant cortisol reactivity
	Galbally *et al*. (2018) [51]	Placenta	Site-by-site; *OXTR* (16 CpGs)	SEQUENOM MassARRAY EpiTYPER platform	Mean methylation percentage of triplicate samples	One-way ANOVAs Significant antidepressant–DNAm associations explored with scatter plots and multiple regression models	$p < 0.05$	1	No	Association
	McLaughlin *et al*. (2017) [34]	Buccal cells (24–72 hours after birth)	Regional; *ABCB1*, *CYP2D6*, and *OPRM1* (mean of all CpG DNAm values within a gene to compare across samples)	Pyromark Q24 Pyrosequencer (Qiagen)	Methylation percentage	One-way ANOVAs	$p < 0.05$	0	No	Association

(*Continued*)

Table 2. (Continued).

Reference	Sample tissue	Individual sites, regional or global level	Method	Epigenetic outcome	Statistical tests	Statistical thresholds	Model covariates[†]	Cell count correction	Authors' inference about exposure–outcome associations[*]
Mansell *et al*. (2016) [45]	Cord blood (mononuclear cells only)	Site-by-site; *NR3C1* promoter (21 CpGs)	SEQUENOM MassARRAY EpiTYPER platform	Log-transformed methylation percentage (mean of triplicate arrays; log base not specified) for regression modelling; mean methylation with no transformation for data presentation	Student's *t*-test, ANOVA or pairwise correlation tests (as appropriate) to investigate the associations between exposures, DNAm, and covariates. Multivariate linear regression models to investigate the association of maternal mental well-being and DNAm of *NR3C1*	Bonferroni corrected $p < 0.00079$ (accounting for three maternal well-being measures and 21 CpG units) Unadjusted $p < 0.05$	10	Determine monocyte and lymphocyte frequencies by FACS, used this as a covariate in the model	Association
Gartstein *et al*. (2016) [38]	Cord blood	Site-by-site; *SLC6A4* promoter (10 CpGs)	PyroMark MD System (Biotage, Qiagen)	Methylation percentages	Identified the most important regions of methylation over the 10 CpGs of *SLC6A4* by PCA and literature searches. Using these CpGs, performed hierarchical multiple regression models including interaction terms	$p < 0.05^b$	2	No	Association

(*Continued*)

Table 2. (Continued).

Reference	Sample tissue	Individual sites, regional or global level	Method	Epigenetic outcome	Statistical tests	Statistical thresholds	Model covariates[†]	Cell count correction	Authors' inference about exposure–outcome associations[*]
Ciesielski *et al*. (2015) [33]	Placenta	Site-by-site; 15 genes (in 27 CpGs)	Infinium Human-Methylation27 BeadChip array (Illumina)	Adjusted[a] β values	Logistic regression to determine if the methylation of CpG sites were associated with low birth weight. Fisher's exact tests and follow-up exact binomial tests were applied to compare the significant CpGs from the regression across the included groups	FDR cut-off < 0.05	1	No	Findings suggest that decreased DNAm of one placental *LEPR* CpG may be part of a biological mechanism linking ongoing maternal psychiatric disease and poor foetal growth. The authors base this proposal on the observation of associations across their variables and state that they rule out other explanations of this apparent DNAm pattern. Notably, the authors also emphasize that the observed associations do not by default suggest causation and that additional validation and characterization studies are needed
Soubry *et al*. (2011) [52]	Cord blood	Regional level; *IGF2* DMR (3 CpGs) and *H19* DMR (4 CpGs)	Pyromark MD System (Biotage, Qiagen)	Mean methylation percentage from triplicate assays	*t*-tests between mean methylation over all samples of the DMRs and the covariates Multiple regression models	$p < 0.05$[b]	7 (and SVA)	Validated that *IGF2* and *H19* DMRs were equal across different cell fractions from cord blood [105]	Association
Devlin *et al*. (2010) [44]	Cord blood	Site-by-site; *SLC6A* (10 CpGs) and *BDNF* (12 CpGs)	PyroMark MD System (Biotage, Qiagen)	Methylation percentage	MANCOVA models	$p < 0.05$	0	No, but the authors recognize this limitation	Association

(*Continued*)

Table 2. (Continued).

Reference	Sample tissue	Individual sites, regional or global level	Method	Epigenetic outcome	Statistical tests	Statistical thresholds	Model covariates†	Cell count correction	Authors' inference about exposure–outcome associations*
Oberlander *et al*. (2008) [36]	Cord blood (mononuclear cells only)	Site-by-site; *NR3C1* (13 CpGs) and global (LINE-1)	PyroMark MD System (Biotage, Qiagen)	Methylation percentage	Multiple regression models	$p < 0.05$	0	No	Findings suggest an association between increased 3rd trimester maternal depressed mood and higher HPA stress responsiveness in the 3-month-old infant. This association may potentially be mediated epigenetically through DNAm of human *NR3C1*. However, the authors acknowledge that to infer any functional consequences of neonatal cord blood *NR3C1* CpG3 DNAm, they will need more direct evidence, and any mediational relationship cannot be deduced from their study

†Includes all covariates adjusted for in the statistical models. Most studies also employed other strategies to adjust for or assess suspected covariates; these are presented in the Supplementary Table S3.
*Full explanation of the authors' reasoning behind a causal relationship is in Supplementary Table S5.
[a]What is meant by 'adjusted' is not specified in the article.
[b]Assumed $p < 0.05$ based on which results are considered significant, but the p was not stated clearly in the article.
(M)AN(C)OVA: (multivariate) analysis of (co)variance; CpG: 5 –Cytosine–phosphate–guanine–3 site; DMR: differentially methylated regions; DNAm: DNA methylation; FACS: fluorescence-activated cell sorting; FDR: false discovery rate; HPA: hypothalamic-pituitary-adrenal; PCA: principal component analysis; (S)SRI: (selective) serotonin reuptake inhibitor; SVA: surrogate variable analysis.

unexposed pregnancies. Using a different study design, Gervin *et al.* [35] compared DNAm in long-term paracetamol-exposed children with ADHD to short-term-exposed children with ADHD (2,089 differentially methylated CpGs), unexposed children with ADHD (192 differentially methylated CpGs), and unexposed children without ADHD (6,211 differentially methylated CpGs). Although the studies report vastly different numbers of significant CpGs, both Gervin *et al.* [35] and Addo *et al.* [48] concluded that prenatal paracetamol exposure may be associated with DNAm in cord blood from susceptible individuals or placentae, respectively.

Yeung *et al.* [30] investigated the association between prenatal acetylsalicylic acid exposure and DNAm in cord blood. In the randomized controlled trial, women were randomly assigned to receive 81 mg of acetylsalicylic acid or placebo every day until conception (within six menstrual cycles) and during pregnancy [49]. The DNAm of one CpG (3,500 base pairs upstream of the *POU4F1* promoter) in cord blood was significantly associated with prenatal exposure to acetylsalicylic acid [30]. However, Yeung *et al.* concluded that the association of prenatal acetylsalicylic acid exposure with DNAm in cord blood is negligible, as only one CpG with a minor effect size (1% increase in CpG DNAm) was discovered in their association study [30].

In the EWAS on prenatal methadone exposure, McLaughlin *et al.* [34] reported a significant increase in buccal cell DNAm of *ABCB1*, *CYP2D6*, and *OPRM1* in neonates of mothers who were methadone-maintained during pregnancy. The authors argued that their results demonstrated that opioids interact with epigenetic mechanisms, and that the altered DNAm of the opioid metabolism-related genes may have a functional significance that needs further investigation [34].

Discussion

In this review, we have systematically summarized the literature investigating associations between prenatal medication exposure and epigenetic differences in neonates. We included a total of 18 studies on DNAm, examining *in utero* exposure to antidepressants, AEDs, paracetamol, acetylsalicylic acid, or methadone. We found substantial inconsistency across studies, including heterogeneity in methodology, materials, design, genome coverage, and statistical modelling, making the interpretation of findings and cross-study comparisons challenging. The novelty of the field combining epidemiological and pharmacoepigenetic methods may partly explain this heterogeneity due to a lack of consensus on how to perform analyses and report findings. Therefore, we discuss the results of the reviewed studies with respect to both epidemiological and epigenetic considerations, and suggest 10 recommendations for future studies in prenatal pharmacoepigenetics, as summarized in Box 1.

Prenatal pharmacoepigenetic candidate gene studies should have a clearly defined hypothesis guided by teratological principles [50] and pharmacological, epidemiological, and biological knowledge (**pt. 1, Box 1**). Founding the research question on a well-informed hypothesis is fundamental for a transparent and well-designed prenatal pharmacoepigenetic study. This was mostly done in the candidate gene studies included in this review, which provided a rationale for selecting the genes being studied, such as the gene being related to psychiatric disorders (the serotonin transporter gene *SLC6A4*) [32,38,44] or stress reactivity (the glucocorticoid and mineralocorticoid receptor genes *NR3C1* and *NR3C2*) [32,36,37,45].

Small molecular and structural differences between drugs are known to cause variations in toxicity and teratogenicity [50]. Although the reviewed studies on analgesics focused on one specific medication [30,34,35,48], the studies on psychotropics investigated the effect of medication classes on neonatal DNAm [29,31–33,36–38,43–47,51,52]. In the two studies on AEDs [46,47], several medications were investigated, which may be too broad considering the various different pharmacological [53,54] and epigenetic mechanisms of action of AEDs [21,55,56]. For example, Smith *et al.* [47] jointly analysed seven different AEDs among 53 women, but also performed a stratified analysis of carbamazepine monotherapy (36 women). In contrast, Emes *et al.* [46] jointly analysed valproate, lamotrigine, and carbamazepine among nine women, and did not stratify

Box 1. (1)HYPOTHESIS: candidate gene studies should use a plausible hypothesis to guide the study design Hypotheses should be defined prior to designing a candidate gene study, and be guided by principles of teratology, knowledge of pharmacological mechanisms, and epidemiological and biological observations. Hypothesis-free EWASs are also important as the field of prenatal pharmacoepigenetic studies is still emerging.

(2)MEDICATION SELECTION: investigate individual medications rather than medication classes Unless the pharmacological and epigenetic mechanisms of action of medications are expected to be similar across the medication class, medications should be analysed on an individual substance level.

(3)STATISTICAL POWER: ensure sufficient sample sizes to detect relevant DNAm differences To detect biologically relevant DNAm associations and to ensure valid interpretation of the results, tools developed for power assessments in epigenetic studies should be used when planning such studies.

(4)STUDY DESIGN: include a disease comparison group to disentangle medication from indicationStudies should include a disease comparison group to better differentiate the effects of exposure to medication from the underlying maternal disease. This may reduce the impact of confounding by indication.

(5)SYSTEMATIC ERROR: assess selection bias, information bias, and confounding Selection bias should be assessed by comparing characteristics of study samples to the target population. The validity of medication exposure, neonatal phenotype, and other covariates should be reported, and information bias and misclassification addressed. Measured confounders of the exposure–outcome association(s) are to be adjusted for and residual confounding investigated. Importantly, cell type heterogeneity should be considered a confounding factor in epigenetic studies.

(6)TISSUE SELECTION: biomarkers and extrapolation of DNAm patterns across tissues If the research aim is not only to report a tissue-independent biomarker, but to extrapolate results to other target tissues, the limitations of such translation should be recognized,, and reduced using software applications or data sets on cross-tissue correlations of modifications.

(7)LONGITUDINAL PERSPECTIVE: assess persistence of DNAm patterns throughout childhoodThe follow-up of epigenetic patterns later in childhood is essential to assess the relevance of these changes over time, as they may suggest a long-term impact on the phenotypic outcome.

(8)DATA INTEGRATION: integrate epigenetic data with complementary omics dataIntegration of complementary omics data, such as genomic and transcriptomic data, can strengthen functional and causal inferences of the findings.

(9) CAUSAL INFERENCE: provides a framework for interpreting exposure-outcome associations Causal inference methods, such as two-step Mendelian randomization, may support the inference of causation from exposure–outcome associations, including how medication may impact phenotypic outcome via DNAm changes. Importantly, the underlying assumptions of causal methods are often untestable and, therefore, such methods should be used carefully.

(10)REPLICATION: replicate findings using different methods and independent cohorts Replication both across methods and in independent cohorts is essential to increase the validity of the findings and the generalizability of the results to enhance clinical relevance.

.

their analyses on individual medications. Analyses on a medication class level may mask effects or give heterogeneous results that are difficult to interpret. Consequently, prenatal pharmacoepigenetic studies should aim to investigate individual medications rather than medication classes and ensure sufficient study power to do this (**pt. 2 & pt. 3, Box 1**).

The median sample size of the reviewed studies was 201 (IQR of 289), with sample sizes as low as

Table 3. Overview of genes examined in more than one candidate gene study on antidepressants.

	NR3C1	*SLC6A4*	*BDNF*	*FKBP5*
	Glucocorticoid receptor	**Serotonin transporter**	**Brain-derived neurotrophic factor**	**Regulator of the glucocorticoid receptor**
Galbally *et al*. (2020) [37]	N.S.			
Mansell *et al*. (2016) [45]	N.S.			
Gartstein *et al*. (2016) [38]		+		
Ciesielski *et al*. (2015) [33]				N.S.
Non *et al*. (2014) [32]	N.S.	–	N.S.	–
Devlin *et al*. (2010) [44]		N.S.	N.S.	
Oberlander *et al*. (2008) [36]	N.S.			
TOTAL STUDIES	**4**	**3**	**2**	**2**

'+': significantly increased DNAm level in medication-exposed group; '–': significantly decreased DNAm level in medication-exposed group; 'N.S.': no significant difference between the medication-exposed and non-exposed groups.

18 [46] and 23 [31]. None of the studies reported a power assessment to justify the selected sample size. The power of an EWAS depends on many variables, including the significance level, effect size, sample size, array technology, tissue type, and distribution of DNAm differences [57]. Therefore, power calculations are challenging, but simulation studies for power estimation [42,58,59], as well as power assessment tools [42,57], may support the investigation of power in epigenetic studies. In epigenetic epidemiology, the effect sizes are expected to be small, ranging from 0.05 to 0.1 and upwards [60,61], as evidenced in studies on prenatal smoking exposure, with effect sizes commonly ranging from 0.02 to 0.1 [62,63]. We recommend that future prenatal pharmacoepigenetic studies perform and report power assessments in order to ensure sufficient power to detect genuine epigenetic differences between comparison groups (**pt. 3, Box 1**).

The indication for medication use is an important potential confounder in prenatal pharmacoepigenetic studies, as the observed outcome may be associated with the underlying maternal illness and not the medication used to treat it [64,65]. Among the 14 studies on psychotropics in this review, only eight studies included such a comparison group [29,32,33,36,37,44,51,52]. Notably, seven of these studies found an association between the underlying maternal depression and DNAm in the neonate [29,32,33,36,37,44,51], emphasizing the importance of including this comparison group in future prenatal pharmacoepigenetic studies (**pt. 4, Box 1**).

When defining medication-exposed comparison groups, more than half of the reviewed studies relied partly or entirely on self-reported medication use during pregnancy [29,35,37,38,43,45–48,51]. This measure does not necessarily reflect the actual medication use [66–68] and is vulnerable to recall bias if reported retrospectively [69]. In five studies, medication exposure was assessed at birth using maternal and/or neonatal blood concentrations of the medication [31,34,37,47,51]. Although informative at birth, this measure does not reflect medication use in earlier stages of pregnancy. Similarly, the eight studies investigating the association between DNAm and neonatal phenotypic outcomes included various outcome

definitions without assessing the validity of the measurement [31,33–38,46]. In five studies, data were measured objectively (birth weight [31,33,46] and cortisol levels [36,37]), whereas two studies relied on diagnoses by specialists [34,35], and one study used parent reports on infant temperament [38]. An assessment of medication exposure, neonatal phenotype, and covariates is crucial to avoid misclassification. Therefore, we recommend that future prenatal pharmacoepigenetic studies perform sensitivity analyses to assess the robustness of the findings, taking into account the validity of the measures (**pt. 5, Box 1**). For example, methods to quantify the impact of exposure and outcome misclassifications, such as probabilistic bias analysis [70], are highly recommended [64].

A wide range of different covariates were considered in the studies (Supplementary Table S3). Selecting an appropriate set of confounders to control for is critical to avoid systematic bias (**pt. 5, Box 1**). Ten studies selected confounders by assessing associations between covariates, exposure, and outcome [31,33,34,36–38,45,46,51,52]. We suggest to control for covariates that are assumed to be confounders, i.e., covariates that are not part of the causal path, and that are both a cause of the exposure and the outcome. Therefore, the covariates to be accounted for should occur upstream of the prenatal exposure, while mediators (which are part of the causal pathway) should not be accounted for when investigating the *total effect* of the exposure on the outcome. The specific covariates to be taken into account need to be assessed for each individual study, as the relevance of the covariates depends on several factors (e.g., study design and tissue type), and include both technical covariates related to laboratory procedures and biological covariates. Examples of biological covariates to be evaluated are maternal age, smoking during pregnancy, infant sex, gestational age, and folate use in pregnancy, which were the covariates most commonly accounted for in the studies included in this review (Supplementary Table S3). To this end, future prenatal pharmacoepigenetic studies may also benefit from implementing causal inference tools, such as directed acyclic graphs (DAGs) [71], to identify a sufficient set of confounders for adjustments [64]. Such investigations can be complemented by assessing whether the selected confounders largely capture the model variability, as was performed by surrogate variable analysis in Gervin *et al.* [35], and by principal component analyses in Addo *et al.* [48] and Cardenas *et al.* [29]. For an excellent overview of a general approach to identify relevant confounders in observational studies, please refer to the review by VanderWeele [72].

The majority of the reviewed studies were based on cord blood, which consists of cells exhibiting cell type-specific DNAm patterns [40]. Therefore, prenatal pharmacoepigenetic studies should consider whether DNAm differences associated with medication exposure reflect variation in constituent cell types, which are known to mediate or confound the exposure associations [40,73]. When investigating cell-type proportions as a mediator, in order to assess the *direct effect* of medication exposure on associated DNAm differences, it may be necessary to adjust for estimated or measured cell-type composition (see, e.g., Liu *et al.* [74] and Gervin *et al.* [75]). However, if the *total effect* of medication exposure on DNAm is more interesting, for instance when searching for potential biomarkers of a phenotypic outcome, the cell-type composition should not be accounted for, as it may remove relevant DNAm–phenotypic outcome associations (see, e.g., Ollikainen *et al.* [76]). Cell type composition may act as a *confounder* when assessing the extent to which DNAm mediate the effect of drug exposure on a phenotypic outcome, and should in such instances be accounted for [73]. In summary, variation in cell-type composition confers an important covariate in epigenetic studies, and should be appropriately evaluated [40,60,73,77]. Surprisingly, only six of the reviewed studies considered cell-type composition in their analyses [29,30,35,45,48,52], emphasizing the need for increased awareness among prenatal pharmacoepigenetic researchers to evaluate cell types in future studies (**pt. 5, Box 1**). There are several different methods to determine and account for the cell-type composition in tissue samples, and these are extensively described in the excellent recent review by Teschendorff and Relton [40].

Among the reviewed records, eight studies hypothesized an association between DNAm and

neonate phenotypes [31,33–38,46], including five studies concerning brain-related phenotypic outcomes [34–38]. However, DNAm in peripheral surrogate tissues does not necessarily resemble DNAm in the target tissue [78–80], which challenges the accuracy of the extrapolation of the findings. Although this limitation was acknowledged in most studies [29–32,35–38,43–45,47,48,51], only one study attempted to reduce the constraint by including a correlation analysis of select CpGs across adult whole blood and brain tissue [29]. Importantly, investigation of peripheral tissues is still considered valuable, since biomarkers of maternal disease or child developmental outcomes do not need to be from the relevant tissue (i.e., do not need to be tissue-specific). However, to realize the ultimate aim of prenatal pharmacoepigenetics of gaining direct mechanistic insights into how medication exposure impacts the foetus with potential phenotypic consequences, future studies should validate tissue extrapolation by, for example, investigating cross-tissue correlations in available databases [61,81–85] (**pt. 6, Box 1**). Yet, current databases are mostly available on adult tissues, limiting the relevance to prenatal pharmacoepigenetic studies. Researchers have been calling for initiatives to develop biobanks of foetal and child brain specimens, while also taking into account the ethical issues of building such biobanks [86].

Only one of the reviewed studies investigated DNAm patterns longitudinally during childhood, finding that DNAm at a CpG in *ZNF575* persisted into early childhood [29]. Though investigating the DNAm at birth provides information on the immediate impact of prenatal medication exposure, the follow-up of epigenetic patterns later in childhood is valuable to assess the persistence over time and increase the clinical relevance of the findings (**pt. 7, Box 1**).

The clinical relevance of prenatal pharmacoepigenetic research may also be strengthened by functional and causal interpretations of the results. Using a multi-omics approach with integration of omics data (e.g., genomics, epigenomics, and transcriptomics data) [87] could substantiate the epigenetic findings (**pt. 8, Box 1**). However, the reviewed studies only used single omics data (i.e., epigenomics). Although single omics data are potentially useful both as biomarkers and in providing insight into biological pathways, this is limited to correlations or associations often reflecting reactive, rather than causative, processes. We recommend that future studies include additional omics data, as this may enable (i) investigation of the functional consequences of DNAm on gene expression [40], (ii) adjustment for the genetic variation associated with DNAm variation [88–90], and (iii) utilization of genomic methylation quantitative trait loci (mQTLs) to implement causal inference methods, such as two-step Mendelian randomization [40,91] (**pt. 9, Box 1**). There are several openly accessible resources making omics data available for integration, as thoroughly reviewed by Walton, Relton and Caramaschi [92].

Causal modelling and reasoning are increasingly being applied in genetic epidemiology to strengthen the ability to make causal inferences about associations, but it is still new to the field of pharmacoepigenetics [40,64]. For example, two-step Mendelian randomization [91,93–95] has been used to assess how DNAm can mediate an association between prenatal exposure and phenotypic outcomes in children [96,97]. Notably, only one reviewed study attempted to make causal inferences about an association between prenatal antidepressant exposure, cord blood DNA, and infant stress reactivity in a mediation analysis [37]. We foresee important advances in future prenatal pharmacoepigenetic studies using the causal inference framework (**pt. 9, Box 1**). Importantly, the causal models rely on assumptions that need to be met for them to be valid [98]. As these assumptions are often untestable [98], careful use of the causal inference framework in pharmacoepigenetic studies is essential.

Lastly, to validate findings, replication using a different technology and in an independent cohort is essential, both to determine the robustness of the associations and to assess the level of technical and biological variation. Notably, only two of the reviewed studies applied more than one method to assess DNAm [31,32], and only one study attempted to validate their results in an independent cohort [29], emphasizing the need for an increased focus on replication in prenatal pharmacoepigenetics (**pt. 10, Box 1**). Several multi-cohort consortia to enable replication of studies are

already in place, such as the Pregnancy and Childhood Epigenetics consortium (PACE) [99].

Conclusion

Investigating the potential effects of pharmacological treatment in pregnancy is essential to establish foetal epigenetic safety, understand the underlying mechanisms, and recognize the clinical consequences for the offspring. However, studies on prenatal medication exposure and epigenetic changes are largely heterogeneous and inconsistent. To improve the quality, comparability, and interpretability of future prenatal pharmacoepigenetic studies, we propose 10 recommendations bridging the fields of prenatal epidemiology and epigenetics. Epidemiological approaches and causal inference frameworks will reduce systematic bias and improve our ability to interpret exposure–outcome associations, including how medications may impact phenotypic outcomes via changes in DNAm. Furthermore, it is essential to consider the persistence of DNAm patterns over time and the potential for cross-tissue extrapolation when assessing the biological relevance of the epigenetic contribution. Importantly, integrating more omics data and implementing two-step Mendelian randomization can strengthen the functional and causal inferences of the findings. In conclusion, a consensus on how to perform and report prenatal pharmacoepigenetic studies will fuel the development of the field and contribute to future high-quality studies of clinical relevance.

List of Abbreviations

ADHD	Attention-deficit/hyperactivity disorder
AED	Antiepileptic drug
ATC	Anatomical Therapeutic Chemical
CpG	5 –Cytosine–phosphate–guanine–3 site
DAG	Directed acyclic graph
DOHaD	Developmental Origins of Health and Disease
DNAm	DNA methylation
EWAS	Epigenome-wide association study
FDR	False discovery rate
IQR	Interquartile range
mQTL	Methylation quantitative trait locus
NSAID	Non-steroidal anti-inflammatory drug
SNRI	Serotonin and noradrenaline reuptake inhibitor
SRI	Serotonin reuptake inhibitor
SSRI	Selective serotonin reuptake inhibito

Acknowledgments

We thank Christian Page and Mollie Wood for their critical appraisal of the article. We also thank the librarians at the Library of Medicine and Science of the University of Oslo, particularly Bente Kathrine Rasch and Hilde Strømme, for excellent support in developing the search strategy for the review.

Author’s contribution

EWO, HMEN, and KG conceived the idea of the systematic literature review. EWO, HMEN, and KG planned the searches. EWO performed the searches. EWO and KG performed the screening and selection of records. EWO extracted the data and drafted the first version of the paper. EWO, HMEN, and KG all revised the paper. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the PharmaTox Strategic Research Initiative, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway and the European Research Council (ERC) Starting Grant “DrugsInPregnancy” [grant number: 678033].

Ethics approval and consent to participate

Not applicable.

ORCID

Emilie Willoch Olstad http://orcid.org/0000-0002-9578-8886
Hedvig Marie Egeland Nordeng http://orcid.org/0000-0001-6361-2918
Kristina Gervin http://orcid.org/0000-0002-9851-1890

References

[1] Griffiths SK, Campbell JP. Placental structure, function and drug transfer. Contin Educ Anaesthesia Crit Care Pain. 2015;15(2):84–89.
[2] Tetro N, Moushaev S, Rubinchik-Stern M, et al. The placental barrier: the gate and the fate in drug distribution. Pharm Res. 2018;35(4):1–16.
[3] Goasdoué K, Miller SM, Colditz PB, et al. Review: the blood-brain barrier; protecting the developing fetal brain. Placenta. 2017;54:111–116.

[4] Ek CJ, Dziegielewska KM, Habgood MD, et al. Barriers in the developing brain and Neurotoxicology. Neurotoxicology. 2012;33:586–604.
[5] Barker DJP. The origins of the developmental origins theory. J Intern Med. 2007;261(5):412–417.
[6] Gillman MW. Developmental origins of health and disease. N Engl J Med. 2005;353(17):1848–1850.
[7] Gluckman PD, Hanson MA, Pinal C. The developmental origins of adult disease. Matern Child Nutr. 2005;1(3):130–141.
[8] Godfrey K. The 'developmental origins' hypothesis: epidemiology. In: Gluckman PD, Hanson MA, editors. Dev Orig Heal Dis. Cambridge: Cambridge University Press; 2006. p. 6–32.
[9] Godfrey KM, Lillycrop KA, Burdge GC, et al. Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease. Pediatr Res. 2007;61(5 Part 2):5–10.
[10] Hjorth S, Bromley R, Ystrom E, et al. Use and validity of child neurodevelopment outcome measures in studies on prenatal exposure to psychotropic and analgesic medications – A systematic review. PLoS One. 2019;14(7):e0219778.
[11] Bromley R, Weston J, Adab N, et al. Treatment for epilepsy in pregnancy: neurodevelopmental outcomes in the child. Cochrane Database Syst Rev J. 2014.
[12] Weston J, Bromley R, Jackson CF, et al. Monotherapy treatment of epilepsy in pregnancy: congenital malformation outcomes in the child. Cochrane Database Syst Rev. 2016;11(11).
[13] El Marroun H, White T, Verhulst FC, et al. Maternal use of antidepressant or anxiolytic medication during pregnancy and childhood neurodevelopmental outcomes: a systematic review. Eur Child Adolesc Psychiatry. 2014;23(10):973–992.
[14] Prady SL, Hanlon I, Fraser LK, et al. A systematic review of maternal antidepressant use in pregnancy and short- and long-term offspring's outcomes. Arch Womens Ment Health. 2018;21(2):127–140.
[15] Tosato S, Albert U, Tomassi S, et al. A systematized review of atypical antipsychotics in pregnant women: balancing between risks of untreated illness and risks of drug-related adverse effects. J Clin Psychiatry. 2017;78(5):e477–89. Physicians Postgraduate Press Inc.
[16] Bauer AZ, Kriebel D, Herbert MR, et al. Prenatal paracetamol exposure and child neurodevelopment: a review. Horm Behav. Academic Press Inc. 2018;101:125–147. .
[17] Yazdy M, Desai R, Brogly S. Prescription opioids in pregnancy and birth outcomes: a review of the literature. J Pediatr Genet. 2015;04(2):056–70. Georg Thieme Verlag KG.
[18] Ornoy A, Weinstein-Fudim L, Ergaz Z. Antidepressants, antipsychotics, and mood stabilizers in pregnancy: what do we know and how should we treat pregnant women with depression. Birth Defects Res. 2017;109(12):933–956. John Wiley and Sons Inc.
[19] Schaefer C, Peters PWJ, Miller RK, editors. Drugs during pregnancy and lactation: treatment options and risk assessment. 3rd ed. Academic Press, London, UK; 2014.
[20] Barouki R, Melén E, Herceg Z, et al. Epigenetics as a mechanism linking developmental exposures to long-term toxicity. Environ Int. 2018;114:77–86.
[21] Lötsch J, Schneider G, Reker D, et al. Common non-epigenetic drugs as epigenetic modulators. Trends Mol Med. 2013;19(12):742–753.
[22] Viuff ACF, Pedersen LH, Kyng K, et al. Antidepressant medication during pregnancy and epigenetic changes in umbilical cord blood: a systematic review. Clin Epigenetics. 2016;8(1). DOI:10.1186/s13148-016-0262-x
[23] Moher D, Liberati A, Tetzlaff J, et al. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med. 2009;6(7): e1000097.
[24] Centre for Reviews and Dissemination University of York; National Institute for Health Research. PROSPERO [Internet].
[25] Page MJ, Shamseer L, Tricco AC. Registration of systematic reviews in PROSPERO: 30,000 records and counting. Syst Rev. 2018;7(1):32.
[26] Counsell C. Formulating questions and locating primary studies for inclusion in systematic reviews. Ann Intern Med. 1997;127(5):380–387.
[27] WHO Collaborating Centre for Drug Statistics Methodology. ATC/DDD Index 2020 [Internet].
[28] Ouzzani M, Hammady H, Fedorowicz Z, et al. Rayyan – a web and mobile app for systematic reviews. Syst Rev. 2016;5(1). DOI:10.1186/s13643-016-0384-4
[29] Cardenas A, Faleschini S, Cortes Hidalgo A, et al. Prenatal maternal antidepressants, anxiety, and depression and offspring DNA methylation: epigenome-wide associations at birth and persistence into early childhood. Clin Epigenetics. 2019;11(1). DOI:10.1186/s13148-019-0653-x.
[30] Yeung EH, Guan W, Zeng X, et al. Cord blood DNA methylation reflects cord blood C-reactive protein levels but not maternal levels: a longitudinal study and meta-analysis. Clin Epigenetics. 2020;12(1). DOI:10.1186/s13148-020-00852-2.
[31] Gurnot C, Martin-Subero I, Mah SM, et al. Prenatal antidepressant exposure associated with CYP2E1 DNA methylation change in neonates. Epigenetics. 2015;10 (5):361–372.
[32] Non AL, Binder AM, Kubzansky LD, et al. Genome-wide DNA methylation in neonates exposed to maternal depression, anxiety, or SSRI medication during pregnancy. Epigenetics. 2014;9 (7):964–972.
[33] Ciesielski TH, Marsit CJ, Williams SM. Maternal psychiatric disease and epigenetic evidence suggest a common biology for poor fetal growth. BMC Pregnancy Childbirth. 2015;15(1). DOI:10.1186/s12884-015-0627-8

[34] McLaughlin P, Mactier H, Gillis C, et al. Increased DNA methylation of ABCB1, CYP2D6, and OPRM1 genes in newborn infants of methadone-maintained opioid-dependent mothers. J Pediatr. 2017;190:180–184.
[35] Gervin K, Nordeng H, Ystrom E, et al. Long-term prenatal exposure to paracetamol is associated with DNA methylation differences in children diagnosed with ADHD. Clin Epigenetics. 2017;9(1). DOI:10.1186/s13148-017-0376-9
[36] Oberlander TF, Weinberg J, Papsdorf M, et al. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics. 2008;3(2):97–106.
[37] Galbally M, Watson SJ, van Ijzendoorn M, et al. The role of glucocorticoid and mineralocorticoid receptor DNA methylation in antenatal depression and infant stress regulation. Psychoneuroendocrinology. 2020;115:104611.
[38] Gartstein MA, Hookenson KV, Brain U, et al. Sculpting infant soothability: the role of prenatal SSRI antidepressant exposure and neonatal SLC6A4 methylation status. Dev Psychobiol. 2016;58(6):745–758.
[39] Weisenberger DJ, van den Berg D, Pan F, et al. Comprehensive DNA Methylation Analysis on the Illumina® Infinium® Assay Platform. Appl Note, Illumina.
[40] Teschendorff AE, Relton CL. Statistical and integrative system-level analysis of DNA methylation data. Nat Rev Genet. 2018;19:129–147.
[41] van Rooij J, Mandaviya PR, Claringbould A, et al. Evaluation of commonly used analysis strategies for epigenome- and transcriptome-wide association studies through replication of large-scale population studies. Genome Biol. 2019;20(1):235. BioMed Central Ltd.
[42] Mansell G, Gorrie-Stone TJ, Bao Y, et al. Guidance for DNA methylation studies: statistical insights from the Illumina EPIC array. BMC Genomics. 2019;20(1):366. BioMed Central Ltd.
[43] Schroeder JW, Smith AK, Brennan PA, et al. DNA methylation in neonates born to women receiving psychiatric care. Epigenetics. 2012;7(4):409–414.
[44] Devlin AM, Brain U, Austin J, et al. Prenatal exposure to maternal depressed mood and the MTHFR C677T variant affect SLC6A4 methylation in infants at birth. PLoS One. 2010;5(8):e12201.
[45] Mansell T, Vuillermin P, Ponsonby AL, et al. Maternal mental well-being during pregnancy and glucocorticoid receptor gene promoter methylation in the neonate. Dev Psychopathol. 2016;28(4pt2):1421–1430.
[46] Emes RD, Clifford H, Haworth KE, et al. Antiepileptic drugs and the fetal epigenome. Epilepsia. 2013;54(1): e16–9.
[47] Smith AK, Conneely KN, Newport DJ, et al. Prenatal antiepileptic exposure associates with neonatal DNA methylation differences. Epigenetics. 2012;7:458–463.
[48] Addo KA, Bulka C, Dhingra R, et al. Acetaminophen use during pregnancy and DNA methylation in the placenta of the extremely low gestational age newborn (ELGAN) cohort. Environ Epigenetics. 2019;5(2). DOI:10.1093/eep/dvz010.
[49] Schisterman EF, Silver RM, Lesher LL, et al. Preconception low-dose aspirin and pregnancy outcomes: results from the EAGeR randomised trial. Lancet. 2014;384(9937):29–36.
[50] Friedman JM. The principles of teratology: are they still true? Birth Defects Res A Clin Mol Teratol. 2010;88(10):766–768.
[51] Galbally M, Ryan J, van Ijzendoorn M, et al. Maternal depression, antidepressant use and placental oxytocin receptor DNA methylation: findings from the MPEWS study. Psychoneuroendocrinology. 2018;90:1–8.
[52] Soubry A, Murphy S, Huang Z, et al. The effects of depression and use of antidepressive medicines during pregnancy on the methylation status of the IGF2 imprinted control regions in the offspring. Clin Epigenetics. 2011;3(1):2.
[53] Cook AM, Bensalem-Owen MK. Mechanisms of action of antiepileptic drugs. Therapy. 2011;8(3):307–313.
[54] White HS, Smith MD, Wilcox KS. Mechanisms of action of antiepileptic drugs. Int Rev Neurobiol. 2007;81:85–110.
[55] Monti B, Polazzi E, Contestabile A. Biochemical, molecular and epigenetic mechanisms of valproic acid neuroprotection. Curr Mol Pharmacol. 2010;2 (1):95–109.
[56] Göttlicher M, Minucci S, Zhu P, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. Embo J. 2001;20 (24):6969–6978.
[57] Graw S, Henn R, Thompson JA, et al. PwrEWAS: a user-friendly tool for comprehensive power estimation for epigenome wide association studies (EWAS). BMC Bioinformatics. 2019;20(1). DOI:10.1186/s12859-019-2804-7
[58] Tsai PC, Bell JT. Power and sample size estimation for epigenome-wide association scans to detect differential DNA methylation. Int J Epidemiol. 2015;44(4):1429–1441.
[59] Saffari A, Silver MJ, Zavattari P, et al. Estimation of a significance threshold for epigenome-wide association studies. Genet Epidemiol. 2018;42(1):20–33. Wiley-Liss Inc.
[60] Heijmans BT, Commentary: MJ. The seven plagues of epigenetic epidemiology. Int J Epidemiol. 2012;41 (1):74–78.
[61] Mill J, Heijmans BT. From promises to practical strategies in epigenetic epidemiology. Nat Rev Genet. 2013;14(8):585–594.
[62] Breton CV, Marsit CJ, Faustman E, et al. Small-magnitude effect sizes in epigenetic end points are important in children's environmental health studies: the children's environmental health and disease prevention research center's epigenetics working group. Environ Health Perspect. 2017;125(4):511–526. Public Health Services, US Dept of Health and Human Services.

[63] Joubert BR, Felix JF, Yousefi P, et al. DNA methylation in newborns and maternal smoking in pregnancy: genome-wide consortium meta-analysis. Am J Hum Genet. 2016;98(4):680–696. Cell Press.

[64] Wood ME, Lapane KL, van Gelder MMHJ, et al. Making fair comparisons in pregnancy medication safety studies: an overview of advanced methods for confounding control. Pharmacoepidemiol Drug Saf. 2018;27(2):140–147.

[65] Medicines Agency E The European Network of Centres for Pharmacoepidemiology and Pharmacovigilance (ENCePP) Guide on Methodological Standards in Pharmacoepidemiology [Internet].

[66] Skurtveit S, Selmer R, Odsbu I, et al. Self-reported data on medicine use in the Norwegian mother and child cohort study compared to data from the Norwegian prescription database. Nor Epidemiol. 2014;24:209–216.

[67] Joseph RM, van Staa TP, Lunt M, et al. Exposure measurement error when assessing current glucocorticoid use using UK primary care electronic prescription data. Pharmacoepidemiol Drug Saf. 2019;28(2):179–186.

[68] Gnjidic D, Du W, Pearson SA, et al. Ascertainment of self-reported prescription medication use compared with pharmaceutical claims data. Public Heal Res Pract. 2017;27.

[69] Rothman KJ, Greenland S, Lash TL. Chapter 9: validity in Epidemiologic Studies. In: Mod Epidemiol. 3rd ed. Philadelphia, PA, USA: Lippincott Williams & Wilkins; 2008. p. 128–147.

[70] Lash TL, Fox MP, Maclehose RF, et al. Good practices for quantitative bias analysis. Int J Epidemiol. 2014;43 (6):1969–1985. Oxford University Press.

[71] Textor J, Hardt J, Knüppel, S. DAGitty, Epidemiology: 2011;22(5):745). DOI: 10.1097/EDE.0b013e318225c2be

[72] VanderWeele TJ. Principles of confounder selection. Eur J Epidemiol. 2019;34(3):211–219. Springer Netherlands.

[73] Houseman EA, Kim S, Kelsey KT, et al. DNA methylation in whole blood: uses and challenges. Curr Environ Heal Reports. Springer. 2015;2(2):145–154.

[74] Liu Y, Aryee MJ, Padyukov L, et al. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. Nat Biotechnol. 2013;31(2):142–147. Nature Publishing Group.

[75] Gervin K, Andreassen BK, Hjorthaug HS, et al. Intra-individual changes in DNA methylation not mediated by cell-type composition are correlated with aging during childhood. Clin Epigenetics. 2016;8(1):110. Springer Verlag.

[76] Ollikainen M, Ismail K, Gervin K, et al. Genome-wide blood DNA methylation alterations at regulatory elements and heterochromatic regions in monozygotic twins discordant for obesity and liver fat. Clin Epigenetics. 2015;7(1). DOI:10.1186/s13148-015-0073-5.

[77] Jones MJ, Moore SR, Kobor MS. Principles and challenges of applying epigenetic epidemiology to psychology. Annu Rev Psychol. 2018;69(1):459–485.

[78] Liu H, Liu X, Zhang S, et al. Systematic identification and annotation of human methylation marks based on bisulfite sequencing methylomes reveals distinct roles of cell type-specific hypomethylation in the regulation of cell identity genes. Nucleic Acids Res. 2016;44 (1):75–94.

[79] Lister R, Pelizzola M, Dowen RH, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature. 2009;462(7271):315–322.

[80] Gu J, Stevens M, Xing X, et al. Mapping of variable DNA methylation across multiple cell types defines a dynamic regulatory landscape of the human genome. G3 Genes, Genomes, Genet. 2016;6:973–986.

[81] Ollikainen M, Smith KR, Joo EJ-H, et al. DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome. Hum Mol Genet. 2010;19(21):4176–4188.

[82] de Carli MM, Baccarelli AA, Trevisi L, et al. Epigenome-wide cross-tissue predictive modeling and comparison of cord blood and placental methylation in a birth cohort. Epigenomics. 2017;9(3):231–240.

[83] Ma B, Allard C, Bouchard L, et al. Locus-specific DNA methylation prediction in cord blood and placenta. Epigenetics. 2019;14(4):405–420.

[84] Herzog EM, Eggink AJ, Willemsen SP, et al. The tissue-specific aspect of genome-wide DNA methylation in newborn and placental tissues: implications for epigenetic epidemiologic studies. J Dev Orig Health Dis. 2020;12(1):113–123.

[85] Relton CL, Gaunt T, McArdle W, et al. Data resource profile: accessible resource for integrated epigenomic studies (ARIES). Int J Epidemiol. 2015;44(4):1181–1190.

[86] Abbott A. Tissue-bank shortage: brain child. Nature. 2011;478(7370):442–443. Nature.

[87] Conesa A, Beck S. Making multi-omics data accessible to researchers. Sci Data. 2019;6(1):251.

[88] van Dongen J, Nivard MG, Willemsen G, et al. Genetic and environmental influences interact with age and sex in shaping the human methylome. Nat Commun. 2016;7(1):1–13.

[89] Gaunt TR, Shihab HA, Hemani G, et al. Systematic identification of genetic influences on methylation across the human life course. Genome Biol. 2016;17 (1). DOI:10.1186/s13059-016-0926-z.

[90] Banovich NE, Lan X, McVicker G, et al. Methylation QTLs are associated with coordinated changes in transcription factor binding, histone modifications, and gene expression levels. PLoS Genet. 2014;10(9): e1004663. DOI: 10.1371/journal.pgen.1004663

[91] Relton CL, Davey Smith G. Two-step epigenetic Mendelian randomization: a strategy for establishing the causal role of epigenetic processes in pathways to disease. Int J Epidemiol. 2012;41(1):161–176.

[92] Walton E, Relton CL, Caramaschi D. Using openly accessible resources to strengthen causal inference in epigenetic epidemiology of neurodevelopment and mental health. Genes (Basel). 2019;10(3):193. MDPI AG.
[93] Kirkbride JB, Susser E, Kundakovic M, et al. Prenatal nutrition, epigenetics and schizophrenia risk: can we test causal effects? Epigenomics. 2012;4(3):303–315.
[94] Pingault JB, O'Reilly PF, Schoeler T, et al. Using genetic data to strengthen causal inference in observational research. Nat Rev Genet. 2018;19(9):566–580.
[95] Relton CL, Smith GD. Mendelian randomization: applications and limitations in epigenetic studies. Epigenomics. 2015;7(8):1239–1243.
[96] Caramaschi D, Sharp GC, Nohr EA, et al. Exploring a causal role of DNA methylation in the relationship between maternal vitamin B12 during pregnancy and child's IQ at age 8, cognitive performance and educational attainment: a two-step Mendelian randomization study. Hum Mol Genet. 2017;26(15):3001–3013.
[97] Allard C, Desgagné V, Patenaude J, et al. Mendelian randomization supports causality between maternal hyperglycemia and epigenetic regulation of leptin gene in newborns. Epigenetics. 2015;10(4):342–351.
[98] Pearl J. An introduction to causal inference. Int J Biostat. 2010;6(2). DOI:10.2202/1557-4679.1203
[99] Felix JF, Joubert BR, Baccarelli AA, et al. Cohort profile: pregnancy and childhood epigenetics (PACE) consortium. Int J Epidemiol. 2018;47(1):22–23.
[100] Addo KA, Bulka C, Dhingra R, et al. Acetaminophen use during pregnancy and DNA methylation in the placenta of the extremely low gestational age newborn (ELGAN) cohort [corrected]. Environ Epigenetics. 2020;6(1). DOI:10.1093/eep/dvaa006.
[101] Gervin K, Salas LA, Bakulski KM, et al. Systematic evaluation and validation of reference and library selection methods for deconvolution of cord blood DNA methylation data. Clin Epigenetics. 2019;11(1). DOI:10.1186/s13148-019-0717-y.
[102] Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13(1). 10.1186/1471-2105-13-86.
[103] Gervin K, Page CM, Aass HCD, et al. Cell type specific DNA methylation in cord blood: a 450K-reference data set and cell count-based validation of estimated cell type composition. Epigenetics. 2016;11(9):690–698.
[104] Hayes AF. Beyond Baron and Kenny: statistical mediation analysis in the New Millennium. Commun Monogr. 2009;76(4):408–420.
[105] Hoyo C, Murtha AP, Schildkraut JM, et al. Methylation variation at IGF2 differentially methylated regions and maternal folic acid use before and during pregnancy. Epigenetics. 2011;6(7):928–936.

II

PAPER II

No impact of prenatal paracetamol and folic acid exposure on cord blood DNA methylation in children with attention-deficit/hyperactivity disorder

Olstad EW, Nordeng HME, Lyle R, Gervin K

Submitted to *Epigenetics*.

No impact of prenatal paracetamol and folic acid exposure on cord blood DNA methylation in children with attention-deficit/hyperactivity disorder

Emilie Willoch Olstad[1,2,*], Hedvig Marie Egeland Nordeng[1,2,3], Robert Lyle[2,4,5] and Kristina Gervin[1,2,6]

[1] Pharmacoepidemiology and Drug Safety Research Group, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway

[2] PharmaTox Strategic Research Initiative, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway

[3] Department of Child Health and Development, Norwegian Institute of Public Health, Oslo, Norway

[4] Department of Medical Genetics, Oslo University Hospital and University of Oslo, Oslo, Norway

[5] Centre for Fertility and Health, Norwegian Institute of Public Health, Oslo, Norway

[6] Department of Research and Innovation, Division of Clinical Neuroscience, Oslo University Hospital, Oslo, Norway

Word count (excl. acknowledgements, declarations and references): 2,344 words

* Corresponding author

Emilie Willoch Olstad

e.w.olstad@farmasi.uio.no

Abstract

Pharmacoepigenetic studies are important to understand the mechanisms through which medications influence the developing fetus. For instance, we and others have reported associations between prenatal paracetamol exposure and offspring DNA methylation (DNAm). Additionally, folic acid (FA) intake during pregnancy has been associated with DNAm in genes linked to developmental abnormalities. In this study, we aimed to: (i) expand on our previous findings showing differential DNAm associated with long-term prenatal paracetamol exposure in offspring with attention-deficit/hyperactivity disorder (ADHD), and (ii) examine if there is an interaction effect of FA and paracetamol on DNAm in children with ADHD, using data from the Norwegian Mother, Father and Child Cohort Study (MoBa) and the Medical Birth Registry of Norway. We did not identify any impact of paracetamol or any interaction effect of paracetamol and FA on cord blood DNAm in children with ADHD.

Keywords: ADHD, DNA methylation, Epigenetics, Epigenetic epidemiology, EWAS, Folic acid, MoBa, MBRN, Paracetamol, Acetaminophen.

Introduction

There is an increasing interest in understanding how maternal medication use during pregnancy may affect epigenetic patterns and impact fetal development [1]. Epigenetics entails modifications to the DNA which may alter gene expression, without changing the DNA sequence [2]. DNA methylation (DNAm) is the most commonly studied epigenetic modification, whereby a methyl group is attached to cytosine-phosphate-guanine dinucleotides (CpGs) [2]. DNAm is reversible and influenced by both genetics and environmental factors, such as medications [2]. Therefore, pharmacoepigenetic studies are useful to better understand the mechanisms through which medications may impact the developing fetus.

Epidemiological studies have suggested adverse neurodevelopmental outcomes of long-term paracetamol use during pregnancy, including increased risks of attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) [3]. In contrast, folic acid (FA) intake during pregnancy has been associated with reduced ADHD and ASD symptoms in the child [4]. Several studies have found associations between prenatal paracetamol exposure and differential DNAm in cord blood [5, 6] and placenta [7], but none of the differences in DNAm overlapped across the studies [1, 5–7]. FA is an essential methyl donor for DNAm. Interestingly, a meta-analysis [8] and a recent FA intervention study [9] found an association of maternal FA intake during pregnancy with differences in DNAm at genes linked to developmental abnormalities.

In this study, we aimed to: (i) expand on our previous findings showing epigenome-wide differences in DNAm associated with long-term prenatal paracetamol exposure (≥20 days) in children with ADHD [5], and (ii) examine if there is an interaction effect of FA and paracetamol on DNAm in children with ADHD. To do this, we selected umbilical cord blood samples from the Norwegian Mother, Father and Child Cohort Study (MoBa), which contains information on maternal use of paracetamol and FA during pregnancy, and conducted an epigenome-wide association study (EWAS).

63 ## Materials and Methods

64 This study is based on umbilical cord blood samples from MoBa, conducted by the Norwegian Institute of
65 Public Health (NIPH) [10]. MoBa is a prospective, population-based birth cohort (n = 114,500 children, n =
66 95,200 mothers and n = 75,200 fathers), including births in Norway between 1998 and 2008 [10]. In 40.6% of
67 pregnancies, parents consented to participate. Throughout pregnancy and childhood, the participants complete
68 multiple questionnaires. MoBa also includes a biobank with approximately 90,000 blood samples collected
69 from both parents during pregnancy, and from mother and child (umbilical cord) at birth [11]. This study is
70 based on Data Version 8 released by MoBa in 2015. All MoBa participants have provided a written, informed
71 consent to participate in the cohort and can retract their consent at any time. The establishment of MoBa and
72 initial data collection was based on a license from the Norwegian Data Protection Agency and approval from
73 The Regional Committees for Medical and Health Research Ethics. The MoBa cohort is currently regulated
74 by the Norwegian Health Registry Act. All the analyzed data were de-identified, and the linking of MoBa to
75 the relevant health registries was handled by NIPH and the respective registries. Our study was approved by
76 the Regional Committee for Medical Research Ethics South East Norway.

77 The selection of individuals for the study was based on observational data from MoBa questionnaires Q1
78 (gestational weeks 0–13), Q3 (gestational weeks 13–29) and Q4 (gestational week 30 to delivery), the Medical
79 Birth Registry of Norway (MBRN) and the Norwegian Patient Registry (NPR). MBRN is a national health
80 registry containing information about all births in Norway and NPR contains diagnoses asserted by specialists
81 in governmental hospitals and outpatient clinics. MoBa was linked to NPR and MBRN using the personal 11-
82 digit identification number unique to every permanent resident of Norway. We included 261 pregnancies from
83 MoBa, divided into three groups: (i) children with ADHD prenatally exposed to paracetamol ≥20 days
84 (exposed group; n = 61), (ii) children with ADHD and unexposed to paracetamol (ADHD-controls; n = 100),
85 and (iii) children without ADHD and unexposed to paracetamol (population controls; n = 100). Pre-term births
86 (<37 weeks) and twins were excluded. The definitions of the different measures and the selection of covariates
87 are presented in the Supplementary methods (Additional File 1).

88 DNAm was assessed using the Infinium HumanMethylation EPIC BeadChip (Illumina). Samples were
89 randomly allocated to sample plates and beadchips, and processed as previously described [5]. All analyses
90 were performed in the R programming language (http://www.r-project.org/). The quality control, filtering,
91 background correction and normalization steps are detailed in the Supplementary methods (Additional File 1).
92 The final data set included 795,515 probes and 261 samples.

93 We used β values (ratio of methylated signal to total signal) for visualizations and M values for statistical tests
94 [12]. Principal component analysis (PCA) on the DNAm data was used to test the strength of association
95 between covariates and DNAm variation (tests applied on categorical and continuous variables included one-
96 way analysis of variance [ANOVA] and Spearman's correlation test, respectively). To identify differentially
97 methylated CpGs associated with paracetamol, we fit linear regression models onto the mean DNAm
98 differences in *limma* [13]. We included the $CD8^+$ T cell proportion as a covariate in all models. Interaction

99 was assessed by including an interaction term in the model. We pairwise compared the exposed group to the
100 ADHD-control and the population control groups. All comparisons were adjusted for multiple testing (false
101 discovery rate [FDR] <0.05) [14]. We performed a surrogate variable analysis to examine unmeasured sources
102 of variation in DNAm [15]. Further details on the statistical analyses are presented in the Supplementary
103 methods (Additional File 1).

104 Results

105 To enable a systematic replication and expansion of our previous findings [5], we selected three groups: one
106 *exposed group*, consisting of children with ADHD prenatally exposed to paracetamol ≥20 days ($n = 61$), and
107 two control groups, the *ADHD-control group*, including children with ADHD and unexposed to paracetamol
108 ($n = 100$), and the *population control group*, including children without ADHD and unexposed to paracetamol
109 ($n = 100$). The samples were selected from MoBa, the same cohort as our previous study was based on, but
110 only 17 (exposed) samples were included in both the original and the current study. We selected model
111 covariates by assessing their contribution to DNAm variation using PCA (Additional file 1: Supplementary
112 methods). These analyses identified that the estimated $CD8^+$ T cell proportion was significantly associated
113 with principal components (PCs) 2 and 3, and differed between the comparison groups (Additional file 1:
114 Figure S1 and Tables S1–2), and therefore, this covariate was included in the regression models.

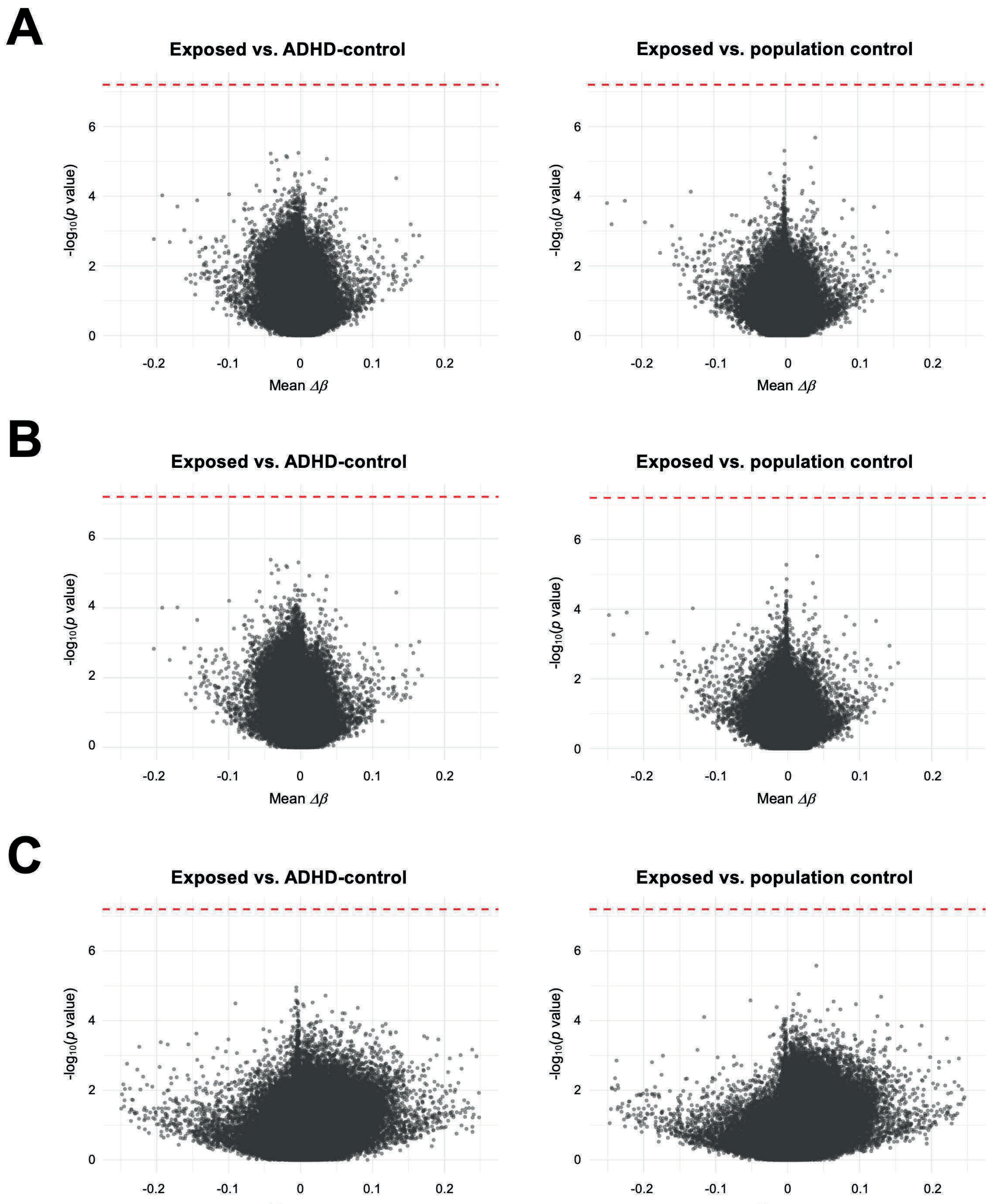
115 We ran three different models to assess the impact of paracetamol and FA on DNAm in children with ADHD
116 (Table 1): (i) a *crude model* to examine whether prenatal paracetamol exposure was associated with DNAm in
117 the cord blood of children with ADHD, (ii) an *adjusted model* where we adjusted the crude model for FA
118 intake during pregnancy to investigate the influence of FA status on the variance in DNAm, and (iii) an
119 *interaction model* where we examined any interaction effect of FA and paracetamol on DNAm. We ran the
120 three models comparing the exposed group to each of the two control groups separately, and also ran sensitivity
121 analyses excluding the $CD8^+$ T cell proportion from the models, which did not change the results (Additional
122 file 1: Figure S2).

123 **Table 1.** Overview of the three models used to assess the association of prenatal paracetamol and folic acid (FA) exposure
124 with cord blood DNA methylation (DNAm) in children with attention-deficit/hyperactivity disorder (ADHD). We ran
125 each model separately for the exposed group compared to each of the two control groups.

Model*	Definition	Group comparisons	Purpose
Crude model**	DNAm ~ group + CD8$^+$ T cells	***Comparison 1*** Exposed vs. ADHD-control ***Comparison 2*** Exposed vs. population control	Replicate our previous results [5] in a different study population from MoBa.
Adjusted model	DNAm ~ group + FA + CD8$^+$ T cells	***Comparison 1*** Exposed vs. ADHD-control ***Comparison 2*** Exposed vs. population control	Address the influence of FA intake on the variance in DNAm.
Interaction model	DNAm ~ group*FA + CD8$^+$ T cells	***Comparison 1*** Exposed vs. ADHD-control ***Comparison 2*** Exposed vs. population control	Assess whether there is an interaction effect of paracetamol and FA intake on DNAm.

126 **Abbreviations:** ADHD: attention-deficit/hyperactivity disorder; DNAm: DNA methylation; FA: folic acid; MoBa: the Norwegian Mother, Father
127 and Child Cohort Study.
128 * Sensitivity analysis excluding CD8$^+$ T cell proportion from the model produced similar results.
129 ** Crude with respect to folic acid intake.

130 To test the association of DNAm with prenatal paracetamol exposure and ADHD, we ran linear regression
131 models comparing the exposed group to the ADHD control group and the population control group (the crude
132 model). This analysis did not identify any significant differences between the exposed group and either of the
133 other two control groups (Figure 1A; Additional file 1: Figure S3A; Additional file 2), and thereby did not
134 replicate our previous findings. We then assessed whether FA could influence the association between
135 paracetamol exposure and DNAm by running an adjusted model, adjusting for FA intake during pregnancy.
136 This analysis did not reveal any significant CpGs and the *p* values were largely similar to those observed in
137 the crude model (Figure 1B; Additional file 1: Figure S3B; Additional file 2). Finally, to understand whether
138 there is an interaction effect of paracetamol and FA intake on DNAm in children with ADHD, we ran an
139 interaction model where the group and FA exposure variables interacted. This analysis neither revealed any
140 significant CpGs (Figure 1C; Additional file 1: Figure S3C; Additional file 2).



141

142 ***Figure 1.*** *Volcano plots comparing mean differences in DNAm (Δβ) for each CpG between the exposed group and either*
143 *the ADHD-control group or the population control group.* ***(A)*** *Crude model, only adjusted for $CD8^+$ T cell proportion.*
144 ***(B)*** *Adjusted model, adjusting for FA intake during pregnancy and $CD8^+$ T cell proportion.* ***(C)*** *Interaction model,*
145 *assessing any interaction effect of prenatal paracetamol and FA exposure on DNAm. Each CpG is plotted by the $-log_{10}$*
146 *of the* p *values against the mean per-CpG difference in DNAm (Δβ) of the compared groups. In (C) mean Δβ reflects the*
147 *interaction term (i.e., mean $\Delta\beta_{no\ FA}$–$\Delta\beta_{FA}$). The red dotted line indicates the FDR-adjusted* p *value significance threshold*
148 *(FDR<0.05).*

149 ## Discussion

150 Overall, we identified no impact of paracetamol nor interaction of paracetamol and FA on cord blood DNAm
151 in children with ADHD in our data. Results from the crude model did not identify any significant differences
152 in DNAm between the exposed group and either of the two control groups. Consequently, we did not replicate
153 our previous results [5]. These findings were surprising, as the present study is based on a larger number of
154 samples from the same cohort (MoBa), using the same study design and similar inclusion criteria as in the
155 original study. However, our studies are performed five years apart and methods have evolved, including the
156 introduction of the Illumina Infinium EPIC platform and novel analyses methods, such as normalization and
157 cell type deconvolution procedures. While we could not explain our replication failure solely by differing
158 DNAm measurements between platforms [16], we cannot exclude that other aspects of the analysis may have
159 contributed to the lack of replication.

160 Our findings also differ from two recent studies on prenatal paracetamol exposure and DNAm, which found
161 differential DNAm at some CpGs associated with paracetamol [6, 7]. However, comparison to these studies is
162 difficult for several reasons, including differing study designs, exposure definitions, phenotypes, tissue types
163 and analysis pipelines. There are also other challenges when comparing current prenatal pharmacoepigenetic
164 studies [1], which may contribute to the lack of replication and overlap of findings.

165 When assessing whether FA contributed to the variance in DNAm or if there was an interaction effect of
166 paracetamol and FA on DNAm, we did not find any significant CpGs, suggesting that there is no effect of
167 paracetamol and FA intake on DNAm in children with ADHD. Previous studies have reported differential
168 DNAm associated with maternal FA intake during pregnancy in genes linked to developmental abnormalities
169 [8, 9]. However, these studies examined FA intake as the main exposure rather than as an interacting factor
170 with medication use [8, 9], and therefore, are not directly comparable to the current study.

171 While we have improved multiple aspects of our previous study [5], there are some limitations to the current
172 study. The sample sizes of the groups are relatively small, albeit larger than in our previous study. Although
173 the MoBa cohort is one of the world's largest prospective birth cohorts, only 61 children with a clinical ADHD
174 diagnosis were exposed to paracetamol ≥20 days. Additionally, we did not assess the dose and timing of FA
175 intake during pregnancy. However, in Norway, the recommended FA supplement dosage is 400 μg/day,
176 starting one month prior to pregnancy and lasting throughout the first trimester of pregnancy.

177 In conclusion, this study did not replicate previous findings in MoBa or other studies investigating the
178 influence of paracetamol on DNAm, and did not identify any interaction effect of paracetamol and FA on
179 DNAm in children with ADHD. Our results contribute to the growing literature on prenatal
180 pharmacoepigenetics, but should be replicated in other cohorts. Replication of pharmacoepigenetic studies is
181 essential to ensure robust findings and to increase the clinical relevance of such studies.

Acknowledgements

The Norwegian Mother, Father and Child Cohort Study is supported by the Norwegian Ministry of Health and Care Services and the Ministry of Education and Research. We are grateful to all the participating families in Norway taking part in this on-going cohort study.

Declarations

Ethics approval and consent to participate

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 (REC). MoBa is currently regulated by the Norwegian Health Registry Act. All MoBa participants have given their written informed consent to participate in the cohort study. The current study has been approved by REC South East Norway (REC reference: 23136, 2014/163). All data are de-identified, and the linkage between MoBa and the different health registries were handled by NIPH along with the relevant registries.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the Norwegian Mother, Father and Child Cohort Study, but restrictions apply to the availability of these data and so are not publicly available. However, data are available from the authors upon reasonable request and with permission from the Norwegian Mother, Father and Child Cohort Study.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the European Research Council (ERC) Starting Grant "DrugsInPregnancy" (grant number: 678033) and the Research Council of Norway (NFR) "Medications during pregnancy: effects on neurodevelopmental disorders in childhood and epigenetic outcomes" (grant number: 241117). MoBa is supported by the Norwegian Ministry of Health and the Ministry of Education and Research, NIH/NIEHS (contract number: NO1-ES-75558), NIH/NINDS (grant numbers: UO1 NS 047537-01 and UO1 NS047537-06A1), and the Norwegian Research Council/FUGE (grant numbers; 151918/S10).

210 **Authors' contribution**

211 EWO, HMEN, RL and KG conceived the idea of and designed the study. EWO conducted the analyses. EWO
212 generated plots and tables and drafted the first version of the paper. EWO, HMEN, RL and KG all revised the
213 paper. All authors read and approved the final manuscript.

214 **Authors' information**

215 Not applicable.

216 ## References

217 1. Olstad EW, Nordeng HME, Gervin K. Prenatal medication exposure and epigenetic outcomes: a
218 systematic literature review and recommendations for prenatal pharmacoepigenetic studies.
219 Epigenetics. 2021.
220 2. Rakyan VK, Down TA, Balding DJ, Beck S. Epigenome-wide association studies for common human
221 diseases. Nature Reviews Genetics 2011 12:8. Nature Publishing Group; 2011;12:529–41.
222 3. Bauer AZ, Swan SH, Kriebel D, Liew Z, Taylor HS, Bornehag CG, et al. Paracetamol use during
223 pregnancy — a call for precautionary action. Nature Reviews Endocrinology 2021 17:12. Nature
224 Publishing Group; 2021;17:757–66.
225 4. Li M, Francis E, Hinkle SN, Ajjarapu AS, Zhang C. Preconception and Prenatal Nutrition and
226 Neurodevelopmental Disorders: A Systematic Review and Meta-Analysis. Nutrients. MDPI AG;
227 2019;11:1628.
228 5. Gervin K, Nordeng H, Ystrom E, Reichborn-kjennerud T, Lyle R. Long-term prenatal exposure to
229 paracetamol is associated with DNA methylation differences in children diagnosed with ADHD.
230 Clinical Epigenetics. 2017;9.
231 6. Eslamimehr S, Jones AD, Anthony TM, Arshad SH, Holloway JW, Ewart S, et al. Association of
232 prenatal acetaminophen use and acetaminophen metabolites with DNA methylation of newborns:
233 analysis of two consecutive generations of the Isle of Wight birth cohort. Environmental Epigenetics.
234 Oxford Academic; 2022;8:1–10.
235 7. Addo KA, Bulka C, Dhingra R, Santos HP, Smeester L, O'Shea TM, et al. Acetaminophen use during
236 pregnancy and DNA methylation in the placenta of the extremely low gestational age newborn
237 (ELGAN) cohort. Environmental Epigenetics. 2019;5.
238 8. Joubert BR, Den Dekker HT, Felix JF, Bohlin J, Ligthart S, Beckett E, et al. Maternal plasma folate
239 impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. Nature
240 Communications. Nature Publishing Group; 2016;7.
241 9. Ondičová M, Irwin RE, Thursby S-J, Hilman L, Caffrey A, Cassidy T, et al. Folic acid intervention
242 during pregnancy alters DNA methylation, affecting neural target genes through two distinct
243 mechanisms. Clinical Epigenetics 2022 14:1. BioMed Central; 2022;14:1–20.
244 10. Magnus P, Birke C, Vejrup K, Haugan A, Alsaker E, Daltveit AK, et al. Cohort Profile Update: The
245 Norwegian Mother and Child Cohort Study (MoBa). Int J Epidemiol. 2016;45:382–8.
246 11. Paltiel L, Haugan A, Skjerden T, Harbak K, Bækken S, Stensrud NK, et al. The biobank of the
247 Norwegian Mother and Child Cohort Study – present status. Norsk Epidemiologi. Norwegian
248 Epidemiological Society; 2014;24:29–35.
249 12. Du P, Zhang X, Huang C-C, Jafari N, Kibbe WA, Hou L, et al. Comparison of Beta-value and M-
250 value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics.
251 2010;11:587.
252 13. Smyth GK. limma: Linear Models for Microarray Data. Bioinformatics and Computational Biology
253 Solutions Using R and Bioconductor. Springer-Verlag; 2005. p. 397–420.
254 14. Benjamini Y. Discovering the false discovery rate. Journal of the Royal Statistical Society Series B:
255 Statistical Methodology. 2010;72:405–16.
256 15. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The SVA package for removing batch effects
257 and other unwanted variation in high-throughput experiments. Bioinformatics. 2012;28:882–3.
258 16. Olstad EW, Nordeng HME, Sandve GK, Lyle R, Gervin K. Low reliability of DNA methylation across
259 Illumina Infinium platforms in cord blood: implications for replication studies and meta-analyses of
260 prenatal exposures. Clinical Epigenetics. BioMed Central; 2022;14:80.

261

262 # Supplementary Material

263 **Additional file 1 (.docx)**

264 **Supplementary methods.** Details on exposures, outcomes and statistical analyses.

265 **Figure S1.** Contribution of covariates to the variation in DNAm.

266 **Figure S2.** QQ plots and histograms of *p* values of models *not* adjusted for $CD8^+$ T cell proportion.

267 **Figure S3.** QQ plots and histograms of *p* values of models adjusted for $CD8^+$ T cell proportion.

268 **Table S1.** Overview of the comparison group characteristics stratified by folic acid status.

269 **Table S2.** Overview of the cell type composition of the comparison groups stratified by folic acid status.

270

271 **Additional file 2 (.xlsx)**

272 Table of CpGs, *p* values and FDR-adjusted *p* values for the models and comparisons performed.

Additional File 1

Supplementary methods

Paracetamol exposure definition

Paracetamol use was retrieved from self-reports in three Mother, Father and Child cohort study questionnaires (MoBa; Q1, Q3, and Q4). Pregnant people report use of paracetamol per every 4-week interval of their pregnancy, in questionnaires distributed during and after pregnancy. Q1 and Q3 are distributed around gestational weeks 15 (0–4; 5–8; 9–12; ≥13 weeks) and 30 (13–16; 17–20; 21–24; 25–28; ≥29 weeks), respectively. Q4 is distributed approximately 6 months after delivery and covers the last weeks of pregnancy (from gestational week 30 until delivery). Women were presented with a number of illnesses and problems, and could report any medications used for the respective indications. The women also reported the total number of days they used the medication for each specific indication. We defined long-term prenatal exposure to paracetamol (Anatomical Therapeutic Chemical [ATC] code: N02BE01), as the use of paracetamol for ≥20 days during pregnancy (any indication), as in our previous study [1]. Paracetamol exposure was coded as a binary "yes"/"no" variable.

Attention-deficit/hyperactivity disorder definition

We retrieved offspring attention-deficit/hyperactivity disorder (ADHD)-diagnosis from the Norwegian Patient Registry (NPR; 2008–2016), which contains all diagnoses asserted by specialists in governmental hospitals and outpatient clinics, in accordance with the 10th revision of the International Classification of Disease (ICD-10). ADHD was defined as an ICD-10 diagnosis of hyperkinetic disorder (HKD; F90.0, F90.1, F90.8, or F90.9) between 2008 and 2016. HKD corresponds to ADHD in the Diagnostic and Statistical Manual (DSM) system [2–5].

Folic acid exposure definition

Folic acid (FA) use is recorded in both MoBa and the medical birth registry of Norway (MBRN). In MoBa, pregnant people report use of FA per every 4-week interval of their pregnancy, in questionnaires Q1 and Q3 described above. Self-reported FA use in MoBa corresponds well with maternal FA serum concentrations at 19 weeks of pregnancy [6, 7]. In MBRN, use of FA is recorded upon interview during the first antenatal consultation (pregnancy weeks 6–12). In this consultation, the pregnant woman is asked whether she has used FA prior to and/or during pregnancy. If she answers "yes", this is recorded as FA use in the corresponding check box (FA prior to pregnancy and/or FA during pregnancy). We defined exposure to FA in pregnancy as pregnant people with recordings of FA use during pregnancy in *both* MoBa and MBRN (n_{FA} = 135; $n_{\mathrm{no\ FA}}$ = 126).

Covariates

Analyses of potential covariates for inclusion in the linear regression models were done in three steps. First, we performed a principal component analysis (PCA) to analyze the association between the first three principal components (PCs), explaining the most DNAm variation across samples, and relevant covariates (Figure S1A–B). We assessed the relevant covariates previously included in prenatal pharmacoepigenetic studies [8]: maternal age, maternal education, marital status, primiparity, alcohol use, smoking, multivitamin use, psychotropic and analgesic use, maternal diseases, *in vitro* fertilisation, Caesarean section, gestational age, infant sex, birth weight, bisulphite conversion and the composition of all cell types ($CD8^+$ T cells, $CD4^+$ T cells, natural killer cells, B cells, monocytes, granulocytes and nuclear red blood cells [nRBCs]). Second, we assessed the individual contribution of the significant covariates to the variation in DNAm (Figure S1C), as described elsewhere [9, 10]. Briefly, we fit multivariable linear regression models for each of the first three PCs. The explanatory variables were the covariates associated with either of the three PCs. For each PC, we found the partial R^2, indicating the variation each covariate contributed to the variation of the PC, when accounting for the contribution of all other covariates in the model. The weighted partial R^2 for the three PC models was reported as the variability contributed to DNAm variation by the respective covariate. We found that all covariates except bisulphite conversion and cell type composition contributed <0.1% of the variation in DNAm. Finally, we tested whether the covariates contributing the most to the DNAm significantly differed between the comparison groups (Tables S1–2), resulting in a model including only the $CD8^+$ T cell proportion as a covariate.

We estimated cell type composition ($CD8^+$ and $CD4^+$ T cells, natural killer cells, B cells, monocytes, granulocytes and nucleated red blood cells [nRBCs]), using the "estimateCellCounts2" function of *minfi* [11] (Table S2). This function deconvolves the data using the Houseman reference-based approach [12] and depends on a recently published, validated cord blood reference data set (*FlowSorted.CordBloodCombined.450k*) [13, 14].

Processing and normalization of the DNA methylation data

The DNAm data was quality controlled using the quality control module implemented in *RnBeads* (v. 2.8.1) [15, 16]. First, samples with >5% low-quality CpGs or low bisulphite intensity were removed (0 samples). CpGs with >5% low-quality values were also removed (n = 8,947). Low-quality probes either exhibited a detection p value $>10^{-6}$ or a bead count <3. We performed background correction with the *ENmix* exponential-truncated-normal out-of-band (oob) method [17], dye bias correction with RELIC (REgression on Logarithm of Internal Control probes) [18] and probe-type correction with RCP (Regression of Correlated Probes) [19]. We then removed probes with SNPs overlapping with the CpG interrogation site or the nucleotide extension site (n = 29,176), cross-reactive probes (n = 14,921) [20, 21] and probes on the sex chromosomes (n = 17,532). The final data set consisted of 795,515 probes and 261 samples.

Statistical analyses

Hypothesis tests

Significant differences of covariates between groups were tested using the Wilcoxon's rank-sum test (continuous variables), and Chi-squared test or Fisher's exact test (categorical variables), as appropriate. To test which covariates were significantly associated with the most DNAm variation, we used Spearman's correlation test (continuous variables) and one-way analysis of variance (ANOVA; categorical variables).

Differential DNA methylation analyses

The β values (the ratio of methylated signal to the sum of methylated and unmethylated signal) were used for visualisation purposes, while M values ($\log_2(\beta/(1-\beta))$) were used for statistical tests, as recommended based on their statistical properties [22]. To identify differentially methylated sites associated with paracetamol, we fit linear regression models onto the mean DNAm differences, using *limma* [23]. Interaction was assessed by including an interaction term in the model. We pairwise compared the exposed group to the ADHD-control and the population control groups. All comparisons were adjusted for multiple testing with a false discovery rate (FDR) cut off <0.05, using the Benjamini and Hochberg method [24].

Surrogate variable analysis

We performed a surrogate variable analysis to examine any unmeasured sources of variation in DNAm. We used the *sva* package with default parameters to estimate surrogate variables [25].

References

1. Gervin K, Nordeng H, Ystrom E, Reichborn-kjennerud T, Lyle R. Long-term prenatal exposure to paracetamol is associated with DNA methylation differences in children diagnosed with ADHD. Clin Epigenetics. 2017;9.
2. World Health Organization. Chapter V: Mental and Behavioural Disorders. The 10th revision of the International Statistical Classification of Diseases and Related Health Problems. 2018.
3. American Psychiatric Association. Neurodevelopmental Disorders. Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Association; 2013.
4. Thapar A, Pine DS, Leckman JF, Scott S, Snowling MJ, Taylor E. Rutter's Child and Adolescent Psychiatry: Sixth Edition. Rutter's Child and Adolescent Psychiatry: Sixth Edition. Chichester, UK: John Wiley & Sons, Ltd; 2015.
5. Wieselberg HM, Rutter M, Thorley G. Which boys respond to stimulant medication? a controlled trial of methylphenidate in boys with disruptive behaviour. Psychological Medicine. Psychol Med; 1987;17:121–43.
6. Roth C, Bjørke-Monsen AL, Reichborn-Kjennerud T, Nilsen RM, Smith GD, Stoltenberg C, et al. Use of folic acid supplements in early pregnancy in relation to maternal plasma levels in week 18 of pregnancy. Molecular Nutrition and Food Research. Mol Nutr Food Res; 2013;57:653–60.
7. Bjørke-Monsen AL, Roth C, Magnus P, Midttun Ø, Nilsen RM, Reichborn-Kjennerud T, et al. Maternal B vitamin status in pregnancy week 18 according to reported use of folic acid supplements. Molecular Nutrition & Food Research. John Wiley & Sons, Ltd; 2013;57:645–52.
8. Bauer AZ, Swan SH, Kriebel D, Liew Z, Taylor HS, Bornehag CG, et al. Paracetamol use during

pregnancy — a call for precautionary action. Nature Reviews Endocrinology 2021 17:12. Nature Publishing Group; 2021;17:757–66.

9. Fages A, Ferrari P, Monni S, Dossus L, Floegel A, Mode N, et al. Investigating sources of variability in metabolomic data in the EPIC study: the Principal Component Partial R-square (PC-PR2) method. Metabolomics. Springer New York LLC; 2014;10:1074–83.
10. Perrier F, Novoloaca A, Ambatipudi S, Baglietto L, Ghantous A, Perduca V, et al. Identifying and correcting epigenetics measurements for systematic sources of variation. Clin epigenetics. 2018;10.
11. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. 2014;30:1363–9.
12. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13.
13. Gervin K, Page CM, Aass HCD, Jansen MA, Fjeldstad HE, Andreassen BK, et al. Cell type specific DNA methylation in cord blood: A 450K-reference data set and cell count-based validation of estimated cell type composition. Epigenetics. 2016;11:690–8.
14. Gervin K, Salas LA, Bakulski KM, Van Zelm MC, Koestler DC, Wiencke JK, et al. Systematic evaluation and validation of reference and library selection methods for deconvolution of cord blood DNA methylation data. Clinical Epigenetics. 2019;11.
15. Müller F, Scherer M, Assenov Y, Lutsik P, Walter J, Lengauer T, et al. RnBeads 2.0: Comprehensive analysis of DNA methylation data. Genome Biol. BioMed Central Ltd.; 2019;20:55.
16. Assenov Y, Müller F, Lutsik P, Walter J, Lengauer T, Bock C. Comprehensive analysis of DNA methylation data with RnBeads. Nat Methods. 2014;11:1138–40.
17. Xu Z, Niu L, Li L, Taylor JA. ENmix: A novel background correction method for Illumina HumanMethylation450 BeadChip. Nucleic Acids Res. Oxford University Press; 2016;44:e20.
18. Xu Z, Langie SAS, De Boever P, Taylor JA, Niu L. RELIC: a novel dye-bias correction method for Illumina Methylation BeadChip. BMC Genomics. BMC Genomics; 2017;18.
19. Niu L, Xu Z, Taylor JA. RCP: a novel probe design bias correction method for Illumina Methylation BeadChip. Bioinformatics. Bioinformatics; 2016;32:2659–63.
20. Pidsley R, Zotenko E, Peters TJ, Lawrence MG, Risbridger GP, Molloy P, et al. Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. Genome Biol. 2016;17.
21. McCartney DL, Walker RM, Morris SW, McIntosh AM, Porteous DJ, Evans KL. Identification of polymorphic and off-target probe binding sites on the Illumina Infinium MethylationEPIC BeadChip. Genom Data. Elsevier Inc; 2016;9:22–4.
22. Du P, Zhang X, Huang C-C, Jafari N, Kibbe WA, Hou L, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics. 2010;11:587.
23. Smyth GK. limma: Linear Models for Microarray Data. Bioinformatics and Computational Biology Solutions Using R and Bioconductor. Springer-Verlag; 2005. p. 397–420.
24. Benjamini Y. Discovering the false discovery rate. J R Stat Soc Series B Stat Methodol. 2010;72:405–16.
25. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The SVA package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics. 2012;28:882–3.
26. Bateman BT, Mhyre JM, Hernandez-Diaz S, Huybrechts KF, Fischer MA, Creanga AA, et al. Development of a comorbidity index for use in obstetric patients. Obstetrics and Gynecology. Obstet Gynecol; 2013;122:957–65.
27. Easter SR, Bateman BT, Sweeney VH, Manganaro K, Lassey SC, Gagne JJ, et al. A comorbidity-based screening tool to predict severe maternal morbidity at the time of delivery. American Journal of Obstetrics and Gynecology. Mosby Inc.; 2019;221:271.e1-271.e10.

Supplementary figures

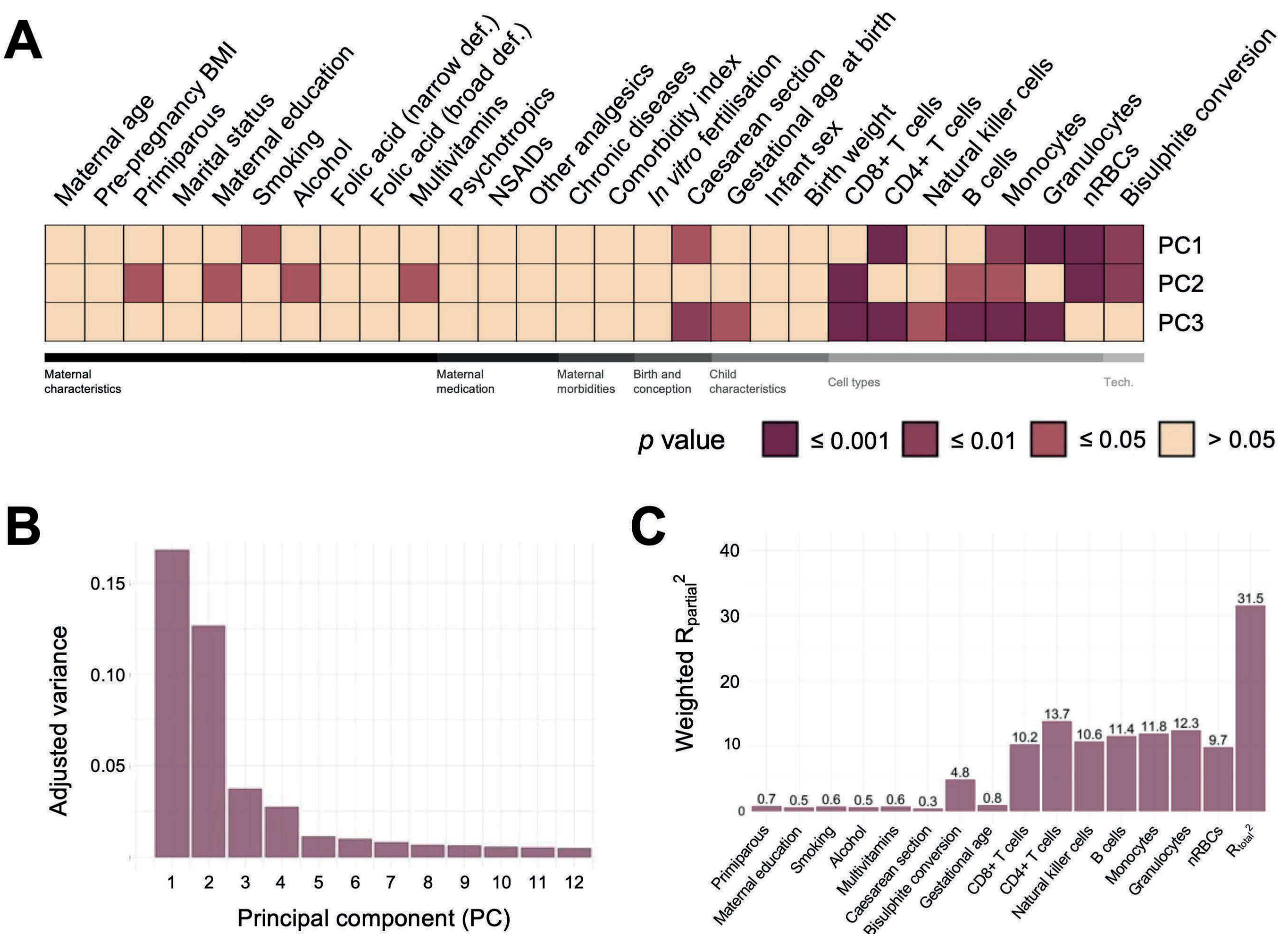


Figure S1. *Principal component analysis (PCA) was used to compute the principal components (PCs), which represent DNA methylation variation.* ***(A)*** *The significance of the association between the top three PCs and various covariates (association tests included the Spearman's correlation test [continuous variables] and one-way analysis of variance [ANOVA; categorical variables]).* ***(B)*** *Scree plot indicating the contribution to DNAm variation by each of the 12 first PCs.* ***(C)*** *The weighted partial R^2 for each covariate (i.e., the variability contributed by the respective covariate to the top three PCs, when accounting for the variability contribution of all other covariates in the model).* ***Abbreviations:*** *BMI: body mass index; nRBC: nucleated red blood cell; NSAID: non-steroidal anti-inflammatory drug; Tech.: technical covariate.*

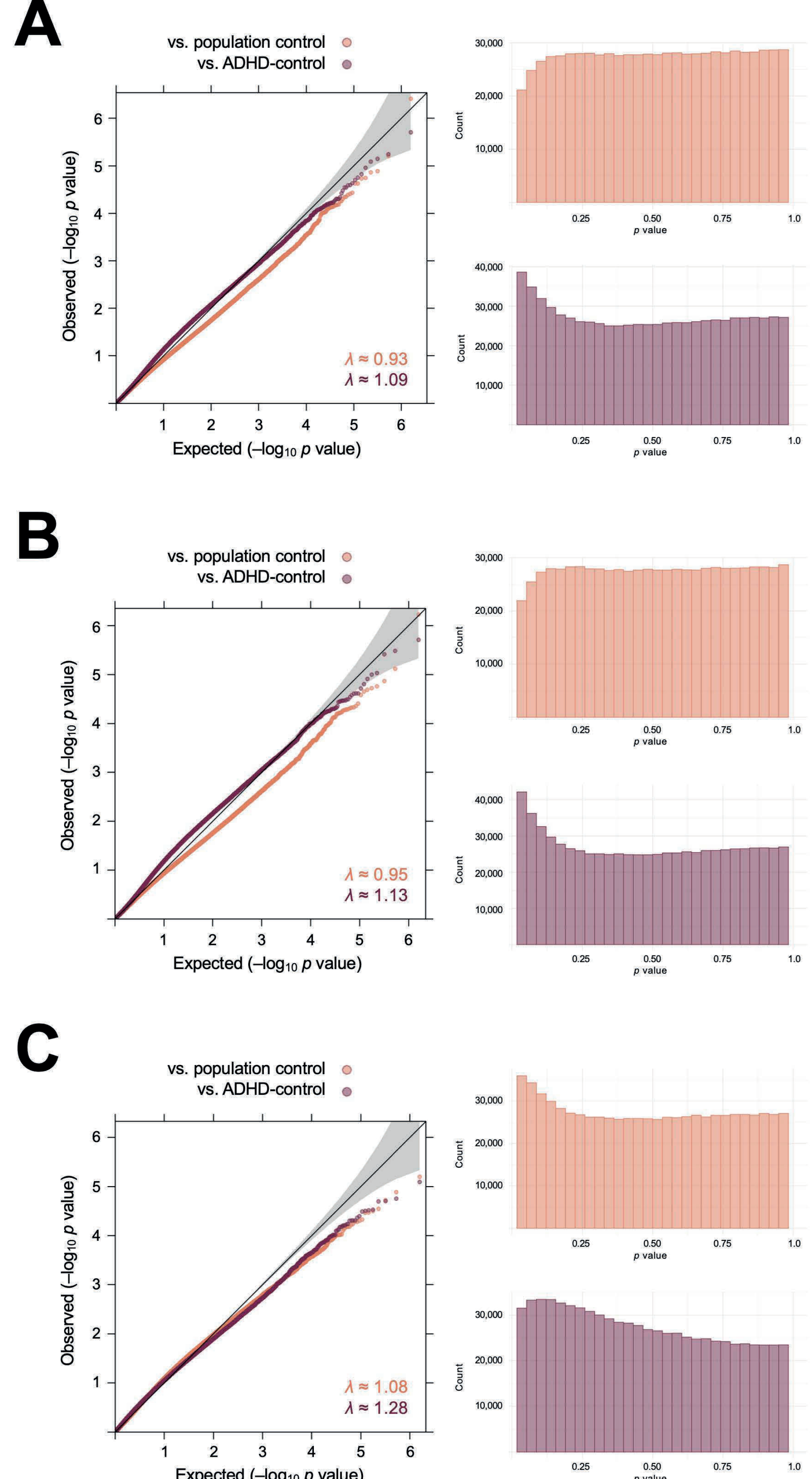


Figure S2. *Quantile-quantile (QQ) plots and histograms of the* p *values of* ***(A)*** *the crude model,* ***(B)*** *the adjusted model, and* ***(C)*** *the interaction model,* not *adjusted for the CD8*$^{+}$ *T cell proportion. The* p *values reflect the pairwise comparisons of the exposed group to the population control group (orange) or the ADHD-control group (purple).* λ *is the genomic inflation factor.*

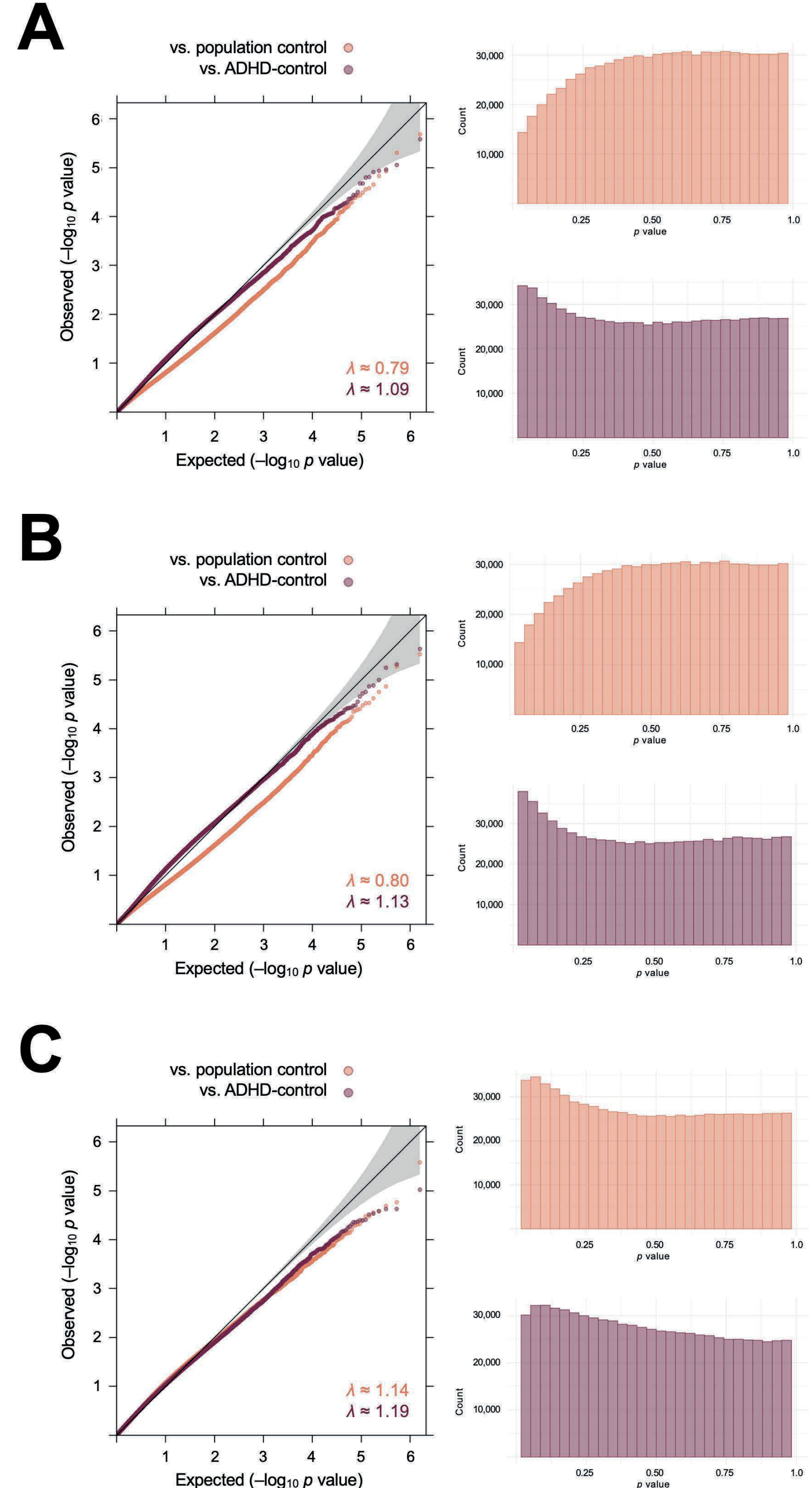


Figure S3. *Quantile-quantile (QQ) plots and histograms of the* p *values of* ***(A)*** *the crude model,* ***(B)*** *the adjusted model, and* ***(C)*** *the interaction model, all adjusted for the CD8⁺ T cell proportion. The* p *values reflect the pairwise comparisons of the exposed group to the population control group (orange) or the ADHD-control group (purple).* λ *is the genomic inflation factor.*

Supplementary tables

Table S1. Overview of the comparison group characteristics stratified by folic acid status. *P* values for significant differences between the exposed group and the two control groups, were calculated using the Wilcoxon rank-sum test (continuous variables), and the Chi-squared test or Fisher's exact test (categorical variables).

	Exposed (*n* = 61)		**ADHD-control** (*n* = 100)		**Population control** (*n* = 100)		***p***
	Folic acid (*n* = 37)	**No folic acid** (*n* = 24)	**Folic acid** (*n* = 45)	**No folic acid** (*n* = 55)	**Folic acid** (*n* = 52)	**No folic acid** (*n* = 48)	
Maternal characteristics							
Maternal age (mean years ± SD)	31.0 ± 4.5	28.8 ± 4.7	29.6 ± 4.6	27.8 ± 5.2	30.2 ± 5.1	29.2 ± 5.3	**N.S.**
Pre-pregnancy BMI (mean BMI ± SD)	27.0 ± 5.9	26.8 ± 5.1 *3 NA*	25.2 ± 4.4 *1 NA*	24.9 ± 5.3 *3 NA*	23.2 ± 3.7 *1 NA*	24.1 ± 3.6 *2 NA*	a,b
Primiparous (yes; *n* (%))	10 (27.0)	8 (33.3)	27 (60.0)	22 (40)	27 (51.9)	21 (43.8)	c,d
Married or cohabiting (yes; *n* (%))	16 (43.2)	13 (54.2)	16 (35.6)	23 (41.8)	27 (51.9)	21 (43.8)	**N.S.**
Maternal education University/college (*n* (%)) High school or lower (*n* (%))	 13 (35.1) 24 (64.9)	 12 (50.0) 11 (45.8) *1 NA*	 24 (53.3) 19 (42.2) *2 NA*	 17 (30.9) 37 (67.3) *1 NA*	 35 (67.3) 15 (28.8) *2 NA*	 23 (47.9) 21 (43.8) *4 NA*	e
Smoking in pregnancy (yes; *n* (%))	6 (16.2)	6 (25.0)	2 (4.4)	11 (20.0)	1 (1.9)	6 (12.5)	f
Alcohol in pregnancy (yes; *n* (%))	8 (21.6)	3 (12.5) *1 NA*	9 (20.0)	16 (29.1) *1 NA*	10 (19.2)	16 (33.3) *1 NA*	**N.S.**
Multivitamins in pregnancy (yes; *n* (%))	18 (48.6)	5 (20.8)	25 (55.6)	9 (16.4)	36 (69.2)	9 (18.8)	**N.S.**
Maternal medications*							
Days of paracetamol (median days; 1st–3rd quartile)	30.0 (25.0–60.0)	27.5 (21.0–37.0)	---	---	---	---	**---**
Psychotropics** (yes; *n* (%))	8 (21.6)	3 (12.5)	5 (11.1)	4 (7.3)	0 (0)	3 (6.3)	g
NSAIDs (yes; *n* (%))	13 (35.1)	9 (37.5)	1 (2.2)	9 (16.4)	4 (7.7)	3 (6.3)	h,i
Other analgesics*** (yes; *n* (%))	7 (18.9)	10 (41.7)	2 (4.4)	1 (1.8)	2 (3.9)	3 (6.3)	j,k
Maternal morbidities							
Comorbidity index**** (mean score ± SD)	0.8 ± 1.6	0.9 ± 1.0 *3 NA*	0.6 ± 1.2 *1 NA*	0.6 ± 1.1 *2 NA*	0.4 ± 1.0 *3 NA*	0.5 ± 1.0 *1 NA*	**N.S.**
Chronic diseases***** None (*n* (%)) 1–2 diseases (*n* (%)) ≥3 diseases (*n* (%))	 32 (86.5) 5 (13.5) 0 (0)	 22 (91.7) 2 (8.3) 0 (0)	 43 (95.6) 2 (4.4) 0 (0)	 49 (89.1) 6 (10.9) 0 (0)	 52 (100) 0 (0) 0 (0)	 45 (93.8) 3 (6.2) 0 (0)	l
Conception and birth							
***In vitro* fertilisation** (yes; *n* (%))	0 (0)	0 (0)	3 (6.7)	0 (0)	2 (3.8)	1 (2.1)	**N.S.**
Caesarean section (yes; *n* (%))	2 (5.4)	5 (20.8)	6 (13.3)	9 (16.4)	7 (13.5)	5 (10.4)	**N.S.**
Child characteristics							
Gestational age at birth (mean weeks ± SD)	39.6 ± 1.4 *1 NA*	39.2 ± 1.4	39.1 ± 1.9	39.5 ± 1.6	39.7 ± 1.7	39.5 ± 1.6	**N.S.**
Infant sex (female; *n* (%))	14 (37.8)	11 (45.8)	14 (31.1)	12 (21.8)	25 (48.1)	19 (39.6)	**N.S.**
Birth weight (mean grams ± SD)	3,601 ± 456	3,564 ± 376	3,468 ± 523	3,672 ± 540	3,603 ± 463	3,495 ± 583	**N.S.**

Technical covariates

Bisulphite conversion							
Plate 1 (*n* (%))	16 (0.43)	11 (0.46)	16 (0.36)	14 (0.26)	20 (0.39)	19 (0.40)	
Plate 2 (*n* (%))	12 (0.32)	9 (0.38)	20 (0.44)	28 (0.51)	14 (0.27)	13 (0.27)	**N.S.**
Plate 3 (*n* (%))	9 (0.24)	4 (0.17)	9 (0.20)	13 (0.24)	18 (0.35)	16 (0.33)	

Abbreviations: ADHD: attention-deficit/hyperactivity disorder; BMI: body mass index; NA: missing value; N.S.: not significant; SD: standard deviation.

* Within-group percentages may add up to more than 100% as one woman may have used medications from several medication groups.
** Includes all medications with the N02 Anatomical Therapeutic Chemical (ATC) code except paracetamol (i.e., opioids, antimigraine preparations, and other analgesics and antipyretics)
*** Includes all antidepressants, antiepileptics and antipsychotics
**** Includes all variables available in MBRN and MoBa from a list provided in Bateman *et al.* (2013) [26, 27]. The different variables are given different weights (weight in parentheses).The variables included in the final score are: asthma (1), cardiovascular disease (3), chronic renal disease (1), congenital heart disease (4), illicit substance use (2), gestational hypertension (1), mild-unspecified preeclampsia (2), severe preeclampsia (5), placenta previa (2), pre-existing diabetes mellitus (1), pre-existing hypertension (1), previous Caesarean delivery (1), lupus (2), alcohol abuse (weekly consumption; 1) and maternal age group (> 44 years: 3; 40–45 years: 2; 35–40 years: 1; < 35 years: 0).
***** Chronic diseases included were asthma, rheumatoid arthritis, epilepsy, Crohn's disease, lupus, multiple schlerosis (MS), cancer and diabetes mellitus. All diseases were weighted equally and each additional disease added 1 to the final score.

[a] $p \approx 0.04$, comparing exposed to ADHD-controls
[b] $p < 0.0001$, comparing exposed to population controls
[c] $p \approx 0.02$, comparing exposed to ADHD-controls
[d] $p \approx 0.03$, comparing exposed to population controls
[e] $p \approx 0.02$, comparing exposed to population controls
[f] $p \approx 0.03$, comparing exposed to population controls
[g] $p \approx 0.01$, comparing exposed to population controls
[h] $p < 0.0001$, comparing exposed to ADHD-controls
[i] $p < 0.0001$, comparing exposed to population controls
[j] $p < 0.0001$, comparing exposed to ADHD-controls
[k] $p < 0.0001$, comparing exposed to population controls
[l] $p \approx 0.05$, comparing exposed to population controls

Table S2. Overview of the cell type composition of the comparison groups stratified by folic acid status. *P* values for significant differences between the exposed group and the two control groups were calculated using the Wilcoxon rank-sum test and any significant comparisons are detailed in the caption.

	Exposed (n = 61)		ADHD-control (n = 100)		Population control (n = 100)		*p*
	Folic acid (n = 37)	**No folic acid** (n = 24)	**Folic acid** (n = 45)	**No folic acid** (n = 55)	**Folic acid** (n = 52)	**No folic acid** (n = 48)	
B cells; *mean proportion ± s.d.*	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	**N.S.**
$CD4^+$ T cells; *mean proportion ± s.d.*	0.14 ± 0.06	0.16 ± 0.05	0.15 ± 0.06	0.15 ± 0.06	0.14 ± 0.05	0.14 ± 0.06	**N.S.**
$CD8^+$ T cells; *mean proportion ± s.d.*	0.04 ± 0.03	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	[a]
Granulocytes; *mean proportion ± s.d.*	0.57 ± 0.10	0.55 ± 0.09	0.57 ± 0.11	0.58 ± 0.09	0.59 ± 0.10	0.58 ± 0.09	**N.S.**
Monocytes; *mean proportion ± s.d.*	0.06 ± 0.04	0.05 ± 0.03	0.05 ± 0.03	0.05 ± 0.03	0.06 ± 0.04	0.05 ± 0.04	**N.S.**
Natural killer cells; *mean proportion ± s.d.*	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.01 ± 0.02	0.02 ± 0.02	0.02 ± 0.03	**N.S.**
nRBCs; *mean proportion ± s.d.*	0.12 ± 0.10	0.14 ± 0.10	0.14 ± 0.12	0.12 ± 0.10	0.12 ± 0.08	0.11 ± 0.09	**N.S.**

Abbreviations: ADHD: attention-deficit hyperactivity disorder; nRBC: nucleated red blood cell; N.S.: not significant.

[a] $p \approx 0.03$, exposed to population controls

PAPER III

Low reliability of DNA methylation across Illumina Infinium platforms in cord blood: implications for replication studies and meta-analyses of prenatal exposures

Olstad EW, Nordeng HME, Sandve GK, Lyle R, Gervin K

Clinical Epigenetics. 2022 Jun 28;14(1):80.
doi: 10.1186/s13148-022-01299-3.

III

Olstad *et al. Clinical Epigenetics* (2022) 14:80
https://doi.org/10.1186/s13148-022-01299-3

Clinical Epigenetics

RESEARCH

Open Access

Low reliability of DNA methylation across Illumina Infinium platforms in cord blood: implications for replication studies and meta-analyses of prenatal exposures

Emilie Willoch Olstad[1,2*], Hedvig Marie Egeland Nordeng[1,2,3], Geir Kjetil Sandve[2,4], Robert Lyle[2,5,6] and Kristina Gervin[1,2,7]

Abstract

Background: There is an increasing interest in the role of epigenetics in epidemiology, but the emerging research field faces several critical biological and technical challenges. In particular, recent studies have shown poor correlation of measured DNA methylation (DNAm) levels within and across Illumina Infinium platforms in various tissues. In this study, we have investigated concordance between 450 k and EPIC Infinium platforms in cord blood. We could not replicate our previous findings on the association of prenatal paracetamol exposure with cord blood DNAm, which prompted an investigation of cross-platform DNAm differences.

Results: This study is based on two DNAm data sets from cord blood samples selected from the Norwegian Mother, Father and Child Cohort Study (MoBa). DNAm of one data set was measured using the 450 k platform and the other data set was measured using the EPIC platform. Initial analyses of the EPIC data could not replicate any of our previous significant findings in the 450 k data on associations between prenatal paracetamol exposure and cord blood DNAm. A subset of the samples ($n = 17$) was included in both data sets, which enabled analyses of technical sources potentially contributing to the negative replication. Analyses of these 17 samples with repeated measurements revealed high per-sample correlations ($\overline{R} \approx 0.99$), but low per-CpG correlations ($\overline{R} \approx 0.24$) between the platforms. 1.7% of the CpGs exhibited a mean DNAm difference across platforms > 0.1. Furthermore, only 26.7% of the CpGs exhibited a moderate or better cross-platform reliability (intra-class correlation coefficient ≥ 0.5).

Conclusion: The observations of low cross-platform probe correlation and reliability corroborate previous reports in other tissues. Our study cannot determine the origin of the differences between platforms. Nevertheless, it emulates the setting in studies using data from multiple Infinium platforms, often analysed several years apart. Therefore, the findings may have important implications for future epigenome-wide association studies (EWASs), in replication, meta-analyses and longitudinal studies. Cognisance and transparency of the challenges related to cross-platform studies may enhance the interpretation, replicability and validity of EWAS results both in cord blood and other tissues, ultimately improving the clinical relevance of epigenetic epidemiology.

*Correspondence: e.w.olstad@farmasi.uio.no

[1] Pharmacoepidemiology and Drug Safety Research Group, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway
Full list of author information is available at the end of the article

© The Author(s) 2022. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

Keywords: Epigenetic epidemiology, Epigenetics, EWAS, MoBa, MBRN, Validity, Replication, Reliability, Illumina Infinium platforms, Microarrays

Background

Epigenetics entails modifications of the DNA that can impact gene expression, but does not involve changes in the underlying DNA sequence. The most commonly studied epigenetic modification is DNA methylation (DNAm), which occurs at cytosine bases of cytosine–phosphate–guanine dinucleotide sites (CpGs). DNAm can be impacted by the DNA sequence, as well as environmental influences [1–4]. There is an increasing interest in the role of epigenetics within epidemiology. Several pharmacoepidemiological studies have reported an association between prenatal psychotropic or analgesic medication exposure and neurodevelopmental outcomes in the offspring [5–13]. Furthermore, multiple epigenome-wide association studies (EWASs) have identified DNAm changes associated with medication exposure during pregnancy (e.g. valproic acid, antidepressants and paracetamol) [14–20]. Recently, we found an association between cord blood DNAm and prenatal long-term exposure to paracetamol in children with attention-deficit/hyperactivity disorder (ADHD) [21]. These initial findings may suggest that DNAm is involved in the relationship between prenatal medication exposure and adverse neurodevelopmental outcomes [3, 4].

Despite a growing interest in epigenetics, and an increasing number of published EWASs, there are several critical biological and technical challenges in epigenetic epidemiology, which have important implications for the interpretation, validity and clinical translation of the findings [1, 22, 23]. One key challenge is the paucity in the replication of findings. For instance, two systematic literature reviews on the association of offspring epigenetic patterns with medication use [20] and maternal well-being in pregnancy [24] uncovered largely inconsistent findings. These reviews suggest multiple origins of the discrepant results, such as small sample sizes resulting in low statistical power and poor study designs [20, 24]. The majority of EWASs are based on DNAm data generated using the Illumina Infinium HumanMethylation BeadChip platforms, including the 27 k ($n > 27{,}000$ CpGs), 450 k ($n > 450{,}000$ CpGs) and the EPIC arrays ($n > 850{,}000$ CpGs) [25]. Recent studies have elucidated technical aspects related to the Infinium platforms, which have significant influences on the analyses and interpretation of results. These studies have shown significant per-CpG differences and poor per-CpG correlation both within [26–35] and across [31, 32, 36–40] microarray platforms, which challenges combined analyses of DNAm data from both platforms (e.g. [41–45]). In cord blood, the median correlation of individual CpGs across platforms was only 0.24 [37]. Furthermore, 2.4% of the CpGs exhibited a mean difference in measured DNAm level between the platforms ≥ 0.1 [37], on the same order as the low effect sizes often observed within epigenetic epidemiology [1, 22, 46]. Furthermore, only 18.0% of CpGs in adult whole-blood exhibit a moderate or better reliability across platforms (intra-class correlation coefficient [ICC] ≥ 0.5) [31]. The technical aspects contributing to low reliabilities of CpGs may affect the power of EWASs [28, 47]. Consequently, poor concordance of measured DNAm levels across platforms may impact both the replicability and validity of EWAS results.

In an ongoing study, we aim to replicate and expand our previous findings showing associations between long-term prenatal exposure to paracetamol (≥ 20 days) and DNAm in children with ADHD [21]. Analyses of DNAm data generated from a larger number of samples selected from the same cohort using the Infinium EPIC platform find no significant CpGs associated with paracetamol exposure. Accordingly, we fail to replicate any of our previous significant findings [21]. Examining a subset of samples with repeated measurements in both data sets has enabled a thorough investigation of potential technical origins of the negative replication. Our findings could not explain the failure to replicate our previous results, but are still important for replication EWASs, as well as studies combining DNAm from different Infinium platforms, such as longitudinal studies or meta-analyses.

Results

Lack of replicability may originate from several technical sources

This study is based on a subset of samples ($n = 17$) included in two datasets and consists of repeated measurements using the Infinium 450 k and EPIC platforms. The samples were selected from the Norwegian Mother, Father and Child Cohort Study (MoBa). In the data set assessed on the 450 k platform ($n = 384$ samples), we have previously published associations between prenatal exposure to paracetamol and DNAm differences in children with ADHD [21]. Analysis of the second data set ($n = 261$ samples), which was designed to expand on these findings using the EPIC platform, has failed to replicate our previous findings (data not shown). This prompted a thorough investigation of whether technical aspects of the Infinium platforms could explain the negative replication. Using

a subset of samples with repeated measurements from both studies ($n=17$ samples), we conducted systematic analyses to assess the integrity and reliability of the DNAm data between the Infinium platforms.

The DNAm data separate into clusters explained by microarray platforms

We performed stringent quality control, normalisation and probe filtering procedures of the DNAm data from the two data sets containing the samples with repeated measurements, to minimise technical variation related to pre-processing of the data. First, we examined DNAm data measured for a set of genotyping probes on each platform ($n=59$ probes). Clustered heatmaps of DNAm values at these genotyping probes showed that the repeated cross-platform measurements of each sample grouped together and hence, excluded potential mix-up of samples (Additional file 1: Fig. S1). Second, we examined whether pre-processing steps such as background and probe-type correction impacted the cross-platform concordance. To do this, we used the intra-class correlation coefficient (ICC), which equals 1 if there is perfect per-CpG concordance between the measured DNAm in the 450 k and EPIC data sets. Generally, an ICC < 0.5 is considered poor [48, 49]. We computed the ICCs after pre-processing the 450 k and EPIC data sets separately, using the default settings of five commonly used pre-processing pipelines *ChAMP* [50, 51], *ENmix* [34], *minfi* [52], *RnBeads* [53] and *wateRmelon* [54] (Additional File 1: Table S1). We also included one pipeline commonly reported in the literature, namely *RnBeads* with the background and probe-type corrections *ENmix.oob* [34] and BMIQ [55], respectively. This analysis revealed that the *ENmix* pipeline exhibited larger ICCs than the other pipelines (Fig. 1). Therefore, we performed the rest of the analyses on data sets normalised using the default settings of the *ENmix* pipeline.

Next, we performed principal component analysis (PCA) to explore technical variation in the DNAm data related to the 450 k and EPIC platforms. As expected, PCA revealed distinct clustering of samples corresponding to the 450 k and EPIC platforms (Fig. 2). Similar plots were observed when pooling all the available 450 k and EPIC samples ($n=628$ samples; data not shown).

DNAm levels differ between the 450 k and EPIC platforms

To further investigate the dissimilarities between the 450 k and EPIC platforms, we computed the difference in and correlation of DNAm at overlapping CpGs on the two platforms ($n=397{,}813$ CpGs). These analyses

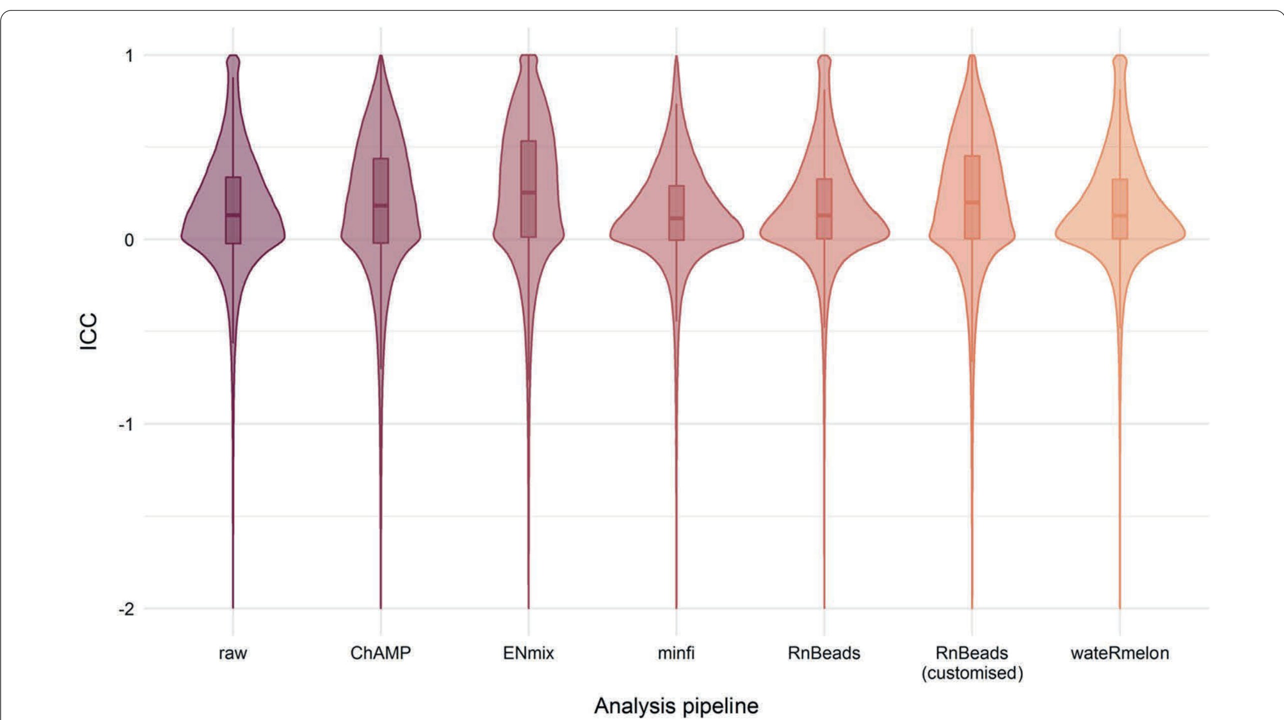


Fig. 1 Overview of the ICC distribution computed from raw data and from data pre-processed using the default settings of five common EWAS analysis pipelines. Additionally, we included one common analysis pipeline ("RnBeads (customised)", including the normalisation methods *ENmix.oob* and *BMIQ*). All pipelines examined also exhibited ICCs lower than −2, but these were removed from the illustration for visualisation purposes. The default settings of each analysis pipeline are detailed in Additional file 1: Table S1

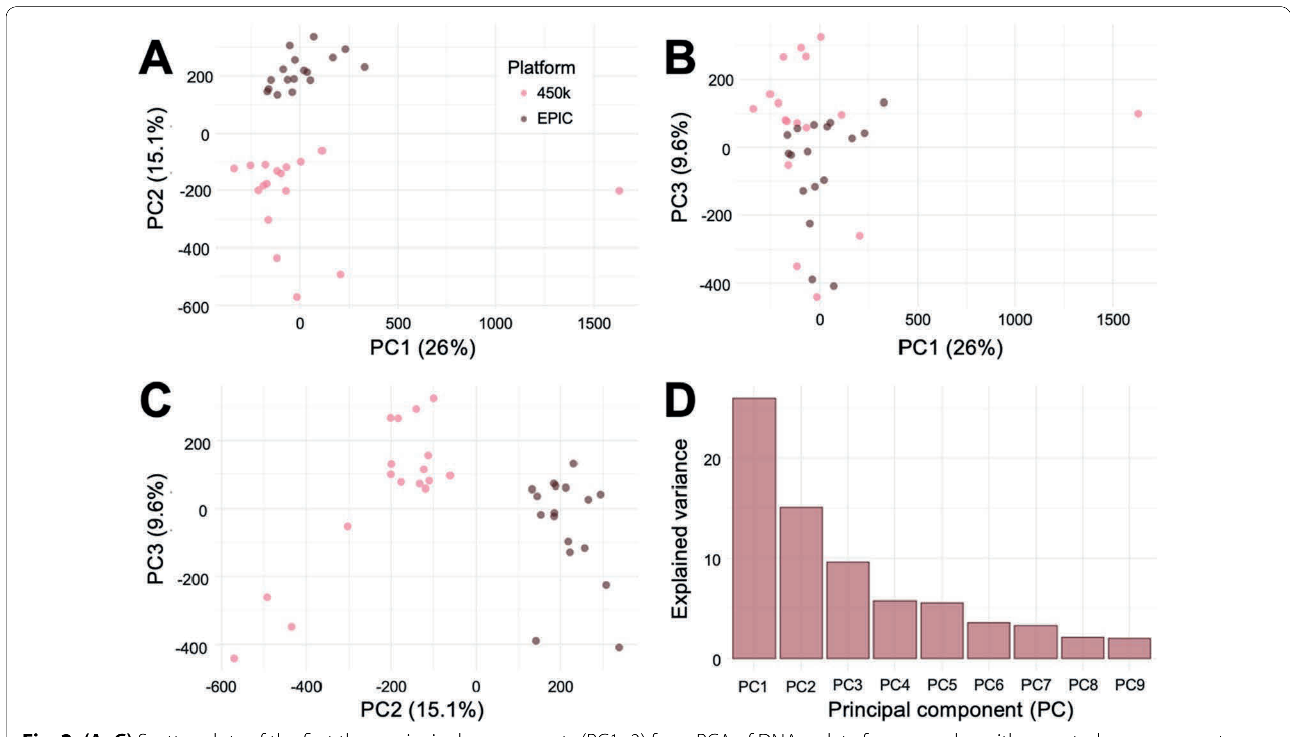


Fig. 2 **(A–C)** Scatter plots of the first three principal components (PC1–3) from PCA of DNAm data from samples with repeated measurements ($n=17$ samples) using the 450 k and EPIC platforms, and **(D)** a scree plot showing the amount of variance explained by the first nine PCs

revealed small per-sample absolute differences in DNAm at overlapping CpGs between the two arrays (median ≈0.008 and mean ≈0.017 absolute DNAm differences). For 0.1% ($n=454$) of CpGs, the mean DNAm difference over all replicates was > 0.25, while 0.007% ($n=27$) of CpGs exhibited a mean DNAm difference > 0.5 (Fig. 3). These numbers are largely in line with previous studies, comparing differences in measured DNAm between the 450 k and EPIC arrays in cord blood [37], whole-blood [31, 32, 36, 37], placenta [38] and cartilage [39]. Furthermore, of the 27 CpGs with an absolute mean DNAm difference > 0.5, 5 of these CpGs also exhibited absolute mean DNAm difference > 0.5 in both cord blood [37], whole-blood [37], placenta [38] and cartilage [39] (Additional file 1: Fig. S2).

We observed a high per-sample correlation of DNAm between the platforms, both when comparing replicates, and when comparing two independent samples across the platforms (Fig. 4A). The median per-sample Pearson's correlation coefficient was 0.996 and the mean was 0.992, with the lowest correlation between any two samples being 0.969 and the highest being 0.998. In contrast, the per-CpG correlations of measured DNAm between the platforms were significantly lower: the median correlation was 0.237 and the mean was 0.238, with the lowest correlation being -0.822 and the highest being 1.00 (Fig. 4B). The per-CpG correlation appeared to be related to the variance of each CpG, which were similar for both platforms; CpGs with high correlation also exhibited larger variance (Fig. 4B). The high per-sample correlation, low per-CpG correlation, and the relationship between CpG variance and correlation, have previously been reported for cord blood [37], and multiple other tissues [31, 32, 36–39].

Few CpGs are reliable between the 450 k and EPIC platforms

In order to examine concordance of cross-platform DNAm levels, we assessed the reliability of the CpGs, reflecting both correlation and agreement. To do this, we computed the ICC, as previously suggested by Sugden et al. (2020) comparing cross-platform DNAm levels in adult whole-blood [31]. Overall, the ICCs of the overlapping CpGs were poor (median = 0.246 and mean = 0.230; Fig. 5A). Approximately 26.7% ($n=106{,}078$) of the CpGs exhibited an ICC ≥ 0.5. This is similar to the findings of the recent study by Sugden et al. in adult whole-blood, where 18.0% of CpGs exhibited an ICC ≥ 0.5 [31]. Approximately 38.6% ($n=40{,}916$) of the CpGs with an ICC ≥ 0.5 in the current study overlapped with the CpGs with an ICC ≥ 0.5 reported by Sugden et al. [31] (Additional File 2). The microarray type II probes exhibited slightly better ICCs and correlation coefficients than type

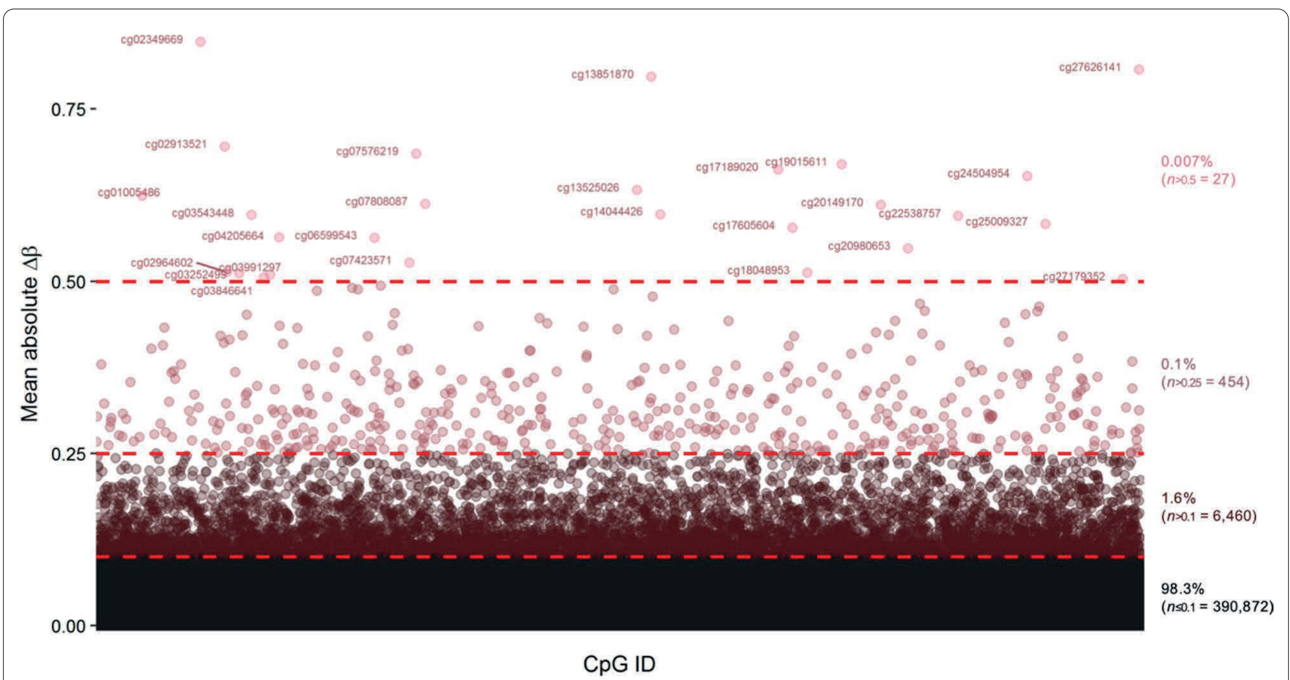


Fig. 3 Mean absolute difference in measured DNA methylation ($\Delta\beta$) per CpG, on the 450 k and EPIC platforms. Red dotted lines indicate a mean $\Delta\beta > 0.1$, > 0.25 and > 0.5. Illumina CpG IDs are named if the mean $\Delta\beta > 0.5$

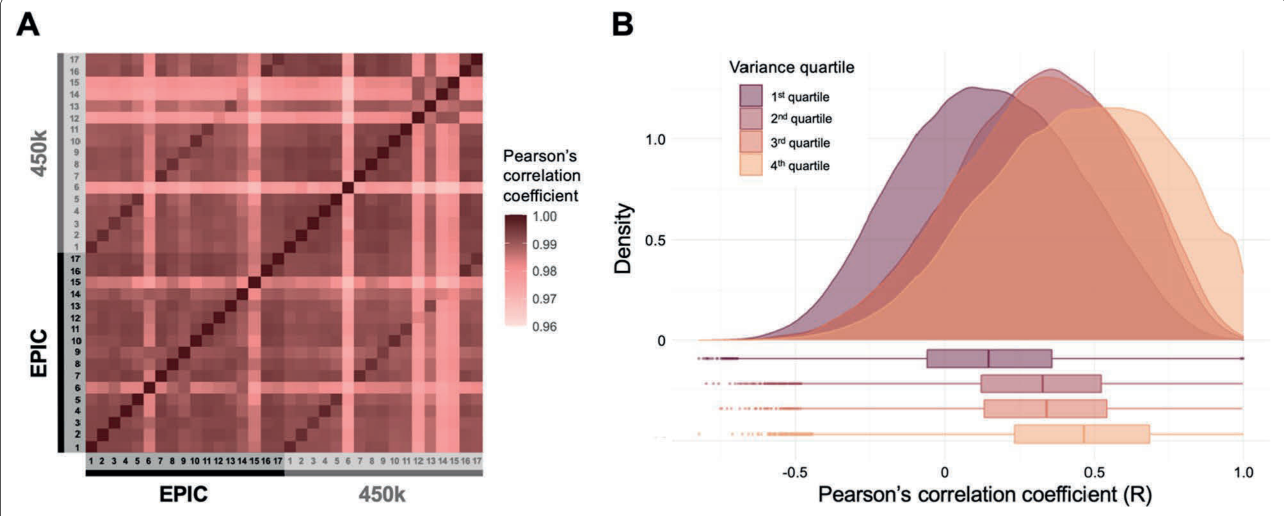


Fig. 4 Pearson's correlation coefficients of DNAm in replicates of the 450 k and EPIC platforms, for **(A)** per-sample correlations in a correlogram, and **(B)** per-CpG correlations as distributions stratified by variance quartiles, based on the variance of the respective CpGs on the EPIC platform

I probes (Additional File 1: Fig. S3). Probes with poor ICCs and correlation coefficients appear more frequently in CpG islands (Additional File 1: Figs. S4 and S5), possibly due to an increased proportion of largely unmethylated CpGs in these regions (Additional File 1: Fig. S6).

Considering the poor CpG reliabilities, we investigated if the ICCs of the repeated measurements were higher than expected for two randomly paired samples. Therefore, we paired each EPIC sample with a randomly selected 450 k sample. The distribution of ICCs computed from the 17 repeated measurements (Fig. 5A) is significantly different from the ICC distributions computed from the 17 random 450 k-EPIC pairs (Kolmogorov–Smirnov test: $p < 2.2*10^{-16}$; Additional file 1: Fig. S7).

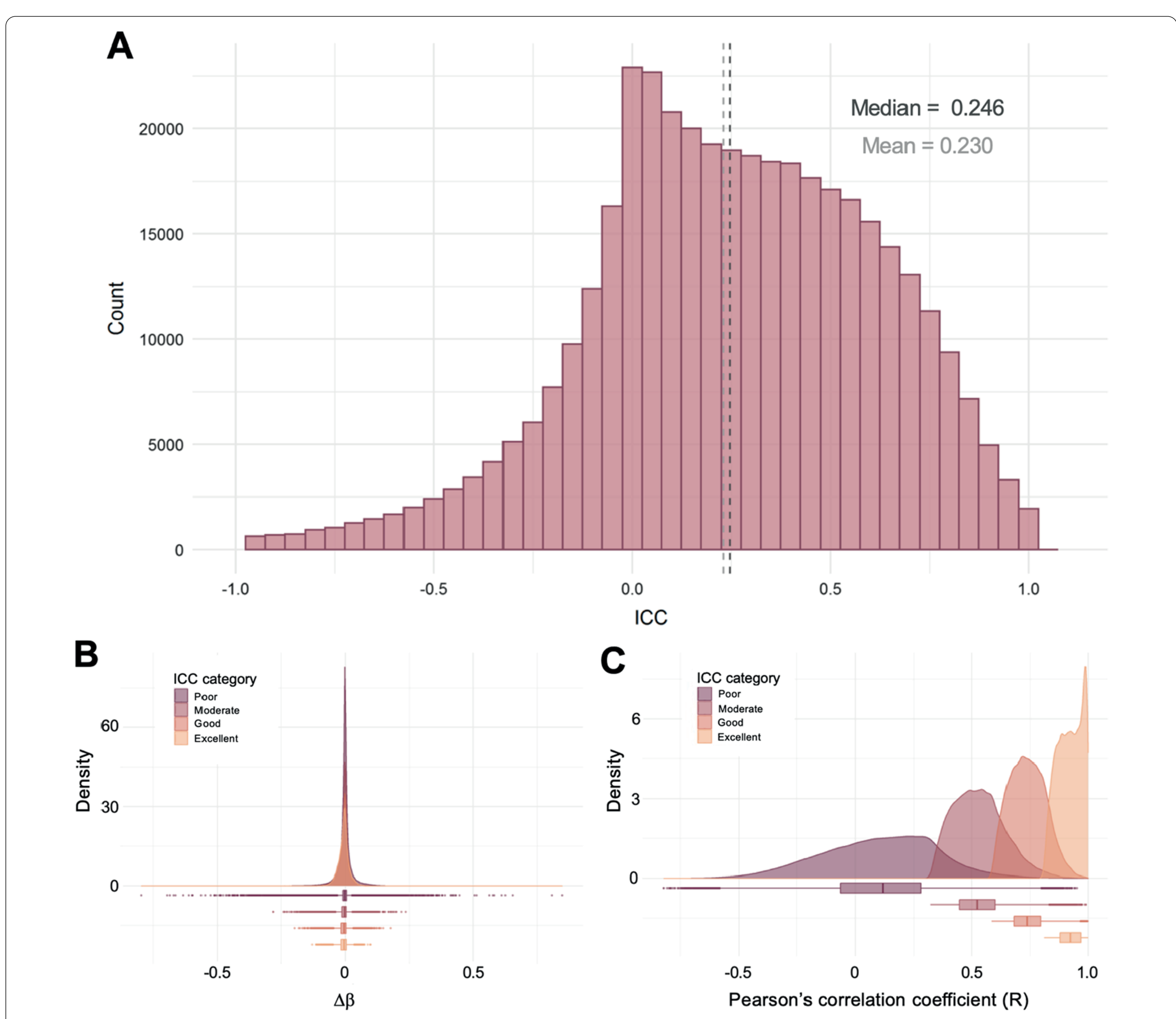


Fig. 5 **(A)** Histogram of the ICCs computed from the 17 samples assessed on both the 450 k and EPIC platforms. **(B)** Density distribution of mean difference in DNAm level, stratified by ICC category. **(C)** Density distribution of Pearson's correlation coefficient, stratified by ICC category. The ICC categories are defined as follows: poor: ICC < 0.5; moderate: 0.5 ≤ ICC < 0.75; good: 0.75 ≤ ICC < 0.9; and excellent: ICC ≥ 0.9. The dark grey, dotted line indicates the median ICC, and the light grey, dotted line indicates the mean ICC. Outlying CpGs with ICCs less than the mean ICC minus three standard deviations were removed for visualisation purposes, but were included for summary statistic calculations

Furthermore, only a small percentage of the CpGs of the random pairs (2.4%–4.8%) exhibited an ICC ≥ 0.5, which are significantly different proportions from the ICCs of the repeated measurements (Pearson's Chi-squared test: $p < 2.2*10^{-16}$).

The ICC reflects both correlation and agreement across microarray platforms

To investigate if the ICCs reflect both agreement and correlation across platforms, we examined the distribution of mean differences in DNAm and Pearson's correlation coefficients, for each of four ICC categories: poor (ICC < 0.5), moderate (0.5 ≤ ICC < 0.75), good (0.75 ≤ ICC < 0.9) and excellent (ICC ≥ 0.9) [48]. The distribution of mean differences in DNAm is relatively similar between the ICC categories. However, there are far more of the poor CpGs displaying large differences in mean DNAm levels across platforms compared to the other ICC categories (Fig. 5B). In contrast, the correlation coefficient increases with improving ICC category; the poor ICC category exhibits a wide range of low correlation coefficients (median ≈ 0.12), while the distribution of the correlation in the excellent category is highly skewed to the right (median ≈ 0.92). The moderate and

good categories exhibit a wider range of correlation coefficients than the excellent CpGs, with a median of 0.52 and 0.74, respectively (Fig. 5C).

These observations demonstrate that the reliability of each CpG depends on both the correlation and the agreement between the two platforms [48]. An excellent CpG will have both a low mean difference in DNAm between platforms and a high correlation, explaining the small range in values of both the mean DNAm differences and the correlation coefficients. In contrast, a poor probe (including a larger range of ICCs) may exhibit an acceptable correlation but have a large mean DNAm difference (Additional file 1: Fig. S8). For instance, 685 of the 5407 CpGs with an $R \geq 0.9$ nevertheless have an $ICC \leq 0.9$, with 22 CpGs even having a poor ICC (< 0.5). Furthermore, of the 395,286 CpGs with a mean DNAm difference ≤ 0.1, 289,327 exhibit a poor ICC (< 0.5). This is likely due to low correlations, as the median R for these CpGs is 0.12, while the median R was 0.59 for the 105,959 CpGs with a mean DNAm difference ≤ 0.1 and an $ICC \geq 0.5$. Hence, the ICC better reflects reliability across platforms than either accuracy or correlation on their own.

The significant CpGs in the 450 k data have low reliabilities

We then asked if our failure to replicate the findings in our original study [21] could be explained by poor-performing probes, by examining the ICCs of the significant CpGs from the 450 k data set. The significant CpGs for the three group comparisons performed in the original study have median ICCs of 0.119, 0.122 and 0.135 (Additional file 1: Fig. S9). These reliabilities are low compared to the overall mean and median of the ICCs including all common CpGs across platforms.

Discussion

Replication of association studies is important to ensure robust and valid findings. In an ongoing study, we aimed to replicate and expand on findings in our previous study, where we found an association between long-term prenatal paracetamol exposure and differences in DNAm in children with ADHD, using the Infinium 450 k platform [21]. Surprisingly, analyses of the follow-up data consisting of a larger sample and use of the Infinium EPIC platform have not replicated the results from our original study. Indeed, a challenge of EWASs is to discern spurious findings from true positives, rendering the replication of significant associations challenging [1, 22, 23]. Recent studies have shown low concordance across 450 k and EPIC platforms in different tissues [31, 32, 36–40]. Therefore, we have conducted a systematic evaluation of technical aspects related to concordance of DNAm data across the Infinium platforms in our studies in cord blood, by using data from a subset of samples with repeated measurements from the 450 k and EPIC platforms.

Technical variation such as batch effects is systematic variation caused by, for example, processing by different technicians, varying reagent batches and differences in the scanner performance. PCA of DNAm data from the samples with repeated measurements demonstrated distinct clustering of samples corresponding to the platform. If these differences in DNAm were independent of the platform and resulted entirely from positioning on the beadchip or bisulphite conversion plate, we would expect the changes to be relative and to not impact the replicability. Considering the general challenge of replication of EWASs [1, 22, 23] and the low per-CpG concordance across platforms reported in several recent studies [31, 32, 36–40], we were encouraged to examine possible cross-platform differences in DNAm. Corroborating previous studies, we observed a high per-sample correlation even between the randomly paired samples [32, 36–40]. In contrast, the per-CpG correlation was significantly lower, and some probes exhibited large differences in mean measured DNAm for overlapping CpGs on the two platforms.

Considering the highly concerning findings by Sugden et al. [31], reporting low reliabilities (measured by ICCs) for most CpGs across the 450 k and EPIC platforms in adult whole-blood, we estimated the ICCs of each CpG across the two platforms in our cord blood samples. Ideally, the ICC will approach 1 if the between-sample variation is much larger than the within-sample variation, suggesting larger biological variation than technical variation. However, most CpGs in our study exhibited poor reliabilities (ICC < 0.5) [31, 48] and we found that only 26.7% of CpGs in cord blood had an acceptable reliability across platforms. Interestingly, 38.6% of these CpGs overlapped with the 18.0% reliable CpGs identified in adult whole-blood [31]. This may suggest that some probes are generally unreliable in different tissues, possibly due to cell-type specific variability in DNAm. In contrast, other CpGs may perform worse in specific tissues, similar to what has been suggested for both per-CpG correlations and differences in DNAm between platforms [37–39]. In future studies, it would be interesting to examine the ICCs between Infinium platforms and other DNAm measuring technologies, such as whole-genome bisulphite sequencing (WGBS) or methylated immunoprecipitation (MeDIP).

We observed a substantial difference in the distribution of ICCs for different pre-processing steps used in common analysis pipelines. The *ENmix* pipeline exhibited the largest median ICC, suggesting that this pipeline may be better to best conserve the similarity of normalised

repeated measurements from different platforms. In contrast, both the default *RnBeads*, *minfi* and *wateRmelon* pipelines have no better ICC distributions than the raw data. Notably, compared to a recent study reporting the ICC distribution of multiple different pipelines for within-platform repeated measurements [35], the distribution of cross-platform ICCs varies more dependent on the analysis pipeline used. However, the analysis pipeline with the highest median ICC is *ENmix* for both cross-platform and within-platform comparisons [35].

Interestingly, some studies have reported that cross-platform differences in DNAm and poor per-CpG correlations do not substantially impact the outcome of EWASs [32, 37]. However, when investigating the relationship of ICCs with the likelihood of replication of CpGs, Sugden et al. observed a positive relationship between increasing ICC and increasing replication rate for the association of DNAm with smoking [31]. Similar associations of ICCs with replicability have been found when ICCs were estimated from 450 k-450 k replicates [26, 49]. For instance, smoking-DNAm associations in whole-blood are highly replicable [56], and in one study, 96% of CpGs associated with smoking exhibit high reliability [26]. Additionally, poor ICCs have been shown to decrease the power of individual CpGs in EWASs, i.e. reducing the positive predictive value (PPV) by decreasing the number of true positives [28, 31, 47]. The median ICC of the significant CpGs in our original study was poor. However, if these findings were explained by the low reliability of the probes, we would expect none or very few significant CpGs. Consequently, based on the calculated ICCs using our 17 samples with repeated measurements, we have no explanation for the lack of replicability of our original findings.

A limitation of the present study is the small sample size used to assess the ICCs. However, ICC calculations generally require relatively small sample sizes [47, 57], and Sugden et al. found that sample sizes as small as 25 would be sufficient to detect 80% of all CpGs with an ICC $\geq$ 0.75 [31]. Furthermore, our results on both per-CpG correlations, differences in mean DNAm and ICCs are in line with other studies reporting one or more of these measurements for various tissues [31, 32, 36–40]. Nevertheless, a study including a larger number of repeated measurements in cord blood across the 450 k and EPIC platforms should be performed to strengthen our findings. Another limitation of our study is our inability to assess which technical variable(s) associated with the platform are contributing to the differences between platforms. Firstly, the DNAm on the 450 k and EPIC platforms was measured three years apart. Yet, this largely emulates the setting in most studies relying on data processed at different times and in different facilities (e.g., longitudinal studies and meta-analyses). Furthermore, all samples included in the current study were processed in the same core facility and by the same technician. Secondly, batches of bisulphite conversion reagents and scanners may also contribute to the cross-platform differences. Nevertheless, we expect that such technical variation is relative within the platforms and, consequently, that probes are mainly affected equally within the platform. Finally, it is challenging to assess the potential contribution of sample plate and beadchip to cross-platform differences, due to the different platform layouts (the 450 k beadchip can load 12 samples, while the EPIC beadchip can load 8 samples). To limit the contribution of variation from sample plate and beadchip in our data, the samples were randomly positioned on plates and beadchips. Accordingly, technical variation contributed by these variables should be random and should not inflict much bias when comparing DNAm between platforms.

The substantial differences across platforms revealed in this and previous studies [31, 32, 36–40] are troubling when trying to replicate findings using a different platform than in the original study. Replication of findings has long been considered a major challenge within epigenetic epidemiology [1, 22, 23], and to our knowledge, only one study has highlighted the potential impact of unreliable CpGs for replication of findings using data from different microarray platforms [31]. Challenges associated with differences in mean DNAm levels across platforms are not necessarily limited to issues of replication. For instance, longitudinal studies based on DNAm measured at multiple timepoints may suffer under the development of new microarray technologies (e.g., [41, 42]). Furthermore, this is also relevant for large meta-analyses combining data from multiple cohorts to increase the power of EWASs (e.g. [43, 44]), often based on large consortia such as the Pregnancy And Childhood Epigenetics (PACE) consortium [45]. Such strategies may be impacted by unreliable probes when combining data sets from different platforms. Similarly, unreliable CpGs across platforms may have implications for current EWAS knowledgebases, such as the EWAS Atlas [58] and the EWAS catalogue [59], which curate EWAS publications to report DNAm-trait associations.

Conclusion

In conclusion, our failure to replicate significant CpGs associated with prenatal paracetamol exposure prompted a thorough investigation of potential technical origins of our null findings. The observation of low cross-platform per-CpG correlation and reliability corroborates previous reports. However, the low-reliability probes could not explain the inability to replicate previous findings in our case. Nevertheless, the poor cross-platform reliabilities

may have important implications for future EWASs, in replication, meta-analyses and longitudinal studies. Therefore, we encourage researchers performing EWASs to examine the reliability of probes within and across tissues and to establish which probes are most comparable across microarray platforms. However, in many cases, the availability of repeated measurements from individual samples may be limited for reasons such as extra cost and limited availability of sample material. To this end, we encourage joint efforts to more extensively outline reliable probes in different tissues. If such investigations reveal common poor-performing probes across or within tissues, other studies may rely on this information when performing cross-platform studies. We hope our findings, supporting the results by Sugden et al. [31], increase awareness of possible challenges in including both 450 k and EPIC data in the same study. Cognisance and transparency of these challenges as well as appropriate precautions when performing cross-platform epigenetic investigations, may enhance the interpretation, replicability and validity of results, and could ultimately improve the clinical relevance of epigenetic epidemiology.

Methods

Sample population

We analysed cord blood samples from the Mother, Father and Child Cohort Study (MoBa). MoBa is a population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health (NIPH) [60–63]. Participants were recruited from all over Norway from 1999–2008 [60, 61]. The women consented to participation in 41% of the pregnancies [60, 61]. The cohort includes approximately 114,500 children, 95,200 mothers and 75,200 fathers [60, 61]. The current study is based on Data Version 8 of the quality-assured data files released for research in 2015. Observational data from MoBa questionnaires Q1 (gestational week 0–13), Q3 (gestational week 13–29) and Q4 (gestational week 30 to delivery) were used to select individuals for the study. The personal, 11-digit identification number, unique to every permanent resident of Norway, was used to link MoBa to the Norwegian Patient Registry (NPR) and the Medical Birth Registry of Norway (MBRN). MBRN is a national health registry containing information about all births in Norway. We also analysed umbilical cord blood samples retrieved from the MoBa biobank [62, 63]. The biobank stores blood samples obtained from both parents during pregnancy, and from mothers and children (umbilical cord) at birth [62, 63].

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 (REC). MoBa is currently regulated by the Norwegian Health Registry Act. All MoBa participants have given their written informed consent to participate in the cohort study. The current study has been approved by REC South East Norway (REC reference: 23,136, 2014/163). All data are de-identified, and the linkage between MoBa and the different health registries was handled by NIPH along with the relevant registries.

Study design and measurements

The MoBa biobank contains 90,000 cord blood samples drawn at birth [63]. In our original study using the 450 k platform, we selected 384 samples from the biobank, and in the study using the EPIC platform, we selected 261 samples. Out of these samples, 611 samples were unique to either the 450 k data set or the EPIC data set, and 17 samples were measured on both the 450 k and EPIC platforms. The samples were selected based on prenatal exposure to paracetamol and child ADHD diagnosis, and all samples were term births (≥ 37 weeks). The 17 samples available in both data sets were all prenatally long-term exposed to paracetamol and had received an ADHD diagnosis.

Long-term prenatal exposure to paracetamol (Anatomical Therapeutic Chemical [ATC] code: N02BE01) was defined as the use of paracetamol for ≥ 20 days during pregnancy (coded as “yes” or “no”), based on a threshold from previous studies [64–68]. Use was self-reported and collected from three MoBa questionnaires (Q1, Q3 and Q4). Offspring diagnosis of ADHD was retrieved from the NPR (2008), containing all individual diagnoses asserted by specialists according to the 10th revision of the International Classification of Disease (ICD-10), as reported by governmental hospitals and outpatient clinics. Children were defined as having ADHD if they had received an ICD-10 diagnosis of hyperkinetic disorder (HKD; F90.0, F90.1, F90.8 or F90.9) between 2008 and 2016. HKD corresponds to ADHD in the Diagnostic and Statistical Manual (DSM) system [69–72], as an HKD diagnosis requires both inattentiveness and hyperactivity symptoms.

DNA methylation

Generation of DNAm data

The 450 k DNAm data from the samples in our original study are described elsewhere [21]. The samples assessed on the Infinium HumanMethylation EPIC BeadChip (Illumina) were processed similar to the 450 k data set [21]. Samples were randomly allocated to sample plates and beadchips, as previously described [21].

Quality control and pre-processing

Analyses were performed in the R programming language (http://www.r-project.org/). Quality control, normalisation and filtering of the data (Table 1) were performed using the default pipeline of *ENmix* [34]. The EPIC and 450 k data sets were pre-processed separately and all samples were included in the pre-processing ($n_{\mathrm{EPIC}} = 261$; $n_{\mathrm{450k}} = 384$). Subsequently, the 17 samples with repeated, cross-platform measurements were used for further analyses.

First, samples with > 5% low-quality CpGs or low bisulphite intensity were removed (7 samples from the 450 k data set and 0 samples from the EPIC data set). Then, CpGs with > 5% low-quality values were also removed (5598 and 8947 CpGs from the 450 k and EPIC data sets, respectively). Background correction was performed using the *ENmix* exponential-truncated-normal out-of-band (oob) method [34], dye bias correction was executed using RELIC (REgression on Logarithm of Internal Control probes) [73], and probe-type correction was achieved using RCP (Regression of Correlated Probes) [74]. We removed probes with SNPs overlapping with the CpG interrogation site or the nucleotide extension site ($n_{\mathrm{EPIC}} = 29{,}176$; $n_{\mathrm{450k}} = 16{,}803$), cross-reactive probes ($n_{\mathrm{EPIC}} = 14{,}921$; $n_{\mathrm{450k}} = 21{,}563$) [36, 75–77] and probes on the sex chromosomes ($n_{\mathrm{EPIC}} = 17{,}532$; $n_{\mathrm{450k}} = 10{,}012$). These pre-processing steps resulted in a total of 795,515 probes in the EPIC data set and 431,536 probes in the 450 k data set. Of these, 397,813 CpGs overlapped between the two platforms.

Pre-processing using the default settings of common analysis pipelines

The raw data were also pre-processed using the default settings of four other common EWAS analysis pipelines: *ChAMP* [50, 51], *minfi* [52], *RnBeads* [53] and *wateRmelon* [54]. Additionally, we used the default *RnBeads* pipeline [53], but changed the background and probe-type correction methods to *Enmix.oob* [34] and BMIQ [55], respectively. The CpGs were annotated based on ilm10b4.hg19 [78].

Table 1 Overview of retained probes upon filtering of data from the EPIC and 450 k microarray platforms

	EPIC probes	450 k probes
Raw data	866,091	485,512
> 5% low-quality values	857,144	479,914
SNP-enriched probe removal	827,968	463,111
Cross-reactive probe removal	813,047	441,548
Sex chromosome removal	795,515	431,536

Statistical analyses

The β values (the ratio of methylated signal to the sum of methylated and unmethylated signal) were used for visualisations and calculation of all concordance measurements. To test for differences in distributions, we used the Kolmogorov–Smirnov test, and to test for differences in proportions, we used the Pearson's Chi-squared test. To examine the correlations between both samples and CpGs from the different microarrays, we estimated the Pearson's correlation coefficient. The ICC of each CpG was computed using the *irr* package [79]. We estimated the ICC by fitting an absolute agreement and mean of k raters ($k = 2$), two-way random effects model, as has previously been suggested for such comparisons [31]. The visualisation of the overlaps between studies of CpGs with mean DNAm differences > 0.5 across platforms was generated using the *UpSetR* package [80]

Abbreviations

ADHD: Attention deficit/hyperactivity disorder; ATC: Anatomical therapeutic chemical; DSM: Diagnostic and statistical manual; CpG: 5′–Cytosine–phosphate–guanine–3′ site; DNAm: DNA methylation; EWAS: Epigenome-wide association study; FDR: False discovery rate; HKD: Hyperkinetic disorders; ICC: Intra-class correlation coefficient; ICD-10: The 10th revision of the International Classification of Disease; MBRN: The Medical Birth Registry of Norway; MoBa: The Norwegian Mother, Father and Child Cohort Study; NIPH: The Norwegian Institute for Public Health; NPR: The Norwegian Patient Registry; PCA: Principal component analysis; PPV: Positive predictive value; REC: The Regional Committees for Medical and Health Research Ethics.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-022-01299-3.

Additional file 1: Fig. S1. Heatmap and clustering of genotyping probes. **Fig. S2.** Overlap of the CpGs exhibiting differences in mean DNAm > 0.5 in five studies. **Fig. S3.** Probe types and density distribution of Pearson's and intra-class correlation coefficients. **Fig. S4.** Annotation groups and relation to Pearson's and intra-class correlation coefficient categories. **Fig. S5.** Histogram of the distribution of the per-CpG island intra-class correlation coefficients. **Fig. S6.** Density plot of DNAm levels stratified by annotation categories. **Fig S7.** Histograms of the distribution of intra-class correlation coefficients for randomly paired samples. **Fig S8.** Scatter plot of the difference in mean DNAm level against the intra-class correlation coefficient. **Fig S9.** ICC distributions for the significant CpGs of our original study. **Table S1.** Overview of common pipelines with default settings for analysing DNA methylation data.

Additional file 2: Table of CpGs with corresponding intra-class correlation coefficients, Pearson's correlation coefficients, mean difference across platforms, and whether the CpG exhibited an ICC ≥ 0.5 both in the current study and in the Sugden et al. study.

Acknowledgements

The Norwegian Mother, Father and Child Cohort Study is supported by the Norwegian Ministry of Health and Care Services and the Ministry of Education and Research. We are grateful to all the participating families in Norway taking part in this ongoing cohort study.

Author contributions

EWO, HMEN and KG conceived the idea of and designed the study. EWO conducted the analyses. EWO generated plots and tables and drafted the first

version of the paper. EWO, HMEN, GKS, RL and KG all revised the paper. All authors read and approved the final manuscript.

Funding
This work was supported by the European Research Council (ERC) Starting Grant "DrugsInPregnancy" (grant number: 678033) and the Research Council of Norway (NFR) "Medications during pregnancy: effects on neurodevelopmental disorders in childhood and epigenetic outcomes" (grant number: 241117). MoBa is supported by the Norwegian Ministry of Health and the Ministry of Education and Research, NIH/NIEHS (contract number: NO1-ES-75558), NIH/NINDS (grant numbers: UO1 NS 047537–01 and UO1 NS047537-06A1) and the Norwegian Research Council/FUGE (grant numbers: 151918/S10).

Availability of data and materials
The data that support the findings of this study are available from the Norwegian Mother, Father and Child Cohort Study, but restrictions apply to the availability of these data and so are not publicly available. However, data are available from the authors upon reasonable request and with permission from the Norwegian Mother, Father and Child Cohort Study.

Declarations

Ethics approval and consent to participate
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 (REC). MoBa is currently regulated by the Norwegian Health Registry Act. All MoBa participants have given their written informed consent to participate in the cohort study. The current study has been approved by REC South East Norway (REC reference: 23136, 2014/163). All data are de-identified, and the linkage between MoBa and the different health registries was handled by NIPH along with the relevant registries.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Pharmacoepidemiology and Drug Safety Research Group, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway. [2]PharmaTox Strategic Research Initiative, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway. [3]Department of Child Health and Development, Norwegian Institute of Public Health, Oslo, Norway. [4]Department of Informatics, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway. [5]Department of Medical Genetics, Oslo University Hospital and University of Oslo, Oslo, Norway. [6]Centre for Fertility and Health, Norwegian Institute of Public Health, Oslo, Norway. [7]Department of Research and Innovation, Division of Clinical Neuroscience, Oslo University Hospital, Oslo, Norway.

Received: 5 March 2022 Accepted: 12 June 2022
Published online: 28 June 2022

References

1. Mill J, Heijmans BT. From promises to practical strategies in epigenetic epidemiology. Nat Rev Genet Nat Rev Genet. 2013;14:585–94.
2. Relton CL, Smith DG. Is epidemiology ready for epigenetics? Int J Epidemiol. 2012;41:5–9.
3. Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD, Hanson MA. Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease. Pediatr Res. 2007;61:5–10.
4. Barouki R, Melén E, Herceg Z, Beckers J, Chen J, Karagas M, et al. Epigenetics as a mechanism linking developmental exposures to long-term toxicity. Environ Int. 2018;114:77–86.
5. Hjorth S, Bromley R, Ystrom E, Lupattelli A, Spigset O, Nordeng H. Use and validity of child neurodevelopment outcome measures in studies on prenatal exposure to psychotropic and analgesic medications—a systematic review. PLoS ONE. 2019;14(7):e0219778. https://doi.org/10.1371/journal.pone.0219778.
6. Kellogg M, Meador KJ. Neurodevelopmental effects of antiepileptic drugs. Neurochem Res Neurochem Res. 2017;42:2065–70.
7. Bromley R, Weston J, Adab N, Greenhalgh J, Sanniti A, McKay AJ, et al. Treatment for epilepsy in pregnancy: neurodevelopmental outcomes in the child. Cochrane Database Syst Rev. 2014. https://doi.org/10.1002/14651858.CD010236.pub2.
8. Bauer AZ, Kriebel D, Herbert MR, Bornehag CG, Swan SH. Prenatal paracetamol exposure and child neurodevelopment: A review. Horm Behav Academic Press. 2018;101:125–47.
9. El Marroun H, White T, Verhulst FC, Tiemeier H. Maternal use of antidepressant or anxiolytic medication during pregnancy and childhood neurodevelopmental outcomes: a systematic review. Eur Child Adolesc Psychiatry. 2014;23:973–92.
10. Prady SL, Hanlon I, Fraser LK, Mikocka-Walus A. A systematic review of maternal antidepressant use in pregnancy and short- and long-term offspring's outcomes. Arch Womens Ment Health. 2018;21:127.
11. Tosato S, Albert U, Tomassi S, Iasevoli F, Carmassi C, Ferrari S, et al. A Systematized review of atypical antipsychotics in pregnant women: balancing between risks of untreated illness and risks of drug-related adverse effects. J Clin Psychiatry J Clin Psychiatry. 2017;78:e477–89.
12. Ornoy A, Weinstein-Fudim L, Ergaz Z. Antidepressants, antipsychotics, and mood stabilizers in pregnancy: what do we know and how should we treat pregnant women with depression. Birth defects Res Birth Defects Res. 2017;109:933–56.
13. Yazdy MM, Desai RJ, Brogly SB. Prenatal exposures and short and long term developmental outcomes: prescription opioids in pregnancy and birth outcomes: a review of the literature. J Pediatr Genet. 2015;4:56.
14. Radhakrishna U, Vishweswaraiah S, Uppala LV, Szymanska M, Macknis J, Kumar S, et al. Placental DNA methylation profiles in opioid-exposed pregnancies and associations with the neonatal opioid withdrawal syndrome. Genomics. 2021;113:1127–35.
15. Kallak TK, Bränn E, Fransson E, Johansson Å, Lager S, Comasco E, et al. DNA methylation in cord blood in association with prenatal depressive symptoms. Clin Epigenetics BioMed Central Ltd. 2021;13:1–14.
16. Wachman EM, Wang A, Isley BC, Boateng J, Beierle JA, Hansbury A, et al. Placental OPRM1 DNA methylation and associations with neonatal opioid withdrawal syndrome, a pilot study. Explor Med. 2020;1:124–35.
17. Czamara D, Dieckmann L, Röh S, Kraemer S, Rancourt RC, Sammallahti S, et al. Betamethasone administration during pregnancy is associated with placental epigenetic changes with implications for inflammation. Clin Epigenetics BioMed Central Ltd. 2021;13:1–17.
18. Vidal AC, Murphy SK, Murtha AP, Schildkraut JM, Soubry A, Huang Z, et al. Associations between antibiotic exposure during pregnancy, birth weight and aberrant methylation at imprinted genes among offspring. Int J Obes (Lond). 2013;37:907.
19. Oni-Orisan OO, Dansereau LM, Marsit CJ, Smith LM, Neal CR, Della Grotta SA, et al. DNA methylation in children with prenatal methamphetamine exposure and environmental adversity. Pediatr Res Pediatr Res. 2021;89:1152–6.
20. Olstad EW, Nordeng HME, Gervin K. Prenatal medication exposure and epigenetic outcomes: a systematic literature review and recommendations for prenatal pharmacoepigenetic studies. Epigenetics. Epigenetics; 2021;
21. Gervin K, Nordeng H, Ystrom E, Reichborn-Kjennerud T, Lyle R. Long-term prenatal exposure to paracetamol is associated with DNA methylation differences in children diagnosed with ADHD. Clin Epigenetics. 2017. https://doi.org/10.1186/s13148-017-0376-9.
22. Heijmans BT, Mill J. Commentary: the seven plagues of epigenetic epidemiology. Int J Epidemiol. 2012;41:74–8.
23. Jones MJ, Moore SR, Kobor MS. Principles and challenges of applying epigenetic epidemiology to psychology. Annu Rev Psychol Annu Rev Psychol. 2018;69:459–85.
24. Ryan J, Mansell T, Fransquet P, Saffery R. Does maternal mental well-being in pregnancy impact the early human epigenome? Epigenomics. 2017;9:313–32.
25. Campagna MP, Xavier A, Lechner-Scott J, Maltby V, Scott RJ, Butzkueven H, Jokubaitis VG, Lea RA. Epigenome-wide association studies: current

knowledge, strategies and recommendations. Clin Epigenetics. 2021. https://doi.org/10.1186/s13148-021-01200-8.
26. Bose M, Wu C, Pankow JS, Demerath EW, Bressler J, Fornage M, et al. Evaluation of microarray-based DNA methylation measurement using technical replicates: the atherosclerosis risk in communities (ARIC) study. BMC Bioinform BioMed Central Ltd. 2014;15:1–10.
27. Shvetsov YB, Song MA, Cai Q, Tiirikainen M, Xiang YB, Shu XO, et al. Intra-individual variation and short-term temporal trend in DNA methylation of human blood. Cancer Epidemiol Biomarkers Prev. 2015;24:490.
28. Dugue PA, English DR, MacInnis RJ, Jung CH, Bassett JK, Fitzgerald LM, et al. Reliability of DNA methylation measures from dried blood spots and mononuclear cells using the HumanMethylation450k BeadArray. Sci Rep. Nature Publishing Group; 2016;6.
29. Forest M, O'Donnell KJ, Voisin G, Gaudreau H, MacIsaac JL, McEwen LM, et al. Agreement in DNA methylation levels from the Illumina 450K array across batches, tissues, and time. Epigenetics. 2018;13:19.
30. Xu Z, Taylor JA. Reliability of DNA methylation measures using Illumina methylation BeadChip. Epigenetics Epigenetics. 2021;16:495–502.
31. Sugden K, Hannon EJ, Arseneault L, Belsky DW, Corcoran DL, Fisher HL, et al. Patterns of Reliability: Assessing the Reproducibility and Integrity of DNA Methylation Measurement. Patterns. 2020;1:100014.
32. Logue MW, Smith AK, Wolf EJ, Maniates H, Stone A, Schichman SA, et al. The correlation of methylation levels measured using Illumina 450K and EPIC BeadChips in blood samples. Epigenomics Future Sci Group. 2017;9:1363–71.
33. Zaimi I, Pei D, Koestler DC, Marsit CJ, De Vivo I, Tworoger SS, et al. Variation in DNA methylation of human blood over a 1-year period using the Illumina MethylationEPIC array. Epigenetics. 2018;13:1056–71.
34. Zongli X, Niu L, Li L, Taylor JA. ENmix: a novel background correction method for Illumina Humanmethylation450 Beadchip. Nucleic Acids Res. 2016;44(3):e20–e20. https://doi.org/10.1093/nar/gkv907.
35. Xu Z, Niu L, Taylor JA. The ENmix DNA methylation analysis pipeline for Illumina beadchip and comparisons with seven other preprocessing pipelines clin epigenetics 2021 131. BioMed Central. 2021;13:1–8.
36. Pidsley R, Zotenko E, Peters TJ, Lawrence MG, Risbridger GP, Molloy P, Van Djik S, Muhlhausler B, Stirzaker C, Clark SJ. Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. Genome Biol. 2016. https://doi.org/10.1186/s13059-016-1066-1.
37. Solomon O, MacIsaac J, Quach H, Tindula G, Kobor MS, Huen K, et al. Comparison of DNA methylation measured by Illumina 450K and EPIC BeadChips in blood of newborns and 14-year-old children. Epigenetics. 2018;13:655–64.
38. Fernandez-Jimenez N, Allard C, Bouchard L, Perron P, Bustamante M, Bilbao JR, et al. Comparison of Illumina 450K and EPIC arrays in placental DNA methylation. Epigenetics. 2019;14:1177–82.
39. Cheung K, Burgers MJ, Young DA, Cockell S, Reynard LN. Correlation of infinium humanmethylation450K and METHYLATIONEPIC beadchip arrays in cartilage. Epigenetics. 2020;15:594–603.
40. Moran S, Arribas C, Esteller M. Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. Epigenomics Future Med Ltd. 2016;8:389–99.
41. Han L, Zhang H, Kaushal A, Rezwan FI, Kadalayil L, Karmaus W, et al. Changes in DNA methylation from pre-to post-adolescence are associated with pubertal exposures. Clin Epigenetics BioMed Central Ltd. 2019;11:1–14.
42. Li S, Nguyen TL, Wong EM, Dugué PA, Dite GS, Armstrong NJ, et al. Genetic and environmental causes of variation in epigenetic aging across the lifespan. Clin Epigenetics BioMed Central Ltd. 2020;12:1–12.
43. Ewing E, Kular L, Fernandes SJ, Karathanasis N, Lagani V, Ruhrmann S, et al. Combining evidence from four immune cell types identifies DNA methylation patterns that implicate functionally distinct pathways during Multiple Sclerosis progression. EBioMedicine. 2019;43:411–23.
44. Neumann A, Walton E, Alemany S, Cecil C, González JR, Jima DD, et al. Association between DNA methylation and ADHD symptoms from birth to school age: a prospective meta-analysis. Transl Psychiatry 101. 2020;10:1–11.
45. Felix JF, Joubert BR, Baccarelli AA, Sharp GC, Almqvist C, Annesi-Maesano I, et al. Cohort profile: pregnancy and childhood epigenetics (PACE) consortium. Int J Epidemiol Oxford Academic. 2018;47:22–23u.
46. Breton CV, Marsit CJ, Faustman E, Nadeau K, Goodrich JM, Dolinoy DC, et al. Small-magnitude effect sizes in epigenetic end points are important in children's environmental health studies: the children's environmental health and disease prevention research center's epigenetics working group. Environ Health Perspect Environ Health Perspect. 2017;125:511–26.
47. Dugué PA, English DR, MacInnis RJ, Joo JE, Jung CH, Milne RL. The repeatability of DNA methylation measures may also affect the power of epigenome-wide association studies. Int J Epidemiol Int J Epidemiol. 2015;44:1460–1.
48. Koo TK, Li MY. A Guideline of selecting and reporting intraclass correlation coefficients for reliability research. J Chiropr Med. 2016;15:155.
49. Chen J, Just AC, Schwartz J, Hou L, Jafari N, Sun Z, et al. CpGFilter: model-based CpG probe filtering with replicates for epigenome-wide association studies. Bioinformatics. 2016;32:469.
50. Morris TJ, Butcher LM, Feber A, Teschendorff AE, Chakravarthy AR, Wojdacz TK, et al. ChAMP: 450k Chip analysis methylation pipeline. Bioinform Oxford Academic. 2014;30:428–30.
51. Tian Y, Morris TJ, Webster AP, Yang Z, Beck S, Feber A, et al. ChAMP: updated methylation analysis pipeline for Illumina BeadChips. Bioinform Oxford Academic. 2017;33:3982–4.
52. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2014;30(10):1363–9. https://doi.org/10.1093/bioinformatics/btu049.
53. Müller F, Scherer M, Assenov Y, Lutsik P, Walter J, Lengauer T, et al. RnBeads 2.0: Comprehensive analysis of DNA methylation data. Genome Biol. BioMed Central Ltd. 2019;20:55.
54. Pidsley R, Y Wong CC, Volta M, Lunnon K, Mill J, Schalkwyk LC. A data-driven approach to preprocessing Illumina 450K methylation array data BMC Genomics. BioMed Central. 2013;14:1–10.
55. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, et al. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinform Bioinform. 2013;29:189–96.
56. Dugué PA, Jung CH, Joo JE, Wang X, Wong EM, Makalic E, et al. Smoking and blood DNA methylation: an epigenome-wide association study and assessment of reversibility. Epigenetics. 2020;15:358.
57. Bujang M, Baharum N. A simplified guide to determination of sample size requirements for estimating the value of intraclass correlation coefficient: a review. Arch Orofac Sci. 2017.
58. Li M, Zou D, Li Z, Gao R, Sang J, Zhang Y, Li R, Xia L, Zhang T, Niu G, Bao Y, Zhang Z. EWAS Atlas: a curated knowledgebase of epigenome-wide association studies. Nucleic Acids Res. 2019;47(D1):D983–8. https://doi.org/10.1093/nar/gky1027.
59. Battram T, Yousefi P, Crawford G, Prince C, Babaei MS, Sharp G, et al. The EWAS Catalog: a database of epigenome-wide association studies. OSF Prepr. OSF Preprints; 2021;4.
60. Magnus P, Irgens LM, Haug K, Nystad W, Skjærven R, Stoltenberg C, et al. Cohort profile: The Norwegian Mother and Child Cohort Study (MoBa). Int J Epidemiol Oxford Academic. 2006;35:1146–50.
61. Magnus P, Birke C, Vejrup K, Haugan A, Alsaker E, Daltveit AK, et al. Cohort profile update: The Norwegian mother and child cohort study (MoBa). Int J Epidemiol Int J Epidemiol. 2016;45:382–8.
62. Paltiel L, Haugan A, Skjerden T, Harbak K, Bækken S, Stensrud NK, et al. The biobank of the Norwegian mother and child cohort study – present status. Nor Epidemiol Norwegian Epidemiol Soc. 2014;24:29–35.
63. Rønningen KS, Paltiel L, Meltzer HM, Nordhagen R, Lie KK, Hovengen R, et al. The biobank of the Norwegian mother and child cohort study: a resource for the next 100 years. Eur J Epidemiol Eur J Epidemiol. 2006;21:619–25.
64. Brandlistuen RE, Ystrom E, Nulman I, Koren G, Nordeng H. Prenatal paracetamol exposure and child neurodevelopment: A sibling-controlled cohort study. Int J Epidemiol. 2013;42:1702–13.
65. Stergiakouli E, Thapar A, Smith GD. Association of acetaminophen use during pregnancy with behavioral problems in childhood: evidence against confounding. JAMA Pediatr Am Med Assoc. 2016;170:964–70.
66. Avella-Garcia CB, Julvez J, Fortuny J, Rebordosa C, García-Esteban R, Riano Galán I, et al. Acetaminophen use in pregnancy and neurodevelopment: attention function and autism spectrum symptoms. Int J Epidemiol. 2016;45:1987–96.

67. Thompson JMD, Waldie KE, Wall CR, Murphy R, Mitchell EA. Associations between acetaminophen use during pregnancy and ADHD symptoms measured at ages 7 and 11 years. PLoS ONE. 2014;9(9):e108210. https://doi.org/10.1371/journal.pone.0108210.
68. Liew Z, Ritz B, Rebordosa C, Lee PC, Olsen J. Acetaminophen use during pregnancy, behavioral problems, and hyperkinetic disorders. JAMA Pediatr Am Med Assoc. 2014;168:313–20.
69. World Health Organization. Chapter V: Mental and Behavioural Disorders. 10th Revis Int Stat Classif Dis Relat Heal Probl. 2018.
70. American Psychiatric Association. Neurodevelopmental Disorders. Diagnostic Stat Man Ment Disord. American Psychiatric Association; 2013.
71. Thapar A, Pine DS, Leckman JF, Scott S, Snowling MJ, Taylor E. Rutter's Child and Adolescent Psychiatry: Sixth Edition. Rutter's Child Adolesc. Psychiatry Sixth Ed. Chichester, UK: John Wiley & Sons, Ltd; 2015.
72. Wieselberg HM, Rutter M, Thorley G. Which boys respond to stimulant medication? a controlled trial of methylphenidate in boys with disruptive behaviour. Psychol Med Psychol Med. 1987;17:121–43.
73. Zongli Xu, Langie SAS, De Boever P, Taylor JA, Niu L. RELIC: a novel dye-bias correction method for Illumina Methylation BeadChip. BMC Genomics. 2017. https://doi.org/10.1186/s12864-016-3426-3.
74. Niu L, Xu Z, Taylor JA. RCP: a novel probe design bias correction method for Illumina Methylation BeadChip. Bioinformatics Bioinformatics. 2016;32:2659–63.
75. Benton MC, Johnstone A, Eccles D, Harmon B, Hayes MT, Lea RA, et al. An analysis of DNA methylation in human adipose tissue reveals differential modification of obesity genes before and after gastric bypass and weight loss. Genome Biol BioMed Central. 2015;16:1–21.
76. Chen Y, Lemire M, Choufani S, Butcher DT, Grafodatskaya D, Zanke BW, et al. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics 8. 2013;8:203–9. https://doi.org/10.4161/epi.23470.
77. McCartney DL, Walker RM, Morris SW, McIntosh AM, Porteous DJ, Evans KL. Identification of polymorphic and off-target probe binding sites on the Illumina Infinium MethylationEPIC BeadChip. Genomics Data. Elsevier Inc; 2016;9:22–4.
78. Hansen KD. IlluminaHumanMethylationEPICanno.ilm10b4.hg19: Annotation for Illumina's EPIC methylation arrays [Internet]. 2017.
79. Gamer M, Lemon J, Fellows I, Singh P. Irr: Various coefficients of interrater reliability and agreement. R package version, 0.84.1. 2019.
80. Conway JR, Lex A, Gehlenborg N. UpSetR: an R package for the visualization of intersecting sets and their properties. Bioinformatics Oxford Academic. 2017;33:2938–40.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

PAPER IV

Effects of prenatal exposure to (es)citalopram and maternal depression during pregnancy on DNA methylation and child neurodevelopment

Olstad EW, Nordeng HME, Sandve GK, Lyle R, Gervin K

Submitted to *Molecular Psychiatry.*

IV

1 # Effects of prenatal exposure to (es)citalopram and
2 maternal depression during pregnancy on DNA
3 methylation and child neurodevelopment

4 **Running title:** Prenatal (es)citalopram, DNAm and neurodevelopment

5 Emilie Willoch Olstad (M.Sc.)[1,2,3,*], Hedvig Marie Egeland Nordeng (Ph.D.)[1,2,3,4], Geir Kjetil
6 Sandve (Ph.D.)[2,3,5], Robert Lyle (Ph.D.)[2,6,7] and Kristina Gervin (Ph.D.)[1,2,3,8]
7

8 [1] Pharmacoepidemiology and Drug Safety Research Group, Department of Pharmacy, Faculty of
9 Mathematics and Natural Sciences, University of Oslo, Oslo, Norway

10 [2] PharmaTox Strategic Research Initiative, Faculty of Mathematics and Natural Sciences, University of Oslo,
11 Oslo, Norway

12 [3] UiO:RealArt Convergence Environment, University of Oslo, Oslo, Norway

13 [4] Department of Child Health and Development, Norwegian Institute of Public Health, Oslo, Norway

14 [5] Department of Informatics, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo,
15 Norway.

16 [6] Department of Medical Genetics, Oslo University Hospital and University of Oslo, Oslo, Norway

17 [7] Centre for Fertility and Health, Norwegian Institute of Public Health, Oslo, Norway

18 [8] Department of Research and Innovation, Division of Clinical Neuroscience, Oslo University Hospital, Oslo,
19 Norway

20

21 **Word count (excl. abstract, acknowledgements and references):** 4,613 words

22

23 * Corresponding author

24 Emilie Willoch Olstad

25 +47 930 60 396
26 e.w.olstad@farmasi.uio.no

27 Department of Pharmacy
28 University of Oslo
29 P.O. 1068, Blindern
30 0316 OSLO
31 Norway

Abstract

Studies assessing associations between prenatal exposure to antidepressants, maternal depression and offspring DNA methylation (DNAm) have been inconsistent. Here, we investigated whether prenatal exposure to citalopram or escitalopram ((es)citalopram), and maternal depression is associated with differences in DNAm. Then, we examined if there is an interaction effect of (es)citalopram exposure and DNAm on offspring neurodevelopmental outcomes. Finally, we investigated whether DNAm at birth correlates with neurodevelopmental trajectories in childhood. We analyzed DNAm in cord blood from the Norwegian Mother, Father and Child Cohort Study (MoBa) biobank. MoBa contains questionnaire data on maternal (es)citalopram use and depression during pregnancy, and information about child neurodevelopmental outcomes assessed by internationally recognized psychometric tests. In addition, we retrieved ADHD diagnoses from the Norwegian Patient Registry and information on pregnancies from the Medical Birth Registry of Norway. In total, 958 newborn cord blood samples were divided into three groups: (i) prenatal (es)citalopram exposed (n = 306), (ii) prenatal maternal depression exposed (n = 308), and (iii) propensity score-matched controls (n = 344). Among children exposed to (es)citalopram, there were more ADHD diagnoses and symptoms, and delayed communication and psychomotor development. We did not identify differential DNAm associated with (es)citalopram or depression, nor any interaction effects on neurodevelopmental outcomes throughout childhood. Trajectory modelling identified subgroups of children following similar developmental patterns. Some of these subgroups were enriched for children exposed to maternal depression, and some subgroups were associated with differences in DNAm at birth. Interestingly, several of the differentially methylated genes are involved in neuronal processes and development. These results suggest DNAm as a potential predictive molecular marker of later abnormal neurodevelopmental outcomes, but we cannot conclude whether DNAm links prenatal (es)citalopram exposure or maternal depression with child neurodevelopmental outcomes.

Introduction

More than 1 in 10 women experience perinatal depression [1], and lasting depressive symptoms during pregnancy may contribute both adverse maternal and child outcomes [2, 3]. To treat moderate to severe depression, pregnant women are increasingly prescribed antidepressants [4–6], with 1–7% of pregnant women using selective serotonin reuptake inhibitors (SSRIs) [4, 5, 7–9]. The structurally similar citalopram and escitalopram (hereafter, (es)citalopram) are collectively the most frequently prescribed SSRIs to pregnant

60 women [4, 5, 9]. Pharmacoepidemiological studies have linked prenatal antidepressant exposure and maternal
61 depression during pregnancy to an increased risk of abnormal neurodevelopmental outcomes in the child [10–
62 12]. The underlying mechanisms are not known, but it has been shown that prenatal antidepressant exposure
63 is associated with epigenetic differences in cord blood (in particular, DNA methylation [DNAm] of cytosine-
64 phosphate-guanine sites [CpGs]) [13–15]. However, studies show conflicting results and are based on small
65 sample sizes, candidate genes, broad exposure definitions and some lack a depression group to control for
66 indication [14]. In five epigenome-wide association studies (EWASs) on prenatal antidepressant exposure and
67 newborn cord blood DNAm, none of the differentially methylated CpGs overlap between any of the studies
68 [15–19].

69 Studies have also investigated associations between prenatal exposure to antidepressants, DNAm in candidate
70 genes and child outcomes related to the central nervous system, without significant findings [20–22]. While
71 these studies are limited to a few candidate genes and investigated short-term outcomes, larger EWASs of
72 long-term neurodevelopmental outcomes are needed. Associations between poor maternal mental health
73 during pregnancy and DNAm differences in the offspring have also been shown, with several CpGs relevant
74 to child neurodevelopment [23, 24]. Therefore, it is equally important to deconvolve the effect of prenatal
75 exposure to antidepressants and unmedicated maternal depression on DNAm and altered neurodevelopment
76 in the offspring.

77 Children with certain neurodevelopmental outcomes such as attention-deficit/hyperactivity disorder (ADHD),
78 show heterogeneity related to both phenotypic presentation and developmental course [25]. Interestingly,
79 prospective studies have shown that DNAm measured at birth before symptom onset is associated with
80 different ADHD symptom trajectories [26, 27]. Such results lend epigenetic insights into neurodevelopmental
81 trajectories in childhood. However, whether prenatal environmental factors like prenatal antidepressant
82 exposure and maternal depression may influence DNAm patterns associated with neurodevelopmental
83 trajectories are not known.

84 In the present study, we have conducted epigenome-wide association analyses and investigated (i) whether
85 prenatal exposure to (es)citalopram or maternal depression is associated with differences in DNAm in newborn
86 cord blood, (ii) the interaction effects of (es)citalopram and DNAm on long-term neurodevelopmental
87 outcomes in the child, and (iii) whether DNAm at birth is associated with later neurodevelopmental

88 trajectories. This enabled a systematic investigation of the different aspects previously linked to neurotoxicity
89 of antidepressants by integrating maternal unmedicated depression and child neurodevelopmental outcomes in
90 our EWAS.

91 Methods

92 Study population

93 This study is based on data and cord blood samples from the Norwegian Mother, Father and Child Cohort
94 Study (MoBa), conducted by the Norwegian Institute of Public Health (NIPH) [28]. MoBa is an ongoing
95 prospective, population-based birth cohort study (*n* = 114 500 children, *n* = 95 200 mothers and *n* = 75 200
96 fathers), and 40.6% of women giving birth in Norway between 1998 and 2008 consented to participate.
97 Participants complete questionnaires throughout pregnancy and in childhood. Cord blood samples were
98 retrieved from the MoBa biobank, which contains blood samples from both parents during pregnancy, and
99 from mothers and children (umbilical cord) at birth [29]. This study is based on data version 12 released by
100 MoBa in 2020. MoBa was also linked to the Norwegian Patient Registry (NPR) and the Medical Birth Registry
101 of Norway (MBRN).

102 The establishment of MoBa and initial data collection was based on a license from the Norwegian Data
103 Protection Agency and approval from the Regional Committees for Medical and Health Research Ethics
104 (REC). The MoBa cohort is currently regulated by the Norwegian Health Registry Act. All data were de-
105 identified and the linking of MoBa to health registries was handled by NIPH and the respective registries. Our
106 study was approved by the REC South East Norway (reference: 23136, 2014/163).

107 Sample selection and study design

108 Samples were selected into three groups: (i) prenatally (es)citalopram exposed, (ii) prenatally maternal
109 depression exposed, and (iii) propensity score-matched controls (unexposed to antidepressants and maternal
110 depression). Selection was based on MoBa questionnaires Q1 (gestational weeks 0–13), Q3 (weeks 13–29)
111 and Q4 (week 30 to 6 months after delivery). Only live, singleton births with cord blood samples available in
112 the MoBa biobank were included. Women using antiepileptics and psycholeptics were excluded due to the
113 potential teratogenic effects of these medications [30–37].

114 In the (es)citalopram group, other antidepressants were allowed, except when used concomitantly with
115 (es)citalopram on the same indication. The indications for (es)citalopram were depression, anxiety and other
116 mental health problems. The depression group included women reporting depression, anxiety or other mental
117 health problems, and exhibiting a mean depression symptom score ≥2.0 on either the Hopkins Symptom
118 Checklist (SCL) -5 or -8. All samples available based on these selection criteria were included in the
119 (es)citalopram or depression groups. The control group included women with no self-reported mental health
120 problems and mean SCL-5 and -8 scores of 1.0 (no depressive symptoms), replying to both Q1, Q3 and Q4.
121 Of the 17 228 women fulfilling these criteria, the final control group was selected by propensity score matching
122 to the (es)citalopram group.

123 Exposures

124 *Prenatal (es)citalopram exposure*

125 Citalopram (Anatomical Therapeutic Chemical [ATC]: N06AB04) is a mixture of the two stereoisomers R-
126 citalopram and S-citalopram, and escitalopram (ATC: N06AB10) contains only the S-citalopram stereoisomer.
127 Maternal use of (es)citalopram was collected from the questionnaires Q1, Q3 and Q4 for four-week intervals
128 (gestational weeks 0–4; 5–8; 9–12; 13–16; 17–20; 21–24; 25–28; 30–birth). Prenatal (es)citalopram exposure
129 was defined as reported use at either of these timepoints (see self-report validity in the Supplementary
130 Methods).

131 *Maternal depression*

132 Depression was assessed by two measures. The first measure was based on self-reported depression and
133 recorded as answering “Yes” to having depression (Q1, Q3), anxiety (Q1), other psychological problems (Q3)
134 or mental health problems (Q4) during pregnancy. Second, for the depression and control groups, we also
135 included selection criteria on mean depression symptom scores from short versions of the SCL (SCL-5 in Q1
136 and SCL-8 in Q3; Supplementary Methods) [38–40]. A mean SCL-5 score ≥2.0 is indicative of depression
137 [41, 42].

138 Outcomes

139 *DNA methylation*

140 DNAm levels were measured using the Infinium MethylationEPIC BeadChip at Life & Brain
141 (www.lifeandbrain.com/en/). Samples were randomly allocated to plates and beadchips, and processed as
142 described previously [43]. The quality of the DNAm data were examined in the quality control module of
143 *RnBeads* [44, 45]. Probes and samples that could bias the normalization and down-stream analyses were
144 removed, including probes with SNPs (n = 17 371), cross-reactive probes (n = 43 463) [46], and poor-
145 performing probes and samples with a detection p value >0.01 (n = 18 435 probes; n = 1 sample). Then,
146 background correction was done using the exponential-truncated-normal (ENmix) out-of-band (oob) method
147 [47], followed by beta-mixture quantile (BMIQ) normalization [48]. After normalization, non-CpG probes (n
148 = 1 033) and probes on the sex chromosomes (n = 16 941) were removed. Finally, if *RnBeads*-estimated and
149 MBRN-registered newborn sex differed, the sample was removed (n = 4). The final, filtered data included 769
150 652 probes and 958 samples.

151 *Neurodevelopmental outcomes*

152 Child neurodevelopment was assessed using parental self-reports on internationally recognized psychometric
153 tests at ages 0.5 years (Q4), 1.5 years (Q5), 3 years (Q6) and 5 years (Q5y). In addition, we retrieved ADHD
154 diagnoses from the NPR recorded by specialists, registered as F90 in the 10th revision of the International
155 Classification of Disease (ICD-10). The psychometric instruments included were the Child Behavior Checklist
156 DSM-oriented (CBCL-DSM) ADHD subscale [49, 50] and the Ages and Stages Questionnaire (ASQ)
157 communication and psychomotor subscales [51] (Table 1). These tests cover different domains of
158 neurodevelopment. The psychomotor subscale covers both the fine and gross motor items of the ASQ. Age-
159 of-onset of independent walking is an important milestone in gross motor development, and therefore, was
160 also included in the analyses. In the CBCL-DSM, higher scores indicate more ADHD symptoms, and in the
161 ASQ subscales, lower scores indicate possible developmental delays (Table 1; Supplementary Tables 1–2).
162 Raw mean scores were standardized to T scores prior to statistical analysis (standardized to the entire MoBa
163 population).

164 **Covariate assessment**

We assessed potential covariates (listed in Supplementary Methods) in three steps. First, we performed principal component analysis (PCA) on the DNAm data, and tested the associations between principal components (PCs) 1–3 and the covariates (one-way analysis of variance [categorical variables] and Spearman's correlation test [continuous variables]; Supplementary Figure 1A–B). Second, the individual contribution of the covariates significantly associated with DNAm variation was assessed by PC-PR^2 (Supplementary methods) [52, 53]. All covariates except bisulfite conversion and cell types contributed <1% of the DNAm variation (Supplementary Figure 1C). Finally, we tested whether the covariates contributing the most to the DNAm variation differed between the comparison groups (Wilcoxon's rank-sum test [continuous variables] and Chi-squared or Fisher's exact test [categorical variables]; Supplementary Tables 3–4).

Cell type composition ($CD8^+$ and $CD4^+$ T cells, natural killer cells, B cells, monocytes, granulocytes and nucleated red blood cells [nRBCs]) was estimated using the *estimateCellCounts2* function implemented in *minfi* [54] and a validated cord blood reference (*FlowSorted.CordBloodCombined.450k*) [55, 56].

Statistical analyses

Propensity score matching

We generated the propensity scores using a logistic regression model to estimate the conditional probability of receiving (es)citalopram given defined pretreatment characteristics (prenatal paracetamol exposure, non-steroidal anti-inflammatory drugs [NSAIDs], opioid and antimigraine medication exposure, siblings, and maternal age, pre-pregnancy body mass index [BMI], education, income, lifetime history of major depression [LTHMD], smoking and alcohol consumption) [57, 58]. From these, we selected the covariates with a p value <0.1 for inclusion in the final model matching the (es)citalopram subjects to controls: maternal income, BMI, LTHMD, smoking and alcohol at the start of pregnancy, and parity. We used nearest neighbor matching with a caliper width of 0.20 of the pooled standard deviation of the regression model (≈ 0.22) [58].

Trajectory analyses

Trajectory analyses of psychometric test scores over multiple timepoints were performed using latent class growth analysis (LCGA; also called group-based trajectory models), which is an unsupervised clustering method for longitudinal data [59]. Models were run using the *lcmm* function in the *lcmm* R package [60], with

191 maximum likelihood estimation (Supplementary Figure 2). We examined 1–5 classes, using a linear or
192 quadratic shape of time, and the *thresholds* link function, as suggested for psychometric test data [61]. Initial
193 values were selected using an automatic grid search of 100 random value vectors. Each model was run for a
194 maximum of 100 iterations, if a model did not converge, we increased to maximum 10,000 iterations. The
195 final models were selected based on the goodness-of-fit and discriminatory power of the models, using the
196 Akaike information criterion (AIC), the Bayesian information criterion (BIC), the sample size-corrected BIC
197 (c-BIC) and entropy (Supplementary Tables 5–7). Lower AIC, BIC and c-BIC indicate better relative model
198 fit, while entropy close to 1 indicates good classification.

199 *Differential DNAm analyses*

200 We used β values for visualization purposes and M values for statistical analyses [62]. Pairwise group
201 comparisons were performed by fitting linear regression models to mean DNAm in *limma* [63], defined by:

202 $$\text{DNAm} \sim \beta_1 * \text{group} + \varepsilon.$$

203 Interaction was assessed by running logistic (ADHD diagnosis) or ordinal logistic regression models (T scores
204 and age-of-onset of walking):

205 $$\text{Neurodevelopmental outcome} \sim \beta_0 + \beta_1 * \text{DNAm} + \beta_2 * \text{(es)citalopram} + \beta_3 * \text{DNAm} * \text{(es)citalopram} + \varepsilon,$$

206 where β_3 represents the interaction between DNAm and (es)citalopram exposure. Ordinal logistic regression
207 was used due to the highly skewed distributions of the T scores for some of the neurodevelopmental outcomes
208 (Supplementary Figure 3).

209 To assess the effect of (es)citalopram and limit the impact of depression, the interaction models were run
210 including the (es)citalopram and depression groups only.

211 Finally, trajectory classes and DNAm associations were assessed by pairwise comparisons of trajectory classes
212 in linear regression models:

213 $$\text{DNAm} \sim \beta_0 + \beta_1 * \text{trajectory class} + \varepsilon.$$

All comparisons were adjusted for multiple testing with a false discovery rate (FDR) <0.05, using the Benjamini and Hochberg method [64].

Analyses of significant CpGs

The annotation of CpGs was performed using the *IlluminaHumanMethylationEPICanno.ilm10b4.hg19* package [65]. Gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed with *missMethyl* [66]. The BECon web application [67] was used to assess the blood-brain correlation of the significant CpGs.

Results

Prenatal exposure to (es)citalopram and maternal depression, and DNA methylation patterns in cord blood

We selected samples into three groups: (i) prenatally (es)citalopram exposed ($n = 306$), (ii) prenatally maternal depression exposed ($n = 308$), and (iii) propensity score-matched controls ($n = 344$). Sample characteristics are presented in Table 2. First, we ran PCA to identify potential covariates associated with variation in DNAm (Supplementary Figure 1). This analysis revealed an association of the estimated nRBC proportion with DNAm variation, which contributed $>5\%$ of the variation explained by PCs 1–3 and was significantly different between the groups (Supplementary Figure 1 and Tables 1–2). However, as the difference in mean nRBC proportion between groups was negligible (0.01–0.02), it was not included as a covariate in our models. Then, we performed pairwise epigenome-wide association analyses between the groups to identify differential DNAm associated with prenatal exposure to (es)citalopram and maternal depression. These analyses did not reveal any significant differences in DNAm associated with prenatal (es)citalopram exposure or maternal depression (Figure 1). However, inspecting the distributions of p values revealed that the comparison of the (es)citalopram group to the control group exhibited lower p values than the other two comparisons. GO analyses of the top 1,000 CpGs ranked according to significance for the pairwise comparison of the (es)citalopram group to the controls did not reveal any enriched GO terms or KEGG pathways. Future studies including more samples will be important to increase the power to replicate and further evaluate the apparent trend in our data.

240 **More ADHD symptoms and delayed communication and psychomotor skills among children**
241 **exposed to (es)citalopram during pregnancy**

242 Studies have reported an association between prenatal antidepressant exposure and child ADHD diagnosis,
243 but results have been conflicting [10–12]. We observed a significantly higher proportion of children with
244 ADHD in this study (*n* = 51, 5.3%) compared to the whole MoBa cohort (*n* = 3 014, 3.0%; Fisher's exact test,
245 *p*<0.00001). This was also evident when comparing the three sample groups in our study, where children
246 prenatally exposed to (es)citalopram (7.5%) were significantly more likely to have an ADHD diagnosis
247 compared to the controls (2.9%, Table 3). Children prenatally exposed to maternal depression also exhibited
248 a higher proportion of ADHD diagnoses (5.8%) than the control group, but this difference was not significant.
249 There were also significant differences between the comparison groups for several parent-reported
250 neurodevelopmental outcomes (Table 3). ADHD symptoms were assessed using the CBCL-DSM, and
251 communication and psychomotor skills were measured with the ASQ and age-of-onset of walking, assessed
252 at 0.5 years, 1.5 years, 3 years and 5 years. The raw mean scores of the questionnaires were standardized to *T*
253 scores based on the entire MoBa population. In the CBCL-DSM higher *T* scores indicate more ADHD
254 symptoms and in the ASQ lower *T* scores indicate possible developmental delays (Table 1).

255 **Interaction effects of DNAm and prenatal exposure to (es)citalopram on neurodevelopmental**
256 **outcomes**

257 While some studies suggest that prenatal exposure to antidepressants is associated with abnormal
258 neurodevelopmental outcomes such as ADHD [10–12], little is known about molecular mechanisms
259 underlying such associations. We investigated the potential interaction of DNAm and prenatal (es)citalopram
260 exposure on several neurodevelopmental outcomes. Specifically, we examined the interaction effect of
261 (es)citalopram exposure and DNAm on ADHD diagnosis and symptoms (CBCL-DSM), and on
262 communication and psychomotor skills, by comparing the children exposed to (es)citalopram and depression
263 only. These analyses did not identify any significant interaction effects of (es)citalopram exposure and DNAm
264 on any of the neurodevelopmental outcomes.

265 **DNAm at birth and later neurodevelopmental trajectories**

266 Children with abnormal neurodevelopmental outcomes often present heterogeneity in developmental course
267 and studies have shown that DNAm is associated with different neurodevelopmental trajectories [26, 27].
268 Hence, we investigated whether DNAm measured in cord blood at birth before symptom onset was associated
269 with later neurodevelopmental trajectories of ADHD symptoms, and communication and psychomotor
270 development. Trajectories were estimated over three or four timepoints from 0.5 to 5 years after birth,
271 depending on the neurodevelopmental outcome (Supplementary Tables 5–7).

272 Children were classified into trajectories following similar developmental patterns (Figure 2). Specifically,
273 trajectory analysis of the CBCL-DSM ADHD subscale classified children into 4 trajectories (Figure 2A).
274 Children in the two trajectories with the lowest CBCL-DSM *T* scores, indicating fewer ADHD symptoms
275 (classes 1 and 2), showed similar developmental courses. A large proportion of children were classified into
276 the class 3 showing a moderate CBCL-DSM *T* score. Children in the highest trajectory (class 4) had a
277 consistently high CBCL-DSM *T* score from 62 to 68 between 1.5 and 5 years of age, indicating more
278 pronounced and slightly increasing ADHD symptomatology. As expected, the class 4 was significantly
279 enriched with ADHD diagnosed children, and notably, also exhibited a significantly higher proportion of
280 children exposed to maternal depression (Supplementary Table 9).

281 The trajectory analyses of the ASQ communication and psychomotor subscales classified children into 3 and
282 5 trajectory classes, respectively (Figure 2B–C, Supplementary Tables 10–11). Of note, the ASQ
283 communication trajectory class 3 contained only 6 children following a very different developmental course
284 compared to the other children (Figure 2B). The children in the three study comparison groups were evenly
285 distributed between the trajectory classes (Supplementary Tables 10–11). In conclusion, these results clearly
286 demonstrate heterogeneity in the developmental course of the different outcome measures between children.

287 Next, to investigate whether DNAm at birth may be a potential biomarker of later developmental trajectories
288 reflecting symptom severity, we performed epigenome-wide analyses and compared DNAm between the
289 identified trajectories. For the CBCL-DSM ADHD subscale trajectories, children in the two classes showing
290 the lowest *T* scores (class 1 and 2; Figure 2) and therefore, unlikely to have ADHD, were grouped together in
291 the analyses. We pairwise compared the three trajectories and found no significant associations between cord
292 blood DNAm at birth and the trajectories.

293 For the ASQ communication subscale trajectories, we excluded trajectory class 3 containing only 6 children,
294 and compared DNAm between classes 1 and 2 (Figure 2B). Multiple CpGs (n = 254) were differentially
295 methylated between the two ASQ communication trajectories (Supplementary Table 8). Interestingly, two
296 CpGs annotated to *PEX10*, involved in peroxisomal processes, have previously been identified in child saliva
297 associated with ADHD [68], in cord blood associated with ADHD trajectories [26] and upon prenatal exposure
298 to paracetamol in children with ADHD [43]. Also, four CpGs are annotated to the *BEGAIN* gene, which is
299 involved in regulation of postsynaptic neurotransmitter receptor activity in the brain. Other genes of interest
300 are 3 CpGs located in *HOXC4*, which is involved in the development of the nervous system, 1 CpG in *KCNJ5*
301 previously associated with ADHD [43], and 1 CpG in *SHANK2* which is involved in transmission in excitatory
302 neurons. Mutations in the *SHANK2* gene has been associated with both ADHD and autism spectrum disorder
303 [69].

304 For the ASQ psychomotor subscale trajectories, pairwise comparisons of DNAm between all 5 trajectory
305 classes (Figure 2C), revealed differentially methylated CpGs between trajectory classes 3 and 4 (n = 32 CpGs
306 annotated to 24 genes). Interestingly, several of these overlapped with differentially methylated CpGs
307 identified between the communication trajectories, which are annotated to the *RFTN1, ERV3-1*, *RBM39*,
308 *SHANK2*, *DYRK2*, *GABPA*, *ATP5J*, *PEX10*, *FAM45A*, *FAM45B*, *RNASEH2C*, *PPP1R12B* and *PRKXP1* genes
309 (n = 16 CpGs annotated to 13 genes; Figure 3A). In addition to the functions of the *PEX10* and *SHANK2* genes
310 described above, several of the overlapping genes are involved in for example cellular growth and development
311 (e.g., *DYRK2* and *TGFB*), neuronal differentiation (*GABPA*) and neurological phenotypes (*RNASEH2C*).

312 **Blood-brain DNAm correlation**

313 To strengthen the mechanistic insights and interpretation of the significant DNAm findings in cord blood, we
314 used BECon [67] to look up the correlation of DNAm in blood and brain tissue for the CpGs associated with
315 the communication or psychomotor trajectories. Data on blood-brain correlations were available for 145 of the
316 254 CpGs associated with the communication trajectories. Of these, most CpGs exhibited relatively weak
317 correlations between –0.5 and 0.5 (n = 141 CpGs), while 4 CpGs were positively correlated (>0.5) between
318 blood and brain (Supplementary Table 12). Of the 32 significant CpGs associated with the psychomotor
319 trajectory classes 3 and 4, 14 had blood-brain correlation data available in BECon. Among these CpGs, 12

showed weak correlation ($-0.5 < R < 0.5$) and 2 CpGs were positively correlated (Supplementary Table 12). In Figure 3B, the 5 CpGs available in BECon of the 16 CpGs associated with both the communication and psychomotor trajectories are shown. A CpG annotated to the *PRKXP1* gene was positively correlated with overall brain DNAm at this CpG (Figure 3B). Further, one of the CpGs annotated to the *PEX10* gene exhibited positive correlation with one brain area (BA10; Figure 3B). In summary, these findings suggest that several of the significant CpGs identified in our study likely reflect DNAm levels in the brain.

Discussion

We performed epigenome-wide association analyses and investigated whether prenatal exposure to (es)citalopram or maternal depression was associated with differences in cord blood DNAm at birth. To explore the role of DNAm on child neurodevelopmental outcomes associated with prenatal (es)citalopram exposure, we investigated the interaction effect on neurodevelopment. We also examined whether DNAm at birth was associated with later developmental trajectories of ADHD symptoms, and communication and psychomotor skills. To our knowledge, this is the largest EWAS to date deconvolving associations of DNAm, and prenatal (es)citalopram exposure and maternal depression, and assessing the potential effects on long-term neurodevelopmental outcomes.

The initial EWAS on (es)citalopram and maternal depression did not identify any differentially methylated CpGs compared to controls. However, inspecting the distributions of *p* values revealed that the comparison of the (es)citalopram group to the controls exhibited lower *p* values than the other two comparisons. Future studies including more samples and DNAm analyses at a single cell level will be necessary to replicate and further interpret this non-significant signal. We did not replicate previous findings showing association between prenatal antidepressant exposure or maternal depression and DNAm [15–19, 70], and there are several possible explanations for this [13, 14]. For example, previous EWASs are based on small sample sizes, varying genome coverage and heterogenous methodologies [13, 14].

In line with previous studies [10–12], we observed differences in proportions of ADHD diagnoses across the study groups, specifically when comparing children prenatally exposed to (es)citalopram to controls. There was also an increased proportion of ADHD diagnoses in the depression group compared to controls, albeit not

346 significant. In MoBa, there are several parent-reported psychometric tests of neurodevelopment, including
347 CBCL and ASQ, assessed between 0.5 and 5 years of age. We chose the CBCL-DSM ADHD subscale to
348 measure ADHD symptoms and symptom heterogeneity [25, 71], and to possibly identify children with
349 subthreshold ADHD. The ASQ communication and psychomotor subscales were included as the ASQ is an
350 internationally recognized and widely used psychometric test, and covers other domains of neurodevelopment
351 which can be, but are not necessarily, related to ADHD [25]. There were significant differences in several of
352 the psychometric test *T* scores between the different groups. Trajectory analyses classified children into
353 developmental trajectories of the CBCL-DSM ADHD subscale, and the ASQ subscales of communication and
354 psychomotor skills. The trajectories of ADHD symptom development are similar to trajectories identified
355 previously [26]. Taken together, our results emphasize the importance of taking symptom heterogeneity and
356 developmental course into consideration when assessing neurodevelopment in the prenatal
357 pharmacoepigenetic context.

358 Whether and how DNAm potentially mediates an increased risk of abnormal neurodevelopment in children
359 prenatally exposed to antidepressants and/or maternal depression is not known. Identification of molecular
360 biomarkers for early risk detection of ADHD and related neurodevelopmental outcomes could potentially aid
361 in the identification of children in need of early intervention and support. In this respect, DNAm patterns in
362 cord blood measured at birth before manifestation of symptoms are potentially particularly useful. Trajectories
363 of communication and psychomotor development were associated with differential cord blood DNAm of genes
364 previously associated with ADHD and autism spectrum disorder trajectories in childhood [26, 68]. Multiple
365 genes were also involved in cellular growth development and neurological phenotypes. Interestingly, several
366 of the differentially methylated genes also overlapped between the communication and psychomotor
367 trajectories, suggesting a common effect. We found differential DNAm of *PEX10*, which encodes a protein
368 functioning in peroxisomal processes. Such processes have been implicated in fatty acid oxidation in ADHD,
369 and have also been reported by Walton *et al.* [26] and Wilmot *et al.* [68]. Although communication and
370 psychomotor trajectories are not specific to ADHD, the complex etiology underlying ADHD is often
371 accompanied by learning problems, and motor and/or speech delays [25]. While only one CpG in *PEX10*
372 appeared to positively correlate between blood and brain, our results nevertheless suggest DNAm at birth as a

373 potential molecular biomarker of later neurodevelopmental trajectories in children prenatally exposed to
374 (es)citalopram and depression.

375 Our study has several limitations and strengths. While this study to our knowledge is the largest prospective
376 EWAS on antidepressants and DNAm, it may still be underpowered to detect DNAm differences associated
377 with (es)citalopram and maternal depression. In particular, interaction models may inherently decrease power
378 and the psychometric tests at higher ages exhibit a pronounced decrease in respondents, mostly due to loss to
379 follow-up [72]. To partly circumvent this limitation, the LCGA handled missing data when score for at least
380 one timepoint was known using maximum likelihood estimation. The loss to follow-up seemed to be
381 differentially distributed among the comparison groups, with more depressed women lost to follow-up. This
382 may bias our results towards the null, as women with more depressive symptoms are missing. We attempted
383 to limit confounding by indication by including a depression group. However, the depression group scored
384 significantly higher on the SCL-5 and -8, suggesting more severe depression symptoms at the time of reporting,
385 likely due to being unmedicated. Therefore, we cannot exclude residual confounding by the severity of
386 depression, as well as other unmeasured confounders. Finally, there is a known genetic component of ADHD,
387 which we could not assess in the present study. Future studies including integrated analyses of genetic
388 information, would enable investigations of a genetic susceptibility to ADHD. The main strengths of the
389 present study include the relatively large sample size, and a focus on one specific antidepressant. Moreover,
390 we also applied propensity score matching to select the unexposed control group, thereby improving inference
391 of causation [57]. Finally, we cover multiple different domains of neurodevelopment at several timepoints
392 throughout early life, and also assess ADHD at both the diagnosis and symptoms level [73].

393 In conclusion, we did not identify significant differences in DNAm associated with prenatal exposure to
394 (es)citalopram or maternal depression. There were more ADHD symptoms, as well as delayed communication
395 and psychomotor skills among children exposed to (es)citalopram compared to the controls. Differences in
396 DNAm were associated with child neurodevelopmental trajectory classes reflecting symptom severity.
397 Consequently, DNAm may be potential predictive molecular markers of later abnormal neurodevelopmental
398 outcomes. Future studies are needed for replication and assessment of a functional impact on neuronal
399 differentiation and developmental processes in model systems. Additionally, it will also be important to
400 improve causal inference by integrating genetic data and simulate causal relationships using machine learning

401 approaches on real-world and artificial data. This can elucidate the properties of causal relationships in
402 observational studies using molecular data.

403 Acknowledgements

404 Availability of data and materials

405 The data that support the findings of this study are available from the Norwegian Mother, Father and Child
406 Cohort Study, but restrictions apply to the availability of these data and so are not publicly available. However,
407 data are available from the authors upon reasonable request and with permission from the Norwegian Mother,
408 Father and Child Cohort Study.

409 Competing interests

410 The authors declare that they have no competing interests.

411 Funding

412 This work was supported by the European Research Council (ERC) Starting Grant "DrugsInPregnancy" (grant
413 number: 678033) and the Research Council of Norway (NFR) "Medications during pregnancy: effects on
414 neurodevelopmental disorders in childhood and epigenetic outcomes" (grant number: 241117). MoBa is
415 supported by the Norwegian Ministry of Health and the Ministry of Education and Research, NIH/NIEHS
416 (contract number: NO1-ES-75558), NIH/NINDS (grant numbers: UO1 NS 047537-01 and UO1 NS047537-
417 06A1), and the Norwegian Research Council/FUGE (grant numbers; 151918/S10).

418 Authors' contribution

419 EWO, HMEN, RL and KG conceived the idea of and designed the study. EWO conducted the analyses. EWO
420 generated plots and tables. EWO and KG drafted the first version of the paper. EWO, HMEN, RL, GKS and
421 KG all revised the paper. All authors read and approved the final manuscript.

422 Acknowledgements

423 The Norwegian Mother, Father and Child Cohort Study is supported by the Norwegian Ministry of Health and
424 Care Services and the Ministry of Education and Research. We are grateful to all the participating families in

425 Norway taking part in this on-going cohort study. We thank Kaja Kristine Selmer for critical appraisal of the
426 article.

427 Supplementary Information

428 Supplementary information is available at *Molecular Psychiatry*'s website.

429 References

430 1. Woody CA, Ferrari AJ, Siskind DJ, Whiteford HA, Harris MG. A systematic review and meta-
431 regression of the prevalence and incidence of perinatal depression. J Affect Disord. J Affect Disord;
432 2017;219:86–92.

433 2. Jarde A, Morais M, Kingston D, Giallo R, MacQueen GM, Giglia L, et al. Neonatal Outcomes in
434 Women With Untreated Antenatal Depression Compared With Women Without Depression: A
435 Systematic Review and Meta-analysis. JAMA Psychiatry. American Medical Association;
436 2016;73:826–37.

437 3. Rogers A, Obst S, Teague SJ, Rossen L, Spry EA, MacDonald JA, et al. Association Between
438 Maternal Perinatal Depression and Anxiety and Child and Adolescent Development: A Meta-
439 analysis. JAMA Pediatr. American Medical Association; 2020;174:1082–92.

440 4. Charlton R, Jordan S, Pierini A, Garne E, Neville A, Hansen A, et al. Selective serotonin reuptake
441 inhibitor prescribing before, during and after pregnancy: a population-based study in six European
442 regions. BJOG An Int J Obstet Gynaecol. Blackwell Publishing Ltd; 2015;122:1010–20.

443 5. Molenaar NM, Bais B, Lambregtse-van den Berg MP, Mulder CL, Howell EA, Fox NS, et al. The
444 international prevalence of antidepressant use before, during, and after pregnancy: A systematic
445 review and meta-analysis of timing, type of prescriptions and geographical variability. J Affect
446 Disord. Elsevier; 2020;264:82–9.

447 6. Mitchell AA, Gilboa SM, Werler MM, Kelley KE, Louik C, Hernández-Díaz S. Medication use
448 during pregnancy, with particular focus on prescription drugs: 1976-2008. Am J Obstet Gynecol.
449 Mosby; 2011;205:51.e1-51.e8.

450 7. Haas DM, Marsh DJ, Dang DT, Parker CB, Wing DA, Simhan HN, et al. Prescription and Other
451 Medication Use in Pregnancy. Obstet Gynecol. NIH Public Access; 2018;131:789.
452 8. Lupattelli A, Spigset O, Twigg MJ, Zagorodnikova K, Mårdby AC, Moretti ME, et al. Medication
453 use in pregnancy: A cross-sectional, multinational web-based study. BMJ Open. 2014;4:4365.
454 9. Zoega H, Kieler H, Norgaard M, Furu K, Valdimarsdottir U, Brandt L, et al. Use of SSRI and SNRI
455 antidepressants during pregnancy: A population-based study from Denmark, Iceland, Norway and
456 Sweden. PLoS One. Public Library of Science; 2015;10.
457 10. Man KKC, Chan EW, Ip P, Coghill D, Simonoff E, Chan PKL, et al. Prenatal antidepressant
458 exposure and the risk of attention-deficit hyperactivity disorder in children: A systematic review and
459 meta-analysis. Neurosci Biobehav Rev. Neurosci Biobehav Rev; 2018;86:1–11.
460 11. Morales DR, Slattery J, Evans S, Kurz X. Antidepressant use during pregnancy and risk of autism
461 spectrum disorder and attention deficit hyperactivity disorder: Systematic review of observational
462 studies and methodological considerations. BMC Med. BioMed Central Ltd.; 2018;16:1–14.
463 12. Sujan AC, Öberg AS, Quinn PD, D'Onofrio BM. Maternal antidepressant use during pregnancy and
464 offspring neurodevelopmental problems – a critical review and recommendations for future research.
465 J Child Psychol Psychiatry. NIH Public Access; 2019;60:356.
466 13. Viuff AC, Pedersen LH, Kyng K, Staunstrup NH, Borglum A, Henriksen TB. Antidepressant
467 medication during pregnancy and epigenetic changes in umbilical cord blood: a systematic review.
468 Clin Epigenetics. 2016;8:94.
469 14. Olstad EW, Nordeng HME, Gervin K. Prenatal medication exposure and epigenetic outcomes: a
470 systematic literature review and recommendations for prenatal pharmacoepigenetic studies.
471 Epigenetics. Epigenetics; 2021;
472 15. Kallak TK, Bränn E, Fransson E, Johansson Å, Lager S, Comasco E, et al. DNA methylation in cord
473 blood in association with prenatal depressive symptoms. Clin Epigenetics. BioMed Central Ltd;
474 2021;13:1–14.
475 16. Cardenas A, Faleschini S, Cortes Hidalgo A, Rifas-Shiman SL, Baccarelli AA, Demeo DL, et al.
476 Prenatal maternal antidepressants, anxiety, and depression and offspring DNA methylation:

477 Epigenome-wide associations at birth and persistence into early childhood. Clin Epigenetics.
478 2019;11 (1) (no.

479 17. Schroeder JW, Smith AK, Brennan PA, Conneely KN, Kilaru V, Knight BT, et al. DNA methylation
480 in neonates born to women receiving psychiatric care. Epigenetics. 2012;7:409–14.

481 18. Gurnot C, Martin-Subero I, Mah SM, Weikum W, Goodman SJ, Brain U, et al. Prenatal
482 antidepressant exposure associated with CYP2E1 DNA methylation change in neonates. Epigenetics.
483 2015;10:361–72.

484 19. Non AL, Binder AM, Kubzansky LD, Michels KB. Genome-wide DNA methylation in neonates
485 exposed to maternal depression, anxiety, or SSRI medication during pregnancy. Epigenetics.
486 2014;9:964–72.

487 20. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to
488 maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and
489 infant cortisol stress responses. Epigenetics. 2008;3:97–106.

490 21. Gartstein MA, Hookenson K V, Brain U, Devlin AM, Grunau RE, Oberlander TF. Sculpting infant
491 soothability: the role of prenatal SSRI antidepressant exposure and neonatal SLC6A4 methylation
492 status. Dev Psychobiol. 2016;58:745–58.

493 22. Galbally M, Watson SJ, van Ijzendoorn M, Saffery R, Ryan J, de Kloet ER, et al. The role of
494 glucocorticoid and mineralocorticoid receptor DNA methylation in antenatal depression and infant
495 stress regulation. Psychoneuroendocrinology. 2020;115:104611.

496 23. Ryan J, Mansell T, Fransquet P, Saffery R. Does maternal mental well-being in pregnancy impact
497 the early human epigenome? Epigenomics. 2017;9:313–32.

498 24. Kallak TK, Fransson E, Bränn E, Berglund H, Lager S, Comasco E, et al. Maternal prenatal
499 depressive symptoms and toddler behavior: an umbilical cord blood epigenome-wide association
500 study. Transl Psychiatry 2022 121. Nature Publishing Group; 2022;12:1–11.

501 25. Cecil CAM, Nigg JT. Epigenetics and ADHD: Reflections on Current Knowledge, Research
502 Priorities and Translational Potential. Mol Diagnosis Ther. Adis; 2022;26:581–606.

503 26. Walton E, Pingault JB, Cecil CAM, Gaunt TR, Relton CL, Mill J, et al. Epigenetic profiling of
504 ADHD symptoms trajectories: a prospective, methylome-wide study. Mol Psychiatry. Mol
505 Psychiatry; 2017;22:250–6.

506 27. Neumann A, Walton E, Alemany S, Cecil C, González JR, Jima DD, et al. Association between
507 DNA methylation and ADHD symptoms from birth to school age: a prospective meta-analysis.
508 Transl Psychiatry. Springer Nature; 2020;10.

509 28. Magnus P, Birke C, Vejrup K, Haugan A, Alsaker E, Daltveit AK, et al. Cohort Profile Update: The
510 Norwegian Mother and Child Cohort Study (MoBa). Int J Epidemiol. Int J Epidemiol; 2016;45:382–
511 8.

512 29. Rønningen KS, Paltiel L, Meltzer HM, Nordhagen R, Lie KK, Hovengen R, et al. The biobank of
513 the Norwegian Mother and Child Cohort Study: a resource for the next 100 years. Eur J Epidemiol.
514 Eur J Epidemiol; 2006;21:619–25.

515 30. Lupattelli A, Chambers CD, Bandoli G, Handal M, Skurtveit S, Nordeng H. Association of Maternal
516 Use of Benzodiazepines and Z-Hypnotics During Pregnancy With Motor and Communication Skills
517 and Attention-Deficit/Hyperactivity Disorder Symptoms in Preschoolers. JAMA Netw open. JAMA
518 Netw Open; 2019;2:e191435.

519 31. Sundbakk LM, Wood M, Gran JM, Nordeng H. Impact of prenatal exposure to benzodiazepines and
520 z-hypnotics on behavioral problems at 5 years of age: A study from the Norwegian Mother and Child
521 Cohort Study. PLoS One. 2019/06/07. 2019;14:e0217830.

522 32. Brandlistuen RE, Ystrom E, Hernandez-Diaz S, Skurtveit S, Selmer R, Handal M, et al. Association
523 of prenatal exposure to benzodiazepines and child internalizing problems: A sibling-controlled
524 cohort study. PLoS One. PLoS One; 2017;12.

525 33. Bromley R, Weston J, Adab N, Greenhalgh J, Sanniti A, Mckay AJ, et al. Treatment for epilepsy in
526 pregnancy: neurodevelopmental outcomes in the child. Cochrane database Syst Rev. Cochrane
527 Database Syst Rev; 2014;2014.

528 34. Betcher HK, Montiel C, Clark CT. Use of Antipsychotic Drugs During Pregnancy. Curr Treat
529 options psychiatry. NIH Public Access; 2019;6:17.

530 35. Poels EMP, Schrijver L, Kamperman AM, Hillegers MHJ, Hoogendijk WJG, Kushner SA, et al.
531 Long-term neurodevelopmental consequences of intrauterine exposure to lithium and antipsychotics:
532 a systematic review and meta-analysis. Eur Child Adolesc Psychiatry. Eur Child Adolesc Psychiatry;
533 2018;27:1209–30.

534 36. Veroniki AA, Rios P, Cogo E, Straus SE, Finkelstein Y, Kealey R, et al. Comparative safety of
535 antiepileptic drugs for neurological development in children exposed during pregnancy and breast
536 feeding: a systematic review and network meta-analysis. BMJ Open. BMJ Open; 2017;7.

537 37. Tomson T, Battino D, Perucca E. Teratogenicity of antiepileptic drugs. Curr Opin Neurol. Curr Opin
538 Neurol; 2019;32:246–52.

539 38. Derogatis LR, Lipman RS, Covi L. The SCL-90: an outpatient psychiatric rating scale.
540 Psychopharmacol Bull. 1973;9:13–28.

541 39. Derogatis LR, Lipman RS, Rickels K, Uhlenhuth EH, Covi L. The Hopkins Symptom Checklist
542 (HSCL): a self-report symptom inventory. Behav Sci. Behav Sci; 1974;19:1–15.

543 40. Hesbacher PT, Rickels K, Morris RJ, Newman H, Rosenfeld H. Psychiatric illness in family practice.
544 J Clin Psychiatry. 1980;41:6–10.

545 41. Tambs K, Røysamb E. Selection of questions to short-form versions of original psychometric
546 instruments in MoBa. Nor Epidemiol. Norwegian Epidemiological Society; 2014;24:195–201.

547 42. Strand BH, Dalgard OS, Tambs K, Rognerud M. Measuring the mental health status of the
548 Norwegian population: a comparison of the instruments SCL-25, SCL-10, SCL-5 and MHI-5 (SF-
549 36). Nord J Psychiatry. Nord J Psychiatry; 2003;57:113–8.

550 43. Gervin K, Nordeng H, Ystrom E, Reichborn-kjennerud T, Lyle R. Long-term prenatal exposure to
551 paracetamol is associated with DNA methylation differences in children diagnosed with ADHD.
552 Clin Epigenetics. 2017;9.

553 44. Müller F, Scherer M, Assenov Y, Lutsik P, Walter J, Lengauer T, et al. RnBeads 2.0: Comprehensive
554 analysis of DNA methylation data. Genome Biol. BioMed Central Ltd.; 2019;20:55.

555 45. Assenov Y, Müller F, Lutsik P, Walter J, Lengauer T, Bock C. Comprehensive analysis of DNA

556 methylation data with RnBeads. Nat Methods 2014 1111. Nature Publishing Group; 2014;11:1138–
557 40.

558 46. Pidsley R, Zotenko E, Peters TJ, Lawrence MG, Risbridger GP, Molloy P, et al. Critical evaluation
559 of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation
560 profiling. Genome Biol. 2016;17.

561 47. Xu Z, Niu L, Li L, Taylor JA. ENmix: A novel background correction method for Illumina
562 HumanMethylation450 BeadChip. Nucleic Acids Res. Oxford University Press; 2016;44:e20.

563 48. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, et al. A beta-
564 mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k
565 DNA methylation data. Bioinformatics. Bioinformatics; 2013;29:189–96.

566 49. Achenbach TM, Ruffle TM. The Child Behavior Checklist and related forms for assessing
567 behavioral/emotional problems and competencies. Pediatr Rev. Pediatr Rev; 2000;21:265–71.

568 50. Nøvik TS. Validity of the child behaviour checklist in a Norwegian sample. Eur Child Adolesc
569 Psychiatry. Eur Child Adolesc Psychiatry; 1999;8:247–54.

570 51. Squires Potter, L., & Bricker, D J. The ASQ User's Guide. ASQ User's Guid. Paul H. Brookes; 1999.

571 52. Fages A, Ferrari P, Monni S, Dossus L, Floegel A, Mode N, et al. Investigating sources of variability
572 in metabolomic data in the EPIC study: the Principal Component Partial R-square (PC-PR2) method.
573 Metabolomics. Springer New York LLC; 2014;10:1074–83.

574 53. Perrier F, Novoloaca A, Ambatipudi S, Baglietto L, Ghantous A, Perduca V, et al. Identifying and
575 correcting epigenetics measurements for systematic sources of variation. Clin Epigenetics. Clin
576 Epigenetics; 2018;10.

577 54. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a
578 flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation
579 microarrays. 2014;30:1363–9.

580 55. Gervin K, Page CM, Aass HCD, Jansen MA, Fjeldstad HE, Andreassen BK, et al. Cell type specific
581 DNA methylation in cord blood: A 450K-reference data set and cell count-based validation of

582 estimated cell type composition. Epigenetics. 2016;11:690–8.

583 56. Gervin K, Salas LA, Bakulski KM, Van Zelm MC, Koestler DC, Wiencke JK, et al. Systematic
584 evaluation and validation of reference and library selection methods for deconvolution of cord blood
585 DNA methylation data. Clin Epigenetics. 2019;11.

586 57. Wood ME, Lapane KL, van Gelder MMHJ, Rai D, Nordeng HME. Making fair comparisons in
587 pregnancy medication safety studies: An overview of advanced methods for confounding control.
588 Pharmacoepidemiol Drug Saf. Pharmacoepidemiol Drug Saf; 2018;27:140–7.

589 58. Austin PC. Optimal caliper widths for propensity-score matching when estimating differences in
590 means and differences in proportions in observational studies. Pharm Stat. Pharm Stat; 2011;10:150–
591 61.

592 59. Wood ME, Lupattelli A, Palmsten K, Bandoli G, Hurault-Delarue C, Damase-Michel C, et al.
593 Longitudinal Methods for Modeling Exposures in Pharmacoepidemiologic Studies in Pregnancy.
594 Epidemiol Rev. Oxford University Press; 2021;43:130.

595 60. Proust-Lima C, Philipps V, Liquet B. Estimation of Extended Mixed Models Using Latent Classes
596 and Latent Processes: The R Package lcmm. J Stat Softw. American Statistical Association;
597 2017;78:1–56.

598 61. Proust-Lima C, Dartigues JF, Jacqmin-Gadda H. Misuse of the linear mixed model when evaluating
599 risk factors of cognitive decline. Am J Epidemiol. Inserm; 2011;174:1077.

600 62. Du P, Zhang X, Huang C-C, Jafari N, Kibbe WA, Hou L, et al. Comparison of Beta-value and M-
601 value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics.
602 2010;11:587.

603 63. Smyth GK. limma: Linear Models for Microarray Data. Bioinforma Comput Biol Solut Using R
604 Bioconductor. Springer-Verlag; 2005. p. 397–420.

605 64. Benjamini Y. Discovering the false discovery rate. J R Stat Soc Ser B (Statistical Methodol.
606 2010;72:405–16.

607 65. Hansen K. IlluminaHumanMethylationEPICanno.ilm10b4.hg19: Annotation for Illumina's EPIC

608 methylation arrays. R package version 0.6.0 [Internet]. 2017.

609 66. Phipson B, Maksimovic J, Oshlack A. missMethyl: an R package for analyzing data from Illumina's
610 HumanMethylation450 platform. Bioinformatics. Bioinformatics; 2016;32:286–8.

611 67. Edgar RD, Jones MJ, Meaney MJ, Turecki G, Kobor MS. BECon: a tool for interpreting DNA
612 methylation findings from blood in the context of brain. Transl Psychiatry 2017 78. Nature
613 Publishing Group; 2017;7:e1187–e1187.

614 68. Bateman BT, Mhyre JM, Hernandez-Diaz S, Huybrechts KF, Fischer MA, Creanga AA, et al.
615 Development of a comorbidity index for use in obstetric patients. Obstet Gynecol. Obstet Gynecol;
616 2013;122:957–65.

617 69. Easter SR, Bateman BT, Sweeney VH, Manganaro K, Lassey SC, Gagne JJ, et al. A comorbidity-
618 based screening tool to predict severe maternal morbidity at the time of delivery. Am J Obstet
619 Gynecol. Am J Obstet Gynecol; 2019;221:271.e1-271.e10.

620 70. Wilmot B, Fry R, Smeester L, Musser ED, Mill J, Nigg JT. Methylomic analysis of salivary DNA
621 in childhood ADHD identifies altered DNA methylation in VIPR2. J Child Psychol Psychiatry Allied
622 Discip. J Child Psychol Psychiatry; 2016;57:152–60.

623 71. Ma SL, Chen LH, Lee CC, Lai KYC, Hung SF, Tang CP, et al. Genetic Overlap Between Attention
624 Deficit/Hyperactivity Disorder and Autism Spectrum Disorder in SHANK2 Gene. Front Neurosci.
625 Frontiers Media SA; 2021;15.

626 72. Inkster AM, Konwar C, Peñaherrera MS, Brain U, Khan A, Price EM, et al. Profiling placental DNA
627 methylation associated with maternal SSRI treatment during pregnancy. Sci Reports 2022 121.
628 Nature Publishing Group; 2022;12:1–14.

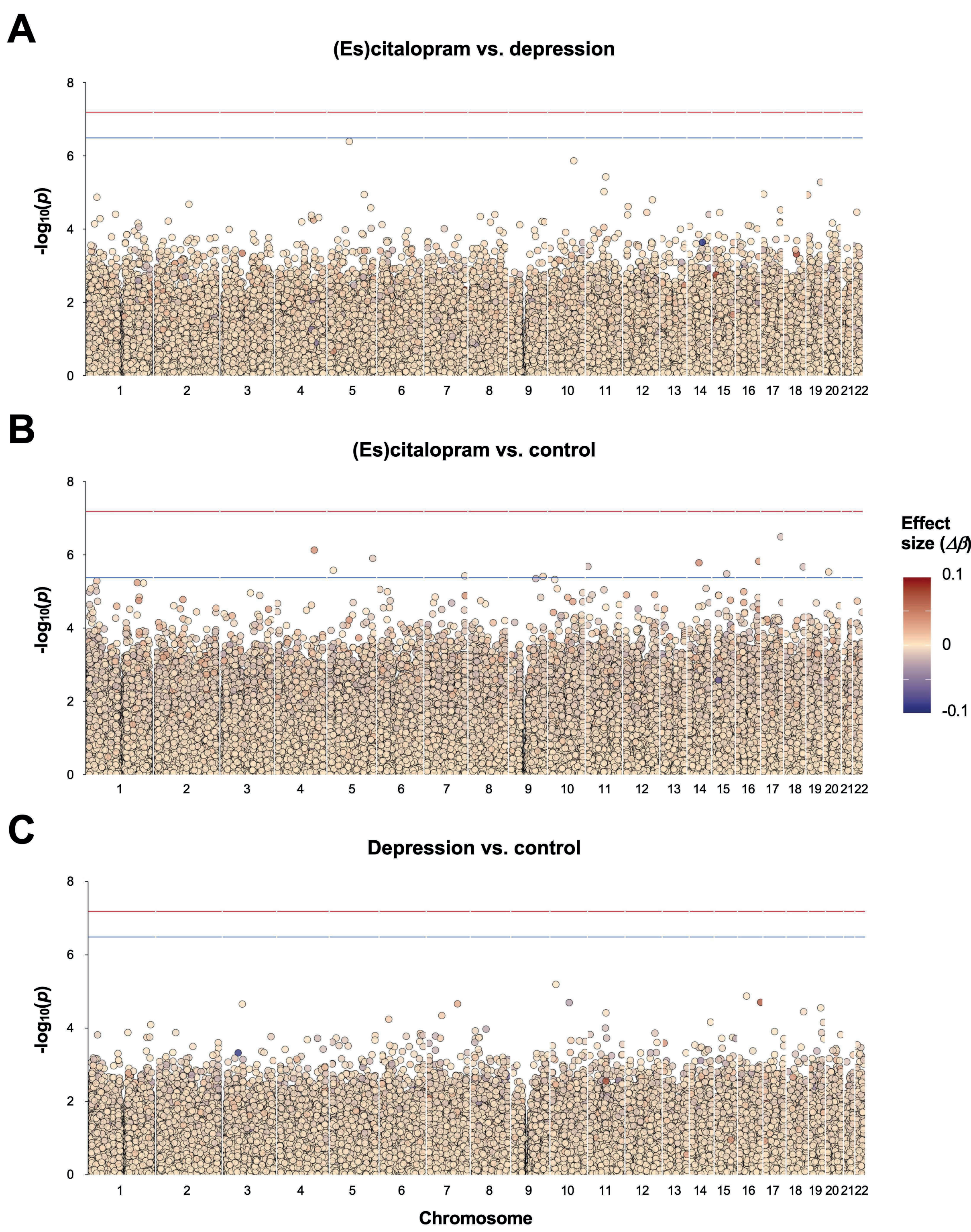
629 73. Franke B, Michelini G, Asherson P, Banaschewski T, Bilbow A, Buitelaar JK, et al. Live fast, die
630 young? A review on the developmental trajectories of ADHD across the lifespan. Eur
631 Neuropsychopharmacol. Eur Neuropsychopharmacol; 2018;28:1059–88.

632 74. Vejrup K, Magnus P, Magnus M. Lost to follow-up in the Norwegian mother, father and child cohort
633 study. Paediatr Perinat Epidemiol. John Wiley & Sons, Ltd; 2022;36:300–9.

634 75. Nordeng H, Lupattelli A, Wood M. Prenatal exposure to antidepressants and increased risk of
635 psychiatric disorders. BMJ. BMJ; 2017;358.

636

637 # Figures



638

639 **Figure 1. Modified Manhattan plots of difference in DNAm between groups.**

640 Log_{10} *p* value against the genomic positions of the CpGs. Each dot represents a CpG, colored according to the
641 DNAm difference between **(A)** the (es)citalopram and depression groups, **(B)** the (es)citalopram and control groups,
642 and **(C)** the depression and control groups. The red lines indicate the FDR significance cutoff (<0.05) and the blue
643 lines indicate a liberal FDR significance cutoff (<0.25).

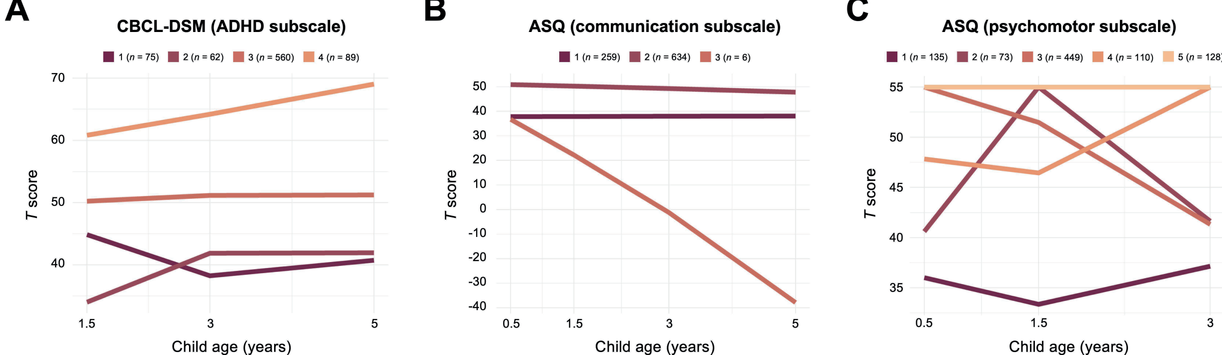


Figure 2. Neurodevelopmental trajectories identified using latent class growth analysis.
Trajectories were identified for **(A)** the CBCL-DSM ADHD subscale, **(B)** the ASQ communication subscale, and **(C)** the ASQ psychomotor subscale.

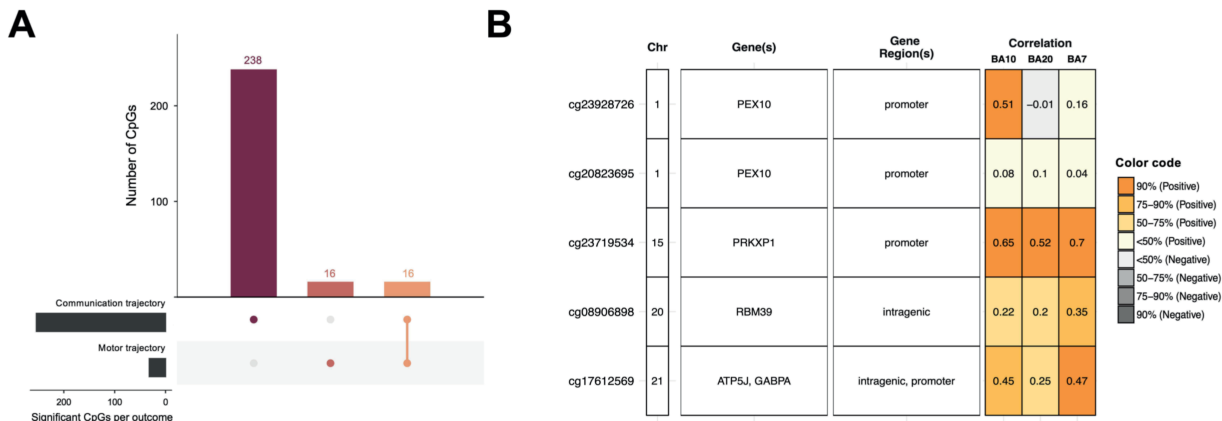


Figure 3. CpGs associated with developmental trajectories and blood-brain correlation of DNAm.
(A) Upset plot [70, 71] showing overlap of significant CpGs associated with communication and/or psychomotor developmental trajectories. Overlapping CpGs are indicated by filled dots for the respective outcomes. The vertical bar plot indicates the number of CpGs for the particular intersection. **(B)** Blood-brain correlation of significant CpGs associated with both communication and psychomotor trajectories. Correlation is reported as the Spearman's correlation coefficient of DNAm between blood and brain. Modified plot from the BECon web application [67].

Table

Table 1. Overview of which questionnaires are used to assess neurodevelopmental outcomes in the children.

<table>
<tr><th></th><th>0.5 years (Q4)</th><th>1.5 years (Q5)</th><th>3 years (Q6)</th><th>5 years (Q5y)</th><th>Score interpretation</th></tr>
<tr><td>ADHD</td><td></td><td colspan="3">CBCL-DSM (ADHD)</td><td>Higher score = more symptoms</td></tr>
<tr><td>Communication</td><td colspan="4">ASQ (communication)</td><td>Lower score = poorer skills</td></tr>
<tr><td>Psychomotor</td><td colspan="3">ASQ (psychomotor)</td><td></td><td>Lower score = poorer skills</td></tr>
</table>

Abbreviations: ADHD: attention-deficit/hyperactivity disorder; ASQ: Ages and Stages Questionnaire; CBCL-DSM: Child Behaviour Checklist DSM-oriented subscale; Q-: MoBa questionnaire.

Table 2. Overview of the comparison group characteristics.

	(Es)citalopram group (*n* = 306)	Depression group (*n* = 308)	Control group (*n* = 344)	*p*
Maternal characteristics				
Maternal age (mean years ± SD)	30.3 ± 5.2	28.4 ± 5.3	30.9 ± 4.6	a,c
Pre-pregnancy BMI (mean BMI ± SD)	24.5 ± 5.1 *8 NA*	24.3 ± 4.8 *7 NA*	23.8 ± 4.2 *2 NA*	N.S.
Maternal education				
University/college (*n* (%))	178 (58.2)	136 (44.2)	247 (71.8)	
High school or lower (*n* (%))	124 (40.5) *4 NA*	163 (52.9) *9 NA*	89 (25.9) *8 NA*	a,b,c
Smoking in pregnancy (yes; *n* (%))	43 (14.1) *2 NA*	52 (16.9)	28 (8.1)	b,c
Alcohol in pregnancy (yes; *n* (%))	36 (11.8) *49 NA*	28 (9.1) *45 NA*	64 (18.6) *15 NA*	c
Folic acid in pregnancy (yes; *n* (%))	182 (59.5)	168 (54.6)	205 (59.6)	N.S.
Maternal medications				
Analgesics[a] (yes; *n* (%))	190 (62.1)	191 (62.0)	178 (51.7)	b,c
Antidepressants except (es)citalopram (yes; *n* (%))	19 (6.2)	---	---	---
NSAIDs (yes; *n* (%))	55 (18.0)	45 (14.6)	30 (8.7)	b,c
Maternal morbidities				
Comorbidity index** (mean score ± SD)	0.5 ± 0.9 *27 NA*	0.5 ± 0.9 *6 NA*	0.4 ± 0.9 *13 NA*	N.S.
Chronic diseases***				
None (*n* (%))	277 (90.5)	280 (90.9)	325 (94.5)	
1–2 diseases (*n* (%))	27 (8.8)	28 (9.1)	19 (5.5)	N.S.
≥3 diseases (*n* (%))	0 (0) *2 NA*	0 (0)	0 (0)	
SCL-5 (mean score ± SD)	1.9 ± 0.8 *16 NA*	2.8 ± 0.5	1.0 ± 0	a,b,c
SCL-8 (mean score ± SD)	1.7 ± 0.6 *44 NA*	2.7 ± 0.5	1.0 ± 0	a,b,c
Life-time history of major depression (yes; *n* (%))	136 (44.4) *7 NA*	101 (32.8) *9 NA*	114 (33.1) *1 NA*	a,b
Child characteristics				
Birth weight (mean grams ± SD)	3 568 ± 501	3 579 ± 512	3 629 ± 503 *1 NA*	b
Gestational age (mean weeks ± SD)	39.4 ± 1.5 *1 NA*	39.4 ± 1.6 *1 NA*	39.7 ± 1.5 *1 NA*	b,c
Infant sex (female; *n* (%))	148 (48.4)	149 (48.4)	181 (52.6)	N.S.

Abbreviations: ADHD: attention-deficit/hyperactivity disorder; ASQ: Ages and Stages Questionnaire; BMI: body mass index; CBCL-DSM: Child Behaviour Checklist DSM-oriented subscale; NA: missing value; N.S.: not significant; SCL: Hopkins Symptom Checklist; SD: standard deviation.

* All medications with ATC N02.

** All variables available in MBRN and MoBa from list by Bateman *et al.* [68,69].

*** Chronic diseases include: asthma, rheumatoid arthritis, epilepsy, Crohn's disease, lupus, multiple sclerosis (MS), cancer and diabetes mellitus.

Significant difference between: [a] the (es)citalopram and depression groups; [b] the (es)citalopram and control groups; [c] the depression and control groups.

657

Table 3. Overview of child neurodevelopmental outcomes by comparison group.

	(Es)citalopram group (*n* = 306)	Depression group (*n* = 308)	Control group (*n* = 344)	*p*
ADHD diagnosis				
ADHD diagnosis (*n* (%))	23 (7.5)	18 (5.8)	10 (2.9)	b
ADHD assessment				
CBCL-DSM (ADHD subscale) – 1.5 years (median *T* score ± SD)	46.3 (12.3) *94 NA*	52.5 (12.3) *106 NA*	46.3 (12.3) *58 NA*	a,c
CBCL-DSM (ADHD subscale) – 3 years (median *T* score ± SD)	52.1 (17.4) *140 NA*	52.1 (17.4) *148 NA*	47.7 (13.1) *103 NA*	b,c
CBCL-DSM (ADHD subscale) – 5 years (median *T* score ± SD)	51.7 (13.4) *178 NA*	56.2 (17.8) *206 NA*	47.3 (17,8) *177 NA*	a,b,c
Communication assessment				
ASQ (communication subscale) – 0.5 years (median *T* score (IQR))	55.3 (0) *47 NA*	55.3 (14.5) *50 NA*	55.3 (14.5) *12 NA*	**N.S.**
ASQ (communication subscale) – 1.5 years (median *T* score (IQR))	51.6 (19.7) *90 NA*	58.1 (13.1) *103 NA*	58.1 (13.1) *54 NA*	b
ASQ (communication subscale) – 3 years (median *T* score (IQR))	55.7 (8.5) *141 NA*	55.7 (8.5) *146 NA*	55.7 (8.5) *106 NA*	**N.S.**
ASQ (communication subscale) – 5 years (median *T* score (IQR))	55.8 (7.3) *189 NA*	48.5 (14.6) *215 NA*	55.8 (7.3) *199 NA*	b,c
Motor assessments				
ASQ (psychomotor subscale) – 0.5 years (median *T* score (IQR))	55.6 (9.0) *47 NA*	55.6 (9.0) *49 NA*	55.6 (9.0) *12 NA*	b
ASQ (psychomotor subscale) – 1.5 years (median *T* score (IQR))	55.4 (7.2) *92 NA*	55.4 (14.5) *102 NA*	55.4 (7.2) *62 NA*	**N.S.**
ASQ (psychomotor subscale) – 3 years (median *T* score (IQR))	51.3 (14.9) *142 NA*	51.3 (14.9) *151 NA*	51.3 (14.9) *108 NA*	**N.S.**
Age-of-onset of independent walking (median number of months (IQR))	13.0 (2.3) *147 NA*	12.6 (2.4) *161 NA*	12.6 (2.1) *114 NA*	**N.S.**

Abbreviations: ADHD: attention-deficit/hyperactivity disorder; ASQ: Ages and Stages Questionnaire; CBCL-DSM: Child Behaviour Checklist DSM-oriented subscale; IQR: interquartile range; NA: missing value; N.S.: not significant; SD: standard deviation.

Significant difference between: [a] the (es)citalopram and depression groups; [b] the (es)citalopram and control groups; [c] the depression and control groups.

658

Supplementary Information

Supplementary Methods

Validity of exposure and outcome measures

Prenatal (es)citalopram exposure

We used maternal self-reports of (es)citalopram use in MoBa. MoBa antidepressant self-reports and redeemed prescriptions registered in the Norwegian Prescription Database (NorPD) have shown good agreement, with 87.0% of reports of antidepressant use in MoBa also found to have filled a prescription for the antidepressant [1].

Maternal depression and depressive symptoms

The Hopkins Symptom Checklist is an internationally recognized instrument to measure symptoms of mental disorders, including depression and anxiety [2, 3]. The original instrument consists of 90 items. However, MoBa includes shorter versions of 5 or 8 items (SCL-5 and -8, respectively), relating specifically to symptoms of depression and anxiety [4]. SCL-5 and -8 have been shown to correlate well with the full-item instrument (0.90 and 0.92 respectively) [4]. The SCL-5 has a sensitivity 82.0% and a specificity of 96.0% [5].

Neurodevelopmental outcomes

The Child Behavior Checklist DSM-oriented (CBCL-DSM) ADHD subscale, and the Ages and Stages Questionnaire (ASQ) communication and psychomotor (covering fine and gross motor) subscales were used to measure different domains of neurodevelopment. When compared to ADHD diagnoses from semi-structured clinical interviews, the CBCL-DSM ADHD subscale exhibits a moderate agreement ($\kappa = 0.51$), a sensitivity of 81% and a specificity of 70% [6]. Only a subset of the original ASQ items were included in MoBa and these items span different age ranges in the ASQ questionnaires, to introduce more variation in scores across individuals (Supplementary Table 2). Overall, the ASQ exhibits good agreement (84%) compared to standardized assessments, having a sensitivity of 72% and a specificity of 86% [7]. The Norwegian version of the ASQ has also been validated [8].

Covariate assessment

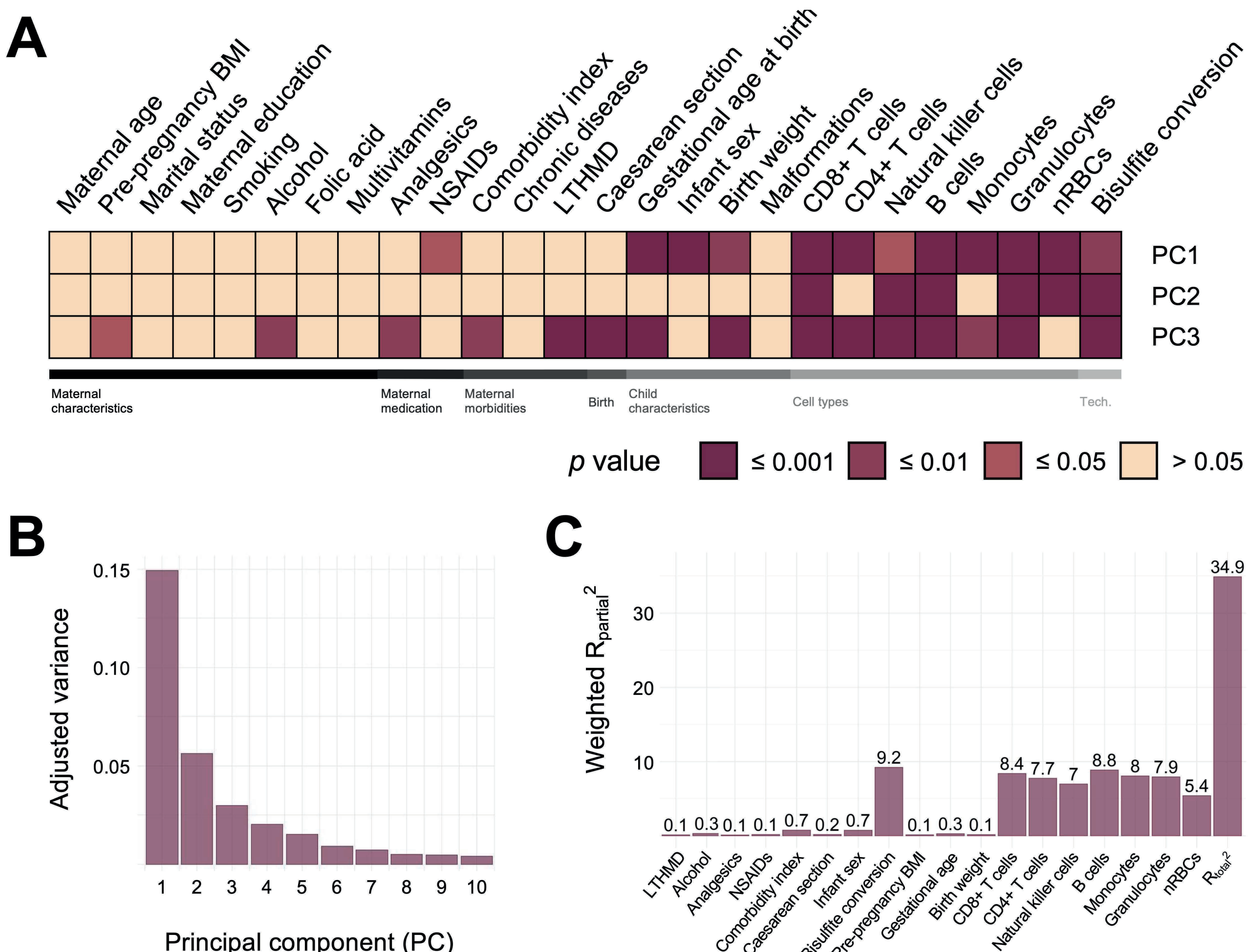
When assessing covariates for inclusion in the regression models, we analyzed covariates previously included in prenatal pharmacoepigenetic studies [9]: maternal age and BMI, marital status, maternal education, alcohol use, smoking, multivitamin use, psychotropic and analgesic use, maternal morbidities, Caesarean section, gestational age, infant sex, birth weight, bisulfite conversion and the estimated composition of white blood cells. The PC-PR^2 method is extensively described elsewhere [10, 11]. Briefly, for each of the PCs 1–3 we fit

linear regression models on the covariates. We computed the partial R^2 for each covariate, reflecting the variation contribution of individual covariates to the total variation in the DNAm PC, when accounting for the contribution of the other covariates.

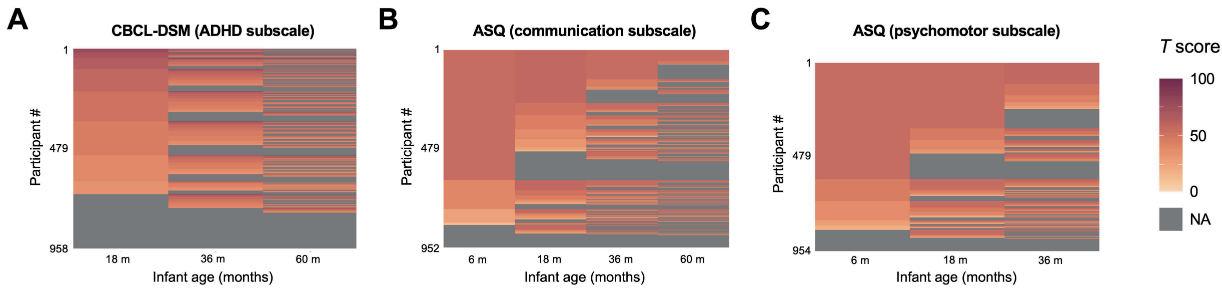
References

1. Skurtveit S, Selmer R, Odsbu I, Handal M. Self-reported data on medicine use in the Norwegian Mother and Child cohort study compared to data from the Norwegian Prescription Database. Nor Epidemiol. Norwegian Epidemiological Society; 2014;24:209–16.
2. Derogatis LR, Lipman RS, Covi L. The SCL-90: an outpatient psychiatric rating scale. Psychopharmacol Bull. 1973;9:13–28.
3. Derogatis LR, Lipman RS, Rickels K, Uhlenhuth EH, Covi L. The Hopkins Symptom Checklist (HSCL): a self-report symptom inventory. Behav Sci. Behav Sci; 1974;19:1–15.
4. Tambs K, Røysamb E. Selection of questions to short-form versions of original psychometric instruments in MoBa. Nor Epidemiol. Norwegian Epidemiological Society; 2014;24:195–201.
5. Strand BH, Dalgard OS, Tambs K, Rognerud M. Measuring the mental health status of the Norwegian population: a comparison of the instruments SCL-25, SCL-10, SCL-5 and MHI-5 (SF-36). Nord J Psychiatry. Nord J Psychiatry; 2003;57:113–8.
6. Skarphedinsson G, Jarbin H, Andersson M, Ivarsson T. Diagnostic efficiency and validity of the DSM-oriented Child Behavior Checklist and Youth Self-Report scales in a clinical sample of Swedish youth. PLoS One. PLOS; 2021;16.
7. Squires Potter, L., & Bricker, D J. The ASQ User’s Guide. ASQ User’s Guid. Paul H. Brookes; 1999.
8. Richter J, Janson H. A validation study of the Norwegian version of the Ages and Stages Questionnaires. Acta Paediatr. Acta Paediatr; 2007;96:748–52.
9. Olstad EW, Nordeng HME, Gervin K. Prenatal medication exposure and epigenetic outcomes: a systematic literature review and recommendations for prenatal pharmacoepigenetic studies. Epigenetics. Epigenetics; 2021;
10. Fages A, Ferrari P, Monni S, Dossus L, Floegel A, Mode N, et al. Investigating sources of variability in metabolomic data in the EPIC study: the Principal Component Partial R-square (PC-PR2) method. Metabolomics. Springer New York LLC; 2014;10:1074–83.
11. Perrier F, Novoloaca A, Ambatipudi S, Baglietto L, Ghantous A, Perduca V, et al. Identifying and correcting epigenetics measurements for systematic sources of variation. Clin Epigenetics. Clin Epigenetics; 2018;10.
12. Achenbach TM, Ruffle TM. The Child Behavior Checklist and related forms for assessing behavioral/emotional problems and competencies. Pediatr Rev. Pediatr Rev; 2000;21:265–71.
13. Bateman BT, Mhyre JM, Hernandez-Diaz S, Huybrechts KF, Fischer MA, Creanga AA, et al. Development of a comorbidity index for use in obstetric patients. Obstet Gynecol. Obstet Gynecol; 2013;122:957–65.
14. Easter SR, Bateman BT, Sweeney VH, Manganaro K, Lassey SC, Gagne JJ, et al. A comorbidity-based screening tool to predict severe maternal morbidity at the time of delivery. Am J Obstet Gynecol. Am J Obstet Gynecol; 2019;221:271.e1-271.e10.

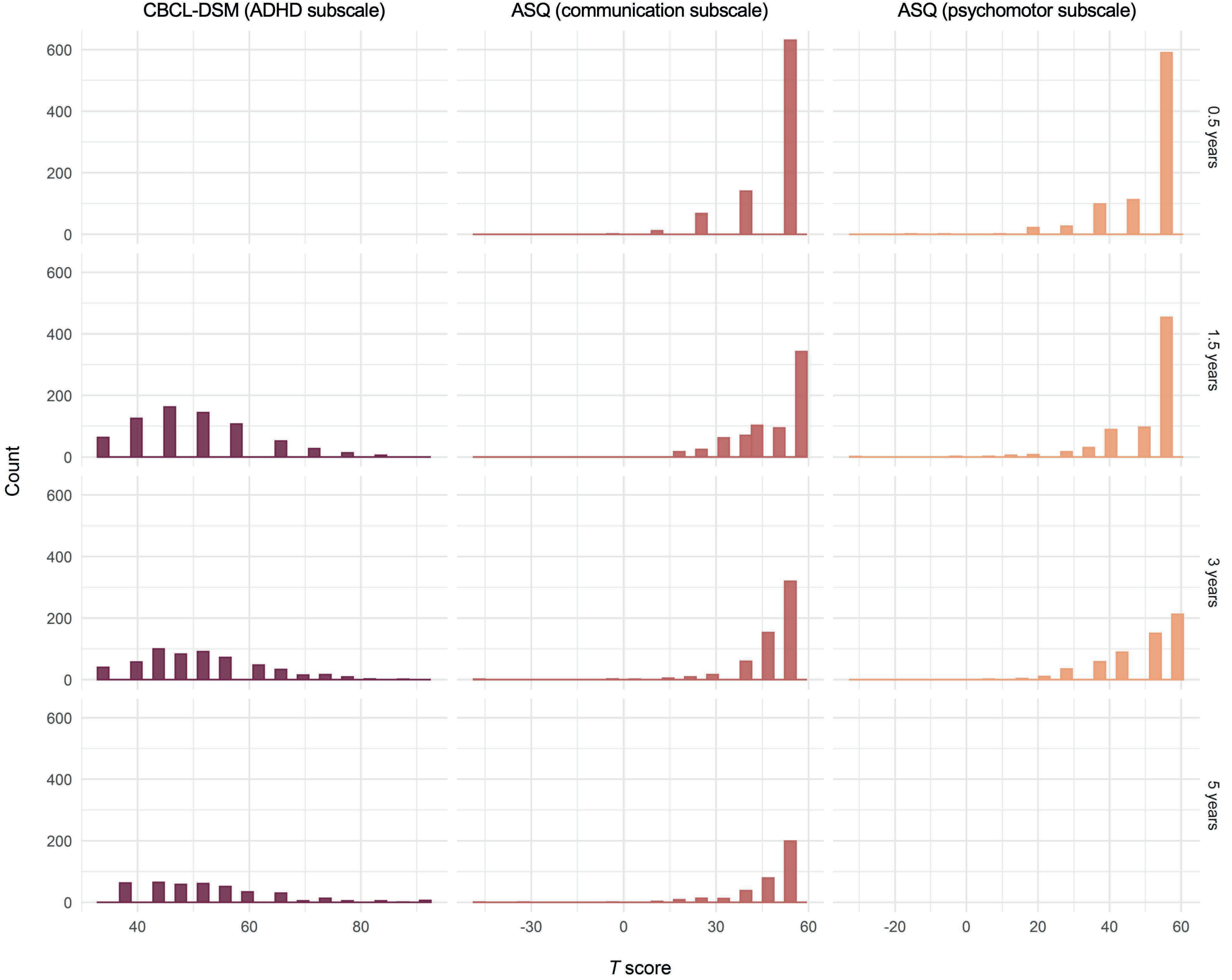
Supplementary Figures



Supplementary Figure 1. Principal component analysis (PCA) and PC-PR^2 to investigate potential covariates. **(A)** Heat map of the association between different covariates and the top three principal components (PCs) representing the most DNAm variation. **(B)** Scree plot indicating the 10 first PCs' contribution to DNAm variation. **(C)** Weighted partial R^2 for each covariate significantly associated with either of the first 3 PCs (i.e., the variability contributed by the respective covariate to the top three PCs, when accounting for the variability contribution of all other covariates in the model). **Abbreviations:** BMI: body mass index; nRBC: nucleated red blood cell; LTHMD: life-time history of major depression; NSAID: non-steroidal anti-inflammatory drug; Tech.: technical covariate.



Supplementary Figure 2. Lasagna plots showing the *T* score for every participant per time point reported. The development of the *T* score for each participant over the time points at which the respective psychometric test was distributed, for **(A)** CBCL-DSM (ADHD subscale), **(B)** ASQ (communication subscale), and **(C)** ASQ (motor subscale). Subjects with *T* scores < 0 were removed for visualization purposes, but were included in statistical analyses ($n_{comm} = 6$ subjects and $n_{motor} = 4$ subjects). **Abbreviations:** ADHD: attention-deficit/hyperactivity disorder; ASQ: Ages and Stages Questionnaire; CBCL-DSM: Child Behavior Checklist, Diagnostic and Statistical Manual of Mental Disorders subscale.



Supplementary Figure 3. Histograms of *T* scores for the psychometric tests per timepoint.
Distribution of *T* scores for subscales of the CBCL-DSM and ASQ questionnaires (columns) across multiple timepoints (rows). **Abbreviations:** ADHD: attention-deficit/hyperactivity disorder; ASQ: Ages and Stages Questionnaire; CBCL-DSM: Child Behavior Checklist, Diagnostic and Statistical Manual of Mental Disorders subscale.

Supplementary Tables

Supplementary Table 1. Overview of the Child Behaviour Checklist (CBCL) items included in the study [12]**. Only items of the ADHD subscale were included.**

Item (Q5/Q6/Q5y)	To what extent are the following statements true of your child's behavior during the last two months?
1 / 2 / 2	Can't concentrate, can't pay attention for long
3 / 3 / 3	Can't sit still, restless or overactive
NA / 4 / 4	Can't stand waiting, wants everything now
NA / 8 / 8	Demands must be met immediately
4 / 15 / 14	Gets into everything
NA / 18 / 24	Poorly coordinated or clumsy
2 / 20 / 19	Quickly shifts from one activity to another

Response options (score): "Not true" (0); "Somewhat or sometimes true" (1); "Very true or often true" (2).

Supplementary Table 2. Overview of the Ages and Stages Questionnaire (ASQ) items included in the study [7]**. Only items of the communication and psychomotor subscales were included.**

	Item	Question
0.5 years	*Communication*	
	4	When you "chat" to your child, does he/she try to "chat" back to you?
	5	Does your child babble and make sounds when he/she is lying on his/her own?
	8	When you call your child, does he/she turn towards you one of the first times you say his/her name?
	Gross motor	
	2	When your child is on his/her tummy, does he/she straighten both arms and push her whole chest off the bed or floor?
	3	Does your child roll over from his/her back onto his/her tummy?
	Fine motor	
	9	Does your child grab a toy you offer and then put it in his/her mouth or hold it?
	10	When your child is sitting on your lap, does he/she stretch out for a toy or something else on the table in front of you?
	11	Does your child hold onto a toy with both hands when he/she is examining it?
1.5 years	*Communication*	
	1	When you ask him/her, does your child go into another room to find a familiar toy or object? (You might ask, "Where is your ball?", or say, "Bring me your coat" or "Go get your blanket")
	2	Does your child say eight or more words in addition to "mama" and "dada"?
	3	Without showing him/her first, does your child point to the correct picture when you say, "Show me the kitty" or ask, "Where is the dog"?
	Gross motor	
	4	Does your child move around by walking, rather than by crawling on his/her hands and knees?
	5	Can your child walk well and seldom fall?
	6	Does your child walk down stairs if you hold onto one of his/her hands?

Age	No.	Item
		Fine motor
	7	Does your child throw a small ball or toy with a forward arm motion? (If he/she drops the ball, mark "Not yet")
	8	Does your child stack a small block or toy on top of another one? (E.g. small boxes or toys about 3 cm in size)
	9	Does your child turn the pages of a book by himself/herself? (He/she may turn more than one page at a time.)
3 years		***Communication***
	5	Without showing him/her first, does your child point to the correct picture when you say, "Where is the cat" or "Where is the dog"? Your child must only point at the correct picture
	6	When you ask your child to point to his/her eyes, nose, hair, feet, ears, and so forth, does he/she correctly point to at least seven body parts? (The child can point to parts of himself/herself, you, or a doll.)
	7	Does your child make sentences that are three or four words long?
	8	Without giving him/her help by pointing or using gestures, ask your child to "Put the shoe on the table" and "Put the book under the chair". Does your child carry out both of these directions correctly?
	9	When looking at a picture book, does your child tell you what is happening or what action is taking place in the picture? (For example, "Barking", "Running", "Eating" and "Crying"?) You may ask, "What is the dog (or boy) doing?"
	10	Can your child tell you at least two things about an object he/she is familiar with? If you say, for example, "Tell me about your ball", will your child answer by saying something like "It is round, I can throw it, it is big."
		Gross motor
	1	Without holding onto anything for support, does your child kick a ball by swinging his/her leg forward?
	2	Can your child catch a large ball with both hands?
		Fine motor
	3	When drawing, does your child hold a pencil, crayon, or pen between his/her fingers and thumb like an adult does?
	4	Can your child undo one or more buttons?
5 years		***Communication***
	1	Can your child tell you at least two things about common object? For example, if you say to your child, "Tell me about the ball", does he say something like, "It is round. I throw it. It is big"?
	2	Without giving your child help by pointing or repeating directions, does your child follow three directions that are unrelated to one another? Give all three directions before your child starts. For example, you may ask your child to "Clap your hands, walk to the door, and sit down" or "Give me the pen, open the book, and stand up."
	3	Does your child use four- and five- word sentences? For example, does your child say, "I want the car"?
	4	When talking about something that already happened, does your child use words that end in "ed" such as walked, jumped or played? Ask your child questions, such as "How did you get to the store?" ("We walked.") "What did you do at your friend's house?" ("We played.")
	5	Does your child use comparison words, such as heavier, stronger or shorter? Ask your child questions, such as "A car is big, but a bus is _____" (bigger); "A cat is heavy, but a man is ____" (heavier); A TV is small, but a book is ____ " (smaller).
	6	Does your child answer the following questions: 1. "What do you do when you are hungry?" (Acceptable answers include: "Get food", "Eat", "Ask for something to eat", and "Have a snack".) 2. "What do you do when you are tired?" (Acceptable answers include: "Take a nap", "Rest", "Go to sleep", "Go to bed", "Lie down", and "Sit down.")
	7	Does your child repeat the sentences shown below back to you, without any mistakes? You may repeat each sentence one time. Mark "yes" if your child repeats both sentences without mistakes or "sometimes" if your child repeats one sentence without mistakes. "Jane hides her shoes for Maria to find." "Al read the blue book under his bed."

Response options (score): "No, not yet" (0); "Yes, but seldom / Sometimes / A few times" (5); "Yes, often" (10).

Supplementary Table 3. Extended overview of the comparison group characteristics.

	(Es)citalopram group (*n* = 306)	Depression group (*n* = 308)	Control group (*n* = 344)	*p*
Maternal characteristics				
Maternal age (mean years ± SD)	30.3 ± 5.2	28.4 ± 5.3	30.9 ± 4.6	a,b
Pre-pregnancy BMI (mean BMI ± SD)	24.5 ± 5.1 *8 NA*	24.3 ± 4.8 *7 NA*	23.8 ± 4.2 *2 NA*	**N.S.**
Maternal education				
University/college (*n* (%))	178 (58.2)	136 (44.2)	247 (71.8)	
High school or lower (*n* (%))	124 (40.5)	163 (52.9)	89 (25.9)	c,d,e
	4 NA	*9 NA*	*8 NA*	
Smoking in pregnancy (yes; *n* (%))	43 (14.1) *2 NA*	52 (16.9)	28 (8.1)	f,g
Alcohol in pregnancy (yes; *n* (%))	36 (11.8) *49 NA*	28 (9.1) *45 NA*	64 (18.6) *15 NA*	h
Folic acid in pregnancy (yes; *n* (%))	182 (59.5)	168 (54.6)	205 (59.6)	**N.S.**
Multivitamins in pregnancy (yes; *n* (%))	109 (35.6)	127 (41.2)	119 (34.6)	**N.S.**
Maternal medications				
Analgesics* (yes; *n* (%))	190 (62.1)	191 (62.0)	178 (51.7)	I,j
Antidepressants except (es)citalopram (yes; *n* (%))	19 (6.2)	---	---	**---**
NSAIDs (yes; *n* (%))	55 (18.0)	45 (14.6)	30 (8.7)	k,l
Maternal morbidities				
Comorbidity index** (mean score ± SD)	0.5 ± 0.9 *27 NA*	0.5 ± 0.9 *6 NA*	0.4 ± 0.9 *13 NA*	**N.S.**
Chronic diseases***				
None (*n* (%))	277 (90.5)	280 (90.9)	325 (94.5)	
1–2 diseases (*n* (%))	27 (8.8)	28 (9.1)	19 (5.5)	**N.S.**
≥3 diseases (*n* (%))	0 (0)	0 (0)	0 (0)	
	2 NA			
SCL-5 (mean score ± SD)	1.9 ± 0.8 *16 NA*	2.8 ± 0.5	1.0 ± 0	m,n,o
SCL-8 (mean score ± SD)	1.7 ± 0.6 *44 NA*	2.7 ± 0.5	1.0 ± 0	p,q,r
LTHMD (yes; *n* (%))	136 (44.4) *7 NA*	101 (32.8) *9 NA*	114 (33.1) *1 NA*	s,t
Birth				
Caesarean section (yes; *n* (%))	48 (15.7)	51 (16.6)	27 (7.9)	u,v
Child characteristics				
Birth weight (mean grams ± SD)	3,568 ± 501	3,579 ± 512	3,629 ± 503 *1 NA*	w
Gestational age (mean weeks ± SD)	39.4 ± 1.5 *1 NA*	39.4 ± 1.6 *1 NA*	39.7 ± 1.5 *1 NA*	x,y
Infant sex (female; *n* (%))	148 (48.4)	149 (48.4)	181 (52.6)	**N.S.**
Malformation (yes; *n* (%))	16 (5.2)	15 (4,9)	9 (2.6)	**N.S.**

Technical covariates

Bisulphite conversion				
Plate 1 (*n* (%))	30 (9.8)	29 (9.4)	31 (9.0)	
Plate 2 (*n* (%))	28 (9.2)	32 (10.4)	34 (9.9)	
Plate 3 (*n* (%))	28 (9.2)	24 (7.8)	38 (11.1)	
Plate 4 (*n* (%))	3 (1.0)	5 (1.6)	5 (1.5)	
Plate 5 (*n* (%))	24 (7.8)	29 (9.4)	35 (10.2)	
Plate 6 (*n* (%))	33 (10.8)	30 (9.7)	28 (8.1)	**N.S.**
Plate 7 (*n* (%))	29 (9.5)	28 (9.1)	35 (10.2)	
Plate 8 (*n* (%))	32 (10.5)	32 (10.4)	27 (7.9)	
Plate 9 (*n* (%))	26 (8.5)	30 (9.7)	30 (8.7)	
Plate 10 (*n* (%))	13 (4.2)	17 (5.5)	12 (3.5)	
Plate 11 (*n* (%))	30 (9.8)	27 (8.8)	32 (9.3)	
Plate 12 (*n* (%))	30 (9.8)	25 (8.1)	37 (10.8)	

Abbreviations: ADHD: attention-deficit/hyperactivity disorder; BMI: body mass index; NA: missing value; N.S.: not significant; SD: standard deviation.

* Includes all medications with the N02 Anatomical Therapeutic Chemical (ATC) code except paracetamol (i.e., opioids, antimigraine preparations, and other analgesics and antipyretics)

** Includes all variables available in MBRN and MoBa from a list provided in Bateman *et al.* (2013) [13, 14]. The different variables are given different weights (weight in parentheses).The variables included in the final score are: asthma (1), cardiovascular disease (3), chronic renal disease (1), congenital heart disease (4), illicit substance use (2), gestational hypertension (1), mild-unspecified preeclampsia (2), severe preeclampsia (5), placenta previa (2), pre-existing diabetes mellitus (1), pre-existing hypertension (1), previous Caesarean delivery (1), lupus (2), alcohol abuse (weekly consumption; 1) and maternal age group (> 44 years: 3; 40–45 years: 2; 35–40 years: 1; < 35 years: 0).

*** Chronic diseases included were asthma, rheumatoid arthritis, epilepsy, Crohn's disease, lupus, multiple schlerosis (MS), cancer and diabetes mellitus. All diseases were weighted equally and each additional disease added 1 to the final score.

[a] $p < 0.0001$, comparing (es)citalopram to depression
[b] $p < 0.0001$, comparing depression to controls
[c] $p \approx 0.001$, comparing (es)citalopram to depression
[d] $p < 0.0001$, comparing (es)citalopram to controls
[e] $p < 0.0001$, comparing depression to controls
[f] $p \approx 0.02$, comparing (es)citalopram to controls
[g] $p \approx 0.001$, comparing depression to controls
[h] $p \approx 0.01$, comparing depression to controls
[i] $p \approx 0.01$, comparing (es)citalopram to controls
[j] $p \approx 0.01$, comparing depression to controls
[k] $p \approx 0.001$, comparing (es)citalopram to controls
[l] $p \approx 0.03$, comparing depression to controls
[m] $p < 0.0001$, comparing (es)citalopram to controls
[n] $p < 0.0001$, comparing (es)citalopram to controls
[o] $p < 0.0001$, comparing depression to controls
[p] $p < 0.0001$, comparing (es)citalopram to depression
[q] $p < 0.0001$, comparing (es)citalopram to controls
[r] $p < 0.0001$, comparing depression to controls
[s] $p \approx 0.004$, comparing (es)citalopram to depression
[t] $p \approx 0.002$, comparing (es)citalopram to controls
[u] $p \approx 0.003$, comparing (es)citalopram to controls
[v] $p \approx 0.001$, comparing depression to controls
[w] $p \approx 0.05$, comparing (es)citalopram to controls
[x] $p \approx 0.001$, comparing (es)citalopram to controls
[y] $p \approx 0.03$, comparing depression to controls

Supplementary Table 4. Overview of the cell type proportions of the comparison groups.

	(Es)citalopram (n = 306)	Depression control (n = 308)	Healthy control (n = 344)	p
B cells; *mean proportion ± SD*	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	**N.S.**
CD4⁺ T cells; *mean proportion ± SD*	0.12 ± 0.05	0.13 ± 0.05	0.13 ± 0.05	**N.S.**
CD8⁺ T cells; *mean proportion ± SD*	0.05 ± 0.02	0.05 ± 0.03	0.05 ± 0.02	**N.S.**
Granulocytes; *mean proportion ± SD*	0.57 ± 0.10	0.57 ± 0.10	0.58 ± 0.10	**N.S.**
Monocytes; *mean proportion ± SD*	0.06 ± 0.03	0.06 ± 0.03	0.06 ± 0.03	**N.S.**
Natural killer cells; *mean proportion ± SD*	0.02 ± 0.02	0.03 ± 0.03	0.03 ± 0.02	**N.S.**
nRBCs; *mean proportion ± SD*	0.12 ± 0.11	0.11 ± 0.10	0.10 ± 0.10	[a,b]

Abbreviations: ADHD: attention-deficit hyperactivity disorder; nRBC: nucleated red blood cell; N.S.: not significant; SD: standard deviation.

[a] $p < 0.01$, (es)citalopram to controls
[b] $p < 0.05$, depression to controls

Supplementary Table 5. Model specification and fit, classification quality and distribution across classes for the latent class growth analysis of the CBCL-DSM ADHD subscale (n = 786). The selected model is shaded in grey.

Model specifications		Goodness-of-fit/classification quality				Classification (% of samples)				
Number of classes	**Time function**	**AIC**	**BIC**	**c-BIC**	**Entropy**	**Class 1**	**Class 2**	**Class 3**	**Class 4**	**Class 5**
1	Linear	9430.7	9547.4	9468.0	1.00	100.0	---	---	---	---
1	2nd degree polynomial	9432.5	9553.8	9471.2	1.00	100.0	---	---	---	---
2	Linear	9243.3	9373.9	9285.0	0.52	63.0	37.0	---	---	---
2	2nd degree polynomial	9247.2	9387.2	9291.9	0.52	63.1	36.9	---	---	---
3	Linear	9193.5	9338.2	9239.8	0.59	66.9	10.6	22.5	---	---
3	2nd degree polynomial	9199.2	9357.8	9249.9	0.59	69.7	19.6	10.7	---	---
4	Linear	9186.3	9344.9	9237.0	0.58	3.7	23.8	63.5	9.0	---
4	**2nd degree polynomial**	**9170.1**	**9347.5**	**9226.8**	**0.69**	**9.5**	**7.9**	**71.2**	**11.3**	**---**
5	Linear	9178.0	9350.7	9233.2	0.64	40.2	0.5	3.7	48.5	7.1
5	2nd degree polynomial	*Did not converge*				---	---	---	---	---

Abbreviations: AIC: Akaike information criterion; BIC: Bayesian information criterion; c-BIC: sample size-corrected Bayesian information criterion; LCGA: Latent class growth analysis.

Supplementary Table 6. Model specification and fit, classification quality and distribution across classes for the latent class growth analysis of the ASQ communication subscale (n = 899). The selected model is shaded in grey.

Model specifications		Goodness-of-fit/classification quality				Classification (% of samples)				
Number of classes	Time function	AIC	BIC	c-BIC	Entropy	Class 1	Class 2	Class 3	Class 4	Class 5
1	Linear	9910.6	10030.6	9951.2	1.00	100.0	---	---	---	---
1	2nd degree polynomial	9904.1	10028.9	9946.3	1.00	100.0	---	---	---	---
2	Linear	9784.6	9919.1	9830.2	0.49	27.6	72.4	---	---	---
2	2nd degree polynomial	9690.4	9834.4	9739.1	0.54	55.2	44.8	---	---	---
3	**Linear**	**9765.2**	**9914.1**	**9815.6**	**0.66**	**28.8**	**70.5**	**0.7**	**---**	**---**
3	2nd degree polynomial	9669.8	9833.0	9725.0	0.68	1.0	43.8	55.2	---	---
4	Linear	9771.2	9934.5	9826.5	0.68	28.8	70.5	0.7	0.0	---
4	2nd degree polynomial	9677.8	9860.2	9739.5	0.55	44.9	1.0	54.1	0.0	---
5	Linear	9777.2	9954.9	9837.4	0.72	28.8	0.0	70.5	0.0	0.7
5	2nd degree polynomial	9685.8	9887.4	9754.0	0.44	61.6	1.0	0.0	37.4	0.0

Abbreviations: AIC: Akaike information criterion; BIC: Bayesian information criterion; c-BIC: sample size-corrected Bayesian information criterion; LCGA: Latent class growth analysis.

Supplementary Table 7. Model specification and fit, classification quality and distribution across classes for the latent class growth analysis of the ASQ motor subscale (n = 895). The selected model is shaded in grey.

Model specifications		Goodness-of-fit/classification quality				Classification (% of samples)				
Number of classes	Time function	AIC	BIC	c-BIC	Entropy	Class 1	Class 2	Class 3	Class 4	Class 5
1	Linear	8091.7	8202.0	8129.0	1.00	100.0	---	---	---	---
1	2nd degree polynomial	8085.9	8201.0	8124.8	1.00	100.0	---	---	---	---
2	Linear	7492.2	7617.0	7534.4	0.69	27.9	72.1	---	---	---
2	2nd degree polynomial	7177.5	7311.8	7222.8	0.66	65.5	34.5	---	---	---
3	Linear	7383.6	7522.7	7430.6	0.66	15.8	30.1	54.2	---	---
3	2nd degree polynomial	7050.8	7204.3	7102.7	0.63	16.9	53.1	30.1	---	---
4	Linear	7374.1	7527.6	7426.0	0.65	18.0	54.0	24.4	3.7	---
4	2nd degree polynomial	7014.1	7186.8	7072.4	0.67	15.6	47.7	7.9	28.7	---
5	Linear	7365.2	7533.1	7421.9	0.70	0.1	53.9	18.0	25.1	2.9
5	**2nd degree polynomial**	**6966.2**	**7158.1**	**7031.1**	**0.68**	**15.1**	**8.2**	**50.2**	**12.3**	**14.3**

Abbreviations: AIC: Akaike information criterion; BIC: Bayesian information criterion; c-BIC: sample size-corrected Bayesian information criterion; LCGA: Latent class growth analysis.

Supplementary Table 8. Listed significant CpGs from the models run in the study (statistics and annotation).

Please see separate .xlsx file ("Supplementary Information, Supplementary Table 8.xlsx").

Supplementary Table 9. Distribution of comparison groups in the trajectory classes identified for the CBCL-DSM ADHD subscale using latent class growth analysis.

	Class 1 (*n* = 75)	Class 2 (*n* = 62)	Class 3 (*n* = 560)	Class 4 (*n* = 89)	*p*
Group					
(Es)citalopram (*n* (%))	21 (28.0)	24 (38.7)	172 (30.7)	23 (25.8)	**N.S.**
Depression (*n* (%))	18 (24.0)	9 (14.5)	162 (28.9)	44 (49.4)	**<0.001**
Control (*n* (%))	36 (48.0)	29 (46.8)	226 (40.4)	22 (24.7)	**<0.01**
ADHD diagnosis (yes; *n* (%))	0 (0)	2 (3.2)	29 (5.2)	12 (13.5)	**<0.001**

n = 786, as women not answering to the CBCL at any of the time points were removed from the analysis.

Abbreviations: ADHD: attention-deficit/hyperactivity disorder; N.S.: not significant.

Supplementary Table 10. Distribution of comparison groups in the trajectory classes identified for the ASQ communication subscale using latent class growth analysis.

	Class 1 (*n* = 259)	Class 2 (*n* = 634)	Class 3 (*n* = 6)	*p*
Group				
(Es)citalopram (*n* (%))	87 (33.6)	188 (29.7)	1 (16.7)	**N.S.**
Depression (*n* (%))	84 (32.4)	191 (30.1)	4 (66.7)	**N.S.**
Control (*n* (%))	88 (34.0)	255 (40.2)	1 (16.7)	**N.S.**

n = 899; women not answering to the ASQ at any of the time points were removed from the analysis.

Abbreviations: N.S.: not significant.

Supplementary Table 11. Distribution of comparison groups in the trajectory classes identified for the ASQ total motor subscale using latent class growth analysis.

	Class 1 (*n* = 135)	Class 2 (*n* = 73)	Class 3 (*n* = 449)	Class 4 (*n* = 110)	Class 5 (*n* = 128)	*p*
Group						
(Es)citalopram (*n* (%))	45 (33.3)	26 (35.6)	129 (28.7)	35 (31.8)	40 (31.3)	**N.S.**
Depression (*n* (%))	49 (36.3)	23 (31.5)	145 (32.3)	29 (26.4)	32 (25.0)	**N.S.**
Control (*n* (%))	41 (30.4)	24 (32.9)	175 (39.0)	46 (41.8)	56 (43.8)	**N.S.**

n = 895; women not answering to the ASQ at any of the time points were removed from the analysis.

Abbreviations: N.S.: not significant.

Supplementary Table 12. BECon output: blood-brain correlation of the significant CpGs identified in the study.

Please see separate .xlsx file ("Supplementary Information, Supplementary Table 12.xlsx").