Faculty of Medicine
University of Helsinki

ASSOCIATIONS BETWEEN PRENATAL AND EARLY LIFE STRESS AND PHYSICAL AND MENTAL HEALTH OUTCOMES IN PROSPECTIVE PREGNANCY AND BIRTH COHORTS OF CHILDREN, ADOLESCENTS AND OLDER ADULTS: THE ROLE OF EPIGENETICS AND GENETICS

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DOCTORAL DISSERTATION

To be presented for public discussion with the permission of the Faculty of Medicine of the University of Helsinki, in Hall 107, Athena, on the 13th of January, 2021 at 16 o'clock.

Helsinki 2020

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ISBN 978-951-51-6929-7 (pbk.)
ISBN 978-951-51-6930-3 (PDF)

Unigrafia
Helsinki 2020

ABSTRACT

Maternal depression and anxiety during pregnancy may present risks for the developing fetus and offspring lifelong physical and mental health. Exposure to postnatal early life stress (ELS) has also been extensively associated with health problems decades later. According to the Developmental Origins of Health and Disease (DOHaD) hypothesis, environmental factors during pregnancy and early childhood may compromise the development of tissue, organs and systems, such as hypothalamic-pituitary-adrenal (HPA) axis. While the underlying biological mechanisms are not fully understood, epigenetic alterations and genetic vulnerability are the promising biomarkers, which have been suggested to mediate the association of antenatal and early adversity with physical and mental health later in life.

The aim of this work was to examine whether exposure to maternal antenatal depression and anxiety was associated with polyepigenetic modifications in their children reflected by the polyepigenetic biomarkers of child's epigenetic gestational age (GA) and glucocorticoid (GC) exposure score. Additionally, it explored whether these modifications were associated with and mediated the effects of antenatal exposures on child mental health outcomes and whether the associations were moderated by child's sex. As epigenetic processes undergo age-related changes, the next aim was to study whether epigenetic modifications reflected by the polyepigenetic biomarker of epigenetic clock were associated with physical growth, neuroendocrine functioning, cognition and mental health in adolescents. Finally, this thesis also examined whether genetic variants in *FKBP5*, the gene that plays a role in the HPA-axis regulation, interacted with exposure to ELS in prediction of type 2 diabetes (T2D), cardiovascular disease (CVD), and quantitative glycemic traits in older adults.

The participants for the studies come from three prospective cohorts.

Studies I and II capitalize on the Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction (PREDO) birth cohort. We had full information on genome-wide methylation and genotype from 817 fetal umbilical cord blood samples. In Study I, 694 mothers provided information on their history of depression diagnosed before pregnancy, 581 completed the Center for Epidemiological Studies Depression Scale (CES-D) throughout pregnancy, and 407 completed the Child Behavior Checklist (CBCL) at child's mean age 3.7 years. DNA methylation (DNAm) GA of fetal cord blood DNA was based on the methylation profile of 148 selected CpG sites. Polyepigenetic biomarker of child's epigenetic GA was calculated as the arithmetic difference between DNAm GA and chronological GA and adjusted for chronological GA. In Study II, we had information on child diagnoses of mental and behavioral disorders and the number of days the child had been receiving in- or

outpatient treatment for these disorders as the primary diagnosis from birth to age 7.1-10.7 years (n=814). Mothers (n=583) reported depressive and anxiety symptoms during pregnancy, using CES-D and State Anxiety Inventory (STAI), respectively. A weighted cross-tissue polyepigenetic GC exposure score was calculated based on the methylation profile of 24 CpGs.

Study III was based on the Glycyrrhizin in Licorice (Glaku) cohort. We had information available on DNA samples, physical growth and pubertal development, cognitive abilities, psychiatric problems assessed by mothers with CBCL questionnaire, and saliva samples to estimate cortisol levels for a subsample adolescents at the mean age of 12.3 (n=239). DNAm age was estimated using the Horvath age estimation algorithm. The polyepigenetic biomarker of epigenetic clock was calculated as the unstandardized residual from a linear regression of DNAm age on chronological age and six cell count types.

For Study IV, a total of 1,728 Helsinki Birth Cohort Study (HBCS) participants born from 1934 to 1944 were genotyped for *FKBP5* SNPs (rs1360780, rs9394309, rs9470080) and were administered a 2-hour (75 g) oral glucose tolerance test (OGTT) and a questionnaire on physician-diagnosed and medication use for chronic diseases at a mean age of 61.5 years. Of them, 273 were exposed to ELS defined as separation from biological parents at a mean age of 4.7 years due to evacuations during World War II.

In Study I we found that lower child's epigenetic GA at birth was significantly associated with maternal history of depression diagnosed before pregnancy and higher antenatal depressive symptoms. It also prospectively predicted child's total and internalizing problems in early childhood, partially mediating the association of maternal antenatal depression with child internalizing problems, although only in boys. It may signal about their developmental vulnerability to maternal depression during pregnancy (Study I). In Study II we show that while polyepigenetic GC exposure score at birth was not predictive of higher risk for any mental and behavioral disorder in childhood, lower score was associated with more days spent in- or outpatient treatment for any mental and behavioral disorder as the primary diagnosis. This finding may contribute to better understanding and identification of children at risk for more severe mental and behavioral disorders already at birth (Study II). Next, we demonstrate that adolescents with epigenetic clock age acceleration (AA) displayed more advanced physical growth and development, had higher salivary cortisol upon awakening and higher odds for displaying borderline clinically significant internalizing problems, which may index risk of earlier aging and age-related diseases (Study III). Finally, Study IV revealed that three selected *FKBP5* polymorphisms moderated the association of ELS on insulin and glucose values at fasting state and/or during an OGTT in late adulthood, supporting the role of gene-environment interaction and HPA axis dysregulation in the development of metabolic disorders.

These study findings provide valuable insights on how the polyepigenetic biomarkers of antenatal adverse exposures and aging and biomarkers of genetic vulnerability in combination with the information about ELS might contribute to early identification of individuals at risk for complex mental and physical disorders enabling timely targeted preventive and therapeutic interventions.

TIIVISTELMÄ

Developmental Origins of Health and Disease (DOHaD)-hypoteesin mukaan raskaudenaikaiset ja varhaislapsuuden ympäristötekijät voivat vaikuttaa kudosten, elinten ja elimistön säätelyjärjestelmien, kuten hypotalamus-aivolisäke-lisämunuainen -akseli (HPAA) toimintaan pitkälläkin aikajänteellä. Esimerkiksi raskaudenaikainen masennus ja ahdistus voivat haitata sikiön kehittymistä ja lisätä terveysongelmien riskiä syntymän jälkeen. Myös varhaislapsuuden stressi voi lisätä terveysongelmien riskiä. Epigeneettiset muutokset ja geneettinen vaihtelu ovat lupaavia biomarkkereita, joiden on ehdotettu välittävän ja muokkaavan sikiöaikaisten ja varhaislapsuuden ympäristövaikutusten yhteyksiä fyysiseen ja henkiseen terveyteen myöhemmässä elämässä.

Tämän työn tarkoituksena oli tutkia, liittyykö altistuminen raskaudenaikaiselle masennukselle ja ahdistukselle kahteen lapsen epigeneettiseen biomarkkeriin: epigeneettiseen gestaatioikään ja glukokortikoidialtistuksesta kertovaan epigeneettiseen indikaattoriin. Lisäksi työssä selvitettiin, välittyikö raskaudenaikaisten altisteiden vaikutus lasten mielenterveyteen näiden biomarkkereiden kautta. Seuraavana tavoitteena oli tutkia kolmannen epigeneettisen biomarkkerin, epigeneettisen kellon, yhteyksiä fyysiseen kasvuun, neuroendokriinisiin vasteisiin, kognitiivisiin kykyihin ja mielenterveyteen murrosikäisillä. Lopuksi tässä opinnäytetyössä tutkittiin myös HPAA säätelyssä olennaisen FKBP5 geenin varianttien ja varhaisen stressin yhteisvaikutusta insuliini- ja glukoositasoihin sekä tyypin 2 diabetekseen ja sydän- ja verisuonitauteihin myöhäisessä aikuisuudessa.

Tutkimuksiin osallistujat tulevat kolmesta prospektiivisesta kohortista. Osatutkimukset I ja II hyödynsivät PREDO syntymäkohortin aineistoa. Tähän kuuluu genominlaajuinen metylaatio- ja genomiaineisto 817:sta napaverinäytteestä. Osatutkimuksessa I, 694:ltä äidiltä oli lisäksi tieto masennusdiagnoosista ennen raskautta, 581 täytti CES-D masennusoirekyselyn raskauden aikana ja 407 täytti CBCL-kyselyn lapsen käyttäytymis- ja tunneongelmista kun lapset olivat keskimäärin 3.7 -vuotiaita. Epigeneettinen gestaatioikä eli määriteltiin 148 napaveren DNA:n metylaatiokohdan (CpG) perusteella. Tutkimuksessa II aineistona oli lasten mielenterveys- ja käyttäytymishäiriöiden diagnoosit sekä niiden päivien lukumäärästä, jolloin lapsi oli ollut näiden sairauksien takia avo- tai sairaalahoidossa syntymästä 7.1 - 10.7 vuoden ikään saakka (n = 814). Äidit raportoivat myös raskaudenaikaisen masennusoireensa CES-D kyselyllä ahdistuksensa STAI kyselyllä (n = 583). Glukokortikoidialtistuksesta kertova epigeneettinen biomarkkeri laskettiin 24 CpG:n metylaatioprofiilin perusteella.

Tutkimus III perustui Glaku -kohortin aineistoon. Aineistona oli DNA-näytteet, fyysinen kasvu ja murrosiän kehitys, kognitiiviset kyvyt, CBCL-kyselylomakkeella raportoidut käyttäytymis- ja tunneongelmat ja sylkinäytteistä määritetty kortisolipitoisuus keskimäärin 12.3 -vuoden iässä (n = 239). Epigeneettinen ikä arvioitiin Horvathin algoritmilla.

Tutkimuksessa IV määritettiin FKBP5 geenin variantit (rs1360780, rs9394309, rs9470080) n=1728 HBCS -tutkimukseen osallistuneelta vuosina 1934–1944 syntyneeltä. Heille tehtiin myös 2-tunnin (75 g) sokerirasitustesti ja he raportoivat lääkärin diagnosoimista kroonisista sairauksista ja lääkkeidenkäytöstä keskimäärin 61.5 vuoden iässä. Tutkittavista 273 oli altistunut varhaiselle stressille, joka määriteltiin eroksi biologisista vanhemmista 2. maailmansodan aikana tapahtuneen evakuoinnin (sotalapsi) vuoksi keskimäärin 4.7 vuoden iässä.

Tutkimuksessa I havaitsimme, että matalampi lapsen epigeneettinen gestaatioikä, suhteessa kronologiseen gestaatioikään syntymähetkellä liittyi ennen raskautta diagnosoituun äidin masennukseen ja raskausaikaisiin masennusoireisiin. Se ennusti myös lapsen käyttäytymis- ja tunneongelmia varhaislapsuudessa sekä välitti osittain äidin masennuksen yhteyttä lapsen tunneongelmiin, erityisesti pojilla. Tutkimuksessa II osoitimme, että glukokortikoidialtistuksesta kertovan epigeneettisen indikaattorin matalmpi taso liittyi lapsen mielenterveyden ja käyttäytymishäiriön vuoksi sairaalassa tai avohoidossa vietetyn hoitojakson pituuteen. Tutkimuksessa III osoitimme, että korkeampi epigeneettinen ikä suhteessa kronologiseen ikään, oli yhteydessä fyysiseen kasvuun ja kehitykseen, korkeampiin syljen kortisolitasoihin heräämisen jälkeen ja lievien internalisoivien ongelmien korkeampaan riskiin. Lopulta Tutkimuksessa IV osoitimme, että kolme FKBP5-varianttia muokkasi varhaislapsuuden stressikokemuksen yhteyttä korkeampiin paaston- ja/tai sokerirasituksen jälkeisiin insuliini- ja glukoosiarvoihin myöhäisessä aikuisiässä.

Nämä tutkimustulokset antavat arvokasta tietoa siitä, kuinka raskauden- tai lapsuuden aikaisten altistusten tai ikääntymisen epigeneettiset biomarkkerit ja geneettiset biomarkkerit yhdessä varhaista stressiä kuvaavan tiedon kanssa voivat auttaa tunnistamaan ajoissa ne henkilöt, joilla on kohonnut riski mielenterveyden ongelmille tai fyysisille sairauksille. Tunnistaminen mahdollistaa ennaltaehkäisevien toimenpiteiden kohdentamisen oikea-aikaisesti jopa vuosikymmeniä ennen oireiden ilmaantumista.

ACKNOWLEDGEMENTS

I have never planned to be a researcher. However, here I am, preparing for my PhD defense. When I set out on this journey, I attended my colleague's defense right away, so I could visualize my public defense in front of all my colleagues, family and friends, followed by a big party. Yet again, life surprised me with the pandemic and many challenges of 2020. "If you want to make God laugh, tell him your plans", Woody Allen once said. It seems like by now I should take this as a motto and appreciate every moment as it is. Therefore, right now I would like to take such a moment and thank the people who have been with me throughout the five years of my academic journey.

First, I would like to thank my supervisor, Academy Professor Katri Räikkönen-Talvitie. Thank you, Katri, for teaching me all the necessary skills for conducting high-quality research, for giving me an opportunity to work with a wide range of topics and datasets and for guiding me through the challenges. Thank you for your immense support upon my return from maternity leave, understanding the struggles of combining motherhood and career and finding the fine line of life-work balance. On the occasions when I felt overwhelmed, you told me that if getting a PhD were easy, everyone would do it, which made me value my work. Your confidence encouraged me and helped to bring us here. Thank you!

I would like to continue with expression of gratitude to my second supervisor, Professor Jari Lahti. Thank you, Jari, for believing in me and taking a chance on a young fresh graduate who was extremely passionate about behavioral genetics, but had no idea about academic work. You helped me with writing my first research plan, introduced me to Katri and gave me space to explore the ideas I came up with during my learning process. Thank you for your positive attitude and support!

I would also like to thank Marius Lahti-Pulkkinen and Polina Girchenko, who contributed tremendously to three of my papers and who took care of finalizing the publication of two of them while I was taking care of my daughter. My road to PhD would have been much longer without you two, I am very grateful for your help!

I am further grateful to the pre-examiners of my doctoral thesis, Dr. David Gyllenberg and Dr. Kirsi Peltonen. Thank you for your insightful comments, which allowed me to reflect on the methodology and impact of my research work, and made this thesis more balanced and clear.

All of my papers were written in collaboration with accomplished researchers, thanks to whom I had access to incredible datasets and the most novel biomarkers. I want to thank my co-authors: Eero Kajantie, Elisabeth Binder, Darina Czamara, Rebecca Reynolds, Hannele Laivuori, Pia Villa, Esa Hämäläinen and other great minds for your thoughtful comments and

expertise, which made collaboration with you a great learning experience and an honor!

This work would have been impossible without funding from the University of Helsinki's Doctoral Programme in Psychology, Learning and Communication (PsyCo). I am extremely grateful for this opportunity! I would also like to acknowledge that this thesis capitalizes on data from three prospective cohorts and such profound work involves input from many people. I would like to thank all the participants of the studies, all the researchers involved in this work and all the institutions that supported these studies: Academy of Finland, University of Helsinki, Alfred Kordelin Foundation, Ella and Georg Ehrnrooths Stiftelse, Yrjö Jahnsson Foundation, British Heart Foundation, Foundation for Pediatric Research, Juho Vainio Foundation, Novo Nordisk Foundation, Signe and Ane Gyllenberg Foundation, Sigrid Jusélius Foundation, Finnish Medical Foundation, Jane and Aatos Erkko Foundation, Päivikki and Sakari Sohlberg Foundation, and European Commission.

A great source of support for me was the knowledge that I am never alone in this work, but part of a wonderful Developmental psychology research group. I would like to thank Rachel Robinson, Kati Heinonen, Anu-Katriina Pesonen, Soile Tuovinen, Riikka Pyhälä-Neuvonen, Elena Toffol, Alfredo Ortega-Alonso, Soili Lehto, Ilona Merikanto, Siddheshwar Utge, and Tuomas Kvist for your help and kindness. My special thanks go to people, with who we were going through PhD process's ups and downs together. Kadri Haljas, Elina Wolford, Satu Kumpulainen, Liisa Kuula-Paavola, Ville Rantalainen, Katri Savolainen, Sara Sammallahti, our time in Siltavuorenpenger and Weekly Wine Meetings will forever stay the happiest memories of this part of my journey.

Doing a PhD is a challenge; doing it as an expat living in a foreign country without speaking the native language of the country is a whole new level of challenge. It might have been a very lonely and desolate experience if it wasn't for my friends who became my Finnish family. My deepest thanks and love go to Alexandra, Diana, Monika, Cristian, Erison, Katy, Hannah and Dasha. I am also blessed with being able to preserve the most precious friendships I have from Russia, which date ten, twenty and more years back. Thank you my dear Sasha, Marta, Liza, Ira, Ira and Ira (it's not a typo, but three wonderful people). Even if you might have zero idea what my research work is about, you are supporting me through life no matter what.

I want to further thank my parents-in-law, Ana Maria and José Carlos, who moved from Brazil to Finland to be closer to our family. Sem a sua ajuda, eu não teria conseguido terminar minha tese nesta primavera, durante a pandemia. Obrigada pela ajuda e carinho!

The person without who none of this would literally be possible is my mom. The only phrase she told me when I said that I want to be a psychologist was "I wouldn't be able to help you with knowledge or connections, but I will support you in all other ways I can". And so she did: she encouraged my every

decision, no matter how hard it was for her. Мамулечка, я не могу представить лучшей мамы, чем ты. Спасибо тебе за все, что ты для меня делаешь. Я тебя очень люблю! I would also like to thank my little sister Nastya, who has always seen me as an example and whose love and admiration is my great source of inspiration. Настяша, быть твоей сестрой – мое счастье, очень тебя люблю.

There are two special small persons I would like to thank. Juju, you came to me in the time when I really needed a break in order to see the perspective of where I am going and what I really want to do in life. You opened a whole new world for me, a whole new me, really. Я тебя очень люблю и буду любить тебя долго-долго, так долго, сколько позволит эта жизнь. My little one, I think you knew that I need you right now, without rushing into new jobs or projects; I will need to take time to just be, and being your Mom is the best task ever. So with your small kicks inside you remind me where my priorities are, you keep me grounded and you fill me with life and love. Я очень жду нашей встречи, мой уже такой любимый малыш.

Finally, my husband, my rock, my life partner and my best friend Vini. When I get into a storm of my emotions, when I get lost on my way, when I lose the perspective of what really matters in life, you are always here to hug me and tell how proud you are of me. Together we've been through long distance relationship, loss of loved ones, birth of our daughter, struggles of my postpartum recovery and early parenthood and now lockdown during pandemic. Now we are almost through over 5 years of journey towards my PhD, and as long as you hold my hand, I know I can do it and so much more. Sunshine, I can't be more excited to see what lies ahead of us! Eu te amo. Я тебя люблю. I love you. Lava you. In all the languages, always.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Suarez, A., Lahti, J., Czamara, D., Lahti-Pulkkinen, M., Knight, A.K., Girchenko, P., Hämäläinen, E., Kajantie, E., Lipsanen, J., Laivuori, H., Villa, P.M., Reynolds, R.M., Smith, A.K., Binder, E.B., and Räikkönen, K. The Epigenetic Clock at Birth: Associations with Maternal Antenatal Depression and Child Psychiatric Problems. *J Am Acad Child Adolesc Psychiatry*. 2018; 57(5):321-8.

II Suarez, A., Lahti, J., Lahti-Pulkkinen, M., Girchenko, P., Czamara, D., Arloth, J., Malmberg, A.L.K., Hämäläinen, E., Kajantie, E., Laivuori, H., Villa, P.M., Reynolds, R.M., Provençal, N., Binder, E.B., and Räikkönen, K. A Polyepigenetic Glucocorticoid Exposure Score at Birth and Childhood Mental and Behavioral Disorders. *Neurobiol Stress*. 2020; 13: 100275.

III Suarez, A., Lahti, J., Czamara, D., Lahti-Pulkkinen, M., Girchenko, P., Andersson, S., Strandberg, T.E., Reynolds, R.M., Kajantie, E., Binder, E.B., and Räikkönen, K. The Epigenetic Clock and Pubertal, Neuroendocrine, Psychiatric, and Cognitive Outcomes in Adolescents. *Clin Epigenetics*. 2018; 10:96.

IV Suarez, A., Lahti, J., Kajantie, E., Eriksson, J.G., and Räikkönen, K. Early Life Stress, *FKBP5* Polymorphisms, and Quantitative Glycemic Traits. *Psychosom Med*. 2017; 79(5): 524-32.

The publications are referred to in the text by their roman numerals. All original publications have been reprinted with the kind permission of the copyright holders.

ABBREVIATIONS

11β-HSD2	11 β Hydroxysteroid Dehydrogenase Type 2 Enzyme
AA	Age Acceleration
ACTH	Adrenocorticotropic Hormone
ADHD	Attention Deficit/Hyperactivity Disorder
AUC	Area Under the Curve
AVP	Arginine Vasopressin
β	Standardised Beta Coefficient
B	Unstandardised Beta Coefficient
BDI-II	Beck Depression Inventory–II
BMI	Body Mass Index
BMIQ	Beta-Mixture Quantile
CBCL	Child Behavior Checklist
CES-D	Center for Epidemiological Studies Depression Scale
CI	Confidence Interval
CNS	Central Nervous System
CpG	Cytosine Linked to Guanine by Phosphate
CRH	Corticotropin-Releasing Hormone
CRHBP	Corticotrophin Releasing Hormone Binding Protein
CRHR1	Corticotrophin Releasing Hormone Receptor 1
CRHR2	Corticotrophin Releasing Hormone Receptor 2
CVD	Cardiovascular Disease
DEX	Dexamethasone
DNA	Deoxyribonucleic Acid
DNAm	Deoxyribonucleic Acid Methylation
DOHaD	Developmental Origins of Health and Disease
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders (4th edition)
ELS	Early Life Stress
FKBP5	FK506 binding protein 51
GxE	Gene – Environment Interaction
GA	Gestational Age
GC	Glucocorticoid
GLAKU	Glycyrrhizin in Licorice Cohort
GLM	Generalized Linear Models
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HbA1c	Hemoglobin A1c Protein
HBCS	Helsinki Birth Cohort Study
HOMA-IR	Homeostasis Model Assessment Method
HPA axis	Hypothalamic-Pituitary-Adrenal Axis
IFG	Impaired Fasting Glucose

IGT	Impaired Glucose Tolerance
IQ	Intelligence Quotient
ISI	Insulin Sensitivity Index
IUGR	Intrauterine Growth Restriction
LD	Linkage Disequilibrium
LDL	Low-Density Lipoproteins
M	Mean
MAF	Minor Allele Frequency
MD	Mean Difference
MDD	Major Depressive Disorder
MDS	Multi-Dimensional Scaling
MPIP	Max Planck Institute of Psychiatry Cohort
MR	Mineralocorticoid Receptor
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1 Gene
NR3C2	Nuclear Receptor Subfamily 3 Group C Member 2 Gene
OGTT	Oral Glucose Tolerance Test
p	Probability
PC	Principal Component
PDS	Pubertal Development Scale
PREDO	Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction Study
PTSD	Posttraumatic Stress Disorder
PVN	Paraventricular Nucleus
SD	Standard Deviation
SES	Socioeconomic Status
SNP	Single-Nucleotide Polymorphism
STAI	State Anxiety Inventory
T2D	Type 2 Diabetes Mellitus
WHO	World Health Organization
WWII	World War II
ZINB	Zero-Inflated Negative Binomial Regression

1 INTRODUCTION

Fetal exposure to prenatal stress, including maternal depression and anxiety, is highly prevalent. It has been estimated that 1 in 10 women has a major depressive disorder diagnosis, 1 in 5 reports clinically relevant depressive symptoms and 1 in 4 reports clinically relevant anxiety symptoms during pregnancy (1–3). Mounting evidence indicates that these maternal mental health problems not only complicate her well-being and health during pregnancy, but they may also present harm for the offspring physical and mental health (4–7).

These findings are compatible with the Developmental Origins of Health and Disease (DOHaD) framework. According to this framework, fetal exposure to environmental adversities may alter the fetal developmental milieu in ways that may harm rapidly developing organs and physiological feedback systems and thereby increase risk for physical and mental health problems in later life (8).

While the biological mechanisms that mediate these associations still remain unclear, it has been suggested that they may become embedded in fetal epigenetic modifications, such as modifications in fetal DNA methylation (DNAm) (9–11). Studies that would have tested prenatal stress exposure and modifications in DNAm or that would have tested associations between these modifications with child mental health are, however, scanty and most of the existing studies are limited to examining DNAm of a few candidate genes. Large-scale epigenome-wide association studies have been emerging. However, they require large sample sizes, with pooling and harmonizing data from various cohorts. An alternative approach is to identify and study polyepigenetic scores of biomarkers of risk. However, only a few studies have exploited this approach in this context (12–14).

In this thesis, I focused on two such novel polyepigenetic risk scores based on fetal cord blood DNAm, namely the child epigenetic gestational age (GA) (15) and the polyepigenetic glucocorticoid (GC) exposure score (13). In Study I, I explored whether maternal depression during pregnancy was associated with child epigenetic GA at birth. I also examined whether it was associated with and mediated the associations of maternal depression during pregnancy with child psychiatric problems and whether the associations were moderated by child's sex. In Study II, I examined whether the polyepigenetic GC exposure score at birth was associated with any mental and behavioral disorder diagnosis in childhood and its severity and whether this polyepigenetic biomarker mediated the associations of maternal depressive and anxiety symptoms during pregnancy and the child mental health outcomes.

As DNAm undergoes age-related changes (16), which are now recognized as a hallmark of the aging process, in Study III I also explored if another polyepigenetic biomarker, namely the epigenetic clock of aging (17) measured

from peripheral blood DNAm, was associated with physical growth, neuroendocrine functioning, cognition and mental health in adolescents.

Apart from prenatal stress, exposure to early life stress (ELS), such as abuse, neglect, maltreatment, and separation from parents, constitutes a major public health and social welfare problem. More than 25% of adults worldwide report being physically abused as a children (18), up to 30% of girls and 15% of boys are exposed to sexual abuse in high-income countries (19). Millions of children get separated from their parents or primary caregivers due to conflict, population displacement and other emergencies worldwide (20).

In a series of studies exposure to ELS has been associated with mental health outcomes (21,22) and this association has been shown to be moderated by genetic variants in the *FKBP5* gene associated with hypothalamic-pituitary-adrenal (HPA) axis functioning (23–25). However, it remains uncertain whether ELS is also associated with physical health outcomes and whether this association may be moderated by variants in the *FKBP5* gene. Hence, in Study IV of this thesis I also explored if ELS, defined here as temporary separation from both biological parents due to child evacuations during World War II (WWII), interacted with three selected common *FKBP5* polymorphisms in predicting cardiovascular disease (CVD), type 2 diabetes (T2D), and quantitative glycemic traits in older adults. As candidate gene – environment interaction (GxE) studies were demonstrated to have significant limitations (26), guidelines of the editorial policy for candidate gene studies (27,28) were followed in this study.

2 REVIEW OF THE LITERATURE

2.1 PRENATAL ADVERSITY AND SUBSEQUENT HEALTH

Experiences during prenatal period and early childhood have a profound lifelong influence on physical and mental health. Deeper understanding of this phenomenon started over 50 years ago, when the British scientists E.M. Widdowson and R.A. McCance discovered that rat pups, who were undernourished for three weeks after birth, gained weight slower than the pups from the control group throughout their lifespan (29). Lack of nutrition for three weeks at a later stage of development, contrarily, had no long-term effect (29). These experiments shed light both on the lifelong effects of early environments and the existence of critical periods of development (30). While decades of animal studies were confirming and expanding the early life programing theory, it was found essential to understand, to which extent these principles could apply to human health and development.

2.1.1 DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE (DOHAD) FRAMEWORK

In the late 1980s, while studying the geographical differences of coronary heart disease mortality rates between England and Wales, Barker and Osmond found that these differences were associated with previous differences in infant and adult mortality (31). It was the first of the three landmark papers published in *The Lancet* between 1986 and 1993 (31–33), which gave rise to fetal origins hypothesis, also known as “Barker’s hypothesis” (8). Following the original findings, Barker and colleagues showed that higher risk of death from coronary heart disease was associated with lower weight at birth and at one year of age (32). The authors suggested that lower birth weight may reflect poorer fetal and infant growth environment, which may lead to poorer adult environment with higher risk for coronary heart disease (32). The authors continued investigating the effects of adverse prenatal environments and further suggested that undernutrition *in utero* may permanently alter glucose and insulin metabolism leading to changes in the body’s structure and function, predisposing individuals to higher risk for coronary heart disease in later life (33).

These findings stimulated interest in epidemiological studies of prenatal adversities in relation to health outcomes across the lifespan. Since reliable measurement of the fetal nutrition and environment is technically complicated in humans, birth weight, intrauterine growth restriction (IUGR), and preterm birth have been extensively used as proxy markers of an adverse prenatal environment. Researchers found intriguing associations of birth

weight and prematurity with physical health problems decades later, such as cardiovascular disease (CVD) (34–37), hypertension (35,37–39), insulin resistance and type 2 diabetes (T2D) (37,40), and asthma (41).

In 2003 at the World Congress on Fetal Origins of Adult Disease in Brighton, United Kingdom, Barker's hypothesis was transformed into Developmental Origins of Health and Disease (DOHaD) framework (8). Although initially, DOHaD framework focused on prenatal nutrition and overlooked other adverse exposures, currently it recognizes a broad scope of developmental cues from *in utero* environment to infancy and beyond with long-term health consequences (8). The early life environmental cues may include stressful life experiences, namely early life stress (ELS).

While initial research within DOHaD framework focused on physical aging-related and chronic illnesses, individual differences in neurodevelopmental, cognitive and mental health outcomes have also been linked with birth weight, IUGR and preterm birth (5,22,42–45).

It is important to note that along with DOHaD, there are several other frameworks suggesting how exposure to stress at different stages of development might affect the individual's health. Lupien et al., for instance, proposed the Life cycle model of stress (46), postulating that the effects of chronic or repeated exposure to stress (or a single exposure to severe stress) at different stages of life depend on the brain areas, which are developing or declining most rapidly at the time of the exposure. Another approach is the Three-hit model (47), providing an alternate avenue to gain insight into the prenatal and ELS pathways to disease. In this model, it is proposed that genetic variability (hit 1) in interaction with priming prenatal and/or early life adversity (hit 2) influences the response of brain and body following significant stress later in life (hit 3). The Three-hit model, hence, emphasizes the importance of both genetic and environmental factors in understanding the vulnerability to stress-induced physical and mental health problems (48). Life History Theory, on the other hand, emphasizes evolutionary perspective of socialization and individual reproductive strategy differences (49). It postulates that experiences in early life can program an individual's developmental trajectory in order to respond most effectively to the environmental demands they are likely to encounter later in life (49,50).

Notably, all these models are not mutually exclusive, and together may give a deeper understanding of the origins of various disorders. However, it is important to acknowledge that both strength and limitation of the Life cycle model of stress, the Three-hit model and the Life History Theory approaches lie in their focus on specific mechanisms and a set of outcomes they address. Contrarily, DOHaD framework is more general and may be applied when studying a wide range of exposures, biological mechanisms and physical and mental health outcomes. Therefore, we selected DOHaD as the principal contextual model for the studies included in this thesis, as they address adverse exposures during pregnancy and in early childhood, mental and physical disorders as outcomes and explore a number of biological

mechanisms that may mediate the associations between those exposures and outcomes.

2.1.2 MATERNAL STRESS DURING PREGNANCY AND OFFSPRING DEVELOPMENT

Maternal stress, including mental health problems, during pregnancy are among the most common adverse environmental intrauterine exposures. The impact of antenatal maternal stress on neurodevelopmental outcomes is well established in animal studies (51). Animal models offer the possibility to manipulate both prenatal and postnatal environments. Therefore, they allow separating the influence of maternal stress during pregnancy on the offspring development from genetic and postnatal environmental factors. Cross-fostering studies, when pups from prenatally stressed dams were placed in non-stressed dams' care, for example, have confirmed the long-term effects of prenatal stress on the offspring health and behavior (52).

The idea that maternal stress during pregnancy might affect the fetus and her subsequent physical and emotional development in humans was introduced in late 1950s-early 1960s (53). Now we have a significant amount of evidence confirming and expanding this idea, despite the challenges of drawing causality conclusions in epidemiological settings (51,54). Multiple studies have linked maternal antenatal stress with offspring poorer cognitive functioning (55), risk for attention deficit/hyperactivity disorder (ADHD) and for anxiety and depression (51,56,57).

Many prospective studies have focused on maternal depression and anxiety as antenatal stress exposures, due to high prevalence of these mental health problems during pregnancy. Prevalence of clinically significant symptoms of depression and anxiety during pregnancy is estimated to vary between 7% to 20% (2,3). Mounting evidence indicates that these maternal mental health problems not only complicate her quality of life and health during pregnancy, but they are associated with increased risk of preterm birth, lower birth weight and neurodevelopmental adversities of the offspring later in life (4–7,46). Infants of mothers with antenatal symptoms of anxiety or depression show more difficult/reactive temperament and a higher incidence of sleeping and feeding problems (58,59), independent of postnatal maternal mental health (60,61).

In line with these findings, a recent meta-analysis shows that for mothers experiencing prenatal depression and anxiety, the odds of having children with behavioral difficulties were almost 1.5 to 2 times greater than for those not experiencing prenatal distress (7). Children born to mothers reporting higher levels of depression and anxiety during pregnancy are at risk for ADHD (62,63), internalizing problems (64,65), and sleep disorders (66), with these effects continuing into adolescence (67,68) and adulthood (69). In addition, neuroimaging studies have shown that maternal depressive and anxiety

symptoms during pregnancy are associated with alterations in offspring structural and functional brain connectivity across various brain regions and networks (9,70).

Interestingly, the effects of maternal stress exposure during pregnancy, and particularly antenatal depression, on offspring developmental outcomes have shown sex specificity (71–73). Rodent studies consistently demonstrate that maternal stress during pregnancy was associated with long-lasting morphological changes in brain structure (74,75) and depression- and anxiety-like behavioral phenotype (73,75) in male but not in female offspring. Evidence in humans, however, indicates that maternal antenatal depression was associated with a higher risk of offspring depression at 18 years of age in girls only (71).

In Studies I-II we contribute to this body of literature by exploring the association between maternal history of depression before pregnancy and antenatal depressive and anxiety symptoms and child psychiatric problems and possible role of child's sex in moderation of these associations.

2.2 EARLY LIFE STRESS (ELS) AND SUBSEQUENT HEALTH

According to DOHaD hypothesis, the roots of adult disease may also lie among disruptions of early stages of development after birth (8,76). The term ELS has been used to describe a broad spectrum of adverse exposures during prenatal and neonatal life, early and late childhood, and continuing into adolescence.

The most common adversities during childhood and adolescence include child physical and sexual abuse, neglect, maltreatment, separation from parents, parental loss, and starvation. Experience of such disrupting early life adversities constitute a major public health and social welfare problem in the general population: according to the World Health Organization (WHO), more than 25% of adults worldwide report being physically abused as a children (18). During childhood, between 15% and 30% of girls and up to 15% of boys are exposed to some type of sexual abuse in high-income countries (19).The prevalence for child neglect was estimated at 163/1,000 for physical neglect and 184/1,000 for emotional neglect, with no clear gender differences (77). Over 700,000 children are reported to be victims of childhood maltreatment nationally each year in the United States (78), while in China the pooled prevalence of childhood maltreatment was estimated at 64.7% among Chinese college students (79).

ELS may also take the form of separation from one or both parents in childhood. In animal studies it is described by maternal separation paradigm, which entails early separation of the pups from dams for a long period during the first two or three weeks (80). Long-term maternally separated rodents consistently show anxiety- and depression-like behaviors, drug-seeking

behaviors and neuroendocrine stress-induced responses (81). In humans, separation from parents due to war, immigration, natural disasters and other life obstacles has been linked with long-term health consequences (82).

ELS during critical phases of brain development is associated with higher levels of imbalance and reduced adaptability to stress in adult life, leading to enhanced vulnerability to diseases (83). Mounting evidence indicates a higher risk of depression (84,85), posttraumatic stress disorder (PTSD) (86,87), personality disorder (88), and overall psychopathology (21,22) in adults exposed to ELS in childhood in retrospective and prospective studies (83). Furthermore, emerging data suggest that ELS is also associated with chronic physical health consequences in adulthood (89,90). Early adversity has been linked with increased risk of cardiometabolic illnesses, such as obesity, CVD and T2D (91–94).

We expand this emerging evidence by exploring the association between ELS defined as temporary separation from biological parents due to evacuation during World War II (WWII) and CVD, T2D, and quantitative glycemic traits in Study IV.

2.3 BIOLOGICAL MECHANISMS MEDIATING PRENATAL AND EARLY LIFE ENVIRONMENTAL ADVERSITIES ON PHYSICAL AND MENTAL HEALTH OUTCOMES

The biological mechanisms underlying the associations of adverse fetal and early life environment with health and development later in life are not fully understood.

During early stages of development, there are critical periods when tissues and organs go through rapid cell division (95). These sensitive periods are also characterized by developmental plasticity, where the developing organism is susceptible to environmental effects, which may result in phenotypic differences between individuals (96). However, the sensitive periods occur within a critical developmental stage and are followed by a reduction of plasticity, which then results in fixed altered anatomy and/or functioning (34).

While some changes may be beneficial for the individual to adapt to their environment and survive until reproductive age, they may also lead to harmful and maladaptive long-term consequences for both mental and physical health, especially when the actual environment does not match the predicted environment of what the individual has adapted to during early life stages (97,98).

2.3.1 HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS AS A MEDIATOR

Studies in animals and humans have shown that brain is highly sensitive to stress across the lifespan, with particular susceptibility to adverse

environmental factors during prenatal and early life (46). It remains uncertain how prenatal or early life stress exerts its impact on the developing brain; however, it is widely shown that stress triggers the activation of the hormonal system known as hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis plays a key role in the regulation of cardiovascular, metabolic, reproductive, systems as well as emotions and behavior (11). It is one of the main stress response pathways and has been studied extensively in relation to physical and mental health (43,46,51,83,86,99).

In order to understand how early life adversity may exert its effect on the offspring health via altering HPA axis functioning, it is important to understand the HPA axis organization (11,46).

When the brain detects a threat, a coordinated physiological response is activated (Figure 1).

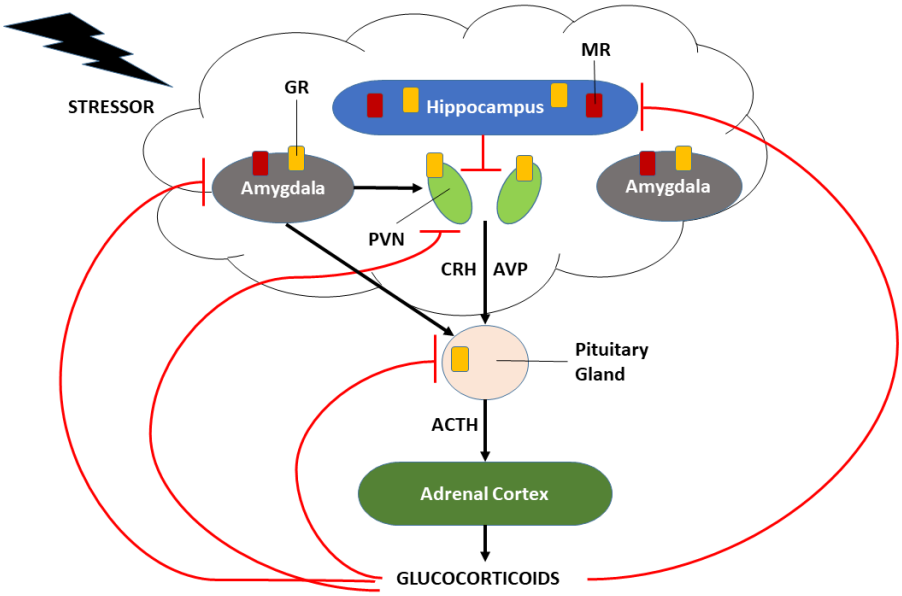

Figure 1 Hypothalamic-pituitary-adrenal axis reaction to stress

The hypothalamic paraventricular nucleus (PVN) initiates an endocrine cascade with the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). They trigger the secretion of adrenocorticotropic hormone (ACTH), which is released from the anterior pituitary gland into the peripheral circulation. When ACTH reaches the adrenal cortex, it responds with the release of glucocorticoids (GCs). GCs are the class of steroid hormones, which are represented by cortisol in humans. When released, GCs act on glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) at various levels within the axis. If the perceived stressor recedes, GR and MR trigger the feedback loops in the hippocampus in order to inhibit the HPA axis activity and return to homeostasis. By contrast, if the GCs activate the

receptors of the amygdala, the brain structure involved in fear processing (100), the HPA axis response is enhanced in order to deal with the perceived stress.

The stress response orchestrated by the HPA axis is a well-choreographed multi-system reaction, involving behavioral, physiological, and metabolic responses, with tightly regulated components that need to become activated and deactivated in the certain circumstances (101). Thus, it is not surprising that disruptions in the biological response to stress can lead to dysregulation of neuronal function, behavior, metabolism, cardiovascular and immune systems (102). Abnormal functioning of the HPA axis is implicated in a wide range of psychiatric illnesses such as depression (23,103), PTSD (104), neurodegenerative diseases (105), and anxiety (106). It is further associated with inflammation (101), skin disorders (107), cardiometabolic disorders (CVD, stroke, hypertension, T2D, and obesity) (108–110), and other chronic illnesses (111).

2.3.1.1 Glucocorticoid (GC) overexposure in utero

Prenatal programming of the offspring's HPA axis functioning has been extensively investigated. Mounting evidence indicates increased fetal exposure to GCs as one of the most plausible underpinning mechanisms mediating the negative effects of prenatal stress and altered HPA axis functioning (11,46,112,113).

GCs play a vital role during normal fetal development. During pregnancy there is a physiological rise of 2- to 4-fold in maternal GCs that is important for proper fetal growth and maturation, particularly for lung function and brain development (13,114). However, fetal exposure to excess levels of maternal endogenous GCs, namely cortisol, have been associated with suboptimal offspring neurodevelopment (43).

Since GCs have such a potent effect on the developing tissues, fetal exposure to GCs is tightly regulated by a number of mechanisms, primarily by high expression of a GC barrier enzyme, 11 β hydroxysteroid dehydrogenase type 2 (11β-HSD2), in placental and fetal tissues (114). Normally, it converts 80–90% of active maternal cortisol to its inactive form cortisone (115), which is translated in up to 10 times lower cortisol levels in fetus as compared to her mother. However, it has been shown that excess maternal GCs due to stress, depression and anxiety during pregnancy may downregulate placental 11β-HSD2, which leads to subsequent fetal overexposure to maternal GCs (Figure 2) (11,116,117).

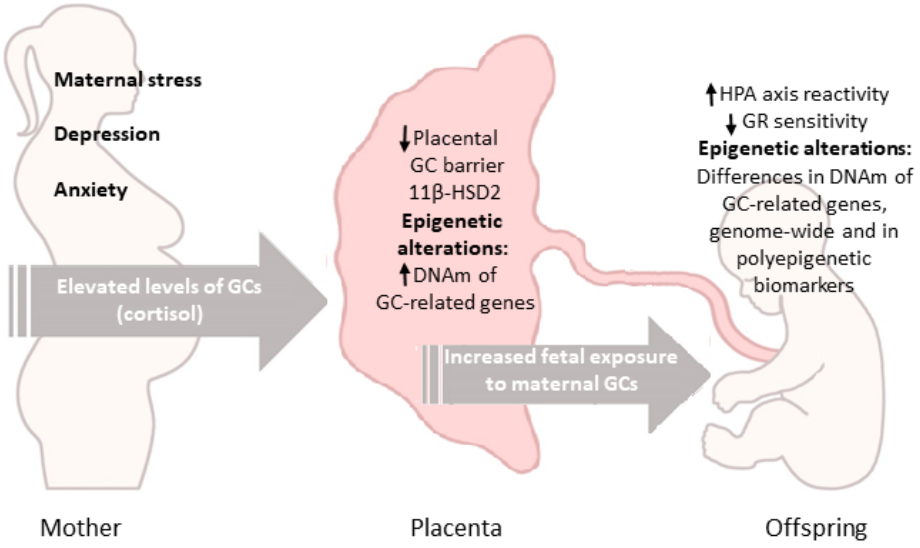

Figure 2 Maternal-to-fetal transfer of glucocorticoids

Exposure of the fetus to high levels of GCs, in turn, can permanently alter the HPA axis functioning, which is clearly observed in animal studies, with similar effects, although less pronounced, described in humans (43,112,117). For instance, inhibition or deficiency of placental 11β-HSD2 has been shown to reduce hippocampal GR expression (118) but, conversely, increases amygdala GR mRNA levels (119). Increased GR expression in the amygdala was associated with anxiety-like behavior in rodents, while a reduction in hippocampal GR may disrupt the GC negative feedback loop and lead to an overactive HPA axis, with both pathways enhancing susceptibility to somatic diseases and mental health problems (5,117,119).

While likely not the sole mechanism explaining the long-term health problems following exposure to prenatal maternal stress and psychopathology, excessive exposure to GCs above the required physiological levels may contribute to the observed adverse physical and mental health outcomes (13). In Studies I and II we explore this question by examining the association between maternal depression and anxiety before and during pregnancy and child psychiatric problems in their offspring.

2.3.2 EPIGENETIC ALTERATIONS: DNA METHYLATION

At the molecular level, epigenetic mechanisms have been suggested to play a key role in explaining how prenatal and early life adversity may exert their effect on physical and mental health across the lifespan (9–11).

The term ‘epigenetics’ refers to heritable changes in gene expression (activation or silencing) that occur without alterations to the DNA sequence.

Epigenetic mechanisms include DNA methylation (DNAm), histone modification, and the presence of noncoding RNA; currently, most studies have focused on DNAm.

DNAm refers to the transfer of a methyl (CH_3) group from S-adenosyl methionine (SAM) to the fifth position of cytosine nucleotides, forming 5-methylcytosine (5mC) (120). In mammals, most 5mC occurs at nucleic sequences in the context of cytosine-phosphate-guanine (CpG) dinucleotides. Up to 80% of CpG sites are methylated in human somatic cells, with most unmethylated CpG sites clustered in the CpG island located on the promoter region of the genes (120). DNAm can change the functional state of regulatory gene regions, but it does not change the DNA sequence, thus, presenting the classic 'epigenetic mark' (121). Accumulating evidence has shown that DNAm is functionally involved in many forms of stable epigenetic repression, such as imprinting, X chromosome inactivation and silencing of repetitive DNA (120,121).

There is now extensive evidence in humans that methylation levels genome-wide in peripheral blood, cord blood as well as in placenta and offspring candidate genes involved in GC action are altered by the early life environment (112).

Placental mildly increased DNAm of GC-related genes, such as *11β-HSD2*, FK506 binding protein 51 gene (*FKBP5*), and Nuclear Receptor Subfamily 3 Group C Member 1 gene (*NR3C1*), has been associated with higher perceived maternal prenatal stress (Figure 2); increased DNAm of *11β-HSD2* and *FKBP5, in turn,* was associated with reductions in a key fetal coupling, indicative of delayed neurobehavioral development (122). Maternal depression has been associated with greater placental DNAm of GR-coding *NR3C1* and *11β-HSD2* and predicted poorer self-regulation, lower muscle tone, and more lethargy in neonates (123).

Prenatal stress was significantly associated with offspring methylation in the *NR3C1* exon 1F CpG site 36 methylation in a meta-analysis across 7 studies (124). Other stress-related genes which have been investigated in the context of prenatal distress and child DNAm include *FKBP5*, gene for CRH binding protein (*CRHBP*), CRH receptors 1 and 2 (*CRHR1* and *CRHR2*), and the MR-coding Nuclear Receptor Subfamily 3 Group C Member 2 (*NR3C2*) (125). Overall, however, recent systematic reviews on the effects of maternal prenatal depression and anxiety on the offspring methylation status of candidate genes indicate that the findings are inconsistent (5,6,124,125).

Epigenome-wide studies of maternal prenatal stress and psychopathology and the offspring DNAm have also yielded mixed findings. While some studies identified CpG sites with significantly different DNAm levels in neonates exposed to maternal non-medicated depression or anxiety (126) and antidepressants in pregnancy (127), others revealed no significant genome-wide association between maternal depressive symptoms and infant DNAm (128).

After birth, epigenetic modifications also play a crucial role in the interaction of ELS with specific genotypes, as they regulate functional expression of genes by decreasing, silencing, or increasing gene expression (129). For example, childhood adversity has been shown to interact with *FKBP5* rs1360780 single nucleotide polymorphism (SNP) and induce its demethylation and moderate the risk for PTSD (24,130).

Epigenome-wide DNAm studies in peripheral tissues following ELS exposure have also been conducted. More than 800 differentially methylated genes implicated in cellular signaling, immune responses and brain function were detected in blood samples from children exposed to institutional placement, compared to children raised by their biological parents (131).

In animals, alterations in DNAm in candidate genes and genome-wide have been found in the hypothalamus and hippocampus of the offspring exposed to prenatal stress or to synthetic GCs, and in primary neuronal cell line in response to synthetic GCs (132,133). While alterations in offspring DNAm in candidate genes and genome-wide have been studied also in humans in response to maternal prenatal depressive and anxiety symptoms, the pattern of findings is highly inconsistent (5,6,125,134). The conflicting findings may reflect small sample sizes and studying DNAm in tissues with uncertain relevance for offspring neurodevelopment, namely cord or peripheral blood, placenta, buccal smear or saliva.

Therefore, both the candidate gene methylation and large-scale epigenome-wide DNAm approaches have their limitations: the former one may not reflect the complexity of the prenatal adversity exposure effects on the developing epigenome, while the latter requires pooling and harmonizing data from multiple cohorts across varying tissue types, exposure and outcome measurements. Furthermore, these findings are usually based on the retrospective data in populations with established physical and mental health problems, limiting the options for prevention and early intervention. However, novel DNAm-based polyepigenetic biomarkers calculated at early stages of development might address these limitations.

2.3.2.1 Polyepigenetic fetal GC exposure score

A recent study identified 496 CpG sites with significant changes in DNAm following *in utero* synthetic GC exposure overlapping between peripheral whole blood and hippocampal progenitor cells in the Max Planck Institute of Psychiatry (MPIP) cohort (13). Based on these CpGs a cross-tissue weighted polyepigenetic GC exposure score was generated, identifying 24 CpG sites in fetal cord blood in the Prediction and Prevention of Pre-eclampsia and Intrauterine Growth Restriction (PREDO) cohort. Maternal depressive and anxiety symptoms during pregnancy were associated with lower polyepigenetic GC exposure score of the fetus suggesting that these fetuses might be vulnerable for neurodevelopmental adversities later in life (13). However, whether the polyepigenetic GC exposure score at birth could predict

the child neurodevelopmental risk or could be the biomarker, mediating the effect of maternal prenatal depression and anxiety on the offspring development, remains unknown and we address this knowledge gap in Study II.

2.3.2.2 Polyepigenetic biomarkers of aging: Epigenetic Gestational Age (GA) and Epigenetic clock

DNAm can also be used to generate aggregate markers of aging, such as the Hannum (135), the Horvath (17) and the Levine (136) epigenetic age predictors. The Hannum age predictor is based on DNAm of 71 CpG sites in whole blood of 19- to 101-year-old individuals, demonstrating a median absolute difference between DNAm age and actual chronological age of up to 4.9 years (135). The Horvath age predictor is based on DNAm of 353 CpG sites of multiple tissues of 0- to 100-year-old individuals with a median absolute difference between DNAm and chronological age of up to 3.5 years (17). Both predictors are highly correlated with an individual's chronological age ($r > 0.91$). The Levine age predictor, also known as 'PhenoAge', is a newer biomarker of aging and is based on the 513 CpG sites in whole blood regressed against chronological age and nine markers of phenotypic aging: albumin, creatinine, glucose, C-reactive protein, lymphocyte percentage, mean cell volume, red blood cell distribution width, alkaline phosphatase and white blood cell count (136). In this way, by tapping into physiological dysregulation, the Levine clock yielded improved predictions for all-cause mortality and age-related diseases compared to the Hannum and the Horvath clocks (136).

The difference between DNAm age and chronological age is called 'epigenetic age acceleration' (AA), which reflects the rate of biological aging, with a positive value suggesting older biological age in comparison to chronological age. AA, based on these molecular aging biomarkers, has been shown to predict disease trajectories and mortality more accurately than chronological age (137). A recent systemic review and meta-analysis of studies in middle-aged and elderly individuals revealed that the Horvath and the Hannum-based measures of AA were associated with an increased risk of cancer incidence, CVD (including stroke and coronary heart disease), and all-cause mortality (138). The meta-analysis also indicated that each 5-year increase in DNAm age was associated with an 8 to 15% increased risk of mortality (138). Furthermore, AA was associated with higher body-mass index (139), menopause (140), chronic inflammation (136,141,142), lower physical and cognitive fitness (143), increased risk for Alzheimer's disease (144) and PTSD (145), and lower longevity (146).

Studies in middle-aged to elderly populations are, however, confounded by the often decade-long processes of aging-related disease and aging in itself. Therefore, studies of aging should focus on younger groups, when inter-individual differences in aging trajectories start to emerge, but before most

age-related diseases become manifest (147). Such studies focusing on AA early in life are scarce. In one study, which tested associations between the Horvath epigenetic age predictor at birth, 7 and 17 years and physical growth and development among 400-1000 UK children, found that higher AA at birth predicted higher fat mass in childhood and adolescence, faster growth in weight and body mass index (BMI), slower growth in fat mass, and higher odds of increasing Tanner stage of testes development between childhood and adolescence (148). The same study also found that AA at age 7 was associated with increased height in childhood and adolescence, but slower growth in height between childhood and adolescence (148), suggesting earlier physiological maturation. The study of pubertal development in 94 Chilean adolescent girls revealed that a five-year average increase in Horvath clock-based AA was associated with a significant decrease in time to menarche and 5% greater percentage of fibro-glandular volume, and revealed an overall stronger inverse association of AA with pubertal tempo (149). In a study of 46 US adolescent girls also using the Horvath epigenetic age predictor, AA at age 13 years was associated with higher salivary cortisol (150). There is, however, an absence of literature of other early life phenotypes well-known to be related to aging-related diseases and/or premature mortality, namely, psychiatric problems and cognitive functioning (151,152). We address this critical knowledge gap in Study III.

Furthermore, it is probable that departure of DNAm age from chronological age starts as early as *in utero*. A recent study demonstrated that a higher epigenetic gestational age (GA) (higher DNAm GA than chronological GA), based on the Horvath and the Hannum epigenetic age predictors of cord blood methylation data, was associated with maternal smoking during pregnancy and delivery by cesarean section (14). These predictors are, however, not well suited for epigenetic age estimation at birth, because their correlation with chronological GA is nearly 0 (14).

To address this problem, two epigenetic clocks were developed to estimate GA of neonates. Knight's clock is based on fetal umbilical cord blood or newborn blood spots and calculates the DNAm GA using 148 CpG sites (15). Bohlin's clock estimates the DNAm GA using 96 CpG sites from the cord blood (153). Both Knight's and Bohlin's DNAm GAs showed a high correlation with ultrasound-based GA in their testing datasets ($r > 0.81$) (15,153). Unlike the wide applications of the Horvath's and Hannum's epigenetic clocks in adults, studies of epigenetic GA are limited. In the Knight's et al. study lower epigenetic GA (lower DNAm GA than chronological GA) at birth was associated with maternal socioeconomic disadvantage and low birth weight (15). In 814 Finnish mother-neonate pairs, Girchenko et al. have extended these analyses by showing that lower epigenetic GA was associated with maternal insulin-treated gestational diabetes mellitus in a previous pregnancy and Sjögren syndrome, and higher epigenetic GA with maternal age over 40 years at delivery, neonate's lower 1-minute Apgar score, and female sex (12). In the study which examined both Knight's and Bohlin's epigenetic GAs,

maternal vitamin D3 supplementation was associated with lower Knight's and Bohlin's epigenetic GAs, but only in African American subgroup (154). In the same study both epigenetic GAs were positively associated with birth weight and head circumference and, additionally, Bohlin's higher epigenetic GA was associated with maternal BMI and birth weight (154).

However, it remains unclear whether maternal depression and anxiety may affect the epigenetic GA and whether it may be the biological mechanism mediating the effects of maternal adversity during pregnancy on the offspring development later in life. We address these questions in Study I.

2.3.3 GENETIC VULNERABILITY

While overexposure to GC due to maternal adversity during pregnancy may have effect on the fetal GR, possibly via epigenetic modifications, and, thus, affect its sensitivity to stress and cortisol exposure later in life, postnatal and childhood stress act on child's HPA axis directly, posing long-term effects on GR sensitivity (83). However, not all individuals, who are exposed to either prenatal or early life adversity, or both, develop stress-related diseases later in life (47,155). In the three-hit model, resilience or vulnerability to develop stress-related disorders across the lifespan has been explained in terms of the interaction between the genetic variation with priming early life adversity, which influences the brain and body response to significant stress later in life. The genetic variant that potentially provides an unfavorable genetic make-up is, therefore, the primary component in this framework (48).

The classic view of how GCs may permanently alter transcription of proteins, hormones and neurotransmitters involved in brain development and function is via genetic activation. The GCs activate intracellular GR and MR which translocate to the nucleus, bind to specific DNA sequences and modulate the messenger RNA (mRNA) regulation (48). While MR is mainly restricted to limbic parts of the brain, GRs are the ones that primarily bind cortisol throughout the body and brain, thus presenting the main focus in the genetic studies (48). The hypersensitivity of the cortisol feedback is at least partly due to SNPs in the *NR3C1*, *CRHR1*, and *FKBP5* (156). These genes are integral to the reactivity and regulation of the HPA axis and therefore cortisol function.

SNPs refer to genetic variation in a single nucleotide at specific DNA loci (alleles) and give rise to different forms of the gene. There are major alleles – the alleles that are encountered in higher proportion of the population, and minor alleles.

A number of specific *NR3C1* SNPs have been found to contribute to asthma (157), elevated stress response (158), and depression (159,160). SNPs in *CRHR1* have been implicated in depression and anxiety (161) and addictive behavior (162). Polymorphisms in *FKBP5* have been extensively associated with elevated recovery cortisol both in adults following Trier Social Stress Test (163) and infants in response to Strange Situation Procedure (164), as well as

with depression, anxiety and PTSD (165–167). *FKBP5* has also been identified as a promising therapeutic target for obesity and related metabolic outcomes, such as adipogenesis, regulation of glucose metabolism, and T2D (168).

Although the candidate gene approach has demonstrated certain results, they are usually hard to replicate. Moreover, the assumption that genes cause diseases and the expectation that direct paths would be found from gene to disease has not proven fruitful for complex psychiatric and somatic disorders (169). Recognizing the shortcomings of candidate gene approach, the focus of studies of complex traits has largely shifted to genome-wide association studies (GWAS) (170) and generation of polygenic risk scores (171). While GWAS studies proved to be reproducible and revealed valuable insights in the genetic makeup of complex phenotypes (170), similarly to large-scale epigenome-wide DNAm approaches, they require pooling and harmonizing data from multiple cohorts across varying exposure and outcome measurements, thus, available for a rather small number of traits. To date, no GWAS has identified genetic variants implicated in stress reactivity, and polygenic risk scores only include a handful of SNPs based on the only genome-wide association meta-analyses of morning plasma cortisol levels published thus far (172,173). Therefore, despite the advantages of GWAS and polygenic risk score studies, it remains inevitable to rely on old-fashioned methodology for studies of genetic vulnerability for stress-related outcomes.

2.3.3.1 Gene x Environment interaction and FKBP5

Seminal paper by Caspi et.al on the serotonin transporter gene (*5-HTT*) has demonstrated that individuals with one or two copies of the short allele in *5-HTT* promoter polymorphisms exhibited more depressive symptoms and suicidality only if they also experienced stressful life events, compared to no such effects in the long allele carriers (174). While later research found no support for any candidate gene polymorphism or any polymorphism by environment associations with depression phenotypes (175,176), this historical study opened the avenue for the gene by environment (GxE) interaction studies.

Among GxE interaction studies, one of the most consistent results come from the examination of *FKBP5* polymorphisms in relation to early life stress (23–25).

FKBP5 codes for the FK506 binding protein 51, a heat shock protein 90 co-chaperone (167). It participates in inhibition of the GR activity, whereas GR activation induces *FKBP5* transcription via steroid hormone response elements (177). *FKBP5* forms an intracellular negative feedback loop that regulates the GR sensitivity and, thus, is responsive to stressful exposures, making it a promising candidate for GxE interaction studies (25).

The best investigated SNPs belong to functional haplotype, a set of SNPs that tend to be inherited together, spanning the whole *FKBP5* gene (from the promoter area to the 3′ UTR in Caucasians) that is tagged by rs3800373,

rs9296158, or rs1360780 and contains up to 18 polymorphisms, which are in strong linkage disequilibrium (LD) with the tagging SNP (25).

The effects of GxE interaction with these polymorphisms are found across the lifespan. In infants, newborns who went through neonatal intensive care unit (NICU) care procedures, and had a minor allele in one of the genotyped *FKBP5* SNPs (rs3800373 (C), rs1360780 (T), rs9470080 (T)) had higher risk for poorer neurobehavioral outcomes compared to protective allele carriers (178). In children, those who experienced an acute medical injury and had at least one minor allele in rs3800373 (C) or rs1360780 (T) showed higher peritraumatic dissociation (179), a well-established risk factor for the development of PTSD (180) in comparison to major allele carriers. The case-control study among adolescents demonstrated that the odds for being in the major depression (MD) group increased to a greater extent among carriers of at least one copy of the *FKBP5* CATT haplotype consisting of minor alleles of rs3800373, rs9296158, rs1360780 and rs9470080 SNPs or at least one minor allele of these SNPs depending on the number of sociodemographic, moderate and total number of stressors (181). A series of studies in adults have demonstrated that individuals who were exposed to ELS and had one or two copies of minor allele in rs1360780 (T), rs9296158 (A), rs9470080 (T), rs9394309 (G), had elevated risk of MD and subclinical depressive symptoms (182–184) and PTSD (130,185). Neuroimaging studies show that among MD patients with ELS experience, the minor rs1360780 T allele carriers displayed lower volumes within the hippocampus-amygdala-transition-area compared to those homozygous for the major C allele (186).

However, to date only a handful of studies has examined whether *FKBP5* SNPs interact with ELS on physical health outcomes. Individuals with increased peritraumatic distress and at least one copy of the *FKBP5* rs3800373 minor allele (G) reported more severe chronic pain relative to individuals with less peritraumatic distress or those with two copies of major T allele (187). Another study of chronic pain showed that in participants, who survived motor vehicle collision (MVC), presence of one minor allele in 6 *FKBP5* SNPs (rs3800373, rs7753746, rs9380526, rs9394314, rs2817032, rs2817040) predicted neck pain severity and overall pain six weeks after the traumatic event (188). The authors also replicated the results among sexual assault survivors: of the six SNPs associated with pain outcomes in the MVC discovery cohort, four (rs3800373, rs9394314, rs2817032, rs2817040) were associated with overall pain severity and three (rs3800373, rs9380526, rs2817032) were associated with neck pain severity six weeks after sexual assault (188). In another national community-based study, carriers of one or two minor T-alleles of rs1360780 were found to be at increased risk for self-reported physician-diagnosed physical problems if they retrospectively reported exposure to physical, emotional, or sexual abuse in childhood, but not if they were homozygous major C-allele carriers (189). However, the extent to which *FKBP5* polymorphisms interact with ELS in predicting CVD, T2D,

and quantitative glycemic traits remains unknown. We address this knowledge gap in Study IV.

3 AIMS OF THE STUDY

To address these critical knowledge gaps in the literature, the overall aim of this work was to explore polyepigenetic biomarkers and markers of genetic vulnerability in association with prenatal and early life adverse exposures and mental and physical health problems in children, adolescents and older adults.The more specific objectives of each study include:

I. The aim of Study I was to examine whether maternal history of depression diagnosed before pregnancy and depressive symptoms during pregnancy, were associated with a polyepigenetic biomarker, namely the child's epigenetic GA at birth based on fetal cord blood DNAm data. Next, Study I explored whether this polyepigenetic biomarker at birth was associated with and mediated the associations of maternal depression with child psychiatric problems in the PREDO cohort in childhood. Additionally, Study I examined whether these associations were moderated by child's sex.

II. The aim of Study II was to examine whether a polyepigenetic biomarker, namely child polyepigenetic GC exposure score at birth, which correlated with maternal depressive and anxiety symptoms during pregnancy in the PREDO cohort, was associated with any child mental and behavioral disorder and its severity measured as the number of days in in- or outpatient treatment in medical care. Additionally, Study II explored whether this polyepigenetic biomarker mediated the associations of maternal prenatal depressive and anxiety symptoms and the child mental health outcomes in the PREDO cohort in childhood.

III. As epigenetic processes undergo age-related changes, the aim of Study III was to examine whether a polyepigenetic biomarker, namely the epigenetic clock of aging based on the Horvath's epigenetic DNAm age predictor, was associated with physical growth, HPA axis functioning, psychiatric problems and cognition in the Glycyrrhizin in Licorice (GLAKU) cohort in adolescence.

IV. The aim of Study IV was to examine whether three selected common SNPs (rs1360780, rs9394309, rs9470080) in *FKBP5*, the gene that plays a role in the HPA-axis regulation, interacted with ELS defined here as temporary separation from both biological parents due to child evacuations during World War II (WWII), in prediction of CVD, T2D, and quantitative glycemic traits in the Helsinki Birth Cohort Study (HBCS) cohort in late adulthood.

4 METHODS

4.1 OUTLINE OF THE STUDY COHORTS

This thesis is based on three prospective cohorts: the Prediction and Prevention of Pre-eclampsia and Intrauterine Growth Restriction (PREDO) cohort, the Glycyrrhizin in Licorice (Glaku) cohort, and the Helsinki Birth Cohort Study (HBCS). The methods are described by the Study numbers (Study I – IV).

The outline of the study cohorts and their representativeness are described in Table 1.

Table 1. Outline of the study cohorts, sample sizes and representativeness of the analytic samples

	Study I	Study II	Study III	Study IV
Cohort	PREDO	PREDO	Glaku	HBCS
Entire Cohort, N	4,777	4,777	1,049	13,345
Representative Cohort, N	1,089	1,089	451	2,003
Analytic Sample, N (%)	814 (74.8%)	814 (74.8%)	239 (53.0%)	1,728 (86.3%)
Representativeness of the Analytic Sample	Children from the analytic sample were more mature at birth, younger at follow-up and had higher externalizing problems in comparison to the representative cohort. The groups did not differ in child sex, 1-min Apgar score, internalizing and total psychiatric problems, nor did their mothers differ in age at birth, pre-pregnancy BMI, gestational diabetes, hypertensive disorders,	Children from the analytic sample were more mature at birth and younger at follow-up, and their mothers less frequently reported having type 1 diabetes, in comparison to the representative cohort. The groups did not differ in child sex and childhood mental and behavioral disorders, nor did their mothers differ in age at birth, pre-pregnancy BMI,	Adolescents from the analytic sample had higher weight, height and BMI, and their mothers had higher weight at delivery, in comparison to the representative cohort. The groups did not differ in chronological age at adolescent follow-up, sex, birth order, body size at birth, GA, nor did their mothers differ in licorice or alcohol consumption or	Adults from the analytic sample were more often women and had lower fasting insulin, 30-min glucose and HOMA-IR indices. The groups did not differ in evacuation status or father's occupational status in childhood, age at clinical examination, BMI, T2D, IFG, IGT, CVD, fasting and 120-min glucose, 30-min and 120-min insulin,

	Sjogren syndrome, maternal education, smoking or alcohol consumption during pregnancy, maternal history of depression or antenatal depressive symptoms.	gestational diabetes, hypertensive disorders, maternal education, smoking during pregnancy, maternal lifetime diagnosis of any mental disorder, antenatal depressive and anxiety symptoms.	smoking during pregnancy, BMI at delivery or mode of delivery, age at menarche; neither their mothers and fathers differ in educational attainment or height.	incremental insulin, ISI and AUCs indices.
Exposure and its timing	Maternal history of depression diagnosed before pregnancy and depressive symptoms during pregnancy (Prenatal)	Maternal depressive and anxiety symptoms during pregnancy (Prenatal)	-	Temporary separation from both biological parents due to child evacuations during WWII (Childhood)
Biomarker and timing of its measurement	Epigenetic GA at birth	Polyepigenetic GC exposure score at birth	Epigenetic clock based on Horvath's epigenetic age predictor in adolescence	Genetic vulnerability represented by *FKBP5* polymorphisms in late adulthood
Outcome and its timing (Mean Age (years))	Psychiatric problems in childhood (3.7)	Mental and behavioral disorder diagnosis and its severity in childhood (8.5)	Physical growth, HPA axis functioning, psychiatric problems and cognition in adolescence (12.4)	T2D, CVD and quantitative glycemic traits in late adulthood (61.5)

Note. PREDO – Prediction and Prevention of Pre-eclampsia and Intrauterine Growth Restriction cohort; Glaku – Glycyrrhizin in Licorice cohort; HBCS – Helsinki Birth Cohort; BMI – Body Mass Index; GA – Gestational Age; HOMA-IR – Homeostasis Model Assessment Method; T2D – Type 2 Diabetes; IFG – Impaired Fasting Glucose; IGT – Impaired Glucose Tolerance; CVD – Cardiovascular Disease; ISI – Insulin Sensitivity Index; AUC – Area Under the Curve GC – Glucocorticoid; WWII – World War II

4.2 STUDY I

4.2.1 PARTICIPANTS

The participants of Study I come from the PREDO study, which comprises altogether 4,777 mothers who gave birth to a singleton live-born offspring in Finland between 2006 and 2010 (Table 1) (190).

The women were recruited in consecutive order when they attended their first ultrasound screening at 12 to 13 weeks of gestation at one of the ten study hospitals in Southern and Eastern Finland.

To enrich the number of women with pre-eclampsia and IUGR in the PREDO sample, 1,079 pregnant women with known risk-factor status were recruited. Among them 969 had one or more risk factors for preeclampsia and IUGR, and 110 had no known risk factors (190). Additional 10 participants come from the epidemiological hand of the study, who also provided placental samples.

In total, we had 817 (75.4% of the sample) fetal umbilical cord blood samples with full information on genome-wide methylation and genotype passing through quality control. Of those, additional 3 samples were excluded for Study I (Figure 3), thus, forming our analytic sample (n=814).

Figure 3 presents the participant flow chart for Studies I and II and Table 1 describes the representativeness of the analytic sample.

The Ethics Committees of the Helsinki and Uusimaa Hospital District and the participating hospitals approved the study protocol. Written informed consents were obtained from all participating women.

4.2.2 MEASURES

4.2.2.1 Prenatal exposure: Maternal antenatal depression

Maternal antenatal depressive symptoms were estimated with the 20-item Center for Epidemiological Studies Depression Scale (CES-D) (191) biweekly up to 14 times throughout pregnancy starting from 12+0-13+6 until 38+0-39+6 weeks+days gestation or delivery. The CES-D comprises 20 questions, rated from none (0) to all of the time (3) on depressive symptoms during the past week, and a sumscore ranges from 0 to 60. For Study I trimester-specific

means of the CES-D raw sum-scores were calculated to represent the level of depressive symptoms at each pregnancy trimester and the mean of these three trimester-specific values to represent the overall level of antenatal depressive symptoms.

Additionally, between 12+0–13+6 weeks+days of gestation participants answered the question "Have you ever been diagnosed by a physician with depression?" followed by a question on timing of the diagnosis. 76 women reported the diagnosis before pregnancy and 12 of them indicated using antidepressant medication during the past year.

4.2.2.2 Polyepigenetic biomarker at birth: Child epigenetic GA

Fetal cord blood samples were collected according to standard procedures. DNA was extracted at the National Institute for Health and Welfare, Helsinki, Finland, and the Institute for Molecular Medicine Finland, University of Helsinki, Finland. Methylation analyses were performed at the Max Planck Institute of Psychiatry (MPIP) in Munich, Germany. DNA was bisulphite converted using the EZ-96 DNA Methylation kit (Zymo Research, Irvine, CA). Genome-wide methylation status of over 485,000 CpG sites was measured using the Infinium Human Methylation 450 BeadChip (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. The arrays were scanned using the iScan System (Illumina Inc., San Diego, CA). The quality control pipeline was set up using the R-package minfi (192). Samples with maternal blood contamination were excluded (n = 9) (193). The final dataset contained 428,619 CpG sites.

DNAm GA was calculated following the Knight et al. method and is based on the methylation profile of 148 selected CpG sites (15). Chronological GA was based on ultrasound scans performed at 12+0-13+6 weeks+days of gestation. We calculated epigenetic GA as the arithmetic difference between DNAm GA and chronological GA and adjusted for chronological GA. Adjustment for chronological GA was necessary to remove the effect of chronological GA entirely (Pearson correlation between DNAm GA-GA arithmetic difference and GA $r = -0.27$, $p < 0.01$).

To control for the potential effects of cell type heterogeneity in fetal umbilical cord blood, cord blood cell composition was estimated for seven cell types (nucleated red blood cells, granulocytes, monocytes, natural killer cells, B cells, CD4(+)T cells, and CD8(+)T cells) following the Bakulski et al. method (194) using the R-package minfi (192).

To control for the potential effects of population structure, genotyping was performed on Illumina Human Omni Express Exome Arrays (Illumina Inc., San Diego, CA). Only markers with a call rate of at least 98%, minor allele frequency of 1% and a p value for deviation from Hardy–Weinberg equilibrium > × 10−06 were kept in the analysis. We performed multidimensional scaling (MDS) analysis on the identity by state matrix of quality-controlled genotypes (195). The first 2 MDS components depicted the population structure.

4.2.2.3 Child outcomes: Internalizing and externalizing problems

The mothers rated their child's psychiatric problems at the child's age of 2.3 to 5.8 years using the Child Behavior Checklist (CBCL 1/ $\frac{1}{2}$ - 5) comprising 99 problem items rated on a scale of "not true" (0) to "very or often true" (2) (196). Based on the 99 problem items we calculated the *t* scores for the total problems and its two subscales, internalizing and externalizing problems.

4.2.3 COVARIATES

All analyses were adjusted for child's chronological GA, cord blood cell type composition and the first two MDS components in order to remove the effect of chronological GA and to control for the potential effects of cell type heterogeneity and the population structure discussed above.

Based on our previous findings in the Study I cohort (12), we thereafter made adjustments for maternal age at delivery (≥40 years/<40 years), insulin-treated gestational diabetes mellitus in a previous pregnancy (yes/no), Sjögren syndrome (yes/no), neonate's 1-minute Apgar score (≤6/>6), and sex (girl/ boy). In the analyses of child psychiatric problems, we made further adjustments for child's age at follow-up (years) and maternal Beck Depression Inventory–II scores (BDI-II <14/BDI-II ≥14) (197) reported at the child follow-up in order to control for concurrent depressive symptoms.

4.3 STUDY II

4.3.1 PARTICIPANTS

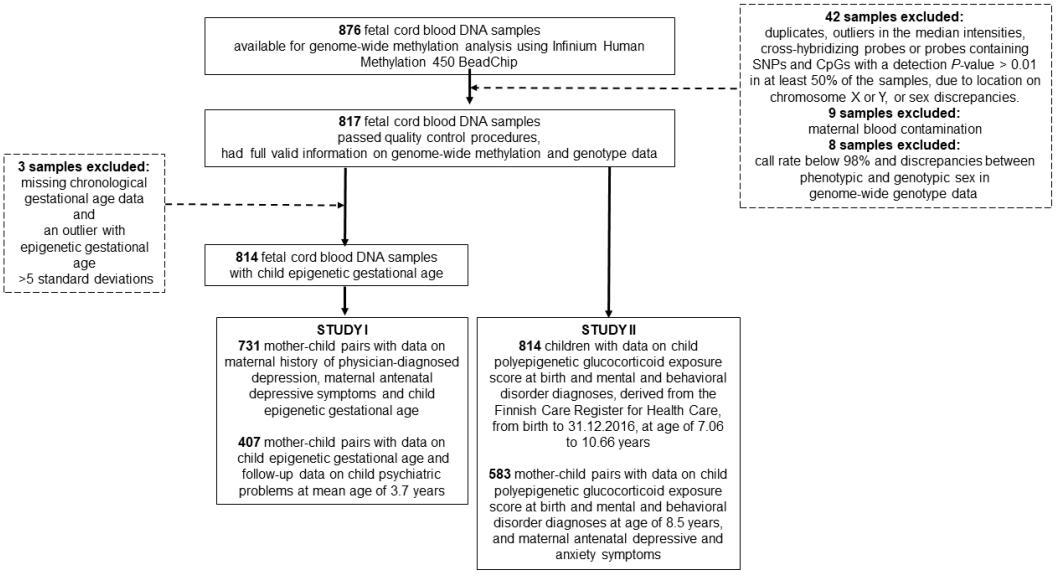

Figure 3 Flow chart of the PREDO study

The participants of Study II come from the same representative cohort as Study I. The analytic sample for this study consisted of 814 participants. For more details on the sample attrition and its representativeness, see the respective section for Study I, Table 1 and Figure 3.

4.3.2 MEASURES

4.3.2.1 Prenatal exposure: Maternal antenatal depressive and anxiety symptoms

Depressive symptoms were measured using the CES-D questionnaire described above (191). For Study II the mean of all 14 raw sum-scores was used to reflect the level of depressive symptoms throughout pregnancy.

Anxiety symptoms were measured using the State Anxiety Inventory (STAI) (198). The STAI comprises 20 items rated from not at all (1) to very much (4), and a sumscore ranges from 20 to 80. We calculated the mean of all 14 raw sum-scores representing the overall level of antenatal anxiety symptoms.

4.3.2.2 Polyepigenetic biomarker at birth: Polyepigenetic GC exposure score

DNAm and genotyping are described in the respective section of Study I description.

Weighted polyepigenetic GC exposure score was calculated from the selected 24 CpG sites as described previously (13). The methylation level of each site was multiplied by the weight and summed to get the score for each sample. The weights represent the coefficients from the elastic-net regression using dexamethasone (DEX) associated changes in DNA methylation of the CpG sites in peripheral blood in the MPIP cohort (13).

4.3.2.3 Child outcomes: Any mental and behavioral disorder and its severity

We identified any mental and behavioral disorder diagnosis in children from the Care Register for Health Care (HILMO) between the child's birth in 11/07/2006-07/24/2010 and 12/31/2016, when the children were 7.06–10.66 years old. The HILMO includes primary and subsidiary diagnoses of all inpatient treatments and of outpatient treatments in public specialized medical care settings in Finland. We included diagnoses coded F00-F99 according to International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10) in the mental and behavioral

disorder category. Validation studies have indicated that HILMO has high validity for psychiatric diagnoses (199).

To study severity of any mental and behavioral disorder diagnosis, we summed up the number of days the child had been receiving in- or outpatient treatment for mental or behavioral disorder as the primary diagnosis. Table 2 shows the number of broad category diagnoses according to the number of days in in- or outpatient hospital treatment dichotomized at the median.

Table 2. Child Mental and Behavioral Disorder Diagnoses. The number and percentage of offspring with any mental and behavioral disorder diagnosis.

	ICD-10 Code	Total Sample (N=99)	Subsample below median (N=49)	Subsample above median (N=50)	p-value
		N(%)	N(%)	N(%)	
Number of days of any inpatient treatment or outpatient treatment in a specialized hospital (range)		1 – 108	1 – 6	7 – 108	
Any childhood mental disorder	F00-F99	99 (100%)	49 (49.5%)	50 (50.5%)	NA
Non-affective psychosis	F2	0	0	0	NA
Mood disorders	F3	1 (1%)	0	1 (2%)	0.32
Neurotic, stress-related, and somatoform disorders	F4	1 (1%)	1 (2%)	0	0.31
Behavioral syndromes associated with physiological disturbances and physical factors	F5	11 (11.1%)	9 (18.4%)	2 (4%)	0.023
Disorders of adult personality and behavior	F6	0	0	0	NA
Mental retardation	F7	2 (2%)	1 (2%)	1(2%)	0.988
Psychological development disorders	F8	64 (64.6%)	23 (46.9%)	41 (82%)	<0.001
Behavioral and emotional disorders	F9 (F90-F98)	49 (49.5%)	19 (38.8%)	30 (60%)	0.035
Unspecified mental disorder	F99	0	0	0	NA
Number of broad category diagnoses	F2 – F9				<0.001
1		75 (75.8%)	46 (93.9%)	29 (58%)	
2		20 (20.2%)	2 (4.1%)	18 (36%)	
3		4 (4%)	1 (2%)	3 (6%)	

Note. ICD-10 refers to International Statistical Classification of Diseases and Related Health Problems, Tenth revision

4.3.3 COVARIATES

All analyses were adjusted for cord blood cell type composition, the first two genetic MDS components, child's sex, gestational age at birth, maternal age at delivery (years) and maternal smoking during pregnancy (yes/no) following the previous study of polyepigenetic GC exposure score in this cohort (13). All analyses were further adjusted for child's birth year and variables related to maternal physical and mental risk factors: maternal education (primary or secondary/tertiary), having at least one of the known maternal metabolic risk factors during pregnancy (hypertensive disorder, gestational diabetes mellitus, type 1 diabetes, pre-pregnancy body-mass index (BMI) ≥25), and maternal lifetime diagnosis of any mental disorder. Maternal lifetime diagnoses of any mental disorder were derived from HILMO in- and outpatient visits (any/no; ICD-9: 290-319, ICD-10: F00-F99 diagnosis codes with inpatient data available between 1987 and 2016 and outpatient data available between 1998 and 2016).

In the analysis of the severity of the mental and behavioral disorders, we made additional adjustments for follow-up time.

4.4 STUDY III

4.4.1 PARTICIPANTS

The participants for Study III come from Glaku, an urban community-based cohort comprising 1,049 infants born between March and November 1998 in Helsinki, Finland (200). Between 2009 and 2011, 920 were invited and 451(49% participation rate) participated in the follow-up at the mean age [standard deviation (SD)] of 12.3 [0.5] years. Of the participating adolescents, 243 provided blood samples, of which 239 DNA samples remained for genetic analyses after quality control procedures. These participants formed the analytic sample for Study III. Its representativeness is described in Table 1.

Ethics Committees of the City of Helsinki and the Uusimaa Hospital District approved the study protocol. Written informed consent was obtained from the mother at birth and from parent/guardian and adolescent at the follow-up.

4.4.2 MEASURES

4.4.2.1 Polyepigenetic biomarker in adolescence: Epigenetic clock based on Horvath's epigenetic age predictor

Blood samples were collected, and DNA was extracted according to standard procedures.

Methylation analyses were performed at the MPIP in Munich, Germany. DNA was bisulphite-converted using the EZ-96 DNA Methylation kit (Zymo Research, Irvine, CA). Genome-wide methylation status of over 850,000 CpG sites was measured using the Illumina Infinium MethylationEPIC arrays (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. The arrays were scanned using the iScan System (Illumina Inc., San Diego, CA). The final dataset contained 812,943 CpGs.

We calculated DNAm age using the Horvath age estimation algorithm (17) with a freely available online tool (http://labs.genetics.ucla.edu/horvath/dnamage/). This calculator also incorporates information on blood cell counts for six cell types (granulocytes, monocytes, natural killer cells, B cells, CD4+ T cells, and CD8+ T cells) based on the Houseman method (201). We calculated epigenetic age as the unstandardized residual from a linear regression of DNAm age on chronological age and six cell count types.

To control for the potential effects of population structure, genotyping was performed on Illumina Human OmniExpress Exome 1.2 bead chip (Illumina Inc., San Diego, CA) at the Tartu University, Estonia according to the standard protocols. After performing MDS analysis on the identity by state matrix of quality-controlled genotypes, we identified the first three components, which depicted the origin admixture and were included as covariates in the statistical analyses (195). This information was available for 221 participants (92.5%) of the analytic sample.

4.4.2.2 Adolescence outcomes: Pubertal, neuroendocrine, psychiatric, and cognitive

To estimate physical growth and pubertal development we used the following measures:

(a) The difference between the child's height-for-age SD score based on Finnish growth charts (202) and midparental target height in SD units (203). This score reflects the remaining growth potential and the timing of the pubertal growth spurt.
(b) Weight in light clothing without shoes, values were transformed into weight-for-age SD scores based on Finnish growth charts.
(c) BMI (weight (kg)/height (m2)), values were transformed into BMI-for-age SD scores based on Finnish growth charts.
(d) The Tanner Staging Questionnaire (204) administered by a research nurse.
(e) The Pubertal Development Scale (PDS) (205), a self-report questionnaire on secondary sex characteristics.

To estimate cortisol measurements, saliva samples were collected on two consecutive days using cotton swabs. On the first day, samples were collected upon awakening and 15, 30, 45, and 60 min thereafter, at 12:00 midday, at

5:00 p.m., and at bedtime. A low dose of DEX (3 µg/kg of total body weight) was administered after the bedtime saliva sample, and a sample was collected upon awakening the next day. Salivary cortisol concentrations were determined by solid-phase, time-resolved fluorescence immunoassay with fluorometric end-point detection (DELFIA; Wallac, Turku, Finland).

Of the diurnal measures, we used the following scores:

(a) Cortisol at awakening;
(b) Cortisol awakening response (peak value after awakening minus value upon awakening);
(c) Nadir (minimum of diurnal values);
(d) Response to DEX suppression test (value upon awakening on day two minus value upon awakening on day one).

To estimate psychiatric problems, mothers rated their child's psychiatric problems using the Child Behavior Checklist (CBCL/6-18) comprising 99 problem items rated on a scale of "not true" (0) to "very or often true" (2) (206). The CBCL yields hierarchically structured scales, presented in Figure 4.

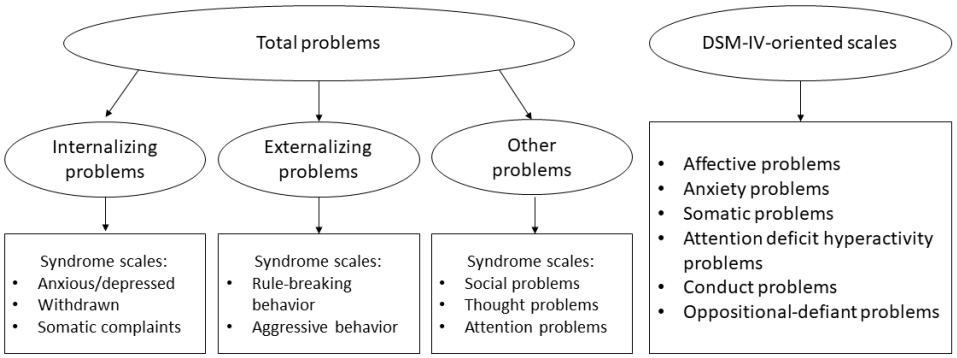

Figure 4 Hierarchical structure of CBCL scales

Following the CBCL manual, we used the 82nd percentile as the cutoff to identify adolescents with borderline clinically significant problems (206).

To measure cognitive abilities, adolescents were administered the short form of the Wechsler-Intelligence-Scale-for-Children-III (207), which included vocabulary, similarities, block design, and picture arrangement subtests. We used age-standardized scores to estimate age-standardized total intelligence, verbal and performance intelligence quotients (IQs) (208).

4.4.3 COVARIATES

All analyses were adjusted for child's sex and the first three MDS components to control for population structure (model 1).

We made further adjustments for covariates previously associated with physical growth and development, salivary cortisol, psychiatric problems, and cognition in this cohort (209): the highest educational level of either parent (secondary or less/vocational/university), maternal age (years), and BMI (kg/m2) at delivery, maternal smoking (no/yes), weekly alcohol (no/yes) and glycyrrhizin in licorice (0–249 mg/week, 250–499 mg/week, ≥ 500 mg/week) consumption during pregnancy, delivery mode (vaginal/cesarean), parity (primiparous/multiparous), gestational age (weeks), and birth weight (grams) of the adolescent (model 2).

In addition, we conducted analyses of pubertal maturation adjusting for maternal self-reported age at menarche (years) as a proxy of the genetic component of pubertal development.

Analyses of HPA axis activity were additionally adjusted for time at awakening and time at DEX intake as well as for child's BMI-for-age SD score.

4.5 STUDY IV

4.5.1 PARTICIPANTS

The participants for Study IV come from the HBCS, which includes 13,345 men and women, who were born in Helsinki, Finland, between 1934 and 1944, and were alive and living in Finland in 1971 when a unique personal identification number was allocated to all Finnish residents (210,211).

Between 2001 and 2004, a random sample of 2,902 HBCS participants was invited and 2,003 of them participated (69% participation rate) in a clinical examination at mean age [SD] = 61.5 [2.9] years. Blood samples for DNA extraction were obtained at the time of this visit. Genotype data were available for 1728 of those participants and they comprised the analytic sample of Study I. The representativeness of the analytic sample is described in Table 1.

The HBCS study protocol was approved by the Ethics Committee of the National Public Health Institute and it was carried out in accordance with the Declaration of Helsinki. All participants have signed a written informed consent form.

4.5.2 MEASURES

4.5.2.1 Childhood exposure: Early life stress

During WWII nearly 80,000 Finnish children were separated from their biological parents due to evacuation from the strains of war to Sweden or

Denmark (212). Information on the separation was gathered from the Finnish National Archives' registry, which was kept by the Ministry of Social Affairs and Health between 1939 and 1946 and which includes detailed data on separations of 48,628 children. We identified 215 participants (12.4% of the analytic sample) from our study cohort who were separated temporarily from their parents in childhood.

In addition, it is estimated that over 20,000 more children were evacuated without their parents either abroad or within homeland through personal ties (212). Therefore, questions relating to wartime separation from both parents were embedded in the psychological survey at mean age 61.5 years when the participants attended the clinical examination, where additional 58 participants (3.4% of the analytic sample) self-reported being separated from both parents during WWII.

4.5.2.2 Genetic vulnerability: FKBP5 polymorphisms

Genotypes of intronal SNPs rs1360780, rs9394309, and rs9470080 were taken from the modified Illumina 610k array (Illumina, San Diego, California). We chose these SNPs using three criteria: (a) their location in *FKBP5* gene within functional haplotype (25); (b) they have been previously reported to have significant G x E associations with risk of depression (182–184) and PTSD (130) in adulthood after ELS, and altered HPA axis reactivity (163,164); and (c) minor allele frequency > 5% in this sample. Genotyping was conducted at the Wellcome Trust Sanger Institute, Cambridge, UK, according to standard protocols.

Genotyping success rate was >99 % in all three SNPs. Observed genotype frequencies did not deviate from the Hardy-Weinberg equilibrium ($p > .39$). Minor allele frequencies were 21.6 % (C > T) for rs1360780, 24.3 % (A > G) rs9394309, and 25.6 % (C > T) for rs9470080. SNPs were in high LD ($r2 = 0.72–0.94$) and, according to the solid spine algorithm with default values, belonged to the same haploblock.

4.5.2.3 Adulthood outcomes: Type 2 Diabetes, Cardiovascular Disease, and Quantitative Glycemic Traits

After a 12-hour overnight fasting period participants were administered a 75-g oral glucose tolerance test (OGTT). Venous samples for plasma glucose and serum insulin were collected at fasting and further at 30 and 120 minutes after the glucose load. Plasma glucose was measured with a glucose dehydrogenase method (HemoCue, Ängelholm, Sweden) and serum insulin with a fluoroimmunoassay (Delphia; PerkinElmer Finland, Turku, Finland). The participants filled in a questionnaire regarding the physician-diagnosed chronic diseases and use of medication for these diseases during the same visit.

We defined the participants as having diabetes if: (a) they reported a physician-diagnosed diabetes; (b) they reported use of antidiabetic medication; (c) if they met the World Health Organization (WHO) (213) criteria for diabetes based on the OGTT (fasting plasma glucose ≥7.0 mM or 120-minutes plasma glucose ≥11.1 mM).

We defined the participants as having CVD if they reported physician-diagnosed coronary heart disease and/or stroke.

We defined the participants as having impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) if they met the WHO criteria (213) (IFG: fasting plasma glucose 6.1–6.9 mM and 120-minutes plasma glucose <7.8 mM; IGT: fasting plasma glucose <7.0 mM and 120-minutes plasma glucose ≥7.8 and <11.1 mM during the OGTT).

In order to estimate insulin sensitivity and insulin secretion we use the homeostasis model assessment method (HOMA-IR) (214), Insulin Sensitivity Index (ISI) (215), incremental insulin (216), and Area Under the Curve (AUC) (216) indices. The formulas of these indices calculated based on the OGTT are listed in Figure 5.

1. $$\text{HOMA} - \text{IR} = \frac{\text{Fasting plasma insulin } \left[\frac{mU}{l}\right] \; x \; \text{Fasting plasma glucose} \left[\frac{mM}{l}\right]}{22.5}$$

2. $$\text{ISI} = \frac{10000}{\sqrt{\begin{array}{c}(\text{Fasting plasma glucose} \left[\frac{mM}{l}\right] x \; \text{Fasting plasma insulin} \left[\frac{mU}{l}\right] x \\ \text{Mean OGTT glucose} \left[\frac{mM}{l}\right] x \; \text{Mean OGTT insulin} \left[\frac{mU}{l}\right])\end{array}}}$$

3. $$\text{Incremental insulin} = 30\text{−minute insulin} \left[\frac{mU}{l}\right] - \text{Fasting plasma insulin} \left[\frac{mU}{l}\right]$$

4. $$\text{AUC insulin} = 15 \; x \; \text{Fasting plasma insulin} \left[\frac{mU}{l}\right] + 15 \; x \; 30\text{−minute insulin} \left[\frac{mU}{l}\right] + 45 \; x \; 30\text{−minute insulin} \left[\frac{mU}{l}\right] + 45 \; x \; 120\text{-minute insulin} \left[\frac{mU}{l}\right]$$

5. $$\text{AUC glucose} = 15 \; x \; \text{Fasting plasma glucose} \left[\frac{mM}{l}\right] + 15 \; x \; 30\text{−minute glucose} \left[\frac{mM}{l}\right] + 45 \; x \; 30\text{−minute glucose} \left[\frac{mM}{l}\right] + 45 \; x \; 120\text{-minute glucose} \left[\frac{mM}{l}\right]$$

Figure 5 OGTT-based quantitative glycemic traits

4.5.3 COVARIATES

All analyses were adjusted for age at the time of clinical examination (years), sex, body mass index (BMI), and father's occupational status (lower, middle, upper) as a proxy of socioeconomic status (SES) in childhood following previous findings in relation to physical health outcomes in this cohort (217,218), and the first three MDS components derived from

multidimensional scaling analyses performed with Plink to control for population stratification (model 1). We further made adjustments for own maximum attained level of education in adulthood (basic/primary or less, lower secondary, upper secondary, or tertiary) as a proxy of SES at the time of testing (model 2). We also made adjustments for depressive symptoms measured with the Beck Depression Inventory (BDI) (219) and stressful life events during the past 12 months measured with the Stressful Life Event Scale (SLE) (220) (model 3) as they have been previously associated with the three selected SNPs in this cohort (183). In the analyses of glycemic traits, we also used self-reported physician-diagnosed diabetes and use of antidiabetic medication as covariates.

4.6 STATISTICAL ANALYSES

4.6.1 STUDY I

We used linear regression analysis to explore the associations of maternal history of depression diagnosed before pregnancy and trimester-weighted mean of antenatal depressive symptoms with child epigenetic GA. We tested whether these effects were additive by adding maternal depression diagnosis × antenatal depressive symptoms interaction term into regression equation following main effects of these variables. Generalized additive mixed model analyses were used to test if there were gestation stage–specific effects of maternal depressive symptoms levels across the 14 biweekly measurements on child's epigenetic GA. For all analyses, depressive symptoms scores were square root–transformed to attain normality; the symptom scores were further standardized to a mean of 0 and an SD of 1 to facilitate interpretation.

We used linear regression analyses to test associations between child epigenetic GA and psychiatric problems. Problem scores were log-transformed to attain normality and standardized to facilitate interpretation. In order to account for multiple testing we also report Bonferroni corrected CIs assuming two multiple tests, as the internalizing and externalizing CBCL subscales were highly intercorrelated (Pearson $r = 0.62$, $p < 0.001$), and together explained 95.3% of the variance in total problems.

When testing whether the associations varied by sex, we entered sex × history of depression before pregnancy/antenatal depressive symptoms interaction term into the regression equation with child epigenetic GA at birth as the outcome, and sex × child epigenetic GA interaction into the regression equation with child psychiatric problems as the outcome.

Finally, we tested whether child epigenetic GA at birth mediated the association between maternal antenatal depression and child psychiatric problems by using the PROCESS macro for SPSS (version 24.0).

We applied the bootstrapping method in all analyses, using unrestricted random sampling method to generate 1,000 samples, and 95% bootstrap CI using the normal distribution theory.

We used IBM SPSS 24.0 and SAS 9.4 to perform statistical analyses.

4.6.2 STUDY II

With Cox Proportional Hazards models, we estimated the associations between the polyepigenetic GC exposure score and any mental and behavioral disorder in children. Time dependency analysis confirmed that the proportional hazards assumption was met (p-value for the time variable =0.87).

We studied the association between the polyepigenetic GC exposure score and severity of the mental and behavioral disorders in children using Zero-Inflated Negative Binomial (ZINB) regression analysis (221) to account for the excessive number of zeros in the outcome count variable.

We pursued to test whether polyepigenetic GC exposure score mediated the association between maternal depressive and anxiety symptoms during pregnancy and any mental and behavioral disorder and its severity in children using Sobel test. Mediation tests were conducted pending that the criteria for mediation were met, i.e., that the predictor, mediator and the outcome variables were significantly interrelated.

Statistical analyses were performed with SAS 9.4 and IBM SPSS Statistics 25.0.

4.6.3 STUDY III

We used generalized linear models (GLM) to explore associations between epigenetic age and outcomes, specifying Gaussian reference distribution for continuous (growth anthropometry, salivary cortisol, cognition), ordinal logistic for categorical (Tanner stages and PDS), and binary logistic reference distribution for dichotomous outcomes (psychiatric problems).

All analyses were adjusted for covariates and confounders as described in Covariates section for Study IV. We further tested if the associations between epigenetic age and outcomes varied by sex, therefore including sex × epigenetic age interaction term into the GLMs following main effects of these variables as adolescent boys and girls may differ in epigenetic age, pubertal maturation, and the prevalence and etiology of psychiatric problems. In order to account for multiple testing within each developmental domain, we also report Bonferroni-corrected p-values.

We used IBM SPSS version 24.0 software to perform statistical analyses.

4.6.4 STUDY IV

We used multiple linear regression analysis to test if the three selected SNPs in *FKBP5* gene, separation status, and their interaction were associated with the continuous outcomes (fasting, 30-minute, 2-hour, and AUC insulin and glucose; incremental insulin; ISI; and HOMA-IR) and logistic regression for the binary outcomes (IFG, IGT, T2D, and CVD). Each SNP was tested in a separate model assuming both additive and dominant genetic effects.

We further performed haplotype analysis, where rs1360780, rs9394309, and rs9470080 were analyzed within one haploblock in one model.

All continuous variables were log-transformed to attain normality.

We made additional adjustments for SNP by covariate and separation status by covariate interactions as suggested by Keller (222).

In order to account for multiple testing we also present Bonferroni corrected p-values assuming two multiple tests, as indicated by principal component analysis with two factors explaining 82.2% of the total variance.

We used IBM SPSS version 24.0, Plink, and R 3.2.2 software for the analyses. Linkage disequilibrium between the SNPs and haploblock structure was evaluated with Haploview 4.2 (223). We performed haplotype analyses using the Haplo.stats package 1.6.11 of the R statistical software (224).

5 RESULTS

5.1 EPIGENETIC GA, MATERNAL ANTENATAL DEPRESSION AND CHILD PSYCHIATRIC PROBLEMS AT THE AGE OF 3 TO 5 YEARS (STUDY I)

Figure 6 shows that maternal history of depression diagnosed before pregnancy (β = −0.25, 95% CI = −0.46 to −0.03; Panel A) and greater antenatal depressive symptoms (β = −0.08, 95% CI = −0.16 to −0.004; Panel B) were associated with lower epigenetic GA after adjusting for model 1 covariates. When adjusted further for the model 2 covariates, the association with history of depression before pregnancy remained significant (β = −0.24, 95% CI = −0.45 to −0.02), while the association with antenatal depressive symptoms became attenuated (β = −0.07, 95% CI = −0.15 to 0.004).

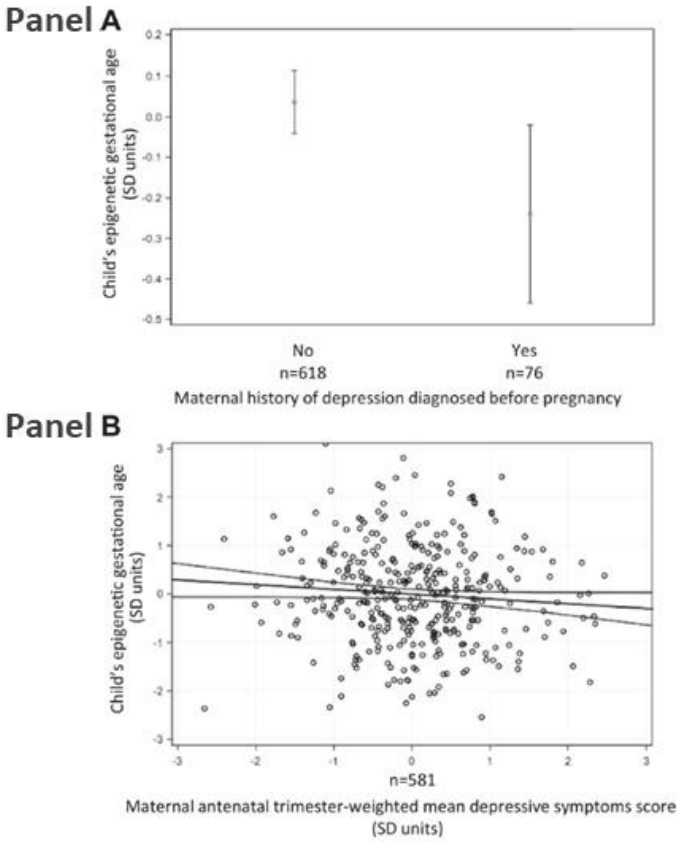

Figure 6 Maternal Antenatal Depression and Child's Epigenetic GA at Birth. Panel A: Means and 95% CIs of child's epigenetic GA in groups divided according to maternal history of depression diagnosed before pregnancy. Panel B: Scatterplot with a regression line displaying the unstandardized regression coefficient (β) and 95% CIs showing associations between maternal trimester–weighted mean antenatal depressive symptoms and child's epigenetic GA

There were neither gestation stage–specific effects of antenatal depressive symptoms on child's epigenetic GA (Figure 7), nor sex × maternal depression diagnosis (95% CI = −0.41 to 0.46 for interaction term) or sex × maternal depressive symptoms (95% CI = −0.07 to 0.22 for interaction term) interactions on child's epigenetic GA.

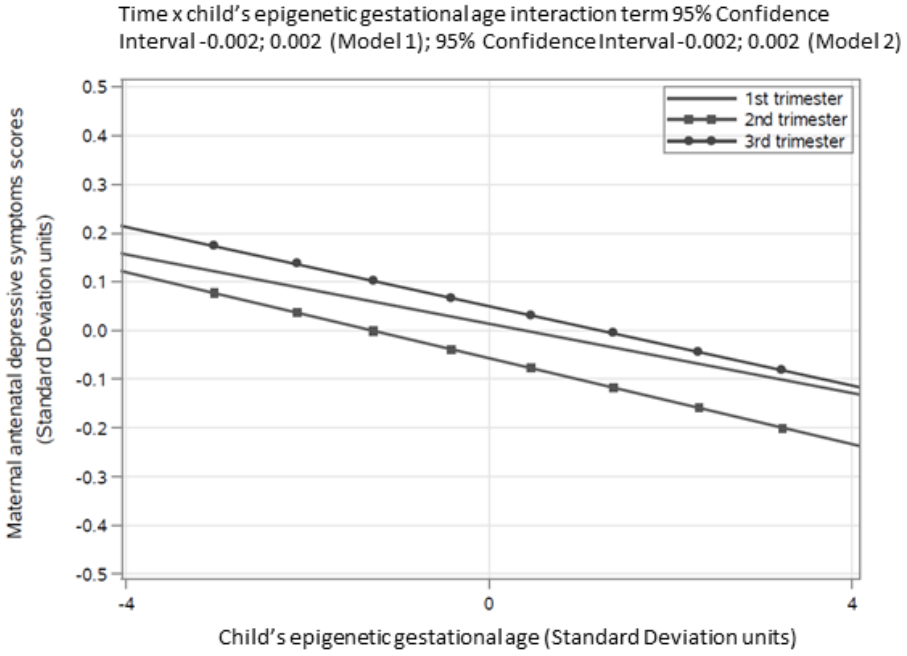

Figure 7 Associations between maternal antenatal depressive symptoms at different stages of pregnancy and child's epigenetic gestational age

Table 3 shows that there were no significant associations between child's epigenetic GA and psychiatric problems in the total sample. However, sex × epigenetic GA interactions were significant for total and internalizing problems in model 1 and for all three main domains in model 2. The interaction between sex and epigenetic GA on internalizing problems remained significant after Bonferroni correction (Bonferroni-corrected (across 2 multiple tests) 95% CI = 0.02–0.48 and 0.01–0.46 in models 1 and 2, respectively), but not on externalizing problems in model 2 (Bonferroni-corrected (across 2 multiple tests) 95% CI = −0.02 to 0.43).

Table 3. Associations between child's epigenetic gestational age at birth and psychiatric problems at 3.7 years.

	Epigenetic Gestational Age in SD units (Independent Predictor Variable)						
	Epigenetic estational Age Main Effects		Sex x Epigenetic Gestational Age Interaction	Boys (n=212)		Girls (n=195)	
Psychiatric problems in SD units (Dependent Outcome Variable)	**β**	**95% CI**	**95% CI**	**β**	**95% CI**	**β**	**95% CI**
Total							
Model 1	-0.06	-0.17;0.05	0.04;0.44	-0.16	-0.31;-0.0003	0.08	-0.08;0.24
Model 2	-0.06	-0.16;0.05		-0.17	-0.32;-0.02	0.10	-0.06;0.26
Internalizing							
Model 1	-0.04	-0.15;0.08	0.05;0.46	-0.16	-0.31;0.001	0.13	-0.05;0.30
Model 2	-0.03	-0.15;0.08		-0.16	-0.31;-0.01	0.14	-0.04;0.31
Externalizing							
Model 1	-0.06	-0.17;0.05	-0.01;0.39	-0.13	-0.29;0.02	0.06	-0.10;0.23
Model 2	-0.06	-0.17;0.05		-0.15	-0.30;0.01	0.08	-0.08;0.24

Note: β refers to unstandardized regression coefficient from linear regression analysis.

Table 3 also shows that lower epigenetic GA at birth was significantly associated with greater total (models 1 and 2) and internalizing problems (model 2) only in boys, while there were no significant associations for externalizing problems in either boys or girls.

When we examined the sex differences further, we found that on average, boys had significantly lower epigenetic GA at birth than girls (mean epigenetic GA (SD) = -1.5 (2.0) *vs* -0.9 (1.7), respectively, p=0.003). They also showed higher scores in total (46.6 (9.6) *vs* 44.4 (7.9), p=0.010) and externalizing problems (48.4 (9.5) *vs* 45.3 (7.8), p<0.001) in comparison to girls. There were no other sex-specific differences in sample characteristics (p>0.07; data not shown).

The mediation analyses were conducted in boys, as epigenetic GA was not associated with psychiatric problems in girls; and only on total and internalizing problems, as they were predicted by child's epigenetic GA (Table 3) and by maternal antenatal depression in boys (p<0.002; data not shown). Figure 8 shows that, in boys, epigenetic GA partially mediated the association between antenatal depression and internalizing problems. Mediation was not significant on total problems (95% CI = −0.001 to 0.14 for indirect effect).

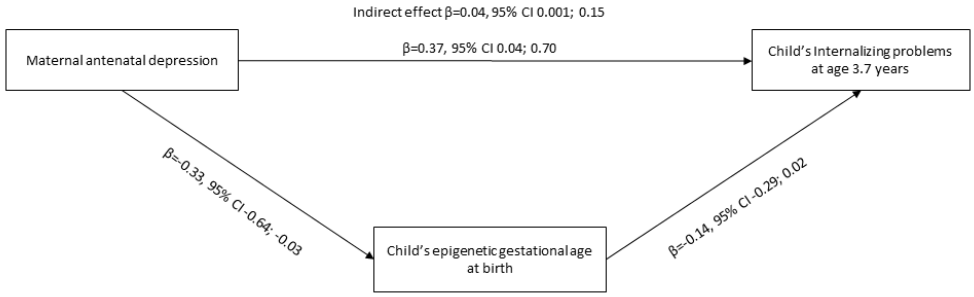

Figure 8 Maternal Antenatal Depression Acts Partly via Child's Epigenetic Gestational Age at Birth on Child's Psychiatric Problems Among Boys

5.2 POLYEPIGENETIC GC EXPOSURE SCORE AT BIRTH, CHILD MENTAL AND BEHAVIORAL DISORDERS AT THE AGE OF 7 TO 11 YEARS, AND MATERNAL ANTENATAL DEPRESSIVE AND ANXIETY SYMPTOMS (STUDY II)

There were 99 (12.2%) children diagnosed with any mental or behavioral disorder during the follow-up. Compared to children with no mental disorders, those with diagnosis were more often boys, their mothers had lower education, higher early pregnancy BMI, higher anxiety symptoms during pregnancy, and more often had lifetime diagnosis of any mental disorder (p-values < 0.05); there were no significant differences in other characteristics. In the 99 children with any mental and behavioral disorder diagnosis, the

median number of days spent in in- or outpatient treatment for any mental and behavioral disorder as the primary diagnosis was 7.00 (Interquartile Range = 16.00) days. Table 2 further shows that those children who had spent 7.00 days or more in the in- or outpatient treatment more often had more than one broad category diagnosis (F2 – F9) (p-values < 0.05).

Polyepigenetic GC exposure score was not significantly associated with the hazard of being diagnosed with any mental and behavioral disorder in children (HR = 0.38, 95% CI 0.05; 2.90, p=0.35). However, lower polyepigenetic score was significantly associated with more days spent in in- or outpatient treatment for any mental and behavioral disorder as the primary diagnosis (hurdle model estimate =-1.08 natural logarithm units per each standard deviation increase in polyepigenetic score; 95% CI -1.70; -0.46, p=0.001). This translated into 2.94 (95% CI 1.59, 5.45, p=0.001) more days spent in in- or outpatient treatment per each SD unit decrease in the polyepigenetic score according to contrast estimate results (Figure 9).

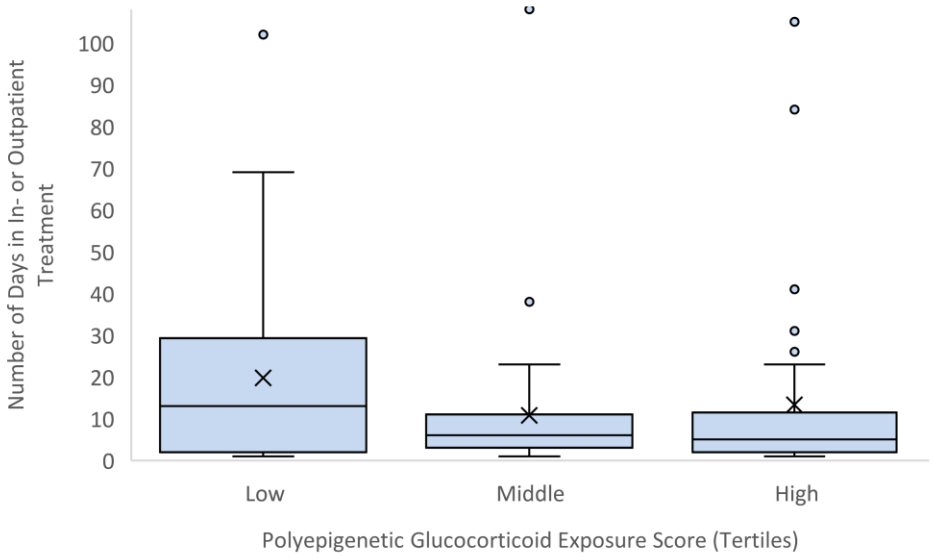

Figure 9 The number of days the child has spent in any inpatient treatment or in outpatient treatment in public specialized medical care with the any mental and behavioral disorder as the primary diagnosis (N=99) according to the polyepigenetic glucocorticoid exposure score at birth categorized into tertiles. Horizontal lines refer to the medians and interquartile ranges and cross marks to the mean values

Before proceeding to mediation analyses, we tested if the criteria for mediation were met. We have previously shown that higher maternal anxiety and depressive symptoms during pregnancy were associated with a lower polyepigenetic score in the offspring (13), and the above analyses showed that the score was also associated with a higher number of days spent in in- or outpatient treatment. However, in the subsample of 583 women who reported depressive and anxiety symptoms during pregnancy, these symptoms were not significantly associated with the number of days the child had spent in the in-

or outpatient treatment (p>0.58). Hence, we did not pursue mediation.

However, in post hoc analyses in the entire PREDO cohort with data available on maternal depressive (n=3,404) and anxiety symptoms (n=3,405) during pregnancy, higher maternal depressive and anxiety symptoms were associated with 1.31 (95% CI 1.07; 1.61; (hurdle model estimate =0.27, 95% CI 0.06; 0.48, p=0.011) and 1.32 (95% CI 1.08; 1.62; hurdle model estimate =0.28, 95% CI 0.08; 0.48, p=0.007) more days spent in in- or outpatient treatment, respectively, per each SD unit increase in the symptomatology. This is suggestive of possible mediation in a cohort with greater statistical power.

5.3 EPIGENETIC CLOCK, PHYSICAL AND NEUROCOGNITIVE DEVELOPMENT IN ADOLESCENTS (STUDY III)

Table 4. Associations between epigenetic clock, physical and neurocognitive development in 11.0 – 13.2-year-old adolescents

Outcome	Epigenetic age acceleration (years) calculated as unstandardized residual regressing DNA methylation on chronological age and blood cell types					
	Model 1			Model 2		
	B / OR	95% CI	*p*	B / OR	95% CI	*p*
Anthropometry						
Weight-for-age (SD)	0.06	0.01; 0.11	0.02	0.05	0.00; 0.10	0.051
Height-for-age (SD)	0.08	0.03; 0.13	0.003	0.07	0.02; 0.12	0.01
Body-mass-index-for-age (SD)	-0.04	-0.01; 0.09	0.15	0.02	-0.02; 0.07	0.31
Tanner Staging Questionnaire						
Pubic hair development (I–IV)	1.09	0.98; 1.21	0.12	1.15	0.99; 1.25	0.07
Breast/Genitals development (I–IV)	1.13	1.02; 1.25	0.018	1.15	1.03; 1.29	0.014
Pubertal development scale						
Stage I – III	1.16	1.02; 1.32	0.015	1.19	1.05; 1.34	0.008
Child Behavior Checklist						
Total problems	1.18	0.98; 1.41	0.089	1.15	0.95; 1.39	0.14
Internalizing problems domain	1.29	1.11; 1.51	<0.001	1.34	1.13; 1.60	<0.001
Anxious/depressed	1.29	1.07; 1.56	0.008	1.39	1.08; 1.78	0.011

Withdrawn	1.34	1.15; 1.56	<0.001	1.37	1.17; 1.61	<0.001
Somatic complaints	1.11	0.97; 1.28	0.13	1.12	0.96; 1.29	0.14
Externalizing problems domain	1.02	0.76; 1.36	0.92	0.99	0.74; 1.32	0.96
Rule-breaking behavior	1.10	0.82; 1.47	0.52	1.05	0.79; 1.40	0.72
Aggressive behavior	1.05	0.78; 1.41	0.74	1.07	0.81; 1.42	0.64
Other problems domain						
Social problems	1.14	0.96; 1.36	0.14	1.13	0.96; 1.34	0.14
Thought problems	1.18	1.01; 1.37	0.035	1.20	1.01; 1.43	0.034
Attention problems	0.98	0.82; 1.17	0.85	0.95	0.79; 1.15	0.61
DSM-IV-oriented scales						
Affective problems	1.27	1.08; 1.48	0.003	1.29	1.08; 1.54	0.004
Anxiety problems	1.25	1.01; 1.57	0.045	1.29	0.98; 1.68	0.07
Somatic problems	1.05	0.91; 1.20	0.50	1.03	0.89; 1.19	0.73
Attention deficit hyperactivity problems	1.05	0.81; 1.36	0.71	0.99	0.81; 1.23	0.98
Conduct problems	1.11	0.85; 1.45	0.44	1.14	0.89; 1.46	0.32
Oppositional-defiant problems	1.07	0.83; 1.38	0.60	1.14	0.87; 1.48	0.34

Note: B refers to unstandardized regression coefficient from generalized model with Gaussian reference distribution; OR refers to odds ratio from generalized linear model with ordinal logistic reference distribution; 95% CI refers to 95% confidence interval.

Table 4 shows that after adjustment for covariates of model 1, increase in AA was associated with higher weight-for-age, taller height-for-age, and less missed units from the target adult height (p values < 0.02; Bonferroni-corrected (across seven tests) p< 0.036). Each year increase in AA was further associated with a more advanced Tanner stage of breast/genitals development and a more advanced pubertal stage on the PDS (p values < 0.018; Bonferroni-corrected (across seven tests) p > 0.05). When adjusted for model 2 covariates the results remained virtually identical, except for weight-for-age SD score, which became attenuated (p = 0.051). Adjustments for maternal self-reported age at menarche did not affect the significant associations (all p-values < 0.042; data not shown).

Table 4 also shows that after model 1 covariate adjustments, each year increase in AA was associated with 29% higher odds for internalizing problems, and 29% and 34% higher odds for anxious/depressed and withdrawn problems on the internalizing problems domain, respectively (p-values< 0.008). Each year increase in AA was further associated with 27 % and

25% higher odds for DSM-IV oriented affective and anxiety problems (p-values< 0.045). Each year increase in AA was also associated with 18% higher odds for thought problems within the domain of other problems (p = 0.035). When adjusted further for the model 2 covariates, the association of AA with anxiety problems became non-significant (p =0.07), while the other significant associations remained unaffected (p-values < 0.034) (Table 4). When corrected for multiple testing (tests across four internalizing, six DSM-IV, and three other problems domains), the associations with internalizing, anxious/depressed, withdrawn, and affective problems remained significant (Bonferroni-corrected p-value < 0.018). There were no other significant associations with child psychiatric problems (p-values> 0.09).

There were no significant associations between AA and assessed cognitive abilities (p > 0.27; data not shown).

Figure 10 shows that in models adjusting for model 1 covariates and time at awakening, for each year increase in AA, salivary cortisol at awakening increased by 4.2% (p = 0.021). This association survived adjustment for model 2 covariates as well as for the adolescents BMI-for-age SD score (p-values < 0.02), but not correction for multiple testing (across four tests; Bonferroni-corrected p = 0.08).

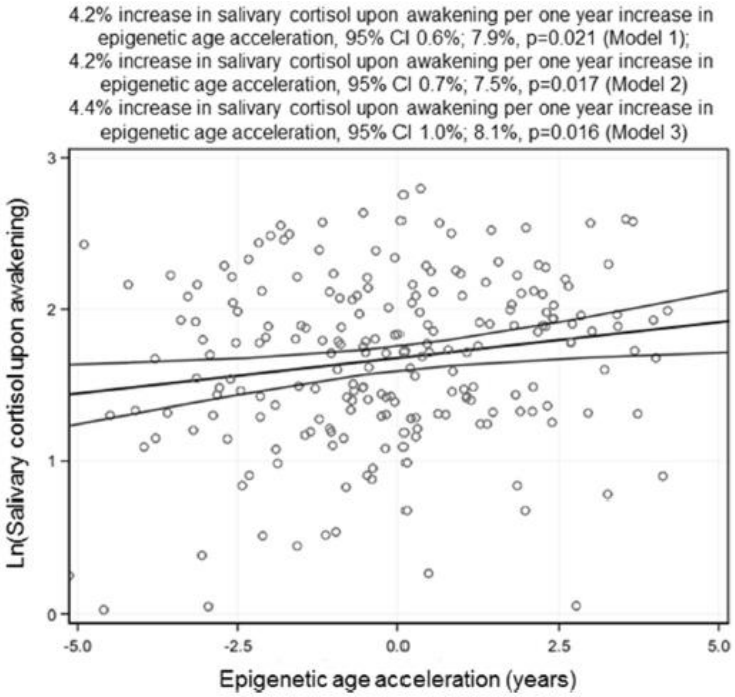

Figure 10 A scatterplot with a regression line and 95% confidence intervals showing associations between epigenetic age acceleration and salivary cortisol upon awakening in 11.0–13.2-year-old adolescents

5.4 GXE INTERACTION OF ELS AND *FKBP5* POLYMORPHISMS IN ASSOCIATION WITH QUANTITATIVE GLYCEMIC TRAITS IN LATE ADULTHOOD (STUDY IV)

Figure 11 shows that after adjustment for model 1 covariates and using additive genetic model we found significant interaction of ELS with rs1360780 in the analyses of fasting, 30-minute, incremental, and AUC insulin (Panel A). It further shows that we found significant interaction of ELS with rs9394309 in the analyses of 30-minute and incremental insulin (Panel B). Figure 11 also shows significant interaction of ELS with rs9470080 in the analyses of incremental insulin (Panel C). All the results remained significant when adjusted for model 2 covariates; the analyses of fasting insulin survived Bonferroni correction ($p<0.025$). Adjustment for model 3 covariates did not affect the results.

When we performed ELS interaction analyses using dominant genetic model, the results remained significant, revealing additional significant association between rs9470080 by separation status interaction with 30-minute insulin.

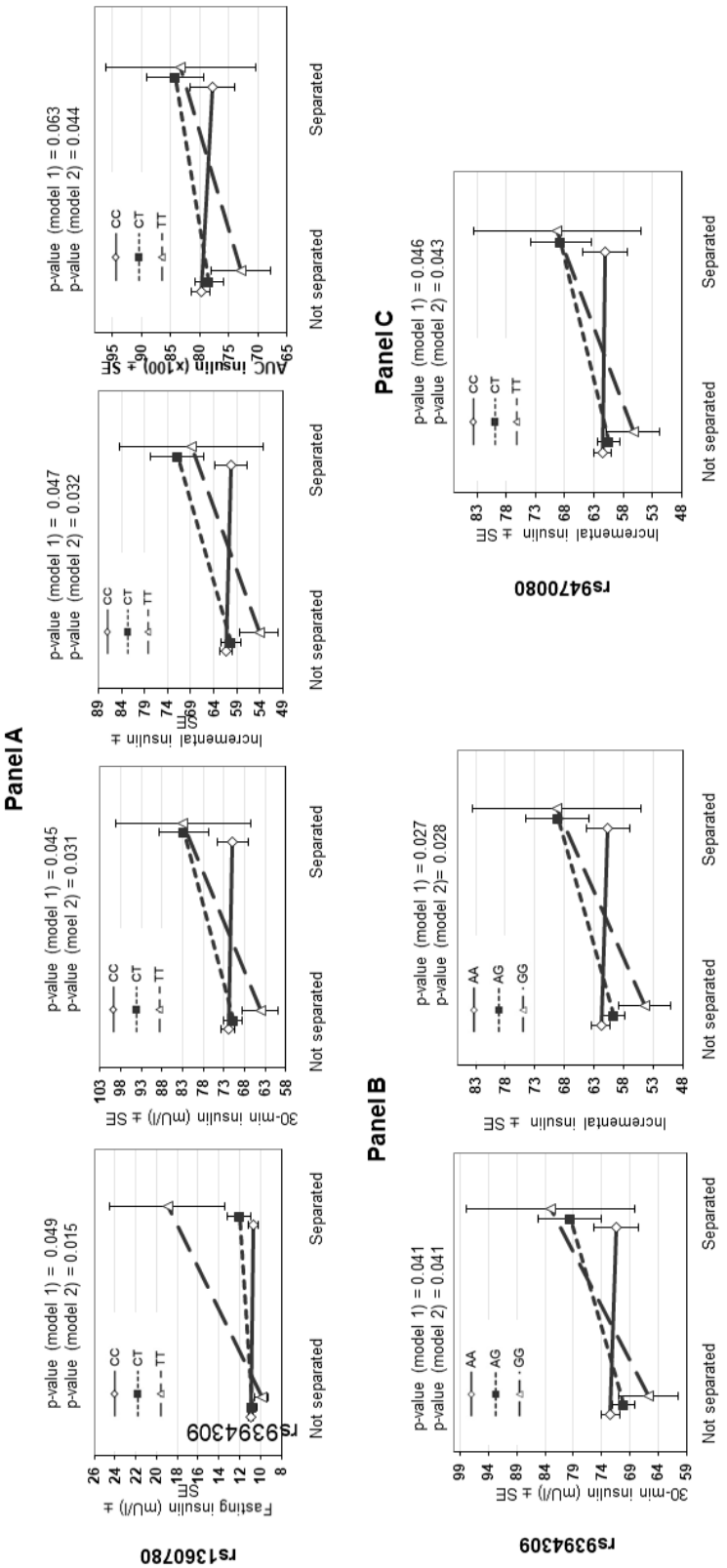

Figure 11 A scatterplot with a regression line and 95% confidence intervals showing associations between epigenetic age acceleration and salivary cortisol upon awakening in 11.0–13.2-year-old adolescents

Table 5 shows that across all the significant interactions, insulin values were higher among carriers of at least one copy of the minor allele if they were separated compared to if they were not; no such differences between separated and non-separated groups existed among homozygous major allele carriers.

In the dominant genetic model analyses we also found a significant interaction between rs9470080 and separation status with IFG (p value for interaction = 0.049; Bonferroni-corrected p-value = 0.098): among carriers of at least one copy of minor allele, the odds for IFG was higher in the separated (n = 87 [20.3%]) in comparison to the non-separated group (n = 535 [13.4%]; odds ratio = 1.84, 95% confidence interval = 0.97 – 3.52, p = 0.06)).

All the significant interactions remained virtually unaffected in the analyses using models 2 and 3. In the additional analyses of quantitative glycemic traits with further adjustment for antidiabetic medication and physician diagnosed diabetes, the only interaction that remained significant was that of rs1360780 by separation status on fasting insulin (p = 0.009, Bonferroni-corrected p = 0.018). Here we also found a previously non-significant interaction between rs1360780 by separation status on HOMA-IR (p = 0.030, Bonferroni-corrected p = 0.059). The findings remained virtually identical when we made adjustments following the Keller model.

Finally, haplotype analysis demonstrated that carriers of the haplotype formed by the minor alleles in rs1360780 (T), rs9394309 (G), and rs9470080 (T) (19.6% of individuals) who were separated from their parents had higher levels of fasting (p values for interaction = 0.048, Bonferroni-corrected p = 0.096), 30-minute (p values for interaction = 0.032, Bonferroni-corrected p = 0.064), and incremental insulin (p values for interaction = 0.021, Bonferroni-corrected p = 0.042), thus, confirming our findings from the single SNPs by separation status analyses. None of the covariate adjustments affected these findings, except when adjusting for antidiabetic medication use and physician-diagnosed diabetes, only the results for fasting insulin remained unaffected (p = 0.021), revealing additional association with higher levels of HOMA-IR (p = 0.049).

Table 5. Mean differences in glycemic traits in individuals who were and were not exposed to ELS according to the genotypes of the three selected *FKBP5* SNPs.

	Fasting insulin			30-minute insulin			Incremental insulin			AUC insulin		
FKBP5 SNPs:	Mean difference (%)	95% CI	p	Mean difference (%)	95% CI	p	Mean difference (%)	95% CI	p	Mean difference (%)	95% CI	p
rs1360780												
CC [1]	-2.5	-11.76 to 6.75	.60	-1.96	-13.04 to 9.12	.73	-3.18	-15.4 to 9.03	.61	-3.06	-12.82 to 6.7	.54
CT / TT [2]	13.95	1.68 to 26.23	.026	17.62	4.07 to 31.16	.011	19.21	3.75 to 34.66	.015	12.52	0.24 to 24.79	.046
rs9394309												
AA [1]	-1.59	-11.08 to 7.9	.74	-3.08	-14.53 to 8.38	.60	-5.38	-17,91 to 7.15	.40	-3.92	-14.09 to 6.24	.45
AG / GG [2]	10.17	-1.45 to 21.79	.086	15.59	2.78 to 28.4	.017	17.98	3.26 to 32.71	.017	9.86	-1.55 to 21.27	.090
rs9470080												
CC [1]	-1.13	-10.83 to 8.57	.82	-2.3	-13.99 to 9.4	.70	-4.78	-17.57 to 8.01	.46	-2.68	-12.98 to 7.63	.61
CT / TT [2]	9.76	-1.7 to 21.22	.095	14.51	1.77 to 27.25	.026	16.83	2.22 to 31.45	.024	8.67	-2.76 to 20.10	.14

Note: Mean differences were log-transformed to attain normality and are presented in percentage differences between values in the separated and nonseparated groups. The results are presented from Model 1.

[1] n= 951-1067, [2] n=649-760

6 DISCUSSION

This work has three major aims. First, to examine whether exposure to prenatal stress was associated with polyepigenetic modifications in fetal cord blood and whether these modifications were associated with and mediated the effects of prenatal stress on child mental health outcomes. In order to attain this aim we used data from the PREDO prospective cohort with well-characterized mother–child data on epigenomic, medical, psychological, and socio-demographic characteristics in the Studies I and II. The main results of Study I demonstrate that lower child's epigenetic GA at birth was significantly associated with maternal history of depression diagnosed before pregnancy and higher antenatal depressive symptoms and it prospectively predicted child's total and internalizing problems in early childhood, partially mediating the association of maternal antenatal depression with child internalizing problems, although only in boys. In Study II we show that polyepigenetic GC exposure score at birth was not associated with higher risk for any childhood mental and behavioral disorder, but it was significantly associated with the severity of these disorders.

Second, as epigenetic processes undergo changes related to age, we set out to study whether polyepigenetic modifications were associated with physical growth, neuroendocrine functioning and mental health in adolescents. In order to obtain this aim we used data from the Glaku prospective cohort with a wide range of epidemiological data available. The results of Study III reveal that adolescents with higher AA, i.e. higher DNAm age compared to chronological age, displayed more advanced physical growth and development, had higher salivary cortisol upon awakening and higher odds for displaying borderline clinically significant internalizing problems.

Third, this study aimed to examine whether exposure not only to prenatal, but also to ELS was associated with physical health outcomes in an elderly population, and whether these associations were moderated by genetic variants that play a role in the regulation of the HPA-axis functioning. In order to obtain this aim we used data from the HBCS prospective cohort with well-defined genetic, medical, psychological, and socio-demographic characteristics. The main results of Study IV show that three selected *FKBP5* polymorphisms moderate the association of ELS on insulin and glucose values at fasting state and/or during an OGTT in late adulthood.

Individual study findings in relation to previous evidence are discussed below.

6.1 MATERNAL ANTENATAL DEPRESSION, POLYEPIGENETIC BIOMARKER OF EPIGENETIC GA AND CHILD PSYCHIATRIC PROBLEMS (STUDY I)

Study I shows that maternal history of depression diagnosed before pregnancy and higher levels of antenatal depressive symptoms were significantly associated with child's lower epigenetic GA at birth, with no additive effect of these conditions and no gestation stage specificity.

This study also shows that child's lower epigenetic GA at birth prospectively predicted child's total and internalizing problems in early childhood, although only in boys. Also in boys, we found partial mediation of the association between maternal antenatal depression and child internalizing problems by child's epigenetic GA at birth.

These findings may seem to contradict the existing evidence in adult populations which point to higher rather than lower DNAm age, in comparison to chronological age, as suboptimal in relation to physical and mental health outcomes (137,138). Importantly, DNAm age is an ongoing readout of molecular processes that play a significant role in development, maintenance of cells, tissues and organs, and, ultimately, their decay. DNAm age increases as stem and progenitor cells undergo differentiation to produce more committed cells for growth during the early developmental years and for replenishment of differentiated cells during the maintenance years (225). Therefore, the biological significance of the DNAm acceleration at early formative stages, and particularly at prenatal stage, may differ from the one at later stages of life. The study conducted in children confirms that children who are epigenetically older at birth are taller and have a higher fat mass throughout childhood and adolescence even after adjusting for sex (14).

In line with our findings, a recent study reported epigenetic GA deceleration in newborns who were exposed to prenatal adversity reflected by the cerebroplacental ratio, a hemodynamic parameter reflecting fetal adaptation to hypoxic conditions (226). The association of lower epigenetic GA with increased risk is congruent with the DOHaD concept: the risk for aging-related diseases and mental problems is higher for individuals exposed to prenatal environmental adversities, which are associated with lower birth weight, preterm birth, and maternal depression during pregnancy (36,43). Because DNAm is a dynamic process, which has been shown to undergo age-related changes (16), both lower and higher epigenetic age might signal risk depending on the life stage.

Although we expected to find sex differences in the associations, it remains unclear why the associations with psychiatric problems were specific to boys. These results disagree with previous evidence of higher vulnerability in girls exposed to maternal depression during pregnancy (71). Furthermore, Graham et.al have showed that in girls, rather than in boys, elevated maternal cortisol during pregnancy was associated with higher internalizing symptoms, and this association was mediated by stronger neonatal amygdala connectivity (100).

However, many studies suggest corresponding adverse effects of antenatal depression on offspring psychopathology in both sexes (5,72) and, corresponding with our findings, a rodent study showed that maternal early pregnancy stress predicted a depression-like phenotype only in male offspring (73). Indeed, evidence exists suggesting that what might be phenotypically adaptive for males and females may be determined by a complex interaction of type, timing, severity, and chronicity of the prenatal stress exposure and the developmental stage of the offspring (72,73). Whether lower epigenetic GA in boys would also predict other outcomes or would become predictive of outcomes in girls in later life stages is the subject of ongoing studies.

Furthermore, our findings of lower epigenetic GA at birth in boys contradict previously demonstrated older epigenetic age in boys compared to girls, with this sex difference persisting into old age where it accompanies higher mortality risk (225). However, as discussed above, the biological meaning of the epigenetic age acceleration and deceleration in prenatal and postnatal environments might differ, with lower epigenetic GA reflecting less maturity, which may pose long-term mental health risk. Moreover, studies at later stages of development are performed using the tissues other than cord blood, which may account for the discovered discrepancies. Thus, the findings on the possible sex specificity of the effects of prenatal stress on offspring developmental outcomes are inconclusive, and a consensus exists that further exploration of the potential sex specificity of prenatal epigenetic programming effects is needed (72,73).

6.2 ASSOCIATIONS BETWEEN POLYEPIGENETIC GC EXPOSURE SCORE AT BIRTH AND CHILD MENTAL AND BEHAVIORAL DISORDERS (STUDY II)

Study II shows that a novel polyepigenetic biomarker reflecting fetal GC exposure at birth was not significantly associated with higher hazard for any childhood mental and behavioral disorder in a follow-up of the children from birth to 7.1-10.7 years of age. However, it was significantly associated with the severity of these disorders, such that lower polyepigenetic GC exposure score at birth was associated with more days spent in in- or outpatient treatment for any mental and behavioral disorder as the primary diagnosis in a public specialized medical care**.** For each SD unit decrease in this score, the child had spent almost three more days in in- or outpatient treatment. These findings, thus, suggest that this novel polyepigenetic biomarker may contribute to identification of children at risk for more severe mental and behavioral disorders already at birth, which may allow alternative avenues for timely targeted preventive interventions, before any manifest symptoms or disorders occur.

While maternal depressive and anxiety symptoms during pregnancy correlated with the fetal polyepigenetic GC exposure score in this study sample (13), they were not correlated with the severity of child mental and behavioral disorders in this study. Hence, we could not test for mediation. However, in a larger sample of the PREDO study, for whom we missed cord blood samples, maternal higher depressive and anxiety symptoms were significantly associated with 1.3 more days spent in in- or outpatient treatment, indicating possible insufficient power in the subset to detect this association, suggesting plausibility for mediation in a larger sample.

The fact that a lower polyepigenetic GC exposure score was associated with higher severity of child mental and behavioral disorders is in line with an increased prenatal GC exposure in the severely affected children. In fact, a lower polyepigenetic GC exposure score was previously shown to be reflective of higher DNA demethylation with GR activation (13) and exposure to GC has been associated with DNA demethylation specifically at glucocorticoid-responsive elements (227). These findings would suggest that children with more severe mental health problems have been exposed to more GC prenatally or are more sensitive to their epigenetic effects. This may be indicative of an increased priming of target genes to subsequent stress exposure, as suggested by previous findings in hippocampal progenitor cells (13). Furthermore, combining GC-exposure of human cerebral organoids as a model of early brain development and single cell sequencing, we could show that GC target genes in the developing brain are enriched for genes that have been associated with neurodevelopmental disorders and psychiatric disease, including major depression, schizophrenia as well as cross disorders risk in large genome-wide association studies (228). The strongest disease enrichments were found for transcripts regulated in late neuronal progenitors and neurons. This would suggest that prenatal GC exposure may contribute to the increased risk for more severe mental and behavioral disorders observed in the offspring (43) via lasting epigenetic changes in relevant neuronal target genes and that this risk maybe exacerbated in with additional genetic risk.

6.3 ASSOCIATIONS OF POLYEPIGENETIC BIOMARKER OF EPIGENETIC CLOCK IN ADOLESCENCE WITH TEMPO OF MARKERS OF PHYSICAL GROWTH AND DEVELOPMENT, HPA AXIS FUNCTIONING, PSYCHIATRIC PROBLEMS AND COGNITION (STUDY III)

In Study III we showed that adolescents with higher AA, i.e. higher DNAm age than chronological age, were heavier- and taller-for-age, and closer to their expected target adult height, suggesting an earlier growth spurt and less remaining growth potential. Their pubertal stage of breast/genital development, measured with Tanner Staging Questionnaire, and of secondary

sex characteristics, measured with PDS, were also at a more advanced stage. The advanced growth and maturation was accompanied by higher salivary cortisol upon awakening and higher odds for displaying borderline clinically significant internalizing problems, namely anxious/depressed and withdrawn problems, affective and anxiety and thought problems. While these associations remained significant after adjustments for a number of important covariates, only the associations with stature and with internalizing/affective problems remained significant after correction for multiple testing. Our results, thus, suggest that adolescents whose biological DNAm age is higher than their chronological age display more advanced physiological development and risk for psychiatric problems that may indicate risk of earlier aging.

Our findings are in line with the life history theory, which suggests that early development and early puberty are meaningful tradeoffs in conditions of environmental adversity (49,50). We suggest that AA and advanced growth and maturation might indicate more advanced tempo of aging processes present from adolescence onwards. Indeed, more advanced physical growth and pubertal development have been shown to predict aging-related diseases, such as cancers (229), cardio-metabolic disorders and their risk factors (230,231), and depression (232). Interestingly, recent studies from the ALSPAC cohort discovered associations of childhood DNAm AA with exposure to adverse childhood experiences (233,234). As pubertal timing has been previously shown to mediate the association between childhood trauma and cardiovascular risk in adulthood (235) and overall early life adversity has been associated with both accelerated pubertal timing and cellular aging, future studies should examine whether the AA might be the biological mechanism explaining these risk avenues.

The findings with physical growth agree with previously reported association of higher AA at age 7 with increased height in childhood and adolescence (148). Yet, in the same study, contrarily to our results, higher AA at age 7 predicted slower, rather than faster height growth between childhood and adolescence and AA at ages 7 and 17 years was not associated with a number of pubertal development markers, including the Tanner Staging Questionnaire (148). However, in line with our findings, higher AA at birth did predict higher odds of increasing Tanner stage of testes development (148). Similarly, our discovered association of higher AA with more advanced pubertal development agrees with the results from a study in Chilean adolescent girls, where a five-year average increase in AA was associated with a significant decrease in time to menarche and revealed an overall stronger inverse association of AA with pubertal tempo, although there was no significant association with the time of thelarche (149). There is evidence that the timing of pubertal development may be sensitive to exogenous factors during different critical exposure windows affecting the hypothalamic-pituitary-gonadal maturation, the biological system orchestrating an increase in gonadal steroid production and initiating puberty (236). As DNAm

undergoes age-related changes (16), a potential explanation for the somewhat discrepant study findings on physical growth and development and pubertal maturation between our and the previous studies is the age at which DNAm and physical and pubertal development were measured.

Cortisol measurements are often used to study the HPA axis functioning, and while the effects of high cortisol concentrations on health vary, they are generally associated with a number of physical and mental health adversities, such as obesity (237), sleep problems (238), anxiety and depression (103,106) in studies in both children and adults. Our findings of the association between AA and elevated salivary cortisol upon awakening might indicate that risk for aging-related disease and dysregulation of the HPA axis may be discovered already in adolescence. These results are in partial agreement with previously discovered association of higher AA measured from salivary DNA at age 13 with higher salivary cortisol measured across two days in a study of 46 adolescent girls (150). That study, however, did not account for a number of covariates, such as genetic population structure, which is strongly associated with methylation profiles (195) or BMI, which is strongly associated with the cortisol levels (237), and, hence, it remains unclear if the findings of that study would have survived adjustments for these important covariates. Our results disagree with a more recent study from the ALSPAC cohort which demonstrated null findings in the study of AA and morning plasma cortisol (233). There have been conflicting evidence of significant discrepancy in the measurements of cortisol from plasma and saliva, which might explain the disagreement of our findings (239). The cortisol system is highly complex; therefore, further research using standardized measures for cortisol is needed to further examine its role in the relationship between epigenetic AA and aging-related disorders.

Our study also revealed novel findings related to psychiatric problems, namely, that higher AA was associated with internalizing/affective-type and thought problems, but not externalizing-type of problems. This finding may reflect statistical power, as internalizing problems in our sample were twice as prevalent as externalizing problems, or adolescent sample, an age period with a marked rise in internalizing problems (240). This pattern is, however, congruent with Study I, where we have demonstrated that polyepigenetic biomarker of GA was inversely correlated with higher internalizing problems in boys. There is evidence that childhood psychiatric problems tend to track into adulthood (241), and even when they do not or are subthreshold (242), these problems increase risk for adverse adulthood outcomes and vulnerability for earlier aging.

In Study III AA was not associated with cognitive abilities, which is somewhat surprising, as poorer childhood cognitive functioning is predictive of aging-related diseases, including dementia (243). However, due to the dynamic nature of DNAm alterations (16), we cannot rule out that AA at later developmental stages will change and become associated with cognitive function and perhaps change its links to behavioral problems.

6.4 MODERATION OF ELS ASSOCIATION WITH QUANTITATIVE GLYCEMIC TRAITS IN LATE ADULTHOOD BY *FKBP5* POLYMORPHISMS (STUDY IV)

Study IV demonstrated that three selected *FKBP5* polymorphisms moderated the association of ELS on insulin and glucose values at fasting state and/or during an OGTT in midlife. Among carriers of at least one copy of the minor allele, those who had been exposed to ELS had higher insulin values at fasting and 30 minutes after the glucose load, higher incremental and the AUC insulin, and higher odds for IFG, compared with carriers not exposed to ELS. There were no such differences in these glycemic traits in carriers homozygous for the major allele. These findings were further confirmed by the haplotype analyses, where a haplotype formed by minor alleles of these three SNPs interacted with ELS in predicting the quantitative glycemic traits: carriers of the haplotype formed by the minor alleles had higher levels of fasting, 30-minute, and incremental insulin if they were separated in comparison to those who were not. These findings were not explained by a number of important covariate; however, when we made adjustments for antidiabetic medication use and physician-diagnosed diabetes, only the ELS by rs1360780 and ELS by haplotype interactions on fasting insulin remained significant; interestingly, in these analyses also, these two interactions became significant on insulin resistance. Our findings, thus, suggest that ELS is associated with higher insulin and glucose values in midlife if we do not take into account their antidiabetic medication use and T2D diagnosis, and with higher insulin and insulin resistance values when we do, in individuals genetically vulnerable to HPA axis dysregulation.

We did not find G x E interactions in the analyses of T2D or CVD, while they have been previously associated with ELS, regardless of the genetic vulnerability in *FKBP5* region. The three selected *FKBP5* polymorphisms, although related to HPA axis reactivity, may offer a perspective that grasps only a surface of the pathways that may explain the ELS–manifest associations on complex polygenic diseases, such as T2D and CVD. Furthermore, we cannot rule out that another explanation for the lack of significant G x E interactions on T2D and CVD relates to the still relatively young age of the sample and, consequently, small number of individuals with established diagnosis decreasing statistical power to detect significant associations. We did, however, find significant G x E interactions on higher levels of quantitative glycemic traits, which predict increased risk of future T2D and CVD (213). Elevated concentrations of insulin, which we observed in the participants with minor alleles in rs1360780, rs9394309, and rs9470080 and a haplotype formed from these alleles who also experienced ELS, may be an indication of compensatory hyperinsulinemia-increased insulin secretion in β cells in an attempt to overcome insulin resistance (244). However, if not controlled, after a period of compensatory hyperinsulinemia with normal glucose tolerance, β-

cell insulin secretion declines, insulin receptors get down-regulated, and IGT and eventually overt T2D may result (245). Therefore, follow-up studies of HBCS participants are essential to see if this transition occurs at later stages of life, particularly in those who carry the environmental and genetic risks.

Several potential pathways may account for our findings, which are related to *FKBP5* expression in the body, with tremendous differences of its expression across tissues (168).

First, hypothalamus, hippocampus and amygdala, the regions controlling the stress response and whole-body metabolism, show the highest *FKPB5* expression in the brain (168). *FKBP5* has a number of upstream and intronic glucocorticoid response elements (GREs) (177), whose function is moderated by both genetic variation and environmental factors, the latter being mediated by epigenetic mechanisms. This has been demonstrated in the study by Klengel et al., where the function of the GRE in intron 2 in the whole blood was moderated by rs1360780 and linked to early adverse experience (24). Interestingly, there were no changes in DNAm after exposure to adult trauma, suggesting that there are developmental periods in which cells are particularly sensitive to epigenetic effects within *FKBP5* (246). In fact, exposure to GCs during proliferation and differentiation was shown to lead to significant reduction in DNA methylation in intronic GREs (246). In line with our findings, increased stress-related epigenetic effects have been shown in risk-haplotype carriers (24,247–249). Chronic or repeated exposure to stress (or a single exposure to severe stress) and associated elevated levels of stress hormones can, in turn, lead to increased portal and peripheral free fatty acids, impaired ability of insulin to translocate intracellular SLC2A4 glucose transporters to the cell surface, and insulin hypersecretion (250,251). These effects lead to long-term metabolic consequences, which include hypertension, metabolic syndrome, insulin resistance, and T2D (251).Thus, stressful experience in the early stages of development may lead to attenuated development of these brain areas due to high density of GRs and persistent postnatal neurogenesis, which, in combination with preexisting genetic vulnerability, may have permanent effects on brain development and endocrine and metabolic systems (46,251).

Second, *FKBP5* shows a strong expression in human adipocytes, skeletal muscle and lymphocytes (252). The first study investigating the effects of *FKBP5* expression in adipose tissue on metabolism revealed that *FKBP5* expression levels were associated with markers of insulin resistance such as higher plasma insulin, HOMA-IR and subcutaneous adipocyte diameter and lower plasma high-density lipoproteins (252). Furthermore, the same study shows that *FKBP5* expression was highly upregulated by the synthetic GC DEX in human subcutaneous and omental adipose tissue, suggesting that *FKBP5* regulation may be implicated in GC-induced insulin resistance (252). Finally, the authors suggest that a number of *FKBP5* polymorphisms may be linked to the susceptibility to develop insulin resistance and dyslipidemia (252), as preclinical studies indicate a regulatory role of *FKBP5* in adipogenesis

(240).These results were confirmed in a follow-up study, where *FKBP5* gene expression was strongly correlated with insulin resistance, driven by glucose AUC during OGTT data, with the strongest associations in non-diabetic obese subjects (254), which may link into previous findings of the rs1360780 polymorphism within *FKBP5* gene association with reduced weight loss following bariatric surgery of obese patients (255). Furthermore, Ortiz and colleagues reported an association between *FKBP5* intron 2 methylation and higher glycated hemoglobin A1c protein (HbA1c), low-density lipoproteins (LDL), BMI and waist circumference, placing the patients with T2D at higher risk for CVD (256). Further studies are needed to identify whether methylation status at intron 2 may explain the elevated *FKBP5* expression in adipose tissues and higher risk for glucose and insulin dysregulation and higher risk for T2D and CVD in individuals with risk alleles of *FKBP5* polymorphisms who were exposed to early adversity. However, taken together, our study corroborates the body of literature demonstrating the significance of *FKBP5* in associations with metabolic dysfunction and dysglycemia, suggesting elevated risk for cardiometabolic disorders in *FKBP5* risk allele carriers, particularly following the GC exposure, which may act via epigenetic dysregulation at *FKBP5* loci.

Finally, *FKBP5* expression was shown to increase with age (257). It has been shown that transcriptional regulation of *FKBP5* over age is mediated by similar epigenetic mechanisms as ELS–induced changes and that these effects could converge to alter the trajectory of *FKBP5* expression, with potentially exacerbated effects in risk genotype carriers (258). Furthermore, a recent study revealed that aging and stress synergistically decrease DNAm at selected regulatory *FKBP5* CpGs, which promotes nuclear factor-κB (*NF-κB*)–driven peripheral inflammation, while *NF-κB* binding to the *FKBP5* enhancer stimulates *FKBP5* expression, which together form a positive feedback loop and potentially contribute to pro-inflammatory states and higher risk for cardiovascular diseases (259). Therefore, *FKBP5* demethylation, particularly in risk allele carriers exposed to ELS, may promote aging-related diseases via reinforcing the inflammatory pathways.

6.5 METHODOLOGICAL CONSIDERATIONS

The main general strengths of all the Studies I – IV relate to their longitudinal design and well-characterized cohorts. This allowed us to account for a large number of important covariates, which have been associated either with the predictors, or with the outcomes, in all of the analyses. Across all the analyses in Studies I – IV we have made adjustments for genetic population structure (195), and across Studies I – III for the effects of cell type heterogeneity (194,201), which are factors strongly influencing genetic and epigenetic profiles (260,261).

General limitation of Studies I through IV relates to the ethnic homogeneity of the cohorts, which were based in Nordic high-income country, which may limit generalizability of our findings to other populations. Another limitation for Studies I – III includes the use of one tissue type for the calculation of the polyepigenetic biomarkers, which may also preclude the generalizability to other tissue types.

Both Studies I and II use data from the PREDO cohort, where in the assessment of DNAm from the umbilical cord blood we applied novel bioinformatics methods to account for any sample contamination by maternal blood (193). Study I strength also includes validation of the findings with the bootstrap method and hierarchically structured analysis strategy of child psychiatric problems to decrease the likelihood of false-positive findings. Strength of Study II is in utilization of the data from a validated nationwide healthcare registry on the child mental and behavioral disorder diagnoses as well as on the number of days the child had been in in- or outpatient treatment for these disorder as the primary diagnosis (199). This is likely to decrease the common-method bias present in studies where both the predictor and the outcome are reported by the same person (262). Furthermore, the polyepigenetic score in Study II has been derived from cross-tissue overlap of GC responsive CpG sites of neuronal cell lines and peripheral blood, which allows for more possible organisms-wide relevance. Yet, our novel findings from Studies I and II must be interpreted with caution, and further studies are needed to confirm or refute them, as they have several limitations. The first one relates to the PREDO recruitment strategy, which was based on women's risk factor status for preeclampsia and IUGR, precluding generalizations to groups that differ from ours. The second limitation is statistical: in Study I the low number of mothers with depression diagnosis limited our statistical power to assess additive effects of depression before and during pregnancy, while in Study II there was comparatively low number of children with mental and behavioral disorders leading to decreased statistical power to study these disorder diagnoses, although in proportion (12.1%) this number was slightly higher in magnitude than the one found in the general population of Finnish children (263). Next, while there is convincing evidence that length of stay in hospital treatment may indicate the severity of psychiatric disorders (264), multiple factors such as SES, family structure, place of residence and others may affect the length of in- and outpatient treatment for these disorders, particularly in children. Therefore, future studies estimating the severity of child mental and behavioral disorders based on the length of in- and outpatient treatment, which control for those factors as well as use alternative indices of severity, such as the degree to which the disorder interferes the daily function, are warranted to confirm the plausibility of our approach. Moreover, as many psychiatric problems manifest in adolescence and later in life (265), further follow-ups of the PREDO cohort as the children age are warranted. Finally, even though we accounted for a number of important covariates, we cannot rule out that some other unmeasured factor might explain our findings,

and hence cannot rule out residual confounding. Hence, other factors operating in the prenatal and postnatal environment may be driving the discovered associations. In Study I prenatal factors, which may affect the epigenetic GA, include depression-related changes in endocrine and neurotransmitter functioning, and levels of inflammatory markers in the mother, fetus, and placenta (266). As for the postnatal environmental factors, there is preliminary evidence suggesting that DNAm, which, in turn, may associate with child psychopathology (267).

A further strength of Study III relates to availability of a number of aging-related phenotypes that we measured decades before the aging-related diseases become manifest. We were also able to account for a number of early life adversities and their proxies, particularly for maternal glycyrrhizin in licorice use during pregnancy, which is a potent inhibitor of the placental GC barrier enzyme 11β-HSD2 and has been previously associated with poorer neurocognitive tests performance and higher odds for having borderline clinically significant externalizing psychiatric problems at ages 8 and 11–13 (209,268), higher diurnal and stress-induced salivary cortisol profiles at age 8 (269), and more advanced pubertal maturation in girls at age 11–13 (209) in a larger sample of this study cohort. The limitations of Study III include the narrow age range of the sample, and hence, the small magnitude of the correlation between DNAm age and chronological age. The small magnitude of this correlation is, however, similar to the other two previous childhood epigenetic age studies (148,150). Further, in Study III we had only one DNA sample collection and, thus, only cross-sectional measurement of the epigenetic clock, which precludes testing developmental changes and causal inferences. Finally, while we measured psychiatric problems with a standardized, validated, and widely used tool (206), we cannot rule out potential information-bias as it was reported by the mother.

Strengths of Study IV relate to objectively recorded ELS augmented by self-report, and clinical measurement of glucose tolerance. Primary limitation of Study IV lies in the shortcomings of candidate GxE approach, which has been largely criticized for the lack of reproducibility, low power and high false discovery rates, among others (26). Although the list of challenges associated with characterizing candidate GxE studies is long, many of these can be addressed by adopting a rigorous research practices (26). Moreover, stricter guidelines for publishing results of candidate GxE studies are now applied by respected peer-review journals in order to preserve the high quality of publications (27,28). In Study IV we have carefully followed these recommendations by using objective measurements of the ELS exposure and the health outcomes, following strong evidence for the choice of the candidate gene and its polymorphisms, correcting our analyses for a number of important covariates (including population heterogeneity), applying Bonferroni method to correct the alpha level, and using prospective cohort with well-characterized data (28). Furthermore, although it remains important to confirm our results in other cohorts with comparable exposure

and outcome data, in addition to testing the associations with single polymorphisms, we confirmed our findings with haplotype analysis that strengthened our approach. Next limitation includes possible lack of statistical power to detect significant G x E interactions for some of our outcomes due to limited sample size and uncertainty on the quality of the ELS experience during evacuation. The latter remains an unavoidable study limitation because we do not have objective data on the evacuation experience and any adulthood recall of childhood events is at least to some extent biased. Furthermore, staying in Finland during the war with one's parent(s) may have been equally or even more stressful than the separation from one's parent(s). However, both the separated and the nonseparated participants were exposed to war in childhood, because none of the children were evacuated before the war broke out, and some of them returned home during the war. Hence, our findings may offer a rather conservative estimate of the differences between the separated and the nonseparated groups.

6.6 IMPLICATIONS OF THE STUDY AND DIRECTIONS FOR FUTURE RESEARCH

The findings of the studies included in this thesis clearly show that prenatal and early life stress have a significant negative long-term impact on physical and mental health. These effects are embedded in human biology and act, at least in part, via genetic and epigenetic mechanisms.

Currently there is an increased general public interest in postpartum depression and its impact on child development, but awareness on the antenatal depression remains insufficient. However, there is multiple evidence, including the studies from this thesis, that maternal mental health during pregnancy may disrupt child neurodevelopment via epigenetic mechanisms and put these children at lifelong risk for mental and physical disorders. Therefore, it is of essence to establish high-quality system to monitor maternal mental health and overall wellbeing during pregnancy, possibly embedding it in regular clinical checkup visits. Furthermore, it is important to popularize the information regarding maternal antenatal depression and anxiety, educate expecting parents and public on the impact of these disorders on the developing fetus and to normalize treatment for mental health problems.

Early life adversity, including separation from parents during childhood, which was investigated as part of the thesis, should be addressed in order to mitigate the long-term impact of ELS on physical and mental health. Following the ideas of the attachment theory developed by John Bowlby (270), in times of war conflicts, cataclysms, immigration and social disputes, it should be a priority for children to stay with their parents, unless there is danger for their health and life under these circumstances.

The findings of this thesis highlight the potential value of the genetic and epigenetic biomarkers in identifying individuals at risk for mental and physical health problems. While the clinical utility of such biomarkers remains to be unraveled in future studies, this work is among the pioneer studies, which demonstrate that this kind of polyepigenetic biomarkers can be generated as early as at birth and may increase understanding of the origins and biological mechanisms of disease manifestation. Furthermore, our findings are a step forward in understanding how a routine collection of umbilical cord blood at birth for DNA sampling may contribute to early identification of children at risk for complex somatic and psychiatric disorders and open new avenues for timely targeted preventive and intervention measures. Furthermore, it may be beneficial to estimate the epigenetic clock and its deviation from chronological age at different time-points throughout the lifespan, in order to see its dynamic and allow for timely interventions. Next, identifying biological risks based on genetic makeup may also provide opportunities for developing therapeutic targets. Finally, more work needs to be done to better understand the clinical applications of DNA methylation and related to it polyepigenetic biomarkers. Interestingly, the insurance industry is already considering the use of epigenetic age acceleration to predict risk of mortality to determine insurance premiums (271).

However, many questions remain unanswered. First, it is yet unclear whether the initial prenatal insult may induce immediate pathophysiological outcomes and/or whether the initial exposure may lead to cellular reprogramming via DNAm and other biological mechanisms that prime differential responses to the same environmental conditions later on that then lead to pathology (11). Therefore, more longitudinal studies with multiple DNAm measurements, including epigenetic age and polyepigenetic GC exposure score estimation, which account for both pre- and postnatal adversities in relation to child neurodevelopment, are needed.

Second, in Study I we observed that epigenetic GA deceleration in cord blood was associated with maternal depression and child psychiatric problems, while in Study III we saw the association of epigenetic age acceleration at the mean age of 12.4 years with more advanced physical growth and development, higher salivary cortisol and higher odds for psychiatric problems. Longitudinal studies with multiple measurements of the DNAm age and tracking of the health outcomes are warranted to establish whether the asynchrony of DNAm in relation to chronological age shifts from deceleration to acceleration in the risk groups and whether epigenetic GA at birth predicts other epigenetic age biomarkers in later life.

Third, although we began to unravel the changes in methylation profiles associated with adverse exposures and mental health outcomes, we do not yet know how we could address these methylation changes, whether they could serve as therapeutic targets and to which extent they could have predictive power. Currently, epigenetic therapies are successfully used in the clinic to treat certain types of cancers and open new avenues to develop personalized

treatments, which may allow for lower dosing, limiting side effects of treatment and improving overall quality of life and treatment compliance (272–274). Future studies focused on deeper understanding of the role of epigenetic modifications in manifestation of psychiatric disorders and development of more precise genetic and epigenetic biomarkers should uncover the potential use of epigenetic therapeutics when addressing mental health problems as well.

Next, while epigenetic age is clearly a strong biomarker for aging, it is a mystery as to what age-related biological process it is measuring (271). Future studies should address this knowledge gap and clarify, whether DNA methylation alterations contribute to pathogenesis of mental and physical health problems or it is a non-causal biomarker.

It is also unclear whether the epigenetic age acceleration can be prevented, slowed, or reversed. However, lifestyle factors appear promising: observational data from the Women's Health Initiative demonstrates that a higher intake of vegetables, fruits and fish is associated with reduced epigenetic age acceleration at a single time point (275).

Finally, similar to emerging genome-wide interaction studies in relation to depression (276,277), genome-wide G x E studies in much larger samples than our Study IV sample are warranted in order to unravel the admittedly complex genetic pathways via which ELS may exert effects on manifest T2D and CVD. They may open avenues for generating more accurate polygenic risk scores that could lead to better understanding of genetic makeup of the complex phenotypes, development of improved prediction and treatment of psychiatric and somatic stress-related illnesses. However, such large-scale studies will need to concur challenges that relate not only to the sample size but also to ELS exposures that are quantitatively and qualitatively comparable across individuals.

6.7 CONCLUSIONS

Overall, the findings from the Studies I – IV that comprise this thesis add to results from previous studies exploring genetic and epigenetic mechanisms, which may mediate the associations between prenatal and early life adversity and lifelong physical and mental health outcomes. In Studies I and II we show that polyepigenetic biomarkers of child GA and GC exposure at birth are associated with both antenatal maternal mental wellbeing and psychiatric problems in children. However, the biological pathways explaining these associations are likely very complex, therefore, in Study I we found only partial sex-specific and in Study II no mediation of these polyepigenetic biomarkers in associations between maternal antenatal depression and anxiety and mental and behavioral disorders in children. We further show in Study III that polyepigenetic biomarker of epigenetic clock of aging predicted more advanced pubertal development, higher cortisol levels and more psychiatric

problems in adolescents. Finally, Study IV reveals that exposure to ELS is moderated by genetic variants that play a role in the regulation of the HPA axis functioning in association with quantitative glycemic traits in older adults.

To sum up, we show that the polyepigenetic biomarkers of antenatal adverse exposures and aging and biomarkers of genetic vulnerability in combination with the information about ELS might contribute to early identification of individuals at risk for complex mental and physical disorders enabling timely targeted preventive and therapeutic interventions.

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