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Epigenetic Signatures of maternal stress phenotypes in newborns: FELICITy study

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विद्या ददाति विनयं विनयात् याति पात्रताम्।

पात्रत्वाद्धनमाप्नोति धनाद्धर्मं ततः सुखम्॥

vidyā dadāti vinayaṁ vinayād yāti pātratām|

pātratvāddhanamāpnoti dhanāddharmaṁ tataḥ sukham||

Knowledge gives humility, from humility, one attains character; From character, one acquires wealth; from wealth good deeds (righteousness) follows and then happiness.

ABSTRACT

Maternal stress is the major known risk factor for alterations in brain structure, cognition and behaviour associated with neurodevelopmental disorders. However, the underlying molecular mechanisms is not known but a mechanistic link between genetic and non-genetic factors causing complex diseases or traits has been provided by epigenetic processes. Epigenetics deals with the heritable changes in gene regulation without any change in the DNA sequence itself. Among several molecular mechanism that mediate epigenetic phenomena DNA methylation and microRNAs are well known markers. We hypothesize that stress-induced alterations can be gauged by the infant epigenetic biomarkers, which can be employed for early detection and follow up of affected children.

Hence, this thesis aims at examining the potential effects of prenatal stress in newborns through epigenetic mechanisms using different study designs and applying statistical approaches.

Our first step was to explore the relationship between DNA methylation and signatures of prenatal stress, using the EWAS (Epigenome Wide Association Studies) approach. EWAS are a rapidly growing area of research, in part due to recent advances in technology that have allowed for a deeper coverage of the human methylome. 738 pregnant women were screened for stress exposure using Cohen Perceived Stress Scale (PSS) and 164 women were enrolled. Prenatal Distress Questionnaire (PDQ) was also administered to assess specific pregnancy worries. Stress levels in the studied mothers were assessed during the third trimester using the Cohen Perceived Stress Scale (PSS) questionnaire, classifying women with PSS ≤ 19 to a control group and those with PSS ≥ 19 to a stress group. Transabdominal fetal Electrocardiograms (taECG) were recorded to derive coupling between maternal and fetal heart rates resulting in a “Fetal Stress Index” (FSI). Upon delivery, we collected maternal hair strands for cortisol measurements and newborn saliva for epigenetic analyses. DNA was extracted from saliva samples and DNA methylation was measured using EPIC Bead-Chip array (850k CpG sites). Linear regression was used to identify associations between PSS/PDQ/FSI/Cortisol and DNA methylation. We found five significant CpGs that were associated with PDQ and cortisol at FDR ≤ 5%. Three CpGs were annotated to genes (Illumina Gene annotation file): YAP1, TOMM20 and CSMD1 and two CpGs were located approximately lay at 50kb from SSBP4 and SCAMP1. In addition, two DMRs (Differentiated Methylation regions) related to maternal stress measures PDQ and cortisol were found: DAXX and ARL4D.

In the second step, we profiled transfer RNA fragments (tRFs) and microRNAs (miRs) in serum samples from umbilical cord blood of newborns assigned to stress and control groups based on maternal stress estimation. At birth, serum RNA was extracted from the umbilical cord blood of newborns and subjected to short RNA-sequencing, followed by differential expression (DE) analyses of tRFs and miRs. We found that tRF profiles revealed significant differences between stressed and control female, but not male newborns. We found in stressed female newborns declined levels of tRFs derived from tRNAs of mitochondrial origin, accompanied by exclusive upregulation of tRFs derived from Aspartate tRNAs of nuclear origin. Furthermore, using cubic SVM classification we identified four tRFs derived from mitochondrial-originated tRNAs which could separate female newborns into stress and control groups at 100% accuracy. The same four tRFs could also separate stress and control groups looking at males and females as one group, at 86.4% accuracy. In contrast, although linear discriminant analysis (LDA) based on miR profiles once again highlighted the stressed female newborns as distinct, we found no specific DE of miRs between the stress and control newborn groups.

In conclusion, this doctoral thesis enhances our understanding of prenatal stress in newborns by integrating methylation data from saliva and RNA data from serum samples and identifying several biomarkers associated with it. Firstly, in the methylation section: Genes annotated to these CpGs were found to be involved in secretion and transportation, nuclear signalling, Hippo signalling pathways, apoptosis, intra-cellular trafficking, and neuronal signalling. Moreover, some CpGs are annotated to genes related to autism, Post Traumatic Stress Disorder (PTSD) and schizophrenia. Secondly, in the RNA section: The findings suggest that female newborns are particularly sensitive to prenatal stress and reflect specific fetal cord blood tRF changes, more than miR changes, as potential biomarkers for attending to the damages caused by maternal stress during pregnancy. These results can help better the molecular mechanisms involved in prenatal stress and allow timelier detection of babies at risk and can thus be used to potentially have a more effective allocation of resources for early intervention programs to improve child development. A biomarker-guided early intervention strategy is the first step in the prevention of future health problems, reducing their personal and societal impact.

ZUSSAMENFASSUNG

Mütterlicher Stress ist der wichtigste bekannte Risikofaktor für Veränderungen der Gehirnstruktur, der Kognition und des Verhaltens in Verbindung mit neurologischen Entwicklungsstörungen. Die zugrundeliegenden molekularen Mechanismen sind zwar nicht bekannt, aber epigenetische Prozesse scheinen bei der Entstehung komplexer Krankheiten und Merkmale eine Rolle zu spielen. Die Epigenetik befasst sich mit den vererbbaren Veränderungen der Genregulation, ohne dass die DNA-Sequenz selbst verändert wird. Unter den verschiedenen molekularen Mechanismen, die epigenetische Phänomene vermitteln, sind DNA-Methylierung und microRNAs bekannte Marker.

Wir stellen die Hypothese auf, dass stressinduzierte Veränderungen durch epigenetische Biomarker bei Kindern gemessen werden können, die in Zukunft zur Früherkennung und Nachverfolgung betroffener Kinder eingesetzt werden können. Ziel dieser Arbeit ist es daher, die potenziellen Auswirkungen von pränatalem Stress bei Neugeborenen durch epigenetische Mechanismen mit Hilfe verschiedener Studiendesigns und unter Anwendung statistischer Verfahren zu untersuchen.

In einem ersten Schritt untersuchten wir die Beziehung zwischen DNA-Methylierung und Signaturen von pränatalem Stress mit Hilfe des EWAS-Ansatzes (Epigenome Wide Association Studies). EWAS umfasst ein schnell wachsendes Forschungsgebiet, was zum Teil auf die jüngsten technologischen Fortschritte zurückzuführen ist, und eine Erfassung des menschlichen Methyloms ermöglicht habt.

In unserer Studie wurden 738 schwangere Frauen mit Hilfe der Cohen Perceived Stress Scale (PSS) auf Stressbelastung untersucht und 164 Frauen wurden in die Studie eingeschlossen. Darüber hinaus wurde der Prenatal Distress Questionnaire (PDQ) eingesetzt, um spezifische Schwangerschaftssorgen zu erfassen. Das Stressniveau der untersuchten Mütter wurde während des dritten Trimesters mit Hilfe des Fragebogens Cohen Perceived Stress Scale (PSS) ermittelt, wobei Frauen mit einem PSS ≤ 19 einer Kontrollgruppe und solche mit einem PSS ≥ 19 einer Stressgruppe zugeordnet wurden. Transabdominale fetale Elektrokardiogramme (taECG) wurden aufgezeichnet, um die Kopplung zwischen mütterlichen und fetalen Herzfrequenzen zu ermitteln, war zur Berechnung eines neuer Parameters- dem

"Fetal Stress Index" (FSI)- führte. Nach der Entbindung sammelten wir Haarsträhnen der Mütter für Cortisol Messungen und Speichelproben der Neugeborenen für epigenetische Analysen. Aus den Speichelproben wurde DNA extrahiert, und die DNA-Methylierung wurde mit einem EPIC Bead-Chip-Array (850k CpG-Stellen) gemessen. Mittels linearer Regression wurden Zusammenhänge zwischen PSS/PDQ/FSI/Cortisol und DNA-Methylierung ermittelt.

Wir fanden fünf signifikante CpGs, die mit PDQ und Cortisol bei FDR ≤ 5 % assoziiert waren. Drei CpGs wurden mit Genen annotiert (Illumina Gene annotation file): YAP1, TOMM20 und CSMD1, und zwei CpGs befanden sich etwa 50kb von SSBP4 und SCAMP1 entfernt. Darüber hinaus wurden zwei DMRs (Differentiated Methylation regions) gefunden, die mit den mütterlichen Stresswerten PDQ und Cortisol in Verbindung stehen: DAXX und ARL4D.

Im zweiten Schritt erstellten wir Profile von Transfer-RNA-Fragmenten (tRFs) und microRNAs (miRs) in Serumproben aus Nabelschnurblut von Neugeborenen, die anhand der mütterlichen Stresseinschätzung in Stress- und Kontrollgruppen eingeteilt wurden. Bei der Geburt wurde Serum-RNA aus dem Nabelschnurblut der Neugeborenen extrahiert und einer RNA-Kurzsequenzierung unterzogen, gefolgt von differenziellen Expressionsanalysen (DE) von tRFs und miRs. Wir fanden heraus, dass die tRF-Profile signifikante Unterschiede zwischen den beiden Gruppen für weibliche Neugeborene aufwiesen, aber nicht für männliche. Bei gestressten weiblichen Neugeborenen fanden wir eine Verringerung der tRFs, die von tRNAs mitochondrialen Ursprungs stammen, begleitet von einer Hochregulierung der tRFs, die von Aspartat-tRNAs nuklearen Ursprungs stammen. Darüber hinaus identifizierten wir mit Hilfe der kubischen SVM-Klassifikation vier tRFs, die von tRNAs mitochondrialen Ursprungs abgeleitet sind und weibliche Neugeborene mit 100 %iger Genauigkeit in Stress- und Kontrollgruppen einteilen konnten. Dieselben vier tRFs konnten auch Stress- und Kontrollgruppen trennen, wenn man Männer und Frauen als eine Gruppe betrachtet, und zwar mit 86,4 % Genauigkeit. Obwohl die lineare Diskriminanzanalyse (LDA) auf der Grundlage von miR-Profilen die gestressten weiblichen Neugeborenen erneut als unterschiedlich hervorhob, fanden wir keine spezifische DE von miRs zwischen den Gruppen der Stress- und Kontroll-Neugeborenen.

Zusammenfassend lässt sich sagen, dass diese Doktorarbeit unser Verständnis von pränatalem Stress bei Neugeborenen durch die Integration von Methylierungsdaten aus Speichel- und RNA-Daten aus Nabelschnurserumproben und die Identifizierung mehrerer damit verbundener Biomarker verbessert. Erstens, im Bereich der Methylierung: Es wurde festgestellt, dass Gene, die an diesen CpGs annotiert sind, an Sekretion und Transport, Kernsignalisierung, Hippo-Signalwegen, Apoptose, intrazellulärem Transport und neuronaler Signalgebung beteiligt sind. Außerdem sind einige CpGs mit Genen verknüpft, die mit Autismus, posttraumatischer Belastungsstörung (PTSD) und Schizophrenie in Verbindung

stehen. Zweitens, im Abschnitt über RNA: Die Ergebnisse deuten darauf hin, dass weibliche Neugeborene besonders empfindlich auf pränatalen Stress reagieren und spezifische tRF-Veränderungen im fetalen Nabelschnurblut mehr als miR-Veränderungen als potenzielle Biomarker für die durch mütterlichen Stress während der Schwangerschaft verursachten Schäden dienen. Diese Ergebnisse können dazu beitragen, die molekularen Mechanismen, die bei pränatalem Stress eine Rolle spielen, besser zu verstehen, und sie ermöglichen eine frühere Erkennung von Risikokindern und damit eine wirksamere Zuweisung von Ressourcen für Frühinterventionsprogramme zur Verbesserung der kindlichen Entwicklung. Eine auf Biomarker gestützte Frühinterventionsstrategie ist der erste Schritt zur Prävention künftiger Gesundheitsprobleme und zur Verringerung ihrer persönlichen und gesellschaftlichen Auswirkungen.

Declarations

Chapter 1:

I conducted the experiments and the study-level data pre-processing, statistical analysis and analyses and drafted the thesis and the manuscript. Dr. Silvia Lobmaier, Dr. Martin Frasch and Dr. Marta Antonelli contributed to data collection management, data analysis and project advice. Sample and data collection for the mothers and their newborns was done at Klinikum rechts der Isar by Dr. Silvia Lobmaier, Dr. Marta Antonelli, Camilla Zelgert and Peter Zimmerman. Laboratory procedures was performed by me in collaboration with Nadine Lindemann. Dr. Melanie Waldenberger and Rory Wilson contributed to the EPIC methylation profiling advice, data analysis and statistical advice which was done at the Helmholtz Zentrum. I did the pre-processing of the data under the guidance of Rory Wilson and Dr. Martin Frasch. James MacDonald and Theo Bammler contributed to the statistical analysis and advice. I drafted the Thesis and the manuscript text under the supervision of Dr. Marta Antonelli and Dr. Melanie Waldenberger. Dr. Silvia Lobmaier and Dr. Marta Antonelli conceptualized the project, protocol and project development, data collection and management, data analysis and funding acquisition.

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Chapter 2:

Samples were collected at Klinikum rechts der Isar by Dr. Silvia Lobmaier, Dr. Marta Antonelli, Camilla Zelgert and Peter Zimmerman. The rational for the experiments was designed by Dr. Martin Frasch, Dr. Hermona Soreq and Shani Vaknine and was conducted by Shani Vaknine

under the supervision of Dr. Hermona Soreq. RNA samples were stored at Helmholtz Zentrum in Dr. Melanie Waldenberger's group. Sample pre-processing, PAX-RNA extraction and bioanalyser measurements were done by Ritika Sharma

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Abbreviations

Abbreviations	Full name
°C	Degree Celsius
%	Percentage
µl	microliter
ACTH	adrenocorticotrophin
ANS	Autonomic nervous system
ASD	Autism Spectrum disorder
BMI	body mass index
CRH	corticotrophin-releasing hormone
CpG	cytosine-phosphate-guanine site
DNA	deoxyribonucleic acid
DMP	Differentially methylated positions
DMR	Differentially methylated regions
EWAS	Epigenome Wide Association Studies
EDTA	ethylenediaminetetraacetic acid
FDR	False discovery rate
FHR	Fetal heart rate
FSI	fetal stress Index
GR	Glucocorticoid receptor
GC	Glucocorticoids
g	Gram
h	Hour
HCL	Hydrochloric acid
HPA	Hypothalamic Pituitary Adrenal axis
kg	Kilogram
L	Liter
$MgCL_2$	Magnesium Chloride
MHR	maternal heart rate
miRNAs	Micro-RNA
mg	Milligram
ml	Milli-liter
mm	Millimeter
mM	millimolar
min	minute
NaOH	Sodium hydroxide
ng	Nanogram
nts	nucleotides
PVN	paraventricular nucleus
PCR	Polymerase chain reaction

PS	Prenatal stress
p-value	Probability value
rpm	Revolutions per minute
RNA	ribonucleic acid
RT	Room temperature
sec	Seconds
TSS	transcription start site
TRIS	tris (hydroxymethyl) aminomethane
UTR	untranslated region
UV	Ultraviolet
V	volt

Chapter I: Epigenetic study

1. Introduction

All living organisms maintain a complex state of equilibrium or homeostasis, which can be continuously challenged by internal or external adverse events, named stressors (Chrousos, Loriaux and Gold, 2013). The concept of stress was first described by Hans Selye as 'non-specific response of the body to any noxious stimulus" (Selye, 1950). Since then, is has been revised in the literature several times, to recently refine it by distinguishing it into 'stressor and 'stress response' where stressor is "any environmental demand that exceeds the physiological regulatory capacity of an organism" (Lucassen *et al.*, 2014). In general, stress is now defined as any stimulus, including an adverse environment, experience, or perceived threat, that disrupts the body's internal balance (i.e., physiological homeostasis), which elicits a physiological response involving both peripheral and central systems (Chrousos, 2009). Stress becomes negative when adaptation or coping mechanisms fail (Danese and McEwen, 2012).

Pregnancy is a very important time in a woman's life and there is now extensive evidence from stress studies indicating that maternal adversity during gestational period can lead to lasting physiological and pathological outcomes in their offspring.

An increasing number of epidemiological studies prove that adversities in early life environment (especially in the mother's womb) can significantly increase the risk for developing neurological and physical diseases later in life (Bale *et al.*, 2010). Association between low birth weight and increased cortisol response to psychosocial maternal stress in children (Jones *et al.*, 2006) and young adults (Wüst *et al.*, 2005) has been reported. Moreover, maternal stress during pregnancy was associated with increased cortisol response (Entringer *et al.*, 2009). Van den Bergh et al, (Van den Bergh *et al.*, 2017) states that prenatal stress predicts behavioural problems in the offspring during the first 10 years of life such as attention, aggressive behaviour, and depressed behaviour.

One of the key questions to ask is what biological processes affect these early fetal experiences leaving permanent marks onto their future behaviour? One theory suggests that maternal stress during gestation affects offspring development through placenta, which controls the offspring's environment and is vulnerable to maternal stress (Bronson and Bale, 2016). A more recent theory posits epigenetics could be one path determining the relation between prenatal stress and fetal programming.

1.1 Prenatal stress

1.1.1 Development Hypothesis

Several developmental models have been proposed based on Barker's hypothesis of "Fetal Basis of Adult Diseases" (FeBAD) also known as the "Developmental origins of Health and Disease" (DOHAD) or the "Fetal Programming hypothesis" (Barker, 1990, 2004). Barker and his group, based on their studies on adult cardiovascular diseases, stated the hypothesis that the predisposition to the non-communicable diseases can be traced back to embryonic development, a sensitive period when plasticity is especially high and susceptible to environmental factors (Barker, 1990).

More recently Van Den Bergh, proposed an extension to the original DOHAD hypothesis known as the "Developmental origins of Behaviour Health and Disease" (DOBHaD) which states that specific imaging and non-invasive recordings might show signs of disorders to come, and this appearance of signatures allows early diagnosis and potential treatment of disorders (Van den Bergh, 2011).

Prenatal stress may thus be the prime time for the fetus's epigenetic signature with lasting effects on stress reactivity and the immune system until long after birth, thus increasing vulnerability to neurodevelopmental disorders.

1.1.2 Definition of Prenatal stress

Prenatal stress refers to the stress reaction experienced by the growing fetus when the pregnant mother is exposed to psychosocial or physical stress (Lemaire *et al.*, 2000). It is known that prenatal stress factors may have lasting effects on the organ development of the growing fetus, thereby influencing the risk for physical and mental health and diseases later in life (Barker, 1990).

True to this assumption, many studies have indicated changes in stress reactivity in association with negative prenatal environments. Maternal stress during pregnancy was associated with increased cortisol response (Entringer *et al.*, 2009). However, possible mediators linking maternal stress and the offspring are not just cortisol and other glucocorticoid receptors (GRs) but also cytokines, reactive oxygen species (ROS), catecholamines, serotonin/tryptophan and maternal microbiota (Rakers *et al.*, 2016). Therefore, early life stress may influence fetal behaviour through processes that affect stress response systems such as the delayed response through activation of the Hypothalamic Pituitary Adrenal axis (HPA) and the quick response that involves the rapid activation of the Autonomic Nervous system (ANS).

1.1.3 Prenatal stress and HPA axis

It has been proposed that dysregulation of the maternal hypothalamic-pituitary adrenal (HPA) axis determines fetal exposure to stress hormones influencing development and birth outcomes and programming the fetal HPA axis, therefore determining responses to stress and susceptibility to physical and mental illness in later life.

HPA axis plays a key role in the regulation of homeostasis and mediates the stress response of the glucocorticoid. Glucocorticoids (GC) are the end products of the HPA-axis activation and are produced by the adrenal glands in response to the adrenocorticotropic hormone secretion

in the pituitary. GC play a key role in the perpetuation of resting and stress related homeostasis affecting the adaptable reaction of the organism towards stressors (Nicolaides *et al.*, 2015).

Psychological (e.g. anxiety and abuse) and physiological (e.g.hypoxia) stress can result in the activation of corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus. Secretion of the CRH and other factors into the hypophyseal portal circulation results in increased synthesis and release of adrenocorticotrophin (ACTH) from anterior pituitary corticotrophs (Ulrich-Lai and Herman, 2009). In turn, ACTH stimulates the adrenal cortex to secrete cortisol into the bloodstream, cortisol feeds back to the glucocorticoid and mineralocorticoid receptors in the pituitary and hypothalamus to regulate its own secretion.

During pregnancy, the regulation of the maternal HPA axis undergoes dramatic changes (Figure 1) . Circulating cortisol levels rise markedly to around threefold non-pregnant levels by the third trimester (Jung *et al.*, 2011). This rise in cortisol is partly due to estrogen stimulation of corticosteroid-binding globulin with a rise in free (or bioavailable) cortisol levels (Demey-Ponsart *et al.*, 1982; Qureshi *et al.*, 2007). In addition, the placenta secretes large quantities of CRH into the maternal bloodstream during the second and third trimesters of pregnancy (Hillhouse *et al.*, 1993; Petraglia *et al.*, 1993). Placental CRH stimulates the maternal pituitary gland, thus further increasing both ACTH and consequently cortisol levels. In turn, maternal cortisol stimulates placental CRH synthesis creating a positive feed-forward drive with resultant higher cortisol levels (Robinson *et al.*, 1988). Despite the increasing circulating levels of cortisol, the diurnal secretion of cortisol is maintained throughout pregnancy (Mastorakos and Ilias, 2003). However, as pregnancy progresses, the increased circulating cortisol downregulates hypothalamic production of CRH and thus the responsiveness of the HPA axis to both physiological and psychological stress is attenuated during late pregnancy (Wadhwa *et al.*, 1997; de Weerth and Buitelaar, 2005).

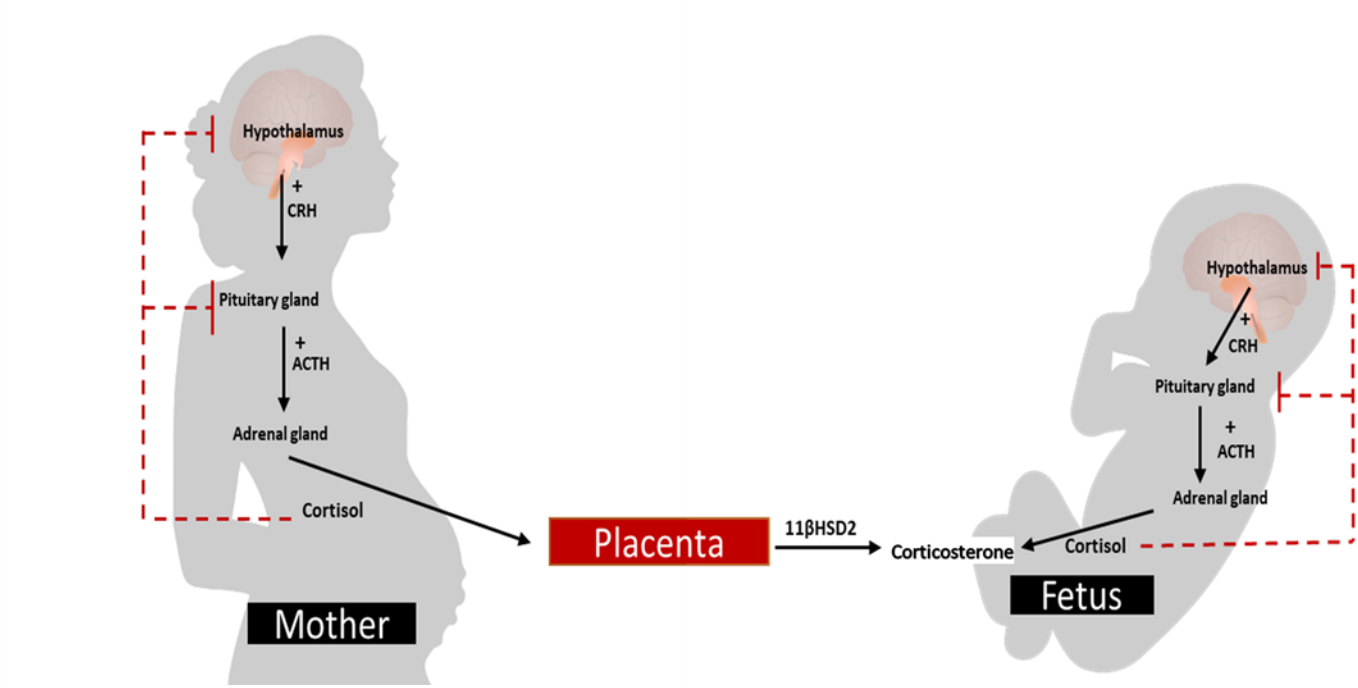


Figure 1: HPA axis in pregnancy (Modified from (Howland, Sandman and Glynn, 2017)*)*

In a study of 100 mothers, at 32 weeks' gestation, at Queen Charlotte's hospital demonstrated a strong correlation between plasma levels of the stress hormone cortisol in the mother and in the fetus (Glover, 1999; Teixeira, Fisk and Glover, 1999). Increased concentrations of cortisol in the brain leads to:

1. Elimination of neurons
2. Cell migration to the wrong destinations resulting in formation of wrong circuits
3. Inhibition of dendritic branches
4. Destruction of synapses
5. Decreases serotonin (5-HT1A) in the brain. Serotonin is known as the 'feel of well-being' neurotransmitter, and stress and anxiety decrease receptors of serotonin
6. Suppresses immune and inflammatory response

1.1.4 Prenatal stress and ANS

Fetal ANS is very sensitive to maternal stress (Monk *et al.*, 2003; Makino *et al.*, 2009; Fink *et al.*, 2010) and common markers of ANS such as FHR reactivity to a stimulus, reflect emerging individual differences in the development of the autonomic- and central nervous systems related to styles of future emotional regulation and risk for psychopathology (Kinsella and Monk, 2009; Gao, Huang and Li, 2017). It is hence likely that the FHR response to MHR changes represents a fetal stress memory and may serve as a novel biomarker to detect PS effects early in utero which may help guide early interventions postnatally.

Fetus at risk for fetal programming especially that of the ANS can be examined by analysis of the FHR patterns. This can be done by the method: phase-rectified signal averaging (PRSA) which is the analysis of the fetal heart rate, measured by electrocardiography (ECG) or cardiotocography (CTG) (Bauer *et al.*, 2006; Kantelhardt *et al.*, 2007). PRSA allows the detection of quasi-periodicities in non-stationery data, and it can eliminate the signal artifacts and extract the areas of interest. Lobmaier et al. first demonstrated the PRSA method for fetuses (Lobmaier *et al.*, 2012). since it was already established in adults by Schmidt and his team (Bauer *et al.*, 2006). To assess the ANS system impact on the FHR the beat-to-beat information (R-R intervals) is analysed. A new multidimensional trans-abdominal fetal ECG monitor allows to detect and record fetal and maternal ECG, non-invasively from the surface on the mother's abdomen. The signal from this examination can be used for analysis of the FHR (Frasch *et al.*, 2014). Recently, couplings between maternal heart rate (MHR) and FHR were assessed on a signal- processing algorithm termed bivariate PRSA (BPRSA) initially described for adults by Bauer et al (2009) and now the first time for the feto-maternal dyad by Lobmaier et al (Lobmaier *et al.*, 2020). This yielded in the fetal stress Index (FSI) values, which jointly assesses changes in MHR and FHR. In this study, the authors could detect periodic MHR decreases that reflect typical patterns of maternal breathing showing that control group fetuses remained stable whereas fetuses of stressed mother showed significant decrease in

FHR. Fetal stress Index (FSI) is envisioned to become a powerful tool to measure of the effects of maternal stress on fetal ANS. Therefore, it is likely that FSI represents fetal stress memory and can serve as a biomarker to detect PS effects early in utero. This will also help guide early interventions postnatally (Lobmaier *et al.*, 2020).

1.2 What is "Epigenetics"?

It is in recent times that the study of early hardships in infants is being viewed through an epigenetic lens leading to a growing attention to the epigenetic link between early stressful experiences and offspring behaviour.

The epigenetic term was coined by Conrad Waddington in 1942 which means "in addition to changes in genetic sequence" (Waddington, 2012). Epigenetics refers to the study of mitotically and/or meiotically heritable changes/alterations of the chromatin network, which does not require a change in DNA sequence but leads to a change in gene expression resulting in altered production of proteins (Jaenisch and Bird, 2003; Bird, 2007).

Several molecular mechanisms mediate epigenetic phenomena: DNA methylation, histone modification, chromatin remodelling and non-coding RNA (Tammen, Friso and Choi, 2013). DNA methylation is the most common studied epigenetic mediator. DNA methylation is the process where a methyl group is added to the DNA molecule and the focus of this thesis.

1.2.1 DNA methylation

As mentioned above, DNA methylation is a chemical modification that works by the addition of a methyl group (-CH3) added by enzymes known as the DNA methyltransferases (DNMTs) on C5 position of cytosine on the DNA molecule resulting in 5- methyl cytosine Figure 2. DNA methylation occurs in the context of CpG dinucleotides where a cytosine nucleotide is next to

a guanine nucleotide. DNA methylation can affect gene activity and phenotype and yet do not alter the DNA sequence (Bird, 2002; Jones and Liang, 2009).

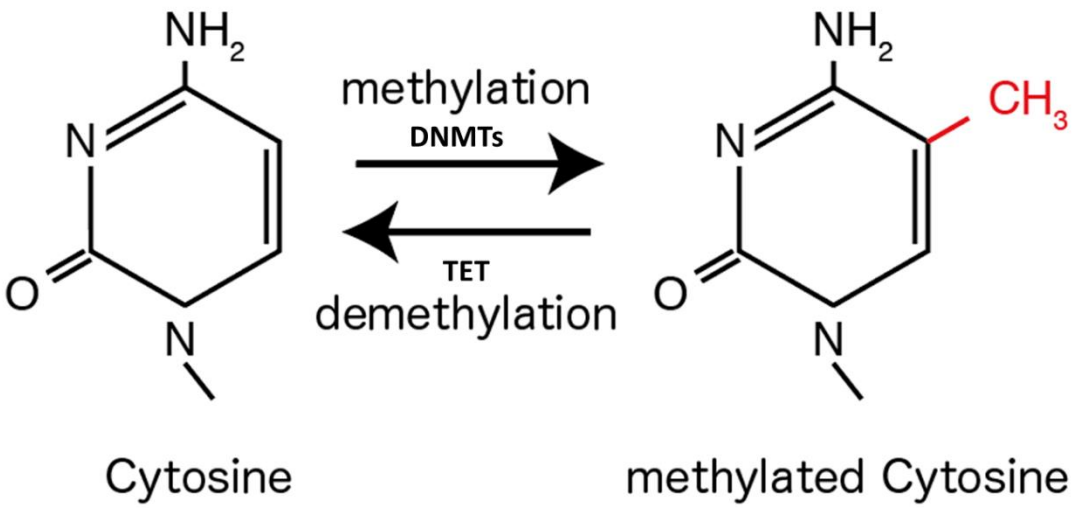


Figure 2: DNA methylation- Addition of methyl group to the C5 position of cytosine (Adapted from: (Pfeifer and Jin, 2018)*)*

In early embryogenesis the DNA is largely free of methylation. The three principal enzymes from the DNMT family responsible for establishing and maintaining DNA methylation are: *DNMT1*, *DNMT3A* and *DNMT3B*. *DNMT1*, known as the maintenance *DNMT*, maintain DNA methylation pattern after cell DNA replication cycle. Whereas *DNMT3A* and *DNMT3B*, the *de novo* DNMTs, mediate the *de novo* CpG methylation principally during embryogenesis when the cell fate is being determined (Rondelet and Wouters, 2017). DNA methylation is stable chemically and genetically compared to other epigenetic marks. But DNA methylation marks can be reversed by passive or active demethylation. Passive demethylation occurs in the absence of *DNMT1* resulting in fully unmethylated strands during successive DNA replication cycles. Alternatively, active methylation is catalysed by Ten -eleven translocation (TET) enzymes which sequentially oxidize 5mc to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fc) to 5-carboxylcytosine (5caC) a subsequent thymine DNA glycosylase mediated base excision repair (He *et al.*, 2011; Ito *et al.*, 2011).

TET proteins are involved in the maintenance of pluripotent stem cells and post-mitotic somatic cells (Kohli and Zhang, 2013). Downregulation of *TET* expression also has been observed in various tumor types including breast cancer and leukaemia (Yang *et al.*, 2013).

The total number of CpGs in the genome is about 28 million, among which 60-80% are generally methylated. CpG islands (CGIs) are regions of DNA where more than 70% of the sequence is comprised of CpG dinucleotides. These are mainly found in the promoter regions of the genes but also appear in gene bodies, often acting as alternative promoters (Saxonov, Berg and Brutlag, 2006). Contrary to the CpG sites, most of the CGIs tend to remain unmethylated to maintain transcription of the active gene implying that methylation of CGIs, lead to gene repression (Mohn *et al.*, 2008). Regions adjacent to CGIs (~2kb) with low density CpGs are called CGI shores, which have been suggested to be related to tissue-specific DNA methylation (Doi *et al.*, 2009; Irizarry *et al.*, 2009).

DNA methylation can affect the gene expression in possibly three ways: - 1) Modification of transcription factors binding affinity to the gene promoter, 2) repression of the spatial accessibility of the transcription factors or DNA binding proteins to promoters by altering the chromatin structure, 3) Affecting the binding between promoter or gene body to methylation - specific recognition factors (Zhang and Pradhan, 2014).

DNA methylation is involved in several important processes such as X chromosome inactivation, genomic imprinting and silencing of germline specific genes and repetitive elements (Bird, 2002; Jones and Liang, 2009). Moreover, DNA methylation has also been shown to be involved in several diseases. Many studies show that at different stages and disease types produce a different epigenetic pattern (Nicoletti *et al.*, 2016; Goel, Karir and Garg, 2017; Leygo *et al.*, 2017; Belzeaux *et al.*, 2018). Therefore, DNA methylation is known not only to be a promising biomarker for diagnosis but also prognosis of different malignancies.

1.3 Epigenetics of stress: Role of Epigenetic mechanisms during early development

The epigenome is very vulnerable to external exposures during early prenatal development, the time when immense reprogramming and programming of epigenetic alterations take place to incorporate cell and tissue specific gene expression.

1.3.1 Evidence from animal studies

Animal studies exhibit compelling evidence linking the effects of PS during different stages of pregnancy with changes in behaviour, physiology, metabolism and brain morphology (Beydoun and Saftlas, 2008; Weinstock, 2008; Frasch *et al.*, 2020). Animal studies have also analysed maternal exposure to drugs, stress and toxicants that can alter epigenetic gene programming and contribute to neurodevelopment and behavioural deficits during later development in offspring and therefore the pregnancy period should not be neglected. However, one should be aware of the differences in pre- and postnatal brain development in different species, when translating the results of PS in animal studies to human studies (Lupien *et al.*, 2009).

Agouti viable yellow (A^{vy}) model were the first animal model to provide the evidence that maternal environment exposure during pregnancy can induce phenotypic changes by epigenetic mechanisms in the offspring. This study shows that maternal dietary supplementation can increase methylation at specific CpG sites and alters the phenotype such as coat color and obesity in A^{vy} mouse model (Cooney, Dave and Wolff, 2002; Bernal and Jirtle, 2010). Many studies have associated prenatal stress with changes in global methylation status of even examining a particular locus (candidate gene approach).

For example, Weaver and his colleagues, 2004, examined the epigenetic effects of differences in maternal care in rats and found that the offspring of mothers who showed reduced care exhibited high-levels of DNA methylation in exon 1 of *NR3C1* which is associated to a decreased expression of the Glucocorticoid receptor (GR) in the rat hippocampus (Weaver *et al.*, 2004, 2005), whereas offspring of high maternal care mothers shows DNA demethylation of this promoter. This paper suggests that maternal behaviour can influence the epigenetic regulation of genes involved in stress response, learning and memory, and social behaviour of offspring. Specially maternal behaviour such as maternal licking and grooming has been shown to increase DNA methylation of *NR3C1* and decrease DNA methylation of the brain-derived neurotrophic factor gene- *BDNF* in offspring, which is associated with increased stress resilience and enhanced learning and memory (Weaver *et al.*, 2004). Even during adulthood, the DNA methylation differences were stable, although they could be reversed by administration of methionine or histone deacetylase (HDAC) inhibitor (Weaver, Meaney and Szyf, 2006).

Research by Mueller and Bale in rodents has shown that two genes related to the HPA axis, the glucocorticoid receptor gene (*Nr3c1*) and corticotrophin-releasing factor (*Crf*) were programmed by DNA methylation during the early life. It was also observed that the effects of maternal stress were sex-specific and were dependent on the timing of stress exposure. The male offspring exposed to chronic stress displayed not only depressive symptoms in adulthood but also showed an increase HPA response to stress (Mueller and Bale, 2008).

Brain-derived neurotrophic factor (*BDNF*) has also been associated with prenatal stress. Rats exposed to maternal separation show lower *BDNF* mRNA and protein expression in the hippocampus (Gray, Milner and McEwen, 2013; Gray *et al.*, 2014), along with higher levels of miR-16, a miRNA that has been shown to be negatively associated with BDNF expression (Bai *et al.*, 2012).

Monteleone et al, also observe an alteration in methylation pattern in a CpG island of the first intron of the gene *gpm6a*. *Gpm6a* gene which encodes for the membrane glycoprotein M6A participating in the establishment of neuronal morphology, is observed too high in the hippocamps of PS rat's brain (Monteleone *et al.*, 2014). Prenatal stress also increases the risk of gestational diabetes, preterm birth, and delayed brain development in multiple future generations. This has been observed in miRNA and mRNA signatures of preterm birth by Yao et al .

In a study published in the journal Epigenetics, by Franklin and colleagues, the group, found that prenatal stress in rats, such as maternal deprivation and poor maternal care in F1, F2 and F3 generations led to changes in DNA methylation and histone modifications in genes related to stress response and anxiety-like behaviour. These responses were observed in form of differences in social recognition and depressive behaviour when compared to offspring from the control group.

Overall, these studies suggest that prenatal stress is related to epigenetic changes in specific brain regions and genes in animals, which may lead to altered behaviour and increased susceptibility to psychiatric disorders later in life. However it is important to remember that animal studies may not necessarily translate directly to humans and more research is needed to fully understand the effects of prenatal stress on epigenetic regulation in humans.

1.3.2 Evidence from human studies

Evidence from epigenetic alterations in rodent models invoked parallel investigations in humans. It is very challenging to establish an epigenetic link between prenatal environment and stress exposure in humans. Firstly, the brain as a target tissue is inaccessible in humans, and therefore we rely on data from peripheral tissues. Secondly, humans are exposed to varying environmental agents and it is tough to clearly dissociate a specific factor leading to

any neurodevelopmental outcome. However, in the past few years there are several well controlled and measured studies that provide evidence that link prenatal maternal stress to newborn epigenetically.

The well-established evidence of an epigenetic link with in-utero environmental conditions comes from the Quebec Ice storm cohort induced by a natural disaster known as the Project ice storm. Cao-Lei et al studied the cohort from Project ice storm which indicated a correlation between the exposure to prenatal stress and differential DNA methylation of 957 genes in 13-year-old offspring. The methylation patterns corresponded with the degree of subjective maternal stress rather than objective stress reported by the mothers during pregnancy. Many of the differentially methylated genes that were reported by this study were related to immune function and metabolism (Cao-Lei *et al.*, 2014, 2016).

In a study by Oberlander et al, newborns born to mothers who had symptoms of depression during pregnancy had high levels of methylation of the glucocorticoid receptor gene in the umbilical cord and increased levels of salivary cortisol concentrations at three months of age (Oberlander *et al.*, 2008).

Deducting from the findings in animal studies by Meaney's group, McGowan and colleagues examined the *GR1F* promoter in the hippocampus of the human suicide victims with childhood abuse, showing that dysfunctional families and childhood adversity are linked to altered HPA stress response and therefore leading to an increased risk for suicide (McGowan *et al.*, 2009). A decreased GR expression and increased GR gene promoter methylation was observed in the hippocampus of suicide victims with a history of childhood abuse when compared to suicide victims without a history of childhood abuse. Yehuda et al, investigated the intergenerational effects of trauma on the epigenetic regulation of the glucocorticoid receptor gene in Holocaust survivor offspring (Yehuda *et al.*, 2014). They examined the *NR3C1* promoter methylation in samples of adult population without PTSD with at least one Holocaust survivor parent (with or without PTSD) (n= 80) and then matched the participants demographically without parental

Holocaust exposure or PTSD (n=15). They found an interaction between maternal and paternal PTSD in prediction of offspring *NR3C1* promoter methylation. Offspring exposed to both maternal and paternal PTSD showed lower levels of *NR3C1* promoter methylation but when seen in absence of maternal PTSD, i.e. exposure to paternal PTSD, offspring had higher levels of promoter methylation. However, there was a negative correlation between *NR3C1* promoter methylation and *NR3C1* expression(Yehuda *et al.*, 2014). Their findings suggest that exposure to trauma can lead to epigenetic modifications that are transmitted across generations and can impact the stress response systems of the offspring. One thing to remember is that replication of these complicated interactions in a relatively small sample size is needed and these results are to be considered as initial hypothesis about intergenerational transmission of post-traumatic stress.

Another pioneer study that demonstrated the propagation of epigenetic marks across generations via gametes changes and gestational uterine environment in humans are in the Holocaust survivors and their offspring by the same group (Yehuda *et al.*, 2016). They showed that parental trauma influenced the methylation of *FKBP5*, which was observed both in the parent and offspring (Yehuda *et al.*, 2016).

Hompes et al, in their study of 83 pregnant women assessed maternal stress in each trimester and found a significant association with methylation at one CpG site on the *NR3C1* promoter in the cord blood of newborns (Hompes *et al.*, 2013). However, Hompes et al, were not able to replicate the results by Oberlander et al. , who found associations with methylation at 3 CpG (not *NR3C1)* sites with some prenatal depression and anxiety indicators. Despite of inconsistencies in these studies, it seems that maternal stress during pregnancy can alter gene expression in offspring leading to increase in risk to their development due to stress. Most of these studies indicate and prove that effects of stress on *NR3C1* promoter methylation initially found in rats, translates into human studies (Oberlander *et al.*, 2008).

In another study by Appleton et al, prenatal socioeconomic adversity during pregnancy was associated with lower levels of placental *HSD11B2* methylation in children (Appleton *et al.*, 2013). The same study further investigated methylation link with *SLC6A4* and *BDNF* in terms of maternal depression in second and third trimester (Devlin *et al.*, 2010). This study showed that maternal depressed mood in the second (not third) trimester was associated with decreased maternal and infant *SLC6A4* promoter methylation, while no association was observed for *BDNF*. Moreover, Braithwaite et al, studied 57 mothers and the association with methylation of *NR3C1* and *BDNF* in 2-month-old offspring with depressive symptoms in the mother between 2nd and 3rd trimester. This group found that decreased methylation of an exon upstream of the brain-derived neurotrophic factor gene (*BDNF*) in both male and female infants, while increased *NR3C1* DNA methylation in male infants was associated with prenatal depressive symptoms (Braithwaite *et al.*, 2015).

Tyrka et al, showed a blunted cortisol and adrenocorticotrophic hormone response in adults who had experienced childhood trauma. It was associated with hypermethylation of the GR promoter in whole blood (Tyrka *et al.*, 2012). They also showed childhood adversities were negatively associated with methylation (Tyrka *et al.*, 2016).

1.4 Methods of epigenome-wide DNA methylation profiling

Most of the previous maternal stress studies have been limited to DNA methylation studies in candidate genes and only in recent years epigenome wide studies of DNA methylation have gained popularity allowing us to evaluate locus-specific methylation across the entire genome (Rakyan *et al.*, 2011). There are many methods to date with different advantages that have been developed to make genome-wide DNA methylation high throughput profiling possible. Human microarray approaches and next generation sequencing are the most popular ones to map on a genome-wide scale (Bell and Spector, 2011; Heyn and Esteller, 2012).

1.4.1 Whole Genome Bisulphite sequencing

Whole Genome Bisulphite Sequencing (WGBS) is currently the gold standard for samples which have a reference genome available (Stirzaker *et al.*, 2014). WGBS provides a comprehensive complete genomic coverage of ~28 billion CpG sites in the human genome (Lister *et al.*, 2009). This is based on the treatment of genomic DNA with sodium bisulphite, which converts unmethylated cytosines to uracils while leaving methylated cytosines unchanged, followed by whole genome sequencing (Clark *et al.*, 1994). However, WGBS requires large amount of DNA, is extremely costly, sequencing depth is rather low (<20x) and data analysis requires intense downstream computational support.

1.4.2 Microarray based approach.

The most popular and cost-effective platform is the microarray-based approach. InfiniumMethylation27 BeadChip (27k) array, first introduced in 2008, is a relatively old version where the coverage is 27,000 CpG sites in the promoter specific regions that are predominantly unmethylated (Bibikova *et al.*, 2009). But it was not until the later version of array Illumina 450k, introduced in 2011, was truly considered as “epigenome-wide” approach as its coverage increased to ~485,000 CpGs, which represented 5% out of approximately 10^7 possible CpG sites across the genome. The 450k combined the original Infinium I assay with the novel Infinium II probes and allows up to 12 samples per array design (Bibikova *et al.*, 2011). The 450k comprised of many genomic categories such as CpG islands, shores, and shelves, the 5’UTR, 3’UTR and bodies of RefSeq genes, FANTOM4 promoters, the MHC region and some enhancer regions (Bibikova *et al.*, 2011). But the 450K still missed important regulatory regions. To improve this limitation Illumina released the EPIC-850K with new regions specifically designed to target the enhancer regions (Illumina support: http://support.illumina.com). The 850k array also uses a combination of the Infinium I and II

assays, like 450k, but achieves additional coverage to >850,000 sites, by increasing the size of each array; a 450k slide contains 12 arrays whilst the 850k has only 8. The principle of EPIC chip is explained in more detail in the methods section.

Regardless of the Illumina array version, each CpG has two measurements: a methylated intensity (M) and unmethylated intensity (U). These intensity values can be used to determine the proportion of methylation at each CpG locus. And methylation values are usually reported as beta (β) or M values. Beta and M values are related through a logit transformation. The raw methylation level at each CpG is quantified using beta value defined as: -

$$\beta = \frac{M}{(M + U + 100)}$$

where constant 100 is to stabilize the beta values when intensities are low. A β-value of 0 represents a completely unmethylated CpG site and a β-value approaching 1 represents a fully methylated CpG site.

Other than being cost effective, one potential benefit is to minimize variation caused during methylation detection and their wide-spread use also allows for meta-analysis across several different studies.

1.5 Epigenome Wide Association Studies (EWAS)

Epigenome- wide association studies (EWASs) are large-scale studies in disease-associated epigenetic variation by capturing non-genetic determinants of human diseases (Rakyan *et al.*, 2011).

Data generated from Illumina array is then used in an epigenome-wide association study (EWAS) analysis. Overall EWAS looks at the association between methylation and phenotype

of interest and can identify loci where methylation may differ either between case and control or associated with a complex trait from the methylome. However, it is not possible to infer anything about causality or the direction of effect. The key concept is that the environmental influences could alter DNA methylation, especially in the critical stages of development, leading to long lasting and negative health outcomes.

1.5.1 Main challenges of epigenetic epidemiology studies (EWAS studies)

While the number of DNA methylation studies are increasing, some important questions regarding the reliability and validity of DNA methylation in human research remain unanswered.

Firstly, it is not clear which markers of DNA methylation are stable over what periods of time and which markers can change rapidly with change in environment and genetics. It has also been shown that certain methylation patterns are different across different ethnicities (Park *et al.*, 2018; Joseph *et al.*, 2019). It is also challenging to draw firm conclusion if CpG sites vary due to environmental pressures. Talen's et al found evidence for stable DNA methylation in CpG sites of eight genes over a period of one to two decades of young to middle ages individuals (Talens *et al.*, 2010). Such studies indicate that methylation can be stable over a long period of time but differences in age range, array methods make it impossible to state solid verdicts.

Confounding is a major concern, if appropriate confounding factors are not considered, they can lead to seemingly significant results, even if the phenotype or exposure of interest isn't associated with epigenetic variation. Common confounders that need to be considered are age, sex, cellular heterogeneity, and substance use such as smoking and medication. Batch effects are linked to experimental procedure are also a common source of false associations. They also should be accounted for by randomizing samples across batches, as well accounting for experimental batches statistically in the analyses.

Tissue remains to be an issue in epigenetic epidemiological studies. The challenge of tissue accessibility is two-fold. In addition to confounding due to cellular heterogeneity (Houseman *et al.*, 2012), the tissue specificity issue indicates that the disease-specific epigenetic alteration could also be tissue-specific and therefore the disease-specific tissue sample is the most informative. However, in humans' internal tissues are more difficult to access, especially brain, the tissue of interest in the psychological-neurobiological context. Because in human brain DNA methylation is inaccessible ante mortem, many studies only investigate peripheral tissues such blood, cord blood, and saliva/buccal. One study by Hannon et al have observed inter-individual methylomic variation across cortex, cerebellum in the brain and found that DNA methylation from whole blood was not a strong predictor of variation in the brain (Hannon *et al.*, 2015).

The reason for this variation between blood and brain is not only because of the blood and brain barrier but also because of the heterogeneity of cellular composition of blood samples that must be controlled for (Houseman *et al.*, 2012). Cellular heterogeneity can also influence the comparison of DNAm across tissues and can lead to varied results. Recently, Buccal/saliva tissues have also been used frequently for information of methylation levels as it is less invasive compared to blood sampling. A study by Smith et al , show that buccal epithelial tissues are less heterogenous than blood cells and closer to brain the developmental sense (Smith *et al.*, 2015).

Causality attribution is one of the major challenges in epigenetic epidemiological studies. In GWAS studies this remains stable i.e., there is no possibility of reverse causation, which means the genome remains stable from conception as it was established first and therefore the direction of relationship is from genome to the phenotype. But this does not hold true for epigenetic studies and there is a possibility of reverse causation (Riancho, Del Real and Riancho, 2016). If there are epigenetic differences observed, the question is if the epigenetic differences are the cause, or the consequence of the trait observed. Causality is critical to

determine the mechanisms that lead to the disease. One of the common means to address the question of causality is through mediation modelling, mostly based on longitudinal study designs. In longitudinal studies, analysis of epigenetic signatures in healthy individuals needs to be observed before the onset of disease (Richmond *et al.*, 2014). Other methods to assess causal inference include Mendelian randomization, a method using genetic polymorphisms as instrumental variables.

Replication or validation seems to be another important concern when it comes to epidemiological studies to confirm preliminary findings. Replication can be defined as “the reproducibility of preliminary results in a cohort that is as similar to, but independent of, the preliminary cohort “ (Campagna *et al.*, 2021). Therefore, it is often difficult to find a replication cohort, especially when sufficient patients are difficult to collect in rare diseases and the recruitment and follow up is tough in human studies.

Given the challenges and caveats to methylation epidemiological study, it is clear a perfect study does not exist. To get an in-depth knowledge of epigenetic epidemiology, combinations of different types of studies and replications across different cohorts is necessary.

2. Hypothesis

Stress and anxiety during gestational period increase the risk for poor infant development and if this lingers during the post-natal period, it will lead to cognitive disabilities. There are many association studies related to prenatal stress with respect to the HPA. But to our knowledge, epigenome wide DNA methylation related to exposure to markers from the HPA axis and ANS measured in saliva have not been considered yet. This PhD thesis aims to contribute to this body of research and aims to fill in the gaps that are currently there.

In this study, we propose that infants affected by prenatal stress and thus epigenetic reprogramming already show alterations of the fetal ANS and HPA. Methylation levels will be altered in salivary DNA from PS-exposed infants. This is important because the detection of such epigenetic signatures, ideally at birth, will help to detect “at-risk children” who can benefit from early stimulation programs and follow-up. It is theorized that this epigenetic trait might correlate with the increased maternal stress and altered cognitive development. Therefore, this could be an approach not only to identify novel candidate genes and to study interactions using biological pathway analysis but also in-utero detection of predisposition to the behavioural and developmental impairment.

We hypothesized that late gestation biophysical alterations in mother-fetus dyads due to PS are reflected in the neonatal epigenetic marks observed at birth. To test these hypotheses, we devised the FELICITy study to obtain a combination of non-invasive multimodal physiological measures of PS.

This first chapter focuses on how DNA methylation could act as a biological mediator between prenatal stress and associated mental health outcomes.

3. Objectives

3.1 General objective

The aim of this study was to examine the association between prenatal exposure to maternal psychosocial stress and offspring genome-wide saliva methylation using different methods. We used the data from the FELICITy cohort, which consists of 114 subjects.

Furthermore, we postulate that children affected by prenatal stress and thus epigenetic reprogramming, already show alterations of the fetal Autonomic Nervous System (ANS). This could be an approach for in-utero detection of predisposition to behavioral and developmental impairment.

3.2 Specific Objective

For the Epigenetic analysis Illumina Infinium MethylationEPIC BeadChip is used, which includes around 850000 methylation loci. We want to test the feasibility of using a biomarker panel from salivary DNA as an early and non-invasive means in the PS-exposed infants.

4. Materials

4.1 Chemicals, Supplements, Media

Name	Manufacturer
Agarose	Biozym (Oldendarf, Germany)
dNTPs	Thermo Fisher Scientific (Waltham, MA, USA)
Ethanol	Merck (Darmstadt, Germany)
Magnesium chloride (25mM)	Qiagen (Hilden, Germany)
Midori green dye	Nippon Genetics
Boric acid	Carl Roth (Karlsruhe, Germany)
EDTA	Merck (Darmstadt, Germany)
Tris	Merck (Darmstadt, Germany)
DNA ladder: EcoRI + HindIII Marker	Thermo Fisher Scientific (Waltham, MA, USA)
Primer 1	Metabion (Planegg, Germany)
Primer 2	Metabion (Planegg, Germany)
Taq-Polymerase	Qiagen (Hilden, Germany)
Buffer for PCR (10x)	Qiagen (Hilden, Germany)

4.2 Laboratory equipment

Name	Manufacturer
Nanodrop ND 8000 spectrophotometer	Peqlab
Illumina microarray sequencer	Illumina (San Diego, CA, USA)
Gel electrophoresis chamber	Biozym
Thermomixer comfort	Eppendorf
Thermomixer compact	Eppendorf
96-well plate	Thermo Fisher Scientific (Waltham, MA, USA)
microcentrifuge tube	Eppendorf
Gel documentation system Felix 2000	Biostep
Illumina iScan reader	Illumina (San Diego, CA, USA)
Falcon tubes 15 ml/ 50 ml	BD
Micropipettes	Mettler Toledo (Gießen, Germany)
Centrifuge Universal 32R	Hettich Centrifuges
Multi Tube Vortexer DVX-2500	VWR

Centrifuge Typ 3500	Hettich Centrifuges
Gel electrophoresis device: Bio-Rad Power Pac 300/3000	BIO-RAD Laboratories
Heat block	SciGene (Sunnyval, CA, USA)
PCR cycler: Thermal Cycler C1000™	BIO-RAD Laboratories, Munich, Germany
Vortex mixer	Velp Scientifica (Bohemia, NY, USA)

4.3 Kits

Name	**Manufacturer**
PrepIT kit	DNA Genotek
EZ-96 DNA Methylation TM Kit	Zymo Research
Infinium HumanMethylation850 BeadChip.	Illumina
Oracollect-DNA kit	DNA Genotek

4.4 Online tools, and databases

Name	**URL**
NCBI (National center of Biotechnology Information)	http://www.ncbi.nlm.nih.gov/
UCSC genome browser	http://genome.ucsc.edu/
R	http://www.r-project.org/
Ingenuity Pathway analysis	http://www.ingenuity.com/products/ipa
SAFRI	https://gene.sfari.org/
STRING-DB	https://string-db.org/
Image-CpG	http://han-lab.org/methylation/default/imageCpG
Scopus	www.scopus.com
Gene ontology	geneontology.org/
Pubmed	pubmed.ncbi.nlm.nih.gov/
R	www.r-project.org

4.5 Software

Computer software
Microsoft office 2015
R software (R foundation)
Genome Studio
NanoDrop version 3.1.0 NanoDrop Technologies

4.6 Gel and Buffer compositions

TBE 5X	54 g Tris Base 27.5 g boric acid 20 ml 0.5 M EDTA pH 8.0 add 1 L H2O, final pH ~8.3
6X Blue Juice gel loading dye for DNA gel electrophoresis	30% Ficoll 0.5 M EDTA 0.025% bromophenol blue
TE Buffer	Tris/HCL EDTA

5. Methods

5.1 Study population

The FELICITy study is a longitudinal, prospective matched control study that was designed to identify epigenetic and stress markers at the newborn stage and at two years old of infants.

This study was conducted at the Department of Obstetrics and Gynecology at the Klinikum rechts der Isar of the Technische Universität München (TUM), a tertiary center of Perinatology of high complexity located in Munich, Germany, that serves about 2000 mothers/newborns per year. TUM obstetricians had identified prospective subjects according to the inclusion criteria of the study, consisting of singleton pregnant women between 18 to 45 years of age in their third trimester (at least 28 weeks gestation). Exclusion criteria is detailed in Table 1. Over a period of two years, 164 women were recruited. Figure 3 displays the recruitment flowchart.

Table 1: Exclusion criteria.

Exclusion criteria of the FELICITy study
• serious placental alterations as fetal growth restriction according to (Gordijn *et al.*, 2016)
• fetal malformations

- maternal severe illness during pregnancy (i.e., preclampsia) (American College of Obstetricians and Gynecologists and the Society for Maternal-Fetal Medicine, Kilpatrick and Ecker, 2016)

- maternal drug or alcohol abuse

- preterm birth (less than 37 weeks)

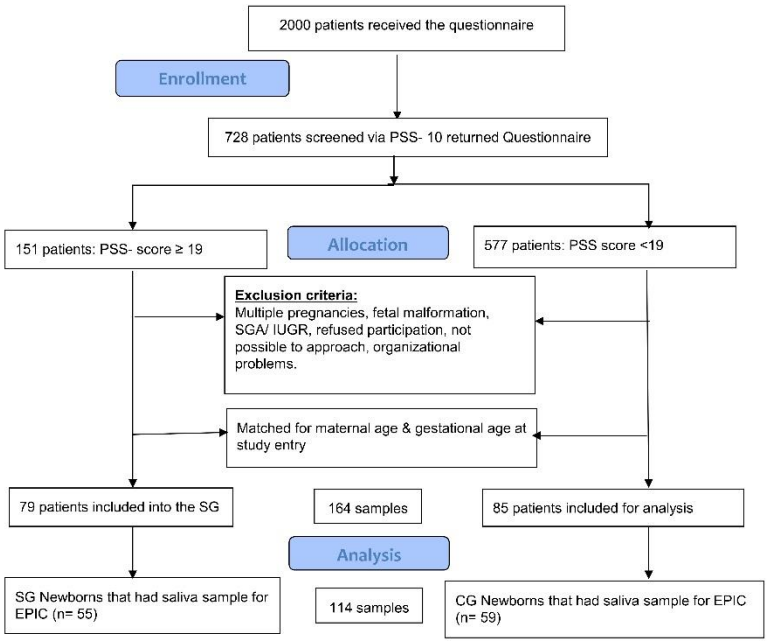


Figure 3: Recruitment flowchart: Schematic flowchart of the population recruitment and selection for the FELICITy study.

This clinical study was started in July 2016 and was completed in July 2019. Prospective women were referred to attend an informational session, where the procedures were explained, formal enrolment was completed and the consent forms from the participants themselves and parental consent for their infants was also obtained. Figure 4 shows the timeline and the study design of the FELICITy cohort.

At the initial stage demographic information was collected from the consented women. Pregnant women were screened for stress exposure using Cohen Perceived stress scale (PSS- 10) and were classified into stressed (SG, PSS-10 ≥ 19, n = 79) and control group (CG, PSS-10 < 19, n =85) matched 1:1 for parity, maternal age, and gestational age. Prenatal Distress Questionnaire was also administered to assess specific pregnancy worries. Maternal and fetal ECGs were recorded to derive coupling between maternal and fetal heart rates resulting in a Fetal Stress Index (FSI). Upon delivery, maternal hair strands were collected for cortisol measurements and newborn's saliva samples were collected.

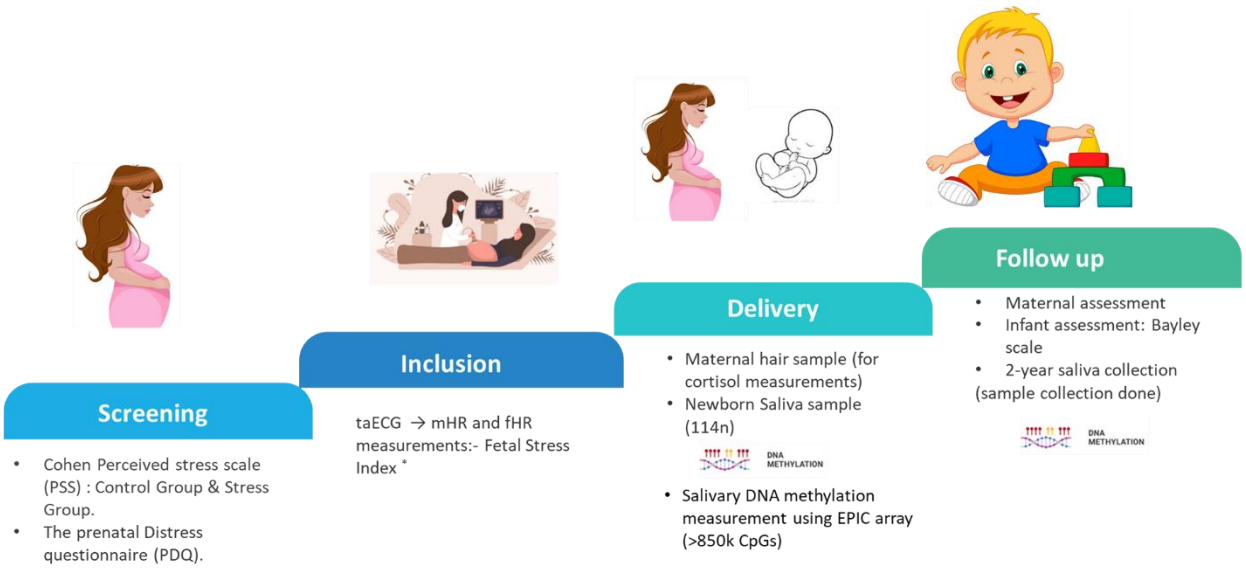


Figure 4: Study design and timeline of FELICITy study.

The follow up or the fourth part of the study is in process and therefore not included in this thesis.

The study in this thesis is a cross- sectional study approach.

5.1.1 Ethics statement

The study protocol is in strict accordance with the Committee of Ethical Principles for Medical Research from TUM and has the approval of the “Ethikkommission der Fakultät für Medizin der Technische Universität München” (registration number 151/16S). ClinicalTrials.gov registration number is NCT03389178. Written informed consent was received from participants prior to inclusion in the study.

Participant data is stored coded and confidential, with only authorized staff having access to the obtained information (data privacy law).

5.2 Phenotype Assessment

Exposure: Maternal stress during pregnancy

Stress can be assessed using general self-report instruments designed for pregnant women and these maybe more predictive of the perinatal outcome than generic stress inventories and able to assess pregnancy-related stress. We used psychosocial stress assessment instrument (PSS and PDQ) and measured stress as chronically accumulated cortisol using maternal hair samples.

5.2.1 Psychosocial stress assessment

Maternal psychosocial stress was measured using Cohen Perceived Stress Scale (PSS-10) (Cohen, Kamarck and Mermelstein, 1983). PSS-10 is a widely used psychological instrument to measure non-specific perceived stress and measures the degree to which a situation in a person´s life is appraised as stressful. It has been validated in German speaking populations and is a quick tool for screening stress among prospective subjects (Klein *et al.*, 2016). The PSS-10 predicts objective biological markers of stress and increased risk for disease among people with higher perceived stress levels. PSS-10 measured high maternal stress was

associated with temperamental variation of young infants and may show a risk for psychopathology later in life (Huizink *et al.*, 2002).

5.2.2 Prenatal distress questionnaire

Prenatal Distress Questionnaire (PDQ) was also administered to the participants to assess specific worries and concerns related to pregnancy (Alderdice and Lynn, 2011; Caparros-Gonzalez *et al.*, 2019; Lobmaier *et al.*, 2020). PSS score and PDQ score were correlated using the Spearman method in R studio (version 4.1.0). R2 is mentioned in the results section.

5.2.3 Hair cortisol assessment

On the day of parturition, maternal hair strands (~ 3mm diameter) were collected form the posterior vertex region on the head as close to the scalp as possible. The hair samples were sent to the Department of Biochemistry (Endocrinology section) of the Faculty of Pharmacy and Biochemistry (University of Buenos Aires, Argentina) for cortisol measurement using an automated chemiluminescent immunoassay. This method was validated and putative confounders such as dye, washing or dandruff shampoo were shown to not interfere with cortisol measurements. Based on the hair growth rate 1cm per month, the 3 cm long hair segment reflects the integrated hormone secretion over the three-month period prior to sampling. For analysis, fifty milligrams of hair from 3 cm closest to roots (equivalent to 3 months of growth), were weighed in an analytical balance as per Society of Hair testing (Cooper *et al.*, 2012). The cortisol was extracted and measured according to Iglesias et al. (Iglesias *et al.*, 2015). This procedure has been validated with the standard method of mass spectrometry and was patented by University of Buenos Aires (Gonzalez *et al.*, 2019).

5.2.4 Fetal Stress Assessment

The detailed fetal assessment is described elsewhere (Lobmaier *et al.*, 2020). In brief, bivariate PRSA (bPRSA) was used to assess the coupling between maternal (MHR) and fetal heart rate (MHR) resulting in Fetal Stress Index: FSI. Bivariate phase-rectified signal averaging allows for identifying and quantifying relationships between two synchronous recorded signals. These two signals in this study are maternal heart rate (MHR) the trigger signal and fetal heart rate (FHR) the target signal.

For the ANS assessment, the FHR was measured by transabdominal electrocardiography (taECG) recording at 900Hz sampling rate for at least 40 min duration using AN24 (GE HC/Monica healthcare, Nottingham Uk). Fetal ECG extraction algorithm SAVER (Li, Frasch and Wu, 2017) was applied to detect the fetal R-peaks and the maternal R-peaks in the taECG separately. With the fetal and maternal R peaks, the fetal and maternal RR interval time series were obtained. Mean FHR and mean maternal heart rate were calculated. Generally, bPRSA identifies and quantifies the relationship between two simultaneously recorded signals. As FSI measures the response of FHR to MHR decreases, it was calculated using MHR and FHR.

5.3 Saliva Sample processing for EPIC Chip

Outcome: DNAm measurement from newborn saliva

Immediately after delivery a study nurse obtained the newborn saliva/buccal sample by gently rubbing the gums on both sides with the sponge of the Oracollect-DNA kit (DNA Genotek, Canada). Samples were stored room temperature. Throughout the thesis and for ease of reading we have referred as “saliva” to the sample containing both the saliva fluid plus leukocytes and squamous epithelial cells from the oral cavity (Theda *et al.*, 2018). Also, at delivery newborn recording were taken and recorded such as including birth weight, length and head circumference, pH, and Apgar score.

5.3.1 DNA extraction

DNA was extracted from 1ml saliva samples using the PrepIT kit (DNA Genotek).

The sample was mixed by inversion and gentle shaking for few seconds. Afterwards, it was incubated at 50°C in an air incubator for a minimum of 2 hours. 500µl of sample was transferred into a microcentrifuge tube. 20µl of PT-L2P was added and mixed well for few seconds. The samples were incubated on ice for 10 minutes and then centrifuged at room temperature (RT) for 7 minutes at 15,000xg. The clear supernatant was transferred to a new microcentrifuge tube, while the pellet was discarded. 600µl of room temperature ethanol (95% to 100%) was added to the clear supernatant and gently mixed by inverting 10 times. DNA was precipitated by letting the sample stand at RT for 10 minutes followed by centrifugation at RT for three minutes at 15,000xg. In the next step the supernatant was discarded without disturbing the pellet and incubated for 1 minute at RT after adding 250µl of 70% ethanol. The ethanol was removed and again 50µl of TEC (TRIS/EDTA/Citrate) solution was added and then mixed for a minimum of 5 seconds. The samples were incubated at RT overnight and finally stored.

5.3.2 Quality control of saliva samples for Bisulphite conversión

The bisulfite conversion and the following EPIC experiments require DNA of high quality. After DNA extraction, DNA quality was therefore assessed via agarose gel electrophoresis. DNA quantification was determined, as well as amelogenin polymerase chain reaction (PCR) was performed.

5.3.2.1 Determination of DNA concentration

DNA concentration was determined by Nanodrop assay kit, based on spectrophotometry measuring reflection or transmission of material. DNA has a maximum absorption of UV light at 260 nm, whereas proteins have their maximum at 280 nm. Therefore, protein contamination

in DNA samples can be estimated due to the A260 nm/A280 nm ratio. For values between 1.7 and 2.0 protein contamination is excluded to the greatest possible extent. In addition, values > 1.5 with salts and phenol contamination were excluded. DNA concentration and potential contamination were measured with the 8-channel Nanodrop system following manufacturer`s instruction. 1µl of DNA was measured on the nanodrop and TE (Tris/EDTA) buffer was used as a blank.

5.3.2.2 Agarose gel electrophoresis

To assess the integrity of the DNA, Agarose gel electrophoresis was performed after DNA extraction. To determine fragmentation and evaluate the quality of DNA, DNA fragments were separated by their size and then compared with commercially available standards with known fragment sizes.

Gel for checking DNA integrity was prepared with 0.8% agarose gel dissolved in 1x tris-borate-EDTA (TBE) buffer in the microwave for 30 seconds. After agarose dissolved in TBE buffer, 2.5 µl/100 ml Midori Green was added and mixed very well. Midori green intercalates with the DNA and can be visualized under UV light. The agarose gel was then poured into the plates with the combs attached. The gel was allowed to sit for 30 mins to solidify before removing the combs and adding the samples.

After the gel was solidified, 4µl 6x Loading Dye were added to 1µl of DNA to sediment them in the gel-well and afterwards loaded on the agarose gel. In addition, control samples containing 50ng, 100ng, and 200ng of DNA were added to the wells of the gel. After adding all the samples and controls, 2µl Lambda DNA/EcoRI + HindIII Marker 3 were loaded to the agarose gel. The DNA fragments were separated under a constant electrical field of 120V for 90 min. After separation, the DNA fragments were visualized and photographed using the Gel documentation system Felix 2000. Based on the properties of the agarose gel shorter fragments move faster than longer ones. Samples with high degradation were excluded.

5.3.2.3 Amelogenin test for sex determination

The gender was determined using a PCR amplifying the amelogenin gene, which encodes for a gene responsible for biomineralization during tooth enamel development (Eng, Ainsworth and Waye, 1994). Two different forms exist on X and Y-chromosomes, so that women have two copies on the X-chromosome and therefore, one fragment appears on the agarose gel, while men have one copy on the X and Y-chromosome each and therefore, two fragments with different sizes appear on the gel. The primer sequence and length are shown in Table 2. A 2µl aliquot of DNA extract from the samples was used to run PCR to check for the sex of the newborn.

Table 2: Primer length of the fragment

5'-CTGATGGTTGGCCTCAAGCCTGTG-3' X	977 bp
5'-TAAAGAGATTCATTAACTTGACTG-3' Y	788 bp

Table 3: Pipetting scheme for master mix for one 96-well plate (20 µl reaction)

10x Buffer	200 µl
dNTPs mix (25 mM)	16 µl
Primer rev (100 pmol)	5 µl
Primer for (100 pmol)	5 µl
MgCl2 (25 mM)	120 µl

Taq-Polymerase	20 µl
H2O	1534 µl

Table 3 shows the master mix for the PCR reaction. 19 µl PCR master mix were added to 1 µl DNA, vortexed, and centrifuged at 1500g for 20 seconds. Table 4 shows the PCR program that was used:

Table 4: Cycling protocol for amelogenin PCR

Temperature	Duration	No. of cycles
95°C	15min	1
95°C	30sec	40
62°C	30sec	
72°C	1min	
72°C	10min	1
20°C	1min	1

Afterwards the PCR product was loaded on a 1.5% agarose gel, diluted at 1 x TBE puffer, to check the bands and furthermore the gender. The gel runs at 90 V for 90 min.

5.3.3 Bisulphite conversión

For bisulphite conversion EZ-96 DNA Methylation TM Kit (Zymo Research) was used.

5.3.3.1 Principle

Bisulphite conversion is a process in which the genomic DNA is denatured (separated into single strands) and unmethylated cytosine are converted to uracil and afterwards into thymine using sodium bisulphite. As seen in Figure 5, during the whole genome amplification process, unmethylated cytosines will be converted into uracil and afterwards into thymine, while 5-methylcytosine (5-mc) or the methylated cytosines remains intact and protected. This allows researchers to differentiate between methylated and unmethylated cytosine residues. (Kint *et al.*, 2018).

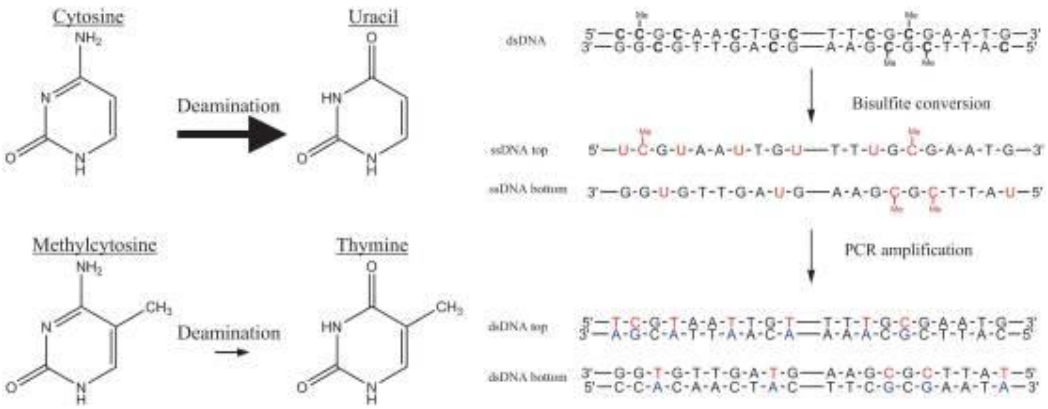


Figure 5: Principle of bisulphite conversion. Source: - (Kint *et al.*, 2018)

5.3.3.2 Laboratory procedure for Bisulphite conversion

For almost all samples, 500ng of the extracted salivary DNA was treated with sodium bisulphite using the EZ –DNA methylation kit following the manufacturer's protocol. For samples with bad DNA quality and low DNA concentration 200g of salivary DNA was used.

Initially, bisulphite conversion reagent was diluted in 7.5 ml water and 2.1 ml dilution buffer and vortexed thoroughly for 10 min at room temperature (RT) till it was completely solved. As the reagent is light sensitive all the steps up till clean-up were performed in the dark. Furthermore, 144 ml 100% ethanol were added to the wash buffer concentrate. For bisulphite conversion 500ng of DNA was suspended in 45µl water. 5µl dilution buffer was added and mixed by pipetting up and down. The conversion plate was incubated at 37°C for 15 min and afterwards centrifuged. 100 µl of conversion reagent was added to each sample, mixed, centrifuged, and incubated at 50°C for 16-20h for the following genome-wide DNA methylation analysis using Infinium HumanMethylation850 BeadChip.

After (overnight) incubation, the samples were immediately incubated for 10 min on ice to stop bisulphite conversion. 400µl binding buffer were pipetted in the binding plate placed on the collection plate. The converted samples were loaded completely in the well and mixed by pipetting up and down till no smears remained visible. Afterwards the plates were centrifuged for 3 min at 4,500 RCF (≥ 3,000 x g), turned around 180°C, and centrifuged again for 3 min, to ensure that the wells of the binding plate are dry. The flow-through was discarded, 500µl wash buffer were added and centrifuged again as described before. 200µl desulphonation buffer were pipetted to the samples and incubated for 15-20 min at RT. Afterwards the plate was again centrifuged as described above, 500µl wash buffer were added, centrifuged, and another 500µl wash buffer were added. Samples were centrifuged now for 6 min as described previously. The binding plate was placed onto an elution plate and 15µl elution buffer were added directly on the filter without touching it. Elution took place using elution buffer. The samples were incubated for 3 min at RT and afterwards centrifuged for 3 min and finally an additional 2 min after turning the plate. Water as well as elution buffer were warmed to 50°C in the heat oven during the desulphonation to ensure an optimum elution rate as DNA solves better in warm solutions. Converted DNA was immediately used for upstream analysis. Additionally, the converted DNA can be stored for short-term at -20°C, but it should not be older than 6 months.

5.4 EPIC chip

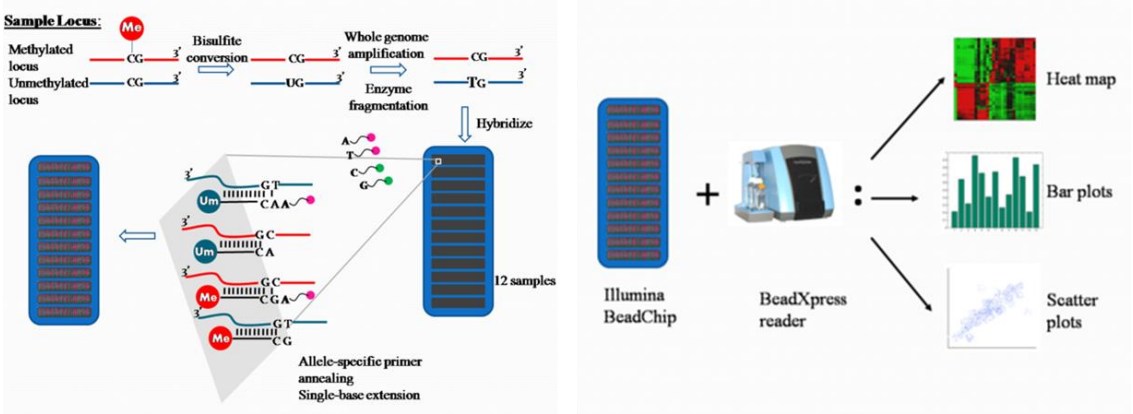
In this section the Illumina platform and the principle behind it are discussed.

5.4.1 Principle

The EPIC DNA methylation interrogates the methylation status of over 850,000 CpG sites. Probes were designed covering multiple sub-regions as defined by Illumina; promoter regions at 200bps and 1500bps blocks upstream of the transcription start site (annotated to TSS200 and TSS1500 resp) as well as the 5' and 3' UTR, first exon and gene body regions are targeted by probes. The BeadChip includes probe types of two different chemistries: (1) Type I probes, in which two different probe types interrogate each CpG site, one for each methylated and unmethylated CpGs. (2) Type II probes bind to the nucleotide just before the target site, and create a single base extension of G or A complementary to the methylated C or unmethylated T. There is a higher proportion of Type II probes on 850K, which reflects the increased coverage of distal regulatory elements, and also take up less physical space on the BeadChip, maximizing the probe number on the 850K BeadChip (Pidsley *et al.*, 2016).

Figure 6: Workflow of Infinium beadchip array. (Source: (Weisenberger *et al.*, 2008)*)*

Figure 6 shows the workflow of the Infinium beadchip array. The Infinium platform uses the bead technology for measurement of DNA methylation at individual CpG loci on the human genome. Individual beads hold oligos of 23 base addresses, to permit identification of their



physical location on the BeadChip. 50bp Probe sequences are also designed complimentary to specific 50 base regions of bisulphite converted genomic DNA with a CpG site at of 3' end of the probe. After hybridization to the bisulphite converted sample DNA, single base extension of the probe adds a fluorescently labelled ddNTPs at the 3'CpG site to allow 'genotyping' of the C/T conversion that results from bisulphite conversion. Then the fluorescent signal is measured. The proportion of DNA methylation at a particular CpG site is given as the methylation beta (β) value (Bibikova *et al.*, 2009).

5.4.2 Laboratory procedure for 850k

DNAm was assessed using the Illumina Infinium Human MethylationEPIC BeadChip array (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. This array measures over 850,000 loci at a single nucleotide resolution. Samples were allocated on the plates and chip in pairwise order (stress and control) and run together.

The following steps describe the day 2 procedure -transfer of bisulphite converted DNA to MSA5 plate.

Amplification of bisulphite converted DNA: -

On the second day, MA1 (Multi sample amplification 1 mix), RPM (random primer mix) and MSM (multiple-sample amplification master mix) were thawed at room temperature (RT). The hybridization oven was preheated at 37°C.

To 4 µl of sample in the MSA5 plate, 20 µl of MA1 were added, followed by 4 µl of 0.1 NaOH and then sealed using a cap mat. The plate Is vortexed for 1 min at 1600 rpm and was pulsed centrifuged at 280*g. The samples were incubated for 10 min at RT, as it is important to prevent DNA degradation by NaOH. 68 µl RPM and 75 µl of MSM was added to the MSA5 plate, then resealed and was vortexed again at 1600 rpm for 1 minute. It was finally pulsed centrifuged at 280xg and incubated in the Illumina hybridization oven for 20-24 hours at 37°C.

Fragmentation

Continuing day three, FMS (fragmentation solution) tubes were thawed at RT and heat block was preheated at 37°C.

The MSA5 plate was removed from the hybridization oven and centrifuged at 280xg. 50ul of FMS was added to the samples, sealed with the cap mat and vortexed at 1600 rpm for 1 min followed by centrifugation at 280xg, and finally incubating for 1h on the heat block at 37°C.

Precipitation

100 µl of PM1 (Precipitation solution- thawed at RT) was added to the sample plates followed by vortexing at 1600 rpm for a minute, and then was incubated for 5 min at 37°C before finally centrifuging at 280xg. 300 µl Isopropanol was added to the plate, sealed with a new and dry cap mat, and inverted 10 times to mix. The plate was incubated for 30 min at 4°C and then centrifuged at 4°C for 20 minutes. The supernatant was discarded (after checking for the pellet) by inverting the plate quickly. It was tapped several times for a minute on an absorbent pad until all wells were completely drained. Finally, the plate was dried upside down on a tube rack for 1hour at RT.

Resuspension (Post-AMP)

RA1 (resuspension, hybridization, and wash solution) was thawed completely (solution has to re-dissolved). The hybridization oven was preheated at 48°C and the heat sealer was switched on.

46 µl RA1 was added to the plate and sealed with an aluminium seal using a heat sealer and incubated for at 48°C for 1h in the hybridization oven, followed by vertexing at 1,800 rpm for 1 min and finally centrifugation at 280xg for 1 min.

Hybridization

12 resuspended samples can be hybridized on one BeadChip and therefore up to 8 BeadChips are used for one plate.

Samples were denatured for 20 min at 95°C using a heat block (preheated beforehand), to enable the annealing of single-stranded samples to locus-specific 50mers, which are linked to one of the around 850,000 CpG sites detectable with the Infinium HumanMethylationEPIC BeadChip. During this incubation step the Hyb Chambers were prepared. BeadChip Hyb Chamber gaskets were placed into the Hyb Chambers. 400 µl PB2 (humidifying buffer used during hybridization) were suspended into the humidifying buffer reservoirs in the Hyb Chamber. The lid was placed right away on the Hyb Chambers to prevent evaporation. After 20 min incubation the plate was cooled down to RT in 30 min and centrifuged at 280xg. BeadChips were removed from the zipper bags and mylar packages and were placed on the Robot BeadChip Alignments Fixtures with the barcode end aligned to the ridges on the fixture. At the robot PC MSA5 tasks | hyb-multi BC2 was selected and the right kind and number of BeadChips were chosen. Robot BeadChip Alignments Fixtures with Robot Tip Alignment Guide were placed onto the robot as well as the sample-plate. 15 µl sample volume were pipetted automatically on the BeadChips. Afterwards the BeadChips were removed from the robot and placed in a Hyb Chamber insert, orienting the barcode at the end (it has to match the barcode symbol to the Hyb Chamber insert). Hyb Chamber inserts were placed together with the BeadChip inside the Hyb Chambers. The closed Hyb chambers were incubated for 16-20 h at 48°C in the hybridization oven using the rocker.

Washing Beadchip

On the next day, the wash rack with the beadchip was washed in the wash dish containing 200ml of PB1(reagent used to prepare BeadChip for hybridisation). The BeadChip was securely ensured in the wash rack and submerged completely. The wash rack is moved up and down for 1 minute. This step is repeated for all remaining BeadChips.

Extension and Staining of the BeadChip

XC4 (Xstain BeadChip solution 4) was prepared by adding 330 ml 100% ethanol. XC1, XC2 (Xstain BeadChip solution 1 and 2), STM (superior two-color master mix), ATM (anti-stain two-color master mix) and TEM (two-color extension master mix) were thawed completely. Formamide was prepared by mixing 1.2 ml water, 23.75 ml formamide, and 50 µl EDTA).

XC1, XC2, STM, ATM, 25 ml formamide, 30 ml RA1, and 145 ml XC3 (Xstain BeadChip solution 3) were loaded at the robot. Last three were placed in a bowl. The program Xstain Task was selected and the thermo block was preheated at 32°C. 150 ml PB1 (reagent used to prepare BeadChip for hybridization) were filled in two washing dishes with washing racks. The cover seal was removed from each BeadChip and they were immediately placed in the washing rack submerged with PB1. Afterwards the full washing rack was moved up and down for 1 min. It was moved into the second washing dish with clean PB1 and the washing step was repeated.

BeadChips were loaded on the multi-sample BeadChip alignment fixture with 150 ml PB1 and black frames. The barcode must align its barcode with the ridges stamped onto the alignment fixture. Afterwards a clear spacer was placed on top of each BeadChip and the alignment bar was placed onto the alignment fixture. A clean glass back plate was put on top of the clear spacer on each BeadChip. The plate reservoir should be at the barcode end of the BeadChip. Metal clamps were attached to the flow-through chambers and end of clear spacers were trimmed using scissors. Final flow-through chambers were loaded on the robot. When the thermo block reached 44°C, the robot was started using the program X stain Tasks | Xstain HD Bead Chip.

A plastic shell was filled with PB1 and BeadChips (without metal clamps, glass back plate, clear spacer and black frames) were placed on a staining rack. BeadChips were moved up and down 10 times and afterwards soaked for 5 min. XC4 was filled in a second plastic shell

and the washing rack was put into it. It was moved up and down 10 times and soaked for 5 min. The staining rack was removed in a smooth, rapid motion and placed directly on a tube rack with the barcodes faced up. Each chip was placed on a rack with the barcode facing up, which was placed in a vacuum desiccator applying vacuum pressure. BeadChips were dried for around 1 h and afterwards the bottom side was cleaned with ethanol to remove any XC4 excess.

Image BeadChip

BeadChips were scanned using the Illumina iScan, which is a two-channel high-resolution laser imager, scanning BeadChips at two wavelengths creating an image file for each channel. Afterwards the intensity values for each bead type were determined via Genome Studio software (Illumina, Inc.) and a data file was created.

5.4.3 Genome studio

The raw Illumina Intensity data (idat files) were used to summarize the data from EPIC beadchip array. For each sample a “Red” and a “green” idat file were available, that presented intensities of the methylated and unmethylated probes. In addition, a manifest file is also available for EPIC array which includes a description of the probes such as probe IDs, chromosome, location, relation to the epigenetically relevant features.

GenomeStudio methylation module was used to analyse the methylome-wide data from the data files obtained by the BeadArray Reader in combination with the Infinium HumanMethylation850 manifest file, including information of annotation of CpG sites.

Initial quality control was done in GenomeStudio using the “Control Dashboard” in the software. This includes the assessment of staining, extension, hybridization, target removal, bisulphite conversion I and II, specificity, and negative control. β-values are continuous variables

between 0 and 1 that represent the methylation degree. They were exported and used for statistical analysis explained further in section 5.5.2.

5.5 Illumina methylation EPIC Beadchip chip array processing

5.5.1 Principle

As also described in the introduction section, for each CpG, there are two measurements: a methylated intensity (M) and an unmethylated intensity (U). These intensity values can be used to determine the proportion of methylation at each CpG locus. As mentioned before (Section:1.4.2), methylation values are either reported as beta values or M-values related via logit transformation.

$$\beta = \frac{M}{M + U}$$

$$M\ value = log2\ \frac{M}{U}$$

Generally, beta values are preferred for describing the level of methylation at a locus or for graphical presentation, as the percentage methylation is easily interpretable. However, for statistical reasons and distributional properties, M values are more appropriate for statistical testing (Du *et al.*, 2010). A normalized M-value near 0, shows a semimethylated locus, a positive M value indicates that more molecules are methylated than unmethylated, whereas negative have a opposite interpretation (Du *et al.*, 2010). But in practice it has been observed that choosing to transform or not does not seriously affect the analysis results (Bell *et al.*, 2011).

This section further outlines DNA methylation data analysis including pre-processing (after Genome Studio), quality control, normalization, and statistical methods for association analysis as well as Pathway analysis used for the FELICITy study

Figure 7.

Figure 7: Methylation array steps for data processing and analysis pipeline used for the FELICITy study.

5.5.2 Quality control of methylation data

The basic analyses were done using the R statistical computing environment.

The raw Intensity data (idat files) were imported into R using the Bioconductor minfi package (Aryee *et al.*, 2014). The Bioconductor R package minfi was used for quality control of methylation data including read outs, normalization, ß- and M- value calculation.

Failed probes were excluded based on detection p-value larger 0.01 in >50% of the samples. X chromosome, Y chromosome, and no-specific binding probes were removed. The remaining

probes were background corrected using the out of bound probes. Further failed samples due to other processing issues were checked for and removed (Chen *et al.*, 2013).

Illumina BeadChip platform is mainly a genotyping technology, and if a SNP is present at or near a CpG site the methylation value may be capturing a SNP and not the CpG methylation. As SNPs closer to CpG methylation are more influential and therefore these SNPs need to be removed. Therefore, probes with common single nucleotide polymorphisms (SNPs) near the methylation binding site were identified and filtered out.

Gender mismatch and outlier were also checked using multi-dimensional scaling plots. Males and females were in distinct separate clusters. Outliers, i.e., samples whose behaviour deviated from that of others, were excluded from the analysis. Concordance between the reported sex and methylation predicted sex was confirmed.

5.5.3 Normalization of methylation data

Normalization helps to remove technical and systematic variability from the data so to make measurements comparable across all samples. As discussed in section 5.4.1 that EPIC methylation array has two probe design types (Type I and Type II) which differ through which they quantify methylation levels (Maksimovic, Gordon and Oshlack, 2012). Therefore, there are many normalization procedures in the Illumina context that work on reducing differences between different probes (Marabita *et al.*, 2013; Wu *et al.*, 2014). We used the functional normalization procedure proposed by Fortin et al, 2014 (Fortin *et al.*, 2014) an extension to quantile normalization. They show that this normalization procedure outperforms all the existing normalization methods (Fortin *et al.*, 2014).

The data were normalized using a functional normalization procedure, an extension of quantile normalization included in the R package minfi, which also uses two principal components of a set of control probes to remove technical variability. Batch effects were identified by inspecting

the association of principal components of the methylation levels with possible technical batches using linear regressions and visual inspection of PCA plots using the Bioconductor R package shinyMethyl (version 0.99.3).

The β-values (proportion of methylated probes at each CpG) were then converted to M-values (logit base 2 of the β-values), which were used for all linear modelling. The final analysis included 808,554 probes.

5.5.4 Confounders

There are several other additional factors that influence data analysis which are not related to the scientific question that we want to answer, but rather due to the methodology itself, that introduces unwanted variability to the data. These additional factors, known as confounders. Confounders are variables that are associated or has a relationship with both the exposure and the outcome of interest Figure 8. Confounders can be classified in two main categories, technical confounders, and non-technical confounders.

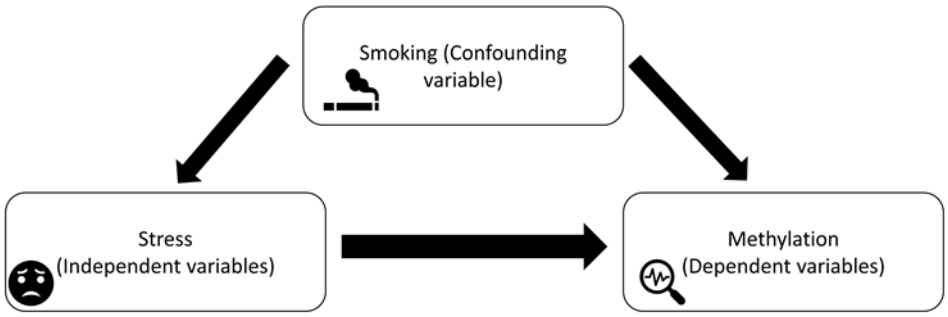


Figure 8: Illustration showing confounding and relationship between independent and dependent variables.

5.5.4.1 Technical Confounders

Batch effects are non-biological experimental variations commonly observed in multiple groups of samples (batches) in high-throughput experiments e.g., microarray experiments, that can 'confound' the results by adding variation to the data, thus decreasing the statistical power to detect biological phenomena.

To adjust for technical variation, we used Surrogate variable analysis (SVA) and Illumina plate factor correction.

There tend to be sources of signal due to factors that are unknown, unmeasured, or too complicated to capture through simple models and this is true even for well-designed, randomized studies (Leek and Storey, 2007).

Surrogate variables are covariates constructed directly from high-dimensional data (like gene expression/RNA sequencing/methylation/brain imaging data) that can be used in subsequent analyses to overcome these problems by adjusting for unknown, unmodeled, or latent sources of noise (Jaffe *et al.*, 2016). Methylation is known to be sensitive to many factors such as age, gender, and environmental exposures. Therefore, SVA can correct for any sources of un-modelled variability and confounding factors (Leek *et al.*, 2010, 2012).

It should also be noted that the cellular DNA source of saliva is heterogeneous. Adult saliva samples contain epithelial cells, leukocytes and bacteria, therefore future studies should estimate the proportion of epithelial cells in saliva samples to control for the variation. While there are many studies that have estimated saliva cell type composition in infants, children, and adults, to date, there are no studies indicating the cell-mixture composition of the saliva of the newborn. Housemann et al, in had introduced the reference-free cell type method to estimate cell types in tissues such as saliva, placenta and adipose tissue, which is closely related to surrogate variable analysis (SVA)(Houseman, Molitor and Marsit, 2014). We have therefore used SVA directly to estimate for all the unobserved variability including cell types. It

has been shown that using SVA increases the biological accuracy and reproducibility by identifying the sources of heterogeneity and correctly accounting for them in the analysis (Leek *et al.*, 2012).

Models were adjusted with three surrogate variables (SV) that were generated from the M-values, using the Bioconductor 'SVA' package. The surrogate variables were used as covariates in the statistical analysis.

5.5.4.2 Non- technical confounders

Furthermore, we included non-technical or biological confounders such as sex of the newborn, gestational age at birth, maternal smoking, autoimmune diseases, and gestational diabetes. The sex of the newborn was obtained from the clinical history of the patient as well as confirmed via experiment using the Amelogenin PCR (Section 3.3). Gestational age was calculated from the first day of the woman's last menstrual cycle to the date of delivery. Maternal smoking was categorized into two categories: 'never smoked' and 'smoking during pregnancy'. Covariates autoimmune diseases and gestational diabetes were categorized into two categories: 'Yes' and 'No'.

5.5.5 Control for Multiple testing

Determining the correct p-value threshold for statistical significance is critical to differentiate true positives from false positives and false negatives. Since we are performing thousands of hypothesis tests, we need to adjust the p-values for multiple testing. To determine the statistical significance in our study two different statistical procedures were used chronologically, accounting for multiple testing.

First, the Bonferroni correction method, which is the most conservative method for selecting p-values due to the assumption that every CpG tested is independent of the rest. This method is used to correct for the inflation of type I error (the probability of obtaining false-positives

associations) when multiple tests are performed. It is estimated by dividing the significance threshold (p=0.05) by the number of tests performed. The Bonferroni threshold for the DMP analysis is 6E-08.

Second the False discovery rate is a popular but less conservative approach, that controls the expected proportion of false positives among the rejected null hypothesis (Benjamini and Hochberg, 1995). It represents the expected proportion of false-positive associations among the associations declared as statistically significant. The FDR threshold is often set to 0.05 or 0.1, which means 5% or 10% of the significant results are assumed to be false. In our study probes were considered significantly differentially methylated at a false discovery rate (FDR) < 0.05. FDR- set thresholds may be suited for exploratory or a hypothesis generating study.

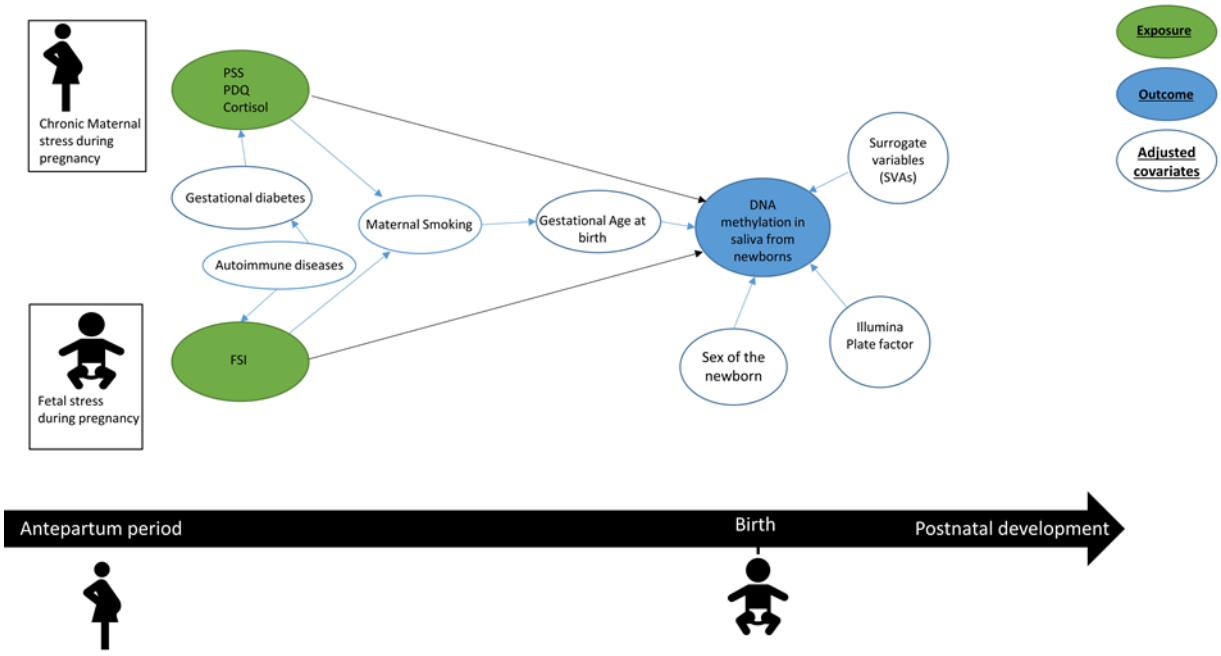


Figure 9: Direct acyclic graph (DAG) displaying the hypothesized associations between maternal and fetal stress and infant salivatory DNA methylation.

5.6 Statistical Analysis

The statistical association between methylation levels (M values) and phenotype are identified either at the level of individual CpG sites (DMPs) or a broader genomic region (DMR). We have used both the approaches to identify for associations between methylation and phenotype. DMP analyses is CpG or site by site regression analysis which analyses each CpG site individually, while DMR analysis (or regional analysis), captures an average pattern of DNA methylation among neighbouring sites.

We conducted a series of analyses on the genome wide microarray data, with each technique designed to capture potentially different patterns of DNA methylation. The first analysis conducted is EWAS analysis for the DMPs (CpG or site by site regression analysis), which analyzed each CpG site individually. Second, we performed a sex-interaction EWAS analysis for the DMPs to compare methylation patterns in terms of sex. The Illumina database ('IlluminaHumanMethylationEPICanno.ilm10b4.hg19') was primarily used for identifying gene annotations for the significant hits. The University of California Santa Cruz genome browser (UCSC) was used to verify genes identified with Illumina database and, where genes were missing in the Illumina database, was searched to augment genes within 50kb of the CpG site. We also performed DNA variability analysis, candidate gene analysis and looked for regions of increased variability.

5.6.1 Differentially methylated positions (DMPs)

DMPs are CpG sites with statistically significant differences in average (usually mean) methylation levels between groups. Each CpG site or DMP was separately tested for association with exposure to stress.

Multivariate fixed-effect linear regression models were used to assess the relationship between DNA methylation and each stress phenotype (PSS; PDQ; Cortisol and FSI, section 5.2) using age, sex, plate number and surrogate variables as covariates in the following model. Figure 9 shows the proposed DAG that displays the covariates used in our analysis.

DNA methylation ~ Stress phenotype + Newborn sex + Gestational age + Smoking + Autoimmune diseases + gestational diabetes + Illumina plate factor +SVAs

Linear regression was chosen because they are generally quite robust to violations in their assumptions. All the statistical analyses were done in R version 3.5.2.

We visualized the epigenome-wide associations study results using Manhattan plots and quantile-quantile (QQ plots). Genomic inflation factor was calculated for each association. We corrected the p-values for inflation if lambda was above 1.1, using a Bayesian method for estimation of empirical null distribution as implemented in R/Bioconductor package 'bacon' (van Iterson *et al.*, 2017).

5.6.1.1 Sex interaction analysis

Sex interaction analysis was performed in the FELICITy cohort for each CpG/DMP site association with the stress measures. The model was identical to the adjusted model, but with a "Sex * methylation" interaction term (male as a reference sex). Statistical significance threshold was at FDR < 0.05.

5.6.2 Differentially methylated regions (DMRs) analysis

DMRs are genomic regions made of several continuous DMPs (Rakyan *et al.*, 2011). They are often associated with a specific region, such as CpG islands in promoter regions, but can also be intergenic regions. Compared to DMPs, DMRs may be biologically relevant and are more likely to be associated with modified gene expression because of the strong correlation among adjacent CpGs (Jaffe, Murakami, *et al.*, 2012).

The DMRs mapped to or near genes that are enriched for "the biological process of the regulation of sequence-specific DNA binding transcription factor activity" suggests that these genes are involved in regulation of gene expression. Several tools provide a programmatic approach for identifying DMRs. DMRcate is the most popular tool for DMR detection as for

2021 (Campagna *et al.*, 2021). It performs a regression of methylation level at each CpG site based on phenotype, while accounting for covariates (Peters *et al.*, 2015).

While not specific to methylation data, Comb-p (Pedersen *et al.*, 2012) is also a popular tool to identify DMRs as its performance is comparable to DMRcate (Peters *et al.*, 2015).

DMRs were initially identified using the 'the Bioconductor DMRcate package (Peters *et al.*, 2015) and verified using comb-p, which are consistently reported to have best sensitivity and high control of false positive rate when compared to other DMR tools (Peters *et al.*, 2015). A significant DMR can be detected even if there is no genome wide significant DMP in the region. DMRcate identifies DMRs from tunable kernel smoothing process of association signals.

DMRcate was used on the results of the limma analysis to test for DMRs. The parameters for DMRcate (lambda = 1000, C = 2) were set and an FDR cut-off of 0.05 was used to determine significance. Further Comb-p was used to verify DMRs identified by DMRcate. For comb-p, identified DMRs consisting of at least two probes and having a Sidak-corrected p-value <0.05 were considered statistically significant (Pedersen *et al.*, 2012). DMRs were annotated to gene symbols according to genome assembly (Hg19). P-value for each DMR was adjusted for multiple testing with Sidak correction method as implemented by default in the 'comb-p' tool.

5.6.3 Differential variability analysis (DMV)

From the previous methods of DMP and DMR analysis there is enough evidence in the literature (Irizarry *et al.*, 2008) that average level of DNA methylation is meaningful, but Feinberg and Irizarry et al (Feinberg and Irizarry, 2010) also propose that increased biological variability in methylation at specific genomic locations may also be highly relevant to common diseases. These regions are known as variably methylated regions (VMRs), regions of interindividual methylation variability within each sample genome wide, done using Differential variability analysis. VMRs are important as in developmentally important genes they identify

stochastic epigenetic variation and secondly VMRs between individuals may serve as an epigenetic signature stable for more than a decade (Feinberg and Irizarry, 2010; Jaffe, Feinberg, *et al.*, 2012). These signatures maybe correlated with the disease status.

Similar to the DMR analysis, DMV analysis was done to test differences in group variances (between stress group and control group) rather than mean methylation. A differential variance model was fit, using the Bioconductor miss methyl package, using the varFit function (Phipson and Oshlack, 2014). The goal here was to find regions where infants from stress group mothers have higher or lower variability. Same covariates were used as in the previous analysis.

5.6.4 Candidate gene-specific analyses

We interrogated DNAm in the vicinity of genes that have been previously implicated in neuronal and neurobiological disorders. A list of 85 genes were prepared by reviewing papers published previously. All probes on the EPIC array annotated to these 85 genes were selected, resulting in a total number of 984 CpGs. We corrected for multiple testing on a gene-level by applying FDR. We subsetted the methylation array data to just those CpGs that are within the promoter region of the gene (2kb upstream of the gene to 200bp downstream of the gene, inclusive).

5.6.5 Regions of Increased Variability

Additional analysis that was done is to look for regions of increased variability. This approach aims at finding regions that have higher variability across all samples as compared to other regions. This is done by first computing the ratio of the variation at a given CpG and the mean variation over all CpGs, saying a CpG is significant if the ratio for a CpG is larger than the 95th quantile of all ratios. We then searched for regions, with a minimum of 20 CpGs that have consistent increased variation.

5.7 Exploratory analysis

Pathways, gene, and disease analysis allow to draw objective means of conclusions and result interpretation. These analyses can identify complex, multi CpG-effects, biological functions between multiple genes, in which CpG have modest effects individually, but large effects when combined.

There are wide range of pathway analysis methods and to investigate possible enrichment for specific pathways, a disease association analysis was done using the publicity available databases STRING-DB, SFARI database, Ingenuity pathway analysis and Web Gestalt. Tested genes for the network analysis were annotated from CpG sites with FDR value < 0.05 in the association study (DMP and DMRs).

a) Protein-protein interaction analysis using STRING-db: -

Protein-protein interactions (PPIs) are the basis of biological function that are affected by several factors including diseases and therapeutics, and they interact with molecules such as DNA (Gonzalez and Kann, 2012). PPI are basically mathematical representations of PPIs and can be used to understand the molecular drivers of disease states or identify potential therapeutic targets (Gonzalez and Kann, 2012). There are many publicly available tools to identify PPINs (Protein-Protein Interaction networks) from a gene list, which utilize publicly available PPI data for which the reliability and level of annotation varies. Two tools are STRING-db (Szklarczyk *et al.*, 2019) and ToppGene (Chen *et al.*, 2009).

The protein encoding the genes that were annotated to significant DMps were analyzed using STRING-db. STRING-DB is a semi-unsupervised statistical network analysis database that has known proteins and their physical and functional interaction networks, which works on computational predictions. We used Kyoto Encyclopedia of Genes and Genomes (KEGG)

database in STRING-DB to explore whether annotated genes have been related to neurobiological and neuronal processes or diseases.

b) SFARI database

We also used **SFARI database** to extract information for the genes annotated to CpGs specific to autism spectrum disorder. SFARI gene is a database centered on genes involved in autism and has up-to-date information on all human genes associated with Autism Spectrum Disorder (ASD).

c) Ingenuity Pathway analysis:

The Ingenuity Pathway Analysis (IPA) software was used to detect potential pathways and networks in the DNA methylation data. The genes that were annotated to significant DMPs in the association analysis were used to check for potential pathways.

d) WEB-gestalt

Enrichment analysis was performed for the DNA methylation results using the tools available in the WEB-based Gene SeT AnaLysis Toolkit (WEB-Gestalt) [30], selecting Gene Set Enrichment Analysis (GSEA) as the enrichment method. We performed two enrichment analyses: one using the DMP results and the other using the DMR results. For both enrichment analyses, we used all the genes for which the DMPs/DMRs were mapped to or near to the genes. We uploaded the gene ID and metric table into WEB-Gestalt, selecting 1000 permutations and setting the minimum and maximum number of genes in the category as 5 and 500, respectively, and the mean between duplicate genes as the collapse method. We performed analyses for enriched GO (gene ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. FDR < 0.05 using the BH method was considered significant.

5.8 Saliva-brain DNAm associations

Many epigenetic studies today rely on saliva, buccal or blood as primary source, sample. We use saliva as the source for our epigenetic study. However, the use of this source is questioned in context of psychiatric disorders where the tissue of interest is brain which is often limited to postmortem samples. Use of saliva as a peripheral source is necessary cause of the ease of sample collection. But it is also vital to know whether DNAm markers in saliva mirror those in the brain. Thus, the Image-CpG database (Braun *et al.*, 2019)was used to determine which loci were informative markers of the brain for measurements of DNAm. The Image-CpG database includes DNAm data derived from four tissues including brain, blood, saliva, and buccal cells from a sample of medically intractable epilepsy patients. Resected brain tissue samples were acquired from multiple brain regions including the temporal cortex, hippocampus, amygdala, frontal cortex, frontal lobe, and insular cortex; sampled brain regions varied by participant. It can be accessed at https://han-lab.org/methylation/default/imageCpG.

Correlations are available for DNAm measurements using the Infinium Human-Methylation EPIC chip. Summary statistics are provided to the public including Spearman rho correlations and p values for tissue pairs. For this paper, we were interested in the saliva–brain tissue correlations for all significant DMPs (FDR < 0.05) and the CpGs located in DMRs. Thus, the median rho for DMPs and CpGs included in each DMR was computed as well as the minimum and maximum rho for CpGs.

6. Results

6.1 Cohort characteristics

Of the 728 subjects who returned the questionnaires, the descriptive socio-demographic characteristics of the participating mothers and their offspring (n=114) are shown in Table 5. The average age of the mother at study entry was 34 years. Most of the mothers in this study are Caucasians, married and having a university degree. Most mothers also had planned pregnancies and 72% of mothers had vaginal deliveries. The sex distribution was almost equal, where 57% of the newborns were males. This table, reflecting the subgroup with stored saliva samples, is consistent with the previous two publications from the FELICITy study (Lobmaier *et al.*, 2020; Zimmermann *et al.*, 2022).

Table 5: Baseline characteristics of study population (n= 114, mother-newborn pairs), FELICITy study.

Characteristics	**n = 114**
Continuous variables	*[mean (SD)], [median(range)]*
Age of mother at study entry ,years	34.6 (4.5)
Birthweight , grams	3544 (431)
Gestational age at screening, weeks	34.1 [33.2, 35.0]
Gestational age at inclusion, weeks	36.7 [35.3, 37.5]
Gestational age at birth, weeks	39.7 [38.8, 40.5]
BMI at study entry, kg/m²	27.6 [25.2, 30.4]
BMI pregestational, kg/m²	21.8 [20.3, 24.9]
Score PSS	17.0 [9.0, 22.0]
Score PDQ	10.5 [6.2, 16.7]
Cortisol in maternal hair ,pg/mg	97.0 [58.0, 161.0]
FSI	0.15 [-0.28, 0.60]
Categorical variables	*n (%)*
Gender, female	52 (45)
European/Caucasian, Others	8 (7)
Married, yes	85 (74)
University degree, yes	80 (70)
Household income> 5000€/month , Yes	53 (46)
Working status at screening, working	4 (3)
Multiparity, Yes	85 (74)
Planned pregnancy , Yes	92 (81)
Cesarean delivery, Yes	28 (24)
Smoking, Yes	7 (6)
IVF / ICSI, Yes	10 (8)
Gestational diabetes, Yes	11 (9)
Autoimmune disease , Yes	15 (13)
Displayed are the population characteristics of the FELICITy study population. Continuous variables are given in mean (SD) and median (range) while categorical variables are shown in proportion n(%). Data are mean (SD) using Chi square test, mean (interquartile) using Wilcox. Test or n (%) using Fischer´s test.	
PSS: Cohen perceived stress scale, PDQ: Prenatal distress questionnaire, BMI: Body-mass index; ICSI: Intracytoplasmic sperm injection; IVF: In-vitro-fertilization	

6.2 Exploratory data análisis

To ensure that the data were of high quality we did some exploratory data analysis. We did Principal component analysis, a method that takes high dimensional data and reduces it to a few dimensions to visualize how similar samples of same type are. Samples that are more like each other should cluster together. In Figure 10 the left bar plot shows that most of the variation in the samples is captured by the first principal component (or dimension one). When we divide our whole cohot in SG (Stress Group) and CG (Control group), the PCA plot on the right shows that there is no variation in the data according to the stress group and that are no groups that really explain any differences between the samples.

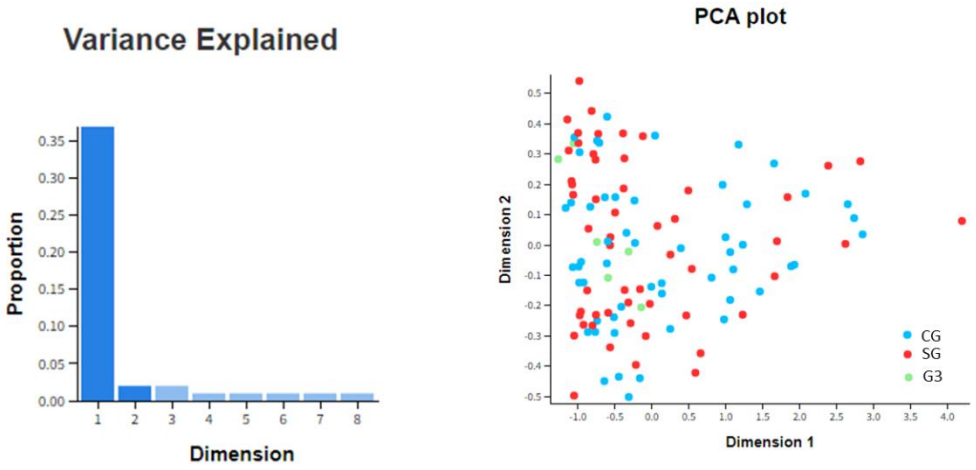


Figure 10: Principal component analysis. A) PCA bar plot showing how much variance is explained by the first principal component. B) PCA plot. The colored points on the plot show stress group. Blue is Control group (CG), red is stress group (SG) and green is group 3, samples that were not in any group.

We further explored the data by doing a correlation plot to check for collinearity between variables. Figure 11 shows the correlation matrix.

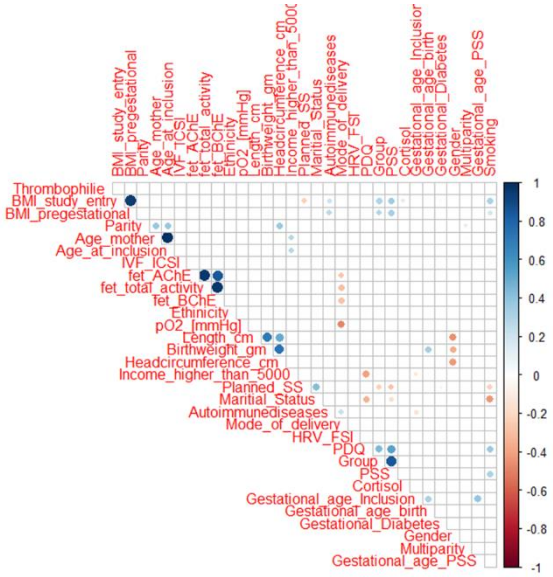


Figure 11: Correlation matrix

Since PSS and PDQ both variables were used to assess the perceived stress in the cohort. We did a correlation plot for the variables. The perceived stress measures (PSS and PDQ scores) were moderately correlated with each other (Spearman R2 = 0.537; p-value = 6.952e-10) (Figure 12).

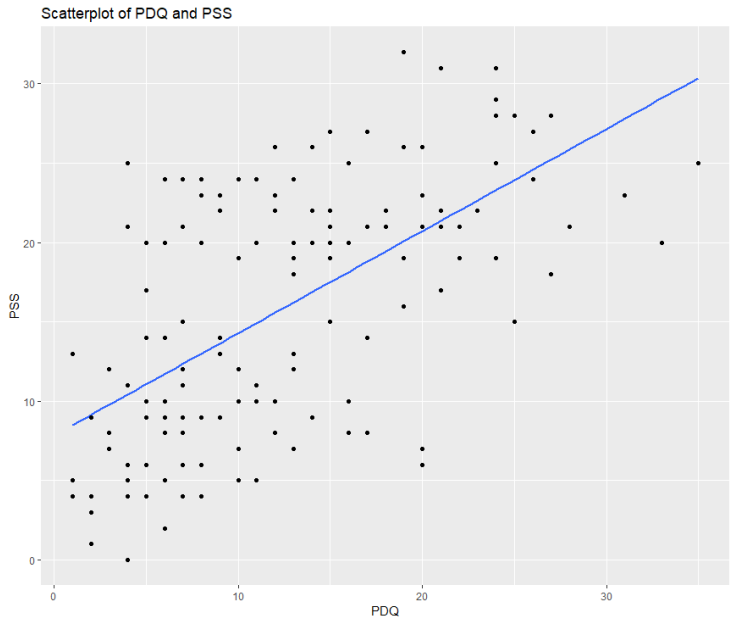


Figure 12: Scatterplot for PSS and PDQ

6.3 Epigenome-wide Association study (EWAS)

We performed an Epigenome – Wide Association Study (EWAS) with the Illumina EPIC Methylation EPIC array in saliva samples obtained from newborns at the time of delivery. We examined the association between prenatal exposure to maternal psychosocial stress and offspring genome-wide saliva methylation using four statistical approaches in association with four complementary maternal and fetal stress measures. The stress measures are: 1) the PSS-10 questionnaire, accounting for the perceived stress of a mother during the third trimester; 2) the PDQ questionnaire accounting for the specific worries related to pregnancy such as pain during labor and delivery, personal appearance after delivery and baby´s health; 3) maternal hair cortisol levels (integrated cortisol levels in hair reflecting three prior months of stress exposure) accounting for the chronic activity of the HPA stress response system of the mother during the third trimester and 4) the FSI, a biophysical ANS biomarker for stress, which accounts for fetal ANS reactivity to maternal heart beat during pregnancy.

6.3.1 Differentially methylated positions (DMPs)

Each CpG site was separately tested for association with exposure to stress (PSS, PDQ, Cortisol and FSI) and separate linear regression models were run, unless otherwise specified. All the models were adjusted as specified in the methods section.

6.3.1.1 **DNA Methylation and PSS score**

In the paper Lobmaier et al, the whole cohort is divided into SG and CG (Lobmaier *et al.*, 2020; Zimmermann *et al.*, 2022). Therefore, we first used the ANOVA model in the limma package to make comparisons between the two groups (Stress Group and Control group), divided by the PSS-10 scale, at each CpG. We saw no evidence for any differentially

methylated CpGs. To ensure that the model was correctly specified- p-value distribution was looked at, which under null distribution follows a uniform distribution. (Figure 13).

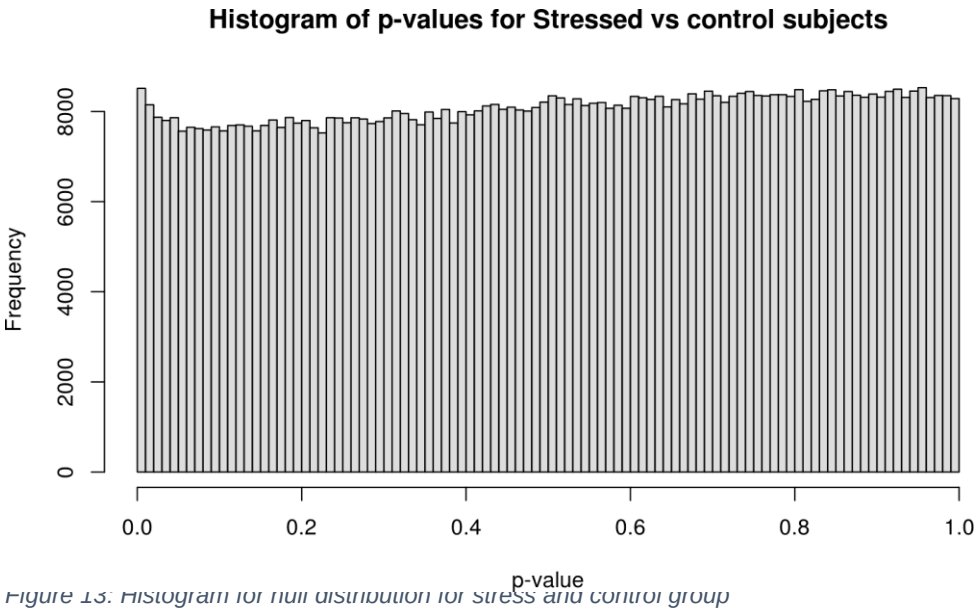


Figure 13: Histogram for null distribution for stress and control group

Moving further, PSS score (the continuous variable) which was used to divide the cohort into SG and CG was used for the association analysis as well, by fitting in a linear regression. Consistent with the ANOVA model, we also did not identify any differentially methylated sites in relation to PSS score. Figure 14 shows the Manhattan and Q-Q plot, while Table 6 shows the top five hits for the association with no significant findings.

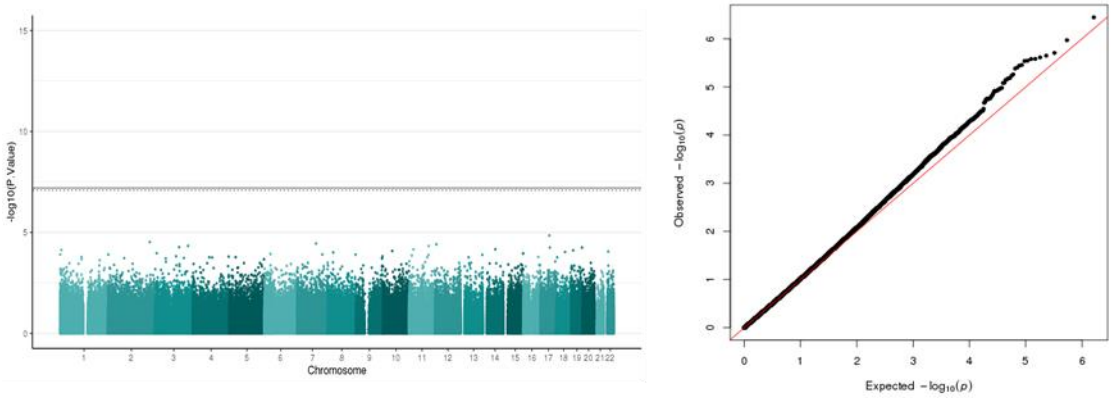


Figure 14: Left: Manhattan plots of salivary DNA methylation associated with PSS (Cohen perceived stress scale). The x-axis represents the genomic loci of the individual CpGs and the y-axis represents the –log10 (p-value). Black line: Bonferroni threshold (p = 6.183879e-08) and the dotted line: Multiple testing correction threshold (FDR <0.05) have been added to the plot. There are no CpGs that cross the significance threshold. Right: Quantile-Quantile plot of the association between PSS and salivary DNA methylation that has a lambda =1.001.

6.3.1.2 DNA methylation and PDQ score

The association analysis with PDQ scores yielded one CpG (cg06542869, p = 4.62E-08) (Figure 16) achieving FDR <0.05. This site has a positive direction of effect, and it is located in the body of the protein coding gene *YAP1* (Yes1 regulated transcription factor) present in chromosome 11 (Table 6). The regression coefficients and values for the next three non-significant hits for this association are reported in Table 6. The Q-Q plot shown in Figure 2 is corrected for inflation which has a lambda value of 1. 04. The uncorrected vs the corrected Q-Q plot is shown in the Figure 15.

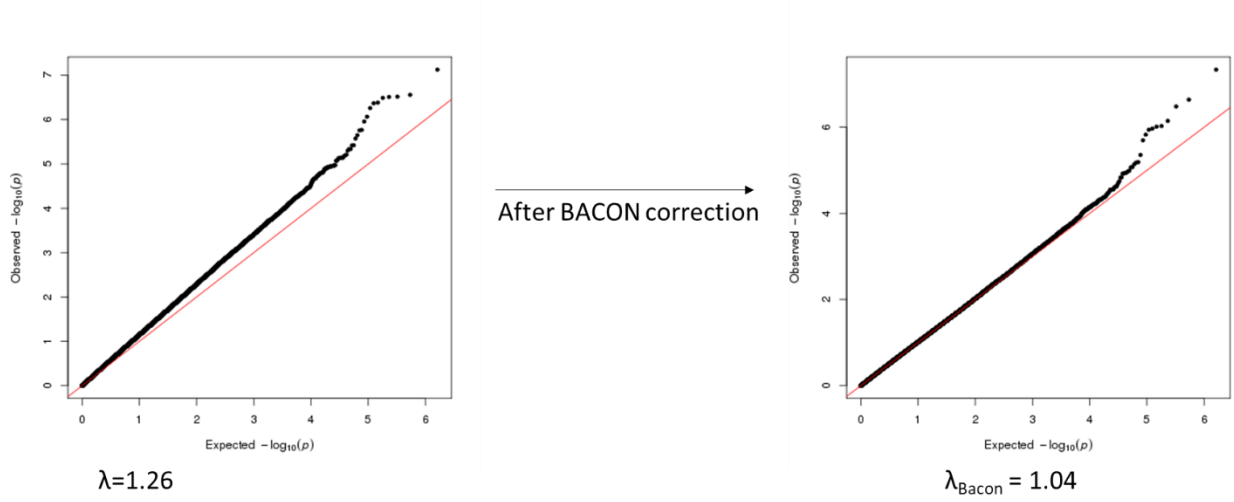


Figure 15: Extended Q-Q plot of salivary DNA methylation associated with PDQ after BACON correction.

Quantile-quantile (QQ) plots of observed and expected distributions of p-values of the association between salivary DNA methylation and PDQ. Lambda (λ) is the genomic inflation factor, and λ Bacon is the genomic inflation factor after correction for inflation, estimated using the method of Van Iterson et al implemented using the bacon R package.

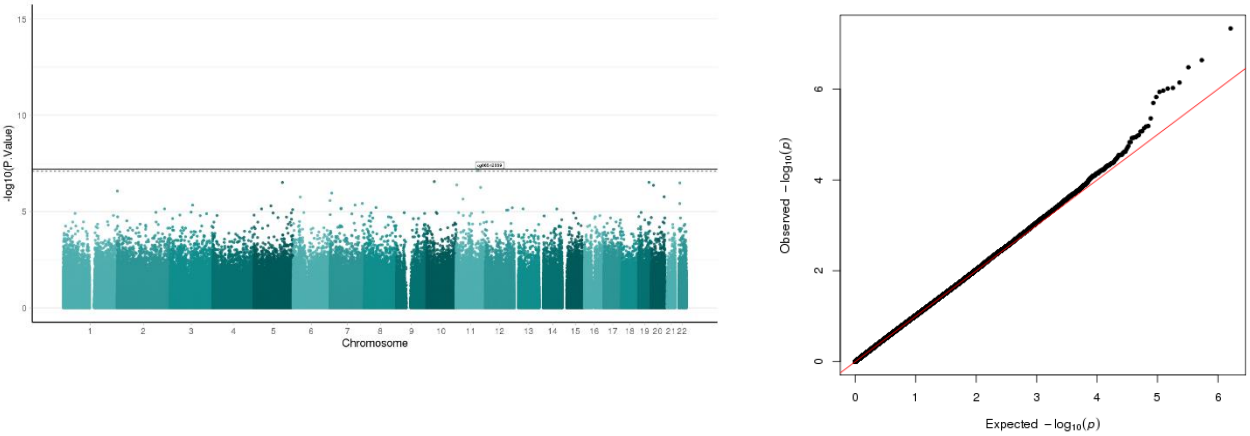


Figure 16: Left: Manhattan plots of salivary DNA methylation associated with PDQ (Prenatal distress questionnaire. The x-axis represents the genomic loci of the individual CpGs and the y-axis represents the –log10 (p-value). Black line: Bonferroni threshold (p = 6.183879e-08) and the dotted line: Multiple testing correction threshold (FDR <0.05) have been added to the plot. CpGs that cross the FDR threshold are marked in the

Manhattan plot. One CpG crosses the FDR threshold. Right: Quantile-Quantile plot of the association between PDQ and salivary DNA methylation that has a lambda = 1.04.

6.3.1.3 DNA methylation and Cortisol

We identified stronger associations with cortisol compared to other stress variables (Figure 17,Table 6). Four CpG sites were identified after controlling for multiple testing using FDR < 0.05 (cg11409463, cg20905655, cg25252839 and cg05306225). The top hit was cg11409463, located on chromosome 5 but did not annotate to any gene via the Illumina gene annotation file. A look up on UCSC browser showed that the nearest genes within 50kb distance to this CpG site are *SCAMP1* (Secretory Carrier Membrane Protein 1) and *AP3B1* (Adaptor Related Protein Complex 3 Subunit Beta 1). This CpG site also overlaps with several transcription factors from the AP-1 family. The second hit was cg20905655 (p = 1.16E-07) located on chromosome 19 which did not annotate to any gene via Illumina platform. According to the UCSC genome browser, the nearest genes within the 50kb region are *SSBP4* (Single Stranded DNA Binding Protein 4) (Castro et al., 2002) and *LRRC25* (Leucine Rich Repeat Containing 25). The third hit was *TOMM20* (Translocase of Outer Mitochondrial Membrane 20) (cg25252839, p = 1.24E-07) located on chromosome 1. All the CpG sites had a positive direction of association except for the fourth hit, cg05306225, which annotates for the gene *CSMD1* (CUB and Sushi Multiple Domains 1) located on chromosome 8 and encoding for Q96PZ7-CSMD1_HUMAN (CUB and sushi domain-containing protein 1). Inspection of quantile-quantile (QQ) plot did not show evidence for inflation or bias (Figure 17 ; lambda = 1.08)

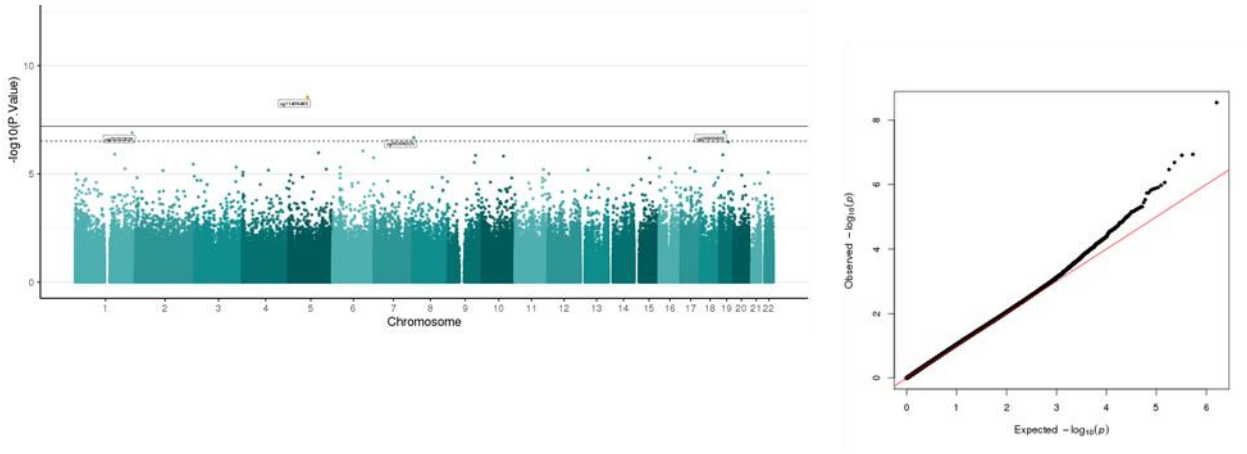


Figure 17: Left: Manhattan plots of salivary DNA methylation associated with cortisol. The x-axis represents the genomic loci of the individual CpGs and the y-axis represents the –log10 (p-value). Black line: Bonferroni threshold (p = 6.183879e-08) and the dotted line: Multiple testing correction threshold (FDR <0.05) have been added to the plot. CpGs that cross the FDR threshold are marked in the Manhattan plot. There are four CpGs that cross the significance threshold. Four CpGs cross the FDR threshold. Right: Quantile-Quantile plot of the association between cortisol and salivary DNA methylation with lambda = 1.08.

6.3.1.4 DNA methylation and FSI

There were no DMPs that survived the correction for multiple testing when the association was performed with FSI, the biophysical biomarker of PS exposure on the fetus. Of interest, the top hit, CpG (cg13547817, p= 8.51E-08) with an FDR: 0.06, very close to the threshold, mapped to the gene *ERP44* (Endoplasmic reticulum protein 44) on chromosome 9, which is a protein coding gene whose related pathways are the Innate immune system and translational control. Table 6 shows the top four hits from the FSI association analysis and Figure 18 shows the Manhattan plot and the Q-Q plot.

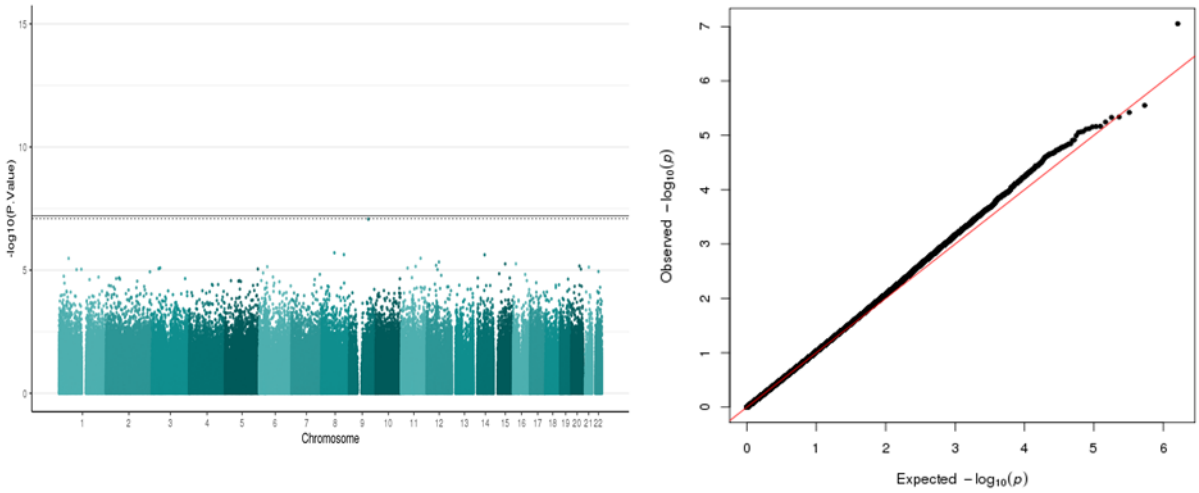


Figure 18: Left: Manhattan plots of salivary DNA methylation associated with FSI (Fetal stress index). The x-axis represents the genomic loci of the individual CpGs and the y-axis represents the –log10 (p-value). Black line: Bonferroni threshold (p = 6.183879e-08) and the dotted line: Multiple testing correction threshold (FDR <0.05) have been added to the plot. CpGs that cross the FDR threshold are marked in the Manhattan plot. There are no DMPs that cross the Bonferroni correction threshold. Right: Quantile-Quantile plot of the association between FSI and salivary DNA methylation with lambda= 1.01.

Table 6: - CpG sites associated with stress measures in DNA methylation analysis.

Stress measure	Probe	Coef[d]	P.Value[e]	FDR[f]	chr[g]	Relation to Island	Illumina Gene annotation	Genes within 50kb of associated CpG
PSS[a]	cg17478679	-0.2	3.59E-07	0.25	chr14	Body	KPNB1	CRHR1
	cg22124215	-0.1	1.07E-06	0.25	chr17	OpenSea	MARCH4	DIRC3
	cg06195987	0.52	1.96E-06	0.25	chr6	N_Shore	NA	LMTK2
	cg15426815	0.31	2.24E-06	0.25	chr4	TSS1500	MIRC200	C1S
PDQ[b]	**cg06542869**	**0.02**	**4.62E-08**	**0.03**	**chr11**	**OpenSea**	**YAP1**	**YAP1**
	cg22861369	0.02	2.30E-07	0.08	chr5	S_Shore	PDLIM4	SLC22A4
	cg01629131	0.03	3.31E-07	0.08	chr20	OpenSea	NA	RP11, RP1
	cg03105159	0.01	7.18E-07	0.11	chr2	S_Shore	ALKAL2	ALKAL2, FAM105B
Cortisol	**cg11409463**	**0.003**	**2.87E-09**	**0.002**	**chr5**	**OpenSea**	**NA**	**SCAMP1**
	cg20905655	**0.004**	**1.16E-07**	**0.03**	**chr19**	**OpenSea**	**NA**	**SSBP4**
	cg25252839	**0.002**	**1.24E-07**	**0.03**	**chr1**	**OpenSea**	**TOMM20**	**SNORA14B**
	cg05306225	**-0.002**	**2.08E-07**	**0.04**	**chr8**	**N_Shore**	**CSMD1**	**CSMD1**
FSI[c]	cg13547817	-0.39	8.85E-08	0.07	chr9	OpenSea	ERP44	ERP44; INVS
	cg07642729	-0.35	2.81E-06	0.49	chr8	TSS1500	ASB15	-
	cg24795351	-0.34	3.79E-06	0.49	chr8	OpenSea	PREX2	PREX2
	cg16692227	-0.21	3.80E-06	0.49	chr14	N_Shore	SAMD12	SAMD12

The table shows top four CpGs from the EWAS that are associated to the respective stress measures. **Marked in bold are significant.**

[a]Cohen perceived stress scale; [b]Prenatal distress questionnaire: [c]Fetal stress Index; [d]Regression coefficients from statistical model; [e]Statistical significant from statistical model

[f]false discovery rate; [g]Chromosome

NA: Not available.

6.3.2 Sex-specificity analysis

The CpG-by-sex interaction analysis did not reveal any significant differences between sexes for the associations (Table 7).

Table 7: Epigenome-wide results of the Interaction analysis between Gender and Stress measures.

Stress measures	Probe	Coef[d]	P.Value[e]	FDR[f]	Chr[g]	Illumina Gene annotation
PSS[a]	cg09723184	0.03	6.23E-06	0.87	chr8	FBXO43
	cg27293447	-0.04	7.25E-06	0.87	chr2	LOC102800447
PDQ[b]	cg03756940	-0.05	1.10E-07	0.08	chr2	NA
	cg00008621	-0.04	2.06E-07	0.08	chr14	HIF1A
Cortisol	cg18197866	0.003	2.55E-07	0.16	chr12	PXN
	cg20460797	-0.006	4.17E-07	0.16	chr4	NSG1
FSI[c]	cg24715106	0.54	2.51E-07	0.2	chr11	AQP11
	cg23782719	-0.42	2.54E-06	0.56	chr6	RNF182

The table shows the top two CpGs from the EWAS of the interaction analysis that are associated with the stress measures.
[a]Cohen Perceived stress scale
[b]Prenatal distress questionnaire
[c]Fetal stress index
[d]Regression coefficients from the statistical model
[e]Significance from the statistical model
[f]False discovery rate
[g]Chromosome
NA: Not available.

6.3.3 Differentially methylated regions (DMRs)

DMRs are genomic regions that have consistently different DNA methylation across multiple adjacent CpGs (Rakyan *et al.*, 2011). The DMRs mapped to or near the genes that are enriched for the biological process of the regulation of sequence-specific DNA binding transcription factor activity suggest that these genes are involved in regulation of gene expression. Regional analysis identified associations with maternal stress measures (PDQ and Cortisol). All DMRs identified by DMRcate as well as the DMPs overlapped with the DMRs identified by comb-p. Results are shown in Table 8.

DMRcate identified two DMRs related to maternal stress measures PDQ and cortisol. One DMR associated with PDQ was found to be in the ***DAXX*** locus (Death-associated protein 6) on chromosome 6 (Figure 19). The other DMR, associated with cortisol, was found to be in the ***ARL4D*** (ADP- Ribosylation factor 4D) on chromosome 17.

Table 8: Differentially methylated regions (DMRs) in salivary DNA associated with stress measures in FELICITy study using DMRcate.

Stress measures	*Chr[b]*	*Start (bp[c])*	*End(bp)*	*CpGs[d]*	*p-value[e]*	*Sidak P[f]*	*Gene*
PDQ[a]	6	33288180	33288600	8	0.000166981	8.67E-08	DAXX
Cortisol	17	41476044	41476457	11	6.50E-10	1.27E-06	ARL4D

[a]Prenatal distress questionnaire

[b]Chromosome

[c]Physical position (basepair)

[d]Number of probes in the region

[e]Statistical significance

[f] p of Sidak multiple-testing correction

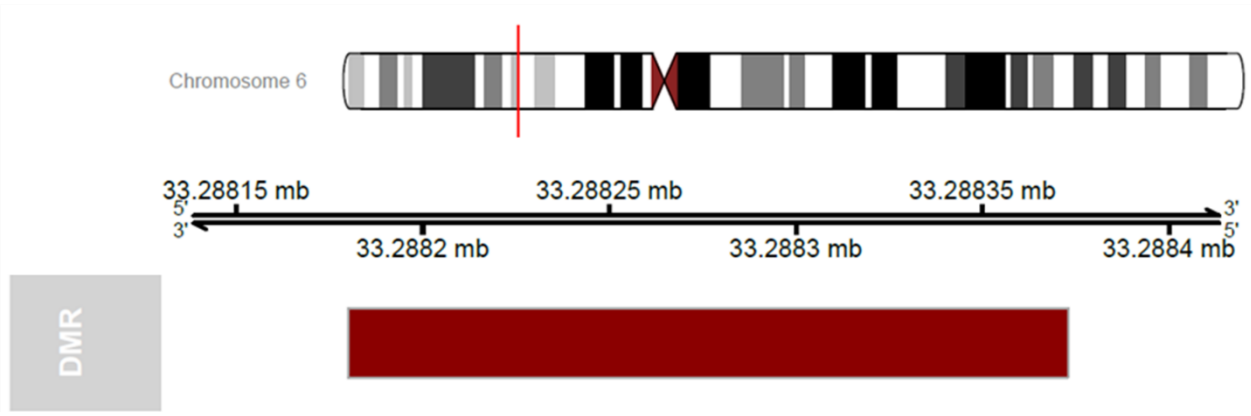


Figure 19: Visualization of the DMR identified by DMRcate in their genomic context: DAXX on chromosome 6

List of DMRs for PDQ and Cortisol, identified by Comb-p is given the following Table 9 and Table 10. All DMRs identified by DMRcate and the DMPs, overlapped with the DMRs identified by comb-p.

Table 9:- DMRs associated with PDQ identified by comb-p

DMR position (hg 19)	No. of probes in DMR	p-value	Sidak P	Gene
chr6: 33288180 – 33288600	**8**	**0,000166981**	**8,67E-08**	**DAXX**
chr19: 52034861 - 52035288	6	2,91E-08	1,54E-11	SIGLEC6
chr17: 41278135 - 41278445	12	0,00000138	5,31E-10	BRCA1; NBR2
chr14: 104315145 - 104315224	3	0,0000217	2,12E-09	LINC00637
chr11:66102055 - 66102353	5	0,000727696	0,000000268	RIN1
chr13: 20751679 - 20751946	3	0,001887053	0,000000624	PPIAP28
chr17: 80847270 - 80847663	4	0,001286623	0,000000626	TBCD
chr11: 113659927 - 113659953	4	0,033131424	0,00000108	ATF4P4
chr17: 152089 - 152351	4	0,009607021	0,00000313	RPH3AL
chr10: 43626330 - 43626562	4	0,013596016	0,00000393	RET
chr17: 202690 - 202989	5	0,025438429	0,00000953	RPH3AL
chr11: 102028101 - 102028102	1	0,036647188	4,62E-08	YAP1*

Marked in bold is the DMR that also overlaps with DMRcate.
* DMRs that overlap with DMPs from the main analysis.

Table 10: DMRs associated with Cortisol identified by comb-p

DMR position (hg 19)	No. of probes in DMR	p-value	Sidak P	Gene
chr17: 41476044 - 41476457	**11**	**6,50E-10**	**1,27E-06**	**ARL4D**
chr18: 54814254 - 54814549	6	8,51E-13	2,33E-09	BOD1L2
chr16: 52581551 - 52582263	9	1,90E-12	2,16E-09	TOX3
chr9: 124989052 - 124989840	7	3,22E-10	3,31E-07	LHX6
chr17: 48912265 - 48912861	12	4,43E-10	6,00E-07	WFIKKN2
chr6: 170190914 - 170191083	4	7,46E-09	3,57E-05	LINC00242
chr3: 79816838 -79817334	11	7,47E-09	1,22E-05	ROBO1
chr6: 169852497 -169852843	4	3,37E-08	7,88E-05	WDR27
chr11: 1948933 -1949131	6	6,07E-08	0,00024786	TNNT3
chr7: 788797 -788990	4	6,95E-08	0,000291025	HEATR2
chr4: 153877907 - 153878056	3	8,33E-08	0,000452166	FHDC1
chr11: 112150832 - 112151122	3	9,70E-08	0,000270525	RP11-356J5.12
chr4: 57548093 -57548291	4	1,17E-07	0,000477071	HOPX
chr13: 114800309 -114800550	3	1,56E-07	0,000521662	RASA3
chr1: 1267117 -1267437	5	1,66E-07	0,000419853	TAS1R3
chr22 : 30902615 -30902897	4	3,60E-07	0,001032888	SEC14L4
chr21: 35831871-35832205	10	5,89E-07	0,001424952	KCNE1
chr14 : 91283338-91283607	3	7,57E-07	0,002273363	TTC7B
chr15 : 74466704 -74466934	5	9,27E-07	0,003253622	ISLR

chr7 : 922775 -923090	6	1,19E-06	0,00305216	GET4
chr12 : 57588171 -57588351	6	1,25E-06	0,005589701	LRP1
chr17 : 37123638 -37123950	9	1,28E-06	0,003315611	FBXO47
chr20 : 748992 -749453	7	1,30E-06	0,002277144	SLC52A3
chr15 : 101728234 -101728452	4	1,39E-06	0,005138224	CHSY1
chr4 : 146099474 -146099545	3	1,51E-06	0,017027847	OTUD4
chr11 : 112131509 -112131714	3	1,68E-06	0,006585914	PTS
chr2 : 219246760 -219247056	5	1,74E-06	0,004745404	SLC11A1
chr11 : 113258223 -113258470	5	3,17E-06	0,010312593	ANKK1
chr20 : 2361344 - 2361565	7	4,37E-06	0,015873854	TGM6
chr7 : 43288757 -43289039	5	5,35E-06	0,01522361	HECW1
chr16 : 3704613-3704802	3	5,36E-06	0,02266864	DNASE1
chr13 : 113776873 -113777161	8	5,50E-06	0,01531352	F10
chr19 :7852051 -7852239	6	6,21E-06	0,026358888	CLEC4GP1
chr16 : 17371751-17372023	2	6,30E-06	0,018555432	XYLT1
chr17 :61515708 -61515855	3	6,99E-06	0,037705573	CYB561
chr22 : 18269793 -18269959	2	7,60E-06	0,036357082	MICAL3
chr6 : 170055155-170055333	2	8,10E-06	0,036120258	WDR27
chr11 : 6426681 -6426895	5	1,14E-05	0,042188137	APBB1
chr5 : 140529738- 140530029	4	1,14E-05	0,03130193	PCDHB6
chr1 : 2398204- 2398401	2	1,16E-05	0,046603166	PLCH2
chr4 :114213914 -114214186	5	1,23E-05	0,03579022	ANK2
chr6 : 31838474- 31838775	8	1,47E-05	0,038661037	SLC44A4
chr5 : 77634319 -77634320	1	2,87E-09	0,002320288	SCAMP1*
chr1 : 235285886 - 235285887	1	1,24E-07	0,095671698	TOMM20*
chr8 : 4848947 -4848948	1	2,08E-07	0,154760323	CSMD1*

Marked in bold is the DMR that also overlaps with DMRcate.

* DMRs that overlap with DMPs from the main analysis.

6.3.4 Differential variability analysis

Traditional Differential variability analysis aims to look for significant changes in variance levels in groups of diseased and non-diseased individuals. It is possible that any differences between groups is reflected by changes in variability rather than methylation. We were interested in CpG sites that are consistently methylated in one group but variably methylated in another group, the group in our cohort refers to the infants from stressed mothers and the respective controls.

Therefore, a differential variance model was fit with a goal to find regions where newborns from the stressed mother have higher or lower variability. Sex, gestational age, Illumina plate factor and the eight surrogate variables were taken as covariates for this analysis as well. There were no differences in variability seen in the FELICITy cohort.

6.3.5 Candidate gene-specific analyses

We also analysed 85 genes related to the stress response systems and subsetted methylation array data to the gene specific CpGs that are within the promoter region of the gene. 984 CpGs within those gene regions were subsetted. There were no CpGs that meet the false discovery rate threshold of 0.05.

6.3.6 Regions of Increased Variability

Another analysis that was done was to look for regions of increased variability. Increased variability intends to find regions that have higher variability across all samples, as compared to other regions. Regions of heightened variability across all samples and not just a group of samples was looked for by computing the ratio of variation at a given CpG and then the mean variation over all CpGs. Regions with a minimum of 20CpGs sites that have consistent increased variation are looked for. 49 such regions of increased variability were found across all samples. Some of the genes mapped to these regions are *HLA-C*, *TMEM9B*, *HOXA3* and *PKP3*.

6.4 Exploratory analysis of DMPs

In a second layer of analysis, the network and pathway interactions for the genes corresponding to the significant CpGs from the association analysis was done.

Firstly, **STRING-db** was used, a database and software application enabling a semi-unsupervised statistical network analysis of known and predicted protein-protein interactions as well as their physical and functional interaction networks based on computational predictions, i.e., enrichment (Szklarczyk *et al.*, 2019). Proteins encoded by the genes that were annotated to significant DMPs were analyzed using STRING-db. Unique URL for the resulting analysis is as follows:

https://version115.stringdb.org/cgi/network?taskId=bMnf9hIgSYrT&sessionId=bl9Pry04KzyB

The protein- protein interaction (PPI) enrichment p-value for this network is 3.47e -06. The top three biological processes identified in Figure 20 were 1) Hippo signaling pathway, 2) Regulation of canonical Wnt signaling pathway and 3) Cell- cell junction assembly.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways map molecular objects such as genes, proteins etc. to molecular interactions or relations. The top pathway identified from the KEGG pathways was the Hippo signaling pathway. Figure shows YAP1 interacting with several proteins of the Hippo-signaling pathway and the ß-Catenin signaling pathways-CTNNB1 (Catenin Beta 1). YAP appears also directly related to TEAD1 and TEAD4 since YAP/TAZ are transcriptional coregulators and partners of the TEAD family transcription factors.

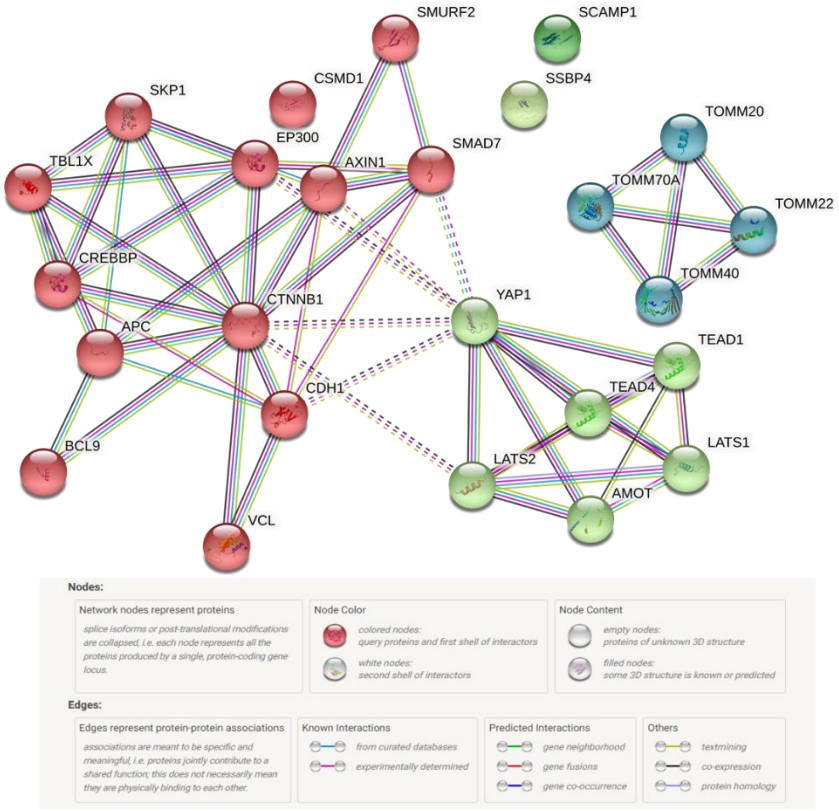


Figure 20: ***Network plot of significant hits from the EWAS analysis.***

STRING-Db network analysis for significant hits from the association for PDQ and cortisol. Protein-protein interaction (PPI) enrichment p-value: 3.47e-06. PPI legend by string-db.org. The permanent link is https://version-11-5.string-db.org/cgi/network?taskId=bvfqNrZYaHe6&sessionId=bjK7XvqNxMXe

As the next step, we used the **SFARI gene database** (Abrahams *et al.*, 2013) to extract information for the genes annotated to CpGs specific to ASD (Frasch *et al.*, 2019). SFARI gene is a database centered on genes involved in autism and has up-to-date information on all human genes associated with ASD. Of all the genes looked up in the SFARI database(Abrahams *et al.*, 2013), only *CSMD1* appeared with a score of three indicating strong relevance to autism spectrum disorders (gene.sfari.org/database/human-gene/*CSMD1*). Many findings indicate that *CSMD1* has been closely related to neurogenesis, cognition, and neuropsychology (Liu *et al.*, 2019). This study by Liu et al also provides evidence that *CSMD1* an important biomarker and is associated with Schizophrenia.

Pathway analysis of the DMPs was also done using the **Ingenuity pathways analysis software**. Consistent with the STRING-db results the top pathways are Hippo signalling and ERBB4 signalling. shows the overlapping canonical pathways from the IPA and highlighted in bright pink are the top pathways.

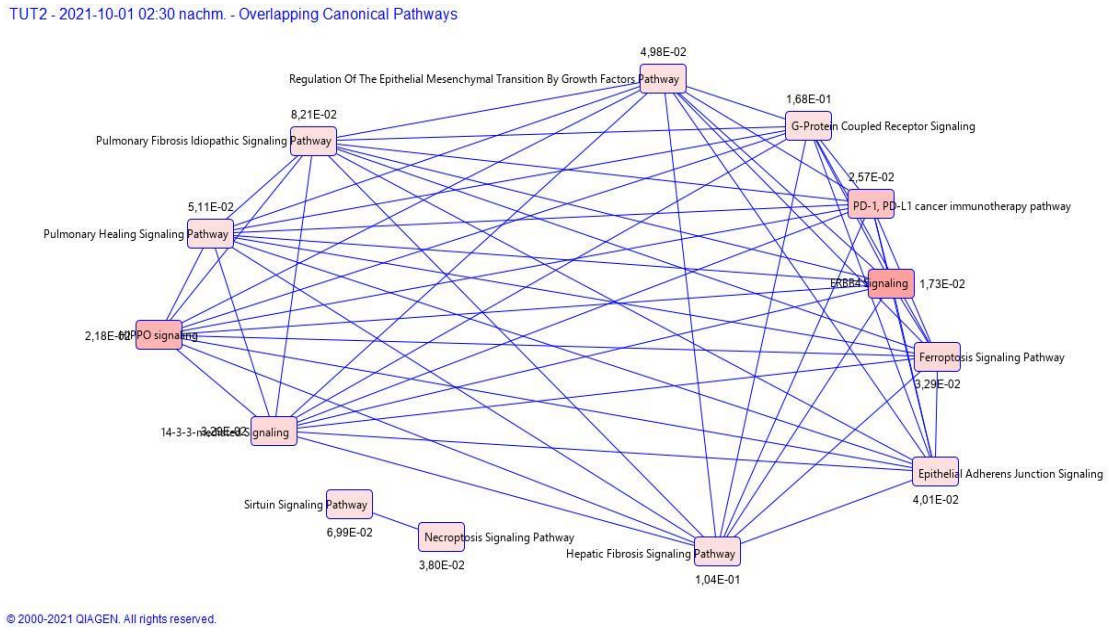


Figure 21: Top Canonical pathway from IPA

Further, the **gene ontology consortium** was also used for enrichment analysis of significant DMPs and DMRs altogether. There were no pathways that crossed the FDR threshold. Nevertheless, the top pathways are listed in the Table 11.

Table 11: Top Gene Ontology enrichment analysis of DMPs and DMRs

1	**Protein transporting ATPase activity**
2	Sumo-dependent protein binding
3	Mitochondrion targeting sequence binding

6.5 Relationship between saliva and brain DNAm markers

Many epigenetic studies today rely on saliva, buccal or blood samples as primary source. Since the primary organ affected by stress is not available in human studies and post-mortem brain tissues samples cannot capture the fluid state of the epigenome, the selection of a peripheral tissue for epigenetic studies of psychiatric traits is often questioned. Binder´s group showed that saliva reflects better DNA methylation patterns of brain than methylation in blood, but it is still crucial to know if DNAm markers in saliva mirror those in the brain. To this end, Braun et al have developed a website IMAGE-CpG, that permits to find the degree of cross-tissue correlation and determine which loci were informative markers of the brain for measurements of DNAm in saliva. The Image-CpG database includes DNAm data derived from four tissues including brain, blood, saliva, and buccal cells from a sample of medically intractable epilepsy patients.

For the FELICITy study a separate analysis was performed of the five genome-wide significant results and the two DMRs, assessing the degree of correlation between DNAm of saliva and brain tissues, using a web-based resource IMAGE-CpG.

Table 12 includes the rho values for the significant CpGs for the DMP analysis. While Table 13 includes the median rho correlation as well as minimum and maximum rho correlation

values for CpGs included within each DMR. Across both the DMRs the median correlation between saliva and brain was 0.24 and 0.21. The minimum rho observed was -0.1 while the maximum rho was 0.53.

We observed non-significant moderate correlation between brain and saliva DNAm for the CpG cg20905655.

Table 12: - Degree of correlation between saliva and brain DNA methylation of the significant CpGs at FDR <0.05.

cgid	Gene	chromosome	Position	rho brain saliva	p value brain saliva
cg06542869	*YAP1*	11	102028101	-0.1	0.60
cg11409463	-	5	77634319	0.32	0.15
cg25252839	*TOMM20*	1	235285886	-0.2	0.22
cg05306225	*CSMD1*	8	4848947	0	0.98
cg20905655	-	*19*	18520786	0.47	0.03

Table 13: - Degree of correlation between saliva and brain DNA methylation of the significant DMRs.

DMR	CpG	chromosome	Position	Rho brain saliva	P brain_saliva
DAXX	cg03477252	6	33288180	0.53	0.01
	cg22904406	6	33288296	0.33	0.14

	cg26500914	6	33288323	0.14	0.53
	cg09365002	6	33288329	0.22	0.33
	cg07905975	6	33288366	0.29	0.19
	cg24498636	6	33288372	0.24	0.27
	cg17251196	6	33288599	-0.1	0.41
ARLD4	cg01065373	17	41476044	0.14	0.53
	cg23635580	17	41476114	0.14	0.52
	cg11029904	17	41476242	0.53	0.01
	cg20985649	17	41476244	-0.0	0.66
	cg12842219	17	41476263	0.09	0.67
	cg21332495	17	41476307	0.31	0.16
	cg14806146	17	41476317	0.18	0.42
	cg20420791	17	41476369	0.27	0.21
	cg25673373	17	41476379	0.23	0.29

7. Discussion

Most of the maternal stress studies in the past have been limited to targeted DNA methylation analyses in candidate genes (Oberlander *et al.*, 2008; Monk *et al.*, 2016). It is only in the recent years that epigenome wide studies of DNA methylation have gained popularity allowing to evaluate locus-specific methylation across the entire genome (Mehta *et al.*, 2013; Cao-Lei *et al.*, 2014; Non *et al.*, 2014; Tobi *et al.*, 2015). These different approaches have been recently summarized in two reviews and a meta-analysis (Sosnowski *et al.*, 2018; Cao-Lei *et al.*, 2020; Sammallahti *et al.*, 2021). When analysing these studies, a general conclusion on what type of epigenetic signature is observed in prenatally stressed infants is difficult to draw since many methodological differences are still observed in terms of the type and timing of the prenatal insult studied, the age of the child and the tissue employed to detect DNA methylation. This makes comparison among studies very difficult leading to inconclusive evidence on the association between PS and DNA methylation in the neonate. To bridge this gap, we have examined the association between psychological, molecular, and biophysical maternal-fetal stress measures and the genome wide methylation profile in newborn saliva. Our findings validate the hypothesis that PS biomarkers are associated with epigenome–wide DNA methylation in newborn saliva across multiple CpG sites those relevant to neuronal, immune and endocrine homeostasis.

7.1 EWAS: DMPs

In our study, PDQ, but not PSS, and cortisol showed a significant association with five CpG sites. Out of these five CpGs, three were annotated to *YAP1*, *TOMM20* and *CSMD1*; two CpGs were not annotated to any gene but lay within 50kb of *SSBP4*, *SCAMP1* and *LRRC25*. We discuss the functional implications of these associations in the following paragraphs.

YAP1 and its related protein WWdomain- containing transcription regulator 1 (*WWTR1*; also known as TAZ) (*YAP/TAZ*) are the main effectors of the Hippo signaling pathway (Ma *et al.*, 2019). This evolutionarily conserved signaling cascade regulates cell proliferation, stemness, organ size control, and regeneration. Its dysregulation has been associated to multiple forms of cancers, the immunity response, and cardiovascular diseases (Ma *et al.*, 2019; Kandilya *et al.*, 2020). Although widely expressed in several tissues, *YAP* is selectively expressed in astrocytes and neural stem cells in the mouse developing brain and its deletion causes reactive astrogliosis and astrocyte-driven microglial activation (Huang *et al.*, 2016). Moreover, Passaro et al , demonstrated that the transient downregulation of *YAP* in mouse embryonic stem cells disrupts cellular homeostasis altering the ability to differentiate properly (Passaro *et al.*, 2021). In our study the hypermethylated CpG cg06542869 annotated to *YAP1* is associated with specific pregnancy worries (PDQ score). The functional consequence of the hypermethylation of one single CpG site in the open sea of the *YAP1* gene is highly speculative without evaluating the translated protein. However, it has been demonstrated that the methylation of one single CpG can impact on the methylation levels of neighbouring CpG sites (Passaro *et al.*, 2021). Any modification of methylation status of the *YAP1* gene might potentially lead to alterations in cell proliferation, cell differentiation and astrogliosis. In fact, the network analysis of the protein encoded by *YAP1* using STRING-db showed an interaction with several proteins of the Hippo signalling pathway such as the TEAD family of transcription proteins. The phosphorylation and inhibition of *YAP/TAZ* activate the Hippo pathway limiting tissue growth and cell proliferation. Upon dephosphorylation, *YAP/TAZ* translocate to the nucleus, binding to TEAD and inducing transcriptional programs related to cell proliferation, survival, and migration (Ma *et al.*, 2019).

TOMM20 (Translocase of outer mitochondrial membrane 20) is involved in glucose/ energy metabolism and deubiquitination. Together with *TOMM22* functions as a transit peptide receptor at the surface of the mitochondrial outer membrane and facilitates the movement of preproteins (Goping, Millar and Shore, 1995; Hernández, Giner and Hernández-Yago, 1999).

Diseases associated with *TOMM20* include Optic Atrophy 1 and 11. Our results show that the hypermethylated CpG site cg25252839 is associated with cortisol levels and annotates to *TOMM20*.

The ***CSMD1*** gene has been proposed to have brain specificity since it encodes a cell adhesion molecule highly expressed in membrane associated proteins in the CNS, with almost no detection in other tissues (Abd El Gayed *et al.*, 2021). The CSMD1 protein is related to immune function playing a crucial role in regulating complement activation and inflammation in the developing brain (Liu *et al.*, 2019; Abd El Gayed *et al.*, 2021) and may also play a role in growth cone function (Kraus *et al.*, 2006). The CSMD1 protein is predominantly expressed in neurons mainly in the cerebral cortex and the hippocampus and has been involved in brain circuits development, neurotransmission, axon guidance, regeneration, and plasticity (Abd El Gayed *et al.*, 2021). *CSMD1* protein coding gene has been previously associated to autism spectrum disorders (ASD) (Cukier *et al.*, 2014; Guo *et al.*, 2017). Corroborating the above statement, *CSMD1* also appears on the SFARI database listing genes associated with ASD. It scored as level 3, meaning there is suggestive evidence from significant but non-replicated association studies. Moreover, *CSMD1* has been associated to posttraumatic stress disorder (Nievergelt *et al.*, 2015; Melroy-Greif *et al.*, 2017), schizophrenia (Håvik *et al.*, 2011; Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011; Liu *et al.*, 2019; Abd El Gayed *et al.*, 2021), and bipolar disorders (Xu *et al.*, 2014).

In our study, we found that the hypo methylated CpG cg05306225 annotates for the gene *CSMD1* and is associated with high maternal cortisol levels. Although it is difficult to predict the functional consequences of this single site hypomethylation as mentioned above, it is interesting to observe that the probable destabilization of the methylation status of flanking CpGs mentioned before, is in a gene with high brain specificity and associated with several neuropsychiatric disorders. In particular, the association of this gene with ASD refers to several

reports showing that the risk for ASD is linked to PS (Beversdorf *et al.*, 2005; Kinney *et al.*, 2008).

The two other CpGs that were significantly associated with cortisol levels but are not annotated to any gene are **cg11409463** and **cg20905655**, both hypermethylated. The nearest gene to the CpG site cg11409463 is *SCAMP1* whose protein is involved in secretion and transportation. Diseases associated with this gene include Childhood Kidney Cell Carcinoma and Branchiootorenal Syndrome 1. This same CpG site overlaps with several transcription factor binding sites from the AP-1 family (the Jun, the Fos and ATF-2 subfamily) such as *JUNB, FOS, SETDB1, ATF3, CBX3, TRIM28, ZNF143*. The AP-1 family is responsible for cell growth, differentiation (Angel and Karin, 1991) and apoptosis (Ameyar, Wisniewska and Weitzman, 2003). The nearest genes to CpG site cg20905655 are *SSBP4* and *LRRC25*, the latter related to autophagic degradation. So far, not much is known about the functional role of *SSB4* and its relation to stress yet.

Of interest, cortisol-associated methylation disbalances in several genes found in neonatal saliva suggest that the transplacental barrier might be impaired and abnormally permeable to steroid hormones. In fact, it has been described that the metabolizing enzymes that lay within trophoblasts and protect the fetus from overexposure to glucocorticoids, are sensitive to maternal stress (Seckl and Holmes, 2007; Aye and Keelan, 2013). For example, the glucocorticoid-inactivating enzyme, 11β-hydroxysteroid dehydrogenase type-2 (*11βHSD2*), showed a reduced placental expression in relation to maternal anxiety and depressed mood in humans (Jensen Peña, Monk and Champagne, 2012; O'Donnell *et al.*, 2012). The reduced placental expression of *11βHSD2* will potentially lead to a fetal glucocorticoid overexposure affecting developmental events such as fetal growth restriction, altered HPA axis development, impaired offspring brain function, permanent changes in the expression of specific transcription factors and early development of proliferative neural precursors (Shams *et al.*, 1998; Seckl and Holmes, 2007). Our observation that the newborn saliva shows cortisol associated epigenetic

changes in genes related to energy metabolism, cell differentiation and function of the developing brain might be highlighting that one of the underlying mechanisms linking maternal stress with childhood outcomes is through transplacental mediated methylation disbalances in specific genes, among other mechanisms, such as transcriptional regulation of placental gene expression as suggested by Aushev et al (Aushev *et al.*, 2021).

7.2 EWAS: DMRs

To expand the search of epigenetic signatures associated with stress measures during pregnancy we considered DMRs. Two DMRs were detected, one associated to **PDQ (*DAXX*)** and the other to cortisol (*ARL4D*). *DAXX* gene encodes for a protein that resides in multiple locations in the nucleus and cytoplasm. Pathways related to Daxx are apoptosis and survival caspase cascade as well as TGF-β signaling pathways (Yang *et al.*, 1997). Diseases associated with *DAXX* include Gastric neuroendocrine neoplasm, intellectual disability, and Alpha-Thalassemia. Interestingly, *ATRX* gene which has been previously linked with ASD, interacts with *DAXX* in histone chaperone complex and influences DNA methylation (Deciphering Developmental Disorders Study, 2015; Hoelper *et al.*, 2017; Tremblay and Jiang, 2019). Moreover, *DAXX* is known to be an extended Class II, non-antigen binding *HLA* (human leukocyte antigen) gene associated with autoimmune diseases that interacts with death receptor Fas related to ASD (Torres, Westover and Rosenspire, 2012). *ARL4D* belongs to ADP-ribosylation factors (ARFs), members of the Ras family of small GTPases, involved in membrane transport, membrane lipid modifications and maintenance of organelle integrity (D'Souza-Schorey and Chavrier, 2006). Interestingly, the transcription of *Arl4d* was found to be consistently regulated by glucocorticoids such as cortisol (Juszczak and Stankiewicz, 2018). So far, not much is known about its function, but it has been shown that *Arl4D* is involved in neurite growth (Yamauchi *et al.*, 2009), adipogenesis (Yu *et al.*, 2011) and actin remodelling (Li *et al.*, 2007). In adult mice, *Arl4d* is expressed in neocortical layer 1 and hippocampus, mostly in cortical interneurons (CIN), whose loss or alteration have been related to neurological

disorders such as autism, schizophrenia, and epilepsy (Rubin *et al.*, 2020). Interestingly, both DMRs are directly and indirectly related to neurological disorders such as ASD. To the best of our knowledge, this is the first report showing significant DMRs in the context of stress measures during pregnancy in newborn saliva samples. Previously Drzymalla et al (Drzymalla *et al.*, 2021), have identified DMRs related to maternal stress but using cord blood.

We found an average of decreased methylation of *DAXX*, which we expect to be associated with increased expression of its protein since the region we assessed is upstream of the *DAXX* promoter.

Previous studies employing EPIC array on neonatal tissues in association with maternal stress and/or anxiety are limited to one study by (Kallak *et al.*, 2021) . These authors investigated DNA methylation in cord blood of newborns exposed to maternal depression and anxiety. They found two DMPs: one upstream of the ATP Binding Cassette Subfamily F Member 1 gene (*ABCF1*) and the other upstream of Homo sapiens integrator complex subunit 10 gene (INTS10). Although the maternal stress model is different from ours, it is interesting to note that ABCF1 was previously associated with ASD in a multi-omics data analysis (Sun *et al.*, 2019). Other comparable studies employing Illumina Infinium 450 BeadChip found mismatching results when studying DNA methylation in infant tissues in relation to maternal stress. Rijlaarsdam et al., showed no associations between PS exposure and neonatal cord blood DNA methylation, whereas Wikenius et al. studying maternal depressive symptoms, found no significant association with 6 weeks infant´s saliva DNA methylation (Rijlaarsdam *et al.*, 2016; Wikenius *et al.*, 2019). In contrast, Non et al. reported the identification of CpGs located in a cluster of genes related to transcription, translation and cell division processes in cord blood of neonates exposed to non-medicated depression or anxiety(Non *et al.*, 2014)

Table 14: General function of protein encoded by annotated genes from the DMPs and DMR analysis

Gene	Function
YAP1	Transcriptional regulator which can act both as a coactivator and a corepressor and is the critical downstream regulatory target in the Hippo signaling pathway which is involved in development, growth, repair, and homeostasis and plays a pivotal role in organ size control and tumor suppression by restricting proliferation and promoting apoptosis
TOMM20	Enables protein-transporting ATPase activity and unfolded protein binding activity. Involved in protein targeting to mitochondrion. Located in mitochondria-associated endoplasmic reticulum membrane and mitochondrial outer membrane.
CSMD1	Predicted to act upstream of or within several processes, including learning or memory; mammary gland branching involved in pregnancy; and reproductive structure development. Predicted to be integral component of membrane. Diseases associated with *CSMD1* include Autism and Schizophrenia.
SSBP4	Predicted to enable single-stranded DNA binding activity. Predicted to be involved in positive regulation of transcription by RNA polymerase II.
SCAMP1	This gene product belongs to the *SCAMP* family of proteins, which are secretory carrier membrane proteins. They function as carriers to the cell surface in post-golgi recycling pathways. Different family members are highly related products of distinct genes and are usually expressed together. These findings suggest that these protein family members may function at the same site during vesicular transport rather than in separate pathways. A pseudogene of this gene has been defined on chromosome 1. Alternative splicing results in multiple transcript variants. Among its related pathways are Innate Immune System.
DAXX	This gene encodes a multifunctional protein that resides in multiple locations in the nucleus and in the cytoplasm. It interacts with a wide variety of proteins, such as apoptosis antigen Fas, centromere protein C, and transcription factor erythroblastosis virus E26 oncogene homolog 1 (*ETS1*). In the nucleus, the encoded protein functions as a potent transcription repressor that binds to sumoylated transcription factors. Its repression can be relieved by the sequestration of this protein into promyelocytic leukemia nuclear bodies or nucleoli. This protein also associates with centromeres in G2 phase. In the cytoplasm, the encoded protein may function to regulate apoptosis. The subcellular localization and function of this protein are modulated by post-translational modifications, including sumoylation, phosphorylation and polyubiquitination.
ARL4D	ADP-ribosylation factor 4D is a member of the ADP-ribosylation factor family of GTP-binding proteins. *ARL4D* is closely like *ARL4A* and *ARL4C* and each has a nuclear localization signal and an unusually high guanine nucleotide exchange rate. This protein may play a role in membrane-associated intracellular trafficking. Mutations in this gene have been associated with Bardet–Biedl syndrome (BBS).

To summarize these results, we conclude that these genes have been related to several regulatory processes of tissue and cellular homeostasis that, when disturbed, can elicit a stress response (Chovatiya and Medzhitov, 2014). Moreover, dysregulation of the expression of *CSMD1* and *YAP1* have been related to disorders of the immune system as well as of the central and autonomic nervous systems.

7.3 EWAS: Biophysical signature of chronic stress in mother-fetus dyad and DNA methylation

No significant CpG sites were observed in association with FSI. This may be either due to insufficient study power or reflect an underlying mechanism. Namely, it is possible that regardless of the non-specific chronic stress perceived by the mother (PSS) and the ANS response of the fetus (FSI), what most impacts the fetal epigenetic profile is the stress generated by specific worries related to pregnancy (captured by PDQ) and the associated high circulating levels of cortisol that is crossing the maternal-fetal placental barrier and impacting the fetal physiology on the scale of epigenome. It is possible that FSI is not the appropriate biophysical correlate of epigenome-level alterations due to PS. In future studies, in order to investigate this relationship further we intend to analyse in more depth the relationship between the neonatal epigenome and the biophysical features of ANS derived from maternal and fetal HRV.

Exploratory Network analysis

To functionally integrate the results, pathways analysis on the significant hits from both the association analysis (DMPs and DMRs) was conducted. Pathway analysis results indicated that the genes whose CpGs presented significant methylation in both the analyses were involved in important biological processes such as Hippo signalling, outer mitochondrial membrane organization. which has been associated with prenatal stress previously.

Borna et al, , found that prenatal stress in rats led to changes in the expression of genes involved in mitochondrial function and biogenesis (Borna *et al.*, 2017). In 2014 a study by Janssen et al found that mitochondrial dysfunction in human placentas was associated with changes in DNA methylation patterns which suggests that altered mitochondrial function could lead to epigenetic modifications that affect placental function and fetal development (Janssen *et al.*, 2014).

While the direct associations between Hippo Signalling outer mitochondrial membrane organisation have not been well understood, these studies do indicate that prenatal stress induced alterations in mitochondrial function and organisation could potentially affect epigenetic regulation, which in turn could affect cellular signalling pathways such as Hippo signalling pathway.

7.4 Strengths

Several strengths are to be highlighted. First, saliva cells are easy and non-invasive to obtain in newborns. Even though epigenetic changes like DNA methylation are cell and tissue-specific, some CpG sites show cross tissue relevance. Changes in peripheral tissues such as saliva could serve as potential biomarkers for disease risk while also giving an advantage of being non-invasively obtainable. Since the primary organ affected by stress is not available in human studies and post-mortem brain tissue samples cannot capture the fluid state of the epigenome (Tylee, Kawaguchi and Glatt, 2013), more accessible samples such as saliva and blood are often used as substitutes. Binder and colleagues showed that saliva reflects better DNA methylation patterns of the brain than methylation in blood, highlighting that saliva is the sample medium of choice for epigenetic studies of psychiatric traits, especially in small children (Smith *et al.*, 2015).

Secondly, we believe that our study's findings can be generalized to the population of pregnant women in most clinics, as this study includes mothers experiencing typical daily stress situations rather than extreme stress exposures.

7.5 Study limitations and methodological issues

Limitations to our study are as follows:

1) Our study has a relatively small sample size, which makes identifying subtle differences in methylation difficult. Originally, we powered the study based on the primary outcome in this project: a difference in the child's mental developmental index at 24 months of age between infants from stressed mothers and controls. Assuming a relevant difference in means of 5 with a SD of 10 (82) (alpha 5% and power (1-beta) 80%), we needed to include 63 stressed mothers in our analyses. To account for 15%, drop out we aimed at for 75 stressed women. Given the figures in the literature (83) we expected around 10% screen-positives on the anxiety screener.

   Since there is no other available study with cohorts of pregnant women and newborn saliva samples obtained, we have not yet been able to verify our findings in an independent cohort. Thus, the novel findings of DMPs and DMRs related to these stress measures should be considered as hypothesis-generating and requiring further validation in larger cohorts.

2) Assessing the DNA methylation levels as soon as the baby is born in association with four stress measures shows the impact of maternal stress on epigenetic marks during the fetal life. However, to serve as early neurodevelopmental biomarkers these marks must be related to the corresponding neurodevelopmental appraisals. Since epigenetic marks are not fixed at birth and methylation patterns change with age, we are presently carrying out a longitudinal study in this cohort. The DNA methylation status at two years of age will allow us to detect the

epigenetic drift defined as the difference in the DNA methylation status over time (Wikenius *et al.*, 2019). Moreover, the 2-years' time point will allow us to test for an association with the neurodevelopmental outcome showing the influence of the environment during the first two years of life on the epigenetic traits and whether the present early neonatal epigenetic differences can serve as biomarkers for early interventions to help restore optimal neurodevelopmental trajectories (Antonelli *et al.*, 2022).

3) The results of the current study are not able to demonstrate if the differences in DNA methylation are a cause of maternal stress or consequence of maternal stress. The causation in this study is not implied and further research using Mendelian randomisation needs to be done to determine if prenatal stress causes changes in epigenetic regulation or vice versa.

8. Conclusion

In this study, we identified novel associations between newborn epigenome-wide methylation levels measured non-invasively in saliva and chronic psychosocial stress experienced by the mother during pregnancy. Our study took advantage of the unique factor of using a non-invasive saliva-based sample for DNA methylation measurement. And at present there is no study analysing the association between salivary DNA methylation from newborns in a genome-wide approach in context with psychosocial prenatal stress. We identified novel associations between newborn epigenome-wide methylation levels measured non-invasively in saliva and chronic psychosocial stress experienced by the mother during pregnancy. We found, in this study that an accumulation of annotated genes in pathways that can be linked to prenatal stress.

The epigenetic changes are mostly related to genes involved in secretion and transportation, nuclear signalling, Hippo signalling pathways, apoptosis, intra-cellular trafficking, and neuronal signalling. Most strikingly, we found that both DMP (such as *CSMD1*) and DMRs (*DAXX* and *ARL4D*) are annotated to genes related to neurological disorders such as ASD, PTSD, and schizophrenia, pointing out to the potential risk of these children to suffer from these disorders.

Taken together, our findings highlight the potential impact of maternal stress during pregnancy on the epigenetic profiles of their offspring and suggests that neonatal saliva maybe a useful non-invasive tool for assessing prenatal exposures and can predict health outcomes. It demonstrates that newborns exposed to chronic stress during gestation show DNA methylation signatures related to neuronal, immune, and endocrine homeostasis. However, further research is necessary to confirm these findings and explore the potential long-term health implications of these epigenetic changes.

Chapter II: Modified profiles of transfer RNA fragments in umbilical cord blood reflect sex-specific stress levels in newborns

1. Introduction

As discussed in the previous chapter it is known that maternal stress during pregnancy is a known risk factor predicting diverse developmental impairments in newborns, but the exact route through which maternal stress affects gene expression in the offspring's brain are not known yet. In Section 1.2 of chapter 1 we had seen that epigenetic changes may one of the mechanisms that mediate transgenerational stress effects (Bale, 2015) and continuing the same line of thought we will look into short non-coding RNAs. MicroRNAs are the most studied short non-coding RNAs and yet another gene regulatory mechanism that are influenced by environmental factors. By studying their potential mis-alterations will help in slimming the gap between prenatal stress and neurodevelopmental disorders.

Non- coding RNA

Non-coding RNAs (ncRNA) have been studied as upcoming biomarkers in various diseases and conditions, including Parkinson's disease and post-traumatic stress disorder (Hanan *et al.*, 2020; Vaknine and Soreq, 2020; Winek, Soreq and Meisel, 2021). There are many types of non-coding RNAs such as small RNAs (18 -200 nts) and long non-coding (>200 nts) RNAs.

Short non-coding RNAs have many roles that involve gene regulation through either RNA interference, RNA modification or spliceosomal involvement. Some types of short non-coding RNAs are microRNAs (miRNAs), small interfering RNAs (siRNAs), transfer RNA (tRNA) and piwi-associated RNAs (piRNAs). Small non-coding RNAs, such as microRNAs (miRs), have been shown to act as transcription regulators by binding to target motifs in hundreds of target genes each, influencing varied biological pathways (O'Connor, Dinan and Cryan, 2012). Short

noncoding RNAs have been seen as promising biomarkers for neurodegenerative diseases (Blanco *et al.*, 2014; Muñoz-Culla *et al.*, 2016).

MicroRNAs

MicroRNAs (miRNAs) are single stranded small non-coding RNA transcripts of 18- 24 nucleotides that act as post-transcriptional gene regulators by either degradation or translation repression and are not translated into proteins (Shukla, Singh and Barik, 2011). miRNAs regulate many different mRNAs as they do not require perfect complementarity to the targets. MiRNA operate at network levels as one miRNA can have many targets and one target can be regulated by many miRNAs. miRNA molecules expression depends on many factors and can be regulated by other epigenetic factors as well. MiRNAs are particularly abundant in the brain and are known to be cell and tissue specific (Kosik, 2006).

MicroRNAs due to their small size are currently known as the means of transgenerational epigenetic inheritance, as they can translocate easily during meiosis and fertilization (Lim and Brunet, 2013). Recently many studies have recognized the importance of differential expression of miRNA-regulated in epigenetic regulation (Bartel, 2004). MiRNA is a promising biomarker for PS studies even though most of the evidence comes from animal studies (Matrisciano *et al.*, 2013; Cao-Lei *et al.*, 2014; Hamada and Matthews, 2019). In a study by Monteleone et al, PS is shown to increase miRNA-133 in the prefrontal cortex and hippocampus in PS male rats (Monteleone *et al.*, 2014). Research suggests that miRNAs are both responsive and susceptible to significant environmental insults such as gestational stress and may increase the offspring vulnerability to stress-related psychopathological conditions (Hollins and Cairns, 2016).

Altered miRNA expression in response to early environment and stress is suggested to prime neuroplasticity and physiological processes (Cohen *et al.*, 2011; Babenko *et al.*, 2012; Babenko, Kovalchuk and Metz, 2012; Zucchi, Yao and Metz, 2012). Therefore, miRNA maybe

critical to mediate the effects of prenatal stress and maternal care on offspring development. However, the exact functional and pathological implications of the dysregulation of these miRNAs in context to prenatal stress are still unknown.

It has been previously shown that altered miRNA expression is associated with many common psychiatric and neurological disorders such as bipolar disorder, schizophrenia, autism, depression and inflammatory conditions (Kocerha *et al.*, 2009; Dinan, 2010; Wu *et al.*, 2010; Voineskos *et al.*, 2011).

Transfer RNA fragments

Transfer RNA fragments (tRFs) are another up-and-coming class and a major source of small non-coding RNAs. They are 16-50nt long non-random cleavage products of transfer RNAs (tRNAs), which differ from one another by their cleavage type, genome origin (nuclear or mitochondrial) and tRNA amino acid family (Loher, Telonis and Rigoutsos, 2017; Magee and Rigoutsos, 2020). tRFs can interact with RNA-binding proteins and/or limit the translation of mRNAs carrying complementary sequence motifs in a similar manner to miRNAs and certain subsets of them were shown to replace miRNAs during extreme stress events (Torres *et al.*, 2019; Winek *et al.*, 2020).

Recently tRFs are being recognized as regulators in many biological processes and modified tRF levels are being shown in different pathologies such as cancer, neurodevelopmental and neurodegenerative disorders (Anderson and Ivanov, 2014; Blanco *et al.*, 2014; Zhu *et al.*, 2018). In animal studies it can be seen that 13 dysregulated tRFS in brain samples of SAMP8 mouse model for AD, from which four were upregulated, indicating the potential role of tRFs in early detection of AD (Zhang *et al.*, 2019). tRFs are generated via the enzymatic degradation of TRNA, independent of the non-transcription.

Relationship between prenatal stress and Acetylcholine

The mechanism of brain response to stress is well studied and described in Chapter 1 of the Introduction. During a stressful event, the brain triggers the release of corticotrophin releasing hormone (CRH) and vasopressin from the paraventricular nucleus of the hypothalamus. This in turn secrets the adrenocorticotrophic hormone (ACTH) which results in the release of glucocorticoids, mainly cortisol in humans, from the adrenals (Groeneweg *et al.*, 2011; Spanagel, Noori and Heilig, 2014). Glucocorticoids is an important regulator of basal and stress-related homeostasis and studies have shown to regulate an array of genes in many organs and tissues (McEwen, De Kloet and Rostene, 1986). But glucocorticoids are not only the sole mediators of stress. Allostasis is the process by which homeostasis is regained after stress and takes place by the interaction between the PFC, amygdala, and the hippocampus via the HPA axis. During this process many neurotransmitters and neuromodulators such as acetylcholine, GABA and glutamate have been observed to be differentially modulated (Sullivan and Gratton, 2002; Herman *et al.*, 2005; Garrido *et al.*, 2013).

In the unavailability of a direct neural connection between the fetus and mother, any alterations in the function of fetal neuroendocrine systems must be mediated by maternal hormones (Rakers *et al.*, 2016). These can be mainly Cortisol, Catecholamines, reactive oxygen species (ROS), ACTH and ß-endorphin. Having discussed the basic pathways of stress such as the HPA axis and ANS system in the previous chapter, we must still not forget the origin of the stress response in the brain per se. This is basically the components of the cholinergic system which plays an important role in cognitive functions and has been shown to be a sensitive indicator of chronic stress exposure in adults (Perry *et al.*, 1999; Gold, 2003; Shenhar-Tsarfaty *et al.*, 2014). But its role as a stress biomarker from the fetal state to the newborns is still not known.

Acetylcholine is an excitatory neurotransmitter and a neuromodulator that plays an important role in the cognitive functions and neural mechanisms of learning and memory formation as

well as increases alertness and attention. It plays a huge part in the central nervous system, peripheral nervous system, and the autonomic nervous system (Hasselmo, 2006; Granger *et al.*, 2016). Acetylcholine releases support and modulates working memory to long term memory and different phases of memory from formation to consolidation and retrieval. The hippocampus, the area of the limbic system within the brain is highly lined with extrinsic and intrinsic inputs from cholinergic neurons (Granger *et al.*, 2016).

ACh is produced by the interaction of the acetyl group from acetyl coenzyme A with choline, which is catalyzed by choline acetyltransferase (ChAT). Vesicular ACh transporter (VAChT) packs ACh in vesicles and is hydrolyzed/inactivated in the serum by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Kalamida *et al.*, 2007; Changeux and Taly, 2008).

Cholinergic signalling (or acetylcholine signalling) is the activation of receptors bound to acetylcholine. Receptors for acetylcholine classify into muscarinic and nicotinic receptors. Nicotinic acetylcholine signalling begins during early development and via acetylcholine it spreads throughout the central nervous system thus activating a range of ligand-gated ion channels (John and Berg, 2015). Therefore, this signalling plays a role not only during development by shaping neural networks but also as an adult where it modulates network function in many ongoing ways (John and Berg, 2015). Wang Y et al , recently showed that cholinergic signalling control excitation and inhibition balance of neuronal networks in the brain(Y. Wang *et al.*, 2021). During development, acetylcholine signalling is necessary for physiological processes which is the formation of the peripheral nervous system (PNS) and central nervous system (CNS), including spontaneous neuronal activity, axon pathfinding and synaptogenesis (Dwyer, McQuown and Leslie, 2009). Thus, any modification or loss in the cholinergic neuron system can lead to major dysfunction of the neural networks and can cause cognitive disorders such as Parkinson’s and Alzheimer’s disease (Ahmed, Knowles and Dehorter, 2019).

It has been previously shown, via human imaging experiments, that depressed individuals have high brain levels of acetylcholine (Ach) compared to control subjects (Saricicek *et al.*, 2012; Esterlis *et al.*, 2013; Hannestad *et al.*, 2013). Exposure to stress also alters the function of cholinergic enzymes such as AChE (Sternfeld *et al.*, 2000). A recent study by Mineur et al, find that the hippocampus is also engaged in acetylcholine signalling modulating stress-related behaviours (Mineur *et al.*, 2022). Additionally, Sailaja et al, , have shown that the acetylcholinesterase gene (AChE) undergoes transcriptional and alternative splicing after stress (Sailaja *et al.*, 2012). The group identified histone deacetylase (HDAC4) as the mediator of stress- inducible changes in AchE promoter in mice hippocampus.

In addition, Kaufer et al , successfully showed that mice exposed to a traumatic event exhibit long lasting changes in the activities of genes involved in acetylcholine metabolism (Kaufer *et al.*, 1998). Their findings help to explain how acute stress in animals could lead to chronic depression, memory loss or other neurodevelopmental disorders as seen in post-traumatic stress disorder and Gulf war syndrome.

But there are not many previous studies that take a deep look into the cholinergic system and prenatal stress. Therefore, we believe that cholinergic system in context of prenatal maternal stress is yet to be examined.

Small RNAs and cholinergic signaling

miRNAs target the cholinergic pathways, and this group of miRNAs are called “CholinomiRs” (Winek, Soreq and Meisel, 2021).

As mentioned above, in recent years it has been highlighted the role of tRFs in health and disease. Many studies at experimental level show that tRFS contribute to ischemic stroke responses (Li *et al.*, 2016), Parkinson disease progression (Magee, Londin and Rigoutsos, 2019) and epilepsy (Hogg *et al.*, 2019). These studies add proof to the current literature

pointing to the role of tRFs in many CNS pathologies but that still needs to be further elucidated.

Considering these exposures to stressful insults, acetylcholine signalling is a major determinant of cognition and is subject to regulation by stressful conditions and therefore makes acetylcholine signalling mechanism crucial mechanism underlying complex behaviours (Vaknine and Soreq, 2020; Winek, Soreq and Meisel, 2021). Measuring activity levels of the circulating acetyl- and butyrylcholinesterases (AChE, BChE) which terminate cholinergic signalling may reflect the global cholinergic tone under chronic stress exposure in adults, as has been shown previously in many diseases and conditions (Assayag *et al.*, 2010; Shenhar-Tsarfaty *et al.*, 2014).

In this chapter we provide a link between maternal prenatal experience and epigenetic reprogramming of the transcriptome by focusing on miRNA and tRFs in serum samples of newborns.

2. Hypothesis and Objective

In the current study, we profiled transfer RNA fragments (tRFs) and microRNAs (miRs) in serum samples from umbilical cord blood of newborns assigned to stress and control groups based on maternal stress estimation, and measured the global cholinergic activity, to assess the possibility that those biomarkers may help to deepen the understanding of the influence of maternal stress during pregnancy on the newborn.

3. Materials and Methods

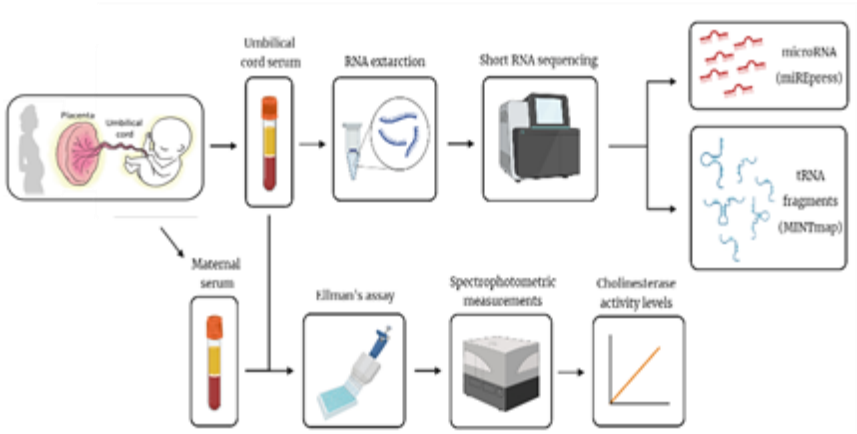


Figure 22:- Flowchart of materials and the methods.

3.1 Serum samples collection

As shown in the Figure 22 maternal and umbilical cord blood serum were collected at birth and stored at -80 ^{o}C. It was then thawed and used to measure the cholinesterase activity and RNA extraction.

3.2 Cholinesterase activity assay

To assess cholinesterase activity, the Ellman's assay (Ellman *et al.*, 1961) was used. Briefly, each serum sample was defrosted in room temperature water and diluted 1:20 in PBS. 10uL of the solution were mixed with 180 µL Ellman's solution, with or without the BChE inhibitor Tetraisopropyl pyrophosphoramide (iso-OMPA), in a 96 flat well plate. All samples were tested in triplicates, and each plate also contained three additional control samples to avoid differences between plates: (1) an "in-house" serum sample, (2) a maternal sample, (3) a

newborn sample. The plates were then incubated for 20 minutes covered in aluminium foil, afterwards 10 µL Acetylthiocholine (ATCh) were added to each well as substrate. Next, the plates were read in kinetic measurements using a Tecan Spark microplate reader (Tecan Group, Männedorf, Switzerland) at room temperature and 405 nm wavelength for 21 Kinetic cycles of one minute each.

The kinetic measurements were retrieved using the SparkControl magellan 1.2 code and were further analysed in MATLAB R2019b (Mathworks, Natick, MA) and R (Team, R Developement Core, 2009) programming software. Average per sample in "mean OD/minute" units was then converted to units of “nmol substrate hydrolysed per minute per ml” using the Beer-Lambert law (A= εlc; molar absorptivity constant ε 2-nitro-5-thiobenzoate= 13,600), multiplied by the serum dilution factor. BChE activity was calculated by subtracting AChE activity from the cholinergic tone (aka cholinesterase activity without the BChE inhibitor).

3.2.1 RNA extraction

120 Umbilical cord blood serum samples were used for extracting of RNA. Each newborn serum sample was defrosted in room temperature and 500uL of the sample were centrifuge at +4^{o}C, 3000g for 5 minutes. Then 200uL taken from the top of the centrifuge sample were used for RNA extraction by the QIAGEN’s miRNeasy Serum/Plasma Advanced Kit, according to manufacture instruction. A subset of 48 newborn RNA samples was chosen from the initial cohort for RNA sequencing, based on their RNA quality and concentration, assessed using NanoDrop and Bioanalyzer, as well as the mother's PSS score. PSS score was selected to reflect extreme stress and control groups, with control < 10 and stress > 19. The samples were than divided into four groups of 12 samples each (shown in Table 15): female newborns from stressed mothers (female_stress), female newborns from control mothers (female_ctl), male

newborns from stressed mothers (male_stress), male newborns from control mothers (male_ctl).

Table 15 Characteristics of RNA sequencing cohort.

	stress		ctl	
sex	*female*	*male*	*female*	*male*
n	12	12	12	12
Age	33	33	33	34.08
PSS	22.92	25.42	6.58	6.83

3.2.2 Sequencing and alignment

Short RNA sequencing and library preparation were done by the National Center for Genomic Technologies at the Hebrew University of Jerusalem using the DIAGENODE D-Plex Small RNA-seq Kit (C05030001), according to manufacturer's instruction. The samples were sequenced with two Illumina NextSeq 500/550 High Output Kit v2.5 (75 Cycles) flow cells (20024906), 24 samples each, on the Illumina NextSeq500 using 1.6ng RNA per sample. Following the sequencing, quality control was assessed again using FastQC (Andrews, 2010) version 0.11.8. Cutadapt (Martin, 2011) was used for extracting the adaptor sequences, followed by Flexbar (Dodt *et al.*, 2012; Roehr, Dieterich and Reinert, 2017) for further bad quality screening and cleaning. tRFs alignment was done using MINTmap(Loher, Telonis and Rigoutsos, 2017) to MINTbase(Pliatsika *et al.*, 2016), whereas for miRs reads which were longer than 25nt or shorter than 16nt were filtered before alignment to miRbase (Griffiths-Jones, 2004; Griffiths-Jones *et al.*, 2006, 2008; Kozomara and Griffiths-Jones, 2011, 2014; Kozomara, Birgaoanu and Griffiths-Jones, 2019) version 21 using miRExpress (Wang *et al.*, 2009) 2.0.

3.2.3 Bioinformatic analysis

Following alignment four samples were disqualified due to low quality, leaving group size accordingly: female_stress (n=10), female_ctl (n=11), male_stress (n=11), male_ctl (n=12). A filtering step to disqualify short RNA with low expression was then done by keeping each short RNA that had reads count higher than zero in at least 4 samples (about 1/3 of a group size), while others which did not match this criterion were filtered out. This resulted in 3442 tRFs and 259 miRs in each downstream analysis. Differential expression analysis was performed using DESeq2 (Love, Huber and Anders, 2014) package in R and findings were deemed statistically significant for $p < 0.05$ after FDR correction using Wald test. Linear discriminant analysis (LDA) was done using MASS (Venables and Ripley, 2002) package in R as well and classification test in the classification learner app in Matlab.

4. Results

4.1 tRF profiles differentiate between stressed/control female but not male newborns

Serum tRF profiles revealed significant differences between stressed and control newborns, while looking at male and female newborns together, due to 23 differentially expressed (DE) tRFs. Looking deeper into the DE tRFs characteristics revealed a pattern of elevated levels of i-tRFs from Aspartate (Asp) tRNAs of nuclear origin and decreased levels of mitochondrial (MT) tRFs (Figure 23).

When looking on DE tRFs in male and female newborns separately, the previous patterns were mimicked and strengthened in the female stress group compared to female control group analysis, which yielded 24 DE tRFs, 11 of them shared with the general stress vs. control analysis described above. Surprisingly, analysis of the male stress group compared to the male control group revealed no DE tRFs, and none of the expression patterns regarding the MT decrease and the nuclear Asp i-tRF increase emerged when investigating males alone (Figure 23 b, c, & d). This result suggests that the patterns seen in the general stress vs. control analysis were a result of the female-specific stress response and may provide evidence for a female-originated stress-specific tRFs pattern shown at birth.

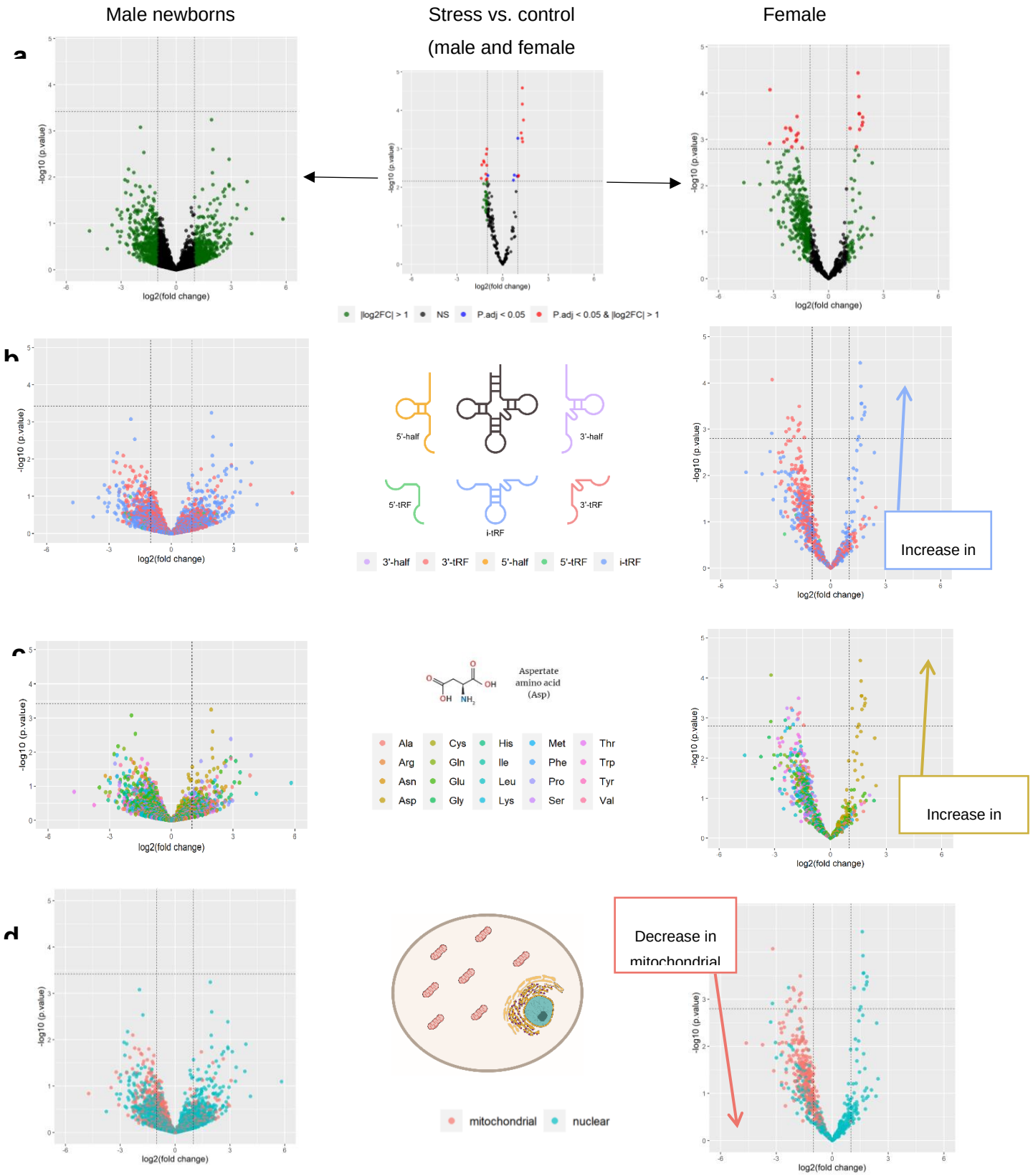


Figure 23 tRFs expression present female specific stress response. (a) volcano plots of the three differential expression contrasts: stress vs. control (male and female together), male stress vs. male control, and female stress vs. female control. (b) volcano plots of the male and female contrasts showing distribution of tRF cleavage type, (c) tRNA family, and (d) genome origin.

4.2 microRNA-based LDA identifies female stress group as distinct

In-depth analysis of miR profilles revealed no DE miRs between the stress and control newborn groups, whether testing male and female together or separately. However, clustering by linear discriminant analysis (LDA) based on the expression of the 50 most variable miRs between the samples highlighted the stressed female newborns as distinct (Figure 24 a). Observing the most influential miRs in each LD component and looking for their targets using miRWalk (Dweep and Gretz, 2015) identified two of the most influential miRs, hsa-miR-223-3p and hsa-miR-23a-3p, as cholino-miRs, meaning that they were predicted to target over 5 cholinergic genes each (Figure 24 b).

hsa-miR-223-3p specifically seemed to contribute to both LD components identifying the female stress group as distinct. This miR displays various connections to inflammation pathways (Huang *et al.*, 2014; Pachathundikandi and Backert, 2018; Yi *et al.*, 2020; Z. Wang *et al.*, 2021), as visible by its cholinergic targets – the inflammation-provoking IL6, and the inflammation-regulating transcription factors STAT1, STAT3, and STAT5a. In addition, hsa-miR-223-3p is known to be upregulated in hepatic differentiation of human umbilical cord Wharton's jelly-derived mesenchymal stem cells (Raut and Khanna, 2017), as well as being elevated in the blood of patients with first-episode schizophrenia (Zhao *et al.*, 2019).

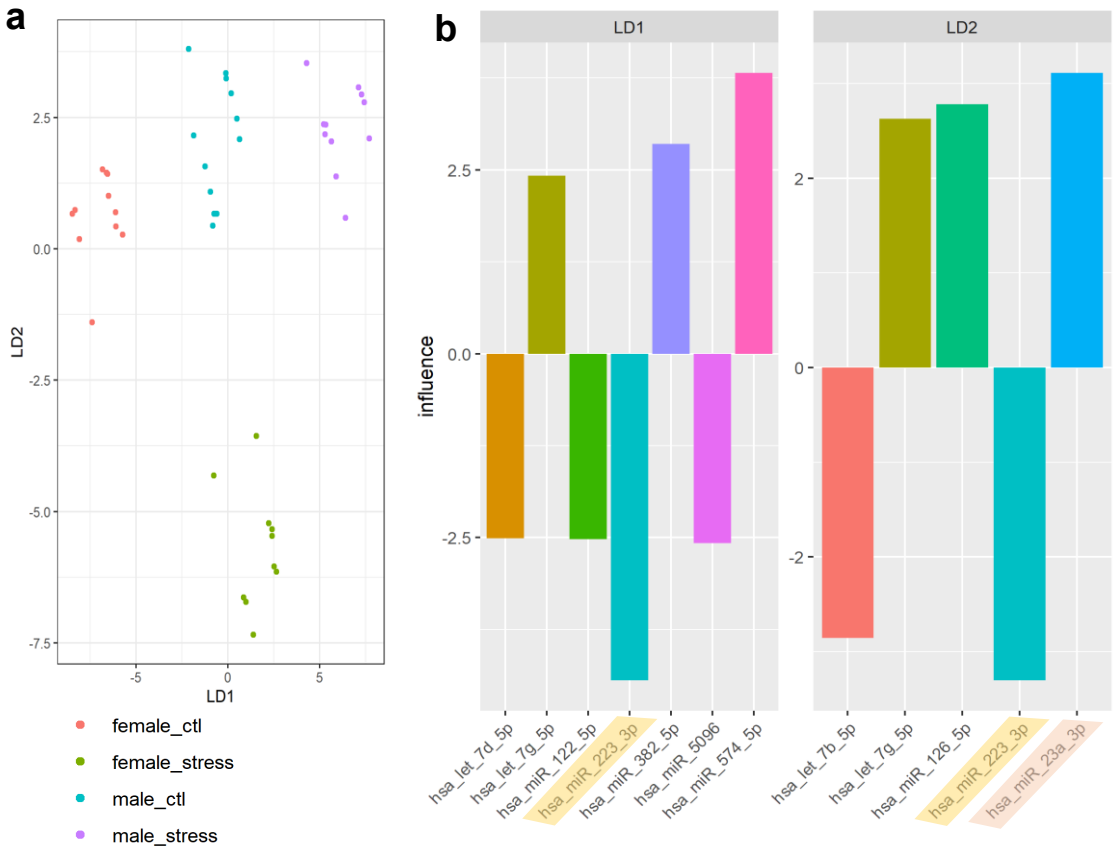


Figure 24 Analysis of miRs expression values identifies female stress groups as distinct. (a) LDA analysis based on the expression of the 50 most variable miRs between samples; (b) barplot of the contribution of the top influencing miRs to LD1 and LD2 LDA components.

4.3 Maternal AChE levels correlate with specific male and female newborn tRFs

Three aspects of cholinesterase activity (AChE activity, BChE activity, and cholinergic tone in total) were tested for both maternal and newborn samples. None of these three cholinergic activity types showed significance differences between maternal and newborn samples of stress and control groups in the RNA sequenced cohort (n=48 maternal and newborn couples; Figure 25 a).

Importantly, however maternal AChE activity of female newborns alone correlated negatively with the levels of the mitochondrial originated cholino-tRF tRF-31-X4J09KZYJMN5D (R = -0.61, p.value = 0.02; Figure 25 b), which was predicted to target over 5 cholinergic genes using

DIANA tools MR-microT (Reczko *et al.*, 2012; Kanellos *et al.*, 2014) too (CHAT, SRSF7, BMPR2, BMPR1A, and CYT1A). Inversely, maternal AChE activity correlated positively with the nuclear Aspartate-originated tRF-25-OSRNLNKSEK levels in male newborns (R = 0.56, p. value = 0.034; Figure 25 c).

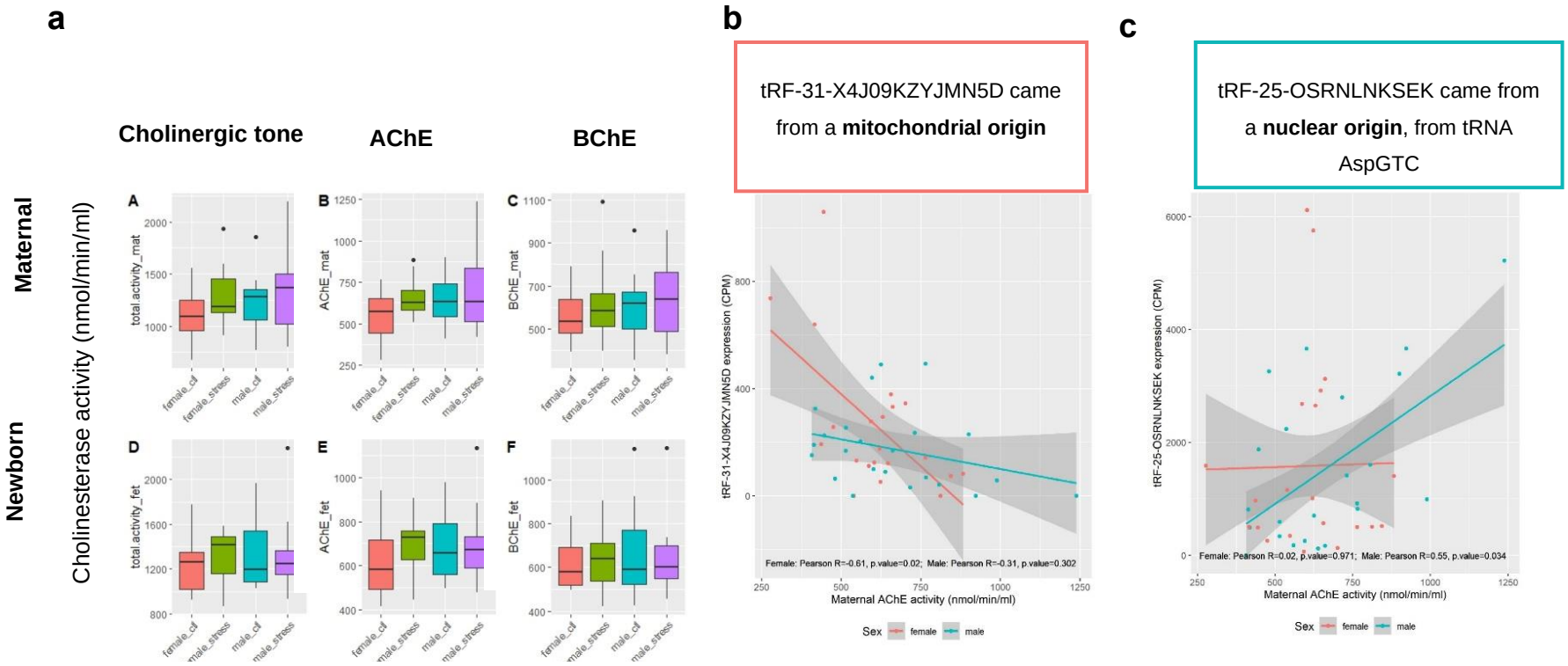


Figure 25 Cholinergic activity correlated with specific tRFs expression patterns. (a) box plot showing the three types of cholinergic activity, AChE, BChE and the general Cholinergic tone which includes both AChE and BChE, in the four sex-specific stress groups for both maternal and newborns serum samples; (b) Pearson corrlation of tRF-31-X4J09KZYJMN5D expression in counts per million (CPM) to the maternal AChE activity in nmol/min/ml; (c) Pearson corrlation of tRF-25-OSRNLNKSEK expression in CPM to the maternal AChE activity in nmol/min/ml;

4.4 Classification to groups based on tRFs expresión

In order to test whether tRFs expression sufficed to classify newborns into stress and control groups based on their mothers' perceived stress during the pregnancy, the Matlab classification learner app was applied to classify the tRFs expression data. Different sets of tRFs were tested in the male and female groups together and apart to determine the best group of tRF markers for the task.

Using cubic SVM classification with 5-fold cross-validation identified four tRFs derived from mitochondrial-originated tRNAs (Figure 26 a), which could separate female newborns into stress and control groups at 100% accuracy (Figure 26 b). The same tRFs were able to differentiate the entire cohort, male and female as one, at 86.4% using the same algorithm (Figure 26 c).

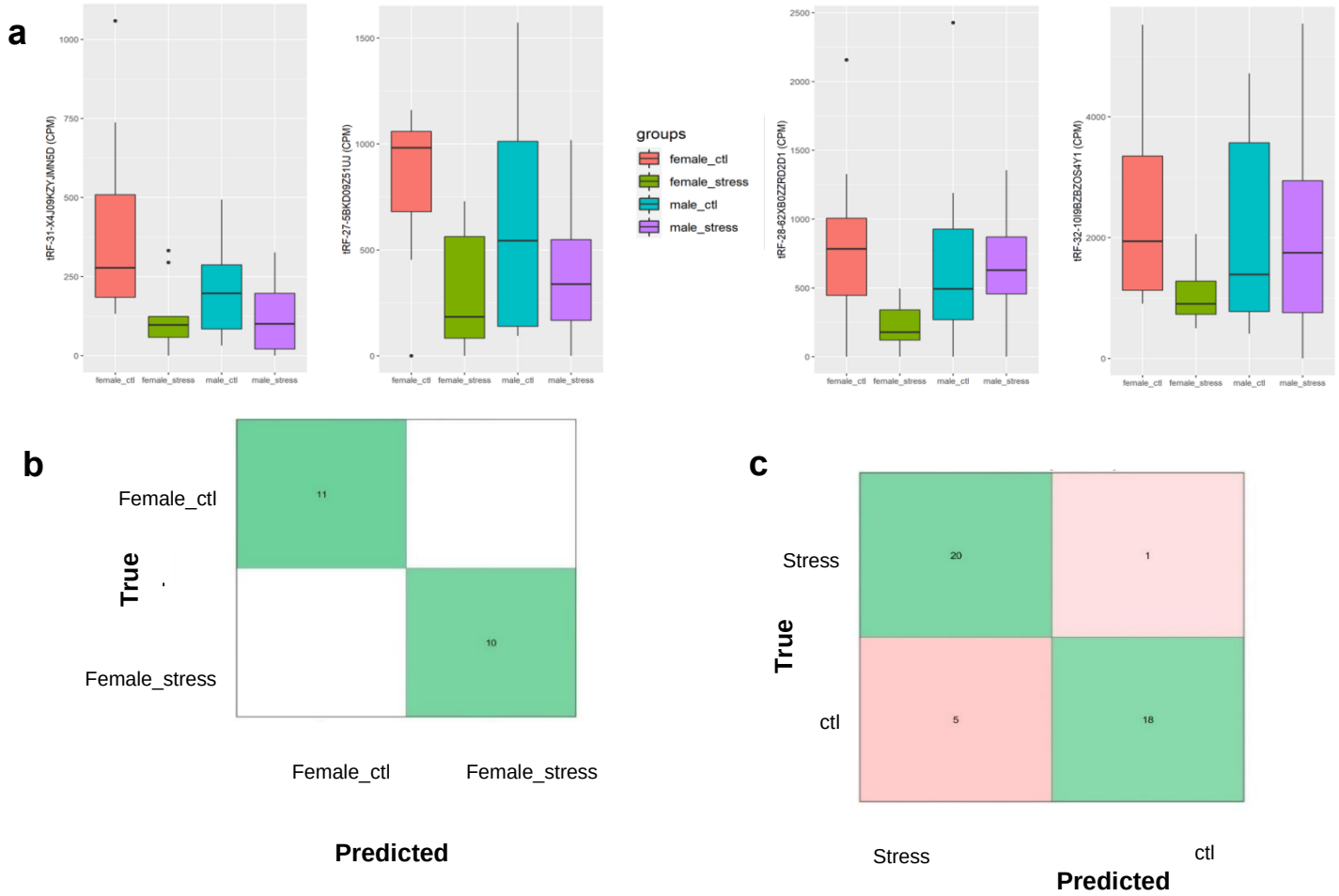


Figure 26 Classification to sex-specific stress groups based on tRFs expression. (a) box plots of the four MT tRFs with expression in CPM. (b,c) showing confusion matrix of cubic SVM algorithm.

5. Discussion

The review by Winek et al is a detailed guided review of how Acetylcholine signaling is related to brain disorders using the molecular fine tuners such as miRNAs and tRFs (Winek, Soreq and Meisel, 2021). But to our knowledge no studies have assessed the combined impact of miRNA and tRF in maternal stress markers specially the septohippocampal cholinergic system which includes acetylcholine. It is further known that, upon stress, there is increase in activity in the neurons including acetylcholine (Gilad, 1987; Mark, Rada and Shors, 1996), and the cholinergic system may be involved in the emotional response (Janowsky, Overstreet and Nurnberger, 1994). It can therefore be hypothesized that prenatal stress-induced changes in this neurotransmitter system might be causally related to neurodevelopmental and behavioural impairments. On a similar note, Day et al, ,1998, (Day *et al.*, 1998) has shown that gestational stress has long-term effects on the development of forebrain cholinergic system and this in turn might be involved in some of the behavioural abnormalities found in PS rats.

From this study, we present a novel picture of sex-specific biomarkers of stress, visible at birth. Female -specific stress was presented by DE tRFs, which showed a specific pattern of downregulation of mitochondrial-originated tRFs and upregulation of nuclear aspartate originated tRFs. This pattern was not visible in the serum of male newborns.

In addition, miRs expression patterns did not differ when subjected to differential expression analysis in either contrast or group. However, using LDA based on the 50 miRs with the biggest variability between samples discovered the female stress group as distinct from the three other groups, male stress, and control as well as female controls.

Finally, cholinesterase activity in maternal and umbilical cord serum from newborns revealed no significant differences between the stress and control groups. However, we discovered a

correlation between cholinergic-related gene targets which inversely linked between tRFs expression and maternal AChE activity, highlighting the cholinergic system's role in sex-specific stress. And possibly indicating a function of the stress-modulated tRFs in regaining cholinergic balance.

It is also already known that biological sex is a strong predictor of many aspects of several neurodevelopmental disorders. This could be due to interactions between several risk factors such as the genetic background and environmental insults and sex-specific development. Our study manages to indicate the sex-specific stress. Nevertheless, given these sex-related differences in cholinergic response system, the miRNA and tRF molecular regulators should be investigated in detail in both males and females.

The results summarized in this chapter should be regarded as an initial step for miRNA/tRF based molecular approach in the cholinergic pathway in the context of prenatal stress.

Investigating the links between prenatal stress and the pathways of the forebrain and behavioural and emotional consequences is vital for gaining deeper insights into the growing infant's psychology and behaviour. These mechanisms have been shown to be significantly affected in prenatal stress models in animal studies and related populations overlap with these models and therefore the consequences.

6. Conclusion

To conclude, our findings identify female newborns as particularly sensitive to prenatal stress and reflect specific cord blood tRF changes, more than miRNA changes, as potential biomarkers and/or therapeutic targets for addressing the damage caused by chronic maternal stress during pregnancy, with potential cholinergic involvement.

General Conclusions

In the first chapter we thought it was important to look into the DNA methylation mechanism and determine the biomarkers of prenatal stress in them. While in the second chapter we investigated yet another epigenetic mechanism the non-coding RNAs. Further understanding of these epigenetic mechanisms and neurodevelopmental phenomena will help determine possible interventions for pregnant women and their infants.

In conclusion despite the varied findings in chapter 1 and chapter 2, overall, both studies provide ample evidence supporting both hypothesis that PS produces changes in the offspring at the newborn stage involving persistent alterations in gene function through changes in DNA methylation and miRNA modifications. These studies show that methylation and small RNA can be correlated with prenatal stress, depression, and anxiety and therefore with newborn's cognitive development.

To assess the long-term stability of these modifications, the upcoming study at two-year old will be very useful and will provide clear evidence.

Implications & Future directions

This thesis contributes to the field of epigenetic epidemiology of mental health in several important ways: First the work presented in this thesis enhances our understanding of tissue-specific epigenetic variation and the utility of surrogate tissue (Saliva) in epigenetic studies of stress phenotypes.

Second this thesis presents novel insights into the field of prenatal stress epigenetics. Thus, it presents one of the first epigenome-wide studies in newborns using saliva as a non-invasive measure.

Albeit these identified findings may represent exciting new targets/markers for early intervention of prenatal stress in newborns, additional research is needed to validate these findings. Further functional studies on the reported genes annotated to the significant CpGs will be useful. Moreover, FELICITy study includes a longitudinal timepoint at two years of age of the same infants. This approach will help answer the question whether newborn epigenetic marks remain constant until 2 years of age, or if DNA methylation levels at these sites change across development. If there is a change in epigenetic mark throughout life it might indicate that it may be responsive to external stimuli, stress in our context, and if there is no change in DNA methylation patterns over time, it could indicate that a regulatory process is acting from birth.

In addition to the longitudinal approach, a meta-analysis of multiple cohorts would strengthen the observed evidence and could also potentially detect further associated CpG sites due to increased sample size.

Illumina Infinium Human Methylation EPIC bead chip only investigates a subset of all the methylated sites in the human genome (from the 28 million known genomic CpG sites). Whole-genome bisulphite sequencing would provide more information on the methylation status. Further developments of statistical methods are necessary for integration of data across multiple platforms and multi 'omics' platforms.

Similarly, tRFs and miRNAs have been implicated in the response to prenatal stress and may serve as potential biomarkers or therapeutic targets. For example, specific tRFs and miRNAs that are differentially expressed in response to prenatal stress could be used as early indicators of prenatal stress-related disorders or as targets for developing new treatments.

In order to identify epigenetic biomarkers for prenatal stress that are both cost-effective and easy to sample, future research should aim to detect and prevent prenatal stress at an early stage using biomarkers identified in our studies and previous research. Lobmaier et al (Lobmaier *et al.*, 2020), have already shown that the differences in FSI in stress and control group, therefore we envision that ANS biophysical markers in combination with HPA axis markers will be powerful tools in predicting the chronic effects of stress on newborns and infants. This will help in early detection of neurodevelopmental trajectories and provide intervention effectively and improve outcomes of pregnancy affected by stress.

List of Publications

1. Sharma, R., Frasch, M.G., Zelgert, C. et al. Maternal–fetal stress and DNA methylation signatures in neonatal saliva: an epigenome-wide association study. Clin Epigenet 14, 87 (2022). PMID: 35836289, https://doi.org/10.1186/s13148-022-01310-x

2. Early Biomarkers and Intervention Programs for the Infant Exposed to Prenatal Stress. Antonelli MC, Frasch MG, Rumi M, Sharma R, Zimmermann P, Molinet MS, Lobmaier SM. Curr Neuropharmacol. 2021. PMID: 33550974, https://doi.org/10.2174/1570159x19666210125150955

3. Zimmermann, P., Antonelli, M.C., Sharma, R. et al. Prenatal stress perturbs fetal iron homeostasis in a sex specific manner. Sci Rep 12, 9341 (2022), PMID: 35662279, https://doi.org/10.1038/s41598-022-13633-z

Oral/Poster Presentations

1. Sharma et al: **FELICITy study: Preliminary insights on the search of biomarkers in blood and saliva.** TUM- IAS General Assembly, Institute of Advanced Study- Technical University Munich, 24 May, 2019.
2. Sharma et al: **FELICITy study:- In search of non-invasive biomarkers of prenatal stress.** Epigenomics of Common Diseases conference, Wellcome Genome Campus, UK, 6-8 November 2019.
3. Sharma et al: **Association between prenatal stress and infant DNA methylation.** European society of Human Genetics 2020 virtual conference, European society of human Genetics, 6-9 June 2020.
4. Sharma et al: **Electrophysiological and Epigenetic Biomarkers of Prenatal stress in a human cohort.** FENS virtual conference 2020, Federation of European Neuroscience Societies. 11-15 July 2020.
5. Sharma et al: **In search of Biomarkers of Maternal stress: FELICITy study.** SAN 2020, Argentinian Society for Neuroscience Research, 7-9 October 2020.
6. Sharma et al: **Epigenetic signatures of Prenatal stress: FELICITy study.** TUM Medical Graduate Center. 2nd Science Day, Technical University Munich, 22. October 2020.
7. Sharma et al: **Autonomic and Epigenetic Signatures of prenatal stress: FELICITy study.** DOHaD ANZ Digital Trainee Conference, The DOHAD society for Australia and New Zealand, 12th November 2020.
8. Sharma et al: **Early Biomarkers in Prenatal stress**, General Assembly of the TUM Institute for Advanced study, Institute of Advanced studies, General Assembly, 24-25th June 2021.
9. Sharma et al: **Effects of maternal stress on neonatal DNA methylation in saliva: FELICITy cohort**; DOHaD society meeting, DOHAD- Canada, 2nd June 2021.
10. Sharma et al: **Epigenome-wide association with maternal stress in newborn saliva: FELICITy study**; SAN 2021, Argentinian Society for Neuroscience Research , 18-22 October 2021.
11. Sharma et al: **Early-Life stress: Molecular mechanisms, cellular effects and Epigenetics**. Society of Neuroscience annual virtual meeting 2021; Society of Neuroscience, 8-11 November 2021

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