Fakultät für Medizin

Geschlechtsspezifische Veränderungen des fetalen autonomen Nervensystems sowie der Eisenhomöostase von Neugeborenen, welche während der Schwangerschaft maternalem Stress ausgesetzt waren

Peter Zimmermann

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Vorsitz: Prof. Dr. Gabriele Multhoff

Prüfer*innen der Dissertation:

1. apl. Prof. Dr. Silvia Lobmaier
2. apl. Prof. Dr. Gerhard Rammes

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Für meine Familie

Inhaltsverzeichnis

Abkürzungsverzeichnis

Abb.	Abbildung
ADHS	Aufmerksamkeitsdefizit-Hyperaktivitätssyndrom
ANS	Autonomes Nervensystem
BMI	Body-Mass-Index
CI	Konfidenzintervall (engl. Confidence Intervall)
CTG	Cardiotokogramm
DAG	Gerichteter azyklischer Graph (engl. Directed acyclic Graph)
fHR	Fetale Herzfrequenz (engl. Heartrate)
FSI	Fetal Stress Index
GEE	Generalized estimating equations
Hb	Hämoglobin
HPA	Hypothalamus-Hypophysen-Nebennierenrinden-Achse (engl. Hypothalamic-Pituitary-Adrenal axis)
ICSI	Intrazytoplasmatische Spermieninjektion (engl. Intracytoplasmic sperm injection)
IDA	Eisenmangelanämie (engl. Iron deficency anemia)
IL	Interleukin
IUGR	Intrauterine Wachstumsretardierung (engl. Intrauterine growth restriction)
IVF	In-Vitro-Fertilisation
mHR	maternale Herzfrequenz (engl. Heartrate)
ML	Machine Learning
NICU	Neugeborenen-Intensivstation (engl. Neonatal intensive care unit)

OR	Odds Ratio
PDQ	Pregnancy Distress Questionnaire
PS	Pränataler maternaler Stress
PSS-10	Perceived Stress Scale-10
SES	Sozioökonomischer (engl. Socioeconomic) Status
SGA	Small for Gestational Age (Geburtsgewicht<10. Perzentile)
SSW	Schwangerschaftswoche
sTfR	Löslicher (engl. Soluble) Transferrinrezeptor
Tab.	Tabelle
taECG	transabdominales Elektrokardiogramm (engl. Electrocardiogram)
TBI	Total Body Iron
ZnPP/H	Zink-Protoporphyrin/Häm-Index

1 Einleitung und Problemstellung

In der Schwangerschaft hat die werdende Mutter ein erhöhtes Risiko für die Entwicklung chronischen Stresses. Physiologische Umstellungen in der neuen potenziell herausfordernden, Situation einer Schwangerschaft können in etwaigen Belastungen für die Frau wie etwa Problemen des Umgangs mit dem eigenen Körperbild, den Hormonschwankungen und Veränderungen der Lebensgewohnheiten resultieren. (Bjelica, Cetkovic, Trninic-Pjevic, & Mladenovic-Segedi, 2018) In der aktuellen Literatur umfasst pränataler mütterlicher Stress (PS) dabei insbesondere: einschneidende Ereignisse im Leben der Mutter, das Durchleben einer Katastrophe und ebenfalls den in dieser Studie betrachteten, rein subjektiv empfundenen psychosozialen Stress in der Schwangerschaft (Van den Bergh et al., 2020).

Schon länger ist bekannt, dass Umwelteinflüsse während der Schwangerschaft wie etwa PS sich auf die Entwicklung des heranreifenden Kindes auswirken können (Edwards, Benediktsson, Lindsay, & Seckl, 1993). Dieses auch als „fetale Programmierung“ (Alyamani & Murgatroyd, 2018; Faa et al., 2016) bezeichnete Phänomen wird insbesondere durch das Autonome Nervensystem (ANS) und die Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA) biochemisch vermittelt (Mulkey & du Plessis, 2019; Rakers et al., 2017). In der Zwischenanalyse dieser Studie konnten Lobmaier et al. (2020) zeigen, dass PS zu einer Beeinflussung der fetalen Herzfrequenz (fHR) führen könnte. Aus diesem Zusammenhang wurde ein nichtinvasiver Biomarker des PS, der sogenannte Fetal Stress Index (FSI), berechnet (Lobmaier et al., 2020). Eine Dysregulation der HPA erhöht nachweislich das Risiko für Beeinträchtigungen des Neugeborenen und für die Entwicklung bestimmter chronischer Erkrankungen wie etwa einer arteriellen Hypertonie, sowie neuropsychiatrischer Auffälligkeiten und Entwicklungsstörungen wie beispielsweise eines Aufmerksamkeitsdefizits-Hyperaktivitätssyndroms (ADHS) (Frasch et al., 2020; Van den Bergh et al., 2020).

Veränderungen während der Schwangerschaft betreffen zudem den weiblichen Eisenstoffwechsel: die Eisen-Absorption ist erhöht und die Konzentration des Eisenregulationshormons Hepcidin erniedrigt, wodurch es zu einer verstärkten Freisetzung von Eisen in die Blutlaufbahn kommt. Innerhalb des zweiten und dritten Trimenons kann sich der mütterliche Eisenbedarf bis auf das Achtfache oder insgesamt 1 g zusätzliches Eisen erhöhen. (Fisher & Nemeth, 2017) Dafür wird vor allem die Zunahme der mütterlichen und fetalen Blutbildung verantwortlich gemacht (Georgieff, 2020). Als Mikronährstoff spielt Eisen, neben seiner Funktion als Bestandteil des Hämoglobins (Hb) für den Sauerstofftransport, auch als Redoxsystem in den oxydierten Eisenformen Fe^{2+}/Fe^{3+} und als Cofaktor vieler enzymatischer Reaktionen des Stoffwechsels eine wichtige Rolle im menschlichen Körper (Fisher & Nemeth, 2017; Mairbaurl & Weber, 2012). Eine Dysregulation der Eisenhomöostase von Mutter oder Kind kann bekanntermaßen zu bleibenden neurologischen Schäden führen (L. Iglesias, Canals, & Arija, 2018). Verschiedene Studien deuten darauf hin, dass auch PS die mütterliche Eisenhomöostase beeinträchtigen könnte (Armony-Sivan et al., 2013; Campbell et al., 2020; Coe, Lubach, & Shirtcliff, 2007; Rendina, Blohowiak, Coe, & Kling, 2018).

Geschlechtsspezifische Unterschiede der Auswirkungen von PS sind gut beschrieben und ihre Berücksichtigung wird in Studien am Menschen empfohlen (Sutherland & Brunwasser, 2018). Die Bedeutung des kindlichen Geschlechts für durch PS induzierte Veränderungen der Eisenhomöostase ist immer noch weitestgehend unklar und wird in der Literatur widersprüchlich diskutiert (Campbell et al., 2020).

Die vorliegende Arbeit befasst sich mit der Hypothese, dass das fetale ANS durch PS beeinflusst wird und dabei geschlechtsspezifische Unterschiede bezüglich des FSIs während des dritten Trimenons, sowie der Eisenhomöostase menschlicher Neugeborener feststellbar sind.

Drei verschiedene Kompartimente der Eisenhomöostase wurden betrachtet:

Erstens wurde Ferritin als klinisch gebräuchlicher Indikator des Speichereisens ausgewählt. Zweitens wurde die Transferrin-Sättigung zur Analyse des Eisentransports untersucht. (Georgieff, 2020) Darüber hinaus wurde drittens das Protein Hepcidin, das aufgrund der direkten Beeinflussung aller anderen Eisenparameter auch als „Master of Regulation" bezeichnet wird, analysiert. Es gilt als potenzieller „Echtzeit"-Biomarker für den momentanen Eisenstatus des menschlichen Körpers. (Hare, 2017) Der Hepcidin-Plazenta-Ferroportin-Achse wird zudem eine Schlüsselrolle bei der Kontrolle des plazentaren Eisentransports zugeschrieben (Lipiński, Styś, & Starzyński, 2013). Mutmaßliche Beziehungen zwischen Eisenparametern, PS und ANS sind in Abb. 1 beschrieben.

Die oben erwähnten Analysen der Eisenhomöostase wurden als sekundäres Studienendprodukt der FELICITy-Studie durchgeführt, einer klinischen Studie mit dem Ziel nichtinvasive Biomarker für Kinder unter dem Einfluss von PS zu ermitteln. Die so gewonnenen Erkenntnisse sollen dabei helfen, betroffene Kinder durch präventive Maßnahmen entsprechend zu fördern, um somit das Risiko späterer Beeinträchtigungen zu reduzieren (Antonelli et al., 2021; Babbar & Shyken, 2016; Hutchon et al., 2019) (Anhang 12).

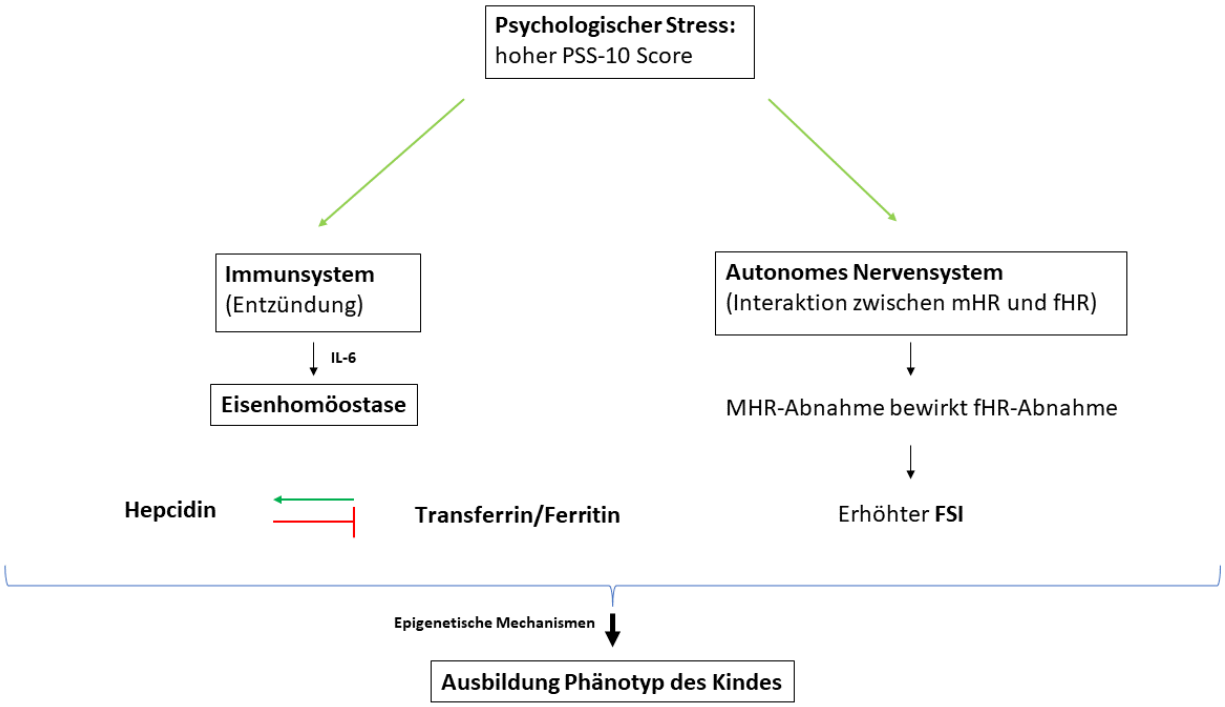

Abbildung 1. Mutmaßlicher Zusammenhang pränatalen maternalen Stresses (PS) mit der Eisenhomöostase und der Interaktion maternaler und fetaler Herzfrequenz

Das vereinfachte Modell der Hepcidin-Plazenta-Ferroportin-Achse basiert auf den Veröffentlichungen von Cortes et al. (2017) und Lobmaier et al. (2020): Hepcidin hat die Schlüsselrolle als Regulator dieses Systems. Es bindet an Ferroportin, verhindert die Freisetzung von Eisen in die Blutbahn und somit auch die Synthese des Transportproteins Transferrin und des Speicherproteins Ferritin. Die Hepcidin-Spiegel im Blut werden stark durch entzündliche Prozesse, insbesondere durch das Zytokin IL-6 (Interleukin-6), beeinflusst. (Hare, 2017) Einen weiteren möglichen Pfad der Prägung des kindlichen Phänotyps stellt die Beeinflussung der Interaktion zwischen mütterlicher und fetaler Herzfrequenz (mHR, fHR), die etwa bei der maternalen Exspiration auftritt, dar (Lobmaier et al., 2020).

Abkürzungen: PSS: Perceived stress scale; FSI: Fetal stress index

2 Methodik

2.1 Ethische Betrachtungen

Das Studienprotokoll der FELICITy-Studie wurde in strenger Übereinstimmung mit den ethischen Prinzipien für medizinische Forschung der Technischen Universität München (TUM) durchgeführt und hat die Zustimmung der „Ethikkommission der Fakultät für Medizin der TUM“ (Registrierungsnummer 151/16S). Die Registrierungsnummer auf ClinicalTrials.gov lautet NCT03389178. Von jeder Studienteilnehmerin wurde ein schriftliches Einverständnis eingeholt (Anhang 1). Dieses erfolgte, nachdem die Probandinnen mittels verschiedener Fragebögen auf das Vorliegen von PS hin selektiert, telefonisch kontaktiert und umfangreich aufgeklärt wurden, sowie zeitlich vor der Erhebung der Daten im dritten Trimenon.

2.2 Studiendesign und -population

Die FELICITy-Studie, eine prospektive Kohortenstudie, wurde von Juni 2016 bis Juli 2019 in der Frauenklinik des „Klinikums rechts der Isar“ der TUM durchgeführt. Das Studienprotokoll wurde von Dr. Silvia Lobmaier der TUM sowie Dr. Marta Antonelli der Universidad de Buenos Aires konzipiert. Von Juni 2016 bis März 2018 wurden die Patientinnen durch die damalige Doktorandin Camilla Zelgert rekrutiert, ab diesem Zeitpunkt durch mich.

Bei der Geburtsanmeldung während des dritten Trimenons (≥ 28. Schwangerschaftswoche (SSW)) erhielten 2000 Patientinnen einen Fragebogen mit der validierten deutschen Version des „Cohen Perceived Stress Scale-10“ (PSS-10) (Klein et al., 2016) (Anhang 2). Der PSS-10 quantifiziert anhand von zehn Items Depressivität, Angst, Abgeschlagenheit und genereller Unzufriedenheit als Symptome generell erfahrenen Stresses (Cohen, Kamarck, & Mermelstein, 1983). Anhand der Ergebnisse des PSS-10 und unter Verwendung eines Cut-off-Scores des PSS

von ≥ 19 wurden die Probandinnen in eine Stress- (SG) und eine Kontrollgruppe (CG) eingeteilt. Der Cut-off-Wert wurde im Rahmen einer Pilotstudie erhoben und analog zu den verwendeten Cut-off-Werten anderer Studiengruppen ausgewählt (Lobmaier et al., 2020).

Folgende Kriterien wurden bei der Gruppeneinteilung angewandt:

Einschlusskriterien:

- Deutschsprachige Frauen mit Einlings-Schwangerschaft, 18 bis 45 Jahre alt, innerhalb des dritten Trimenons (≥ 28. SSW)

In der SG galt zusätzlich folgendes Kriterium:

- PSS-Wert ≥ 19

In der CG galten zusätzlich folgende Kriterien:

- PSS-Wert < 19
- Matching der nächsten gescreenten Patientin mit SG nach mittlerem Gestationsalter, maternalem Alter und Parität (Multi vs. Nulli)

Ausschlusskriterien:

- Minderjährigkeit
- Mehrlingsschwangerschaften
- Schwerwiegende plazentare Störungen (z.B. Intrauterine Wachstumsretardierung)
- Fehlbildungen des Kindes (z.B. Chromosomenaberration)
- Schwere mütterliche Erkrankungen in der Schwangerschaft (American College of, Gynecologists, the Society for Maternal-Fetal, Medicine, & Kilpatrick, 2016)
- Mütterlicher Drogen-/Alkoholabusus
- Frühgeburt (< 37. SSW)

- Nabelschnurblut pH < 7,10

2.3 Datenerhebung

Über den PSS-10 Fragebogen hinaus erhielten die Patientinnen des Weiteren die validierte deutsche Version (Pluess, Bolten, Pirke, & Hellhammer, 2010) des „Prenatal Distress Questionaire“ (PDQ) (Yali & Lobel, 1999) zur Messung schwangerschaftsspezifischen Stresses (Anhang 3). Anhand von zwölf Items wurden schwangerschaftsspezifische Sorgen und Ängste bezüglich der Veränderung des Körpergewichts, Schwangerschaftsbeschwerden, der Gesundheit des Kindes, der Geburt sowie Auswirkungen auf die Beziehung abgefragt. Ein weiterer Fragebogen erfasste sozialdemografische und medizinische Daten (Anhang 4 und 5).

Es folgte die Aufnahme eines Oxford-CTGs sowie zeitgleich eines transabdominalen Elektrokardiogramms (taECG) des Typs AN24 (GE HC/Monica Health Care, Nottingham, UK) bei einer Frequenz von 900 Hz über mindestens 40min. Dr. Hau-Tieng Wu der Duke University unterstützte die Arbeitsgruppe bei der Extraktion der mütterlichen und fetalen RR-Werte aus dem taECG. Dr. Alexander Müller der TUM ermittelte daraufhin die Kopplung von fHR und maternaler Herzfrequenz (mHR) mittels bivariater phasengleichgerichteter Signalmittelung („bivariate phase-rectified signal averaging“) und berechnete den FSI, als Maßzahl für die fetale Antwort auf Abfälle der mHR. FSI-Werte konnten für n = 139 Probandinnen erhoben werden (SG: n = 74; CG: n = 65). Die exakte Berechnung des FSIs wurde durch eine Publikation von Lobmaier et al. (2020) beschrieben.

Es wurden folgende Gewebeproben nach Protokoll (Anhang 6) entnommen:

1. **Serum-Blutproben von Nabelschnur und Mutter:** Entsprechend geschulte Hebammen entnahmen bei der Geburt Nabelschnurblutproben von n = 107 Patientinnen (SG: n = 53; CG: n = 54). Außerdem wurde eine mütterliche Blutprobe extrahiert. Die Serumröhrchen

von Nabelschnur und Mutter wurden zunächst zentrifugiert, die Serumproben in zwei 1.8 ml Cryovial-Röhrchen umgefüllt und mit einem entsprechenden Patientencode pseudonymisiert. Diese wurden zunächst im Kreissaal des Klinikums rechts der Isar für ≤ 1 Jahr bei ≤ (–20 °C) und anschließend im Helmholtz Zentrum München bei –80 °C bis zur Analyse der zu bestimmenden Eisenbiomarker gelagert (Jansen, Beekhof, & Schenk, 2013; LifeSciences, 2022; Pfeiffer & Looker, 2017).

1.1. Die Serum-Eisen-Werte des Nabelschnurblutes wurden photometrisch, Transferrin und Ferritin über Turbidimetrie durch das interne klinische Labor des „Klinikums rechts der Isar " bestimmt.

1.2. Die Hepcidin-Konzentration wurde von mir eigenständig unter Anleitung der PhD-Studentin Ritika Sharma am Helmholtz Zentrum München mit dem kompetitiven „Hepcidin 25 (bioactive) HS ELISA" (DRG Instruments GmbH, Marburg) erhoben. Die Durchführung der Messung erfolgte nach dem beiliegenden Protokoll (Anhang 7).

2. **EDTA-Blutblutproben von Nabelschnur und Mutter:** Zusammen mit dem Serum wurde von Nabelschnur und Mutter ebenfalls EDTA-Blut entnommen. Eines der Nabelschnurblut-EDTA-Röhrchen wurde unmittelbar nach Entnahme direkt durch das interne klinische Labor zur Erstellung eines großen Blutbildes analysiert. (Tab. 1, 4) Die weiteren Proben wurden für zukünftige DNA-Analysen tiefgefroren in der Biobank aufgehoben.

3. **Speichelprobe des Kindes:** Ein Abstrich der Mundhöhle der Neugeborenen wurde durch eine entsprechend geschulte Hebamme unter Verwendung eines Oracollect-DNA-Kits (DNA Genotek, Canada) durchgeführt. Die Aufbewahrung erfolgte bei Raumtemperatur zur späteren epigenomweiten Analyse von DNA-Methylierungsmustern durch Sharma et al. (2020) am Helmholtz Zentrum München (Sharma et al. (2022), Anhang 11).

4. **Haarprobe der Mutter:** Während des Wochenbetts erfolgte die Entnahme einer etwa 3 mm dicken Haarsträhne. Diese wurde entsprechend der Empfehlungen der „Society of Hair Testing“ (SoHT, 2003) an der posterioren Vertex-Region nahe der Kopfhaut entnommen. Die Haarproben lagerten bis zur Analyse in einem Umschlag lichtgeschützt und trocken bei Raumtemperatur. Die Cortisolspiegel der Haare wurden in der Klinischen Biochemie der Universidad de Buenos Aires mittels „Auto-Analyzern“ gemessen (Gonzalez et al., 2019). Eine 3 cm lange Haarprobe misst dabei ungefähr die Cortisol-vermittelte Exposition mit PS innerhalb eines Zeitraums von drei Monaten (S. Iglesias et al., 2015).

5. **Plazentaproben:** Aus der abgenabelten Plazenta wurden zwei ca. 1 cm^3 Proben entnommen und ebenfalls in der Biobank für weitere Analysen tiefgefroren gelagert.

Nach der Geburt erfolgte die Evaluation klinisch relevanter Informationen der Mutter sowie des Neugeborenen wie beispielsweise der Körpermaße, des APGAR-Scores und der Blutgasanalyse (Anhang 5). Außerdem wurden Routinelaborparametern wie etwa das maternale Hb und der Anämie-Status während des Krankenhauseintritts unmittelbar vor der Geburt gemessen (Centers for Disease, 1989).

2.4 Auswertung

2.4.1 Statistik für Gruppenvergleiche

Kontinuierliche Größen wurden mittels des Shapiro-Wilk-Tests auf das Vorliegen einer Normalverteilung überprüft. T-Tests für unabhängige Stichproben wurden für

Gruppenvergleiche zwischen SG und CG durchgeführt, die einer Gaußschen Verteilung unterliegen. Für nicht-normalverteilte Daten wurde der Mann-Whitney-U-Test benutzt. Der Chi-Quadrat-Test wurde für nominale Größen verwendet. Stetige Variablen wurden nach Spearman korreliert und für den Body Mass Index (BMI) wurde mittels einer bivariaten logistischen Regression adjustiert. Dr. Martin Frasch der University of Washington modellierte mittels „Generalized Estimating Equations“ (GEE) die Effekte der Variablen „Geschlecht“ und „Gruppe“ und deren Interaktion (Geschlecht*Gruppe) auf die Eisenbiomarker. Alle statistischen Tests wurden zweiseitig durchgeführt und ein Signifikanzniveau (α) von 0.05 angenommen. Für die statistische Analyse und Visualisierung wurden IBM SPSS Statistics für Windows, Version 25 (IBM Corp., Armonk, NY, USA) und Exploratory Version 6.2.2 benutzt.

2.4.2 Kausale Graphische Analyse

In Kooperation mit Dr. Martin Frasch und Dr. Natasha Wenzel der University of Washington wurde mit DAGitty (www.dagitty.net) (Textor, van der Zander, Gilthorpe, Liskiewicz, & Ellison, 2016) ein gerichteter azyklischer Graph (DAG) erstellt. Dieser beschreibt die kausale Beziehung zwischen der Ausgangsvariablen (PS) und den drei resultierenden Variablen: den Eisenbiomarkern, dem FSI und der deutschen Adaption der „Bayley Scales of Infant and Toddler Development – Third Edition” (Bayley Score), eines pädiatrischen Entwicklungstests (Abb. 8). Als Teil des DAGs erlaubt die Verwendung des Bayley-Scores, welcher für die aktuelle Kohorte noch nicht berechnet ist, die Adjustierung für unbeobachtete Variablen, um deren möglichen Einfluss auf die resultierenden Eisenparameter-Variablen zu verhindern. Der DAG dient der Visualisierung struktureller Beziehungen zwischen den Variablen und der Minimierung des Bias der statistischen Analyse (Greenland, Pearl, & Robins, 1999).

3 Ergebnisse

3.1 Rekrutierung der Studienteilnehmerinnen

728 Studienteilnehmerinnen gaben ihren PSS-10 Fragebogen zurück. Unter Berücksichtigung oben genannter Ein-/ und Ausschlusskriterien wurden nachfolgend 164 schwangere Frauen für die Studie rekrutiert sowie eine SG mit n = 79 Patientinnen und eine CG mit n = 85 Patientinnen gebildet. (Abb. 2)

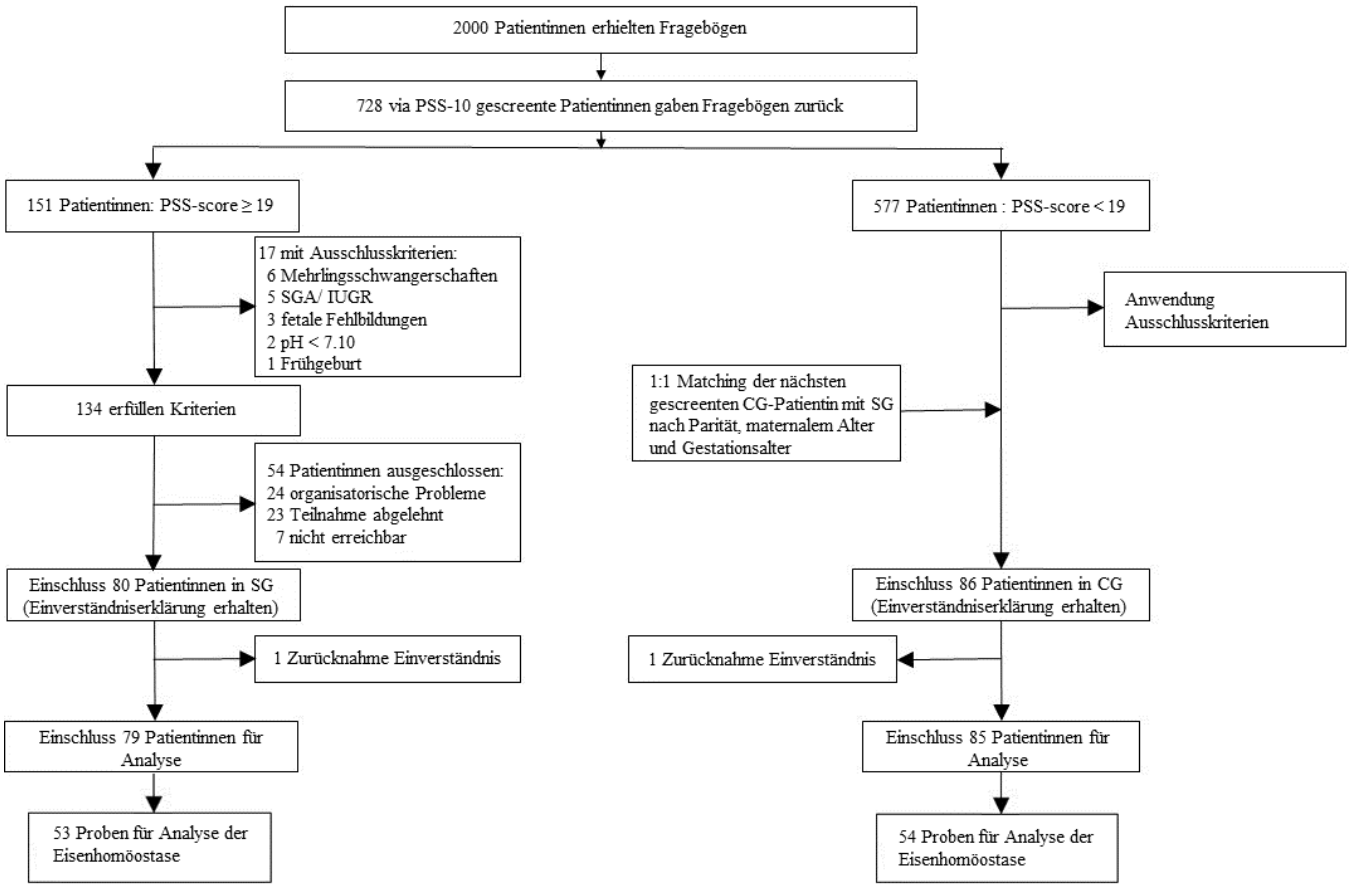

Abbildung 2. Flussdiagramm der Patientenrekrutierung

Abkürzungen: SG: Stressgruppe; CG: Kontrollgruppe; PSS: "Perceived stress scale"; SGA: Small for gestational age; IUGR: Intrauterine Wachstumsretardierung

3.2 Sozialdemografische Daten und perinatales Outcome

Das Alter der Studienteilnehmerinnen betrug durchschnittlich 33.0 (±4.4) Jahre (Abb. 3) und das mediane Gestationsalter 36.4 (35.3–37.4) SSW (Abb. 4).

Folgende charakteristische Unterschiede ließen sich zwischen SG und CG feststellen: In der SG gab es mehr Raucherinnen und Patientinnen mit Gestationsdiabetes als in der CG. Im Vergleich mit der CG waren Schwangerschaften von Probandinnen der SG zudem öfters nicht geplant und es wurde seltener eine in-vitro Fertilisation angewendet. Ähnlich wie in der Zwischenanalyse der vorherigen Studie von Lobmaier et al. (2020) hatten Probandinnen in der SG im Vergleich zu Frauen in der CG einen höheren BMI und seltener einen Universitätsabschluss sowie seltener ein monatliches Einkommen von mehr als 5000€. In der SG wurde häufiger ein Kaiserschnitt durchgeführt als in der CG. Dieser Gruppenunterschied war nach Adjustierung für den BMI allerdings nicht mehr feststellbar (OR 1.82; 95% CI 0.87–3.78; p = 0.11).

Die finalen Ergebnisse dieser Arbeit zeigten keine Gruppenunterschiede bezüglich mütterlicher Haar-Cortisol-Konzentration und arteriellen Nabelschnurblut pO2. Zwischen SG und CG gab es keine Unterschiede bezüglich der angewandten Matching-Kriterien. (Tab. 1) Die demografischen Daten der ausgeschlossenen Patientinnen unterschieden sich im Mittel nicht von denen der Studienteilnehmerinnen.

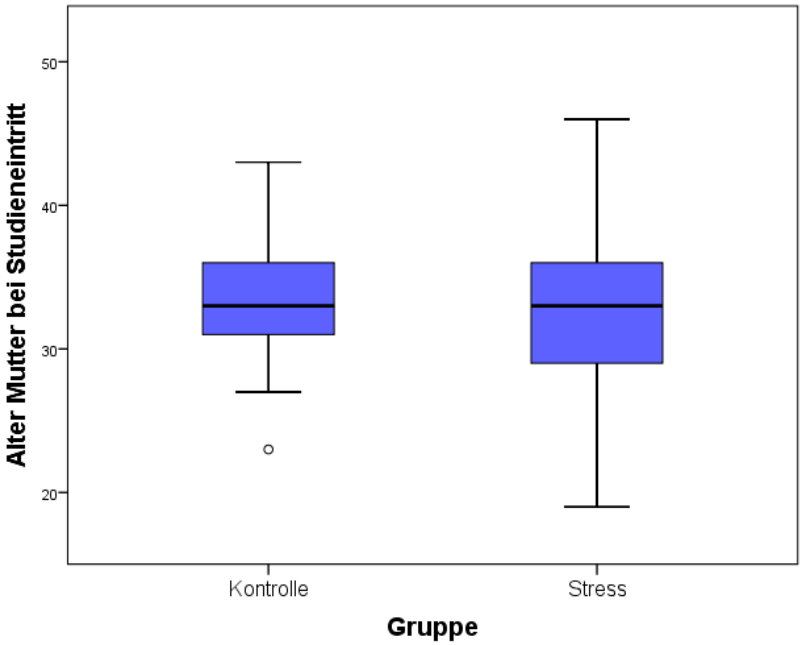

Abbildung 3. Boxplot des Matching-Kriteriums: „Alter der Mutter bei Studieneintritt“

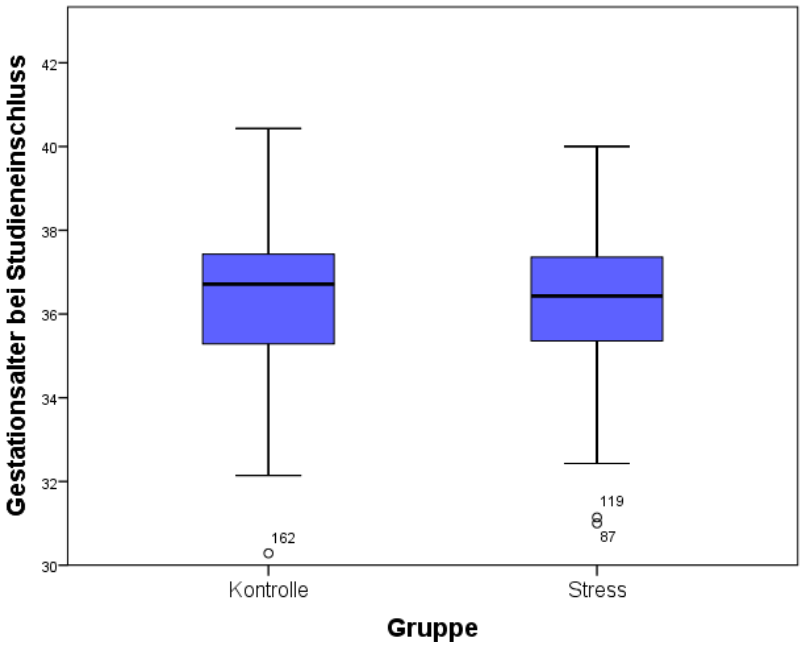

Abbildung 4. Boxplot des Matching-Kriteriums „Gestationsalter bei Studieneintritt“

Tabelle 1. Charakteristika der Studienkohorte

Parameter	**CG**	**SG**	
	n=85	n=79	p
Baseline			
Gestationsalter beim Screening [Wochen]	34.0 (33.3–35.0)	34.0 (32.6–34.9)	0.304
Gestationsalter beim Studieneinschluss [Wochen]	36.7 (35.2–37.6)	36.4 (35.3–37.4)	0.612
Alter der Mutter beim Studieneinschluss [Jahre]	33.4 (±3.7)	32.7 (±5.1)	0.307
BMI bei Studieneinschluss [kg/m²]	26.3 (24.4–28.9)	27.8 (25.3–34.6)	**0.010**
Prägestationaler BMI [kg/m²]	21.5 (20.2–23.5)	23.3 (20.7–27.5)	**0.013**
PSS-10 Score	9 (6–12)	22 (20–24)	**<0.001**
PDQ Score	7 (5–11)	15 (11–21)	**<0.001**
Mütterliches Haar-Cortisol [pg/mg]	88 (40–133)	97 (61–165)	0.104
Europäisch/Kaukasisch	78 (92)	73 (92)	0.879
Verheiratet	67 (80)	55 (70)	0.136
Universitätsabschluss	65 (77)	46 (58)	**0.013**
Einkommen > 5000€/Monat	49 (58)	28 (35)	**0.004**
Rauchen	1 (1)	7 (9)	**0.022**
Multiparität	37 (44)	38 (48)	0.557
Geplante Schwangerschaft	75 (93)	53 (67)	**0.001**

IVF / ICSI	9 (11)	2 (3)	**0.039**
Gestationsdiabetes	2 (2)	12 (15)	**0.003**
Autoimmunerkrankungen	6 (7)	13 (16)	0.060
Arbeitsverhältnis beim Screening	3 (4)	4 (5)	0.502
Eisensubstitution	31 (36)	35 (44)	0.307
FSI*	–0.01 ((–0.36)–0.34)	0.38 ((–0.22)–0.75)	**0.024**
Perinatales Outcome			
Gestationsalter bei Geburt [Wochen]	39.9 (39.0–40.6)	39.5 (38.6–40.6)	0.148
Geburtsgewicht [g]	3526.9 (395.1)	3484.0 (463.0)	0.526
Perzentile Geburtsgewicht [%]	49.0 (28.3–71.8)	55.0 (28.0–74.3)	0.863
Länge [cm]	52.9 (±2.5)	52.8 (±2.6)	0.919
Kopfumfang [cm]	35 (34–36)	35 (34–36)	0.412
Sectio Caesarea	17 (20)	27 (35)	**0.035**
Geburtseinleitung	15 (18)	19 (24)	0.310
Weibliches Geschlecht	41 (48)	30 (38)	0.137
5-min Apgar < 7	3 (4)	2 (3)	0.691
NICU	3 (4)	3 (4)	0.912
Ergebnisse der arteriellen Nabelschnurblut-Analyse			

Base Excess [mmol/L] (n=78 CG, n=72 SG)	–5.5 (±3.3)	–5.2 (±3.0)	0.557
Lactat [mmol/L] (n=53 CG, n=50 SG)	4.4 (3.0–5.3)	3.8 (3.0–4.8)	0.317
Glukose [mg/dL] (n=57 CG, n=51 SG)	84.0 (64.0–98.0)	71.0 (63.5–91.5)	0.338
pH (n=81 CG, n=77 SG)	7.26 (±0.09)	7.28 (±0.08)	0.203
PO2 [mmHg] (n=66 CG, n=57 SG)	21.1 (16.7–26.6)	18.4 (13.6–23.5)	0.102
PCO2 [mmHg] (n=69 CG, n=64 SG)	50.8 (±10.2)	49.4 (±9.2)	0.382
Leukozyten [G/L] (n=53 CG, n=49 SG)	14.6 (11.9–17.4)	13.3 (10.2–17.6)	0.291
Neutrophile [%] (n=53 CG, n=48 SG)	51.0 (46.5–56.0)	54.0 (47.0–61.0)	0.249

Die Daten beschreiben Mittelwerte (Standartabweichung) mittels t-Test, den Median (Interquartilsabstand) mittels Mann-Whitney-U-Test oder n (%) mittels Chi-Quadrat-Test. Gruppenunterschiede mit p-Wert < 0.05 sind **fett** markiert.

Abkürzungen: CG: Kontrollgruppe; SG: Stressgruppe; PSS: Perceived stress scale; PDQ: Prenatal distress questionnaire; BMI: Body-Mass-Index; NICU: Neonatal intensive care unit; ICSI: Intracytoplasmic sperm injection; IVF: In-Vitro-Fertilisation

* Fehlende Werte für 11 CG und 14 SG

3.3 Genereller und schwangerschaftsspezifischer Stress

Der mediane PSS der SG betrug 22 (Interquartilsabstand: 20–24) und in der CG 9 (6–12) ($p < 0.001$). In der SG zeigte sich nur eine schwache bis keine Korrelation zwischen PSS-10 und PDQ ($R^2 = 0.04$; CI $-0.04 \leq R^2 \leq 0.12$; $p < 0.001$) im Vergleich zur Betrachtung der gesamten Kohorte ($R^2 = 0.35$; CI $0.23 \leq R^2 \leq 0.47$; $p < 0.001$) (Abb. 5).

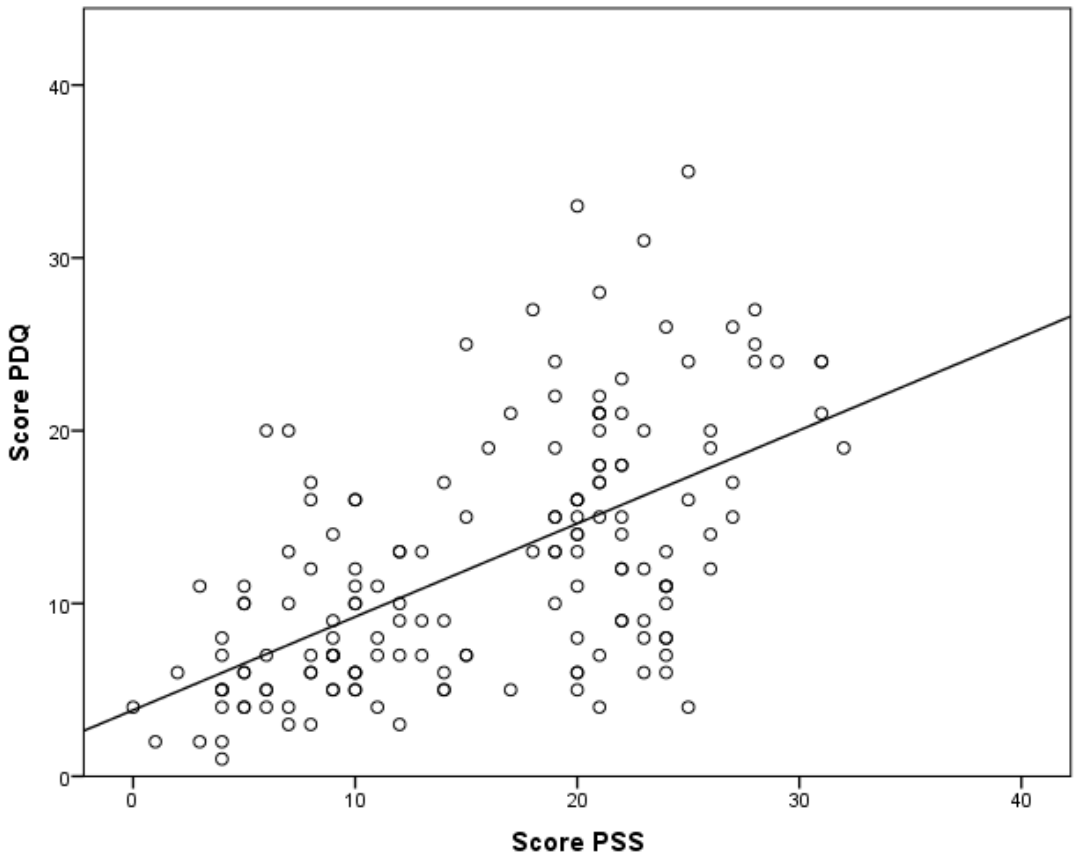

Abbildung 5. Korrelation zwischen PSS-10 und PDQ

Moderate positive Assoziation zwischen dem allgemeinen Stress-Fragebogen PSS-10 mit dem schwangerschaftsspezifischen Fragebogen PDQ ($R^2 = 0.35$; CI $0.23 – 0.47$; $p < 0.001$)

In der SG erhielten im PDQ insbesondere Ängste und Sorgen um physische Stressfaktoren „mäßig“ bis „sehr starke“ Zustimmung. Häufig genannte Stressoren waren dabei insbesondere

der Geburtsprozess (57 %), körperliche Symptome wie Übelkeit und Erbrechen (55%) sowie die Ernährung des Babys (47%). Psychische und soziale Bedenken wie eine mögliche problematische Entwicklung der Beziehung zu anderen Menschen (29%), Vater (24%) und Kind (11%) erhielten in unserer Kohorte vergleichsweise selten eine „mäßige" bis „sehr starke" Zustimmung. Etwa ein Drittel der Frauen fühlten sich durch die Gewichtszunahme und die Veränderung des Körperbildes in der Schwangerschaft „mäßig" bis „sehr stark" beeinträchtigt. (Tab. 2)

Im PSS-10 gaben Schwangere der SG besonders häufig „manchmal" bis „sehr oft" das subjektive Gefühl von Nervosität und Stress an (94%). Hohe Werte in den gleichen Kategorien erreichten zudem das Gefühl (89%) und ebenso der Ärger (87%) darüber wichtige Dinge im Leben nicht beeinflussen zu können. (Tab. 3)

Tabelle 2. Items des PDQs in der Stressgruppe (n = 79) mit Score > 1 nach Häufigkeit

Item Sorgen und Ängste während der Schwangerschaft über:	**Absolute Häufigkeit**	**Prozentuelle Häufigkeit (%)**
Wehen und Geburt	45	56.9
Körperliche Symptome	43	54.5
Ernährung	37	46.8
Geburt eines kranken Kindes	36	45.6
Gefühlsmäßige Höhen und Tiefen	35	43.7
Frühgeburt	31	39.3
Gewichtszunahme	29	36.8
Veränderung Figur und Körperumfang	29	36.8
Versorgung des Babys nach Geburt	26	32.9
Veränderung von Beziehungen mit anderen Menschen	23	29.2

Veränderung Beziehung zu Vater des Kindes	19	24.0
Kein emotionaler Bezug zu Baby	9	11.4

Tabelle 3. Items des PSS-10 in der Stressgruppe (n = 79) mit Score > 1 nach Häufigkeit

Item	Absolute Häufigkeit	Prozentuelle Häufigkeit (%)
Gefühl nervös und "gestresst" zu sein	74	93.7
Gefühl wichtige Dinge nicht beeinflussen zu Können	70	88.6
Ärger über fehlende Beeinflussung wichtiger Dinge	69	87.3
Erregung über Eintritt etwas völlig Unerwarteten	67	84.8
Gefühl mit anstehenden Aufgaben und Problemen nicht richtig umgehen zu können	64	81.0
Gefühl mit Ärger im Leben nicht klar zu kommen	60	76.0
Gefühl alles nicht mehr im Griff haben	61	77.3
Dinge entwickeln sich nicht nach eigenen Vorstellungen	58	73.5
Unsicherheit im Umgang mit anstehenden Aufgaben und Problemen	54	68.4
Probleme haben sich so angestaut, dass Sie nicht mehr bewältigbar sind	53	67.1

3.4 Maternale und fetale Eisenhomöostase

10.4% der eingeschlossenen Frauen waren bei der Blutbildkontrolle kurz vor der Geburt anämisch (Hb < 11 g/dL). Ohne Berücksichtigung des kindlichen Geschlechts konnten keine Unterschiede zwischen SG und CG bezüglich der gemessenen fetalen Eisenparametern, mütterlicher Eisensubstitution, fetaler und mütterlicher Hämoglobinkonzentration sowie MHC, MVC und Anämie-Status festgestellt werden (Tab. 4). Die Ergebnisse, welche die Eisenhomöostase betreffen, wurden als Poster auf der „2020 DOHaD ANZ Digital Trainee Conference“ präsentiert und diskutiert (Anhang 8).

Tabelle 4. Eisenparameter

Charakteristika	**CG** n=54	**SG** n=53	p
Nabelschnurblut Serum-Eisen [µg/dL]	151.5 (±37.3)	141.4 (±38.5)	0.172
Nabelschnurblut Serum-Transferrin [mg/dL]	176.3 (162.2–205.9)	186.6 (165.8–217.0)	0.348
Nabelschnurblut Serum-Transferrinsättigung [%]	59.5 (±17.6)	54.8 (±19.3)	0.189
Nabelschnurblut Serum-Ferritin [µg/L]*	242.4 (140.6–329.6)	176.0 (106.4–267.0)	0.134
Nabelschnurblut Serum-Hepcidin [ng/dL]	23.6 (13.4–39.24)	18.9 (9.2–36.9)	0.184
Nabelschnurblut Plasma-Hämoglobin [g/dL]**	15.6 (±1.6)	15.7 (±1.6)	0.832
Nabelschnurblut Plasma-MCV [fL]**	104 (101–106)	104 (100–107)	0.734
Nabelschnurblut Plasma-MCH [pg]**	35 (34–35)	35 (34–35)	0.605

	n=74	n=71	p
Maternales pränatales Plasma-Hämoglobin [g/dL]	12.3 (1.0)	12.2 (1.1)	0.376
Maternales pränatales Plasma-MCV [fL]	87 (84–90)	88 (83–90)	0.766
Maternales pränatales Plasma MCH [pg]	30 (28–31)	30 (28–31)	0.862
Maternales pränatale Anämie: Hb<11g/dL	8 (10.8)	9 (12.7)	0.537
Maternales postnatales Plasma-Hämoglobin [g/dL]***	11.1 (1.5)	10.8 (1.3)	0.164
Maternales postnatales Plasma-MCV [fL]***	87 (85–91)	88 (85–91)	0.669
Maternales postnatales Plasma-MCH [pg]***	30 (29–31)	30 (28–31)	0.683

Die Daten beschreiben Mittelwerte (Standartabweichung) mittels t-Test, den Median (Interquartilsabstand) mittels Mann-Whitney-U-Test oder n (%) mittels Chi-Quadrat-Test.

* fehlende Werte für 1 SG

** fehlende Werte für 1 CG und 4 SG

*** fehlende Werte für 3 SG

3.5 FSI

Die Analyse der Interaktion von mHR und fHR ergab einen höheren FSI in der SG als in der CG (0.38 ((–0.22)–0.75) versus –0.01 ((–0.36)–0.34); p = 0.024) (Tab. 1). Der FSI zeigte keine Korrelation zu den gemessenen fetalen Eisenparametern, auch nicht unter Berücksichtigung des Geschlechts (Tab. 5). Die FSI-Ergebnisse wurden auf der „Second International Summer School on TSPPM-2021“ innerhalb des Zeitraums 16. bis 23.07.2021 als Poster präsentiert und diskutiert. (Anhang 9)

Tabelle 5. Geschlechtsspezifische lineare Regression von FSI und Serum-Eisenparametern

Charakteristika	**Männliche Neugeborene** (n=58)		**Weibliche Neugeborene** (n=49)	
	R^2	p	R^2	p
Eisen [µg/dL]	0.001	0.78	<0.001	0.96
Transferrinsättigung[%]	0.002	0.89	<0.001	0.94
Ferritin [µg/L]*	0.003	0.96	0.048	0.13
Hepcidin [ng/dL]	0.028	0.23	0.004	0.84

Die Korrelationen wurden mittels Spearman-Korrelation berechnet.

* fehlende Werte für 1 SG mit männlichem Geschlecht

3.6 Geschlechtsspezifische Unterschiede

In männlichen Neugeborenen wurden geschlechtsspezifische Gruppenunterschiede der Eisenhomöostase nachgewiesen. Die Transferrinsättigung des Nabelschnurblutes männlicher Neugeborener der SG war im Vergleich zu dem Wert männlicher Neugeborener der CG niedriger, selbst unter Berücksichtigung der Eisensubstitution. Für die Ferritin-Konzentration konnte eine Tendenz zu niedrigeren Werten männlicher Neugeborener der SG festgestellt werden (p =0.069, Tab. 6). Durch das GEE-Model konnte gezeigt werden, dass das Geschlecht ein signifikanter Effekt-Modifikator ist, der die Gruppenunterschiede bei Ferritin maßgeblich hervorbringt (p = 0.038, Abb. 6). Eine Tendenz ließ sich mit der gleichen Methode auch für die Transferrin-Sättigung zeigen (p = 0.070, Abb. 7). Für Hepcidin konnten keine signifikanten geschlechtsspezifischen Unterschiede gezeigt werden.

Das maternale Haar-Cortisol in der SG von Müttern weiblicher Neugeborener wies eine Tendenz zu höheren Werten auf (p = 0.073, Tab. 6). Die Gruppenunterschiede des FSIs lassen

sich beinahe ausschließlich durch den Gruppenunterschied der männlichen Neugeborenen erklären. (Tab. 6) Die Ergebnisse geschlechtsspezifischer Unterschiede der Eisenhomöostase wurden als Journal-Artikel publiziert. ((Zimmermann, 2022), Anhang 10)

Tabelle 6. Geschlechtsspezifische Interaktion pränatalen Stresses mit Eisenbiomarkern

Charakteristika	CG	SG	
Männliche Neugeborene	n=26	n=32	p
FSI (n=35 CG, n=43 SG)	–0.13 ((–0.45)–0.31)	0.30 ((–0.18)–0.61)	**0.050**
Mütterliches Haar-Cortisol [pg/mg] (n=35 CG, n=36 SG)	115 (14–146)	124 (40–161)	0.466
Nabelschnurblut Ferritin [µg/L]*	229.7 (113.9–429.6)	149.6 (96.8–234.0)	**0.069**
Nabelschnurblut Transferrinsättigung [%]	63.4 (±17.7)	52.9 (±20.2)	**0.041**
Nabelschnurblut Hepcidin [ng/dL]	26.1 (11.8–41.8)	17.0 (10.5–30.7)	0.184
Weibliche Neugeborene	n=28	n=21	p
FSI (n=39 CG, n=22 SG)	0.10 (±0.55)	0.27 (±0.84)	0.394
Mütterliches Haar-Cortisol [pg/mg] (n=32 CG, n=21 SG)	88 (46–119)	122 (67–180)	**0.073**
Nabelschnurblut Ferritin [µg/L]	218.2 (±84.8)	243.1 (±130.0)	0.423
Nabelschnurblut Transferrinsättigung [%]	55.9 (±17.0)	57.8 (±17.8)	0.703
Nabelschnurblut Hepcidin [ng/dL]	22.7 (14.1–37.7)	20.9 (6.0–37.1)	0.599

Die Daten beschreiben Mittelwerte (Standartabweichung) mittels t-Test, den Median (Interquartilsabstand) mittels Mann-Whitney-U-Test. Gruppenunterschiede mit p-Wert < 0.1 sind in **fett** gekennzeichnet.

* fehlende Werte für 1 SG

Abbildung 6. Violine Plot des geschlechtsabhängigen Unterschiedes des Ferritin-Spiegels

GEE-Modellierung der Effekte von Geschlecht und Gruppe und deren Interaktion (Geschlecht*Gruppe) auf Ferritin. GEE Ferritin: Geschlecht*Gruppe **p = 0.038**

Abkürzungen: GEE: Generalized estimating equations; SG: Stressgruppe; CG: Kontrollgruppe

Abbildung 7. Violine Plot des geschlechtsabhängigen Unterschiedes der Transferrinsättigung

GEE-Modellierung Effekte von Geschlecht und Gruppe und deren Interaktion (Geschlecht*Gruppe) auf die Transferrinsättigung. GEE Transferrinsättigung: Geschlecht*Gruppe p = 0.070

Abkürzungen: GEE: Generalized estimating equations; SG: Stressgruppe; CG: Kontrollgruppe

3.7 Abschätzung kausaler Beziehungen der Variablen

„Mütterliches Alter“ und „Sozioökonomischer Status“ (SES) oder „Mütterliches Alter“ und „Bildung“ konnten innerhalb des DAGs als Störgrößen, bezogen auf den Pfad *PS* → *Nabelschnurblut Ferritin* and *PS* → *Bayley Score* identifiziert werden. In unseren Daten wird der SES durch die binäre Variable „Einkommen > 5000€/ Monat“ und die Bildung der Mutter durch „Universitätsabschluss“ beschrieben. (Abb. 8) ((Zimmermann, 2022), Anhang 10)

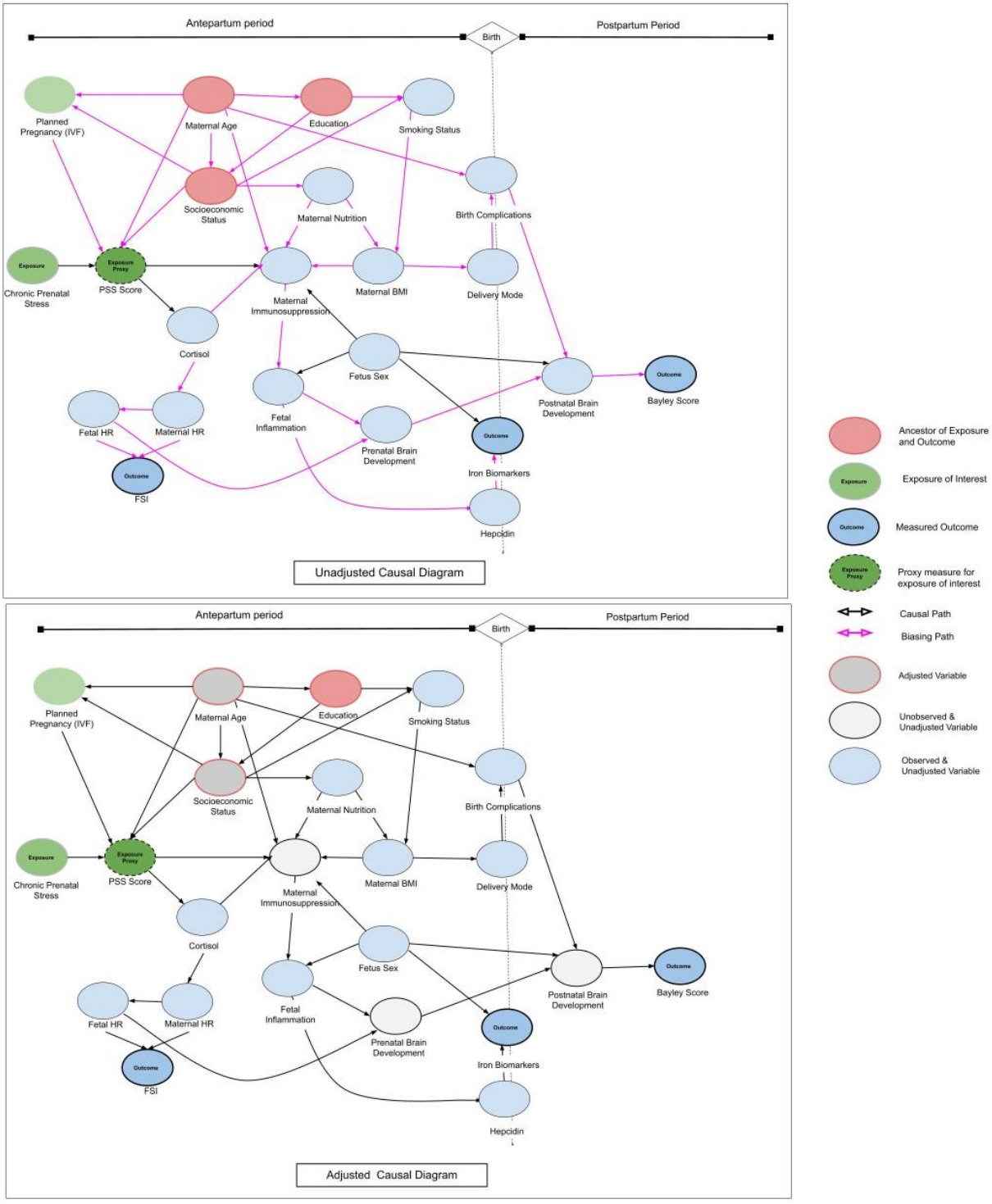

Abbildung 8. Mütterliches Alter, Sozioökonomischer Status und Bildung als Störgrößen im Pfad des pränatalen maternalen Stresses (PS)

"Directed acyclic graph"-Analyse der Beziehungen zwischen maternalen und fetalen pränatalen, perinatalen und postnatalen Einflüssen, Kovariablen und Outcomes.

Abkürzungen: PSS: Perceived stress scale; FSI: Fetal stress index; HR: Herzfrequenz; BMI: Body-Mass-Index; IVF: In-vitro-Fertilisation

4 Diskussion

Diese Arbeit bekräftigt die Annahme einer geschlechtsabhängigen Assoziation zwischen PS und den untersuchten Eisenbiomarkern sowie dem FSI in einer ansonsten gesunden Kohorte. Das männliche Geschlecht scheint dabei der treibende Faktor zu sein. Darüber hinaus konnten mittels einer DAG-Analyse die Variablen „SES“ und „Bildungsabschluss“ als zusätzliche Störfaktoren identifiziert werden. Der erneute Nachweis eines Unterschieds des FSIs zwischen SG und CG bekräftigt zudem die Resultate der Zwischenanalyse von Lobmaier et al. (2020). Auch die soziodemographischen Daten zeigten sich weitestgehend konkordant mit Ergebnissen vorheriger Studien, vor allem dahingehend, dass der PS stark mit Einkommen und Bildung korreliert (Klein et al., 2016).

Generell ist der Einfluss von PS auf die fetale Eisenhomöostase nur schlecht verstanden. Eine Publikation von Coe et al. (2007) kam zu dem Schluss, dass Kinder gestresster Rhesusaffen-Mütter mit höherer Wahrscheinlichkeit einen Eisenmangel entwickeln. Weitere Studien am Menschen deuteten auf eine Korrelation zwischen PS und dem Zink-Protoporphyrin/Häm-Index, sowie dem Ferritin-Spiegel des Nabelschnurblutes hin (Armony-Sivan et al., 2013; Campbell et al., 2020; Rendina et al., 2018). (Tab. 7)

Während der Schwangerschaft beeinflussen mütterliche Stresshormone wie etwa das Cortisol den heranwachsenden Fetus mutmaßlich über epigenetische Mechanismen (Alyamani & Murgatroyd, 2018; Rakers et al., 2017). Cortes et al. (2017) vermuteten in ihrer Veröffentlichung eine Auswirkung chronischen Stresses auf das Eisen-Regulationssystem fetaler Mikroglia-Kulturen des Schafes, vermittelt durch die Expression einer Stress-induzierten Variante des Enzyms Acetylcholinesterase. Sie mutmaßten, dass afferente, cholinerge, antiinflammatorische Signale über den nikotinergen α7-Acetylcholin-Rezeptor

verschiedene Metallionentransporter und Ferroportin herunterregulieren. Ferroportin agiere dabei als Hepcidin-Rezeptor.

Tabelle 7. Aktuelle Studien über den Einfluss von PS auf die Eisenhomöostase

Autor	Studiendesign und -population	Experimentelles Design	Analyse	Haupt-Studienergebnisse
Campbell et al. (2020)	**Design**: Studie am Menschen, prospektive Kohortenstudie **Ort**: Mexico City, Mexiko **Hypothese**: PS und psychologische Störungen und BMI sind mit niedrigerem Nabelschnurblut-Hb und -Ferritin assoziiert	**Einschlusskriterien**: <20 SSW, ≥18 Jahre, langfristig in Mexiko **Ausschlusskriterien**: Herz-, Nierenerkrankungen, Corticosteroid-/Antiepileptika-Einnahme, Alkoholabusus **Stress-Messung**: Prospektiv mit Fragebögen CES-D, STI, PSS-4, ETV, NLEs, CRISYS im 2. Und 3. Trimenon	**Kohorte:** n=455 **Proben:** Nabelschnurblut- Hb und -Ferritin **Zeitpunkt**: Geburt (Nabelschnur)	Nabelschnurblut-Ferritin war niedriger in Kindern von Müttern mit hohem PS (−23%; 95% CI: −35%, −9%), and mütterlicher Adipositas (−17%; 95% CI: −31%, 0.2%) Keine Assoziationen mit Nabelschnurblut-Hb nachweisbar
Rendina et al. (2018)	**Design**: Studie am Menschen, prospektive Kohortenstudie **Ort:** Meriter Hospital (Madison, USA) **Hypothese**: Nicht erwähnt	**Einschlusskriterien**: Frauen mit gesunden Neugeborenen und Risikofaktoren für kindliche Eisenmangelanämie, Einlings-Schwangerschaft, Englisch oder Spanisch sprechend, mütterliches Alter >18 **Ausschlusskriterien:** fetale Chromosomenaberrationen, weitere schwere fetale Anomalien, schwere Geburtskomplikationen, neonatologische Intensiv-betreuung, Geburt <35 SSW **Stress-Messung**: retrospektiver Fragebogen <48h nach Geburt, Fokus auf Schwangerschafts-bezogenen Stress	(1) **Kohorte:** n=245 **Proben:** Nabelschnurblut EDTA Zink-Protoporphyrin/Häm (ZnPP/H) und Retikulozyten angereicherter ZnPP/H), Plasma-Ferritin **Zeitpunkt**: Geburt (Nabelschnur) (2) **Kohorte**: n=79 **Proben**: Plasma-Ferritin **Zeitpunkt:** Alter Kind 1 Jahr	PS korreliert mit Nabelschnurblut ZnPP/H- Index (r=0.21, P<0.01) Hoher PS und ein hoher ZnPP/H Index erhöhen Wahrscheinlichkeit eines Eisenmangels (Plasmaferritin <12µg/L) im Alter von 1 Jahr. Keine signifikanten Effekte für Nabelschnurblut-Ferritin nachweisbar
Armony-Sivan et al. (2013)	**Design:** Studie am Menschen, retrospektive Fall-Kontroll-Studie **Ort**: Barzilai Medical Center (Ashkelon, Israel) **Hypothese**: PS im ersten Trimenon korreliert mit niedrigeren Nabelschnurblut-Ferritin-Konzentrationen	**Einschlusskriterien:** Unkomplizierte Einlings-Schwangerschaft, Alter Mutter > 18 Jahre, Kein Drogen/Alkohol-Konsum, Keine Psychischen Erkrankungen, Keine Frühgeburt (<37SSW), Normales Geburts-gewicht, 5min APGAR >8, Nabelschnurblut-Ferritin <370 ng/mL **SG**: Schwangerschaft im ersten Trimester während Raketenangriff auf Region **CG**: Schwangerschaft 4-5 Monate nach Raketenangriff	**Kohorte:** n=140 SG: n=63; CG: n=77 **Probe**: Nabelschnurblut-Ferritin **Zeitpunkt**: Geburt (Nabelschnur)	Nabelschnurblut-Ferritin-Konzentration in SG verglichen zu CG niedriger (145.7±62.0 vs. 169.3±85.4 ng/mL, P<0.05)
Coe et al. (2007)	**Design**: Tierstudie (Rhesusaffen), Retrospektive Interventionsstudie **Hypothese**: Nicht erwähnt	**SG**: Verursachung von Stress durch tägliche akustische Stress-Reize während erster 20 Wochen der Schwangerschaft (insgesamt 24 SSW) **CG**: keine Intervention	**Kohorte**: n=64 SG: n=40; CG: n=24 **Proben**: Plasma-Cortisol, großes BB, Immunhistochemie **Zeitpunkt:** 2-wöchiges Intervall	Hb-Konzentration, MCV, NK-Aktivität signifikant niedriger in SG

Abkürzungen: PS: maternaler pränataler Stress; SG: Stressgruppe; CG: Kontrollgruppe; BB: Blutbild; SSW: Schwangerschaftswoche; BMI: Body-Mass-Index; ZnPP/H: Zink-Protoporphyrin/Häm-Index; Hb: Hämoglobin; CI: Konferdenzintervall

4.1 Der geschlechtsspezifische Einfluss von PS

In verschiedenen Tierstudien konnten Stress-abhängige geschlechtsspezifische Veränderungen der Neurotransmitter sowie eine reduzierte dendritische Dichte des Hippocampus und des präfrontalen Kortex beobachtet werden (Bowman et al., 2004; Weinstock, 2001). Auch den Menschen betreffend gibt es Evidenz zu geschlechtsabhängigen Auswirkungen von PS. Bale and Epperson (2015) schlussfolgerten in einem aktuellen Review, dass von PS betroffene männliche Feten ein erhöhtes Risiko für das Auftreten von Verhaltens- und Entwicklungsstörungen wie etwa einer Schizophrenie oder eines ADHS hätten (Fineberg et al., 2016; Zhu et al., 2015). Auffälligkeiten wurden hierbei bereits früh in schlechteren Ergebnissen von Entwicklungstests sichtbar (Gerardin et al., 2011; Glasheen et al., 2013). Bezüglich des weiblichen Nachwuchses sei PS insbesondere mit affektiven Störungen wie z.B. Depressionen assoziiert (Quarini et al., 2016). (Bale & Epperson, 2015; Sutherland & Brunwasser, 2018)

Campbell et al. (2020) fanden kürzlich Hinweise auf geschlechtsspezifische Veränderungen der Eisenhomöostase durch PS. Dabei verwendeten sie sechs spezifische PS-Fragebögen jeweils im zweiten und dritten Trimenon bei durchschnittlich 28 Jahre alten Müttern. In ihrer Kohorte von 493 Patientinnen stellten sie fest, dass männliche Kinder im Vergleich zu weiblichen Neugeborenen schwangerer Frauen, die Gewalt ausgesetzt waren, öfters Assoziationen mit niedrigeren Ferritinspiegeln des Nabelschnurblutes aufwiesen. Im Gegensatz dazu waren einschneidende negative Lebensereignisse sowie Angst- und depressive Störungen der Mutter eher in Mädchen als in Jungen mit niedrigeren Ferritinspiegeln des Nabelschnurblutes assoziiert. (Tab. 7)

Die Mechanismen des Mutter-Fetus-Transfers verschiedener Arten des PS sind weitgehend unklar. Es besteht daher die Notwendigkeit einer weiteren Erforschung der oben erwähnten

geschlechtsspezifischen Einflüsse von PS auf die Eisenhomöostase, sodass diese Zusammenhänge besser verstanden werden können.

4.2 Die Beziehung der Eisenhomöostase zu PS

Die vorliegenden Ergebnisse dieser Arbeit konnten keine Assoziationen zwischen mütterlicher Anämie und fetalem Eisenmangel sowie PS nachweisen. Diese Erkenntnisse stimmen mit Resultaten vorheriger Veröffentlichungen, welche zeigten, dass der Fetus widerstandsfähig gegenüber moderaten Veränderungen in der maternalen Eisenhomöostase sein könnte, überein (Bencaiova & Breymann, 2014; Sifakis & Pharmakides, 2000).

Die Vermutung liegt nahe, dass die von uns gezeigten relativ kleinen Störungen der fetalen Eisenhomöostase durch PS vor allem bezüglich der Ferritinspiegel bereits geschlechtsspezifische Entwicklungsstörungen verursachen könnten (Tamura et al., 2002). Zudem könnte der Zeitpunkt der pränatalen Stressbelastung entscheidend für den durch mütterliches Cortisol vermittelten Einfluss von PS sein (Howland, Sandman, & Glynn, 2017). Der fehlende Nachweis von Unterschieden der Eisenparameter zwischen SG und CG ohne Berücksichtigung des Geschlechts könnte Anpassungen widerspiegeln, die insbesondere in weiblichen Feten auftreten, wenn die Schwangerschaft fortschreitet (Hodes & Epperson, 2019).

Diesbezüglich sind dringend weitere Untersuchungen notwendig, da den oben erwähnten neurologischen Störungen therapeutisch durch eine spezifische präventive Eisensubstitution begegnet werden könnte (Georgieff, 2020).

4.3 Eisensubstitution in der Schwangerschaft

Etwa ein Drittel der Patientinnen der hier untersuchten Kohorte nahm bereits regelmäßig Eisenpräparate ein. Generell ist sich die Wissenschaft in der Empfehlung einig, eine Eisenmangelanämie in der Schwangerschaft zu behandeln (Brannon & Taylor, 2017; Dumrongwongsiri et al., 2021; Georgieff, 2020). Aufgrund einer hohen Prävalenz der Eisenmangelanämie schwangerer Frauen und Kinder in sogenannten „Low and Middle Income Countries“ tritt die World Health Organisation zudem für eine präventive Eisen-Supplementation während der Schwangerschaft ein (Armitage & Moretti, 2019; WHO, 2016). Die Europäische Behörde für Lebensmittelsicherheit empfiehlt allerdings keine generelle pränatale Gabe von Eisenpräparaten, solange kein Eisenmangel festgestellt worden ist (EFSA, 2015; Shao et al., 2012). Auch in Deutschland besteht die Indikation für eine Eisensubstitution derzeit nur bei Vorliegen eines labordiagnostisch gesicherten Eisenmangels (AWMF, 2021). Für eine eher restriktive Nahrungsergänzung mit Eisen sprechen pränatale Anpassungen, wie die Zunahme des Plasmavolumens und eine damit einhergehende milde physiologische Anämie sowie das Ausbleiben der Menstruation in der Schwangerschaft (Brannon & Taylor, 2017). Zudem besteht die Gefahr einer Überladung mit Eisen, welche aufgrund dessen reaktiven Potentials für den Organismus ein erhöhtes Risiko zellulärer Schäden sowie einer Hyperglykämie birgt (Cantor, Bougatsos, Dana, Blazina, & McDonagh, 2015; Feng, Qi, Xu, Shen, & Li, 2012; Wessling-Resnick, 2017).

4.4 Das Immunsystem, PS und die Eisenhomöostase

Die vorliegenden Daten dieser Studie konnten kein Auftreten erhöhter inflammatorischer Prozesse in Neugeborenen der SG nachweisen (Tab. 1). Trotzdem wäre es denkbar, dass akute Entzündungsreaktionen, die üblicherweise während der Geburt auftreten, einen Einfluss auf

unsere Nabelschnurblut-Ergebnisse hatten (Fisher & Nemeth, 2017). Zelluläre Botenstoffe wie z.B. das Zytokin Interleukin (IL)-6 vermitteln entzündliche Ereignisse auf biochemischer Ebene (Fig 1). In der Literatur finden sich Hinweise auf höhere IL-Konzentrationen des Nabelschnurblutes chronisch gestresster Mütter (Andersson et al., 2016). Zusätzlich wird die Expression des Akute-Phase-Proteins Ferritin durch entzündliche Prozesse hochreguliert, wodurch auch dessen Funktion als klinischer Biomarker des körpereigenen Eisenspeichers beeinträchtigt werden kann (Comes, Modreira, Mesquita, & Gomes, 2018; Georgieff, 2020).

Zusammenfassend lässt sich sagen, dass der Einfluss von PS wohlmöglich auch durch inflammatorische Prozesse vermittelt wird, welche zusätzlich die Eisenhomöostase beeinflussen können.

Dieser Zusammenhang sollte in zukünftigen Studien, beispielsweise durch die Ergänzung laborchemischer Analysen mit der erweiterten Bestimmung inflammatorischer neonataler Parameter wie IL-6 genauer untersucht werden (Farajdokht, Soleimani, Mehrpouya, Barati, & Nahavandi, 2015). In Zukunft könnte als verlässlicher Marker für den Einfluss von PS auf die Eisenhomöostase zudem der lösliche Transferrin-Rezeptor (sTfR) dienen (Akesson, Bjellerup, Berglund, Bremme, & Vahter, 1998). Der sTfR ist spezifisch und sensibel genug, einen pränatalen Eisenmangel unabhängig von akuten inflammatorischen Prozessen zu detektieren und könnte kombiniert mit Ferritin in der Maßzahl „Total Body Iron“ (TBI) verlässliche Auskunft über den Eisenvorrat des Organismus geben (Mei et al., 2017). (Tab. 8)

Tabelle 8. Vergleich verschiedener Eisen-Biomarker
(adaptiert nach Brannon and Taylor (2017), Restrepo-Gallego, Diaz, and Rondo (2021))

Eisen-Indikator	Was wird gemessen?	Vorteile	Nachteile
Hämoglobin (Hb)	Anämie	Klinischer Routine-parameter, einfach zu bestimmen	Geringe Spezifität, Sensibilität, Beeinflusst durch Entzündung, Zunahme Plasmavolumen in Schwangerschaft
Ferritin (SF)	IDA-Diagnostik: Eisenspeicher	Klinischer Standard bei Eisenmangel, WHO-Standardisierung	Beeinflussung durch entzündliche Prozesse (akute Phase Protein)

Löslicher Transferrin-Rezeptor (sTfR)	Aktueller Eisenbedarf	Wenig durch entzündliche Prozesse beeinflusst	Begrenzte Verfügbarkeit, variierende laborspezifische Referenzbereiche, Von Erythropoese abhängig
sTfR/SF Total body iron (TBI)	Genaues Bild Eisenstatus	Umfassendes Bild vom Eisenstatus	Benötigt zwei Parameter
Transferrinsättigung	IDA-Diagnostik: Verhältnis Serum-Eisen zu Transferrin-Konzentration	Weit verbreitet	Von Nahrungsaufnahme abhängig, Beeinflussung durch entzündliche Prozesse
Zink-Protoporphyrin	IDA-Diagnostik	Günstig, einfach zu bestimmen	Beeinflussung durch entzündliche Prozesse, Thalassämie, Blei-Vergiftung
Hepcidin	Regulation der Eisenhomöostase, Aktueller Eisenbedarf	Sehr sensitiv	Experimentell, Beeinflussung durch entzündliche Prozesse, Vitamin A-Mangel

4.5 Adipositas als Auslöser und Folge von PS

Die Auswertung des PDQs ergab, dass die Gewichtszunahme sowie die Veränderung des Körperbildes viele schwangere Frauen zusätzlich belasten. Umgekehrt gibt es in der Literatur Hinweise darauf, dass Stress durch das ANS selbst ebenfalls einen Einfluss auf die Pathogenese einer Adipositas haben könnte (Guarino, Nannipieri, Iervasi, Taddei, & Bruno, 2017). Übergewicht könnte somit Auslöser und Folge zugleich von PS sein. Des Weiteren decken sich unsere Ergebnisse mit der Literatur dahingehend, dass Adipositas den Geburtsmodus zu Gunsten einer erhöhten Sectio-Rate beeinflussen kann (Marchi, Berg, Dencker, Olander, & Begley, 2015).

Darüber hinaus heben aktuelle Studien den Einfluss von Übergewicht auf den Eisenstoffwechsel hervor. Ein erhöhter BMI kann über Mediatoren wie IL-6 und Erythropoetin die Serum-Hepcidin-Konzentration des Nabelschnurblutes beeinflussen. (Andrews, Soto, & Arredondo-Olguin, 2015; Dosch et al., 2016; Korlesky et al., 2019) Adipositas könnte somit ebenfalls eine wichtige Rolle als Vermittler der Folgeschäden durch PS spielen.

4.6 Die Verwendung von Haar-Cortisol als Nachweis von PS

Der vorliegenden vollständigen Datensatz der FELICITy-Studie unterschied sich bezüglich der Haar-Cortisol-Konzentration zwischen SG und CG nur innerhalb des weiblichen Geschlechts. Eine kürzlich publizierte Metaanalyse kam sogar zu dem Fazit, dass Stress-Scores, die über Selbsteinschätzung erhoben wurden, nur unzureichend mit der Haar-Cortisol-Konzentration korrelieren (Stalder et al., 2017). Trotz fehlenden Nachweises eines direkten biochemischen Korrelats könnten die Auswirkungen von PS allerdings auch erst später im Erwachsenalter auftauchen (Brunson et al., 2005; Mastorci et al., 2009; Weinstock, 2008).

4.7 Der FSI als PS-Biomarker in der späten Schwangerschaft

Die Erkenntnisse dieser Arbeit bestätigten das Auftreten eines erhöhten FSIs in der SG während des dritten Trimenons (Lobmaier et al., 2020). Aufgrund der schwachen Assoziation des FSIs zu den gemessenen Eisenparametern lässt sich vermuten, dass PS die Interaktion von mHR mit fHR auf unterschiedlichen Signalwegen beeinflusst. Wie bereits oben erwähnt ist es darüber hinaus auch denkbar, dass PS-induzierte FSI-Veränderungen eventuell Langzeitschäden ohne direktes biochemisches Korrelat verursachen könnten (Brunson et al., 2005; Mastorci et al., 2009; Weinstock, 2008).

4.8 Machine Learning zur Vorhersage von PS

Dr. Martin Frasch von der University of Washington simulierte mittels eines „Machine Learning“ (ML)-Ansatzes aus den soziodemografischen und biophysikalischen Daten der FELICITy-Studie ein realitätsnahes Szenario, um von PS betroffene Mutter-Fetus-Dyaden zu identifizieren ((Zimmermann, 2022), Anhang 10). Bereits die alleinige Verwendung

soziodemografischer Krankenakten-Daten zeigte dabei ein hohes Vorhersagepotenzial (Topol, 2019). Die Ergänzung um den FSI als biophysikalisches Charakteristikum verbesserte das ML-Modell zusätzlich, sodass eine Verwendung der taECG-Technologie einen Beitrag zur Verbesserung der Früherkennung schädigender Einflüsse auf die Gesundheit wie etwa PS leisten könnte (Frasch et al., 2020; Sarkar et al., 2021).

4.9 Stärken und Schwächen

Eine Stärke der FELICITy-Studie ist insbesondere das prospektive Design, welches mögliche Fehler des Stress-Screenings, die aufgrund falscher Erinnerungen der Patientinnen hätten entstehen können, verhinderte. Eine weitere Qualität dieser Arbeit ist die Definition bestimmter „Matching-Kriterien“, wodurch die Daten für potenziell verzerrende Faktoren adjustiert wurden. Darüber hinaus zeigten mutmaßliche Störfaktoren wie die Einnahme von Eisenpräparaten und die Ethnie keine Unterschiede zwischen SG und CG. Außerdem befragte der PSS-10 die Patientinnen über Alltagsstress, sodass unsere Ergebnisse auch für die Allgemeinbevölkerung repräsentativ und vergleichbar sein könnten.

Andererseits existieren auch gewisse Limitationen. Aufgrund der Sprachbarriere konnten nur deutschsprachige Patientinnen in die Studie eingeschlossen werden. Daher wurden fremdsprachige Immigrantinnen der multikulturellen Stadt München von vornhinein ausgeschlossen. Aussagekräftige biochemische Marker wie IL-6 und sTfR konnten aus finanziellen Gründen nicht mitbestimmt werden. Aufgrund limitierter Ressourcen wurden zudem nicht alle gescreenten CG-Patientinnen eingeschlossen und stattdessen ein Matching-System angewendet. Außerdem waren die Patientinnen-Zahlen für die unterschiedlichen Subanalysen aufgrund des Drop-outs (Entbindung in anderer Klinik,

fehlende Nabelschnurblutentnahme, ...) teilweise deutlich niedriger als in der Gesamt-Kohorte (Tab. 1, Abb. 2).

Diese Studie unterschied nicht bezüglich arteriellen und venösen Ursprungs der analysierten Nabelschnurblut-Proben. In der aktuellen Literatur findet sich kein Hinweis darauf, dass dieses Problem bislang adressiert worden ist. Generell sind der plazentare Eisentransfer und die Erhebung des fetalen Eisenstatus nur schlecht verstanden (Delaney et al., 2019; Sangkhae & Nemeth, 2019). Zu dem Zeitpunkt der Verfassung dieser Arbeit existierten keine allgemein gültigen Referenzwerte für die Eisenparameter des Nabelschnurblutes. Die etablierten Referenzwerte beginnen zwar mit der Geburt (WHO, 2020), sind allerdings nicht ohne Weiteres auf Nabelschnurblut übertragbar, da die Eisenparameter des Nabelschnurblutes üblicherweise höher sind (Lorenz, Peter, Poets, & Franz, 2013). Die obigen Aspekte bedürfen weiterer Erforschung, sodass potenziell verzerrende Einflüsse auf die Analyse des Nabelschnurblutes identifiziert werden können.

Des Weiteren gibt es in der Literatur Anhaltspunkte dafür, dass schwangerschaftsspezifische Stress-Skalen stärker mit dem Verlauf der Geburt und der Entwicklung des Kindes assoziiert sein könnten als allgemeine Stress-Scores (Davis & Sandman, 2010; DiPietro, Novak, Costigan, Atella, & Reusing, 2006; Sandman, Davis, Buss, & Glynn, 2012; Wadhwa, Entringer, Buss, & Lu, 2011). Insbesondere spezifische Ängste in der Schwangerschaft scheinen mit der Entwicklung des heranreifenden Kindes signifikant assoziiert zu sein (Bayrampour et al., 2016; Davis & Sandman, 2010; Huizink et al., 2017; Khalesi & Bokaie, 2018; Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011). Auch in dieser Studie erhielt das Item „Ich habe Angst vor den Wehen und der Geburt“ des PDQs hohe Zustimmungswerte. Allerdings korrelierten PDQ und PSS-10, auf Basis dessen SG und CG eingeteilt worden sind, lediglich moderat miteinander. Ähnlich wie in der vorliegenden Arbeit zeigte der PSS-10 in einigen weiteren Studien zudem keine Assoziation mit maternalen Cortisol-Spiegeln und

Neugeborenen-Kenndaten (Baibazarova et al., 2013; Wing et al., 2017). (Tab. 2). Dies könnten Hinweise darauf sein, dass möglicherweise sensitivere Instrumente als der PSS-10 existieren um schwangerschaftsspezifische Stressoren zu erfassen.

Da die FELICITy-Studie PS ausschließlich im dritten Trimenon maß, wurde notwendigerweise die zeitliche Dynamik des auftretenden Stresses in der Schwangerschaft vernachlässigt. Der Cortisol- und Corticotropin-Releasing-Hormon-Spiegel des mütterlichen und fetalen Blutes nimmt bekanntermaßen während der Schwangerschaft, insbesondere in der zweiten Hälfte, deutlich zu (Lockwood et al., 1996; Mastorakos & Ilias, 2000). Es wird vermutet, dass sich die physiologische Stressachse im Verlauf der Schwangerschaft verändert, sodass die Stressantwort je nach Zeitpunkt der Schwangerschaft unterschiedlich ausfallen könnte (Entringer et al., 2010; Schulte, Weisner, & Allolio, 1990). Einige Studien konnten zeigen, dass diese Dynamik der Stressantwort auch mit der postpartalen Entwicklung des Kindes korrelierte (Davis & Sandman, 2010; Tollenaar et al., 2011; Vedhara et al., 2012).

4.10 Ausblick

Der letzte Abschnitt der FELICITy-Studie wird ein Augenmerk auf die neurologische Entwicklung der eingeschlossenen Kinder im Alter von 2 Jahren legen. Dadurch werden sich auch Schlüsse darüber ziehen lassen, ob eine Assoziation zwischen den in dieser Arbeit festgestellten Veränderungen der Eisenhomöostase der SG mit kindlichen Entwicklungsdefiziten, die potenziell durch PS-induzierte Fetale Programmierung entstanden sind, existiert.

Außerdem führte eine PhD-Studentin (Sharma et al., 2020) im Helmholtz-Zentrum München eine genomweite Analyse epigenetischer Veränderungen, die aufgrund von PS aufgetreten sein könnten, durch (Sharma et al. (2022), Anhang 11).

Selbstverständlich wäre es für zukünftige Studien, welche den Einfluss von PS auf die Eisenhomöostase untersuchen, wünschenswert, eine größere Kohorte miteinzubeziehen, sodass auch kleinere, signifikante Unterschiede zwischen den Gruppen detektieren werden können. Darüber hinaus wäre es interessant, die Kohorte einen längeren Zeitraum über zu beobachten. So könnten mögliche durch PS verursachte Langzeitfolgen, die eventuell kein direktes biochemisches Korrelat besitzen, nachgewiesen werden. Außerdem wäre zur Berücksichtigung der zeitlichen Dynamik in der Stressantwort ein wiederholtes Stress-Screening während der Schwangerschaft wünschenswert. Für die Untersuchung des Einflusses von PS als weitgehend unabhängigen Faktor, wäre ein zusätzliches Matching der Gruppen für den SES zu empfehlen. Weitere Studien könnten zudem schwangerschaftsspezifische Stressinstrumente, welche insbesondere schwangerschaftsspezifische Ängste messen, stärker miteinbeziehen.

In zukünftigen Untersuchungen des Eisenstoffwechsels könnte ebenfalls die Bestimmung des sTfRs und Ferritins zur Berechnung des TBIs, eines verlässlichen Markers des körpereigenen Eisenvorrats, erfolgen. Da die Rolle Hepcidins sowohl im Rahmen der Stressreaktion als auch in der Regulation des plazentaren Eisentransfers nicht vollständig verstanden ist, sollte sich zukünftige Forschung zudem mit den molekularen Mechanismen eines etwaigen Einflusses von PS auf die Eisenhomöostase beschäftigen (Farajdokht et al., 2015; Fisher & Nemeth, 2017).

Des Weiteren bleiben folgende Fragen bislang unbeantwortet:

Erstens, stellen die in dieser Studie beobachteten Veränderungen eine physiologische oder maladaptive Antwort auf PS dar?

Zweitens, welche Bedeutung haben therapeutische Möglichkeiten bei der Entdeckung, dass PS die fetale Eisenhomöostase beeinflussen kann?

Drittens, welche Rolle spielt die Eisensubstitution als korrektive therapeutische Option in diesem Kontext (Dumrongwongsiri et al., 2021; Georgieff, 2020)?

Viertens, kann das nichtinvasive fetale Monitoring mithilfe des FSIs das Auftreten von PS-assoziierten Entwicklungsabnormitäten im zeitlichen Verlauf der Schwangerschaft exakt identifizieren?

Fünftens, können weibliche Feten PS erfolgreicher kompensieren als Feten männlichen Geschlechts?

Die Ergebnisse dieser Arbeit deuten darauf hin, dass die Berücksichtigung des Geschlechts bei der Durchführung potenzieller Therapien zur Kompensation des Einflusses von PS notwendig sein könnte (Antonelli et al., 2021; Babbar & Shyken, 2016; Hutchon et al., 2019) (Anhang 12).

5 Zusammenfassung

Einleitung: Die schädigende Auswirkung mütterlichen pränatalen Stresses (PS) auf die neuronale Entwicklung des Kindes bedarf der Entwicklung von Biomarkern, welche frühzeitige therapeutische Interventionen ermöglichen.

Methodik: Die FELICITy-Studie, eine prospektive Fall-Kontroll-Studie, wurde im Zeitraum von Juni 2016 bis Juli 2019 am Klinikum rechts der Isar der Technischen Universität München durchgeführt. 2000 schwangere Frauen wurden mittels *Cohen Perceived Stress Scale-10*-Fragebogen im dritten Trimenon gescreent und in eine Stress- (SG) und Kontrollgruppe (CG) eingeteilt. Insgesamt wurden 164 Patientinnen nach Parität sowie Gestations- und maternalem Alter bei Studieneinschluss gematcht, rekrutiert.

In dem Serum des Nabelschnurblutes wurden bei der Geburt Hepcidin, Ferritin, Transferrin und Eisen gemessen. Mittels transabdominalen Elektrokardiogramms (taECG) wurde der Fetal Stress Index (FSI), der die Interaktion von mütterlicher und fetaler Herzfrequenz widerspiegelt, berechnet. Der Beitrag des Geschlechts zu den Gruppenunterschieden zwischen SG und CG wurde ermittelt. Zur Untersuchung möglicher kausaler Zusammenhänge wurde ein gerichteter azyklischer Graph (DAG) erstellt.

Ergebnisse: In dieser Arbeit konnten geschlechtsspezifische Assoziationen zwischen PS und der fetalen Eisenhomöostase sowie des fetalen autonomen Nervensystems quantifiziert durch den taECG-basierten FSI, festgestellt werden.

Die Transferrinsättigung ($p = 0.041$) männlicher Neugeborenen der SG war niedriger im Vergleich zur CG. Durch das GEE-Model konnte gezeigt werden, dass das Geschlecht ein signifikanter Effekt-Modifikator ist, der die Gruppenunterschiede bezüglich des Ferritins maßgeblich hervorbringt ($p = 0.038$, Abb. 6). Die DAG-Analyse identifizierte die Variable

„Sozioökonomischer Status“ als zusätzliche Störgröße. Der mittlere FSI war in der SG höher als in der CG (0.38 ((–0.22)–0.75) versus –0.01 ((–0.36)–0.34); p = 0.024)).

Diskussion: Die Ergebnisse der vorliegenden Studie betonen die unterschätzte Rolle des multifaktoriellen potenziell durch Veränderungen der Eisenhomöostase verursachten Einflusses von PS auf die postnatale neuronale Entwicklung. Die betrachteten Biomarker dieser Arbeit eröffnen neue Einblicke in die Assoziation von PS mit kindlichen Entwicklungsstörungen. Diese könnten zu der Entwicklung neuer präventiver Strategien beitragen (Antonelli et al., 2021; Babbar & Shyken, 2016; Hutchon et al., 2019) (Anhang 12).

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Abbildungsverzeichnis

Tabellenverzeichnis

Informationsblatt und Einwilligungserklärung für die Teilnehmerinnen

Studie: *FELICITY - Einfluss von maternalem Stress in der Schwangerschaft auf die kindliche Entwicklung in Perinatal- und Kleinkindperiode*

Liebe Frau .....,

Wir laden Sie zur Teilnahme an einer klinischen Studie ein. Bevor Sie sich entscheiden, ist es wichtig, den wissenschaftlichen Hintergrund und was diese Studie beinhaltet zu verstehen. Die Studie wird Ihnen detailliert von Ihrem Arzt erklärt, diese Information führt nur die wesentlichen Punkte aus und soll Ihnen helfen, sich an die Dinge zu erinnern, die Sie den Arzt noch fragen wollen.

Bitte nehmen Sie sich die Zeit, die folgende Information genau zu lesen und wenn Sie dies wünschen mit anderen Personen (z.B. Ehemann) zu diskutieren. Bitte fragen Sie uns, wenn Ihnen etwas unklar ist, bzw. wenn Sie noch mehr Informationen benötigen. Bitte lassen Sie sich Zeit und entscheiden Sie dann, ob Sie teilnehmen möchten, oder nicht.

Worum geht es in dieser klinischen Untersuchung?

Im Rahmen dieser Studie werden Kinder untersucht, deren Mütter während der Schwangerschaft unter vermehrtem Stress leiden. Dazu benötigt man auch eine Kontrollgruppe von Müttern ohne Anzeichen für erhöhten Stress. Bisherige Daten haben gezeigt, dass ein Teil der Kinder durch den Einfluss des mütterlichen (maternalen) Stresses ein höheres Risiko für die Entwicklung von Erkrankungen wie Depression, Aufmerksamkeits-Defizit-Hyperaktivitäts-Syndrom und andere psychischen Erkrankungen im Kindes- und Erwachsenenalter trägt. Ziel dieser Studie ist es, Marker zu finden, die diese Kinder frühzeitig entdecken. Mithilfe von unterschiedlichen Untersuchungsmethoden und Messwerten sollen gefährdete Kinder bereits während der Schwangerschaft sowie kurz nach der Geburt erkannt werden um somit rechtzeitig weitere Folgen zu verhindern.

Es besteht die Annahme, dass Neugeborene von gestressten Müttern Veränderungen des autonomen Nervensystems (Sympathikus/Parasympathikus), bestimmter der DNA aufgesetzter Merkmale (Epigenetik) und des Mikrobioms (bakterielle Besiedelung) aufweisen. Das autonome

Nervensystem stellt dabei das nicht durch das Bewusstsein gesteuerte System, welches es unserem Körper ermöglicht sich an äußere Einflüsse anzupassen (z.B. schnellerer Herzschlag bei Aufregung...).

Unser Ziel ist es nun, herauszufinden welche genauen Veränderungen beim Kind im Mutterleib, bei der Geburt und im Alter von 24 Monaten bei mütterlichem Stress stattfinden.

Wie sieht die Studienteilnahme konkret aus?

Während der Schwangerschaft: Bei der ersten Vorstellung im Rahmen der Studie wird über Fragebögen, ein genaues psychologisches Stress-Profil der Schwangeren erstellt. Sie werden dadurch in die Gruppe „gestresster" Mütter oder in die Kontrollgruppe eingeteilt. Im Anschluss wird eine CTG Aufzeichnung durchgeführt, so wie Sie es von den normalen Vorsorgeuntersuchungen her kennen, wobei ein spezielles Gerät unserer Klinik verwendet wird und zusätzlich dazu eine Aufzeichnung Ihres und des kindlichen EKGs erfolgen. Diese Untersuchung dauert etwa 40 min. Die Aufzeichnungen (CTG/EKG) werden zu einem späteren Zeitpunkt hinsichtlich verschiedener Charakteristika analysiert.
Ein Einfluss auf die weitere Betreuung oder Konsequenzen für die Behandlung ergeben sich aus den zusätzlichen Untersuchungen nicht. **Es wird auch bei den Schwangeren, die im Rahmen der Studie betreut werden so verfahren wie bei allen Schwangeren**.

Geburt: Bei Aufnahme in den Kreissaal möchten wir Ihnen gerne eine kleine Haarprobe entnehmen (an einer verdeckten Stelle am Hinterkopf werden ca. 3 mm Haare entfernt). Dadurch können wir über ein Hormon (Cortisol) den „Stress-Status" der letzten 2 Monate bestimmen. Außerdem wird Ihnen nach Legen eines intravenösen Zugangs, den jede Patientin unter der Geburt bei uns bekommt, ca. 15 ml Studienblut abgenommen. Somit kann ein „extra Stechen" vermieden werden. Nach der Abnabelung des Neugeborenen erfolgt eine Blutentnahme aus den Nabelschnurgefäßen (ca. 20 ml) sowie die Entnahme einer Speichelprobe des Neugeborenen. Aus diesem Blut/ aus der Speichelprobe werden DNA-Merkmale der Blutzellen /Mundschleimhautzellen bestimmt. Ein kleines Stück des Mutterkuchens (Plazenta) (ca. 2 mal 1 cm) wird entnommen und für weiteren Analysen verarbeitet.

Es kann sein, dass aufgrund bestimmter Gegebenheiten sie aus der Studie ausgeschlossen werden und nicht zu den Folgeuntersuchungen eingeladen werden. Darüber werden wir Sie natürlich nach der Geburt informieren.

24 Monate: Im Alter von 24 Monaten werden Sie erneut von uns kontaktiert und bekommen einen Fragebogen mit Fragen zu Ihrem Stillverhalten und Symptomen einer möglichen postpartalen Depression zugeschickt. Zudem werden Sie von uns zu weiterführenden

Untersuchungen eingeladen, bei welchen Sie den Fragebogen bitte ausgefüllt mitbringen. Bei Ihnen und Ihrem Kind wird erneut eine EKG-Untersuchung durchgeführt. Es erfolgt die Messung von Blutdruck und Puls. Außerdem wird anhand eines etablierten Entwicklungstests (Bayley Test) ein neurologisches sowie motorisches Entwicklungsprofil Ihres Kindes erstellt und eine Speichelprobe bei Ihrem Kind entnommen um DNA-Merkmale der Blutzellen /Mundschleimhautzellen zu bestimmen.

Welche Vorteile ergeben sich aus der Teilnahme an der klinischen Prüfung ?

Ihre Schwangerschaft wird nach den besten derzeit üblichen Untersuchungsmethoden überwacht, die durch Experten festgelegt wurden. Außerdem werden neuere Untersuchungen durchgeführt, die bisher nicht Standard sind. Zusätzlich wird ihr Kind auch noch in den ersten Lebensmonaten im Rahmen der Studie Folgeuntersuchungen erhalten, die normalerweise nicht durchgeführt würden. **Falls dabei tatsächlich Auffälligkeiten bei Ihrem Kind** festgestellt werden, können Sie frühzeitig bei Spezialisten hierfür eine **kostenlose Mitbetreuung** erhalten („Babysprechstunde“ der Institutsambulanz der Abteilung Kinder- und Jugendpsychosomatik, Klinikum rechts der Isar).

Welche Risiken ergeben sich aus der Untersuchung ?

Durch die CTG-Aufzeichnung, wie auch durch die EKG-Aufzeichnung selbst gibt es **keine unerwünschten Begleiteffekte**. Auch die Entnahme von Nabelschnurblut, Plazenta und Speichel ist ohne jegliches Risiko für Ihr Kind und wird aus anderen Gründen häufig routinemäßig durchgeführt. Die Betreuung in der Klinik ist durch die Studienteilnahme nicht verändert. Falls Sie sich entschließen sollten, aus Gründen, die Sie uns **nicht** mitteilen müssen, von der Teilnahme an dieser Untersuchung zurückzutreten, werden Ihnen **keine Nachteile** in der weiteren Betreuung in unserer Klinik entstehen.

Gab es eine ethische Überprüfung dieser Studie ?

Das Studienprotokoll wurde von einer unabhängigen Ethikkommission (Ethikkommission der Technischen Universität München) geprüft und im Rahmen der berufsrechtlichen Beratung wurden keine Einwände erhoben.

Wie wird der Datenschutz, die Vertraulichkeit bei der Überprüfung der Originaldokumente gewährleistet ?

Ihr schriftlich dokumentiertes Einverständnis erlaubt es uns, dass Ihre persönlichen Daten registriert werden. Nur die Prüfer, sowie autorisierte Personen in- und ausländischer Gesundheitsbehörden haben im Rahmen der entsprechenden gesetzlichen Vorschriften Zugang zu den vertraulichen Daten, in denen Sie namentlich genannt werden. Diese Personen unterliegen der Schweigepflicht und sind zur Beachtung des Datenschutzes verpflichtet. Die Weitergabe der Daten im In- und Ausland erfolgt ausschließlich zu statistischen und wissenschaftlichen Zwecken und Sie werden ausnahmslos darin nicht namentlich genannt. Auch in etwaigen Veröffentlichungen der Daten dieser klinischen Prüfung werden Sie nicht namentlich genannt. Selbstverständlich können Sie jederzeit ohne Nennung von Gründen Ihr schriftliches Einverständnis zurückziehen.

Haben Sie noch Fragen ?

Sie haben das Recht, sich jederzeit über diese Studie zu informieren. Falls Sie irgendwelche Fragen zu dieser Studie haben, so wenden Sie sich bitte an Ihren Prüfarzt:

Oberärztin PD Dr. Silvia Lobmaier

Tel.: 089 4140 5417

Ist die Teilnahme an dieser Studie freiwillig ?

Die Teilnahme an dieser Studie ist freiwillig und Sie dürfen jederzeit ohne Begründung die Teilnahme beenden. Dies wird Ihnen keinerlei Nachteile bringen. Ihr Prüfarzt kann Sie jederzeit aus der Studie ausschließen. Er wird Ihnen dazu die Gründe mitteilen.

Rahmenbedingungen

Die Studie wird gemäß den Grundsätzen der Deklaration von Helsinki des Weltärztebundes sowie den Vorschriften des deutschen Arzneimittelgesetzes und der Leitlinie zur Guten

Klinischen Praxis durchgeführt. Diese Dokumente können jeweils bei Ihrem Prüfarzt eingesehen werden.

Studie: *FELICITY - Einfluss von maternalem Stress in der Schwangerschaft auf die kindliche Entwicklung in Perinatal- und Kleinkindperiode*

Schriftliche Einwilligung und datenschutzrechtliche Erklärung

Ich, ……..…….…….. (Name der Patientin – in Druckbuchstaben**)**

wurde von ……..…….…….. (Name der/-s aufklärenden Ärztin/Arztes - in Druckbuchstaben)

über Wesen, Bedeutung und Tragweite der Studie eingehend aufgeklärt.

Ich wurde darüber informiert und bin damit einverstanden, dass meine erhobenen Daten aufgezeichnet werden. Es ist mir bewusst, dass der Zugang zu meinen persönlichen Daten nur Personen gestattet ist, die der Schweigepflicht und der Begutachtung des Datenschutzes verpflichtet sind. Die Weitergabe der Daten im In- und Ausland erfolgt ausschließlich zu statistischen und wissenschaftlichen Zwecken.

Ich wurde darauf hingewiesen, dass ich meine Einwilligung jederzeit ohne Angabe von Gründen widerrufen kann, ohne dass mir dadurch Nachteile für meine weitere medizinische Versorgung entstehen.

Hiermit erkläre ich mich freiwillig bereit, an der Studie teilzunehmen.

München, den ……..……..	München, den……..……..……..
……..…	……..……..
Unterschrift d. Patientin	Unterschrift der/-s aufklärenden Ärztin/Arztes

Anhang 1. Einverständniserklärung FELICITy-Studie

Fragebogen 1 zur Stresserfassung

Bitte tragen Sie hier Ihren vollständigen Namen ein: Vorname, Nachname: _____

Die folgenden Fragen beschäftigen sich damit, wie häufig Sie sich während des letzten Monats durch Stress belastet fühlen. (Bitte kreuzen Sie **pro** Aussage **eine** Antwort an)	**Nie**	**Selten**	**Manchmal**	**Häufig**	**Sehr oft**
1. Wie oft hatten Sie sich im letzten Monat darüber aufgeregt, dass etwas völlig Unerwartetes eingetreten ist?					
2. Wie oft hatten Sie im letzten Monat das Gefühl, wichtige Dinge in Ihrem Leben nicht beeinflussen zu können?					
3. Wie oft hatten Sie sich im letzten Monat nervös und „gestresst“ gefühlt?					
4. Wie oft hatten Sie sich im letzten Monat sicher im Umgang mit persönlichen Aufgaben und Problemen gefühlt?					
5. Wie oft hatten Sie im letzten Monat das Gefühl, dass sich die Dinge nach Ihren Vorstellungen entwickeln?					
6. Wie oft hatten Sie im letzten Monat das Gefühl, mit all den anstehenden Aufgaben und Problemen nicht richtig umgehen zu können?					
7. Wie oft hatten Sie im letzten Monat das Gefühl, mit Ärger in Ihrem Leben klar zu kommen?					
8. Wie oft hatten Sie im letzten Monat das Gefühl, alles im Griff zu haben?					
9. Wie oft hatten Sie sich im letzten Monat darüber geärgert, wichtige Dinge nicht beeinflussen zu können?					
10. Wie oft hatten Sie im letzten Monat das Gefühl, dass sich die Probleme so aufgestaut haben, dass Sie diese nicht mehr bewältigen können?					

Fragebogen 2 zur Stresserfassung

Bitte tragen Sie hier Ihren vollständigen Namen ein: Vorname, Nachname: _____

Kreuzen Sie jeweils die **eine** Antwortalternative an, die Ihnen als beste Schätzung am Zutreffendsten erscheint.	**Trifft gar nicht zu**	**Trifft ein wenig zu**	**Trifft mäßig stark zu**	**Trifft stark zu**	**Trifft sehr stark zu**
1. Mich stören Gewichtszunahmen während der Schwangerschaft.					
2. Körperliche Symptome wie Übelkeit, Erbrechen, geschwollene Füße oder Rückenschmerzen, die im Zusammenhang mit der Schwangerschaft auftreten, belasten mich.					
3. Ich bin besorgt darüber, wie ich mein Baby richtig versorge, wenn ich nach der Krankenhausentlassung wieder nach Hause komme.					
4. Mich plagen die gefühlsmäßigen Höhen und Tiefen während der Schwangerschaft.					
5. Ich mache mir Sorgen darüber, dass sich meine Beziehungen zu anderen Menschen, die mir wichtig sind, während meiner Schwangerschaft verändern.					
6. Ich mache mir Sorgen, ob ich mich für mein Baby gesund und ausgewogen genug ernähre.					
7. Insgesamt belasten mich die Veränderungen meiner Figur und meines Körperumfangs.					
8. Ich mache mir Sorgen darüber, dass sich durch das Baby die Beziehung zum Vater des Kindes ändern wird.					
9. Ich mache mir Sorgen darüber, ein krankes Baby zur Welt zu bringen.					
10. Ich habe Angst vor den Wehen und der Geburt.					
11. Ich habe Angst vor einer möglichen Frühgeburt.					
12. Ich mache mir Sorgen darüber, dass ich keinen emotionalen Bezug zu meinem Baby finde.					

Code: _____

Allgemeiner Fragebogen (von der Patientin auszufüllen)

Datum:_____/_____/_____

Name und Vorname:_____
Geburtsdatum:_____Geburtsland:_____
Geburtsland der Eltern: Mutter_____Vater_____
Adresse:_____
Telefonnummer (Mobil u/o Festnetz):_____
Email:_____
Familienstand:_____
Beruf:_____
Aktueller Arbeitsstatus (Mutterschutz/arbeitend):_____
Nachname des Kindes_____

Letzte Periode:_____Entbindungstermin:_____
Aktuelle Schwangerschaftswoche:_____
Aktuelles Alter:_____Größe(cm):_____
Aktuelles Gewicht (kg):_____
Gewicht vor der Schwangerschaft (kg):_____
Wieviele Schwangerschaften insgesamt:_____
Wieviele Kinder:_____
Aktuelle Medikamente/Vitamine (z.B. Femibion, Eisen, Zentrum...):_____
_____

Bitte kreuzen Sie das Zutreffendsten an:

Rauchen während der Schwangerschaft: ☐ JA ☐ NEIN
Zigaretten/Tag:_____
Regelmäßiger Alkoholkonsum in der Schwangerschaft: ☐ JA ☐ NEIN
Drogen während der Schwangerschaft: ☐ JA ☐ NEIN
Künstliche Befruchtung (IVF/ICSI): ☐ JA ☐ NEIN
Geplante Schwangerschaft: ☐ JA ☐ NEIN
Höchster Schulabschluß: ☐ Hauptschule ☐ Realschule
☐ Abitur ☐ Hochschule/Universität

Monatliches Einkommen/Haushalt (Netto): ☐ < 1000€ ☐ 1000-2500€
☐ 2500-5000€ ☐ 5000-10000€ ☐ >10000 €

Anhang 4. Soziodemografischer Fragebogen

Klinische Anamnese (vom Studienbeauftragten auszufüllen)

Resultat Stresstest: Test 1 (PSS)_____Test 2 (PDS)_____
Frühere Schwangerschaften:

Plazentalösung		Frühgeburt	
SIH/Präeklampsie/Eklampsie/HELLP		IUGR/SGA	
Perinatale Mortalität			

_____
_____

Aktuelle Schwangerschaft:

Gestationsdiabetes		Arterielle Hypertonie	
Autoimmune Erkrankung		Thrombophilie	
Schätzgewicht <10. Perzentile		RR	/

_____
_____

Perinatales Outcome:

PE/E/HELLP		SIH	
Kindsgewicht		Perzentile	
Länge		Kopfumfang	
Geschlecht		Gestationsalter bei Geburt	
Geburtsdatum		Geburtsmodus	
Ind. zur EL		Ind. zur Sectio	
Lungenreife		APGAR	
Nabelschnur-pH		BE	
pO2		pCO2	
Laktat		Glucose	
Postpartale Aufnahme Neo		Grund	

_____
_____

Anhang 5. Klinischer Fragebogen

FELICITy Studie - Checkliste

Das Abnahmeset befindet sich in einer Plastiktüte mit der Beschriftung: **FS_____** und befindet sich in der Kartonbox mit der Aufschrit "FELICITy". **Bitte die bereits abgenommen Proben ankreuzen.**

Schwarz geschriebene Punkte werden von der Hebamme entnommen/durchgeführt.
Gelb angezeichnete Stellen werden vom Studienarzt durchgeführt.

Zuerst die Tüte mit dem **Patientenetikett** versehen.

☐

1. Vor der Geburt / Maternales Blut:

- Haarprobe entnehmen (siehe das gesonderte Abnahmeprotokoll). **FS_____** Code auf die Alufolie schreiben. Bei Raumtemperatur lagern. Sammelbox liegt bei Prof. Antonelli.

  ☐

- 9ml **EDTA-Blut** (=großes, rotes Röhrchen) mit **"M"** mit Patientenetikett bekleben. Nach der Abnahme ca. 10-mal über Kopf drehen. Bei Raumtemperatur lagern.
  -> **"mat_ FS____ "** draufkleben und Patientenetikett entfernen**.** Nun erneut durchgemischt in den -20° Tiefkühler

  ☐

- 9ml **Serum-Blut** (= großes, braunes Röhrchen) mit **"M"** mit Patientenetikett bekleben.
  Stehen lassen bei Raumtemperatur für 15-30 min. Dann Zentrifugieren der Probe für 10 min bei 2500 U/min. Lagerung des Röhrchens dann in stehender Position (Röhrchenhalter) im **Kühlschrank**.
  Abgetrenntes Serum in weniger als 12 Stunden! in 2-4 x 2ml leere Cryovials umfüllen.
  -> **"mat_ FS____ "** draufkleben! Nun entweder zwischenzeitlich in den -20° Tiefkühler oder/dann Einfrieren der Proben bei -80°C.

  ☐

  Datum: _____ Zeit:_____(Abnahme)

  Datum: _____ Zeit:_____(Umfüllen)
  Anzahl befüllter Cryovials:

2. Nach der Geburt / Nabelschnurblut (am besten vor der Placentalösung):

- 9ml **EDTA-Blut** (=großes, rotes Röhrchen) mit **"Fet"** mit Patientenetikett bekleben. Nach der Abnahme ca. 10-mal über Kopf drehen. Bei Raumtemperatur lagern.
  -> **"fet_ FS____ "** draufkleben und Patientenetikett entfernen**.** Nun durchgemischt in den -20° Tiefkühler ☐

- 9ml **Serum-Blut** (=großes, braunes Röhrchen) mit **"Fet"** beschriftet mit Patientenetikett bekleben. Stehen lassen bei Raumtemperatur für 15-30 min. Dann Zentrifugieren der Probe für 10 min bei 2500 U/min. Lagerung des Röhrchens dann in stehender Position (Röhrchenhalter) im **Kühlschrank**.
  Abgetrenntes Serum in weniger als 12 Stunden! in 2-4 x 2ml leere Cryovials umfüllen.

-> **“fet_ FS____ “** draufschreiben! Nun entweder zwischenzeitlich in den -20°Tiefkühler oder/dann Einfrieren der Proben bei -80°C.

☐

Datum: _____ Zeit:_____(Abnahme)

Datum: _____ Zeit:_____(Umfüllen)
Anzahl befüllter Cryovials:

- 2,5 ml Blut in **PAXgene-Röhrchen.** !Gesondertes Abnahmesystem in der Plastiktüte enthalten!. (Falls Vakuum aus dem Röhrchen gelassen wurde, ist auch die Abnahme durch eine separate Spritze sowie Einspritzen in das Paxgene-Röhrchen möglich. Nach der Abnahme ca. 10-mal über Kopf drehen. Patientenetikett drauf Bei **Raumtemperatur** ! **aufrecht** ! lagern. Danach ! aufrecht ! im Tiefkühler (bei -20°C) für 10-16 Stunden lagern.
  -> **“fet_ FS____ “** draufschreiben und Patientenetikett entfernen**.** Dann mittels Kühlbox zum Labor bringen und im -80°C-Schrank lagern.

  ☐
- 1ml **EDTA-Blut** (=kleines, rotes Röhrchen) als Differentialblutbild in die Klinische Chemie “bomben” (Auftragszettel ausgefüllt in der Tüte -> Säuglingsetikett drauf!).
  !!! Bitte unbedingt ankreuzen, wenn durchgeführt !!!

  ☐

3. Nach der Geburt / Placentaprobenentnahme:

- Mithilfe von eingetütetem Skalpell und Pinzette ein ca. 0,5 cm^2 (= halber Fingernagel vom kleinen Finger) kleines Stück zentral von der Placenta (am besten von der mütterlichen Seite) herausschneiden. Probe in 2ml leerem **CryoVials.** Mit Patientenetikett bekleben. Sofort im **Tiefkühler** (bei -20°C) lagern.
  ->“**mat_DNA_FS_____”** draufschreiben und Patientenetikett entfernen**.**

  ☐
- Zweite Placentaprobe wie bei vorherigem Punkt entnehmen. Probe in 2ml **CryoVials.** Mit Patientenetikett bekleben. Sofort im **Tiefkühler** (bei -20°C) lagern.
  -> **“mat_microbiom_FS____”** draufschreiben und Patientenetikett entfernen**.**

  ☐

  Datum: _____ Zeit:_____(Abnahme)

4. Speichelprobe Kind:

- Dafür **Oracollect** benutzen. Anleitung zur Abnahme auf der Rückseite des Sets. Beide inneren Wangen abstreichen. Lagerung bei Raumtemperatur. Patientenetikett draufschreiben

  ☐

  -> **“fet_ FS____ “** draufschreiben und Patientenetikett entfernen**.** Aufbewahrungsbox bei Prof. Antonelli.

  Datum: _____ Zeit:_____

Besonderheiten während der Geburt (z.B.. PDA, Analgesie)/ Probleme bei der Probenentnahme:

_____

_____

_____

_____

_____

Material pro Patientin:

2 x 9 ml Serumröhrchen (S-Monovette® 9 ml, Serum Gel mit Clotting Activator, 92x16 mm, brown EU/US code, transparent label, 50/inner box sterile) Ref # 02.1388

2 x 9 ml EDTAröhrchen (S-Monovette® 9 ml, K3 EDTA, 92x16 mm, red EU code, paper label, 50/inner box sterile) Ref# 02.1066.001

1 x 2.5 ml Paxgene tubes (PreAnalytiX/Qiagen, Cat.no. 762165) mit Vacutainer adapter system (BD Bioscience, Cat.no. 364880)

1 x 1ml EDTA-Blut für Differentialblutbild (S-Monovette® 2.7 ml, K3 EDTA, 75x13 mm, red EU code, transparent label, 50/inner box sterile) Ref # 04.1917

1 x steriles Skalpell (Feather Disposable Scalpel)

1 x sterile Plastikpinzette (Polystyrol-Pinzette)

6 x 2 ml leere CryoVials

1 Oracollect Kit (DNAGenotek OC-175)

1 Schnurschlaufe für Haarprobe

Anhang 6. Protokoll zur Probenentnahme

HEPCIDIN PROTOCOL

TRIAL EXPERIMENT (8/7/19)

MATERIALS NEEDED

- Erlenmeyer with deionized water.
- Graduated Cylinder with 1170 ml of deonized labelled Wash Solution.
- Plastic or glass flask to store the remainder Wash Solution.
- Automatic pipettes various volumes and tips.

REAGENTS

- ✓ Bring all reagents and required number of strips to **room temperature** prior to use.
- ✓ Reconstitute the lyophilized contents of each **standard vial** with 0.5 mL deionized water and let stand for 10 minutes in minimum. Mix several times before use.
- ✓ Reconstitute the lyophilized content of **Controls** with 0.5 mL deionized water and let stand for 10 minutes in minimum. Mix the control several times before use.
- ✓ Dilute 30 mL of concentrated **Wash Solution** with 1170 mL deionized water to a final volume of 1200 mL.

TRIAL (Follow template)

1. Secure ONE STRIP of Microtiter wells in the frame holder.
2. Dispense 20 µL of Standard 0, 1, 3 & 5 into first four wells.
3. Dispense 20 µL of Control LOW into fifth well.
4. Dispense 20 µL of Control HIGH sixth well.
5. Dispense 20 µL Serum sample from an excluded patient into seventh and eighth wells.
6. Dispense 50 µL Enzyme Conjugate into each well. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
7. Incubate for 60 minutes at room temperature

8. Briskly shake out the contents of the wells.
9. Rinse the wells with 4 x 300 µL diluted Wash Solution per well for manual washing.
10. Strike the wells sharply on absorbent paper to remove residual droplets.

    Important note: The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!

11. Dispense 100 µL of Enzyme Complex into appropriate wells.
12. Incubate for 30 minutes at room temperature.
13. Briskly shake out the contents of the wells.
14. Rinse the wells with 4 x with 300 µL diluted Wash Solution per well for manual washing.
15. Strike the wells sharply on absorbent paper to remove residual droplets.
16. Add 100 µL of Substrate Solution to each well.
17. Incubate for 20 minutes at room temperature.
18. Stop the enzymatic reaction by adding 100 µL of Stop Solution to each well.
19. Determine the absorbance (OD) of each well at 450 ± 10 nm with a microtiter plate reader.

    It is recommended that the wells be read within 10 minutes after adding the Stop Solution.

Anhang 7. Protokoll für Messung der Hepcidin-Konzentration im Nabelschnurblut

Klinikum rechts der Isar
Technische Universität München

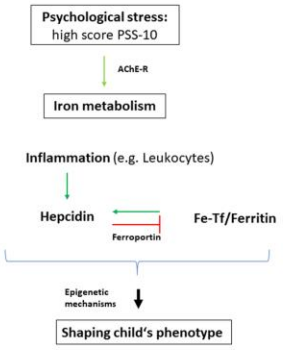
INFLUENCE OF PRENATAL STRESS ON IRON METABOLISM: FELICITy STUDY

Peter Zimmermann[1,3], Ritika Sharma[1,2,3], Camilla Zelgert[3], Martin G. Frasch[4], Marta C. Antonelli[3, 5], Silvia M. Lobmaier[3]

[1]TUM School of Medicine, TUM Graduate School. [2]Research Unit of Molecular Epidemiology, Institute of Epidemiology, Helmholtz Zentrum Munich, Neuherberg, Germany. [3]Department of Obstetrics and Gynecology, Technical University of Munich, Munich, Germany. [4]Dept. of Obstetrics and Gynecology & Center on Human Development and Disability (CHDD), University of Washington, Seattle, WA, USA. [5]IBCN, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.

Introduction

Facing a new and challenging situation influencing emotional state, body shape and self-concept, pregnant women are vulnerable to chronic prenatal stress (PS). Moreover, during pregnancy, women's iron needs rises which increases the risk for iron deficiency.



Objectives

We aimed to assess the influence of PS on the sensitive fetal iron metabolism. We hypothesized that PS impacts fetal serum iron biomarkers in a sex-dependent manner.

Methods

Design and Populations	Experimental Design	Cohort
design: human study, prospective, observational cohort study **site:** Klinikum rechts der Isar, TU München, Germany	**inclusion criteria**: women with singleton pregnancy, 18-45 years old, German speaking, 1:1 matching for gestational and maternal age and parity **exclusion criteria**: fetal malformations, IUGR, maternal severe illness, maternal drug abuse **measures of stress:** retrospective with questionnaire in the beginning of 3rd trimester. Grouping as Stress (SG) or Control (CG) based on the PSS-10 score	**sample:** SG: n = 55 CG: n= 52 **analytes:** cord blood, ferritin, transferrin, iron, hepcidin

Results

Cord blood transferrin saturation (p=0.044) and serum iron were lower in stressed male neonates (p=0.008) regardless of iron supplementation. For serum ferritin, the difference was a trend (p=0.075); hepcidin showed no significant difference. The generalized estimating equations (GEE) model examining the contribution of sex to the group difference of the iron serum biomarkers revealed a significant interaction driven by male sex and CG. Not accounting for neonatal sex, the comparison of the analysed values of the iron metabolism showed no significant differences between SG and CG.

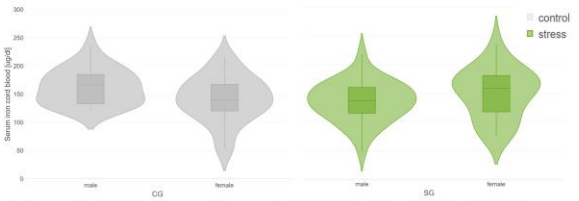


Figure A. GEE Iron: group*sex p=0.016. Outlier threshold is 1.5 IQR.

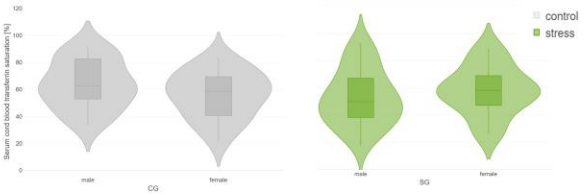


Figure B. GEE transferrin: group*sex p=0.070. Outlier threshold is 1.5 IQR.

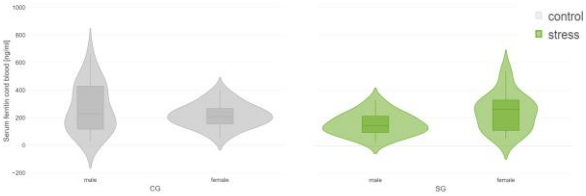


Figure C. GEE ferritin: group*sex p=0.038. Outlier threshold is 1.5 IQR.

Discussion

Influence of PS during third trimester of pregnancy on iron metabolism seems to be sex-dependent. We may observe even stronger group differences regarding iron outcome measures at birth if a future study were to focus on an earlier stage of pregnancy (e.g., first trimester). These findings underscore the importance of early detection of PS effects to enable timely support of the affected children through preventive measures and reduce the risk of later cognitive impairments.

Anhang 8. Poster: 2020 DOHaD ANZ Digital Trainee Conference (12.11.2020)

Klinikum rechts der Isar
Technische Universität München

Hans Fisher Senior Fellowship

IAS TUM Institute for Advanced Study Technische Universität München

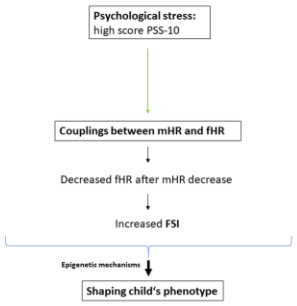
Prenatal stress perturbs fetal heart rate variability in a sex specific manner

Peter Zimmermann[1]; Marta C. Antonelli[1,2]; Ritika Sharma[1,3]; Alexander Müller[4]; Camilla Zelgert[1]; Bibiana Fabre[5]; Natasha Wenzel[6]; Hau-Tieng Wu[7,8]; Martin G. Frasch[9]; Silvia M. Lobmaier[1]

[1]Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technical University of Munich, Germany
[2]Instituto de Biologia Celular y Neurociencias "Prof. E. De Robertis," Facultad de Medicina, UBA, Buenos Aires, Argentina
[3]Helmholtz Zentrum Munich, Neuherberg, Germany
[4]Innere Medizin I, Department of Cardiology, Klinikum rechts der Isar, Technical University of Munich, Germany
[5]Facultad de Farmacia y Bioquimica, Instituto de Fisiopatologia Y Bioquimica Clinica (INFIBIOC), Universidad de Buenos Aires, Buenos Aires, Argentina
[6]Department of Epidemiology, University of Washington, Seattle, WA, USA
[7]Dept. of Mathematics and Dept. of Statistical Science, Duke University, Durham, NC, USA
[8]Mathematics Division, National Center for Theoretical Sciences, Taipei, Taiwan;
[9]Department of Obstetrics and Gynecology and Center on Human Development and Disability (CHDD), University of Washington, Seattle, WA, USA

Background

Facing a new and challenging situation influencing emotional state, body shape and self-concept, pregnant women are vulnerable to chronic prenatal stress (PS). The effect of PS manifests particularly in lasting changes to a child's stress response through the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal system, also known as a process of "fetal programming".



Aims

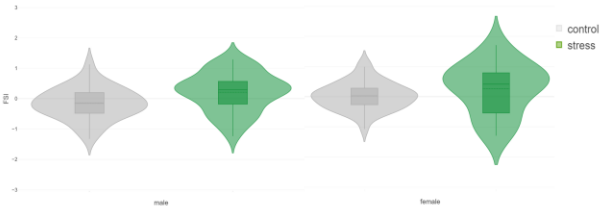
We aimed to assess the influence of PS on the child's ANS. Therefore we evaluated fetal and maternal heart rate (fHR, mHR) coupling and calculated a measure we refer to as fetal stress index (FSI).

Materials and Methods

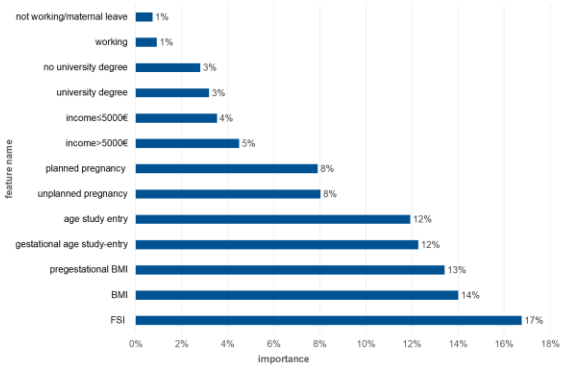
Design and Populations	Experimental Design	Cohort
design: human study, prospective, observational cohort study **site:** Klinikum rechts der Isar, TU München, Germany	**inclusion criteria**: women with singleton pregnancy, 18-45 years old, German speaking, 1:1 matching for gestational and maternal age and parity **exclusion criteria**: fetal malformations, IUGR, maternal severe illness, maternal drug abuse **measures of stress:** retrospective with questionnaire in the beginning of 3rd trimester. Grouping as Stress (SG) or Control (CG) based on the PSS-10 score	**sample:** CG: n = 74 SG: n = 66 **analyte:** mHR and fHR coupling via taECG

Results

Mean FSI was higher among SG than among CG (0.38 ((–0.22)–0.75) versus –0.01 ((–0.36)–0.34); p = 0.024) respectively). FSI group differences were explained by male neonates only. FSI, and demographic data predicted the groups at an AUROC=0.759 (±0.082). Use of clinical and demographic data reduced the classification performance to AUROC = 0.688 (±0.142), similar to using FSI alone (AUROC = 0.665 ± 0.126).



Sex-specific violine plots of FSI group differences. FSI difference was explained by male neonates only.
Male newborns: –0.13 ((–0.45)–0.31) versus 0.30 ((–0.18)–0.61); p = 0.050
Female newborns: 0.10 ((±0.55) versus 0.27 ((±0.84); p = 0.394
Outlier threshold is 1.5 IQR.



Machine learning (ML) feature importance ranking contributing to classification of stressed group and control group participants

FSI: Fetal stress index; BMI: Body-mass index

Conclusion

PS affects coupling between mHR and fHR detectable non-invasively a month prior to birth. Early detection of PS can support neurodevelopmental follow-up to prevent long-term sequelae. Notably, adding biophysical characteristics improves ML model performance, thus emphasizing the potential of antepartum mother–child monitoring using taECG to improve the early detection of health abnormalities such as PS.

Anhang 9. Poster: 2. International Summer School on TSPPM-2021 (16. - 23.07.2021)

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Prenatal stress perturbs fetal iron homeostasis in a sex specific manner

Peter Zimmermann[1], Marta C. Antonelli[1,2], Ritika Sharma[1,3], Alexander Müller[4], Camilla Zelgert[1], Bibiana Fabre[5], Natasha Wenzel[6], Hau-Tieng Wu[7,8,9], Martin G. Frasch[10,11]✉ & Silvia M. Lobmaier[1,11]✉

The adverse effects of maternal prenatal stress (PS) on child's neurodevelopment warrant the establishment of biomarkers that enable early interventional therapeutic strategies. We performed a prospective matched double cohort study screening 2000 pregnant women in third trimester with Cohen Perceived Stress Scale-10 (PSS-10) questionnaire; 164 participants were recruited and classified as stressed and control group (SG, CG). Fetal cord blood iron parameters of 107 patients were measured at birth. Transabdominal electrocardiograms-based Fetal Stress Index (FSI) was derived. We investigated sex contribution to group differences and conducted causal inference analyses to assess the total effect of PS exposure on iron homeostasis using a directed acyclic graph (DAG) approach. Differences are reported for $p < 0.05$ unless noted otherwise. Transferrin saturation was lower in male stressed neonates. The minimum adjustment set of the DAG to estimate the total effect of PS exposure on fetal ferritin iron biomarkers consisted of maternal age and socioeconomic status: SG revealed a 15% decrease in fetal ferritin compared with CG. Mean FSI was higher among SG than among CG. FSI-based timely detection of fetuses affected by PS can support early individualized iron supplementation and neurodevelopmental follow-up to prevent long-term sequelae due to PS-exacerbated impairment of the iron homeostasis.

In the second and third trimester of pregnancy maternal iron requirements can increase up to eightfold or a total of 1 g of additional iron, due to expanding maternal and fetal erythropoiesis[1,2]. Iron homeostasis dysregulation of pregnant mothers and/or children is known to induce lasting neurological damage in the offspring[3].

Prenatal maternal stress (PS), including both pregnancy-specific and general psychosocial stress and anxiety, can jeopardize the balance of the maternal iron homeostasis[4–7]. Pregnant women are especially vulnerable to chronic stress as they face new and potentially challenging situations such as body image issues, lifestyle changes, and fluctuating hormones[8]. PS induces lasting changes to fetal stress response, in a process known as "fetal programming"[9] which might be partly transmitted by the autonomic nervous system (ANS) and the hypothalamic–pituitary–adrenal (HPA) system. In an interim analysis of pregnant women with PS and controls, we showed that PS results in entrainment of fetal heart rate (fHR) by maternal heart rate (mHR), thus yielding a non-invasively obtainable PS biomarker in mother–fetus dyads that we refer to as Fetal Stress Index (FSI)[10]. HPA dysregulation increases the risk of newborn impairment and higher vulnerability toward certain chronic diseases and neurobehavioral disorders[11,12].

[1]Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany. [2]Instituto de Biología Celular y Neurociencias "Prof. E. De Robertis," Facultad de Medicina, UBA, Buenos Aires, Argentina. [3]Helmholtz Zentrum Munich, Neuherberg, Germany. [4]Innere Medizin I, Department of Cardiology, Klinikum Rechts Der Isar, Technical University of Munich, Munich, Germany. [5]Facultad de Farmacia y Bioquímica, Instituto de Fisiopatología y Bioquímica Clínica (INFIBIOC), Universidad de Buenos Aires, Buenos Aires, Argentina. [6]Department of Epidemiology, University of Washington, Seattle, WA, USA. [7]Department of Mathematics, Duke University, Durham, NC, USA. [8]Department of Statistical Science, Duke University, Durham, NC, USA. [9]Mathematics Division, National Center for Theoretical Sciences, Taipei, Taiwan. [10]Department of Obstetrics and Gynecology and Center on Human Development and Disability (CHDD), University of Washington, Seattle, WA, USA. [11]These authors contributed equally: Peter Zimmermann and Marta C. Antonelli as well as Martin G. Frasch and Silvia M. Lobmaier. ✉email: mfrasch@uw.edu; silvia.lobmaier@tum.de

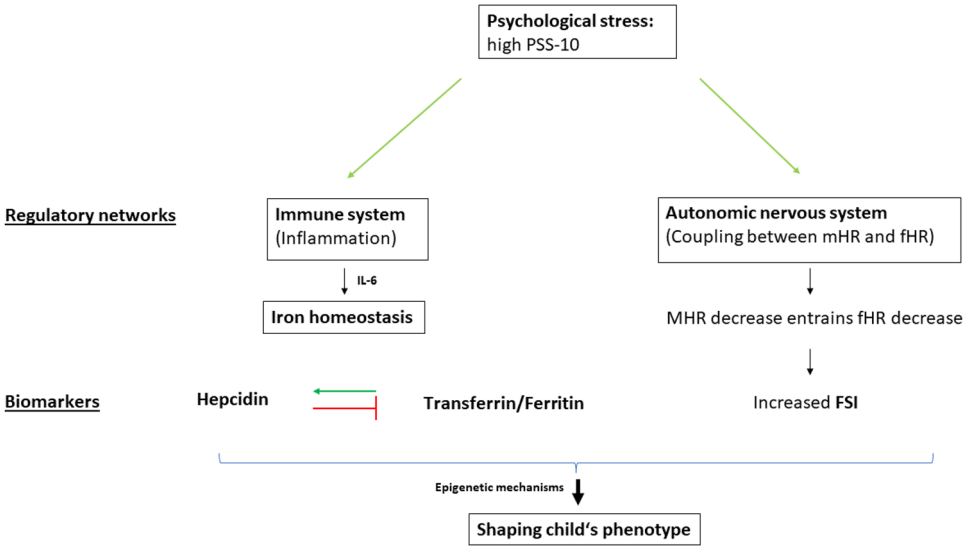

Figure 1. Putative link of PS with iron homeostasis and maternal–fetal heart rate coupling. A proposed simplified model of the hepcidin–placental–ferroportin axis based on the articles of Cortes et al.[15] and Lobmaier et al.[10]: Hepcidin is the key regulator within this system. By binding to ferroportin, it prevents the release of iron into the bloodstream and therefore the synthesis of the transport protein transferrin and the storage protein ferritin. Hepcidin levels are influenced strongly by inflammatory processes, especially the cytokine IL-6. Another possible pathway influencing the child's stress phenotype is assumed to be realized by interference in the coupling between maternal and fetal heart rate (mHR, fHR) as seen during maternal expiration. *PSS* perceived stress scale, *FSI* fetal stress index.

Sex-specific PS effects are well described and recommended for the general consideration as part of human PS studies[13]. However, the contribution of child's sex to the PS-induced alterations in iron homeostasis of the neonate is unclear and suffers from contradictions[4].

Consequently, we tested the hypothesis that PS influences the fetal ANS which results in sex-specific changes to FSI during third trimester and the iron homeostasis in human neonates.

To gauge the fetal iron homeostasis, we assessed the following iron biomarkers in umbilical cord blood serum: ferritin, transferrin, hepcidin[14] and iron[1]. The putative relationships between hepcidin, PS and ANS are summarized in Fig. 1.

Results

Sociodemographic parameters and perinatal outcomes. Women enrolled in the study had a mean age of 33.0 (± 4.4) years and a median gestational age of 36.4 (35.3–37.4) weeks of gestation at study entry. Demographics of the excluded participants did not differ from those with all measures. Statistical comparison of SG and CG showed no differences in the used matching criteria (Table S1).

Maternal and fetal iron homeostasis. 10.4% of the included women were anemic prior to delivery (Hb < 11 mg/dL). However, we found no differences in fetal iron parameters, maternal intake of iron supplements, fetal and maternal hemoglobin, RBC indices and anemia status between SG and CG (Table S2).

FSI. MHR and fHR coupling analysis revealed a higher FSI among SG than among CG (0.38 (− 0.22 to 0.75) versus − 0.01 (− 0.36 to 0.34); p = 0.024) (Table S1). FSI showed no correlation to any measured fetal iron biomarker for either sex (Table S3).

However, within the automatically binned ranges of cord blood serum iron biomarker values, we observed FSI differences. FSI was higher in SG than in CG for ferritin levels between 153 and 279 µg/L ((n = 42; 24 CG; 18 SG); 0.40 (± 0.57) versus 0.01 (± 0.47); p = 0.03, transferrin saturation of 32–47% ((n = 24; 12 CG; 12 SG); 0.30 (± 0.66) versus − 0.24 (± 0.27); p = 0.045), and hepcidin values between 0 and 57 ng/mL ((n = 92; 47 CG; 45 SG) (0.34 (± 0.68) versus − 0.01 (± 0.56); p = 0.01). Using current newborn guidelines and validated ranges the above-mentioned values of iron markers would be normal[16–18]. Overall, FSI at ~ 36 weeks of gestation was higher in SG fetuses averaging 0.34 compared with − 0.10 in CG fetuses within these cord blood iron biomarker ranges.

Sex-specific differences. We identified sex-specific differences in iron homeostasis among male infants and showed that the PS effect on iron homeostasis depends on the neonates' sex.

Cord blood transferrin saturation was lower in SG male neonates compared with those in male CG, regardless of iron supplementation. For ferritin levels, we observed a trend towards lower values in male SG (Table 1).

Characteristics	CG	SG	
Male newborns	**n = 26**	**n = 32**	**p**
FSI (n = 35 CG, n = 43 SG)	− 0.13 (− 0.45 to 0.31)	0.30 (− 0.18 to 0.61)	**0.050**
Maternal hair cortisol [pg/mg] (n = 35 CG, n = 36 SG)	115 (14 to 146)	124 (40 to 161)	0.466
Cord blood ferritin [µg/L]*	229.7 (113.9 to 429.6)	149.6 (96.8 to 234.0)	**0.069**
Cord blood transferrin saturation [%]	63.4 (± 17.7)	52.9 (± 20.2)	**0.041**
Cord blood hepcidin [ng/dL]	26.1 (11.8 to 41.8)	17.0 (10.5 to 30.7)	0.184
Characteristics	**CG**	**SG**	
Female newborns	**n = 28**	**n = 21**	**p**
FSI (n = 39 CG, n = 22 SG)	0.10 (± 0.55)	0.27 (± 0.84)	0.394
Maternal hair cortisol [pg/mg] (n = 32 CG, n = 21 SG)	88 (46 to 119)	122 (67 to 180)	**0.073**
Cord blood ferritin [µg/L]	218.2 (± 84.8)	243.1 (± 130.0)	0.423
Cord blood transferrin saturation [%]	55.9 (± 17.0)	57.8 (± 17.8)	0.703
Cord blood hepcidin [ng/dL]	22.7 (14.1 to 37.7)	20.9 (6.0 to 37.1)	0.599

Table 1. Sex-specific effect of PS on biomarkers. Data are mean (SD) using t-test or median (interquartile range) using Mann–Whitney U test. Sample size is indicated as applicable. Differences with p-value < 0.1 are in bold. *Missing values for 1 SG.

The GEE model revealed that sex is a significant effect modifier that exhibited differences for ferritin (p = 0.038, Fig. 2), and a trend for transferrin saturation (p = 0.070, Fig. S1). For hepcidin, we found no significant sex-driven differences.

Interestingly, maternal hair cortisol tended to increase in SG mothers of female neonates (Table 1). FSI group differences were explained by male neonates only.

Estimated causal effect. We conducted causal inference to investigate the effects of the aforementioned relationships more explicitly. We identified certain variables as adjustment sets in blocking all non-causal paths between the treatment and outcome variables while leaving all causal paths unblocked (Fig. 3). Examination of the causal model on the *PS* → *Cord Blood Ferritin* and *PS* → *Bayley Score* pathway demonstrated two minimum adjustment sets: "Maternal Age" and "SES" or "Maternal Age" and "Education." Either set could be used to obtain an estimate for the causal effect. Our SES data are represented by "Household income > 5000 €/month," and maternal education by "University Degree." Controlling for the minimum adjustment set "University Degree" and "Maternal Age" revealed an estimated average exposure effect of lowered cord blood ferritin at the alpha = 0.10 level at − 38.06 µg/L (95% CI − 79.91 to 3.78) in SG compared with that in CG (Table S4). This average exposure effect became obscured when fetal sex was included (p-value increased from 0.07 to 0.19) demonstrating that sex is a strong effect modifier on the causal pathway between *PS* → *Fetal Iron Biomarker*.

ML for group classification. First, we considered iron biomarkers and FSI, which predicted the groups at an AUROC = 0.706 (± 0.194). Next, we added the salient clinical and demographic data (gestational and maternal age, BMI at study entry, pre-pregnancy BMI, planned/non-planned pregnancy, higher education yes/no, income over 5000€ yes/no). These are the features that we also used in the DAG approach and that were available at the time of the taECG measurement at study entry. Doing so, we achieved an AUROC = 0.759 (± 0.082). The corresponding feature importance ranking is shown in the supplement (Fig. S2). Use of clinical and demographic data reduced the classification performance to AUROC = 0.688 (± 0.142), similar to using FSI (AUROC = 0.665 ± 0.126) or iron parameters (AUROC = 0.587 ± 0.183) alone. In general, sex ranked in the lower 5% of variable importance, yielding only a slight improvement.

Discussion

PS disrupts fetal iron homeostasis in a sex-specific manner. This study indicates a sex-dependent difference in fetal iron homeostasis and FSI due to PS in an otherwise healthy cohort, mainly driven by the male sex. Causal inference approach allowed us to independently verify fetal sex as an important effect modifier on the causal pathway between PS and cord blood ferritin. The findings strengthen previous published FSI results[10].

The PS effect on the fetal iron biomarkers has been poorly understood. Rhesus monkey infants born to stressed mothers were more likely to develop iron deficiency[7]. Likewise, several studies in humans have shown a correlation between PS and cord blood zinc protoporphyrin/heme index as well as PS and ferritin levels[4–6].

During pregnancy, maternal stress hormones such as cortisol influence the growing fetus and its neurodevelopment, presumably via epigenetic mechanisms[9,19]. Cortes and colleagues proposed an influence of chronic stress through a stress-induced altered expression of a variant of the enzyme acetylcholinesterase on the iron-regulating system in fetal sheep brain-derived primary microglia cultures[15]. They assumed the afferent cholinergic anti-inflammatory pathway signaling on microglial α7 nicotinic acetylcholine receptors to down-regulate metal ion transporter and ferroportin, which acts as a hepcidin receptor (Fig. 1).

Animal studies observed stress-dependent cognitive deficits mainly seen in males[20,21]. In humans, sex-specific PS effects are reflected by lower scores in conduct assessments and higher test scores for emotional disturbance in

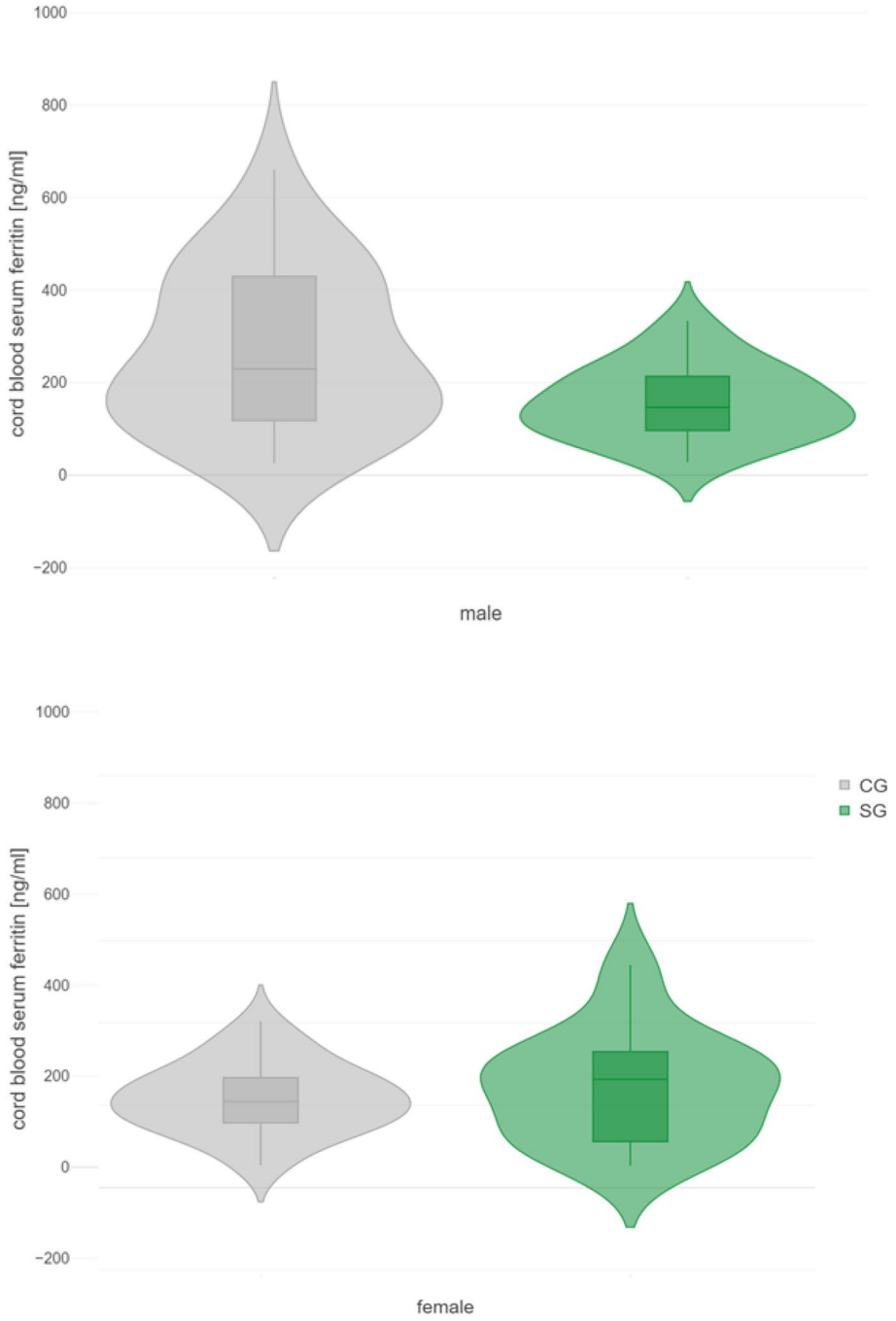

Figure 2. Sex-dependent group difference in cord blood serum ferritin levels. GEE model the main effects of sex and study group and their interaction (sex*group) on ferritin. GEE ferritin: group*sex p = 0.038. *GEE* generalized estimating equations, *SG* stressed group, *CG* control group.

males compared to females[13,22,23]. Campbell et al. applied six specific PS questionnaires, each twice in the second and third trimester, to 428 ~ 28-years-old mothers and found newborns of pregnant women exposed to violence to be stronger associated with cord blood ferritin levels lower in boys than in girls[4].

The relation of iron homeostasis biomarkers to PS. Our results show no relationship between the presence of maternal anemia, fetal iron deficiency and PS. These findings are in agreement with literature suggesting that the fetus is robust against moderate changes of the maternal iron homeostasis[24,25].

Within the DAG framework, we estimated that PS reduced the cord blood serum ferritin levels by approximately 15%. These findings are exceeding the adaption factor for inflammatory processes in infants the WHO uses in a current guideline[26]. We assume that during pregnancy even relatively small additional shifts in fetal iron homeostasis, especially in ferritin levels, may induce sex-specific neurodevelopmental effects[27]. Our observations

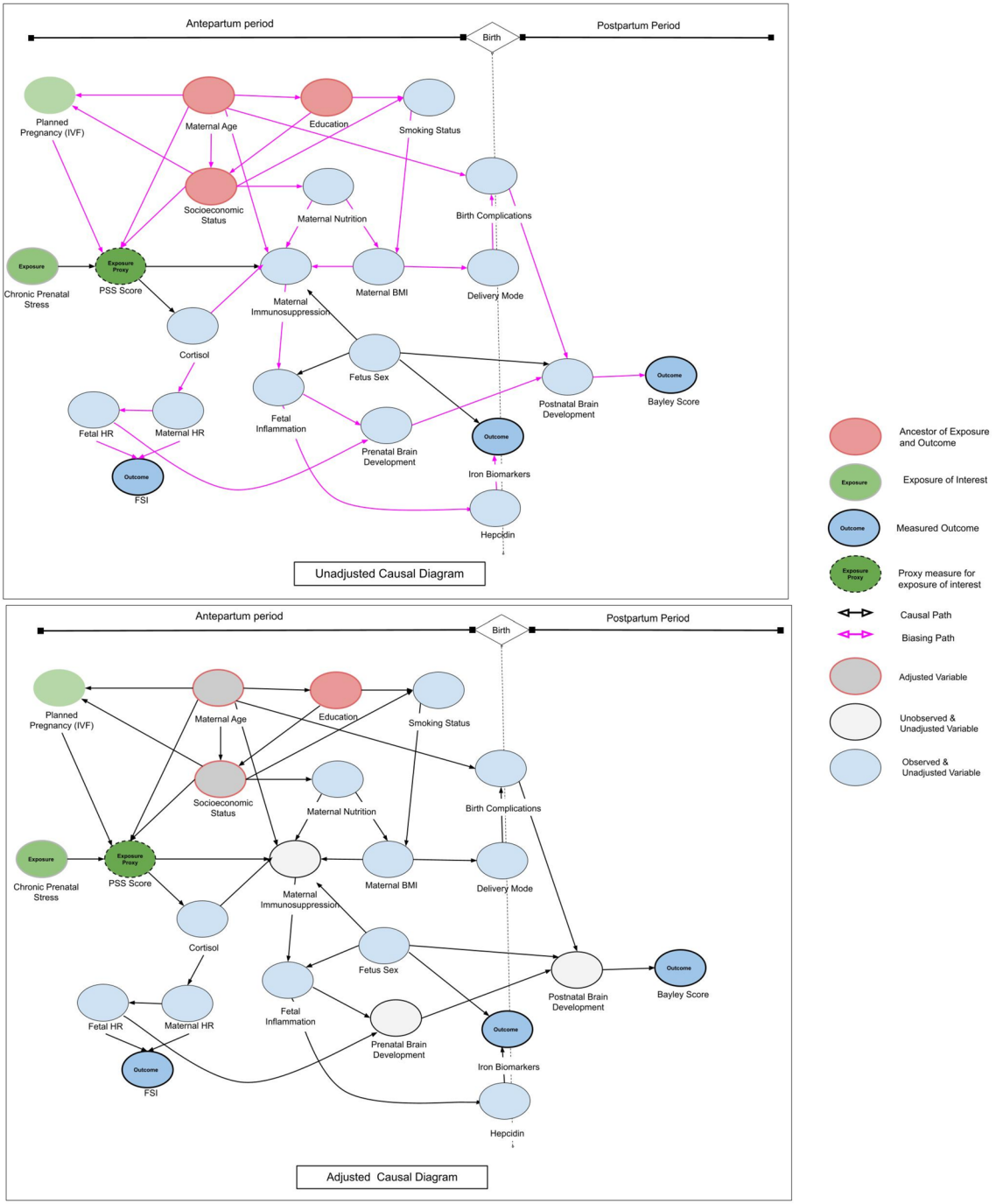

Figure 3. Maternal age, socioeconomic status, and education as confounding factors within the prenatal stress trajectory. Directed acyclic graph analysis of the relationships between maternal and fetal antenatal, perinatal, and postnatal exposures, covariates, and outcomes. *PSS* perceived stress scale, *FSI* fetal stress index, *HR* heart rate, *BMI* body-mass index, *IVF* in-vitro-fertilization.

regarding the link between PS, fetal iron homeostasis and the postnatal neurodevelopmental trajectories warrant further investigations, because this condition may be corrected therapeutically via targeted prenatal and/or postnatal iron supplementation[2].

The PS effect transmitted by maternal cortisol on the fetal neurodevelopment may depend on the time course of exposure[28,29]. Hypothetically, taking our explanation further antepartum, i.e., to ~ 3.5 weeks earlier at the time of taECG recording, we speculate that PS-induced differences in hepcidin at that time may lead to the reported changes in iron parameters that could still be detected in the cord blood[1]. Our exploratory findings of higher FSI within certain ranges of at-birth iron biomarkers support this notion. The absence of group differences of cord blood iron parameters including the whole cohort may reflect adaptions (more pronounced in females) that occur as pregnancy progresses[20].

The role of the immune system. Our data in leukocytes showed no evidence of increased inflammatory processes in SG neonates (Table S1). Nevertheless, acute inflammatory processes, a common phenomenon during delivery, may have had an effect on our cord blood findings transmitted by other cellular messengers such as the cytokine IL-6 (Fig. 1). In general, inflammation upregulates the acute phase protein ferritin influencing its role as a biomarker of the iron storage[30]. Inflammation also upregulates hepcidin levels leading to an intestinal sequestration of iron[14]. Cord blood interleukin levels were increased in chronically stressed mother's infants[31]. Taken together, the effects of PS can be mediated by inflammatory processes and this link should be investigated further in future studies including a broader characterization of the maternal and neonatal inflammatory profiles[32].

FSI as a potential biomarker of PS in late gestation. The present findings confirm that FSI is increased in PS during the third trimester of pregnancy[10]. Because the FSI showed poor association with the measured iron biomarkers, we assume that PS influences fHR and mHR coupling by different pathways. Moreover, in our DAG framework it is conceivable that FSI may serve as an indicator of subsequent altered neurodevelopmental trajectories, even in the absence of biochemical PS correlates such as alterations in iron homeostasis[33,34].

ML-based predictions of PS. With our ML approach, we mimicked a real-life scenario to identify mother–fetus dyads affected by PS. Our results are consistent with findings in other clinical settings where electronic medical record mining identified patients at risk even without additional biophysical assessments, such as ECG[35]. Notably, adding biophysical characteristics improves ML model performance, thus emphasizing the potential of antepartum mother–child monitoring using taECG to improve the early detection of health abnormalities such as PS.

Strengths and limitations. Strengths of the FELICITy study are the prospective design preventing recall bias and the definition of criteria for a matching system to exclude possible confounders. Additionally, eventual confounding factors such as the intake of iron supplement and ethnic group showed no group differences (Fig. S1). This is the first prospective longitudinal study starting in utero aiming to assess PS and fetal biomarkers. Additionally, it is the first study to use causal inference and machine learning approaches to investigate sex-dependent influence of PS on the fetal iron homeostasis. There are certain limitations. Our inclusion criteria prevented us from enrolling non-German-speaking patients. This may have biased how the PS effects are represented in the multicultural Munich population. Also, we used a matching system that could not include every screened CG patient. Due to the uncertainties of a human study, several subject numbers for different sub-analyses were lower. Furthermore, we focused on measuring PS in the third trimester which necessarily neglected earlier stages of pregnancy and a possible temporal dynamic of PS over the entire course of pregnancy.

We chose not to include other potential effect modifiers on the causal pathway of the DAG such as inflammatory processes as they are difficult to define quantitatively and were not the focus of this study. However, future studies could further refine estimates of *PS → Iron Biomarker* average exposure effect by adjusting for these covariates.

This study did not differentiate between arterial or venous origin of the analyzed cord blood samples. To our knowledge this issue has not been addressed in literature so far. In general, the placental iron transfer and the assessment of the fetal iron status using cord blood parameters are poorly understood[36,37]. As of the date of the manuscript's submission no commonly used normal ranges of cord blood iron parameters exist. The established ranges start with the child's birth[26] but are not applicable to cord blood ranges since in cord blood usually, iron parameters are higher[38]. These issues warrant further research to identify potential biasing effects on cord blood analysis.

Conclusions

We show that during third trimester PS exerts a sex-dependent effect on fetal iron homeostasis and on the fetal ANS measured by FSI, an ECG-derived measure of chronic stress transfer from mother to fetus. The reported biomarkers open novel avenues of research into the association between PS and adverse neurodevelopmental outcomes. They can contribute to development of novel therapeutic intervention strategies[39,40].

We propose the following aspects of future research: First, do the changes we report represent a healthy or maladaptive response to PS? Second, will applying FSI monitoring during pregnancy permit to track a "deviating neurodevelopmental trajectory" and when exactly during gestation do these changes occur? The non-invasive fetal monitoring with FSI tracking may help answer these questions. Third, what is the significance and therapeutic opportunity of the discovery that PS can impact fetal iron homeostasis? Do female fetuses possess more successful compensatory mechanisms in response to PS than males do? The sex-specific effects of this impact warrant further investigations. Fourth, which role may iron supplementation play in this context as a corrective therapeutic option[2,41]? Our findings indicate that we will need to consider the sex when devising therapeutic strategies to compensate for the intrauterine adversity due to PS[42].

Methods

Ethical considerations. The study protocol is in strict accordance with the Committee of Ethical Principles for Medical Research from Technical University of Munich (TUM) and has the approval of the "Ethikkommission der Fakultät für Medizin der TUM" (registration number 151/16S). ClinicalTrials.gov registration number is NCT03389178. Written informed consent was obtained from each subject for participation in this

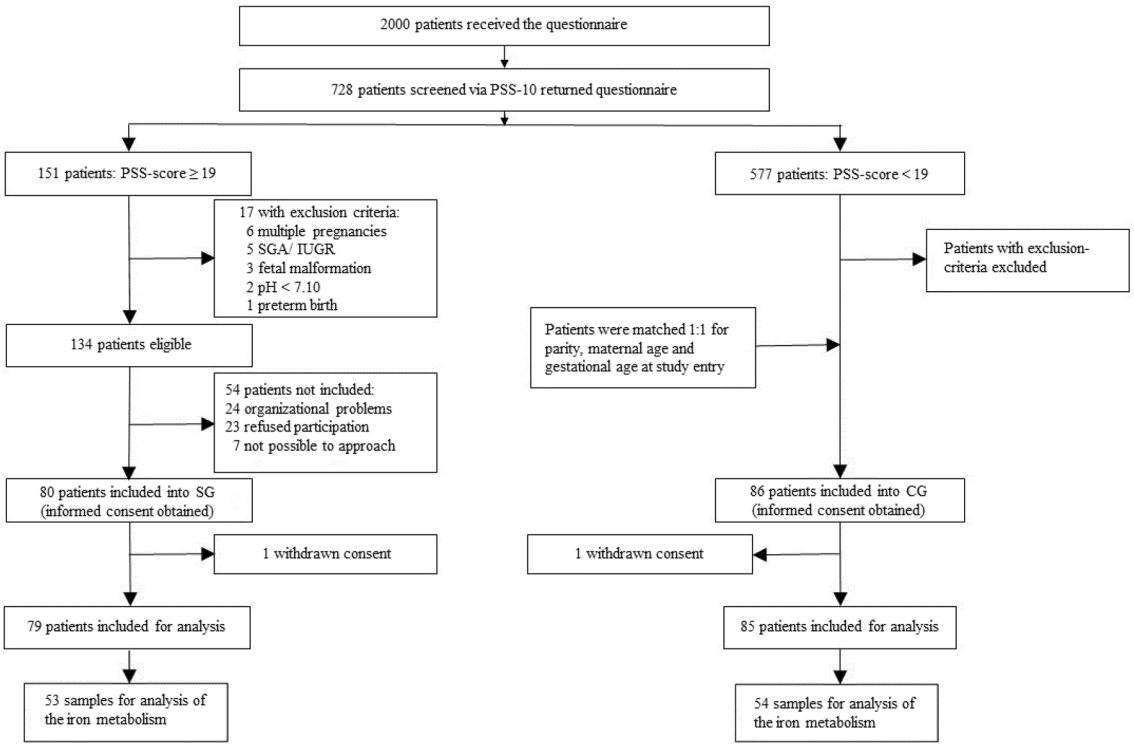

Figure 4. Recruitment flow chart. *SG* stressed group, *CG* control group, *PSS* perceived stress scale, *SGA* small for gestational age, *IUGR* intrauterine growth restriction.

study after having read an informative brochure and after PS screening via questionnaires and before data collection in the third trimester.

Procedures. *Study design and study population.* A prospective matched double cohort study was performed between June 2016 and July 2019 at the Department of Obstetrics and Gynecology at "Klinikum rechts der Isar" of the TUM, Germany (Fig. 4). We screened 2000 women using the validated German version of "Cohen Perceived Stress Scale-10" (PSS-10) questionnaire[43]. This test quantifies the overall chronic stress based on 10 items including anxiety, depression, abnormal fatigue, and general dissatisfaction as symptoms of a generally perceived stress[44]. By means of PSS-10 we classified the patients into either stressed group (SG) or control group (CG) using a cutoff PSS-10 score of ≥ 19[10].

Singleton pregnant women between 18 and 45 years of age in their third trimester (at least 28 weeks gestation) were included. Exclusion criteria were serious placental alterations (e.g., IUGR), fetal malformations, maternal severe illness during pregnancy[45], preterm birth, cord blood pH < 7.10, and maternal drug or alcohol abuse. The CG (n = 85; PSS-10 < 19) was additionally matched with SG patients (n = 79; PSS-10 ≥ 19) for parity and gestational and maternal age at study entry (Fig. 4). 728 participants returned the PSS-10 questionnaire and a total of 164 pregnant women were recruited.

Data collection. Participants received a sociodemographic and medical history questionnaire including a bivariate question regarding iron supplementation of usually 100 mg per day. Additionally, we recorded a transabdominal electrocardiogram (taECG) at 900 Hz sampling rate of at least 40 min duration using AN24 (GE HC/Monica Health Care, Nottingham, UK). Maternal and fetal ECGs were extracted from taECG signal as described before[10]. FHR and mHR coupling was estimated using the bivariate phase-rectified signal averaging method yielding the FSI[46] which provided a measure for the fetal response to mHR decreases[10]. FSI data was evaluable from n = 139 study subjects (SG: n = 74; CG: n = 65).

During delivery, cord blood samples from a total of n = 107 patients (SG: n = 53; CG: n = 54) were extracted (Fig. 4). EDTA tubes were directly analyzed to receive an adequate hemogram and serum samples were stored at − 80 °C until analysis[47,48]. Serum iron, transferrin, and ferritin were measured at the internal clinical laboratory of "Klinikum rechts der Isar," and hepcidin was determined using the commercial competitive "Hepcidin 25 (bioactive) HS ELISA" (DRG Instruments GmbH, Marburg, Germany).

Maternal hair samples were taken during postnatal hospitalization at the posterior vertex region of the scalp[49] for cortisol measurement using auto-analyzers[50]. Cortisol levels in 3-cm hair samples reflect chronic stress exposure of approximately 3 months prior to delivery.

After childbirth, the CG and SG perinatal outcomes were assessed. Covariates reviewed were gestational age, maternal age, gravidity, body-mass index (BMI), ethnicity, nicotine use, socioeconomic status (SES), birth weight, sex, Apgar score, and cord blood gas analysis. Additionally, clinical routine laboratory parameters were recorded including maternal hemoglobin and anemia status at the moment of hospital admission for delivery[51].

Statistics for between-group comparison. Continuous data were tested via Shapiro–Wilk test for normal distribution. We used t-tests for independent samples to compare SG and CG when data followed a Gaussian distribution. For non-normally distributed data, the Mann–Whitney U test was performed. Pearson's Chi-squared test compared binary coded data, and Spearman's rank-order correlation examined the relations between two variables. To assess exploratively differences in FSI in relation to iron biomarkers due to PS, we binned the value distribution of each iron biomarker automatically into five categories of equal width and compared the corresponding FSI values within each small subset. We used generalized estimating equations (GEE) to model the main effects of sex and study group and their interaction (sex*group) on iron biomarkers. All statistical tests executed were two-sided, and we assumed a significance level (α) of 0.05. IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, NY, USA) and Exploratory version 6.2.2 were used for modeling, statistical analysis, and visualization.

Causal graph analysis. We used DAGitty (www.dagitty.net)[52] to construct a directed acyclic graph (DAG) which defined the causal relationships between exposure (PS) and the two outcome measures: Iron biomarkers and a composite measure of neurodevelopment using the German adaptation of "Bayley Scales of Infant and Toddler Development—Third Edition" (Bayley Score) (Fig. 3). The Bayley score has not yet been calculated for the present cohort, however we included it as an outcome measure in the causal inference framework to allow for adjustment of unobserved variables and to prevent conflict with the iron biomarker outcome. The purpose of the DAG was to visualize the structural relationships between variables and minimize bias in our statistical analysis[53] (additional Explanation in Supplement N1). The estimation of the total PS effect on both outcomes was performed in SAS version 3.8 using the "CAUSALGRAPH" function with robust error estimation.

Machine learning (ML). To test the clinical utility and relative contribution of the measured parameters we classified the participants as SG or CG using ML approaches on the collected demographic, clinical, biophysical (i.e., FSI) and biochemical features (scikit-learn on Dataiku DSS 8.0.2)[54]. Binary features were expressed as categorical variables and dummy-encoded. No imputation was undertaken; rather, the missing rows were dropped. Using the conventional 80 : 20 data split for training : validation, we tested the classification performance of the following algorithms: random forest, gradient tree boosting, logistic regression, decision tree, K nearest neighbors (grid), extra trees, artificial neural network, LASSO-LARS, SVM and SGD. Threefold cross-validation with randomized grid search was used for hyperparameter optimization and fivefold cross-validation to rank the models created by each algorithm on AUROC (area under receiver operating curve). The highest-ranking model was selected and reported for each test.

Data availability

The datasets generated and/or analyzed during the current study are not publicly available due to possible links to patients and especially newborns identities but are available from the corresponding author on reasonable request.

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Author contributions
P.Z.: patient recruitment, data collection and management, data analysis, and manuscript writing. M.F.: data collection and management, data analysis, machine learning analysis and manuscript writing and editing. S.M.L. and M.C.A.: protocol and project development, data collection and management, data analysis, and manuscript writing and editing. A.M. and H.T.W.: machine learning analysis and manuscript writing and editing. N.W.: DAG analysis, manuscript reviewing and editing. R.S.: manuscript reviewing and editing, CZ: patient recruitment and data collection. B.F.: cortisol analysis in hair samples and manuscript editing.

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MGF holds patents on fetal ECG technologies. No other disclosures were reported.

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Correspondence and requests for materials should be addressed to M.G.F. or S.M.L.

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Supplementary Online Content

Table S1. Study outcome parameters

Characteristics	CG	SG	
	n=85	n=79	p
Baseline			
Gestational age at screening [weeks]	34.0 (33.3–35.0)	34.0 (32.6–34.9)	0.304
Gestational age at inclusion [weeks]	36.7 (35.2–37.6)	36.4 (35.3–37.4)	0.612
Age mother at study entry [years]	33.4 (±3.7)	32.7 (±5.1)	0.307
BMI at study entry [kg/m²]	26.3 (24.4–28.9)	27.8 (25.3–34.6)	**0.010**
BMI pregestational [kg/m²]	21.5 (20.2–23.5)	23.3 (20.7–27.5)	**0.013**
Score PSS	9 (6–12)	22 (20–24)	**<0.001**
Cortisol in maternal hair [pg/mg]	88 (40–133)	97 (61–165)	0.104
European/Caucasian	78 (92)	73 (92)	0.879
Married	67 (80)	55 (70)	0.136
University degree	65 (77)	46 (58)	**0.013**
Household income> 5000€/month	49 (58)	28 (35)	**0.004**
Smoking	1 (1)	7 (9)	**0.022**
Multiparity	37 (44)	38 (48)	0.557
Planned pregnancy	75 (93)	53 (67)	**0.001**
IVF / ICSI	9 (11)	2 (3)	**0.039**
Gestational diabetes	2 (2)	12 (15)	**0.003**
Autoimmune disease	6 (7)	13 (16)	0.060
Working status at screening	3 (4)	4 (5)	0.502
Iron supplement	31 (36)	35 (44)	0.307
FSI*	–0.01 ((–0.36)–0.34)	0.38 ((–0.22)–0.75)	**0.024**
Perinatal outcome			
Gestational age at birth [weeks]	39.9 (39.0–40.6)	39.5 (38.6–40.6)	0.148
Birthweight [g]	3526.9 (395.1)	3484.0 (463.0)	0.526
Birthweight percentile [%]	49.0 (28.3–71.8)	55.0 (28.0–74.3)	0.863
Length [cm]	52.9 (±2.5)	52.8 (±2.6)	0.919
Head circumference [cm]	35 (34–36)	35 (34–36)	0.412
Cesarean delivery	17 (20)	27 (35)	**0.035**
Labor induction	15 (18)	19 (24)	0.310

Gender female	41 (48)	30 (38)	0.137
5-min Apgar<7	3 (4)	2 (3)	0.691
Admission to NICU	3 (4)	3 (4)	0.912
Arterial plasma cord blood analysis results			
Base Excess [mmol/L] (n=78 CG, n=72 SG)	–5.5 (±3.3)	–5.2 (±3.0)	0.557
Lactate [mmol/L] (n=53 CG, n=50 SG)	4.4 (3.0–5.3)	3.8 (3.0–4.8)	0.317
Glucose [mg/dL] (n=57 CG, n=51 SG)	84.0 (64.0–98.0)	71.0 (63.5–91.5)	0.338
pH (n=81 CG, n=77 SG)	7.26 (±0.09)	7.28 (±0.08)	0.203
PO2 [mmHg] (n=66 CG, n=57 SG)	21.1 (16.7–26.6)	18.4 (13.6–23.5)	0.102
PCO2 [mmHg] (n=69 CG, n=64 SG)	50.8 (±10.2)	49.4 (±9.2)	0.382
Leukocytes [G/L] (n=53 CG, n=49 SG)	14.6 (11.9–17.4)	13.3 (10.2–17.6)	0.291
Neutrophils [%] (n=53 CG, n=48 SG)	51.0 (46.5–56.0)	54.0 (47.0–61.0)	0.249

Data are mean (SD) using t-test, median (interquartile range) using Mann-Whitney U test or n (%) using Pearson's Chi-squared test. Sample size is indicated as applicable. Differences with p-value < 0.05 are in bold.

PSS: Perceived stress scale; PDQ: Prenatal distress questionnaire; BMI: Body-mass index; NICU: Neonatal intensive care unit; ICSI: Intracytoplasmic sperm injection; IVF: In-vitro-fertilization

*missing values for 11 CG and 14 SG

Table S2. Iron parameters

Characteristics	CG	SG	
	n=54	n=53	p
Cord blood serum iron [µg/dL]	151.5 (±37.3)	141.4 (±38.5)	0.172
Cord blood serum transferrin [mg/dL]	176.3 (162.2–205.9)	186.6 (165.8–217.0)	0.348
Cord blood serum transferrin saturation [%]	59.5 (±17.6)	54.8 (±19.3)	0.189
Cord blood serum ferritin [µg/L]*	242.4 (140.6–329.6)	176.0 (106.4–267.0)	0.134
Cord blood serum hepcidin [ng/dL]	23.6 (13.4–39.24)	18.9 (9.2–36.9)	0.184
Cord blood plasma hemoglobin [mg/dL]**	15.6 (±1.6)	15.7 (±1.6)	0.832
Cord blood plasma MCV [fL]**	104 (101–106)	104 (100–107)	0.734
Cord blood plasma MCH [pg]**	35 (34–35)	35 (34–35)	0.605
	n=74	n=71	p
Maternal prenatal plasma hemoglobin [mg/dL]	12.3 (1.0)	12.2 (1.1)	0.376
Maternal prenatal plasma MCV [fL]	87 (84–90)	88 (83–90)	0.766
Maternal prenatal plasma MCH [pg]	30 (28–31)	30 (28–31)	0.862
Maternal prenatal anemia: Hb<11mg/dL	8 (10.8)	9 (12.7)	0.537
Maternal postnatal plasma hemoglobin [mg/dL]***	11.1 (1.5)	10.8 (1.3)	0.164
Maternal postnatal plasma MCV [fL]***	87 (85–91)	88 (85–91)	0.669
Maternal postnatal plasma MCH [pg]***	30 (29–31)	30 (28–31)	0.683

Data are mean (SD) using t-test, median (interquartile range) using Mann-Whitney U test or n (%) using Pearson's Chi-squared test.

*missing values for 1 SG

**missing values for 1 CG and 4 SG

***missing values for 3 SG

Table S3. Sex-specific linear regression of FSI and serum iron parameters

Characteristics	**Male newborns** (n=58)		**Female newborns** (n=49)	
	R^2	p	R^2	p
Iron [µg/dL]	0.001	0.78	<0.001	0.96
Transferrin saturation [%]	0.002	0.89	<0.001	0.94
Ferritin [µg/L]*	0.003	0.96	0.048	0.13
Hepcidin [ng/dL]	0.028	0.23	0.004	0.84

Correlations were performed using Spearman's rank correlation.

*missing values for 1 SG with male sex

Table S4. Study outcome parameters for minimum adjustment sets: effect on serum ferritin and role of sex

Parameter	Exposure Group	Estimate of Ferritin (µg/L)	Robust Std. Error	95% Confidence Limits		Z	Pr > |Z|
Adjustment for "Household income > 5000€/month" and "Maternal Age."							
POM	CG	245.12	21.00	209.28	280.95	13.41	<.0001
POM	SG	212.08	21.00	170.92	253.24	10.10	<.0001
Average Exposure Effect		–33.04	27.93	–87.79	21.71	–1.18	0.2369
Adjustment for "University Degree" and "Maternal Age."							
POM	CG	246.76	17.08	213.28	280.25	14.44	<.0001
POM	SG	208.70	16.85	175.68	241.72	12.39	<.0001
Average Exposure Effect		–38.06	21.35	–79.91	3.78	–1.78	**0.0746**
Adjustment for "Household income > 5000€/month", "Maternal Age" and "Fetus Sex."							
POM	CG	245.15	19.45	207.03	283.28	12.60	<.0001
POM	SG	212.48	16.44	180.27	244.69	12.93	<.0001
Average Exposure Effect		–32.68	24.31	–80.32	14.96	–1.34	0.1788
Adjustment for "Household income > 5000€/month", "University Education" and "Fetus Sex."							
POM	CG	248.07	20.48	207.93	288.21	12.11	<.0001
POM	SG	213.01	20.61	172.60	253.41	10.33	<.0001
Average Exposure Effect		–35.06	26.73	–87.44	17.33	–1.31	0.1896

Differences in Average Exposure Effect with p-value < 0.1 are in bold.

POM: Potential Outcome Model

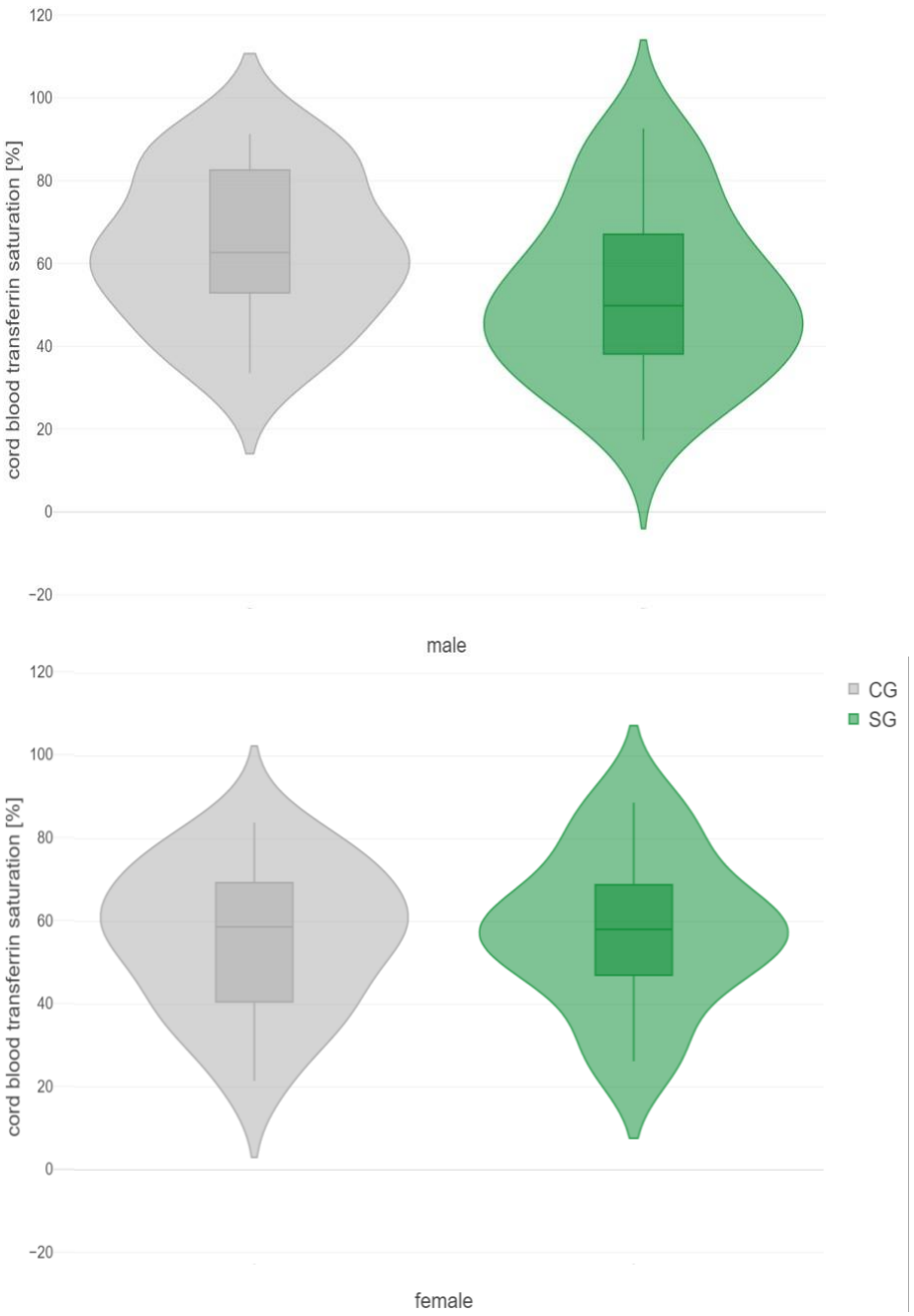

Fig S1. Sex-dependent group difference in cord blood serum transferrin sat. levels
GEE model the main effects of sex and study group and their interaction (sex*group) on transferrin sat. levels.
GEE transferrin: group*sex p = 0.070

SG: Stressed group; CG: Control group; GEE: Generalized estimating equations

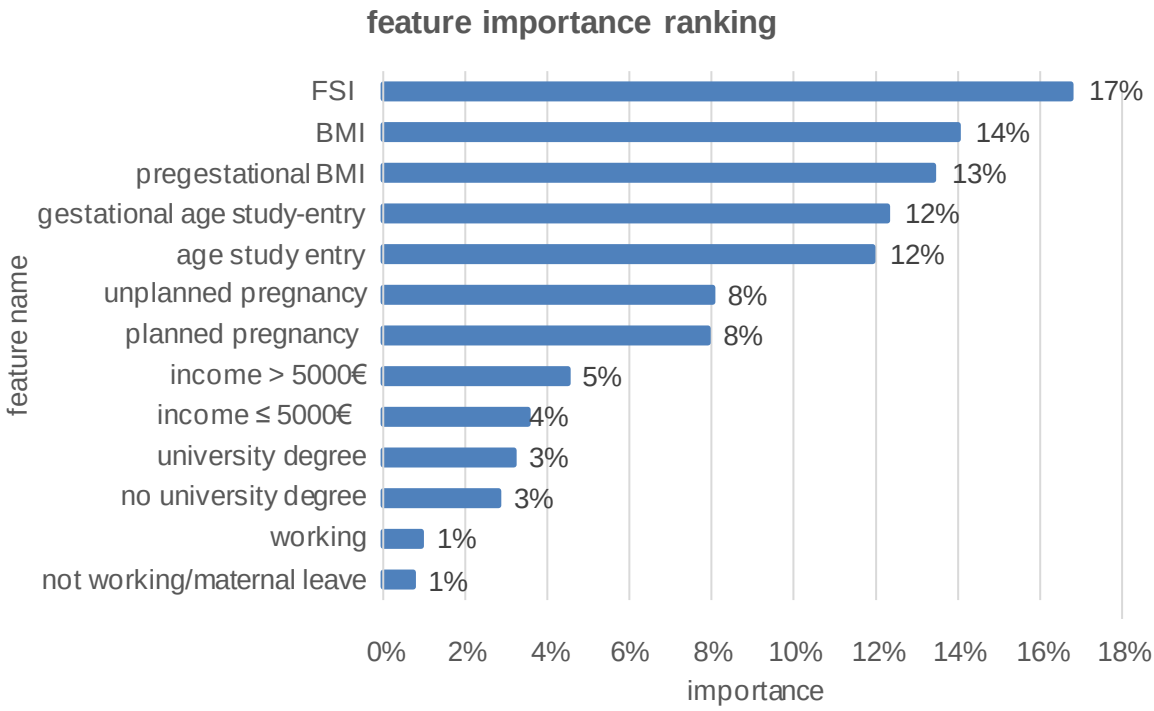

Fig S2. Machine learning feature importance ranking contributing to classification of stressed group and control group participants

FSI: Fetal stress index; BMI: Body-mass index

Supplement N1. Additional information about causal inference analysis and causal diagrams

The causal diagram shown in Fig. 3 was built as a conceptual model to demonstrate the authors' assumptions about the factors influencing the neonates' health outcome. As work by Greenland et al.[1] has demonstrated, if these diagrams are constructed according to certain rules they can provide a rigorous way to address confounding. A correctly drawn causal diagram can be used to determine whether controlling for a certain combination of variation would be sufficient to remove confounding from the exposure-outcome association, or to identify variables that should not be controlled or that need not be controlled.

As this cohort study examined multiple maternal exposures, determining a causal diagram was necessary to aid in communication with our audience *and* to ensure that we controlled for any confounding, especially as confounding can also depend on what other variables have already been controlled for.

In terms of this study, we made use of the online software "dagitty", a browser-based environment for creating, editing, and analyzing causal diagrams that makes use of the aforementioned causal diagram rules defined by Greenwood et al.

- An arrow denotes a direct causal effect: that is X→ Y implies that with all other variables held constant,
  changing X would change Y.
- While traditional approaches focus on individual variables, causal diagram theory focuses on how variables relate to causal paths and which set of control variables are sufficient to block those paths. In the case of Figure 3, pink arrows represent a direct causal effect along a biased path, while black arrows represent a direct causal effect along a non-biased path.
- The minimal adjustable set discussed in the manuscript is the minimum number of variables to 'close' all biasing paths (turn all pink lines to black) shown in the latter half of the new Fig. 3.

1 Greenland, S., Pearl, J. & Robins, J. M. Causal diagrams for epidemiologic research. Epidemiology 10, 37-48 (1999).

Anhang 10. Zimmermann et al. Prenatal stress perturbs fetal iron homeostasis in a sex specific manner

Clinical Epigenetics

RESEARCH

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Maternal–fetal stress and DNA methylation signatures in neonatal saliva: an epigenome-wide association study

Ritika Sharma[1,3†], Martin G. Frasch[2†], Camila Zelgert[1], Peter Zimmermann[1], Bibiana Fabre[4], Rory Wilson[3], Melanie Waldenberger[3], James W. MacDonald[5], Theo K. Bammler[5], Silvia M. Lobmaier[1†] and Marta C. Antonelli[1,6*†]

Abstract

Background: Maternal stress before, during and after pregnancy has profound effects on the development and lifelong function of the infant's neurocognitive development. We hypothesized that the programming of the central nervous system (CNS), hypothalamic–pituitary–adrenal (HPA) axis and autonomic nervous system (ANS) induced by prenatal stress (PS) is reflected in electrophysiological and epigenetic biomarkers. In this study, we aimed to find non-invasive epigenetic biomarkers of PS in the newborn salivary DNA.

Results: A total of 728 pregnant women were screened for stress exposure using Cohen Perceived Stress Scale (PSS), 164 women were enrolled, and 114 dyads were analyzed. Prenatal Distress Questionnaire (PDQ) was also administered to assess specific pregnancy worries. 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's saliva for epigenetic analyses. DNA was extracted from saliva samples, and DNA methylation was measured using EPIC BeadChip array (850 k CpG sites). Linear regression was used to identify associations between PSS/PDQ/FSI/Cortisol and DNA methylation. We found epigenome-wide significant associations for 5 CpG 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 50 kb from ***SSBP4*** and ***SCAMP1***. In addition, two differentiated methylation regions (DMR) related to maternal stress measures PDQ and cortisol were found: ***DAXX*** and ***ARL4D***.

Conclusions: Genes annotated to these CpGs were found to be involved in secretion and transportation, nuclear signaling, Hippo signaling pathways, apoptosis, intracellular trafficking and neuronal signaling. Moreover, some CpGs are annotated to genes related to autism, post-traumatic stress disorder (PTSD) and schizophrenia. However, our results should be viewed as hypothesis generating until replicated in a larger sample. Early assessment of such noninvasive PS biomarkers will allow timelier detection of babies at risk and a more effective allocation of resources for early

†Ritika Sharma, Martin G. Frasch, Silvia M. Lobmaier and Marta C. Antonelli are equally contributing authors

*Correspondence: mca@fmed.uba.ar

[1] Department of Obstetrics and Gynecology, Klinikum Rechts der Isar, Technical University of Munich, Munich, Germany
Full list of author information is available at the end of the article

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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.

Keywords: Pregnancy, Prenatal stress, Perceived stress, Biomarkers, Newborn saliva, DNA methylation, Cortisol, Epigenetics, EWAS

Background

Compelling evidence from both animal and human studies indicates that adversities in the perinatal environment significantly increase the risk for developing neurocognitive disorders later in life [1–10].

Human studies provide substantial evidence that maternal stress during the gestational period (namely PS) can lead to behavioral, cognitive and temperamental disorders in the infant, increasing child morbidity and neurological dysfunction. For example, maternal psychosocial stress (general and specific stress and anxiety) increases the risk for the growing infant to develop disorders such as attention-deficit hyperactivity disorder (ADHD) [10], autism spectrum disorders (ASD) [11] and sleep disturbance that can result in depression and other psychiatric disorders. Severe PS is associated with increased cortisol response after a behavioral challenge paradigm in young adults [12]. Moreover, several studies have indicated that PS and glucocorticoid exposure may reprogram the cardiovascular system, including aberrations in cardiac and kidney development [13].

It is important to understand the mediators that connect the mother' stress with the fetus. In this regard, Rakers et al. [8] proposed that the causal pathway lies not only via cortisol but also includes catecholamines, reactive oxygen species, cytokines, serotonin/tryptophan and maternal microbiota. The stress response system has been traditionally linked to the hypothalamic–pituitary–adrenal (HPA) axis that is responsible for the production and secretion of corticosteroids under basal and stressed conditions [14]. However, the autonomic nervous system (ANS) also plays a key role through its rapid (within seconds) activation enabling fine adjustments of the target organs. This aspect has been often neglected. In this regard, Monk et al. [15] have shown that the fetal ANS is very perceptive of maternal anxiety. This study shows that during women's recovery following a stress-eliciting task, fetal heart rate (fHR, a biomarker of ANS) changed in association with the mother's acute cardiovascular activity [15]. In addition, fHR sensitivity to a stimulus reflects emerging individual differences in the development of ANS [16, 17].

Responding to environmental factors, these stress-mediating pathways are assumed to leave permanent epigenetic signatures that may affect the neurobehavioral outcomes of the child. In fact, it has been shown that the epigenome is vulnerable to external exposures during the early prenatal development, a crucial period when intense programming of gene expression is taking place [18, 19]. Among these mechanisms, DNA methylation is recognized as the most well-characterized epigenetic signature.

While some knowledge has been gained linking the scale of epigenetic organization of phenotypical information with the psychological behavior [20, 21], the connection between the epigenetic signatures of adversity and the scale of biophysical organization of human integrative physiology has remained unexplored. This is of great interest because biophysical behavior, such as the properties of ANS describable noninvasively by mathematical HRV analyses, is highly accessible, now more than ever, with the rise of the wearable technologies and remote health monitoring [22]. As such, linking these scales of physiological organization may aid in early detection of unhealthy developmental trajectories and timely intervention while also providing a more mechanistic multi-scale framework for understanding these complex relationships [7]. With the present 'FELICITy' study, we aimed to address this knowledge gap.

First, we hypothesized that early epigenetic signatures of PS are detectable in neonatal saliva. 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 [23]. Second, 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 noninvasive multimodal physiological measures of PS.

We recruited a cohort of third trimester pregnant women screened for exposure to chronic psychosocial stress during pregnancy. The biophysical signature of the PS exposure has been validated during late gestation using noninvasive feto-maternal transabdominal electrocardiogram (taECG). This approach yielded fetal stress index (FSI), a joint maternal–fetal biomarker of PS [24]. At the same time, we also acquired quantitative psychological maternal chronic stress scores (details in Results and Methods) [24]. Using maternal behavioral data and the biophysical FSI assessment on the one hand and the epigenetic analysis of the newborn's saliva on the other

hand, we aimed to validate the presence of linkages between the mother–fetus behavior and the neonatal saliva's epigenetic modifications. To test for persistence of such multimodal multi-scale linkages we are currently following up this cohort for a second time point at two years of age. Here, we report the existence of such anticipated linkages at birth. The study represents a first report of a well-controlled, prospective study to investigate epigenome-wide methylation changes in > 850.000 loci in newborn saliva samples in association with behavioral and biophysical maternal–fetal stress measures.

Results

We performed an Epigenome-Wide Association Study (EWAS) with the Illumina MethylationEPIC BeadChip array, which includes around 850,000-methylation loci 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.

Cohort characteristics

Of the 728 subjects who returned the questionnaires, the socio-demographic characteristics of the participating mothers and their offspring (n = 114) are shown in Table 1. The average age of the mother at study entry was 34 years. Most of the mothers in this study were Caucasians, married and having a university degree. Pregnancies were mostly planned, and 72% of mothers had vaginal deliveries. The perceived stress measures (PSS and PDQ scores) were moderately correlated (Spearman R2 = 0.537; *p* value = 6.952e-10).

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.

Table 1 Baseline characteristics of study population (n = 114, mother–newborn pairs), FELICITy study

Characteristics	n = 114
Maternal characteristics—Baseline	
Age of mother at study entry, years	34.61 (± 4.52)
Gestational age at screening, weeks	34.07 [33.18, 34.96]
Gestational age at inclusion, weeks	36.71 [35.32, 37.54]
Score PSS	17.00 [9.00, 22.00]
Score PDQ	10.50 [6.25, 16.75]
BMI at study entry, kg/m^2	27.61 [25.20, 30.42]
BMI pregestational, kg/m^2	21.82 [20.30, 24.96]
Ethnicity, Caucasians/Europeans	106 (92.98)
Married, yes	85 (74.6)
University degree, yes	80 (70.2)
Household income > 5000€/month, Yes	53 (46.5)
Working status at screening, working	4 (3.5)
Multiparity, Yes	85 (74.6)
Planned pregnancy, Yes	92 (81.4)
Cesarean delivery, Yes	28 (24.6)
Smoking, Yes	7 (6.1)
IVF / ICSI, Yes	10 (8.8)
Gestational diabetes, Yes	11 (9.6)
Autoimmune disease, Yes	15 (13.2)
Cortisol in maternal hair, pg/mg	97.00 [58.00, 161.00]
Infant characteristics—Perinatal outcome	
FSI	0.15 [−0.28, 0.60]
Gestational age at birth, weeks	39.71 [38.86, 40.57]
Gender, female	52 (45.6)
Birthweight, grams	3544.96 (± 431.78)

Data are mean (SD) using chi-square test, mean (interquartile) using Wilcox. test or n (%) using Fisher's test. All the continuous variables are shown as [mean (SD)], [median(range)] and the categorical variables as n (%)

PSS Cohen Perceived Stress Scale, *PDQ* Prenatal Distress Questionnaire, *BMI* body mass index; *ICSI* intracytoplasmic sperm injection; *IVF* in vitro fertilization; *FSI* Fetal Stress Index

DNA methylation and stress measures

DNA Methylation and PSS score PSS score (the continuous variable) was used for the association analysis. We did not identify any differentially methylated sites in relation to PSS score. Figure 1 shows the Manhattan plot and the Q–Q plot. Table 2 shows the top four hits for the association with no significant findings.

DNA methylation and PDQ score The association analysis with PDQ scores yielded one CpG (cg06542869, p = 4.62E-08) (Fig. 2) 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 2). The regression coefficients and values for the next three nonsignificant hits for this association are reported in

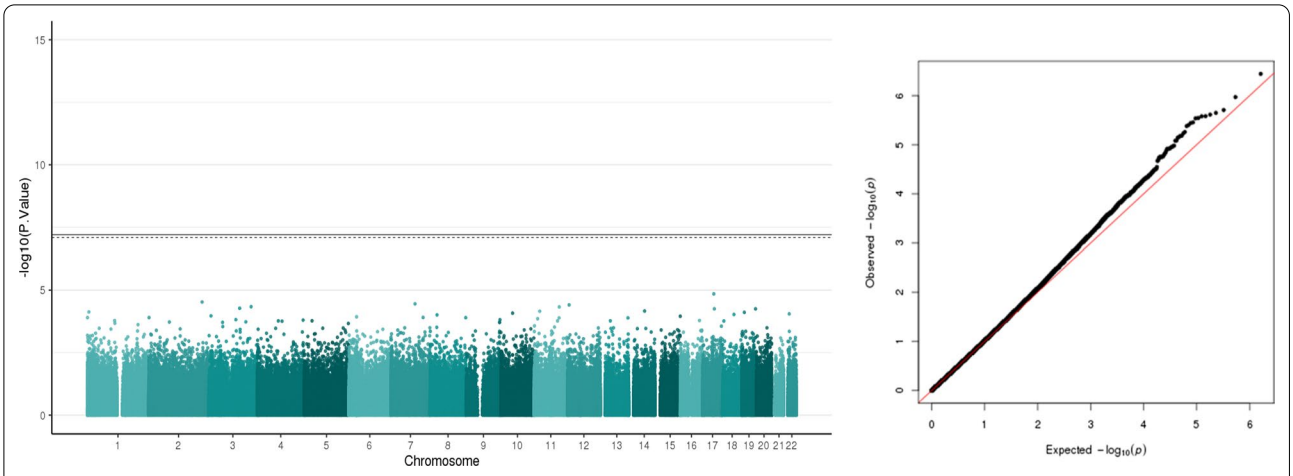

Fig. 1 Manhattan plot and Q–Q plot of salivary DNA methylation associated with PSS. Manhattan plots of salivary DNA methylation associated with PSS. The x-axis represents the genomic loci of the individual CpGs and the y-axis represents the –log10 (p value). Black line: Bonferroni threshold (p = 6.183879e-08) and the dotted line: Multiple testing correction threshold (FDR < 0.05) has been added to the plot. There are no CpGs that cross the significance threshold. Quantile–quantile (QQ) plot shows the expected and the observed quantiles

Table 2 CpG sites associated with stress measures in DNA methylation analysis

Stress measure	Probe	Coef[d]	P.Value[e]	FDR[f]	chr[g]	Illumina gene annotation	Genes within 50 kb of associated CpG
PSS[a]	cg17478679	−0.2	3.59E-07	0.25	chr17	KPNB1	CRHR1
	cg22124215	−0.1	1.07E-06	0.25	Chr2	MARCH4	DIRC3
	cg06195987	0.52	1.96E-06	0.25	Chr7	NA	LMTK2
	cg15426815	0.31	2.24E-06	0.25	Chr12	MIR200C	C1S
PDQ[b]	**cg06542869**	**0.02**	**4.62E-08**	**0.03**	**chr11**	**YAP1**	**YAP1**
	cg22861369	0.02	2.30E-07	0.08	chr5	PDLIM4	SLC22A4
	cg01629131	0.03	3.31E-07	0.08	chr20	NA	RP11, RP1
	cg03105159	0.01	7.18E-07	0.11	chr2	ALKAL2	ALKAL2, FAM105B
Cortisol	**cg11409463**	**0.003**	**2.87E-09**	**0.002**	**chr5**	**NA**	**SCAMP1**
	cg20905655	**0.004**	**1.16E-07**	**0.03**	**chr19**	**NA**	**SSBP4;**
	cg25252839	**0.002**	**1.24E-07**	**0.03**	**chr1**	**TOMM20**	**SNORA14B**
	cg05306225	**−0.002**	**2.08E-07**	**0.04**	**chr8**	**CSMD1**	**CSMD1**
FSI[c]	cg13547817	−0.39	8.85E-08	0.07	chr9	ERP44	ERP44; INVS
	cg07642729	−0.35	2.81E-06	0.49	chr8	ASB15	-
	cg24795351	−0.34	3.79E-06	0.49	chr8	PREX2	PREX2
	cg16692227	−0.21	3.80E-06	0.49	chr14	SAMD12	SAMD12

The table shows top four CpGs from the EWAS that are associated with 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 the statistical model

[e] Significance from the statistical model

[f] False discovery rate

[g] Chromosome

NA: Not available

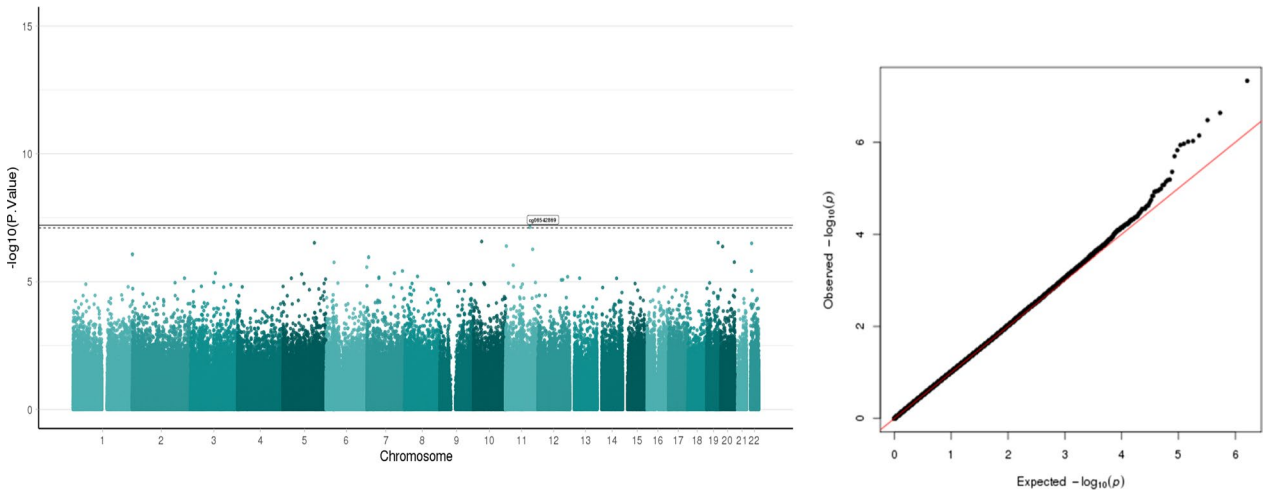

Fig. 2 Manhattan plot and Q–Q plot of the association between PDQ and salivary DNA methylation. Manhattan plots of salivary DNA methylation associated with PDQ. The x-axis represents the genomic loci of the individual CpGs and the y-axis represents the –log10 (p value). Black line: Bonferroni threshold (p = 6.183879e-08) and the dotted line: Multiple testing correction threshold (FDR < 0.05) has been added to the plot. CpGs that cross the FDR threshold are marked in the Manhattan plot. There is 1 CpG that crosses the significance threshold. Quantile–quantile (QQ) plot shows the expected and the observed quantiles

Table 2. The Q–Q plot shown in Fig. 2 is corrected for inflation which has a lambda value of 1. 04. The uncorrected vs the corrected Q–Q plot is shown in Additional File 1: Fig. S1.

DNA methylation and cortisol We identified stronger associations with cortisol compared to other stress variables (Fig. 3, Table 2). 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 University of California Santa Cruz (UCSC) browser showed that the nearest genes within 50 kb distance to this CpG site are *SCAMP1* (Secretory Carrier Membrane Protein 1) and ***AP3B1*** (Adaptor

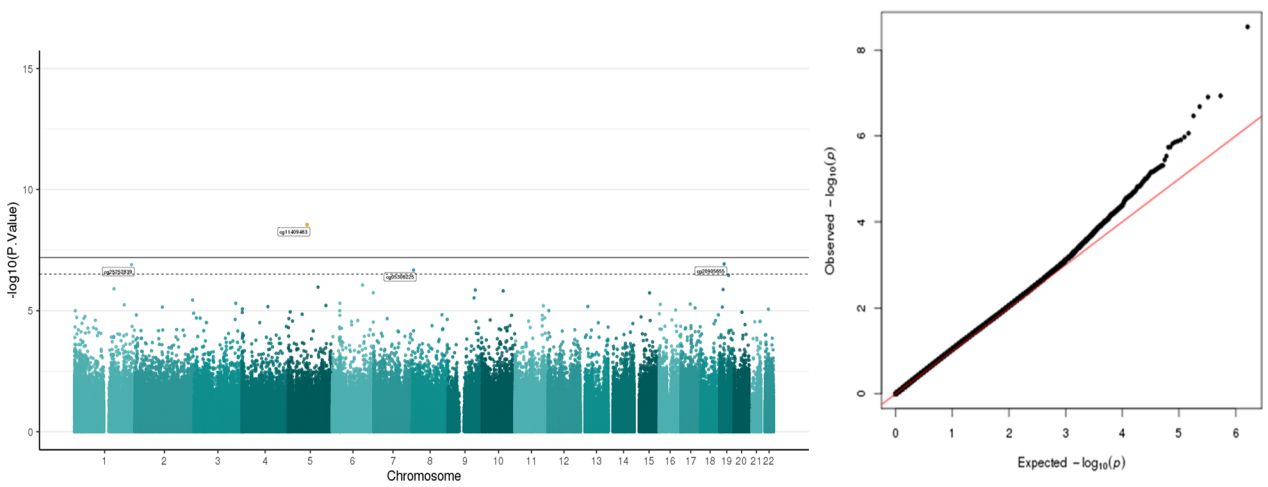

Fig. 3 Manhattan plot and Q–Q plot of the association between cortisol and salivary DNA methylation. The lambda value for the Q–Q plot is 1.08. Manhattan plots of salivary DNA methylation associated with cortisol. The x-axis represents the genomic loci of the individual CpGs and the y-axis represents 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 FDR multiple correction threshold. Quantile–quantile (QQ) plot shows the expected and the observed quantiles

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 50 kb 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 (Fig. 3; lambda = 1.08).

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. 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 2 shows the top four hits from the FSI association analysis and Fig. 4 shows the Manhattan plot and the Q–Q plot.

Sex specificity analysis

The CpG-by-sex interaction analysis did not reveal any significant differences between sexes for the associations (Table 3).

Exploratory analysis of DMPs

In a second layer of analysis, the network interactions between the proteins encoded by the genes that were annotated to significant DMPs were analyzed using STRING-db, a database and software application enabling an 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 [25]. Unique URL for the resulting analysis is as follows:

https://version-11-5.string-db.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 were 1) Hippo signaling pathway, 2) regulation of canonical Wnt signaling pathway and 3) cell–cell junction assembly. Figure 5 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.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways map molecular objects such as genes and proteins to molecular interactions or relations. The

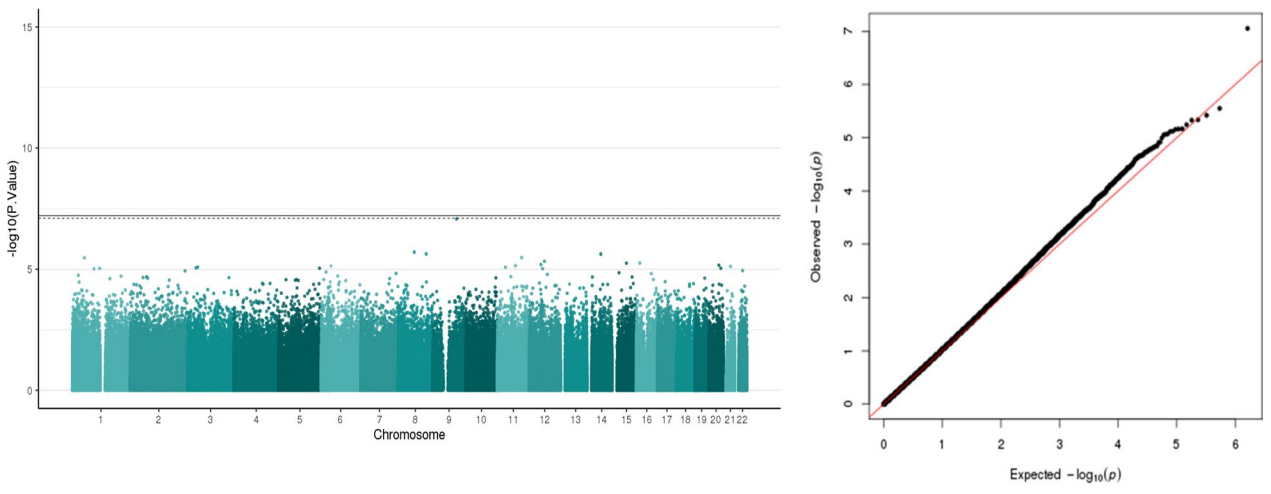

Fig. 4 Manhattan plot and Q–Q plot of the association between FSI and salivary DNA methylation. Manhattan plots of salivary DNA methylation associated with FSI (Fetal Stress Index). The x-axis represents the genomic loci of the individual CpGs and the y-axis represents the –log10 (p value). Black line: Bonferroni threshold (p = 6.183879e-08) and the dotted line: Multiple testing correction threshold (FDR < 0.05) has 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. Quantile–quantile (Q–Q) plot shows the expected and the observed quantiles and has a lambda 1.01

Table 3 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 top two CpGs from the EWAS of the interaction analysis that are associated with the stress measures

NA Not available

[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

top pathway identified from the KEGG pathways was the Hippo signaling pathway.

As the next step, we used the SFARI gene database [26] to extract information for the genes annotated to CpGs specific to ASD [27]. 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 this database [26], only *CSMD1* appeared with a score of three indicating strong relevance to ASD (gene.sfari.org/database/human-gene/*CSMD1*).

Differentially methylated regions (DMRs)

DMRs are genomic regions that have consistently different DNA methylation across multiple adjacent CpGs [28]. 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 4.

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

Discussion

Most of the maternal stress studies in the past have been limited to targeted DNA methylation analyses in candidate genes [29, 30]. 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 [31–34]. These different approaches have been recently summarized in two reviews and a meta-analysis [18, 35, 36]. When analyzing 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, in particular, those relevant to neuronal, immune and endocrine homeostasis.

Maternal stress measures and DNA methylation patterns

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

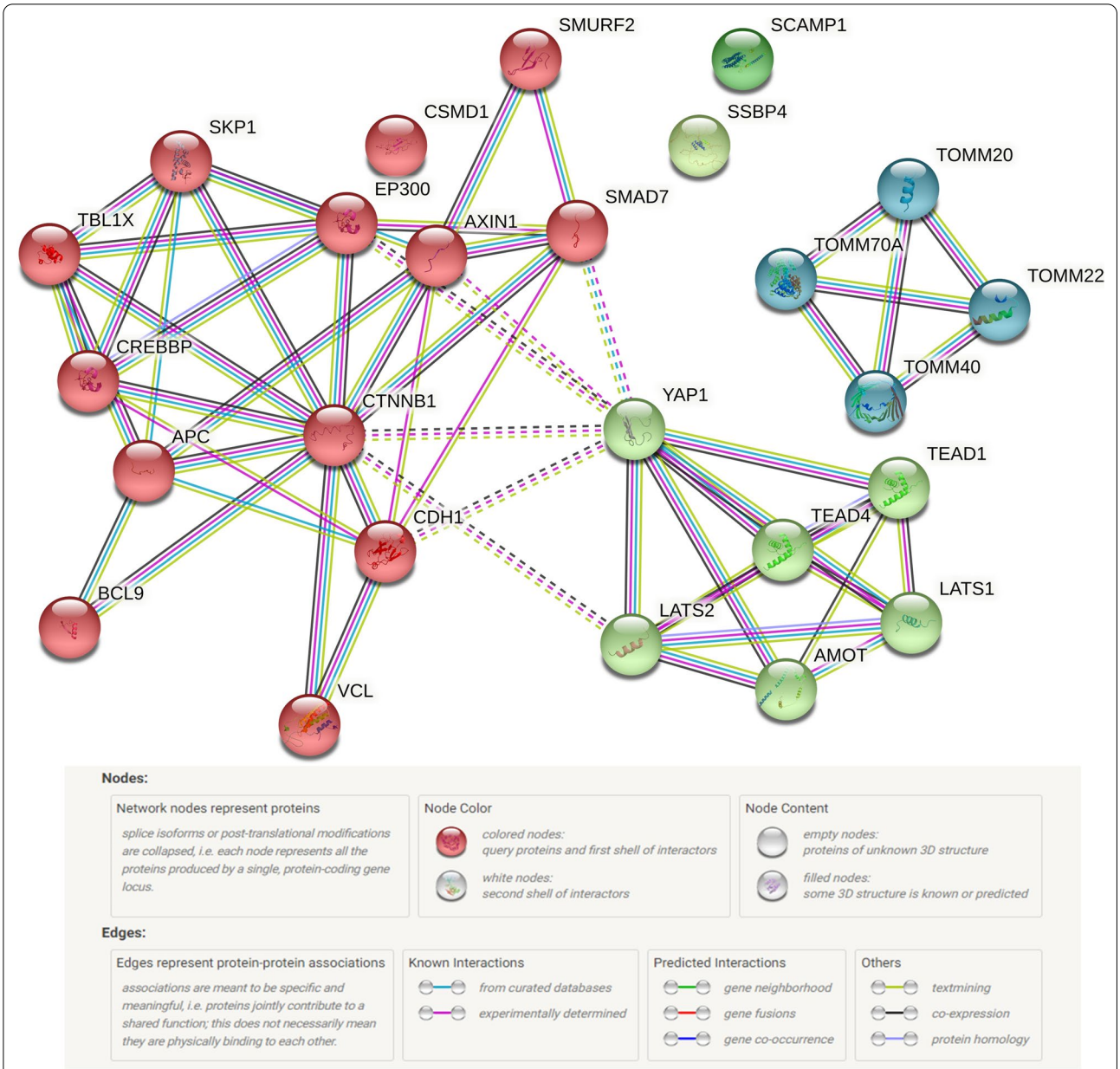

Fig. 5 Network plot of significant hits from the EWAS analysis. STRING-Db network analysis for significant hits from the association for PDQ and cortisol. Protein–protein interaction (PPI) enrichment p value: 3.47e-06. PPI legend by string-db.org. The permanent link is: https://version-11-5.string-db.org/cgi/network?taskId=bvfqNrZYaHe6&sessionId=bjK7XvqNxMXe

CSMD1; two CpGs were not annotated to any gene but lay within 50 kb 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 [37]. This evolutionarily conserved signaling cascade regulates cell proliferation, stemness, organ size control and regeneration. Its dysregulation has been associated with multiple forms of cancers, the immunity response and cardiovascular diseases [37, 38]. 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 [39]. Moreover, Passaro et al. [40] demonstrated that the transient downregulation of YAP in mouse embryonic

Table 4 Differentially methylated regions (DMRs) in salivary DNA associated with stress measures in FELICITy study

Stress measures	Chr[b]	Start (bp[c])	End(bp)	CpGs[d]	p value[e]	Sidak P[f]	Gene
PDQ[a]	**6**	**33,288,180**	**33,288,600**	**8**	**0.000166981**	**8.67E-08**	**DAXX**
Cortisol	**17**	**41,476,044**	**41,476,457**	**11**	**6.50E-10**	**1.27E-06**	**ARL4D**

Marked in **bold** are significant

[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

stem cells disrupts cellular homeostasis altering the ability to differentiate properly. 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 neighboring CpG sites [41]. Assuming that hypermethylation is generally associated with transcriptional silencing of genes, the 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 signaling 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 [37].

TOMM20 (translocase of outer mitochondrial membrane 20) is involved in glucose/energy metabolism and deubiquitination. Together, TOMM22 functions as a transit peptide receptor at the surface of the mitochondrial outer membrane and facilitates the movement of preproteins [42, 43]. 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 [44]. The CSMD1 protein is related to immune function playing a crucial role in regulating complement activation and inflammation in the developing brain [44, 45] and may also play a role in growth cone function [46]. 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 [44]. ***CSMD1*** protein coding gene has been previously associated with autism spectrum disorders (ASD) [47, 48]. 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 with post-traumatic stress disorder [49, 50], schizophrenia [44, 45, 51, 52], and bipolar disorders [53].

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 back to several reports showing that the risk for ASD is linked to PS [11, 54].

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 [55] and apoptosis [56]. 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 [57, 58]. 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 [59, 60]. 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 [57, 61]. 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. [62].

To expand the search of epigenetic signatures associated with stress measures during pregnancy we considered DMRs. Two DMRs were detected, one associated with 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 [63]. 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 [64–66]. 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 [67]. ***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 [68]. Interestingly, the transcription of Arl4d was found to be consistently regulated by glucocorticoids such as cortisol [69]. So far, not much is known about its function, but it has been shown that Arl4D is involved in neurite growth [70], adipogenesis [71] and actin remodeling [72]. 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 [73]. Interestingly, both DMRs are directly or indirectly related to neurological disorders such as ASD. To the best of our knowledge, this is the first report showing significant DMRs in the PS context in newborn saliva samples. Previously, Drzymalla et al. [74] have identified DMRs related to maternal stress but using cord blood.

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. [75]. 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 [76]. 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. [77] showed no associations between PS exposure and neonatal cord blood DNA methylation, whereas Wikenius et al. [78], studying maternal depressive symptoms, found no significant association with 6-week infant's saliva DNA methylation. In contrast, Non et al. [33] 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.

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 [79]. Moreover, dysregulation of the expression of ***CSMD1*** and ***YAP1*** has been related to disorders of the immune system as well as of the central and autonomic nervous systems.

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, to investigate this relationship further we intend to analyze in more depth the relationships between the neonatal epigenome and the biophysical features of ANS derived from maternal and fetal HRV.

Strengths and limitations

In the present study, we report the findings of the largest prospectively followed cohort of its kind to date. Several strengths are to be highlighted. Firstly, saliva cells are easy and noninvasive 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 noninvasively obtainable. Since the primary organ affected by stress is not available in human studies and postmortem brain tissue samples cannot capture the fluid state of the epigenome [80], 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 [81].

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.

Limitations to our study are as follows. 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. With this in mind, 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% dropout, we aimed at for 75 stressed women. Given the figures in the literature [83], we expected around 10% screen-positives on the anxiety screener. As we show in Additional File 1: Fig. S2, 2000 patients received the questionnaires and 728 patients were screened upon returning the questionnaires.

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. The only way to validate that one EWAS study compares to another is to use the same psychological tests in a similar population. Comparison with other studies is difficult since we have used a more narrowly defined chronic stress paradigm, saliva medium, and a newer Infinium array that may have together increased our chances in discovering meaningful CpG associations. However, the novel findings of DMPs and DMRs related to these stress measures in newborn saliva should be considered as hypothesis-generating and requires further validation in larger cohorts.

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 have to 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 2 years of age will allow us to detect the epigenetic drift defined as the difference in the DNA methylation status over time [78]. Moreover, the 2 years of 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 [23].

Conclusions

In this study, we identified novel associations between newborn epigenome-wide methylation levels measured noninvasively in saliva and chronic psychosocial stress experienced by the mother during pregnancy. The epigenetic changes are mostly related to genes involved in secretion and transportation, nuclear signaling, Hippo signaling pathways, apoptosis, intracellular trafficking and neuronal signaling. 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 demonstrate that newborns exposed to chronic stress during gestation show DNA methylation signatures related to neuronal, immune and endocrine homeostases.

Materials and methods

Study design

Women with singleton pregnancies, between ages 18 and 45 in their third trimester (at least 28-week gestation) were recruited at the Department of Obstetrics and Gynecology at 'Klinikum rechts der Isar' of the Technische Universität München (TUM). Exclusion criteria were serious placental alterations, fetal malformations and maternal severe illness during pregnancy or use of recreational drugs [24]. Between June 2016 and July 2019, 164 women were recruited. Due to methodological problems with saliva sampling, methylation data was available for 114 subjects.

Measures

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 also measured stress as chronically accumulated cortisol using maternal hair samples.

(a) Psychosocial stress assessment Maternal psychosocial stress was measured using the validated German version of Cohen Perceived Stress Scale (PSS-10) [84]. PSS-10 is a widely used psychological instrument to measure nonspecific perceived chronic 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 population and is a quick tool for screening chronic stress among prospective subjects [85]. In addition, the validated German version of the Prenatal Distress Questionnaire (PDQ) was also administered to the participants to assess specific pregnancy worries and concerns [24, 86–89]. PSS score and PDQ score were correlated using the Spearman method in R studio.

(b) Hair cortisol assessment After delivery, maternal hair strands (~3 mm diameter) were collected from 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 [90] Based on the hair growth rate of 1 cm per month, the 3 cm long hair segment reflects the integrated hormone secretion over the three-month period prior to sampling. The cortisol was extracted and measured according to Iglesias et al. [91]. This procedure has been validated with the standard method of mass spectrometry and was patented by University of Buenos Aires [90].

Exposure: fetal stress

Fetal stress assessment

The detailed fetal assessment is described elsewhere [24]. In brief, bivariate PRSA (bPRSA) was used to assess the coupling between maternal (mHR) and fHR resulting in Fetal Stress Index (FSI). The fHR was measured by taECG. Fetal ECG extraction algorithm SAVER [92] 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. Here, the two signals are mHR as the trigger signal and fHR as the target signal. FSI measures the response of fHR to decreases in mHR.

Outcome: DNAm measurement from newborn saliva

Sample and data acquisition

Newborn saliva sampling Immediately after delivery, the midwife 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) and stored it at room temperature. Throughout the manuscript 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 [93].

DNA extraction DNA was extracted from 1 ml saliva samples using the PrepIT kit (DNA Genotek). A 1 µl aliquot of the extracted samples was checked on 0.8% Agarose gel and measured via NanoDrop for quality. A 2 µl aliquot of DNA extract from the samples was used to run PCR to check for the sex of the newborn.

Illumina MethylationEPIC BeadChip array Bisulfite conversion of DNA and processing of methylation arrays were accomplished in collaboration with the Institute of Epidemiology (Complex diseases group) at Helmholtz Zentrum Munich. For each sample, 500 ng of the extracted salivary DNA was treated with sodium bisulfite using the EZ–DNA methylation kit following the manufacturer's protocol. DNAm was assessed using the Illumina Infinium Human MethylationEPIC BeadChip array (Illumina Inc., San Diego, CA, USA) according to the manufactur-

er's instructions. This array measures over 850,000 loci at a single-nucleotide resolution. The BeadChip includes probe types of two different chemistries: [1] Type I probes, in which two different probe types interrogate each CpG site, one which targets methylated DNA and one that targets unmethylated DNA. [2] Type II probes binding 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.

Data processing

Quality control

The arrays were scanned using an Illumina iScan reader and processed using GenomeStudio software (Illumina, Inc.). The raw data (idat files) were imported into R using the Bioconductor minfi package [94] and CpGs that have below-background expression levels in more than six samples were filtered out. Concordance between the reported sex and methylation predicted sex was confirmed. Probes with common single-nucleotide polymorphism (SNPs) near the methylation binding site were identified and filtered out. To simplify the analysis, probes were restricted to those on autosomal chromosomes. The remaining probes were background-corrected using the out-of-bound probes [95], and normalized using a functional normalization procedure, which uses two principal components of a set of control probes in order to remove technical variability [96]. 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 modeling. To account for unobserved variability or potential batch effects, models were additionally adjusted with three surrogate variables (SV) that were generated from the M-values, using the Bioconductor 'SVA' package [97]. The surrogate variables were used as covariates in the statistical analysis. The final analysis included 808,554 probes. Linear regression was used to examine the associations of each CpG site with stress measures. Probes were considered significantly differentially methylated at a false discovery rate (FDR) < 0.05 [98].

Covariates Figure 6 shows the proposed DAG that displays the covariates used in our analysis. We included covariates such as newborn sex, 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. 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 signifying autoimmune dis-

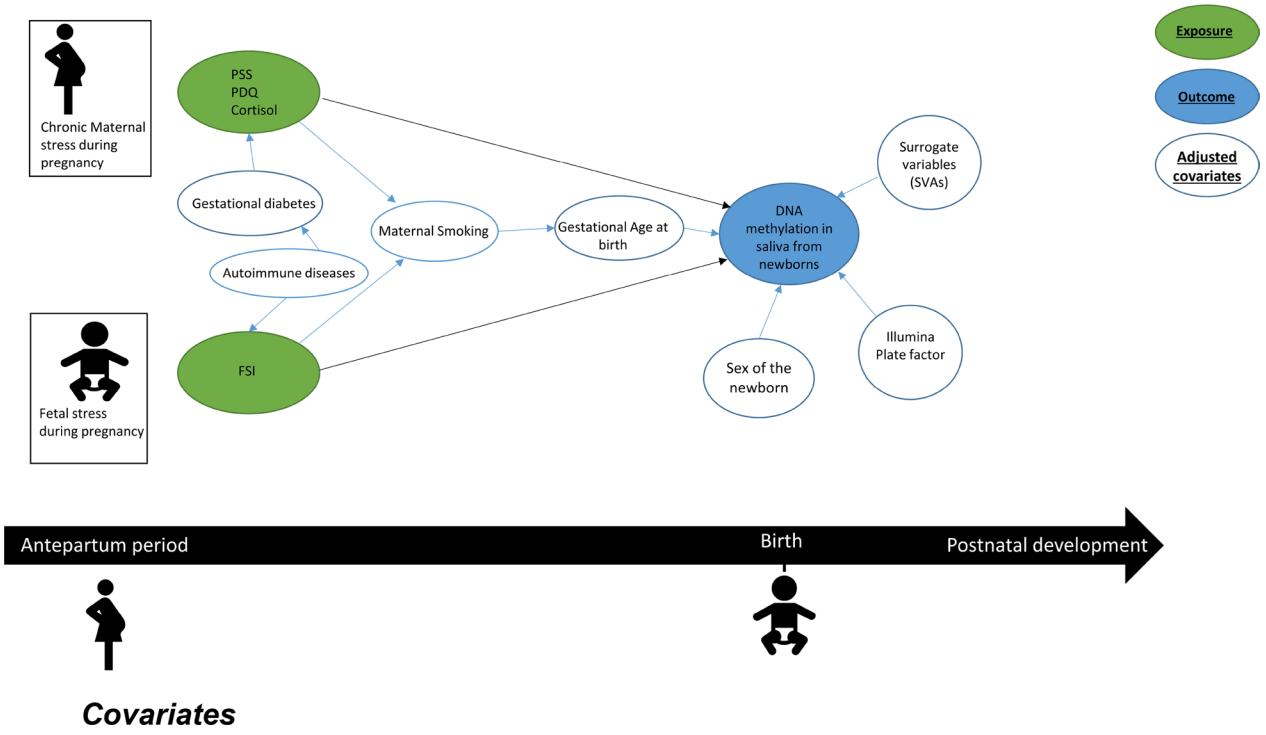

Fig. 6 Direct acyclic graph (DAG) displaying the hypothesized associations between maternal and fetal stress and infant salivatory DNA methylation

eases and gestational diabetes were categorized into two categories: 'Yes' and 'No.'

We adjusted for technical covariate, i.e., Illumina plate factor. It should be noted that the cellular DNA source of saliva is heterogeneous. While there is much literature dealing with saliva cell type composition in infants, children and adults [78, 93, 99], to date, there are no studies indicating the cell mixture composition of the saliva of the newborn. Housemann et al., in 2014 [100], introduced the reference-free cell type method to estimate cell types in tissues such as saliva, placenta and adipose tissue [100], which is closely related to surrogate variable analysis (SVA) [101]. 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 [101]. Three surrogate variables that were generated in the quality control step, using the *SVA* package, were also used as covariates in the main model.

Data analysis

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 was 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 UCSC genome browser was used to verify genes identified with Illumina database and, where genes were missing in the Illumina database, was searched to augment genes within 50 kb of the CpG site. Third, the biological exploration and network analysis for each CpG annotating to specific genes was conducted using the online software STRING-DB [25] and SFARI [26]. Fourth, we conducted DMR analysis (or regional analysis), which captures an average pattern of DNA methylation among neighboring sites. Figure 7 shows a summary of the methodological study design.

Differentially methylated positions (DMPs) analysis

Each CpG site or DMP was separately tested for association with exposure to stress. Three sets of EWAS analyses were run to identify CpG sites associated with either PSS, PDQ, FSI or cortisol. All the statistical analyses were done in R version 3.5.2. We used the following linear regression model in 'limma' R package to test for DMPs:—DNA methylation ~ Stress measures + Newborn

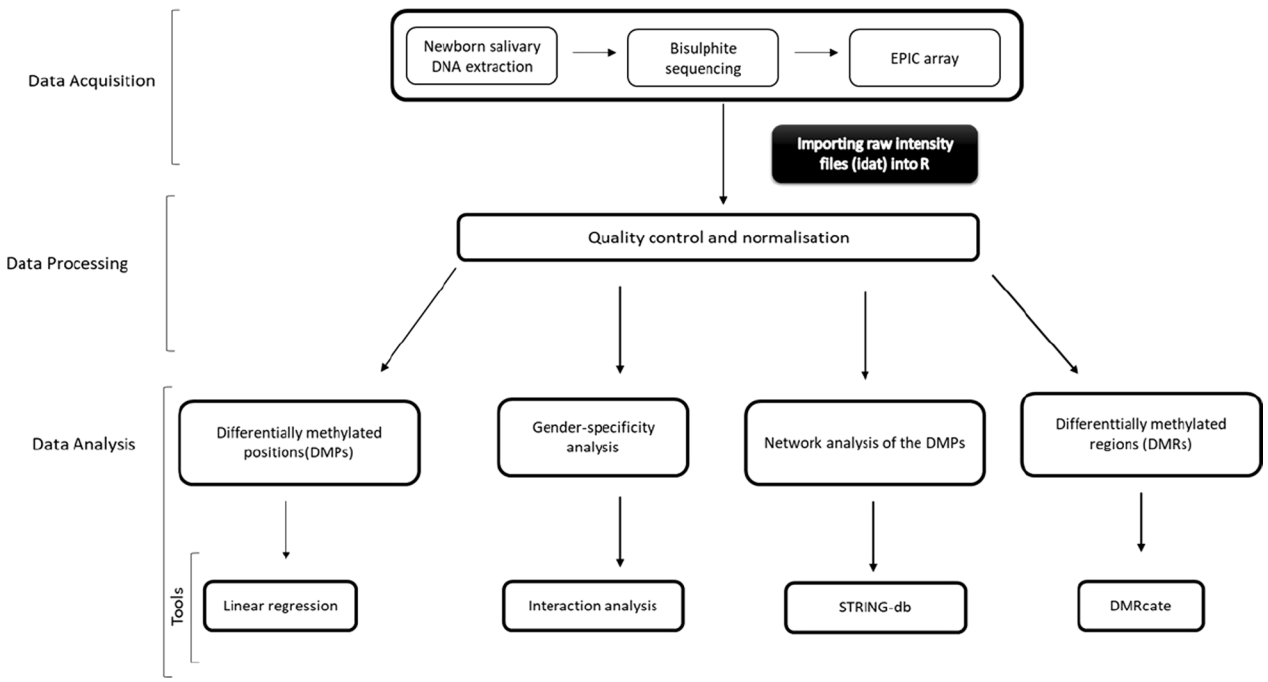

Fig. 7 Overall methodological study design. Illumina measured salivary DNA methylation using the EPIC microarray platform. The raw data were processed and quality-controlled using array-specific algorithms in R studio. Data visualization and statistical analysis identified relevant associations and derived a list of differentially methylated positions and regions

sex + Gestational age + Smoking + Autoimmune diseases + gestational diabetes + Illumina plate factor + SVAs. 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' [102].

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 set at FDR < 0.05.

Exploratory analysis of DMPs

To investigate whether specific biological processes and networks are overrepresented in our EWAS results, network analyses were performed for the DMPs using STRING-db. The protein encoding genes that were annotated to significant DMPs were analyzed using STRING-DB. STRING-DB is an unsupervised statistical network analysis database that has known proteins and their physical and functional interaction networks [25]. We used the KEGG database in STRING-DB to explore whether annotated genes have been related to neurobiological and neuronal processes or diseases. We also used the SFARI gene database [26] to extract information for the genes annotated to CpGs specific to ASD [27].

Differentially methylated regions (DMRs) analysis

DMRs were initially identified using the Bioconductor DMRcate package [103] and verified using comb-p [104]. These packages are consistently reported to have the best sensitivity and highest control of false positive rate when compared to other DMR tools [105]. A significant DMR can be detected even if there is no genome-wide significant DMP in the region. DMRcate identifies DMRs from the tunable kernel smoothing process of association signals [103].

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 a FDR cutoff 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 [104]. 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.

Abbreviations

ADHD: Attention-deficit hyperactivity disorder; ANS: Autonomic nervous system; *ARL4D*: ADP-ribosylation factor 4D; ASD: Autism spectrum disorder; CNS: Central nervous system; *CSMD1*: CUB and Sushi multiple domains 1; CTG : Cardiotocography; *DAXX*: Death-associated protein 6; DMPs: DNA-methylated positions; DMR: DNA-methylated regions; ECG: Electrocardiogram; EWAS: Epigenome-wide association study; FHR: Fetal heart rate; FSI: Fetal stress index; HPA axis: Hypothalamic–pituitary–adrenal axis; MHR: Maternal heart rate; PDQ: Prenatal Distress Questionnaire; PRSA: Phase-rectified signal averaging; PS: Prenatal stress; PSS: Perceived Stress Scale; PTSD: Post-traumatic stress disorder; QQ: Quantile–quantile; SNPs: Single-nucleotide polymorphism; SV: Surrogate variables; TaECG: Transabdominal electrocardiography; *TOMM20*: Translocase of outer mitochondrial membrane 20; UCSC: University of California Santa Cruz; *YAP1*: Yes1-regulated transcription factor.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-022-01310-x.

Additional file 1. S1. Uncorrected and BACON corrected Quantile-quantile plot. **Additional file S2**. Enrollment flowchart for FELICITy study.

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Author contributions

RS conducted the experiments and the study-level data processing, statistical analysis and analyses and drafted the manuscript. RS, SML, MGF and MCA contributed to data collection management, data analysis, and manuscript writing and editing. CZ and PZ contributed to patient recruitment and data collection. BF and DG performed cortisol analysis in hair samples. RW and MW contributed to EPIC methylation profiling advice, data analysis and statistical advice. JWM and TKB contributed to statistical analysis and advice. SML and MCA conceptualized the project, protocol and project development, data collection and management, data analysis and funding acquisition. All authors read and approved the final manuscript.

Author's Information

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Availability of data and materials
The datasets supporting these findings are not publicly available. Instead, the datasets used and/or analyzed for the current study are available from the corresponding author on reasonable request and after Institutional Review Board review and approval. Scripts used in data processing and statistical analyses have been made publicly accessible at https://ascgitlab.helmholtz-muenchen.de/ritika.sharma/felicity-project/-/tree/master/

Declarations

Ethics approval and consent to participate
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.

Consent for publication
Not applicable.

Competing interests
MGF holds a pending US patent on fetal ECG technology (20210330236); US patent on fetal EEG (9215999), equity in Delfina and Vitalink AI. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author details
[1]Department of Obstetrics and Gynecology, Klinikum Rechts der Isar, Technical University of Munich, Munich, Germany. [2]Department of Obstetrics and Gynecology and Center On Human Development and Disability (CHDD), University of Washington, Seattle, WA, USA. [3]Research Unit of Molecular Epidemiology, Institute of Epidemiology, Helmholtz Zentrum Munich, Munich, Germany. [4]Instituto de Fisiopatología y Bioquímica Clínica (INFIBIOC), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina. [5]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA, USA. [6]Instituto de Biología Celular y Neurociencia "Prof. E. De Robertis", Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.

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Early Biomarkers and Intervention Programs for the Infant Exposed to Prenatal Stress.

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Marta C. Antonelli*[1,2#], Martin G. Frasch[3#], Mercedes Rumi[1], Ritika Sharma[2,4], Peter Zimmermann[2], Maria Sol Molinet[2] and Silvia M. Lobmaier[2].

[1]Instituto de Biología Celular y Neurociencia "Prof. E. De Robertis", Facultad de Medicina, UBA, Buenos Aires, Argentina; [2]Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technical University of Munich, Germany; [3]Department of Obstetrics and Gynecology, University of Washington, Seattle, WA, USA; Center on Human Development and Disability, University of Washington, Seattle, WA, USA; [4]Research Unit of Molecular Epidemiology, Institute of Epidemiology, Helmholtz Zentrum Munich, Neuherberg, Germany.

 # these authors contributed equally to this work

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Corresponding author:

Dr. Marta C. Antonelli. Laboratory of Perinatal Programming of Neurodevelopment, Instituto de Biología Celular y Neurociencias "Prof.E. De Robertis", Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155 3er piso, 1121 Buenos Aires, Argentina. Phone: +54 11 5285-3266. and Frauenklinik und Poliklinik (Hans Fischer Senior Fellow IAS-TUM). Klinikum rechts der Isar, Ismaninger Str.22. 81675, München, Germany. Phone: +49 894140-6671.

E-mail: mca@fmed.uba.ar

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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MCA has conceived and designed the concept and road map of the manuscript, drafted it, searched the literature and coordinated the edition of the final version. MGF greatly contributed to the final version of the manuscript by thoroughly reviewing and editing all versions of the manuscript and helping with the design of the illustration. MR prepare and managed the bibliography and review the manuscript. RS and PZ critically review the manuscript. MSM helped in the literature search and critically review the manuscript. SLM helped in the preparation of the manuscript and critically reviewed it. The authors gratefully acknowledge Hermona

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ABSTRACT

Functional development of affective and reward circuits, cognition and response inhibition later in life exhibits vulnerability periods during gestation and early childhood. Extensive evidence supports the model that exposure to stressors in the gestational period and early postnatal life increases an individual's susceptibility to future impairments of functional development. Recent versions of this model integrate epigenetic mechanisms of the developmental response. Their understanding will guide the future treatment of the associated neuropsychiatric disorders. A combination of non- invasively obtainable physiological signals and epigenetic biomarkers related to the principal systems of the stress response, the Hypothalamic-Pituitary axis (HPA) and the Autonomic Nervous System (ANS), are emerging as the key predictors of neurodevelopmental outcomes. Such electrophysiological and epigenetic biomarkers can prove to timely identify children benefiting most from early intervention programs. Such programs should ameliorate future disorders in otherwise apparently healthy children. The recently developed Early Family-Centered Intervention Programs aim to influence the care and stimuli provided daily by the family and improving parent/child attachment, a key element for healthy socio-emotional adult life. Although frequently underestimated, such biomarker-guided early intervention strategy represents a crucial first step in the prevention of future neuropsychiatric problems and in reducing their personal and societal impact.

1. GESTATIONAL ENVIRONMENT AND EARLY PARENTING

The spread and depth of evidence from both animal and human studies leave nowadays little doubt about the impact of the gestational environment on the fetal brain development; a concept that has been named “fetal programming” [1, 2]. Prenatal stress (PS) impacts early behavioural, cognitive development and temperament in human infants and increases child morbidity and neurological dysfunction such as attention-deficit hyperactivity disorder (ADHD) and sleep disturbance during infancy which if persistent in adulthood might result in depression and vulnerability to psychotic disorders [3, 4]. In their review, Van Den Bergh *et al.* [3] concluded that numerous epidemiological and case-control studies of the past decade show neurodevelopmental disorders in offspring exposed to maternal stress during pregnancy. Reviewed studies mainly refer to pregnant women exposed to psychosocial stress including states of anxiety, depressive symptoms, major life events experienced by the mother, experience of a disaster and subjective distress during the third trimester of pregnancy. These pregnant women have infants that show: less affective reactivity at 5 months [5], higher temperamental reactivity at 6 months [6], positive association with high respiratory sinus arrhythmia at 8-10 months [7], higher negative affectivity at 24 months [8], higher reaction intensity at
24-30 months [9], and decreased cognitive functions at the age of 24 months [10]. Together, these findings point to the main outcome of the PS exposure being at the cognitive and emotional regulatory levels. More recently, Persson and Rossin-Slater [11] showed that *in utero* exposure to bereavement has long-lasting effects on the consumption of psychiatric medication both during childhood and adulthood. Children are 25% more prone to taking medication to treat ADHD and experience a 13% and 8% increase in the consumption of prescription drugs for anxiety and depression, respectively, when they reach adulthood. These data highlight the serious economic burden of pregnancy-associated mental illnesses generating important private and societal costs. For example, in the United States the market for antidepressant drugs totaled $9.6 billion in 2008. The consumption of prescription drugs for treating ADHD increased five times in a decade from $1.7 billion in 2003 to $9 billion in 2013. Persson and Rossin-Slater [11] also pointed out that in Sweden, ³*mental illness accounts for a larger share of health expenditures on prescription drugs that any other therapeutic drug*´. Despite these insights, we observe that the long-term health-economic impact of pregnancy- associated psychiatric

illness remains highly underappreciated, especially considering that early interventions during pregnancy and/or nursing periods can recover these high costs to individuals and society.

It has also been acknowledged that not only intrauterine adversities due to maternal stress influence the development of the child, but also a low maternal involvement during upbringing will influence the child´s neurodevelopmental outcome [9, 12-15]. Several studies have shown that high levels of pregnancy-specific anxiety and trait anxiety during pregnancy may persist postnatally predicting higher levels of parenting stress three months after birth and leading to a lower parenting self-efficacy and negative perceptions of parenting-related issues [16] suggesting that anxiety during pregnancy interferes with an optimal preparation for parenting. This means that maternal stress during pregnancy and during early parenting can program physiological responses and lifetime trajectories of the infant, which in interaction with genetic liabilities and early-life challenges, will determine the ultimate health status. The concepts derived from these studies contributed to the emphasis on maternal health as a global priority for the World Health Organization (http://www.who.int/pmnch) and the International Monetary Fund (http://www.imf.org/external/np/exr/facts/mdg.htm). WHO (2016) states that: [3]*"The burden of mental disorders continues to grow with significant impacts on health and major social, human rights and economic consequences in all countries of the world."* This reinforces the idea that nurturing care in early life is essential to enable children to become healthy and productive citizens with adequate intellectual skills, creativity and wellbeing [17, 18]. This means that investing in childhood as early as possible will impact the development of a healthy society.

1.1 Developmental Hypothesis

Several developmental models have been reported based on Barker's Fetal Basis of Adult Diseases (FeBAD) hypothesis. Since 1987 and based on their studies on adult cardiovascular diseases, Barker and his group elaborated the hypothesis that the *in utero* environment may permanently program the structure and physiology of the offspring [19,
20]. The original FeBAD hypothesis evolved to include adaptive responses or phenotypic plasticity [21]. The term FeBAD was later changed to include the term Health, introducing the notion that not only diseases can be shaped during the perinatal period but also the health outcomes. The name of the hypothesis was then agreed to become the Developmental Origins of Health and Disease (DOHaD) [22, 23]. An extension of this hypothesis was recently developed by Van den Bergh *et al* [24] who proposes the "Developmental Origins of Behavior, Health and Disease" (DOBHaD) hypothesis, introducing two fundamental concepts: a) specific signs may announce disorders to come before they appear and 2) in spite of the negative impact of perinatal adversities, developmental plasticity allows timely changes for reversion. The DOBHaD hypothesis integrates concepts from the epigenetic field and reinforces the idea that understanding the process by which development responds to an insult can guide the future treatment of the disorder.

1.2 Biological mechanisms

In recent years, much effort has been devoted to understanding the biological mechanisms that underlie the basis of neurodevelopmental disorders triggered by PS. Two questions arise sequentially: 1) What are the potential mediators that connect the stressed mother with the fetus? and 2) How do these mediators leave "permanent" signatures in the baby? The first question has been thoroughly reviewed by Rakers *et al* [4] and they conclude that the main mediators of maternal-fetal stress are not only the well-known cortisol but also catecholamines, reactive oxygen species (ROS), cytokines, serotonin/tryptophan and maternal microbiota. This implies that not only the re-programming of the Hypothalamic-Pituitary axis (HPA) is involved but the Sympathetic-Adrenal Medullary System (SAMS) as well, among other systems.

The second question is still a matter of intense study but there is a general consensus that epigenetic mechanisms are the most probable link between prenatal environmental exposures and the disruption of normal brain function [25, 26]. Most of these mechanisms and signals have been proposed as predictive biomarkers of neurobehavioral outcomes but in spite of much promising research efforts, we still lack well-designed longitudinal studies of maternal/fetal dyads with or without stress exposure that will definitively and unequivocally correlate biological signals with the child behavioral outcome. These biological signals might help to predict adverse outcomes and to involve the child in early stimulation programs.

The importance of finding reliable early predictive biomarkers is highlighted by the fact that the stress situation is often not detected on time or the pregnant/nursing mother is unaware of the potential effect of stress on the disadvantageous outcome for her baby. It is well known that the brain is particularly sensitive to changes in the perinatal environment during early development, but the consequences of prenatal damage may not necessarily be apparent until a critical age when neurodevelopmental defects may be precipitated by a subsequent exposure to other insults. Most frequently, negative outcomes in children are discovered many years later missing many opportunities and years of adequate stimulation programs. In fact, several studies reported that various developmental disorders were detected at school age in apparently healthy children. Screening trials in healthy children from 0-5 years old showed a 16-20% prevalence of global developmental and communication disorders [27-29].

As stated by Rakers *et al*. [4], we believe that the effects of maternal stress on fetal development are mediated by a *"multiple stress-transfer mechanisms acting together in a synergistic manner"*. We propose that a combination of multimodal biomarkers will help to detect "at-risk children" as early as possible in order to make the decision to face early stimulation programs, whenever no prevention measures in the mother can be timely taken [30]. In this review, we will describe some of the combinations of signals and biomarkers as well as early intervention programs that have proven effective in reversing or ameliorating neurodevelopmental disorders in children exposed to prenatal stress.

2. BIOMARKERS FOR EARLY DETECTION

The importance of early detection of children at risk of future neurodevelopmental sequelae has been clearly delineated by Braun *et al* [31]. Although much work is still needed, there are clear hints from animal and humans studies that after controlling for time of stress exposure, early interventions in children can ameliorate, "reverse" or "repair" the cognitive deficits induced by PS. As mentioned before, when the mother is unaware of her stress or the newborn has no apparent behavioral symptoms, the availability of predictive biomarkers is urgently needed.

Gene-environment interactions generally involve epigenetic changes and these changes can leave stable marks in the genome in response to the environment, potentially altering the gene expression for life and even transgenerationally [32]. Indeed, this phenotypic stability and reversal under intervention are features that give these epigenetic changes the potential to become predictive biomarkers that allow early intervention and prevention of neurobehavioral risk. This hypothesis has been proposed and developed in numerous studies both in humans and in animal models and most of them found several genes with alterations in the methylation profile that could be identified as biomarkers. However, we believe these studies have two important limitations: a) most of the studies were carried out using candidate genes interrogating the HPA axis, on the basis that stress alters this axis, disregarding the multigenic nature of the stress response genes; and b) few studies have assessed altered sympathetic and vagal activity during fetal life as a possible consequence of prenatal stress. In the following paragraphs we will describe the most studied potential biomarkers, the

epigenetic signatures and the emerging candidate biomarkers related to the Autonomic Nervous System (ANS). We refer to ANS as including SAMS and the parasympathetic (i.e., mostly vagus nerve) activities.

2.1. Epigenetic biomarkers

The process of 'fetal programming" is mediated by the impact of prenatal experience on the developing HPA axis. The HPA is a dynamic metabolic system that regulates homeostatic mechanisms, including the ability to respond to stressors [33] and which is highly sensitive to adverse early life experiences [34]. It has been suggested that the latency between the exposure to stress and the occurrence of the disease is pointing out to the fact that the environment triggers stable changes that have the potential to manifest later in life [35].

Several recent reviews support the hypothesis that fetal programming is mediated by epigenetic mechanisms that persistently alter gene transcription affecting physiology and behavior [25, 36]. Epigenetic mechanisms change the gene activity or expression altering the chromatin organization without modifying the genetic code. Several processes have been described that stably alter the gene accessibility to the transcriptional machinery such as DNA methylation/hydroxymethylation, histone modifications (acetylation, methylation, ubiquitination and sumoylation) and microRNAs modifications. However, the most highly studied and best characterized epigenetic mark, DNA methylation, involves a direct covalent, chemical modification of a cytosine base lying sequentially adjacent to a guanine base (thus a CpG dinucleotide); such methylation is a relatively stable epigenetic tag, catalyzed by a group of enzymes called DNA methyltransferases (DNMTs) [37]. CpG dinucleotides are relatively infrequent in the genome, and areas of comparatively high CpG content have been termed 'CpG islands'. CpG islands tend to be hypomethylated compared to other CpG sites and are found associated with approximately 70% of known gene promoters, i.e., the regulatory, non-coding portion of a gene that plays a role in transcription control. Promoter DNA methylation (often in CpG islands) and gene body DNA methylation generally show opposite associations with gene expression. The presence of 5-methylcytosine is usually associated with the transcriptional silencing of the underlying DNA sequence [38, 39]. The first evidence for an epigenetic mechanism mediating exposure to a perinatal insult was provided by examining the epigenetic effects of

differences in maternal care in the rat [40]. These authors and others found that the offspring of mothers who exhibited reduced litter care (low licking and grooming) present in adulthood increased methylation in exon 1_7 of NR3C1 (Nuclear Receptor Subfamily 3 Group C Member 1, that encodes the glucocorticoid receptor), a change associated with a decreased expression of glucocorticoid receptor (GR) in the hippocampus and with an exacerbated response to stress [40-
42]. Moreover, epigenetic variations have been reported, mostly changes in DNA methylation in several brain regions, in animal models after prenatal exposure to stress [43], maternal separation [44] and response to variations in the mother-offspring interaction [45, 46]. In humans, higher levels of methylation were detected in exon 1F of NR3C1 (homologous exon 1_7 in rat) in the hippocampus of suicide victims who were, in turn, victims of child abuse [47], in umbilical cord blood cells of children whose mothers were diagnosed with depression and anxiety during pregnancy [48] and in pregnant mothers undergoing chronic and war-related stress [49].

Biomarkers are molecules that characterize the particular signature of a physiological/pathological process and that can be easily accessed and quantified. To evaluate PS outcomes in newborns, biomarkers are particularly needed to reveal epigenetic reprogramming that occurs in the brain, an inaccessible organ in humans [50]. DNA methylation in blood, saliva or tissues such as placenta is, therefore, a promising biomarker candidate since: a) it is easily detectable and quantifiable; b) it is chemically stable and not affected by cyclic

fluctuations as cortisol; c) it is an early response mechanism, making it a sensitive indicator. At present, changes in DNA methylation of certain genes can be monitored accurately in samples of human placenta and umbilical cord blood [51]. Alternatively, saliva and buccal epithelium cells represent an additional easily accessible source of DNA and provide information on the systemic condition of an individual. The procedures for obtaining saliva have the advantage of being simple, inexpensive and less invasive since they do not involve any side effects such as bruising, infections, etc. In addition, sampling can be done from early childhood onwards [48, 51]. In a recent study, Essex *et al*, [52] examined buccal epithelial cells in a cohort of adolescents and found differences in DNA methylation in those adolescents whose parents reported experiencing stress and depression during the adolescent childhood.

The methods for analyzing gene-specific DNA methylation can be divided into ***"candidate gene"*** and ***"genome-wide"*** studies. The ***'candidate gene'*** approach directly tests the effects of genetic variants of a potentially contributing gene in an association study [53] thus excluding a large number of genes. In contrast, the ***Epigenome-Wide Association Studies (EWAS)*** approach allows an unbiased analysis of locus-specific methylation across an entire genome. Thanks to the incorporation of microarray-based methylation profiling platforms, this technique is now relatively inexpensive. All studies summarized in the preceding paragraphs were performed interrogating candidate genes based on the hypothesis that DNA methylation in brain regions of the fetus is related with the fetal HPA axis response to maternal stress.

However, the multigenic nature of the stress response and neuropsychiatric disorders, is manifested through small and simultaneous changes in the expression of several genes [54]. Accordingly and beyond the HPA axis genes, Vidal *et al* [55], found alterations in the methylation in the differentially methylated region (DMR) of MEST gene (Mesoderm Specific Transcript) and more recently, Vangeel et al [56], found an association between methylation of a gene IGF2 DMR (Insulin family of Growth Factors) with pregnancy-associated anxiety. However, recent publications employing the EWAS approach have shown contradictory results. Combining data from two independent population- based samples in an EWAS meta-analysis (n=1740 dyads), Rijlaarsdam *et al.* [57], showed no large effects of prenatal maternal stress exposure on neonatal cord blood DNA methylation. More recently, Wikenius *et al.* [58] studying prenatal maternal stress (n=184 dyads), in the form of maternal depressive symptoms, found no significant genome-wide association between maternal depressive symptoms and infant DNA methylation. In contrast, Non *et al.* [59] reported the identification of 42 CpG sites with significantly different cord blood DNA methylation levels in neonates (n=36) exposed to non-medicated depression or anxiety relative to controls. Interestingly, they report that after a gene ontology analysis they found a significant clustering of genes related to transcription, translation, and cell division processes, but no genes were related to the HPA axis. Employing a cross-tissues/cross-species study of the effects of early life stress (ELS) using a genome-wide approach, Nieratschker *et al.* [60] found that *MORC1* (MORC family CW-type zinc finger 1, a protein

required for spermatogenesis) was differentially methylated in humans (n=180 dyads), monkey and rat both in peripheral tissues as well as in the brain and at different time points throughout life-span. Other studies investigated extreme conditions of ELS, such as war situations or natural disasters and found broad effects of ELS on methylation in several genes of the HPA axis in a war-related stress situation (n=24 dyads) [49], while Cao-Lei *et al.* [61] reported that PS in the form of a natural disaster (Ice-Storm in Quebec) was correlated with DNA methylation (in T-cells of 13 years old children) in 1675 CGs associated with 957 genes related to immune function. It is probably too early to draw general conclusions based on these studies since many differences are still observed in terms of the type and timing of prenatal insult, tissue employed and the children´s age.

Histone and miRNA modifications are also promising biomarkers of PS. Even though most of these studies come from animal models, several biomarkers were shown to be related to neurological diseases in humans [62-65]. In our studies, PS was shown to increase miRNA-133 in prefrontal cortex and hippocampus of in PS male rats [66] and an increase in histone methyltransferase *suv39h1* in hippocampus of PS offspring,

with no changes in histone deacetylases *hdac2* and *hdac3* mRNA levels [67]. Zucchi *et al.* [65] showed that several miRNA profiles were up-regulated in PS offspring brains (miR145 and miR103) and several were down-regulated (miR323, miR98 and miR219). At the protein level and employing a mouse model, Benoit *et al.* [68] showed that adult offspring exposed to unpredictable chronic stress during pregnancy exhibited decreased acetylated histone H3 (AcH3) in the hippocampus with sex-specific changes. In accordance with the gender differences for miRNA-133 in male rats, Van Den Hove *et al.* [69] showed an up regulation of histone deacetylase 4 protein (HDAC4) in the hippocampus of prenatal restraint stress (PRS) male offspring, while it was down regulated in frontal cortex of PS female offspring.

In summary and in spite of the disparity of these findings, overall there is ample evidence supporting the hypothesis that PS produces stable and long-term phenotypic changes in the offspring involving persistent alterations in gene function through changes in DNA methylation, histone and miRNA modifications. Although further use of the epigenome-wide approach will clarify this association, it seems plausible to envision epigenetic marks as biomarkers for children at-risk of suffering developmental diseases.

2.2. Biomarkers of the Autonomic Nervous System

HPA-axis oriented studies have overlooked other physiological signs. For example, corticosteroid administration during pregnancy has shown to affect autonomic balance in utero [70-73]. This effect is transient but repeated fetal administration of betamethasone alters nervous system maturation. In fact, Braithwaite *et al.* [74] found no association between maternal cortisol and infant DNA methylation suggesting that the effects of maternal depression may not be mediated directly by glucocorticoids; instead, sympathetic nervous system activity, a component of the fetal ANS, may be the mediating pathway.

The fetal ANS has proven to be very sensitive to maternal stress [75-77]. Among other authors, Kinsella and Monk [78] and Gao *et al.* [79] have indicated that common biomarkers of ANS such as Fetal Heart Rate (FHR) reactivity to a stimulus, or heart rate variability, reflect emerging individual differences in the development of the autonomic and central nervous systems related to styles of future emotional regulation and the risk for psychopathology.

Advanced analysis of FHR patterns specifically assessing changes in the autonomic regulation of FHR may identify the fetus at increased risk for fetal programming. This can be assessed by a relatively new promising method , phase-rectified signal averaging (PRSA) analysis of FHR, measured by electrocardiography (ECG) or cardiotocography (CTG) [80, 81]. PRSA can eliminate signal artifacts and extract areas of interest from FHR. In contrast to other methods analyzing FHR variability, PRSA permits the detection of quasi-periodicities in non-stationary data, an important benefit of the PRSA approach in dealing with an often-noisy FHR signal. Schmidt and his team already reported the excellent performance of PRSA in adult cardiology for predicting mortality after myocardial infarction [80]. This method was first described for fetuses by our own research group [82-84]. PRSA has then been successfully applied in fetal medicine also

by other teams, despite the challenges of a non-stationary signal, with more intrinsic disturbances in the signal than in the adult after a myocardial infarction. The novel parameter referred to as cardiac average acceleration and deceleration capacity is more specific than the conventional FHR analyses in identifying intrauterine growth restriction (IUGR) antepartum [83-85], predicting IUGR outcome and strongly correlates with acid-base biomarkers during acute hypoxic stress in humans during labor [86, 87] and the fetal sheep model of labor [88]. Newer data also show an activation of ANS in fetuses affected by maternal gestational diabetes which could not be seen using conventional techniques [89]. To evaluate the ANS influence on FHR using PRSA approach, the beat-to-beat information (R-R intervals of ECG) should be analyzed, although the conventional Doppler CTG signal can also be used for the analysis. The new generation of the trans-abdominal

fetal ECG monitors (such as Monica AN24, Monica Healthcare, Nottingham, UK or Invu by Nuvo-Group, Tel Aviv, Israel) allows for a completely non-invasive and passive recording of fetal and maternal ECG: it only records electrophysiological signals from the women's abdomen without hampering mobility or other diagnostic procedures. This signal can then be used for a more sophisticated analysis of FHR such as PRSA or multidimensional FHR variability analysis [90].

We have recently assessed couplings between maternal heart rate (MHR) and FHR as a new biomarker for PS based on a signal-processing algorithm termed bivariate PRSA (bPRSA) yielding fetal stress index (FSI) values by jointly assessing changes in MHR and FHR. In a prospective case-control study matched for maternal age, parity and gestational age, we used the Cohen Perceived Stress Scale-10 (PSS-10) questionnaire to categorize women in the 3rd trimester of pregnancy as stress group and control group. We could detect periodic MHR decreases reflecting typical pattern of maternal breathing (sinus bradycardia during expiration). Interestingly, control group fetuses remained "stable" during these periods whereas fetuses of stressed mothers showed significant decreases of FHR. The proposed FSI provides unique insights into the relationship between two connected biological systems: mother and fetus. 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 prenatally and postnatally [91].

After birth, ANS activity can also be assessed by measuring the baroreceptor reflex sensitivity (BRS). To improve the signal to noise ratio and hence the quality of BRS estimation, non-invasive continuous blood pressure monitoring with Finapres is used to obtain systolic blood pressure information, combined with a simultaneous ECG recording to extract a high-quality R-R signal and compute the pulse. A bivariate PRSA method, akin to FSI, is then applied for BRS calculation [92]. This method allows the correlation of blood pressure with ECG giving complementary insights into ANS. This approach has never been deployed in neonates and infants affected by fetal programming before and seems to be a very promising tool for this patient group.

Human studies pose various challenges in terms of confounding factors and accessibility to brain tissues. Future studies will hence require a combination of non-invasive physiological measures of the stress response system that are unequivocally linked to PS and that could be deployed as predictive biomarkers of the child neurodevelopmental outcomes. By integrating multiple non-invasively obtainable sources of information this framework could yield progress in maternal, fetal and child health, offering a more precise and personalized prediction and new possibilities for designing interventions to improve neurodevelopmental outcomes of pregnancy affected by PS.

3. EARLY INTERVENTION PROGRAMS IN CHILDREN

The biological rationale for designing Early Intervention Programs (EIP) is based on the well-known property of the brain known as cerebral plasticity. As elegantly synthesized by Brenhouse and Andersen [93]: *[3]While genes provide the blueprint to construct the brain, experience sculpts that brain to match the needs of the environment'*. Assuming that the conditions are favorable, there is a time window during which brain functions develop normally; however, during anomalous conditions, there is a sensitive period during which the structure or function can be modified. This adaptation

to the needs of the environment implies that environmental influences such as stress have a significant impact on brain development. One of the roles of cerebral plasticity is correcting anomalous cognitive development and may have favorable effects even if the change it induces does not reproduce the normal sequence of normal development. Since the young brain has a higher potential towards plasticity, intervention programs are devised

as early as possible aiming at stimulating the brain during the periods of its plasticity so that such neuroplasticity processes may correct the abnormal cognitive development [94-96].

Although it seems logical to assume that maternal stress during pregnancy would lead to parenting stress during nursing, few studies have addressed this important topic. Among these studies, Huizink *et al.* [16] observed that pregnancy-specific anxiety and trait anxiety predict higher levels of parenting stress 3 months after birth. As observed by Mc Quillan *et al.* [97], postnatal stress leads to poor and insufficient sleep which in turns leads to to less observed positive parenting practices and more self-reported dysfunctional parenting. More recently, Hagaman *et al* [98] showed that, in a peri-urban cohort in Pakistan, depression and stress during pregnancy were significantly associated with poor maternal functioning in everyday life during the first year after birth. It is, therefore, plausible to predict that the neurodevelopmental outcome in terms of affective and reward circuits, cognition, mood, emotional regulation, personality and learning abilities of the infant would be shaped by the antenatal exposure to maternal stress and subsequent less than optimal parenting. Inversely, it has been demonstrated that engaging in positive parenting practices, such as reading to children, engaging in storytelling or singing and eating meals together as a family may influence a child's future success in the educational system and reduce the risk for delays at the developmental, social and behavioral levels [99]. According to Garg *et al.* [100], ³*The quality of the early care environment can buffer some of the negative effects of prenatal adversity on child development´* and at the molecular level. These authors demonstrated that the early care environment co-varies with variation in genome-wide DNA methylation in middle childhood meaning that there is an association between infant attachment at 36 months of age and DNA methylome variation in later childhood.

Excluding pathological outcomes, there is again little research directed at correlating the influence of both prenatal and postnatal unfavorable environments on the offspring´s behavior, particularly on how the environment will impact on the infant personality and character. Two recent studies show that environmental harshness and hawk character during the first two years of life predicted worse visual-problem solving at 4 years of age [101] and that children´s temperamental predispositions, paired with a history of regulatory problems in infancy and maternal depressive symptoms contribute to an increased risk of behavioral problems [15], pinpointing the influence of early disadvantageous environments and character on behavioral and cognitive abilities in later life.

In the face of this body of evidence, the development of EIP is urgently needed to ameliorate these undesired behavioral and cognitive outcomes and to aid parents whose babies were exposed to PS. The history of "Early Stimulation Programs", as were originally called, dates back to 1965 when a publicly funded program was created in the United States for vulnerable children in low-income families [102]. Since then, most of the early stimulation programs have been targeted to neurodevelopmentally impaired or preterm or low-birth-weight infants. Even though there are no specific programs designed for babies that were exposed to PS, the wealth of programs developed to stimulate children and to improve parenting abilities are extremely valuable antecedents that will help to choose the appropriate intervention strategy for an early stimulation program oriented toward prenatally stressed babies. As mentioned above, these babies develop as "apparently healthy children" until global developmental and communication disorders are diagnosed many years later, sometimes at school age.

Since summarizing the existing literature on the EIP is out of the scope of this review, we will briefly outline the main benefits reported in selected studies. According to Bonnier [95], EIP have been developed for three different target populations: 1) Low socioeconomic status children at-risk, 2) Children with disorders that induce developmental delays (e.g. Down Syndrome, Cerebral Palsy) and 3) Preterm or low weight babies. Cognition improvements are greater than motor skills [95] although other studies point out that there are moderate effects on cognitive and behavioral outcomes [103] and improved visual and motor skills during

infancy [104] but that cognitive benefits persist into preschool age [105]. In spite of the different targeted populations and the heterogeneity between studies, most reviews concur that EIP

produce the greatest positive effects when the programs involve both parents and the child and that long-term stimulation programs improved child-parent interactions [95, 96].

In this sense, a recent review [106] and a meta-analysis [107] report the results of several studies employing different programs involving parents and children known as: *Early Interventions focused on the family* or *Early Family- Centered Interventions*. This approach aims at improving the parent-infant interactions and relationship resulting in an improvement in the child´s development and increased resilience. Although its effectiveness has yet to be fully proven, parental psychosocial support is a sensible approach to increase parent´s ability and knowledge to care for their child. Parent´s support is intended to reduce stress, anxiety and depression as well as improving the parent´s attunement and capacity to interact with their child, which will eventually improve the developmental outcomes. Intuitively, it is clear that building a strong parent-infant relationship will help the infant learn self-regulatory skills such as crying but being consolable, self-soothing, sleep-wake changes and feeding [106]. As mentioned before, most of these programs targeted a cohort of preterm babies and only one study assessed children at social risk excluding preterm children. In any case, and although the age of the babies, the assessment tools and the programs differed among studies, all interventions involved components of guidelines for parents to stimulate child development through improving reciprocity in parent/baby relationship and a better understanding of the child´s needs. In spite of the mentioned heterogeneities in study design, the overall conclusion was that *Early Family-Centered Intervention Programs* improved the cognitive and motor development in preterm infants when compared to the standard care.

4. PERSONALIZED AND PREVENTIVE MEDICINE: FROM EARLY LIFE HEALTH MONITORING TO EARLY LIFE PREVENTION PROGRAMS

Figure 1 summarizes how we envision the connection between prospective studies and the identification of biomarkers with the administration of EIP that will lead to a personalized and preventive medicine for babies exposed to PS.

In the **Prospective Study** section of the Figure we illustrate that stressed pregnant women in their third trimester are categorized as stressed with PSS (Cohen Perceived Stress Scale questionnaire, PSS-10) and controls matched for 1:1 for parity, maternal age, and gestational age at study entry. Two and a half weeks after screening, a transabdominal ECG (taECG) recording at 900 or 1000 Hz sampling rate of at least 40 min is performed to compensate for inevitable signal artifacts. From fetal and maternal ECG, fetal and maternal R-peaks are detected and to derive the FHR and MHR. The bPRSA method is used to quantify interactions between FHR and MHR as a measure of transfer of maternal stress onto fetus (Fetal Stress Index, FSI). This measure uniquely captures biophysical dynamics of mother-fetus dyad. Maternal serum is obtained to detect total cholinergic status as the total capacity for acetyl choline (ACh) hydrolysis (that is, the summation of ACh esterase, AChE, and butyl choline esterase, BChE, activities). Cholinergic status, especially the ratio of AChE/BChE, has been shown to be a sensitive indicator of chronic stress exposure in adults, but its role as stress biomarker in a developing organism remains to be fully elucidated [108-110]. AChE activity levels are assessed in the maternal serum and cord blood samples with a specific BChE inhibitor, by using a microtiter plate assay (MPA). On the day of parturition, hair strands are collected from the posterior vertex region on the head, as close to the scalp as possible, for cortisol measurement using Automated Chemiluminescent ImmunoAssay (CLIA). Based on an approximate hair growth rate of 1 cm per month, the proximal 3 cm long

hair segment is assumed to reflect the integrated hormone secretion over the three-month-period prior to sampling [111].

Soon after birth, a neonatal specialized midwife collects a saliva/buccal sample from the newborn by gentle rubbing the gums on both sides with a special device and stores it at room temperature. DNA from the infant saliva sample is extracted and methylation is measured using the Infinium HumanMethylation EPIC beadchip array or similar technologies. Cord blood serum for miRNA detection and AChE/BChE analysis is also collected.

At 24 months of age, infants' development is assessed by the german version of Bayley Scale of Infant Development Third Edition (BSID-III). The BSID-III is composed from a series of subtests aimed at evaluating cognitive, language and motor skills on infants from 16 days to 42 months. A specialized professional, who is blind regarding maternal stress categorization, administers the tests, which lasts about 120 minutes. A new saliva sample is collected from the infant at this final visit for epigenetic analysis. Based on the assumption that PS imprints these phenotypic modalities permitting a mutual inference, we examine putative relationships between the measures derived from all epigenetic biomarkers, FHR analyses techniques and maternal and child cognitive assessments. One key challenge is the integration of heterogeneous datasets, such as multidimensional HRV indices, the epigenetic information, and biochemistry indices. With the entire multimodal cohort data, the machine learning tools build predictive models of the relationship between PS and the different multimodal biomarkers. With the generated model, clinicians can make predictions for the effects of PS on later neurodevelopment, e.g., by using ta ECG and cord-blood samples to predict BSID at 2 years of life.

In the **Personalized and Preventive Medicine** section of the Figure, we highlight the significance of this proposal stressing the importance of identifying early non-invasive pre- and postnatal biomarkers of brain programming due to intrauterine stress exposure. This will help to predict adverse postnatal brain developmental trajectories, which is a prerequisite for designing EIP. EIP improve long-term developmental trajectories and may prevent development of health abnormalities entirely. Using an unprecedented multimodal combination of epigenomic predictors and clinical health outcomes such as prenatal stress, FHR analyses in utero and epigenetic alterations in newborns promises new insights into the early causes of neuropsychological dysfunction during early childhood.

CONCLUSION

Stress and anxiety during pregnancy increase risk for poor child neurodevelopment. If stress persists during the nursing period, it will lead to deficient parenting interfering with the mother-infant attachment. This implies that during critical periods of brain development, i.e., pregnancy and nursing periods, the baby is subjected to environmental negative influences known to shape developmental trajectories, including neuronal connections. This apparently healthy baby, if exposed to a repeated stressful situation later in life, may show impairments in the functional development of affective and reward circuits, cognition and response inhibition.

There is now a general consensus that both stress systems, namely the HPA and the SAMS, leave permanent epigenetic marks in selected genes that if adequately linked to the neurodevelopmental disorders can emerge as reliable predictive biomarkers. In addition, simultaneous maternal-fetal heart rate monitoring is emerging as a novel early PS biomarker. Since the pregnant and nursing mother can be unaware of her stress situation or of the harmful consequences to her child, the identification and characterization of biomarkers that can timely predict adverse postnatal brain developmental trajectories, is urgently needed and a prerequisite for designing therapeutic interventions. The recently developed Early Family-Centered Intervention programs

aim to support family dynamics in the domestic environment and are highly recommended due to the possibility of improving the care and stimuli offered daily to the infant.

By integrating multiple non-invasively obtainable sources of information using novel epigenetic, electrophysiological and machine learning methods, unambiguously linked to the disorders, this approach could yield progress in maternal-fetal medicine, pediatrics and developmental psychology, offering a more precise and personalized prediction and new possibilities for designing early interventions to improve neurodevelopmental outcomes of pregnancy affected by PS. This is an important first step in preventing neuropsychological problems and in reducing their personal and societal impact.

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FIGURE LEGENDS

FIGURE 1

a) **PROSPECTIVE STUDY**

Schematic representation of a Prospective Matched Case/Control study to derive biomarkers of prenatal stress. Pregnant women in their third trimester are screened and a transabdominal electrocardiogram (taECG) is perfomed two and a half week after screening. The bivariate Phase Rectified Signal Averaging (bPRSA) method is used to quantify interactions between fetal and maternal Heart Rate (fHR and mHR) as a measure of transfer of maternal stress onto the fetus (Fetal Stress Index, FSI). Maternal serum is obtained to detect total cholinergic status as the total capacity for ACh hydrolysis by Acetyl and Butyryl Cholinesterase (AChE and BChE activities) using a microtiter plate assay (MPA). On the day of parturition maternal hair strands are collected for cortisol measurement using automated Chemoluminiscent ImmunoAssay (CLIA). Upon delivery, saliva and serum samples are collected from the newborn. DNA from the infant saliva sample is extracted and methylation is measured, e.g., using the Infinium HumanMethylation EPIC beadchip array. Cord blood serum for miRNA detection and AChE/BChE analysis, is also collected. At two years of age, the mother is invited to return with the toddler to evaluate the neurocognitive development assessed by Bayley Scale III of Infant development (BSID). Machine learning analysis will examine putative relationships between the measures derived from all epigenetic and molecular biomarkers, FSI and maternal and child cognitive assessments.

b) ***PERSONALIZED AND PREVENTIVE MEDICINE***

Timely assessment of non-invasive biomarkers for pregnant women and newborns will allow early detection of the babies-at-risk. This will help choosing the appropiate Early Intervention Program (EIP) and improve child development. Ultimately, such approach will help prevent long-term neuropsychological problems. Such intervention programs should ameliorate future disorders in otherwise apparently healthy children reducing the personal and societal impact of such disorders.

Anhang 12. Antonelli et al. Early Biomarkers and Intervention Programs for the Infant Exposed to Prenatal Stress

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