Psychoneuroendocrinology 119 (2020) 104754

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

Maternal prenatal psychological distress and hair cortisol levels associate with infant fecal microbiota composition at 2.5 months of age

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ARTICLE INFO

Keywords:
Microbiota
Stress
Cortisol
HPA axis
Gut
Psychological distress

ABSTRACT

Background: Maternal prenatal stress associates with infant developmental outcomes, but the mechanisms underlying this association are not fully understood. Alterations in the composition and function of infant intestinal microbiota may mediate some of the observed health effects, a viewpoint that is supported by animal studies along with a small human study showing that exposure to prenatal stress modifies the offspring's intestinal microbiota. In the current study, we aim to investigate the associations between maternal prenatal psychological distress (PPD) and hair cortisol concentration (HCC) with infant fecal microbiota composition in a large prospective human cohort.

Methods: The study population was drawn from FinnBrain Birth Cohort Study. Maternal PPD was measured with standardized questionnaires (EPDS, SCL, PRAQ-R2, Daily Hassles) three times during pregnancy (n = 398). A measure addressing the chronicity of PPD was composed separately for each questionnaire. HCC was measured from a five cm segment at gestational week 24 (n = 115), thus covering the early and mid-pregnancy. Infant fecal samples were collected at the age of 2.5 months and analyzed with 16S rRNA amplicon sequencing.

Results: Maternal chronic PPD (all symptom measures) showed positive associations (FDR < 0.01) with bacterial genera from phylum *Proteobacteria*, with potential pathogens, in infants. Further, chronic PPD (SCL, PRAQ-R2, and Daily Hassles negative scale) associated negatively with *Akkermansia.* HCC associated negatively with *Lactobacillus*. Neither maternal chronic PPD nor HCC associated with infant fecal microbiota diversity.

Conclusion: Chronic maternal PPD symptoms and elevated HCC associate with alterations in infant intestinal microbiota composition. In keeping with the earlier literature, maternal PPD symptoms were associated with increases in genera from*Proteobacteria* phylum. Further research is needed to understand how these microbiota changes are linked with later child health outcomes.

1. Introduction

Prenatal exposure to maternal psychological distress and/or alterations in the hypothalamus-pituitary-adrenal (HPA) axis functioning has been linked to the suboptimal emotional, behavioral, and cognitive development of the offspring (Monk et al., 2019; Weinstock, 2008). Further, maternal prenatal stress predisposes some of the offspring to an increased risk of somatic conditions such as asthma (van de Loo et al., 2016). The dimensions of prenatal stress can be assessed e.g. by using self-report questionnaires that measure maternal prenatal psychological

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https://doi.org/10.1016/j.psyneuen.2020.104754
Received 15 November 2019; Received in revised form 6 May 2020; Accepted 29 May 2020
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distress (PPD) or reactions to stressful life events or daily hassles (Weinstock, 2008). It seems that even mild or moderate stress exposure can impact the development of the offspring, when the exposure is recurrent or chronic (Graignic-Philippe et al., 2014; Pervanidou and Chrousos, 2018; Soares-Cunha et al., 2018). Additionally, HPA axis functioning measures are frequently used to measure prenatal stress (Weinstock, 2008), and maternal HPA axis functioning is suggested to signal environmental cues to the developing fetus and thus mediate prenatal stress exposure. Maternal physiological adaptations or responses to stress during pregnancy, such as HPA axis activation and immune activation, may lead to physiological alterations in the fetus or the infant (Monk et al., 2019), but the exact mechanisms mediating the often adverse effects of prenatal stress in the progeny are still not fully understood.

In addition to the HPA axis and immune activation, maternal prenatal stress has been shown to alter the pregnant dam's gut microbiota in mice (Jašarević et al., 2017). One exploratory study conducted in a human population suggested that maternal PPD has an association with maternal fecal microbiota composition, suggesting that mechanisms similar to those observed in rodents might also take place in humans (Hechler et al., 2019). The results from animal studies suggest that prenatal stress exposure might influence offspring outcomes via altering the offspring microbiota (Jašarević et al., 2017). Furthermore, alterations in maternal vaginal microbiota may also partially mediate the effects of prenatal stress on the offspring's gut and hippocampus development (Jašarević et al., 2018). However, distortions in the balance of early gut microbiota have been linked with similar child outcomes as prenatal stress exposure, including asthma (Stokholm et al., 2018) and suboptimal neurodevelopment (Carlson et al., 2018). Regarding the composition of the fecal microbiota of infants, the genera of *Bifidobacterium* and *Bacteroides* are of special interest as typically the fecal microbiota of a breastfed and vaginally born infant is characterized by high abundances of these genera (Bäckhed et al., 2015; Stewart et al., 2018). Interestingly, it has been reported that both *Bifidobacterium* and *Bacteroides* would be affected by prenatal stress exposure (Bailey et al., 2004; Gur et al., 2019). Infant microbiota serves as an interesting intermediate phenotype, and investigating the possible influences of prenatal exposures on its composition in humans is of high relevance.

There is one previous human study suggesting that maternal prenatal stress, as assessed by a composite score covering both self-reported symptoms and a saliva cortisol measure, is linked to an altered developmental pattern of infant fecal microbiota during the first 110 days of life (Zijlmans et al., 2015). This study used a measure of maternal stress during pregnancy that comprised the mean of two maternal noon saliva cortisol measures and a composite score yielded from a range of self-reported PPD symptom scales (Zijlmans et al., 2015). However, saliva cortisol concentration is highly fluctuating, and obtaining a stable cortisol measure, such as hair cortisol concentration (HCC), is potentially more relevant for assessing the long-term homeostasis of the HPA axis during pregnancy. Similar to saliva, serum, or urinary cortisol, HCC is related to some but not all types of PPD symptoms. Reportedly, HCC has been positively associated with depressive symptoms when they were consistently elevated throughout pregnancy (Mustonen et al., 2019) and also with other types of PPD measures, albeit the associations appear rather weak especially with cross-sectional questionnaire assessments (Hoffman et al., 2016; Mustonen et al., 2018).

The current study aimed to determine the associations between chronic maternal prenatal stress and infant fecal microbiota composition and diversity parameters in a large prospective human cohort. We were especially interested in analyzing the potential influence of chronic PPD on infant fecal microbiota composition and diversity and aimed to assess the overall level of PPD exposure across pregnancy by composing a chronic variable for each domain of PPD (depression, general and pregnancy-related anxiety, and daily hassles). With a smaller population and with data available on maternal mid-pregnancy HCC as a measure of long-term cortisol levels in early and mid-pregnancy (Mustonen et al., 2018), we also aimed to investigate the association between maternal HCC levels and infant fecal microbiota composition and diversity. We expected that HCC levels and chronic maternal PPD would be related to infant fecal microbiota profile. More specifically, based on the existing animal studies (Bailey et al., 2004; Jašarević et al., 2017) and one human study (Zijlmans et al., 2015), we hypothesized that infants exposed to chronic maternal self-reported PPD across pregnancy or elevated HCC cortisol levels in early and mid-pregnancy would have lower abundances of potentially health-promoting genera *Bifidobacterium* and *Lactobacillus* in their gut.

2. Materials and methods

2.1. Study subjects

The available target study population with fecal microbiota composition at the infant age of 2.5 months (n = 446) was drawn from the FinnBrain Birth Cohort Study (Karlsson et al., 2018), which aims to study the influences of early life stress exposures on later child developmental and health outcomes. Based on specific study designs, biological samples were collected from subpopulations of the original cohort of 3,808 families (Karlsson et al., 2018). The study has been approved by the Ethics Committee of the Hospital District of Southwest Finland. Written informed consent was obtained from all families, and mothers provided written informed consent on behalf of their infants. The study is conducted in South-West Finland.

Self-report prenatal questionnaires were obtained at gestational weeks (gwk) 14 (mean = 15, SD = 1.2, range 13–21), 24 (mean = 25, SD = 1.3, range 23–30), and 34 (mean = 35, SD = 1.1, range 33–40). At gwk 14, parental background data, including the level of education (categorized as (i) university education, i.e., tertiary level academic/general education; (ii) vocational tertiary education; (iii) secondary level education) and the intake of selective serotonin reuptake inhibitor or serotonin-norepinephrine reuptake inhibitors (SSRI/SNRI intake) were reported (Karlsson et al., 2018). Information on the duration of exclusive and partial breastfeeding was collected through postnatal follow-up questionnaires, and infant medication intake was reported at fecal sample collection. Breastfeeding at 2.5 months was categorized as never breastfed, breastfeeding ceased, partial breastfeeding, and exclusive breastfeeding in accordance with Stewart et al. (Stewart et al., 2018). Information about maternal pre-pregnancy body mass index (BMI; kg/m^2), the duration of gestation (preterm < 37 gwk; term 37–41; post term ≥ 41 gwk), antibiotic intake during the neonatal period, birth weight (g) and height (cm), and the mode of delivery [all caesarian (C)-section vs. all vaginal] was collected from the National Birth Registry provided by the National Institute for Health and Welfare (www.thl.fi).

As a part of the larger FinnBrain Birth Cohort study, a project focusing on the development of the gut-brain axis has been established. This project aims to investigate the role of early life exposures specifically on infant fecal microbiota. For the current study, 446 mother-infant dyads with infant stool sample taken at the age of 2.5 months were included. Out of the initial population, complete information on breastfeeding, birth mode, and infant age and sex was available from 404 dyads. Further, out of these 404 dyads, 398 dyads had at least 50 % of the maternal prenatal questionnaire data, thus forming the population in the analyses regarding PPD (Table 1). Out of the 398 dyads, a total of 312 subjects had responded to at least 90 %, 348 at least to 80 %, 356 at least to 70 %, and 360 at least to 60 % of the questionnaires. The average response rate was 90.4 %. The Pregnancy-Related Anxiety Questionnaire-Revised2 (PRAQ-R2) was introduced to the study protocol only later during the course of the main cohort data collection leading to a higher rate of unavailable data in the first timepoint (71 %) (Table 2.)

Out of the target population of the current study (n = 446), HCC at

Table 1
Infant and mother characteristics included in the statistical analyses that had the complete background information as well as adequate prenatal questionnaires (n = 398) and/or HCC (n = 115, total population in the analyses n = 399).

		Whole population n = 399	Boy n = 204	Girl n = 195
Mother's age	mean (SD), years	30.8 (4.4)	30.9 (4.3)	30.8 (4.5)
Mother's education, count (%)	secondary or primary level	91 (22.8 %)	42 (20.6 %)	49 (25.1 %)
	vocational tertiary	126 (31.6 %)	66 (32.4 %)	60 (30.8 %)
	university	165 (41.4 %)	86 (42.2 %)	79 (40.5 %)
	missing data	17 (4.3 %)	10 (4.9 %)	7 (3.6 %)
SSRI/SNRI use during 1 st trimester, count (%)	no	361 (90.5 %)	185 (90.7 %)	176 (90.3 %)
	yes	18 (4.5 %)	8 (3.9 %)	10 (5.1 %)
	missing data	20 (5%)	11 (5.4 %)	9 (4.6 %)
Mother's prepregnancy BMI	mean (SD), kg/m^2	24.4 (4.6)	23.9 (4.3)	25.0 (4.8)
Gestational age	mean (SD), gwks	40.1 (1.4)	40.0 (1.5)	40.2 (1.2)
Prematurity, count (%)	preterm	14 (3.5 %)	11 (5.4 %)	3 (1.5 %)
	term	281 (70.4 %)	143 (70.1 %)	138 (70.8 %)
	post term	104 (26.1 %)	50 (24.5 %)	54 (27.7 %)
Birth weight	mean (SD), grams	3,626.7 (464.7)	3,699.1 (490.0)	3,551.1 (425.0)
Mode of delivery, count (%)	C-section	68 (17 %)	33 (16.2 %)	35 (17.9 %)
	vaginal	331 (83 %)	171 (83.8 %)	160 (82.1 %)
Categorical breastfeeding, count (%)	none	5 (1.3 %)	2 (1%)	3 (1.5 %)
	ceased	19 (4.8 %)	10 (4.9 %)	9 (4.6 %)
	partial	62 (15.5 %)	35 (17.2 %)	27 (13.8 %)
	exclusive	313 (78.4 %)	157 (77 %)	156 (80 %)
Infant antibiotic courses, count (%)	no	355 (89 %)	174 (85.3 %)	181 (92.8 %)
	yes	44 (11 %)	30 (14.7 %)	14 (7.2 %)
Age during fecal sampling	Mean (SD), days	64.3 (13.4)	63.8 (12.7)	64.9 (14.2)

gwk 24 and the key covariates (breastfeeding, birth mode, infant age and sex) were available from 115 dyads (Table 1). Further, both successfully measured HCC and adequate PPD questionnaire data with key covariates were available from 114 dyads, and either HCC or adequate PPD questionnaire data with the key covariates were available from 399 dyads (Table 1). It should be noted that infant stool samples and maternal hair samples were derived from two partially distinct recruitment processes resulting in the reported discrepancy in the number of study subjects providing each sample type.

2.2. Maternal prenatal psychological distress

2.2.1. Questionnaires

Prenatal maternal depression and anxiety symptoms were measured with the Edinburgh Postnatal Depression Scale (EPDS) (Cox et al., 1987; Gibson et al., 2009) and Symptom Checklist-90, anxiety subscale (SCL-90) (Derogatis et al., 1973; Holi et al., 1998), which were also reported at three months postpartum. Stress related to everyday life was measured with Daily Hassles (Korpela et al., 2008), which consists of both a positive and a negative scale and where using four points the subjects rate their worries (a-scale, "negative") or delights (b-scale, "positive") related to social relationships, work, finances, household matters, news and media, and substance use (tobacco, alcohol, drugs). Only the negative scale was included in the study and a total score was calculated for it. PRAQ-R2 (Huizink et al., 2016) was used to measure pregnancy-related worries and anxiety related to the fear of giving birth, worries about bearing a physically or mentally handicapped child, and concerns about the mother's own appearance.

In line with our aim, which was to assess the influence of the chronic symptom level across pregnancy on infant fecal microbiota, a categorical variable of each symptom scale (chronic EPDS, PRAQ-R2, SCL, and Daily Hassles negative subscale) was built. Subjects that scored above the selected cut-offs in two measurement points were classified as having chronically elevated scores for each variable. The total score of 10 or more was used as a cut-off point for EPDS in order to present more clinically significant symptoms (Gibson et al., 2009), while median split was used for the other questionnaires. The median scores of the questionnaires in this subpopulation corresponded with those of the whole FinnBrain Birth Cohort population [(Korja et al., 2018); Table 2].

2.2.2. Hair cortisol concentration assessment

HCC samples were collected during a study visit at gwk 24 as a part of the sample collection protocol among the pregnant female cohort participants (mean = 24.6 gwk, SD = 1.15, range 22.3–27.9 gwk) (Karlsson et al., 2018). The aim is to study the effects of HPA axis

Table 2
Mean questionnaire sum scores and proportion of missing data for each measurement point and number of subjects scoring above cut-off in at least two measurement points. N = 398.

		EPDS	SCL	PRAQ	Daily Hassles negative scale
gwk 14	median (range)	4 (0–26)	2 (0–30)	21 (10–45)	12 (6–19)
	missing data	5 %	5 %	71 %	11 %
gwk 24	median (range)	4 (0–21)	2 (0–28)	21 (10–44)	12 (6–18)
	missing data	2 %	2 %	2 %	8 %
gwk 34	median (range)	3 (0–20)	2 (0–25)	21 (10–47)	11 (6–19)
	missing data	4 %	4 %	4 %	10 %
postnatal	median (range)	4 (0–19)	1 (0–17)	–	–
	missing data	11 %	11 %		
Cut-off		10	median	median	median
Chronically high*	count(%)	30 (7.5 %)	120 (30.2 %)	41 (10.3 %)	140 (35.2 %)

* Subjects scoring above the cut-off in two or more prenatal measurements.

functioning and the long-term cortisol secretion on health and developmental outcomes. There were no differences in the maternal PPD questionnaires (chronic EPDS, PRAQ-R2, SCL, and Daily Hassles negative subscale) between the mothers with or without available hair samples (Wilcoxon rank-sum test, W 1874-16469, FDR 0.30-0.92). Hair samples were cut from a standardized area in the posterior vertex region of the head most proximal to the scalp. For analyses, a five cm segment weighing 5–15 mg was used to cover the past five months of pregnancy. HCC assessment was performed by ELISA using an IBL International Cortisol Saliva kit, as previously described (Mustonen et al., 2019).

2.3. Fecal sample collection and analysis

Parents collected the infant fecal samples at 2.5 months of age at their homes. Parents were instructed both orally and by written tutorials to collect the fecal material into sterile collection tubes, to immediately store the samples in their household refrigerators or freezers, and to deliver the samples to the study center as soon as possible after the collection using coolers. Parents were also requested to mark the date and time of the sample taking. Samples were immediately homogenized, divided into aliquots, and frozen in the research facilities. Only those delivered within 48 h were included in the analyses. Due to variation in sample collection times, age as months during sample collection was included in the adjusted analyses. DNA extraction, amplification of variable region V4 of the bacterial 16S rRNA gene, as well as next-generation sequencing with Illumina MiSeq system were performed as previously reported (Rintala et al., 2018). Of the delivered samples (n = 517), only the samples that were collected and preprocessed according to the instructions given and successfully sequenced were included in the analyses (n = 446, 86 %).

2.4. Data analysis

Quality of the raw sequences was checked with the FastQC program (v. 0.10.1; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), after which the downstream analyses were performed using QIIME pipeline (v. 1.9) (Caporaso et al., 2010). The sequence reads were filtered with a quality score acceptance rate of ≥ 20, resulting in 41k–1,052k reads per sample (total: 73,222k, mean: 178,156, sd: 109k). Chimeric sequences were filtered out using USEARCH (v. 6.1), and operational taxonomic units (OTUs) were picked using UCLUST algorithm with 97 % sequence similarity (Edgar, 2010). OTUs representing less than 0.05 % of the total sequence count were removed. Annotations for the OTUs were derived from the GreenGenes database (DeSantis et al., 2006).

Statistical analyses were performed with R 3.5.0 software using *phyloseq* (McMurdie and Holmes, 2014) and *microbiome* R/Bioconductor packages. *Microbiome* package was used to calculate diversity (Shannon Index) and richness indexes which describe the intra-individual bacterial diversity or richness. As the HCC values are highly skewed, natural logarithm of the continuous HCC values was used in the analyses according to common practice.

Potential confounders [the mode of delivery, breastfeeding status, infant age during sampling (months), and infant sex] (Bäckhed et al., 2015; Stewart et al., 2018) were selected *a priori* based on earlier research. To further evaluate the possible importance of the background variables in this study population, descriptive statistics were performed. Associations between the background variables (the mode of delivery, breastfeeding, infant age and sex, infant antibiotic use, mother's SSRI/SNRI use, birth weight and height, mother's education, the number of previous deliveries, and maternal pre-pregnancy BMI) and the independent variables PPD (chronic EPDS, PRAQ-R2, SCL, and Daily Hassles negative subscale) and HCC were calculated. These analyses included Chi-squared test for two categorical variables, Wilcoxon's rank sum test, Kruskall-Wallis H test for categorical and continuous variables, and Spearman correlation coefficient for two continuous variables.

Additionally, as infant antibiotic use may have substantial impact on the infant microbiota composition, a graded adjustment was performed by controlling additionally antibiotic use if some significant associations with microbiota and independent variables were detected. Moreover, graded adjustment was performed with the available postnatal psychological distress measures by including the corresponding postnatal (of children aged three months) maternal symptom score whenever available. Thus, the model which included prenatal EPDS as the main predictor was initially controlled for the mode of delivery, breastfeeding status, infant age during sampling, and infant sex, and in the next step also by using postnatal EPDS. Likewise, the SCL model was additionally controlled by using the postnatal maternal score of SCL. For PRAQ, Daily Hassles, or HCC, there were no corresponding postnatal measures.

The R package *DESeq2* (Love et al., 2014), which uses shrinkage estimation for dispersions and fold changes ($\log_2$ Fold-Changes are reported) to perform the quantitative analysis of differential expression, was used to analyze the associations between the PPD measures (chronic EPDS, PRAQ-R2, SCL, and Daily Hassles negative subscale) or HCC and the abundance of each classified or unclassified bacterial genus in the infant fecal samples. The DESeq2 analyses were adjusted for the *a priori* chosen confounders (the mode of delivery, breastfeeding, infant age during sampling, and infant sex) by including them as covariates in the model. The intra-individual diversity i.e. beta diversity was analyzed with the Permutational Analysis of Variation (PERMANOVA) using the function adonis in the R package *vegan* (Oksanen et al., 2017), which uses Bray-Curtis dissimilarity. The beta diversity analyses were controlled for the selected covariates and 999 permutations were used. The associations between alpha diversity or richness and independent variables were analyzed first with unadjusted linear models. Linear models were adjusted for the covariates only if the unadjusted associations between chronic PPD symptoms or HCC and alpha diversity or richness were statistically significant. Graded adjustment models for both antibiotic use and maternal postnatal psychological distress as described above were performed, if significant associations were detected in either the alpha diversity linear models, beta diversity, or differential expression analyses.

Missing PPD questionnaire data (n = 398, population with at least 50 % PPD questionnaire data available as described in Section 2.1) was imputed with R package *missForest* (Stekhoven and Bühlmann, 2011) and function missForest with default parameters. The method uses random forest algorithm and takes into account multiple variable types and non-linear interactions as well as averages over multiple imputations. Missing data replaced by imputation comprised 9% of the entire questionnaire matrix (see Section 2.1 and Table 2). All analyses were adjusted for multiple comparisons using the Benjamini-Hochberg method separately for each response variable, and findings with False Discovery Rate (FDR) ≤ 0.01 and when applicable, absolute $\log_2$ Fold-Change > 1 were considered as statistically significant.

3. Results

3.1. Descriptive analyses on maternal chronic PPD, HCC, and the background variables

The maternal mean HCC of approximately the first five months of pregnancy was 19.2 pg/mg (SD = 35.2, median = 11.0 pg/mg) ranging between 0.3 and 269.6 pg/mg. C-section was more prevalent in the chronically elevated SCL group (FDR 0.02, χ^2 = 9.8; Supplementary Table 1). None of the other selected background variables (breastfeeding, infant age and sex, infant antibiotic use, mother's SSRI/SNRI use, gestational age at birth, birth weight and height, mother's education, the number of previous deliveries, maternal pre-pregnancy BMI) differed from the other maternal PPD groups (chronic EPDS, PRAQ-R2,

SCL, and Daily Hassles negative subscale, FDR ≥ 0.1 for all; Supplementary Table 1) or HCC (FDR 0.70–1, Supplementary Table 2).

3.2. Infant fecal microbiota composition

Five different bacterial phyla (*Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes,* and *Verrucomicrobia*) and 50 different bacterial genera were detected in the population. *Bacteroides, Bifidobacterium,* and *Veillonella* were the most abundant among the identified genera (12–20 % average relative abundance, Supplementary Table 3). In addition, the representatives of family *Enterobacteriaceae* were highly abundant in a vast majority of the samples, and genus level identification for 20 % of the taxa could not be reached.

3.3. Associations between the maternal chronic PPD, HCC, and infant fecal microbiota composition

3.3.1. Chronic PPD symptoms

Abundances of genera from gram-negative *Proteobacteria* phylum showed associations with chronically elevated maternal chronic PPD symptoms when adjusting for selected covariates[1]: Daily Hassles negative subscale associated positively with *Erwinia, Haemophilus, and Serratia*; SCL associated positively with *Campylobacter, Citrobacter, and Serratia*; EPDS associated negatively with *Desulfovibrio* and positively with *Citrobacter* and *Serratia,* and PRAQ-R2 associated positively with *Camplylobacter, Serratia,* and *Haemophilus* (FDR < 0.01, Supplementary Table 4, Fig. 1)[1] .

Genera *Veillonella, Finegoldia, Dialister, Dorea,* and *Coprococcus* belonging to gram-positive *Firmicutes* as well as *Actinomyces* and *Rothia* belonging to gram-positive *Actinobacteria* showed positive associations with maternal chronic PPD measures (Supplementary Table 4, Fig. 1) [1]. *Akkermansia* (the only genus belonging to phylum *Verrucomicrobia*), *Pseudoramibacter, Phaslarctobacterium, Megamonas, Megasphera, Eubacterium, Epulopiscium, Anaerotruncus, Pseudoramibacter_Eubacterium* (phylum *Firmicutes*), *Paraprevotella, Parabacteroides, Odoribacter* (gram-negative *Bacteroidetes*), as well as *Slackia, Actinobaculum,* and *Propionibacterium* (phylum *Actinobacteria*) showed only negative associations with maternal chronic PPD symptoms (Supplementary Table 4, Fig. 1)[1]. *Butyricimonas* and *Prevotella* (phylum *Bacteroidetes*) were positively associated with EPDS (Supplementary Table 4, Fig. 1). *Staphylococcus* (phylum *Actinobacteria*) was positively associated with SCL and negatively with EPDS (Supplementary Table 4, Fig. 1).[1]

Graded adjustment with infant antibiotic intake lead to only minor alterations (Supplementary Table 5): the association between *Dialister* and EPDS as well as *Megasphaera* and PRAQ-R2 attenuated (FDR > 0.01 and when applicable, absolute $\log_2$ Fold-Change ≤1, Supplementary Table 5). Adjusting for postnatal maternal EPDS attenuated the EPDS associations with *Eubacterium, Citrobacter,* and *Dialister* (Supplementary Table 6), however, the majority of the observed associations between EPDS and genera remained unchanged regarding the direction of the $\log_2$ Fold-Change and the significance level. Adjusting for postnatal maternal SCL attenuated the observed SCL associations with *Actinomyces, Rothia, Serratia,* and *Dialister* (Supplementary Table 6).

3.3.2. Hair cortisol concentration

Maternal HCC reflecting the cortisol levels of the first five months of pregnancy was associated with the abundance of several bacterial genera in the infant fecal samples: maternal HCC was associated negatively with genera *Slackia* and *Actinobaculum* (phylum *Actinobacteria*), *Paraprevotella* and *Butyricimonas* (phylum *Bacteroidetes*), *Citrobacter* (phylum *Proteobacteria*), as well as *Ruminococcus, Phascolarctobacter, Anaerotruncus, Enterococcus,* and *Lactobacillus*

[1] Adjusted for infant age and sex, the mode of delivery, and breastfeeding by including the variable as a covariate.

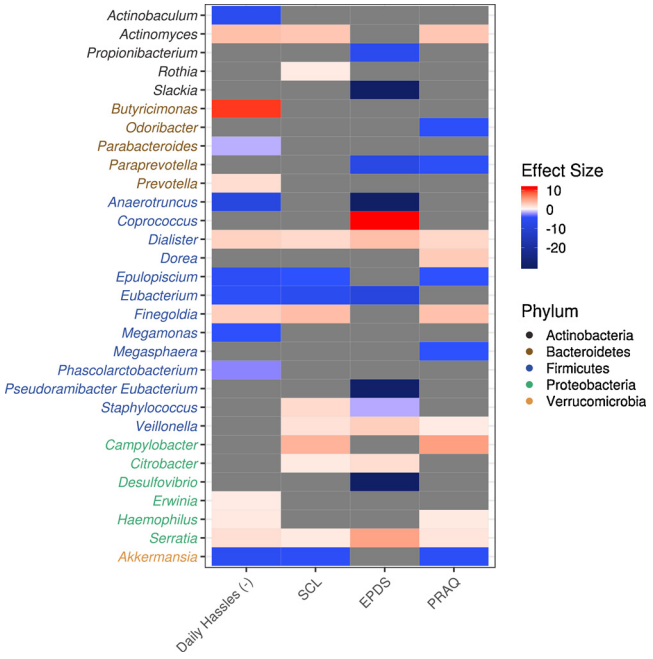

Fig. 1. $\log_2$ Fold-Changes of genera significantly (FDR < 0.01, absolute $\log_2$ Fold Change > 1) associated with chronic PPD symptoms. Gray depicts non-significant findings.

(phylum *Firmicutes*) (Supplementary Table 4, Fig. 2.)[1]. Graded adjustment with infant antibiotic intake did not change the abovementioned associations as indicated by the direction of the $\log_2$ Fold-Change and the significance level (Supplementary Table 5).

3.4. Associations between maternal prenatal stress and infant fecal microbiota diversity

Neither maternal HCC of approximately the past five months of pregnancy nor any of the chronic PPD symptom variables were associated with the infant fecal microbiota alpha (Shannon Index and richness, FDR ≥ 0.27) or beta diversity[1] (FDR ≥ 0.97, Supplementary Table 7).

4. Discussion

Exposure to prenatal stress, including maternal PPD or elevated cortisol levels, may predispose infants to suboptimal development and various adverse health outcomes (Monk et al., 2019; van de Loo et al., 2016), but the exact biological mechanisms mediating these health outcomes are largely unknown. This large cohort study showed that infant fecal microbiota composition, but not the diversity of this ecosystem, may be linked to maternal self-reported PPD levels as well as maternal HCC reflecting the cortisol levels in early and mid-pregnancy. As hypothesized, our results suggest that higher levels of prenatal cortisol exposure are related to decreased abundances of potentially health promoting bacteria such as *Lactobacillus* spp. (Mu et al., 2018). On the other hand, maternal chronic PPD is associated with increased abundances of potentially opportunistic gram-negative genera within the *Proteobacteria* phylum (Jiang et al., 2015). Although we failed to observe associations with the *Bifidobacterium* genus, our findings corroborate some results from existing animal models (Bailey et al., 2004) and human studies (Naudé et al., 2019; Zijlmans et al., 2015). The findings endorse the need to evaluate the longitudinal development of infant microbiota and developmental outcomes as well as the maternal phenotypes such as diet and medication intake in future studies.

In this study, infants exposed to the maternal PPD symptoms had increased abundances of genera that include several pro-inflammatory

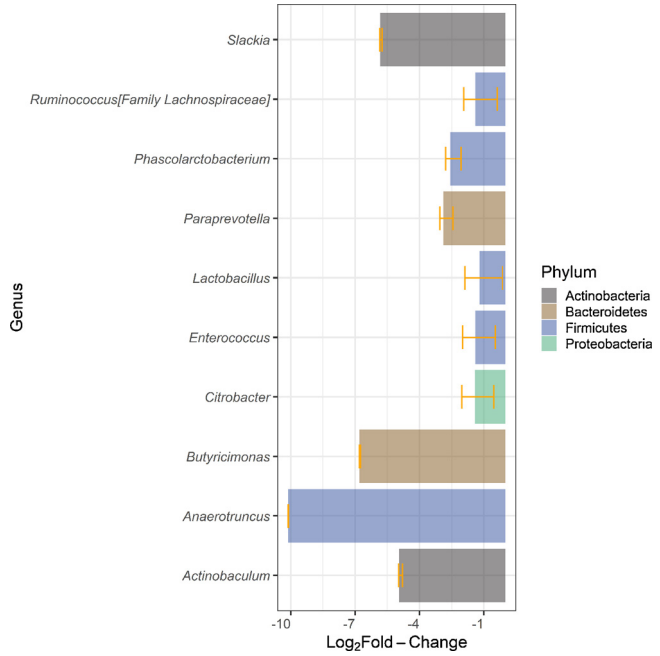

Fig. 2. Log_2 Fold Changes of genera abundances of genera associated with natural logarithm of HCC as a continuous variable (FDR < 0.01, absolute log_2 Fold Change > 1). Error bars show unadjusted 95 % confidence intervals of the Log_2 Fold-Changes derived from normally distributed log_2 Fold Change standard errors from DESeq2 model.

and potentially pathogenic bacteria, such as *Serratia, Haemophilus, Citrobacter,* and *Campylobacter* from the *Proteobacteria* phylum and *Veillonella* and *Finegoldia* from the *Firmicutes* phylum. These findings are in line with previous evidence, since increased abundances of genera belonging to gram-negative *Proteobacteria* have been associated with exposure to maternal prenatal stress (Naudé et al., 2019; Zijlmans et al., 2015). *Proteobacteria* as well as *Veillonella* and *Dialister* (despite being taxonomically classified in *Firmicutes* phylum) (Antunes et al., 2016) are gram-negative bacteria and harbor lipopolysaccharides (LPS) in their outer membrane. LPS is one of the most extensively studied virulence factor of gram-negative bacteria, and it has been suggested to influence the development of psychopathology (Rudzki and Szulc, 2018). Increased *Proteobacteria* levels have been reported for example in patients with depression (Jiang et al., 2015), and further rodent models show that colonization with pathogens such as *Campylobacter jejuni* may increase anxiety-like behavior (Goehler et al., 2008). This could serve as an interesting indicator that the observed alterations in fecal microbiota in this study could mediate to some extent the observed health effects of PPD in the offspring, although this remains to be investigated by further longitudinal research.

According to the hypothesis, we found evidence that infants with low levels of exposure to maternal PPD had increased abundances of potentially health-promoting bacteria, including *Akkermansia* (Cani and de Vos, 2017), and infants exposed to low levels of maternal HCC had increased abundances of *Lactobacillus* (Mu et al., 2018). Intervention studies have shown that probiotic treatments with *Bifidobacterium* and *Lactobacillus* species are able to alleviate self-reported distress and affect cortisol excretion (Messaoudi et al., 2010). Experimental models support the associations found in humans, as the colonization with *Lactobacillus* and/or *Bifidobacterium* have been demonstrated to normalize both the HPA axis functioning (Sudo et al., 2004) and depressive-like behavior (Messaoudi et al., 2010). Our observations are in line with the previous human study (Zijlmans et al., 2015) which indicated that lactic acid bacteria abundance may serve as a potential marker for healthy infant microbiota and optimal prenatal conditions regarding PPD.

Additionally, in this study various associations were detected between the maternal early and mid-pregnancy HCC and the members of infant fecal microbiota. Existing associations were partially similar to those found between the maternal chronic PPD symptoms and the infant fecal microbiota, while self-reported PPD symptoms did not directly translate to HCC levels, for example due to the relatively low degree of variance in PPD symptoms in this general population based study (Mustonen et al., 2018). Previously, maternal prenatal HCC has been linked to child HCC (Karlén et al., 2013), supporting the view that maternal prenatal HPA axis functioning is related to the programming of the child stress regulation systems. It is of note that in a study population of 8–16-year-olds, a child's HCC was not related to their fecal microbiota characteristics (Nathalie et al., 2019). However, to the best of our knowledge, HCC has not been used in the context of maternal prenatal stress and infant fecal microbiota before. While the exact mechanisms of how the prenatal cortisol levels influence fetal development remain poorly understood, our study can offer guidance to setting hypotheses for later experimental studies. In humans, antenatal corticosteroid treatment may increase infant gut peptide production (Costalos et al., 2003) and inflammatory responses in preterm infants (Rautava et al., 2016). Hence, despite the fact that literature on prenatal cortisol exposure is scarce, it can be interpreted that prenatal glucocorticoid exposure may affect gut physiology and immunity, and this may be reflected in the fecal microbiota composition.

There are several noteworthy differences compared to the previous study on this topic (Zijlmans et al., 2015). First, Zijlmans et al. measured maternal saliva cortisol at around gwk 37 for two days as opposed to the current HCC measurement, which covers approximately the first five months of pregnancy. As saliva cortisol depicts momentary cortisol levels, it should be noted that while there is an increase in overall cortisol levels towards the end of pregnancy, the HPA axis reactivity is attenuated, and therefore saliva cortisol may have limited value as an indicator of maternal prenatal cortisol homeostasis (de Weerth and Buitelaar, 2005). Thus, obtaining a stable cortisol measure, such as HCC, is potentially a relevant approach when we aim to assess cortisol secretion during a developmentally important time such as pregnancy (Hoffman et al., 2016; Mustonen et al., 2019, 2018). Second, Zijlmans et al. did not report specific associations between infant fecal microbiota characteristics and different domains of prenatal stress or cortisol levels separately, but they only applied a combined measure comprising of both saliva cortisol and self-report questionnaires from late pregnancy (Zijlmans et al., 2015). Our paper provided novel information by including prenatal depressive symptoms in addition to stress related to daily hassles, as well as general and pregnancy-specific anxiety. This is noteworthy as the distinction between symptom domains may be important to the infant outcomes (Nolvi et al., 2016). Differences in the assessment of domains of prenatal stress as well as microbiota sampling and characterization (limited to a single sample in the current study) preclude straight-forward comparison between our study and the previous paper with a similar topic (Zijlmans et al., 2015).

Recently, Kang et al. suggested a potential mechanism linking maternal PPD with infant fecal microbiota, as they reported that maternal depressive symptoms are associated with reduced fecal secretory IgA content in infants (Kang et al., 2018). IgA is a crucial factor in mucosal immunity, taking part in the modulation of bacterial colonization in the gut (Planer et al., 2016). It can be speculated that maternal stress could result in immune-dysregulation and concurrent IgA depletion in the infant gut alongside with a bloom of certain opportunistic bacterial taxa such as members within *Proteobacteria*. Additionally, maternal transmission of bacteria from the mother to the infant may have a role to play in this. As shown in a human study (Hechler et al., 2019) and rodent studies, stress can alter the maternal vaginal and gut microbiota composition (Jašarević et al., 2017), which may affect the vertical transmission of bacteria and in this way mediate the effects of prenatal stress on the offspring gut microbiota (Jašarević et al., 2018). Thus, future studies should take maternal microbiota characterization into

consideration to offer insight into the potential mechanisms.

In our study, longitudinal assessment of the maternal prenatal stress is strengthened by using a biological stress measure along with the questionnaire data. Mothers are assessed for multifaceted chronic psychological distress symptoms. The imputation of maternal PPD questionnaires may cause minor bias to the results, albeit PPD symptoms are reportedly relatively stable during pregnancy (Korja et al., 2018) and imputation is limited to mothers with at least 50 % of the PPD questionnaire data available. The population was relatively healthy with low variance in the PPD measures, which may dilute the observed effects. Clinical populations including subjects with pathological conditions might yield more distinct and extreme cases of phenotypes absent from the current study population. We did not assess exposure to stressful major life events with the self-reports, but the alterations in HCC can be related to multiple types of chronic stress such as caregiving stress or stress caused by unemployment (Mustonen et al., 2018; Stalder et al., 2017). The study could have benefited from additional assessment of maternal cortisol concentrations covering the late pregnancy, and ideally, the logistics of the study would have enabled a comprehensive coverage of both infant stool samples and maternal prenatal HCC samples from the entire study population.

Additionally, while the scope of our study did not cover the assessment of some of the possibly important mediators/moderators, it seems that postnatal antibiotic intake or postnatal maternal psychological distress in our graded adjustment approach did not change the overall conclusions. This may be due to low number of infants receiving antibiotic treatment and low levels of postnatal maternal psychological distress as well as the relatively young infant sample with relatively brief exposure to the postnatal environment. However, studies with longer follow-ups of infants and longer potential postnatal exposures could provide different results. Further research is needed to address the role of potentially important mediators/moderators such as breastmilk composition (Di Benedetto et al., 2019) or maternal medication intake, nutrition, or physical activity (Monk et al., 2019) in the association between maternal prenatal distress and infant fecal microbiota.

Only a single sampling of fecal microbiota was analyzed with 16S rRNA sequencing, offering a picture of infant fecal microbiota profiles at one time-point. 16S rRNA sequencing is prone to biases that may explain some of the observed discrepancies in the results, such as the high proportion of unidentified members of the *Enterobacteriaceae* family (20 % relative abundance). Updating the data processing pipeline from QIIME (Caporaso et al., 2010; Kuczynski et al., 2012) to QIIME2 (Bolyen et al., 2019) and from Greengenes (DeSantis et al., 2006) to the SILVA (Quast et al., 2012) database, which are currently more common choices than at the time of conducting this study, can be expected to improve the coverage and accuracy of read mapping. We see no reason, however, to expect *a priori* that the overall qualitative conclusions of this study would be affected by such updates in the amplicon sequence data processing pipeline. In addition, the amplicon sequencing based gut microbiota profiles do not necessarily describe the functional potential of the gut bacteria, and it should be mentioned that the method does not allow a species- or strain-level resolution. Thus, far-reaching interpretations cannot be made based on an individual genus but rather on the repeated association signatures. Additionally, some of the statistically significant genera had low abundances, and thus these results should be always interpreted with caution. However, it is equally important to consider that also the low abundance genera may be significant for the functioning of the microbiota.

Further, it seems that the genera compositions, rather than the cruder indicators such as alpha diversity, are associated with the prenatal exposures. It is noteworthy that microbiota alpha diversity is low in infancy especially in vaginally born and breastfed infants, but the diversity increases especially after the cessation of breastfeeding (Stewart et al., 2018). Additionally, there are some preliminary results showing that the lower early life alpha diversity associates with better cognitive development in 1–2-year-olds (Carlson et al., 2018). This indicates that the interpretation of the results related to microbiota alpha diversity in infants is not straightforward.

To conclude, our study showed that maternal chronic PPD and early and mid-pregnancy HCC levels are associated with distinct infant fecal microbiota composition in a large, prospective human cohort. The observed fecal bacterial signatures in the infants with exposure to maternal chronic PPD, such as increased abundances of potentially inflammatory genera from *Proteobacteria* phylum, warrant future follow-up of these children, since similar fecal microbiota alterations have been previously associated with future adverse health outcomes such as asthma (Stokholm et al., 2018). The results of this study describe only associations yet corroborate certain interesting findings reported in earlier literature and offer hypotheses for future mechanistic studies.

Data availability statement

Due to Finnish federal legislation, the research data cannot be made available online, but data can potentially be shared with Material Transfer Agreement as part of research collaboration. Collaboration requests can be sent to the Board of the FinnBrain Birth Cohort Study, please contact Linnea Karlsson (linnea.karlsson@utu.fi).

Acknowledgements

LK was funded by the Academy of Finland (grant number 308176), Waterloo Foundation (2110-3601), Yrjö Jahnsson Foundation (grant numbers 6847, 6976), Signe and Ane Gyllenberg Foundation, Finnish State Grants for Clinical Research (P3654), and Jalmari and Rauha Ahokas Foundation. LL was supported by the Academy of Finland (grant numbers 295741 & 307127). VL was supported by the UTUGS/MATTI graduate school. Finnbrain Birth cohort Study (HK) has been funded by the Academy of Finland (grant numbers 253270, 134950), Jane and Aatos Erkko Foundation, as well as Signe and Ane Gyllenberg Foundation. AJR and BC are supported by FCT project PTDC/MED-NEU/29071/2017.

HK, LK, HMU, EM, AKA, AK, and PH conceptualized the study. HMU, EM, AKA, AK, SK, and PM participated in the data collection. SK, PM, and BC performed the hair sample preprocessing. BC and AJR performed the HCC analyses. Fecal samples were analyzed by EM and AK. The original draft was written by AKA and AK and reviewed and edited by HK, LK, HMU, EM, LL, VL, PM, SK, PH, BC, and AJR. Statistical analysis and visualizations were done by VL and AKA, supported by LL. Project administration was done by LK and HK. Funding was provided by HK, LK, and HMU. All authors commented and accepted the final version of the manuscript.

We want to thank all the participating families and the FinnBrain staff and assisting personnel including Heidi Kunnasranta, Minna Lamppu, and Katri Kylä-Mattila. We acknowledge Juha Pursiheimo for the collaboration regarding the 16S rRNA gene sequencing and Sami Pietilä for raw sequencing data processing. All authors declare that they have no conflicts of interest.

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