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DOI
10.1016/j.clnu.2022.09.013

Publication date
2022

Document Version
Final published version

Published in
Clinical nutrition

Clinical Nutrition 41 (2022) 2517–2528

Contents lists available at ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Original article

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ARTICLE INFO

Article history:
Received 24 May 2022
Accepted 23 September 2022

Keywords:
Lactating women
Breastfeeding
Postpartum stress
Breast milk
Nutrition
Fatty acid

SUMMARY

Background & aims: Maternal stress in the postpartum period affects not only the mother, but also her newborn child who is at increased risk for a wide range of disorders later in life. The mechanisms underlying transmission of maternal stress to the child remain elusive. Human milk (HM) is a potential candidate and is an important source of fatty acid (FA), which are crucial for child (neuro)development. This study aims to investigate whether maternal psychological and biological stress influences HM FA composition over the first month postpartum.

Methods: The Amsterdam Mother's Milk study is a prospective cohort study. We included lactating women who delivered at term with a large range of stress levels: a high stress (HS) group, women whose child was hospitalized for a minimum of 2 days (n=23) and a control (CTL) group, women who gave birth to a healthy child (n=73). HM was collected three times a day at postpartum days 10, 17 and 24. Perceived psychological stress was measured using multiple validated questionnaires, while biological stress measures were based on cortisol in hair, saliva and HM. HM FAs were analyzed by gas-chromatography and compared between groups.

Results: Maternal perceived stress scores were significantly higher in the HS group ($p < 0.01$), whereas cortisol measurements did not differ between groups. The absolute concentrations of total FA in HM (p=0.023), including the total amount of poly unsaturated fatty acids (PUFAs) (p=0.022) and omega-6 PUFAs (p=0.018), were lower in the HS group compared to the CTL group. Relative values of FAs did not differ between groups.

Conclusion: Maternal stress in the first month postpartum was associated with overall lower levels of FA in HM. This possibly indicates a route of transmission of maternal stress signals to the infant. Future research should investigate if these stress-induced changes in HM FAs have consequences for child development.

1. Introduction

Maternal stress during the postpartum period is highly prevalent, takes many different forms, ranges in intensity and personal perception, and can be exacerbated in case of stressful events, such as for example hospitalization of the child, during the postnatal period [1,2]. Notably, maternal postpartum stress affects not only the mother, but also her newborn child. In fact, stress exposure during this early sensitive developmental period increases the infant's risk to develop a wide range of disorders, including metabolic and mental diseases [3,4]. Because stress exposure of a mother is often unavoidable, understanding the mechanisms underlying the transmission of maternal stress to her infant is key in order to find targets to prevent these long-lasting detrimental effects. Several mechanisms have been suggested, one of them being changes in human milk composition [5,6], with a key role for fatty acids (FA)

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https://doi.org/10.1016/j.clnu.2022.09.013

Abbreviations

ALA	α-Linoleic acid
ARA	arachidonic acid
AUC	Area under the curve
C19:0	Nonadecanoic acid
CTL group	Control group
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
EPDS	Edinburgh Postnatal Depression Scale
FA	Fatty acid
FFQ	Food Frequency Questionnaire
FID	Flame Ionization Detector
HM	Human milk
HS group	High stress group
IQR	Interquartile range
JTV	Youth Trauma Checklist
LA	Linoleic acid
LC PUFA	Long chain polyunsaturated fatty acid
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LSC-r	Life Stressor Checklist-revised
MUFA	monounsaturated fatty acid
N3 PUFA	omega-3 polyunsaturated fatty acid
N6 PUFA	omega-6 polyunsaturated fatty acid
P	postpartum day
PSS	Perceived Stress Scale
PUFA	polyunsaturated fatty acid
S1 and S2	saliva collection moment 1 and saliva collection moment 2
SD	Standard deviation
SFA	Saturated fatty acid
SLE+	Supported Liquid Extraction
STAI	State-Trait Anxiety Inventory
STAI-s	STAI-state
STAI-t	STAI-trait

[7]. When infants are exclusively breastfed, human milk (HM) is the sole source of early-life nutrition, and thus of FA [8–10]. There is evidence from human research that early-life FA intake is critical for infant development and health [11,12] and that suboptimal FA levels early in life are a risk factor for later-life disease vulnerability [13–17]. In addition, evidence from animal experimental studies suggests that early-life stress exposure alters plasma and brain PUFA status in rodents [7,18] and that improving FA composition in the early-life diet is able to protect against the early-life stress induced cognitive dysfunction [7].

Notably, a large number of factors influence the FA composition of HM, for example the maternal diet, BMI and genetic profile [19]. There is emerging evidence that maternal stress and stress related psychopathologies may also influence the FA composition of HM, although the evidence so far is not conclusive [20–26] and how maternal stress, specifically during the sensitive early postpartum period, affects the FA composition of HM has not yet been addressed. In fact, in the currently published studies, stress measurements were mostly performed during pregnancy and not at the time of milk sampling and mostly investigated the influence of maternal stress on HM FAs at later stages of lactation.

Therefore, we investigated the hypothesis that maternal stress during the early postpartum period affects the FA composition of HM by looking at associations between maternal psychological and biological stress measures and HM FA composition over the first month postpartum. A better understanding of stress induced changes in HM FA composition is important in order to be able to develop optimal nutritional advice/supplementation for mothers and children in stressful circumstances.

2. Subjects and Methods

2.1. Research design and study population

The Amsterdam Mother's Milk study is a prospective observational cohort study. Women were recruited during pregnancy or within the first ten days after giving birth. Recruitment took place in the neonatal and maternal wards, via social media and flyers at midwife practices. Mothers were eligible to participate when they met the following inclusion criteria; 18 years of age or older, and if they had the intention to breastfeed their infant for at least the first month after birth. Exclusion criteria were; maternal (gestational) diabetes mellitus, maternal use of psychopharmaceuticals or glucocorticoid medication, major congenital disease of the neonate or a life expectancy of the neonate of less than one month. To ensure the inclusion of a large enough range of stress levels among the included participants, two groups of women who delivered at term, were included; a high stress group (HS group) and a control group (CTL group). Women were included in the high stress group when they gave birth to an infant at term who was admitted to the hospital for a minimum of two days, where the hospitalization of the infant was considered the maternal stressor. Women who gave birth at term to a healthy infant were included in the CTL group. Women were screened and asked to participate in the study between November 2017 and December 2019. Written informed consent was obtained from all participants prior to participation. This study was approved by the Ethics Committee of the Amsterdam University Medical Centre, AMC on the 2nd of May 2017 (METC 2017 025, NL59994.018.16) and conducted in accordance with the Declaration of Helsinki.

2.2. Data collection and storage

2.2.1. Study timeline

Inclusion took place before day 10 after birth either in the hospital or at home. During this visit a strand of hair was cut for cortisol measurement and the participants completed a questionnaire about their general health, pregnancy and life-time stress experiences (Fig. 1, item 1 and 2). Thereafter, the study had three collection days: at postpartum (P) day 10, 17 and 24, women collected two saliva samples (morning cortisol) and three milk samples (FAs and cortisol). At the end of the study, participants filled out four questionnaires about their stress experience during the study period (Fig. 1, item 3–5) and a questionnaire to measure their food intake (Fig. 1, item 6). Details on sample collection, measurements and questionnaires are given below.

2.2.2. Hair sample collection and storage

At inclusion, a hair strand (approximately 100 hairs, 3 mm diameter) was cut as close as possible to the scalp from a posterior vertex position by the researcher. Hair was stored in the dark at room temperature until analysis. Information about hair characteristics was obtained via a short questionnaire to be able to correct for factors influencing hair cortisol and cortisone levels, including sun exposure, use of hair products or certain types of medication. Hair samples were used to assess cortisol and cortisone in hair as a

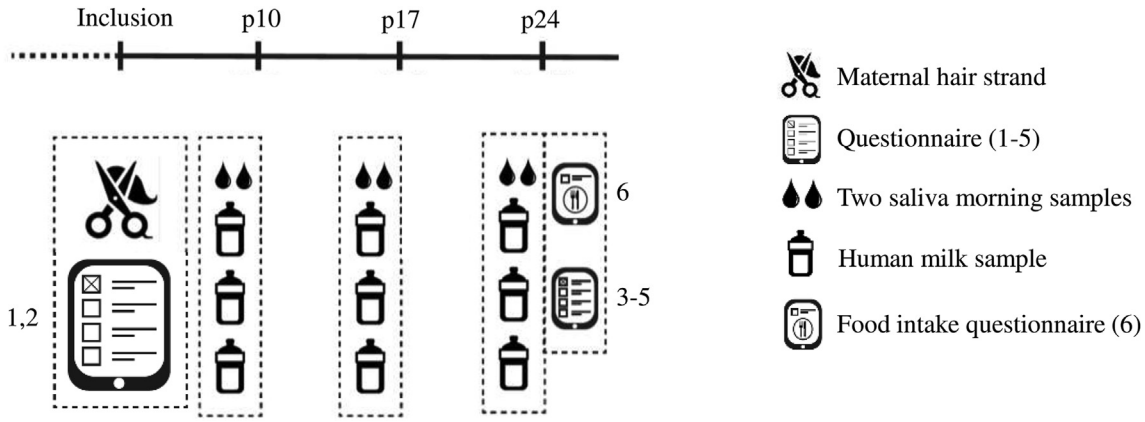


Fig. 1. Study design.
1=Life Stressor Checklist-revised.
2=Youth Trauma Checklist.
3=Perceived Stress Scale.
4=Edinburgh Postnatal Depression Scale.
5=State-Trait Anxiety Inventory.
6=Food Frequency Questionnaire.
Abbreviations: p=postpartum day.

baseline biological stress measurement reflecting levels during the last trimester of pregnancy.

2.2.3. Saliva sample collection and storage

To measure the cortisol awakening peak, saliva was collected two times in the morning at every collection day by chewing on a swab (Salivette, Sarstedt, Rommelsdorf, Germany) for 1 min. To have the highest chance of finding the cortisol morning peak level [27], two saliva samples were collected. The first (S1) was obtained within 0–10 min after awakening and the second sample (S2) was obtained 30–45 min after awakening. Participants were requested to write down at what time they woke up and the date and time of saliva collection on the tube and on a preprinted form. After collection, saliva samples were sent to the study site where they were centrifuged and stored at −20 °C until analysis.

2.2.4. Milk sample collection and storage

Participants collected three HM samples at every collection day (p10, p17 and p24), to measure concentrations of FAs and cortisol. HM cortisol is a non-invasive marker of expressed glucocorticoid levels, as it has been shown to correlate with plasma cortisol levels [28]. The HM collection was instructed to be one sample in the morning, one in the afternoon and one in the evening. This was done to be able to take into consideration the circadian rhythm of HM cortisol and to make sure that circadian variation in HM FAs was represented in the samples [29,30]. Because the fat content of HM may vary considerably during the same feeding [31], participants were requested to fully empty one breast before feeding their infant (so the sample would contain a mixture of foremilk and hindmilk), mix the milk and thereafter donate 5 ml of HM in a sterile propylene container (Sarstedt, Germany). Since no difference in HM FA composition was found between the left and right breast [32], participants were free to choose from which breast the milk was collected. Immediately after collection, participants were requested to write down the date and time of milk collection, the total amount of milk that was collected and the way they pumped the milk (i.e. manually or electric pump etc). Participants stored the milk samples in their freezer (approximately −20 °C) up until collection by the researcher. At the study site, HM samples were thawed once to prepare aliquots. Thereafter, HM samples were stored at −20 °C until analysis.

2.3. Questionnaires

The stress questionnaires used in this study are described below. For the analyses, per questionnaire the total scores and its ranges were used.

1. *Life Stressor Checklist-revised (LSC-r):* Participants filled out the Dutch version of the LSC-r questionnaire to report on their lifetime history of stress exposure. The checklist is a 26-item scale to identify the exposure to traumatic events or other stressful life events [33]. Each item questions whether a certain event happened in the participants' life [34].
2. *Youth Trauma Checklist (JTV):* The Dutch version of the Childhood Trauma Questionnaire- Short Form (25 items), is a self-report inventory that provides brief and relatively non-invasive retrospective assessment of child abuse and neglect [35]. The JTV discriminates five clinical domains of abuse/neglect (physical, sexual and emotional abuse, physical and emotional neglect). This questionnaire was used to provide a measure of the participants early-life stress exposure history.
3. *Perceived Stress Scale (PSS):* The Perceived Stress Scale is a validated 14-item questionnaire developed by Cohen et al. (1983,1988). The questionnaire aims to determine the degree to which certain situations are being experienced as stressful [36]. The questions examine to what extent participants rate their lives as being unpredictable, uncontrollable or overloaded [37]. Questions pertain to the past month, reflecting on perceived stress levels during the study period. Each question is scored on a 5-point Likert Scale.
4. *Edinburgh Postnatal Depression Scale (EPDS):* Participants filled out the Dutch version of the well-validated EPDS [38], a 10-item self-inventory to assess symptoms of depression and/or anxiety in women who recently delivered a child.
5. *State-Trait Anxiety Inventory (STAI):* The Dutch version of the State-Trait Anxiety Inventory (STAI) was filled out by the participants. The STAI is a well-established measure of trait and state anxiety. The first part of this inventory, the STAI-State (STAI-s) contains 20 items to assess personal state anxiety ("anxiety at this moment"), rated on a four-point intensity scale. The second part, the STAI-trait (STAI-t) contains

20 items and assesses personal state anxiety ("anxiety in general") rated on a four-point intensity scale [39].

6. *Food Frequency Questionnaire (FFQ):* This questionnaire was developed by the department of Human Nutrition of Wageningen University (the Netherlands) to assess the average intake (in grams per day) of nutrients in the past month. Using a web form, participants reported the intake of foods consumed during the previous month. This FFQ was specifically designed for the Dutch population. The FFQ is expected to include foods that cover the daily intake of each nutrient of food of interest for at least 90%.

2.4. Laboratory analysis

Hair cortisol/cortisone: The proximal 3-cm hair segment was used for analyses. Wash and steroid extraction procedures followed the protocol described in Stalder et al. [40] with some changes being made to allow analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The lower limits of quantification of this assay were below 0.1 pg/mg for cortisol and cortisone, and the inter- and intra-assay coefficients of variance were between 3.7% and 8.8%.

2.4.1. Saliva cortisol/cortisone

Cortisol and cortisone in saliva were measured using Supported Liquid Extraction (SLE+) followed by LC-MS/MS detection. Quantification was done using an isotope dilution, with a limit of quantification of 0.3 nmol/L. The mean intra-assay variation of cortisol was 6%, the mean intra-assay variation of cortisone was 7%.

2.4.2. HM cortisol/cortisone

For each milk sample, 0.5 ml of HM of each time point was used to determine the concentrations of cortisol and cortisone by Liquid extraction followed by SLE+ and LC-MS/MS detective as described in earlier research by Van der Voorn et al. [41]. Quantification was done using an isotope dilution.

2.4.3. HM fatty acids

For determination of fatty acids in HM samples, the three milk samples of one collection day were mixed to have a good representation of FA levels during the whole day. These mixed samples were analyzed using gas-chromatography. Forty different kinds of FAs were analyzed. During the procedure, a known amount of Nonadecanoic acid (C19:0) was added as an internal standard to a 20 μl milk sample. FAs were extracted according to Bligh and Dyer [42], adding dichloromethane, methanol and water. The dichloromethane layer was evaporated to dryness, and the extracted lipids were converted to FA methyl esters with methanol +2% sulphuric acid at 100 °C for 60 min. The FA methyl esters were extracted with 1 ml hexane. 1 μl of the hexane was injected into the gas-chromatograph. The FA methyl esters were separated on a CP-Sil 88 column and detected with a FID detector. FA methyl ester identification was based on retention time. The relative concentration was based on the peak area and the absolute concentration was calculated after normalization with the C19:0 peak.

2.5. Statistical analysis

Characteristics were described as frequencies, means with standard deviations (SD) or medians with interquartile ranges (IQR), depending on the variable/distribution. To test differences between study groups in maternal-infant characteristics and stress measurements, unpaired student T-tests, Chi-square tests, Mann–Whitney U tests or Linear Mixed Models were used as appropriate.

As cortisol levels vary over the course of a day [29], we calculated the HM cortisol area under the curve (AUC) to provide a more reflective value of HM cortisol throughout the day. To this purpose, HM cortisol values were standardized to 7:00 AM, 14:00 PM and 22:00 PM using the following formula: HM cortisol in mmol/L −/+ unstandardized regression coefficient of all HM cortisol values * (new (standardized)) time point − real time point). Subsequently, the HM cortisol AUC for each collection day was calculated from these three time points, as described by Pruessner et al. [43]. As cortisol levels in HM are the highest in the morning around 7:00 AM [29], the cortisol value at 7:00 AM was considered the HM cortisol peak. The highest cortisol value of the two morning saliva samples was considered the saliva cortisol morning peak. When time of S1 collection was >30 min after waking up, saliva values were excluded. Correlations between stress measurements were determined using Pearson or Spearman correlation, depending on the distribution.

Participants were excluded from the final analysis when they completed less than one full day of sample collection. FAs in HM were categorized in the most important FA groups and most important single FAs, namely: total FA, saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), omega-6 (n6) PUFA, omega-3 (n3) PUFA, linoleic acid (LA), arachidonic acid (ARA), α-linoleic acid (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). All outcome variables were checked for normal distribution. When variables were not normally distributed, a log transformation of the data was performed. To analyze the relation between stress and FAs in HM, the different HM FAs were compared between the HS and CTL group. As FA levels considerably differ between the different weeks and stages of lactation [44] and thereby possibly also factors influencing HM FAs, we stratified the analysis for lactation stage. The analyses were performed separately for transitional milk samples (p10) and all the mature milk samples (p17 and p24). For all analyses containing within-person repeated measures, a Linear Mixed Model was used. To test for differences in HM FAs between the HS and CTL group in transitional milk (p10), a linear regression model was used, controlling for factors differing between study groups. To test for differences in HM FAs between the HS and CTL group in mature milk (all samples from p17 and p24), linear mixed models were used to control for within-person repeated measures.

As participants were included into the HS group or the CTL group based on a predefined stressor, and it could be possible that mothers in the CTL group also experience high levels of stress, a post hoc exploratory analysis was conducted to investigate the relationship between the various perceived stress scores as continuous variables and cortisol values, and the FA composition of HM independently of the study group. For this post hoc exploratory analysis, Linear Mixed Models were used.

2.5.1. Confounding

In our primary outcome analysis, where we tested if HM FAs differ between the HS group and the CTL group, we corrected our analyses for all maternal and infant baseline factors that statistically differed between study groups. In addition, we tested if maternal dietary intake of specific FAs statistically differed between study groups and when this was the case, the comparison was corrected for the maternal intake of this specific FA.

In our post hoc exploratory analysis, where we tested if there was an association between maternal perceived stress scores or cortisol values and HM FAs, independent of study group, we corrected our analysis for factors that were expected to influence HM FA composition based on previous literature [19] including FA dietary intake, maternal education and maternal BMI. Due to our

relatively limited sample size we choose to correct in the statistical model for the three most relevant confounders within our study.

Due to the explorative nature of the study, the statistical analyses were not corrected for multiple testing. To reduce the number of statistical tests and the likelihood of a type 1 error, the separate FAs were categorized into groups and analyzed as such. Statistical analyses were tested two-sided. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 27. GraphPad Prism 9 for Windows was used to display results in graphs.

3. Results

3.1. Maternal characteristics and food intake

In total, 116 lactating women were included in the study, of whom 86 in the CTL group and 30 in the HS group. Twenty women dropped out during the study: 11 in the CTL group and 7 in the HS group, due to various reasons (Fig. 2). Maternal characteristics are shown in Table 1. Maternal baseline characteristics did not differ between study groups, with the exception of infant sex (mothers in the HS group gave birth to a male infant in 74% of the cases, whereas mothers in the CTL group gave birth to a male infant in 48% of the cases (p=0.029). Hence, all group comparisons were corrected for infant sex. Way of HM pumping (manually, electric) and storage time of the samples did not differ between study groups.

As shown in Table 2, there was no difference in absolute FA dietary intake between both study groups. However, the ratio between the intake of n6 and n3 PUFA was lower in the HS group (p=0.013). Hence, the comparison of the HM n6/n3 PUFA ratio between study groups, was corrected for maternal intake of these FAs. There was no difference in FA supplement intake between the CTL group (21%) and the HS group (15%).

3.2. Maternal stress measures

Table 3a shows the stress measurements that were collected during the study period for both study groups. The questionnaire based life-time stress exposure scores, collected at the start of the study, did not differ between study groups. This was also the case for hair cortisol levels which reflect cortisol during the last trimester of pregnancy. Perceived stress measured over the study period was higher in the HS group compared to the CTL group, indicated by higher scores on the PSS, EPDS, STAI-s and STAI-t. There were no differences in HM or saliva cortisol or cortisone levels between study groups. Cortisone levels strongly correlated with cortisol levels in all sample types (hair, saliva and HM, Pearson's $r > 0.7$ and $p < 0.0001$).

All questionnaire based stress scores correlated with each other (PSS, EPDS, STAI-s, STAI-t). No correlations were found between questionnaire based stress scores and maternal hair, saliva or milk cortisol levels (Table 3b).

3.3. FAs in HM differ between study groups

Table 4 shows the absolute concentrations of FAs per study group in transitional milk (p10) and mature milk (p17 + p24). In transitional milk, there were no differences in the levels of FAs between the study groups. However, the total amount of FAs in mature milk was lower in the HS group. This difference was also apparent when looking at the three main groups of FAs specifically: the levels of SFA, the levels of MUFA and the levels of (LC) PUFAs were all lower in the HS (Fig. 3). The difference in (LC) PUFA concentration between groups was due to lower (LC) n6 PUFA levels in mature milk as shown in Fig. 3b. Additionally, the concentrations of the two most abundant n6 PUFAs, ARA and LA, were lower in the HS group. The levels of (LC) n3

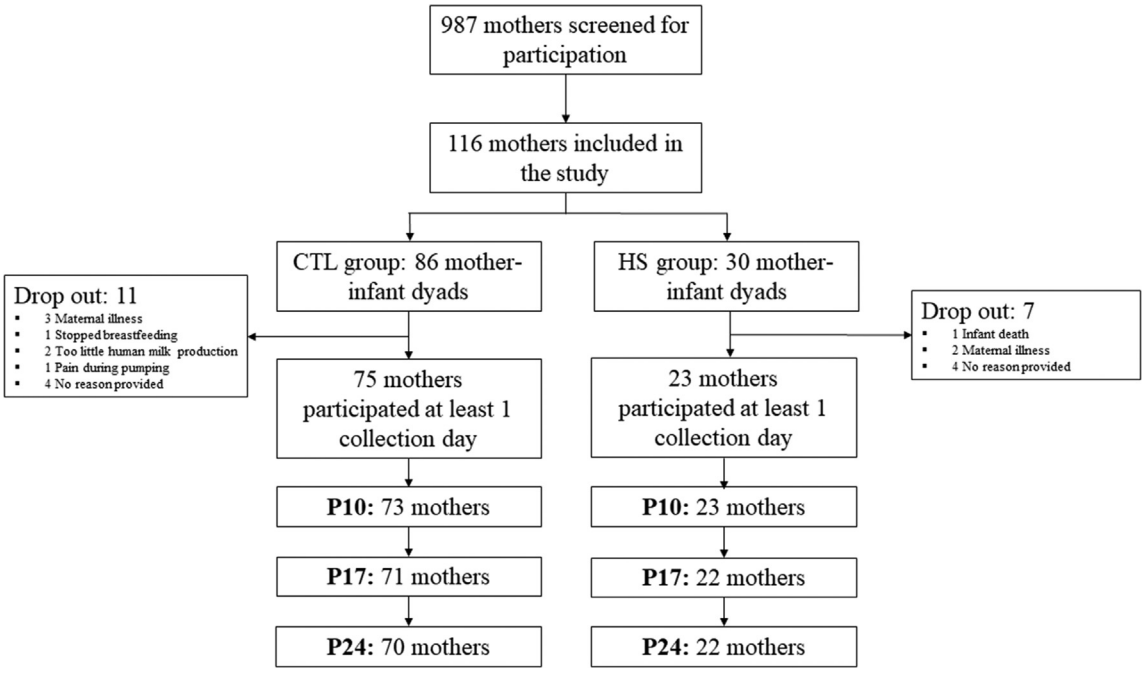


Fig. 2. Flowchart of study population.
Drop out indicates the mothers that did not complete human milk sample collection of one single study collection day.
Participants per collection day are not exactly the same mothers at each time point.
Abbreviations: CTL group=control group, HS group=High stress group, p=postpartum day.

Table 1
Maternal-infant characteristics.

Characteristics	Total *n.* Per characteristic	CTL group (n=75)	HS group (n=23)	p-value
Maternal age in years *mean (SD)*	96	32.4 (3.3)	32.3 (3.4)	0.681
Maternal ethnicity *%*	96			0.064
Dutch		80.8%	60.9%	
Turkey		2.7%	0%	
Morocco		1.3%	0%	
Surinam		1.3%	13.0%	
Netherlands Antilles		1.3%	4.3%	
Other Western[a]		9.6%	8.7%	
Other non-Western[b]		2.7%	13.0%	
Maternal education *%*	95			0.142
Lower educational level[c]		6.9%	26.1%	
Middle educational level[d]		2.8%	4.3%	
High educational level[e]		90.3%	70.1%	
Maternal BMI (kg/m^2) *median (IQR)*	96	22.2 (3.6)	22.3 (4.3)	0.764
Mode of delivery *% of caesarean*	96	27.4%	17.4%	0.423
Smoking *% of*	93			0.369
Non smoker		70.0%	56.5%	
Past smoker		28.6%	43.5%	
Current smoker		1.4%	0%	
Parity *% of primiparous*	96	60.3%	69.6%	0.422
Season of human milk collection *%*	98			0.132
Winter		26.0%	15.0%	
Spring		23.3%	10.0%	
Summer		17.8%	40.0%	
Fall		32.9%	35.0%	
Birth weight child (gr) *mean (SD)*	96	3564 (487)	3359 (562)	0.093
Infant sex *% of male*	96	47.9%	73.9%[f]	0.029[f]

Difference between groups was tested using an unpaired student T-test, Chi-square test or Mann–Whitney U test as appropriate.
Abbreviations: CTL group=control group, HS group=high stress group, IQR=interquartile range, SD=standard deviation.
[a] Includes: Europe, North America, Oceania, Japan, Indonesia.
[b] Includes: Latin-America, Africa, Asia, other.
[c] International Standard Classification of Education 2 or below.
[d] International Standard Classification of Education 3-4.
[e] International Standard Classification of Education 5 or higher.
[f] =significantly higher in the stressed group ($p < 0.05$).

PUFAs were the same in both study groups, as were the separate n3 PUFAs ALA, DHA and EPA. Relative values of fatty acids did not differ between study groups. All values were corrected for sex of the infant.

Table 2
Maternal fatty acid intake over the study period measured by Food Frequency Questionnaire.

	FA intake in grams per day		p-value
	CTL group n=71	HS group n=19	
Total fat *median (IQR)*	91.8 (30.2)	87.1 (33.2)	0.345
SFA *mean (SD)*	36.7 (14.9)	33.2 (16.6)	0.377
MUFA *mean (SD)*	33.9 (12.2)	33.0 (14.3)	0.783
PUFA *mean (SD)*	18.7 (7.5)	17.1 (7.5)	0.412
n6 PUFA *mean (SD)*	14.3 (5.9)	12.7 (5.8)	0.314
LA *mean (SD)*	15.1 (6.2)	13.8 (6.2)	0.414
n3 PUFA *mean (SD)*	2.23 (1.07)	2.30 (1.02)	0.804
ALA *mean (SD)*	1.76 (0.72)	1.67 (0.71)	0.650
EPA *median (IQR)*	0.10 (0.12)	0.16 (0.16)	0.124
DHA *median (IQR)*	0.15 (0.18)	0.26 (0.28)	0.067
n6/n3 PUFA ratio *mean (SD)*	6.71 (1.85)	5.49 (1.77)	0.012[a]
LA/ALA ratio *median (IQR)*	8.5 (1.79)	8.2 (1.58)	0.258

All values are in grams per day and include supplement intake.
Difference between groups was tested using an unpaired student T-test or Mann–Whitney U test as appropriate.
Abbreviations: CTL group=control group, HS group=high stress group, FA=fatty acids, SFA=saturated fatty acids, MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids, LC=long chain, n6 PUFA=omega-6 polyunsaturated fatty acids, n3 PUFA=omega-3 polyunsaturated fatty acids, LA=linoleic acid, ARA=arachidonic acid, ALA=alpha-linolenic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid.
[a] =significantly lower in the stressed group ($p < 0.05$).

3.4. Post hoc exploratory analyses: relationship between maternal cortisol levels and FAs in HM

A post hoc exploratory analysis was conducted to investigate the relationship between the various perceived stress scores and cortisol values, and the FA composition of HM independently of the study group. As the association between stress and FAs seemed to be only present in mature milk, post hoc exploratory analyses were only performed on mature milk values (p17 and p24). As shown in Table 5 and Fig. 4, the HM cortisol AUC was negatively associated with total FAs, MUFA (LC) PUFA (LC) n6 PUFA, n3 PUFA and the separate FAs LA, ARA, EPA and DHA. However, these associations disappeared when correcting for maternal FA intake, maternal education and maternal BMI (Table 5), where maternal FA intake seemed to influence the association the most.

3.5. Post-hoc exploratory analyses: association between life-time stress exposure scores and FAs in HM

PSS, EPDS or STAI scores were unrelated to FAs in HM. However, higher life-time stress exposure scores (LSC-r) were associated with lower n6 PUFAs, LA and a lower n6/n3 ratio in mature milk, even after correction for maternal FA intake, maternal education and maternal BMI (−61.7 [−123.8, 0.3]; p=0.05, −53.2 [−106.5, −0.01]; p=0.05 and −0.1 [−0.2, −0.05]; p=0.001 respectively). These changes were both seen in the absolute as well as the relative values. The complete results of this post hoc exploratory analysis are shown in Supplementary Table 1.

Table 3a
Maternal perceived stress scores and cortisol values.

	Total *n.* Per stress measure	Questionnaire based stress scores		p-value
		CTL group (n=75)	HS group (n=23)	
Life-time stress (test score)				
JTV *median (IQR)*	96	28 [10]	29 [10]	0.459
LSC-r *median (IQR)*	96	6.0 [7]	6.5 [8]	0.432
Perceived stress during study period (test score)				
PSS *mean (SD)*	96	16 [6]	21 [5]	0.001*
EPDS *median (IQR)*	96	4.0 [5]	7.5 [6]	0.004*
STAI-s *median (IQR)*	96	26 [11]	36 [16]	0.005*
STAI-t *median (IQR)*	96	29 [9]	37.5 [9]	0.004*
Biological measures of Stress				
Stressover the last 3 months of pregnancy				
Hair cortisol *median (IQR)*	94	6.42 (11.53)	7.07 (6.81)	0.280
Stress on collection days				
Saliva cortisol (morning peak) *median (IQR)*		5.30 (4.20)	5.80 (5.30)	0.490
p10	90	5.50 (4.30)	5.90 (7.10)	0.811
p17	93	4.90 (4.40)	6.60 (4.00)	0.260
p24	91	5.30 (3.40)	5.00 (4.30)	0.169
Human milk cortisol (morning peak) *median (IQR)*		6.60 (6.37)	9.58 (12.56)	0.329
p10	80	6.63 (5.86)	16.16 (12.47)	0.202
p17	83	5.41 (4.65)	12.91 (13.67)	0.345
p24	79	6.75 (8.11)	7.57 (8.67)	0.395
Human milk cortisol AUC *median (IQR)*		51.26 (35.00)	64.02 (55.31)	0.074
p10	85	59.88 (42.05)	69.28 (46.47)	0.545
p17	87	43.68 (34.96)	72.52 (57.87)	0.080
p24	84	50.09 (31.69)	63.94 (77.91)	0.264

*=significantly different between groups ($p < 0.05$).

4. Discussion

Maternal postpartum stress was associated with lower absolute concentrations of total FA, SFA, MUFA (LC) PUFAs and specifically of (LC) n6 PUFA in mature milk, while HM FA levels in transitional milk were unaffected. We did not detect a relationship between maternal stress and the relative values of FAs in HM.

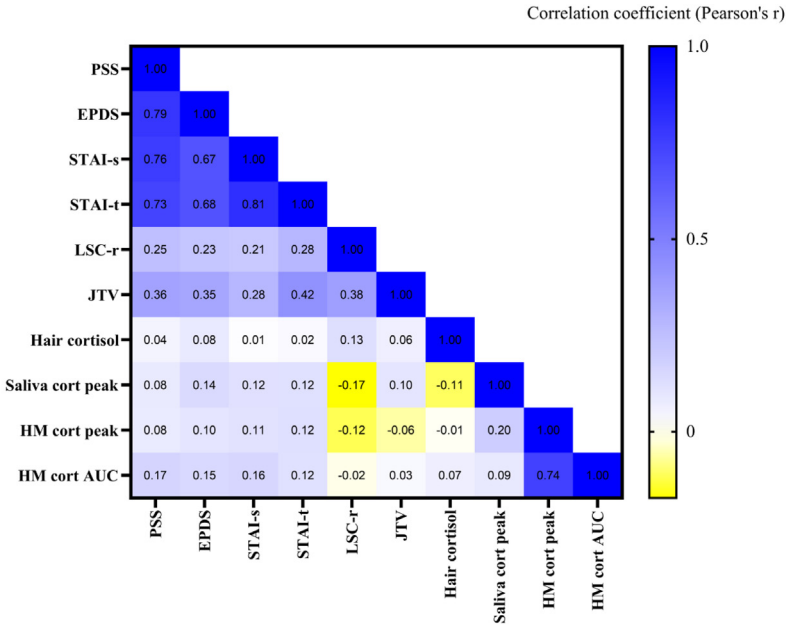
The HM FA values in our study were comparable to values described previously [19,45,46]. Associations between maternal perinatal stress and HM FA composition have been reported before, although the direction of association and the particular FAs affected differed between studies [21,23,25,26]. These differences may be due to heterogeneity in study designs, including type and timing of stress information, timing and method of HM collection, as well as assessment of relative *vs*. absolute FA values, hindering comparability. Nonetheless, taken the current evidence together we can conclude that maternal stress is associated with HM FA composition. While a positive association between maternal stress and total fat and SFAs in HM has been shown before [23,26], we here observed a negative association with the total amount of FAs, the SFAs, the MUFAs and the n6 (LC) PUFAs. While a positive association between maternal stress and HM FA could be due to an influence of stress on maternal eating habits and specifically higher maternal fat intake [47,48], in our cohort the stress exposure was not associated with maternal FA intake. The lack of such compensatory increase in FA intake, to meet the higher requirements associated with stress, could potentially explain the observed lower availability of substrates necessary for milk fat synthesis. We observed no differences between study groups in the HM n3 PUFAs. It can be hypothesized that due to the critical function of n3 PUFAs in infant growth and brain development [12,16,49,50], the levels of n3 PUFAs are more tightly regulated and maintained at a required level as much as possible [51,52].

There are several processes that might be influenced by stress, which may lead to the observed effects of maternal stress on HM FA composition. FAs in HM can originate directly from maternal FAs in the circulation, from release from adipose tissue, or from *de novo* lipogenesis and lipid metabolism in the liver and mammary gland [15,53,54]. Acute stress may influence these processes directly or may influence the transport of FAs into HM. In addition, stress may influence the type of FAs being utilized from maternal stores [55]. Which of these mechanisms might be at play for the effects observed in our study remains to be determined. When thinking of what could mediate the stress induced changes in HM, the stress hormone cortisol is a plausible candidate. Interestingly, while cortisol levels did not differ between our groups, we found a negative association between HM cortisol and HM FAs, which however disappeared after correction for maternal FA intake, BMI and education. Normally, in response to stress, cortisol is released in the maternal circulation, acting to mobilize maternal energy and fat resources and would thus be expected to increase FAs in the maternal circulation and in HM [56,57]. However, during breastfeeding, oxytocin is released, counteracting the release of cortisol [58,59], which could explain the lack of significantly elevated cortisol levels in our HS group. Another possible explanation for the fact that cortisol values were not elevated in the HS group but were negatively associated with HM FAs, might be that the sample size of the HS group was not sufficient to detect a statistically significant difference. In fact the median cortisol values (of both saliva and HM) are higher (although not significant) in the HS group compared to the CTL group, and there is a 'trend' towards a higher HM cortisol AUC in the HS group (p=0.074).

In post-hoc analyses, we observed that lifetime stress exposure was negatively related to n6 PUFAs, LA and n6/n3 PUFA ratio, while recent perceived stress, depression and anxiety scores were unrelated to HM FAs. Chronic high stress levels may have more pronounced consequences due to an overall effect on maternal physiology and metabolism. Indirect pathways may include long-term effects on dietary intake or other life style factors [47,48]. Other, more direct pathways may include the gradual depletion of substrates for HM FA synthesis under the influence of chronic stress [60,61].

Strengths of this study are its longitudinal design, and the timing and frequency of HM sample collection. Moreover, the first

Table 3b
Correlations between the different maternal stress measurements.



* = significantly different between groups ($p<0.05$)
Differences between groups in figure 3a were tested using an unpaired student T-test, Mann-Whitney U test or Linear Mixed Models as appropriate. Correlations between stress measures in figure 3b were tested using Pearson or Spearman correlations as appropriate.

Abbreviations: CTL group = control group, HS group = high stress group, JTV = Dutch version of the Youth Trauma Questionnaire, LSC-r = Life stressor checklist revised, PSS = perceived stress scale, EPDS = Edinburgh Postnatal Depression Scale, STAI-s = State and Trait Anxiety Inventory (state), STAI-t = State and Trait Anxiety Inventory (trait), IQR = interquartile range, SD = standard deviation, HM = human milk, AUC = Area under the curve

Table 4
Absolute concentrations of fatty acids in human milk between study groups.

	Concentrations in transitional milk in mg/L *Mean (SD)*			Concentrations in mature milk in mg/L *Mean (SD)*		
	CTL group	HS group	Estimate (95% CI)	CTL group	HS group	Estimate (95% CI)
Total FA	34 960 (11 749)	32 882 (9570)	−2445 (−8029, 3139)	38 405 (12 599)	32 988 (9450)	−6124 (−10927, −1321)[a]
SFA	14 788 (5350)	13 457 (4822)	−1500 (−4091, 1091)	15 603 (5062)	13 675 (4529)	−2141 (−4083, −199)[a]
MUFA	14 490 (5278)	13 976 (3889)	−729 (−3188, 1730)	16 399 (5803)	14 115 (4030)	−2661 (−4861, −461)[a]
PUFA	5230 (1703)	5040 (1459)	−155 (−967, 658)	5717 (2034)	4811 (1490)	−881 (−1681, −80)[a]
LC PUFA	757 (244)	752 (284)	−18 (−143, 107)	738 (240)	629 (199)	−125 (−219, −31)[a]
n6 PUFA	4655 (1543)	4456 (1294)	−163 (−894, 569)	5114 (1849)	4263 (1333)	−818 (−1546, −90)[a]
n3 PUFA	576 (215)	584 (268)	7.1 (−105, 119)	595 (227)	548 (218)	−62 (−161, 37)
LC n6 PUFA	539 (179)	516 (190)	−31 (−121, 59)	534 (182)	443 (141)	−101 (−173, −30)**
LC n3 PUFA	219 (91.3)	237 (125)	13 (−37, 62)	206 (97.0)	186 (84.9)	−29 (−68, 11)
LA	4116 (1394)	3940 (1166)	−131 (−791, 528)	4534 (1627)	3819 (1212)	−717 (−1348, −86)[a]
ARA	153 (54.5)	152 (62.4)	−6.7 (−34, 21)	149 (56.1)	125 (43.2)	−27.1 (−50.1, −4.08)[a]
ALA	347 (134)	347 (168)	0.2 (−70, 70)	391 (175)	362 (154)	−32 (−104, 41)
EPA	23.3 (14.1)	25.5 (15.4)	4.6 (−24, 33)	26.7 (16.2)	24.6 (15.0)	−15 (−38, 8.9)
DHA	124 (55.0)	131 (69.0)	1.2 (−6.0, 8.4)	110 (57.5)	100 (49.3)	−3.8 (−9.8, 2.3)
n6/n3 PUFA ratio[b]	8.3 (2.0)	8.6 (3.2)	0.6 (−0.6, 1.8)	8.8 (2.4)	8.2 (2.4)	−0.3 (−1.5, 8.6)
LA/ALA ratio	12.5 (4.1)	13.1 (5.4)	0.9 (−1.1, 2.9)	12.8 (5.1)	11.3 (3.9)	−1.2 (−2.9, 0.5)

Transitional milk includes the milk samples collected at postpartum day 10. Mature milk includes all the samples collected at postpartum day 17 and 24.
Total *n* per time point: p10=96, p17=93, p24=92.
Abbreviations: CTL group=control group, HS group=high stress group, CI=confidence interval, SD=standard deviation, FA=fatty acids, SFA=saturated fatty acids, MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids, LC=long chain, n6 PUFA=omega-6 polyunsaturated fatty acids, n3 PUFA=omega-3 polyunsaturated fatty acids, LA=linoleic acid, ARA=arachidonic acid, ALA=alpha-linolenic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid.
[a] =significantly lower in the stressed group ($p < 0.05$). **=significantly lower in the stressed group ($p < 0.01$). Group comparisons are corrected for infant sex.
[b] comparison both corrected for infant sex and maternal fatty acid intake.

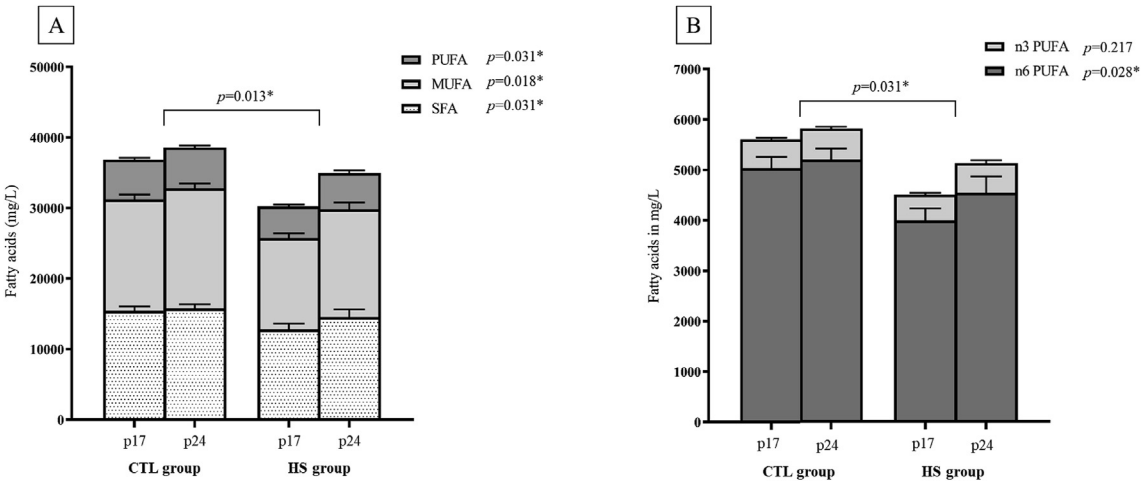


Fig. 3. a) absolute concentrations of total FAs in mature milk divided in PUFA, MUFA and SFA. **b)** absolute concentrations of total PUFA, divided in n3 and n6 PUFA. Mature milk includes all the samples collected at postpartum day 17 and 24.
*=significantly lower in the HS group ($p < 0.05$). Group comparisons are corrected for infant sex. Error bars indicate the standard deviation.
Abbreviations: CTL group=control group, FA=fatty acids, HS group=high stress group, LC=long chain, MUFA=monounsaturated fatty acids, n3=omega-3, n6=omega-6, PUFA=polyunsaturated fatty acids, SFA=saturated fatty acids.

month postpartum is a sensitive time window, frequently missed in earlier HM research, in which breastfed infants are dependent on HM as their only source for nutrients. Therefore, knowledge of the factors influencing HM during these first weeks after birth is extremely important. The fact that mothers were exposed to a stressor (infant hospitalization) ensured that the study population contained participants with high levels of stress. Extensive information about maternal factors and stress was collected, which allowed detailed investigation of effects of psychological and biological stress on HM FAs. Limitations are the relatively small sample size, especially in the HS group. Due to this relatively small sample size, when comparing both study groups, we only corrected our analysis for baseline factors that statistically differed between study groups. However, it is important to realize that some other baseline factors, although not statistically different between groups, could have influenced our results, for example maternal ethnicity, maternal education or season of milk collection [19]. In addition, the influence of unmeasured confounders, for example maternal genetic profile, cannot be ruled out. Another potentially stress inducing maternal factor is a caesarean section as mode of delivery. A caesarean section can be a physical as well as a psychological stressor and has been linked to breastfeeding rates, initiation and duration [62]. Also, it has been linked to an altered HM microbiome [63] and some first results suggest an association with altered human milk nutrients, including FAs [64]. We collected information on caesarian section, but as the caesarean section rate did not differ between our study groups, we do not expect it to have affected differences in HM FAs between groups in our study. Because of the exploratory nature of this study we decided not to correct for multiple testing, even though we did perform a

Table 5
Correlation between human milk fatty acids and human milk cortisol AUC in mature milk.

	Concentration of human milk FAs in total study population in mg/L	Correlation with human milk cortisol AUC *Unadjusted model*	Correlation with human milk cortisol AUC *Adjusted model*
	Mean (SD)	**Estimate** (95% CI)	**Estimate** (95% CI)
Total FA	37 118 (12 124)	−75.3 (−140.0, −10.6)[a]	−44.9 (−119.3, 29.5)
SFA	15 142 (4996)	−25.1 (−51.8, 1.5)	−18.7, −49.8, 12.3)
MUFA	15 856 (5511)	−33.1 (−62.3, −3.9)[a]	−17.8 (−51.1, 15.4)
PUFA	5502 (1953)	−13.6 (−24.0, −3.2)[a]	−9.80 (−21.5, 1.90)
LC PUFA	712 (235)	−1.53 (−2.72, −0.33)[a]	−0.89 (−2.23, 0.45)
n6 PUFA	4912 (1775)	−12.3 (−21.7, −2.92)[a]	−9.10 (−19.7, 1.47)
N3 PUFA	584 (225)	−1.30 (−2.59, −0.01)[a]	−0.85 (−2.30, 0.60)
LC n6 PUFA	513 (177)	−1.09 (−2.00, −0.17)[a]	−0.64 (−1.67, 0.40)
LC n3 PUFA	202 (94.4)	−0.43 (−0.88, 0.01)	−0.24 (−0.75, 0.27)
LA	4363 (1565)	−9.74 (−17.9, −1.53)[a]	−6.85 (−16.2, 2.45)
ARA	143 (54.2)	−0.28 (−0.55, −0.01)[a]	−0.26 (−0.54, 0.02)
ALA	385 (170)	−0.79 (−1.69, 0.12)	−0.58 (−1.58, 0.42)
EPA	26.2 (15.9)	−0.08 (−0.15, −0.01)[a]	−0.49 (−0.13, 0.03)
DHA	108 (55.7)	−0.27 (−0.53, −0.00)[a]	−0.16 (−0.45, 0.13)
n6/n3 ratio	8.7 (2.4)	−0.003 (−0.014, 0.008)	−0.002 (−0.014, 0.09)
LA/ALA ratio	12.4 (4.9)	−0.01 (−0.03, 0.01)	−0.01 (−0.03, 0.01)

Transitional milk includes the milk samples collected at postpartum day 10. Mature milk includes all the samples collected at postpartum day 17 and 24.
Total *n* per time point: p17=93, p24=92.
Analysis is adjusted for maternal fatty acid intake, maternal BMI and maternal education.Abbreviations: ALA=alpha-linoleic acid, ARA=arachidonic acid, CI=confidence interval, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, FA=fatty acids, LA=linoleic acid, LC=long chain, MUFA=monounsaturated fatty acids, n3=omega-3, n6=omega-6, PUFA=polyunsaturated fatty acids, SD=standard deviation, SFA=saturated fatty acids.
[a] =significant association ($p < 0.05$).

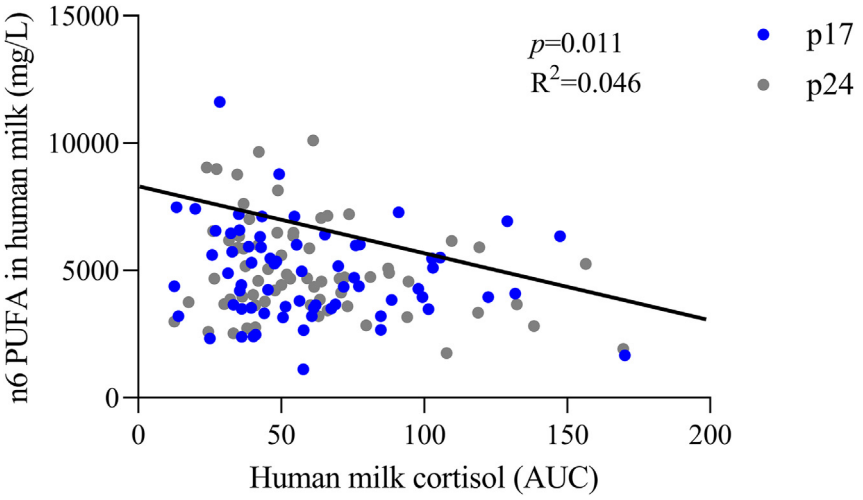


Fig. 4. Relationship between n6 PUFA and cortisol AUC in mature milk.
Mature milk includes all the samples collected at postpartum day 17 and 24.
The depicted association is uncorrected for confounding.
Abbreviations: AUC=area under the curve, n6 PUFA=omega-6 poly unsaturated fatty acids.

relatively large number of statistical tests, which increases the likelihood of a type 1 error. Therefore, our results should be interpreted with caution and future studies should demonstrate whether these findings can be replicated. Also, current perceived stress scores were only measured once at the end of the study. We were therefore not able to investigate whether the different effects of maternal stress on FAs in transitional and mature milk, were related to changes in the amount of stress experienced at that exact moment. Lastly, selection bias might have contributed to the fact that our cohort mostly consisted of healthy and highly educated women. Also, the majority of our participants were of a Western ethnic background. This limits generalizability of the results, as it is known that stress perception and responses can differ between people from different ethnic backgrounds and with different socioeconomic statuses [65–68].

The absolute differences in HM FAs of mothers in the HS group compared to the mothers in the CTL group may seem relatively small. However, as infants drink this milk multiple times a day, for a period of at least two weeks, this may result in a cumulative effect. As we do not know the HM volume consumed by the infant, we are unable to determine the exact transfer of FAs to the infant, but considering the fact that mothers who experience high levels of stress produce less milk [69–71], this poses infants of mothers with high stress at an even higher risk of receiving less FAs. At this point we can only speculate on how these stress related changes might influence infant development. Previous research showed that suboptimal nutritional FA status during early-life is associated with adverse developmental outcomes [13–16]. Moreover, there is evidence that adverse developmental outcomes were associated with lower intake of n6 PUFA levels, specifically of ARA, which was also decreased in the HS group in our cohort [12,72–74]. In contrast, a lower intake of n6 PUFAs has been described to be beneficial [17,75,76].

Our findings emphasize the importance of the maternal psychological state in the postpartum period and the need to identify mothers at risk for high stress levels. Over the last years, there has been increasing attention for the prevention of the detrimental consequences of stressful experiences in early-life. Stress reduction programs for both parents and children have been developed [77,78] and advantages of family integrated care are being increasingly acknowledged [79–81]. Mothers who listened to relaxing music were found to produce milk with a higher fat content [82]. As HM FA status is association with the maternal diet, nutritional advice/supplementation may be considered to support lactating women with high stress levels, as nutritional interventions are relatively safe, cheap and easy to implement. In animal studies, nutritional supplementations under stressful conditions have shown promising results and seem to be able to modulate the lasting adverse consequences of early-life stress [7,83].

In conclusion, findings from this prospective cohort study suggest that there is a relationship between maternal stress in the postpartum period and the FA profile of mature milk. To what extent these differences in FA concentrations are of physiological importance for infant development remains to be examined in future research.

Funding statement

This research was funded by the NWO Food and Cognition and Behavior program (057-14-003) and by Amsterdam Brain & Cognition.

Author contributions

EFGN, PJL, JBvG, SRdR and AK designed research. HGJ and EFGN conducted research. LS, JBvG, PJL and AK provided essential materials. HGJ and SRdR analyzed data or performed statistical analysis. HGJ, EFGN, SRdR and AK wrote paper. AK had primary responsibility for final content.

Conflict of interest

JBvG is the founder and director of the Dutch National Human Milk Bank and a member of the National Health Council. JBvG has been a member of the National Breastfeeding Council from March 2010 to March 2020. L.S. is employed by Danone Nutricia Research. The other authors have no relevant interests to disclose.

Acknowledgements

Authors gratefully acknowledge the skillful assistance of Martin Balvers and Gerrit Witte for the chemical analyses performed on

the samples at Danone Nutricia Research Utrecht and Bernd Stahl for his help in the discussions around the study design and the interpretation of the HM FA data. Lastly we want to thank the Dutch Human Milk Bank for helping with the transport of study samples and all of the parents who participated in the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2022.09.013.

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