A human milk perspective on the transmission of maternal factors to her child

Focus on stress, nutrition and immunity

Juncker, H.G.

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CHAPTER

3

Maternal stress is associated with higher protein bound amino acid concentrations in human milk

Hannah G. Juncker[1,2,6], Eva F.G. Naninck[1,2], Britt J. van Keulen[2,6], Jolinda E. Harinck[2], Lidewij Schipper[3], Paul J. Lucassen[1], Johannes B. van Goudoever[2,6], Susanne R. de Rooij[4,5,6], Aniko Korosi[1]

1 Brain Plasticity group, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands
2 Amsterdam UMC, University of Amsterdam, Vrije Universiteit, Emma Children's Hospital, Amsterdam, The Netherlands
3 Danone Nutricia Research, Utrecht, The Netherlands
4 Amsterdam UMC location University of Amsterdam, Department of Epidemiology and Data Science, Meibergdreef 9 Amsterdam, The Netherlands
5 Amsterdam Public Health research institute, Aging and Later Life, Health Behaviors and Chronic Diseases, Amsterdam, The Netherlands
6 Amsterdam Reproduction and Development, Amsterdam, The Netherlands

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Abstract

Background

Maternal stress in the postpartum period affects not only the mother, but also her newborn child, who is at increased risk of developing metabolic and mental disorders later in life. The mechanisms of how stress is transmitted to the infant are not yet fully understood. Human milk (HM) is a potential candidate as maternal stress affects various components of HM, e.g. fat and immunoglobulin concentrations. So far, it is unknown whether maternal stress also affects the amino acids (AAs) in HM, even though this nutrient is of extreme importance to child health and development. The aim of this study was to investigated if and how maternal stress is associated with the AA composition of HM.

Methods

In this observational cohort study (Amsterdam, The Netherlands), lactating women were recruited in two study groups: a high stress (HS) group; women whose child was hospitalized (n=24), 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 validated questionnaires, while biological stress measures were based on hair, saliva and HM cortisol concentrations. HM protein-bound and free AAs were analyzed by liquid chromatography and compared between groups.

Results

Maternal perceived stress scores were higher in the HS group ($p<0.01$). The concentrations of protein-bound AAs in HM were higher in the HS group compared to the CTL group ($p=0.028$) and were positively associated with HM cortisol concentrations ($p=0.024$). The concentrations of free AAs did not differ between study groups and were unrelated to cortisol concentrations.

Conclusion

Findings from this prospective cohort study suggest that maternal stress in the postpartum period is associated with an altered human milk amino acid composition which could play a role in the transmission of maternal stress effects to her child. The physiological implications of these stress-induced changes for infant development await future research.

Introduction

Maternal stress in the postpartum period not only affects the mother, but may also have consequences for her newborn child. It has been shown that maternal stress occurring during this sensitive developmental period is associated with the infant's risk of developing a wide range of disorders, including metabolic and mental health disorder (1-3). Because prevention of stressful maternal circumstances in the early postnatal period is generally difficult, a better understanding of the processes underlying these detrimental consequences for the infant is needed. Several mechanisms by which transmission of maternal stress to her infant occurs have been suggested, one of them being stress-induced changes in human milk (HM) composition (4, 5).

HM is the optimal source of nutrition for new-born infants (6-8). It is a highly complex fluid, consisting of over a hundred different components that are influenced by many different factors (9). It has been demonstrated previously that maternal psychopathology, and maternal stress, in the postpartum period is associated with an altered composition of fatty acids in HM (10-16). However, whether maternal stress is also associated with changes in other HM nutrients, for example the amino acids (AA), is so far unknown. As AAs in HM are critical for infant growth and development and are necessary for almost all infant body processes (17), it is important to understand how they are affected by maternal stress. In addition, previous human studies point towards an effect of stress on AAs in plasma and previous animal experimental studies even suggest a change in milk composition under the influence of stress.

In both human and animal plasma, AAs are decreased as a result of stress (18-20). As maternal plasma is the source from where AAs are transported into milk, lower maternal plasma AA levels, e.g. due to stress, may likely result in lower AA levels in milk (21). Whereas human studies addressing this aspect are lacking, two studies in mice have shown that while maternal stress resulted in lower concentrations of AA in maternal plasma and reduced growth of the offspring, the concentrations of the AAs asparagine and alanine were increased in milk (22, 23). In another animal study, maternal stress during lactation lowered methionine levels in offspring brain and plasma and induced cognitive deficits later in life, which, notably, could be partly counteracted by methionine supplementation of the diet of the dam (24). While this suggests that maternal stress lowers methionine levels in milk, which could have important programming effects for brain health, the AA concentrations in milk were not determined in this study (24).

The aim of this study was to investigate whether maternal stress in the first month postpartum is associated with the fraction of protein-bound AA (BAA), making up for 90-95% of the AAs, and the free AA (FAA), comprising a relatively small fraction of the

AA in HM (17). We further focused on methionine as a key AA that may play a role in long-term consequences of early life stress and thereto also investigated the associations between maternal stress and this specific AA. A better understanding of potential stress-induced changes in HM AA composition will contribute to our knowledge on how maternal stress can be transferred to the infant.

Material and Methods

1 Research design and study population

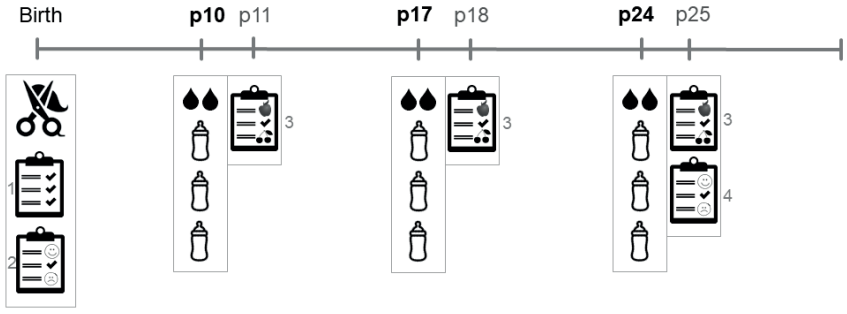
We studied a prospective observational cohort of lactating women that were followed over their first month postpartum, who experienced various amounts of stress. Participants were recruited during pregnancy or within the first ten days after giving birth, via social media, flyers at midwife practices or at the maternal or neonatal ward of the Amsterdam University Medical Center (Amsterdam, The Netherlands). Mothers were eligible to participate when they were 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; 1) maternal (gestational) diabetes mellitus, as the glucocorticoid system could be regulated differently (25, 26), 2) maternal use of psychopharmaceuticals or glucocorticoid medication, as this might interfere with scoring of the questionnaires, the regulation of the glucocorticoid system and maternal cortisol concentrations (27), 3) major congenital disease of the neonate and/or a life expectancy of the neonate of less than one month (duration of the study).

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 CTL group when they gave birth to a healthy infant at term. Women were included in the HS group when they gave birth to an infant at term who was admitted to the hospital for a minimum of two days. Hospitalization of the infant was considered the maternal stressor.

Recruitment took place in The Netherlands 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 Data collection and storage

Study timeline: Figure 1 shows the study time-line. Recruitment took place within the first ten days postpartum. After recruitment of the participant, a strand of hair was collected for glucocorticoid measurements and the participants completed a questionnaire about their general health, their pregnancy and about life-time stress experiences. The study had three collection days; at postpartum (P) day 10, 17 and 24. On these collection days, women collected two saliva samples and three HM samples. After each collection day, the participants filled out a 24-hour food recall questionnaire. At the end of the study, participants filled out three questionnaires about their stress experience during the study period. See for further details on sample collection, measurements and questionnaires, the items below.



= Maternal hair strand. Collected to measure baseline (cumulative) cortisol concentrations.

= Two saliva samples. Collected in the morning for measurement of cortisol awakening response.

= Human milk sample. Collected in at three times during the day for measurement of amino acids and cortisol.

= Questionnaires.

- 1: General questionnaire about mother-infant characteristics.
- 2: Two questionnaires to measure maternal life time stressors as a baseline of maternal stress: the Life Stressor Checklist- revised and the Youth Trauma Checklist
- 3: 24-hour food intake questionnaire to assess maternal food intake.
- 4: Three questionnaires to measure maternal stress over the study period: the perceived stress scale, the Edinburgh Postnatal Depression Scale and the State-Trait Anxiety Inventory

Figure 1: Study time-line
Time-line of study procedures. Abbreviations: p = postpartum day

Hair sample collection and storage: A strand of hair (approximately 100 hairs, 3mm diameter) was cut by the researcher as close to the scalp as possible at the posterior vertex position. Hair was stored in the dark at room temperature until analysis. A short questionnaire was filled out by the participants in order to correct for factors influencing hair glucocorticoid concentrations. Hair samples were analyzed for cortisol and cortisone as a baseline biological stress measurement reflecting the last trimester of pregnancy (28, 29).

Saliva sample collection and storage: Saliva was collected two times in the morning at every collection day to measure the cortisol awakening response. Participants were instructed to chew on a swab (Salivette, Sarstedt, Rommelsdorf, Germany) for one minute. The first sample (S1) was obtained within 0-10 minutes after waking and the second sample (S2) was obtained 30-45 minutes after waking (30). Participants were requested to write down their wake-up time and the time of saliva collection. After collection, saliva samples were sent to the study site where they were centrifuged, aliquotted and stored at -20°C until analysis.

Milk sample collection and storage: At every collection day (P10, P17 and P24), participants collected three HM samples in which concentrations of AAs and cortisol/cortisone were measured. HM cortisol/cortisone was measured to be able to directly correlate this with the HM AAs . To take into consideration the circadian rhythm of HM cortisol and to make sure that circadian variation in HM AAs was represented in the samples (32, 33), participants were instructed to collect one HM sample in the morning, one in the afternoon and one in the evening on each day that they collected milk. To make sure the HM sample would contain a mixture of both foremilk and hindmilk, participants were requested to fully empty one breast before feeding their infant, mix the milk and thereafter put 5 ml of HM in a sterile polypropylene container (Sarstedt, Germany) for analysis. Participants were free to choose from which breast the milk was collected. Participants were requested to write down the date and time of milk collection, the pumping method used (i.e. manually or electric pump etc) and the total amount of milk that was collected. Participants stored the milk samples in their freezer (-20°C) up until collection by the researcher. At the study site, HM samples were stored at -20°C until analysis.

3 Questionnaires

The questionnaires used in the study are described below. For the analyses, the total questionnaire scores and its ranges were used. To establish the participant's life-time stress exposure, the participants filled out two questionnaires, at the start of the study:

The Life Stressor Checklist- revised (LSC-r): i.e., the Dutch version of the LSC-r questionnaire. This checklist is a 26-item scale to identify exposure to traumatic events or other stressful life events (34). Each item questions whether a certain event happened in the participant's life (35).

The Youth Trauma Checklist (JTV): The Dutch version of the JTV questionnaire (25 items) is a self-report inventory that provides brief and relatively non-invasive retrospective assessment of early-life traumatic experiences (36). The JTV discriminates five domains of abuse/neglect (physical, sexual and emotional abuse, physical and emotional neglect).

24-hour food recall questionnaire (24h-recall): After each collection day, participants received a 24h-recall using the digital program Compl-eat™ developed by the department of human nutrition of Wageningen University (the Netherlands) (37). This questionnaire assesses the exact food intake (in grams per day) on the collection day. The Compl-eat™ web-based module was specifically designed for the Dutch population and guides participants to accurately report all foods and drinks consumed during the previous 24 hours. Information on AA supplement intake was taken into account in the 24h-recall. The mean intake for protein and all AAs of the three collection days was calculated and used as a measure of maternal intake over the study period.

A measure of psychological stress levels *during* the study period, was obtained by three questionnaires that the participants filled out at the end of the study, concerning levels of stress they experienced during the past month:

Perceived Stress Scale (PSS): The Perceived Stress Scale is a validated 14-item questionnaire. The questionnaire determines the degree to which certain situations are experienced as stressful (38, 39). Each question is scored on a 5-point Likert Scale.

Edinburgh Postnatal Depression Scale (EPDS): The Dutch version of the well-validated EPDS is a 10-item self-inventory to assess symptoms of depression and/or anxiety in women who recently gave birth (40).

State-Trait Anxiety Inventory (STAI): The Dutch version of the STAI is a well-established measure of trait and state anxiety and consists of two parts. The first part of this inventory, the STAI-State (STAI-s), contains 20 items to assess anxiety at this moment, rated on a four-point intensity scale. The second part, the STAI-trait (STAI-t), contains 20 items and assesses anxiety in general rated on a four-point intensity scale (41).

4 Laboratory analysis

Hair cortisol/cortisone: For analyses, the proximal 3-cm hair segment was used. Wash and steroid extraction procedures were performed as described by Stalder et al, with some changes being made to allow analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (42). The lower limits of quantification were below 0.1 pg/mg for cortisol and cortisone. The inter- and intra-assay coefficients of variance were between 3.7% and 8.8%.

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

HM cortisol/cortisone: For each HM sample, cortisol and cortisone were determined using Liquid Liquid extraction followed by SLE+ and LC-MS/MS detective as described earlier by Van der Voorn et al (43). Quantification was done using an isotope dilution.

Protein bound AA in HM: For determination of BAA in HM samples, the three milk samples of one collection day were mixed to have a good representation of BAA concentrations during the whole day. Thereafter, 0.20ml of diluted hydrochloric acid containing 0.5% 2-mercaptoethenol was added to the HM sample and mixed. The present oxygen was removed by flushing the headspace of the tube with nitrogen for 60 seconds. The protein was hydrolyzed by heating the mixture during 20-22 hours. When the mixture was cooled down, 0.20 ml of sodium hydroxide solution, 2.0ml demi water, and 0.2ml internal standard (Norvaline 20ug) were added and mixed. The mixture was centrifuged and a small part of the liquid was filtered over a 0.45 mm Polyvinylidene difluoride - filter. The peak area of each AA was calculated using the Labsolutions software of Shimadzu and compared to the peak area of the internal standard (Sigma).

Free AA in HM: The mixed HM samples were also used to determine the free AA in HM. An Ultra Fast Liquid Chromatography (UFLC) based protocol was used. Each 50ul milk sample was mixed with 1.0ml internal standard solution (2.5mg/ml L-norvaline). This mixture was centrifuged and 25ul of the supernatant was transferred into a sample vial. A pre-column derivatization process was carried out by adding 30ul of o-phthalaldehyde (OPA)-reagent to the vial and mixing 3 times with a mixing volume of 45ul. One ul of this OPA-derivatized sample was injected and analyzed in a UFLC system with fluorimetry to detect the signal.

Standard AA solution Sigma AA-S-18 was used for calibration. To prepare the calibration AA solution, asparagine and tryptophan were added into Sigma AA-S-18 stock solution to reach a concentration of 2.5uM/ml of each AA. Next, 0.50ml, 1.0ml, 2.0ml, and 5.0ml of this solution was mixed with 1.6ml of perchloric acid and further diluted to 50ml. The calibration AA solution was prepared to OPA-derivate as described previously and measured in an UFLC system. The calibration curve was constructed from peak areas and AA concentrations. Response factors of each AA were obtained by an extra analysis on standard AA solution containing internal standards.

5 Statistical analysis

Sample characteristics were described as mean with standard deviation (SD), median with 25th and 75th percentile (Q1-Q3) or frequencies. To test differences in maternal and infant characteristics (including maternal BMI and infant sex), dietary AA intake over the study period and stress measurements (questionnaires and cortisol) between both study groups, unpaired student T-tests (for continuous normally distributed data), Chi-square

tests (for binary of categorical data), Mann-Whitney U tests (for continuous not normally distributed data) or Linear Mixed Models (for data that were measured at multiple time points) were used. The mean of the three 24-h recalls was used to compare the dietary intake of AA between groups as this reflects the overall intake of the participants during the study period.

The HM cortisol area under the curve (AUC) was calculated to provide a value that better reflects HM cortisol throughout the day, which is known to follow a circadian rhythm (32). Therefore, all HM cortisol values were standardized to 7:00 AM, 14:00 PM and 22:00 PM, by regressing the time of collection to cortisol values. For each participant, we then calculated the estimated cortisol value at 07:00h, 14:00h and 22:00h. To do this, the following formula was used: HM cortisol in mmol/L -/+ unstandardized regression coefficient of all HM cortisol values * (new (standardized)) time point – real time point) (44). Subsequently, the HM cortisol AUC for each collection day was calculated as described by Pruessner et al. (45). The cortisol value at 7:00 AM was considered the HM cortisol morning peak (32). The highest cortisol value of the two morning saliva samples was considered the saliva cortisol morning peak. When time of S1 collection was >30 minutes after waking up, saliva values were excluded.

Analyses were performed separately for BAA and FAA. Before analyses, the HM AAs were categorized into different outcome variables: total AA, essential AA and non-essential AA. Because AA from one precursor family can be converted into other members of this family, AA were also grouped into precursor groups; the glutamate precursor group (glutamic acid, glutamine, arginine), the aspartate precursor group (aspartic acid, methionine isoleucine, threonine, lysine), the serine precursor group (serine, glycine), the pyruvate precursor group (valine, leucine, alanine), the aromatic precursor group (phenylalanine, tyrosine, tryptophan) and the histidine precursor group (histidine). Methionine was analyzed separately (24).

All outcome variables were checked for whether they were normally distributed. When variables were not distributed normally, a log transformation was performed. When participants completed less than one full day of sample collection, they were excluded from the final analysis.

To answer the research question if and how maternal stress affects the BAA and FAA concentrations in HM, the AA outcomes as described above were compared between the HS and CTL group. As all study time-points were taken along in the comparison, linear mixed models were used to analyze the group differences to control for within-person repeated measures. The analysis was corrected for factors differing between study groups. In addition, we tested if maternal dietary AA intake during the study

period differed between study groups. If maternal dietary intake of a specific AA statistically differed between groups, the comparison of that specific HM AA, was corrected for the maternal intake. As HM AA concentrations differ between the different weeks and stages of lactation, the interaction between the study time-point and study group was investigated and reported.

To answer the research question whether HM cortisol concentrations (cortisol AUCs) are related to HM AA concentrations, we performed a secondary analysis. The relationship between HM AA and HM cortisol AUCs was investigated, independent of the study group. This relationship was only investigated for total AA, essential AA, non-essential AA and for methionine in HM. As all study time-points were taken along in this secondary analysis, Linear Mixed Models were used to control for within person repeated measures. The analysis was corrected for potential confounding factors that have shown to influence the AA composition of HM in previous literature: AA dietary intake and maternal BMI (17, 46-48). 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, most of the separate AAs were categorized into their precursor families 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 the results.

Results

6 Maternal characteristics, food intake and stress measures

In total, 86 lactating women were included in the CTL group and 30 in the HS group. Nineteen women stopped participating before the end of the study due to various reasons (see Figure 2 for drop out reasons), 13 women (15%) in the CTL group and 6 women (20%) in the HS group (Figure 2). Characteristics and dietary protein and AA intake of the participants are shown in Table 1 and Table 2 respectively. Maternal baseline characteristics, including maternal age, BMI, ethnicity, education level, alcohol consumption, smoking, dietary habits and mode of delivery did not differ between the study groups. Maternal protein/AA intake during the study period was also similar between both study groups. In addition, the HM pumping method used and storage time of the samples did not differ between study groups. The only difference between study groups was that mothers in the HS group gave birth to a male infant more often than mothers in the CTL group, 75% and 49% respectively ($p=0.048$), the birthweights did not differ between study groups. In the HS group, hospitalization duration ranged between 2 and 12 days,

with a median of 7 days. Duration of hospitalization in this group was not associated with maternal stress scores (p=0.282 for PSS score).

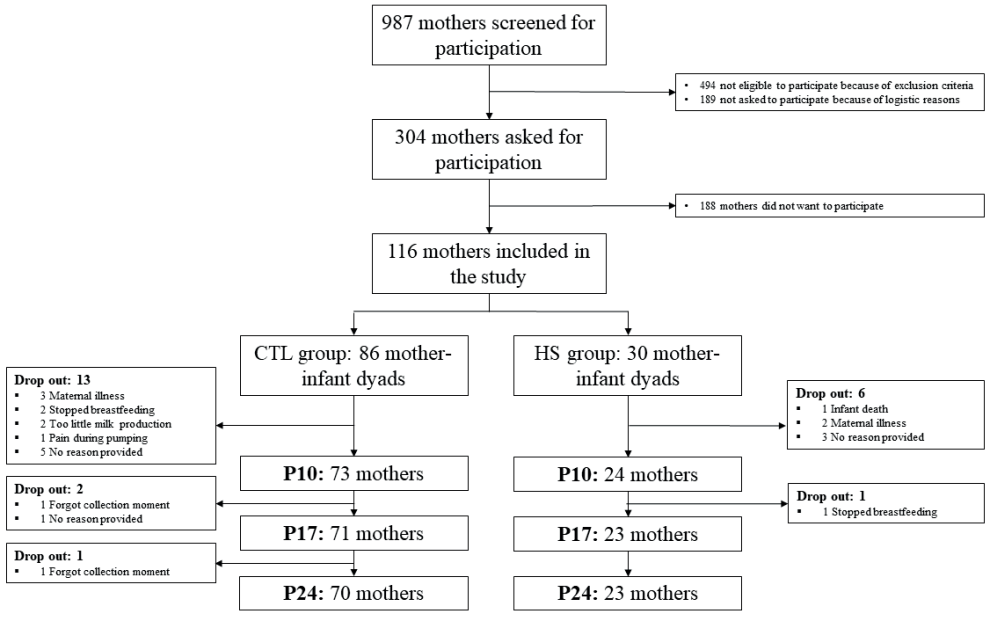


Figure 2: Flowchart of the study population
Flowchart of the study population. Abbreviations: CTL group = control group, HS group = High stress group, p = postpartum day.

Lifetime psychological (JTV and LSC-r questionnaire) and biological stress (hair cortisol) measurements were the same between study groups (Table 3). Perceived stress during the study period was higher in the HS group, and women in the HS groups scored higher on the PSS, EPDS, STAI-s and STAI-t (p<0.01) (Table 3). There were no differences in HM cortisol AUCs or the HM/saliva cortisol morning peak concentrations between study groups.

7 BAA concentrations in HM are higher in the HS group

Table 3 shows the concentrations of BAAs in HM per study group on all collection days, and Figure 3A+B depicts the BAA dynamics over the study period per study group. Total concentrations of BAAs were higher in the milk of women in the HS group compared to the CTL group (819 [92.4, 1547]; p=0.028). This was also the case for essential BAAs (476 [55.8, 896]; p=0.027), non-essential BAAs (363 [55.9, 669]; p=0.021) and for the concentrations of the BAA precursor groups (p<0.035), except for the glutamate precursor group, which showed the same concentrations in both study groups. There were no interactions between study group and study time point (Table 3 and Figure 3). All statistical analysis were corrected for infant sex.

Table 1: Maternal and infant baseline characteristics

	Maternal characteristics		
	CTL (n=75)[1]	**HS** (n=24)[2]	**p-value**
Age *Mean (SD)*[a]	32.4 (3.3)	32.5 (3.6)	0.815
BMI (kg/m^2) *Mean(SD)*[a]	23.1 (3.7)	24.3 (5.5)	0.246
Ethnicity %[b]			0.338
Dutch	88.0 %	83.3 %	
Surinamese	0%	4.2%	
Antillean	1.3%	0%	
Other Western[1]	8.0%	12.5%	
Education *%*[b]			0.090
Low education[2]	1.3%	0%	
Medium education[2]	8.0%	29.2%	
High education[2]	86.7%	70.8%	
Smoking *%*[b]			0.333
Never	65.3%	58.3%	
In the past	26.7%	41.8%	
Yes	1.3%	0%	
Alcohol use *%*[b]			0.281
Never	82.7%	83.3%	
1x/ month or less	4.0%	12.5%	
2-4x/ month	8.0%	4.2%	
Vegan *%*[b]	2.7%	0%	0.513
Vegetarian *%*[b]	8.0%	4.2%	0.576
Mode of delivery[b] *% Caesarean*	29.4%	16.7%	0.453
Infant characteristics			
Birthweight (gr) *Mean(SD)*[a]	3564,2 (487.5)	3399.7 (584.6)	0.176
Sex *% of male*[b]	49.3%	75.0%	0.048

[1] Europe, North America, Oceania, Indonesia, Japan
[2] Based on the International Standard Classification of Education (ISCED) 2011
· Low education (ISCED 2011) : Levels 0-2
· Medium education (ISCED 2011): Levels 3-4
· High education (ISCED 2011): Levels 5-8
Statistical difference between groups tested using: [a]Student T-test, [b]Chi-square test
Abbreviations: CTL = control group, HS = high stress group, SD = Standard deviation, BMI = Body Mass Index, gr = Grams,

8 FAA concentrations in HM did not differ between study groups

Table 4 shows the concentrations of FAA in HM per study group on all collection days, and Figure 3C+D depicts the FAA dynamics over the study period per study group. Total

Table 2: Maternal energy, protein and amino acid dietary intake

	Maternal dietary intake		
Intake component *mean (SD)*	**CTL (n = 73)**	**HS (n = 21)**	**p-value**
Energy total Kcal	2163 (572)	1950 (664)	0.192
Energy total Kilojoule	9073 (2396)	8183 (2780)	0.194
Total protein (gr)	77.1 (22.9)	70.7 (20.6)	0.227
Plant protein (gr)	36.2 (10.4)	30.9 (11.0)	0.059
Animal protein (gr)	41.1 (20.6)	39.9 (15.2)	0.773
Amino Acids (mg):			
Total	74883 (23438)	67295 (19226)	0.138
Essential	33033 (11068)	30133 (9001)	0.225
Non-essential	41850 (12514)	37162 (10522)	0.093
Glutamate family	26222 (7599)	22824 (6744)	0.056
Aspartate family	18519 (6632)	17125 (5234)	0.319
Methionine	1642 (603)	1513 (450)	0.292
Serine family	7684 (2320)	7030 (2076)	0.224
Pyruvate family	13382 (4406)	12226 (3574)	0.224
Aromatic family	7093 (2298)	6298 (1778)	0.100
Histidine family	1982 (659)	1792 (577)	0.207

Food intake values are the mean of the three 24-hour recall questionnaires, which reflects the overall intake of the participants during the study period.
Abbreviations: Kcal = kilocalories, CTL = control group, HS = high stress group, SD = standard deviation, gr = gram, mg = milligram
All statistical differences between groups were tested using a student t-test.Glutamate family members contain: glutamine, arginine, proline. Aspartate family contains: asparagine, methionine, threonine, isoleucine, lysine. Serine family members contains: serine, glycine, cysteine. Pyruvate family members contain: valine, alanine, leucine. Aromatic family members contain: phenylalanine, tyrosine. Histidine family members contains: histidine.

concentrations of FAA over the entire study group did not differ between the HS and CTL group, neither did the concentrations of essential FAA, non-essential FAA and the concentrations of the FAA precursor family groups. All statistical analysis were corrected for infant sex.

9 Bound methionine does not differ between study groups, free methionine is higher in the HS group

Despite higher concentrations of BAA of the aspartate precursor family in the HS group (255 [46.0, 465]; p=0.017), to which protein-bound methionine belongs, protein-bound methionine concentrations did not differ between the HS and CTL group. In contrast, free methionine concentrations, were higher in the HS group compared to the control group (0.49 [0.21, 0.76]; p=0.001). All statistical analysis were corrected for infant sex.

Table 3. Maternal perceived stress scores and cortisol values between study groups

	Questionnaire based stress scores		
	CTL group (n=73)	**HS group (n=24)**	**p-value**
Life-time stress (test score)			
JTV *median (Q1-Q3)*[a]	28.0 (25.0 – 35.5)	29.5 (27.3 – 38.8)	0.459
LSC-r *median (Q1-Q3)*[a]	6.0 (3 - 10)	5.5 (2 - 10)	0.432
Perceived stress during study period (test score)			
PSS *mean (SD)*[b]	16.52 (6.4)	20.48 (6.7)	0.001
EPDS *median (Q1-Q3)*[b]	5.0 (2 - 7)	8.0 (5.8 - 11)	0.004
STAI-s *median (Q1-Q3)*[b]	27.0 (23 - 34)	36.0 (25 - 40)	0.005
STAI-t *median (Q1-Q3)*[b]	29.0 (26 - 36)	37.0 (31 - 40)	0.004
	Biological measures of stress: cortisol		
Stress over the last 3 months of pregnancy			
Hair cortisol *median (Q1-Q3)*[b]	6.0 (3.1 – 14.4)	7.1 (5.3 – 11.6)	0.280
Stress on collection days			
Saliva cortisol (morning peak) *median (Q1-Q3)*[c]	5.4 (3.4 – 8.0)	5.7 (3.3 – 8.9)	0.490
p10[b]	5.5 (3.6 – 8.3)	5.9 (3.0 – 10.5)	0.811
p17[b]	5.0 (3.3 – 8.0)	6.2 (4.4- 8.3)	0.274
p24[b]	5.4 (4.0 – 7.6)	5.2 (2.1 – 6.1)	0.268
Human milk cortisol AUC *median (Q1-Q3)*[c]	52.0 (36.9 – 72.1)	64.0 (41.0 – 91.2)	0.074
p10[b]	59.1 (41.2 – 81.4)	69.3 (40.7 – 86.4)	0.465
p17[b]	46.2 (35.3 – 70.9)	57.1 (44.6 – 93.2)	0.088
p24[b]	51.0 (36.7 – 70.6)	62.9 (41.0 – 112.8)	0.282

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), Q1-Q3 = 25th to 75th percentile, SD = standard deviation, HM = human milk, AUC = Area under the curve

Statistical difference between groups tested using: [a]Mann-Withney U test, [b]Student T-test, [c]Linear Mixed Effects model (correction for within-person repeated measures)

10 Dynamics of FAAs in HM over the first month of lactation differ between study groups

In general, the BAA concentrations in HM decreased over the study period (p<0.001), while the FAA concentrations increased (p=0.019). As indicated in Table 4**,** for total FAA, there was an interaction between study group and study time point. This was the same for the essential FAAs, non-essential FAAs and the glutamate precursor group, pyruvate precursor group and aromatic precursor group. The different dynamics over the study period between the HS and CTL group are depicted in Figure 3C+D. All statistical analysis were corrected for infant sex.

Table 4: Protein-bound amino acids in human milk over the study period per study group

Amino acids in ug/L *mean + SD*	CTL group			HS group			Difference between study groups
	P10 (*n*=73)	**P17** (*n*=71)	**P24** (*n*=70)	**P10** (*n*=24)	**P17** (*n*=23)	**P24** (*n*=23)	**Estimate (CI)**
Total	11539 (1534)	10238 (1158)	9792 (1337)	12442 (2739)	11103 (2917)	10311 (2547)	819 (92, 1547)*
Essential	5815 (842)	5247 (649)	4960 (708)	6357 (1632)	5753 (1639)	5234 (1564)	476 (55, 896)*
Non-essential	5576 (704)	4952 (515)	4779 (636)	5976 (1134)	5309 (1272)	5023 (987)	363 (55, 669)*
Glutamate family	2636 (331)	2398 (210)	2357 (293)	2737 (365)	2500 (377)	2412 (287)	92.4 (-15, 200)
Aspartate family	3142 (432)	2702 (337)	2601 (397)	3387 (806)	2950 (872)	2799 (707)	255 (46, 465)*
Methionine	205 (44.9)	180 (28.1)	163 (28.4)	213 (52.1)	200 (58.1)	164 (36.1)	7.07 (-7, 21)
Serine family	1038 (154)	925 (121)	868 (136)	1173 (419)	1044 (414)	951 (399)	124 (23, 225)*
Pyruvate family	3264 (473)	2997 (373)	2797 (389)	3570 (826)	3226 (835)	2893 (811)	235 (17, 454)*
Aromatic family	980 (151)	883 (110)	831 (122)	1103 (340)	992 (334)	899 (329)	110 (26, 194)*
Histidine family	331 (49.8)	293 (37.9)	284 (42.6)	363 (93.2)	320 (93.5)	303 (85.4)	27.6 (3, 52)*

Protein-bound amino acids in human milk per study group at all collection time points in ug/L.
Statistics: Group comparisons are corrected for infant sex. *= significantly higher in the HS group ($p<0.05$). No study group – time point interactions were found. Group differences and interaction were tested using Linear Mixed Effects Models to correct for within-person repeated measures.
Glutamate family members contains: glutamine, arginine, proline. Aspartate family members contains: asparagine, methionine, threonine, isoleucine, lysine. Serine family members contains: serine, glycine, cysteine. Pyruvate family members contains: valine, alanine, leucine. Aromatic family members contains: phenylalanine, tyrosine. Histidine family members contains: histidine.
Abbreviations: CTL = control, HS = high stress, p = postpartum day, SD = standard deviation.

Table 5: Free amino acids in human milk over the study period per study group

Amino acid in ug/L *mean+SD*	CTL group			HS group			Difference between study groups
	P10 (*n*=73)	**P17** (*n*=71)	**P24** (*n*=70)	**P10** (*n*=24)	**P17** (*n*=23)	**P24** (*n*=23)	**Estimate (CI)**
Total	292 (91.2)	334 (74.3)	338 (79.2)	321 (78.8)	324 (91.4)	300 (73.8)	-16.1 (-48.4, 16.3)[1]
Essential	50.8 (15.4)	50.4 (13.9)	45.3 (12.9)	62.2 (36.1)	56.6 (32.8)	43.8 (17.4)	2.1 (-4.0, 8.3)[1]
Non-essential	207 (80.5)	251 (66.5)	262 (73.1)	225 (62.9)	235 (79.0)	227 (68.1)	-16.5 (-46.0, 13.1)[1]
Glutamate family	167 (68.8)	203 (59.0)	215 (64.9)	179 (57.1)	190 (71.5)	184 (64.9)	-13.8 (-40.2, 12.6)[1]
Aspartate family	20.1 (8.5)	25.0 (11.2)	21.0 (5.7)	23.4 (9.8)	29.8 (12.9)	20.9 (7.2)	1.6 (-1.1, 4.3)
Methionine	1.1 (0.4)	4.1 (5.5)	1.2 (0.8)	1.5 (1.3)	6.6 (7.9)	1.5 (2.1)	0.5 (0.2, 0.8)*
Serine family	14.6 (5.6)	18.3 (4.9)	18.4 (7.2)	17.4 (5.4)	18.8 (5.4)	18.2 (4.3)	1.0 (-1.0, 3.0)
Pyruvate family	41.8 (11.0)	35.1 (12.1)	33.0 (8.9)	49.0 (22.7)	33.2 (19.1)	29.7 (8.5)	-2.2 (-6.3, 1.9)[1]
Aromatic family	7.2 (2.8)	11.5 (4.0)	11.5 (4.0)	10.1 (7.7)	12.2 (8.7)	11.1 (4.2)	0.9 (-0.7, 2.5)[1]
Histidine family	3.9 (1.7)	5.1 (1.3)	4.8 (2.4)	4.6 (1.7)	5.4 (2.6)	4.3 (1.4)	0.2 (-0.4, 0.9)

Free amino acids in human milk per study group at all collection time points in ug/L.
*= significantly higher in the HS group ($p<0.05$). Group comparisons are corrected for infant sex.
[1]= significant study group – time point interaction ($p<0.05$).
Statistics:Group differences and interactions were tested using Linear Mixed Effects Models to correct for within-person repeated measures.
Glutamate family members contains: glutamine, arginine, proline. Aspartate family members contains: asparagine, methionine, threonine, isoleucine, lysine. Serine family members contains: serine, glycine, cysteine. Pyruvate family members contains: valine, alanine, leucine. Aromatic family members contains: phenylalanine, tyrosine. Histidine family members contains: histidine.
Abbreviations: CTL = control, HS = high stress, p = postpartum day, SD = standard deviation.

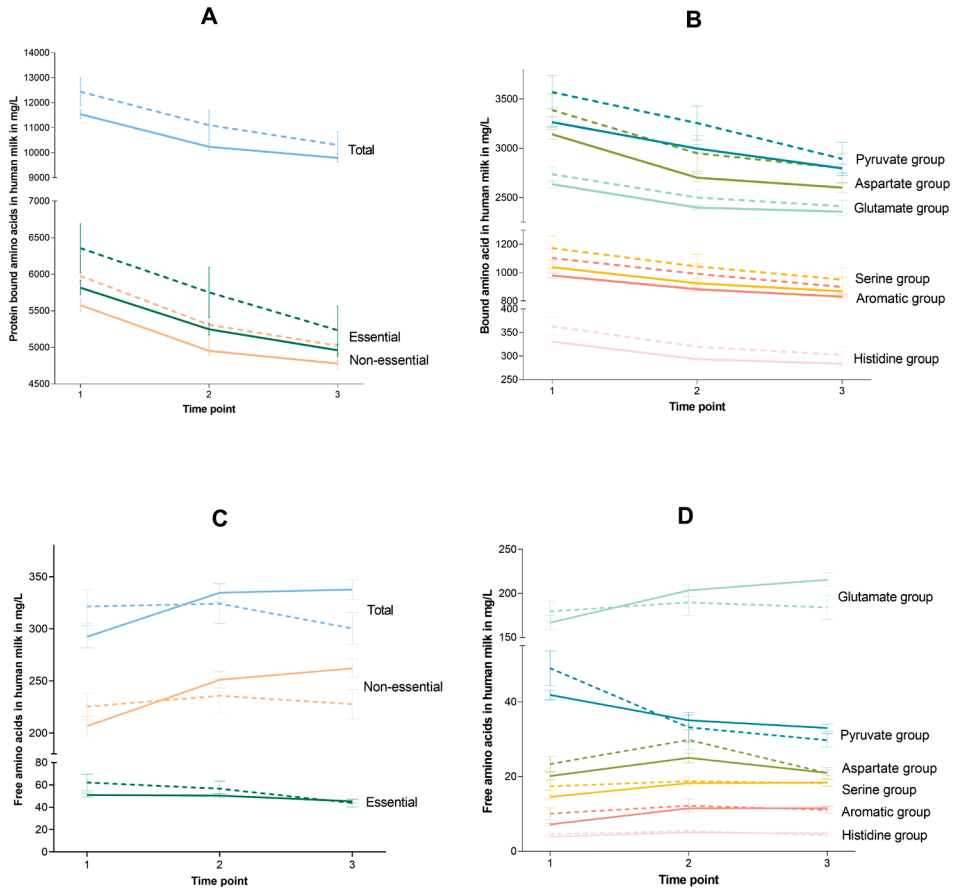


Figure 3: Dynamics of amino acids over the study period per study group
Graphs show the concentrations of the protein-bound amino acids (panel A + B) and the free amino acids (panel C + D) at the different collection moments. The continued line indicates the Control Group and the dotted line indicates the High Stress Group. Error bars indicate the standard error of the mean.
Glutamate family contains: glutamine, arginine, proline. Aspartate family contains: asparagine, methionine, threonine, isoleucine, lysine. Serine family contains: serine, glycine, cysteine. Pyruvate family contains: valine, alanine, leucine. Aromatic family contains: phenylalanine, tyrosine. Histidine family contains: histidine.

11 HM BAAs are positively associated with HM cortisol levels

Total BAA concentrations, as well as essential BAAs and non-essential BAAs, were positively associated with the HM cortisol AUC (5.54 [0.73, 10.35]; p=0.024, 2.66 [0.01, 5.30]; p=0.049 and 3.07 [0.85, 5.29]; p=0.007 respectively). Protein-bound methionine was not associated with the HM cortisol AUC. Total FAA, essential FAA, non-essential FAA and free methionine were also not associated with the HM cortisol AUC, also not at the separate study time points.

Discussion

In this study, we explored associations between maternal stress and the AA composition of HM. We demonstrated that perceived as well as biological maternal stress in the first month postpartum was positively associated with the concentrations of BAAs in HM. In particular, BAA concentrations were increased in the HM of mothers with high stress levels, with the exception of bound methionine. Methionine was, contrary to our hypothesis, not associated with maternal stress. However, while the overall concentrations of FAAs in HM did not differ between study groups, free methionine was higher in the HM from mothers with high levels of perceived stress.

Our results are in line with previous animal experimental studies (22, 23). In stressed dams, an increase in some of the milk BAAs was observed, while milk FAAs were overall not affected by stress exposure. To date, human studies on the effect of maternal stress on AA composition of HM are scarce. One study investigated the influence of maternal postpartum stress on the metabolome of HM, including three AAs, and found that high stress levels were positively associated with the concentrations of tryptophan and tyrosine. However, after correction for multiple testing, these associations disappeared (16). Another study by Ziomkiewicz et al. found that proteins in HM were not associated with maternal psychological nor biological stress (15). This difference may be attributed to the fact that Ziomkiewicz et al. measured whole proteins instead of specific AAs.

The biological mechanism behind the observed associations between maternal stress and HM AA composition is not yet clear. In fact, the process behind the transport of AAs from the maternal blood stream into the mammary gland and into the HM, is complex and not yet fully understood (21). Both BAAs and FAAs in HM can be transported from the maternal circulation and BAAs can be synthesized out of FAAs by the mammary gland itself (21). It has further been demonstrated, in contrast to our current results in HM, that almost all AAs in the maternal plasma are decreased as a result of maternal stress (18-20). The origin of such a reduction is not fully elucidated. It might be due to the influence of catecholamines produced during stress, which exert an anti-insulin effect on the metabolism of AAs (18-20). As we observed higher concentrations of HM BAAs in the HS group, but no association between stress and FAAs in HM, this may indicate an increased active transport of AAs from the maternal circulation into HM and/or an increased synthesis of BAAs in the mammary gland.

Exactly how and to what extent stress influences transport processes also remains to be elucidated. As previous research showed associations between glucocorticoid levels and the expression of certain AA transporters in the mammary gland, e.g. the system L and the system y^+ amino acid transporters, one could hypothesize that cortisol is

involved in the mechanisms leading to these stress-induced changes in HM (21, 49). Indeed, we observed a positive association between cortisol levels and BAA levels in HM, which was also found in previous animal experimental research (22). A possible explanation for the fact that that cortisol values were not elevated in the HS group but were positively associated with HM BAAs, might be that the sample size of the HS group was not sufficient to detect a statistically significant difference. In fact there was a 'trend' towards a higher HM cortisol AUC in the HS group (p=0.074).

We further focused specifically on methionine, as in mice, maternal stress has led to reduced methionine in brain and plasma of the offspring which was associated with later-life cognitive deficits (24). Contrary to our hypothesis, bound methionine levels were not decreased in the HM samples in our HS group and free methionine levels were even higher in mothers with high perceived stress levels. This was an unexpected result but it can be speculated that the absence of an increase in protein-bound methionine is the result of stress-induced breakdown into free methionine, which would result in an increase in the free form of this essential AA. In addition, another possible explanation for the absence of an increase in protein-bound methionine is that while some transporters of AAs in the mammary gland seem to be upregulated under the influence of cortisol, one of the three transporters that facilitates the transport of methionine into HM is rather downregulated by glucocorticoids (21, 49). Unfortunately, the influence of cortisol on the other two methionine-transporters remains so far unknown (21).

Increased concentrations of BAA in the HM of mothers in the HS group may be due to the fact that mothers with high levels of stress produce less milk in general, compared to mothers with lower levels of stress (50-52). When AA transport into milk is maintained at the same level, the concentrations of BAA in HM will then increase. This might lead to a maintenance of an appropriate transmission of these important nutrients to the infant. On the other hand, this would also mean that the infant might be at risk of receiving inappropriate amounts of methionine, which was not increased in HM under stressful circumstances as was shown in our results. As we did not measure the total milk volume production during the day or AA concentrations in the infant, this remains speculation and awaits future research.

During lactation, milk specific proteases break down HM proteins into FAAs. HM further contains protease activators and protease inhibitors (53). Over the first month of lactation, protease inhibitors decrease which leads to an increase in total FAAs, but subsequently, this also contributes to a decrease in HM BAAs over time (53). Indeed, a decrease of BAA and an increase in total FAA over time was observed for the mothers in our study, except for the concentrations of HM FAAs of the mothers in the HS group, where the total FAA levels decreased over time. The different dynamics of FAA observed

in the HS group might be due to a different regulation, or production of proteases under the influence of stress. Another explanation for the decrease of total HM FAA under stressful circumstances might be that the FAAs are more often bound in response to stressful circumstances resulting in the increase of HM BAA observed in the HS group.

The strengths of our current study are its longitudinal design and the timing and frequency of HM sample collection. The first month postpartum is a sensitive time window, that has been frequently missed in earlier HM research, but since breastfed infants depend on HM during this period as their only source of nutrition, these first weeks after birth represent a very important period. Furthermore, the fact that mothers were exposed to a stressor (i.e. infant hospitalization) ensured that the study population contained participants with established high levels of perceived stress. Additionally, extensive information on both maternal psychological and biological stress was collected, and the HS and CTL groups were similar at baseline with regard to life-time stress levels or other important characteristics that could have influenced the relationship between stress and HM AA levels. Limitations of this study are, firstly, the relatively small sample size, especially in the HS group. Secondly, current perceived stress scores were only measured once, i.e. at the end of the study. Therefore, we were not able to investigate whether the different dynamics of FAA in the HS group were related to changes in the amount of stress experienced at that exact moment. Moreover, because of the exploratory nature of this study we decided not to correct for multiple testing, even though we did perform a 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. In addition, no reliable information was collected about infant feeding mode and additional feeding practices. The relatively high loss to follow up and the fact that this was higher in the HS group compared to the CTL group (19% versus 23% respectively) may have caused selection bias. Drop out of women who may have been more stressed, especially in the HS group, may have led to underestimation of associations between maternal stress and AA. 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 may limit generalizability of the results.

The results of our study suggest that mothers with high stress levels have increased concentrations of BAA in their milk. The increased concentrations of BAA that we found, may partially compensate for the higher nutritional requirements of infants under stressful circumstances. However, as impaired growth and development have been observed in children of mothers with high amounts of stress in the postnatal period, this increase in AA in the HM might not be sufficient to maintain optimal growth and

development. Moreover, the increase was not observed for protein-bound methionine and total HM FAA.

In conclusion, findings from this unique prospective cohort study suggest that there is a relationship between maternal stress in the first month postpartum and the AA concentrations of HM during this time. Our findings emphasize the importance of the maternal psychological state during lactation. In the last years, attention for the prevention of the detrimental consequences of stressful experiences in early-life has increased. Stress reduction programs for children and parents have been developed, such as family integrated care and relaxation therapy using relaxing music (56-62), with one study demonstrating effects on milk composition (62). Future research should replicate our findings, investigate the entire period of lactation and focus on to what extend these stress-induced changes in human milk composition are of clinical importance for short- and long-term infant development and health.

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