



Original article

Maternal influences on the glucocorticoid concentrations of human milk: The STEPS study



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SUMMARY

Background & aims: Human milk (HM) contains a wide array of non-nutritive bioactive elements, including glucocorticoid hormones (glucocorticoid; cortisol and cortisone). The relationship between milk-borne glucocorticoids, measures of maternal health and patterns of breast-feeding is not yet established. This study was conducted to determine the influence of maternal and infant related biological and socio-demographic factors on the levels of glucocorticoids hormones in HM.

Methods: Samples were obtained from lactating mothers (n = 656) participating in the Finnish cohort the STEPS study (Steps to the Healthy Development and Well-being of Children) when the infants were 11.29 (± 2.6) weeks of age. Glucocorticoids (both cortisol and cortisone) concentrations were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Maternal demographics, biological and social factors were obtained using hospital records and self-reported diaries and questionnaires. **Results:** The majority of women reported that they were exclusively breastfeeding at the time of sample donation (51.2%). For all collected samples, cortisone (9.55 ± 3.44 ng/ml) was the predominant hormone and cortisol (7.39 ± 5.97 ng/ml) was present in all samples. Strong and positive correlation was found between cortisol and cortisone ($r = 0.60, p < 0.0001$). Cortisone was statistically different between overweight, normal and underweight women ($p = 0.01$) for cortisol no difference was seen ($p = 0.96$). Whilst, preterm birth (born before 37 week gestation) was positively associated with both cortisol ($p = 0.04$) and cortisone ($p = 0.01$). There was also a significant but weaker negative relationship between mothers educational status and cortisol ($p = 0.05$) and no effect was seen for cortisone ($p = 0.82$). Interestingly, no significant differences was found in glucocorticoid concentrations between exclusive and partial breastfeeding women.

Conclusion: HM contains glucocorticoids hormones. The concentrations are influenced by the varying maternal factors including maternal weight, preterm birth and maternal educational status, suggesting the possible role of maternal biological and social influences on milk hormonal composition. Interestingly, there was no influence of feeding patterns on HM glucocorticoids. Further analysis is required to fully explore the relationship with measures of maternal stress, including mother's glucocorticoid status.

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Introduction

Human breast milk and breastfeeding is the optimal source of nourishment for a newborn infant. Exclusive breast-feeding for the

first six months of life is the normative standard of infant feeding [1,2]. Unlike standardised manufactured infant formula milk, HM composition is dynamic. It differs between women, over the course of a single feed and its composition adaptively changes to meet the needs of a growing infant [3].

HM is a primary carrier of a wide variety of nutrients, and also includes non-nutritive bioactive factors such as glucocorticoids.

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Cortisol and cortisone are the primary glucocorticoids produced from the adrenal glands, in response to physiological and psychological stress [4]. Beyond these, cortisol plays a key role in gluconeogenesis, lipolysis and energy metabolism [5–8]. Glucocorticoids in human milk are reflective of the circulating abundance in the maternal blood and saliva [9]. The mechanism of transport from circulation into HM is not fully established. It is also not established, whether glucocorticoids in HM are biologically active in either the digestive tract or absorbed into the infants circulatory system. However, throughout the first year of life, breastfed children exhibit 40% greater salivary cortisol, than formula fed infants [10]. There is some evidence that milk borne glucocorticoids may influence on the psychological maturation of the child [11,12]. Higher glucocorticoid levels in milk were linked with increased fearful temperament in infants, however these effects were more prominent in female children [13]. Unlike other bioactive components of milk, glucocorticoids have diverse functions; hence will have the potential to exert significant effect on offspring phenotype, which are still unknown.

Currently there is limited understanding of the predominant determinants of HM glucocorticoids [14,15]. It is however plausible that maternal stress and the subsequent systemic stimulation of hypothalamic–pituitary axis (HPA) axis is likely to be a significant key regulatory mechanism, that elevates glucocorticoid levels in both animals and human milk [16–18]. The period before and in the subsequent, months after childbirth are associated with marked changes and variation in maternal physiological and psychological status. Several prenatal and postnatal factors may have an immediate effect on maternal stress level. These include maternal health status prior to and during pregnancy [19,20], nature [21] and timing of the delivery [22], social/familial environment mother finds herself (post-natal depression) in [23,24] and varied pattern of breast feeding may have an immediate effect on maternal stress levels [25]. Numerous studies have demonstrated the inverse relationship between breastfeeding and maternal stress, exclusive breastfeeding is associated with blunted HPA axis response thereby reducing cortisol release to psychological response [26]. Furthermore, long term–chronic stress or depressive symptoms throughout pregnancy can have a negative influence on the duration of breastfeeding [27]. However, not much is known about the impact of maternal and infant associated factors on milk stress hormonal level and how this would affect infant's development.

In the current study, we hypothesised that maternal factors such as maternal age, BMI or education and infant factors such as sex, gestational age, could possibly influence the glucocorticoid concentrations of HM. Thus, the main aim of the study was to identify which maternal or infant related biological, social and environmental factors are associated with HM glucocorticoid levels around infant's age of 3 months. Moreover, we aimed to analyse the influence of breast-feeding patterns on glucocorticoid levels. To establish the predominant determinants influencing HM glucocorticoid concentrations, analysis was performed on the HM samples collected as a part of large population based study conducted in Turku, Finland.

Methods

Study design and subjects

The present study is based on data from mothers and children participating in a longitudinal Finland cohort, Steps to healthy development of Children (the STEPS Study), which has been described in detail elsewhere [28]. Briefly, all Finnish- and Swedish-speaking mothers who delivered a living child between 1 January 2008 and 31 April 2010 in the Hospital District of

Southwest Finland formed the cohort population (in total 9811 mothers and their 9936 children). Of them, 1797 mothers (18.3%) volunteered as participants for the intensive follow-up group of the STEPS study during the first trimester of pregnancy (1387 mothers recruited at maternity health-care clinics) or soon after delivery (410 mothers recruited at delivery wards). Together with these mothers, their 1658 partners and 1827 children (including thirty pairs of twins) enrolled in the follow-up group.

Of all mothers ($n = 1797$) enrolled in the STEPS study, 812 (45.2%) mothers provided HM samples. Written informed consent were obtained from the participants. The study protocol was approved by the Ethics Committee of the Hospital District of South West Finland in 2007. To be able to use milk samples for hormonal analysis, additional ethics were obtained and the study protocol was approved by Ethics Committee of Hospital district of Southwest Finland in March 2015.

Of the 812 HM samples, 650 (80.04%) were analysed including mothers for six pairs of twins (children $n = 656$) and 162 were excluded for various reason [(no response for ethics ($n = 118$) or declined for ethics ($n = 4$), unclear labelling ($n = 25$), empty tube ($n = 1$) or unsuccessful analytical analysis ($n = 14$). The children mean age at the time of milk collection was 11.29 (± 2.6 weeks).

Sociodemographic, familial and infant characteristics

Analyses were adjusted for various potential confounding factors. Information regarding mother's age, marital status, education, occupation class, living in urban area, total family income and number of siblings were obtained from self-administered questionnaires during the prenatal period. In addition, self-reported height and weight before pregnancy were also collected upon recruitment for an additional analysis of pre-pregnancy BMI (kg/m^2) and classified as underweight ($<18.5 \text{ kg}/\text{m}^2$), normal weight ($18.5\text{--}24.9 \text{ kg}/\text{m}^2$), while overweight ($25.0\text{--}29.9 \text{ kg}/\text{m}^2$) and obese ($\geq 30.0 \text{ kg}/\text{m}^2$) were combined as single category.

Maternal education status was classified into either advanced or low based on the information provided. Those who had the highest levels of vocational training (such as a 4-year program at a polytechnic institute) or any academic degree (bachelors, masters, licentiate or doctoral degree) were regarded as advanced education. Occupational class was classified into two categories: (i) professionals (in high positions, e.g. managerial, and nurses); and (ii) others (blue-collar workers in industry or agriculture and service workers). Family income was classified into two categories: high income ($\geq 3000 \text{ €/month net}$) and low income ($<3000 \text{ €/month net}$). Family structure was classified into three categories (i) single (mothers being single parent); (ii) nuclear (families with children from the current relationship); and (iii) blended (families with children from current and previous relationships). Parental marital status was categorised into three groups as married (legally or traditionally), common law and others (including single, divorced or widow). Seasonal variation was classified into two categories, milk collected during winter (Oct–Feb) with shorter photoperiod and summer (Mar–Sep) with longer photoperiod. Data regarding maternal distress was assessed using these questions. First, 1) how happy you feel at the moment; 2) how anxious you feel at the moment and 3) how depressed you feel at the moment, all scaled from 0 (very unhappy, anxious or depress) to 10 (not at all). Life satisfaction was asked with three question: Are you satisfied with your 1) life situation, 2) relationship and 3) housework at the moment all scaled from 0 (not satisfied) to 5 (very satisfied). Furthermore, sum variable of anxiety, depression and life satisfaction were calculated.

Information regarding pregnancy duration, delivery and gestational diabetes as well as children's sex, birth weight, length, and

possible twin brother/sisters were obtained from the Longitudinal Census Files. Delivery was defined as premature if the pregnancy lasted ≤ 37 weeks (Yes/No).

Milk collection and infant feeding information

Mothers were asked to collect breast milk at children's age of 3 months. The collection procedure was standardized by the following written instructions. The mothers collected the samples by manual expression in the morning from single breast, first milking a few drops to waste before collecting the actual sample (10 ml) into a plastic container. The mothers brought the samples to the research centre, or the samples were collected from their homes on the day of sampling. All samples were frozen and stored at -70 °C until further analysis.

Information about breast-feeding (BF) and complimentary feeding (CF) were obtained with self-administered follow-up diary. Follow-up diary consisted of detailed information about duration of exclusive BF (date when started and date when ended), partial BF, referred as total duration of BF (date when started and when ended) and initiation of CF and also information about which specific foods or food groups were given and at what age the foods were given for the first time. In addition, the use of formula milk was recorded. Exclusive BF was defined as infant receiving no other food than mother's breastmilk, except for water, drops or syrups consisting of vitamins, mineral supplements or medicines. Partial breastfeeding was defined as infant receiving HM and any liquid or food including non-human milk and formula. Families were instructed to record information about feeding to the follow-up diary in real-time to avoid memory related bias.

Milk sample preparation

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for sample analysis. Sample preparation was initiated by heating milk samples at 37 °C for 10 min and vortexing them for 20 s, before adding 100 μ l of internal standard to 100 μ l of HM. The internal standard consisted of 12 ng/ml cortisol d4, 60 mg/ml corticosterone d8, 20 pg/ml of Estradiol and 20 pg/ml testosterone-d3, prepared in milli-Q water. Steroids were then extracted using 1 ml ethyl acetate (Merck, Darmstadt, Germany); and the top organic layer was removed and vacuum dried (Savant, SC250 EXP, Thermo Scientific, United States) for ~ 2 h. The dried residues were reconstituted with 80 μ l of 50/50% methanol (Merck, Germany)/water and transferred to HPLC injector vials.

Liquid chromatography-tandem mass spectroscopy

The HPLC Mass Spectrometer used a Surveyor MS pump and auto sampler followed by an Ion Max APCI source on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer, all controlled by FinniganX caliber software. (Thermo Electron Corporation, San Jose, CA.) The mobile phase was methanol-water gradient starting at 50:50 (v/v) (peaking at 80:20 before returning to 50:50) at flow the rate of 500 μ l/min. The chromatography was performed at 40 °C. The instrument was set up at selection reaction monitoring (SRM) mode; m/z 363.1 \rightarrow 121 at 24 V for cortisol; 363.1 \rightarrow 163 at 24 V for cortisone; 289.17 \rightarrow 97.2 at 28 V for testosterone, 255.14 \rightarrow 159.2 at 18 V for estradiol, 315.18 \rightarrow 109.2 at 26 V for progesterone. Argon gas was used as the collision gas at 1.2 mTorr for all the steroids. Steroids concentrations were calculated from standard curves generated for each steroid from the injection of standards; cortisol and progesterone 0.05–100 ng/ml, cortisone 0.025–50 ng/ml, testosterone and estradiol 5–10,000 pg/ml in methanol for each assay. Mean of inter

and intra-assay coefficient of variation for cortisol were 12.87% and 4.93%; cortisone 12.40% and 6.08%. All samples were measured in blinded fashion.

Statistical analysis

The concentration of cortisol and cortisone in milk were calculated and reported in ng/ml. Descriptive statistics on continuous variables are presented as mean and SD, while counts and percentages are used for categorical variables. Descriptive summaries were produced for three breast-feeding groups (BF), to compare the differences between BF groups. The chi-square test was used for categorical variables while one-way analysis of variance (ANOVA) was used for continuous variables. Univariate analyses were carried out to determine relationships between baseline characteristics and HM glucocorticoids.

The Pearson correlation coefficient was used to analyse the association between the calculated average value of cortisol and cortisone of BM samples. Linear regression models were used to investigate the effect of maternal biological, social and environmental factors on the glucocorticoids levels of HM. Explanatory variables included in the linear regression were family structure, marital status, mode of infant delivery, gestational diabetes, maternal happiness, anxiety, depression, life satisfaction and total duration of breastfeeding. These variables were selected based on previous literature and from our univariate analysis. The beta coefficients, 95% confidence interval (CI), and p value are shown from the linear regression. Statistical significance from the linear regression was defined as p value < 0.05 . SPSS V.21 (IBM SPSS Statistic 21.1nK Chicago, Illinois, USA) was used for data analysis and all graphs were created using GraphPad Prism version 7.0.

Results

Participant characteristics

Maternal, child and human milk characteristics are reported in [Table 1](#).

Glucocorticoids variation of human milk and correlation between cortisol and cortisone

Of the major steroid hormones measured, cortisol and cortisone were present in all of the BM samples. Both hormones differ widely among mothers and within, however cortisone was the predominant glucocorticoid (9.55 ng/ml) and cortisol was less abundant (7.39 ng/ml). A positive correlation was found between cortisol and cortisone concentration in milk, which was statistically significant ($r = 0.60$ $p < 0.001$) ([Fig. 1](#)).

Glucocorticoids variation of human milk with maternal biological and socio-demographic factors

Maternal biological and socio-demographic factors affecting the milk glucocorticoid concentrations are illustrated in [Table 2](#). Maternal BMI (prior to pregnancy) was positively associated with cortisone concentration in milk; cortisone was significantly higher in normal weight mothers (9.82 ng/ml) compared to overweight (8.93 ng/ml) and underweight women ($p = 9.33$ ng/ml) ($p = 0.01$) ([Fig. 2](#)). Furthermore, no differences were detected between the concentration of cortisol and cortisone ($p = 0.45$ and $p = 0.26$, after excluding unknown samples) in the milk samples obtained from women reporting either exclusive or partial feeding practices. Breast-feeding patterns showed no effect on the glucocorticoids concentration (cortisol & cortisone) of the breastmilk at infant's age

Table 1
Characteristics as mean \pm SD or % (n) of the mothers, children and breast milk in the STEPS Study.

Variable	% of n	Mean \pm SD
Maternal Biological Factors (n = 650)		
Mothers age, years		31.24 \pm 4.30
BMI before pregnancy		24.02 \pm 4.44
Overweight (including obese),	28.31 (186)	
Gestational diabetes,	8.81 (59)	
Mode of delivery, C section,	11.94 (78)	
Infant Gender, boys,	53.51 (351)	
Premature births (\leq 37 gestation weeks)	4.27 (28)	
Siblings, none (Siblings)	58.08 (381)	
Twins (n = 6 pairs), twins	1.83 (12)	
Maternal Social and Environmental Factors		
Marital status, married,	63.65 (412)	
Length of relationship (months)		84.57 \pm 56.80
Family structure, nuclear family	89.8 (589)	
Education, advanced	71.12 (477)	
Occupational class at least professional	69.96 (393)	
Living in urban area (city of Turku)	48.69 (319)	
Family incomes, high income	47.09 (304)	
Happiness (scale 0–10)		8.32 \pm 1.34
Anxiety (scale 0–10)		7.58 \pm 2.61
Depressiveness (scale 0–10)		8.08 \pm 2.71
Satisfaction, life situation (scale 0–5)		4.40 \pm 0.72
Satisfaction, relationship (scale 0–5)		4.26 \pm 0.79
Satisfaction, housework at home (scale 0–5)		3.88 \pm 0.97
Breast feeding and HM		
Infant age HM sample collected		2.60 \pm 0.60
Milk samples collected during Winter season (Oct–Feb),	56.86 (373)	
Exclusive BF at the HM sample collected,	51.07 (335)	
Exclusive BF, months		2.90 \pm 2.05
Age introduction of solid foods, months		4.30 \pm 0.95
Total BF, months		10.00 \pm 4.48
Cortisol (ng/ml)		7.39 \pm 5.97
Cortisone (ng/ml)		9.55 \pm 3.44

Advanced education (highest level of vocational training (such as a 4-year programme of a polytechnic institute) or any academic degree (bachelor's, master's, licentiate or doctoral degree) vs. lower education Classification: Professionals (in high positions, e.g. managerial, but also in intermediate positions, such as nurses) vs others [blue-collar workers (in industry or agriculture) and service (e.g. clerical and sales workers)]. Aggregation of income of family members: high income, \geq 3000 €/month net; low income, $<$ 3000 €/month net.

of 3 months (Fig. 3). Also, maternal age, weight, gestational diabetes, mode of delivery were the non-significant predictors of glucocorticoids composition of HM (data shown in Table 2).

Maternal social and environmental factors including family structure, marital status, education, occupation, family income and living in city or rural area had no effect on milk glucocorticoid composition (data for both cortisol and cortisone is shown in

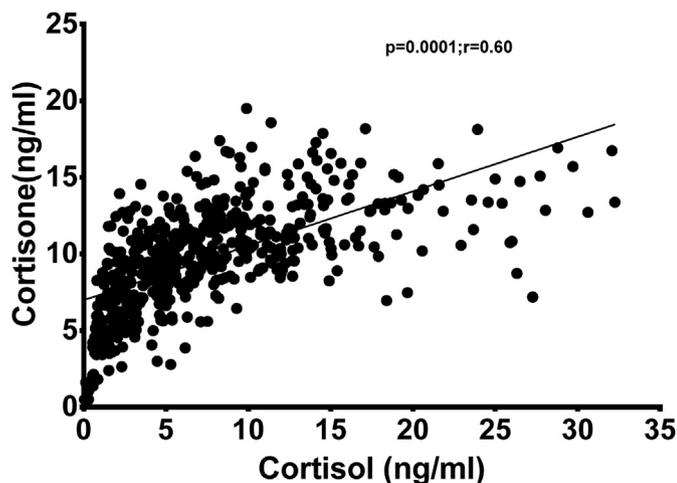


Fig. 1. Residual scatter plot and fitted regression line for the HM cortisol versus HM cortisone concentration at 3 months of established lactation.

Table 2). Furthermore, maternal happiness, anxiety, depression and life satisfaction score collected four months postpartum were non-significant predictor of glucocorticoids variation in HM samples. In order to examine the effect of environment, we investigated the effect of seasonal variations on milk glucocorticoid composition between winter (October–February) and summer (March–September) season, and no significant association was found for the seasonal variation. Results of the association between glucocorticoid concentration and maternal social and environmental factors are presented in Table 2.

Glucocorticoid variation of human milk with infant characteristics

Cortisone levels between infant gender showed trend towards significance ($p = 0.08$); cortisone levels were higher among milk samples taken from mothers with a girl child (9.80 ng/ml) compared to mothers who had a boy child (9.33 ng/ml). Infant birth weight, height, gestational weeks, being born as twin and number of siblings were the non-significant predictor of the glucocorticoid variation in HM.

Based on the results from Table 2 and from literature, we conducted regression models. In model 1, we compared the effect of premature birth and gestational diabetes on milk glucocorticoid concentration. After adjusting for gestational diabetes, we found that premature birth was a significant predictor of both cortisol ($p = 0.04$) and cortisone ($p = 0.01$) in HM. In model 2, education ($p = 0.05$) was found to be the significant predictor of cortisol increase in HM after adjusting for occupation class, family structure and family income. None of these predictors was found to be

Table 2
Univariate association of maternal and infant characteristics and milk glucocorticoids (cortisol and cortisone).

Categorical Variable	Cortisol ng/ml (mean ± SD)	p value	Cortisone ng/ml (mean ± SD)	p value
Maternal demographics				
Maternal weight				
Overweight/obese	7.40 ± 6.13	0.96	8.93 ± 3.32	0.01
Normal weight	7.36 ± 5.86		9.82 ± 3.44	
Under weight	7.68 ± 6.77		9.33 ± 3.79	
Gestational diabetes				
No	7.34 ± 5.91	0.39	9.57 ± 3.43	0.77
Yes	8.04 ± 6.56		9.43 ± 3.46	
Mode of delivery				
Normal	7.29 ± 6.00	0.16	9.49 ± 3.42	0.19
Caesarean	8.30 ± 5.77		10.03 ± 3.51	
Family structure				
Single	7.30 ± 6.06	0.80	10.20 ± 3.86	0.52
Nuclear	7.59 ± 5.91		9.50 ± 3.43	
blended	4.74 ± 1.12		9.76 ± 3.15	
Marital status				
Married	7.29 ± 6.06	0.80	9.51 ± 3.45	0.49
common law	7.59 ± 5.90		9.54 ± 3.33	
others/single	6.93 ± 4.74		10.50 ± 4.28	
Education				
Low	7.76 ± 6.38	0.33	9.56 ± 3.70	0.95
Higher	7.25 ± 5.81		9.54 ± 3.34	
Occupational class				
Others	7.46 ± 6.03	0.45	9.62 ± 3.81	0.30
Professionals	7.05 ± 5.84		9.30 ± 3.32	
Living in urban area				
Living in city	7.60 ± 6.07	0.42	9.74 ± 3.38	0.19
Not living	7.22 ± 5.89		9.39 ± 3.48	
Family incomes				
Low < 3000e	7.61 ± 6.51	0.34	9.50 ± 3.59	0.54
Higher, >3000e	7.16 ± 5.37		9.67 ± 3.31	
Happiness				
Not happy, below 6	5.60 ± 4.44	0.16	9.25 ± 2.79	0.59
Happy	7.47 ± 6.03		9.56 ± 3.43	
Anxiety				
Yes, below 6	7.76 ± 5.79	0.49	9.56 ± 3.39	0.98
No	7.33 ± 6.02		9.56 ± 3.43	
Depression				
Yes, below 6	7.13 ± 5.24	0.63	9.39 ± 3.34	0.60
No	7.47 ± 6.08		9.60 ± 3.44	
Life satisfaction (sum of 3 measures)				
No	7.19 ± 5.50	0.77	9.71 ± 3.21	0.71
Yes	7.44 ± 6.04		9.53 ± 3.45	
Infant and human milk demographics				
Seasons HM sample taken				
Winter (dark)	7.25 ± 6.03	0.49	9.41 ± 3.40	0.24
Summer	7.57 ± 5.90		9.73 ± 3.49	
Breast feeding group				
Exclusive	7.57 ± 6.22	0.70	9.73 ± 3.45	0.36
Partial	7.12 ± 5.89		9.39 ± 3.47	
Unknown	7.37 ± 5.36		9.29 ± 3.31	
Gender				
Boys	7.20 ± 5.61	0.38	9.33 ± 3.31	0.08
Girls	7.61 ± 6.37		9.80 ± 3.57	
Premature births				
≤37 weeks	8.37 ± 7.54	0.38	8.71 ± 3.84	0.19
Normal	7.35 ± 5.90		9.59 ± 3.42	
Siblings				
None	7.40 ± 6.02	0.97	9.49 ± 3.30	0.62
1 or more	7.38 ± 5.92		9.63 ± 3.63	
Twin sister/brothers				
No	7.41 ± 5.99	0.49	9.54 ± 3.44	0.49
Yes	6.22 ± 5.16		10.23 ± 3.62	
BMI before pregnancy				
Birth weight (g)	–	0.62	–	0.004
Birth length (cm)	–	0.20	–	0.81
Exclusive BF, months	–	0.12	–	0.73
Exclusive BF, months	–	0.45	–	0.13
Total BF, months	–	0.45	–	0.94

Statistical significance was defined as p-value <0.05.

significant for cortisone. Table 3 presents the results of linear regression analysis, with cortisol and cortisone in HM as the outcomes.

Discussion

In this large study population, we investigated the influence of breastfeeding practices and several maternal and infant related factors affecting the HM glucocorticoid composition. Our results showed a positive correlation between cortisol and cortisone, with lower concentration of cortisone in overweight women. We also found that premature birth and maternal socio-demographic factors, particularly education status plays a critical role in altering the glucocorticoid composition of HM. Interestingly, our results confirms that exclusive and partial breastfeeding behaviour had no effect on the milk glucocorticoid concentrations.

A major strength of the study is the large sample size with huge statistical databases related to both mother and infant. Several maternal and infant related biological factors were compared. Of all the biological determinants, only maternal weight and preterm birth were found to be positive predictors of glucocorticoid variations in HM. Our results showed that overweight women (mother with higher BMI at the time of pregnancy) had less cortisone in their milk, compared to normal and underweight mothers and cortisol level showed no differences. In the past, several studies have demonstrated significant associations between maternal weight and altered HM composition [29–31]. For instance, leptin concentration in HM increases with maternal adiposity [32], whilst adiponectin levels are inversely correlated with maternal degree of adiposity [33]. To our knowledge, this is the first study, which has investigated the impact of maternal body composition parameters on the milk glucocorticoid profile. Glucocorticoids are known to play an important role in regulating body fat distribution, and often obesity is characterised by increased secretion of glucocorticoids [34,35]. However, majority of these studies have only looked at the cortisol secretion in relation to anthropometry measures of adiposity, BMI (body mass index) and WHR (waist to hip ratio), and have yielded mixed results [36,37]; surprisingly, not much is known about the circulating concentration of cortisone. Unlike plasma, this study suggest cortisone as the predominant hormone in milk samples, since cortisone is not secreted in measurable amounts in maternal plasma, 11 βHSD (hydroxysteroid dehydrogenase) type-2 isoenzyme may be acting in the breast tissue or milk to convert cortisol to cortisone. However, the exact mechanism behind this phenomenon is still unknown. Recently, other studies in stress related disorders has suggested that chronic stress with prolonged hyperactivity of HPA axis, can evolve into hypo-secretion of glucocorticoids [38]. We also found that milk from overweight women had lower level of cortisol compared to normal weight women. However, this observation should be greeted as caution because these measurements were made in milk collected three months after the infants were born.

Preterm birth/delivery is the major cause of infant morbidity and mortality in the industrialised countries [39]. In the past decade, rate of premature birth has increased steadily, and mothers whose babies are born prematurely are often more stressed [40]. Several studies have investigated the relationship between maternal stress and child outcome, and linked maternal prenatal cortisol, as the positive precursor of preterm birth [41]. Yet, very few have investigated the role of premature birth on milk composition. Because human milk is highly dynamic, it is important to consider the factors that may alter its composition after established

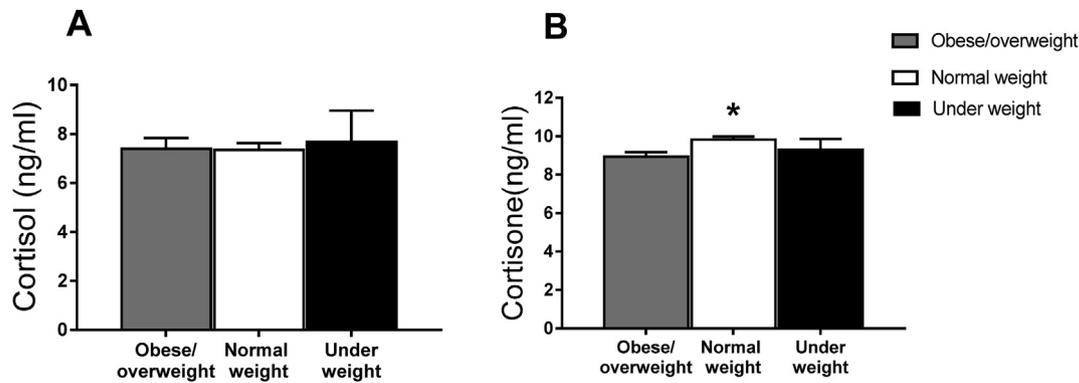


Fig. 2. Glucocorticoids, (A) cortisol and cortisone (B) concentration in the milk of overweight, normal and underweight lactating women at 3 months of established lactation. Difference between the groups are expressed as * $p < 0.01$.

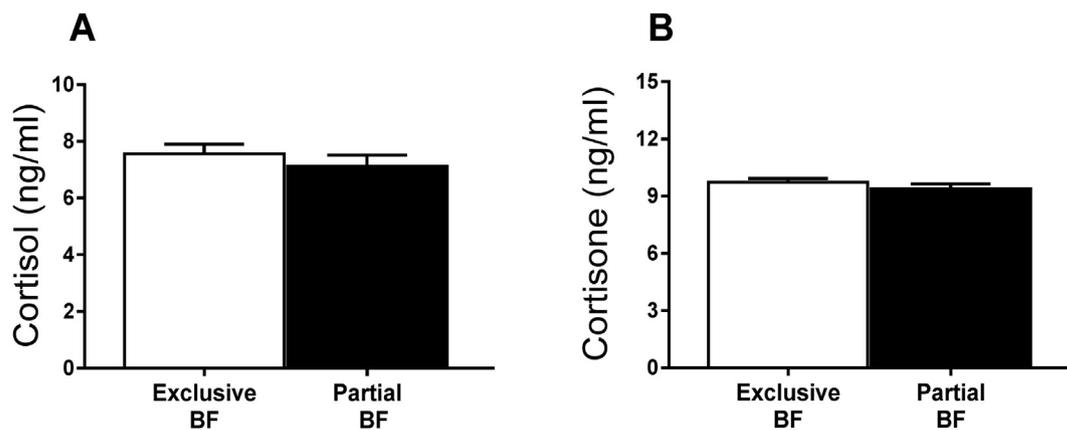


Fig. 3. Glucocorticoids concentration of HM (A) cortisol in HM with exclusive and partial breastfeeding patterns, (B) cortisone in HM with exclusive and partial breastfeeding patterns.

Table 3
Bivariate association of maternal factors with milk glucocorticoids (cortisol and cortisone).

	Cortisol ng/ml			Cortisone ng/ml		
	β	se	p value	β	se	p value
Model 1						
Premature births						
Preterm (≤ 37 weeks)	8.56	4.29	0.04	5.92	2.45	0.02
Normal	ref					
Gestational diabetes						
No	-0.43	0.82	0.6	0.39	0.47	0.40
yes	ref					
Model 2						
Education						
Low	12.21	6.2	0.05	-0.83	3.65	0.82
Higher	ref					
Occupational class						
others	9.105	6.2	0.14	1.23	3.65	0.73
Professionals	ref					
Family structure						
single	1.094	6.2	0.86	2.26	3.65	0.53
nuclear	0.436	1.91	0.82	-0.42	1.12	0.70
blended	ref					
Family incomes						
Low < 3000e	2.15	3.24	0.5	0.40	1.9	0.83
Higher, >3000e	ref					

breastfeeding. In the current study, cortisol was measured in HM samples taken 12–16 week post-birth. Our analysis showed that mother of premature infants, had significantly higher level of cortisol and cortisone in their milk, after adjusting for gestational

diabetes. These results indicate the influence of preterm birth trauma on mother milk glucocorticoid concentration. On contrary, a study by Voorn et al. [9] showed mothers giving birth to preterm infant had lower concentration of milk glucocorticoids compared to the mothers, who delivered full term. However, the significance of our results disappeared when adjusted for other maternal factors. The fact that significance disappeared, showed that preterm birth may not be an independent factor responsible for changing milk glucocorticoid composition, three months after infant birth but could be used for designing larger studies targeting mainly at exploring whether the preterm birth independently affect milk cortisol and cortisone concentration and its relevant effect on breastfeeding infants.

In addition to several biological factors, numerous social and psychological factors are also known to increase cortisol secretion by enhancing HPA axis activity. Different lifestyle factors and social status demands on breastfeeding mothers, further influencing their HPA axis activity pattern. Previous studies have examined the relation between socio-demographic factors and glucocorticoids. However, these results are often with mixed findings. This is the first study to show the effect of maternal social and psychological factors on both cortisol and cortisone level in mothers' milk. Our results showed a positive association between low maternal education and milk cortisol level. Lower maternal education was significantly associated with higher cortisol even after adjusting for several other social factors including maternal occupation, family structure and family income. However, no effect was seen for cortisone. Our results are in accordance with other studies, which

suggest that education plays a critical role in individual stress biology. Briag et al. [42] showed positive association between hair cortisol and low maternal education. On contrary, some previous reports suggests that lower education is associated with lower levels of early morning salivary cortisol [43]. Whereas, working mother with children at home, independent of marital status and social support showed increased urinary cortisol compared to working mothers with no children measurement of 24 working mothers [44], indicating overexpressed HPA axis. Our study, also showed similar result, and demonstrated that mothers either working in industries or agriculture or as a service worker had higher level of cortisol and cortisone in their milk; however, these differences were not statically significant.

Despite all the benefits of breastfeeding, rates of exclusive breastfeeding declines rapidly, particularly within the first three months of infant feeding [45,46]. Numerous factors can influence breastfeeding duration, and establishment of mixed feeding practices. Yet, very little is known about the impact of breastfeeding practices on the composition of mother's milk, particularly glucocorticoids levels of the breastmilk. To our knowledge, this is the first study that has investigated the impact of exclusive versus partial breast-A on the glucocorticoid concentration of mother's milk. Contrary to the hypothesis, our results showed that feeding practices had no impact on milk glucocorticoid concentration. Both cortisol and cortisone were similar between exclusive and partial breastfeeding groups, indicating no role of breastfeeding practices on the hormonal composition of the milk. Exclusive breastfeeding is known to reduces maternal stress (both physiological and psychological) and enhance more positive mood in mothers, compared to the formula feeder [47]. Previous studies have shown no significant effect of breastfeeding on the serum cortisol concentration. However, stress hormones are higher in lactating mothers compared to non-lactating healthy women, 4–6 weeks postpartum [26,47]. Our study provides a better insight about the influence of common breastfeeding practices might have on the glucocorticoids composition of HM, around three months post-birth. This study could be an extension for several other studies, who failed to explain the impact breastfeeding practices on the stress level of mother's milk, few months into already established breastfeeding.

A major strength of the study is its large sample size ($n = 650$), with overall inclusion of 80% collected milk samples [28], allows for high power in our statistical analyses. Furthermore, the use of highly sensitive and reliable LC-MS/MS techniques enabled precise measurement of both cortisol and cortisone in HM. However, our study has some limitations. Being assessed from a large population based cohort, milk was only collected at a single time point and do not reflect resting glucocorticoids expression. Nevertheless, data of child anthropometry at 13 months and 2 year of age, that can be further analysed and have the possibility to further explore the effect of HM hormones on the children who were breastfed for longer periods post-birth.

Determining the physiological variation of glucocorticoid in mothers' milk might be suitable for assessing the extent to which maternal social, biological and environmental factors causes stress in breast-feeding mothers', affecting child's welfare. From this study, we can conclude that HM cortisol and cortisone levels within this cohort are not altered by differences in feeding patterns; but preterm birth and maternal education status along with other social-arrangement appear to play an overlooked role in milk glucocorticoid composition. Although, the mechanisms is still unknown but these findings provide insight into the unappreciated roles of social and biological factors in orchestrating the hormonal composition of mothers' milk. This study gives us more understanding about how and when maternal stress may affect her milk composition, helping us further to acknowledge the role

of HM and glucocorticoids in optimising infant growth and development.

Conflicts of interest

The authors declare no potential conflict of interest with respect to the research, authorship and publication.

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S.P. carried out laboratory analysis and data interpretation and drafted the manuscript. J.M assisted with statistical analysis and contributed to the manuscript development. A.N provided assistance with laboratory analysis. N.J conceptualized and designed the study, coordinated and supervised data collection (psychological data). C.R.W provided with valuable feedback on the manuscript. K.L assisted in developing this collaboration and sample collection. D.C.S designed research question and supervised all aspect of the study. H.L designed samples and background information collection, assisted with data analysis and manuscript development. All authors approve the submission of this manuscript for peer review. KL assisted in developing this collaboration and samples collection.

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