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Exploring role of maternal and infant determinants on glucocorticoid composition of human milk

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Master of Science (Food Science & Biochemistry)

A thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy in Health Sciences, the University of Auckland, 2017.
Abstract

Human milk (HM) is a dynamic and multifaceted complex mammary secretion, optimised for the nourishment and immune protection of infants. The exact nutritional composition of HM is highly variable. The health, stages of development, physiological and psychological status of both mother and infant are major contributors to this variability, and may coincide with the needs of the feeding infant.

HM is enriched with non-nutritive maternally originated hormones. Their biological roles in infants are not well defined, although they may alter infant physiological and psychological functions. Emerging evidence shows maternal originated milk-borne glucocorticoids play critical roles in affecting offspring metabolic programming and behavioural development. To date, studies have focused on the impact of maternal social, biological and environmental factors on macronutrient content of milk such as lipids, proteins and carbohydrates. Despite the importance of HM bioactive compounds, studies investigating milk glucocorticoids are quite limited, with focus on a small number of factors influencing its levels in mother’s milk.

The object of this thesis was to investigate glucocorticoids composition of HM with respect to maternal biological and social factors. Given the current void in understanding the roles of these glucocorticoids on infant health, some analysis of the relationship between infant characteristics and HM glucocorticoids was also undertaken.

The first study involved a large collaborative analysis of selected maternal factors, potentially important in determining the concentration of HM glucocorticoids. This study analysed the impact of a wide range of maternal social, biological, and environmental related factors on milk glucocorticoids composition in HM samples, collected at 3 months of established lactation. From 650 milk samples, we demonstrated that glucocorticoid concentrations were influenced by maternal weight, maternal educational status and preterm birth. Interestingly, HM glucocorticoids composition did not differ based on the individual preference of mothers feeding their infants exclusively or partially.

The second study examined the glucocorticoid concentrations in a cohort of women who gave birth to premature infants. This study also demonstrated that gestational age has a significant impact on milk cortisone concentration, but showed no relation to infant postnatal age. With further method development, it was possible to separate the free versus conjugated (or bound) glucocorticoids. It was demonstrated that the majority of glucocorticoids (cortisol 76% and cortisone 84.2%) in these samples were in the free (and hence biologically available) form.

Given the results of the above studies, we may conclude that there are many factors that affect the variation of milk glucocorticoids. And as far as concentration of cortisol is concerned, the time of the day (circadian rhythms) could be a major predictor. Therefore, a third study was done to examine the HM glucocorticoid concentration over a period of 24-hours, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Both predominant glucocorticoids, cortisol (1.60 ± 0.71 ng/ml) and cortisone (3.40 ± 1.18 ng/ml), exhibited a pronounced 24 hour pattern. This was characterised by the rapid increase in the cortisol and cortisone levels in the early morning hours followed by a gradual
decline throughout the day, mirroring the well-established plasma glucocorticoids circadian pattern. Furthermore, duration of a feed, and difference between feeding breast (breast sides) seemed to have no impact on glucocorticoid concentration in the milk samples collected prior to and again immediately upon the cessation of infant feeding.

The last study of this thesis investigated the glucocorticoids composition of HM during the first 12 months of lactation, with a further analysis of the relationship between glucocorticoids and infant growth. Milk samples were collected at 2, 5, 9 and 12 months of infants’ age. We saw no significant relationship between HM glucocorticoids (cortisol and cortisone) and maternal characteristics (maternal weight, height, BMI and percentage fat mass) and infant growth outcomes (infant height, weight, BMI head circumference and percentage fat mass).

These studies collectively demonstrated the fluctuating profile of HM glucocorticoids. Presence of glucocorticoids in milk demonstrated a circadian pattern, implying that infant feeding time might be an important factor. Additionally, the glucocorticoids concentration in HM was not influenced by lactational stage. Interestingly, maternal and infant related biological and social factors, including preterm birth contributed to the alteration of milk glucocorticoids levels. Cortisone was shown to consistently be the predominant glucocorticoid in HM. This is in contrast to what is found in maternal plasma, suggesting a selective modification or concentration in the human breast milk. Only few studies have measured cortisone levels and hence little is known of its biological function. Findings of this thesis emphasise the need for further investigation on HM hormonal composition and how it may impact infant growth and development, in later life.
Dedication

This thesis is dedicated to my parents

Dr C.S. Pundir and Snehlata Pundir
Acknowledgements

While my name may be alone on the front cover of this thesis but there are many people behind this piece of research, who deserve to be thanked and acknowledged – lactating mothers; collaborators; encouraging supervisors; generous academic and philanthropic funders; supporting family; and an inspiring daughter and incredibly supportive partner.

I am extremely grateful to my supervisor Professor David Cameron-Smith for his continuous support right from my master's through to my PhD. He always gave me the opportunity to make independent decisions and challenged me to be more rigorous and a professional researcher. I would always be thankful to him for his compassionate and optimistic outlook throughout the process. I would also like to thank my co-supervisor Associate Prof. Clare Wall, for constantly encouraging and fostering my interest in human milk research.

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<tbody>
<tr>
<td>AA</td>
<td>Amino acids</td>
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<tr>
<td>Amino acid</td>
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<tr>
<td>AAP</td>
<td>American Association of Paediatrics</td>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>ALA</td>
<td>$\alpha$-linolenic acid</td>
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<tr>
<td>AME</td>
<td>Available milk energy</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BAC</td>
<td>Biologically active compounds</td>
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<td>BF</td>
<td>breast-feeding</td>
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<td>BMI</td>
<td>Body Mass index</td>
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<td>BMIP</td>
<td>Body mass index percentile</td>
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<tr>
<td>C-section</td>
<td>Caesarean section</td>
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<tr>
<td>CBG</td>
<td>Corticosteroid-binding globulin</td>
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<td>Complimentary feeding</td>
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<td>CoA</td>
<td>Coenzyme A</td>
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<td>CRH</td>
<td>Corticotrophin releasing hormone</td>
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<tr>
<td>CRH</td>
<td>Corticotropic releasing hormone</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<tr>
<td>DOHaD</td>
<td>Developmental Origin of Health and disease</td>
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<tr>
<td>EAA</td>
<td>Essential amino acids</td>
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<td>EAA</td>
<td>Essential amino acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>EPA</td>
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<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
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<td>GH</td>
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<td>Glc</td>
<td>D-glucose</td>
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<td>GlcNAc</td>
<td>N-acetylg glucosamine</td>
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<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
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<td>GR</td>
<td>Glucocorticoids receptor</td>
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<td>Abbreviation</td>
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<tr>
<td>HM</td>
<td>Human milk</td>
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<td>HMOs</td>
<td>Human milk oligosaccharides</td>
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<td>HPA</td>
<td>Hypothalamic pituitary adrenal axis</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>HSD</td>
<td>11 β hydroxysteroid dehydrogenase</td>
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<tr>
<td>IgA,</td>
<td>Immunoglobulin A</td>
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<tr>
<td>IGF</td>
<td>Insulin like growth factor</td>
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<tr>
<td>LA</td>
<td>Linoleic acid</td>
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<tr>
<td>LBW</td>
<td>Low birth weight</td>
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<tr>
<td>LCFAs</td>
<td>Long chain fatty acids</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
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<tr>
<td>LCPUFAs</td>
<td>Long chain poly unsaturated fatty acids</td>
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<td>LSE</td>
<td>Low socio-economic standard</td>
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<tr>
<td>LSES</td>
<td>Low socio-economic standard</td>
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<td>MFGM</td>
<td>Milk fat globule membrane</td>
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<td>mins</td>
<td>Minutes</td>
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<td>ml</td>
<td>Millilitre</td>
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<td>MR</td>
<td>Mineralocorticoids receptor</td>
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<td>NAMA</td>
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<td>NEC</td>
<td>Necrotizing enterocolitis</td>
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<tr>
<td>NeuAc</td>
<td>N-acetylneuraminic acid</td>
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<tr>
<td>ng</td>
<td>Nano gram</td>
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<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>NPN</td>
<td>Non protein nitrogen</td>
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<td>NZ</td>
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<td>NYBA</td>
<td>New Zealand Breastfeeding Authority</td>
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<td>ORS</td>
<td>Oral rehydration solution</td>
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<td>Oxt</td>
<td>Oxytocin</td>
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<td>PC</td>
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<td>PROBIT</td>
<td>Promotion of Breastfeeding Intervention Trial</td>
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<td>PS</td>
<td>Phosphatidylserine</td>
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<td>PTH</td>
<td>Parathyroid hormone</td>
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<td>PTHrP</td>
<td>Parathyroid hormone related protein</td>
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<td>PUFAs</td>
<td>Poly unsaturated fatty acids</td>
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<td>RDA</td>
<td>Required Dietary Association</td>
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<td>RER</td>
<td>Rough endoplasmic reticulum</td>
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<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>SES</td>
<td>Socio-economic standard</td>
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<tr>
<td>SHBG</td>
<td>Sex hormone binding globulin</td>
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<tr>
<td>sIgA</td>
<td>Secretory IgA</td>
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<tr>
<td>SRM</td>
<td>Selective reaction monitoring</td>
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<tr>
<td>TAAAs</td>
<td>Total amino acids</td>
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<td>TAG</td>
<td>Triacylglyceride</td>
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<tr>
<td>TCA</td>
<td>Tricarboxylic cycle</td>
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<td>TSST</td>
<td>Trier social stress test</td>
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<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Last updated: 19 October 2015
Chapter 1: Introduction
1.1 Introduction

Over 160 million years ago, mammals appeared with a key-defining feature of synthesising milk to nourish their offspring. In humans, most mothers nourish their infants using maternal milk throughout the first critical stages of their life. Human milk (HM) is a highly complex mixture of proteins, lipids, carbohydrates, minerals and vitamins (1). These compounds act in a synergistic manner to potentially influence the growth, survival and behaviour of an infant (2). Evolutionary success of HM is largely based on its ability to remarkably change its composition at every stage of lactation, within feeds, diurnally and between mothers, to tailor the individual needs of a growing infant (3–5). Interestingly, consumption of HM via breastfeeding protects infants against several infections such as diarrhoea and pneumonia, while also significantly reducing the rates of infant mortality and morbidity (5–8). Therefore, health agencies around the world such as the World Health Organisation (WHO), and the American Association of Paediatrics (AAP) endorses exclusive breastfeeding for the first six months of infants’ life, to optimise the exact benefits of HM, and continue to do so for the next two years, along with the introduction of complementary foods (9). Thus, it is widely believed that human breast milk and breastfeeding are the ideal source of infant nutrition (10–12).

Given the importance of HM in infant survival, significant amount of research has focused on understanding the complexities of HM. Despite the extensive existing knowledge of milk complexities there remains unanswered, particularly about its dynamically regulated composition and the impact of compositional variations on the growth and health of a breastfeeding infant. With the advent of new and modern technologies, HM has been increasingly recognised to contain many biologically active compounds, that are not traditionally regarded as nutrients. This include the major class of hormone - glucocorticoids (2,13,14). HM glucocorticoids have been demonstrated to affect infant behaviour, physiology, and metabolic programming, discussed later in this thesis.

Therefore, the purpose of this review is to examine the complexities of HM and explore the impact of maternal and infant related factors may have on milk profile. This includes initially addressing the mechanisms governing lactation and the physiology of milk secretion. Further, this literature review will examine the existing status of glucocorticoids in HM, which has helped to construct the scope of this thesis.
1.2 Breastfeeding

For centuries, women around the world have been nurturing their babies with HM, as the most natural source of nutrition. HM is a personalised biomaterial, uniquely suited to the needs of human infants, making it superior for feeding new born infants.

Breastfeeding refers to the process of feeding milk to an infant directly from the mother’s breast and is beneficial for both mothers and infants, with improvements evident in metabolic health outcomes in later life (15). The importance of breastfeeding for growing infants has become a major area of research associated with Developmental Origins of Health and Disease (DOHaD) (16).

The WHO, divides breastfeeding into three main categories: exclusive breastfeeding, partial breastfeeding and artificial feeding (17). Exclusive breastfeeding is a process, where an infant receives all his or her nutrition from mothers’ milk or any other liquid is given, except prescribed medications. During partial breastfeeding infants receive other fluids such as infant formula, water or any other solid supplements along with HM (18). Table 1-1 describes the definitions of other categories including predominant breastfeeding and artificial breastfeeding.

Exclusive breastfeeding is the normative standard, against which all other preferred infant feeding methods must be measured, and according to public health standards, direct breastfeeding is at the top of hierarchy followed by expressed milk, donor breast milk and formula feeding (19,20). In the past few decades, evidence behind the importance of these breastfeeding recommendations has advanced prominently, indicating the benefits of breastfeeding. These are described in the section below, explaining the short and long-term benefits of breastfeeding along with the controversies associated with the duration and exclusiveness of breastfeeding.
Table 1-1: Classification of breast-feeding categories

<table>
<thead>
<tr>
<th>Category of feeding</th>
<th>Basic diet of infant</th>
<th>Possible additives</th>
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<tbody>
<tr>
<td>Exclusive breastfeeding</td>
<td>Human breast milk (including expressed breast milk or donor milk)</td>
<td>Medications</td>
</tr>
<tr>
<td>Predominant breastfeeding</td>
<td>Predominantly human breast milk (including expressed breast milk or donor milk)</td>
<td>Water, water based juices, oral rehydration solution (ORS), medications, supplements</td>
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<tr>
<td>Complementary breastfeeding</td>
<td>Human breast milk (including expressed breast milk or donor milk) and baby food</td>
<td>Baby food or liquid including formula milk</td>
</tr>
<tr>
<td>Artificial feeding</td>
<td>Feeding infant with other than mothers milk</td>
<td>Infant formula milk</td>
</tr>
<tr>
<td>Bottle feeding</td>
<td>Baby food (liquid or semi-solid food) either from a bottle or direct</td>
<td>Human breast milk</td>
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1.2.1 Benefits of breastfeeding

Numerous epidemiological studies support the importance of exclusive breastfeeding for both mother and infant, including reduced rate of mortality and morbidity in breastfed children (11). Therefore, the health agencies around the world including the WHO, NHMRC and AAP endorses exclusive breastfeeding for the first six months, followed by breastfeeding supplemented with complementary foods, as the ideal source of infant feeding (11,21).

1.2.1.1 Infant health and well being

Breastfeeding has well established short- and long-term benefits for infants. The short-term health benefits include reduced risk of respiratory tract infections, sepsis, asthma, atopic dermatitis, gastroenteritis, and inflammatory bowel diseases (22–26). Long-term benefits include reduced risk for obesity and diabetes (26–30).

Shorter duration of breastfeeding is associated with reduced risk of mortality and morbidity in infants. Infants’ breastfed exclusively for 3 – 6 months showed 50% reduction in the risk of developing otitis media, 64% reduction in gastrointestinal infection and 72% reduction in the risk of hospitalisation compared to formula fed infants (22). Evidence suggests that breastfeeding reduces the risk of sudden infant death syndrome by almost 50% at all ages (31,32). A pooled analysis of studies carried out in less developed or developing nations, demonstrated the positive impact of promoting breastfeeding on initiation, duration, and exclusivity of feeding, particularly in the first 12 months of life. They showed that exclusively breastfed infants had reduced risk of gastrointestinal infection and atopic eczema (23,33).

Building upon the evidence of short-term benefits of breastfeeding, many researchers explored possible benefits of long-term breastfeeding, which are modest but consistent. Breastfeeding for more than three months provides protection against childhood obesity (28), type-1 diabetes (34),
type-2 diabetes (30) and cardiovascular diseases (35). Furthermore, studies suggest that innate immune factors in HM provides enhanced protection against infectious disease or infections and further reduces the risk of chronic diseases in later life, such as autoimmune disorders, compared to the formula fed infants (36,37). Additionally, exclusively breastfed infants for 6 months or longer consistently demonstrate significant improvement in academic performance at 10 years of age, however gender biases and benefits were more obvious in boys (38).

1.2.1.2 Maternal health and well being

In addition to providing benefits to breastfeeding infants, lactation is also capable of improving maternal health outcomes. Women who breastfeed, have been shown to experience lower risk of postpartum haemorrhaging (39) and lower rates of ovarian (40) and breast cancer later in life (25,41,42). Longer duration of breastfeeding reduces the risk of ovarian cancer in lactating women by 30%, while exclusive and predominant breastfeeding increased the period of lactational amenorrhea (43).

In addition to being a biological process, breastfeeding is also influenced by complex array of social, emotional and psychological factors; while establishing a strong bond between mother and her infant (44). Evidence suggests that breastfeeding plays a vital role in enhancing maternal sensitivity and responsiveness to the cues of their infants, and aid in establishing a strong bond between mother and infant duo (45). Maternal depression during pregnancy contributes to cessation of breastfeeding. Whilst, other studies demonstrated a significant association between breastfeeding and improvement in postpartum depression, by the increased involvement of mother with infant and self-efficacy (46,47).

Breastfeeding also provides economic and environmental benefits (12,48). Economic benefit includes potential reduction of medical expenses and an economical and microbiologically safe way of infant feeding, compared to bottle-feeding. It also cuts back the cost of infant formula and other alternate feeding methods and reduces the risk of contamination (18,49).

In conclusion, all the health experts’ consultations globally conclude that exclusive breastfeeding confers several short and long-term benefits to both mother and infant. Recommendations therefore suggest continuance of breastfeeding for longer periods post infant birth. However, for a detailed analysis of breastfeeding benefits associated with mothers and infants have been reviewed elsewhere (12,50,51).

1.2.2 Breastfeeding trends

1.2.2.1 Global breastfeeding trends

While the rate of exclusive breastfeeding remains low globally, current trends indicate modest improvements in exclusive breastfeeding rates from 33% in 1995 to 37% in 2014 (25). According to UNICEF, South Asia has demonstrated the most significant progress with more than 17% increase between 2000 and 2015. Similar trends were observed in India reporting 46.4% of exclusive
breastfeeding rates between 0-6 months (52), Pakistan with 37.1 % (53), Bangladesh with 42.5% and Nepal with 53.1% (54). Despite the well-recognised benefits of breastfeeding, exclusive breastfeeding is below the expectations of WHO (55). Widespread use of HM substitutes have had a massive impact on breastfeeding rates, particularly in China, where breastfeeding rates fell to its lowest from 80% in 1980 to 44% in 1990 (56).

According to the latest breastfeeding information from the Centre for Disease Control and Prevention (CDC), prevalence of breastfeeding initiation rates in United States were high with more than 75% of newborn received HM at birth, but only 49% were exclusively breastfed at 6 months (57). The United Kingdom (UK) has had the lowest breastfeeding rates in Western world with only 34% of infants’ breastfed. Furthermore, only a small portion of women (1%) adhered to the global recommendation of six months exclusive breastfeeding (58). In Australia, 60% received HM at six months, and only 15% were exclusively breastfed at five months (59); alternatively in Canada, most women initiate breastfeeding (88% initiate breastfeeding) with only 26% of women breastfeeding exclusively at six months (60).

1.2.2.2 New Zealand breastfeeding trends

In the past three decades, rates of breastfeeding have risen significantly throughout the world. According to the data collected by New Zealand Breastfeeding Authority (NZBA), approximately 80% of infants received some HM during the first six-weeks of their life (61). Even though increased rates of breastfeeding initiation are encouraging, this number reduces considerably before reaching the landmark of six months exclusive breastfeeding, recommended by Ministry of Health, New Zealand. Only 17% of infants were exclusively breastfed, until the first six months of their life in contrast to 56% ever breastfed infants (62). A significant decline was observed in the prevalence of exclusive breastfeeding within the first six months, particularly in Maori and Pacific Island women. Maori women discontinue breastfeeding earlier than European women, due to ongoing effects of increased urbanisation and current socio-economic status (63). However, prevalence of breastfeeding in the Pacific Island community is slightly higher (12% at six months) than Maori population (9% at six months) (18).

1.2.2.3 Optimal duration of exclusive breastfeeding

Considerable uncertainty exists about the optimal duration of exclusive breastfeeding. Some researchers maintain that exclusive breastfeeding for six months may lead to maternal nutritional burden and weight loss, particularly in malnourished mothers (64). Before 2001, recommendation for exclusive breastfeeding was between 4 and 6 months. However mothers, based on their perception about milk supply, fed their babies with complementary foods after four months (65). Following a systematic review by Kramer and Kakuma, the WHO made a global recommendation of promoting exclusive breastfeeding for the first six months of the infant’s life (66). This systematic review included 20 studies, and concluded that infants breastfed exclusively for six months were at the lower risk of adverse health effect and no deficit in growth, however these infants showed iron deficiency or were at the risk of being anaemic or iron deficient (66). Additional evidence suggests that the estimated iron intake of exclusively breastfed infants is not sufficient to meet their daily iron requirement (67)
and hence, according to some researchers introduction of complementary foods after 3-4 months is advisable (26).

Besides, prolonged breastfeeding can also delay the return of menses and lead to a rapid decrease in postpartum weight. This may be harmful for a malnourished mother, but considered a benefit in a well-nourished mother or obese women in developed countries (66,68). Furthermore, another systematic review by Lanigan et al (69) found no strong indication to either support or change to the existing recommendation for introducing solid food at six months. This clearly indicates the need for additional high quality randomised trials in different settings and selected population, to review the optimal duration of breastfeeding and formulate evidence-based recommendations (70).

1.2.3 Barriers to breastfeeding

Despite the known benefits of breastfeeding, prevalence of exclusive breastfeeding is still less than international recommended guidelines. Over the last century, breastfeeding has evolved from being the only viable option, to being one largely dictated by maternal preferences. Numerous socio-economic, physiological and biological factors contribute to the decisions made in favour of discontinuing breastfeeding, particularly within the first six months postpartum (71–74). However, maternal attitude towards breastfeeding is the key predictor of breastfeeding patterns. Mothers with strong beliefs and dedications are more likely to establish and maintain breastfeeding for long duration (75,76).

1.2.3.1 Biological factors affecting breastfeeding patterns

A decision around the duration and prevalence of breastfeeding is multifactorial in nature, however many biological factors play a significant role in the cessation of breastfeeding. For instance, multiple births, premature birth, infants with a special need, low birth weight infants', insufficient milk supply, nipple pain or mastitis (77). Numerous studies have revealed the reduction in the rate and duration of exclusive breastfeeding among the mothers with multiple births (78,79). Interestingly, reasons for discontinuing breastfeeding in multiples births were similar to those of singletons (single childbirth) such as sore nipples, limited time, and tiredness from continuous breastfeeding (80). Similar to the singleton mothers, mothers with twins have expressed concern about sufficiency of milk supply. However, evidence suggests that mothers of twins produce twice the amount of milk compared to singletons mothers (80).

Despite the benefit of HM for the survival and growth of premature or low birth weight (LBW) infants, only a small number of mothers sustain successful breastfeeding for longer duration (81). Mothers of preterm infants face numerous barriers to breastfeeding. These includes initiating and maintaining milk supply to switching from gavage feeding to breastfeeding (82,83). However, maternal physical and mental distress during the neonatal period, along with longer physical separation from frequent hospital visit were the most commonly cited reasons for unsuccessful establishment of continued breastfeeding of the preterm infants (84,85).
Unfortunately, certain medical conditions are considered contraindications to breastfeeding. These include viral and infectious disease states such as hepatitis C, if the nipples are bleeding or have cracks. Also if mothers are exposed to high levels of harmful chemicals including usage of recreational drugs, these can pose potential risks to infants (86).

1.2.3.2 Socio-economic and psychological factors affecting breastfeeding patterns

In addition to maternal biological factors, many socio-economic factors significantly affect the duration and preference towards breastfeeding. Longitudinal cohort studies have consistently established a negative correlation between maternal employment, anxiety, postpartum stress, and breastfeeding routines (87,88). Mothers in employment or those who have financial pressure to return to work are more likely to introduce formula milk feeding or premature weaning (89). In some cases, maternal desire to follow parent led routines or early demand for infant independence, are sometimes seen as a threat to direct breastfeeding, while bottle-feeding is seen as a more desirable alternative feeding method. These practices might have a negative impact on the duration of exclusive breastfeeding (90). Furthermore, lifestyle factors, including educational achievements, marital status and socio-economic status play a crucial role in determining breastfeeding patterns (91). Evidence indicates a significant positive association between breastfeeding and well-educated mothers from high socio-economic standard (92).

1.2.3.3 Postpartum depression and breastfeeding

The relationship between postpartum depression and breastfeeding is equivocal (46). In New Zealand, approximately 20-30% of mothers experience postpartum depression within the first three months of delivering an infant, impairing their ability to initiate or continue breastfeeding for recommended duration (47,93). Some studies suggest that breastfeeding may have a positive influence on the postpartum depressed mothers. Breastfeeding facilitates the regulation of hypothalamic-pituitary-adrenal (HPA) system, which gets deregulated during depression (47,94). Mother’s with high symptoms of depression are more likely to combine breastfeeding with infant formula milk feeding, and follow partial breastfeeding between 1-5 months (95). In contrast, several studies have linked unsuccessful breastfeeding as a potential risk factor for postpartum depression (96). Clearly, a better understanding of physiological steps involved in the mechanisms related to postpartum depression are required to be studied in detail (97).

1.2.3.4 Human milk substitutes

Invention of the first infant formula milk brought a revolution in the world of breastfeeding and made partial breastfeeding and infant formula milk feeding a popular choice. Although, breastfeeding rates are comparatively high in New Zealand, around 80% of mothers’ breastfeed their infants at hospital discharge. However, these rates tend to decline significantly within the first six months of infant’s life. There are several clinical and social factors responsible for this change (62,98,99). The most commonly cited reason is the misconception among lactating mothers about insufficient milk supply, further initiating infant formula milk feeding or early weaning (100,101). However, the invention of
formula milk and artificial breastfeeding are the biggest contributors for the shift in paradigm from breast to bottle.

Throughout history, mothers who were unable to breastfeed their infants employed wet nurses or used HM substitutes such as animal milk to nourish their babies. However, increased infant mortality due to various medical issues, such as mastitis, eventually made wet nursing, and HM substitutes a less popular choice among breastfeeding mothers. This created a void, and led to the invention of infant formula milk (102). Easy availability and smart advertising of infant formula milk played a critical role in the decline of breastfeeding (103).

Infant formula manufacturing companies targeted vulnerable groups of women, especially 2-4 weeks postpartum, by proclaiming a message that infant formula milk is a convenient, safe and reliable alternative to breastfeeding (104,105). Daily repetition of this message led to the rapid decline in breastfeeding rates, and reinforced the perception that bottle-feeding is the standard way of feeding an infant (106).
1.3 Anatomy and physiology of lactating mammary glands

Amongst mammals, offspring born to humans are highly dependent on their mothers for their nutritional needs. HM is the only biomaterial evolved to nourish human infants, exerting a strong and selective pressure on the biochemical evolution of lactation, which is characterised by the copious secretion of milk from mammary epithelial cells (107). Lactation represents the most energetically expensive process for all mammalian mothers, accounting for 30% of total energy intake (2). These demands are met by mobilising nutrient transfer from mammary glands tissue or circulation, which varies with maternal diet and her ability to reduce energy output (108).

1.3.1 Anatomy of the human breast

Lactation is defined as the cessation of reproductive period and beginning of lactogenesis. From the onset of pregnancy, mammary glands undergo numerous developmental changes and prepare to take over the role of nourishing a new born (109).

The female breast is a highly specialised apocrine sweat gland, located in the anterior thoracic wall. The base of the breast is situated vertically between second and sixth rib, and horizontally between sternum (medial) to the mid axillary line (lateral) (110). Development of mammary glands is divided into multiple stages, including embryogenesis (prenatal development) which begins in utero at the 6th week of gestation; infant breast; and pubertal development, later development during pregnancy; and finally involution. At birth, the mammary gland only consists of a rudimentary duct with small bud like ends. Though breast and ductal system branches develop with rest of the body growth, it only reaches it functional capacity during pregnancy and at childbirth (107).

The lactating breasts consists of glandular and adipose tissue, supported by flexible fibrous connective tissue called the Coopers' ligament as shown in Figure 1-1. Glandular tissue is comprised of nine lobes, which consist of lobules and alveoli. Each alveoli is lined with a layer of mammary epithelial secretory cells called lactocytes (111). They are the smallest cuboidal to columnar cells, responsible for synthesising and secreting milk into lumen of alveolus. These lumen drains into larger ducts that extend into the nipple in each lactating breast (107,110).
Anatomy of the exocrine milk producing glands, located in the anterior thoracic wall. Each mammary gland contains 15-20 lobes and each lobe is made up of many lobules. Alveoli are the basic unit of mammary gland (balloon shaped type cavity) lined with milk secreting epithelial cells that fills alveoli and connects to lactiferous milk ducts.

### 1.3.2 Human milk synthesis

During late pregnancy, milk synthesis is initiated in the lactocytes of the mammary gland. Several hormonal regulatory steps are involved in the initiation of milk synthesis and are categorised into two stages – lactogenesis stage I (secretory differentiation) and stage II (secretory activation) (112).

During lactogenesis stage I, alveolar cell are differentiated into secretory cells, and mammary epithelial cells, develop the capacity to synthesise milk constituents, such as lactose, casein and alpha-lactalbumin. Whereas stage II is characterised by the initiation of copious milk production at birth (30-40 hours postpartum). Milk produced during lactogenesis stage II is defined as colostrum, a yellowish thick fluid rich in antibodies, and other immunoglobulins. The milk produced during the later stages becomes thinner and pale, containing adequate levels of lactose and fats (113,114).

#### 1.3.2.1 Hormonal regulation of milk secretion

Various hormones including estrogen, prolactin (PL), progesterone (PG), glucocorticoids, insulin and oxytocin (Oxt), are secreted during pregnancy and throughout lactation. These hormones control various stages of lactogenesis involved in the maintenance of milk synthesis and production (Figure 1-2).
Sex steroid hormones, such as estrogen, initiates embryogenesis at 12 weeks before birth, and the lactation process starts under the influence of prolactin, placental lactogen, estrogen, PG and adrenocorticotrophic hormone (ACTH). At delivery, expulsion of the placenta causes a sudden drop of PG and the release of PL that triggers the secretion of milk. The rapid reduction of PG allows anterior pituitary glands to release more PL, essential for initiating and maintaining milk production. After lactogenesis II, milk production switches from endocrine control to the autocrine control system, where mammary papilla stimulation activates the hypothalamus, and inhibits dopamine, a prolactin inhibiting factor, resulting in increased levels of PL (109).

Lactation is an automatic process that starts with the progression of pregnancy, and is critically controlled by breastfeeding duration. Suckling causes sensory stimulation of nipples, which further signals posterior pituitary gland to release Oxt, a hormone primarily responsible for the ejection of milk. Oxt travels via the bloodstream to the breast, where it interacts with its receptors on myoepithelial cells causing the contraction of secretory cells within alveoli, and forcing the release of milk into ducts (111,115). However, reduced or no breastfeeding up to 24 to 48 h postpartum or failure to remove sufficient quantity of milk causes the shrinkage of mammary glands and initiates the involution process. Involution results in the death of alveolar tissue and secretory cells of the mammary glands, thus preventing the required hormones from reaching the breast and maintaining proper lactation (109).

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**Figure 1-2: Physiology of lactation. Adapted version from Neville MC, 2001.**

- **Embryogenesis**: Progesterone (PG), estorgen (E), prolactin (PL), steroids, growth hormone (GH) promotes lobular and alveolar duct system
- **Lactogenesis**: Insulin, glucocorticoids, thyroid, PL, GH and withdrawal of estrogen and PG initiates mammary development
- **Galactokinesis**: Infant suckling induces smooth muscle contraction and ejection of milk from mammary gland
  - PL and oxytocin helps in the maintenance of milk production and milk let down
- **Involution**: Cessation of milk secretion and apoptosis of mammary epithelial cells, due to withdrawal of PL
1.3.2.2 Milk synthesis and milk secretory pathways

Milk synthesis and secretion occurs in all mammals, and the presence of the mammary gland makes mammals a distinguishing class. Milk nutrients are synthesised either de novo in lactocytes or else are transferred into the mammary gland alveoli from the maternal circulation. These components remain stored in alveolar lumen until the suckling and ejection of milk is initiated by breastfeeding (16). Despite considerable variation in milk composition, the main secretory processes for the majority of milk components are highly conserved between mammals. This thesis is mainly focused upon glucocorticoids composition of HM; however, it is essential to consider the synthetic and secretory pathways for other nutrients.

Evidence from electron microscopy studies revealed that the rough endoplasmic reticulum (RER) and Golgi bodies in lactocytes are mainly involved in the synthesis and secretion of major milk nutrients such as fats, protein and lactose (116). Secretion of milk components are accomplished mainly through five secretory pathways (I) Exocytosis of Golgi derived secretory vesicles containing protein and lactose, (II) lipid synthesis and secretion involving apocrine secretion of endoplasmic reticulum (ER) derived lipid droplets, (III) transcytosis of proteins, hormones and immunoglobulins, (IV) transmembrane secretion of ions, water and small molecules such as glucose, and (V) paracellular transport allows for direct transfer of plasma components, leukocytes and interstitial substances (117,118). However, the adherence of cell junction inside the mammary gland epithelium is vital for milk secretion, and any disruption causes reduced synthetic activity and apoptosis of lactocytes.
Figure 1-3: Schematic overview of milk secretory pathways in lactocyte (mammary epithelia secretory cell). Redrawn and adapted from Molinari C, 2012.

Abbreviation: IgA, immunoglobulin A; ATP, Adenosine triphosphate; Ca, calcium; TAG, triacylglycerides; CoA, coenzyme A; UDP, uridine diphosphate
1.3.2.2.1 Lipid synthesis

HM fats mainly consist of 98% triacylglycerol (TAG), which consists of three saturated or unsaturated fatty acids esterified to a glycerol backbone. Evidence suggests that substrates for TAG are obtained from three main sources - de novo synthesis (10%), maternal diet (30%), and adipose tissue (60%). Humans are capable of synthesising small or short chain fatty acids, (up to C-14), while the essential polyunsaturated fatty acids (PUFAs) such as linoleic acid (18:2n-6) and alpha-linolenic acid (18:2n-3) are imported from maternal circulation depending on maternal diet and body stores (119). The relative percentage of milk fatty acids (FAs) obtained from de novo or maternal circulation are dependent on carbon chain length of the FAs. Typically, the short chain FAs are synthesised in the cytoplasm of mammary gland epithelial cells by de novo synthesis. Glucose is used as the primary precursor for fat synthesis, which after phosphorylation enters the glycolysis pathway, leading to the formation of pyruvate and glyceraldehyde-3-phosphate. Pyruvate then enters tricarboxylic cycle (TCA) to produce acetyl coenzyme A (acetyl CoA). Acetyl CoA is then catalysed by acetyl CoA carboxylase and synthetase complex, and converted into specific FAs (Figure 1-4) (120,121).

TAGs are synthesised, and accumulate in the cytoplasm and smooth endoplasmic reticulum (SER) of alveolar cells. They are secreted and released as micro-lipid droplets, coated with a phospholipid monolayer. These droplets then fuse together with other lipid droplets, to form bigger and larger lipid globules, which migrate towards apical cell region and are released from the cell. The exact process is unknown, but thought to be similar to reverse pinocytosis, where droplets get enveloped by the membrane bilayer (120). However, nothing is known about the exact molecular mechanism involved in the formation, transport, and secretion of milk fat droplets from mammary gland lactocyte (122).

![Figure 1-4: Schematic representation of fatty acid synthesis in mammary glands.](image-url)
1.3.2.2 Carbohydrate synthesis

Lactose is the most abundant carbohydrate in HM. Lactose is synthesised in Golgi bodies of mammary alveolar cells utilizing glucose and imported into lactocytes via facilitated diffusion. In Golgi bodies, some of the maternal blood-derived glucose combines with Uridine diphosphate galactose (UDP galactose) to form lactose (16,116). Remaining glucose is converted into glucose-6-phosphate, which further enters one of the three pathways - glycolysis, pentose phosphate pathway, and UDP galactose formation. Sugar molecules are packaged into secretory vesicles, which are then transferred into the alveolar lumen along with other milk constituents. Compared to other mammals, HM contains the highest concentration of lactose, comprising approximately 40% of total milk energy (123).

1.3.2.2.3 Protein and peptides synthesis

The process of protein synthesis is highly dependent on maternal circulatory stores. Casein, α-lactoalbumin, lactoferrin and immunoglobulins are the major proteins in milk, whereas hormones and enzymes make minor contributions (124). Numerous mechanisms are involved in the generation of milk proteins, but the majority are produced via de novo synthesis. The endoplasmic reticulum is the major site of protein synthesis, and after synthesis proteins are exported into the alveolar lumen of lactocyte via Golgi body exocytosis (125). Not all proteins in the milk are synthesised in mammary cells, but rather enter directly from maternal circulation via transcytosis or paracellular pathways, such as immunoglobulins, albumins and prolactin (125).

Of hormones and growth factors present in HM, some are synthesised in the mammary glands, while others are rapidly transported into milk via passive diffusion from maternal circulation (113). In mammals, many hormones including estrogen, GnRH, PTH related peptide (PTHrP), PL, Insulin like growth factors (IGF-I and IGF-II), epidermal growth factor, leptin and prostaglandins are synthesised in the mammary glands (126). Whereas, the source of other hormones, especially glucocorticoids is still unclear. Evidence suggests that hormones present in HM influence the growth and function of mammary glands. For instance, maintenance and cessation of lactation is influenced by a variety of hormones present in HM. Furthermore, many biologically active hormones may function in the transfer of nutrients and regulation of tissue development in breastfeeding infants. However, the function of many hormones present in HM is largely unknown and remains to be elucidated.
1.4 Human milk nutritional composition

HM composition is a highly complex biomaterial with a variety of components. It provides optimal nutrition (fats, carbohydrates, proteins, vitamins and minerals), protection (lactoferrin, immunoglobulins and lysozymes), and developmental components (cytokines, growth factors, oligosaccharides and hormones) to breastfeeding infants. Significant advances have been made to explore the physiology of lactating mother in constructing the comprehensive biochemical profile of milk.

1.4.1 Major fractions of human milk

Since 1960, our understanding of HM and its composition is increasing. HM is synthesised in mammary glands of a pregnant woman. Depending on the stages of lactation HM is differentiated into three categories: colostrum, transitional milk, and mature milk.

1.4.1.1 Colostrum

The first fluid produced by mammary glands immediately after birth is known as colostrum. It is a thick and yellowish fluid, which appears during pregnancy and may last a few days after parturition. Colostrum consist of a high concentration of essential immunologic factors including secretory IgA, lactoferrin and lysozyme (127). Several important growth factors such as epidermal growth factors, and insulin growth factors are also found in colostrum (128). Colostrum is rich in sodium, chloride, and magnesium, but contains a lower concentration of lactose. The onset of copious milk secretion causes the closure of the tight junctions in epithelial cells of the mammary tissue, reducing concentrations of sodium, potassium and increasing lactose concentration in colostrum (5,129). The average energy value of colostrum is 67 kcal/dL; however, its volume varies between 2-20 ml feeding within the first three days of feeding (109).

1.4.1.2 Transitional and mature milk

Transition milk is produced after colostrum and lasts for 7-10 days postpartum. The content of immunoglobulins and protein concentration decreases in transitional milk; whereas the concentration of fat, lactose and overall energy content increases to the level of mature milk. Milk produced around the third week postpartum is regarded as the mature milk (127). Mature milk is the most predominant form of human milk. About 90% of mature milk is water and the rest is composed of carbohydrates, lipids, protein and micronutrients (124). Mature milk is further categorised into fore milk (milk produced towards the beginning of feeding) and hind milk (milk produced towards the end of feeding) (130). The fat content of hind milk is 2-3 times higher than foremilk, and hence provides more energy (25-35 kcal) than foremilk (109,131). The hind milk produced by mothers of very low birth weight infants prior to 28 weeks gestation carries a higher concentration of fat-soluble vitamins A and E (132). Therefore, hind milk has been successfully used and recommended for very preterm infants to improve their growth and nutritional management.
1.4.2 Nutritional components of human milk: macro and micro nutrients

HM contains a wide variety of essential nutrients, including lipids, protein, carbohydrates, minerals and vitamins. The nutritional components of HM are categorised into four major categories—macronutrients, micronutrients, bioactive components and growth factors, essential to fulfil the nutritional need of a growing infant (5).

The macronutrient composition of mature HM is composed of approximately 3.2-3.6 g/L (~5.0%) lipids, 0.9-12 g/l (~0.9%) proteins, and 6.7-8 g/L (~7.2%) carbohydrates (5,133), and minerals (0.02%) are usually expressed as ash (124). It also contains all of the vitamins (both water and fat soluble vitamins) in nutritionally significant amounts (127). Its overall energy content is 60-75 kcal/100 ml with more than 85% of water.

1.4.2.1 Lipids

HM provides a complex array of FAs that serve as the main source of energy, providing up to 40-50% of infant caloric requirement (134). The milk fat primarily exists as membrane bound fat globules or droplets ranging in size from 1-12 µm. The core of the fat globule is mainly composed of triglycerides, representing 98-99% of total fat, surrounded by a structural membrane, composed of cholesterol, phospholipids, and proteins (135). Milk fat globule triglycerides are synthesised from the outer membrane of RER, coated with polar lipids and the proteins associated with membrane lactocyte, are released as distinct lipid droplet in cytoplasm. Their secretion process is distinct and has been explained earlier in Section 1.3.2.

Triacylglycerides (TAGs) are the most abundant fraction of milk fat, accounting for 98% of total lipid content and 40-50% of total milk energy (136). Besides triglycerides, HM contain small quantities of other lipids such as non-esterified FAs, cholesterol, phospholipids, glycoprotein di and monoaerylglyceride are also found (124,137,138). HM contains smaller percentage of phospholipids such as sphingomyelin, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS). The FAs composition of HM is a highly complex mixture of saturated, unsaturated, poly-unsaturated, branched, and cyclic FAs. Over 200 different FAs have been identified in HM with varied chain length and unsaturation (139), including long chain unsaturated fatty acids (LCPUFAs) such as n-3 and n-6 FAs, α-linolenic acid (ALA), linoleic acid (LA), docosahexaenoic acid (DHA) and Eicosapentaenoic (EPA) (127), reflective of maternal diet and stores.

FAs with the chain lengths greater than C-12 account for 85% of milk fats. Of these long chain FAs, approximately 45% are saturated and approximately 40% are monounsaturated, and approximately 15% are PUFAs. Palmitic acid is the most common saturated FAs, while oleic acid and linoleic are the most common unsaturated FAs, contributing to >60% of total FAs content (140). Detailed description of FAs profile and related carbon atom length is given in Table 1-2.
Table 1-2: Fatty acid profile of human milk.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Human breast milk (% W/W)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated fatty acids</strong></td>
<td></td>
</tr>
<tr>
<td>Capric acid (10:0)</td>
<td>1.1</td>
</tr>
<tr>
<td>Lauric acid (12:0)</td>
<td>6.2</td>
</tr>
<tr>
<td>Myristic acid (14:0)</td>
<td>7.8</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>22.1</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>6.7</td>
</tr>
<tr>
<td><strong>Mono saturated fatty acids</strong></td>
<td></td>
</tr>
<tr>
<td>Palmitoleic acid (16:1)</td>
<td>3.1</td>
</tr>
<tr>
<td>Oleic acid (18:1)</td>
<td>35.5</td>
</tr>
<tr>
<td>Gadoleic acid (20:1)</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>Polyunsaturated fatty acids</strong></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>8.9</td>
</tr>
<tr>
<td>Linolenic acid (18:3)</td>
<td>1.2</td>
</tr>
<tr>
<td>Arachidonic acid (20:4)</td>
<td>0.72</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (20:5)</td>
<td>Traces</td>
</tr>
</tbody>
</table>
By far, fat is the most variable component of HM causing significant nutritional variations between fore milk and hind milk, and colostrum and mature milk (109). Hind milk (last milk of the feed) contains 2-3 times more fats compared with foremilk, consequently supplying 25-35 kcal/100 ml more energy to breastfed infant (131). Furthermore, lipids in HM demonstrate the circadian variation over the duration of 24 hour period, with higher concentration of milk fat in the morning and evening feeds compared to night (141). In contrast, milk expressed by the mother of preterm infant demonstrated higher concentration of fats in the evening compared to the milk produced in the morning. Only recently, has it been realised that maternal diet and nutritional health status are major predictors of the HM lipid profile (139,142,143).

About 25% of HM lipid composition is more sensitive to maternal factors, particularly maternal lipid intake (144,145). Maternal diet rich in long chain polyunsaturated FAs influence early weight gain in breastfed infants during first year of infancy in breastfed infants, compared to formula fed infants (105,146). Details of other factors responsible for the variation in milk composition in relation to maternal diet are explained later in Section 1.6.

1.4.2.2 Carbohydrates

Carbohydrates are the second largest constituents of HM. Colostrum contains more than 27% carbohydrates, but this decreases by 15-16% in mature milk (147). Lactose is the most abundant carbohydrate of HM, with fairly consistent level of approximately 60 g/L (148,149). HM contains a high concentration of lactose and is positively correlated with milk yield (150). Thus exclusively breastfed infants are known to receive about 10-14 g of lactose each day per kilogram body weight (133,151). In breastfed infants, lactose exerts beneficial effect on mineral absorption, particularly calcium absorption. Intestinal flora converts milk lactose into lactic acid, which lowers the pH, further increasing the solubility of calcium salts (151). Additionally, lactose is a readily available source of galactose, which is required for the production of galactolipids such as cerebrosides, and is essential for the development of the central nervous system (152).

1.4.2.3 Oligosaccharides

Human milk oligosaccharides (HMOs) are the complex carbohydrate of HM. To date more than 130 different kinds of HMOs have been identified in HM (153). After lactose and TAG, they are the third largest components of HM, ranging from 23 g/l in colostrum to 7 g/l in mature milk (154,155). HMOs are mainly synthesised in the mammary gland, by the sequential addition of diverse monosaccharide units such as D-glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), L-Fucose (Fuc), and N-acetylneuraminic acid (NAMA). These monosaccharide units combine differently to form different HMOs and use lactose as a core unit. Compared to bovine milk, the concentration and complexity of HM oligosaccharides are much greater (156). They vary among individuals and change significantly during different stages of lactation. Colostrum provides approximately 24% of HMOs, gradually declining to 19% in the first month and reduce to 15% by the second month postpartum (157,158).

Oligosaccharides are known as the non-digestible components of milk that can withstand the low pH of infant gut, and resist enzymatic degradation, hence are known as non-digestible components of
milk. They are beneficial for the infant gut system, by acting as a metabolic substrate for gut beneficial bacteria (153,155,159). Several strains of *Bifidobacterium infantis*, have shown their ability to consume and metabolise milk oligosaccharides, and grow vigorously *in vitro* as the sole source of carbon (160,161). Furthermore, strains of *Bifidobacterium bifidum* and to a lesser extent *Bifidobacterium breve* and *Bifidobacterium longum* also display their preference for HMOs (162,163). For years, it was discussed that milk oligosaccharides are crucial for the development of gut microbiomes. However, recently HMOs have gained particular attention as the antibacterial, anti-infective component of mothers’ milk. Lower rates of diarrhoea and respiratory infections in breastfed infants led to the possible role of oligosaccharides in protecting infants against infectious pathogens. Some oligosaccharides present in HM act as a soluble glycan receptor mimicking bacterial binding sites, and act as an analogue for entero-pathogenic bacteria and their toxins. Thus preventing pathogen adhesion on infant mucosal surface of the gastrointestinal and respiratory tract by acting as decoy receptors (159). Through this action oligosaccharides, inhibit the binding of several different types of bacteria such as *C. jejuni, E coli* and *streptococcus pneumonia* (163).

Oligosaccharide present in HM can directly interact with host epithelial cells and reduces the ability of certain pathogens, such as *Campylo-bacter jejuni, Escherichia coli, Vibrio cholera* and *Salmonella* strains to adhere (164). They modulate host epithelial cell gene expression, which significantly modify cell surface glycans, essential for invading intestinal epithelial cells (165). Furthermore, HMOs play an important role in contributing protection to breastfed infants by protecting them against inflammatory diseases. HMOs inhibit the adhesion of leukocytes at inflammation sites and reduces the formation of selectin–initiated Platelets-Neutrophils Complexes (PNCs) (155,159).

In addition to oligosaccharides, HM also contain glycolipids (carbohydrates bound to lipids) and glycoproteins (carbohydrates bound to proteins). Both glycolipids and glycoproteins serve as a functional food for beneficial gut bacteria, building blocks for healthy growth, and provide protection against anti-pathogenic defence (166,167). Glycolipids exist in the outer layer of milk fat globule membrane, and mainly exist as glycosphingolipids. They play a central role in cell-cell communication, cell matrix- interactions; aimed at affecting growth and differentiation of cells, especially neurons (168).

So far, all the potential benefits of complex milk sugar are for breastfed infants. However, very little is known about their effect on lactating mothers. There are a few researchers who argue about the role of milk oligosaccharides in promoting maternal disease. HM is a non-sterile biological fluid, containing complex bacteria, regarded as prebiotics; essential for infant gut health. These bacteria present in milk could be responsible for causing mastitis, and oligosaccharides in mother’s milk may influence the growth of these bacteria by acting as a prebiotics or anti-adhesive (159,169,170). However, not much is known about HMOs interactions with milk bacteria. Hence, further studies are required to study the effect of HMOs not only in the breastfeeding infant, but also in lactating mothers.

### 1.4.2.4 Protein

HM is a rich source of proteins, with a considerable variation throughout lactation. The protein concentration of HM is usually higher during early lactation period, due to high concentration of
immunoglobulin's and lactoferrin, before gradually declining down to a constant value in mature milk (131). However, the concentration of milk produced by the mothers of preterm infants is marginally higher (2.01 g/100 ml) than the full term milk (1.98 g/100 ml) (171,172).

HM proteins are usually categorised into two major categories - whey and casein protein isolates. Caseins exist in micellar structure, accounting for 10-50% of total milk protein, whereas the whey fraction is water soluble and constitutes about 50-90% of protein, with a distinct variation throughout the lactation (127). HM contains β casein, kappa casein, but lacks alpha casein (173). However, β casein is the most dominant component of the HM, a highly phosphorylated protein (173,174). Whilst, the whey isolates of HM include alpha-lactalbumin (10-20%), lactoferrin (7%), secretory IgA (6-16%) and lysozymes (0.7-7.0%) (175). These proteins are involved in the development of infant's immune system that protects them against infectious disease.

The quantity and quality of proteins are the key contributors of HM, and amino acids profile is the key indicator of the milk’s quality (131,173). The WHO endorses amino acids (AAs) pattern of HM as the most suitable estimate of AAs required for the optimal growth of an infant, particularly during the first six months of their life (176). Of the total nitrogen in human milk, non-protein nitrogen (NPN) accounts for roughly 25% of the total nitrogen (173). Major component of NPN includes urea, uric acid, free amino acids, nucleotides, taurine, peptides, polyamines and nucleic acids (5). It appears that some of the NPN plays an important role in the growth and development of a new born child; however, the true potential of their significance is not fully known.

HM is comprised of free amino acids (FAAs) and total amino acids (TAAs). FAAs are protein–unbound amino acids that account for 8-22% of NPN, whereas TAAs accounts for 5-10% of NPN (177). The FAAs from HM enters infant circulation sooner than protein bound AAs, resulting into rapid absorption. The major changes in the AAs concentration are mainly caused by the different stages of lactation (171). TAA content declines significantly during the first 3-4 months of lactation, but the ratio of essential and non-essential AAs remains constant (178,179). However, few FAAs such as glutamic acid, serine, glycine, alanine and cysteine, increase with the advancement in lactation (180). Mature milk has 20 times more glutamine and significantly high amount of glutamic acid, than colostrum (171). Glutamine and glutamic acid play vital role in protecting enteral mucosa and also act as a neurotransmitter (151,181).

### 1.4.2.5 Vitamins

Water-soluble vitamins present in milk include thiamine (vitamin B1), riboflavin (vitamin B2), pyridoxine (vitamin B6), vitamin B12, folate, niacin, pantothenic acid and ascorbic acid (vitamin C)(182). These vitamins are not synthesised in mammary glands. They are mainly derived from the maternal diet, indicating an active role of mammary glands in transporting and metabolising the vitamins (152). HM is a sufficient source of water-soluble vitamin, but the stages of lactation and the maternal vitamin sufficiency are the key regulators for maintaining their level in milk (183).

Thiamine and riboflavin are essential cofactors required for general metabolic reactions (183). The concentration of thiamine in early milk is 0.21mg/l, which increases to 2mg/l in mature milk. However,
the concentration of riboflavin is much higher, 3.5 mg/l, and remains constant throughout the lactation (184). Evidence suggest that the maternal intake of 2 mg/l riboflavin is sufficient to raise optimal milk levels of vitamin B2 in mother milk (151). Furthermore, the concentration of vitamin B6 in early milk is low, ranging between 0.9mg/l to 3.1 mg/l (129). One of the primary concern for vitamin B6, is that HM does not provide a sufficient amount to meet United States Required Dietary Association (RDA) requirement for young infants (184). The concentration of vitamin B12 is higher in colostrum and early milk, but reduces with the duration of lactation from 1.2μg/L to only 5 μg/l (185).

Evidence suggests that supplementation of multivitamins for lactating mothers can increase vitamins in their milk only when mothers are not severely deficient (184). It has been reported that 2.5mg/day of vitamin supplementation increases milk level by almost 50% (182). In contrast to vitamin B12, folate and folic acid concentration in HM increases with the progression of lactation, ranging from 15-20 μg /l in early lactation to 50-100 μg/L at three months (186). However, during pregnancy mothers are recommended to take an additional intake of folic acid, to prevent placenta abruption, intrauterine growth failure and neural tube defects in new-born (109).

HM is an exceptional source of Vitamin C, ranging from 50 to 100 mg/l. Interestingly, concentration of vitamin C in mothers milk is 8-10 times higher than maternal plasma (185). However, maternal supplementation of vitamin C does not make a significant difference in milk vitamin levels, particularly for well-nourished mothers, indicating the limitation related to the transfer of vitamin C. Although HM is an ideal source of nutrition, but maternal dietary intake and her body stores throughout pregnancy to lactation influences its nutritional composition. The maternal nutritional status is not always optimal and hence the majority of health agencies recommend multivitamins to pregnant and lactating mothers, particularly for undernourished or malnourished mothers.

In addition to water-soluble vitamins, HM is an outstanding source of vitamin A and E, containing around 0.053 mg/dl of vitamin A and 1.0 mg/dl vitamin E. However, the concentration of Vitamin E declines with the progression of lactation, but number of births is directly related to increased concentration (109,187) The duration of lactation and poor maternal nutrition has a huge impact on vitamin A levels, but evidence suggests that early maternal supplementation can hugely increase their level in mature milk (188). In contrast to vitamin A and E, HM has relatively low level of vitamin K and D. and their level remains low throughout the lactation. HM contains 1-9 μg/L of vitamin K and only 0.1-1.0 μg/l of vitamin D, much less than the usual recommendation. Therefore, it is recommended that vitamin K should be given to all newborn infants, and all breastfeeding infants should be supplemented with vitamin D regardless of maternal diet and breastfeeding pattern (5,189,190).

1.4.2.6 Minerals

Sodium (Na), potassium (K), calcium (Ca) and phosphorus (P) are major components of minerals in HM whereas, copper (Cu), zinc (Zn) and iron (Fe) are found in lower concentrations (191–193). Evidence suggests that concentrations of minerals in milk vary with the duration of the day for example, afternoon milk has higher concentration of minerals than morning (194). On average mother’s milk provides approximately 300 mg/L of Ca every day and 150 mg/L of P within the first week of postpartum; and the ratio of Ca/P in HM is (2:2), which is greater than cow’s milk (1:4) (184).
Unlike other minerals, the level of Ca in HM is independent of maternal age, parity, and rate of milk production.

For optimal growth, infants require around 8-10 mg of Fe per day, but HM only provides 0.3-0.6mg/L, far below the recommended amounts. However, the bioavailability of Fe from HM is 49% greater than cows’ milk (19.5%) or infant formula (4%)(195–197). There are several other factors, which influence the absorption of Fe from mothers’ milk. Ascorbic acid and lactose are known to stimulate iron absorption, but in contrast, calcium concentration (within the range of reported median values) tends to have an inhibitory effect on the absorption of Fe from milk (195,198). Most micronutrients/minerals are absorbed more efficiently from HM than formula milk, but surprisingly very little is known about the impact different breastfeeding practices on the bioavailability of these minerals. Given the high proportion of mothers, who attempt (and those who sustain) partial breastfeeding practices, it is important to determine the influences on the micronutrient composition of HM.

1.4.2.7 Emerging complexities of human milk

Besides nutrition, HM contains varieties of bioactive components such as biologically active proteins, peptides, hormones, and recently discovered genetic material, miRNAs, that affects infant’s biological processes and thus have an impact on infant survival and health. Bioactive components in milk come from a variety of sources. They are either synthesised by mammary epithelium, or else are migrated from maternal serum; whereas some membrane bound proteins are carried into milk via MFG (199,200). In order to have the complete understanding of HM bioactive components, the comprehensive information about their sources and their characterization is critical, however only selected bioactive components that change with maternal characteristics or lactation period are discussed in this review.

HM contains a wide variety of bioactive proteins, such as lactoferrin, lysozyme, secretory IgA, bile stimulated lipase and haptocorrin, which protects infants against numerous infections by stimulating an appropriate inflammatory response (201). They act as an effective communication tool between a mother and her infant, by actively guiding and educating the infant’s immune and metabolic system. Among these, lactoferrin, lysozymes and secretory IgA play an important role as anti-infective and antibacterial components of HM (202). Moreover, they provide diverse protection against pathogenic organisms and hence offer both passive and active immunity to a new born infant (203). Lactoferrin is a major multifunctional globular glycoprotein belonging to the transferrin family. It exhibits high affinity for iron, thus competes with bacteria for iron and prevents their growth (204,205). While, lysozymes lyse the cell wall of bacteria and work in a synergistic way with lactoferrin to kill bacteria (206). Secretory IgA is the predominant antibodies in the HM, accounts for 90% of total immunoglobulins (207). These antimicrobial proteins are highest in colostrum and decrease throughout lactation; maternal nutritional status exerts no direct effect on the antibacterial components of HM (202).
1.5 Hormones: the biochemical messengers

Hormones are the biochemical messengers secreted by glands into the circulatory system (208). They play a critical role in communication between organs and cells, by stimulating the receptor at local or distant target organs. Hormones employ four possible systems to communicate between organelles such as systemic (endocrine), paracrine, autocrine, and neurotransmitters (209,210).

Based on the chemical structure, hormones are classified into three main classes: peptides and protein hormones; steroid hormones; and amino acid related hormones. Hormones are usually classified into distinct classes, most of them exhibit complex interaction and cross talk between them, which span across the majority of physical functions. Physiological variations are the result of alterations in the rate of production, clearance, and bioavailability. Any disruption in the production and secretion of a hormone in circulation is the most common cause of hormonal dysfunction. Clearance rate of hormones, or removal by the liver is also critical in regulating hormone levels. Lastly, bioavailability of hormones i.e. if the hormones are already bound to a carrier protein or are in the free state, where they can quickly bind to hormone receptors (210). Majority of sex steroid hormones, including testosterone are bound to a carrier protein known as sex hormone binding globulin (SHBG), which allows steroids to travel freely in circulation until required (211). However, the mechanism that frees the hormones from their carrier proteins is still unclear for the majority of the hormones.

Hormones are important for multiple functions, including reproduction, growth, metabolism, and senescence. To evaluate body functions, measurement of hormones can be done in blood, saliva, cerebrospinal fluid (CSF), hair, urine, faeces, and milk. Each medium has its own advantages and limitations, depending upon the type of hormone, species, and most importantly the research question (211). Blood is the most commonly used bio-fluid for diagnostic analysis in laboratory facilities, while urine, faeces and saliva are used more in research facilitates (212). While blood provides the most obvious snapshot of individual hormonal status, saliva and urine are non-invasive and non-manipulative samples, but only a few hormones such as steroids can be assessed in this way.
1.5.1 Hormones in human milk

HM is a heterogeneous, non-uniform biological fluid, containing variety of non-nutritive compounds. Over the past few decades, progress in new methodology and improved techniques has led to the determination of various biologically active hormones in milk. Many of the hormones present in milk are either synthesised in mammary glands or originate from maternal circulation, which can easily migrate from maternal plasma to child via mothers’ milk (201). It is believed that hormones from HM survive the infant gut environment and get absorbed into their circulation, and acts as secondary hormones for breastfeeding infants. These secondary hormones then trigger a variety of signalling pathways and modulate infant physiological and behavioural phenotypes (126). To date, many bioactive hormones from hypothalamic, thyroid, parathyroid, adrenal glands, and gut have been identified in HM. Some of the major classes of hormones identified in HM are listed in Table 1-3.

Insulin was the first bioactive hormone to be identified in HM (213). With the advent of new technology, the presence of other hormones such as leptin, ghrelin, adiponectin resistin and obestatin, have also been identified in HM. These are virtually absent in formula milk (214,215). After entering infant circulation, these hormones can exert a meaningful outcome on infant physiology by regulating energy metabolism and food intake; and any imbalance could cause the development of obesity, diabetes or metabolic disorders in offspring (216). However, the mechanism of action for each hormone in HM is still unclear.

Leptin is another widely studied hormone in HM. It is an anorexigenic polypeptide hormone involved in the regulation of food intake and energy regulation (217). Leptin has been detected in both term and preterm milk, with the highest concentration in colostrum compared to transitional or mature milk (214,215,218). Evidence suggests that leptin in HM comes from two sources, firstly it is secreted into milk by mammary epithelial cell in milk fat globules, secondly, it is transferred into milk from maternal circulation (219,220). An inverse relation has been demonstrated between milk leptin concentration and infant weight gain and length during the first year of life (221). However some reported no association between milk leptin and infant growth (222). A recent study by Field et al. (216) demonstrated that maternal BMI, infant sex and stages of lactation, can significantly affect the compositional make up of HM, particularly, insulin and leptin level of milk.

In addition to leptin, there are other hormones, such as ghrelin and adiponectin that are reported to be synthesised in mammary glands. Ghrelin concentration in colostrum (70±18 ng/ml) and mature milk (97±13 ng/ml) is lower than maternal plasma (95±15 and 135±16 ng/ml) (223). The concentration of ghrelin in milk increases progressively with lactation and tends to be higher in fore milk than hind (224). However, not much is known about the impact of maternal related factors on milk ghrelin concentration, and existing data on ghrelin effects on breastfeeding infants are also rare.

Given the role of adiponectin in energy metabolism, several studies have looked at its presence in HM and identified its effect on infant growth. Adiponectin is the most abundant adipose specific hormone found in HM with a concentration ranging between 4.2-87.9 ng/ml. Colostrum tends to contain the highest concentration of adiponectin before gradually declining throughout lactation (223,225). Similar to other hormones, variation in HM adiponectin was mainly attributed to maternal
factors including length of gestation, parity, ethnicity and mode of delivery (225,226). Infants exposed to higher levels of adiponectin in milk were found to be associated with increased weight gain trajectory during the second year of their life, compared to the slower growth during the first year of active breastfeeding (227).

Only a few studies have investigated the impact of hormones in HM on infant growth; and have demonstrated their involvement in regulating metabolic programming by influencing infant food intake and energy balance (228–230). However, more studies are warranted to investigate the impact of maternal related factors on milk hormonal composition; and demonstrates a better understanding on the long-term consequences of milk-borne hormones on infant development.

This review will focus on steroid hormones, in particular glucocorticoids, and their regulation. There are a number of excellent reviews on other biologically active components that are beyond the scope of this review (126,201–204,231–233). Milk-borne glucocorticoids have been the subject of recent research due to their impact on infant growth and behavioural development. However, the exact mechanism behind how these milk-borne hormones are transferred into milk and how they would influence infant development is still unclear.
Table 1-3: Major hormones in human milk.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adrenal and steroid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.2- 2.0</td>
<td>(234)</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.2- 4.0</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td></td>
<td>(127)</td>
</tr>
<tr>
<td>Estrogen</td>
<td>10-40</td>
<td></td>
</tr>
<tr>
<td>Estradiol</td>
<td>15-840</td>
<td></td>
</tr>
<tr>
<td><strong>Metabolic hormones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td>0.01 -0.65</td>
<td>(235)</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>4.2-87.9</td>
<td>(225)</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>70-90</td>
<td>(224)</td>
</tr>
<tr>
<td>Obestatin</td>
<td>0.53-0.52</td>
<td>(236)</td>
</tr>
<tr>
<td><strong>Growth factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-I</td>
<td>2.3-1.9</td>
<td>(237)</td>
</tr>
<tr>
<td>EGF (epidermal growth factor)</td>
<td>33.3 184.3</td>
<td>(238)</td>
</tr>
<tr>
<td>VEGF (vascular endothelial growth factor)</td>
<td>12.6- 155.0</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>0-80μU/ml</td>
<td>(126)</td>
</tr>
<tr>
<td>Prolactin</td>
<td>73-114</td>
<td>(126,239)</td>
</tr>
<tr>
<td><strong>Gastrointestinal and thyroid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrin</td>
<td>0.001-0.03</td>
<td>(127,240)</td>
</tr>
<tr>
<td>GIP</td>
<td>33-59</td>
<td></td>
</tr>
<tr>
<td>GRP</td>
<td>0.03-0.05</td>
<td></td>
</tr>
<tr>
<td>PYY</td>
<td>0.01-0.03</td>
<td>(241)</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>0.22-4.2</td>
<td></td>
</tr>
</tbody>
</table>
1.5.2 Steroid hormones

Steroids are lipid soluble molecules derived from cholesterol and through several enzymatic transformations are converted either into active or inactive form (Figure 1-5) (211). In mammals, the endocrine system is the major source of all steroid hormones, including pituitary glands, adrenal glands, gonads and liver that play an important role in bio-regulatory mechanisms and behaviour (242).

Steroid hormones are classified into five main categories- estrogen (female sex steroids), androgen (male sex steroid), progesterone, mineralocorticoids, and glucocorticoids (210). Sex steroid hormones provide the physiological basis for sex dimorphism and structural differences between sexes. Estrogen regulates ovary functioning, breast development and fat distribution in females; while androgens particularly testosterone, regulate hair, vocal cord, fat catabolism, genital development and muscle anabolism in males (211). The principal mineralocorticoid in humans is aldosterone, along with deoxycorticosterone and corticosterone, plays a critical role in regulating fluid and electrolyte balance in kidney, colon, sweat and salivary glands (243). Cortisol and cortisone are the main glucocorticoids, which are required for the regulation of energy flux, lipid metabolism, anti-inflammatory and immune-suppressive and essential for behaviour and stress regulation (244,245).
Figure 1-5: Steroid hormone synthesis from cholesterol. Redrawn and adapted version from Bribiescas & Muehlenbein 2010.
1.5.3 Glucocorticoids

Glucocorticoids are produced from the adrenal cortex of the adrenal gland. These hormones affect almost every organ and are involved in a wide range of physiological processes, including inflammation, metabolism, and the body’s response to stress and starvation (244,246–249). The circulating concentration of corticosteroids (both mineralocorticoids and glucocorticoids) is reflective of their synthesis rates, and are released into circulation soon after synthesis, as very little can be stored in the adrenal glands (244,250).

During stress, the responses made by mammals are largely governed by glucocorticoids, which are natural anti-inflammatory steroid hormones produced by adrenal glands that perform multiple functions in body homeostasis (251). They exert their effect by binding to high affinity receptors such as glucocorticoid receptor (GR) or mineralocorticoids receptor (MR) in the target tissue (252). Glucocorticoids cross the plasma membrane of the target cell and combines with receptor in the cytoplasm. The activated GR translocate to the nucleus and alters gene expression (253). The adrenal cortex of humans secrete two primary glucocorticoids, cortisol, and cortisone. Secretion of glucocorticoids is dependent on hypothalamus, anterior pituitary, and adrenal glands as shown in Figure 1-6: Regulatory pathway of cortisol secretion.
Figure 1-6: Regulatory pathway of cortisol secretion.

Schematic presentation of cortisol secretion from adrenal glands regulated by hypothalamic-pituitary-adrenal (HPA) axis. In response to physiological or psychological stimulus, hypothalamus secretes corticotrophin releasing hormone (CRH), which acts on anterior pituitary gland to induce the release of adrenocorticotropic hormone (ACTH). The ACTH in turn stimulates the secretion of cortisol from the adrenal cortex glands into the bloodstream.
1.5.4 Regulation of glucocorticoids

Glucocorticoids display diurnal release patterns. Consistent with many other physiological processes that follow circadian rhythm, glucocorticoid secretion is also under the control of HPA axis, modulating 12 secretory episodes of cortisol over 24-hour period (254). Plasma cortisol level peaks before awakening in the morning, with levels decreasing progressively throughout the day to a minimum at the onset of sleep. These circadian rhythms are distinct and are regulated by the autonomic nervous system and endogenous processes that co-ordinate several physiological processes in response to the environmental changes (255). Additionally, HPA axis activity can be stimulated by physical or emotional stress (256). The stress response is instigated by numerous neural terminals, originating from both brain and limbic region (257). The HPA axis receives signals from central endogenous clocks also known as the pacemaker in the suprachiasmatic nucleus (SCN), stimulating the diurnal release of Corticotrophin Releasing Hormone (CRH) from the hypothalamus. This derives the release of AdrenoCortico–Trophic Hormone (ACTH) from anterior pituitary and finally releasing glucocorticoids from the adrenal cortex of the adrenal glands (258).

1.5.4.1 Free and bound glucocorticoids

A traditional explanation behind the bioavailability of a steroid hormone is that only free hormone can diffuse through the cell surface and reinforce a biological effect (259). According to the free hormone hypothesis, protein bound hormones are physiologically inactive. The biological activity of glucocorticoids are also dependent on the concentration of free cortisol availability (260) i.e. the fraction of hormone not bound to any protein (244). In plasma, the majority of cortisol is bound and only a small fraction is free, which passes from capillaries into tissue by passive diffusion. More than 80% of cortisol is bound to high affinity low capacity corticosteroid-binding globulin (CBG) and 10-15 % is bound to low affinity high capacity protein such as albumin (261,262). Surprisingly, the uptake of free cortisol by liver is three times higher than the amount of circulating cortisol, indicating the rapid dissociation of cortisol from their corresponding binding proteins to replace the pool of free cortisol, further obscuring the difference between free and bound cortisol. Though, bound and unbound forms of cortisol remain in equilibrium, they are affected by several factors including pH and temperature. CBG bound to cortisol is temperature sensitive and releases cortisol in response to variation in temperature such as fever (263), suggesting the pivotal role of CBG in regulating the concentration of cortisol in plasma (264), along with other mechanism such as 11-β hydroxysteroid dehydrogenase (HSD) enzyme, which regulates tissue and systemic levels of glucocorticoids.

1.5.5 Glucocorticoids bioavailability: cortisol and cortisone

In order to understand the significance of measuring free or biologically active hormone, it is important to understand the factors responsible for defining bioavailability. This include inter conversion of cortisone and cortisol by 11 β HSD enzyme.
Cortisol and cortisone are the primary lipophilic glucocorticoids secreted by the adrenal glands under the systematic control of HPA axis. Cortisol is well recognised as a prominent and active hormone, however cortisone is an endogenous glucocorticoids with minimal glucocorticoid activity and is secreted in minimal quantities (265,266). In humans, cortisol accounts for 95% of glucocorticoid activity, with about 15-20 mg secreted daily, whereas only 5% is cortisone. Cortisone is an inactive hormone, and hence requires metabolic inter-conversion to cortisol. 11 βHSD type-1 is a intercellular enzyme, which catalyses the conversion of hormonally inactive cortisone to hormonally active cortisol in the metabolically relevant tissues such as liver, skeletal muscle and adipose tissue (267). However the tissues expressing 11 βHSD type-2, converts cortisol back to cortisone (268) as shown in Figure 1-7.
Figure 1-7: Inter-conversion of cortisol into cortisone

Inter-conversion of cortisol into cortisone and vice versa is initiated by 11 β hydroxysteroid dehydrogenase (HSD). Significant amount of cortisol regenerated in liver and peripheral tissue from circulatory cortisone. 11 βHSD type-1 enzyme converts cortisone into cortisol and 11 βHSD type-2 catalyses the conversion of cortisol into cortisone. Redrawn and adapted version from Cooper, 2009 (269).
1.5.6 Physiological and metabolic functions of glucocorticoids

Glucocorticoids are essential for several physiological and metabolic functions. After being released into circulation, glucocorticoids can access target tissues and exert well-coordinated effects (249). They exert both anabolic (gluconeogenic and glycogenic) and catabolic (proteolytic, lipolytic) effects on numerous tissues including liver, connective tissue, lymphoid tissue, adipose tissue, and muscle tissue (242,248,270).

1.5.6.1 Immune and mental activity

Increasing evidence suggests that glucocorticoids are essential for the development and maintenance of normal immunity (271). They are known to exert anti-inflammatory and immune-suppressive effect by downregulating the inflammatory response and cytokine cascade (272). They affect several psychological processes, including behaviour and cognitive development (273). During chronic disease, cortisol acts as a powerful anti-inflammatory agent and suppresses the expression of increased inflammatory mediators (247). Furthermore, glucocorticoids are essential for maintaining normal cognitive function and mental health. Glucocorticoids, being lipophilic in nature, can easily cross the blood brain barrier and binds to its receptors situated in the hippocampus, amygdala and frontal lobes. These regions of the brain are known to regulate memory and learning function (274–276). Many studies in psycho-neuroendocrinology have investigated the effect of circulating glucocorticoids on human brain and behaviour and postulated the effects of stress might have on individual cognitive neuropsychology (262,277).

1.5.6.2 Growth and energy metabolism

Glucocorticoids are the essential hormones, in regulating normal metabolic reactions. Cortisol has a marked effect on energy metabolism by inducing gluconeogenesis, hyperglycaemia, and hyperinsulinemia. It blocks glucose utilization and preserves blood glucose for energy dependent tissue such as brain and skeletal muscle (278,279). Experimental evidence suggests that excess glucocorticoids promote obesity by increasing positive energy balance and energy consumption. In animals, glucocorticoids induce upper body obesity by increasing lipoprotein lipase activity (an enzyme used for hydrolysis of triglycerides) (280). While in humans, it majorly affects abdominal visceral fat distribution by increasing lipid accumulation in adipocytes. Altogether, these events cause lipid accumulation and retention of visceral adipose tissue suggesting its role in the development of various metabolic disorders. (281,282).

1.5.6.3 Stress regulation

Stress is a common phenomenon consisting of several psychological and physiological reactions. Glucocorticoids and catecholamines are the two important stress hormones secreted in humans. Considering the role of glucocorticoids as stress hormone regulators, cortisol causes a temporary increase in mental alertness and increases cognitive processes, while inhibiting other unnecessary psychological processes (271,283). Glucocorticoids are known to play a significant
role in detecting fear and increasing memory for emotionally relevant events (271), suggesting the positive role of glucocorticoids to respond in regards to changing and challenging environmental conditions and prepare them for fight or flight response. However, long term exposure to cortisol is associated with memory impairment and 14% reduction in hippocampus volume (283). Also, glucocorticoids being catabolic in nature antagonize insulin action and elevate blood pressure, further increasing the risk of hypertension and diabetes (284).

1.5.6.4 Glucocorticoids and their role in intrauterine utero environment

The in utero growth environment is an intricate and dynamic process. During pregnancy, any adverse environmental conditions such as prenatal maternal hypoxia, malnutrition, exposure to chemicals or any psychological stress, influences fetus or infant HPA axis permanently (285). Fetal response to these disturbances lead to increased risk for premature birth, low birth weight, and metabolic or neuroendocrine disorders in adulthood. Association between prenatal and postnatal stress are mainly the result of nutritional or hormonal programming through the over exposure of glucocorticoids (286,287).

In human pregnancy, exposure to glucocorticoids is important for the development and maturation of fetal tissue and organs such as lungs (288). Mothers at the risk of preterm delivery are often given synthetic glucocorticoids (289,290). During fetal development, maternal circulatory glucocorticoids are greater than fetal glucocorticoids (285) Placental 11βHSD-2 plays a crucial role in regulating the access of glucocorticoids to the fetus and converts about 90% of maternal cortisol into cortisone and the remaining 10% of maternal glucocorticoids enter fetal circulation for organ development (291). Expression of 11βHSD-2 correlates with infant gestation age, but in order to maintain appropriate levels of fetal glucocorticoids, 11βHSD-2 expression drops radically around 38th - 40th week of gestation and increases transfer of maternal glucocorticoids into the fetus (292). As maternal glucocorticoids are higher than fetus, any alterations in placental 11βHSD-2 could influence fetal glucocorticoid sensitivity. In addition to maternal stress, maternal under nutrition and lifestyle habits such as alcohol intake down regulates 11βHSD-2 activity and increases the fetuses exposure to glucocorticoids. This influences infants metabolic activity by permanently modulating the physiological functioning of the HPA axis (289,293,294).

1.5.7 Glucocorticoids in human milk

The presence of glucocorticoids in HM was first reported almost 60 years ago (295). With the recent advancement and development of sensitive immunoassays and chromatographic techniques, detection of hormones in milk has become common and expanded. However, the concentrations reported in earlier studies were higher compared to what is reported in the current studies.

Since the discovery of hormones in milk, many studies have been performed to identify their presence in bovine and human milk. As explained earlier, biologically active hormones can either be synthesised in mammary glands or are transferred from maternal circulation to the child via
mother’s milk. Given the importance of breastfeeding over formula feeding, especially with respect to obesity, the majority of human research is focussed on understanding the presence of appetite regulating hormones in milk such as leptin, adiponectin and ghrelin (296). However, their concentration in milk varies greatly with the stages of lactation, maternal and metabolic abnormalities (216,297). Considerable evidence details the effects of circulatory glucocorticoids on adults and children, but little is known about the milk-borne glucocorticoids and their influence on breastfed infants. Lately, glucocorticoids, have gained much needed attention, due to their importance in predicting infant behavioural development and metabolic functioning (14,298). Also, their role in inter-disciplinary science has made it a popular hormone, particularly in developmental studies, where they act as developmental switch causing the change in gene expression essential for infant growth and development (299–301). Yet there remains an incomplete understanding of how the offspring development response to milk-borne glucocorticoids is inherently altered.

In adults, hormones are released from appropriate glands, in response to nervous or metabolic stimuli (201,265), but for breastfeeding infants, HM could act as an additional source of maternal originated hormones, including glucocorticoids (14,302). Studies comparing the difference between formula feeding and breast feeding has shown significant differences on infant body composition (303). In contrast to formula feeding, infants breastfed throughout the first year of life demonstrate a 40% increase in their salivary cortisol levels (304), suggesting the presence of milk-borne cortisol being transmitted via breastfeeding (305,306). Several studies have investigated the relation between maternal and infant cortisol levels. These demonstrate the influence of breastfeeding on infant cortisol levels (14,307).

To date only few studies have investigated the milk-borne glucocorticoids and have postulated their effect on infant biochemistry and physiology. But nothing is known about the regulatory mechanism and how and what possible factors could influence its concentration in milk.

1.5.7.1 Glucocorticoids and infant behaviour development

Since the discovery of glucocorticoids in milk and their association with infant temperament, there has been a sudden upsurge of studies, that are investigating the association of HM glucocorticoids with infant development (298,308,309). Grey et al., (14) was the first human study to confirm that mother’s milk as the potential predictor for influencing offspring phenotype by transferring biologically active hormones through milk. They showed direct relationship between HM cortisol concentration and infant temperament, and further demonstrated that higher level of cortisol in milk was positively associated with more negative temperament in female infants at three months of age. These findings were consistent with one other human study that linked maternal plasma cortisol with infant fearful temperament (310). Infants exposed to prenatal maternal stress showed increased shyness and negative (fearful) behaviour; and this behavioural change was more prominent in female offspring. Another study showed that, higher levels of milk glucocorticoids were associated with enhanced performance of infants on neonatal behavioural scale (233). It has been suggested that association between milk cortisol and infant behaviour could also be influenced by parental behaviour. For instance, mothers with higher cortisol level showed
enhanced accuracy identifying their infants’ odour and were more vigilant and affectionate, indicating the reason for stimulating infants’ superior behaviour. Furthermore, the data from animal studies also demonstrated that cortisol concentration in three to four month old monkeys was positively related to infant temperament in males, but not among female monkeys (309). In contrast, a study conducted in HM was unable to find any sex biased differential sensitivity to milk cortisol (14). Therefore, it would be too early to draw conclusion on milk glucocorticoids differentiation based on sex sensitivity.

In recent years, our understanding of the contents of milk-borne glucocorticoids has improved with technical advancement. To date, only a single study has used chromatographic technique to identify glucocorticoids in HM and has shown the presence of both cortisol and cortisone in HM (234). A recent study has demonstrated that milk glucocorticoids follow diurnal rhythms mimicking maternal HPA axis. Furthermore they also demonstrated the association between hormonal variations and gestational age and showed that preterm milk samples compared to term samples had lower concentration of both cortisol and cortisone (307). There are many other factors, which affect circulatory levels of cortisol in adults including diurnal rhythms, situational factors, and sleep patterns. Yet not much is known about the factors influencing the glucocorticoids composition of HM and how it may differ between individual lactating mothers. Our current understanding about the presence of glucocorticoids in HM and their effect on offspring is limited. Little is known about maternal and infant related factors influencing the stress hormone composition in milk and how it could interfere with infant physiology. Thus, further studies are required to specify biochemical or molecular pathways affecting milk hormonal composition.
1.6 Variations in human milk composition in relation to maternal and infant health

As our ability to measure and identify a variety of novel compounds increases, researchers unravel the intricacies of HM. Hundreds of compounds in milk have been identified, along with their nutritive roles. However, we are still far from predicting the exact composition of mothers’ milk. Its composition is progressively changing over the course of lactation to compensate for the dietary needs of a growing infant (306). There are several maternal and infant related factors, responsible for dictating the composition of human milk. Some of these factors are summarized in Table 1-4. Some of the major factors responsible for changing milk composition such as the type and stages of breastfeeding, maternal age, diet, weight, mode of infant delivery, parity, health condition (maternal stress or depression), socio-status and living conditions, are discussed in detail in the section below.

Table 1-4: Factors associated with the alterations in composition of human milk

<table>
<thead>
<tr>
<th>Factors associated with changes in milk composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breastfeeding</strong></td>
</tr>
<tr>
<td>Breast physiology</td>
</tr>
<tr>
<td>Stages of lactation</td>
</tr>
<tr>
<td>Duration of breastfeeding (frequency and volume)</td>
</tr>
<tr>
<td><strong>Maternal related factors</strong></td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Diet</td>
</tr>
<tr>
<td>Weight and BMI</td>
</tr>
<tr>
<td>Mode of delivery</td>
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<tr>
<td>Premature birth and degree of prematurity</td>
</tr>
<tr>
<td>Parity</td>
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<tr>
<td>Health status (infection, metabolic disorder or depression)</td>
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<tr>
<td>Medication</td>
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<tr>
<td>Menstrual cycle</td>
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<tr>
<td>Social status (education, job marital status, and relationship status)</td>
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<tr>
<td>Living condition (living in urban or rural area and region)</td>
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<tr>
<td><strong>Infant related factors</strong></td>
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<tr>
<td>Infant sex</td>
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<td>Infant birth weight</td>
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</table>
1.6.1 Breast related factors affecting milk composition

1.6.1.1 Lactating breast physiology

Breast storage capacity, emptying rates (breast fullness before and after breastfeeding) breastfeeding frequency and the rate of milk synthesis could have a significant influence on milk production and its composition (311,312). For instance, 70% of fat variation in HM is due to the difference in the rate of milk synthesis, which is affected by the degree of breast fullness (313). Fat content of the milk increases more quickly if the breast is completely empty, but decreases with breast fullness, thereby causing enhanced variation in fat content of fore and hind milk, particularly in the women with larger breast size (149).

There is a lack of literature on the effect of breast anatomy and storage capacity on the hormonal composition of milk. Of all the hormones identified in milk, leptin is the most studied hormone. Average concentration of leptin in HM is not influenced by breast preference (right and left), but is affected by maternal adiposity (314). Considering research on milk-borne hormones and factors influencing their levels in HM is still in its infancy, it could be interesting to have more studies deciphering the association between breast physiology and milk hormones.

1.6.1.2 Stages of lactation

Unlike standardised formula, composition of HM is highly variable and its composition changes at every stage of lactation. So far, lipids are the most variable component of HM followed by proteins and carbohydrates. The concentration of fat in HM changes profoundly over the course of lactation, it reduces considerably during the first and fourth month of lactation, but it gradually increases between ninth and twelfth month (149,315,316).

Total protein concentration decreases in the first six month of lactation and remains constant thereafter, (315,317–319), however, concentration of casein increases, with a simultaneous reduction in whey fraction. This results in a low whey / casein ratio (90: 10) during early lactation; which further increases to 60:40 in mature milk and 50:50 in late lactation (320). Unlike lipids and proteins, lactose stays constant throughout the first year of lactation. Yet only a few studies have investigated the impact of breastfeeding preferences and lactation stages on the concentration of oligosaccharides in HM. Significant reduction has been observed in milk oligosaccharides during the first three months of lactation. (321) However, the research in this area is still in its infancy, and thus requires further studies.

Furthermore, the concentrations of hormones such as leptin, adiponectin, lysozyme, lactoferrin, osteoprotegerin, and beta endorphin, reduces (from transitional to mature milk) during the first month of lactation in both preterm and full term milk (322). The concentration of leptin in HM has previously been shown to be higher in colostrum compared with mature milk and was found to be inversely related with the duration of lactation (323). Though, adiponectin concentration is significantly higher than leptin, the concertation of adiponectin decreases with the duration of lactation (225). Lactational stages are known to contribute greatly to the alteration of milk
composition, yet there are limited studies that examined the impact of lactation stages on milk-borne hormones, particularly glucocorticoids.

1.6.1.3 **Frequency and duration of breastfeeding**

Breastfeeding causes a significant variation in both composition and production of milk. Factors responsible for these changes include breastfeeding volume, frequency, daily milk removal, infant daily milk intake, and distribution of milk throughout the day. In a healthy exclusively breastfeeding population, infant breastfeeds vary between 6-18 times a day, consuming almost 67% of total milk volume available in mothers breast (141). However, the total volume of milk varies greatly between mothers, volume ranged between 500 – 1300 ml/day. This indicates the role of infant demand in determining milk production, instead of being regulated by maternal milk supply (311).

The change in milk composition is well established. Major factors responsible for these variations includes breastfeeding frequency and the time interval between each breastfeed (311). For instance, lactose changes significantly with the number of breastfeeds each day. Although fat is the most variable component of the milk, with hind milk containing more fat than fore milk (324), it does not vary considerably with breastfeeding frequency and feeding patterns (325). However, changes in the amount of total protein intake and consumption of whey and casein protein correlates inversely with breastfeeding frequency (326), suggesting the role of milk protein in imparting a satiating effect among exclusive breastfeeding infants compared to formula feeding (327). Unlike protein, hormones present in HM such as leptin are not influenced by breastfeeding patterns, time within feeds and feed volume. Furthermore, a larger feed is known to reduce leptin concentration in post feed milk (milk after breastfed) (314). Despite all this information about breastfeeding frequency, storage capacity, milk removal before and after breastfeeding, not much is known about the impact of breast physiology, stages of lactation and breastfeeding frequency on the glucocorticoid composition of milk. This area requires further investigation.

1.6.2 **Maternal related factors affecting milk composition**

1.6.2.1 **Maternal age**

Advancing age is often associated with detrimental effect on maternal fertility and has deleterious effects on pregnancy outcomes such as increased cases of preterm birth, gestational diabetes, and neonatal deaths (328). Nevertheless, little is known about the impact of maternal age on the milk composition. A study by Kedem et al (329) showed that fat concentration of colostrum milk was significantly higher in older lactating women compared to younger mothers. They suggested that these differences could be the result of increased fat synthesis in older lactating mothers and reduced water content. Furthermore, another study showed that compared to older lactating women, transition milk from younger women had lower concentration of fat, but ten times higher level of PUFA, such as omega 6 and eicosadecanoic acid (330).
In plasma, concentrations of cortisol vary with age. Cortisol increases significantly with advancing age in both depressed men and women (331,332). Report on cortisol variation due to age is inconclusive. As some suggest, that following a stressor, older adults have limited ability to regulate the HPA axis and have lower capacity to return to baseline and difficulty in maintaining the negative feedback loop (333). Whereas, others suggest only moderate or no change in the concentration of cortisol with advancing age (334,335). It is fairly established that advancing age has a considerable effect on plasma cortisol level, yet, little is known about the impact of maternal age on the hormonal composition of the milk, particularly glucocorticoids.

1.6.2.2 Maternal diet and nutrition

HM composition is highly dynamic and enormously dependent on maternal diet. The available information on the influence of maternal diet on milk composition is complex. Influence of maternal diet varies depending upon the types of nutrients, while some nutrient content remain the same, others can have a significant variation. HM lipids, water-soluble vitamins and hormones are most sensitive to maternal nutrition, while protein levels are only affected under certain conditions (5,336–338). Consumption of high fat diet during lactating period can have adverse effect on milk production and increases the level of leptin in milk (215,339,340).

Stress and socio-economic status are considered the major predictors of hormonal changes and they may affect individual dietary intake and appetite regulation. It is well established that increased glucocorticoids levels, such as cortisol, disturb the normal energy balance. Cortisol triggers the intake of low quality and high sugar foods, and compromises the health of an individual by dysregulating their HPA axis (341,342). A study by Prieto et al (343) demonstrated that women following a Mediterranean diet had lower levels of plasma cortisol compared to the participants consuming higher saturated fats. Although there has been a significant progress in the area of stress research, it is still unclear whether the difference in maternal diet can have an effect on the biologically active components present in milk, particularly glucocorticoids.

1.6.2.3 Maternal weight and body mass index

Maternal weight also significantly affects milk composition, particularly lipids and protein concentration. The fatty acid profile of overweight women’s milk showed increased saturated fat concentration and reduced n-3 fatty acids compared with normal weight women (344). Overweight mothers produced milk with less protein compared to normal weight women (345). Some studies suggest that high pre pregnancy weight and maternal weight gain during pregnancy influence the microbiota and immunomodulatory potential of HM (346). Additionally, maternal BMI and body fat are strong predictor of milk-borne hormones such as leptin and adiponectin (339). Several animal and human studies have linked obesity with the HPA axis and cortisol production (277,347). Greater abdominal fat is associated with hyper responsive HPA axis, however the relationship between weight and cortisol up-regulation in adipocytes and hepatic tissue is still unclear (277). Pre-pregnancy overweight is known to exhibit blunted HPA axis response and increase circulatory
cortisol concentration during late pregnancy, but little is known about the impact of maternal weight on the cortisol levels in HM (348).

1.6.2.4 Mode of delivery

Infant deliveries employing caesarean section (C-section) with and without medical emergencies are increasing around the world. C-section is the result of several complications including maternal obesity, age and pre-eclampsia. Previously, mode of delivery has been linked with the alterations in milk protein composition. Compared to C-section, lactating mothers, who delivered vaginally had significantly higher level of milk protein concentration (349). Furthermore, volume of the milk transferred to infants born via C-section was significantly lower compared to normal birth (350). Whereas, Rubio et al (351) showed that the milk microbiome from mothers who delivered their infant via elective caesarean section differed in composition to mother’s who had caesarean section, indicating the effect of physiological stressful situation on the microbiome of HM.

Exposure to prenatal or postnatal stress in both mother and infant is often associated with altered hormonal responses such as increased secretion of epinephrine, norepinephrine, ACTH, CRH and cortisol. However, the elective C-section was found to be associated with lower stress response in both mother and infant compared with unassisted, emergency, and normal vaginal delivery (352). Much is known about the effect of C-section on delaying the breastfeeding process but nothing is known about the effect of mode of delivery on the milk glucocorticoids composition.

1.6.2.5 Preterm birth

Preterm birth is defined as the birth before 37 weeks of gestation. It is a global health problem with an estimate of 15 million premature births annually (353). It can further be classified into extremely preterm (<28 weeks) and very preterm (between 28-32 weeks) births; and of all the preterm birth, 5% accounts for preterm and about 15% are born as very preterm infants (354).

HM is the preferred source of nutrition for all term and preterm infants. Its composition however is highly dynamic, and degree of prematurity and gestational age are important predictors of milk composition (355,356). It is well established that composition of HM is significantly different between the mothers delivering full term and preterm infants, with 35% more protein in the milk produced for preterm infants. Milk produced by mothers who deliver premature infants are often high in nitrogen, and medium chain fatty acids, essential for infant survival (177,357). Additionally, preterm milk seems to have higher concentration of immunoglobulins, growth factors and hormones compared to the term milk (358,359). However, the concentration of these components reduces gradually with the progression of lactation. Moreover, some of the appetite regulating hormones such as leptin and ghrelin have been detected in the milk produced for preterm infants. Leptin in preterm milk was highly variable and was found at a similar level to full term milk (360). Similarly, levels of ghrelin did not differ between term and preterm milk (224). But to date not much is known about the presence of adiponectin and resistin in the milk of mothers delivering preterm or very preterm infants (218).
Much has been written about the effect of premature birth on the milk energy components, particularly macronutrients. To date, few studies have demonstrated the impact of premature birth on the hormonal composition of the milk. A recent study by Van der voorn et al (2016) showed that mothers giving birth to a very preterm infant had significantly lower levels of cortisol and cortisone than the milk of mothers who delivered full term (307). However, there is minimal literature on the factors influencing the hormonal composition of milk, particularly glucocorticoids. In addition, how these alterations could influence the development of a breastfeeding infant remains unclear.

1.6.2.6 Parity

Increased parity is known to affect circulating level of hormones. However, little is known about the influence of parity on milk glucocorticoids levels. Only a few studies have demonstrated that the concentration of cortisol was markedly reduced in plasma (361) and hair of mothers with low parity (362). Mothers with multiparity are often associated with increased physical and psychological risk factors compared to mothers with a single child (363). A study of rhesus monkey demonstrated that mothers with lower parity produced milk with higher cortisol concentration (308). Whereas primiparous mother experienced lack of sleep and perinatal trauma, compared with experienced multiparous mothers (363). Although much is known about the influence of parity on breastfeeding status, the central question of how it could contribute to the dynamics of milk hormonal composition remains unclear.

1.6.2.7 Socio-economic factors

Numerous social and environmental factors such as maternal economic status and seasonal variation are associated with significant changes in HM, particularly the hormonal composition of milk. Maternal social-economic statuses, or psychosocial factors such as job stability, education, and marital status, are known to alter the HPA axis response and hence circulating cortisol concentration (364). In comparison with the influence of socioeconomic factors on breastfeeding cessation and initiation, little is known about the influence of maternal social factors on milk composition. Evidence suggests significant differences in the macro and micronutrient composition of HM in women from different socioeconomic background (178). The lipid profile of HM is directly associated with maternal living conditions and her economic status (365). Milk from the mothers of higher socioeconomic status had higher concentration of long chain fatty acids, compared with mother from low economic status (365).

Cortisol has received much attention as an important biomarker of socio-economic standard (SES). Low socio-economic standard (LSE) is consistently associated with increased and blunted diurnal secretion of cortisol (364). Role of maternal psychological stress and low economic status is well established in predicting low birth weight, preterm delivery, and significant alteration in infant neurological and behavioural development (366,367). Several studies have examined the relationship between socioeconomic status and individual stress and have shown that individual of lower socio-economic status or lower education have increased level of cortisol (368). Multiple
maternal psychological studies, conducted during pregnancy and lactation, have described maternal education, multiple job holdings, and relationship status as the major predictors of plasma and hair cortisol variations (369–371). These may affect HM composition. However, this has not been studied yet, and thus require further studies that investigate the effect of maternal living conditions on biologically active components milk, particularly glucocorticoids.

1.6.2.8 Postpartum depression

The relationship between postpartum depression and breastfeeding is equivocal. Evidence suggests that depressed mothers are unlikely to start or continue breastfeeding for longer duration (47,93), thus affecting the frequency and duration of breastfeeding and further influencing milk production. Often, unsuccessful breastfeeding has been studied as one of the predictor for postpartum depression. This appears to have an adverse effect on mother’s mood (93,96). Breastfeeding has a positive influence on mothers with postpartum depression. It regulates the HPA system, which is deregulated during depression (47,94). To date, much of the literature is focused upon the impact of depression on breastfeeding frequency and vice versa. The effect of maternal depression on circulating concentration of cortisol in both saliva and plasma are well described but the impact of maternal depression on its milk composition is still in its infancy. The relationship between maternal depression and offspring development is complex (262,372). The onset and duration of maternal depression during and post pregnancy, significantly alters infant biochemical profile and physiological development (372).

1.6.2.9 Environmental and seasonal variation

HM is the first source of nutrition for a newborn infant, hence any disparity in HM composition due to environmental and seasonal variations is of concern. Because milk composition is not pristine, anything not natural in the milk is considered a contaminant. Unfortunately, environmental contaminants can transfer into milk via passive diffusion from lactating women’s plasma. The classic examples includes DDT, polychlorinated biphenyls dioxins, dibenzofurans, polybrominated diphenyl ethers, and heavy metals are considered as the toxic environmental chemicals present in HM (373,374). Lack of complete data on contaminants and toxicokinetics makes it difficult to examine the issue related with chemicals present in the milk and their impact on offspring health, both in the short and long term.

There is a massive amount of literature exploring the impact of seasonal variation on the composition of bovine and diary milk, but nothing is known about the influence of seasonal variation upon hormones, particularly glucocorticoids. Glucocorticoid secretion is often considered the obligatory response to a stressful situation and hence can be affected by several factors including seasonal variations. Persson et al (375) reported that in healthy adults, showed the higher salivary cortisol during the spring season, whereas another study showed lowest concentration of cortisol during spring and summer (376). In contrast, most others found no effect of seasonal variation on salivary cortisol (377). Despite the accumulating amount of information
on salivary and plasma glucocorticoids, little is known about the impact of seasonal factors on the glucocorticoid composition of HM and how it may affect the infant’s development.

1.6.2.10 Diurnal variation

Cortisol has well-established circadian patterns in human plasma, with the levels lowest during the evening between 2000h – 0200h; and highest during the morning shortly after awakening (251). In HM, fat and energy changes from the beginning to end, and follows diurnal patterns. The concentration of fat changes significantly during the 24 hour period. Its concentration increases during the afternoon and evening and decreases during the night (141). Limited information is available on the diurnal variation of the biologically active components in HM. Recently, diurnal variation have been observed in milk glucocorticoids levels (307). The concentration of cortisol and cortisone peaked in the morning, followed by a gradual decrease throughout the day. This suggests the importance of infant feeding time and how it could influence the nutrient intake of breastfeeding infants.

1.6.3 Infant related factors affecting milk composition

Although it has been recognised for decades that HM composition is highly dynamic; and different maternal related factors are important predictors of milk composition, little is known of how infant related factors can influence the biochemical composition of milk. There are only few infant related factors, which have been studied in detail such as infant sex and birth weight or body composition in relation to milk composition.

1.6.3.1 Infant sex

There has been no consensus if milk composition varies according to infant sex. Several animal studies have shown notable sex based difference in milk composition, particularly in fat and energy content. For example, wallabies and red deer displays sex-biasedness, with more postnatal investment in sons over daughter, and produced milk with more protein in favour of sons (378,379). However, biasedness was not observed for micronutrients (380). Similar results were observed in the milk of rhesus macaques. Milk produced for male offspring was more energy dense, while higher milk yield was for female offspring (381). Fat content of the milk produced for first-born male offspring was higher, compared to fat produced for second birth irrespective of infant sex. However, minimal sex-based comparable data was identified for humans. So far, only two studies have reported the sex-based milk differences. In 2010, Powe et al (382) was the first human study to identify the sex-based difference in milk energy content, followed by Fujita et al in 2012 (383). They showed economically sufficient mother produced richer milk for sons, while mothers in poor condition produces richer milk for female offspring (383).
1.6.3.2 Other infant characteristics

Various factors are known to influence infant’s milk intake and potentially influencing its composition and production. HM composition can be influenced by infant related factors such as stomach storage capacity, body weight, growth related metabolic requirements and gastric emptying rate. Evidence suggests that high-energy meal delays the gastric emptying rates in both adults and children. However, gastric emptying rate are much faster in infants after consuming HM compared to cows’ milk or any other meal. These variations of stomach emptying rates suggest the role of HM in changing breastfed frequency, especially for exclusively breastfeeding infants, indicating its role in affecting the composition and production of HM.

Despite the vast amount of information about milk composition, surprisingly, the data regarding the impact of infant related factors on milk composition is limited. Currently, the majority of literature in milk science has mainly concentrated on milk macronutrients and factors influencing their concentration in milk. Remarkably, little is known about the presence of glucocorticoids in HM and the factors influencing its concentration in milk and how various concentration could affect the growth and development of a breastfeeding infant.
1.7 Analysis of human milk components

Continuous enhancement in the chromatographic techniques and advanced statistical and mathematical tools has led to the development of complete human and serum metabolome. The development of nuclear magnetic resonance (NMR) spectroscopy and gas chromatography mass spectrometry (GC-MS), has helped to revealed several complexities of milk metabolites in both bovine and HM (384,385).

Traditionally, HM proteins have been identified using conventional biochemical methods such as protein purification, antibody-detection, or 1-dimensional PAGE based proteomic methods. However, these techniques have major disadvantages and limitations of producing low throughput information on only a few target proteins. The advent of proteomics and high throughput techniques has led to the discovery of various new proteins or peptides, providing a holistic approach for detecting many proteins in a single experiment. It is important to identify these proteins and recognise their function as an insight into exploring the mechanism behind mammary gland function and metabolism.

In a study by Smilowitz (386) which used NMR spectroscopy on milk samples. They identified 65 metabolites from 52 healthy women. HM metabolome revealed a diverse group of sugars, amino acids, oligosaccharides, nucleotides, fatty acids, and other energy metabolites. Of all the metabolites, sugar and lactose were the most dominant (386). Furthermore, GC-MS analysis of full term mothers milk versus formula milk expressed 80% variability, indicating a high amount of lactose in mothers’ milk and high biochemical variability within individuals (385).

Mass spectrometry acts as a foundation for the majority of proteomics and lipidomics study in human milk derived components. Significant efforts have been made to identify host defence protein N-linked glycoprotein and milk fat globule membrane (MFGM) proteins (387,388). In terms of identification of the milk proteome, Palmer et al (389) characterized skim colostrum and identified 151 new proteins. Whereas, ProteoMiner, an advanced protein enrichment technology, identified 115 low abundance proteins in whey fraction of milk protein, which changed quantitatively throughout the entire duration of lactation (390).

1.7.1 Technological advancement of hormonal analysis

In endocrinology, quantitative laboratory methods have been used to identify and characterize important metabolites in biological fluids (391). Hormones are the regulatory products initiating or inhibiting several downstream biochemical reactions occurring inside the cell, tissue or organism (392). Studying these metabolites in biological samples uncovers useful information related to the biochemical alterations at cellular level in response to gene, diet or environmental factors (393–395).

Previously, immunoassays were the preferred methodology for analysing steroid hormones in bio-fluids as shown in Table 1-5, but recent advances in mass spectrometry were a major milestone
that revolutionized the analytical practices in endocrinology. Compared to mass spectrometry, immunoassay delivers restricted accuracy and in some cases gave exaggerated readings (396). Differences in the concentration were mainly attributed to cross reactivity of the interfering antibodies or restricted detection limits (397).

For hormone analysis, LC-MS/MS has become the technique of choice of due to its highly innovative and accurate methodology for analysing wider analytes (398). These powerful analytical tools used to identify and characterize chemical and biological entities in physiological fluids, require minimum sample preparation (399). However, the method development for MS is complex and requires standardization, and technical expertise. LC-MS/MS is a highly precise and sensitive analytical technique that can measure multiple analytes and require fewer samples (400,401). For all these reasons, measurement of steroids using LC-MS/MS has become the method of choice for variety of clinical diagnostic and screening tests in endocrine analysis.

All these discoveries have enriched our knowledge about the HM complexities, which has helped to optimise innovations aimed at improving milk supply, composition and health outcomes for compromised infants (402). Given the fact, it has now repeatedly shown that biochemical makeup of HM is influenced by variety of maternal and infant related factors, further exploration of these factors is still warranted. There is a lack of comprehensive understanding on the HM metabolome, but with the advancement in technologies, more methods can detangle the complex mixture of HM and improve our understanding about the synthesis and regulation of milk volume and composition and its effects on infants’ health.
Table 1-5: Summary of published studies on the presence of glucocorticoids in human milk.

<table>
<thead>
<tr>
<th>References</th>
<th>Year</th>
<th>Method</th>
<th>Cortisol ng/ml</th>
<th>Cortisone ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kulski and Hartman</td>
<td>1981</td>
<td>RIA</td>
<td>Range 0.21-31</td>
<td>-</td>
</tr>
<tr>
<td>Sahlberg and Axelsson</td>
<td>1986</td>
<td>GC-MS after enzyme deconjugation</td>
<td>0-1.44</td>
<td>0-6.1</td>
</tr>
<tr>
<td>Ost et al</td>
<td>1985</td>
<td>HPLC</td>
<td>Range ≤14.85</td>
<td>-</td>
</tr>
<tr>
<td>Xu et al</td>
<td>2011</td>
<td>LC-MS after enzyme deconjugation</td>
<td>1.23</td>
<td>10.14</td>
</tr>
<tr>
<td>Grey et al</td>
<td>2013</td>
<td>ELISA</td>
<td>2.17</td>
<td>-</td>
</tr>
<tr>
<td>Van der Voorn et al</td>
<td>2016</td>
<td>LC-MS</td>
<td>1.44-8.33</td>
<td>3.98-11.95</td>
</tr>
<tr>
<td>Pundir et al</td>
<td>2017</td>
<td>LC-MS/MS</td>
<td>7.64</td>
<td>9.55</td>
</tr>
</tbody>
</table>
1.8 Summary

In summary, mother’s milk is an essential and complete source of nutrition for the survival of a newborn infant. It is a remarkably complex biomaterial, typified by adaptive changes that occur over the course of lactation. As the infant develops, HM adapts in both nutrient composition and volume tailoring to the physiological requirements of the developing infant. Further, any alterations in milk composition have the potential to impact an infant’s health, both short term and long term. Breastfeeding mothers face many physiological and psychological changes over the course of lactation that significantly defines the biochemical composition of milk. A breastfed infant’s behavioural and metabolic programming is affected by the presence of milk-borne hormones, elicited by glucocorticoids, mainly cortisol, whose level in turn is affected by the alterations in maternal social and biological environment.

Given the paucity of data, more research is required to bridge the gap between the effect of maternal and infant related determinants on the glucocorticoids (both cortisol and cortisone) composition of milk. Understanding the factors that influence maternal stress or confounding infant related factors during breastfeeding may help health professionals give appropriate recommendations to lactating mothers and thus improve the long-term effects of breastfeeding on infants.

1.9 Significance

Much progress has been made in the past century on our understanding of the composition of HM. Evidence exists to suggest that HM is a highly complex secretion typified by adaptive changes that occur over the course of lactation to meet an infant’s needs. Maternal and infant related factors play an important role in dictating the composition of milk. Despite all of the sophistication and the current knowledge about HM, there is an incomplete understanding about changing milk composition, with an emerging interest in biologically active compounds. Specifically, the link between maternal conditions and the presence of milk glucocorticoids is unclear. Therefore, it is a promising area of research to expand on. Further, whether maternal conditions alter milk glucocorticoids composition and if these have any influence on infant growth and development remains unknown. Despite the known information on the factors influencing circulatory glucocorticoids, their role in HM has not been explored. Future research must focus on understanding the possible determinants driving the changes in milk composition to assist in developing nutritional strategies for growing infants.
1.10 Thesis Objective

This thesis aims to examine the role of maternal and infant factors affecting the glucocorticoid levels of HM at different stages of lactation.

The specific aims and objectives of the experimental studies comprising this thesis include the following -

- To investigate the impact of maternal associated biological, social and environmental factors on the glucocorticoid concentrations (both cortisol and cortisone) of human milk, at three months of established lactation.

- To investigate the relationship between human milk glucocorticoid concentrations and preterm birth.

- To analyse the concentration of glucocorticoid hormones (both cortisol and cortisone) in human milk over a 24 hour period and explore if glucocorticoids follow a circadian pattern in the human milk.

- Longitudinal analysis of glucocorticoids in human milk over the first 12 months of lactation and its relationship on infant’s development.
1.11 Hypothesis

It was hypothesised that-

- Maternal social and biological conditions will influence the hormonal composition (both cortisol and cortisone) of HM.

- Preterm birth will be associated with higher cortisol concentration and will be attenuated with duration of birth postpartum duration and infant sex.

- Glucocorticoid concentrations of HM will demonstrate diurnal patterns similar to plasma, and duration of breastfeeding (pre and post breastfeeding) will be associated with reduced steroid hormones in milk.

- Glucocorticoids in HM will change substantially throughout the first year of lactation and would cause and effect infant growth outcomes.
Chapter 2: Maternal influences on the glucocorticoid concentrations of human milk – the STEPS study
2.1 Summary

Human breast milk and breastfeeding are the optimal source of infant nutrition, during the first six months of life. In addition to providing nutrients, HM contains a wide array of non-nutritive bioactive elements, including glucocorticoid hormones (glucocorticoids: cortisol and cortisone). Milk-borne glucocorticoids regulate infant stress-mediated responses and may influence infant development. Numerous factors are known to influence maternal stress, and hence may potentially alter the glucocorticoid composition of HM. This study demonstrated that patterns of breastfeeding have no impact on milk hormonal composition, related to the differences in glucocorticoid concentrations within HM. However, multiple regression analysis revealed preterm birth and maternal education as significant predictors of cortisol and cortisone variation in HM.

The samples used in this study were collected from Adjunct Professor Hanna Lagström, from Turku Institute for Child and Youth Research, University of Turku, Turku, Finland. However, the hormonal and data analysis was conducted at the Liggins institute, University of Auckland, Auckland, New Zealand.

The following section contains a manuscript entitled “Impact of maternal factors on the glucocorticoid concentrations of human milk: the STEPS study” co-authored by Shikha Pundir, Johanna Mäkelä, Anu Nuora, Clare Wall, Kaisa Linderborg, Hanna Lagström, David Cameron-Smith. This article has been drafted for submitting in *European Journal of Nutrition*. 
Maternal influences on the glucocorticoid concentrations of human milk – the STEPS study

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Running Title: Maternal factors affecting milk glucocorticoid composition
2.2 Abstract

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 are not yet established. The main objective of this study was to determine the influence of breastfeeding patterns, maternal and infant related biological and social factors may have on the glucocorticoid concentration of HM. HM samples were obtained from lactating mothers (n=656) participating in the Finnish cohort STEP study (Steps to the Healthy Development and Well-being of Children) when the infants were 11.29 (±2.6) weeks of age. Glucocorticoids 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.

The majority of women reported that they were exclusively breastfeeding at the time of HM sample donation (51.2%). For all collected samples, cortisone (9.55 ± 3.44 ng/ml) and cortisol (7.39 ± 5.97 ng/ml) was present in all samples. A positive correlation existed in the HM between both hormones (r=0.60, p<0.0001). Cortisone was significantly lower in overweight/obese women, compared to normal and underweight women (p= 0.01). 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 weaker negative relationship between mothers educational status and cortisol (p=0.05). Interestingly, there was no influence of feeding patterns on HM glucocorticoids. Thus, HM glucocorticoid concentrations are influenced by maternal weight, preterm birth, and maternal educational status, suggesting the possible role of maternal biological and psychosocial influences on the milk hormonal composition. Further analysis is required to fully explore the relationship with measures of maternal stress, including mother’s glucocorticoid status.
2.3 Introduction

Human breast milk and breastfeeding is the optimal source of nourishment for a new born infant. Exclusive breast-feeding for the first six months of life is the normative standard of infant feeding (25). Unlike standardised manufactured infant formula milk, the composition of HM 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 (403,404).

HM is a primary carrier of a wide variety of nutrients, and also includes non-nutritive bioactive factors such as glucocorticoids. Cortisol and cortisone are the primary glucocorticoids produced from the adrenal glands, in response to physiological and psychological stress (246). Beyond these, cortisol plays a key role in gluconeogenesis, lipolysis and energy metabolism (248,306,405,406). Glucocorticoids in human milk are reflective of the circulating abundance in the maternal blood and saliva (307). 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 infant's circulatory system. However, throughout the first year of life, breastfed children exhibit 40% more salivary cortisol, then formula fed infants (304). Animal research has shown that milk borne glucocorticoids are transported into offspring circulation and bind to receptors in infant tissues such as hippocampus (407). Additionally, in human infants the GC receptor expression is upregulated during infancy but only if babies are consuming breast milk, not formula (impoverished for GC) (408): There is some evidence that milk borne glucocorticoids may influence the psychological maturation of the child (308,310). Higher cortisol level in milk was linked with increased fearful temperament in infants, however these effects were more prominent in female child (14). Unlike other bioactive components in milk, glucocorticoids have diverse functions; hence may exert a significant effect on offspring phenotype, which are still unknown.

Currently there is limited understanding of the predominant determinants of HM glucocorticoids. It is however plausible that maternal stress and subsequent systemic stimulation of the hypothalamic-pituitary-axis (HPA) axis is likely to be a significant key regulatory mechanism, that elevates glucocorticoid levels in both animals and human milk (126,233,409). The period prior to and, months following 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 levels. These include, maternal health status prior to and during pregnancy (410,411), method of infant delivery (352) gestational age (412), social/familial surroundings of pregnant or lactating mother (post-natal depression) (364,413), and varied patterns of breast feeding may have an immediate effect on maternal stress levels (414). Numerous studies have demonstrated the inverse relationship between breastfeeding and maternal stress, exclusive breastfeeding is associated with blunted HPA axis responses, thereby reducing cortisol release to psychological response (415). Furthermore, long term–chronic stress or depressive symptoms throughout pregnancy can have a negative influence on the duration of
breastfeeding (416). However, not much is known about the impact of maternal and infant associated factors on milk stress hormone levels and how this would affect infant 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 this study was to analyse which maternal or infant related biological, social and environmental factors are associated with HM glucocorticoid levels around infant age of 3 months. Moreover, we aimed to analyse the influence of breast-feeding patterns on glucocorticoid concentration. 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.
2.4 Methods

2.4.1 Study design

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), has been described in detail elsewhere (417). 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 (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%) 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), unsystematic shipping or labelling (n=25), empty tube (n=1) or unsuccessful analytical analysis (n=14). Mean infant age at the time of milk collection was 11.29 (±2.6 weeks).

2.4.2 Socio-demographic, 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 or city, 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/m²) and classified as underweight (<18.5 kg/m²), normal weight (18.5-24.9 kg/m²), while overweight (25.0-29.9 kg/m²) and obese (≥30.0 kg/m²) 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 (≥3000 €/month net) and low income (<3000 €/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 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 unhappy/ very happy). 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.

### 2.4.3 Feeding information and milk collection

Information about breast-feeding (BF) and complimentary feeding (CF) was obtained using a self-administered follow-up diary. This consisted of detailed information about duration of full 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. Full BF was defined as infant receiving no other food than mother’s milk, except for water, drops, or syrups consisting of vitamins, mineral supplements, or medications. 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 bias.

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 from single breast by manual expression in the morning, 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.
2.4.4 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 mins and vortexing them for 20 seconds, before adding 100 µl of internal standard to 100 µl of milk. The internal standard consisted of 12ng/ml cortisol d4, 60mg/ml corticosterone d8, 20pg/ml of estradiol and 20pg/ml testosterone-d3, prepared in milli-Q water. Steroids were then extracted using 1ml ethyl acetate (Merck, Darmstadt, Germany); and the top organic layer was removed and vacuum dried (Savant, SC250 EXP, Thermo Scientific, United States) for ~2hours. The dried residues were reconstituted in 80µl of 50/50% methanol (Merck, Germany)/water and transferred to HPLC injector vials.

2.4.5 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 quadruple 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 the flow rate of 500 µl/min. The chromatography was performed at 40°C. The instrument was set up at selection reaction monitoring (SRM) mode; m/z 363.1→121 at 24 V for cortisol; 363.1→163 at 24 V for cortisone; 289.17→97.2 at 28 V for testosterone, 255.14→159.2 at 18 V for estradiol, 315.18→109.2 at 26 V for progesterone. Argon gas was used as the collision gas at 1.2mTorr 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 5-10,000pg/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.

2.4.6 Statistical analysis

The concentrations of cortisol and cortisone in milk were calculated and reported in ng/ml. Descriptive statistics on continuous variables are presented as mean ± 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 was carried out to determine relationships between baseline characteristics and HM glucocorticoids.

The Pearson correlation coefficient was used to analyse association between the calculated average value of cortisol and cortisone of HM samples. Linear regression models were used to
investigate the effect of maternal biological, social, and environmental factors on glucocorticoid 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.InK Chicago, Illinois, USA) was used for data analysis and all graphs were created using GraphPad Prism version 7.0.
2.5 Results

2.5.1 Participant and sample characteristics

Maternal, child and HM characteristics are reported in Table 2-1. From the total consenting cohort of 650 mothers, eighty-three percent of (n= 542) mothers provided self-reported data on breastfeeding patterns. Out of 650 mothers, 51.53% (n=335) maintained exclusive breastfeeding and 31.61% (n= 212) reported the adoption of a partial breastfeeding until the time of milk collection. The remaining 17.76% (n=109) failed to report the details of breast feeding habits. Should delete it.
Table 2-1: Characteristics as mean ± SD or % (n) of the mothers, children and breast milk in the STEPS Study.

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

Advanced education (highest level of vocational training (such as a 4-year program 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, ≥3000 €/month net; low income, <3000 €/month net.
2.5.2 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 HM samples. Both hormones differ widely among mother 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 ($r=0.60 \ p=<0.0001$), shown in Figure 2-1.

Figure 2-1: Residual scatter plot and fitted regression line for the HM cortisol versus HM cortisone concentration at 3 months of established lactation.
### 2.5.3 Glucocorticoids variation of human milk with maternal biological and socio-demographic factors

Maternal biological and socio-demographic factors affecting the milk glucocorticoid concentration are illustrated in Table 2-2.

**Table 2-2: Univariate association of maternal and infant characteristics and milk glucocorticoid (cortisol and cortisone)**

<table>
<thead>
<tr>
<th>Categorical Variable</th>
<th>Cortisol (mean ±SD)</th>
<th>p value</th>
<th>Cortisone (mean ±SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal demographics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overweight/obese</td>
<td>7.40 ± 6.13</td>
<td>0.96</td>
<td>8.93 ± 3.32</td>
<td>0.01</td>
</tr>
<tr>
<td>Normal weight</td>
<td>7.36 ± 5.86</td>
<td></td>
<td>9.82 ± 3.44</td>
<td></td>
</tr>
<tr>
<td>Under weight</td>
<td>7.68 ± 6.77</td>
<td></td>
<td>9.33 ± 3.79</td>
<td></td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>7.34 ± 5.91</td>
<td>0.39</td>
<td>9.57 ± 3.43</td>
<td>0.77</td>
</tr>
<tr>
<td>Yes</td>
<td>8.04 ± 6.56</td>
<td></td>
<td>9.43 ± 3.46</td>
<td></td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>7.29 ± 6.00</td>
<td>0.16</td>
<td>9.49 ± 3.42</td>
<td>0.19</td>
</tr>
<tr>
<td>Caesarean</td>
<td>8.30 ± 5.77</td>
<td></td>
<td>10.03 ± 3.51</td>
<td></td>
</tr>
<tr>
<td>Family structure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>7.30 ± 6.06</td>
<td>0.80</td>
<td>10.20 ± 3.86</td>
<td>0.52</td>
</tr>
<tr>
<td>Nuclear</td>
<td>7.59 ± 5.91</td>
<td></td>
<td>9.50 ± 3.43</td>
<td></td>
</tr>
<tr>
<td>blended</td>
<td>4.74 ± 1.12</td>
<td></td>
<td>9.76 ± 3.15</td>
<td></td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>7.29 ± 6.06</td>
<td>0.80</td>
<td>9.51 ± 3.45</td>
<td>0.49</td>
</tr>
<tr>
<td>common law</td>
<td>7.59 ± 5.90</td>
<td></td>
<td>9.54 ± 3.33</td>
<td></td>
</tr>
<tr>
<td>others/single</td>
<td>6.93 ± 4.74</td>
<td></td>
<td>10.50 ± 4.28</td>
<td></td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>7.76 ± 6.38</td>
<td>0.33</td>
<td>9.56 ± 3.70</td>
<td>0.95</td>
</tr>
<tr>
<td>Higher</td>
<td>7.25 ± 5.81</td>
<td></td>
<td>9.54 ± 3.34</td>
<td></td>
</tr>
<tr>
<td>Occupational class</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>7.46 ± 6.03</td>
<td>0.45</td>
<td>9.62 ± 3.81</td>
<td>0.30</td>
</tr>
<tr>
<td>Professionals</td>
<td>7.05 ± 5.84</td>
<td></td>
<td>9.30 ± 3.32</td>
<td></td>
</tr>
<tr>
<td>Living in urban area</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Living in city</td>
<td>7.60 ± 6.07</td>
<td>0.42</td>
<td>9.74 ± 3.38</td>
<td>0.19</td>
</tr>
<tr>
<td>Not living</td>
<td>7.22 ± 5.89</td>
<td></td>
<td>9.39 ± 3.48</td>
<td></td>
</tr>
<tr>
<td>Family incomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low &lt; 3000e</td>
<td>7.61 ± 6.51</td>
<td>0.34</td>
<td>9.50 ± 3.59</td>
<td>0.54</td>
</tr>
<tr>
<td>Higher, &gt;3000e</td>
<td>7.16 ± 5.37</td>
<td></td>
<td>9.67 ± 3.31</td>
<td></td>
</tr>
<tr>
<td>Happiness</td>
<td>Not happy, below 6</td>
<td>5.60 ± 4.44</td>
<td>0.16</td>
<td>9.25 ± 2.79</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------------</td>
<td>--------------</td>
<td>------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>Happy</td>
<td>7.47 ± 6.03</td>
<td></td>
<td>9.56 ± 3.43</td>
</tr>
<tr>
<td>Anxiety</td>
<td>Yes, below 6</td>
<td>7.76 ± 5.79</td>
<td>0.49</td>
<td>9.56 ± 3.39</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>7.33 ± 6.02</td>
<td></td>
<td>9.56 ± 3.43</td>
</tr>
<tr>
<td>Depression</td>
<td>Yes, below 6</td>
<td>7.13 ± 5.24</td>
<td>0.63</td>
<td>9.39 ± 3.34</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>7.47 ± 6.08</td>
<td></td>
<td>9.60 ± 3.44</td>
</tr>
<tr>
<td>Life satisfaction (sum of 3 measures)</td>
<td>No</td>
<td>7.19 ± 5.50</td>
<td>0.77</td>
<td>9.71 ± 3.21</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>7.44 ± 6.04</td>
<td></td>
<td>9.53 ± 3.45</td>
</tr>
<tr>
<td>Infant and human milk demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Season HM sample taken</td>
<td>Winter (dark)</td>
<td>7.25 ± 6.03</td>
<td>0.49</td>
<td>9.41 ± 3.40</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>7.57 ± 5.90</td>
<td></td>
<td>9.73 ± 3.49</td>
</tr>
<tr>
<td>Breast feeding group</td>
<td>Exclusive</td>
<td>7.57 ± 6.22</td>
<td>0.70</td>
<td>9.73 ± 3.45</td>
</tr>
<tr>
<td></td>
<td>Partial</td>
<td>7.12 ± 5.89</td>
<td></td>
<td>9.39 ± 3.47</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>7.37 ± 5.36</td>
<td></td>
<td>9.29 ± 3.31</td>
</tr>
<tr>
<td>Gender</td>
<td>Boys</td>
<td>7.20 ± 5.61</td>
<td>0.38</td>
<td>9.33 ± 3.31</td>
</tr>
<tr>
<td></td>
<td>Girls</td>
<td>7.61 ± 6.37</td>
<td></td>
<td>9.80 ± 3.57</td>
</tr>
<tr>
<td>Premature births</td>
<td>≤ 37 weeks</td>
<td>8.37 ± 7.54</td>
<td>0.38</td>
<td>8.71 ± 3.84</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>7.35 ± 5.90</td>
<td></td>
<td>9.59 ± 3.42</td>
</tr>
<tr>
<td>Siblings</td>
<td>None</td>
<td>7.40 ± 6.02</td>
<td>0.97</td>
<td>9.49 ± 3.30</td>
</tr>
<tr>
<td></td>
<td>1 or more</td>
<td>7.38 ± 5.92</td>
<td></td>
<td>9.63 ± 3.63</td>
</tr>
<tr>
<td>Twin sister/brothers</td>
<td>No</td>
<td>7.41 ± 5.99</td>
<td>0.49</td>
<td>9.54 ± 3.44</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>6.22 ± 5.16</td>
<td></td>
<td>10.23 ± 3.62</td>
</tr>
<tr>
<td>BMI before pregnancy</td>
<td>-</td>
<td>-</td>
<td>0.62</td>
<td>-</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>-</td>
<td>0.20</td>
<td>-</td>
<td>0.81</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>-</td>
<td>0.12</td>
<td>-</td>
<td>0.73</td>
</tr>
<tr>
<td>Exclusive BF, months</td>
<td>-</td>
<td>0.45</td>
<td>-</td>
<td>0.13</td>
</tr>
<tr>
<td>Total BF, months</td>
<td>-</td>
<td>0.45</td>
<td>-</td>
<td>0.94</td>
</tr>
</tbody>
</table>
Maternal BMI (prior to pregnancy) was positively associated with cortisone concentration in milk; whereas cortisone was significantly higher in normal weight mothers (9.82 ng/ml) in comparison with overweight (8.93 ng/ml) and underweight women (9.33 ng/ml) (p=0.01) (Figure 2-2). 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 sample obtained from women reporting either exclusive or partial feeding practices. Breast-feeding patterns showed no effect on the glucocorticoid concentrations (cortisol & cortisone) in HM at infant’s age of 3 months (Figure 2-3 A & B). In addition, maternal age, weight, gestational diabetes, mode of delivery were the non-significant predictors of glucocorticoids composition of HM (Table 2-2).

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. Furthermore, maternal happiness, anxiety, depression, and life satisfaction score collected four months postpartum, were non-significant predictor of glucocorticoids variation in HM samples. Because these measures were not taken at the time of sample collection, could refer to as study design limitation. In order to examine the effect of environment, we investigated the seasonal variations of milk glucocorticoid composition and found no significant difference between winter (October- February) and summer (March- September). Results of the association between glucocorticoid concentration and maternal social and environmental factors are presented in Table 2-2.
Figure 2-2: Glucocorticoids, (A) cortisol and (B) cortisone concentration in the milk of overweight, normal and underweight lactating women at 3 months of established lactation.

Figure 2-3: Glucocorticoids concentration of HM (A) cortisol in HM with exclusive and partial breastfeeding patterns, (B) cortisone in HM with exclusive and partial breastfeeding patterns.
2.5.4 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 non-significant predictors of the glucocorticoid variation in HM.

Based on the results from Table 2-2 and previously cited studies in literature(368,369) we conducted regression models. In model 1, we compared the effect of premature birth and gestational diabetes on milk glucocorticoid concentrations. 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 were found to be significant for cortisone. Table 2-3 presents the results of linear regression analysis, with cortisol and cortisone in HM as the outcomes.
Table 2-3: Bivariate association of maternal factors with milk glucocorticoids (cortisol and cortisone)

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

From this cohort (417), 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, 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 variation 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. In the past, several studies have demonstrated significant associations between maternal weight and altered HM composition (215,344,418). For instance, leptin concentration in HM increases with maternal adiposity (222), whilst adiponectin levels are inversely correlated with maternal degree of adiposity (419). To our knowledge, this is the first study that investigated the impact of maternal body composition parameters on HM glucocorticoid profiles. Glucocorticoids are known to play an important role in regulating body fat distribution. Obesity is characterised by increased secretion of glucocorticoids (420,421). However, most studies have only assessed cortisol secretion related to anthropometric measures of adiposity, BMI (body mass index) and WHR (waist to hip ratio), and have reported mixed results (347,422). Recently, another studies of stress related disorders indicates chronic stress with prolonged hyperactivity of the HPA axis, can evolve into hypo-secretion of glucocorticoids (423). However, the mechanisms that govern this phenomenon is still unknown. Surprisingly, not much is known about circulating concentrations of cortisone. We also found that milk from overweight women had lower cortisol content compared to normal weight women. However, these measures ought to be treated with caution because results were obtained from the samples collected at three months after infant birth.

Preterm birth/delivery is a major cause of infant morbidity and mortality in industrialised countries (354). During the past decade, rate of premature birth has increased steadily, and mothers whose babies are born prematurely are often more stressed (424). Several studies have investigated the relationship between maternal stress and child outcome, and linked maternal prenatal cortisol, as a positive precursor of preterm birth (425). Yet, very few have investigated the role of premature birth on milk composition. Since, HM is highly dynamic, it is essential to consider factors that may alter its composition after breastfeeding is established. The current study measured cortisol and cortisone in HM samples taken 12-16 week post-birth. Our analysis revealed that mothers of premature infants had significantly increased cortisol and cortisone concentrations in their milk, after adjusting for gestational diabetes. In patients with type-2 diabetes, glucocorticoids are suggested to influence insulin sensitivity and impair β-cell functioning (426), however nothing is known about the impact of gestational diabetes on milk glucocorticoid levels. Findings of the current study indicate an ability for
preterm birth to influence mother’s milk glucocorticoid concentrations. Contrarily however, Voorn et al (307) showed mothers giving birth to preterm infants had decreased concentration of milk glucocorticoids compared to mothers who delivered full term infants. However, the significance of our results disappeared when adjusted for other maternal factors including maternal age and weight. Loss of significance may indicate that preterm birth may not be an independent factor responsible for altering glucocorticoid composition at three months after infant birth. However, these findings warrants larger studies targeting mainly at exploring whether preterm birth independently affect milk cortisol and cortisone concentration and its relevant effects on the development of breastfeeding infants.

In addition to several biological factors, numerous social and psychological factors like lifestyle factors may increase cortisol secretion by enhancing HPA axis activity. Previous studies have examined the relation between socio-demographic factors and glucocorticoids. However, these studies are often reported with mixed findings. To our knowledge, this is the first study to show the effect of maternal social and psychological factors on both cortisol and cortisone concentration in HM. We showed a positive association between low maternal education and milk cortisol level, where 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 relationship between maternal education and cortisone was seen. Our results are in accordance with other studies, which suggest that education plays a critical role in individual stress biology. Briag et al (369) reported positive association between hair cortisol and low maternal education, whilst, previously it was suggested that lower education is associated with lower levels of early morning salivary cortisol (427). Working mothers with children at home, independent of marital status and social support showed elevated urinary cortisol compared to working mothers with no children over analysis of 24 hour collected samples (428). This was reflective of overexpressed HPA axis activity. Similarly, our study demonstrated that mothers either working in industries, agriculture or as service workers had increased level of cortisol and cortisone in their milk; however, these differences were not identified as statically significant.

Despite all reported benefits of HM and breastfeeding, rates of exclusive breastfeeding declines rapidly, particularly within the first three months of infant feeding (429,430). Numerous factors including maternal surroundings can influence breastfeeding duration, and establishment of mixed feeding practices. Yet, very little is known about the impact of breastfeeding practices on their ability to alter HM composition, particularly glucocorticoids abundances. To our knowledge, this is the first to investigate the impact of exclusive versus partial breast-feeding at three months post-birth on glucocorticoid concentrations of HM. Contrary to our hypothesis; the results showed feeding practices had no impact on HM glucocorticoid concentration. Since, both cortisol and cortisone were equally abundant between exclusive and partial breastfeeding groups. Exclusive breastfeeding is known to reduces maternal stress (both physiological and psychological) and enhance positive mood in mothers, compared to the formula feeders (431). Previous studies report no 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 (415,431). Our study provides insights into the influence of common breastfeeding practices might have on glucocorticoid composition within HM at three months post-birth. This study could be an extension for several other studies, who failed to explain the impact of breastfeeding practices might have on glucocorticoid composition of HM, a few months into already established breastfeeding.

The large sample size, with overall inclusion of 80% milk samples (417), 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 glucocorticoids in HM might be suitable for assessing the extent to which maternal social, biological and environmental factors causes stress hormone expression in breast-feeding mothers’, and impacts they may have on 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 variables appear to play an overlooked role in milk glucocorticoid composition. Although, the mechanisms are unknown, but these findings provide novel insights into the unappreciated roles of social and biological factors in orchestrating HM hormonal composition. This study enables a better understanding of how and when maternal stress may affect HM glucocorticoid composition allowing enhanced acknowledgement of their roles in optimising infant growth and development.
Chapter 3: Variability of cortisol and cortisone in the milk of mothers delivering preterm infants
3.1 Summary

Preterm birth is associated with significant maternal and infant stress. This chapter presents the work investigating the impact of preterm birth on concentrations of cortisol, cortisone and their free/total ratio in HM samples following preterm birth. Evidence suggests potential roles for milk-borne glucocorticoids on infant growth and behavioural development. We demonstrate the concentration of glucocorticoids is influenced by birth gestational age and is not related to postnatal age.

The samples analysed were collected from Adjunct Professor Donna Geddes from School of Molecular Science, the Western Australia University, Perth, Australia. However, the hormonal and data analysis was done in the Liggins institute, the University of Auckland, Auckland, New Zealand.

The following section contains manuscript ‘Impact of preterm birth on glucocorticoid variability in human milk.’ co-authored by Shikha Pundir, Cameron J. Mitchell, Eric B. Thorstensen, Clare R. Wall, Sharon L Perrella, Donna T Geddes, and David Cameron-Smith. This article has been accepted for the Journal of Human Lactation.
Variability of cortisol and cortisone in the milk of mothers delivering preterm infants

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⁴Riddet Institute, Palmerston North, New Zealand
⁵Food & Bio-based Products Group, AgResearch, Palmerston North, New Zealand

Running Title: Impact of preterm birth on glucocorticoid variability in human milk.
3.2 Abstract

Preterm birth is a stressful event for both the mother and infant. Whilst the initiation of breastfeeding is important for preterm infant health, little is known of the glucocorticoid hormones (cortisol and cortisone) in human milk (HM) following premature birth. Therefore, the present study aimed to investigate the relationship between HM glucocorticoid concentrations and preterm birth. HM was sampled weekly for up to 6 weeks from 22 women who delivered a preterm infant at 28–32 weeks gestation. The HM was analysed for total and free cortisol and cortisone concentrations using liquid chromatography-tandem mass spectrometry. Our results showed that HM sampled from mothers of preterm infants had more cortisone than cortisol (p<0.001), with a strong correlation between both hormones (p=0.001, r=0.85). HM cortisone was significantly higher in mothers who delivered infants after 30 weeks compared with those who delivered before 30 weeks of gestation (p=0.02). Glucocorticoid concentrations did not change over the sampling time (weeks 1 to 6 postpartum), and did not differ by infant sex. Thus, present study demonstrates the presence of glucocorticoids in all milk samples following preterm birth. Cortisone concentration tended to be higher in those who delivered after 30 weeks gestation but did not increase further over the weeks following birth.
3.3 Introduction

Preterm birth affects more than 15 million infants worldwide (432). These infants are at significantly increased risk of acute adverse health outcomes, with the persistence of lifelong metabolic health complications (433,434). The early intake of human milk (HM) confers significant benefits to the health of a preterm infant, with a reduced risk of serious acute events such as sepsis and necrotizing enterocolitis (435,436). It also improves long-term health with reduced rates of re-hospitalisation and evidence of protection against metabolic diseases, including diabetes (356,437,438).

There are several unique compositional differences in the HM produced by mothers of preterm infants. Compared to HM produced following term birth, the HM produced after a preterm birth is higher in protein, long chain fatty acids, sodium and chloride (177,439). Beyond simply supplying nutrients, HM also contains a complex array of non-nutritive components. Given the influences of birth gestation and postpartum time on the nutritive composition of HM (5), it is hypothesised that non-nutritive components may also be altered by degree of infant prematurity.

To date there is very limited data on the presence of biologically active hormones, including the glucocorticoids i.e. cortisol and cortisone, in the milk of mothers who have delivered preterm infants. Studies suggest that glucocorticoids transfer from maternal plasma into the milk, and hence may be influenced by maternal stress (14,310). Given that preterm birth is a time of increased maternal stress and anxiety, during and after discharge from NICU (Neonatal Intensive Care Unit) (440), it is hypothesised that this would impact on glucocorticoids hormone concentrations in the secreted HM.

Currently, there is limited understanding of the psychological and biological importance of glucocorticoids in HM. Of the available data, milk-borne glucocorticoids are known to influence infant temperament in both animal and human infants (14,310). This may however be sex-specific, as elevated milk glucocorticoids were found to be associated with a more confident temperament in mammalian male infants, but not in females (309). Furthermore, the impact of glucocorticoids may extend to influence infant growth. Animal studies demonstrate a relationship between increased levels of milk cortisol and greater infant growth during early and established lactation (308,441). To date, only single study has demonstrated the impact of preterm birth on milk glucocorticoids. It was reported that very preterm birth (birth at 28-32 weeks gestation) was associated with reduced HM glucocorticoids concentrations, relative to term birth (307). However, this study failed to report the difference between free and bound glucocorticoids. It is unknown whether the glucocorticoids in HM exist in two forms- free (active) form or bound (inactive) form complexed to either corticosteroid-binding globulin (CBG) or albumin. However, corticosteroid bound to any of these protein limits their biological activity (442). Thus, the present study aimed to examine the impact of birth gestation on glucocorticoid concentration in HM, the ratio of free to total glucocorticoids as well as subsequent changes during postnatal period.
3.4 Methods

3.4.1 Study design and sample characteristics

Participants for this study were recruited from the neonatal nurseries of King Edward Memorial Hospital, Western Australia, Australia (443). In brief, study population was derived from neonatal nurseries of King Edward Memorial Hospital, Western Australia. The sample for the present study was a subgroup of a larger longitudinal observational study of gastrointestinal function in preterm infants. Stable preterm infants born at 28-32 week gestation that were receiving full enteral feeds of mother’s own expressed breast milk and/or pasteurised donor human milk were recruited. Infants with congenital anomalies, gastrointestinal disease or symptoms of feeding intolerance within the previous 24 hours were excluded. In cases of multiple birth, only one sibling was recruited to the study. The sample for the present study consisted of twenty-two women who delivered preterm infants between 28-32 weeks. Birth gestation was divided into two groups, according to the equal distribution of sample size in each group. Recorded infant characteristics included birth gestation, birth weight, infant gender, and postnatal age at the time of sample collection.

For sample size, were conducted using the effect size and variance in fatty acid composition of the HM due to preterm birth as reported by (444) as previous reports of GCs in preterm birth were not available. Eight participants were required to yield a statistical power of 80% and eleven were included in each group for possible attrition.

Written and verbal informed consent were taken from parents prior to milk collection. All HM samples were collected from February 2011 through April 2014 using a hospital grade electric breast pump (Medela AG, Symphony, Switzerland). On the morning of the study, samples were collected from pooled expressed breast milk and aliquoted into sterile polypropylene-capped tubes and rapidly stored at -20°C, until further analysis (443).

The study was approved by the Ethics Committee of the Women and Newborn Health Service, registration number 1749/EW.

3.4.2 Sample preparation

Glucocorticoids were assayed quantitatively from 100µl of HM with the addition of 100 µl of internal standard. Briefly, the internal standard consisted of 12 ng/ml cortisol d4, 60 ng/ml corticosterone d8 (Sigma Aldrich, Darmstadt, Germany) prepared in water. The assay was initiated by warming the milk samples at 37°C for 5-7 min and vortexed prior to the addition of the internal standard. Glucocorticoids were extracted using 1ml ethyl acetate (Merck, Darmstadt, Germany); the top organic layer was removed to a separate tube and then dried in vacuum drier (Savant SC250EXP, Thermo Scientific, Asheville, NC, USA) for ~ 2 hours. The dried residues reconstituted with 80 µl of methanol (Merck, Darmstadt, Germany) and water (50:50 v/v) and transferred to HPLC injector vials.
The samples were analysed using a HPLC mass spectrometer system consisting of an Accela 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 Finnigan Xcalibur software (Thermo Electron Corporation, San Jose, CA, USA).

The mobile phase was methanol-water gradient starting at 50:50 (v/v) (peaking at 80:20 before returning back to 50:50) at a flow rate of 500 µl/min. The chromatography was performed at 40°C. The following selective reaction monitoring (SRM) parameters were used; m/z 363.1→121 at 24 V for cortisol; 363.1→163 at 24 V for cortisone and 367.1→121.0 at 24V for cortisol d4. To calculate the concentrations of glucocorticoids in HM samples standard curves were generated in blank human plasma spiked with increasing amounts of each steroid; cortisol 0.05-100 ng/ml, cortisone 0.025-50 ng/ml. The area ratio of each steroid to the internal standard was used for quantitation purposes. Steroid concentration in HM was expressed in ng/ml.

3.4.3 Sample preparation of total milk cortisol and cortisone

To detect the total concentration of milk glucocorticoids, a deconjugation enzyme assay was undertaken and to measure the concentration of free glucocorticoids, non-enzymatic previously described assay was used (445). Briefly, 100 µl of warm, vortexed milk was mixed in a glass tube with internal standard (Cortisol d4). This solution was then buffered with 200 µl of 0.5N sodium acetate buffer (pH 5.0) and glucourinadase (255 units) enzyme (Sigma Aldrich, Darmstadt, Germany). The solution was incubated at 37°C in a water bath for 3 hours. The enzyme reaction was stopped by heating the tubes at 70°C for 5 min. Then 1 ml of ethyl acetate (Merck, Darmstadt, Germany) was added and solution was vortexed for 30 s. The top organic layer was removed and transformed to another glass tube and dried using vacuum drier (Savant SC250EXP, Thermo Scientific, Asheville, NC, USA). The dried extracts were reconstituted in 80 µl of mobile phase of methanol (Merck, Darmstadt, Germany) and water 50:50, and were transferred into HPLC vials. 12 µl of sample was injected into HPLC mass spectrometer system, and concentration was analysed as mentioned above.

3.4.4 Statistical Analysis

Statistical analysis were performed using Statistical Package for the Social Science version 21 (SPSS, IBM Corporation, Armonk, NY, USA). The difference between glucocorticoids (cortisol, cortisone), free/total ratios and infant sex and birth gestation groups were analysed using paired student t test. Birth gestation was divided into two groups, according to the equal distribution of sample size in each group. The first group consisted of milk samples from the mothers who delivered < 30 weeks of gestation and the second group consisted of milk samples from mothers who delivered >30 weeks of gestation. Further, mixed model was used to investigate the change in milk glucocorticoid concentrations over the first six weeks following birth; infant sex and gestational age were added to the model as covariates. Pearson correlation was used to examine the relationship
between cortisol and cortisone concentrations. Data are presented as mean ± SD, unless indicated otherwise. Alpha value was set at p<0.05. Graphs were created using Graph Pad Prism version 7.0 (Graph Pad software Inc., La Jolla, CA, USA).
3.5 Results

3.5.1 Cortisol and cortisone in human milk

Infant birth gestation was 29.9 ± 1.54 weeks and infant sex distribution was equal between two groups with 11/22 (50%) males. Non-enzymatic method demonstrated the presence of both cortisol (1.88 ± 1.34 ng/ml) and cortisone (4.48 ± 1.73 ng/ml). There was a significant positive linear correlation between the cortisol and cortisone concentrations (p=0.001, r=0.85), as shown in Figure 3-1. Enzymatic method demonstrated the presence of total glucocorticoids, and after subtracting free glucocorticoids from total, free form (biologically available state) of cortisol accounted for 76.2% and 84.2% of cortisone, with less than 30% of both cortisol and cortisone detected in the bound form. The mean concentration of total cortisol (free and protein bound) was 2.56 ± 1.78 ng/ml and total cortisone was 7.81 ± 2.24 ng/ml.

Figure 3-1: Correlation between HM cortisol and HM cortisone concentration.

Demonstrates the correlation between HM cortisol and HM cortisone concentration in the milk samples collected from mothers who delivered preterm infants ranged between 28-32 weeks of gestational age.
### 3.5.2 Relationship between effect of gestational age, sex and infant characteristics and human milk glucocorticoids

Both mean cortisone concentration and free/total cortisone ratio were significantly different between birth gestation groups ($p=0.02$ and $p=0.04$), as shown in Figure 3-2 (a and b). Cortisone concentration was higher in the milk samples of those who delivered >30 weeks (5.38 ± 2.12 ng/ml) compared with those who delivered <30 weeks gestation (3.73 ± 0.99 ng/ml). The HM free/total ratio of cortisone in the <30 weeks gestation birth group was 0.94 ± 0.08 ng/ml, compared to the > 30 weeks gestation birth group (0.73 ± 0.20 ng/ml).

![Figure 3-2: Distribution of HM glucocorticoids and their free/total ratios between gestational groups](image)

**Figure 3-2: Distribution of HM glucocorticoids and their free/total ratios between gestational groups**

HM glucocorticoids concentration, cortisol, cortisone and their free/total ratio distribution between gestational age groups. Gestational age was categorised into two groups, one group includes milk samples taken from the mother who delivered infants <30 week (n=12) and other group includes milk from the mothers who delivered infants >30 weeks of gestational period (n=10). Difference between gestational age was expressed as *$p<0.01$. Error bar represent standard error of mean ± SEM.
No significant difference was found between birth gestational groups in regard to HM cortisol concentration and free/total cortisol ratio (p= 0.36 and p=0.92). However, the mean cortisol concentration in the milk samples of those who delivered >30 weeks was 2.18 ± 1.79 ng/ml compared to other group who delivered before <30 week (1.63 ± 0.91 ng/ml); whereas free/total ratio of cortisol in the <30 weeks gestation birth group was 0.71 ± 0.24 ng/ml, similar to 0.70 ± 0.20 ng/ml in > 30 weeks gestation birth group.

Furthermore, no significant differences were observed between infant sexes for the average value of cortisol (p=0.36), cortisone (p=0.57), and their ratios free/total cortisol (p=0.96) and free/total cortisone (p=0.51). Table 3-1 shows the mean concentration of hormones in HM from mothers who gave birth to males and females.

**Table 3-1: Glucocorticoid concentrations in human milk of mother’s who delivered preterm infants.**

<table>
<thead>
<tr>
<th>Glucocorticoids (ng/ml)</th>
<th>Female (n=11)</th>
<th>Male (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cortisol</td>
<td>1.61 ± 1.49</td>
<td>2.16 ± 1.25</td>
</tr>
<tr>
<td>Free cortisone</td>
<td>4.26 ± 1.59</td>
<td>4.70 ± 1.59</td>
</tr>
<tr>
<td>Free/total cortisol ratio</td>
<td>0.79 ± 0.16</td>
<td>0.63 ± 0.24</td>
</tr>
<tr>
<td>Free/total cortisone ratio</td>
<td>0.88 ± 0.19</td>
<td>0.85 ± 0.16</td>
</tr>
</tbody>
</table>

Data shown as (mean ± SD).

Furthermore, the HM concentrations of cortisol (p=0.19), cortisone (p=0.48), free/total cortisol (p=0.66) and free/total cortisone (p=0.26) did not change significantly over the time (post birth period ranging between week 1 through to week 6 (Figure 3-3 A and B), after being adjusted for infant sex and birth gestation weeks.
Figure 3-3: Alteration of HM glucocorticoids over a period of six weeks after birth.

Changes in the HM glucocorticoids over a period of 6 weeks after birth. (A) Cortisol changes and (B) cortisone changes in the milk samples following the preterm birth, between week 1 and 6. Error bar represent standard error of mean ± SEM.
3.6 Discussion

There is an emerging evidence for the role of milk glucocorticoids as a regulator of infant growth and behavioural development (308,441). Yet, little is understood regarding the factors that regulate glucocorticoid concentrations in HM. In the current study, the free and total concentration of cortisol and cortisone were measured in HM samples collected from the cohort of women following preterm birth at 28-32 weeks gestation. It was demonstrated that cortisone was the predominant glucocorticoid and that the majority of both cortisone and cortisol were free. Further, the cortisone concentrations tended to be lower in the HM of mothers who delivered < 30 weeks of gestation, but was not influenced by the time since birth.

The average HM cortisol concentration was found to range between 0.2 and 5.60 ng/ml, while cortisone concentration ranged between 2.26 and 9.40 ng/ml. These concentrations are broadly consistent with prior analysis in preterm HM (307). The predominance of cortisone is also similar to that reported in HM in the 4 weeks following term birth (307). Unlike milk and saliva, cortisol is the major glucocorticoid in adult serum (446). The glandular tissue of the breast converts cortisol into cortisone, presumably via the actions of 11βHSD type-2 enzyme (447). The significance of this interconversion, including whether the developing infant has a preferential requirement for milk-derived cortisone, is not yet known. Interestingly, in this context, higher concentrations of cortisone are found within foetal tissues, also due to the actions of placental 11βHSD type-2 enzyme (448,449).

In rat pups associated with infant growth, cortisone exposure demonstrated an increased sensitivity to bacterial enterotoxin and enhance gut maturation (450). Also, cortisone was associated with enhanced intestinal permeability and Necrotizing Enterocolitis (NEC) and thymic activity (greater size thymus in breastfed infants compared to formula fed) (451–453).

Preterm infants have underdeveloped adrenal functioning, suppressed HPA axis responsiveness (454,455), increasing vulnerability to bronchopulmonary dysplasia and cardiovascular instability (456). However the HPA axis in preterm infants adapts rapidly, with a recovery of pituitary-adrenal response by the 14th day of postnatal life (457,458). The present study demonstrates that HM is not sufficiently regulated in relation to the stages of infant development to meet these rapid adaptations in adrenal function. Indeed, the lowest HM concentrations of cortisone was found in mothers who delivered at earlier gestations.

Glucocorticoids are the lipophilic steroids, with a variable proportion bound to large carrier proteins, including corticosteroid-binding globulin (CBG) or albumin (442). It is generally assumed that the biological activity of the cortisol depends on the free concentrations of glucocorticoid hormones in the circulation. It has previously been reported that in circulation, bound cortisol accounts for almost 95% of total cortisol (262). This study demonstrated that more than 70% of cortisol and cortisone in HM were in their free or hormonally active form. Furthermore, the ratio between free cortisone/total cortisone in human milk was higher (0.94) in the milk of mothers who delivered at >30 weeks of
gestation. These findings suggest that free cortisone level increased in comparison to total cortisone, and these ratios may be an index of the maternal adrenal gland (459). There are very few studies that have measured free/total concentration of cortisol and cortisone in plasma or urine samples; but little is known about their ratios and the clinical significance of these ratios in HM.

One of the major strengths of the study is the highly sensitive measurement of cortisol and cortisone. However, the limited sample size, particularly for infant's postnatal age was a limiting factor. Furthermore, due to the lack of term samples, birth gestation was divided into two groups, before and after 30 weeks of gestation. Nevertheless, result of this pilot study could be used to design a future study, aimed at exploring the relation of preterm birth on milk glucocorticoid concentrations and their free/total ratio across the weeks of lactation.

In conclusion, HM from mothers following preterm delivery contains appreciable levels of predominately free (unbound) glucocorticoids. Interestingly cortisone is the predominant glucocorticoid. Currently there is a limited understanding about the influence of glucocorticoids may have on the health and development of breastfed infant. For this study, levels of glucocorticoid tended to be lower in the women who gave birth < 30 weeks of gestation. Yet, there was little evidence of regulation over the subsequent 6 weeks of lactation. This study, then provides further insights into the supply and bioavailability of glucocorticoids in HM fed to preterm infants.
Chapter 4: Glucocorticoids circadian rhythm in human milk: variation of cortisol and cortisone in human milk over the period of 24 hour
4.1 Summary

This chapter presents the work to investigate the variations in HM cortisol and cortisone concentrations over the period of 24 hour and investigate the relation between HM cortisol and cortisone concentrations. There is evidence that glucocorticoids are found in both maternal circulation and in HM, and are known to play critical role in mammary growth and maintenance of lactation. Moreover, they are required for the maintenance of delicate hormonal balance that controls metabolism and stress regulation. Recently, the role of glucocorticoids in HM has been elucidated: HM cortisol can influence developing neonate’s growth and behaviour.

Despite evidences that cortisol exhibit strong circadian rhythm, being highest around morning and lowest around mid-night, surprisingly not much is known about the circadian pattern of HM glucocorticoids. Furthermore, variability of milk composition during and within feeds and between breasts, provoke the question about whether the difference between duration of breastfeeding and breast sides would influence milk hormonal composition.

The samples used in this study were collected from Adjunct Professor Donna Geddes from School of Molecular Science, the Western Australia University, Perth, Australia. However, the method and data analysis was done in the Liggins institute, University of Auckland, Auckland, New Zealand.

The following section contains manuscript “Variation of human milk glucocorticoids over 24 hour period” co-authored by Shikha Pundir, Clare R. Wall, Cameron J. Mitchell, Eric B. Thorstensen, Ching T. Lai, Donna T Geddes, David Cameron-Smith. This article has been accepted for the Journal of Mammary Gland and Neoplasia.
Glucocorticoid circadian rhythm in human milk: variation of cortisol and cortisone in human milk over the period of 24 hour

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Running Title: Variation of human milk glucocorticoids over 24 hour period.


4.2 Abstract

HM contains a complex array of hormones, including members of the glucocorticoid family. The predominant glucocorticoids, cortisol and cortisone may influence the growth and behaviour of the breastfed infant. However, little is understood of the factors regulating the levels of these hormones within HM. The aim of the study was to examine HM cortisol and cortisone concentrations, measured in samples collected at each feed during a 24 hour period. Twenty three exclusively breastfeeding mothers collected milk, prior to and after each breastfeeding session over 24 hour period at 3.2 ±1.60 months. HM cortisol and cortisone levels were measured using high pressure liquid chromatography mass spectroscopy. Cortisone was the predominant glucocorticoid (3.40 ng/ml), and cortisol was detected in all samples (1.62 ng/ml). A positive correlation was found between cortisone and cortisol (r=0.61, y= 1.93± 0.24, p<0.01). Cortisol and cortisone concentrations were significantly higher in morning feeds (2.97 ng/ml and 4.88 ng/ml), compared to afternoon (1.20 ng/ml and 3.54 ng/ml), evening (0.69 ng/ml and 2.13 ng/ml) and night (1.59 and 3.27 ng/ml). No difference was found between glucocorticoid levels of the milk expressed for collection either before or immediately after the breastfeed, or between milk collected from the left or right breast. In conclusion, this study shows that HM glucocorticoid concentrations exhibit a 24 hour pattern, with highest peak levels in the early morning, reflecting the circadian pattern as previously reported in plasma. Thus, HM glucocorticoid concentrations are likely to reflect those in the maternal circulation.
4.3 Introduction

Human milk (HM) is a complex and dynamic biological fluid, which is adaptively altered throughout lactation to tailor to the individual and unique needs of a developing infant (5). HM enables nutrient transfer between mother and infant, with compositional regulation of the macro- and micro-nutrients influencing infant growth and development (308,318). Much recent research has highlighted the complexity of HM, revealing the extent to which developmental needs of the growing infant is paired with HM composition, and the significant knowledge gaps that exist (460). In addition to nutritive components, HM also contains non-nutritive bioactive ingredients, including the glucocorticoid family of steroid hormones (461). Cortisol and cortisone are typically the predominant glucocorticoids found in the maternal circulation (461,462) and in HM (14).

The actions of circulatory glucocorticoids are diverse, with a critical role in classic stress responsiveness (462). Beyond this, glucocorticoids also mediate aspects of physiological adaptations required for mammary function, including contributing to mammary growth, secretory differentiation and activation, maintenance of lactation and milk ejection (231,318,405,463). The functions of HM derived glucocorticoids are less well understood, although they may exert an influence in shaping the infants behaviour, physiology and metabolic programming (14,298,464). Higher milk cortisol levels have been associated with greater nervous and less confident behaviour in both, human infants (14) and rhesus macaques (309). Available data demonstrates beneficial actions of milk cortisol. Higher level of cortisol was significantly correlated with a higher score on the autonomic stability on the Neonatal Behavioural Assessment Scale (233). Further, in the rhesus macaque elevated milk cortisol at peak lactation (3-4 months lactation) is also positively associated with infant weight gain (308). More recently, another study reported the presence of cortisol in HM to be associated with early metabolic programming, and suggested its importance in providing protection against obesity (298). Together, these findings suggest that milk glucocorticoids are bioactive signalling agents, by which maternal physiology may modulate offspring growth and behavioural development. Despite the potential significance of HM glucocorticoids, only a few studies have examined factors influencing their abundance. One key aspect contributing to the complexity of HM is the circadian variation in its composition (465).

Evidence suggest that HM fat exhibits significant 24 hour variation, with total fat content varying at different times of the day (141). It was reported that higher lipid concentrations during the day and evening, compared to the milk expressed during early morning and night. However these changes could reflects higher degree of fullness of the breast, rather than circadian variations (141,149,326). Although many other components of milk including amino acids, melatonin and trace elements have shown temporal correlation with maternal rhythms (465,466). A recent study has identified the presence of circadian regulation of glucocorticoids in HM. This study demonstrates the presence of peak concentrations of cortisol and cortisone around 0700h, declining through the remainder of the day (307). The purpose of the current study is to further analyse the concentrations of cortisol and cortisone over a 24 hour period, in HM obtained from exclusively breastfeeding women. This study
aimed to additionally identify whether glucocorticoids differed over the duration of a feed, with analysis in HM donated immediately before and after infant feeding, across both breasts. We hypothesised that the described circadian regulation of blood glucocorticoid hormones would be reflected in the levels measured in HM, with concentrations falling from pre- to post-feeding samples.
4.4 Methods

4.4.1 Study design and subjects

Mothers were recruited through the Western Australian branch of the Australian breastfeeding Association and through the Child and Adolescent Community Health Nurses of the Oceanic Region. Written informed consent was obtained from the participants. The study protocol was approved by the Human Research Ethics Committee of the University of Western Australia.

4.4.2 Milk collection

All participating mothers were asked to collect the milk samples (1-2 ml) either by using manual expression or by using an electric breast pump into 5 ml polypropylene vials (Disposable Products Pty Ltd, Australia). Milk samples were taken immediately before (pre) and after (post) each breastfeeding session, from each breast at each feed over an entire 24 hour period. All of the samples were initially stored in the mother's home freezer, prior to transport to the laboratory where they were stored at -20°C. Samples were shipped to Auckland (New Zealand) for subsequent glucocorticoid analysis on dry ice and were kept at -80°C frozen until further analysis.

Milk samples were divided into four intervals of six hours as described by Khan et al (2013) and were classified as morning (0401-1000 hours), afternoon (1000–1600 hours), evening (1601-2200 hours) and night (2201-0400 hours). These time periods were used in subsequent statistical analysis.

4.4.3 Sample preparation

Milk steroids were measured by using liquid chromatography mass spectrometry (LC-MS/MS). The internal standard consisted of 12 ng/ml cortisol d4, 60 mg/ml corticosterone d8, prepared in water. Preparation was initiated by adding 100 µl of internal standard to 100 µl HM. All milk samples were heated at 37°C for 10 min and vortexed for 20 seconds before adding 100 µl of sample to glass tubes with internal standards. Steroids were then extracted using 1 ml ethyl acetate (Merck, Germany); the top organic layer was removed into a separate tube and then vacuum dried (Savant, SC250EX, Thermo Scientific, United States) for ~2 hours. The dried residues were reconstituted with 80 µl of 50% methanol (Merck, Germany) water and transferred to HPLC injector vials. All samples were run in duplicates and average values are reported.

4.4.4 Liquid chromatography- tandem mass spectroscopy

The HPLC Mass Spectrometer (MS) 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 Finnigan X caliber software (Thermo Electron Corporation, San Jose, CA.) The mobile
phase was a methanol-water gradient starting at 50:50(v/v) (peaking at 80:20 before returning back to 50:50) at 500 µl/min. The chromatography was performed at 40°C. The instrument was set up in selective reaction monitoring (SRM) mode with the following mass transitions: m/z 363.1→121 for cortisol, 361.1→163 for cortisone, 367.1→121 for cortisol d4 and 355.2→125.2 for corticosterone d8. Dissociation voltage was 24V and the collision gas (Argon) was set at 1.2 mTorr for all steroids.

Steroid concentrations were calculated from a standard curve generated for each steroid relative to its internal standard as (cortisol d4 for cortisol and corticosterone d8 for cortisone) from the injection of standards; cortisol 0.05-100 ng/ml, cortisone 0.025-50 ng/ml, diluted into stripped human plasma and extracted in the same way as the samples for each assay.

4.4.5 Statistical Analysis

Linear mixed models were used to investigate differences between glucocorticoid concentrations over the 24 hour period with the inclusion of time of the day, and pre and post breastfeed as repeated measures fixed factors and infant gender as a non-repeated fixed factor and maternal BMI and parity as covariates. The 24 hour difference between pre and post feed samples were investigated using Univariate analysis and its effect at each time point was tested using student T-test. The 24 hour differences between right and left breast milk samples were also tested using a students paired T-test. Between group differences were assessed by using Sidak post hoc method. Pearson correlation was used to analyse the association between the calculated average value of cortisol and cortisone over the 24 hour period. Univariate relationships between glucocorticoids and milk intake variables such as breastfed volume, frequency of breastfeeding were performed using linear regression. Statistical analysis was performed using SPSS (IBM SPSS Statistic 2 pg1.InK United States). All the data are reported in text and tables as mean ± SD and mean ± SE in figures. P values less than 0.05 were considered statistically significant. Lattice plot was created using R software version 2.15.2 and GraphPad Prism version 7.0 was used to create all other graphs.
4.5 Results

4.5.1 Participant characteristics

A total of 502 human milk samples from twenty-three mothers were measured and analysed for glucocorticoid concentrations. Table 4-1 shows the demographic characteristics of mother and infant. The mean cortisol and cortisone concentrations for the 24 hour period were 1.63 (0.75) ng/ml and 3.42 (1.18) ng/ml, respectively, and individual glucocorticoid profile is presented in Figure 4-1.

Table 4-1: Subject and sample characteristics (n=23).

<table>
<thead>
<tr>
<th>Mother characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years (mean ± SD)</td>
<td>31 ± 8.14</td>
</tr>
<tr>
<td>BMI before pregnancy (mean ± SD)</td>
<td>31 ± 3.92</td>
</tr>
<tr>
<td>Parity</td>
<td>1.3 ± 0.57</td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
</tr>
<tr>
<td>Married n (%)</td>
<td>22 (95.6)</td>
</tr>
<tr>
<td>Unmarried n (%)</td>
<td>1 (4.3)</td>
</tr>
<tr>
<td>Maternal BMI</td>
<td></td>
</tr>
<tr>
<td>Normal weight n (%)</td>
<td>13 (56.5)</td>
</tr>
<tr>
<td>Overweight n (%)</td>
<td>2 (8.7)</td>
</tr>
<tr>
<td>Underweight n (%)</td>
<td>7 (30.4)</td>
</tr>
<tr>
<td>Infant characteristics</td>
<td></td>
</tr>
<tr>
<td>Age in months (mean ± SD)</td>
<td>3.2 ± 1.6</td>
</tr>
<tr>
<td>Male n (%)</td>
<td>18 (78.2)</td>
</tr>
<tr>
<td>Female n (%)</td>
<td>5 (21.7)</td>
</tr>
<tr>
<td>HM and feeding characteristics</td>
<td></td>
</tr>
<tr>
<td>24 hour breast milk production of both breast (ml), (mean ±SD)</td>
<td>740 ± 274.75</td>
</tr>
<tr>
<td>Right breast milk production (ml)</td>
<td>389 ± 144.64</td>
</tr>
<tr>
<td>Left breast milk production (ml)</td>
<td>340 ± 154.57</td>
</tr>
<tr>
<td>Total feeds during 24 hour period (range)</td>
<td>8-21</td>
</tr>
</tbody>
</table>
Figure 4-1: HM glucocorticoids (cortisol and cortisone) over 24 hour in individual mothers (n=23).

Each box of the lattice plot indicates a single mother milk glucocorticoid profile with symbol (○) representing cortisol and (△) representing cortisone. Each solid line indicates cortisol and dashed line cortisone. Time points are categorised as morning (0401-1000h), afternoon (1001-1600h), evening (1601-2200h) and night (2201-0400h).
4.5.2 Temporal changes in HM glucocorticoid composition

HM glucocorticoid (cortisol and cortisone) concentration demonstrated a circadian response over the analysed period. Post-hoc analysis identified that 24 hour averaged morning samples, cortisol was significantly higher than afternoon (p<0.001), evening (p<0.001) and night (p=0.001) samples. Furthermore, the evening cortisol level was significantly lower than afternoon (p=0.03) and night (p=0.02) (Figure 4-2 A). Similarly, a significant difference was observed in the cortisone levels, with morning cortisone higher than afternoon (p=0.01), evening (p<0.001) and night (p<0.001), whereas evening was significantly lower than afternoon cortisone levels (p=0.001) (Figure 4-2 B).

Figure 4-2: HM glucocorticoids concentration, cortisol (A) cortisone (B) concentration for pre and post breastfeed throughout the day.

Solid horizontal lines at top of each bar represent a main effect of time.* Represent significant difference from morning milk cortisol levels when collapsed across pre and post breastfeeding time points. # Represent significant difference from evening milk cortisol levels, when collapsed across pre and post breastfeeding time points. Error bars represent SEM (standard error of means). Difference over time are expressed as * p<0.01**p<0.001, ***p<0.0001 and #p<0.01 ## p<0.001, p<0.01.
4.5.3 Effect of breastfeeding on HM glucocorticoid composition

The 24 hour HM cortisol and cortisone concentrations were not significantly different between pre and post feed samples (cortisol: p = 0.97; cortisone: p = 0.46). The cortisol and cortisone did not differ significantly between pre and post breastfeed samples at any time points, morning (p=0.61 and 0.26), afternoon (p=0.49 and 0.28), evening (p=0.50 and 0.92) and night (p=0.58 and 0.86) Figure 4-2. There was no significant correlation between the 24 hour pre and post feed glucocorticoid levels and breastfeed volume (cortisol pre feed p=0.88, cortisol post feed p=0.94, cortisone pre feed p= 0.83, cortisone post feed p= 0.86). Furthermore, on average each infant had 15 ± 3.29 breastfeeds during 24 hour period, however no significant difference was found between the frequency of breastfeeding and glucocorticoid levels (cortisol p= 0.21 and cortisone p= 0.19).

The HM cortisol concentration did not differ significantly between male and female infants at any time-point, morning (p=0.12), afternoon (p=0.27), evening (p=0.70) and night (p=0.42). Similarly, the HM cortisone concentration did not differ significantly between male and female infants at morning (p=0.07), afternoon (p=0.10), evening (p=0.82) or night (p=0.61). Furthermore, no significant interaction effect was seen between pre and post breastfeed samples, infant gender, parity, maternal BMI, and time of the day.
4.5.4 Between-breast difference on HM Glucocorticoid composition

Overall the 24 hour average concentration of HM glucocorticoid (cortisol and cortisone) for the left and right breast was not significantly different (cortisol: p=0.37; cortisone: p=0.15). The 24 hour average cortisol concentration of milk from left breast was 1.73 (0.70) ng/ml and right was 2.0 (1.01) ng/ml. The cortisone 24 hour average concentration from the left breast was 3.83 (1.27) ng/ml and right breast 3.56 (1.35) ng/ml (Figure 4-3).

Figure 4-3: Human milk glucocorticoids between breast sides.

Distribution of HM glucocorticoids (cortisol and cortisone) in left and right breast milk collected during the 24 hour period. Data is expressed in Mean (SEM).
4.5.5 Relationships between HM cortisol and cortisone

The average HM cortisol concentration over 24 hour was significantly lower than that of cortisone ($p<0.001$). A significant positive correlation was observed between cortisol and cortisone concentrations in HM ($r=0.606$, $y= p<0.0005$)

Figure 4-4. Furthermore, a moderate correlation between two hormones was observed in the afternoon ($r=0.75$, $p<0.001$) and night ($r=0.62$, $p<0.01$), whereas a weak correlation was found in the evening ($r=0.46$, $p<0.01$) and morning ($r=0.20$, $p=0.34$).

Figure 4-4: Residual scatter plot and fitted regression line for the HM glucocorticoids.

A correlation between HM Cortisol and cortisone concentration over the 24 hour period (averaged data).
4.6 Discussion

Glucocorticoids are components of the complex non-nutritive hormonal fraction of HM. Currently there is limited data on the regulation of glucocorticoids in HM, and the circadian pattern of these hormones. Thus this study examined the glucocorticoid concentration of HM samples collected from a cohort of women who were exclusively breast feeding throughout the course of a single 24 hour period. Both cortisol (1.60 ± 0.71 ng/ml) and cortisone (3.40 ± 1.18 ng/ml), were present in all analysed samples. The presence of these glucocorticoids, exhibited a pronounced 24 hour pattern, characterized by the rapid increase in the cortisol and cortisone levels in the early morning hours followed by the gradual fall throughout the day. This mirrors the well described circadian pattern of glucocorticoids in the maternal circulation (467). Further, we found no impact of sampling before or after feeding or between breasts indicating time of sampling to be the most critical factor in investigating glucocorticoids in HM.

In humans, nearly all bodily functions exhibit circadian rhythms and glucocorticoid displays one of the most distinct circadian rhythms. The circadian rhythm of glucocorticoid release is regulated by the endogenous biological clock, the suprachiasmatic nucleus in the anterior hypothalamus. It exerts control over the hypothalamic–pituitary–adrenal (HPA) axis response and thus dictates the pulsatile release of cortisol (251). Typically plasma cortisol concentrations demonstrate circadian rhythms, with elevated levels in the early morning followed by a gradual decline to around midnight, completing the 24 hour cycle, throughout the day (248,251,329,461). This circadian pattern of secretion has been speculated to be an important contributant to the effective actions of glucocorticoids in immunity, growth and metabolism, although the precise mechanisms are not well understood (242). Our data demonstrates replication of this typical circadian pattern, with both cortisol and cortisone concentrations peaking in the early morning, before declining throughout the day to reach the lowest point in the evening. These findings are consistent with a recent study that examined the diurnal rhythm patterns of HM in 10 women, 4 weeks postpartum. In this study, glucocorticoid levels were shown to peak at 0700h when milk was collected 2 hourly for a complete 24 hours, imitating saliva analysis that was conducted at the same time (307). Thus, the rhythmic behaviour of HM glucocorticoid concentrations found in the current study is likely to be reflective of the concentrations in the maternal circulation. This reinforces the past observations that maternal physiological environment plays a critical role in dictating the hormonal composition of the human milk (5,308).

There is currently limited insight into how glucocorticoids are transferred from the maternal circulation to the synthesised HM. Animal studies have shown that cortisol can passively diffuse across the cell membrane and maintains a dynamic equilibrium between milk and plasma cortisol levels. In a study of dairy cows, milk cortisol levels were used as a reliable indicator of stress response, because a rise in plasma cortisol was reflected in milk (468). However, in the current study, glucocorticoids were measured in the milk samples and not in maternal or infant plasma samples, so direct comparison between milk and plasma glucocorticoid concentration was not possible.
As HM composition is dynamic and constantly changing, it is therefore important to further investigate the milk glucocorticoids content with respect to timing of milk secretion (in this study measured in milk obtained before and after the infant fed) and the frequency of feeding. Many studies have reported the effect of breastfeeding on the milk composition by analysing pre- and post-feed samples (5,141,314,469). For instance, milk expressed prior to feed has a lower lipid content when breast is full, compared to immediately after feed when breast is empty (141,149). Interestingly we found that milk removal from the breast by the breastfeeding infant had no influence on glucocorticoid profiles. This is in contrast to what might be expected with the positive effect of lactation in regulating the HPA axis response to both physical and psychological stressed state (415). Breastfeeding by the new born infant tends to reduce plasma cortisol levels and increase both oxytocin and prolactin release (470,471). Indeed, this is consistent with the calming effect of oxytocin released into the maternal circulation at milk ejection which occurs multiple times throughout a breastfeed (472). Further, the results from the current study also demonstrate that milk glucocorticoid levels were independent of breastfeeding frequency and feed volume, indicating no difference between frequent and non-frequent breast feeder; despite the considerable variation among the frequency of breastfeeding between mother and infant dyad.

It is well established that milk output from each breasts may not be equal between breast, as output from one breast is always greater than other, however milk composition between breasts remains independent of the breast sides (141,469). In this study, no differences in the glucocorticoid profiles of the milk were found between the left or right breast. These results are comparable to the evidence that the macro-nutrient composition of the milk including protein, lactose and leptin have also been shown not to differ between breasts (314,325). Unlike macronutrient, HM volume is highly sensitive to maternal condition, and its hormonal composition is associated with maternal circulation (309,473,474). Hence, the sample collection techniques, timings, and volumes are critical determinants pertaining to maternal health and environment. However, lack of standardised sample collection technique reflects the variability in milk macronutrients, challenging researchers to collect a representative milk sample. Clearly, no single sampling technique could be ideal, but our study could be an indicator for selecting an appropriate time for milk collection, enabling better hormonal analysis in future lactation studies.

In humans, two iso-enzymes of 11βHSD catalyse the interconversion of biologically active cortisol into its inactive metabolite, cortisone. We found a moderate positive correlation between the 24 hour cortisol and cortisone concentration (r=0.66), and found strong positive relationship at afternoon (r=0.75) and night (r=0.62). Many plasma and serum based studies have suggested strong correlation between the two hormones under normal physiological conditions, and any alteration in this equilibrium is usually associated with adrenal insufficiency or diseases (446,475). Equilibrium between these hormones may be involved in the regulation of glucocorticoid levels in milk as well. Cortisol, as a classical regulator of stress, and a permissive hormone plays an important role in milk secretion and onset of lactation (476). Chen et al (477) identified maternal stress, long labour and primiparity, as the predictors of delayed onset of lactation. However, it would be interesting to identify
the role of HM cortisol in predicting absence or delayed onset of lactation in mothers with chronic stress or depression.

Recent research suggests that HM composition differs depending upon infant gender, and tends to favour greater nutritional investment for male offspring (382). Additionally, mothers of male offspring had higher cortisol in their milk compared to the mothers of female offspring (2). One limitation of the current sample cohort was that there were few female infants, thus it is not possible to report with any confidence on the presence of any potential gender differences in the glucocorticoids. However, previous studies have shown that infant sex has very little effect on the glucocorticoid levels in plasma and serum (478,479). Secondly, the absence of maternal plasma samples collected at the time of breastfeeding makes it impossible to establish that the relationship in HM glucocorticoids correlates with maternal plasma concentrations. As such, additional analysis of potentially important variables including parity, maternal social and environmental circumstances are required.

One key feature of the current study was the measurement of glucocorticoids in milk using liquid chromatography mass spectrometry (LC-MS). In our study, milk cortisol concentration ranged between 0.1–11 ng/ml and cortisone was between 1.7-14.0 ng/ml, which was either lower/similar to previous reports for both milk (0–218 ng/ml) and plasma (50-230 ng/ml) (234). Variation between present and previous studies could be due to the differences in detection methodology, particularly the use of immunoassay analysis, which lacks specificity of analysis due to cross-reactivity with other related glucocorticoids (14,308,309,396,480). Compared to immunoassay, the method used in current study has both simplified sample preparation workflow and the ability to quantify both cortisol and cortisone simultaneously (481,482).

This study was further strengthened by sample homogeneity, at the time of milk collection infant’s age was within the narrow range of 3.2 ±1.2 months (data not shown). This provides a critical measurement of the duration of maternal lactation, further affecting milk composition. Therefore, the mothers recruited in the current study, produced a homogenous set of milk samples with minimal variation.

In conclusion, the present study demonstrates the fluctuating glucocorticoid profile of human milk samples throughout the day, closely aligning with the circadian rhythm of plasma cortisol that has been previously reported (20). The milk glucocorticoids levels were highest during morning and lowest prior to sleep; however further studies are required to elucidate the mechanistic and functional consequences of the milk glucocorticoids on infant growth and development.
Chapter 5: Longitudinal analysis of glucocorticoids in human milk over the first 12 months of lactation and its impact on infant’s development
5.1 Summary

This chapter presents the work wherein we investigated the variations in HM cortisol and cortisone concentrations over the first 12 months of lactation. We also studied the impact of milk-borne glucocorticoids on infant development. Despite the evidences that milk composition changes during the different stages of lactation to meet infant demand, it is not is known whether the glucocorticoid composition is altered during the lactation period. Further, the influence of altered milk hormonal composition on infant development remains unclear.

The samples used in this study were collected from Adjunct Professor Donna Geddes from School of Molecular Science, The University of Western Australia, Perth, Western Australia. The method was developed and data analysis was done in the Liggins institute, The University of Auckland, Auckland, New Zealand.

The following section contains the manuscript “Longitudinal study of glucocorticoids in human milk from lactating mothers at 2, 5, 9 and 12 months and exposure assessment of infants’ co-authored by Shikha Pundir, Cameron J. Mitchell, Eric B. Thorstensen, Clare R. Wall, Sharon L Perrella, Donna T Geddes, David Cameron-Smith. This article has been drafted for publishing in the journal Hormone Research in Paediatrics.
Chapter 5: Longitudinal analysis of glucocorticoids in human milk over the first 12 months of lactation and its impact on infant’s development

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Running: Variation of cortisol and cortisone in human milk throughout the first year of lactation.
5.2 Abstract

Exposure to glucocorticoids through human milk (HM) is associated with infant temperament, and may be involved in metabolic functioning and infant growth. The aim of the current study was to investigate the glucocorticoids composition of HM over the first 12 months of lactation and to determine how this may influence infant growth. Milk samples were collected from healthy breastfeeding women at four major time points at 2, 5, 9 and 12 months of lactation. Mean cortisol and cortisone concentrations remained constant throughout all measurements, although there was considerable individual variation at each time point. Infant percent fat mass (%FM) and head circumference were significantly correlated with cortisol and cortisone concentrations. No significant differences in glucocorticoid concentrations were found between infants based on sex. Further, a significant correlation was found between maternal body mass index and HM cortisol. This study demonstrated that milk glucocorticoid levels remain constant over the first 12 months of lactation and that infant adiposity and cranial growth are related to HM glucocorticoids.
5.3 Introduction

Over and under nutrition during the first 1000 days of life has a major impact on infant growth and development, particularly on body composition. Breastfeeding for a longer period has several benefits to both mother and infant pair, including providing nourishment, providing immune protection and reducing the risk of obesity (484).

Human milk (HM) contains many proteins and peptides, possessing biological activity, that directly or indirectly affect with infant body functions (5). Presence of hormones, such as leptin, insulin, ghrelin, adiponectin, insulin like growth factor (IGF-1), among others, in HM are of great importance, due to their functional and developmental roles in the infant (214,216,227,228,235,485–487). Hormones in HM, particularly leptin, have been shown to be involved in satiety and energy regulation and metabolism (222,235). Identification of these components highlight the critical role of milk-borne hormones in infant growth and development.

The HM glucocorticoids (cortisol and cortisone) are intriguing steroid hormones involved in the complex regulation of metabolic homeostasis and inflammation. The levels of these hormones in HM are predominately governed by maternal circulating levels (258) and differences exist in salivary cortisol concentration between breastfed infants and formula fed infants, over the first year of life (304,305). Whilst the role of HM cortisol has not been fully elucidated, plasma cortisol strongly correlates with maternal breast milk cortisol. Exposure to milk-borne cortisol has further been associated with infant mood and behavioural development (14). In a very recent study by Hahn-Holbrook and colleagues (298), it has been suggested that milk-bone cortisol may protect against obesity in later life, with higher HM cortisol being associated with lower body mass index percentile (BMIP) at 2 years of age. Together, these findings suggest a significant role of milk glucocorticoids in early metabolic and behavioural programming, however, the intricacies are not well described.

A great deal is known about the nutritional composition of HM and its dynamicity to meet infant demands at every stage of lactation (5,325,329,488). Studies have predominately analysed the alterations of HM macronutrients, occurring over the first 12 months of lactation. Despite this, there is little documentation of the variation of HM glucocorticoids during this period. To the best of our knowledge, there has been only one human study (295) that has analysed the concentrations of cortisol over an extended period of 12 months of lactation. In this study, milk samples (n = 4) from four lactating mothers were analysed to determine the changes in cortisol concentration at different stages of lactation, largely between late pregnancy and after the cessation of breastfeeding. In the current study, we used liquid chromatography mass spectrometry (LC-MS/MS), to analyse the concentration of both, cortisol and cortisone, over the first 12 months of lactation and investigate their association with infant growth and body composition.
5.4 Methods

5.4.1 Study design and subjects

Between 2013 and 2015, breastfeeding mothers ($n=18$) of English-speaking, predominantly Caucasian, mothers of higher social-economic status were recruited from the community, primarily from the Australian Breastfeeding Association. Inclusion criteria were: healthy singletons, gestational age $\geq 37$ weeks, fully breastfed at two and five months (World Health Organization, 2015) and maternal intention to breastfeed until 12 months. Exclusion criteria were: infant factors that could potentially influence growth and development of body composition (BC), maternal smoking and low milk supply. All mothers provided written informed consent to participate in the study, which was approved by The University of Western Australia Human Research Ethics Committee (RA/1/4253, RA/4/1/2639) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616000368437).

5.4.2 Milk collection

All samples were collected by the researchers at Hartmann Human Lactation Research Group laboratory at King Edward Memorial Hospital for Women (Subiaco, Perth, WA) at 2 and/or 5, 9 and 12 months postpartum. Small amounts of (1–2 mL) pre- and post-feed milk samples were collected into 5 mL polypropylene vials (Disposable Products, Adelaide, Australia) from the breast/s that the infant was fed from and samples were frozen at -20°C. Samples were shipped to Auckland, New Zealand for glucocorticoid analysis on dry ice and were kept at -80°C frozen until analysis.

Milk samples were divided into four intervals and were classified as T2 (samples collected between 1.0-2.9 months), T5 (samples collected between 4.0-5.9), T9 (samples collected between 7.0-9.9 months), and T12 (samples collected between 10.0-12.9 months). These time periods were used in subsequent analysis as four major time points.

5.4.3 Maternal and infant characteristics

Up to 4 monitored breastfeeding sessions were organised for each mother/infant dyad between March 2013 and September 2015. At each study session the infants were weighed pre-feed. Infant bioelectrical spectroscopy (BIS) measurements were also taken pre-feed, unless impractical, in which case they were taken post-feed (489). Anthropometric measurements were taken post-feed. Clothing was removed for the measurements except for a dry diaper and a singlet.
**Anthropometric measurements**

Infant’s weight was determined before breastfeeding using Medela Electronic Baby Weigh Scales (±2.0 g; Medela AG, Switzerland), whereas maternal weight was measured using Seca electronic scales (±0.1 kg; California, USA). Maternal and infant BMI was calculated according to the following formula:

\[
BMI = \frac{\text{Body weight (kg)}}{(\text{Height (m)})^2}.
\]

Infant crown-heel length was measured to the nearest 0.1 cm using non-stretch tape and a headpiece and a footpiece, both applied perpendicularly to the hard surface. Infant head circumference was measured with a non-stretch tape to the nearest 0.1 cm. Maternal height was self-reported by participants or measured against a calibrated marked wall (accuracy~0.1cm). Maternal BMI was calculated as shown above.

**Bioimpedance spectroscopy measurements**

Whole body bioimpedance (wrist to ankle) of infants and mothers was measured using the Impedimed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia) according to the manufacturer’s instructions. Mothers were measured in supine position on a non-conductive surface. A series of ten consecutive measurements of percentage fat mass (%FM) were taken within 1−2 minutes and averaged for data analysis. Within participants coefficient of variation (CV) for maternal %FM was 0.21%. Infants’ whole body bioimpedance was measured by applying an adult protocol, as used previously, and data was analysed using settings customized for infants (489). Values of resistance (ohm) at a frequency of 50 kHz (R50) were determined from the curve of best fit, averaged for analysis purposes, and used in BIS age-matched equations for FFM. BIS-based prediction equations for body composition (490–492) were sourced from the literature, evaluated (493) and selected according to the following criteria: absence of significant difference from the reference distribution, closest age match, predominantly Caucasian population.

**5.4.4 Sample preparation: glucocorticoids extraction**

Milk steroids were measured by using liquid chromatography mass spectrometry as described previously (445). The internal standard consisted of 12 ng/ml cortisol d4 and 60 mg/ml corticosterone d8, prepared in water. Preparation was initiated by adding 100 µl of internal standard to 100 µl HM. All milk samples were heated at 37°C for 10 min and vortexed for 10 s. Steroids were then extracted using 1 ml ethyl acetate (Merck, Germany); the top organic layer was removed into a separate tube and then vacuum dried (Savant, SC250EX, Thermo Scientific, United States) for ~2 hours. The dried residues were reconstituted with 80 µl of 50% methanol (Merck, Germany) /water and transferred to HPLC injector vials. All samples were run in duplicates and average values are reported.
5.4.5 Liquid chromatography- tandem mass spectroscopy

The HPLC Mass Spectrometer (MS) used a Vanquish MS pump and auto sampler followed by an Ion Max APCI source on a Thermo Scientific Quantiva Quantum triple quadrupole mass spectrometer, all controlled by Finnigan Xcaliber software (Thermo Electron Corporation, San Jose, CA). The mobile phase was a methanol-water gradient starting at 50:60 (v/v) (peaking at 80:20 before returning back to 60:40) at 300 µl/min. The chromatography was performed at 40°C. The instrument was set up in selective reaction monitoring (SRM) mode with the following mass transitions: m/z 363.2→121.09 for cortisol, 361.1→163.04 for cortisone, 367.1→121.04 for cortisol d4 and 355.2→125.10 for corticosterone d8. Dissociation voltage was 24V and the collision gas (Argon) was set at 1.2 m Torr for all steroids. Steroid concentrations were calculated from a standard curve generated for each steroid relative to its internal standard (cortisol d4 for cortisol and corticosterone d8 for cortisone).

5.4.6 Statistical analysis

Results are expressed as mean ± SD, unless mentioned otherwise. Spearman correlation was run to assess the relationship between HM glucocorticoids and maternal and infant characteristics. ANOVA was used to compare difference in cortisol and cortisone concentration at different stages of lactation. Linear mixed models were employed to investigate associations between HM glucocorticoids concentration and both maternal and infant characteristics. Linear regression was used to investigate predictors of HM glucocorticoids. A p-value less than or equal to 0.05 was considered significant. Statistical analysis was carried out using SPSS software (SPSS version 23.0 for windows, IBM SPSS Inc, IL USA) and GraphPad Prism 7.0 software (California, USA). Lattice plots were produced using R software version 2.15.2.
5.5 Results

5.5.1 Participants

The demographics and characteristics of the study participants are described in Table 5-1. Infant weight (p<0.00), length (p<0.00), head circumference (p<0.00) and percentage fat mass (p<0.00) all increased significantly over the first year of life. Infant BMI (p=0.07) showed a trend towards significance. The concentration of cortisol and cortisone in each individual and at each time point, demonstrating the considerable differences in measured values between samples is shown in

Figure 5-1

Table 5-1: Maternal (M) and infant (Inf.) characteristics of longitudinal study measuring the concentration of glucocorticoids in HM samples at T2, T5, T9, and T12 months postpartum.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mother (n=18)</strong></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>33.88 ± 4.88 (24-43)</td>
</tr>
<tr>
<td><strong>Lactation stage (months)</strong></td>
<td><strong>T2.0</strong></td>
</tr>
<tr>
<td>M weight (kg)</td>
<td>73.06 ± 17.35</td>
</tr>
<tr>
<td>M percentage fat mass (BIS)</td>
<td>35.04 ± 5.27</td>
</tr>
<tr>
<td>Infant sex</td>
<td>Female (n=8)</td>
</tr>
<tr>
<td>Inf. Length (cm)</td>
<td>57.64 ± 1.95</td>
</tr>
<tr>
<td>Inf. Weight (kg)</td>
<td>5.59 ± 0.88</td>
</tr>
<tr>
<td>Inf. BMI</td>
<td>16.30 ± 1.38</td>
</tr>
<tr>
<td>Inf. Head circum.(cm)</td>
<td>39.62 ± 1.35</td>
</tr>
<tr>
<td>Inf. percentage fat mass (BIS)</td>
<td>21.74 ± 2.17</td>
</tr>
</tbody>
</table>


Figure 5-1: HM glucocorticoids (cortisol and cortisone) during the first 12 months of established lactation.

Each box of the lattice plot indicates a single mother HM glucocorticoid profile with symbol (o) representing cortisol and (Δ) representing cortisone. Each solid line indicates cortisol and dashed line cortisone. Time points are categorised as T2 (2 months), T5 (5 months), T9 (9 months), and T12 (12 months).
5.5.2 Changes in HM glucocorticoid concentration throughout the year

The concentration of cortisol ranged between 0.01-5.82 ng/ml and cortisone ranged between 2.60-13.23 ng/ml (Table 2.) For all analysed samples, cortisone was the predominant hormone at all time points (p<0.001), as shown in Table 5-2. Mean concentration of cortisol (p=0.10), cortisone (p=0.06) and cortisol/cortisone ratio (p=0.39) did not differ significantly over the period of 12 months. However, Tukey post hoc analysis showed significant difference between T2 and T5 months for cortisol (p=0.01) and cortisone (p=0.008).

![Graph 1](image1)

![Graph 2](image2)

Figure 5-2: HM glucocorticoids concentration, cortisol and cortisone concentration over the first 12 months of lactation at four time points.

Time points are categorised as T2 (2 months), 5M (5 months), 9M (9 months), and 12M (12 months). Error bars represent SEM (standard error of means).
Table 5-2: Summary of glucocorticoids in HM samples (n=63) collected from 18 breastfeeding Western Australian mothers at different stage of lactation (2, 5, 9 and 12 months).

<table>
<thead>
<tr>
<th>Glucocorticoids</th>
<th>Mean ± SD (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2M</td>
</tr>
<tr>
<td>Cortisol (ng/ml)</td>
<td>1.52 ± 0.96</td>
</tr>
<tr>
<td>Cortisone (ng/ml)</td>
<td>6.19 ± 2.00</td>
</tr>
<tr>
<td>Cortisol/cortisone ratio</td>
<td>0.22 ± 0.13</td>
</tr>
</tbody>
</table>
5.5.3 Relation between maternal characteristics and milk glucocorticoids

An overall significant positive correlation was found between HM cortisol/cortisone ratio and maternal BMI ($r_s=0.33$, $p=0.009$). Furthermore, cortisol ($r_s=-0.29$, $p=0.02$), cortisone ($r_s=0.27$, $p=0.03$) and cortisol/cortisone ratio ($r_s=-0.24$, $p=0.05$) showed a weak negative correlation with maternal height. In addition, maternal %FM showed a positive trend with cortisol ($r_s=0.21$, $p=0.09$) and cortisol/cortisone ratio ($r_s=0.24$, $p=0.06$). The mixed model analysis, identified a significant relationship between HM cortisol and maternal height ($p=0.02$), and maternal BMI ($p=0.04$). However, no significant association was found between HM cortisone and maternal BMI ($p=0.81$) and maternal height ($p=0.60$). In addition, cortisol/cortisone ratio showed no association with maternal BMI ($p=0.19$) and maternal height ($p=0.08$).

5.5.4 Relation between infant characteristics and milk glucocorticoids

An overall, weak, but significant positive correlation was found between cortisol and infant head circumference ($r_s=0.25$, $p=0.05$), and %FM ($r_s=0.27$ $p=0.03$). Furthermore, the HM cortisol/cortisone ratio showed positive correlation with infant %FM ($r_s=0.34$, $p=0.01$) and BMI ($r_s=0.28$, $p=0.32$) and cortisone showed a trend with infant head circumference ($r_s=0.23$, $p=0.07$).

The mixed model analysis showed significant relationship between HM cortisol and infant %FM ($p=0.008$), and head circumference ($p=0.05$); and showed no significant association with infant length ($p=0.37$), infant weight ($p=0.56$) and infant BMI ($p=0.26$). Whereas, cortisone showed significant relationship with infant head circumference ($p=0.01$) and showed no significant association with infant %FM ($p=1.00$), infant length ($p=0.61$), infant weight ($p=1.00$) and infant BMI ($p=1.0$). Furthermore, cortisol/cortisone ratio showed significant relationship with infant %FM ($p=0.04$) and no significant association was found between cortisol/cortisone ratio and infant length ($p=0.50$), infant weight ($p=0.81$), infant BMI ($p=0.72$) and head circumference ($p=0.11$).

Follow-up analysis using linear regression established that %FM and infant head circumference are significantly associated with cortisol ($p=0.03$) and cortisone ($p=0.03$) concentrations in HM, respectively.

Table 5-3: Association between milk glucocorticoids and infant characteristics

<table>
<thead>
<tr>
<th>Infant factors</th>
<th>Unstandardized $\beta$</th>
<th>Standardised $\beta$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant fat mass % (with cortisol)</td>
<td>0.08</td>
<td>0.24</td>
<td>0.05</td>
</tr>
<tr>
<td>Infant head circumference (with cortisone)</td>
<td>0.21</td>
<td>0.26</td>
<td>0.03</td>
</tr>
</tbody>
</table>
5.6 Discussion

This is the largest study to date to analyse HM cortisol and cortisone concentrations in a longitudinal manner over the first year of lactation. We found the concentrations of both hormones remained relatively consistent throughout the first year of life. Further, higher concentrations of HM cortisol were related with greater infant adiposity (%FM) and head circumference. Higher concentrations of cortisone were also associated with larger infant head circumferences indicating a possible role for HM glucocorticoids in infant growth and body composition.

HM is the optimal source of nourishment for the first six months of an infant’s life. Thereafter, mothers are recommended to continue breastfeeding for up to 2 years of age, with timely introduction of complementary feeding (55). Composition of HM is influenced by variety of maternal, infant and environmental factors (5,216,323), and also varies depending on the stage of lactation. Results from our study demonstrate that levels of cortisol, cortisone, and their ratio did not change significantly over the first 12 months of lactation. This is in accordance to a previous study, which only examined the concentration of cortisol, periodically, from 1 to 12 months of lactation, and found no change in concentrations over time (295). Interestingly, the apparent concentration of cortisol measured in the earlier study ranged between 0.2-32 ng/ml and was up to 5.5 fold higher than that of our study. These difference could be due to the difference in assay techniques. Compare to current study previous study used an immunoassay, which are more likely to deliver restricted accuracy and exaggerated readings mainly attributed due to the cross reactivity of the interfering antibodies or restricted detection limit, whereas our study was based on a more accurate method, mass detection technology using a triple quad.

Maternal mental health state, particularly depression, is a critical predictor of infant growth status (494). In addition, psychological distress during pregnancy plays a pivotal role in regulating infant birth weight, whilst postnatal stress causes a significant variation in child’s behaviour and metabolic programming (14,298,495). During infancy or early childhood, head circumference is commonly used as an indicator of infant brain size (496), and is also used for measuring infant’s neurological development, cognitive function and intracranial volume (497). Numerous prospective studies indicate that chronic prenatal maternal stress influences infant brain development and smaller head circumference, although the underlying mechanisms of action are less well described (498–501). Previous studies have mentioned the impact of prenatal maternal stress on infant outcome, such as low birth weight and premature birth. However, only a few studies have investigated the relationship between maternal perceived stress and infant neurodevelopmental outcomes (502). Studies testing these associations have pronounced mixed results in humans, some authors found negative association between maternal stress and infant head circumference (424,501), whereas Obel et al in his follow up study failed show any association between maternal stress and infant head circumference, however have postulated that sever stress may play a significant role in infant brain development and may cause subtle brain impairment (499). The relationship between maternal stress, maternal circulating cortisol levels and impaired fetal development is already established (424), however far less is known of the effects during the first year of life. Chronic prenatal stress
disrupts cognitive performance and reduces brain volume in the area related to learning and memory (500). A negative association between prenatal maternal stress and fetal head growth development has also been reported (501). Recent studies have demonstrated the presence of glucocorticoids in HM, as potential stress biomarkers influencing infant growth and behavioural development. In the current study, regression modelling analysis identified that increasing head circumference was predicted by concentration of HM glucocorticoids. To our knowledge, this is the first study to identify the association between HM glucocorticoids and infant body composition over the first 12 months of lactation. Although, the correlation does not imply causation, further studies are required to extend this observation, and to examine possible relationships between HM glucocorticoids and infant head growth and cognitive development.

Furthermore, the current study demonstrated that higher HM cortisol as a significant predictor of higher %FM in infants at 12 months of age. Elevated cortisol is most likely a potent stimulator of body fat mass gain (503). Evidence suggests that visceral body fat is particularly influenced by cortisol level (504). A recent study by Hahn et al (298) suggested HM cortisol as the predictor of infant obesity. In this study, infants exposed to higher milk cortisol concentration had reduced infant body mass index percentile (BMIP) at two years of age. Again, not much is known about the impact of milk-ingested glucocorticoids on infant's growth. Having further studies, in a larger cohort over a longer period, focused more on infant weight gain, height and BMI will be required to more accurately analyse this relationship.

More recently some studies have suggested the importance of milk borne glucocorticoids and its impact on infant’s growth and behavioural development (2,289,505). Association between early glucocorticoid exposure and infant metabolism is complex, regulated by many maternal related factors such as maternal circulating levels. Excessive exposure to both endogenous and exogenous glucocorticoids during pregnancy and lactation have been previously linked to obesity risk factors (506,507). This study has shown a significant association between maternal BMI and HM cortisol levels. There are several factors that may contribute to the constantly changing composition of HM, including maternal BMI. This may further affect the developmental trajectories of infants fed on HM (508). Numerous evidences suggest a positive association between HM leptin concentration and maternal BMI. Also, presence of leptin in HM has been linked with lower infant weight gain, suggesting a key role in regulating infant body composition. Evidence suggests that individuals with higher BMI are more likely to have increased levels of cortisol in their circulation (509). The current study is the first to demonstrate an association between maternal BMI and HM cortisol concentration. Although the correlation was weak, it was in accordance with previous studies that show obesity or higher BMI is an indicator of increased circulatory or saliva cortisol levels (510,511). Though, BMI is a poor indicator of adiposity as it fails to dissociate lean mass and adiposity. Hence, more robust studies are required to confirm the role of maternal BMI or adiposity in altering milk glucocorticoid composition and its impact on infant development.

Results of the current study underscore the need for future investigation to elucidate the maternal risk factors affecting milk glucocorticoid concentrations and how these variations could influence...
infant growth and development at different stages of lactation. One limitation of the current sample cohort was the lack of maternal plasma samples, thus, it is not possible to report with confidence if these variations in HM glucocorticoids are the results of maternal hypothalamic variabilities that proceed higher BMI or consequences of the overweight state. However, the findings of this study are noteworthy because of the limited information available about the composition of glucocorticoids in HM and their effect on infant growth and body composition.

In conclusion, HM cortisol and cortisone levels remained constant throughout the first 12 months of lactation. Furthermore, infant head circumference and %FM can be significantly predicted by the cortisol and cortisone in HM, respectively, at one year of age.
Chapter 6: General Discussion
6.1 General discussion

6.2 Introduction

Glucocorticoids are the primary stress hormones present in human milk (HM). There is a limited understanding about their presence in milk, but a few studies have shown that cortisol present in milk plays a significant role in depicting an infant’s behaviour and metabolic programing (14,298). The composition of HM varies considerably in response to numerous maternal and infant factors (512), yet, many aspects of maternal biology and environmental factors affecting milk glucocorticoid composition remain unclear. Therefore, this thesis sought to examine the effects of different maternal conditions and infant characteristics on the glucocorticoid profile of HM, and understand the effect of milk glucocorticoid content on infant growth outcomes. Together, the findings from this thesis provide an insight into the source and magnitude of variations in milk glucocorticoid composition over the course of lactation. The method developed in this thesis can also be used in future analysis of HM steroid hormones.

The specific aims of this thesis were:

- To investigate the impact of maternal associated biological, social, and environmental factors on the glucocorticoid concentrations (both cortisol and cortisone) of human milk, at three months of established lactation.

- To investigate the relationship between human milk glucocorticoid concentrations and preterm birth.

- To analyse the concentration of glucocorticoid hormones (both cortisol and cortisone) in human milk over a 24 hour period and explore if glucocorticoids follow a circadian pattern in the human milk.

- Longitudinal analysis of glucocorticoids in human milk over the first 12 months of lactation and its impact on infant’s development.
6.3 Summary of major findings

To quantify the presence of glucocorticoids in HM, a technologically advanced, sensitive, accurate, and reliable LC-MS/MS method was developed. Throughout the thesis, 2131 (n=727) HM samples were analysed. It was demonstrated that all HM samples contained considerable amounts of both cortisol (3.30 ng/ml) and cortisone (6.30 ng/ml), within the range of 0.01-32.0 ng/ml and 0.04-20.0 ng/ml, respectively. Besides cortisol and cortisone, other steroid hormones, including testosterone, estradiol, and progesterone were also identified in HM samples. However, only 23% (n=132) of samples had detectable testosterone, varying between 1.5-30 pg/ml (mean 13.05 pg/ml). Estradiol and progesterone were largely absent in the majority of HM samples (<1% had levels above the detection threshold) and thus were not investigated further in detail.

To identify the major maternal variables affecting HM glucocorticoids, four studies were conducted. The first study was part of a large cohort conducted in Finland, and found that maternal and infant related factors play an important role in dictating the hormonal composition of HM. The study described in chapter 3 suggests that birth gestation period and maternal education may influence the concentration of milk glucocorticoids. Furthermore, our data demonstrated that maternal preference to exclusive and partial breastfeeding behaviour had no influence on the glucocorticoids concentration of HM at three months of established breastfeeding.

The study described in chapter 4 examined the implications of preterm birth and subsequent alteration of milk glucocorticoids during a period of six weeks after birth. Unlike plasma, HM obtained from mothers who delivered prematurely had higher cortisone concentration than cortisol, with a strong correlation between both hormones. This chapter also demonstrates that most of the glucocorticoids available in milk were in the free form, and mothers who delivered infants before 30-weeks of gestation had a lower concentration of glucocorticoids in their milk.

An analysis of glucocorticoids in HM, over a 24 hour period was also conducted (chapter 5). Glucocorticoid levels exhibited a pronounced 24 hour circadian pattern, characterized by the peak cortisol and cortisone concentration during morning hours, followed by a gradual decline throughout the remaining period. Glucocorticoid concentrations in HM samples donated prior to feeding and again immediately upon the cessation of infant feeding did not change. Glucocorticoid concentrations did not differ between left and right breasts.

After investigating the changes in milk composition in a period of 24 hours, the influence of different lactational stages on milk glucocorticoid profile was studied. Its impact on infant growth and development was also observed. This study showed that concentration of glucocorticoids did not differ significantly throughout the first 12 months of lactation. However, significant association was observed between milk glucocorticoids, infant head circumference, and fat mass, implicating milk glucocorticoids in infant brain development and metabolic programming.

The results from this thesis provide an insight into the complex glucocorticoid profile of HM, and the influence that maternal biological, social and environmental factors could have on HM composition.
These results highlight the effects of maternal health and life style conditions, during pregnancy and lactation, on milk composition, and how that ultimately may affect the development of a breastfeeding infant.

6.4 Implications and future directions

Maternal environment and physiology during the prenatal development and postpartum period can predispose fetus and new born to various health related conditions later in life(299). Because HM is an essential link between a mother and her breastfeeding infant, understanding its complexities would give us an insight into the maternal associated determinants dictating the biochemical profile of milk. Moreover, implementing this knowledge to manipulate the conditions around breastfeeding infants could help to optimise the composition of HM.

This thesis serves an important role as an exploratory study and provides significant evidence to support the influence of maternal determinants in dictating the glucocorticoid composition of milk. Findings from this thesis elicit many questions about (i) how a lactating mother’s health condition can influence the profile of HM glucocorticoids; (ii) what are the implications of the altered composition and (iii) how this may impact infant growth and development in later life. Therefore in order to inform lactating mothers, it is important to design a studies which further elicits the impact of maternal phenotypic characterstics on milk glucocorticoids, including maternal biological, social and environmental factors. Moreover, investigating the origin, significance and implication of these difference could aid in providing long term interventions for improving maternal quality of life and hence breast milk quality.

6.4.1 Glucocorticoids in mammalians

Among mammals, quantity and quality of milk composition is highly dependent on several maternal related variables such as development of mammary glands, their health; nutritional and social status are also significant predictor of milk composition (513).Mammalian milk contains significant amounts of glucocorticoids, however, the type of glucocorticoids varies with in the species. For instance, cow milk has no cortisone but contains cortisol (10-18 ng/ml), while rodents have only corticosterone (88-144 ng/ml) (514,515). Evidence suggests that cortisol in HM provides an excellent example of how mothers can influence infant development. The data presented in this thesis demonstrate considerable amount of both cortisol and cortisone in HM and highlights the importance of several maternal and infant related factors, from social surroundings to the time of infant feeding, in altering milk glucocorticoid composition. These findings could be used as a potential target to optimise the quality of HM.

Measurement of plasma cortisol concentration is used as a diagnostic biomarker for many pathologic diseases and is the primary glucocorticoid in humans (516,517). Hypothalamic stimulation causes the release of cortisol into circulation from the adrenal glands, while secretion of cortisone is negligible. In plasma, the balance between cortisol and cortisone concentration is mainly maintained
by the activity of the 11 βHSD isoenzymes (446). The concentration of cortisol in body fluids and tissues is controlled by the 11 βHSD enzymes. 11 βHSD type-1, catalyses the conversion of cortisone into cortisol, whereas, 11 βHSD type-2, catalyses the conversion of cortisone into cortisol. In contrast to plasma, findings of this thesis consistently showed cortisone as the predominant hormone in HM samples. Since cortisone is not secreted in measurable amounts in maternal plasma, 11 βHSD type-2 may be acting in the breast tissue, milk, or other tissue to convert cortisol to cortisone, but the exact mechanism remains unknown (518). Alternatively, there could be increased penetrance of serum cortisone into HM or an increased rate of cortisone clearance from maternal circulation into HM, as compared to that of cortisol. The increase of cortisone levels could be a way of protecting infants from increased concentration of biologically active cortisol. Throughout the thesis, a strong and positive correlation was observed between the concentrations of cortisol and cortisone, indicative of the conversion of cortisone into cortisol or vice a versa. This result further suggests the role of 11 βHSD isoenzymes in the mammary gland or milk. Therefore, further research is required urgently to identify the source of glucocorticoids in HM, in order to understand the impact of its presence on infant feeding and on mother’s milk.

6.4.2 Factors influencing milk glucocorticoid composition and effective strategies to improve the biochemical makeup of human milk

In humans, the process of lactation is a critical phase that imposes a great demand on a mother’s nutritional and psychological state. Most of these maternal conditions are critical in dictating milk composition (519). Findings from this thesis suggest that greater emphasis should be given to the maternal condition during pregnancy and lactation to optimise the milk hormonal composition. For instance, pregnant mothers, well informed on the benefits of breastfeeding are more likely to breastfed their infants and continue it for a longer duration (24). Similarly, keeping mothers well informed about the effect of their social and environmental conditions may help to improve milk hormonal composition. Therefore, strategies need to be developed to address the issues related to maternal social and physiological conditions that may affect the composition of milk.

The association between circulatory cortisol levels and individual social support status are well established. It has been demonstrated that social support attenuates cortisol stress response, via its effect on HPA axis pathway. Maternal education and household prosperity have been shown to alter milk fat composition (520). Furthermore, lower education standards and low quality of life are associated with higher stress levels (521), however the strength of this association varies between individuals. The observations from our study (Chapter 2:), now provide further insight that maternal social conditions such as education may contribute to the glucocorticoid variations in milk. In the STEPS study population, mothers with lower education showed higher level of glucocorticoids in their milk samples, suggesting a potential role of maternal social status in dictating milk composition. Indeed, social factors do get transferred into biological risk (282,522), though the exact mechanism behind this is unclear, but additional support from the government and family is well described to have a positive effect on breastfeeding frequency and maternal mood (233,414,431). Besides,
emotional support such as empathy and huggable conversations have been shown to enhance maternal positive mood and reduce circulatory cortisol levels (523). Maternal plasma cortisol levels have been shown to be positively associated with milk cortisol concentration, indicating that any change in maternal mood while lactating, may reflect into the mother’s milk. Although the study of the impact of milk-borne glucocorticoids on infant growth and development is still in its infancy, it may be preferable to reinforce increased public and individual efforts to improve maternal surroundings and maternal mood, which would further influence milk glucocorticoids composition.

In addition to maternal associated social factors affecting milk composition, maternal stress during pregnancy is associated with poor birth outcomes such as low birth weight, increased miscarriages and premature birth (524,525). Findings from this thesis showed that gestational age could have a relationship with milk glucocorticoid composition. Additionally, we observed that the majority of glucocorticoids in the preterm milk samples were in free form and cortisone concentration was positively linked to the gestational age, with more cortisone was seen in milk of those who delivered after 30 weeks of gestation, compared to those who birthed before 30 weeks. Previous research reports that adrenal gland function is more closely related to infant gestational age, rather than birth, indicating that preterm infants may have immature adrenal inactivity and HPA axis response (456). Thus, premature infants may have limited ability to produce glucocorticoids, particularly cortisol (454,488). Interestingly, we observed that only cortisone changed with gestation age and cortisol showed minimal variation. Our results showed no correlation between milk cortisol concentration and birth gestation and are consistent with other studies which suggest that cortisol levels show minimal variation in relation to birth gestation (454). These variations may further be indicative of the difference in 11βHSD enzyme activity or better diffusion of cortisone into HM. Importantly, this work was only done in the milk taken after birth, and lack of plasma samples was a significant limitation.

This thesis includes one of the few studies done in HM that investigated the influence of birth gestation on milk glucocorticoid composition. Indeed, more robust studies are required to identify the exact mechanism underlying these variations, understand the origin of milk-borne glucocorticoids and their mode of transfer from maternal plasma to milk, particularly during stressful birth. Furthermore, maternal socioeconomic and education status, marital status and age are major prognosticators of stress status, which in turn is a predictor of preterm birth. These social experiences affect maternal plasma cortisol and cortisone levels, which may further dictate milk glucocorticoid composition. Therefore, it is important to identify the factors that alleviate maternal stress and thus improve our understanding on optimising the quality of HM.

This thesis further highlights the daily rhythmic variations in HM, seen in components such as fats, amino acids and some nucleotides. Indeed, concentration of milk fat is highly variable and exhibits significant circadian rhythm throughout the day. Likewise, results from this thesis showed that concentration of glucocorticoids also vary over the course of a 24-hour period, with higher concentrations in the morning and lower in the evening; suggesting the possibility of mimicking maternal plasma circadian patterns. The exact mechanism behind this variation is still unclear, however one explanation could be that the milk glucocorticoids impersonate maternal plasma
hormones, emphasising the direct transfer of maternal hormones into milk, instead of mammary synthesis, as shown in Figure 6-1.

Several studies have shown the importance of meal timing in energy metabolism, however for infants immense emphasis is given on demand feeding, compared to schedule feeding. It is recommended that infants should be breastfed according to their appetite, particularly for the first six months of life. While the importance of on demand feeding is well established, not much is known about the importance of time of feed and how this may dictate infant intake of certain milk components. In this thesis, we observed significant variation of milk glucocorticoids at different times of the day, suggesting that infants, who are ingesting the majority of their feed during morning and evening, may receive a higher quantity of milk-borne glucocorticoids, compared to the infant whose majority milk intake is only at night or evening. These findings highlight the importance of feed timing, particularly for mothers in employment with nonstandard work schedules (526). Although, we still lack information about the impact of HM on infant growth and development, may be it would be logical to cautiously inform lactating mothers about the importance of time of the feed and encourage on demand breastfeeding. Having more studies where Chrono biologists collaborate with infant nutritionists may further clarify the interaction between circadian patterns; milk biologically active hormones and infant development. These alterations may further suggest the importance of standardizing milk collection protocols for research and milk biobanks.

In addition to high degree of variability over the course of day, milk variation during the first 12 months of lactation is of considerable importance. The fascinating fact that HM composition is never constant and always in transition, giving rise to speculation whether these alterations are benefiting infants or if these are the result of maternal physiological changes associated with synthesis of milk. Several new studies now proclaims that these alterations in HM are more extensive than once thought. In most studies, these changes were measured in lipids, proteins and minerals, altering throughout the year to meet the growing needs of an infant (315). However, little is known about the influence of lactational stages on the hormonal composition of HM. This thesis has revealed that glucocorticoids in HM fluctuates throughout the day and in response to various maternal and infant related factors. We further showed the impact of lactational stages on HM glucocorticoid concentrations. Our results showed that cortisol and cortisone remained constant throughout the first year of lactation. Till now, there have been only a handful of studies which have explored the influence of HM glucocorticoids on breastfed infants and none of these studies have measured glucocorticoid variations throughout the first 12 months of lactation, which is the most crucial period for infant nutrition (14,308). Growing evidence suggests that prenatal and early nutrition has a significant impact on adult health by influencing developmental programming (527). Research into developmental origins consider that carefully planned nutrition during pregnancy through to early childhood is a critical window of opportunity and preventive interventions (528). Provision of HM during the early stages of life is generally referred, as an important element for determining later life health, thus deciphering the exact composition of HM, particularly bioactive hormones, is critical in understanding the physiologic significance of dynamic nature of HM.
Figure 6-1: Postulated pathway of maternal stress dictating milk glucocorticoid composition.
6.4.3 Health outcomes for offspring feeding on human milk

Glucocorticoids are the steroid hormones produced by adrenal glands, play an important role in maintaining pregnancy and fetal development. During the last week of pregnancy, glucocorticoid concentration increases rapidly to prepare fetus for birth (529). However the deficiency of glucocorticoids results in poor lung development. On the contrary, excessive prenatal exposure to glucocorticoids are linked with detrimental consequences on infant development (530). Excessive glucocorticoid during pregnancy may suppress fetal and infant growth and programme the infant for life long disease (531). Most of the research is focused upon prenatal glucocorticoid programming during early development and little is known about the influence of milk ingested glucocorticoid on infant development.

After studying the factors responsible for altering milk composition, we attempted to identify the impact of these milk-borne hormones on infant development. Series of rodent studies and a few recent rhesus and human studies have demonstrated the impact of milk-borne glucocorticoids on offspring development (464,532). Previous studies on rodents have shown that mother’s corticosterone supplementation was associated with reduced anxious behaviour in offspring. Furthermore, corticosterone intake was also linked with increased glucocorticoid receptor in the hippocampus region (533). Nevertheless, research on exploring the relation between milk-borne glucocorticoids and infant development is still in its infancy. Only a few studies have linked HM cortisol with infant behavioural development and metabolic programming. A study in rhesus monkeys showed that mothers of male offspring’s had higher concentration of cortisol as compared to mothers of females. Further, the higher concentration of cortisol demonstrated a positive correlation with confident temperament in male offspring’s (309). In humans, presence of cortisol was found to be associated with negative infant temperament, in female offspring’s, however, these results were not statistically significant (14). These two studies suggest that concentration of cortisol influences infant behavioural development; however, they investigated the role of cortisol only at 3 months postpartum. Recently, a study by Hahn et al., demonstrated the long term impact of milk ingested cortisol. Infant’s exposed to higher milk cortisol at three months, demonstrated reduced Body Mass Index Percentile (BMIP) over the period of first two years, suggesting its role in preventing obesity (298). The data shown in this thesis is the first study to demonstrate the positive relationship between milk glucocorticoids (cortisol and cortisone) and infant fat mass (%FM) and head circumference. The relationship between milk hormones and infant growth patterns is complex and modulated by various factors, are still unclear. This thesis shows that glucocorticoids are biologically active hormones present in HM, capable of altering early infant development as well as later life health. There are only a few longitudinal studies exploring the relationship between lactational stages and infant development. Therefore, this thesis has opened up an avenue for future studies, as described in section 6.5, required to identify the factors influencing glucocorticoid concentrations in HM and their impact on breastfed infants.
6.4.4 Limitations

This thesis was a part of many different studies, and thus has a few limitations. Lack of maternal and infant plasma or serum or salivary samples and detailed follow-up on infant breastfeeding frequency, duration and total milk intake for each infant may have helped us to better understand the complexities of milk glucocorticoids. Nevertheless, results of this study could be used to design future studies, aimed at exploring the impact of milk glucocorticoids on infant development in later life.
6.5 Proposed research opportunities

The data presented in this thesis provides an insight into the complex biochemical characteristics of HM, which may contribute to the health of breastfeeding infants and ultimately, how maternal and infant environment can manipulate milk composition and subsequent health development. Further studies should be conducted to identify and characterise vital physiological and psychological determinants, for understanding the complexities of milk composition. To do this, it is suggested that following studies should be performed to investigate and explain maternal hormonal signalling through maternal breast milk and breastfeeding, to improve specific health outcomes for both mother and her infant.

6.5.1 Activity of 11β-hydroxysteroid dehydrogenase in adipose and epithelial cells of mammary gland and human milk

In humans, nearly all bodily functions exhibit circadian rhythm, cortisol displays one of the most distinct circadian patterns, with minimum circulating level at midnight peaking early in the morning, before reducing back to zero to complete the 24-hour cycle. Findings from this thesis indicate that HM glucocorticoids demonstrate a significant circadian pattern. Cortisol and cortisone levels were highest during the morning and lowest in the evening. Unlike in plasma and saliva, cortisone in HM was much higher and was a predominant hormone compared to cortisol. Strong correlations were observed between cortisol and cortisone, indicating the possibility of dynamic regulation between both hormones. Cortisone is a lipophilic glucocorticoid that gets converted into bioactive cortisol by 11βHSD (hydroxysteroid dehydrogenase) enzyme. 11βHSD enzyme has two isoforms 11βHSD type-1 and type-2, responsible for tissue specific conversion of cortisol and cortisone. 11βHSD type-1 is mainly expressed in liver, kidney, brain, and adipose tissue, whereas type-2 is expressed in kidney and salivary glands (534). Accumulation of cortisol in tissues such as adipose or liver could be either due to the enzymatic activity of 11βHSD type-1 enzyme or passive diffusion from plasma. Evidence from animal studies suggest that during pregnancy and lactation, the activity of 11βHSD type-1 enzyme is 20-fold higher in adipose compared to the epithelial cells of mammary glands (447). Activity of the enzyme in adipose cells was 3 times higher in pregnant rats than lactating rats, suggesting the role of 11βHSD enzyme (447). Therefore, we theorise that 11βHSD activity in human mammary gland could be responsible for milk glucocorticoids variations.

Determining the functioning of 11βHSD enzyme in mammary tissue or HM will allow us to better understand the circadian variation of glucocorticoid in HM. Whether the activity of 11βHSD in HM is measurable or not; and if yes, how this influences the glucocorticoids composition through the 24 hour period. The following table describes a proposed study aimed at assessing the activity of 11βHSD enzyme in mammary tissue and HM.
### Table 6-1: Activity of 11 βHSD enzyme in HM and mammary tissue

<table>
<thead>
<tr>
<th><strong>Background</strong></th>
<th>11 βHSD converts cortisol into cortisone by the 11 β-dehydrogenase activity of 11 βHSD enzyme type-2. While, 11 β HSD enzyme type-1, isoform oxidises the reverse action of converting cortisone to cortisol.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Question</strong></td>
<td>If present in epithelia cells of maternal mammary glands, is the level of glucocorticoids in HM regulated by the 11 βHSD enzyme?</td>
</tr>
<tr>
<td><strong>Aim</strong></td>
<td>To assess the presence/activity of 11 βHSD enzyme in mammary tissue and HM.</td>
</tr>
<tr>
<td><strong>Design</strong></td>
<td>Collect mammary tissue of healthy lactating mothers and milk samples at four time points – morning, afternoon, evening and night throughout the 24 hour period.</td>
</tr>
</tbody>
</table>
| **Measures**   | • Maternal BMI  
• Age  
• Health status  
• Plasma insulin, glucose  
• Plasma glucocorticoids (cortisol and cortisone)  
• HM glucocorticoids (cortisol and cortisone)  
• Measure the activity of 11 βHSD enzyme(type 1 and type 2) and compare it both qualitatively and quantitatively.  
• Extract RNA  
• Study gene expression of 11 βHSD mRNA in mammary tissue. |
| **Anticipated results** | • Expected to show the presence of 11 βHSD enzyme in epithelial cells of human mammary gland. The activity of 11 βHSD is likely to vary according to the length of the day. |
6.5.2 Chronic maternal stress and human milk hormonal composition

Extensive evidence from animal and human studies has shown that maternal stress, during pregnancy and after birth can dysregulate normal activity of HPA axis. This dysregulation can further lead to epigenetics effects (300,301), generalised anxiety, panic disorder (506), depression and other stress related physiological functions(448,535). Exposure to stressful events during pregnancy are linked with increases incidences of intrauterine growth restriction (448), low birth weight and premature birth (536,537) as well as hyper responsive HPA axis (538). It is usually observed that women who are stressed during pregnancy tend to remains stressed after birth as well (46,47,539). Although, studies examining the effect of postpartum depression on child development is largely based on correlation studies. Most of the studies so far have focused on measuring cortisol as an index of maternal HPA axis. Maternal cortisol concentration increases by two – three folds during the gestation, however the presence of 11 βHSD type-2 placental enzymes converts excess cortisol into cortisone. This provides enhanced protection to infants against negative impact of increased cortisol level (540,541). More recent evidence suggest that stressed mother, while lactating can represent an important perspective in terms of transferring milk-borne glucocorticoids to infants via ingested milk. Effect of maternal mental health or postpartum depression on breastfeeding frequency is well established. In addition to lower breastfeeding frequency among stressed mothers, those who attempted to breastfeed exhibited negative breastfeeding behaviours and altered milk volume and composition (539). Despite, not much is known about the impact of prenatal stress ad postpartum depression on maternal breast milk quality, particularly glucocorticoids. Investigating mother milk hormonal profile, who are diagnosed with chronic stress could offer an opportunity to understand HM as a means of biochemical transfer of additional glucocorticoids. In addition, analysis of infant growth patterns after ingesting milk from chronically stressed mother could provide a valuable insight into the complex relation between stress and lactation.
Table 6-2: Chronic maternal stress and milk hormonal composition.

| Background | Prenatal chronic stress or maternal postpartum depression is associated with negative health outcomes for infants’ health and later life developments. Majority of studies done so far has focused on measuring cortisol in plasma or saliva as an index of maternal stress or dysregulated HPA axis. However, it can be argued that stressed mother, while lactating can represent an important perspective in terms of transferring circulatory glucocorticoids into milk and influencing infant development. |
| Question | Are maternal psychological stresses associated with altered milk glucocorticoid composition in lactating mother? |
| Aim | To assess the effect of maternal stress on the cortisol, cortisone and ACTH concentration in HM. |
| Design | Collect HM and plasma samples from healthy and psychologically stressed lactating mothers at different stages of lactation. |
| Measures | • Maternal health status  
• Available milk energy (AME) of HM  
• Trier social stress test (TSST) for measuring psychobiological stress  
• Carefully constructed private interview questions to obtain stress and trauma inventories  
• Plasma and HM glucocorticoids (cortisol and cortisone) and ACTH  
• Plasma and HM and HM inflammatory cytokine markers  
• Urinary steroid metabolite ratio of cortisol and cortisone ratio  
• Child plasma and urine metabolic profile (such as cortisol, cortisone and weight gain) at different stages of development |
| Anticipated results | • Increased level of stress hormones in HM  
• Strong correlation between maternal stress levels and milk hormonal concentration and infant circulatory glucocorticoids |
6.5.3 Life style factors affecting glucocorticoids

Maternal life-style factors including recreational drugs such as alcohol, nicotine, caffeine, and marijuana have effect on milk quality and quantity. Aside from other harmful consequences, consumption of these recreational drugs is responsible for modifying the composition of HM (542). During the lactation period, consumption of alcohol by a breastfeeding mother affects the overall quality and quantity of milk (543). Ingestion of alcohol during breastfeeding causes the transfer of alcohol from maternal plasma to milk, which is then consumed by infants, affecting their sleep and gross motor development. Overtime alcoholism can have an adverse effect on milk, and reduce reflex and milk yield by disrupting the hormonal response of the lactating mother. After immediate hours of alcohol consumption, a significant reduction in oxytocin levels, and increase in prolactin has been shown, causing reduction in overall milk supply. Whilst acute alcoholism disrupts hormonal milieu of the lactating mother, maternal smoking reduces the quality of milk by decreasing the pro-inflammatory marker and appetite regulating hormone of HM (544,545). However, the pathophysiological mechanisms behind these effects are still unclear.

Furthermore, effects of other recreational drinks such as coffee intake during pregnancy have been associated with negative health outcomes on the fetus, whereas its effect on milk quality and breastfeeding frequency are not clear and hence require further research. Much of the current research focuses on understanding the impact of these drugs on biochemical composition of milk but little is known about the correlation between maternal stress and her lifestyle choices. The studies done so far suggest that stress and activation of HPA axis can increase the consumption of alcohol and smoking (546). Understanding how maternal stress may influence the consumption of recreational drugs and thus milk glucocorticoids consumption is still unclear.

Therefore, studying maternal social environment in context to her lifestyle behaviour and examining the effect of acute social stress on the composition of HM will allow for better understanding of the relation between maternal conditions and milk composition.

Based on these evidences, the following table outlines a proposed study that will evaluate the effect of anticipated maternal stress on her milk composition.
Table 6-3: Implications of acute stress on maternal consumption of alcohol, smoking, and caffeine on the human milk hormonal composition.

| Background | There has been renewed interest in maternal stress hormones and their effect on infant development. Evidences suggest that consumption of recreational drugs can modify milk composition, however the process by which acute stress would lead to the increased consumption of alcohol, smoking and caffeine and thus affect levels of milk stress hormone, is still unclear. |
| Question | Does anticipated or perceived acute stress increase alcohol, smoking, and caffeine consumption in lactating mothers and how this would affect the hormonal composition of HM? |
| Aim | To assess the effects of acute stress on the consumption of alcohol, smoking and caffeine in healthy lactating mothers and to identify its influence on milk hormonal composition. |
| Design | Collect milk and plasma samples from healthy and alcohol/ other recreational drugs addicted lactating mothers. Also, collect plasma and saliva from breastfeeding infants. |
| Measures | • Body composition of mother and infant  
• Maternal social and biological character tics  
• Trier social stress test (TSST) for measuring psychobiological stress  
• Blood pressure, heart rate  
• Alcohol, nicotine and caffeine concentration in plasma (both mother and infant) and milk  
• Volume of alcohol and coffee intake number of smoked cigarette  
• HM, saliva and plasma glucocorticoids (cortisol and cortisone)  
• HM, saliva and plasma inflammatory markers (IL-6)  
• Subjective self report measures and physiological effect |
| Anticipated Results | • Increased concentration of plasma and milk alcohol, nicotine and caffeine  
• Decreased concentration of antioxidant and pro-inflammatory marker in milk |
6.5.4 Obesity, stress and human milk hormonal composition

The window of early nutrition has a significant influence on adult health, by stimulating functional programming (299). Obesity epidemic is a major public health challenge and the leading cause of morbidity and mortality in developed countries (547). Obesity that begins in early life generally persists into adulthood with an increased risk for cardiovascular disease and diabetes later in life (548,549). The pathophysiology of obesity is far more complex than a simple energy balance equation, growing evidence suggests that prenatal nutrition and social environment in early life has a programming impact on an individual’s predisposition to develop obesity in later life (550).

Human breast milk and breastfeeding are undoubtedly the best source of nutrition for new born infants. HM contains several hormones, including glucocorticoids, leptin, adiponectin, ghrelin, resistin and obestatin, which are involved in the regulation of metabolism (215,216,306,421). Evidence suggests that obese individuals are more responsive to stress (509). Psychological stress is associated with activated SNS (sympathetic nervous system), elevated glucocorticoids, and blood glucose levels to ensure adequate supply of energy for brain and muscles (277,282). Glucocorticoids are majorly concerned with energy intake and its mobilization and thus are known to alter individual feeding (551). A close relationship has been identified between circulatory glucocorticoids and stress-induced obesity. Furthermore, circulating levels of cytokine leptin were found to be elevated in obese individuals and may play a vital role between obesity and stress (552,553).

Most nutrients are absorbed more efficiently from HM than formula milk (based on bovine milk), but surprisingly little is known regarding the impact of maternal obesity and stress on the bioavailability of milk hormones. Several etiological factors concerning maternal obesity have been identified whereas other factors indicating the impact of maternal obesity on milk hormonal composition is still unclear (318,554,555). Thus determining the role of milk-borne hormone in stressed and overweight lactating mother will allow for a better understanding of the endocrinological events regulating early programming and obesity in later life.
Table 6-4: Effect of maternal stress and obesity on milk hormonal composition.

<table>
<thead>
<tr>
<th><strong>Background</strong></th>
<th>It has been suggested that obese individuals are more responsive to stress. Increased stress levels are associated with increased consumption of high calorie food and higher BMI. Evidence suggests that maternal stress and obesity influence milk-borne glucocorticoids and appetite regulating hormones.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Question</strong></td>
<td>Are maternal obesity and psychological stress associated with attenuation in milk stress and appetite regulating hormones of lactating mother?</td>
</tr>
<tr>
<td><strong>Aim</strong></td>
<td>To assess the effect of maternal obesity and stress on the cortisol, cortisone and leptin concentration in HM.</td>
</tr>
<tr>
<td><strong>Design</strong></td>
<td>Collect HM and plasma samples from healthy and obese lactating mothers at four different time points (after every four hours).</td>
</tr>
</tbody>
</table>
| **Measures**   | • Maternal health status  
• Maternal age and BMI  
• Available milk energy (AME) of HM  
• Insulin sensitivity and glycaemic control – HOMR-IR, HbA1C  
• Trier social stress test (TSST) for measuring psychobiological stress  
• Plasma and HM glucocorticoids (cortisol and cortisone)  
• Plasma and HM appetite regulating hormones (leptin, adiponectin and ghrelin)  
• Plasma and HM inflammatory cytokine markers |
| **Anticipated results** | • Increased level of glucocorticoids and appetite regulating hormones in HM  
• Strong correlation between obesity, stress and milk hormonal profile. |
6.5.5 Impact of maternal originated milk-borne glucocorticoids on infant metabolism.

Glucocorticoids are stress regulating hormones known to influence several physiological processes including metabolic, inflammatory and behavioural processes (534). They play an important role in regulating glucose availability via gluconeogenesis. However, the role of cortisol in promoting lipolysis and lipogenesis is controversial, with most studies suggesting that it as a potential stimulator of lipolysis (556). Glucocorticoids also influence the metabolism of fatty acids (FAs), amino acids (AAs), and carbohydrates. Although, the relationship between cortisol secretion and metabolism is complex, and closely linked to the maintenance of circulatory levels (421,557). Infant ingesting HM glucocorticoids, which are possibly transferred from maternal plasma could enter infant’s plasma and brain, causing physiological and behavioural alterations (201). Cortisol was first discovered as a metabolic hormone, with its most important role being the regulation of stress. Cortisol and cortisone ingested by an infant via HM, are implicated in the development of a child’s behaviour and metabolic programming, however, further studies are required in this regard.

The following table outlines a proposed study aimed at assessing the impact of milk-borne glucocorticoids on infant metabolism.
Table 6-5: Effect of human milk ingested cortisol and cortisone on infant metabolism

<table>
<thead>
<tr>
<th>Background</th>
<th>Cortisol is an important metabolic hormone, and has been suggested to maintain the delicate balance of hormonal equilibrium. Knowledge about the metabolic effect is mainly based upon the impact of prenatal exposure of glucocorticoids in predicting the birth weight and weight pattern in later life. However, much less is known about the impact of milk-borne glucocorticoids on infants’ metabolism.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Question</td>
<td>If maternal originated milk-borne glucocorticoids affect the metabolism of carbohydrates, fats and amino acids in infants?</td>
</tr>
<tr>
<td>Aim</td>
<td>To assess the effect of maternal originated milk ingested glucocorticoids on the infant metabolism.</td>
</tr>
<tr>
<td>Design</td>
<td>Collect HM, plasma, saliva, and urine samples from healthy lactating mothers and infants.</td>
</tr>
</tbody>
</table>
| Measures | - Maternal social and biological characteristics  
- Body composition of both mother and infant at the time of milk collection  
- Plasma, saliva, milk and urine glucocorticoid concentrations (cortisol and cortisone) in both mother and infant samples  
- Blood lipid profile of plasma and milk – total cholesterol, LDL, HDL, TAG  
- Insulin sensitivity and glycaemic control in plasma – HOMR-IR, HbA1C  
- Serum glucose, insulin, phosphofructokinase (PFK)  
- Assay the activity of FAs dehydrogenase and fattyacyl-CoA carboxylase in infant plasma  
- PEP carboxylase  
- Available milk energy (AME) HM |
| Anticipated Results | - Expected to influence infant metabolism  
- Elevated cortisol levels in milk may increase urine glucocorticoids |
6.6 Summary

Research in developmental origin shows that better nutrition from pregnancy through to early childhood is a critical period for improving preventive interventions in a child and HM is the most natural source of nutrition for new born infants (5,299). Milk composition is dynamic and changes its composition to variety of maternal and infant related factors as shown in Figure 6-2. This thesis highlights the role of several maternal and infant related factors in dictating the glucocorticoid composition of HM. This research emphasises the importance of highly variable milk composition and its impact on infant development. Data presented here bridges the gap between possible factors altering HM hormonal composition. It provides a new knowledge on how it may vary within an individual, between mothers and with time. More information and mechanistic explanation are required to link specific health outcomes for infants ingesting milk-borne additional hormones. These differences in milk composition may influence infants differently, emphasising the importance of maternal environment for lactating mothers. This research has also given rise to further questions on the long-term effect of breast feeding and ingestion of milk-borne hormones, on infants and thus opened up an avenue for future research in this field.

![Figure 6-2: Factors affecting the composition of human milk.](image-url)
Chapter 7: Appendix
7.1 Protocol for quantification of steroid hormones in human milk

7.1.1 Internal standard

Internal standard (IS) stocks in MeOH was prepared by adding 30 µl of Cortisol d4, 30µl of Corticosterone d8 (1mg/ml stock solution in MeOH), 10µl of Testosterone d3 and 10µl of Estradiol d3 (1mg/ml stock solution in MeOH) was added and volume was made up by adding MeOH. Internal standard was kept at 4°C for up to three months and then diluted on the day of the experiment 1/100 with H2O (10µl of internal standard+ 990µl of H2O).

7.1.2 Standard curve stock solution

A stock solution of standard curve was prepared by mixing were prepared by adding 20 µl of 1mg/ml of Cortisol, 20µl of 1mg/ml of Progesterone, 10 µl of 11-Deoxycortisol, 10µl of 1mg/ml of Corticosterone, 10µl of 1mg/ml of Cortisone, 20 µl of 1mg/ml of Testosterone (used 1:10 dilution of testosterone stock) , 20 µl of 1mg/ml of Estradiol (used 1:10 dilution of estradiol stock) and add methanol, to obtain a final concentration of 10µg/ml of Cortisol, 5000ng/ml of 11-Deoxycortisol, Corticosterone and Cortisone, and 1000ng/ml of Testosterone in methanol. Standard curve from A-I was prepared as shown in Table 7-1..All these standards were kept in refrigerator at 4°C and were diluted on the day of the experiment by adding 10 µl of each standard and 990 µl of stripped human plasma.
Table 7-1: Standard curve for steroid hormones in human milk

<table>
<thead>
<tr>
<th></th>
<th>Cortisol, Progesterone,</th>
<th>Corticosterone, Cortisone, 11-Deoxycortisol</th>
<th>Testosterone, Estradiol</th>
<th>Methanol Stock Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc in water ng/ml</td>
<td>Conc in MeOH ng/ml</td>
<td>Conc in MeOH ng/ml</td>
<td>Conc in water pg/ml</td>
</tr>
<tr>
<td>A</td>
<td>0.2</td>
<td>20</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>0.5</td>
<td>50</td>
<td>0.25</td>
<td>25</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>100</td>
<td>0.5</td>
<td>50</td>
</tr>
<tr>
<td>D</td>
<td>2.5</td>
<td>250</td>
<td>1.25</td>
<td>125</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>500</td>
<td>2.5</td>
<td>250</td>
</tr>
<tr>
<td>F</td>
<td>12.5</td>
<td>1250</td>
<td>6.25</td>
<td>625</td>
</tr>
<tr>
<td>G</td>
<td>25</td>
<td>2500</td>
<td>12.5</td>
<td>1250</td>
</tr>
<tr>
<td>H</td>
<td>50</td>
<td>5000</td>
<td>25</td>
<td>2500</td>
</tr>
<tr>
<td>I</td>
<td>100</td>
<td>10000</td>
<td>50</td>
<td>5000</td>
</tr>
<tr>
<td>From 1 mg/ml stocks</td>
<td>20µl x 2</td>
<td>10µl x 3</td>
<td>20µl x 2</td>
<td></td>
</tr>
</tbody>
</table>
7.1.3 Sample preparation

Samples were first thawed and kept on heating block at 37°C for 7-10 mins and vortexed for roughly 10s.

1. The samples were prepared by liquid extraction the following way:
   - Blank without IS : 200µl of H2O + 100µl of H2O
   - Blank with IS : 200µl of H2O + 100µl of IS in H2O
   - Standards, quality controls and samples : 200µl + 100µl of IS in H2O

2. 1 ml of Ethyl Acetate was added to each tube and the mixtures were vortexed for 40s.

3. All samples were centrifuged for 3 min in Hetovac.

4. After settling, the upper organic phase were transferred to a clean tube and dried down in Savant vacuum concentrator for 1 hour and 20 minutes at 0.08 pressure (on auto run).

5. Dried residues were reconstituted in 80µl of 50/50% MeOH/H2O mixture and transferred to injection vials.

6. 12µl were injected on TSQ Quantum AM.

7.1.4 HPLC and Mass Spectrometry

The HPLC Mass Spectrometer system consists of a Surveyor MS pump and auto sampler followed by an Ion Max APCI source on a Finnigan TSQ Quantum Ultra AM triple quadruple mass spectrometer, all controlled by Finnigan Xcaliber software. (Thermo Electron Corporation, San Jose, CA.)

7.1.4.1 HPLC Conditions

- Column Specifications: Phenomenex Luna 2.5u C18(2)-HST 100 x 3mm 2.5 micron
- Column temperature: 40oC
- Flow rate: 400µl/min
- Mobile phase composition: 60/40% MeOH/H2O isocratic elution
- Run time: 10.0 min
7.1.4.2 Mass spectrometry

Table 7-2: Tune parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
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<tr>
<td>Discharge Current</td>
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<tr>
<td>Vaporizer Temp</td>
<td>420</td>
</tr>
<tr>
<td>Sheath Gas Pressure</td>
<td>20</td>
</tr>
<tr>
<td>Ion Sweep Gas Pressure</td>
<td>2</td>
</tr>
<tr>
<td>Auxiliary Gas Pressure</td>
<td>10</td>
</tr>
<tr>
<td>Capillary temperature</td>
<td>350</td>
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<tr>
<td>Tube Lens Offset</td>
<td>107</td>
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<tr>
<td>Skimmer Offset</td>
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</tr>
</tbody>
</table>

Table 7-3: Selected Reaction Monitoring (SRM) for Cortisone and Cortisol

<table>
<thead>
<tr>
<th>Steroid</th>
<th>SRMs</th>
<th>Retention time Rt (min)</th>
<th>Ionization Mode</th>
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</thead>
<tbody>
<tr>
<td>Cortisone</td>
<td>361.1 - 163.1</td>
<td>3.14</td>
<td>Positive</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>347.2 - 109.1</td>
<td>3.46</td>
<td>Positive</td>
</tr>
<tr>
<td>Cortisol</td>
<td>363.1 - 121.1</td>
<td>2.89</td>
<td>Positive</td>
</tr>
<tr>
<td>Cortisol-d4</td>
<td>367.1 - 121.2</td>
<td>2.89</td>
<td>Positive</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>347.2 - 121.2</td>
<td>4.90</td>
<td>Positive</td>
</tr>
<tr>
<td>Corticosterone-d8</td>
<td>355.2 - 125.2</td>
<td>3.43</td>
<td>Positive</td>
</tr>
<tr>
<td>Testosterone</td>
<td>289.1 - 97.1</td>
<td>4.8</td>
<td>Positive</td>
</tr>
<tr>
<td>Testosterone-d3</td>
<td>292.2 - 97.1</td>
<td>4.83</td>
<td>Positive</td>
</tr>
<tr>
<td>Progesterone</td>
<td>315.1-</td>
<td>8.22</td>
<td>Positive</td>
</tr>
<tr>
<td>Estradiol</td>
<td>255.1-159.2</td>
<td>6.10</td>
<td>Positive</td>
</tr>
<tr>
<td>Estradiol d3</td>
<td>258.2-159.2</td>
<td>6.10</td>
<td>Positive</td>
</tr>
</tbody>
</table>
7.2 Pundir et al. 2016

The following section contains an unaltered reproduction of the article “HM glucocorticoids alterations”, published in the *Journal of mammary gland and neoplasia*. 
Variation of Human Milk Glucocorticoids over 24 hour Period

Shilpa Pandir1 · Clare R. Wall1 · Cameron J. Mitchell1 · Eric B. Thorstensen1 · Ching T. Lai2 · Donna T. Geddes3 · David Cameron-Smith1

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Abstract Human milk (HM) contains a complex array of hormones, including members of the glucocorticoid family. The predominant glucocorticoids, cortisol and cortisone may influence the growth and behaviour of the breastfed infant. However, little is understood of the factors regulating the levels of these hormones within HM. The aim of the study was to examine HM cortisol and cortisone concentrations, measured in samples collected at each feed during a 24 hour period. Twenty three exclusively breastfeeding mothers collected milk, prior to and after each breastfeeding session over 24 hour period at 3.2(±0.60) months. HM cortisol and cortisone levels were measured using high pressure liquid chromatography mass spectrometry. Cortisone was the predominant glucocorticoid (5.40 ng/ml), and cortisol was detected in all samples (1.62 ng/ml). A positive correlation was found between cortisone and cortisol ($r = 0.61, \gamma = 1.93 \pm 0.24, p < 0.0001$). Cortisol and cortisone concentrations were significantly higher in feeds in the morning (2.97 ng/ml and 4.88 ng/ml), compared to afternoon (1.20 ng/ml and 3.54 ng/ml), evening (0.69 ng/ml and 2.13 ng/ml) and night (1.59 and 3.27 ng/ml). No difference was found between glucocorticoids levels of the milk expressed for collection either before or immediately after the breastfeed, or between milk collected from the left or right breast. This study shows that HM glucocorticoid concentrations exhibit a 24 hour pattern, with highest peak levels in the early morning, reflecting the circadian pattern as previously reported in plasma. Thus, HM glucocorticoid concentrations are likely to reflect those in the maternal circulation.

Keywords Glucocorticoids · Human milk · Cortisol · Cortisone · High-performance liquid chromatography

Introduction

Human milk (HM) is a complex and dynamic biological fluid, which is adaptively altered throughout lactation to tailor to the individual and unique needs of a developing infant [1]. HM enables nutrient transfer between mother and infant, with compositional regulation of the macro- and micro-nutrients influencing infant growth and development [2, 3]. Much recent research has highlighted the complexity of HM, revealing the extent to which developmental needs of the growing infant is paired with HM composition, and the significant knowledge gaps that exist [4]. In addition to nutritive components, HM also contains non-nutritive bioactive ingredients, including the glucocorticoid family of steroid hormones [5]. Cortisol and cortisone are typically the predominant glucocorticoids found in the maternal circulation [5, 6] and in HM [7]. The actions of circulating glucocorticoids are diverse, with a critical role in stress and disease responses [6]. Beyond this, glucocorticoids also mediate aspects of physiological adaptations required for mammary function, including contributing to mammary growth, secretory differentiation and activation, maintenance of lactation and milk ejection [5, 8–10] in lactating women.
The functions of HM derived glucocorticoids are less well understood, although they may exert an influence in shaping the infant’s behaviour, physiology and metabolic programming [7, 11, 12]. Higher milk cortisol levels have been associated with greater nervous and less confident behaviour in both human infants [7] and rhesus macaques [13]. In contrast data demonstrates beneficial actions of milk cortisol with higher levels being correlated with a higher score on the autonomic stability on the Neonatal Behavioural Assessment Scale [14]. Further, in the rhesus macaque elevated milk cortisol at peak lactation (3–4 months lactation) is also positively associated with infant weight gain [2]. More recently another study showed on cortisol in HM showed its role towards early metabolic programming, and suggested its importance in providing protection against obesity [12]. Together, these findings suggest that milk glucocorticoids are bioactive signalling agents by which maternal physiology may modulate offspring growth and behavioural development. Despite the potential significance of HM glucocorticoids few studies have examined factors influencing their abundance. One key aspect contributing to the complexity of HM is the circadian variation in its composition [15].

HM fat has exhibited significant 24 h variation, with total fat content varying at different times of the day. Kent et al. [18] found higher lipid concentrations during the day and evening, compared to the milk expressed during early morning and night. However these changes could be higher degree of fullness of the breast, rather than circadian variations [16–18]. Although many other components of milk (including amino acids, melatonin and trace elements have shown temporal correlation with maternal rhythms [15, 19]. A recent study has identified the presence of circadian regulation of glucocorticoids in HM. This study demonstrates the presence of peak concentrations of cortisol and cortisone around 0700 h, declining through the remainder of the day [20]. The purpose of the current study is to further the analysis of concentrations of cortisol and cortisone over a 24 h period, in HM obtained from exclusively breastfeeding women. This study aimed to additionally identify whether glucocorticoids differed over the duration of a feed, with analysis in HM donated immediately before and after infant feeding, across both breasts.

We hypothesised that the described circadian regulation of blood glucocorticoid hormones would be reflected in the levels measured in HM, with concentrations falling from pre- to post-feeding samples.

Methods

Study Design and Subjects

Mothers were recruited through the Western Australian branch of the Australian Breastfeeding Association and through the Child and Adolescent Community Health Nurses of the Australian Region. Written informed consent was obtained from the participants. The study protocol was approved by the Human Research Ethics Committee of the University of Western Australia.

Milk Collection

All participating mothers were asked to collect the milk samples (1–2 ml) either by using manual expression or by using an electric breast pump into 5 ml polypropylene vials (Disposable Products Pty Ltd., Australia). Milk samples were taken (immediately before feed and after feed) each breastfeeding session, from each breast at each feed over an entire 24 h period. All of the samples were initially stored in the mother’s home freezer, prior to transport to the laboratory where they were stored at −20 °C. Samples were shipped to Auckland (New Zealand) for subsequent glucocorticoid analysis on dry ice and were kept at 80 °C frozen until analysis.

Milk samples were divided into four intervals of six hours as described by Khan et al. [17] and were classified as morning (0400–1000 h), afternoon (1001–1600 h), evening (1601–2200 h) and night (2201–0400 h). These time periods were used in subsequent statistical analysis.

Sample Preparation

Milk steroids were measured by using liquid chromatography mass spectrometry. The internal standard consisted of 12 mg/mL cortisol d4, 60 mg/mL corticosterone d8, prepared in water. Preparation was initiated by adding 100 μl of internal standard to 100 μl HM. All milk samples were heated at 37 °C for 10 min and vortexed for 20 s before adding 100 μl of sample to glass tubes with internal standards. Steroids were then extracted using 1 ml ethyl acetate (Merek, Germany); the top organic layer was removed into a separate tube and then vacuum dried (Savant, SC230EX, Thermo Scientific, United States) for ~2 h. The dried residues were reconstituted with 80 μl of 50% methanol (Merek, Germany) /water and transferred to HPLC injector vials. All samples were run in duplicates and average values are reported.

Liquid Chromatography- Tandem Mass Spectrometry

The HPLC Mass Spectrometer (MS) used a Surveyor MS pump and auto sampler followed by an Ion Max APCl source on a Finnigan TS Quant Ultra AM triple quadrupole mass spectrometer, all controlled by Finnigan Xcalibur software (Thermo Electron Corporation, San Jose, CA). The mobile phase was a methanol-water gradient starting at 50:50(v/v) (peaking at 80:20 before returning back to 50:50 at 500 μl/
min. The chromatography was performed at 40 °C. The instrument was set up in selective reaction monitoring (SRM) mode with the following mass transitions: m/z 363.1 → 121 for cortisol, 367.1 → 163 for cortisone, 367.1 → 121 for cortisol d4 and 355.2 → 125.2 for corticosterone d8. Dissociation voltage was 24 V and the collision gas (Argon) was set at 1.2 mTorr for all steroids.

Steroid concentrations were calculated from a standard curve generated for each steroid relative to its internal standard (cortisol d4 for cortisol and corticosterone d8 for corticosterone) from the injection of standards; cortisol 0.05–100 ng/ml, corticosterone 0.025–50 ng/ml, diluted into stripped human plasma and extracted in the same way as the samples for each assay.

**Statistical Analysis**

Linear mixed models were used to investigate differences between glucocorticoid concentrations over the 24 h period with the inclusion of time of the day, and pre and post breastfed as repeated measures fixed factors and infant gender as a non-repeated fixed factor and maternal BMI and parity as covariates. The 24 h difference between pre and post feed samples were investigated using Univariate analysis and its effect at each time point was tested using student T-test. The 24 h differences between right and left breast milk samples were also tested using a students paired T-test. Between group differences were assessed by using Stata post hoc method. Pearson correlation was used to analyse the association between the calculated average value of cortisol and cortisone over the 24 h period. Univariate relationships between glucocorticoids and milk intake variables such as breastfed volume, frequency of breastfeeding were performed using linear regression. Statistical analysis was performed using SPSS (IBM SPSS Statistical 2 pg Ltd, United States). All the data are reported in text and tables as mean ± (SD) and mean ± (SE) in figures. P values less than 0.05 were considered statistically significant. Lattice plot was created using R software version 2.15.2 and GraphPad Prism version 7.0 was used to create all other graphs.

**Results**

**Participant Characteristics**

A total of 502 human milk samples from twenty-three mothers were measured and analysed for glucocorticoid concentrations. Table 1 shows the demographic characteristics of mother and infant. Mean cortisol and cortisone concentrations for the 24 h period were 1.63 (0.75) ng/ml and 3.42 (1.18) ng/ml, respectively; these are presented in Fig. 1.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Subject and sample characteristics (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mother characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Age in years, mean (SD)</td>
<td>31 (8.14)</td>
</tr>
<tr>
<td>BMI before pregnancy, mean (SD)</td>
<td>31 (3.92)</td>
</tr>
<tr>
<td>Parity</td>
<td>1.3 (0.57)</td>
</tr>
<tr>
<td><strong>Maternal status</strong></td>
<td></td>
</tr>
<tr>
<td>Married n (%)</td>
<td>22 (95.6)</td>
</tr>
<tr>
<td>Unmarried n (%)</td>
<td>1 (4.3)</td>
</tr>
<tr>
<td><strong>Maternal BMI</strong></td>
<td></td>
</tr>
<tr>
<td>Normal weight n (%)</td>
<td>13 (56.5)</td>
</tr>
<tr>
<td>Overweight n (%)</td>
<td>2 (8.7)</td>
</tr>
<tr>
<td>Underweight n (%)</td>
<td>7 (30.4)</td>
</tr>
<tr>
<td><strong>Infant characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Male n (%)</td>
<td>18 (78.2)</td>
</tr>
<tr>
<td>Female n (%)</td>
<td>5 (21.7)</td>
</tr>
</tbody>
</table>

**Temporal Changes in HM Glucocorticoid Composition**

HM glucocorticoid (cortisol and cortisone) concentration demonstrated a circadian response over the analysis period. Post-hoc analysis identified that 24 h averaged morning samples, cortisol was significantly higher than afternoon (p < 0.001), evening (p < 0.001) and night (p = 0.001) samples. Furthermore, the evening cortisol level was significantly lower than afternoon (p = 0.03) and night (p = 0.02) (Fig. 2a). Similarly, a significant difference was observed in the cortisone levels, with morning cortisone higher than afternoon (p = 0.01), evening (p < 0.001) and night (p < 0.001), whereas evening was significantly lower than afternoon cortisone levels (p = 0.001) (Fig. 2b).

**Effect of Breastfeeding on HM Glucocorticoid Composition**

The 24 h HM cortisol and cortisone concentrations were not significantly different between pre and post feed samples (cortisol: p = 0.97; cortisone: p = 0.46). The cortisol and cortisone did not differ significantly between pre and post breastfed samples at any time points, morning (p = 0.61 and 0.26), afternoon (p = 0.49 and 0.28), evening (p = 0.50 and 0.92) and night (p = 0.58 and 0.86). There was no significant correlation between the 24 h pre and post feed glucocorticoid levels and breastfed volume (cortisol) (pre feed: p = 0.94, cortisol post feed: p = 0.83, cortisone pre feed: p = 0.88, cortisone post feed: p = 0.86). Furthermore, each infant had 15 (3.29)
Fig. 1 1H34 glucocorticoids (cortisol and corticosterone) over 24 h in individual mothers (n = 23). Each box of the lattice plot indicates a single mother milk glucocorticoid profile with symbol (○) representing cortisol and (△) representing corticosterone. Each solid line indicates cortisol and dashed line corticosterone. Time points are categorised as morning (0600–1000 h), afternoon (1000–1600 h), evening (1600–2200 h), and night (2200–0600 h).

Fig. 2 1H34 glucocorticoids (a) and Corticosterone (b) concentrations throughout the period of 24 h at four different time points. Time points are categorised as morning (0600–1000 h), afternoon (1001–1600 h), evening (1601–2200 h) and night (2201–0600 h). * Represent significant difference from morning milk cortisol levels with remaining time points. # Represent significant difference from evening milk cortisol levels with remaining time points. Error bars represent SEM (standard error of mean). Differences over time are expressed as * p < 0.01 | ** p < 0.001, and ### p < 0.001.
breastfeeds during 24 h period, however no significant difference was found between the frequency of breastfeeding and glucocorticoid levels (cortisol \( p = 0.21 \) and cortisone \( p = 0.19 \)).

The HM cortisol concentration did not differ significantly between male and female infants at any time-point, morning \( (p = 0.12) \), afternoon \( (p = 0.27) \), evening \( (p = 0.76) \) and night \( (p = 0.42) \). Similarly, the HM cortisone concentration did not differ significantly between male and female infants at morning \( (p = 0.07) \), afternoon \( (p = 0.10) \), evening \( (p = 0.82) \) or night \( (p = 0.61) \). Furthermore, no significant interaction effect was seen between pre and post breastfeed samples, infant gender, parity, maternal BMI and time of the day.

### Between-Breast Difference on HM Glucocorticoid Composition

Overall the 24 h average concentration of HM glucocorticoid (cortisol and cortisone) for the left and right breast was not significantly different (cortisol: \( p = 0.37 \); cortisone: \( p = 0.15 \)). The 24 h average cortisol concentration of milk from left breast was 1.73 (0.70) ng/ml and right was 2.0 (1.01) ng/ml (Fig. 3a). The cortisone 24 h average concentration from the left breast was 3.83 (1.27) ng/ml and right breast 3.56 (1.35) ng/ml (Fig. 3b).

### Relationships between HM Cortisol and Cortisone

The average HM cortisol concentration over 24 h was significantly lower than that of cortisone \( (p < 0.001) \). A significant positive correlation was observed between cortisol and cortisone concentrations in HM \( (r = 0.606, p < 0.0005) \) (Fig. 4). Furthermore, a moderate correlation between two hormones was observed in the afternoon \( (r = 0.75, p < 0.001) \) and night \( (r = 0.62, p < 0.01) \), whereas a weak correlation was found in the evening \( (r = 0.46, p < 0.01) \) and morning \( (r = 0.20, p = 0.34) \).

### Discussion

Glucocorticoids are components of the complex non-nutritive hormonal fraction of HM. Currently there is limited data on the regulation of glucocorticoids in HM, and the circadian pattern of these hormones. Thus this study examined the glucocorticoid concentration of HM samples collected from a cohort of women who were exclusively breast feeding throughout the course of a single 24 h period. Both cortisol \( (1.60 \pm 0.71 \text{ ng/ml}) \) and cortisone \( (3.40 \pm 1.38 \text{ ng/ml}) \), were present in all analysed samples. The presence of these glucocorticoids, exhibited a pronounced 24 h pattern, characterized by the rapid increase in the cortisol and cortisone levels in the early morning hours followed by the gradual fall throughout the day. This mirrors the well described circadian pattern of glucocorticoids found in the maternal circulation and reported in lactating women [21]. Further we found no impact of sampling before or after feeding or between breasts indicating time of sampling to be the most critical factor in investigating glucocorticoids in HM.

In humans nearly all bodily functions exhibit circadian rhythms and glucocorticoid displays one of the most distinct circadian rhythms. The circadian rhythm of glucocorticoid release is regulated by the endogenous biological clock, the suprachiasmatic nucleus in the anterior hypothalamus exerts control over the hypothalamic-pituitary-adrenal (HPA) axis response and thus dictates the pulsatile release of cortisol [22]. Typically plasma cortisol concentrations demonstrate circadian rhythms, with elevated levels in the early morning followed by a gradual decline to around midnight to complete the 24 h
cycle, throughout the day [5, 23–25]. This circadian pattern of secretion has been speculated to be an important contributor to the effective actions of glucocorticoids in immunity, growth, and metabolism. Although precise mechanisms are not well understood [23–25]. Our data demonstrates replication of this typical circadian pattern, with both cortisol and cortisone concentrations peaking in the early morning before progressively declining throughout the day to reach the lowest point in the evening. These findings are consistent with a recent study that examined the diurnal rhythm patterns of HM in 10 women, 4 weeks postpartum [20]. In this study glucocorticoids levels were shown to peak at 07:00 h when milk was collected 2 hourly for a complete 24 h, mirroring saliva analysis that was conducted at the same time [20]. Thus the rhythmic behavior of HM glucocorticoid concentrations found in the current study is likely to be reflective of the concentrations in the maternal circulation. This then reinforces the past observations that maternal physiological environment plays a critical role in dictating the hormonal composition of the human milk [1, 2].

There is currently limited insight into how glucocorticoids are transferred from the maternal circulation to the synthesised HM. Animal studies have shown that cortisol can passively diffuse across the cell membrane and maintain a dynamic equilibrium between milk and plasma cortisol levels. In a study of dairy cows, milk cortisol levels were used as a reliable indicator of stress response, because a rise in plasma cortisol was reflected in milk [26]. However, in the current study, glucocorticoids were measured in the milk samples and not in maternal or infant plasma samples, so direct comparison between milk and plasma glucocorticoid concentration was not possible.

As HM composition is dynamic and constantly changing, it is therefore important to further investigate the milk glucocorticoids content with respect to timing of milk secretion (this study measured in milk obtained before and after the infant feed) and the frequency of feeding. Many studies have reported the effect of breastfeeding on the milk composition by analysis of pre- and post-fed samples [1, 7, 27, 28]. For instance, milk expressed prior to feed has a lower lipid content, when breast is full, compared to immediately after feed, when breast is empty [16, 18]. Interestingly we found that milk removal from the breast by the breastfeeding infant had no influence on glucocorticoid profiles. This is in contrast to what might be expected with the positive effect of lactation in regulating the HPA axis response to both physical and psychological stressful state [29]. Breastfeeding by the newborn infant tends to reduce plasma cortisol levels and increase both oxytocin and prolactin release [30, 31]. Indeed, this is consistent with the calming effect of oxytocin released into the maternal circulation at milk ejection which occurs multiple times throughout a breastfeed [32]. Further, the results from the current study also demonstrate that milk glucocorticoid levels were independent of breastfeeding frequency and feed volume, indicating no difference between frequent and non-frequent breastfeeders; despite the considerable variation among the frequency of breastfeeding between mother and infant dyad.

It is well established that milk output from each breasts may not be equal with output from one breast greater than other; however milk composition between breasts remains independent of the breast [18, 27]. In this study, no differences in the glucocorticoid profiles of the milk were found between the left or right breast. These results are comparable to the evidence that the macro-nutrient composition of the milk including protein, lactose and leptin have also been shown not to differ between breasts [29, 33]. Unlike macronutrients, HM volume is highly sensitive to maternal condition, and its hormonal composition is associated with maternal circulation [13, 34, 35]. Hence, the sample collection techniques, sample timing and volume are the critical factors, which must be characterized pertaining to maternal health and its environment. However, lack of standardised sample collection technique reflects the variability in milk macronutrients, and thus it is challenging for researchers to collect a representative milk sample. Clearly, no single sampling technique could be ideal but our study may enable the selection of an appropriate time for collecting milk samples, for hormonal analysis in future lactation studies.

In humans, two iso-enzymes of 11-β-hydroxysteroid dehydrogenase catalyse the interconversion of biologically active cortisol into its inactive metabolite, cortisone. We found a moderate positive correlation between the 24 h cortisol and cortisone concentration (r = 0.66), and found strong positive relationship at afternoon (r = 0.75) and night (r = 0.62). Many plasma and serum based studies have suggested closely correlated dynamics between the two hormones under normal physiological conditions, and any alteration in this equilibrium is usually associated with adrenal insufficiency or diseases [36, 37]. Equilibrium between these hormones might be involved in the regulation of glucocorticoids levels in milk as well. Chen et al. [36] identified maternal stress, long labour and pruritus as the predictor of delayed onset of lactation. Cortisol is a classical regulator of stress and is a permissive hormone which plays an important role in milk secretion and onset of lactation [39]. However, it would be interesting to further identify, the role HM cortisol plays as a predictor of lack of delayed onset of lactation in lactating mother with chronic stress or depression.

Recent research suggests that HM composition differs depending upon infant gender, and tends to favor greater nutritional investment for male offspring [40]. Additionally, mothers of male offspring had higher cortisol in their milk compared to the mothers of female offspring [41]. One limitation of the current sample cohort was that there were few female infants, thus it is not possible to report with any confidence on the presence of any potential gender differences in
the glucocorticoids. However, previous studies have shown that infant sex has very little effect on the glucocorticoid levels in plasma and serum [42, 43]. Secondly, the absence of maternal plasma samples collected at the time of breastfeeding makes it impossible to establish that the relationship in HM glucocorticoids correlates with maternal concentrations. As such, additional analysis is required, including the analysis of potentially important variables including parity, maternal social and environmental circumstances.

One key feature of the current study was the measurement of glucocorticoids in milk using liquid chromatography mass spectrometry (LC-MS/MS). In our study, milk cortisol concentrations ranged between 0.1-11 ng/ml and cortisone was between 1.7-14.0 ng/ml, which was either lower or similar to previous reports for both milk (0-218 ng/ml) and plasma (50-250 ng/ml) [44]. Variation between present and previous studies could be due to the differences in detection methodology, particularly the use of immunoenassay analysis, which lacks specificity of analysis due to cross-reactivity with other related glucocorticoids [3, 17, 45, 46]. Compared to immunoenassay, the method used in current study has both a simplified sample preparation workflow and the ability to quantify both cortisol and cortisone simultaneously [47, 48].

This study was further strengthened by sample homogeneity, at the time of milk collection infants' age were within the narrow range of 5.2 (1.2) months, which provides a critical measurement of the duration of maternal lactation, further affecting the milk composition. Therefore, the mothers recruited in the current study, produced a homogenous set of milk samples with minimal variation.

In conclusion, the present study demonstrates the fluctuating glucocorticoid profile of human milk samples throughout the day, closely aligns with the circadian rhythm of plasma cortisol that has been previously reported [23, 49]. The milk glucocorticoids levels were highest during morning and lowest prior to sleep; however further studies are required to elucidate the mechanistic and functional consequences of the milk glucocorticoids on infant growth and development.

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Compliance with Ethical Standards

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Conflict of Interest CBW is employed through, Faculty of Medical and Health Sciences, University of Auckland. CIM, EBT and DCS are employed through Liggins Institute, University of Auckland, CIL, and DC received salary from an unrestricted research grant from Medica AB, administrated by University of Western Australia.

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7.3 Pundir et al. 2016

The following section contains an unaltered reproduction of the article "Impact of Preterm Birth on Glucocorticoid Variability in Human Milk"
Impact of Preterm Birth on Glucocorticoid Variability in Human Milk

Shilka Pundir, MSc1, Cameron J. Mitchell, PhD1,2, Eric B. Thorstensen, BSc1, Clare R. Wall, PhD1,2, Sharon L. Perrella, PhD1, Donna T. Geddes, PhD1, and David Cameron-Smith, PhD1,4

Abstract

Background: Preterm birth is a stressful event for both the mother and infant. Whereas the initiation of breastfeeding is important for preterm infant health, little is known of the glucocorticoid hormones (cortisol and cortisone) in human milk following preterm birth.

Research aim: The aim of this study was to investigate the relationship between human milk glucocorticoid concentrations and preterm birth.

Methods: Human milk was sampled weekly for up to 6 weeks from 22 women who delivered a preterm infant at 20 to 32 weeks' gestation. Human milk was analyzed for total and free cortisol and cortisone concentrations using liquid chromatography-tandem mass spectrometry.

Results: Milk sampled from mothers of preterm infants had more cortisone than cortisol (p < 0.001), with a strong correlation between both hormones (p = 0.001, r = 0.89). The cortisone was significantly higher in the milk of mothers who delivered infants after 30 weeks compared with those who delivered before 30 weeks of gestation (p = 0.02). Glucocorticoid concentrations did not change over the sampling time (weeks 1 to 6 postpartum) and did not differ by infant gender.

Conclusion: Glucocorticoids were present in all milk samples following preterm birth. Cortisone concentration tended to be higher in those who delivered after 30 weeks' gestation but did not increase further over the weeks following birth.

Keywords
breastfeeding, hormones, human milk, maternal health, prematurity

Background

Preterm birth affects more than 15 million infants worldwide (Filmerowicz et al., 2013). These infants are at significantly increased risk of acute adverse health outcomes, with the persistence of lifelong metabolic health complications (Kostialis, Oudijk, Nijman, Moi, & Pijper, 2016). The early intake of human milk (HM) offers significant benefits to the health of a preterm infant, with a reduced risk of serious acute events such as sepsis and necrotizing enterocolitis (Henneman & Carroll, 2014; Patel et al., 2013). It also improves long-term health with reduced rates of rehospitalization and evidence of protection against metabolic diseases, including diabetes (Moxon & Williams, 2013; Underwood, 2013; Voel et al., 2006).

There are several unique compositional differences in the HM produced by mothers of preterm infants compared with HM produced following term birth, the HM produced after a preterm birth is higher in protein, long chain fatty acids, sodium, and chloride (Gidewicz et al., 2014; Zhang, Adelman, Rai, Boetescher, & Lüdemard, 2013). Beyond simply supplying nutrients, HM also contains a complex array of non-nutritive components. Given the influences of birth gestation and postpartum time on the nutritive composition of HM (Ballard & Morrow, 2013), it is hypothesized that non-nutritive components may also be altered by degree of infant prematurity.

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To date, there are very limited data on the presence of biologically active hormones, including the glucocorticoids (GCs, i.e., cortisol and cortisone), in the milk of mothers who have delivered preterm infants. Based on work by Davis and colleagues (2007) and Greer, Davis, Sandman, and Glynn (2013), one could infer that GCs transfer from maternal plasma into the milk and, hence, may be influenced by maternal stress. Given that preterm birth is a time of increased maternal stress and anxiety, during and after discharge from the neonatal intensive care unit (Pichl-Matulas et al., 2016), it is hypothesized that this would impact GC hormone concentrations in the secreted HM.

Currently, there is limited understanding of the psychological and biological importance of GCs in HM. Of the available data, milk-borne GCs are known to influence infant temperament in both animal and human infants (Davis et al., 2007; Grey et al., 2013). This may, however, be gender specific, as elevated milk GCs were found to be associated with more confident temperament in mammalian infants but not in females (Sulivan, Hindie, Mercede, & Capitano, 2011). Furthermore, the impact of GCs may extend to influencing infant growth where animal studies demonstrate a relationship between increased levels of milk cortisol and greater infant growth during early and established lactation (Dauter et al., 2013; Hindie, et al., 2015). To date, one study has demonstrated that very preterm birth (birth at 28 to < 32 weeks’ gestation) was associated with reduced HM GC concentrations, relative to term birth (van der Veen et al., 2016). However, this study reported only total GCs. It is unknown whether the GCs in HM exist in two forms—free (active) form or bound (inactive) form, which is complexed to either corticosteroid-binding globulin or albumin (Peragimvros, Ray, & Trainer, 2012). Thus, the present study aimed to examine the impact of birth gestation on GC concentration in HM, the ratio of free to total GCs, and subsequent changes during the postnatal period.

Method

Design

The authors conducted a longitudinal, two-group observational study designed to evaluate the impact of preterm birth severity on milk GC concentrations. The Women and Newborn Health Service and the University of Western Australia ethics committee board reviewed and approved the study protocol with registration number 1749/EW.

Setting

The study was conducted in a large neonatal intensive care unit at Special Care Nursery of King Edward Memorial Hospital, Perth, Western Australia, the sole tertiary obstetric hospital for the state (Perrella et al., 2015). The public government-funded hospital provides services for women from all socioeconomic groups.

Key Messages

- Whereas the initiation of breastfeeding is important for preterm infant health, little is known of the glucocorticoid hormones (cortisol and cortisone) in human milk following preterm birth.
- Unlike plasma, human milk demonstrated a higher concentration of cortisone, and the majority of glucocorticoids in milk were in the free form (biologically active) with a strong correlation between cortisol and cortisone.
- Cortisone was lower in the mothers who delivered before 30 weeks of gestation.
- This study provides further insight into the birth gestation impact on milk glucocorticoid composition.

Sample

Participants for this study were recruited from the neonatal nurseries of King Edward Memorial Hospital, Western Australia, Australia (Perrella et al., 2015). The sample for the present study was a subgroup of a larger longitudinal observational study of gastrointestinal function in preterm infants. Convenience sampling was used with no attempt to recruit equal numbers of males and females, as there is no evidence to suggest differences between genders with regard to infant gastrointestinal function.

Stable preterm infants born at 28 to 32 weeks' gestation who were receiving milk for the infant’s own expressed milk or pasteurized donor HM were recruited. Infants with congenital anomalies, gastrointestinal disease, or symptoms of feeding intolerance within the previous 24 hr were excluded. In cases of multiple births, only one sibling was recruited to the study.

The larger study (from which the participants in the current study were recruited) required weekly monitoring of gastric emptying (two consecutive feeds in the first 3 weeks and single feeds thereafter) until discharge transfer from the study hospital. Recruitment was paced to meet the capacity to perform the monitoring sessions (3-6 hr duration) and, hence, the longer duration of data collection. No infants were withdrawn from the study. The sample for the present study consisted of 22 women who delivered preterm infants between 28 and 32 weeks. Birth gestation was divided into two groups, according to the equal distribution of sample size in each group: Recorded infant characteristics included birth gestation, birth weight, infant gender, and postnatal age at the time of sample collection.

For sample size, a priori size calculations were conducted using the effect size and variance in fatty acid composition of the HM due to preterm birth as reported by Gurzel-Borowiadzny, Walkie, and Koleszko (1997), as previous reports of GCs in preterm birth were not available. Eight participants were required to yield a statistical power of 80%, and 11 were included in each group for possible attrition.
Data Collection

Patients provided written and verbal informed consent prior to milk collection. All HM samples used in the current study were collected using a hospital-grade electric breast pump from February 2011 through April 2013. On the morning of the study, samples were collected from pooled expressed HM, aliquoted into sterile polypropylene-capped tubes, and rapidly stored at −20°C until further analysis (Perrella et al., 2015). Hormonal analysis of samples was conducted at the Ligand Institute, The University of Auckland, New Zealand, from May 2016 through August 2016.

Measurement

Glucocorticoids were assayed quantitatively from 100 µl of HM with the addition of 100 µl of internal standard. Briefly, the internal standard consisted of 12 ng/ml cortisol d4 and 60 ng/ml corticosterone d8 (Sigma-Aldrich, Darmstadt, Germany), prepared in water. The assay was initiated by warming the milk samples at 37°C for 5 to 7 min and vortexing prior to the addition of the internal standard. Glucocorticoids were extracted using 1 ml ethyl acetate (Merck, Darmstadt, Germany); the top organic layer was removed to a separate tube and then dried in a vacuum dryer (Savant SC250EXP, Thermo Fisher Scientific, Asheville, NC, USA) for ~2 h. The dried residues were reconstituted with 80 µl of methanol (Merck) and water (50:50) and transferred to high-performance liquid chromatography (HPLC) injector vials.

To detect total and free concentrations of cortisol and corticosterone in milk, a different assay was employed. Total GC concentration in milk was detected using a deconjugation enzyme assay. Briefly, 100 µl of warm, vortexed milk was mixed in a glass tube with internal standard (cortisol d4 was used as an internal standard in HM samples). This solution was then buffered with 200 µl of 5 N sodium acetate buffer (pH 5.0) and glucuronidase (255 units) enzyme (Sigma-Aldrich). The solution was incubated at 37°C in a water bath for 3 h. The enzyme reaction was stopped by heating the tubes at 70°C for 5 min. Then, 1 ml of ethyl acetate (Merck) was added and solution was vortexed for 30 s. The top organic layer was removed, transferred to another glass tube, and dried using a vacuum dryer (Savant SC250EXP, Thermo Fisher Scientific). The dried extracts were reconstituted in 80 µl of a mobile phase of methanol (Merck) and water 50:50 and transferred into HPLC vials, 12 µl of the sample were injected into the HPLC mass spectrometer system, and the concentration was analyzed as mentioned above.

The samples were analyzed using a HPLC mass spectrometer system consisting of an Accela MS pump and autosampler, followed by an Ion Max API source on a Finnigan TSQ Quantum Ultra XE triple quadrupole mass spectrometer, all controlled by Finnigan Xcalibur software (Thermo Electron Corporation, San Jose, CA, USA). The mobile phase was methanol-water gradient starting at 50:50 (v/v) (peaking at 80:20 before returning to 50:50) at a flow rate of 500 µl/min. The chromatography was performed at 40°C. The following selective reaction monitoring parameters were used: m/z 363.1→121 at 24 V for cortisol, 363.1→163 at 24 V for cortisone, and 367.1→121 0 at 24 V for cortisol-d4. To calculate the concentrations of GCs in HM samples, standard curves were generated in blank human plasma spiked with increasing amounts of each steroid: cortisol 0.05–100 ng/ml and cortisone 0.025–50 ng/ml. The area ratio of each steroid to the internal standard was used for quantitative purposes. Steroid concentration in HM was expressed in ng/ml.

Data Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences version 21 (SPSS, IBM Corporation, Armonk, NY, USA). Normality was assessed using Shapiro-Wilk test, when the assumption of normality was not met, nonparametric tests were used. The differences between GCs (cortisol, cortisone), free to total ratios, and infant gender and birth gestation groups were analyzed using Mann-Whitney U test. Samples were divided into two groups, according to the equal distribution of sample size in each group; the first group consisted of milk samples from the mothers who delivered before 30 weeks of gestation and the second group consisted of milk samples from mothers who delivered after 30 weeks of gestation. Furthermore, the mixed model was used to investigate the change in milk GC concentrations over the first 6 weeks following birth; infant gender and gestational age were added to the model as covariates. Pearson correlation was used to examine the relationship between cortisol and cortisone concentrations. Data are presented as mean (standard deviation [SD]), unless indicated otherwise. Alpha value was set at p < .05. Graphs were created using Graph Pad Prism version 7.0 (Graph Pad Software, Inc., La Jolla, CA, USA).

Results

Infant Characteristics

Mean (SD) infant birth gestation was 29.9 (1.34) weeks, and infant gender distribution was equal between the two groups with 11 of 22 (50%) males.

Cortisol and Cortisone in Human Milk

All HM samples contained both cortisol (1.88 [1.34] ng/ml) and cortisone (4.48 [1.73] ng/ml). There was a significant positive linear correlation between the cortisol and cortisone concentrations (p = 0.01, r = .85), as shown in Figure 1. The free form (biologically available state) accounted for 76.2% of cortisol and 84.2% of cortisone, with less than 30% of both cortisol and cortisone detected in the bound form. The
mean (SD) concentration of free cortisol was 1.95 (1.45) ng/ml and cortisone was 6.88 (1.70) ng/ml, whereas mean (SD) concentration of total cortisol (free and protein bound) was 2.56 (1.78) ng/ml and total cortisone was 7.81 (2.24) ng/ml.

Relationships Between Effect of Gestational Age, Gender and Infant Characteristics, and Human Milk Glucocorticoids

Both mean cortisone concentrations and free to total cortisone ratio were significantly different between birth gestation groups (p < 0.02 and p = 0.02, respectively), as shown in Figure 2. Cortisone concentration was higher in the milk samples of those who delivered at 30 to 32 weeks (5.38 [2.12] ng/ml) compared with those who delivered before 30 weeks' gestation (5.73 [0.99] ng/ml). The IIM free to total ratio of cortisone in the < 30 weeks' gestation birth group was 0.64 (0.08) ng/ml, compared with the > 30 weeks' gestation birth group (0.73 [0.20] ng/ml). No significant difference was found between birth gestation groups with regard to IIM cortisol concentration and free to total cortisol ratio, with gestational age (p = 0.82 and p = 0.95, respectively). However, the mean (SD) cortisol concentration in the milk samples of those who delivered at 30 to 32 weeks was 2.18 (1.79) ng/ml compared with the group who delivered before 30 weeks (1.63 [0.91] ng/ml), whereas free to total ratio of cortisone in the < 30 weeks' gestation birth group was 0.71 (0.24) ng/ml, compared with 0.70 (0.20) ng/ml in the > 30 weeks' gestation birth group.

No significant differences were observed between infant gender for the average value of cortisol (p = 0.15), cortisone (p = 0.56), and their ratio of free to total cortisol (p = 0.23) and free to total cortisone (p = 0.31). Table 1 shows the mean concentrations of hormones in HM by infant gender. Furthermore, the HM concentrations of cortisol (p = 0.19) and cortisone (p = 0.48), shown in Figure 3, and free to total cortisol (p = 0.66) and free to total cortisone (p = 0.26) did not change significantly over time (postbirth period ranging from week 1 to week 6 [data not shown]), after being adjusted for infant gender and birth gestation weeks.

Discussion

There is emerging evidence for the role of milk GCs as a regulator of infant growth and behavior development (Dantzer et al., 2013; Hinde et al., 2015). Yes, little is understood regarding the factors that regulate GC concentrations in HM. In the current study, the free and total concentrations of cortisol and cortisone were measured in HM samples collected from the cohort of women following preterm birth at 28 to 32 weeks' gestation. It was demonstrated that cortisone was the predominant GC and that the majority of both cortisone and cortisol were free. Furthermore, the cortisone concentrations tended to be lower in the HM of mothers who delivered before 30 weeks' gestation but not influenced by the time since birth.

In the current study, the average concentrations of HM cortisol and cortisone were broadly consistent with the prior analysis in preterm HM (van der Veer et al., 2016). The predominance of cortisone is also similar to that reported in HMs in the 4 weeks following term birth (Pandur et al., 2017; van der Veer et al., 2016). Unlike in milk, cortisol is the major GC in adult serum (Hwang, Fry, Fry, & Prossman, 2013; Raff & Trivedi, 2013). The glandular tissue of the breast converts cortisol into cortisone, presumably via the actions of 11β-hydroxysteroid dehydrogenase type 2 enzyme (Chapman, Holmes, & Seckl, 2013; Quirk, Slatter, & Funde, 1990). The significance of this interconversion, including whether the developing infant has a preferential requirement for milk-derived cortisone, is not yet known. It is interesting that, in this context, higher concentrations of cortisone were found within fetal tissues, also due to the actions of placental 11β-hydroxysteroid dehydrogenase type 2 enzyme (Cottrell & Seckl, 2009). Furthermore, Gianonc, Nakanishi, Sanger, Schonbucher, and Bauer (2014) reported the association of cortisone with enhanced intestinal permeability, anesthetizing enterocolitis, and rhinologic activity (greater size thymus in breastfed infants compared with formula-fed). Contrary to serum, our results demonstrate a higher concentration of cortisone in milk, but a higher concentration does not imply any significance for infant development. Therefore, further research is required to evaluate the functional significance of GC concentration in breastfed infants.

Preterm infants are reported to have underdeveloped adrenal functioning and an impaired hypothalamic–pituitary–adrenal (HPA) axis response (Flanker et al., 2017), impeding the adequate adrenocortical response to stress or illness. However, the HPA axis in preterm infants adapts rapidly, with a recovery of pituitary–adrenal response by the 14th
Table 1. Glucocorticoid Concentrations in Human Milk of Mothers Who Delivered Preterm Infants, by Infant Gender

<table>
<thead>
<tr>
<th>Gestational age (weeks)</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coriolin (ng/ml)</td>
<td>1.46 (1.49)</td>
<td>2.16 (1.28)</td>
</tr>
<tr>
<td>Cortisone (ng/ml)</td>
<td>4.36 (1.59)</td>
<td>4.70 (1.59)</td>
</tr>
<tr>
<td>Free to total cortisol ratio (ng/ml)</td>
<td>0.79 (0.16)</td>
<td>0.63 (0.24)</td>
</tr>
<tr>
<td>Free to total cortisone ratio (ng/ml)</td>
<td>0.88 (0.19)</td>
<td>0.85 (0.16)</td>
</tr>
</tbody>
</table>

Note. Data are presented as mean (standard deviation).

week of postnatal life (Bolt et al., 2002; Ng et al., 2004). Yet, emerging evidence suggests that later in life, these infants are predisposed to increased HPA axis activity, which increases their vulnerability to bronchopulmonary dysplasia and cardiovascular instability (Rines et al., 2016). The present study demonstrates that GCs in HM are not sufficiently dynamically regulated in relation to the stages of infant development to meet these rapid adaptations in adrenal function. Indeed, the lowest HM concentrations of cortisol were found in mothers who delivered at earlier gestations. These findings could have significant implications for donor milk banks who provide preterm infants with milk from mothers who delivered at term. More research is required to elucidate the potential role of milk-borne GCs in protecting infants against renal adrenal insufficiency.

GCs are lipophilic steroids with a variable proportion bound to large carrier proteins, including corticosteroid-binding globulin or albumin (Perogamsva et al., 2012). It is generally assumed that the biological activity of GCs is highly dependent on the free concentrations of the hormones in the circulation and these binding proteins are required to mount a normal stress response (Perogamsva et al., 2012). It has previously been reported that, in blood, bound cortisol accounts for almost 95% of total cortisol (Levine, Zageory-Sharon, Feldman, Lewis, & Weller, 2007). This current study demonstrated that more than 97% of cortisol and cortisone in HM were in their free or hormonally active form. Furthermore, the ratio of free cortisol to total cortisone in HM was higher (0.94) in the milk of mothers who delivered at 30 to 33 weeks' gestation. These findings demonstrate that the free cortisol level increased in comparison with total cortisone, and these ratios may be an index of the maternal adrenal gland (Lee et al., 2014; Morelli et al., 2016; Nomura, Fujitaka, Juno, Saito, & Ueda, 1996). There are very few studies that have measured free to total concentrations of cortisol and cortisone, determined mainly in plasma or urine samples, but little is known about their ratios and the clinical significance of these ratios in HM. However, results of this pilot study could be used to design a future study aimed to further explore the relation between preterm birth and its impact on milk GC concentration and its bioavailability.

Limitations

This study employed a highly sensitive technique to measure GC concentration in HM. The current study also has limitations. Due to the small sample size and single sourced population, these findings cannot be generalized to the entire population. The small sample size, particularly for infants' postnatal age, may limit the ability to identify whether there are gender differences in the milk GC concentration. In addition, the limited clinical and physiological data on both the maternal characteristics and infant birth may have confounded the interpretation of results. This clearly indicates the need for a larger multicenter study with sampling that can be generalized to the broader population of preterm births.
Conclusion

HM from mothers following preterm delivery contains appreciable levels of predominantly free (unbound) GCs. It is interesting that cortisone was the predominant GC. Currently, there is a limited understanding of the influence that these GCs have on the health and development of the breastfed infant. However, this study provides further insights into the supply and bioavailability of GCs to HM fed to preterm infants. As our study spans only a short period, further research should focus more on understanding the biological implications of milk-borne GCs on the later life health of an infant.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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