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

Focus on stress, nutrition and immunity

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CHAPTER 14

General discussion

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Introduction: human milk as a biological system within the mother-milk-infant triad

The early postpartum period is a critical time window in which maternal influences can lastingly affect the infant's development and health (1). The mechanisms underlying this transmission are complex, multi-dynamic and not yet completely understood. One of the mechanisms suggested to contribute to this transmission is maternal effects on human milk (HM) composition, which in turn affect the infant and its development (2). Three important and intertwining maternal influences in this context are maternal stress, nutrition and immunity (3-10).

HM is a live and adaptive body fluid, consisting of thousands of components, including live cells, an immune system and microbes, that has evolved to optimally nourish and protect an infant (11, 12). To date, the most common way in which HM has been studied, including in this thesis, has been to investigate single components of, or influences on, HM. This approach however overlooks the importance of HM as a complex biological system in which different components change, interact with and evolve in response to a fluctuating environment (13). HM does not stand on its own as a system but is intricately linked to the co-adapting systems of maternal physiology, as well as infant physiology and development, with variations in each, influencing the other, emphasizing the importance of acknowledging the mother-milk-infant triad and its (socio cultural) environment (14). Over the last years, research into individual aspects of this triad has expanded, however, an integrated investigation of how each axis of the triad influences the other is so far scarce (14). Notably, to fully understand the clinical significance of HM variability in different environments and its role as an important mediator of associations between mother and infant, HM should be considered as a 'system within a system' (**Figure 1**) (13). In this general discussion, we intend to discuss the answers to the aims of the thesis within this context.

The main aims of this thesis were to gain more insight into; if maternal stress is associated with a different human milk composition (**Part I**). In **Part II** we investigated how stress during lactation is associated with maternal dietary intake, and how maternal dietary intake is associated with the composition of human milk. In **Part III**, we described how the COVID-19 pandemic has affected maternal stress during lactation and how SARS-CoV-2 infection, vaccination and stress are associated with human milk immunoglobulins. **Part IV** examined if stress-associated differences in human milk nutrient composition mediate the association between maternal stress and infant (neuro)development and we reviewed the evidence for effectiveness of nutritional interventions to combat the consequences of early-life stress.

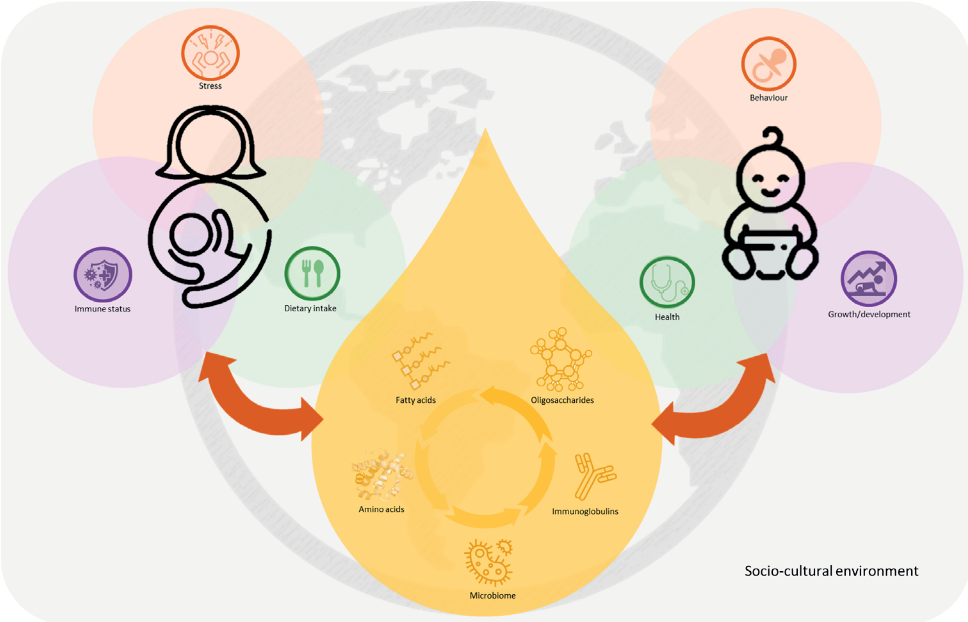

Figure 1. Human milk: a system within a system.
Human milk as a biological system within the 'mother-milk-infant triad' and its socio-cultural environment. The different aspects as described in this thesis are depicted.

In this general discussion, we will consecutively discuss each part of this thesis with its corresponding aim. The answer to each aim will be formulated and discussed within the broader context of HM as a biological system within the mother-milk-infant triad.

Part I: The interplay between maternal stress and human milk composition

In **Part I** of this thesis (**chapters 2-5**), we set out to explore **Aim 1**, i.e. to investigate the association between maternal stress and HM composition. We demonstrated lower HM fatty acid (FA) concentrations and higher protein-bound amino acid (AA) concentrations in lactating women with high levels of stress, compared to women with low levels of stress. These alterations were further associated with HM cortisol concentrations (**Chapter 2, Chapter 3**). Maternal stress-associated differences in HM oligosaccharide (HMO) concentrations only manifested in participants with the non-secretor status, one of the two genetic subtypes that result in distinct HMO compositional patterns. In this subgroup, total HMO concentrations were higher, the specific HMO lacto-N-tetraose (LNT) was higher (and positively associated with HM cortisol) and sialyllacto-N-tetraose c (LSTc) was lower in milk of women with high levels of stress compared to low levels of stress (**Chapter 4**). In addition, maternal stress in the postpartum period, but not HM cortisol concentration, was associated with an altered composition of the HM microbiome (**Chapter 5**).

How do the maternal-stress associated human milk alterations come about?

We can only speculate as to how these stress-associated alterations in HM can emerge. The stress hormone cortisol might be a plausible candidate, given its associations with the HM nutrients that were affected by stress (**Chapter 2-4**). Under the influence of stress, the lactating mother produces cortisol which enters the circulation. Here, it might have influences on maternal body stores of certain nutrients or bio actives, or can affect the process of nutrient or bioactive synthesis or breakdown, influencing the availability of these constituents for HM (15-17). It could also be suggested that cortisol directly influences the mammary gland and its transporters, membranes or *de novo* synthesis and as such, exerting its influence on HM composition (18, 19). The HM microbiome was the sole HM component we investigated that was not associated with HM cortisol. It can be suggested that the observed differences in the HM microbiome in women with high levels of stress compared to low levels of stress might be sensitive to other stress hormones, such as catecholamine (20). An alternative explanation lies in the fact that while the other HM components studied in this thesis are derived from the maternal bloodstream, or are synthesized in the mammary gland, the HM microbiome is thought to originate from the maternal digestive tract as well as from the infant's and mother's mouth and skin microbiome. These distinct sources and pathways might hence be differentially affected by stress (21, 22).

The interplay between the different human milk components

Although research on HM as a biological system and the interplay between its different components is scarce, it is interesting to speculate how different elements studied in this thesis might interact with each other within the HM system. HMO composition shows substantial intra- and inter-individual variations, primarily dependent on maternal polymorphisms in Secretor and Lewis genes (23, 24). For example, lactating mothers with the secretor status produce milk rich in 2'-fucosyllactose (2'-FL), while non-secretors lack this specific HMO (25, 26). Interestingly, stress effects on HMO composition only manifested in participants with the non-secretor status (**Chapter 4**). Notably, research suggests that not only HMOs vary by secretor status, but also the milk microbiome and metabolome, including HM FA and AA (27, 28). For example, (free) FAs but also the AAs pyruvate, choline, leucine and isoleucine were higher in the milk of women with the non-secretor status, whereas alanine, taurine, glutamine and glutamate were lower (27). Moreover, it has been demonstrated that milk of women with the non-secretor status has less *Actinobacteria* and more *Staphylococcaceae* (28), two genera we also found to be related to stress (**Chapter 5**). In addition, emerging evidence suggests correlations between HMO and milk microbiome composition (28, 29) and between milk microbiome and metabolome (30-32). This indeed strengthens the view of HM as a system, where multiple components are influenced by maternal factors and by each other.

Actinobacteria, which we found to be more abundant with higher levels of stress, have shown to be negatively associated with the HMOs LNFPI and 2'FL (28). Moreover, *Veionella*, which we showed to be less abundant in cases with high levels of stress (**Chapter 5**), was negatively correlated to 6'SL, whereas *Staphylococcus* and *Streptococcus* in HM were positively correlated to LNFPII and LNFPIII (28). In addition, we found lower levels of FAs and higher levels of *Staphylocuccus* in HM after stress (**Chapter 2, Chapter 5**), which have been shown to be negatively correlated to one another (28). It could be hypothesized that due to an increase in certain HM bacteria, the HMOs or FAs they utilize decrease or, the other way around, that due to an increased concentration of HMOs or FAs, the utilizing microbial genus will increase. In this way, effects of stress on one HM component can affect other related HM components.

Part II: The interplay between maternal dietary intake, stress and human milk composition

The above underlines the importance of considering HM as a system in which all different components are interrelated. As we observed different associations between stress and HM depending on maternal secretors status (**Chapter 4**), this indicates that maternal genetics are also important when investigating the effect of maternal stress on HM composition. Next to maternal genetic factors, many other intrinsic and extrinsic maternal factors are interrelated with the association between stress and the HM system, including maternal BMI, physical activity and ethnicity (33). One key factor in this context is maternal dietary intake. The association between stress and the nutrient composition of HM, as described in **Part I** of this thesis, already emphasizes the interaction between stress and nutrition (**Chapter 2, Chapter 3**), although this is only a small part of an intricate interplay (2, 34, 35). Only limited research exists on the association between maternal stress, dietary intake and HM quality during lactation. This is despite the fact that stress in the post-partum period is common and alterations in maternal dietary intake may significantly impact the composition of HM, thereby affecting the breastfed infant (36-42). As such, exploring the interplay between maternal food intake, stress and HM composition seems important to better understand the mother-milk-infant triad and its implications for health.

In **Part II** of the thesis (**chapters 6-8**), we set out to explore how maternal stress during lactation was associated with her food intake and dietary quality. Subsequently, we investigated how maternal dietary intake (specific AAs and a vegan diet) affected HM composition. We demonstrated that high levels of stress were associated with lower energy intake and lower diet quality, as well as with a lower intake of vegetables, fruits, and nuts (**Chapter 6**). We also demonstrated that the intake of maternal protein and amino acids was not associated with the specific AA composition of HM (**Chapter 7**). Similarly, lactating mothers consuming a vegan diet, typically low in carnitine and vitamin B6, had similar concentrations of carnitine and vitamin B6 in their milk compared to omnivorous lactating women (**Chapter 8**).

Clinical implications in a broader perspective

The fact that stress affects maternal dietary intake during lactation may raise concerns about the nutritional adequacy of HM of lactating women under stressful conditions. However, for the components we investigated in this thesis (AA, carnitine and vitamin B6, although not investigated during stress), this does not seem to be of concern as their concentrations in the diet do not seem to reflect milk concentrations and should therefore have no consequences for breastfed infants (**Chapter 7, Chapter 8**). Given our results, it can be suggested that HM nutrient concentrations remain relatively stable independent of the maternal nutritional status. However, it is known that certain important HM components, such as the FAs and certain fat soluble vitamins, are affected by maternal diet (38, 43, 44) and it should also be noted that our research was conducted in an affluent environment. In contexts where maternal diets are inadequate and endemic undernutrition and infection are common, it can be suggested that the nutritionally demanding period of breastfeeding (45, 46), may result in suboptimal lactation and possibly HM composition or volume, unless an optimal maternal nutrition is supported (47).

This also touches upon another concern. Reference values for optimal nutrient concentrations in HM are lacking. As a result, accurate information on dietary intake during lactation, with optimal ranges by geography and ethnicity, remains limited. For example, research has shown that FA and vitamin concentrations in HM vary by country, and adequate intakes for infants and lactation are so far only based on sparse data from small studies (48). Reference values for HM, also for specific (vulnerable) populations and subsequent maternal dietary intake recommendations are needed to better understand the clinical importance of certain changes in milk and to optimize milk fortification and formula for vulnerable populations or infants that cannot receive mother's own milk. This emphasizes the importance of not only considering the mother-milk-infant triad, but also its geographic, economic and socio-cultural environment.

Part III: The maternal immune system, stress and human milk composition

To highlight the complexity even more, maternal nutrition is not the only important maternal factor intersecting the association between maternal stress and HM composition.

Maternal stress and nutrition bi-directionally interact with the maternal immune system. The maternal immune system is affected by stress and nutrition, and the immune system has an impact on how much and what you eat, and on your stress response (49-53). Maternal immune activation in the perinatal period can affect infant (neuro)development via immune stimulants and cytokines, possibly transmitted via the placenta or HM (54). Maternal perinatal stress and nutrition can in turn influence this process (54). In addition, maternal perinatal stress and (mal)nutrition have shown to have an impact on programming of the infant immune system, with possible long-term consequences (54). During the COVID-19 pandemic, the importance of this interplay became more prominent and a frequent topic of research (55), as in part discussed below.

In **Part III** of this thesis (chapters 9-11), we set out to explore this interplay by investigating how the COVID-19 pandemic affected maternal stress during lactation and how SARS-CoV-2 infection, vaccination and stress are associated with HM immunoglobulins. We demonstrated that stress levels in lactating women during the first six months postpartum were similar during the COVID-19 pandemic, as compared to before the pandemic (**Chapter 11**). We also demonstrated that SARS-CoV-2 infection and vaccination elicit a SARS-CoV-2 specific immunoglobulin A (IgA) antibody response in HM and that one year into the pandemic, in 23% of Dutch lactating women these antibodies can be detected in their milk (**Chapter 9**). In addition, we showed that all four types of SARS-CoV-2 vaccines elicited a SARS-CoV-2 IgA response in HM, with the highest antibody titers after mRNA vaccination (**Chapter 10**). Furthermore, acute maternal perceived stress was not associated with SARS-CoV-2 specific antibodies in HM, while maternal life-time (chronic) stress was negatively associated with HM SARS-CoV-2 antibodies (**Chapter 11**).

Clinical implications and interplay between stress and the immune system

Although maternal stress levels did not appear to increase during the COVID-19 pandemic, the negative correlation between HM antibodies and maternal life-time stress levels underscores the significance of the maternal psychological state within the mother-milk-infant triad in the context of the immune system (**Chapter 9**). Indeed, several studies have linked maternal stress in the perinatal period to an increased incidence of infectious disease in infants, including necrotizing enterocolitis, meningitis and airway infections (56, 57). Maternal stress in the perinatal period has also been associated with increased risks of immune system related later disorders, such as asthma and allergic disease, in their children (58). While the placenta has been proposed as an important prenatal route of transmission of these maternal stress effects to her infant, our results and those of previous studies suggesting that stress alters the immune profile of HM, indicate a potential biological route of transmission during the postnatal period (59-61).

The interplay between stress, immunity and nutrition, and its consequences for human milk

Maternal stress, immunity and nutrition are all interrelated and affected by each other. Separately *and* together, these three factors may influence the composition of HM. An optimal immune response depends on an adequate diet and nutrition in order to keep infection at bay (55). For example, sufficient protein intake is crucial for optimal antibody production (62). Low micronutrient status, such as of vitamin A or zinc, has been associated with increased infection risk (55, 63, 64). Changes in maternal dietary intake may not only affect her own immune system, but also the (immune) composition of HM milk. Interestingly, research during the COVID-19 pandemic showed that a maternal SARS-CoV-2 infection not only affected the HM immune components, but also HM nutrients. For example, selenium, nickel, iron, copper and aluminum were found to be lower in HM from SARS-CoV-2 positive mothers compared to controls, while zinc and some minority elements were higher (65). If this is a direct result of the maternal immune response, or an indirect result via an altered maternal nutrient intake or a combination of both, remains unclear.

Part IV: Consequences for the infant and a window for opportunity?

As described above, maternal stress is associated with changes in HM, and interrelated with other factors such as nutrition and the immune system. Recognizing the tight interplay among these factors and their mutual influences, it can be assumed that it is the impact of all of these factors that leads to the observed alterations in HM. This prompts the question of whether stress-associated changes in HM composition mediate the association between maternal stress and infant outcomes. We explored this question in **Part IV** of this thesis (**chapters 12-14**) for the FAs and AAs in HM. We observed that maternal stress in the first month post-partum was positively associated with the infant temperament domain of surgency/extraversion at three months of age and that this association was partly mediated by the HM FA, specifically lower n6PUFA levels, but not by stress-associated changes in HM AA composition (**Chapter 12**).

Clinical consequences for the infant

We found that mothers experiencing high stress levels had infants displaying higher surgency levels. High levels of surgency in children may especially be beneficial in a stressful environment, as it may promote active coping and approaching novel situations. This may be in line with the 'predictive adaptive response' hypothesis (66), a theory that states that cues received in early life influence the subsequent development of a specific phenotype that is optimally adapted to the predicted environmental conditions. However, on the longer term, these changes might not always be beneficial (66). High levels of surgency in children have been associated with lower levels of effortful control and may later in life predict a higher risk of psychopathology (57-60). In this light, it is interesting to note that all the stress-associated changes we observed in HM in **Part I** of the thesis, namely higher AA concentrations, lower omega-6 FAs, and higher concentrations of immunity related HMOs, are considered anti-inflammatory (55). In line with the predictive adaptive response hypothesis, it can be speculated that this is a protective mechanism from mother to her child, aimed to protect the infant in the predicted stressful conditions in the future, that might often be associated with a state of inflammation (67-69). However, on the longer term in a healthy situation or environment, these changes can become detrimental if they suppress inflammation and the immune system too strongly.

Acute versus chronic maternal stress and its consequences for the infant
Contradictory to our findings, previous research into the association between maternal psychopathology and infant outcomes often showed a negative association between maternal depression/anxiety and the infant temperament domain of negative affectivity (4, 6, 70). Depression and anxiety are mental health disorders and are often characterized by chronic stress, which is different from the more acute stress that was induced by the infant hospitalization that we studied. Chronic and acute maternal stress may be differently associated with infant behavioural outcomes, including infant temperament. This has also been shown in preclinical research (71, 72).

Interestingly, in this thesis we also see differences when looking into the effects of acute stress (in the form of infant hospitalization and acute stress scores) versus chronic stress (in the form of maternal lifetime stress) on HM FAs (**Chapter 2**), immunoglobulins (**Chapter 13**) and the HM microbiome (**Chapter 5**). These different effects on HM composition might partially be explained by the extending effect of chronic stress on mammary gland development and its function during the postnatal period, as well as by the different physiological responses that both types of stress induce in the human body on a shorter term (73-75). The different effects of acute and chronic stress on HM composition suggests that both types of stress may be differently transmitted from mother to her infant, at least partly via HM. In this context, it is important to realize that numerous previous reports refer to stress, anxiety, trauma and depression as if these are interchangeable constructs (76), which might be too simplistic. Many researchers today consider acute stress to be pro-inflammatory, preparing the immune system for an injury (67), which is in line with the fact that we do not observe diminished antibody levels in HM (**Chapter 13**), and with previous research showing an increase in IgA in HM due to acute stress (77, 78). Chronic stress, however, seems to have both pro and anti-inflammatory components, with a blunted response to cortisol and impaired cellular and anti-viral immunity as main characteristics (79), in line with our observations of a negative association between life time stress scores and HM SARS-CoV-s IgA levels (**Chapter 13**). Since the overall effects of chronic stress on the immune system appear to be detrimental (dependent on the context a child is raised in), it is usually interpreted as maladaptive, but in an evolutionary perspective it may also be seen as a general adaptation of the immune system to an unpredictable environment (80).

The bi-directional links within the mother-milk-infant triad
The biological composition of HM not only depends on the myriad of different maternal inputs, including her diet, as discussed above, but also on the recipient infant (81). In particular, the bidirectional cross-talk between the microbiota and their metabolites in the infant oral cavity, and in HM is an example of direct signalling from the infant to the mother (82, 83). Moreover, the physical act of suckling may lead to changes in mammary gland intraductal pressure which has implications for HM composition (13). In addition, the specific receptors and the permeability of the gut epithelium will determine the absorption and hence mediate the response of the infant to various HM components. It can be suggested that this bi-directional link also mediates the parent-offspring conflict (84). Moreover, there appear to be sex-specific associations between maternal factors and HM composition (85). For example, the protein concentration of HM differed by birth mode among male but not female infants (85). Interestingly, a sex specific association with HM composition might also be present for maternal stress; a previous study shows that Kenyan mothers with a high social economic status produced milk with a higher fat content for male infants, while mothers living in adversity produced milk with a higher fat content for female infants (86). These examples also demonstrate bidirectionality within the mother-milk-infant triad.

A window for opportunity?
When considering strategies to prevent or mitigate the detrimental effects of (maternal) stress on the infant, stress reduction programs seem a plausible intervention. Studies have shown that yoga, Tai Chi and other relaxation techniques such as music therapy or physical activity, provoke significant changes both in hormonal levels as well as in cellular and humoral immunity, with a decrease in plasma cortisol levels and an increase of IgA levels (55, 87). These physiological effects of stress reduction programs on the mother might be transmitted to the infant, for example via HM. Indeed, stress reduction programs for lactating mothers have been developed (88, 89) and, for example, it was demonstrated that stress reduction interventions in lactating mothers increased milk production volume and fat content (90).

A better understanding of the complex interplay between stress, immune status, nutrition and HM composition within the mother-milk-infant triad opens a window for opportunity. Given the converging mechanisms, it can be suggested that nutritional interventions to the lactating stress-exposed mother and child may be used to counteract the adverse stress-related consequences. These nutritional interventions may work via different converging biological pathways, amongst others the immune system. In **Part IV**, we set out to explore the available evidence for such nutritional interventions and the pathways via which they might work. We demonstrate a great potential of nutritional strategies for mental health of individuals exposed to early-life stress as underscored by 60 preclinical studies that show a positive effect of a nutritional intervention to ameliorate the consequences of early-life stress in a majority of the cases (**Chapter 13**). However, clinical evidence on this topic is very scarce, calling for more clinical studies in this promising field (**Chapter 13**).

Stress reduction programs, nutritional interventions and dietary advise can support the lactating mother and her breastfed infant in vulnerable circumstances. As HM is a transmitting pathway from mother to child, a nutritional supplement to the lactating mother can be a relatively easy, safe and cheap intervention, to influence HM nutrient and immune composition, and thereby transfer of nutrients and immune factors to the infant. To date, few interventions exist for influencing HM composition, despite strong evidence from the dairy and breeding animal literature that shows that dietary inputs can optimize milk volume, density, and nutrient composition (13). Systematic reviews of intervention studies have consistently identified the gap in our knowledge and understanding of interventions in promoting optimal HM composition. This can in part be attributed to the lack of understanding of optimal ranges (13). Maternal supplementation with micronutrients and omega-3 FA has been examined but the consequences for maternal and child health remain largely unknown (91, 92). In addition, little is known about effective dose, frequency of dosing, and timing (stage of lactation) for supplementation. The timing of nutritional interventions may be critical, as distinct developmental programs are affected differently depending on the postnatal stage of its occurrence. This creates a sensitive window of vulnerability that needs to be elucidated in future research.

Other future research directions

The rapid advancement of multi-omics technologies presents new opportunities for characterizing HM composition, elucidating the interactions between milk constituents and its associations with the mother and infant. The application of these modern technologies in HM research provides a powerful means to understand HM as a complex biological system (13). To date, many HM studies are cross-sectional, presenting only a snapshot of HM composition, ignoring the temporal variability during lactation. To advance our understanding, there is a need to shift HM research to more longitudinal designs. Despite the emergence of multi-omics studies, still mostly each subsystem of the mother-milk-infant triad is examined in isolation and often focused on a single element or component. This limits our ability to comprehensively understand the interactions between the subsystems and study their response to external influences. Multiple

HM researchers have described and acknowledged these challenges, yet computational methods to effectively capture and decipher the complex ecosystem of the mother-milk-infant triad and its environment are still lacking (13).

In a critical review, Shenhav et al. state that in HM research, it is essential to take an integrative and "multi-layer" approach that accounts for the interactions among individual components and their collective functions (93). This requires defining various components of the ecosystem, such as the HM proteome, lipidome, microbiome and metabolome. Quantifying their levels and describing their dynamics, richness, diversity, and other characteristics, such as their likely interactions, are crucial aspects of this approach. The authors advocate that a possible strategy for defining functionally important interactions within and between triad components is to use knowledge from studies conducted in different fields, but with similar systems, such as microbial community ecology and computational microbiology (93). This could help to understand this complex adaptive "system within a system" that is so vital to maternal and infant health.

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