Free amino acids in human milk

A potential role in early life growth and immunity

Joris van Sadelhoff

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Free amino acids in human milk: a potential role in early life growth and immunity

Vrije aminozuren in moedermelk: een potentiële rol in de groei en immuniteit in het vroege leven

(met een samenvatting in het Nederlands)

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‘Within one linear centimetre of your lower colon there lives and works more bacteria than all humans who have ever been born. Yet many people continue to assert that it is we who are in charge of the world.’

Neil deGrasse Tyson

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Chapter 1

General introduction

Breastfeeding is the natural way by which a newborn is fed. In ancient times, the special nature of human milk (HM) was already well recognized. HM was considered sacred and crucial for the survival of the infant. Though the importance of breastfeeding was appreciated, attempts were made throughout the ages to use alternative sources of nutrition if needed, mostly animal's milk [1, 2]. It was noted that infants fed animal's milk had a higher mortality rate and were prone to indigestion compared to infants fed HM, but compositional differences were not understood until the 18th century, when HM and animal's milk were first subjected to chemical analyses [2]. With this knowledge, scientists began trying to formulate HM substitutes, using animal's milk, mostly cow's milk, as basis [1]. With the invention of evaporated milk, which allowed for a longer storage of the milk, many infant milk formulas (IMFs) became commercially available in the 19th century [3]. As IMFs more and more closely resembled HM through modifications in the carbohydrate, lipid, protein and vitamin content, global breastfeeding rates rapidly declined in the 20th century [4]. In the last decades, however, it has become evident that despite the compositional improvements of IMFs over the years, formula-feeding does not provide the infant with the same health benefits as HM feeding [3]. It is therefore currently being advised by the World Health Organization (WHO) that infants should be exclusively breastfed in the first 6 months of life [5].

The benefits of breastfeeding for infant health warrant an understanding of the factors in HM that mediate these benefits. This not only helps to understand how breastfeeding contributes to optimal infant development, but could also provide valuable insights for further advancing the functionality of IMFs, which is important as exclusive breastfeeding for 6 months is not always feasible for mothers due to practical or medical reasons.

The benefits associated with breastfeeding

HM contains a wide variety of nutrients that change in concentration as the infant ages [6]. This dynamic nature of HM nutrient composition is indicated to be uniquely suited to the changing nutritional requirements of the growing infant [6]. As such, it is generally accepted that exclusive breastfeeding in the first months of life supports optimal infant growth, or in recent years more commonly referred to as healthy infant growth [7]. Compared to infants fed an IMF, exclusively breastfed infants tend to gain more length and weight in the first 2-3 months after birth and then have a slower growth rate up to 12 months of age [8, 9]. This growth pattern of breastfed infants has been associated with a reduced risk for overweight and obesity in infancy and adulthood [10, 11], indicating that early life growth patterns are essential for optimal health throughout life.

Beyond the growth-related benefits, exclusive breastfeeding for the first months of life is also associated with the protection against infections in infancy and may reduce the risk of developing certain immunological conditions in early life, such as Crohn's disease and certain atopic diseases [12-17]. An example of the latter is cow's milk allergy (CMA), which is an adverse immune reaction to protein present in cow's milk and is the most common food allergy in infancy. The incidence of CMA in the first year of life is lower in exclusively breastfed infants compared to formula-fed infants, and clinical reactions are also mostly milder in breastfed children [16-19]. Although most of the affected children outgrow CMA throughout childhood, CMA in infancy can have long-lasting consequences, including delays in growth and an increased risk of atopic diseases later in life [20, 21]. As such, the prevention of CMA in early life is considered of great importance for an optimal health in infancy and later in life

It is widely recognized that the changing composition of macronutrients (*i.e.* protein, lipids and carbohydrates) in HM throughout lactation plays an important role in ensuring healthy growth of breastfed infants, and that the lower exposure to cow's milk protein of exclusively breastfed infants contributes to their lower risk of developing CMA. Besides these factors, bioactive components (*i.e.* extra-nutritional components with biological activity) that are uniquely present in HM can be important contributors to these benefits. This is evidenced by the ability of certain bioactive components in HM to influence growth and inhibit allergic sensitization to cow's milk and other foods in animals [22-28], and is further supported by correlations observed between their levels in HM and outcomes of growth and food allergy in infancy [29-39]. Given the importance of these outcomes for optimal health throughout life, it is of great interest to continue research on the composition of bioactive components in HM and their potential roles in these benefits associated with HM feeding. To understand which components could be of relevance in this context, it is essential to first highlight the key biological mechanisms by which such components can contribute to growth and the protection against food allergy in infancy.

Key biological mechanisms relevant for growth and the protection against food allergy in early life: a pivotal role for the developing intestines

It is well known that the developing intestines play a central role both in regulating early life growth and in the protection against food allergy. While to some extent genetically determined, the development, and thereby also the functionality, of the intestines is influenced to a considerable degree by environmental factors, notably early life nutrition [40]. A key mechanism by which HM and its bioactive constituents can contribute to healthy growth and the protection against food allergy in early life is thus through shaping intestinal development.

Intestinal developmental processes associated with early life growth

In the first months of life, the digestive capacity of the infant is limited as enzymatic functions responsible for protein, carbohydrate and lipid degradation are still developing [41, 42]. During this period, the intestines grow rapidly and undergo structural and functional changes to ensure optimal digestion and absorption of nutrients required for growth [43]. These changes include morphological alterations of intestinal villi and crypts, changes in the expression and activities of intestinal nutrient transporters, and the acquisition of a healthy and diverse gut microbiome. These processes can directly impact growth in early life [44-47] and, importantly, are influenced significantly by early life feeding [7, 48-52]. Bioactive components in HM that could contribute to these critical processes during intestinal development can thus be considered of potential relevance to healthy growth in early life.

Intestinal mechanisms underlying food allergy development and the protection thereof in early life

Besides regulating food digestion and absorption, the intestines are home to the majority of the body's immune cells and play a critical role in directing immune responses to food antigens [53]. The intestinal immune cells, which mainly reside in the gut-associated lymphoid tissues (GALT), are separated from the luminal contents by a layer of epithelial cells (*Figure 1*). This layer forms a physical barrier that limits direct contact of luminal antigens with the underlying immune cells, thereby preventing uncontrolled immune responses to harmless food antigens. While the integrity of this barrier is strictly controlled by intercellular junctions and by microbes that colonize the mucosal surface, disruptions can occur due to physical insults, dysregulated immune responses or pathogens. This allows food antigens to cross the barrier and to come in contact with the underlying intestinal immune compartment. Here, antigens are taken up and processed by mucosal dendritic cells (DCs), which present processed antigens to naïve T-helper (T_H0) cells localized in lymph nodes associated with the gut, such as the mesenteric lymph nodes (MLN). Under physiological conditions, by mechanisms that are not fully understood but involve the production of immunosuppressive factors, DCs instruct T_H0 cells to develop into regulatory T (T_{reg}) cells. These cells actively suppress adaptive immune responses through cell-cell contact and through the secretion of immunoregulatory cytokines (*e.g.* interleukin-10 (IL-10) and transforming growth factor beta (TGF-β)), ultimately leading to oral tolerance, defined as immune unresponsiveness to an ingested food antigen (*Figure 1*). Immunoglobulin A (IgA) antibodies, produced by mucosal B cells, and T-helper 1 (T_H1) cells also play a vital role in maintaining tolerance, through the neutralisation of luminal and mucosal food antigens and through the inhibition of T-helper 2 (T_H2) cells that drive allergic sensitization, respectively [54]. Food allergy results from a failure in tolerance

induction. This can be triggered by intestinal micro-environmental conditions, such as the presence of high levels of TH2 cytokines (*e.g.* IL-4, IL-5 and IL-13) relative to TH1 cytokines (*e.g.* interferon gamma (IFNγ) and IL-2) at the time of antigen presentation [53]. When this happens, DCs instruct TH0 cells to develop into TH2 effector cells, which are key players in the development and pathophysiology of food allergy (*Figure 1*). These cells stimulate B cells to produce immunoglobulin E (IgE) antibodies, which bind to high-affinity receptors present on the cell surface of basophils and mast cells, leading to allergic sensitization to the food antigen. Upon re-exposure, the antigen binds to the cell surface-bound IgE antibodies, which causes cell activation and the subsequent release of allergic mediators that contribute to the clinical manifestations of food allergy.

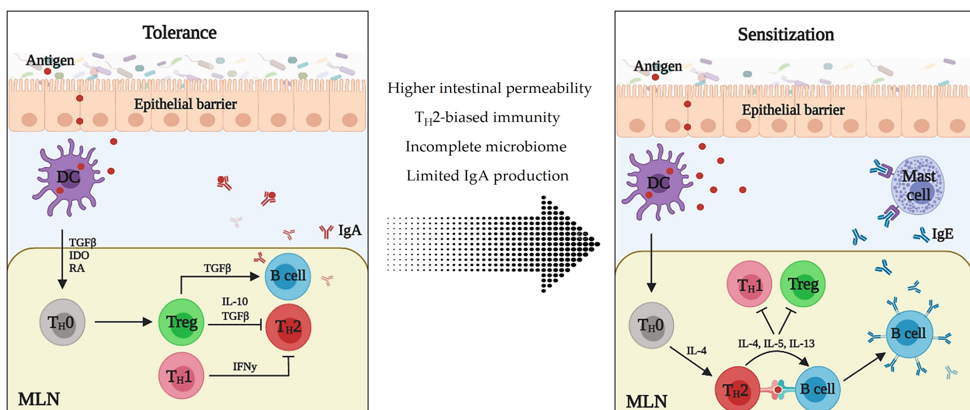

Figure 1. An overview of the mechanisms underlying tolerance and allergic sensitization to food antigens. Mucosal dendritic cells (DCs) process antigens that breached the epithelial barrier and present them to naïve T (TH0) cells localized in gut-associated lymph nodes, such as the mesenteric lymph nodes (MLN). In tolerant responses, by mechanisms that involve the production of immunosuppressive factors such as retinoic acid (RA) and indoleamine 2,3-dioxygenase (IDO), DCs drive the differentiation of TH0 cells into regulatory T (T_{reg}) cells. T_{reg} cells suppress T-helper 2 (TH2) cells through secretion of immunoregulatory factors such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β), ultimately leading to a tolerant state. Immunoglobulin A (IgA), produced by B cells, and T_H1 cells support in the maintenance of tolerance through antigen neutralization and through inhibition of T_H2 cells, respectively. A failure in this mechanism, which can be triggered by micro-environmental factors such as the presence of high levels of TH2 cytokines at the time of antigen presentation, can induce differentiation of TH0 cells towards TH2 cells. These cells suppress T_{reg} and T_H1 cells and instruct B cells to produce immunoglobulin E (IgE), which bind to receptors on the surface of mast cells, leading to allergic sensitization to the food antigen. A higher permeability of the epithelial barrier, TH2-biased immunity, limited IgA production and an incomplete microbiome contribute to the higher susceptibility to food allergic sensitization in the neonatal period.

The neonatal period is characterized by a higher susceptibility to food allergic sensitization because various factors relevant for the development and maintenance of oral tolerance are not yet fully matured in infants (*Figure 1*). Firstly, the immature epithelial barrier of

newborns has a high permeability, leading to a suboptimal protection against the uncontrolled passage of food antigens from the lumen into the immune cell-populated GALT [55, 56], where the antigen can elicit an immune response. Secondly, intestinal adaptive immunity in newborns is often defined as immature as it is functionally different from that of adults. More specifically, the neonatal immune system tends to be biased towards T_H2 immune responses when exposed to antigens, partly due to the T_H2-biased milieu in the fetoplacental unit and likely as a result of the limited T_H1 immunity in early life [57-59]. The resulting T_H2-dominant intestinal micro-environment can trigger allergic sensitization to antigens that breached the epithelial barrier. The limited capacity of newborns to produce IgA antibodies further restricts the ability to induce a tolerant state upon food antigen exposure [60]. Finally, the colonization of microbes in the intestines is far from completed at birth [61]. Intestinal microbes not only play a role in food digestion and absorption, but also play vital roles in the homeostatic maintenance of the immune system, including oral tolerance [62]. Hence, the still developing microbiome in early life is considered a critical contributing factor to the enhanced susceptibility to food allergic sensitization during this period [63]. Interestingly, accumulating evidence shows that the maturation and functionality of the neonatal intestinal factors described above, which all relate to a higher risk of developing food allergy, are modifiable through nutrition [40, 64]. Bioactive components in HM that are capable of compensating for these neonatal intestinal immune deficiencies, or those that support the maturation thereof, may thus play an active role in the protection against food allergy in early life.

A potential role for free amino acids in human milk in early life growth and in the protection against food allergy

In the last decade, preclinical studies have identified a plethora of bioactive components in HM that can directly or indirectly support the intestinal functions relevant for early life growth and the protection against food allergy [65, 66]. For several of these components, correlations have been found between their levels in HM and the outcomes of growth and/or food allergy in infancy, highlighting the potential of such extra-nutritional components to make significant contributions to healthy infant development. Examples include specific carbohydrates (*e.g.* various HM oligosaccharides), fatty acids (*e.g.* butyrate and n-3 long-chain polyunsaturated fatty acids) and proteins (*e.g.* lactoferrin and osteopontin) [29-39]. Currently, some of these components are already being tested in clinical studies for their safety to be added as functional ingredients to future generations of IMFs [67-69].

Another group of components in HM that may be relevant in this perspective, but have hardly been investigated, are free amino acids (FAAs). These are amino acids (AAs) that, in contrast to conjugated AAs, are not part of protein or peptides and thus are readily available for absorption and utilization by the body. These readily available AAs, which make up 5-10% of the total amino acid (TAA, *i.e.* the sum of FAAs and conjugated AAs) content in HM [70], have a shared basic chemical structure but differ in their side chains and thereby in their unique biological properties (*Figure 2*). Traditionally, AAs have been classified as being either nutritionally essential AAs (EAAs) or non-essential AAs (NEAAs), the latter being defined as AAs that can be synthesized in sufficient quantities by the body to maintain growth and health and thus do not need to be provided via the diet. This concept, however, is being increasingly challenged, as accumulating evidence also demonstrates a need for dietary intake of NEAAs to achieve optimal growth and health, especially during critical periods of development, such as the neonatal period [71-73]. Data on FAAs in HM are scarce, but initial evidence indicates that their levels display unique dynamics across lactation and are largely similar between mothers from different geographical regions [70]. This suggests that FAAs in HM may have important functions in infant development. FAAs are best known for their function as building blocks for protein synthesis, however, the low abundance of FAAs relative to the TAA content in HM has led researchers to attribute bioactive rather than nutritional functions for these readily available AAs [70, 74]. This is supported by numerous studies in young animals, in which dietary supplementation of specific FAAs led to a wide range of bioactive effects, particularly within the developing intestines. Depending on the AA, effects range from the stimulation of overall intestinal growth and integrity, to the promotion of intestinal villi growth, nutrient transport, microbiota colonization, and barrier function [75-84]. Additionally, specific dietary FAAs are capable of modulating the functions of the intestinal immune system in animals. Immunomodulatory effects observed include the stimulation of intestinal IgA production, the selective inhibition of T_H2-type immune responses and/or the promotion of T_H1-type immunity in the MLN, and the stimulation of immunoregulatory cytokine secretion by various cells of the GALT [81-87]. As explained, components in HM with such bioactive capacities could be of relevance for early life growth and for the protection against (food) allergic sensitization in infancy. Hence, it is of great interest to better understand the composition of FAAs in HM, and to investigate their potential contributions to a healthy growth and to the protection against allergy development in early life. This may not only increase our understanding of the link between breastfeeding and infant health, but could also provide novel insights for optimizing the functionality of IMFs, which currently contain considerably lower levels of FAAs than HM [88, 89].

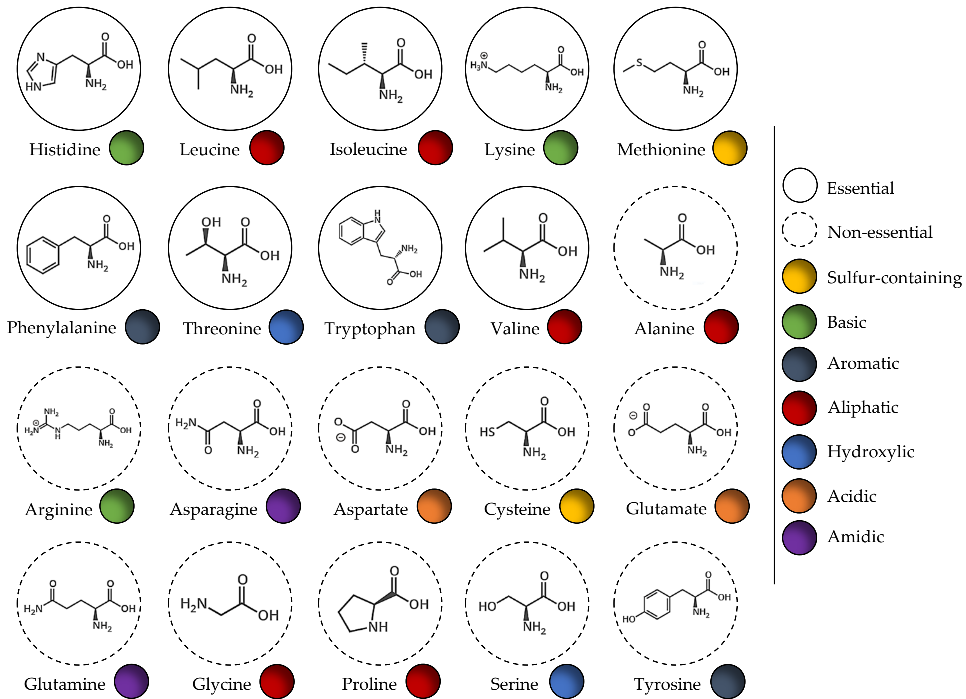

Figure 2. The chemical structure and side chain characteristics of the 20 genetically encoded, standard amino acids.

Aims and outline of this thesis

FAAs in HM and their potential contributions to infant growth and immunity have been scarcely studied. To better understand the potential biological relevance of FAAs in HM, the first aim of this thesis was to provide a detailed analysis of the FAA composition in HM, by assessing the levels, longitudinal changes and within-feed variations (*i.e.* variations within a single HM feeding session) of FAAs throughout lactation. This characterization was further extended by investigating whether FAA levels in HM are influenced by infant sex and maternal characteristics. Secondly, we aimed to evaluate whether FAAs could play a role in early life growth and immunity, by assessing correlations between FAA levels in HM and infant anthropometrics and immunological conditions, respectively. Finally, we investigated experimentally whether FAAs could play a role in the prevention of food allergic sensitization, by evaluating the effects of partial and complete substitution of dietary protein by FAAs on the development of CMA in mice.

In **Chapter 2**, the levels and the longitudinal variation of FAAs and TAAs (*i.e.* the sum of FAAs and conjugated AAs) in HM during the first 6 months of lactation were studied. It was shown that levels of each TAA decreased in the first 3 months of lactation, while levels of several FAAs increased during this period. The highest absolute and relative increases were observed for free glutamate and glutamine, respectively, which were also the most abundant FAAs in HM. This suggests important roles for these FAAs in early life development.

In **Chapter 3**, the within-feed variation of FAAs and protein in HM was investigated. It was found that the sum of FAAs and most individual FAAs were more abundant in foremilk (*i.e.* milk that is expressed at the beginning of a feed), whereas the total protein content was higher in hindmilk (*i.e.* the remainder of the expressed milk). This finding may have implications in situations where hindmilk is fed exclusively, and highlights the importance of strictly controlled HM sample collection methods in studies investigating FAAs and proteins in HM.

Chapter 4 reviews the current knowledge of the composition and secretion of FAAs in HM. Based on the literature reviewed, the FAAs glutamine and glutamate were selected for an in-depth discussion of their effects on intestinal growth, functionality and immunity. This chapter provided rationale to study the levels of these FAAs in HM in relation to infant growth and immunological conditions.

In **Chapter 5**, the levels of FAAs and TAAs in HM were investigated in relation to maternal characteristics, and in relation to early life growth and immunological conditions. Positive associations were observed between levels of free glutamine, glutamate and serine in HM and infant growth in the first 6 weeks of life. Moreover, levels of free glutamine were found to be lower in HM of allergic mothers. Finally, preliminary negative associations were found between several FAAs in HM and the risk of food allergy in early life. These findings suggest a role for specific FAAs in early life growth and warrant further investigations on the potential roles of FAAs in the protection against food allergic sensitization.

In **Chapter 6**, it was investigated whether dietary supplementation with the FAA glutamine could inhibit the development of food allergy in a validated murine model for CMA. It was shown that mice fed a diet containing 2% free glutamine prior and during allergic sensitization developed less severe allergic symptoms following intradermal allergen challenge than mice fed a control diet. This effect coincided with higher levels of allergen-specific IgA in the serum and an increased percentage of activated T_H1 cells in the MLN, which may underlie the lower severity of clinical symptoms observed in the glutamine-supplemented mice. These findings highlight the capacity of dietary glutamine to modulate

immunity, and further indicate that an adequate intake of free glutamine in early life might be important for an optimal protection against food allergy.

When breastfeeding is not possible, infants suffering from severe CMA may require an AA-based formula (AAF) to effectively resolve the allergic symptoms. In AAFs, the protein fraction is substituted for FAAs. In **Chapter 7**, the modulatory effects of complete substitution of dietary protein for FAAs on the development of CMA was studied in mice. It was shown that intake of the FAA-based diet partially prevented the development of allergen-induced allergic symptoms. This preventive effect coincided with a lowered production of allergen-specific IgG1 and IgG2a and a higher count of FoxP3-positive cells in the jejunum, suggesting that the lower clinical severity could be mediated by anti-inflammatory and T_{reg} cell activities. These findings indicate that, next to being effective in the dietary management of CMA, AAFs may also have the potential to inhibit the development of CMA.

Chapter 8 provides a summarizing discussion of the findings of this thesis and describes opportunities for future research.

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Chapter 2

Longitudinal variation of amino acid levels in human milk and their associations with infant gender

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ABSTRACT

It is discussed that specific amino acids (AAs) have functional roles in early life. Understanding the AA composition in human milk (HM) during lactation assists in specifying these roles. To this end, we assessed the levels of free AAs (FAAs), total AAs (*i.e.* the sum of free and bound AAs; TAAs) and protein in HM in the first 6 months of lactation, and evaluated possible associations with infant gender. HM samples of 25 healthy Dutch mothers participating in the PreventCD study were collected monthly during the first 6 months of lactation. Of the participating mothers, 12 gave birth to a boy and 13 gave birth to a girl. Analyses of the HM samples revealed that levels of free glutamate, glutamine, threonine, aspartate, glycine and serine significantly increased during months 1-3 of lactation, both in absolute sense and relative to TAA levels. In contrast, the protein content and levels of each of the individual TAAs decreased during this period. Evaluation of gender differences by mixed model analyses revealed an association between female infant gender and higher protein content ($p = 0.0465$) and TAA content ($p = 0.0362$) in HM during the first 3 months of lactation. Furthermore, there was a tendency for an association of male infant gender with higher levels of free glutamine ($p = 0.0948$) in HM during the first 3 months of lactation. These results show that FAA, TAA and protein levels in HM display a time-specific occurrence during lactation. Moreover, although confirmation is necessary in view of the small sample size, this study indicates that the AA composition in HM shows differential effects of the infant's sex.

INTRODUCTION

Human milk (HM) is considered the best source of infant nutrition, providing all the energy and nutrients necessary for an optimal growth and development. Besides providing optimal nutrition, breastfeeding offers numerous health benefits to the developing infant. It is known to support gastrointestinal development and it provides immediate protection against infectious diseases [1,2]. Moreover, recent studies have shown that breastfeeding reduces mortality rates among infants and can decrease the risk for developing certain types of atopic diseases, autoimmune diseases and (late) metabolic diseases [3-5]. The significant effects of breastfeeding on infant health and development urge the need to define and understand the composition of HM.

Proteins make up a significant part of the macronutrient composition of HM and contribute to its unique activity. A proportion of the proteins in HM is digested to provide the infant with an adequate supply of amino acids (AAs), which is critical for a normal growth and development [6]. Although AAs are released from milk proteins, HM also contains readily available free AAs (FAAs, *i.e.* AAs not part of protein), for reasons not well understood [7].

The composition of FAAs in HM has been only limitedly investigated. As revealed by the systematic review by Zhang *et al.* [7], the few available studies collectively indicate that FAA levels in HM change along lactation and are largely similar between mothers from diverse ethnic backgrounds and geographical locations. These findings suggest that FAAs may have specific roles in early life development. The most abundant FAAs in HM are glutamate and glutamine [7]. These FAAs are important energy substrates for intestinal cells and immune cells [8-10]. Moreover, dietary supplementation of these FAAs has been shown to support intestinal growth and development and to modulate intestinal immunity in various neonatal animals [9, 10]. Thus, FAAs in HM may contribute to the unique health benefits provided by breastfeeding, urging the need for a better characterization and understanding of the FAA composition in HM.

Studies have shown that the HM composition can differ according to infant sex. Sex-specific differences have been reported for among others the energy, fat and hormone content in HM [11, 12]. It is discussed that such differences may relate to the sex-specific requirements for optimal growth and possibly to the sex-specific differences in the occurrence of immune-related conditions, such as asthma, wheeze and coeliac disease (CD) [13-17]. Whether the levels of FAAs in HM are dependent on the infant's sex has never been studied, but is of interest to investigate as this may provide new insights on how FAAs can contribute to early life development.

To increase our understanding of the FAAs in HM and their potential roles in infant development, the present study assessed the temporal changes of FAA levels in HM, for the first time using a longitudinal study design that extends up to 6 months of lactation, and investigated the effect of infant sex on FAAs in HM. To this end, we measured FAA and total amino acid (TAA, *i.e.* the sum of conjugated AAs and FAAs) levels in HM sample series of 25 Dutch women during the first 6 months of lactation, and tested whether AA levels differed between milk intended for male and female infants.

MATERIALS AND METHODS

Subjects

During 2007-2010, HM samples were collected from women participating in the PreventCD study (Current Controlled Trials number is ISRCTN74582487), a dietary intervention study which has been described in detail elsewhere [15]. Briefly, term infants from 0 to 3 months of age and genotypically at risk of developing CD (*i.e.* the presence of DQ2 and DQ8 haplotypes) and with at least one family member with CD were recruited. Participating infants were randomly assigned to receive either 200 mg of wheat (intervention) or 200 mg of lactose (placebo) daily for 8 weeks starting at the age of 16 weeks. The primary outcome was the frequency of CD at 3 years of age. Infant health, height and weight were assessed periodically using standardized questionnaires [15]. For the analyses reported in the present study, HM samples were selected from 25 Dutch women participating in the PreventCD project. Inclusion criteria were that the participating mothers did not have CD and were consuming a normal, gluten-containing diet. Their infants (13 girls and 12 boys) had not developed CD at the age of 5-8 years. Infant weight and growth characteristics are represented in *Table 1*.

Sample collection

Participating women were instructed to collect 10-30 mL of HM at home manually or with an electric pump once a month, around the same day. After sampling, milk samples were frozen immediately at −20°C overnight at the participant's home and were transferred the day after on ice to the hospital, where samples were stored at −80°C until they were analysed. The average milk sampling time in month 1 though month 6 of lactation was 24, 52, 81, 116, 148 and 178 days after delivery.

Table 1. Characteristics of the participating infants

	Male (n = 12)	Female (n = 13)	Statistical analysis of the difference (*p*-value)
		Weight (g)	
Birth	3511.3 ± 144.3 (12)	3655.0 ± 104.1 (13)	0.422
Δ 0 - 2 months	1996.7 ± 202.4 (12)	1406.5 ± 122.6 (10)	0.028 *
Δ 2 - 4 months	1375.5 ± 187.5 (10)	1107.8 ± 78.8 (9)	0.223
Δ 4 - 6 months	1033.6 ± 140.9 (7)	798.4 ± 97.6 (9)	0.179
Δ 0 - 6 months	4156.7 ± 279.7 (9)	3392.8 ± 273.1 (9)	0.068 ⁰
		Length (cm)	
Birth	50.0 ± 0.7 (4)	50.8 ± 0.4 (9)	0.333
Δ 0 - 2 months	6.6 ± 1.0 (4)	7.7 ± 0.6 (6)	0.388
Δ 2 - 4 months	5.9 ± 0.5 (9)	4.4 ± 0.6 (8)	0.056 ⁰
Δ 4 - 6 months	4.0 ± 0.6 (6)	3.6 ± 0.5 (8)	0.631
Δ 0 - 6 months	17.2 ± 1.8 (4)	14.2 ± 0.9 (6)	0.134

Values are given as mean ± SEM (n). Statistical differences in weight or height gain between male and female infants are indicated as follows: $^{0}p < 0.10$, $^{*}p < 0.05$.

Amino acid analyses

A total of 135 HM samples were analysed, comprising 25 series of monthly collected HM samples up to 6 months of lactation. Whereas most longitudinal sample series were complete, some samples were missing, explaining the discrepancy in the number of milk samples analysed in each month of lactation. Prior to AA and protein level analyses, the HM samples were thawed overnight at 4°C and gently vortexed. Levels of FAAs (*i.e.* unbound AAs) and TAAs (*i.e.* the sum of free and bound AAs) were analysed by the liquid chromatography method described in detail elsewhere [18], using an ultra-fast liquid chromatography system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a fluorescence detector and an Acquity UPLC BEH C18 column (1.7m, 100 × 2.1 mm) (Waters, Milford, MA, USA). This method does not allow the detection of proline and cysteine, yielding a total of 18 detectable FAAs, and taurine. The measurements of TAA levels required total protein hydrolysis using 6M HCl, which enabled the detection of 15 TAAs and disabled the detection of tryptophan, cysteine and proline. The acidic hydrolysis process also transformed asparagine into aspartate and glutamine into glutamate, disabling the detection of these TAAs individually. For AA peak identification, AA standards (Sigma, Zwijndrecht, The Netherlands) were used. The protein content per sample was estimated by multiplying the N-content by the conversion factor 6.25 [19]. The N-content in the HM samples was analysed by the Dumas method, described elsewhere [20]. As opposed to the total TAA content, the total protein content also comprises the non-detectable AAs, as well as non-protein N (including taurine and nucleotides).

Statistical analysis

Regression coefficient analysis was applied to analyse the effect of the lactation time on protein, TAA and FAA levels in HM. Briefly, for each subject, the protein, TAA and FAA levels were regressed against time through months 1-3 of lactation, months 4-6 of lactation or the entire 6-months study period. Mean values of the slopes of the regression lines were tested against a hypothetical mean of 0 by means of a one-sample t-test. Differences in levels of protein, TAAs and FAAs between milk for boys and milk for girls in month 1-3 and month 4-6 of lactation were assessed by using a mixed model with subjects as random effect and the sampling time in days after delivery and infant sex as fixed effects. Missing data (n = 3 in months 1-3 of lactation) were considered missing at random. Unbiased effect sizes (Hedges' *g*) of infant sex on AA levels in HM were estimated by the method described in detail elsewhere [21]. Statistical significance was defined as $p < 0.05$ and where applicable trends were indicated when $p < 0.10$. As we consider the present study to be explorative, *p*-values were not adjusted for multiplicity. All data are reported as means ± SEM, unless indicated otherwise. Statistical analyses were performed using GraphPad Prism (version 7.03, GraphPad Software Inc, San Diego, CA) and JMP (version 11.0.0, SAS Institute Inc, Cary, NC).

Ethics

The PreventCD trial, registered as ISRCTN74582487, was approved by the medical ethics committee of the Leiden University Medical Center (LUMC) in the Netherlands on the 1st December 2006 and complied with the Good Clinical Practices (ICH-GCP) guidelines. The parents of the children provided their written informed consent for the study. The study was conducted according to the Declaration of Helsinki.

RESULTS

Changes in the FAA levels in human milk during lactation

Table 2 summarizes the FAA levels in HM in the first 6 months of lactation. Glutamate was the most abundant FAA, increasing from 53.7% to 56.9% of the total FAA content during month 1-6 of lactation. Regression coefficient analysis revealed that the sum of all FAAs significantly increased in the first 3 months of lactation (*Figure 1A*), also when the abundant FAA glutamate was excluded from the analysis (*Table 2*). Significant increases of individual FAAs were found for non-essential AAs (NEAAs) aspartate, glutamate, glutamine, glycine and serine in the first 3 months of lactation (*Table 2)*. Levels of most of the essential AAs (EAAs) remained stable along the study period, only threonine and histidine showed a slight but significant increase and decrease in the first 3 months of lactation, respectively.

Changes in the TAA levels and in the FAA/TAA ratios in human milk during lactation

In contrast to the sum of FAAs, the sum of TAAs decreased in the first 3 months of lactation, followed by a gradual non-significant decline thereafter (*Figure 1B*). Similar findings were observed for the total protein content and for the TAA levels of each of the individual AAs in HM (*Table S1*). Consequently, the ratio of the sum of FAAs to the sum of TAAs significantly increased in the first 3 months of lactation (*Figure 1C*). With respect to individual AAs, the FAA/TAA ratio for Glx (glutamate + glutamine), Asx (asparagine + aspartate), serine, glycine, alanine, threonine and histidine increased significantly in month 1-3 of lactation, and the ratios remained stable thereafter (*Table 3*). For all other AAs the increase of the FAA/TAA ratio was more gradual but also reached significance, or at least showed a trend, when regression coefficient analysis was performed over the entire study period (*Table 3*). Remarkably, while the contribution of FAA to TAA was less than 2% for most of the studied AAs, for Glx this contribution ranged from 9.1% in month 1 to 18.3% in month 6 of lactation (*Table 3*). Of note is also that compared to the TAA content in HM (*Figure 1B*), the FAA content showed a high inter-individual variability at all stages of lactation (*Figure 1A*).

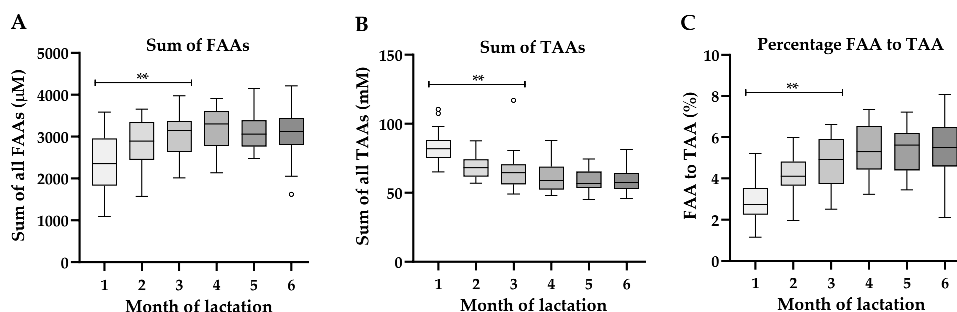

Figure 1. Time-course of amino acid levels in human milk during the first 6 months of lactation. The sum of free amino acids (FAAs) (**A**), total amino acids (TAAs) (**B**), and the relative contribution of the sum of FAAs to the sum of TAAs (**C**) in human milk during the first 6 months of lactation. Data are represented as box-and-whisker Tukey plots in which outliers are plotted as separate data points. Significant changes in the first 3 months of lactation are indicated by $^{**}p < 0.01$, as analysed by regression coefficient analysis.

Table 2. Free amino acid levels in human milk during month 1-6 of lactation

FAA (μmol/L)	Month 1 (n = 23)	Month 2 (n = 25)	Month 3 (n = 24)	Month 4 (n = 18)	Month 5 (n = 23)	Month 6 (n = 22)	Statistical analysis of changes
EAA							
Histidine	23.3 ± 1.4	21.8 ± 1.2	20.9 ± 1.1	19.2 ± 1.2	17.7 ± 0.7	18.5 ± 1.1	*b***, *d***
Isoleucine	12.5 ± 2.2	12.4 ± 1.4	10.4 ± 1.1	12.3 ± 1.9	10.4 ± 0.8	14.4 ± 2.7	
Leucine	35.4 ± 5.1	36.0 ± 3.4	31.8 ± 2.2	40.8 ± 7.0	31.5 ± 2.3	40.1 ± 7.3	
Lysine	31.2 ± 4.1	28.5 ± 4.9	28.9 ± 3.1	28.7 ± 7.6	24.5 ± 2.0	26.9 ± 3.3	
Methionine	2.3 ± 0.5	3.0 ± 0.4	2.6 ± 0.2	3.2 ± 0.5	3.4 ± 0.3	3.9 ± 0.7	
Phenylalanine	18.8 ± 2.5	19.3 ± 1.6	17.4 ± 1.9	18.2 ± 1.8	15.5 ± 0.8	21.0 ± 3.9	
Threonine	66.6 ± 4.7	80.2 ± 6.0	75.4 ± 3.9	81.7 ± 6.4	85.1 ± 4.4	80.5 ± 5.2	*a**, *c**
Tryptophan	23.8 ± 1.3	23.3 ± 1.4	21.8 ± 1.5	22.3 ± 1.8	20.8 ± 1.2	24.1 ± 2.6	
Valine	54.2 ± 4.6	57.9 ± 3.5	53.3 ± 3.3	56.3 ± 3.9	45.6 ± 2.4	49.6 ± 3.7	
NEAA							
Alanine	251.5 ± 22.0	236.2 ± 13.6	241.7 ± 13.2	256.2 ± 15.8	245.3 ± 11.9	227.7 ± 16.4	
Arginine	16.4 ± 1.6	15.9 ± 2.5	13.4 ± 1.2	20.6 ± 5.9	15.3 ± 1.8	16.8 ± 2.0	
Asparagine	9.1 ± 1.9	9.5 ± 1.9	11.4 ± 2.4	12.9 ± 3.4	13.2 ± 2.3	10.1 ± 2.2	
Aspartate	39.8 ± 3.8	50.4 ± 4.0	61.8 ± 7.4	63.6 ± 5.9	74.4 ± 7.7	67.0 ± 7.0	*a***, *c***
Glutamate	1267.4 ± 84.3	1634.3 ± 62.1	1713.4 ± 54.6	1825.4 ± 66.1	1776.5 ± 60.4	1769.2 ± 70.9	*a***, *c***
Glutamine	84.1 ± 23.9	159.3 ± 32.9	265.6 ± 43.6	290.1 ± 43.8	293.4 ± 37.6	281.0 ± 43.3	*a***, *c***
Glycine	82.7 ± 7.9	94.7 ± 6.6	102.5 ± 6.4	104.1 ± 5.4	105.2 ± 5.2	111.5 ± 7.3	*a***, *c***
Serine	79.6 ± 6.7	97.4 ± 6.1	107.0 ± 7.1	107.3 ± 5.8	104.8 ± 6.4	100.0 ± 6.2	*a**, *c***
Taurine	244.1 ± 14.2	240.1 ± 16.6	250.0 ± 20.7	232.1 ± 19.6	218.0 ± 12.8	245.9 ± 26.9	
Tyrosine	16.8 ± 1.9	15.8 ± 1.5	13.8 ± 1.0	14.7 ± 1.7	14.0 ± 1.2	17.3 ± 1.8	
SUM							
EAA	268.1 ± 21.5	282.4 ± 20.0	262.5 ± 12.2	282.7 ± 29.8	254.5 ± 11.3	279.0 ± 25.7	
NEAA	2091.5 ± 129.1	2553.6 ± 102.6	2780.6 ± 94.1	2927.0 ± 107.4	2860.1 ± 81.7	2846.5 ± 118.6	*a***, *c***
FAA	2359.7 ± 140.2	2836.1 ± 111.5	3043.1 ± 100.9	3209.7 ± 117.8	3114.5 ± 88.1	3125.5 ± 133.6	*a***, *c***
FAA - Glutamate	1092.3 ± 64.9	1201.8 ± 66.7	1329.7 ± 60.5	1385.1 ± 70.2	1338.0 ± 54.8	1356.2 ± 81.6	*a***, *c***

FAA: free amino acid; EAA: essential amino acid; NEAA: non-essential amino acid. Values are given as mean ± SEM. Significant changes ($^{*}p < 0.05$, $^{**}p < 0.01$) of FAA levels over time are indicated as follows: *a*: Significant increase from month 1-6 of lactation; *b*: Significant decrease from month 1-6 of lactation; *c*: Significant increase from month 1-3 of lactation; *d*: Significant decrease from month 1-3 of lactation; *e*: Significant increase from month 4-6 of lactation; *f*: Significant decrease from month 4-6 of lactation.

Table 3. Percentage of free amino acids relative to total amino acids in human milk in month 1-6 of lactation

FAA/TAA ratio (%)	Month 1 (n = 23)	Month 2 (n = 25)	Month 3 (n = 24)	Month 4 (n = 18)	Month 5 (n = 23)	Month 6 (n = 22)	Statistical analysis of changes
EAA							
Histidine	1.26 ± 0.083	1.45 ± 0.093	1.47 ± 0.095	1.41 ± 0.074	1.39 ± 0.058	1.50 ± 0.10	*c**
Isoleucine	0.24 ± 0.040	0.30 ± 0.038	0.25 ± 0.022	0.33 ± 0.021	0.30 ± 0.025	0.36 ± 0.039	*a*°
Leucine	0.36 ± 0.047	0.46 ± 0.048	0.42 ± 0.031	0.58 ± 0.10	0.47 ± 0.037	0.60 ± 0.10	*a**
Lysine	0.50 ± 0.067	0.57 ± 0.10	0.45 ± 0.041	0.63 ± 0.16	0.58 ± 0.052	0.64 ± 0.082	*a*°
Methionine	0.17 ± 0.038	0.28 ± 0.041	0.26 ± 0.026	0.44 ± 0.10	0.39 ± 0.038	0.40 ± 0.044	*a***
Phenylalanine	0.61 ± 0.074	0.80 ± 0.071	0.74 ± 0.050	0.87 ± 0.084	0.76 ± 0.044	0.85 ± 0.066	*a*°
Threonine	1.41 ± 0.11	2.09 ± 0.17	2.13 ± 0.16	2.38 ± 0.17	2.58 ± 0.16	2.50 ± 0.20	*a***, *c***
Valine	0.95 ± 0.076	1.27 ± 0.092	1.19 ± 0.075	1.35 ± 0.11	1.12 ± 0.057	1.20 ± 0.080	*c**
NEAA							
Alanine	4.54 ± 0.41	5.28 ± 0.31	5.84 ± 0.35	6.39 ± 0.32	6.35 ± 0.037	5.81 ± 0.39	*a**, *c***
Arginine	0.60 ± 0.054	0.75 ± 0.12	0.68 ± 0.060	1.11 ± 0.31	0.86 ± 0.12	0.84 ± 0.072	*a**
Asx	0.55 ± 0.064	0.84 ± 0.079	1.14 ± 0.17	1.21 ± 0.14	1.49 ± 0.17	1.33 ± 0.18	*a***, *c***
Glx	9.12 ± 0.77	13.81 ± 0.66	15.69 ± 0.76	17.50 ± 0.3	17.97 ± 0.69	18.33 ± 0.90	*a***, *c***
Glycine	2.04 ± 0.19	2.86 ± 0.18	3.37 ± 0.20	3.63 ± 0.16	3.77 ± 0.20	3.88 ± 0.27	*a***, *c***
Serine	1.39 ± 0.14	2.08 ± 0.15	2.50 ± 0.21	2.60 ± 0.12	2.59 ± 0.17	2.57 ± 0.21	*a***, *c***
Tyrosine	0.59 ± 0.064	0.71 ± 0.076	0.64 ± 0.047	0.72 ± 0.076	0.70 ± 0.059	0.85 ± 0.070	*a**
TOTAL							
FAA to TAA	2.56 ± 0.20	3.81 ± 0.19	4.36 ± 0.23	4.91 ± 0.26	4.97 ± 0.20	5.04 ± 0.29	*a***, *c***

FAA: free amino acid; TAA: total amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; Asx: aspartate + asparagine; Glx: glutamate + glutamine. Values are given as mean ± SEM. Significant changes ($^{0}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$) of the percentage of FAA to TAA over time are indicated as follows: *a*: Significant increase from month 1-6 of lactation; *b*: Significant decrease from month 1-6 of lactation; *c*: Significant increase from month 1-3 of lactation; *d*: Significant decrease from month 1-3 of lactation; *e*: Significant increase from month 4-6 of lactation; *f*: Significant decrease from month 4-6 of lactation.

Sex differences in the TAA and FAA levels in human milk during lactation

Analyses of sex differences by fitting a mixed model (girls: n = 13, boys: n = 12) revealed a significantly higher total TAA content ($p = 0.036$) (*Figure 2A*) and total protein content ($p = 0.047$) in HM intended for girls in the first 3 months of lactation. HM intended for girls contained significantly higher TAA levels of each individual AA in the first 3 months of lactation, except for Glx ($p = 0.144$), histidine ($p = 0.051$), valine ($p = 0.061$) and isoleucine ($p = 0.092$) (*Figure 2C*). Effect sizes of infant sex were moderate (Hedges' $g = 0.5 – 0.8$) for TAA levels of Glx, histidine, valine and isoleucine and were large (Hedges' $g \geq 0.8$) for all other measured TAAs (*Figure 2C*). Infant sex had no significant effect on FAA levels in HM, however, a trend for higher levels in milk intended for boys was observed for free glutamine ($p = 0.095$, Hedges' $g = 0.68$) in the first 3 months of lactation (*Figure 2B* and *2D*). No sex differences were found in levels of any FAA or TAA during month 4 to 6 of lactation.

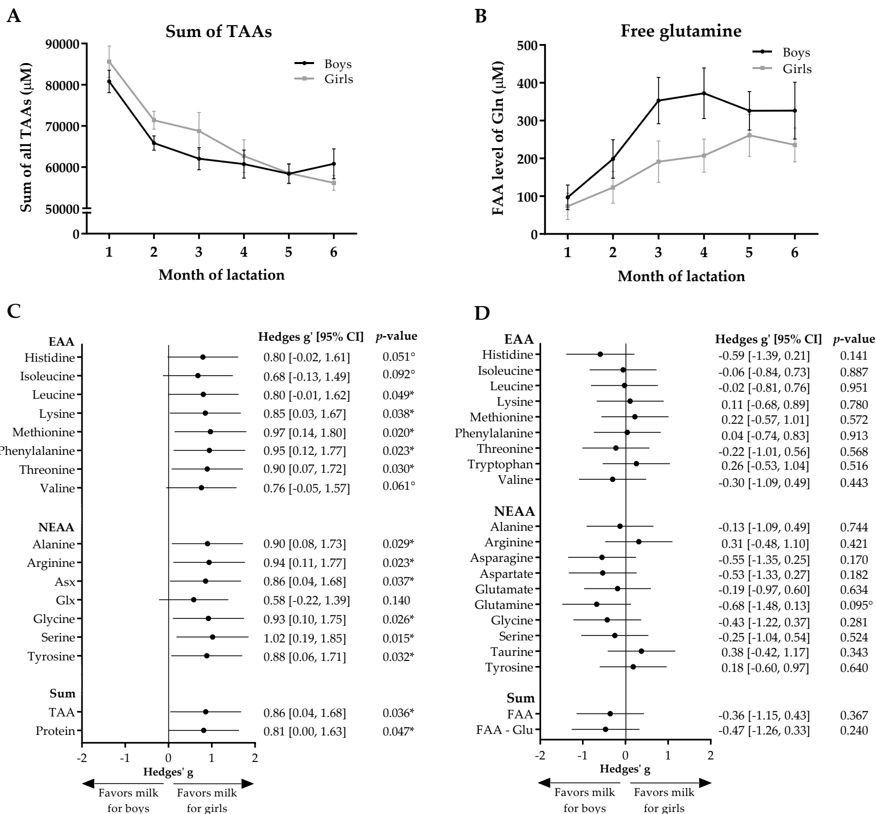

Figure 2. Amino acid levels in milk intended for boys and milk intended for girls. Levels over the course of lactation are shown for the sum of all total amino acids (TAAs) (**A**) and for free glutamine (**B**). Forest plots represent the effect sizes (Hedges' *g*) of infant sex on levels of individual TAAs (**C**) and free amino acids (FAAs) (**D**) in human milk during the first 3 months of lactation. $^{0}p < 0.10$, $^{*}p < 0.05$. EAA: essential amino acid; NEAA: non-essential amino acid; Gln: glutamine, Asx: aspartate + asparagine; Glx: glutamate + glutamine; Glu: glutamate.

DISCUSSION

The composition of HM is considered optimal for supporting a healthy development of the newborn in the first months of life. In recent years, FAAs in HM have gained increasing attention, and the emerging evidence that the FAA composition in HM changes along lactation has led researchers to discuss functional roles of FAAs in infant development. To contribute to this discussion, we studied the temporal changes of FAA levels in HM along lactation, for the first time in a longitudinal study that extends up to 6 months of lactation. Moreover, we reported for the first time on associations between FAA, TAA and protein levels in HM and infant sex.

The levels of FAAs, TAAs and protein found in the present study were comparable to those reported in mature HM in a systematic review by Zhang and colleagues [7]. We have previously confirmed earlier findings that the total protein and TAA content in HM decreases in the first 2 to 3 months of lactation, and that levels remain relatively stable thereafter [22]. The decreasing protein and TAA levels in HM are indicated to correlate with the infant's changing requirements for growth and might prevent overfeeding of AAs, as milk volume intake increases with progressing lactation [23].

While TAA levels of each of the individual AAs decreased in the first 3 months of lactation, levels of the FAAs aspartate, glutamate, glutamine, glycine, serine and threonine were found to increase during this period. Interestingly, previous studies found the same FAAs to increase in the first months of lactation [7, 24, 25]. These studies were performed in different ethnic groups and geographical locations, indicating a globally consistent FAA time course pattern in HM. Our results further indicated that the FAA composition in HM may only be dynamic in the first few months of lactation, as levels of all FAAs remained stable from month 4 to 6. Additional longitudinal studies investigating the FAA levels in HM during even longer periods of time are warranted to confirm this.

Consistent with the findings of other studies, the contribution of FAAs to TAAs in this study was 2.5% to 5%, depending on the time point during lactation [7, 24, 25]. Therefore, it is expected that the contribution of FAAs to the infant's nutritional requirements is low. Despite their low abundance, FAAs in HM contribute significantly to initial changes in the infant's plasma FAA levels following a feed [26]. It is proposed that FAAs are more rapidly absorbed after ingestion than protein-derived AAs, appear more readily in the circulation and thus might reach peripheral organs and tissues faster [8, 26, 27]. Receptors recognizing specific FAAs are found on a wide variety of cells in various tissues, supporting the concept that specific FAAs in HM may be of physiological importance for the infant [9, 10].

Our results showed that glutamate was by far the most abundant FAA and showed the highest absolute increase in concentration, followed by free glutamine. In addition, we observed that the relative contribution of FAA to TAA was remarkably high for glutamate and glutamine, suggesting an important role of these FAAs in the developing neonate. Studies in animals have demonstrated a wide range of functions of these FAAs in the developing intestines [28-30]. For instance, they are important energy substrates for intestinal cells to grow [31] and can stimulate the generation of glutathione and epithelial tight junctions, which are vital components for the protection and maturation of the neonatal gut barrier [30, 32-34]. It is also postulated that glutamine and glutamate may be involved in the infant's development of the immune system, as both AAs are able to modulate the differentiation and functions of a wide range of immune cells. For example, at concentrations comparable to those in HM, glutamine can stimulate T cell proliferation, increase the production of T helper type 1 (TH1) cytokine IL-2 by T cells, inhibit inflammatory cytokine production by various immune cells, and modulate the differentiation of B cells [29, 35-39]. Glutamate has similarly been shown to regulate T cell proliferation and to induce TH1 cytokine production by T cells following antigen stimulation, and has additionally been demonstrated to inhibit the production of inflammatory cytokines IL-6 and IL-23 by dendritic cells, thereby driving the development of regulatory T cells [29, 39, 40]. Given the important role of TH1 immunity and regulatory T cells in the inhibition of food allergic sensitization, it can be speculated that free glutamine and glutamate could, in conjunction with other factors in HM, play a protective immunomodulating role against early life TH2-driven (food) allergy.

The current study reports several associations between infant sex and the AA content in HM. Milk for girls was associated with higher levels of protein and TAAs than milk for boys when evaluated over the first 3 months of lactation. In line with this, Thakkar *et al.* also found that the protein content in milk for girls was slightly higher than that in milk for boys during at least the first 60 days of lactation [41]. They, however, did not report significant differences, possibly due to them analysing sex differences per time point measured, in contrast to our statistical method which includes the first 3 months of lactation simultaneously. Contrary to protein and TAA levels, we found that free glutamine levels tended to be higher in milk for boys in the first 3 months of lactation. A recent study reported a positive association between free glutamine levels in HM and infant length at 4 months of age [42]. In compliance with this association, male infants in the present study tended to gain more length ($p = 0.056$) during months 2 to 4 after birth than female infants. Post-hoc Pearson correlation analysis revealed a positive association between free glutamine levels in HM and infant length gain during this period in the present study (data not shown), but this was not found to be statistically significant, possibly due to the relatively small number of mother-infant pairs included.

The present research has several limitations. First, participating mothers gave birth to children genotypically at risk of CD (*i.e.* the presence of DQ2 and DQ8 haplotypes) and thus the present study is subject to selection bias. However, we expect that the results of the present study are not only limited to this subgroup of children, as participating mothers did not have CD, participating infants had not developed CD at 5-8 years of age, and as the prevalence of DQ2 and DQ8 haplotypes in the general population is high (up to 30%) [43]. Another limitation is that milk sample collection was not strictly controlled as participants could sample from either breast and throughout the day. Sanchez *et al.* reported that levels of some AAs in HM might vary throughout the day, but the variations were only small [44]. In the present study, detailed information regarding maternal diet, other than that participating mothers consumed a free-living, gluten-containing diet, is lacking. Maternal diet might influence the HM composition. However, the influence of maternal diet to protein, FAA and TAA levels in HM is expected to be minor, as those levels appear to be well preserved among mothers across different geographical locations consuming different diets [7, 45, 46]. This is reinforced by a study in China that reported an absence of a link between the AA concentrations in the maternal diet and those in HM [47]. A final limitation of the present research is that it lacks colostrum milk samples (0-5 days) and mainly analyses mature HM (>15 days). The lack of colostrum milk samples is unfortunate, as changes in the composition of HM are generally best observed in the very early stages of lactation.

CONCLUSIONS

The present study demonstrated that levels of FAAs, TAAs and protein in HM vary during lactation. For almost all individual AAs, the abundance of FAA increased relative to bound AA in the first 3 months of lactation. Significant increases in the absolute levels of FAAs in the first 3 months of lactation were found for glutamine, glutamate, serine, threonine, aspartate and glycine. This increase was best observed for the abundant FAAs glutamate and glutamine, which have been implicated in supporting intestinal and immune development in early life. Furthermore, although confirmation is necessary in view of the relatively small sample size, our study found differential effects of infant sex on the TAA and free glutamine levels in HM in the first 3 months of lactation. This provides opportunities to study the AA composition in HM in relation to sex-specific infant outcomes, including growth trajectories and risk for (immune-related) diseases.

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SUPPLEMENTARY DATA

Table S1. Total amino acid levels in human milk during month 1-6 of lactation

TAA (μmol/L)	Month 1 (n = 23)	Month 2 (n = 25)	Month 3 (n = 24)	Month 4 (n = 18)	Month 5 (n = 23)	Month 6 (n = 22)	Statistical analysis of changes
			EAA				
Histidine	1872.9 ± 53.9	1530.9 ± 31.6	1462.9 ± 62.2	1368.1 ± 58.6	1278.0 ± 40.0	1259.7 ± 38.8	*b***, *d***
Isoleucine	5112.8 ± 145.9	4235.2 ± 98.1	4071.5 ± 162.7	3825.6 ± 167.7	3544.6 ± 109.6	3339.8 ± 79.2	*b***, *d***
Leucine	9774.1 ± 276.8	8094.0 ± 190.7	7748.7 ± 314.5	7299.3 ± 318.2	6813.3 ± 208.4	6667.9 ± 191.1	*b***, *d***
Lysine	6281.0 ± 169.4	5154.2 ± 123.1	4902.8 ± 202.9	4578.6 ± 195.9	4281.1 ± 128.7	4259.6 ± 136.7	*b***, *d***
Methionine	1352.4 ± 41.6	1068.5 ± 25.8	1028.2 ± 52.1	944.2 ± 43.2	879.8 ± 31.0	832.6 ± 25.3	*b***, *d***
Phenylalanine	3021.2± 91.9	2452.1 ± 61.3	2309.4 ± 107.9	2154.0 ± 93.3	2050.6 ± 62.3	2089.5 ± 89.3	*b***, *d***
Threonine	4800.2 ± 142.3	3932.4 ± 94.4	3717.7 ± 166.6	3470.8 ± 144.1	3317.5 ± 96.5	3393.9 ± 146.2	*b***, *d***
Valine	5727.5 ± 181.5	4691.9 ± 103.8	4546.9 ± 204.6	4289.8 ± 187.3	4072.3 ± 121.2	4041.1 ± 141.5	*b***, *d***
			NEAA				
Alanine	5573.0 ± 173.6	4539.4 ± 108.2	4280.9 ± 200.3	4015.8 ± 169.5	3891.3 ± 111.7	4068.7 ± 205.9	*b***, *d***
Arginine	2730.2 ± 104.5	2127.9 ± 64.6	2007.9 ± 117.4	1847.8 ± 87.7	1840.2 ± 64.1	1993.2 ± 140.6	*b***, *d***
Asx	9174.8 ± 278.4	7383.2 ± 165.1	6932.7 ± 315.1	6475.7 ± 274.2	6171.5 ± 182.2	6189.0 ± 244.3	*b***, *d***
Glx	15044.7 ± 397.7	13123.7 ± 244.1	12885.6 ± 433.4	12331.7 ± 443.1	11573.0 ± 294.3	11274.9 ± 238.1	*b***, *d***
Glycine	4115.1 ± 130.9	3331.5 ± 95.2	3105.4 ± 161.5	2886.6 ± 121.0	2808.1 ± 87.1	3023.6 ± 180.1	*b***, *d***
Serine	5870.4 ± 180.6	4769.1 ± 111.4	4480.4 ± 202.7	4177.7 ± 172.4	3990.0 ± 118.2	4122.6 ± 200.5	*b***, *d***
Tyrosine	2848.9 ± 89.2	2285.1 ± 54.1	2204.0 ± 98.7	2044.1 ± 92.7	1949.8 ± 59.9	1929.7 ± 72.7	*b***, *d***
			SUM				
TAA (mmol/L)	83.3 ± 2.4	68.7 ± 1.5	65.7 ± 2.8	61.7 ± 2.5	58.5 ± 1.7	58.5 ± 2.0	*b***, *d***
Protein (g/L)	14.4 ± 0.4	11.8 ± 0.2	11.3 ± 0.4	10.5 ± 0.4	10.0 ± 0.3	9.9 ± 0.3	*b***, *d***

TAA: total amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; Asx: aspartate + asparagine; Glx: glutamate + glutamine. Values are given as mean ± SEM. Significant changes (**$p < 0.01$) of TAA levels over time are indicated as follows: *a*: Significant increase from month 1-6 of lactation; *b*: Significant decrease from month 1-6 of lactation; *c*: Significant increase from month 1-3 of lactation; *d*: Significant decrease from month 1-3 of lactation; *e*: Significant increase from month 4-6 of lactation; *f*: Significant decrease from month 4-6 of lactation.

Chapter 3

Differences in levels of free amino acids and total protein in human foremilk and hindmilk

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ABSTRACT

Free amino acids (FAAs) in human milk (HM) are indicated to have specific functional roles in infant development. Studies have shown differences between HM that is expressed at the beginning of a feed (*i.e.* foremilk) and the remainder of the milk expressed (*i.e.* hindmilk). For example, it is well established that human hindmilk is richer in fat and energy than foremilk. Hence, exclusively feeding hindmilk is used to enhance weight gain of preterm, low birthweight infants. Whether FAAs occur differently between foremilk and hindmilk has never been reported, but given their bioactive capacities, this is relevant to consider especially in situations where hindmilk is fed exclusively. Therefore, this study analysed and compared the FAA and total protein content in human foremilk and hindmilk samples donated by 30 healthy lactating women. The total protein content was found to be significantly higher in hindmilk ($p < 0.001$), whereas foremilk contained a significantly higher total content of FAAs ($p = 0.015$). With regards to individual FAAs, foremilk contained significantly higher levels of phenylalanine ($p = 0.009$), threonine ($p = 0.003$), valine ($p = 0.018$), alanine ($p = 0.004$), glutamine ($p < 0.001$) and serine ($p = 0.012$) than hindmilk. Although statistical significance was reached, analysis of the effect size of the milk fraction on the FAA levels revealed that the observed differences were only small. To what extent these differences are of physiological importance for infant development remains to be examined in future research.

INTRODUCTION

Human milk (HM) contains a nutrient composition that supports optimal infant growth and development. Besides nutrients, HM contains a variety of bioactive components that can contribute to the development of the infant's immune system and can play a role in the protective effect of HM against the development of certain types of allergies, auto-immune diseases and metabolic disorders [1-3]. Free amino acids (FAAs), which make up 5-10% of the total amino acid (*i.e.* the sum of protein-bound amino acids and FAAs) content in HM, are increasingly recognized as potential bioactive factors [4]. It was previously shown that levels of specific FAAs, including glutamine and glutamate, increase in HM in the first months of lactation, indicating that there is a need for an adequate intake of FAAs in early life [4, 5]. This, combined with the demonstrated capacity of dietary FAAs to modulate the functions of a wide variety of cells, including intestinal and immune cells, has led researchers to assign functional roles of FAAs in the developing infant.

Studies have demonstrated that the HM composition is different between milk expressed at the beginning of a feed (*i.e.* foremilk) and that expressed at the end of a feed (*i.e.* hindmilk). It is well established that hindmilk contains a higher fat and energy content compared to foremilk [6-8]. Hence, exclusively feeding hindmilk is postulated to be the optimal choice for feeding preterm, low birthweight infants, for whom energy requirements are high but in whom HM volume tolerance is only limited [8-10]. Apart from differences in the fat and energy content, a recent study indicated differences in the peptide content between the two milk fractions as well [11]. Specifically, hindmilk contained a higher number of peptides that corresponded to peptides with sequential removal of amino acids from the N- and C-termini than foremilk, possibly due to the fact that hindmilk resides longer in the mammary gland in the presence of native milk exo- and endoproteases than foremilk [11]. This finding suggests a differential release of FAAs from proteins or peptides in foremilk and hindmilk, which could lead to differences in the FAA content between the two milk fractions. Based on these findings, we hypothesized that FAAs might be higher in human hindmilk compared to foremilk. Differences in FAA levels between the two milk fractions might alter the availability of bioactive FAAs for the developing infant, which is particularly important to consider in situations where either of the two milk fractions is fed exclusively.

METHODS

Subjects

Thirty healthy lactating mothers, aged 27 to 43 years, participated in the present study. Participating women were on average 23.5 ± 11.3 weeks postpartum (range: 6 - 44 weeks), were non-smokers, and were either primiparous or multiparous. The average body mass index (BMI) score of the mothers was 23.8 ± 5.2 kg/m² (range: 18.6 – 38.8 kg/m²). Twenty-eight mothers were of Caucasian ethnicity and two mothers were of another ethnicity. Each of the thirty mothers gave birth to term infants. On average, participants attained a tertiary education level.

Milk sample collection

Participating mothers were instructed to express 30 mL of foremilk and at least 30 mL of hindmilk by pump using only one breast, considering the first 30 mL of milk after milk flow began as foremilk and the remaining milk as hindmilk. The milk samples were placed on ice immediately after collection and samples of 0.5 mL foremilk and 0.5 mL hindmilk were stored at -20°C to be analysed for the FAA content. The remainder of the milk samples were analysed for the total protein content immediately after the end of the session.

Milk sample analysis

A total of 60 milk samples (30 foremilk and 30 hindmilk samples) were analysed for total protein and FAA content. Total protein content was measured by infrared transmission spectroscopy using the MIRIS Human Milk Analyzer (Mid-IR, MIRIS, Uppsala, Sweden), as described and validated in detail elsewhere [12]. For the analyses of the FAA content, 0.5 mL of each foremilk and hindmilk sample was pre-treated with perchloric acid. Levels of FAAs were then measured by an ultra-fast liquid chromatograph system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with an Acquity UPLC BEH C18 column (1.7 m, 100 × 2.1 mm) (Waters, Milford, MA, USA). Amino acid standards (Sigma, Zwijndrecht, The Netherlands) were used for amino acid peak identification. This method did not permit the detection of proline and cysteine, yielding a total of 18 detectable FAAs, as well as the non-proteinogenic FAA taurine.

Statistics

Differences in FAA and protein levels between foremilk and hindmilk samples were analysed by means of paired sample t-tests. Pearson correlation analyses were conducted to evaluate wether potential differences could be dependent on maternal BMI. Unbiased effect sizes (Hedges' *g*) of the milk fraction on the amino acid and protein content in HM

were calculated as described in detail elsewhere [13]. Statistical significance was defined as $p < 0.05$ and trends were indicated when $p < 0.10$. As we consider the present study to be explorative, *p*-values were not adjusted for multiple testing. All data are reported as means ± SEM. Data were processed and computed for statistics using GraphPad Prism (version 7.03, GraphPad Software Inc, San Diego, CA) and Microsoft Office Excel 2016 (version 16.0.8431.2110, Microsoft Corporation, Redmond, WA).

Ethics

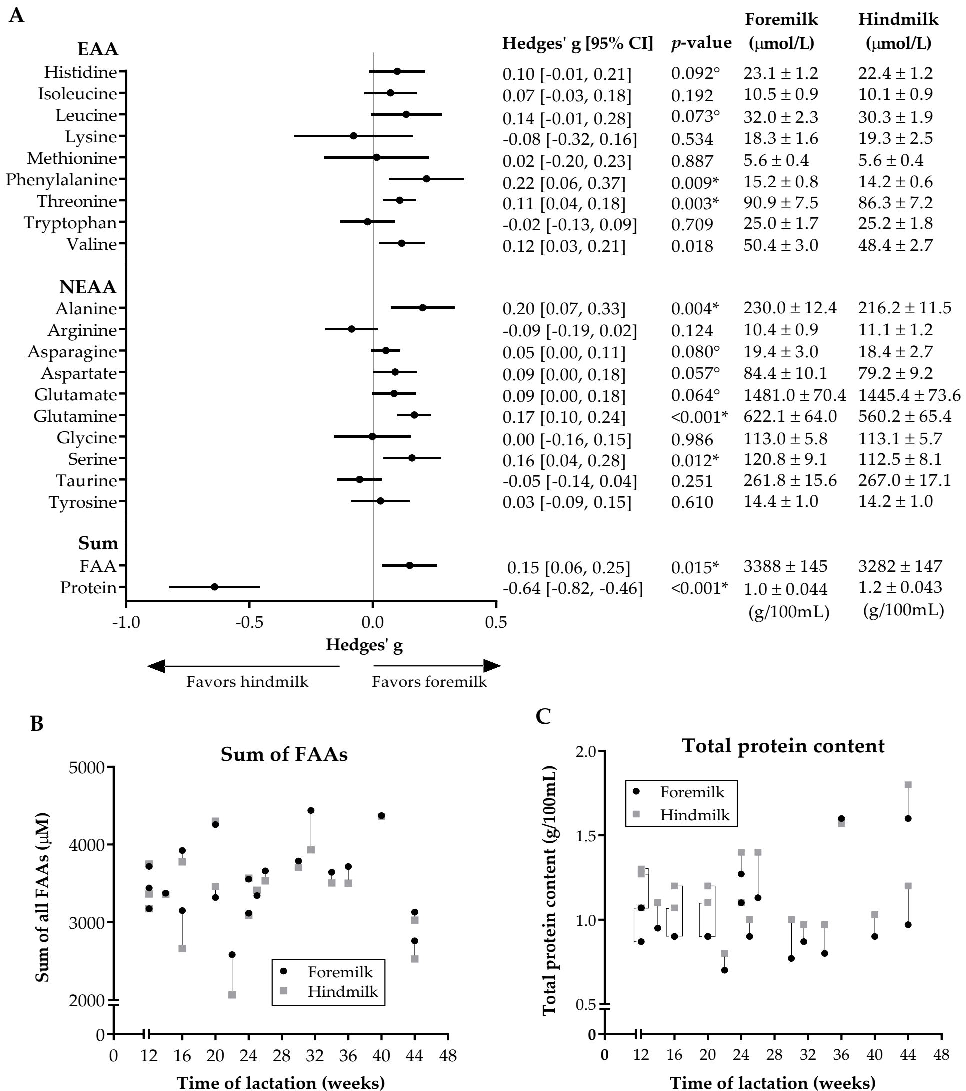
The project proposal, including the consent form used in this study, were submitted to an accredited Medical Research Ethics Committee (the Independent Review Board Nijmegen (IRBN)). The IRBN confirmed that this study did not need a formal ethical committee review according to the Dutch law. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. Written informed consent was obtained from all mothers prior to the study.

RESULTS

Levels of FAAs and total protein in human foremilk and hindmilk samples are shown in *Figure 1*. Whereas the total protein content was significantly higher ($p < 0.001$) in hindmilk compared to foremilk, the total FAA content was significantly higher ($p = 0.015$) in foremilk (*Figure 1A*). With regards to individual FAAs, foremilk contained significantly higher levels of essential amino acids (EAAs) phenylalanine ($p = 0.009$), threonine ($p = 0.003$) and valine ($p = 0.018$), and of non-essential amino acids (NEAAs) alanine ($p = 0.004$), glutamine ($p < 0.001$) and serine ($p = 0.012$) compared to hindmilk (*Figure 1A*). Additionally, foremilk had a tendency towards higher levels of the EAAs histidine ($p = 0.092$) and leucine ($p = 0.073$) as well as the NEAAs asparagine ($p = 0.080$), aspartate ($p = 0.057$) and glutamate ($p = 0.064$). As represented by the forest plot (*Figure 1A*), the difference in protein content between the two milk fractions can be considered moderate (Hedges' $g = 0.64$), whereas the differences in the FAA levels were small (Hedges' $g < 0.5$). No correlation ($p > 0.05$) was observed between maternal BMI and the effect sizes of the observed differences.

Figures 1B and *1C* show the total content of FAAs and protein in foremilk and hindmilk samples of each subject, plotted against the subjects' timepoint of lactation at the time of milk sampling. Differences in the total FAA and protein content between foremilk and hindmilk samples seemed to be independent on the timepoint of lactation. However, within-subject differences in the FAA and protein content between foremilk and hindmilk samples appeared to be smaller than between-subject differences (*Figure 1B* and *1C*). At the time of milk sampling, the different subjects were at varying stages of lactation. To prevent

this from being a driver for between-subject differences, *Figure 1B* and *1C* only show data of subjects who were at 12 weeks postpartum or later at the time of milk sampling, as FAA and protein levels in HM are reported to remain relatively stable after 3 months of lactation [5].



	Hedges' g [95% CI]	*p*-value	Foremilk (μmol/L)	Hindmilk (μmol/L)
EAA				
Histidine	0.10 [-0.01, 0.21]	0.092°	23.1 ± 1.2	22.4 ± 1.2
Isoleucine	0.07 [-0.03, 0.18]	0.192	10.5 ± 0.9	10.1 ± 0.9
Leucine	0.14 [-0.01, 0.28]	0.073°	32.0 ± 2.3	30.3 ± 1.9
Lysine	-0.08 [-0.32, 0.16]	0.534	18.3 ± 1.6	19.3 ± 2.5
Methionine	0.02 [-0.20, 0.23]	0.887	5.6 ± 0.4	5.6 ± 0.4
Phenylalanine	0.22 [0.06, 0.37]	0.009*	15.2 ± 0.8	14.2 ± 0.6
Threonine	0.11 [0.04, 0.18]	0.003*	90.9 ± 7.5	86.3 ± 7.2
Tryptophan	-0.02 [-0.13, 0.09]	0.709	25.0 ± 1.7	25.2 ± 1.8
Valine	0.12 [0.03, 0.21]	0.018	50.4 ± 3.0	48.4 ± 2.7
NEAA				
Alanine	0.20 [0.07, 0.33]	0.004*	230.0 ± 12.4	216.2 ± 11.5
Arginine	-0.09 [-0.19, 0.02]	0.124	10.4 ± 0.9	11.1 ± 1.2
Asparagine	0.05 [0.00, 0.11]	0.080°	19.4 ± 3.0	18.4 ± 2.7
Aspartate	0.09 [0.00, 0.18]	0.057°	84.4 ± 10.1	79.2 ± 9.2
Glutamate	0.09 [0.00, 0.18]	0.064°	1481.0 ± 70.4	1445.4 ± 73.6
Glutamine	0.17 [0.10, 0.24]	<0.001*	622.1 ± 64.0	560.2 ± 65.4
Glycine	0.00 [-0.16, 0.15]	0.986	113.0 ± 5.8	113.1 ± 5.7
Serine	0.16 [0.04, 0.28]	0.012*	120.8 ± 9.1	112.5 ± 8.1
Taurine	-0.05 [-0.14, 0.04]	0.251	261.8 ± 15.6	267.0 ± 17.1
Tyrosine	0.03 [-0.09, 0.15]	0.610	14.4 ± 1.0	14.2 ± 1.0
Sum				
FAA	0.15 [0.06, 0.25]	0.015*	3388 ± 145	3282 ± 147
Protein	-0.64 [-0.82, -0.46]	<0.001*	1.0 ± 0.044 (g/100mL)	1.2 ± 0.043 (g/100mL)

Figure 1. Free amino acid (FAA) levels and total protein content in foremilk and hindmilk of thirty women. (**A**) *Left*: Forest plot indicating the effect sizes (Hedges' *g*) of the milk fraction on the FAA and protein content in human milk. *Right*: average levels of FAAs and the protein content in foremilk and hindmilk samples, expressed as mean ± SEM. For each subject, total levels in hindmilk and foremilk are shown for FAAs (**B**) and protein (**C**), according to the stage of lactation of the subjects at the time of milk sampling. $^{0}p < 0.10$, $^{*}p < 0.05$. EAA: essential amino acid; NEAA: non-essential amino acid.

DISCUSSION

It is discussed that FAAs in human milk (HM) have functional roles in infant development besides serving as building blocks for protein synthesis. Studies revealed that human hindmilk is richer in fat and energy than foremilk [6-8], and recommendations are made to exclusively feed hindmilk to preterm, low birthweight infants to enhance weight gain [8-10]. Whether the FAA content differs between the two milk fractions has never been reported. This, however, might be relevant to consider given the demonstrated bioactive capacities of specific FAAs present in HM. The current study therefore analysed and compared the FAA content in human hindmilk and foremilk samples of 30 healthy women, and reported significant differences in both the total content of FAAs as well as several individual FAAs between the two milk fractions.

The present study found significantly higher protein levels in hindmilk compared to foremilk. Intriguingly, almost all study participants had higher protein levels in their hindmilk than in foremilk. This finding is consistent with several previous reports [14-16], though there are also studies that found no difference in the protein content between the milk fractions [17, 18]. A possible explanation for this inconsistency is the inter-study variation in the definition of foremilk and hindmilk; while some define foremilk as the first 30 mL of milk collected and the remainder as hindmilk, others define foremilk as the milk collected during the first 3 minutes of the milk flow and define hindmilk as the milk collected afterwards [14-18]. With regards to FAAs, we revealed that hindmilk contained a lower total content of FAAs than foremilk. This is surprising, as hindmilk resides longer in the mammary gland in the presence of active endo- and exopeptidases that can cleave of amino acids from milk peptides, a process which may lead to an increase in FAA levels in hindmilk [19, 20]. Of note, HM also contains protease inhibitors which might inhibit the endo- and exopeptidases, however, the interplay between protease inhibitors and proteases in HM is not well understood [21]. The FAAs that were most responsible for the observed differences were alanine, glutamine, phenylalanine, serine, threonine and valine, which are all uncharged amino acids. However, given the tendency of most of the other FAAs to also be more abundant in foremilk, the difference is unlikely to be amino acid-specific.

The nutrient composition of HM is considered insufficient for preterm infants with a low birthweight [22, 23]. As human hindmilk is more energy-dense and contains more fat than foremilk, exclusively feeding hindmilk to preterm infants is used in practice to obtain a higher rate of weight gain [23, 24]. The present study indicates that this practice provides the preterm infant with higher levels of protein, which might suit the increased protein requirements of preterm infants to achieve improved growth and neurodevelopment [25]. However, we demonstrated that this practice may also reduce the supply of FAAs for

preterm infants. An adequate supply of amino acids is critical for an optimal infant development [26-28], and a consistent body of evidence indicates that, besides protein, preterm infants also require increased intakes of FAAs for optimal growth and neurodevelopmental outcomes [25]. The increased FAA needs of preterm infants may be partially explained by their limited capacity to fully digest HM proteins, compared to term infants [25]. The largest absolute and relative difference between the milk fractions was found for the FAA glutamine, which was lower in hindmilk. Studies in young animals indicate a role for dietary free glutamine in promoting the growth, development and functionality of the intestines and its associated immune system [29]. Proposed mechanisms by which free glutamine could contribute to immune development include the provision of energy for immune cells, the modification of T cell and B cell proliferation and activation, and the stimulation of phagocytic capacities of neutrophils and macrophages [29-33]. Mechanisms by which glutamine could support intestinal growth and development involve the promotion of epithelial cell growth and the stimulation of the synthesis of proteins that are critical for the maturation of the neonatal gut barrier, such as tight-junction proteins and glutathione [34-37]. The importance of an adequate availability of free glutamine in early life has also been highlighted by studies showing that an insufficient supply of glutamine can lead to increased gut permeability and decreased functioning of the mucosa and its associated immune functions [38-40]. Preterm, low birthweight infants, which have an underdeveloped gastrointestinal tract and generally have a higher intestinal permeability than term infants [41, 42], might need a higher bioavailability of free glutamine to support adequate intestinal functionality and a timely intestinal maturation [38, 43, 44]. Feeding hindmilk exclusively may not meet these enhanced FAA demands. However, although the levels of free glutamine and other FAAs were statistically lower in hindmilk compared to foremilk, it is important to note that the differences were relatively small. Moreover, inter-individual differences in FAA levels in the milk fractions were generally larger than the intra-individual differences between the milk fractions. Whether the observed differences between the milk fractions could be of physiological relevance for infant development needs to be studied in future research. In addition, the findings of this study warrant the investigation of levels of other bioactive components in foremilk and hindmilk, and call for strict standardization of HM sampling procedures in studies investigating the levels and biological roles of FAAs and protein in HM.

CONCLUSIONS

The present study demonstrates that the levels of FAAs in HM can change during a single feed. Contrary to the protein content, which was significantly higher in hindmilk, the total FAA content was significantly higher in foremilk. Most of the individual FAAs were more

abundant in foremilk, but statistical significance was only reached for alanine, serine, glutamine, threonine, phenylalanine and valine, some of which have been discussed to play important physiological roles in infant development. Although statistically significant, the observed differences were small and between-subject differences appear to be larger than within-subject differences. Whether these differences are of physiological relevance in infant development should be studied further. In addition, these findings call for robust standardization of HM sampling procedures in studies on FAAs and proteins in HM to minimize variation among subjects.

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Chapter 4

Free amino acids in human milk: a potential role for glutamine and glutamate in the protection against neonatal allergies and infections

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ABSTRACT

Breastfeeding is indicated to support neonatal immune development and to protect against infections and certain allergies in early life. The composition of human milk (HM) is widely studied in relation to these unique abilities, which has led to the identification of various immunomodulating components in HM, including various bioactive proteins. In addition to proteins, HM contains free amino acids (FAAs), which have not been well studied. Of those, the FAAs glutamate and glutamine are by far the most abundant. Levels of these FAAs in HM sharply increase during the first months of lactation, in contrast to most other FAAs. These unique dynamics are consistent among different studies, suggesting that their levels in HM are tightly regulated throughout lactation and, consequently, that they may have specific roles in the developing neonate. Interestingly, free glutamine and glutamate are reported to exhibit immunomodulating capacities, indicating that these FAAs could contribute to neonatal immune development and to the unique protective effects of breastfeeding. This review describes the current understanding of the FAA composition in HM. Moreover, it provides an overview of the effects of free glutamine and glutamate on immune parameters relevant for allergic sensitization and infections in early life. The data reviewed provide rationale to study the role of free glutamine and glutamate in HM in the protection against early life (food) allergies and infections.

INTRODUCTION

Human milk (HM) is widely recognized as the best source of infant nutrition, providing the infant with a highly diverse mix of nutrients that supports optimal development. The benefits of HM, however, go beyond that of providing nutrients. An increasing body of evidence suggests that HM feeding provides the infant protection against various immune-related conditions. For example, it is shown that exclusively breastfed infants are less likely to develop respiratory and gastrointestinal infections than infants who fully or partially received an infant milk formula (IMF) [1-5]. This protective effect of breastfeeding against infections may extend well beyond infancy and is indicated to be enhanced upon prolonged breastfeeding [6, 7]. Additionally, evidence suggests that breastfeeding also has the potential to protect against certain allergic diseases, including atopic dermatitis [8, 9], asthma [9-11] and potentially food allergy [12-15], especially if there is a family history of atopy [16]. For cow's milk allergy, which is one of the most common food allergies in infancy, the incidence rate is reported to be up to seven times lower in exclusively breastfed infants compared to IMF-fed infants [17-19]. These protective capacities of HM have driven scientific research into the underlying mechanisms in the past decades [15, 20, 21].

At birth, the immune system is immature [22]. Compared to adults, the neonatal immune system is characterized by diminished innate effector cell functions, limited T-helper 1 (T_H1) immune responses, and skewed T cell responses to antigens towards T-helper 2 (T_H2) immunity. These characteristics correlate with an increased susceptibility to infections and allergies in the neonatal period [23, 24]. This susceptibility is further enhanced by an immature intestinal barrier and an incomplete intestinal microbial colonization at birth [23]. Various factors in HM have been identified that can compensate for these immunological and intestinal immaturities, or that support the development thereof. Such factors may play an active role in the protection against infections and allergies in breastfed infants. Examples include the proteins immunoglobulin A (IgA), which confers protection through neutralizing antigens [25, 26], and lactoferrin, which is implicated to promote a balanced T_H1/T_H2 immunity, to shape intestinal microbial colonization, and to promote the intestinal barrier function [27-32]. In addition to proteins, HM contains free amino acids (FAAs, *i.e.* amino acids not part of protein). Accumulating preclinical evidence indicates that certain dietary FAAs have immunomodulating capacities and can support the maturation and functionality of developing intestines [33, 34]. Thus, FAAs in HM may play an active part in an optimal intestinal and immune development of the infant. Yet, FAAs are typically overlooked as components that may contribute to the immune-related benefits provided by breastfeeding.

Of the total content of amino acids (AAs) in HM, 5-10% is present in free form. The FAAs

glutamate and glutamine are by far the most abundant, together comprising more than 60% of all FAAs present in HM [35]. Their levels display unique and consistent patterns over the course of lactation, suggesting important functions for these FAAs in the developing neonate [35, 36]. Interestingly, these FAAs have been widely associated with intestinal development and immunomodulation, including the modulation of immune mechanisms relevant for the protection against allergies and infections. This review aims to describe the current understanding of the FAA composition in HM, and to provide an overview of the effects of free glutamine and glutamate on immune parameters relevant for the protection against allergic and infectious diseases in early life. For this, we focus on effects observed in young animals and in cells derived thereof. Ultimately, a better understanding of the FAAs in HM and their immunomodulating capacities in early life may open new avenues for the prevention of allergies and infections in infancy.

AMINO ACIDS IN HUMAN MILK: PROTEIN-BOUND AND FREE AMINO ACIDS

It is well known that protein quality and quantity are key aspects of the nutritional value of HM. The total amino acid (TAA) composition of HM, which includes both protein-bound AAs and FAAs, is used to evaluate the quantity and the quality of milk proteins and hence is investigated in numerous studies [36, 37]. Many of these studies, however, only report the TAA composition and do not distinguish between protein-bound AAs and FAAs. As a result, data on FAAs in HM are relatively scarce.

The few studies that have simultaneously investigated FAAs and TAAs in HM have shown that FAAs account for only 5-10% of the TAA content, suggesting that the nutritional value of FAAs in HM may be limited [35, 36]. The potential of FAAs in HM to be of physiological relevance for the infant, however, should not be underestimated, as their levels are up to 100 times higher than the FAA pool in tissues and up to 30 times higher than the plasma FAA levels in infants [38-39]. Dietary FAAs are indicated to be more readily absorbed, appear sooner in the circulation and thus might reach peripheral organs and tissues faster than protein-derived AAs [40-44]. Indeed, a differential plasma FAA profile has been observed between infants receiving an IMF containing FAAs and infants receiving an equivalent portion of AAs in the form of intact protein, suggesting differences in absorption kinetics between FAAs and protein-derived AAs [45-47]. In contrast to their protein-bound counterpart, dietary FAAs can interact with specific receptors present on a wide range of cells in various parts of the body, including the intestines, where they can activate intra-cellular pathways and exert physiological effects [34, 48]. Thus, FAAs in HM have different abilities and hence may serve different functions in the neonate than protein-bound AAs.

While HM directly supplies infants with FAAs, proteins in HM could also provide the infant with FAAs via proteolysis in the neonatal gastrointestinal tract. The efficacy of this process, however, may be relatively limited in the first months of life, due to low concentrations of gastric and luminal enzymes, a high gastric postprandial pH which leaves gastric proteases largely inactive, and a high degree of glycosylation of HM proteins [49-52]. This indicates that an adequate intake of readily available FAAs via the diet may be of particular relevance in early life.

The unique characteristics and abilities of dietary FAAs and the relatively limited proteolytic capacity of neonates underline the importance of understanding the FAA composition in HM, separate from the TAA composition.

The free amino acid composition in human milk is dynamic and seemingly regulated throughout lactation

The composition of HM is known to be dynamic over the course of lactation. The protein content has been consistently shown to decrease in the first 3 months of lactation [35, 36]. It is argued that this decrease relates to the infant's changing requirements for growth and that it prevents overfeeding of AAs, as milk volume intake increases during this period [53, 54]. Not surprisingly, similar dynamics are found for the protein-bound AA content in HM. The extent by which the protein-bound form decreases during lactation is highly similar for each AA, indicating that the dynamics of protein-bound AAs in HM are not AA-specific [35]. In contrast, levels of FAAs in HM display dynamics during lactation that are highly AA-specific; while levels of some FAAs decrease in the first 3 months of lactation, that of others remain stable or sharply increase [35, 36]. Remarkably, these FAA dynamics appear to be consistent across studies in various ethnic groups and geographical regions, indicating that these dynamics are globally consistent and thus seemingly regulated [35, 36, 55, 56].

The underlying mechanisms regulating the dynamics of FAAs in HM are poorly understood. Cells of the mammary gland secrete proteases and anti-proteases into HM that together regulate the cleavage of AAs from HM proteins, thereby generating peptides and FAAs [57]. It can thus be hypothesized that temporal changes in net proteolytic activity in HM contribute to the FAA dynamics, though this is unlikely as levels of all major HM proteases and anti-proteases decrease during lactation, along with levels of their substrates [50, 58]. Mammary gland cells can also directly secrete FAAs into HM via AA transporters present on their cell membranes. Interestingly, animal studies have shown that the expression of certain AA transporters in the mammary gland increases with progressing lactation, while that of others remains unchanged [59-62]. These expression dynamics appear to be tightly controlled by intracellular signalling pathways [63]. Thus, it is plausible

that the changing expression of AA transporters in mammary gland cells along lactation contributes to the FAA dynamics in HM.

To better understand the secretion of FAAs in the mammary gland, several studies have examined the relation between maternal characteristics and FAA levels in HM. Whereas these levels seem to be independent of the mothers' age [64], maternal body-mass index (BMI), and more specifically obesity, may influence levels of several FAAs [65, 66]. Mechanisms underlying this potential relation are not understood but may involve the hormone prolactin, as prolactin regulates the expression of AA transporters in the lactating mammary gland and prolactin secretion is closely related to maternal BMI [67-69]. Studies investigating the relation between maternal diet and the AA content in HM indicate that the TAA composition in HM is largely independent of dietary AA intakes [70, 71]. For FAAs, this relation remains to be examined in humans. However, as studies across different geographical locations where different diets are consumed show largely similar levels and ratios of FAAs in HM, it is likely that maternal diet is not of major influence [35, 36, 55, 56]. This is supported by the finding that oral glutamate supplementation to lactating women did not alter levels of any FAA in their breastmilk [72], and is further endorsed by the absence of associations between FAA levels in plasma of lactating women and the FAA levels in their breastmilk [73, 74]. In fact, levels of some FAAs are up to 15-fold higher in plasma compared to HM, while the level of free glutamate is 40-fold higher in HM.

All together, these findings indicate that selective FAA transport occurs in lactating mammary tissues, and that levels of FAAs in HM might be strictly regulated over the course of lactation.

Correlations of human milk free amino acids with lactation stage and infant anthropometrics: a special role for glutamine and glutamate?

The FAAs glutamine, glutamate, glycine, serine and threonine in HM have consistently been shown to increase in the first months of lactation, whereas levels of most other FAAs remain relatively stable [35, 36, 55, 56]. Of these FAAs, glutamate is by far the most abundant, accounting for up to 50% of the total FAA content in HM. In addition, glutamate shows the highest absolute increase in concentration along lactation, increasing from approximately 1.25 mM to 1.75 mM from month 1 to 6 of lactation [35]. Glutamine, the second-most abundant FAA in HM, shows the highest relative increase in concentration, increasing almost 350% from month 1 to 6 of lactation and reaching a concentration of up to 0.6 mM [35, 64, 75].

To date, only two studies have examined the relation between FAAs in HM and infant anthropometrics, both of which included only a limited number of mother-infant pairs [65, 76]. Despite this, the findings reported are largely consistent. Baldeon *et al.* reported that levels of free glutamate and glutamine were higher in HM intended for infants that had faster weight gain [76]. Consistently, Larnkjaer *et al.* found a positive association between free glutamine levels in HM and infant length gain in the first 4 months of life [65]. These findings are in line with studies showing that milk for boys tends to contain higher levels of free glutamine and glutamate than milk for girls in the first 3 to 4 months of lactation [35, 76], a period during which boys are known to gain more weight and length than girls [77].

The observation that levels of free glutamine and glutamate in HM are relatively high, display unique dynamics along lactation, and may be associated with infant anthropometrics urges the need to understand the functions that these FAAs could have during infant development.

THE DIVERSITY IN PHYSIOLOGICAL FUNCTIONS OF FREE GLUTAMINE AND GLUTAMATE

In the last decade, it has been increasingly recognized that glutamine and glutamate are essential AAs at key times in life, including the neonatal period when rapid growth occurs [78, 79]. Although these FAAs are structurally related, they appear to be different in terms of absorption by the infant. This is evidenced by the finding that oral glutamine supplementation in infants leads to a slight but evident increase in the plasma level of this AA [80, 81], whereas plasma levels of glutamate are unaffected by oral glutamate supplementation [82, 83]. This suggests that free glutamate in HM is almost entirely used by splanchnic tissues, whereas free glutamine might also exert effects elsewhere in the body. Despite these differences, most of the dietary free glutamine and glutamate provided to neonates is shown to be absorbed and used by the intestines [84, 85]. The intestines are not only important for food digestion and absorption, but are also home to the largest immune organ of the body (*i.e.* the gut-associated lymphoid tissue (GALT)) and play vital roles in the protection against infections and allergies, particularly food allergies. Interestingly, glutamine and glutamate have been shown to exert a wide range of physiological effects within developing intestines and cells derived thereof. These include both growth- and immune-related effects, many of which could be relevant for an optimal protection against infections and (food) allergies. These effects are described in detail below and are summarized in *Figure 1*.

Metabolism of glutamine and glutamate in intestinal epithelial cells and immune cells: their function as energy substrates and protein precursors

It is well known that glutamine and glutamate are important energy substrates for intestinal epithelial cells (IECs) and immune cells, especially during periods of rapid growth [86]. In fact, studies in young animals and infants have shown that approximately half of the dietary glutamate and glutamine is oxidized by intestinal cells, ultimately leading to the generation of energy for these cells to adequately function and grow [87]. Glutamine is extensively converted into glutamate within the intestines, which is crucial for the usage of glutamine for energy purposes [88]. The ability to convert glutamate into glutamine, which is the main pathway of *de novo* synthesis of glutamine, is however highly limited in the intestines due to low glutamine synthetase activity [89, 90]. In early life, this ability may be further diminished as intestinal glutamine synthetase activity appears to be particularly low in the pre-weaning period [91, 92]. Given that IECs and immune cells cannot function properly without sufficient availability of free glutamine [93], it is plausible that adequate functioning of these cells in early life might be partially dependent on dietary-derived glutamine.

Besides serving as energy substrates, free glutamine and glutamate are essential precursors for glutathione, which is the main antioxidant of IECs and immune cells and important for the protection against intestinal oxidative stress [94]. In line with this, dietary supplementation of free glutamine and glutamate enhanced glutathione production and reduced oxidative stress in the intestines of piglets [95, 96]. Oxidative stress is indicated to play an important role in the development and maintenance of allergic disorders, including but not limited to food allergies [97-99]. It is not surprisingly therefore that dietary supplementation of antioxidants, or of components that enhance the production thereof, is considered a promising strategy for allergy prevention [99]. In addition to being a precursor for glutathione, glutamine, but not glutamate, is an important precursor for the synthesis of N-acetylglucosamine and N-acetylgalactosamine, which play critical roles in intestinal mucin synthesis. Accordingly, oral glutamine supplementation enhanced the production of secretory mucins and increased the number of mucin-secreting goblet cells in the small intestine of healthy piglets and young mice [100, 101]. Granted that mucins play pivotal roles in the prevention of microbial invasion, these effects of glutamine may be favourable in the protection against intestinal infections [102-104].

Effects of glutamine and glutamate on intestinal growth and barrier function

In the rapidly growing neonate, it is crucial to achieve and maintain rapid growth of IECs to ensure optimal intestinal development and functionality. One of the intestinal functions that is critical for the protection against infections and food allergic sensitization is the barrier function, as it prevents allergen and bacterial translocation from the gut lumen into the immune cell-populated lamina propria and mesenteric lymph nodes (MLNs) [105-107]. In neonates, which are characterized by an immature intestinal barrier with a high permeability, proper availability of nutrients and other factors that contribute to the growth of IECs and to the maturation or functionality of the intestinal barrier is critical to support this protective effect. It is well known that specific dietary AAs can influence these processes. Among these AAs are free glutamine and glutamate, as described in detail below.

Impact of glutamine on intestinal functions

Glutamine is the most widely examined AA in relation to the growth and functionality of IECs. This FAA is able to stimulate IEC proliferation through multiple mechanisms, as shown by numerous *in vitro* experiments. For instance, glutamine dose-dependently enhanced cell proliferation and differentiation of neonatal porcine and rat IECs through activating multiple mitogen-activated protein kinases (MAPKs) [108-110]. Glutamine can also promote IEC growth through augmenting the effects of growth factors, such as insulin-like growth factor 1 and epidermal growth factor, as demonstrated in neonatal porcine and adult human IECs [109, 111-113]. Besides promoting growth, glutamine has also been shown to protect IECs against inflammation-, endotoxin- and oxidant-induced cell damage and cell death, in a highly dose-responsive manner [114-116]. Remarkably, glutamine completely blocked inflammation-induced apoptosis in the adult human IEC line HT-29 when supplied at 0.5 mM [115], indicating that glutamine could have a role in the preservation of intestinal health already at concentrations present in HM.

Multiple lines of *in vitro* evidence indicate that glutamine also plays an important role in the functioning of the intestinal barrier. For instance, studies in neonatal porcine and human adult IECs have shown that glutamine restriction reduces the expression of the major tight junction proteins, including claudin and occludin, which are vital proteins for proper intestinal barrier function [110, 117, 118]. This was accompanied by a reduced distribution of these proteins at the plasma membrane and an increase in paracellular permeability, indicative of diminished barrier functionality. Remarkably, glutamine supplementation in these *in vitro* models fully reversed these processes, suggesting that adequate availability of

free glutamine is essential for an optimal epithelial barrier function. These effects were mediated through enhanced AMP-activated protein kinase signalling and diminished PI3K/Akt signalling, indicating that glutamine supports intestinal barrier function via modulation of specific intracellular pathways [110, 118].

A role for glutamine in the preservation of intestinal health and in the promotion of intestinal growth and barrier function is further supported by the findings of studies in young animals. For instance, in piglets, dietary glutamine supplementation protected against *E.coli*- and endotoxin-induced intestinal damage [114, 119, 120]. Moreover, dietary deprivation of glutamine led to diminished intestinal health and barrier function in rat pups and piglets, among others through shortening of villi and breakdown of epithelial junctions [121, 122]. Conversely, dietary supplementation of glutamine has been consistently shown to increase intestinal villus height and tight junction protein expression, and to promote the epithelial barrier function in piglets [98, 123-125]. Consistent with the ability of dietary glutamine to promote the epithelial barrier, oral glutamine supplementation prevented bacterial translocation in various adult animal models of intestinal obstruction [126-131]. Whether dietary glutamine can also prevent bacterial translocation in neonatal animals, however, remains to be examined.

Impact of glutamate on intestinal functions

A growing body of evidence indicates that, next to glutamine, also glutamate can influence IEC growth and intestinal barrier function. This is among others demonstrated by a recent *in vitro* study in neonatal porcine IECs, in which glutamate dose-dependently enhanced cell proliferation and viability, and enhanced the barrier function through promoting tight junction protein expression [132]. The ability of glutamate to promote intestinal barrier function is also shown in adult human IECs, in which glutamate reduced phorbol-induced hyperpermeability [133]. Remarkably, these effects were observed at a glutamate concentration 3 times lower than that in HM, highlighting the potency of free glutamate in HM to exert physiological effects in the developing intestines.

The capacity of glutamate to promote intestinal growth and functionality is also demonstrated *in vivo*, by various studies in piglets. In these studies, dietary glutamate supplementation enhanced the weight of the small intestine, increased the expression of cell division-related genes by IECs and enhanced overall intestinal health, as evidenced by a higher villus height and enhanced mucosal thickness and integrity [99, 124, 134]. Moreover, in mycotoxin-challenged piglets, dietary glutamate prevented impairments in intestinal morphology and barrier function [135], suggesting that, similar to glutamine, also

glutamate may play a role in the preservation of intestinal health and functionality upon exposure to harmful substances.

As glutamate can be converted into glutamine by IECs, although at limited rates, the observed effects of glutamate in the intestines may be attributable to the functions of glutamine. However, studies examining effects of both glutamine and glutamate within the intestines have found differential effects of these FAAs. For instance, piglets supplemented with dietary glutamine alone were found to have higher villi than piglets supplemented with a combination of glutamate and glutamine, whereas the combination led to the deepest crypts [136]. Moreover, glutamine was found to have protective effects against oxidant- and endotoxin-induced death of porcine neonatal IECs *in vitro*, whereas glutamate had no effect [114]. This suggests that the effects of glutamate are not solely exerted through conversion into glutamine.

Effects of glutamine and glutamate on immune cell functions

In addition to IECs, the immune cells of the GALT also play a crucial role in the prevention of (food) allergies and infections. During early life, immunity in the GALT tends to be biased towards T-helper 2 (TH2) responses, partly due to the TH2-biased immune milieu in the fetoplacental unit [137, 138]. In contrast, T-helper 1 (TH1) immunity is highly limited and gradually develops postnatally [139, 140]. The resulting TH2-dominant intestinal immune milieu is known to increase the susceptibility to food allergic sensitization, whereas the minimal TH1 function correlates with the increased susceptibility of neonates to infections [140, 141]. Moreover, various processes relevant for maintaining intestinal immune homeostasis upon antigen exposure, such as IgA production and anti-inflammatory responses, have not been fully developed yet in infants, which may further contribute to the enhanced susceptibility to (food) allergic disease in early life [142-144]. Extensive research has revealed that immunity in the GALT is influenced to a considerable degree by early life nutrition [144, 145]. Thus, components in HM with the capacity to promote a more balanced intestinal TH1/TH2 immunity, or those that could contribute to the maintenance of intestinal immune homeostasis upon antigen exposure may play a mediating role in the protection against allergies and infections in infancy. Though not often investigated in relation to early life immunity, glutamine and glutamate are immunologically active components that have been associated with these immunomodulatory capacities, as substantiated below.

Impact of glutamine on immune cell functions

The importance of glutamine for the functionality of the immune system is well recognized. While *in vitro* studies using neonatal cells are lacking, numerous studies using adult cells have shown that immune cells fail to develop and function without adequate glutamine availability [146]. For instance, glutamine restriction impaired the growth and differentiation of human B and T cells [147] and diminished antigen presentation and phagocytotic capacities of macrophages and neutrophils [148, 149]. Conversely, glutamine supplementation dose-dependently enhanced phagocytotic capacities of human neutrophils [150, 151]. Consistent with the ability of glutamine to affect immune cell growth and differentiation *in vitro*, *in vivo* studies have shown that dietary glutamine supplementation modulates immune cell populations in developing intestines. Notably, in piglets, dietary glutamine significantly increased the number of intestinal macrophages, which have emerged as essential players in intestinal immune homeostasis through their antimicrobial activities [123, 152]. Moreover, dietary glutamine decreased the proportion of naïve T cells while increasing memory T cells in the MLN of piglets [153]. This shift is thought to be favourable for maintaining immune homeostasis during antigen exposure, as memory T cells have a shorter lag time to proliferate upon antigen stimulation and are less dependent on co-stimulation [154, 155]. Finally, dietary glutamine increased the number of IgA-secreting B cells in the small intestine of young mice [156] and enhanced in various young animals the levels of intestinal IgA [157-160]. As mentioned before, intestinal IgA contributes to the protection against food allergic sensitization and infectious diseases through allergen and pathogen clearance [25, 26]. Together, these findings highlight the potency of dietary free glutamine to shape intestinal immune cell populations in early life, in a manner that may influence antimicrobial and anti-allergic capacities.

A consistent body of evidence shows that glutamine can also exert anti-inflammatory effects within the intestines. *In vitro* studies showed that glutamine supplementation decreased the production of pro-inflammatory cytokines IL-6, IL-8 and/or TNFα, while increasing the production of immunoregulatory cytokine IL-10 in cultured human intestinal biopsies and in various activated adult human intestinal cells, including intra-epithelial lymphocytes (IELs), IECs and mast cells [161-164]. Similar findings have been reported in healthy piglets, in which dietary glutamine reduced levels of IL-8 and IL-1 in the small intestine, while increasing levels of IL-10 [125, 165]. Furthermore, in LPS-challenged piglets and infantile rats, dietary glutamine reduced intestinal expression of various inflammatory markers, including Toll-like receptor-4, NF-κB and TNFα, suggesting that glutamine might also have potent anti-inflammatory effects during immune-compromised conditions [114, 166].

Besides its anti-inflammatory properties, glutamine has also been associated with modulations in TH1 and TH2 immune responses, though data are relatively limited. It has

been shown that murine naïve T cells can differentiate into T_H2 cells under glutamine-restricted conditions, but not into functional T_H1 cells, suggesting that glutamine deprivation favours T_H2 differentiation [167]. Conversely, glutamine supplementation has been reported to enhance T_H1 and/or diminish T_H2 responses in various activated immune cells *in vitro*. For instance, glutamine increased the production of T_H1 cytokines IL-2 and IFNγ by activated murine intestinal IELs and human lymphocytes, while T_H2 cytokines were unaltered [168-171]. In activated human intestinal mast cells, glutamine did not alter the release of T_H1 chemokines, but reduced the release of T_H2 chemokine ligand 2 and leukotriene C4, which are both involved in the pathogenesis of allergic diseases [163, 172]. In line with these *in vitro* findings, dietary glutamine supplementation in healthy piglets lowered the production of T_H2 cytokine IL-4 and increased the IFNγ/IL-4 production ratio by MLN cells [153]. Similarly, dietary glutamine enhanced IFNγ expression and reduced IL-4 expression by IELs in septic mice [173]. Finally, dietary glutamine enhanced IL-2 expression by IELs in rabbits kits while inhibiting expression of IL-6, an inducer of T_H2 differentiation of naïve T cells [174, 175]. Together, these initial findings provide evidence for T_H1-polarizing effects of free glutamine in the intestines, which could possibly help in promoting a more balanced intestinal T_H1/T_H2 immunity in early life. Additional studies investigating glutamine in relation to T_H1/T_H2 immunity in young animals, and in a variety of clinical settings, are necessary to better understand this potential immunomodulatory capacity of glutamine.

Impact of glutamate on immune cell functions

Despite dietary glutamate being almost completely absorbed by the intestines, studies investigating the effects of glutamate on intestinal immune cells are lacking. Yet, receptors for glutamate are found on a variety of intestinal immune cells, including lymphocytes and dendritic cells, suggesting that glutamate has a role in intestinal immune cell functioning [176]. Studies in human peripheral T cells demonstrated that glutamate at low concentrations (<100 μM) dose-dependently increases the proliferative response of T cells to various stimuli [176, 177]. At higher concentrations (>1 mM), however, this effect reversed, indicating that glutamate tends to have immunosuppressive properties at higher concentrations [176, 178]. Accordingly, it is postulated that the high concentration of free glutamate in the intestinal micro-environment, which may reach the millimolar range, could prevent inappropriate responses to dietary antigens by exerting immunosuppressive effects on intestinal T cells [34, 178].

Besides regulating T cell proliferation, there is some evidence that glutamate is involved in regulating T_H1 cytokine production by T cells. Glutamate is released by dendritic cells during T cell interaction, where it has dual roles [179]. In cases of non-specific antigen

presentation, glutamate inhibits T cell activation, while during specific antigen presentation glutamate stimulates T cell proliferation and the production of IL-2 and IFNγ, potentiating a T_H1-polarized response [179]. This latter process depends on glutamate released from dendritic cells, but also on extracellular glutamate concentrations, suggesting that this process could potentially be influenced by dietary glutamate [179]. In line with this, glutamate supplementation of up to 1-2 mM, which is within the range of glutamate levels in HM, enhanced IL-2 and IFNγ secretion by activated adult human peripheral T cells *in vitro* [180]. When supplied at even higher concentrations (>5 mM), however, glutamate inhibited the secretion of these cytokines. Unfortunately, whether glutamate can exert similar modulations in neonatal intestinal T cells *in vitro* and *in vivo* has not yet been investigated. Nevertheless, the available data suggest an immunoregulating role for glutamate that is highly dependent on the immunological context and the concentration of glutamate. At levels present in HM, glutamate could be involved in promoting T_H1-polarized responses during antigen-specific T cell activation, which may help in the maintenance of oral tolerance, though this remains speculative due the lack of evidence in neonatal cells or animals.

Effects of glutamine and glutamate on the intestinal microbiota

In recent years, it has become apparent that the gut microbiota also play vital roles in tolerance induction to dietary antigens [181-183]. This is evidenced among others by clinical studies showing links between the microbiota composition in the neonatal period and the development of allergic diseases in infancy. For instance, a higher gut bacterial diversity in early life has been associated with a lower risk for various allergic diseases, including food allergy [184-187]. Moreover, infants with an increased colonization of *Firmicutes* and a decreased colonization of *Bacteroidetes* (corresponding to an increased *Firmicutes*-to-*Bacteroidetes* ratio), or a decreased colonization of *Bifidobacteria* are reported to be at increased risk of developing food allergies [188-191]. Mechanisms by which gut microbes modify the susceptibility to allergies are still poorly understood, but may involve modulation of T_H1 and T_H2 immunity through secreting bioactive metabolites [192, 193]. The colonization of gut microbiota in early life is shown to be influenced by feeding mode and breastfeeding duration [189]. Thus, HM components that shape the neonatal gut microbiota composition may play an active part in modifying the susceptibility to allergic sensitization. Although data are scarce, several studies have shown that dietary free glutamine and glutamate, among many other components present in HM, can modulate the abundance of gut bacteria associated with the protection against allergic diseases in infancy, as described below.

Impact of glutamine on the gut microbiota composition

A recent study has demonstrated that dietary glutamine supplementation increased the abundance of *Bifidobacteria* in the jejunum of young mice, while the abundance of *Firmicutes* and the *Firmicutes*-to-*Bacteroidetes* ratio was decreased in various intestinal sections of these mice [194]. Similar modulations in the *Firmicutes*-to-*Bacteroidetes* ratio have been reported in glutamine-supplemented adult pigs and human [195, 196]. Moreover, in weaning rabbits, dietary glutamine specifically reduced the intestinal abundance of *Clostridium spp*, of which colonization in early life has been associated with an increased risk of allergic diseases [197, 198]. The mechanisms underlying the effects of glutamine on the gut microbiota composition are poorly understood. It is postulated that glutamine supplementation regulates utilization and metabolism of a variety of AAs in a niche-specific manner, affecting the activity and number of specific microbes [157, 199].

Impact of glutamate on the gut microbiota composition

To our knowledge, only two studies have examined the effects of dietary glutamate on the gut microbiota composition to date, both of which used animals in their post-weaning phase. In pigs, dietary glutamate supplementation markedly enhanced the bacterial diversity in the intestinal flora [200]. Moreover, the glutamate-enriched diet decreased the *Firmicutes*-to-*Bacteroidetes* ratio in the ileum, though this effect was only seen when given in combination with a high fat diet and was not observed in other intestinal sections. Perhaps more interestingly, dietary glutamate specifically promoted the colonization of *Roseburia* and *Faecalibacterium prausnitzii* in pigs [200, 201]. The colonization of *Roseburia* in early life has been positively associated with the acquisition of tolerance to cow's milk [202], and *Faecalibacterium prausnitzii* has similarly been indicated to play a role in the prevention of food allergy [203-205]. These gut microbes are some of the main producers of the short-chain fatty acids butyrate and propionate. Accordingly, a glutamate-enriched diet increased colonic concentrations of these fatty acids in pigs [206]. Butyrate and propionate are immunologically active metabolites associated with the protection against food allergy in animal models [207-208], and, in line with this, a recent study found that high faecal levels of these fatty acids in early life associated with a decreased risk of various allergies in infancy, including food allergies and asthma [209].

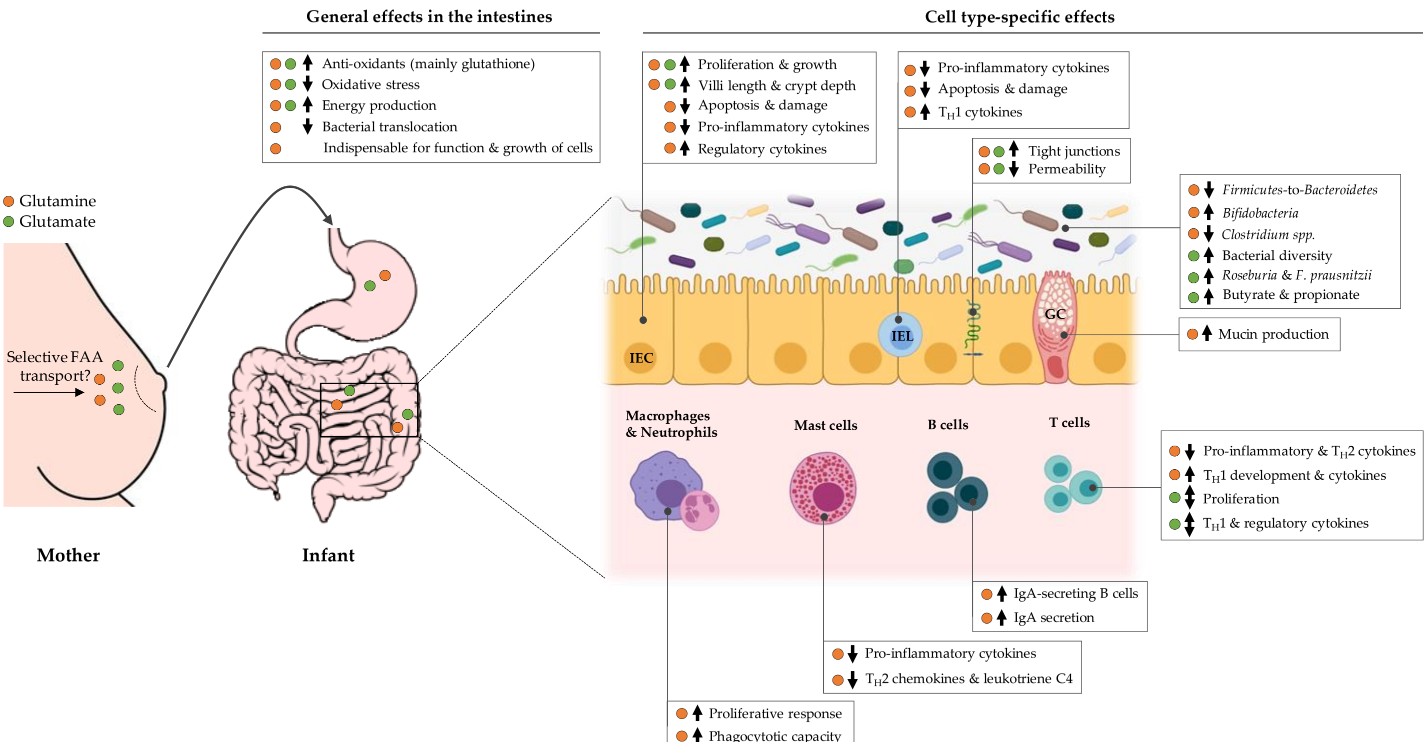

Figure 1. Overview of the potential effects of free glutamine and glutamate, selectively secreted in human milk by mammary gland cells, in the developing infant gut. The ↑ and ↓ indicate an upregulation and downregulation, respectively, of the corresponding target following in *vitro* and/or *in vivo* supplementation with glutamine (●) or glutamate (●). Effects are limited to those that are relevant in the context of allergic sensitisation and infections. FAA: free amino acid; IEC: intestinal epithelial cell; IEL: intraepithelial lymphocyte; GC: goblet cell; T_H1: T-helper 1 cell; T_H2: T-helper 2 cell; IgA: immunoglobulin A; *F. prausnitzii*: *Faecalibacterium prausnitzii*.

CONCLUDING REMARKS

The reported protective effects of breastfeeding against infections and certain atopic diseases in early life has sparked interest in understanding the factors in HM that could contribute to these effects. One of the factors that may be relevant in this perspective, but are typically overlooked as possible contributors to the health benefits of breastfeeding, are FAAs. As described, these AAs are more readily absorbed than protein-derived AAs and can be recognized by specific receptors present on a wide variety of cells. FAAs in HM display unique dynamics along lactation, which are seemingly globally consistent and thus likely to be strictly regulated, suggesting that FAAs in HM may have important functions in the developing infant. In this perspective, the FAAs glutamine and glutamate may be of particular interest, as they account for almost 70% of the FAA content in HM and their levels drastically increase in the first 3 months of lactation, indicating a need for an adequate intake of these FAAs in early life. In neonates, most of the dietary glutamine and glutamate is absorbed by the intestines. Studies in young animals and cells derived thereof demonstrate that these FAAs can have a wide range of physiological effects within developing intestines (*Figure 1*), already at concentrations present in HM. In short, these FAAs are reported to enhance the growth of intestinal epithelial cells, promote intestinal barrier function, influence intestinal immune cell populations, exert anti-inflammatory and potentially TH1-polarizing effects, and modify the abundance of gut bacteria that might play a role in the protection against allergic sensitization. Combined, these effects could possibly support neonates in the protection against infections and atopic diseases, particularly intestinal infections and food allergies.

Altogether, the findings described in this review warrant further research into the potential role of free glutamine and glutamate in HM in the protection against neonatal allergies and infections. Levels of free glutamine and glutamate, among many other HM bioactive factors, are considerably higher in HM than in standard IMFs, leading to significant differences in the intake of these FAAs between breastfed and formula-fed children [210-212]. As many of the effects of glutamine and glutamate described in this review were concentration-dependent, future studies should address whether this differential intake could contribute to the differential occurrence of immune-related conditions between formula-fed and breastfed children.

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Chapter 5

Free and total amino acids in human milk in relation to maternal and infant characteristics and infant health outcomes: the Ulm SPATZ Health Study

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ABSTRACT

Free amino acids (FAAs) are important regulators of key pathways necessary for growth, development and immunity. Data on FAAs in human milk (HM) and their roles in infant development are limited. We investigated the levels of FAAs and total amino acids (TAA, *i.e.* the sum of conjugated amino acids and FAAs) in HM in relation to infant and maternal characteristics and immunological conditions. FAA and TAA levels in HM sampled at 6 weeks (n = 671) and 6 months (n = 441) of lactation were determined using high-performance liquid chromatography. Child growth was ascertained at 4-5 weeks and at 6-7 months of age. Child allergy and lower respiratory tract infections were assessed in the first years of life. Associations of amino acid (AA) levels in HM with child growth and health outcomes were determined by Spearman correlation and modified Poisson regression, respectively. Free glutamine, glutamate and serine in 6-week HM positively correlated with infant weight gain in the first 4-5 weeks of age. Maternal pre-pregnancy weight and body mass index (BMI) were negatively correlated with free glutamine and asparagine in 6-week and 6-month HM and positively correlated with the sum of TAAs in 6-month HM, but significance was lost following confounder adjustment. Free glutamine was lower in 6-month HM of mothers with an allergy (either active or non-active). No consistent associations were found between FAAs in HM and child health outcomes. However, potential negative associations were observed between specific FAAs and the risk of food allergy. These results suggest that specific FAAs play a role in infant growth. Moreover, these findings warrant further investigations in the relation of FAAs in HM with infant health outcomes and maternal allergy.

INTRODUCTION

Human milk (HM) is the preferred source of nutrition during early infancy. It supports infant growth and development and provides protective effects against several immunological conditions in early life, including various infectious [1-3] and allergic diseases [4-7]. HM contains various bioactive components that could contribute to these abilities, such as specific proteins and peptides [8]. Besides these chains of conjugated amino acids (AAs), HM contains free amino acids (FAAs), which account for 4-10% of the total amino acid (TAA, *i.e.* the sum of FAAs and conjugated AAs) content in HM [9, 10]. An adequate AA intake in early life is recognized to be critical for an optimal infant growth and health. However, the current recommended AA intakes are based mostly on the average TAA intake of breastfed infants, without making a distinction between conjugated AAs and FAAs. Absorption kinetics for FAAs and conjugated AAs are different and, in contrast to conjugated AAs, FAAs can be readily recognized by receptors present on a wide variety of cells. Hence, FAAs may serve different functions in the neonate than conjugated AAs. Thus, it is of great interest to separately investigate levels of FAAs and TAAs supplied through HM and their associations with infant growth and health. These data could be used for enhancing other infant feeding regimens such as standard infant milk formulas, which currently contain 10% or less of the FAAs present in HM.

Levels of FAAs in HM are dynamic, with levels of some FAAs increasing while others decrease during the first months of lactation [9-11]. These dynamics are consistent in studies across various ethnic groups and geographical locations, suggesting that FAA levels in HM are regulated and that FAAs might have physiological roles in infant development. In support, specific FAAs have been shown to exert profound physiological effects *in vitro* and in young animals [12-17]. These effects range from the promotion of intestinal development and overall growth to the direct and indirect modification of immunological mechanisms relevant for the protection against allergic sensitization and infections. Based on these findings, it can be hypothesized that FAAs in HM may play a role in infant growth, as well as in shaping defensive mechanisms against early life immunological conditions.

To date, no studies are available on the relation between FAAs in HM and immunological conditions in early life. A few studies have examined associations of FAAs in HM with infant characteristics and have found some evidence for associations of specific FAAs with infant growth [18, 19], as well as with infant sex [10, 18]. However, these studies had less than 80 mother-infant pairs, tested associations only at one timepoint during lactation, did not strictly control sample collection times and methods and/or did not consider maternal characteristics as potential confounding factors. To overcome these research limitations, we investigated associations of infant anthropometrics, infant sex, and maternal characteristics

with FAA levels in HM samples, collected in a strictly controlled sampling procedure at 6 weeks and 6 months postdelivery. Importantly, similar associations were investigated for TAA levels of each AA. These outcomes were compared to the results obtained for FAAs to evaluate whether such associations were simply AA-specific rather than determined by the AA being a FAA. Moreover, this study investigated for the first time whether FAAs in HM could be associated with the occurrence of several commonly occurring immune-related conditions in early life, including atopic dermatitis, wheeze, lower respiratory tract infections and food allergy.

MATERIAL AND METHODS

Study design and population

Data were obtained from the Ulm SPATZ Health Study, an ongoing birth cohort study which, at baseline, included 1006 newborns of 970 mothers (49% of all 1999 eligible families). Details are described elsewhere [20]. Mothers were recruited from the general population shortly after delivery in the University Medical Center Ulm, southern Germany in the period 04/2012 – 05/2013. Exclusion criteria were outpatient delivery, maternal age <18 years, transfer of the newborn or the mother to intensive care immediately after delivery, stillbirth and/or insufficient knowledge of the German language. The ethics board of Ulm University (No. 311/11) approved the Ulm SPATZ Health study, and all mothers gave written informed consent before participating.

Human milk sample collection

HM samples were collected at 6 weeks and 6 months postdelivery from mothers who were still actively breastfeeding at the time and willing to provide a HM sample. Mothers were instructed to manually express or pump the first ~10 mL of HM (*i.e.* foremilk) between 9am and 12pm, after breakfast and before lunch but at least one hour after the last feeding. Mothers stored the HM samples in the refrigerator until study nurses collected the samples on the same day or the day after and delivered them to the study centre. Milk samples were then aliquoted and frozen at -80°C.

Amino acid analyses

HM samples were stored at -80°C until analysis of AAs in 2019. Levels of FAAs (*i.e.* unbound AAs) and TAAs (*i.e.* unbound + conjugated AAs) were analysed by liquid chromatography as described in detail elsewhere [10]. This method omits the detection of proline and cysteine, yielding a total of 18 detectable FAAs and the non-coded AAs taurine, ornithine and citrulline. The measurements of TAAs required acidic hydrolysis of proteins,

which enabled the detection of 15 TAAs and disabled the detection of tryptophan, cysteine, and proline. The acidic hydrolysis process also transformed asparagine into aspartate (combined referred to as Asx) and glutamine into glutamate (combined referred to as Glx), disabling the detection of these TAAs individually. All measurements were done in the same lab and by the same technician in blinded fashion, thereby ruling out systematic bias in sample analysis and/or preparation.

Infant and maternal characteristics and anthropometric measurements

Infant and child body weight and length were obtained from examination documentation recorded during scheduled appointments at 4-5 weeks and 6-7 months of age. Infant birth weight and length were subtracted from these measurements to obtain infant weight gain and length gain, respectively, in the first 4-5 weeks and 6-7 months of age. Maternal pre-pregnancy weight was ascertained from paper-based records of the obstetrics visit at which pregnancy was established if this took place within the first 15 weeks of pregnancy, or was self-reported for mothers whose first appointment took place later in pregnancy. This information was used to calculate maternal body mass index (BMI; calculated as mass(kg) / height(m)2). Other demographic, lifestyle and birth-related data including infant sex, delivery mode, birth weight and length, maternal age, education and smoking status (within one year prior to delivery) were collected using self-administered standardized questionnaires. Maternal allergy was self-reported and classified as mothers who reported a history of hay fever, atopic dermatitis or asthma. These mothers could either have an active or a non-active allergy. Non-allergic mothers reported the absence of allergic disease.

Infant health outcomes

A reported doctor's diagnosis of wheeze in the past 12 months was assessed at 1 year, 2 years and at each yearly follow-up by self-administered questionnaires from parents. Wheezing phenotypes were classified into three categories: transient (*i.e.* report of wheeze at age 1 but not at age 2 and 3), intermediate (*i.e.* report of wheeze at age 2 but not age 1 and 3) and persistent (*i.e.* report of wheeze at age 1, 2 and 3). Atopic dermatitis (AD) was assessed by separate parent and paediatrician reports of doctor-diagnosed AD assessed at 1 year. AD diagnosis was classified as parent-reported (*i.e.* a positive report of doctor-diagnosed AD by the parents), paediatrician-reported (*i.e.* a positive report of doctor-diagnosed AD by the paediatrician) and parent- and paediatrician-reported (*i.e.* a positive report of doctor-diagnosed AD by both the paediatrician and the parents). Children with at least one positive report of AD were defined as cases in the relevant outcome category. A reported doctor's diagnosis of lower respiratory tract infections (including pneumonia, bronchitis, pertussis, tracheobronchitis, Krupp, bronchiolitis and flu) and food allergy was assessed at 1 year of age, by self-administered questionnaires from the children's primary care paediatricians.

Statistical analyses

Data were checked for normal distribution using Shapiro Wilk tests and visual inspection of histogram plots. Wilcoxon rank-sum tests were used to identify differences in AA levels between 6-week and 6-month HM samples. For this analysis, a subset of 6-week samples restricted to mothers with a 6-month sample was used. Principal component analysis (PCA) was used to evaluate correlations between individual FAAs in HM. Centered log ratio (CLR) transformation was applied to FAA data prior to PCA analysis to account for compositionality. CLR was calculated as the natural log of the quotient of concentrations of individual FAAs over the geometric mean of all FAA concentrations within a HM sample. Following PCA, a factor analysis was used to determine specific FAA patterns in HM. Visual inspection of the scree plot and the Kaiser criterion (eigenvalue ≥ 1) were used to determine the number of factors to be retained. Based on this, we retained factors with variable loadings ≥|0.5| [21]. Infant sex differences in AA levels in 6-week and 6-month HM samples were assessed by unpaired t-tests. Unbiased effect sizes (Hedges' *g*) of infant sex on AA levels in HM were calculated as described in detail elsewhere [22]. The potential impact of infant sex on changes of AA levels in HM between 6 weeks and 6 months of lactation were assessed by mixed model analyses, with subjects as random effect and infant sex and the difference in AA levels between 6-week and 6-month HM as fixed effects.

Associations of AAs with infant and maternal anthropometrics were assessed by Spearman correlation. Associations were further investigated using partial correlations that were adjusted for all maternal factors that were associated with levels of any FAA or TAA in 6-week or 6-month HM samples. Differences in AA levels in HM between mothers with or without an allergy (either active or non-active) were assessed by Welch's t-tests. Modified Poisson regression analyses [23] were used to estimate the effects of FAA levels in HM on infant health outcomes. Risk ratios (RR) were modelled with individual FAA levels as continuous independent variables. Models were adjusted for maternal pre-pregnancy BMI, maternal age, maternal education, maternal smoking status and infant sex as these putative confounders were associated with ≥ 5% change in the crude estimate of the effect of any individual FAA on any infant health outcome.

Bonferroni adjustment of the level of significance was applied to account for multiple testing, using an initial limit of $\alpha = 0.05$. We investigated 21 individual FAAs as well as the sum of FAAs and therefore the Bonferroni-adjusted level of significance for FAA data was α threshold = 0.05/22 = 0.002. A total of 15 individual TAAs were investigated as well as the sum of TAAs, hence the Bonferroni-adjusted level of significance for TAA data was α threshold = 0.05/16 = 0.003. As we consider the present study to be explorative, results with a *p*-value of < 0.01 but higher than the Bonferroni-adjusted level of significance were specifically indicated in tables and figures. All *p*-values are shown unadjusted for multiple

comparison. Statistical analyses were done using SAS (version 9.4, The SAS Institute, Cary, NC, USA) and R (version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria). Figures were generated using GraphPad Prism (version 7.03, GraphPad Software Inc., San Diego, CA, USA) and R.

RESULTS

Characteristics of participating mothers and their infants

A total of 741 and 483 mothers were actively breastfeeding at 6 weeks and 6 months postdelivery, respectively. HM samples for AA analyses were available from 671 (90.6% of breastfeeding mothers) and 441 (79.8% of breastfeeding mothers) lactating women at 6 weeks and 6 months of lactation, respectively. In total 411 lactating women provided a HM sample both at 6 weeks and at 6 months of lactation. The characteristics of lactating women and their infants included in the present study are shown in *Table 1*.

Table 1. Characteristics of the lactating women who had amino acid data available at 6 weeks and 6 months of lactation and their infants in the Ulm SPATZ Health Study

	All 6-week samples (n = 671)		All 6-month samples (n = 441)	
	n	% or Mean	n	% or Mean
Maternal characteristics				
Age (years)	671	33.1	441	33.5
Pre-pregnancy BMI				
Underweight (BMI <18.50)	15	2.3%	8	1.9%
Normal (BMI 18.50-24.99)	411	63.3%	274	64.6%
Overweight (BMI 25.00-29.99)	135	20.8%	88	20.8%
Obese (BMI ≥ 30.00)	88	13.6%	54	12.7%
Weight (kilograms)	617	70.5	417	69.9
Height (centimetres)	653	167.6	430	167.7
Maternal education				
Low	40	6.1%	14	3.2%
Intermediate	182	27.5%	104	24.0%
High	439	66.4%	316	72.8%
History of smoking (ever in life)				
Yes	288	43.2%	169	38.5%
No	378	56.8%	270	61.5%
Maternal Allergy (active or non-active)				
Yes	225	33.7%	166	37.7%
No	443	66.3%	274	62.3%
Exclusive breastfeeding				
Yes	502	74.8%	366	83.0%
No	169	25.2%	75	17.0%
Infant characteristics				
Sex				
Boys	354	52.8%	232	52.6%
Girls	317	47.2%	209	47.4%

Table 1 (continued).

Gestational age at birth				
Early (37-38 weeks)	239	35.6%	148	33.6%
Full (39-40 weeks)	352	52.5%	240	54.4%
Late (41-42 weeks)	80	11.9%	53	12.0%
Method of birth				
Vaginal spontaneous	455	67.9%	312	70.7%
Elective caesarean	67	10.0%	36	8.2%
Emergency caesarean	86	12.8%	52	11.8%
Vaginal assisted	62	9.3%	41	9.3%
Weight				
Weight at birth (grams)	670	3212.5	441	3250.1
Weight at 4-5 weeks (grams)	594	4365.5	416	4372.6
Weight at 6-7 months (grams)	554	7852.0	385	7849.1
Length				
Length at birth (centimetres)	669	51.1	440	51.1
Length at 6 weeks (centimetres)	592	62.9	416	62.9
Length at 6 months (centimetres)	553	68.8	384	68.9
Infant immunological conditions				
Atopic dermatitis (AD)				
Parent-reported diagnosis	44	7.9%	27	7.0%
Paediatrician-reported diagnosis	64	12.6%	37	10.5%
Parent- and paediatrician-reported diagnosis	35	6.2%	20	5.1%
Wheeze phenotypes				
Transient wheeze	47	12.1%	30	11.2%
Persistent wheeze	31	8.0%	18	6.7%
Intermediate wheeze	31	8.0%	27	10.0%
Lower respiratory tract infections	151	30.0%	108	31.0%
Food allergy	13	2.6%	7	2.0%

Note that sums may not add up to the total number of participants as percentages exclude missing data.

Amino acid levels in human milk at 6 weeks and 6 months of lactation

The mean levels of FAAs and TAAs in 6-week and 6-month HM samples are reported in *Table 2*. Wilcoxon rank-sum tests showed that of the 21 FAAs only levels of histidine, glycine, arginine and lysine were statistically similar at 6 weeks and 6 months of lactation (*Table 2*). Glutamine, aspartate and citrulline were prominently higher while valine and tyrosine were markedly lower (all $p < 0.001$) at 6 months of lactation. All of the individual TAAs were significantly lower at 6 months compared to 6 weeks of lactation (all $p < 0.001$).

The sum of FAAs accounted for 3.7% and 5.8% of the sum of TAAs in 6-week and 6-month HM, respectively (*Figure 1A* and *1C*). Large differences were observed between the relative contribution of FAA to TAA of individual AAs (*Figure 1B* and *1D*). The percentage of FAA to TAA was the highest for the combination of glutamine + glutamate (Glx), both in 6-week HM (11.5%) and 6-month HM (18.1%). Concentrations of free glutamine plus free glutamate accounted for approximately 56.3% and 61.5% of the sum of FAAs in 6-week and 6-month HM, respectively (*Figure 1A* and *1C*).

Compositional biplots from PCA were used to evaluate potential FAA patterns based on their correlational properties. Factor analyses suggested two FAA patterns in 6-week HM, defined based on factor loadings of ≥|0.5| (*Table S1, Figure S1A*). The first pattern was characterised by positive scores of histidine, alanine, glutamate, glycine, threonine, serine and citrulline and a negative score of ornithine. The second pattern comprised positive scores of lysine, leucine, phenylalanine and a negative score of ornithine. No distinct patterns were observed at 6 months of lactation (*Figure S1B*).

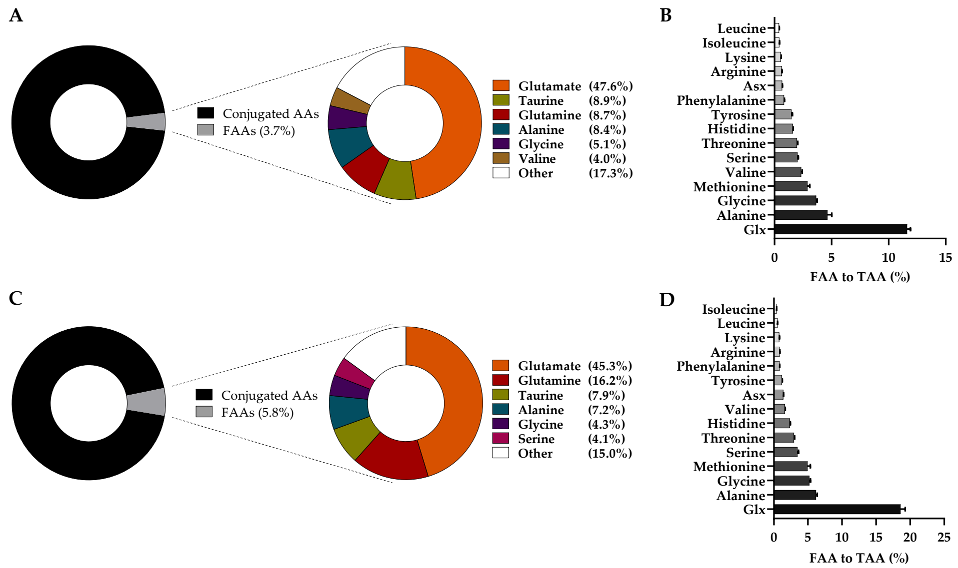

Figure 1. Relative abundance of individual free amino acids (FAAs) in human milk to the sum of FAAs and to total amino acid (TAA) levels of the corresponding amino acid in 6-week human milk (HM) (**A** and **B**, respectively) and 6-month HM (**C** and **D**, respectively). Error bars show the 95% CI of the means. AAs: amino acids; FAAs: free amino acids; TAA: total amino acid; Asx: asparagine + aspartate; Glx: glutamate + glutamine.

Table 2. Free amino acid and total amino acid levels in human milk sampled at 6 weeks and 6 months of lactation

AA	FAAs				TAAs			
	6 weeks (n = 671)	6 weeks (restricted) (n = 411)	6 months (n = 441)	*p*-value[1]	6 weeks (n = 671)	6 weeks (restricted) (n = 411)	6 months (n = 441)	*p*-value[2]
EAAs								
Histidine	28.2 (9.1)	28.0 (9.1)	29.7 (13.9)	0.944	1795.2 (264.3)	1803.3 (255.6)	1326.2 (201.4)	< 0.001*b*
Isoleucine	16.5 (10.3)	15.9 (9.8)	12.7 (5.9)	< 0.001*b*	4034.8 (599.5)	4094.8 (609.8)	3381.8 (461.0)	< 0.001*b*
Leucine	33.9 (17.8)	32.8 (15.7)	34.9 (11.4)	< 0.001*a*	8690.4 (1232.6)	8728.5 (1188.1)	6791.8 (922.6)	< 0.001*b*
Lysine	34.9 (24.1)	32.3 (20.4)	30.2 (15.0)	0.333	6359.2 (949.1)	6371.2 (895.8)	3963.3 (626.2)	< 0.001*b*
Methionine	27.8 (24.2)	29.5 (22.7)	34.6 (22.7)	< 0.001*a*	1010.6 (200.7)	1019.2 (208.4)	750.4 (220.2)	< 0.001*b*
Phenylalanine	21.4 (10.1)	21.3 (10.1)	16.5 (5.6)	< 0.001*b*	2610.9 (422.4)	2594.4 (382.6)	2057.7 (305.0)	< 0.001*b*
Threonine	71.0 (29.3)	70.8 (30.6)	97.1 (37.2)	< 0.001*a*	3707.5 (578.2)	3688.9 (552.9)	3367.3 (507.9)	< 0.001*b*
Tryptophan	14.6 (7.5)	14.5 (7.4)	29.0 (11.8)	< 0.001*a*	-	-	-	-
Valine	109.7 (58.2)	110.5 (54.9)	66.7 (27.4)	< 0.001*b*	4669.9 (755.3)	4678.7 (740.3)	4198.7 (628.1)	< 0.001*b*
NEAAs/conditionally EAAs								
Alanine	231.7 (64.8)	228.2 (62.6)	242.7 (72.2)	< 0.001*a*	5354.5 (988.8)	5294.8 (864.6)	3974.9 (639.3)	< 0.001*b*
Arginine	17.0 (11.3)	15.9 (9.5)	17.0 (6.4)	0.029	2924.9 (1048.9)	2925.1 (1006.3)	2141.1 (589.8)	< 0.001*b*
Asparagine	24.9 (14.9)	25.7 (14.8)	17.7 (8.6)	< 0.001*b*	8558.3 (1324.2)[3]	8527.1 (1221.1)[3]	6240.4 (932.3)[3]	< 0.001*b*
Aspartate	30.9 (18.2)	30.0 (17.4)	65.3 (39.1)	< 0.001*a*	-	-	-	-
Glutamate	1307.0 (410.3)	1268.8 (395.3)	1530.1 (328.5)	< 0.001*a*	13477.7 (1630.0)[4]	13533.9 (1589.8)[4]	11478.3 (1550.2)[4]	< 0.001*b*
Glutamine	239.0 (161.0)	249.9 (162.9)	548.5 (268.6)	< 0.001*a*	-	-	-	-
Glycine	139.1 (40.4)	138.7 (37.1)	146.3 (48.5)	0.056	3902.8 (709.3)	3837.5 (583.5)	2868.4 (516.4)	< 0.001*b*
Serine	100.2 (34.6)	100.0 (33.1)	137.8 (61.7)	< 0.001*a*	5120.4 (817.2)	5068.5 (719.1)	4094.4 (626.5)	< 0.001*b*
Tyrosine	27.5 (20.1)	28.0 (21.0)	17.5 (7.8)	< 0.001*b*	1897.6 (296.9)	1896.1 (281.8)	1506.3 (224.8)	< 0.001*b*
Non-coded AA								
Citrulline	10.7 (4.7)	10.7 (5.0)	20.8 (6.1)	< 0.001*a*	-	-	-	-
Ornithine	14.8 (16.7)	14.1 (15.1)	15.7 (27.0)	< 0.001*a*	-	-	-	-
Taurine	243.6 (93.1)	242.9 (94.3)	266.2 (106.1)	< 0.001*a*	-	-	-	-
Sum								
All AAs	2744.5 (645.1)	2708.5 (627.4)	3377.1 (676.4)	< 0.001*a*	74114.8 (11817.4)	74062.2 (11099.8)	58140.9 (8951.5)	< 0.001*b*

FAA: free amino acid; TAA: total amino acid; EAA: essential amino acid; NEAA: non-essential amino acid. Data are expressed as μmol/L and reported as mean (SD). Significant differences, assessed by Wilcoxon rank-sum tests, between amino acid levels at 6 weeks and at 6 months are defined as follows: *a* Increase from 6 weeks to 6 months; *b* decrease from 6 weeks to 6 months. For this comparison, the 6-month data was restricted to mothers who also provided a sample at 6 weeks ('6 weeks restricted'). [1] Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/22 = 0.002$; [2] Bonferroni adjusted level of statistical significance is $\alpha = 0.05/16 = 0.003$; [3] asparagine + aspartate; [4] glutamate + glutamine.

Infant sex differences in amino acid levels in human milk

HM for girls contained significantly higher levels of taurine at 6 weeks than HM for boys ($p < 0.001$; Hedges' $g = 0.23$; *Figure 2A*). This difference was also observed after adjustment for maternal pre-pregnancy BMI, maternal age and maternal allergy ($p = 0.004$), although the Bonferroni-adjusted level of significance was not reached (α threshold = 0.002). With regards to TAAs, HM for boys tended to contain higher levels of Glx (glutamine + glutamate) at 6 weeks ($p = 0.007$; Hedges' $g = 0.25$; *Figure 2C*), but statistical significance was lost following Bonferroni Correction (α threshold = 0.003). Absolute levels of each FAA and TAA in HM for boys and girls are shown in *Table S2A* and *S2B*.

At 6 weeks, all TAAs and 15 of the 21 measured FAAs were slightly higher in HM for boys (*Figure 2A* and *2C*). At 6 months, all TAAs and 18 of the 21 measured FAAs were slightly higher in HM for girls (*Figure 2B* and *2D*). Results from post-hoc mixed model analyses showed that the increase in the sum of FAAs from 6 weeks to 6 months of lactation tended to be higher for girls ($p = 0.091$). There were no infant sex differences in the changes of the sum of TAAs over lactation ($p = 0.185$).

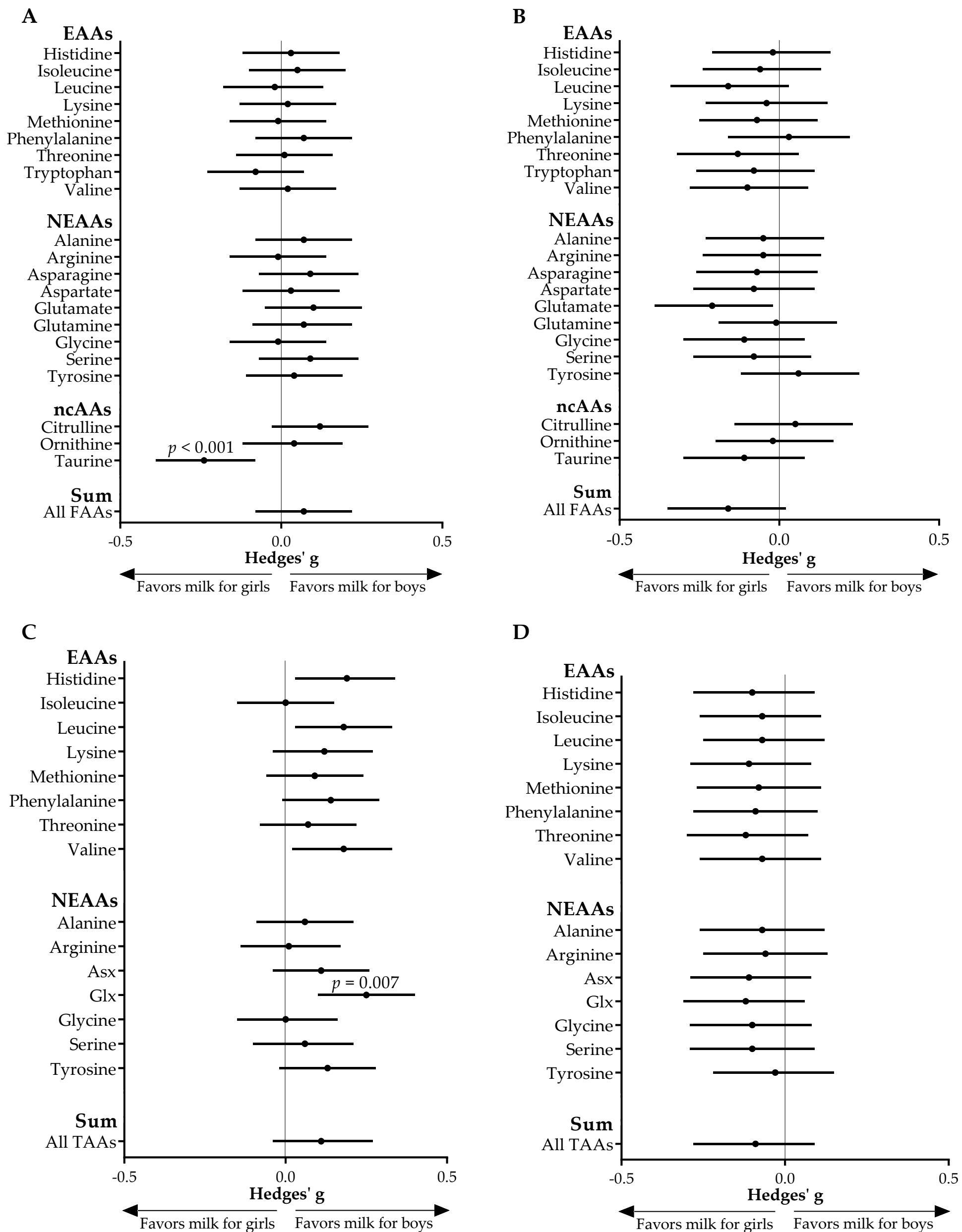

Figure 2. Forest plots indicating the effect sizes (Hedges' g and 95% CI) of infant sex on free amino acid (FAA) levels in 6-week human milk (HM) (n = 354 for boys, n = 317 for girls) **(A)** and 6-month HM (n = 232 for boys, n = 209 for girls) **(B)**, and on total amino acid (TAA) levels in 6-week HM **(C)** and 6-month HM **(D)**. Sex differences in amino acid levels in HM were assessed by unpaired t-tests. The Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/22 = 0.002$ for FAA data, and $\alpha = 0.05/16 = 0.003$ for TAA data. EAA: essential amino acid; NEAA: non-essential amino acid; ncAAs: non-coded amino acids; Asx: asparagine + aspartate; Glx: glutamate + glutamine.

Correlations between maternal characteristics and amino acids in human milk

Negative correlations were found between maternal pre-pregnancy weight and free glutamine and asparagine in 6-week HM (r = -0.118 and r = -0.124, respectively; $p \leq 0.002$) and in 6-month HM (r = -0.183 and r = -0.279, respectively; $p < 0.001$) (*Figure 3*, *Table S3A*). Similarly, maternal pre-pregnancy BMI negatively correlated with free glutamine and asparagine in 6-week HM (r = -0.151 and r = -0.142, respectively; $p \leq 0.001$) and 6-month HM (r = -0.235 and r = -0.328, respectively; $p < 0.001$) (*Figure 3*, *Table S3A*). These associations, however, lost significance after adjustment for infant sex, maternal age and maternal allergy (*Table S4*), except for the association of pre-pregnancy weight with free asparagine in 6-week HM (β = -0.089, $p = 0.031$). Moreover, a positive association was found between maternal age and free tryptophan in 6-month HM (r = 0.158, $p = 0.001$) (*Figure 3*). This association was also observed after adjusting for infant sex, maternal pre-pregnancy BMI and maternal allergy (β = 0.298, $p = 0.034$), but it did not reach the Bonferroni-adjusted level of significance (α threshold = 0.002).

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Maternal pre-pregnancy weight and BMI were positively correlated with the sum of TAAs (r = 0.145 and r = 0.143, respectively; $p \leq 0.003$) and with most individual TAAs in 6-month HM (*Figure 4*, *Table S3B*). However, statistical significance was lost after adjustment for infant sex, maternal age and maternal allergy (*Table S4*).

Levels of free glutamine were lower in 6-month HM of mothers with an allergy, compared to mothers without an allergy (mean difference = 81.1 μM, 95% CI: 30.0 – 132.3 μM; $p < 0.001$) (*Figure 5, Table S5A* and *S5B*). This difference was also observed after adjustment for pre-pregnancy BMI, maternal age and infant sex ($p = 0.006$), although statistical significance was lost after additionally adjusting for multiple testing (α threshold = 0.002).

Correlations between amino acids in human milk and infant anthropometrics

For FAAs, positive associations were found between infant weight gain in the first 4-5 weeks of life and free glutamate, glutamine and serine in 6-week HM (r = 0.123, r = 0.216, and r = 0.192, respectively; $p < 0.001$) (*Figure 3*, *Table S3C*). For serine and glutamine these positive associations were also observed after adjustment for infant sex, maternal pre-pregnancy BMI, maternal age and maternal allergy (β = 0.004, $p = 0.047$ and β = 0.015, $p = 0.005$, respectively) (*Table S4*), though these adjusted associations did not reach the Bonferroni-adjusted level of significance (α threshold = 0.002). In addition, free glutamine in 6-week HM tended to correlate with infant length gain in the first 4-5 weeks of life (r = 0.104, $p = 0.003$), also after adjusting for said confounders (β = 5.436, $p = 0.088$).

For TAAs, infant weight gain and length gain in the first 4-5 weeks of life was negatively associated with the sum of TAAs in 6-week HM (r = -0.152 and r = -0.132, respectively; $p < 0.001$) and with most individual TAAs (*Figure 4*, *Table S3D*). Following adjustment for infant sex, maternal pre-pregnancy BMI, maternal age and maternal allergy, the associations of the sum of TAAs in 6-week HM with infant weight and length gain remained significant (β = -0.212 and -8.568, respectively; $p \leq 0.003$), even after also adjusting for multiple testing (α threshold = 0.003).

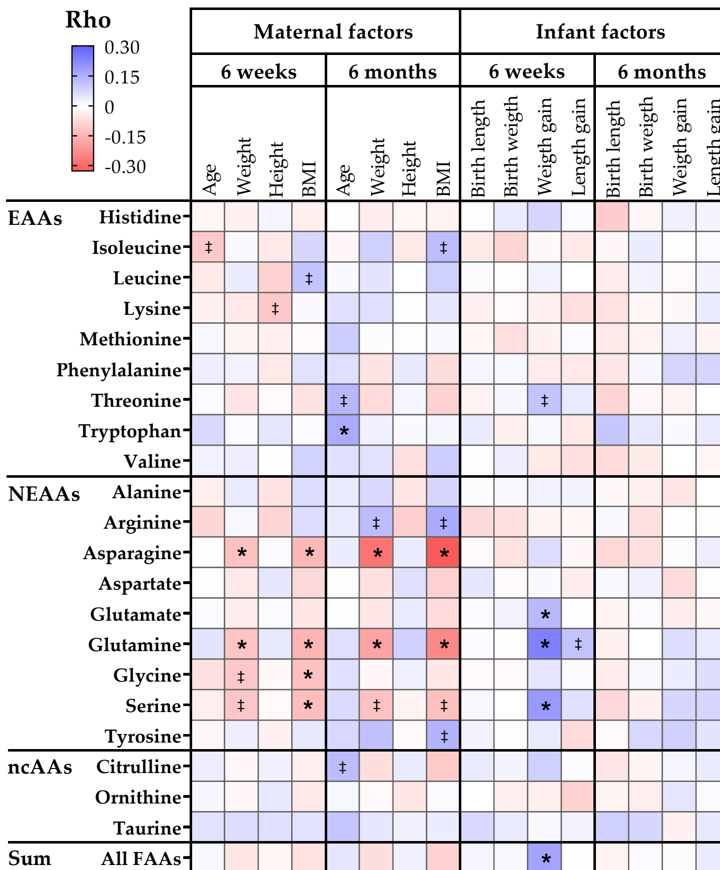

Figure 3. Heatmap of correlations of free amino acids (FAAs) in human milk with maternal and infant anthropometrics. Colouring reflects direction and magnitude of the Spearman correlation coefficients. EAA: essential amino acid; NEAA: non-essential amino acid; ncAA: non-coded amino acid; BMI: body mass index. ‡ $p < 0.01$, * *p*-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/22 = 0.002$.

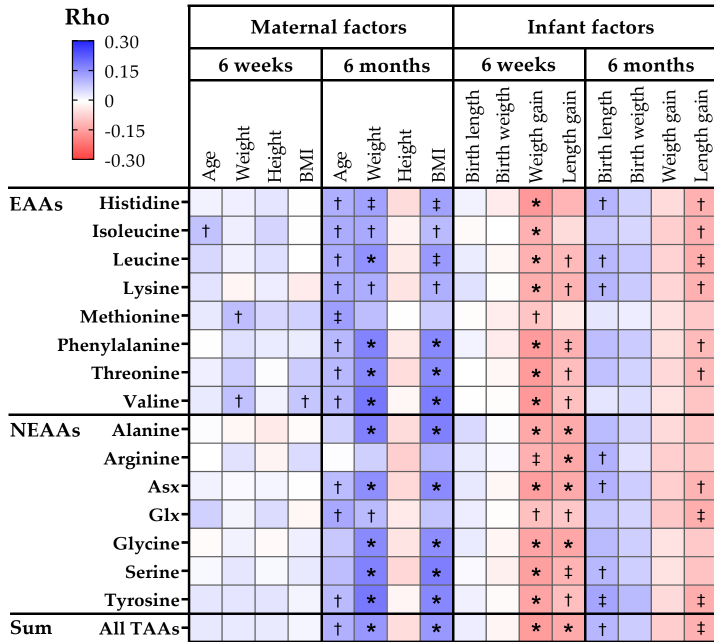

Figure 4. Heatmap of correlations of total amino acids (TAAs) in human milk with maternal and infant anthropometrics. Colouring reflects direction and magnitude of the Spearman correlation coefficients. EAA: essential amino acid; NEAA: non-essential amino acid; Glx: glutamate + glutamine; Asx: aspartate + asparagine; BMI: body mass index; † $p < 0.05$, ‡ $p < 0.01$, * p-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.003$.

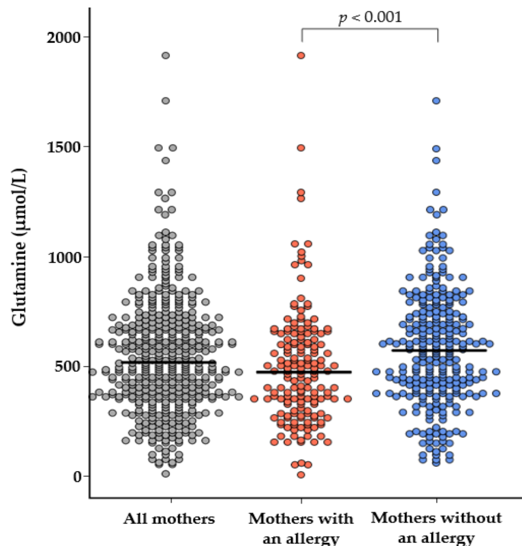

Figure 5. Levels of free glutamine in 6-month human milk samples from all mothers (n = 441) who provided a human milk sample and from mothers with (n = 166) or without (n = 274) an allergy (either active or non-active).

Associations of free amino acids in human milk and infant health outcomes

A higher level of asparagine in 6-week HM was associated with a lower risk of intermediate wheeze (RR = 0.95, 95% CI: 0.92 – 0.98; $p < 0.001$) (*Table S6A*). In 6-month HM, a higher level of histidine and lysine was associated with a higher risk of intermediate wheeze (both RR = 1.04, 95% CI: 1.02 – 1.06; $p < 0.002$). These associations were not consistent for other wheeze outcomes (*Table S6A*). In addition, a higher level of free asparagine in 6-week HM was associated with a lower risk of food allergy (RR = 0.70, 95% CI: 0.58 – 0.84; $p < 0.001$) (*Table S6C*). A higher level of arginine (RR = 0.92, 95% CI: 0.86 – 0.99; $p = 0.029$) and citrulline (RR = 0.85, 95% CI: 0.71 – 0.98; $p = 0.047$) in 6-week HM also associated with a lower risk of food allergy (*Table S6C*), but these associations lost statistical significance following Bonferroni correction (α threshold = 0.002). No significant associations were found for FAAs in HM with lower respiratory tract infections and AD outcomes (*Table S6B*).

DISCUSSION

The present study contributes to the understanding of AAs in HM and their potential association with infant growth and health outcomes. To our knowledge, this is the largest study to date investigating AA levels in HM in relation to infant characteristics, and the first to investigate this for FAAs and TAAs separately. Moreover, this is the first study to evaluate potential associations of FAAs with commonly occurring immunological conditions in the first years of life.

The present study showed that the FAAs glutamine and glutamate are highly abundant in HM, both relative to other FAAs and relative to their TAA levels. This may reflect the high free glutamine and glutamate needs associated with rapid cell division in neonates, particularly in the small intestines and its associated tissues [16, 24]. In line with previous studies [9, 10], we observed that levels of all individual TAAs were lower at 6 months compared to 6 weeks of lactation, while temporal changes of FAAs were AA-specific. However, we observed significant temporal changes of more FAAs in comparison to the previously reported [9-11] limited number of FAAs. This inconsistency could be due to the larger sample size of the present study, which allows to detect smaller changes, or to differences in the sample collection times and intervals. Interestingly, we observed temporal changes in the levels of the non-coded AAs citrulline and ornithine which, to our knowledge, have not been reported previously. These changes were most prominent for citrulline, of which levels doubled from 6 weeks to 6 months of lactation. As citrulline is a potent immunomodulator and shown to beneficially influence intestinal morphology and barrier function when supplied via the diet [25], this apparent temporal regulation of

citrulline secretion in HM could be important for early life intestinal and immune development.

Our results further demonstrated that some FAAs in 6-week HM could be combined into two groups based on correlational properties. Remarkably, the standard FAAs of one group were all glucogenic AAs, whereas the standard FAAs of the other group were all ketogenic AAs. Glucogenic and ketogenic AAs differ in their catabolic pathways. Glucogenic AAs can be converted into glucose, whereas ketogenic AAs can be converted into ketone bodies, which are both crucial metabolites for normal growth and development in the neonatal period [26, 27]. The clustering of these FAAs in HM may suggest a mechanism of selective FAA secretion in HM to ensure adequate glucogenic and ketogenic AAs during this critical period of growth. However, these clusters may only be relevant in early stages of lactation, as factor analysis did not identify these same clusters in 6-month HM.

Besides the stage of lactation, various other factors have been indicated to possibly influence the AA composition in HM, including infant sex. Results from the present study suggest potential sex differences in the temporal changes of the sum of FAAs in HM over lactation. In line with our findings, previous studies indicated that levels of most FAAs were slightly higher in HM for boys in the first 3 months of lactation, but not at later stages of lactation [10, 18]. These results call for replication in larger studies with a smaller time interval of longitudinally collected HM samples over lactation, to better represent the time course. In addition, the present study showed significantly higher levels of taurine in HM for girls at 6 weeks of lactation. At 6 months, taurine levels also tended to be higher in HM for girls. As infants have relatively low capacity to synthesize taurine, dietary intake of this AA in early life is considered essential for normal development [28]. Taurine intake though HM has been associated with perinatal neurodevelopment [29] and with the production of taurine-conjugated bile acids in infants [28, 30]. These bile acids appear to be important regulators of the gut microbiota composition, which is reported to be different for male and female infants [31, 32]. Whether the observed sex differences in taurine levels in HM can contribute to such physiological differences between male and female infants warrants further investigation.

In line with previous findings [19, 33-36], we observed that maternal pre-pregnancy BMI and weight positively correlated with the sum of TAAs, indicative of the total protein content, and negatively correlated with free glutamine and asparagine in HM. Though the direction of these associations did not change following adjustments for confounders, statistical significance was lost. This suggests that these associations may not be solely driven by maternal pre-pregnancy BMI and weight. Interestingly, we observed significantly lower levels of free glutamine in 6-month HM samples of mothers with an allergy. During

inflammatory conditions, such as allergic inflammation, plasma and tissue levels of free glutamine significantly drop, possibly as a result of an increased demand for glutamine by the immune system [37-39]. This may provide a biological explanation for the observed lower glutamine content in HM of allergic mothers. Glutamine is known to have dose-dependent immunomodulating effects in neonatal animals, including effects that could support the protection against allergies [16, 40, 41]. Granted that maternal allergic disease history is considered a risk factor for allergy development in the offspring [42], future studies should address whether a lower intake of glutamine through HM in children of allergic mothers could contribute to this association. As we could not distinguish between mothers with an active or non-active allergy, further investigation of AAs in HM of mothers with an active allergy is warranted.

Free serine, glutamine and glutamate in 6-week HM were positively correlated with infant weight gain. Additionally, free glutamine in 6-week HM tended to be positively associated with infant length gain. Whereas associations with serine have not been previously reported, similar associations of glutamine and glutamate with infant weight and length gain have been reported in smaller studies [18, 19]. Our results are in line with the findings of studies in piglets, which show increased daily body weight gain following dietary supplementation of free glutamine, glutamate and serine [43-45]. Combined, these results suggest a causal relation between intake of these FAAs and weight gain during early life. Serine, glutamine and glutamate have been associated with the promotion of intestinal health, growth and development in various animals, often in a concentration-dependent manner [12, 16, 43, 44]. It is plausible that these FAAs in HM contribute to early life growth via these mechanisms. Contrary to the positive associations of specific FAAs with infant growth, most individual TAAs and the sum of TAAs negatively correlated with infant weight and length gain in the first 4-5 weeks of life. This is somewhat inconsistent with previous studies [46, 47], which have reported either positive associations of HM protein intake with infant weight and/or length gain in the first 3-6 months of life or no associations up to 12 months of age [48]. This inconsistency may be explained by differences in HM sample collection times throughout lactation.

We observed that associations of FAAs with infant and maternal factors were highly AA-specific, in contrast to associations of TAAs. Interestingly, for several AAs, including glutamine and serine, it was observed that the FAA variant positively associated with infant weight and/or length gain in the first 4-5 weeks of life, while their TAA variants revealed an inversed association. This suggests that FAAs may have different roles in infant growth and development than their conjugated forms. Therefore, it may be important to consider the combination of conjugated AAs and FAAs in defining an adequate AA intake in early life.

Newborns are susceptible to developing food allergies, among others due to an immature epithelial barrier function and a bias towards T-helper 2-type (T_H2) immune responses, which drive allergic sensitization. We observed a negative association between free asparagine in 6-week HM and the risk of food allergy. Although non-significant following correction for multiple testing, similar negative associations were suggested for free arginine and citrulline in 6-week HM. These three FAAs have been reported to beneficially affect the intestinal barrier function, and arginine and citrulline have also been shown to enhance the functions of regulatory T cells *in vivo*, which play vital roles in controlling T_H2-biased immune responses [49-51]. These effects can support tolerance induction to dietary antigens and thus could potentially explain the observed negative associations with the risk for food allergy. We also explored correlations of FAAs with asthma, but as asthma cannot be reliably diagnosed until the age of 6 years [52] the incidence was very low (n = 3) in this study. Nonetheless, negative correlations were found between the risk of asthma and citrulline levels in 6-week HM samples (RR = 0.64; 95% CI: 0.53 – 0.79; $p < 0.001$) and 6-month HM samples (RR = 0.57, 95% CI: 0.45 – 0.72; $p < 0.001$). This is interesting to note because citrulline has been used effectively to prevent and control asthma in both animals and human [53-55]. However, granted that a small number of infants were diagnosed with food allergy (n = 13) and supposedly diagnosed with asthma (n = 3) in the present study, these results should be interpreted with caution. Therefore, future studies including larger sample sizes of children with asthma and food allergy to investigate these associations more comprehensively are warranted.

The present study has several strengths and limitations. The strengths of this study include the relatively large study population and the strictly controlled protocol of HM sample collection, which reduced the potential effect of within-feed and circadian rhythm variations on FAA levels in HM [56, 57]. Another strength was that HM was the only source of AAs for most infants, as the majority of mothers exclusively fed HM at 6 weeks and 6 months postpartum (~75% and ~83%, respectively). Limitations of the current study include the lack of available information regarding the maternal diet and the lack of exclusion criteria concerning maternal chronic diseases and medication intake. These maternal factors can influence the HM composition and therefore may have confounded the results of the present study, though it remains to be elucidated whether these factors also influence the AA composition of HM. With regards to maternal diet, this influence may be limited as FAA and TAA levels in HM are highly similar in mothers across different geographical locations with varying dietary habits [9]. Another limitation is that our analytical method did not permit to quantify the concentration of cysteine in HM samples. This is unfortunate, as some studies indicate that cysteine has anti-oxidative and anti-inflammatory roles in neonates and thus may influence infant health outcomes [58, 59]. Further limitations include the relatively few wheeze, AD and food allergy cases, despite the large study population.

Therefore, the power of the present study to detect significant associations between levels of FAAs and clinical outcomes is limited. The application of Bonferroni correction also reduced the probability of finding a significant result based on the large number of tests performed. However, this adjustment for multiple testing also reduces the risk of making extreme inferences in observational or clinical research [60]. To increase the power to detect associations with infant health outcomes, future studies could opt for high-risk cohorts consisting only of children born to allergic mothers or first-degree family members with an allergy. Furthermore, other studies could benefit from using a higher sampling frequency to better represent the time course of AAs in HM and their associations with maternal and infant characteristics. Finally, as other HM components like fatty acids and oligosaccharides may also play a role in infant growth and development [61-63], future studies should focus on modelling the effects of multiple HM components simultaneously.

CONCLUSIONS

In summary, the present study showed that changes of FAAs in HM over lactation are AA-specific, in contrast to changes of TAAs. Positive associations were observed between infant growth in the first 4-5 weeks of life and free glutamine, glutamate and serine in HM, while TAA levels of these AAs revealed an inversed association. No statistically significant associations were observed between AAs in HM and maternal anthropometrics following confounder adjustments. However, lower levels of free glutamine were observed in HM of mothers with an allergy (either active or non-active). This finding warrants further investigations of AA levels in HM of mothers with an active allergy. The present study observed no consistent associations between FAAs in HM and infant AD, wheeze and lower respiratory tract infection outcomes, but suggested potential negative associations between specific FAAs and the risk of food allergy. These associations need confirmation ideally in high-risk cohorts. Together, our findings support the hypothesis that FAAs in HM have physiological functions in early life. Moreover, our results suggest differential physiological effects of FAAs and conjugated AAs in early life, indicating that intake of an appropriate ratio of conjugated AAs and FAAs may be relevant for optimal infant feeding.

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SUPPLEMENTARY DATA

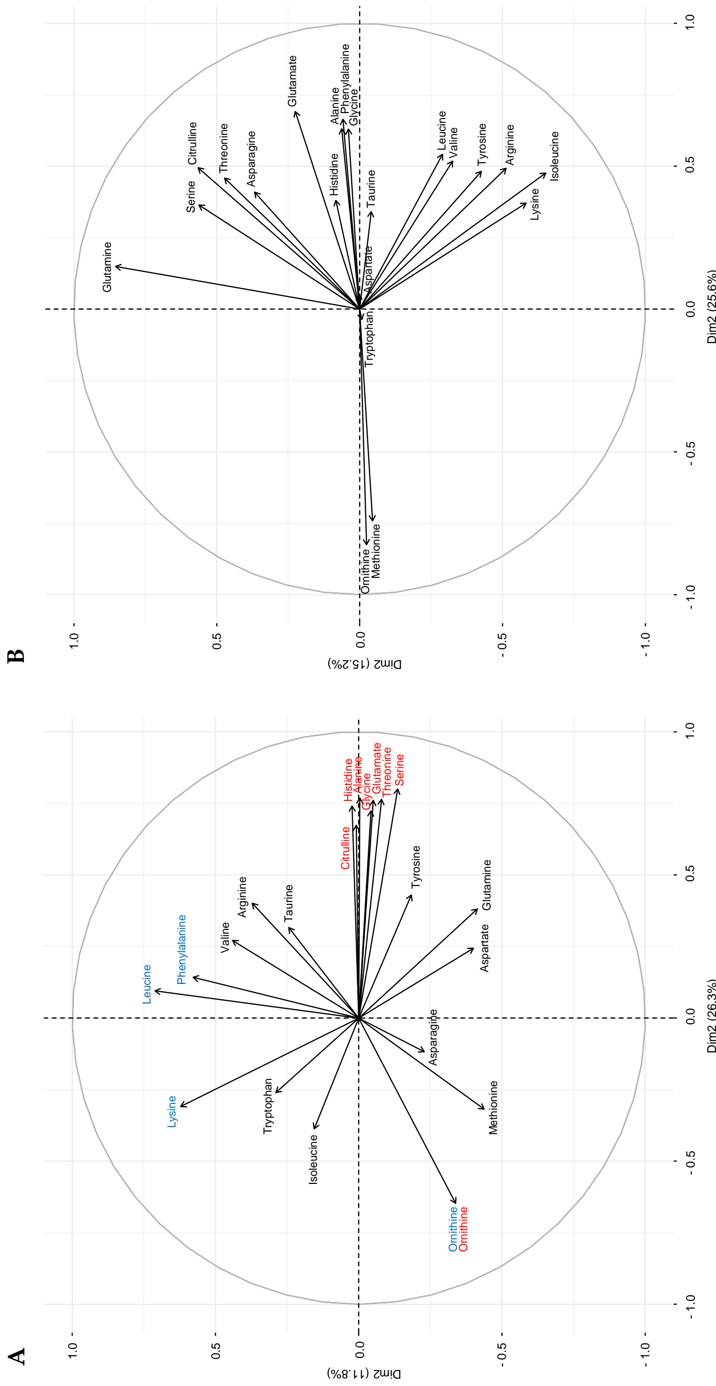

Figure S1. Principle component analysis (PCA). Compositional biplots from PCA of free amino acid (FAA) levels in 6-week human milk (HM) **(A)** and 6-month HM **(B)**. Patterns of FAAs as revealed by factor analyses are coloured (Pattern 1: red; pattern 2: blue).

Table S1. Free amino acid patterns in 6-week human milk obtained from factor analyses.

FAA	Factors loadings	
	Factor 1	Factor 2
EAAs		
Histidine	0.64	-
Isoleucine	-	-
Leucine	-	0.75
Lysine	-	0.51
Methionine	-	-
Phenylalanine	-	0.59
Threonine	0.76	-
Tryptophan	-	-
Valine	-	-
NEAAs/conditionally EAAs		
Alanine	0.66	-
Arginine	-	-
Asparagine	-	-
Aspartate	-	-
Glutamate	0.68	-
Glutamine	-	-
Glycine	0.66	-
Serine	0.83	-
Tyrosine	-	-
Non-coded AAs		
Citrulline	0.58	-
Ornithine	-0.54	-0.56
Taurine	-	-

FAA: free amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; AA: amino acid; HM: human milk. Values are factor loadings from principal component analysis. Only loadings < -0.50 and > 0.50 are shown.

Table S2A. Levels of free amino acids in human milk for boys and girls

FAA (μmol/L)	6 weeks Mean (SD)		6 months Mean (SD)		Analysis of differences at 6 weeks (*p*-value)	Analysis of differences at 6 months (*p*-value)
	HM for boys (n = 354)	HM for girls (n = 317)	HM for boys (n = 232)	HM for girls (n = 209)		
EAAs						
Histidine	28.4 (9.3)	28.1 (8.9)	29.6 (14.6)	29.9 (13.2)	0.661	0.397
Isoleucine	16.7 (10.5)	16.2 (10.1)	12.6 (6.4)	12.9 (5.3)	0.624	0.136
Leucine	33.7 (16.6)	34.2 (19.1)	34.0 (12.0)	35.8 (10.8)	0.930	0.038
Lysine	35.1 (25.2)	34.6 (22.8)	29.9 (16.3)	30.5 (13.6)	0.708	0.104
Methionine	27.7 (23.9)	28.0 (24.6)	33.8 (26.9)	35.5 (27.6)	0.875	0.588
Phenylalanine	21.7 (10.0)	21.0 (10.2)	16.5 (6.1)	16.4 (5.0)	0.283	0.829
Threonine	71.2 (29.7)	70.9 (29.0)	94.8 (38.0)	99.6 (36.3)	0.863	0.081
Tryptophan	14.3 (7.4)	14.9 (7.6)	28.6 (11.8)	29.5 (11.8)	0.381	0.227
Valine	110.4 (58.1)	109.0 (58.4)	65.4 (26.0)	68.1 (28.9)	0.710	0.277
NEAAs/conditionally EAAs						
Alanine	233.8 (65.1)	229.3 (64.5)	241.2 (75.0)	244.5 (69.0)	0.348	0.391
Arginine	17.0 (12.0)	17.1 (10.5)	16.9 (6.9)	17.2 (5.8)	0.468	0.169
Asparagine	25.6 (14.9)	24.3 (15.0)	17.4 (7.7)	18.0 (9.5)	0.153	0.998
Aspartate	31.1 (18.0)	30.6 (18.4)	63.8 (37.3)	66.9 (40.9)	0.551	0.537
Glutamate	1326.8 (415.7)	1284.9 (403.7)	1498.3 (320.6)	1565.3 (334.3)	0.196	0.023
Glutamine	244.0 (160.2)	233.5 (162.0)	547.9 (258.4)	549.2 (280.0)	0.244	0.713
Glycine	138.9 (41.3)	139.3 (39.5)	143.8 (49.9)	149.1 (47.0)	0.792	0.072
Serine	101.6 (34.0)	98.6 (35.3)	135.4 (62.7)	140.5 (60.7)	0.224	0.207
Tyrosine	27.9 (20.2)	27.0 (20.2)	17.7 (8.3)	17.2 (7.3)	0.297	0.760
Non-coded AAs						
Citrulline	11.0 (4.9)	10.4 (4.4)	20.9 (5.8)	20.6 (6.3)	0.199	0.300
Ornithine	15.1 (17.9)	14.4 (15.3)	15.5 (29.8)	16.0 (23.4)	0.563	0.176
Taurine	233.2 (90.3)	255.2 (95.0)	260.8 (108.3)	272.3 (103.7)	**< 0.001***	0.076
Sum						
All FAAs	2765.0 (642.1)	2721.5 (648.6)	3324.6 (652.9)	3435.1 (698.4)	0.353	0.126

FAA: free amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; AA: amino acid; HM: human milk. * *p*-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/22 = 0.002$. *P*-values < 0.002 are indicated in bold.

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Table S2B. Levels of total amino acids in human milk for boys and girls

TAA (μmol/L)	6 weeks Mean (SD)		6 months Mean (SD)		Analysis of differences at 6 weeks (*p*-value)	Analysis of differences at 6 months (*p*-value)
	HM for boys (n = 354)	HM for girls (n = 317)	HM for boys (n = 232)	HM for girls (n = 209)		
EAAs						
Histidine	1818.4 (279.1)	1769.3 (244.7)	1317.0 (191.0)	1336.3 (212.4)	0.054	0.592
Isoleucine	4087.7 (637.6)	4087.7 (549.0)	3365.5 (443.1)	3399.8 (480.4)	0.833	0.463
Leucine	8796.3 (1309.8)	8572.2 (1130.6)	6761.9 (889.3)	6824.8 (959.1)	0.056	0.563
Lysine	6411.4 (983.4)	6301.1 (907.3)	3931.2 (584.5)	3998.8 (668.7)	0.206	0.517
Methionine	1019.0 (217.1)	1001.2 (180.7)	742.1 (133.4)	759.6 (287.1)	0.455	0.831
Phenylalanine	2639.3 (449.1)	2579.2 (388.7)	2044.7 (292.6)	2072.0 (318.2)	0.140	0.446
Threonine	3726.5 (579.8)	3686.2 (576.6)	3339.1 (487.3)	3398.6 (529.1)	0.371	0.307
Valine	4732.5 (794.9)	4600.1 (703.2)	4176.7 (621.3)	4223.0 (636.2)	0.050	0.334
NEAAs/conditionally EAAs						
Alanine	5381.9 (980.8)	5323.9 (998.2)	3954.1 (628.6)	3997.8 (651.5)	0.525	0.561
Arginine	2932.3 (1054.6)	2916.6 (1044.0)	2124.3 (559.5)	2159.6 (622.4)	0.879	0.938
Asx	8629.1 (1366.4)	8479.1 (1272.9)	6192.6 (917.7)	6293.0 (947.6)	0.237	0.431
Glx	13667.2 (1730.6)	13266.1 (1484.2)	11387.5 (1603.9)	11578.4 (1485.9)	0.007‡	0.405
Glycine	3904.4 (695.2)	3901.1 (725.7)	2842.6 (505.1)	2896.8 (528.2)	0.945	0.336
Serine	5141.9 (814.7)	5096.2 (820.6)	4064.3 (605.2)	4127.5 (648.8)	0.517	0.367
Tyrosine	1916.1 (307.8)	1877.0 (283.2)	1502.6 (220.6)	1510.4 (229.8)	0.187	0.783
Sum						
All TAAs (mmol/L)	74.8 (12.2)	73.5 (11.3)	57.7 (8.7)	58.6 (9.2)	0.129	0.435

TAA: total amino acid; Glx: glutamate + glutamine; Asx: aspartate + asparagine; HM: human milk. ‡ $p < 0.01$. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.003$.

Table S3A. Spearman correlation coefficients between free amino acids in human milk and maternal anthropometrics

FAA	Maternal anthropometrics							
	Age		Weight		Height		BMI	
	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)
EAAs								
Histidine	-0.02 (-0.09; 0.06)	0.00 (-0.09; 0.10)	-0.03 (-0.10; 0.05)	-0.04 (-0.13; 0.06)	0.02 (-0.06; 0.09)	-0.02 (-0.11; 0.07)	-0.03 (-0.11; 0.04)	-0.03 (-0.12; 0.07)
Isoleucine	-0.11 (-0.18; -0.03)‡	-0.02 (-0.11; 0.08)	0.01 (-0.07; 0.09)	0.09 (-0.01; 0.18)	-0.05 (-0.12; 0.03)	-0.05 (-0.14; 0.05)	0.08 (-0.01; 0.16)	0.12 (0.03; 0.21)‡
Leucine	-0.04 (-0.12; 0.03)	0.01 (-0.08; 0.10)	0.03 (-0.04; 0.11)	0.06 (-0.04; 0.15)	-0.09 (-0.17; -0.02)	-0.01 (-0.10; 0.09)	0.11 (0.04; 0.19)‡	0.09 (-0.01; 0.18)
Lysine	-0.03 (-0.11; 0.05)	0.06 (-0.04; 0.15)	-0.04 (-0.12; 0.03)	0.06 (-0.03; 0.15)	-0.11 (-0.18; -0.04)‡	-0.02 (-0.12; 0.07)	0.01 (-0.07; 0.09)	0.05 (-0.03; 0.13)
Methionine	0.02 (-0.06; 0.09)	0.09 (0.00; 0.19)	-0.03 (-0.10; 0.05)	0.00 (-0.09; 0.10)	-0.03 (-0.11; 0.04)	0.00 (-0.09; 0.10)	-0.01 (-0.09; 0.07)	0.01 (-0.08; 0.11)
Phenylalanine	0.03 (-0.04; 0.11)	0.06 (-0.03; 0.15)	0.03 (-0.05; 0.10)	-0.06 (-0.15; 0.04)	-0.05 (-0.12; 0.03)	0.04 (-0.05; 0.14)	0.05 (-0.02; 0.13)	-0.07 (-0.16; 0.02)
Threonine	0.01 (-0.07; 0.08)	0.14 (0.04; 0.23)‡	-0.05 (-0.13; 0.02)	-0.11 (-0.20; -0.01)	-0.01 (-0.09; 0.07)	0.02 (-0.08; 0.11)	-0.06 (-0.13; 0.02)	-0.12 (-0.21; -0.03)
Tryptophan	0.07 (-0.01; 0.15)	**0.16 (0.07; 0.25)***	0.01 (-0.07; 0.08)	0.03 (-0.07; 0.12)	0.05 (-0.03; 0.12)	0.01 (-0.08; 0.10)	0.01 (-0.07; 0.08)	0.02 (-0.08; 0.11)
Valine	0.03 (-0.05; 0.10)	0.06 (-0.04; 0.15)	0.03 (-0.04; 0.10)	0.05 (-0.02; 0.12)	0.00 (-0.08; 0.07)	-0.07 (-0.16; 0.03)	0.08 (-0.01; 0.17)	0.09 (-0.02; 0.19)
NEAAs/conditionally EAAs								
Alanine	-0.03 (-0.11; 0.04)	0.04 (-0.06; 0.13)	0.04 (-0.04; 0.12)	0.07 (-0.02; 0.16)	-0.06 (-0.13; 0.02)	-0.05 (-0.14; 0.04)	0.07 (-0.01; 0.14)	0.08 (-0.02; 0.17)
Arginine	-0.08 (-0.16; -0.01)	0.04 (-0.06; 0.13)	0.02 (-0.06; 0.09)	0.13 (0.03; 0.22)‡	-0.09 (-0.16; -0.01)	-0.10 (-0.19; 0.00)	0.07 (-0.01; 0.14)	0.16 (0.06; 0.24)‡
Asparagine	0.00 (-0.08; 0.07)	0.04 (-0.06; 0.13)	**-0.12 (-0.20; -0.05)***	**-0.28 (-0.36; -0.19)***	0.01 (-0.07; 0.08)	0.03 (-0.06; 0.13)	**-0.14 (-0.22; -0.07)***	**-0.33 (-0.41; -0.24)***
Aspartate	0.00 (-0.08; 0.07)	-0.01 (-0.08; 0.07)	-0.05 (-0.12; 0.02)	-0.06 (-0.14; 0.02)	0.05 (-0.01; 0.11)	0.06 (0.00; 0.12)	-0.08 (-0.17; 0.02)	-0.10 (-0.20; 0.01)
Glutamate	0.01 (-0.07; 0.08)	0.00 (-0.09; 0.10)	-0.04 (-0.10; 0.02)	-0.05 (-0.12; 0.02)	0.01 (-0.04; 0.06)	0.04 (-0.03; 0.11)	-0.05 (-0.12; 0.03)	-0.08 (-0.18; 0.02)
Glutamine	0.05 (-0.03; 0.13)	0.06 (-0.04; 0.15)	**-0.12 (-0.19; -0.04)***	**-0.18 (-0.27; -0.09)***	0.04 (-0.03; 0.12)	0.09 (-0.01; 0.18)	**-0.15 (-0.22; -0.08)***	**-0.24 (-0.32; -0.15)***
Glycine	-0.06 (-0.14; 0.01)	0.06 (-0.04; 0.15)	-0.11 (-0.19; -0.04)‡	-0.02 (-0.11; 0.08)	-0.01 (-0.09; 0.06)	0.03 (-0.06; 0.12)	**-0.13 (-0.20; -0.05)***	-0.05 (-0.14; 0.05)
Serine	-0.03 (-0.11; 0.04)	0.07 (-0.03; 0.16)	-0.11 (-0.19; -0.04)‡	-0.12 (-0.21; -0.03)‡	-0.01 (-0.09; 0.06)	-0.03 (-0.12; 0.07)	**-0.13 (-0.20; -0.06)***	-0.13 (-0.22; -0.04)‡
Tyrosine	-0.02 (-0.09; 0.06)	0.07 (-0.02; 0.17)	0.03 (-0.05; 0.11)	0.12 (0.03; 0.21)	-0.03 (-0.10; 0.05)	-0.01 (-0.1; 0.08)	0.04 (-0.03; 0.12)	0.14 (0.05; 0.23)‡
Non-coded AAs								
Citrulline	0.03 (-0.04; 0.11)	0.13 (0.03; 0.22)‡	-0.02 (-0.09; 0.06)	-0.07 (-0.16; 0.02)	0.03 (-0.05; 0.10)	0.04 (-0.06; 0.13)	-0.03 (-0.11; 0.04)	-0.11 (-0.20; -0.01)
Ornithine	0.02 (-0.06; 0.09)	0.01 (-0.08; 0.11)	-0.02 (-0.09; 0.06)	-0.01 (-0.11; 0.08)	0.04 (-0.03; 0.12)	-0.05 (-0.14; 0.04)	-0.04 (-0.12; 0.03)	0.01 (-0.09; 0.10)
Taurine	0.05 (-0.02; 0.13)	0.11 (0.02; 0.20)	0.06 (-0.01; 0.14)	0.05 (-0.05; 0.14)	0.06 (-0.02; 0.14)	0.03 (-0.07; 0.12)	0.05 (-0.02; 0.13)	0.04 (-0.06; 0.13)
Sum								
All FAAs	0.01 (-0.06; 0.09)	0.05 (-0.05; 0.14)	-0.05 (-0.12; 0.03)	-0.07 (-0.17; 0.03)	-0.02 (-0.08; 0.05)	0.03 (-0.05; 0.10)	-0.06 (-0.14; 0.03)	-0.09 (-0.19; 0.02)

FAA: free amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; BMI: body mass index. ‡ $p < 0.01$, * p-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/22 = 0.002$. P-values < 0.002 are indicated in bold.

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Table S3B. Spearman correlation coefficients between total amino acids in human milk and maternal anthropometrics

TAA	Maternal anthropometrics							
	Age		Weight		Height		BMI	
	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)
EAAs								
Histidine	0.02 (-0.06; 0.09)	0.11 (0.02; 0.20)	0.02 (-0.05; 0.10)	0.13 (0.04; 0.22)‡	0.04 (-0.04; 0.11)	-0.06 (-0.15; 0.04)	0.00 (-0.08; 0.07)	0.13 (0.04; 0.22)‡
Isoleucine	0.08 (0.01; 0.16)	0.12 (0.02; 0.21)	0.02 (-0.05; 0.10)	0.12 (0.03; 0.21)	0.06 (-0.02; 0.14)	-0.02 (-0.11; 0.07)	0.00 (-0.08; 0.07)	0.10 (0.00; 0.19)
Leucine	0.05 (-0.02; 0.13)	0.12 (0.02; 0.21)	0.02 (-0.06; 0.10)	**0.15 (0.06; 0.24)***	0.04 (-0.03; 0.12)	-0.03 (-0.13; 0.06)	0.00 (-0.08; 0.08)	**0.14 (0.05; 0.23)***
Lysine	0.04 (-0.04; 0.12)	0.12 (0.02; 0.21)	-0.02 (-0.09; 0.06)	0.11 (0.02; 0.21)	0.02 (-0.05; 0.10)	-0.04 (-0.14; 0.05)	-0.03 (-0.11; 0.04)	0.11 (0.02; 0.20)
Methionine	0.03 (-0.05; 0.11)	0.14 (0.04; 0.23)‡	0.09 (0.01; 0.17)	0.09 (0.00; 0.18)	0.06 (-0.02; 0.13)	0.00 (-0.09; 0.09)	0.06 (-0.01; 0.14)	0.07 (-0.02; 0.16)
Phenylalanine	0.00 (-0.07; 0.08)	0.10 (0.01; 0.19)	0.04 (-0.03; 0.12)	**0.17 (0.08; 0.26)***	0.03 (-0.05; 0.10)	-0.04 (-0.13; 0.06)	0.03 (-0.05; 0.10)	**0.17 (0.07; 0.26)***
Threonine	0.02 (-0.06; 0.10)	0.10 (0.01; 0.19)	0.07 (-0.01; 0.14)	**0.16 (0.07; 0.25)***	0.00 (-0.07; 0.08)	-0.05 (-0.15; 0.04)	0.07 (-0.01; 0.15)	**0.16 (0.07; 0.25)***
Valine	0.03 (-0.05; 0.10)	0.10 (0.01; 0.19)	0.09 (0.01; 0.16)	**0.19 (0.10; 0.28)***	0.02 (-0.06; 0.09)	-0.02 (-0.11; 0.08)	0.08 (0.00; 0.15)‡	**0.18 (0.09; 0.27)***
NEAAs/conditionally EAAs								
Alanine	0.00 (-0.07; 0.08)	0.06 (-0.03; 0.16)	-0.01 (-0.09; 0.06)	**0.18 (0.08; 0.27)***	-0.04 (-0.11; 0.04)	-0.05 (-0.15; 0.04)	-0.01 (-0.08; 0.07)	**0.18 (0.09; 0.27)***
Arginine	0.00 (-0.08; 0.08)	0.00 (-0.09; 0.10)	0.04 (-0.04; 0.12)	0.07 (-0.03; 0.16)	-0.02 (-0.09; 0.06)	-0.07 (-0.17; 0.02)	0.05 (-0.03; 0.12)	0.10 (0.00; 0.19)
Asx	0.02 (-0.06; 0.09)	0.09 (0.00; 0.19)	0.01 (-0.07; 0.08)	**0.16 (0.07; 0.25)***	0.01 (-0.06; 0.09)	-0.06 (-0.15; 0.03)	0.00 (-0.08; 0.07)	**0.16 (0.07; 0.25)***
Glx	0.06 (-0.01; 0.14)	0.12 (0.03; 0.21)	0.01 (-0.06; 0.09)	0.10 (0.00; 0.19)	0.05 (-0.03; 0.12)	-0.04 (-0.13; 0.06)	-0.01 (-0.09; 0.06)	0.08 (-0.01; 0.17)
Glycine	-0.01 (-0.08; 0.07)	0.08 (-0.02; 0.17)	0.02 (-0.06; 0.09)	**0.16 (0.07; 0.25)***	-0.01 (-0.09; 0.07)	-0.04 (-0.14; 0.05)	0.02 (-0.05; 0.10)	**0.16 (0.07; 0.25)***
Serine	0.01 (-0.07; 0.09)	0.09 (0.00; 0.18)	0.03 (-0.04; 0.11)	**0.17 (0.08; 0.26)***	0.01 (-0.07; 0.08)	-0.06 (-0.16; 0.03)	0.03 (-0.04; 0.11)	**0.18 (0.09; 0.27)***
Tyrosine	0.03 (-0.04; 0.11)	0.10 (0.00; 0.19)	0.04 (-0.04; 0.11)	**0.19 (0.10; 0.28)***	0.04 (-0.04; 0.11)	-0.02 (-0.11; 0.08)	0.02 (-0.06; 0.09)	**0.17 (0.08; 0.26)***
Sum								
All TAAs	0.03 (-0.04; 0.11)	0.11 (0.02; 0.20)	0.03 (-0.05; 0.11)	**0.15 (0.05; 0.24)***	0.03 (-0.05; 0.10)	-0.05 (-0.15; 0.04)	0.01 (-0.06; 0.09)	**0.14 (0.05; 0.23)***

TAA: total amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; Glx: glutamate + glutamine; Asx: aspartate + asparagine; BMI: body mass index. ‡ $p < 0.01$, * p-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.003$. P-values < 0.003 are indicated in bold.

Table S3C. Spearman correlation coefficients between free amino acids in human milk and infant anthropometrics

FAA	Infant anthropometrics							
	Birth length		Birth weight		Gain in length		Gain in weight	
	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)
EAAs								
Histidine	0.00 (-0.08; 0.08)	-0.10 (-0.20; -0.01)	0.04 (-0.04; 0.12)	-0.02 (-0.12; 0.08)	0.01 (-0.07; 0.09)	0.02 (-0.08; 0.11)	0.08 (0.00; 0.16)	0.03 (-0.07; 0.12)
Isoleucine	-0.04 (-0.12; 0.04)	-0.02 (-0.11; 0.08)	-0.08 (-0.16; 0.00)	0.03 (-0.06; 0.13)	-0.04 (-0.12; 0.04)	0.01 (-0.09; 0.10)	-0.01 (-0.09; 0.07)	-0.01 (-0.1; 0.09)
Leucine	0.01 (-0.07; 0.08)	-0.04 (-0.13; 0.06)	-0.01 (-0.09; 0.07)	0.03 (-0.07; 0.12)	0.00 (-0.08; 0.08)	0.02 (-0.08; 0.12)	0.03 (-0.05; 0.11)	-0.01 (-0.11; 0.08)
Lysine	-0.03 (-0.11; 0.05)	-0.06 (-0.16; 0.03)	-0.01 (-0.09; 0.07)	-0.02 (-0.11; 0.08)	-0.07 (-0.15; 0.01)	0.04 (-0.06; 0.13)	-0.03 (-0.11; 0.05)	-0.01 (-0.11; 0.08)
Methionine	-0.02 (-0.10; 0.06)	-0.05 (-0.14; 0.05)	-0.07 (-0.14; 0.01)	-0.03 (-0.12; 0.07)	0.01 (-0.07; 0.09)	-0.03 (-0.12; 0.07)	-0.03 (-0.11; 0.05)	0.03 (-0.07; 0.12)
Phenylalanine	0.02 (-0.06; 0.10)	-0.05 (-0.15; 0.05)	0.01 (-0.07; 0.09)	0.02 (-0.08; 0.11)	-0.05 (-0.13; 0.03)	0.08 (-0.02; 0.17)	-0.04 (-0.12; 0.04)	0.08 (-0.01; 0.17)
Threonine	-0.03 (-0.11; 0.05)	-0.08 (-0.18; 0.01)	0.02 (-0.06; 0.10)	-0.02 (-0.11; 0.08)	0.04 (-0.04; 0.12)	0.00 (-0.10; 0.09)	0.11 (0.03; 0.19)‡	-0.02 (-0.12; 0.07)
Tryptophan	0.04 (-0.04; 0.12)	0.11 (0.01; 0.20)	-0.03 (-0.11; 0.05)	0.05 (-0.05; 0.14)	-0.05 (-0.13; 0.03)	0.04 (-0.06; 0.13)	0.01 (-0.06; 0.08)	0.01 (-0.08; 0.11)
Valine	0.00 (-0.08; 0.08)	-0.07 (-0.17; 0.02)	0.03 (-0.05; 0.11)	-0.04 (-0.14; 0.05)	-0.07 (-0.15; 0.01)	-0.02 (-0.11; 0.08)	-0.04 (-0.12; 0.04)	0.00 (-0.10; 0.09)
NEAAs/conditionally EAAs								
Alanine	0.00 (-0.08; 0.08)	-0.01 (-0.11; 0.08)	0.02 (-0.06; 0.09)	-0.03 (-0.12; 0.07)	0.02 (-0.06; 0.10)	0.00 (-0.10; 0.10)	0.03 (-0.05; 0.11)	-0.05 (-0.15; 0.04)
Arginine	-0.08 (-0.16; 0.00)	0.01 (-0.08; 0.11)	-0.07 (-0.14; 0.02)	-0.06 (-0.16; 0.03)	-0.02 (-0.10; 0.06)	-0.01 (-0.10; 0.09)	-0.02 (-0.10; 0.06)	-0.01 (-0.10; 0.09)
Asparagine	-0.01 (-0.09; 0.07)	-0.08 (-0.17; 0.02)	-0.06 (-0.14; 0.02)	-0.06 (-0.16; 0.03)	-0.02 (-0.10; 0.06)	0.03 (-0.06; 0.13)	0.06 (-0.02; 0.14)	0.01 (-0.09; 0.10)
Aspartate	0.05 (-0.03; 0.13)	0.01 (-0.08; 0.11)	-0.01 (-0.09; 0.07)	0.03 (-0.07; 0.12)	-0.09 (-0.17; -0.01)	-0.01 (-0.1; 0.09)	-0.02 (-0.10; 0.06)	-0.07 (-0.16; 0.03)
Glutamate	0.01 (-0.07; 0.09)	-0.02 (-0.12; 0.07)	0.02 (-0.06; 0.10)	0.01 (-0.09; 0.10)	0.00 (-0.08; 0.08)	-0.02 (-0.11; 0.08)	**0.12 (0.04; 0.20)***	-0.04 (-0.13; 0.06)
Glutamine	0.01 (-0.07; 0.09)	-0.03 (-0.12; 0.07)	0.00 (-0.08; 0.08)	0.00 (-0.10; 0.09)	0.10 (0.05; 0.15)‡	0.04 (-0.06; 0.13)	**0.22 (0.14; 0.29)***	0.06 (-0.03; 0.16)
Glycine	-0.01 (-0.09; 0.07)	-0.04 (-0.13; 0.06)	-0.01 (-0.09; 0.07)	0.01 (-0.08; 0.11)	0.01 (-0.07; 0.09)	0.06 (-0.03; 0.16)	0.05 (-0.03; 0.13)	0.03 (-0.06; 0.13)
Serine	0.02 (-0.07; 0.09)	-0.08 (-0.17; 0.02)	0.00 (-0.08; 0.08)	-0.03 (-0.13; 0.06)	0.06 (-0.03; 0.13)	0.08 (-0.02; 0.17)	**0.19 (0.11; 0.27)***	0.08 (-0.02; 0.17)
Tyrosine	0.02 (-0.06; 0.10)	-0.01 (-0.11; 0.08)	-0.01 (-0.08; 0.08)	0.08 (-0.02; 0.17)	-0.07 (-0.15; 0.01)	0.05 (-0.05; 0.15)	0.04 (-0.04; 0.12)	0.09 (-0.01; 0.18)
Non-coded AAs								
Citrulline	0.04 (-0.04; 0.12)	-0.05 (-0.15; 0.05)	0.02 (-0.06; 0.10)	-0.03 (-0.12; 0.07)	0.01 (-0.07; 0.09)	0.04 (-0.06; 0.13)	0.09 (0.01; 0.17)	0.02 (-0.08; 0.11)
Ornithine	0.00 (-0.08; 0.08)	-0.02 (-0.12; 0.07)	-0.03 (-0.11; 0.05)	-0.03 (-0.13; 0.06)	-0.09 (-0.17; -0.01)	0.01 (-0.09; 0.10)	-0.03 (-0.11; 0.05)	0.05 (-0.05; 0.14)
Taurine	0.07 (-0.01; 0.15)	0.09 (-0.01; 0.18)	0.04 (-0.04; 0.12)	0.08 (-0.02; 0.17)	0.06 (-0.02; 0.14)	0.04 (-0.05; 0.14)	0.05 (-0.03; 0.13)	-0.03 (-0.12; 0.07)
Sum								
All FAAs	0.02 (-0.06; 0.10)	-0.03 (-0.12; 0.07)	0.02 (-0.06; 0.10)	0.01 (-0.09; 0.10)	0.00 (-0.08; 0.08)	0.04 (-0.06; 0.13)	**0.16 (0.08; 0.24)***	0.00 (-0.09; 0.10)

FAA: free amino acid; EAA: essential amino acid; NEAA: non-essential amino acid. ‡ $p < 0.01$, * p-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/22 = 0.002$. P-values < 0.002 are indicated in bold.

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Table S3D. Spearman correlation coefficients between total amino acids in human milk and infant anthropometrics

TAA	Infant anthropometrics							
	Birth length		Birth weight		Gain in length		Gain in weight	
	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)	6 weeks Rho (95% CI)	6 weeks Rho (95% CI)	6 weeks Rho (95% CI)	6 months Rho (95% CI)
				NEAAs				
Histidine	0.02 (-0.06; 0.10)	0.10 (0.01; 0.20)	-0.03 (-0.11; 0.05)	0.06 (-0.03; 0.16)	-0.11 (-0.19; -0.04)‡	-0.12 (-0.22; -0.03)	**-0.16 (-0.23; -0.08)***	-0.06 (-0.15; 0.04)
Isoleucine	-0.01 (-0.09; 0.07)	0.08 (-0.02; 0.17)	0.00 (-0.08; 0.08)	0.06 (-0.04; 0.15)	-0.06 (-0.13; 0.03)	-0.12 (-0.21; -0.03)	**-0.12 (-0.20; -0.04)***	-0.08 (-0.17; 0.02)
Leucine	0.03 (-0.05; 0.11)	0.10 (0.00; 0.19)	-0.01 (-0.09; 0.07)	0.07 (-0.02; 0.17)	-0.11 (-0.19; -0.03)‡	-0.13 (-0.22; -0.03)‡	**-0.12 (-0.20; -0.04)***	-0.07 (-0.16; 0.03)
Lysine	0.04 (-0.04; 0.12)	0.10 (0.01; 0.19)	-0.01 (-0.09; 0.07)	0.07 (-0.02; 0.17)	-0.12 (-0.2; -0.04)‡	-0.12 (-0.22; -0.03)	**-0.13 (-0.20; -0.05)***	-0.07 (-0.16; 0.03)
Methionine	-0.01 (-0.08; 0.07)	0.03 (-0.06; 0.13)	-0.03 (-0.11; 0.05)	0.03 (-0.07; 0.12)	-0.03 (-0.11; 0.05)	-0.08 (-0.18; 0.01)	-0.09 (-0.17; -0.01)	-0.05 (-0.14; 0.05)
Phenylalanine	0.02 (-0.06; 0.10)	0.09 (0.00; 0.18)	-0.03 (-0.11; 0.05)	0.07 (-0.02; 0.17)	-0.12 (-0.20; -0.04)‡	-0.11 (-0.20; -0.01)	**-0.16 (-0.23; -0.08)***	-0.05 (-0.15; 0.04)
Threonine	0.00 (-0.08; 0.08)	0.09 (-0.01; 0.18)	-0.01 (-0.09; 0.07)	0.06 (-0.03; 0.16)	-0.10 (-0.18; -0.02)	-0.11 (-0.20; -0.01)	**-0.16 (-0.23; -0.08)***	-0.06 (-0.16; 0.04)
Valine	0.00 (-0.08; 0.08)	0.04 (-0.06; 0.13)	-0.01 (-0.08; 0.08)	0.05 (-0.05; 0.14)	-0.10 (-0.18; -0.02)	-0.09 (-0.18; 0.00)	**-0.16 (-0.24; -0.08)***	-0.04 (-0.14; 0.05)
				NEAAs/conditionally EAAs				
Alanine	0.05 (-0.03; 0.13)	0.09 (0.00; 0.19)	0.00 (-0.08; 0.08)	0.06 (-0.04; 0.15)	**-0.13 (-0.21; -0.05)***	-0.09 (-0.18; 0.01)	**-0.13 (-0.21; -0.06)***	-0.06 (-0.15; 0.04)
Arginine	0.03 (-0.05; 0.11)	0.11 (0.01; 0.20)	0.01 (-0.07; 0.09)	0.04 (-0.05; 0.14)	**-0.13 (-0.21; -0.06)***	-0.09 (-0.18; 0.01)	-0.12 (-0.20; -0.04)‡	-0.08 (-0.17; 0.02)
Asx	0.03 (-0.05; 0.11)	0.11 (0.01; 0.20)	-0.02 (-0.10; 0.06)	0.07 (-0.03; 0.16)	**-0.13 (-0.21; -0.05)***	-0.12 (-0.21; -0.02)	**-0.15 (-0.23; -0.07)***	-0.08 (-0.17; 0.02)
Glx	0.02 (-0.06; 0.10)	0.08 (-0.02; 0.17)	-0.01 (-0.09; 0.07)	0.06 (-0.04; 0.15)	-0.09 (-0.17; -0.01)	-0.13 (-0.22; -0.03)‡	-0.08 (-0.16; 0.00)	-0.09 (-0.18; 0.01)
Glycine	0.03 (-0.05; 0.11)	0.10 (0.00; 0.19)	-0.02 (-0.10; 0.06)	0.07 (-0.03; 0.16)	**-0.14 (-0.21; -0.06)***	-0.08 (-0.18; 0.01)	**-0.14 (-0.22; -0.06)***	-0.05 (-0.14; 0.05)
Serine	0.01 (-0.07; 0.09)	0.10 (0.00; 0.19)	-0.02 (-0.10; 0.06)	0.07 (-0.03; 0.16)	-0.12 (-0.20; -0.04)‡	-0.09 (-0.19; 0.00)	**-0.15 (-0.22; -0.07)***	-0.04 (-0.14; 0.05)
Tyrosine	0.02 (-0.06; 0.10)	0.13 (0.03; 0.22)	-0.01 (-0.09; 0.07)	0.10 (0.00; 0.19)	-0.10 (-0.18; -0.02)	-0.13 (-0.22; -0.03)‡	**-0.14 (-0.22; -0.06)***	-0.06 (-0.15; 0.04)
				Sum				
All TAAs	0.03 (-0.06; 0.10)	0.10 (0.01; 0.19)	-0.02 (-0.10; 0.06)	0.07 (-0.03; 0.16)	**-0.13 (-0.21; -0.05)***	-0.11 (-0.22; -0.00)	**-0.15 (-0.23; -0.07)***	-0.08 (-0.17; 0.02)

TAA: total amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; Glx: glutamate + glutamine; Asx: aspartate + asparagine. ‡ $p < 0.01$, * p-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.003$. P-values < 0.003 are indicated in bold.

Table S4. Partial correlation coefficients for associations identified by Spearman correlation

Anthropometric measurement	Sample collection time	Amino acid	Beta estimate (95% CI)	*p*-value	Putative confounders included[1]
Maternal characteristics					
Pre-pregnancy weight	6 weeks	Free glutamine	-0.676 (-1.556; 0.205)	0.132	Infant sex, maternal age, history in allergic disease
		Free asparagine	-0.089 (-0.171; -0.008)	0.031†	
	6 months	Free glutamine	-2.686 (6.783; -1.410)	0.198	
		Free asparagine	-0.029 (-0.160; 0.102)	0.667	
		Sum of all TAAs	8.340 (-7.430; 24.111)	0.299	
Pre-pregnancy BMI	6 weeks	Free glutamine	-1.801 (-4.752; 1.150)	0.188	Infant sex, maternal age, history in allergic disease
		Free asparagine	-0.167 (-0.396; 0.063)	0.155	
		Free serine	-0.403 (-0.935; 0.128)	0.136	
		Free glycine	-0.513 (-1.135; 0.103)	0.102	
	6 months	Free glutamine	-1.916 (-5.101; 1.269)	0.209	
		Free asparagine	-0.099 (-0.268; 0.069)	0.245	
		Sum of all TAAs	9.938 (-10.177; 30.053)	0.332	
Age	6 months	Free tryptophan	0.298 (0.022; 0.574)	0.034†	Infant sex, pre-pregnancy BMI, history in allergic disease
Infant characteristics					
Gain in weight	6 weeks	Free glutamate	0.032 (-0.008; 0.072)	0.102	Infant sex, maternal age, pre-pregnancy BMI, history in allergic disease
		Free glutamine	0.015 (0.008; 0.023)	0.005‡	
		Free serine	0.004 (0.001; 0.008)	0.046†	
		Sum of all TAAs	-0.212 (-0.364; -0.060)	**0.003***	
	6 months	Sum of all TAAs	-0.155 (-0.267; -0.043)	0.007‡	
Gain in length	6 weeks	Free glutamine	5.436 (-0.811; 11.683)	0.088	
		Sum of all TAAs	-8.568 (-13.554; -3.582)	**<0.001***	

TAAs: total amino acids; BMI: body mass index. [1]All maternal and infant characteristics that were associated with levels of any free amino acid (FAA) or TAA in 6-week or 6-month human milk were included as putative confounders in partial correlation analyses. † $p < 0.05$, ‡ $p < 0.01$, * *p*-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance for FAAs is $\alpha = 0.05/16 = 0.002$ and for TAAs is $\alpha = 0.05/16 = 0.003$.

Table S5A. Free amino acid levels in human milk of mothers with or without an allergy

FAA (µmol/L)	6 weeks Mean (SD)		6 months Mean (SD)		Analysis of differences at 6 weeks (*p*-value)	Analysis of differences at 6 months (*p*-value)
	Mothers without allergy (n = 443)	Mothers with allergy (n = 225)	Mothers without allergy (n = 274)	Mothers with allergy (n = 166)		
EAAs						
Histidine	28.1 (9.3)	28.6 (8.8)	28.6 (12.1)	31.2 (16.0)	0.456	0.068
Isoleucine	16.8 (10.8)	15.8 (9.1)	12.1 (5.1)	13.6 (6.8)	0.179	0.017
Leucine	34.1 (16.7)	33.6 (19.9)	34.0 (10.8)	36.3 (12.3)	0.740	0.045
Lysine	36.0 (25.6)	32.5 (20.6)	28.9 (12.9)	32.1 (17.6)	0.058	0.043
Methionine	27.9 (23.8)	27.9 (25.2)	33.1 (26.7)	36.8 (27.8)	0.997	0.162
Phenylalanine	21.3 (9.9)	21.3 (10.6)	16.2 (5.2)	16.8 (6.1)	0.972	0.325
Threonine	72.2 (30.7)	69.0 (26.4)	97.6 (38.8)	96.0 (34.7)	0.160	0.644
Tryptophan	14.4 (7.3)	14.9 (7.8)	29.3 (11.9)	28.7 (11.7)	0.444	0.613
Valine	108.6 (59.6)	111.4 (55.7)	65.2 (27.4)	69.0 (27.4)	0.546	0.157
NEAAs/conditionally EAAs						
Alanine	231.9 (65.1)	230.4 (62.4)	238.4 (70.3)	250.0 (75.0)	0.780	0.108
Arginine	16.9 (12.0)	17.2 (10.0)	16.6 (5.8)	17.7 (7.3)	0.755	0.091
Asparagine	24.1 (14.4)	26.8 (15.8)	18.1 (8.6)	16.9 (8.6)	0.130	0.146
Aspartate	31.1 (18.0)	30.6 (18.6)	62.5 (36.5)	69.6 (42.7)	0.709	0.176
Glutamate	1325.2 (418.8)	1273.5 (391.3)	1531.4 (323.1)	1526.6 (338.6)	0.116	0.882
Glutamine	238.4 (160.6)	241.3 (162.4)	579.4 (268.0)	498.3 (263.4)	0.828	**<0.001***
Glycine	140.0 (41.4)	137.3 (38.3)	145.5 (46.5)	146.6 (49.9)	0.403	0.817
Serine	101.6 (35.7)	97.8 (32.3)	137.4 (58.5)	136.9 (63.5)	0.165	0.928
Tyrosine	27.2 (19.8)	28.0 (21.0)	16.7 (7.0)	18.7 (8.9)	0.658	0.074
Non-coded AAs						
Citrulline	10.6 (4.7)	10.9 (4.8)	20.9 (6.2)	20.5 (5.8)	0.410	0.424
Ornithine	14.4 (16.3)	15.6 (17.5)	13.6 (23.1)	18.6 (31.1)	0.370	0.071
Taurine	244.4 (94.3)	242.9 (91.3)	264.6 (103.2)	269.2 (111.3)	0.840	0.668
Sum						
All FAAs	2765.3 (668.7)	2707.3 (593.2)	3390.2 (681.8)	3350.0 (666.6)	0.253	0.543

FAA: free amino acid; EAA: essential amino acid; NEAA: non-essential amino acid. * *p*-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/22 = 0.002$. *P*-values < 0.002 are indicated in bold.

Table S5B. Total amino acid levels in human milk of mothers with or without an allergy

TAA (µmol/L)	6 weeks Mean (SD)		6 months Mean (SD)		Analysis of differences at 6 weeks (*p*-value)	Analysis of differences at 6 months (*p*-value)
	Mothers without allergy (n = 443)	Mothers with allergy (n = 225)	Mothers without allergy (n = 274)	Mothers with allergy (n = 166)		
EAAs						
Histidine	1790.6 (262.2)	1805.2 (270.1)	1322.1 (197.3)	1334.9 (207.3)	0.646	0.221
Isoleucine	4013.1 (575.6)	4077.0 (646.8)	3380.2 (457.9)	3389.2 (464.2)	0.078	0.930
Leucine	8684.2 (1227.8)	8710.3 (1250.2)	6772.1 (902.8)	6833.8 (949.9)	0.759	0.127
Lysine	6367.2 (940.1)	6354.0 (969.2)	3948.8 (607.3)	3993.6 (653.1)	0.386	0.851
Methionine	1009.9 (207.3)	1011.5 (189.1)	749.7 (256.0)	752.5 (144.4)	0.763	0.292
Phenylalanine	2615.1 (439.4)	2605.0 (389.8)	2043.9 (282.3)	2082.9 (337.5)	0.506	0.132
Threonine	3700.5 (581.6)	3722.3 (576.0)	3345.0 (465.8)	3409.0 (567.5)	0.763	0.213
Valine	4654.0 (760.9)	4695.0 (747.8)	4164.4 (603.9)	4259.4 (663.0)	0.797	0.501
NEAAs/conditionally EAAs						
Alanine	5349.3 (989.4)	5374.2 (990.5)	3938.8 (569.7)	4040.4 (733.9)	0.799	0.503
Arginine	2874.7 (1038.3)	3028.0 (1070.0)	2145.7 (545.6)	2140.4 (653.0)	0.805	0.567
Asx	8571.5 (1329.0)	8543.8 (1319.9)	6219.5 (858.6)	6283.7 (1039.3)	0.876	0.160
Glx	13469.5 (1622.0)	13502.7 (1655.9)	11517.4 (1448.8)	11426.8 (1702.5)	0.505	0.521
Glycine	3913.7 (720.3)	3888.6 (689.1)	2838.8 (451.1)	2921.1 (605.3)	0.662	0.130
Serine	5125.9 (828.5)	5115.5 (799.8)	4062.1 (571.6)	4152.9 (703.3)	0.918	0.884
Tyrosine	1895.6 (296.7)	1903.0 (298.9)	1498.3 (215.0)	1522.1 (238.3)	0.211	0.843
Sum						
All TAAs	74.0 (11.8)	74.3 (11.9)	57.9 (8.4)	58.5 (9.7)	0.867	0.473

TAA: total amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; Glx: glutamate + glutamine; Asx: aspartate + asparagine. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.003$. *P*-values < 0.003 are indicated in bold.

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Table S6A. Adjusted model results for associations between free amino acids in human milk and infant wheeze outcomes

FAA	Wheeze					
	Transient wheeze		Persistent wheeze		Intermediate wheeze	
	6 weeks (n = 47) RR (95% CI)	6 months (n = 30) RR (95% CI)	6 weeks (n = 31) RR (95% CI)	6 months (n = 18) RR (95% CI)	6 weeks (n = 31) RR (95% CI)	6 months (n = 27) RR (95% CI)
NEAAs						
Histidine	0.98 (0.95; 1.02)	1.00 (0.97; 1.03)	1.02 (0.98; 1.06)	1.02 (0.98; 1.07)	0.95 (0.89; 1.02)	**1.04 (1.01; 1.06)***
Isoleucine	0.98 (0.94; 1.02)	1.04 (0.97; 1.11)	1.00 (0.97; 1.04)	1.01 (0.91; 1.13)	0.97 (0.92; 1.02)	1.07 (0.99; 1.14)
Leucine	0.98 (0.95; 1.00)	1.02 (0.99; 1.05)	0.99 (0.96; 1.02)	1.02 (0.98; 1.06)	1.02 (1.00; 1.03)	1.04 (1.00; 1.09)
Lysine	0.99 (0.97; 1.00)	1.02 (0.99; 1.05)	0.98 (0.97; 1.00)	1.00 (0.96; 1.03)	1.00 (0.99; 1.03)	**1.04 (1.02; 1.06)***
Methionine	1.00 (0.98; 1.01)	1.00 (0.99; 1.02)	1.00 (0.99; 1.02)	1.01 (0.99; 1.02)	0.99 (0.97; 1.01)	1.02 (1.00; 1.03)
Phenylalanine	0.98 (0.93; 1.03)	1.09 (1.03; 1.15)‡	1.00 (0.97; 1.04)	1.05 (0.95; 1.16)	0.98 (0.93; 1.02)	1.04 (0.96; 1.12)
Threonine	1.00 (0.98; 1.01)	1.00 (0.99; 1.00)	1.00 (0.99; 1.01)	1.01 (1.00; 1.02)	1.00 (0.98; 1.01)	1.01 (1.01; 1.02)‡
Tryptophan	0.96 (0.92; 1.01)	1.01 (0.98; 1.04)	1.02 (0.98; 1.07)	0.99 (0.92; 1.06)	0.98 (0.91; 1.04)	0.99 (0.96; 1.03)
Valine	1.00 (0.99; 1.00)	1.01 (1.00; 1.03)	1.00 (0.99; 1.01)	1.00 (0.98; 1.02)	1.00 (0.99; 1.01)	1.01 (1.00; 1.02)
NEAAs/conditionally EAAs						
Alanine	1.00 (0.99; 1.01)	1.00 (1.00; 1.01)	1.00 (0.99; 1.01)	1.00 (0.97; 1.01)	1.00 (0.99; 1.01)	1.01 (1.00; 1.01)
Arginine	0.96 (0.92; 1.00)	1.06 (1.00; 1.11)	0.97 (0.93; 1.01)	0.93 (0.85; 1.00)	1.02 (0.98; 1.07)	1.04 (0.98; 1.10)
Asparagine	1.00 (0.98; 1.02)	1.00 (0.95; 1.05)	1.00 (0.98; 1.03)	0.96 (0.92; 1.00)	**0.95 (0.92; 0.98)***	1.04 (0.99; 1.11)
Aspartate	1.00 (0.98; 1.02)	0.99 (0.97; 1.00)	1.02 (1.00; 1.05)	1.00 (0.99; 1.02)	0.97 (0.95; 0.99)‡	1.00 (0.99; 1.01)
Glutamate	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)
Glutamine	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (0.99; 1.00)	1.00 (1.00; 1.00)
Glycine	1.00 (0.99; 1.01)	1.00 (0.99; 1.01)	1.01 (1.00; 1.02)	1.01 (1.00; 1.02)	0.99 (0.98; 1.00)	1.01 (1.00; 1.02)
Serine	0.99 (0.98; 1.01)	1.00 (0.99; 1.01)	1.00 (1.00; 1.01)	1.01 (1.00; 1.02)	0.98 (0.96; 1.00)‡	1.01 (1.00; 1.01)‡
Tyrosine	1.00 (0.98; 1.02)	1.01 (0.97; 1.06)	1.01 (0.99; 1.03)	1.02 (0.93; 1.10)	0.96 (0.94; 0.99)‡	1.06 (1.00; 1.12)
Non-coded AAs						
Citrulline	0.96 (0.91; 1.02)	1.05 (0.97; 1.12)	1.01 (0.94; 1.09)	0.99 (0.92; 1.08)	0.88 (0.78; 0.99)	1.04 (0.95; 1.14)
Ornithine	0.99 (0.96; 1.02)	0.99 (0.97; 1.01)	1.02 (1.00; 1.04)	1.02 (1.00; 1.03)	0.98 (0.94; 1.02)	1.01 (1.01; 1.02)
Taurine	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (0.99; 1.00)	1.00 (0.99; 1.01)	1.00 (1.00; 1.01)	1.00 (0.99; 1.00)
Sum						
All FAAs	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)

FAA: free amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; RR: risk ratio. ‡ $p < 0.01$, * *p*-value remains statistically significant following Bonferroni adjustment. Bonferroni adjusted level of statistical significance is $\alpha = 0.05/22 = 0.0023$. *P*-values < 0.0023 are indicated in bold. Models were adjusted for maternal pre-pregnancy body mass index (BMI), maternal age, infant sex, maternal education and smoking status.

Table S6B. Adjusted model results for associations between free amino acids and infant atopic dermatitis and respiratory tract infections

FAA	Atopic dermatitis (AD)						Lower respiratory tract infections	
	Parent-reported		Paediatrician-reported		Parent- and paediatrician-reported			
	6 weeks (n = 44) RR (95% CI)	6 months (n = 27) RR (95% CI)	6 weeks (n = 64) RR (95% CI)	6 months (n = 37) RR (95% CI)	6 weeks (n = 73) RR (95% CI)	6 months (n = 44) RR (95% CI)	6 weeks (n = 151) RR (95% CI)	6 months (n = 108) RR (95% CI)
NEAAs								
Histidine	0.98 (0.95; 1.02)	0.98 (0.92; 1.04)	0.98 (0.95; 1.01)	1.01 (0.98; 1.04)	0.97 (0.93; 1.02)	0.98 (0.92; 1.05)	0.99 (0.96; 1.01)	1.01 (1.00; 1.03)
Isoleucine	1.01 (0.98; 1.05)	0.91 (0.78; 1.06)	1.01 (0.98; 1.04)	0.99 (0.91; 1.07)	1.02 (0.98; 1.06)	0.89 (0.72; 1.10)	0.97 (0.95; 0.99)	1.03 (0.99; 1.06)
Leucine	1.01 (0.98; 1.03)	0.93 (0.87; 1.00)	1.00 (0.98; 1.02)	0.97 (0.92; 1.02)	1.00 (0.97; 1.04)	0.92 (0.84; 1.01)	1.00 (0.98; 1.01)	1.01 (0.99; 1.03)
Lysine	1.01 (1.00; 1.02)	0.99 (0.96; 1.02)	1.01 (0.99; 1.02)	1.00 (0.98; 1.03)	1.01 (1.00; 1.03)	0.97 (0.95; 1.00)	1.00 (0.99; 1.01)	1.01 (1.00; 1.02)
Methionine	1.00 (0.98; 1.02)	0.98 (0.96; 1.00)	1.00 (0.98; 1.01)	0.99 (0.98; 1.01)	1.01 (0.99; 1.02)	0.98 (0.95; 1.00)	0.99 (0.98; 1.00)	1.00 (0.99; 1.01)
Phenylalanine	1.00 (0.97; 1.04)	0.95 (0.81; 1.11)	0.99 (0.96; 1.02)	0.96 (0.87; 1.07)	1.00 (0.96; 1.04)	0.95 (0.78; 1.15)	1.00 (0.97; 1.04)	0.98 (0.96; 1.00)
Threonine	1.01 (0.99; 1.02)	0.98 (0.96; 1.01)	1.00 (0.99; 1.02)	1.00 (0.99; 1.01)	1.00 (0.99; 1.02)	0.98 (0.96; 1.01)	0.99 (0.98; 1.00)	1.00 (1.00; 1.01)
Tryptophan	1.04 (1.00; 1.08)	1.00 (0.96; 1.04)	1.02 (0.98; 1.06)	1.00 (0.97; 1.04)	1.04 (0.99; 1.08)	1.00 (0.96; 1.05)	0.99 (0.96; 1.01)	0.99 (0.97; 1.02)
Valine	0.99 (0.99; 1.00)	0.97 (0.95; 1.00)	1.00 (0.99; 1.00)	0.99 (0.98; 1.01)	0.99 (0.98; 1.00)	0.97 (0.94; 1.01)	1.00 (1.00; 1.00)	1.01 (1.00; 1.02)
NEAAs/conditionally EAAs								
Alanine	1.00 (0.99; 1.00)	1.00 (0.99; 1.00)	1.00 (0.99; 1.00)	1.00 (1.00; 1.01)	1.00 (0.99; 1.00)	1.00 (0.99; 1.00)	1.00 (0.99; 1.00)	1.00 (1.00; 1.01)
Arginine	1.04 (1.00; 1.08)	0.95 (0.89; 1.01)	1.01 (0.97; 1.04)	1.00 (0.95; 1.06)	1.03 (0.98; 1.08)	0.93 (0.85; 1.02)	1.00 (0.98; 1.02)	1.03 (1.00; 1.05)
Asparagine	1.00 (0.97; 1.02)	0.94 (0.87; 1.01)	0.99 (0.96; 1.01)	0.96 (0.91; 1.02)	0.99 (0.96; 1.02)	0.93 (0.85; 1.01)	0.98 (0.97; 1.00)	1.02 (0.99; 1.04)
Aspartate	1.01 (0.98; 1.03)	1.00 (0.99; 1.01)	1.00 (0.98; 1.01)	1.00 (0.99; 1.01)	1.01 (0.99; 1.04)	0.99 (0.98; 1.01)	0.99 (0.98; 1.00)	1.00 (1.00; 1.01)
Glutamate	1.00 (1.00; 1.00)	0.99 (0.99; 0.99)‡	1.00 (1.00; 1.00)	0.99 (0.99; 0.99)	1.00 (1.00; 1.00)	0.99 (0.99; 0.99)‡	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)
Glutamine	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)
Glycine	1.00 (0.99; 1.01)	0.99 (0.97; 1.01)	0.99 (0.99; 1.00)	1.00 (0.99; 1.01)	1.00 (0.99; 1.00)	0.99 (0.97; 1.01)	0.99 (0.99; 1.00)	1.00 (1.00; 1.01)
Serine	1.01 (1.00; 1.02)	1.00 (0.99; 1.01)	1.00 (0.99; 1.01)	1.00 (0.99; 1.01)	1.01 (0.99; 1.02)	1.00 (0.99; 1.01)	1.00 (0.99; 1.00)	1.00 (1.00; 1.01)
Tyrosine	1.01 (0.99; 1.03)	0.91 (0.81; 1.02)	1.00 (0.98; 1.02)	0.96 (0.90; 1.03)	1.01 (0.99; 1.03)	0.90 (0.79; 1.03)	0.99 (0.98; 1.00)	1.01 (0.98; 1.03)
Non-coded AAs								
Citrulline	1.03 (0.97; 1.10)	0.96 (0.88; 1.05)	0.99 (0.92; 1.07)	0.97 (0.90; 1.04)	1.05 (0.96; 1.14)	0.99 (0.89; 1.09)	0.97 (0.93; 1.02)	1.00 (0.96; 1.04)
Ornithine	0.99 (0.95; 1.04)	1.00 (0.99; 1.02)	0.98 (0.95; 1.02)	1.01 (1.00; 1.02)	1.00 (0.96; 1.05)	1.00 (0.99; 1.02)	1.00 (0.98; 1.02)	1.01 (1.00; 1.01)
Taurine	1.00 (1.00; 1.00)	1.00 (0.99; 1.01)	1.00 (1.00; 1.01)	1.00 (1.00; 1.01)	1.00 (1.00; 1.01)	1.00 (0.99; 1.01)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)
Sum								
All FAAs	1.00 (1.00; 1.00)	0.99 (0.99; 0.99)‡	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)	0.99 (0.99; 0.99)‡	1.00 (1.00; 1.00)	1.00 (1.00; 1.00)

FAA: free amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; RR: risk ratio. ‡ $p < 0.01$, * p-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/22 = 0.002$. P-values < 0.002 are indicated in bold. Models were adjusted for maternal pre-pregnancy body mass index (BMI), maternal age, infant sex, maternal education and smoking status.

Table S6C. Adjusted model results for associations between free amino acids in human milk and infant food allergy

FAA	Food allergy	
	6 weeks (n = 13) RR (95% CI)	6 months (n = 7) RR (95% CI)
	EAAs	
Histidine	1.02 (0.80; 1.29)	1.00 (0.99; 1.01)
Isoleucine	0.99 (0.82; 1.17)	1.00 (0.97; 1.03)
Leucine	0.99 (0.86; 1.14)	1.00 (0.99; 1.01)
Lysine	0.98 (0.83; 1.15)	1.00 (0.99; 1.01)
Methionine	0.93 (0.85; 1.02)	1.00 (0.99; 1.01)
Phenylalanine	1.01 (0.86; 1.18)	1.00 (0.97; 1.03)
Threonine	0.95 (0.89; 1.03)	1.00 (1.00; 1.00)
Tryptophan	0.97 (0.78; 1.22)	1.00 (0.99; 1.01)
Valine	1.00 (0.97; 1.03)	1.00 (0.99; 1.01)
	NEAAs/conditionally EAAs	
Alanine	0.99 (0.96; 1.02)	1.00 (1.00; 1.00)
Arginine	0.92 (0.86; 0.99)‡	1.00 (0.98; 1.02)
Asparagine	**0.70 (0.58; 0.84)***	1.00 (0.98; 1.02)
Aspartate	0.97 (0.92; 1.03)	1.00 (1.00; 1.00)
Glutamate	0.94 (0.83; 1.04)	1.00 (1.00; 1.00)
Glutamine	0.85 (0.69; 1.02)	1.00 (1.00; 1.00)
Glycine	0.98 (0.94; 1.02)	1.00 (1.00; 1.00)
Serine	0.97 (0.93; 1.01)	1.00 (1.00; 1.00)
Tyrosine	0.93 (0.80; 1.07)	1.00 (0.98; 1.02)
	Non-coded AAs	
Citrulline	0.85 (0.71; 0.98)‡	1.00 (0.98; 1.03)
Ornithine	0.98 (0.82; 1.18)	1.00 (0.99; 1.01)
Taurine	0.99 (0.97; 1.02)	1.00 (1.00; 1.00)
	Sum	
All FAAs	0.94 (0.84; 1.05)	1.00 (1.00; 1.00)

FAA: free amino acid; EAA: essential amino acid; NEAA: non-essential amino acid; RR: risk ratio. ‡ $p < 0.05$, * p-value remains statistically significant following Bonferroni adjustment. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/23 = 0.002$. P-values < 0.002 are indicated in bold. Models were adjusted for maternal pre-pregnancy body mass index (BMI), maternal age, infant sex, maternal education and smoking status.

Chapter 6

Dietary glutamine supplementation attenuates allergic symptoms in a murine model for cow's milk allergy

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ABSTRACT

The conditionally essential amino acid glutamine (Gln) has been described to support the intestinal barrier function and to exert immunomodulating effects in the gut-associated lymphoid tissues (GALT). The intestinal barrier and the GALT play vital roles in the protection against food allergic sensitization. Whether Gln is able to modulate the development of food allergy is not known. The present study investigated the effects of Gln on the development of food allergy, using a murine model for cow's milk allergy (CMA). C3H/HeOuJ mice were orally sensitized to whey protein weekly for five consecutive weeks. Mice were fed either a control diet or a diet supplemented with 0.5% or 2% L-Gln (w/w), starting two weeks before the first sensitization. Following an intradermal challenge with whey, the acute allergic skin response and systemic anaphylaxis were assessed. Whey-specific immunoglobulins were determined in serum collected 30 min after an oral whey challenge. Spleens and mesenteric lymph nodes (MLN) were isolated for analysis of T cell populations and cytokine production after restimulation. Compared to mice fed the control diet, mice fed the 2% Gln diet developed a significantly lower allergen-induced ear swelling, which coincided with elevated levels of whey-specific IgA and a lowered ratio of whey-specific IgE to IgA in serum. Moreover, these mice tended to have a higher ratio of whey-specific IgG2a to IgG1 in serum and had a higher percentage of activated T_H1 cells in the MLN. Effects observed in mice fed the 0.5% Gln diet were similar in direction but mostly non-significant. These findings indicate a protective effect of dietary free Gln against the development of allergic symptoms to whey protein. Underlying mechanisms may involve increased release of allergen-specific IgA in serum and improvements in the mucosal T_H1/T_H2 immune balance. These findings may ultimately have implications for developing novel nutritional strategies for the prevention of CMA.

INTRODUCTION

Food allergies have become a significant paediatric health issue in developed countries. The most common form of food allergy in infancy is cow's milk allergy (CMA), affecting up to 3% of children at 1 year of age in Westernized countries [1]. In affected infants, ingestion of cow's milk can provoke a variety of symptoms ranging from mild itching to severe systemic anaphylaxis. Although most of the affected children outgrow CMA by the age of 4 years, CMA in infancy can have long-lasting health effects, such as a delay in growth and an increased risk of atopic manifestations and gastrointestinal disorders later in life [2, 3]. Because no effective treatment for CMA exists other than avoidance of the culprit food, the prevention of CMA in infancy is critical.

The incidence rates of CMA are the lowest in exclusively breastfed infants [1, 4]. A factor considered to contribute to this is that human milk provides the infant with bioactive components that protect against food allergic sensitization [5-8], among others through supporting the maturation and functionality of the intestinal barrier and the gut-associated lymphoid tissues (GALT) [9, 10]. One of the human milk components widely associated with the functionality and maturation of the intestines and the GALT are free amino acids (FAAs) [11, 12], the levels of which are considerably higher in human milk than in most infant formula. Despite this, few studies have investigated FAAs in the context of food allergy.

One of the most abundant FAAs in human milk is glutamine (Gln), which is an important energy substrate for immune cells and epithelial cells. Previous studies have shown that Gln supplementation has a wide variety of effects within the developing intestines and the GALT, many of which are related to mechanisms relevant for food allergic sensitization and the prevention thereof [13-15]. For instance, in healthy and immunocompromised neonatal animals, Gln supplementation can improve intestinal morphology and barrier function, modulate intestinal lymphocyte populations, exert anti-inflammatory and anti-oxidative effects, and stimulate the intestinal secretion of various components that support the prevention of allergic diseases, such as immunoglobulin A (IgA) [16]. Moreover, Gln supplementation has been shown to prevent or suppress allergic symptoms in animal models for various atopic diseases, including asthma and allergic dermatitis, among others through inhibiting allergy-inducing T helper 2 (TH2)-type immune responses [17-20]. Based on these findings, we hypothesized that Gln supplementation may prevent the development of food allergy. Gln has previously been suggested to be effective in the prevention or treatment of food allergies [16, 21, 22], but this has, to the best of our knowledge, never been actually investigated. To this end, the current study investigated the effects of dietary Gln supplementation on the development of CMA in mice, using an extensively validated murine model of orally induced CMA [23].

MATERIAL AND METHODS

Animals

Three- to four-week old female C3H/HeOuJ mice that were bred and raised for at least two generations on a cow's milk protein-free diet were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice were housed in groups of three in Makrolon Type II L cages (365 x 207 x 140 mm; Tecniplast, Italy) with standard chip bedding, wood wool, plastic tubes and a plastic shelter at the Animal Research Centre (Poonawalla Science Park, Bilthoven, The Netherlands) in a temperature- and humidity-controlled room on a regular 12h light/dark cycle. Mice were given ad libitum access to food and drinking water throughout the study and were allowed to habituate to the laboratory conditions for 7 days prior to the first animal procedure. This study was conducted following the principles of good laboratory animal care, and all experimental procedures were approved by an external, independent Animal Ethics Committee (DEC consult, Soest, The Netherlands) under license of the national competent authority (CCD, Centrale Commissie Dierproeven), securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes.

Diets

During the entire study period mice received one of three diets (SSniff Spezialdiaten GmbH, Soest, Germany) (*Table 1*). The control diet was a semi-purified AIN-93G soy protein-based diet. In the two experimental diets part of the soy protein was replaced by either 5.0 g/kg (0.5% w/w) or 20.0 g/kg (2% w/w) free L-Gln (Ajinomoto, Tokyo, Japan). The diets contained equal amounts of all other ingredients and were isocaloric and largely iso-nitrous (2.86, 2.88 and 2.96 g/kg nitrogen for the control, 0.5% Gln and 2% Gln diet, respectively).

Animal procedures and experimental design

A schematic representation of the experimental study design is shown in *Figure 1*. Upon arrival, 60 mice were randomly assigned to one of the following groups: a sham-sensitized control group fed the control diet (n = 6), a whey-sensitized control group fed the control diet (n = 21), a whey-sensitized experimental group fed the 0.5% Gln diet (n = 15) and a whey-sensitized experimental group fed the 2% Gln diet (n = 18). Sample-sizes are justified in the Statistical analysis section below. On study days 14, 21, 28, 35 and 42 mice were orally sensitized via a gavage with 20 mg of homogenized whey (WPC60, Milei, Friesland Campina) dissolved in 0.5 mL phosphate-buffered saline (PBS) containing 10 μg cholera toxin (CT; List Biological Laboratories) as an adjuvant. Sham-sensitized mice received 10 μg CT in 0.5 mL PBS, without whey. On study day 49, all mice received an intradermal (i.d.)

challenge with 10 μg homogenized whey in 10 μL PBS in the pinna of both ears to determine the acute allergic skin reaction and anaphylactic symptoms. On study day 50, the mice were orally challenged with 100 mg homogenized whey dissolved in 0.5 mL PBS. Thirty minutes after the oral challenge, blood samples were taken via orbital extraction under terminal anaesthesia (isoflurane/air), followed by cervical dislocation. Blood samples were centrifuged at 3000x g for 10 min and serum was stored at -20°C until further analysis.

Table 1. Composition of the diets

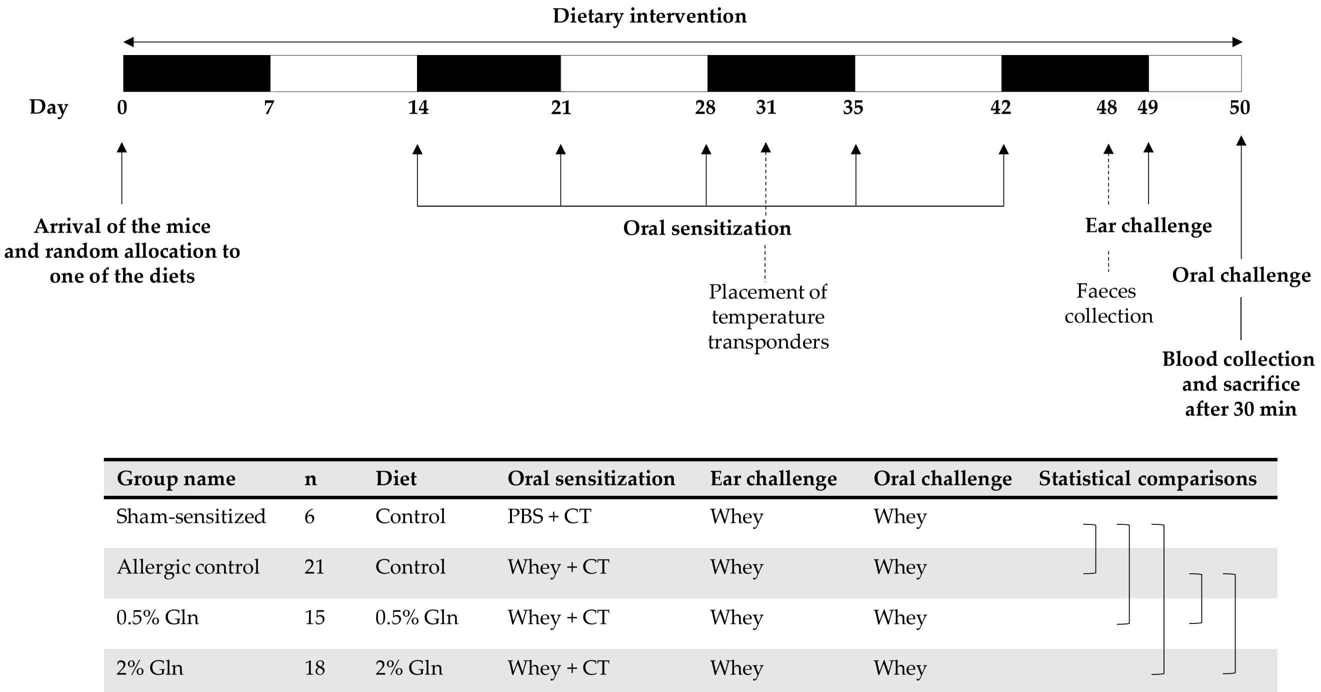
Components	Control diet (g/kg)	0.5% Gln diet (g/kg)	2% Gln diet (g/kg)
Carbohydrates			
Corn starch	397.5	397.5	397.5
Dextrinized corn starch	132.0	132.0	132.0
Sucrose	100.0	100.0	100.0
Fiber			
Cellulose	50.0	50.0	50.0
Protein / Amino acids			
Soy protein	200.0	195.0	180.0
Free amino acids	3.0	8.0	23.0
L-Gln	–	5.0	20.0
L-Cys	1.0	1.0	1.0
DL-Met	2.0	2.0	2.0
Fat			
Soybean oil	70.0	70.0	70.0
Other			
Mineral mix	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5
Tert-butylhydroquinone	0.014	0.014	0.014
Total nitrogen	2.9	2.9	3.0

Gln: glutamine; Cys: cysteine; Met: methionine.

6

Assessment of the allergic skin response and anaphylactic shock severity

To determine the acute allergic skin response to whey, the thickness of each ear was measured in duplicate and in randomized and blinded fashion before and 1 h after i.d. whey challenge in the ear pinnae, using a digital micrometer (Mitutoyo). Whey-induced ear swelling was calculated by subtracting the basal ear thickness from the ear thickness measured 1 h after the challenge. Additionally, the body temperature of the mice was measured and the anaphylactic shock severity was scored 15 min, 30 min and 1 h after the i.d. challenge. Body temperature was measured using temperature transponders (IPTT-300, Bio medic data systems Inc.), which were injected subcutaneously on study day 31 (*Figure 1*). Anaphylactic shock symptoms were scored using a validated, previously described 0- to 4-point scoring system [24]. Mice reaching a shock score of 4 (n = 2), which was considered a humane endpoint, were euthanized and not included in further analysis.



Group name	n	Diet	Oral sensitization	Ear challenge	Oral challenge	Statistical comparisons
Sham-sensitized	6	Control	PBS + CT	Whey	Whey	
Allergic control	21	Control	Whey + CT	Whey	Whey	
0.5% Gln	15	0.5% Gln	Whey + CT	Whey	Whey	
2% Gln	18	2% Gln	Whey + CT	Whey	Whey	

Figure 1. Schematic representation of the experimental design. CT: cholera toxin; Gln: glutamine.

Measurement of allergen-specific immunoglobulins and mMCP-1 in serum

Levels of whey-specific immunoglobulins in serum were analysed by means of ELISA as described previously [25], with few modifications. Briefly, microcolon plates (Greiner) were coated for 18 h at 4°C with 20 μg/ml whey in carbonate/bicarbonate coating buffer (0.05 M, pH = 9.6; Sigma-Aldrich). After washing, the plates were blocked for 1.5 h with 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS. Plates were then incubated with serum samples for 2 h at room temperature (RT), washed, and incubated with 1 μg/ml biotin-labelled rat anti-mouse IgE, IgG1, IgG2a or IgA (BD Pharmingen) in PBS for 1.5 h at RT. After washing, plates were incubated with 0.5 μg/ml streptavidin-horseradish peroxidase (Sanquin) in PBS for 1 h at RT and washed again. The reaction was developed with tetramethyl benzidine substrate (Pierce, Fisher Scientific) and stopped with 4 M H_2SO_4. Absorbance was measured at 490 nm with a microplate reader (BioTek, Powerwave HT). The results are expressed as arbitrary units (AU), calculated based on an internal standard curve of pooled sera of whey-immunized mice. The concentration of mucosal mast cell protease-1 (mMCP-1) in serum was determined using a commercially available ELISA kit (eBioscience), according to the manufacturer's instructions.

Allergen-specific IgA in faeces

Faeces was collected at study day 48 and stored at −80°C until the samples were homogenized in PBS for further analysis. Homogenized faecal samples were centrifuged for 10 min at 10.000 rpm and supernatants were collected for the measurement of whey-specific IgA concentrations by ELISA, following the procedure described above using pooled faeces of whey-immunized mice as an internal standard.

Spleen and mesenteric lymph node cell isolation

Mesenteric lymph nodes (MLN) collected from mice were homogenized by crushing tissues through a 70 μm nylon cell strainer using a syringe. Spleens were homogenized semi-automated by means of a GentleMACS Dissociator (Miltenyi Biotech) and were subsequently incubated with lysis buffer (8.3 g NH_4Cl, 1 g $KHCO_3$ and 37.2 mg Na_2EDTA dissolved in 1 L demi-H_2O, filter-sterilized, pH 7.3) to lyse the red blood cells. Obtained MLN and spleen single cell suspensions were resuspended in RPMI1640 medium (Gibco), containing 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin, prior to flow cytometric analysis and *ex vivo* restimulation assays.

Flow cytometric analysis of immune cells

For flow cytometric analysis, MLN and splenic single cell suspensions (0.6×10^6 cells/mL) were washed with PBS and subsequently blocked for 15 min at 4°C by incubating with 5 μg/mL rat anti-mouse CD16/CD32 (BD Biosciences) dissolved in PBS containing 1% BSA

and 2% FCS. Next, cells were stained extracellularly with CD69-PE-Cy7, CXCR3-PE, CD25-PerCP-Cy5.5 (all from eBiosciences), CD4-BV510, CD196-PE (both from Biolegend), CD127-PE-Cy7 (Miltenyi Biotec) and T1ST2-FITC (MD Bioproducts) for 1 h at RT. Viable cells were distinguished using Fixable Viability Dye eFluor™ 780 (eBiosciences). For intracellular staining, cells were fixated and permeabilized overnight at 4°C using the FoxP3/Transcription Factor Staining Buffer Set (eBiosciences) and subsequently stained with FoxP3-FITC (eBiosciences). Fluorescently stained cells were measured with the BD FACSCanto II flow cytometer (Becton Dickinson) and results were analysed using the FlowLogic Software (Inivai Technologies). Gates were identified and set on the basis of fluorescence-minus-one controls. The panel used in this study and the analyses strategies are shown in *Figure S1*.

Cytokine measurements after *ex vivo* stimulation of splenocytes and MLN cells

For the *ex vivo* restimulation assay, single cell MLN and splenocyte suspensions (0.3×10^6 cells/mL) were cultured in U-bottom culture plates (Greiner) in RPMI1640 medium (Gibco) containing 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were stimulated with 1 μg/mL anti-CD3 (BioLegend) for 2 days or with 500 μg/mL whey for 5 days, after which cell culture supernatants were harvested. Supernatants were stored at -20°C until concentrations of IL-2, IL-5, IL-6, IL-10, IL-13 and IFNγ were determined by ELISA (R&D Systems), according to the manufacturer's instructions.

Short-chain fatty acid concentrations in caecum

Caecum content was collected, snap-frozen in liquid nitrogen and stored at −80°C until the measurement of short chain fatty acid (SCFA) concentrations. Samples were homogenized by vortexing, diluted in 10x volumes of PBS and subsequently centrifuged for 10 min at 14.000 rpm. Concentrations of acetate, butyrate, propionate, and the branched short-chain fatty acids (BCFAs) valerate, iso-valerate and iso-butyrate in the supernatants were analysed by gas chromatography (Shimadzu GC2010, Shimadzu Corporation), using 2-ethylbutyric acid as an internal standard.

Measurement of free amino acid levels in serum

The concentration of free amino acids (FAAs) in serum collected on study day 50 were determined by an ultra-fast liquid chromatography system (Shimadzu Corporation) equipped with a fluorometric detector and an Acquity UPLC BEH C18 column (1.7 m, 100 × 2.1 mm) (Waters Corporation). For amino acid peak identification, amino acid standards (Sigma) were used.

Measurement of food intake and body weight

Food intake was measured per cage with intervals of 3 to 4 days when food was refreshed. Intake was calculated as the difference in weight between food put in the cage and food remaining after the 3- to 4-day time interval. The body weight of all mice was measured weekly. Based on a specific protocol, mice with a body weight >30% lower than the average body weight of the other mice within the group (n = 1), predefined as a humane endpoint, were euthanized and not considered for further analysis. In addition, the welfare of the mice was carefully monitored through daily observation of appearance and behaviour throughout the study.

Statistical analysis

With the indicated number of mice in each group and based on the estimated effect size of dietary intervention on the primary study outcome parameter (*i.e.* ear swelling), the present study was powered (1) to confirm the validity of the CMA model by demonstrating that allergic control mice have higher ear swelling than sham-sensitized mice, and (2) to investigate whether the ear swelling of mice in the 2% Gln group is different from that of allergic control mice. To this end, for the ear swelling, we first tested these two comparisons at a significance level of $\alpha = 0.05$, by means of unpaired t-tests. Only when these two comparisons were positive, the ear swelling of mice in the 0.5% Gln group was compared to that of allergic control mice, using an unpaired t-test at a significance level of $\alpha = 0.05$.

For all study parameters other than the ear swelling, differences between preselected pairs (n = 5; *Figure 1*) were analysed by means of a one-way analysis of variance (ANOVA) with a post hoc Bonferroni test if data were normally distributed (tested by Shapiro-Wilk normality tests). Log-transformation was applied to obtain normality prior to ANOVA testing, if required. For the anaphylactic shock score and for all non-normally distributed parameters, a Kruskal-Wallis test with a post hoc Dunn's test was applied. Spearman Rho correlation tests were used to evaluate correlations between parameters. Statistical analyses were performed using GraphPad Prism (version 8.4.2., GraphPad Software Inc.). *P*-values < 0.05 were considered statistically significant. Trends were indicated when $p < 0.10$. Normally distributed data are presented as mean ± SEM and non-normally distributed data are presented by Tukey box-and-whisker plots.

RESULTS

Food intake and body weight are not affected by Gln supplementation

As the addition of L-Gln to the diet can have an impact on the taste of the chow [26] and thus may influence the feeding behaviour of the mice, food intake and body weight of the animals were monitored throughout the study. No significant differences in either parameter were observed between the groups at any of the timepoints (*Figure 2*), nor in total food intake and weight gain throughout the study (data not shown). One mouse in the 2% Gln group reached a body weight of >30% lower than the average body weight of the other mice within this group, and was hence euthanized and not included in the analyses.

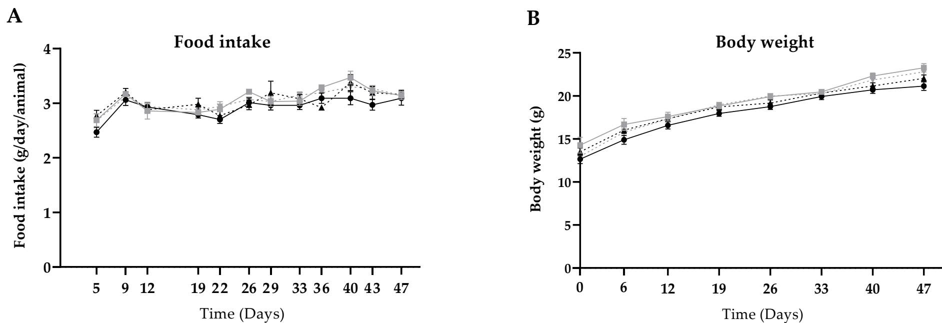


Figure 2. The food intake and body weight of the mice. Food intake of the mice as measured every 3-4 days **(A)** and body weight of the mice as measured weekly **(B)**. Groups are as follows: sham-sensitized mice (n = 6); Allergic control (n = 21); 0.5% Gln (n = 15); 2% Gln (n = 17). Values are expressed as mean ± SEM. Food intake was measured per cage (n = 2-7). Reliable data on food intake at study day 15 is lacking, as on study day 14 all mice received mashed pellets for 1 day to support the recovery from the first exposure to cholera toxin. Differences are analysed with a Kruskal-Wallis test with a post hoc Dunn's test **(A)** or a one-way ANOVA followed by a Bonferroni post hoc test (**B**) for selected groups. Values are expressed as mean ± SEM.

Gln supplementation attenuates the acute allergic response to whey protein

The ear swelling as measured 1 h after the i.d. whey challenge was significantly higher in allergic control mice compared to sham-sensitized mice ($p < 0.001$) (*Figure 3A*). Mice fed the 2% Gln diet had a lower ear swelling than allergic control mice ($p = 0.007$) (*Figure 3A)*.

Anaphylactic shock severity and the body temperature of the mice were determined at 15 min, 30 min and 1 h after the i.d. whey challenge. One mouse in the 0.5% Gln group and one mouse in the 2% Gln group reached an anaphylactic shock score of 4 at 1 h, and were hence euthanized and excluded from further analysis. At all timepoints, the allergic control mice had a higher anaphylactic shock score ($p < 0.05$; *Figure 3B*, *S2A-B*) and a lower body

temperature ($p < 0.05$; *Figure 3C*) than sham-sensitized mice. No significant differences in body temperature or anaphylactic shock scores were observed at any of the timepoints between allergic control mice and mice fed the 0.5% or 2% Gln diet. However, the shock scores and the body temperature among the Gln-supplemented groups followed a similar pattern as observed for ear swelling (*Figure 3A-C*). Accordingly, ear swelling correlated with the anaphylactic shock score (r = 0.730, $p < 0.001$; n = 57; *Figure S2C*) and the body temperature (r = -0.718, $p < 0.001$; n = 57; *Figure S2D*) at 1 h after the i.d. challenge.

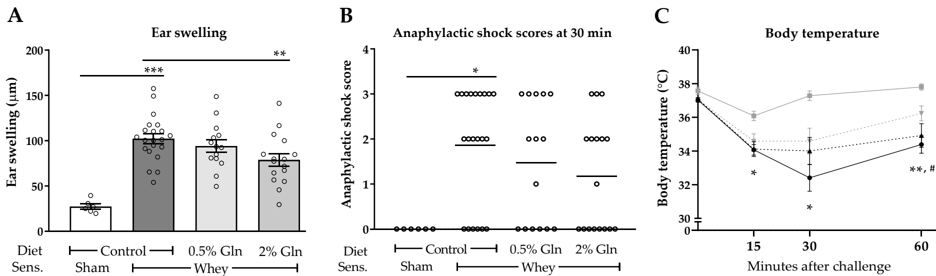


Figure 3. The allergic response to whey protein after intradermal challenge. Ear swelling at 1 h **(A)**, anaphylactic shock scores at 30 min **(B)**, and body temperature **(C)** at 15 min, 30 min and 1 h after i.d. whey challenge. Groups in figure **C**) are as follows: ■ sham-sensitized mice (n = 6); ● Allergic control mice (n = 21); ▲ mice fed the 0.5% Gln diet (n = 15); ▼ mice fed the 2% Gln diet (n = 17). Values are expressed as mean ± SEM. In figure **A**-**B**, significant differences are indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. In figure **C**, asterisks (*$p < 0.05$, **$p < 0.01$) indicate a difference between the sham-sensitized group and the allergic control group, and a hashtag ($^{\#}p < 0.05$) indicates a difference between the sham-sensitized group and the 0.5% Gln group. Differences are analysed by sequential t-tests **(A)**, or a Kruskal-Wallis test with a post hoc Dunn's test for selected groups **(B-C)**. Sens.: sensitization; Gln: glutamine.

The lower allergic skin response in mice fed 2% Gln is associated with higher levels of whey-specific IgA and a lower ratio of whey-specific IgE/IgA in serum

To investigate the humoral immune response and mucosal mast cell activation in the mice, levels of whey-specific immunoglobulins and mMCP-1 were measured in serum collected 30 min after oral whey challenge. Levels of mMCP-1 and each of the immunoglobulins were higher in whey-sensitized mice compared to sham-sensitized mice, irrespective of the diet ($p < 0.05$, *Figure 4A-E*). Dietary intervention had no significant effect on levels of mMCP-1, IgE, IgG1 and IgG2a (*Figure 4A-D*). Levels of IgA were higher in mice fed the 2% Gln diet compared to allergic control mice ($p = 0.014$; *Figure 4E*). Moreover, compared to allergic control mice, mice fed the 2% Gln diet tended to have a higher ratio of IgG2a to IgG1 ($p = 0.069$; *Figure 4F*) and had a lower ratio of IgE to IgA ($p = 0.002$; *Figure 4G*). The latter ratio positively correlated with the ear swelling in whey-sensitized mice (r = 0.634, $p < 0.001$; n = 51; *Figure 4H*). Levels of IgA also correlated with ear swelling, though only moderately (r = -0.362, $p = 0.010$; n = 51; data not shown).

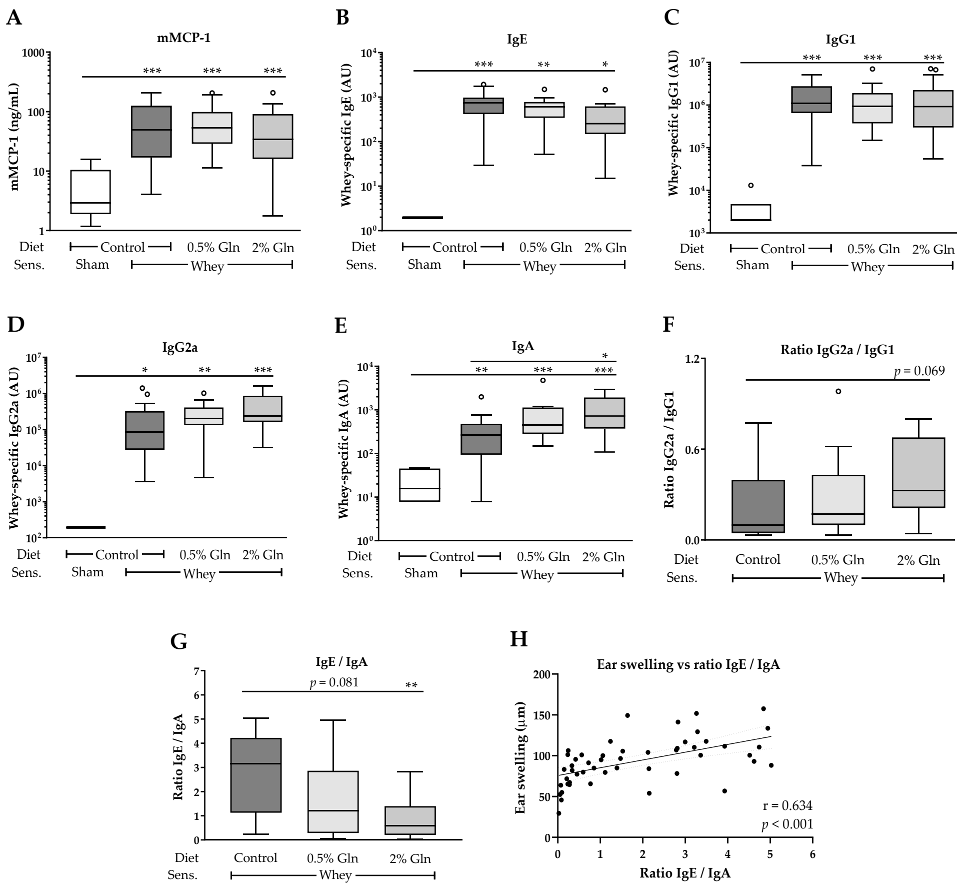


Figure 4. Levels of mMCP-1 and whey-specific immunoglobulins in serum. Serum of all mice was collected 30 min after the oral whey challenge. Serum levels are shown for mMCP-1 **(A)** and for whey-specific IgE **(B)**, IgG1 **(C)**, IgG2a **(D)** and IgA **(E)**. Ratios are shown for whey-specific IgG2a/IgG1 **(F)** and IgE/IgA **(G)**. Spearman correlation between the ratio of whey-specific IgE/IgA and ear swelling **(H)**. Data are represented as box-and-whisker Tukey plots in which outliers are plotted as separate data points. Significant differences are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Differences were analysed by one-way ANOVA followed by a Bonferroni post hoc test when data obtained normality after log-transformation (**A, C, E, F**), or otherwise with a Kruskal-Wallis test with a post hoc Dunn's test (**B, D, G**) for selected groups. AU: Arbitrary Unit; Sens.: sensitization; Gln: glutamine.

Serum whey-specific IgA levels correlate with faecal whey-specific IgA levels

To evaluate whether changes in serum IgA levels were associated with changes in mucosal IgA levels, faecal whey-specific IgA was measured as an indicator of mucosal whey-specific IgA. Faecal IgA levels of mice fed the 0.5% or 2% Gln diet were higher than those of sham-sensitized mice (p = 0.003 and 0.002, respectively), while faecal IgA levels were similar between sham-sensitized mice and allergic control mice (p = 0.171) (*Figure 5A*). Compared to allergic control mice, mean faecal IgA levels were 2.5- and 2.2-fold higher in mice fed the 0.5% Gln and the 2% Gln diet, respectively, but these differences were not statistically significant (p = 0.130 and 0.102, respectively). Faecal IgA correlated with serum IgA in whey-sensitized mice (r = 0.598, $p < 0.001$; n = 49; *Figure 5B*).

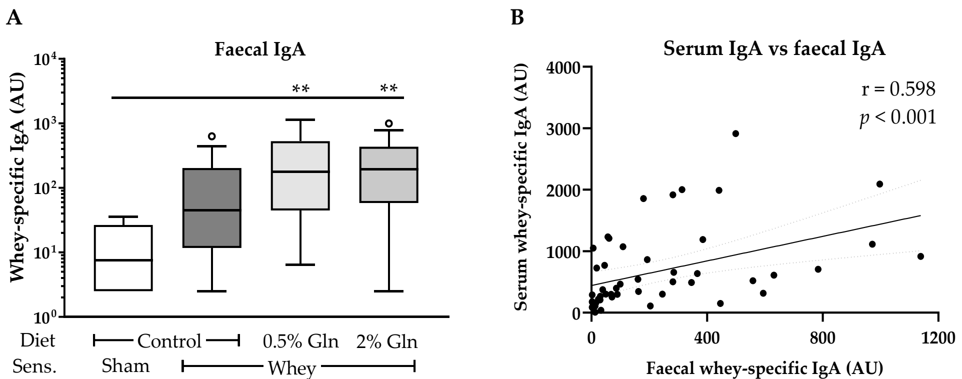


Figure 5. Levels of whey-specific IgA in faecal samples. Levels of whey-specific IgA in faeces, collected 6 days after the last sensitization **(A)**. Spearman correlation between whey-specific IgA levels in serum and faeces **(B)**. Data are represented as box-and-whisker Tukey plots in which outliers are plotted as separate data points. Significant differences are indicated by **$p < 0.01$. Differences were analysed with a one-way ANOVA followed by a Bonferroni post hoc test after log-transformation. AU: Arbitrary Unit; Sens.: sensitization; Gln: glutamine.

Mice fed 2% Gln have a higher percentage of activated T_H1 cells in the MLN than allergic mice, but T_H2 and T_{reg} cell frequencies are not affected

To investigate whether the Gln diets affected splenic and intestinal T cell populations, single cell suspensions of isolated spleens and MLNs were analysed by flow cytometry. In the MLNs, no significant differences were found in T cell populations between sham-sensitized mice and allergic control mice (*Figure 6*). Mice fed the 2% Gln diet had a higher percentage of activated T_H1 cells compared to allergic control mice (p = 0.014; *Figure 6A*). A similar effect was observed for the ratio of activated T_H1 to activated T_H2 cells, but significance was not reached (p = 0.110; *Figure 6B*). Frequencies of activated T_H2 cells, total T_H1 and T_H2 cells, and $FoxP3^+$ regulatory T (T_{reg}) cells in the MLN were similar in all groups (*Figure 6C-F*). No differences in splenic T cell populations were found between the groups (*Figure S3*).

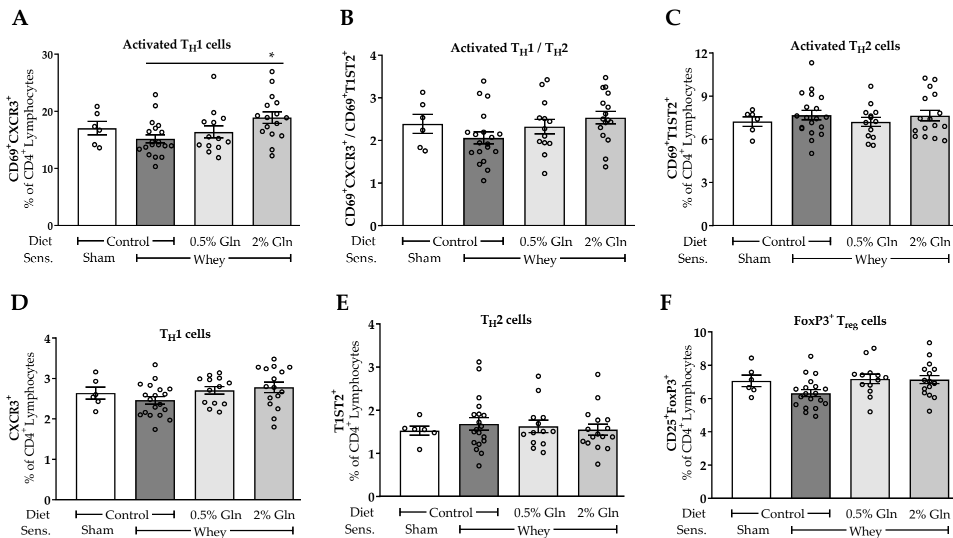


Figure 6. Percentage of T cell subsets in the mesenteric lymph nodes. The percentage of activated T$_H$1 cells ($CD69^+CXCR3^+$ of $CD4^+$ cells) **(A)**, the ratio of activated T$_H$1 cells to activated T$_H$2 cells ($CD69^+T1ST2^+$ of $CD4^+$ cells) **(B)**, the percentage of activated T$_H$2 cells **(C)**. The percentage of total T$_H$1 cells ($CXCR3^+$ of $CD4^+$ cells) **(D)**, total T$_H$2 cells ($T1ST2^+$ of $CD4^+$ cells) **(E)**, and $FoxP3^+$ Treg cells ($CD25^+FoxP3^+$ of $CD4^+$ cells) **(F)**. Values are expressed as mean ± SEM. Differences are indicated by *$p < 0.05$, as analysed by one-way ANOVA followed by a Bonferroni post hoc test for selected groups. Sens.: sensitization; Gln: glutamine; T$_H$1: T helper type 1; T$_H$2: T helper type 2; T$_{reg}$: regulatory T cells.

MLN cells of Gln-supplemented mice produce more IFNγ and IL-2 following *ex vivo* stimulation with anti-CD3 than MLN cells of allergic mice

To assess the stimulatory response of immune cells in the spleen and MLN, splenocytes and MLN cells were restimulated *ex vivo* with whey or anti-CD3. Upon stimulation with whey, splenocytes of allergic control mice produced higher levels of IL-10, IFNγ, IL-2, IL-5 and IL-13 than those of sham-sensitized mice (all $p < 0.05$; *Figure 7A*). The levels of these cytokines did not significantly differ between allergic control mice and mice fed a Gln-enriched diet (*Figure 7A*). MLN cells produced no detectable levels of cytokines following whey stimulation. IL-6 was undetectable in all MLN and splenocyte cultures.

Cytokine production by anti-CD3-stimulated splenocytes was largely similar among all groups, except for IL-10 (*Figure 7B*), which was higher in splenocyte cultures of mice fed 0.5% or 2% Gln compared to those of sham-sensitized mice ($p = 0.003$ and $p = 0.042$, respectively), and higher in cultures of mice fed 0.5% Gln compared to those of allergic control mice ($p = 0.016$). Similarly, anti-CD3-stimulated MLN cells of mice fed 0.5% Gln produced more IL-10 than those of allergic control mice ($p = 0.045$; *Figure 7C*). Moreover,

compared to MLN cells of allergic control mice, MLN cells of mice fed 0.5% Gln produced more IFNγ ($p = 0.013$; *Figure 7C*), and MLN cells of mice fed 2% Gln produced more IFNγ ($p = 0.020$; *Figure 7C*) and IL-2 ($p = 0.004$; *Figure 7C*). The latter effects were not observed in splenocyte cultures (*Figure 7B*).

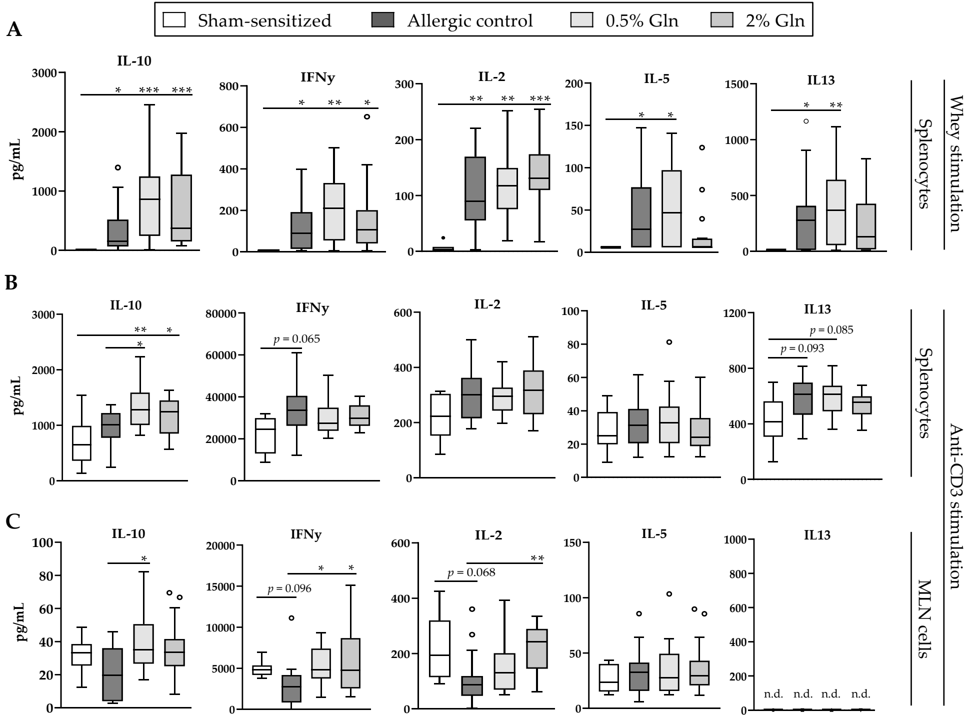


Figure 7. Concentrations of cytokines produced by restimulated splenocytes and MLN cells. Concentrations of IL-10, IFNγ, IL-2, IL-5 and IL-13 as measured in supernatants after *ex vivo* stimulation of splenocytes with whey for five days **(A)**, splenocytes with anti-CD3 for two days **(B)**, and MLN cells with anti-CD3 for two days **(C)**. Data are represented as box-and-whisker Tukey plots in which outliers are plotted as separate data points. Significant differences are indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Differences were analysed with a one-way ANOVA followed by a Bonferroni post hoc test when data were normally distributed or obtained normality after log-transformation (**B**), or otherwise with a Kruskal-Wallis test with a post hoc Dunn's test (**A, C**) for selected groups. Gln: glutamine; n.d.: not detectable.

Caecal branched-chain fatty acids are elevated in allergic mice but not in Gln-supplemented mice

As food allergy has been associated with modulations in the intestinal microbiota, caecal SCFAs were measured as an indicator of microbial metabolic activity. Compared to sham-sensitized mice, allergic control mice had higher levels of iso-valerate (p = 0.040) and valerate (p = 0.011), and also had a higher sum of all BCFAs (p = 0.046) (*Figure 8*). Mice fed the 2% Gln diet had significantly lower levels of these BCFAs (all $p < 0.05$) than allergic control mice (*Figure 8*). A similar pattern among the groups was observed for levels of the BCFA iso-butyrate (*Figure 8*).

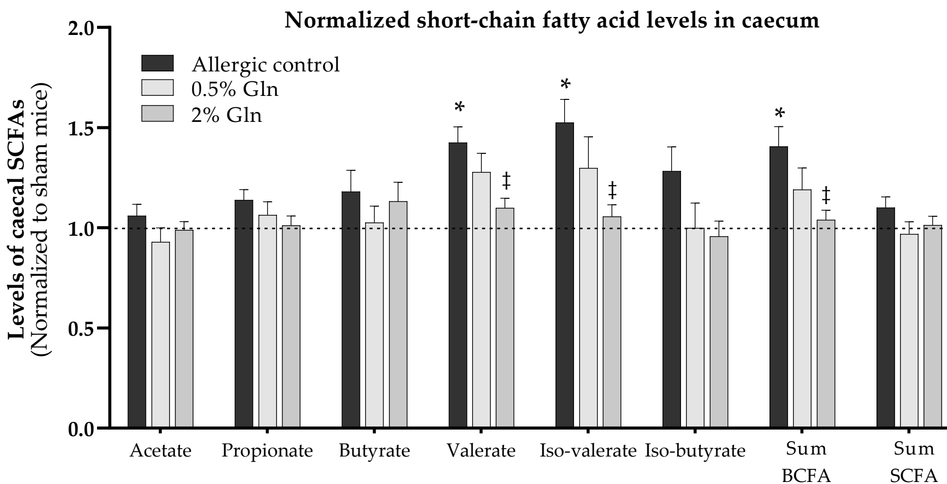


Figure 8. Levels of short chain fatty acids (SCFAs) in caecum. Data are normalized to sham-sensitized mice and expressed as mean ± SEM. Differences compared to sham-sensitized mice are indicated by *$p < 0.01$, and differences compared to allergic control mice are indicated by ‡$p < 0.05$, as analysed with a one-way ANOVA followed by a Bonferroni post hoc test for selected groups using non-normalized data. SCFA: short-chain fatty acids; BCFA: branched short-chain fatty acids (valerate + iso-valerate + iso-butyrate).

Serum levels of most free amino acids are highest in allergic mice

Levels of 12 out of 18 serum FAAs were highest in allergic control mice, and approached sham levels in mice fed the 2% Gln diet (*Figure S4*). Compared to allergic control mice, mice fed the 2% Gln diet had significantly lower levels of His, Leu, Ile and Val, and had lower sums of BCAAs and FAAs in serum (all $p < 0.05$; *Figure S4*).

DISCUSSION

Glutamine (Gln) is known to support intestinal health and development [13-15] and supplementation with this FAA has been effective in preventing and treating various atopic diseases in animals [17-20]. The present study investigated for the first time the effects of dietary Gln supplementation on the development of food allergy, using an extensively validated murine model for cow's milk allergy (CMA). We found that mice fed a diet containing 2% Gln had a lower allergen-induced acute allergic skin response than mice fed a control diet. Elevated levels of allergen-specific IgA and a lowered ratio of allergen-specific IgE to IgA in the serum of the Gln-fed mice may underlie the lower severity of clinical symptoms. Moreover, a higher frequency of activated T_H1 cells in the mesenteric lymph nodes (MLN), a higher production of IFNγ and IL-2 by MLN cells following *ex vivo* restimulation, and a tendency towards a higher ratio of allergen-specific IgG2a to IgG1 in serum suggest enhanced mucosal T_H1 immunity in these mice, albeit without a clear indication of suppressed T_H2 immunity.

The lower ear swelling in mice fed the 2% Gln diet was accompanied by higher serum levels of allergen-specific IgA and a lowered ratio of allergen-specific IgE to IgA in serum. Gln supplementation has been previously associated with increased serum levels of total IgA in healthy and immune-compromised animals [27-30], and with enhanced serum levels of specific IgA in humans following vaccination [31, 32]. The role of serum IgA in allergic disease is relatively unexplored. It has been suggested that serum IgA can act as a blocking antibody that neutralizes allergens that breach the mucosal surface [33], which may limit the ability of allergens to provoke an allergic response. In line with this, intravenous injection of allergen-specific IgA prior to oral allergen challenge effectively inhibited IgE-mediated anaphylaxis in mice [34]. Moreover, in murine models for food allergy [35, 36], as well as in infants with CMA [37, 38], a higher level of allergen-specific IgA in serum has been associated with improved tolerance and less severe allergic symptoms. The present study found a similar association between serum IgA and allergic symptoms, but showed a stronger correlation between the IgE/IgA ratio and allergic symptoms. Studies have reported an increase in this ratio in children allergic to cow's milk or other food antigens [39, 40], while children who developed tolerance to cow's milk showed a functional shift in this ratio towards IgA dominance [41, 42]. Similarly, successful desensitization to cow's milk in children through oral immunotherapy has been associated with increased specific IgA in parallel to decreased specific IgE in serum [43, 44]. Together, these findings suggest that a higher level of allergen-specific IgA and a lower IgE/IgA ratio in serum support tolerance to food allergens, and hence could contribute to the lower severity of clinical symptoms of Gln-supplemented mice as observed in the current study.

Studies suggest a close relation between serum and mucosal IgA, by showing that a considerable portion of the IgA-secreting plasma cells induced in the intestines migrate to the bone marrow to secrete IgA systemically [45, 46]. Gln supplementation has been consistently shown to increase mucosal IgA production in various animals, among others by enhancing the number of IgA-secreting plasma cells in the intestines [47, 48]. To evaluate whether the increased levels of serum IgA in Gln-supplemented mice could be a result of alterations in mucosal IgA, faecal IgA, a commonly used indicator of mucosal IgA in mice [49], was measured and tested for correlations with serum IgA. We observed that faecal IgA levels were the highest in Gln-supplemented mice, suggesting that Gln may have had a stimulating effect on intestinal IgA production. Consistent with previous observations [50-52], faecal IgA correlated with serum IgA in the present study. Thus, the elevated levels of serum IgA in Gln-supplemented mice could, at least in part, be mediated by local effects of Gln within the intestines.

Extensive research supports a role for intestinal IgA in the maintenance of clinical tolerance to food antigens, through allergen neutralization within the intestines [53, 54]. Consistent with this, enhanced faecal IgA levels have been correlated to the establishment of clinical tolerance to food allergens [54, 55]. Though our results indicate that Gln supplementation enhanced allergen-specific faecal IgA levels, we did not find a correlation between these IgA levels and the severity of allergic symptoms (data not shown). This might be explained by the fact that we induced allergic symptoms via a local, dermal allergen challenge. Suppressive effects of intestinal IgA on the development of allergic symptoms may be better observed following an oral allergen challenge, which enables a direct interaction between the allergen and intestinal IgA. Thus, future studies could complement our work by investigating the effects of Gln supplementation on allergic symptoms induced via oral allergen application.

The lower ear swelling of Gln-supplemented mice was not accompanied by a reduction in any of the tested markers for T_H2 immunity, nor by changes in MLN and splenic T_{reg} cell frequencies. However, a higher frequency of activated T_H1 cells in the MLN of mice fed 2% Gln was observed, which coincided with a trend towards a higher ratio of T_H1-associated IgG2a to T_H2-associated IgG1 in serum. Moreover, anti-CD3-stimulated MLN cells of these mice produced higher levels of the T_H1 cytokine IFNγ and of IL-2, a critical regulator of immune homeostasis shown to be effective in preventing food allergy in mice through driving T_{reg} cell-dependent modification of the intestinal T_H1/T_H2 immune balance [56, 57]. We did not observe such effects in the spleen. Combined, these data may suggest that Gln promoted a local modification in the T_H1/T_H2 immune balance in favour of T_H1 immunity. Dietary Gln has previously been associated with similar modifications in the T_H1/T_H2 immune balance. For instance, mice fed a Gln-enriched diet had higher IFNγ and lower IL-

4 expression in intraepithelial lymphocytes, and an increased ratio of TH1 to TH2 cells in blood after induction of sepsis [58, 59]. Similarly, MLN cells of Gln-fed piglets produced more IFNγ and had a higher IFNγ/IL-4 production ratio following stimulation with phytohemagglutinin (PHA) [60]. It has been suggested that Gln may regulate TH1 immunity through activation of mechanistic target of rapamycin complex 1 (mTORC1) or through metabolic conversion into α-ketoglutarate, which are both implicated in the induction of TH1 differentiation [61-63].

We found that allergic control mice had a higher sum of caecal BCFAs than sham-sensitized mice. Dietary supplementation with Gln limited this increase in a dose-dependent manner. The enhanced levels of BCFAs in allergic mice are consistent with the higher BCFAs levels found in faeces of CMA infants [64, 65] and in faeces and caecum of food allergic mice [66]. BCFAs are often used as a biomarker for microbial protein fermentation [67], which is enhanced in cases of impaired gut health and intestinal inflammation [68, 69]. Thus, the higher caecal BCFAs in allergic mice could be related to the mucosal inflammatory processes associated with food allergy. Gln is known to improve gut health and suppress intestinal inflammation [14], which may underlie the lower caecal BCFA content observed in Gln-fed mice. Further research is required to investigate the exact mechanism underlying this observation.

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We observed that levels of most serum FAAs followed a similar pattern among the groups as caecal BCFA levels. The slightly higher levels of serum FAAs in allergic control mice may reflect our previous suggestion of enhanced microbial protein breakdown in these mice, as microbial protein catabolism can contribute significantly to changes in plasma FAAs [70]. Recent studies also suggest a role for enhanced autophagy in allergic disease [71], which may further contribute to an increase in serum FAAs. However, as serum FAA levels are affected by many other factors, such as FAA uptake and excretion by tissues, alterations in protein breakdown may only partially explain our findings. Additional studies investigating serum FAAs in food allergic disease are warranted to better understand the biological basis of this finding.

The results of the present study should be interpreted in light of its limitations. It is important to note that there are differences between the murine and human IgA system. In human, receptors for the Fc region of IgA (FcαR), present on various innate immune cells, are key mediators of the effector function of serum and intestinal IgA [72]. Binding of IgA to FcαR can provoke both anti- and pro-inflammatory responses, depending on the context [73]. Analogous receptors have been found in various rodents but until now not in mice, indicating that effects induced by IgA may differ between mice and humans. This may potentially confound clinical translation of our findings, although studies suggest similar

beneficial effects of allergen-specific IgA in the protection against food allergy in mice and human. Another limitation is that the present study used a design in which part of the dietary protein was substituted for free Gln. As such, the levels of all protein-bound AAs were slightly lower in the Gln diets compared to the control diet. Thus, the reported effects may in part reflect a reduced availability of certain AAs. However, as levels of all AAs in the Gln diets were sufficient for maintaining rodents [74], and as we observed similar growth patterns among the groups, we expect that this hypothesized reduced availability will be negligible.

CONCLUSIONS

The current study demonstrates that a diet in which part of the protein content is substituted by free Gln (2% w/w), and which is provided before and during allergic sensitization, inhibits acute cow's milk allergic symptoms in mice. Underlying mechanisms of the observed clinical benefits may involve enhanced secretion of serum and intestinal allergen-specific IgA. Improvements in the intestinal T_H1/T_H2 immune balance and suppressed intestinal inflammation may further contribute to the clinical improvements. While dietary Gln has been previously associated with the prevention of intestinal inflammation and allergic asthma in various preclinical models, the present study shows for the first time a link between dietary Gln and the protection against food allergy. Our findings warrant further investigations into the effects of dietary Gln on food allergy development in early life, as this may ultimately provide opportunities for developing novel preventive strategies for CMA and possibly other food allergies in infancy.

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6

SUPPLEMENTARY DATA

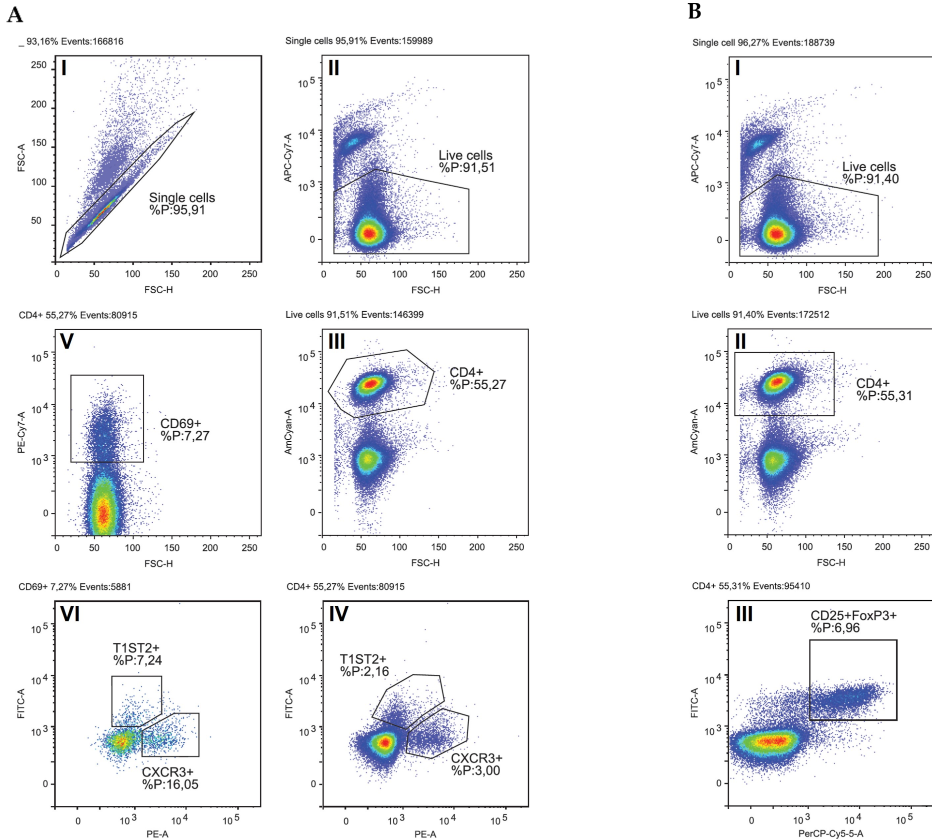

Figure S1. Flow cytometric analysis of T cell populations in the mesenteric lymph nodes and spleen. (A) Single cells are identified by plotting forward scatter-area (FSA) against forward scatter-height (FSH) (plot I). Live cells were distinguished from dead cells using fixable viability dye eFluor 780 (plot II). $CD4^+$ T cells were discriminated on the basis of the surface expression of CD4 (plot III). $CD4^+$ cells were further gated for total (activated and non-activated) T_H1 and T_H2 cells based on the surface expression of CXCR3 and T1ST2, respectively (plot IV), and for activated cells based on the surface expression of CD69 (plot V). The $CD4^+CD69^+$ population was further gated for activated T_H1 and T_H2 cells based on the surface expression of CXCR3 and T1ST2, respectively (plot VI). **(B)** In a separate analysis, after isolating single and live cells (plot I), the $CD4^+$ population (plot II) was gated for regulatory T cells on the basis of surface expression of CD25 and intracellular expression of FOXP3 (plot III).

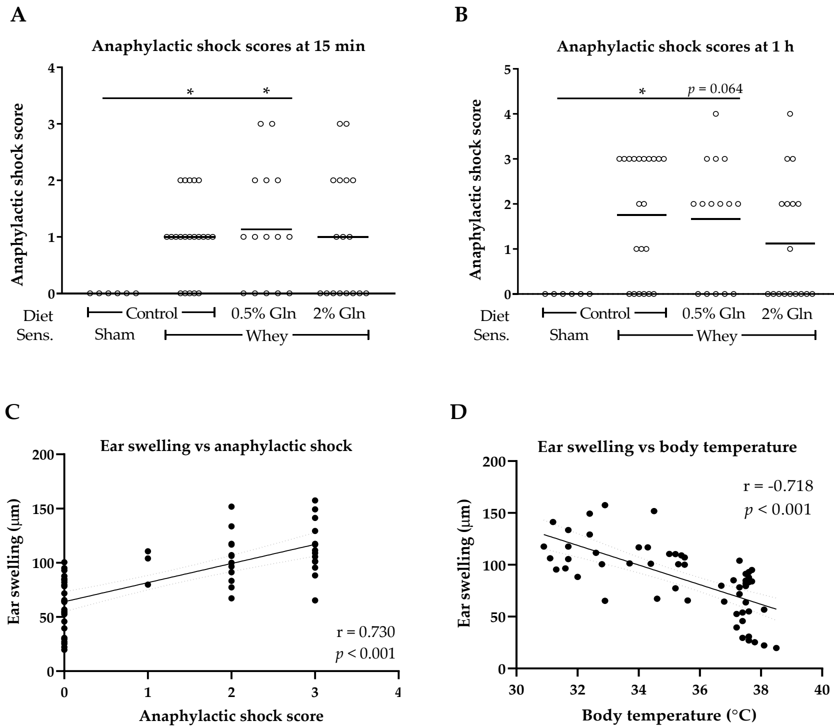

Figure S2. Anaphylactic shock scores after intradermal whey challenge and correlations between allergic symptoms. The anaphylactic shock symptoms are shown as scored at 15 min **(A)** and 1 h **(B)** after the i.d. challenge with whey. The line represents the group mean. Significant differences are indicated by $*p < 0.05$. Differences were analysed by a Kruskal-Wallis test with a post hoc Dunn's tests for selected groups. Spearman correlations are shown between the ear swelling and the anaphylactic shock score at 1 h after i.d. challenge **(C)** and between the ear swelling and the body temperature at 1 h after i.d. challenge **(D)**. Sens.: sensitization; Gln: glutamine.

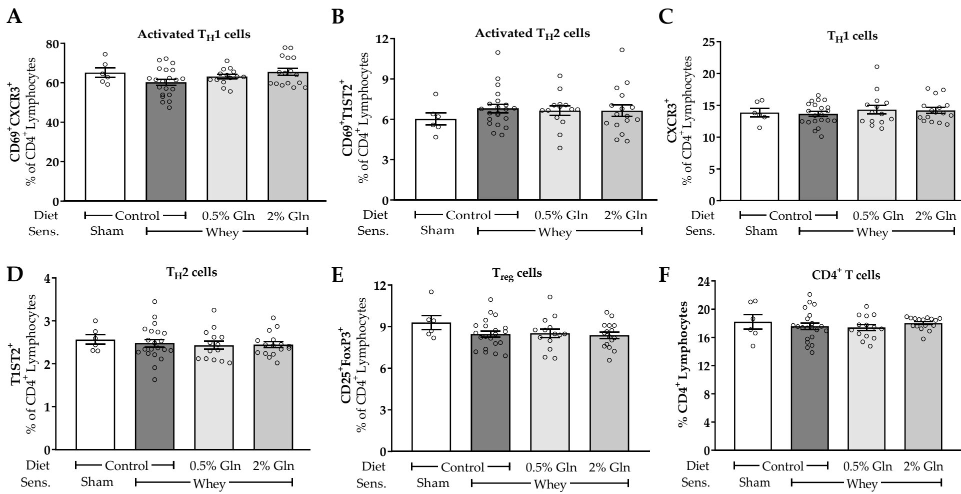

Figure S3. Percentage of T cell subsets in the spleen. The percentage of activated T_H1 cells ($CD69^+CXCR3^+$ of $CD4^+$ cells) **(A)**, activated T_H2 cells ($CD69^+T1ST2^+$ of $CD4^+$ cells) **(B)**, total T_H1 cells ($CXCR3^+$ of $CD4^+$ cells) **(C)**, total T_H2 cells ($T1ST2^+$ of $CD4^+$ cells) **(D)**, $FoxP3^+$ Treg cells ($CD25^+FoxP3^+$ of $CD4^+$ cells) **(E)** and $CD4^+$ cells **(F)**. Values are expressed as mean ± SEM. Differences were analysed with a one-way ANOVA followed by a Bonferroni post hoc test when data were normally distributed or obtained normality after log-transformation **(B-E)** or otherwise with a Kruskal-Wallis test with a post hoc Dunn's test **(A, F)** for selected groups. Sens.: sensitization; Gln: glutamine; T_H1: T helper type 1; T_H2: T helper type 2; T_{reg}: regulatory T cells.

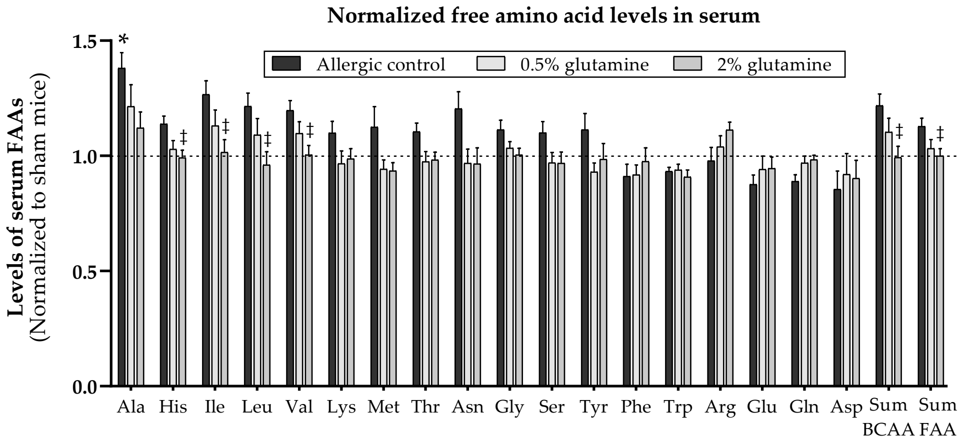

Figure S4. Levels of free amino acids (FAAs) in serum collected at the end of the study. Data are normalized to sham-sensitized mice and expressed as mean ± SEM. Differences compared to sham-sensitized mice are indicated by *$p < 0.01$, and differences compared to allergic control mice are indicated by ‡$p < 0.05$, as analysed with a one-way ANOVA followed by a Bonferroni post hoc test for selected groups using non-normalized data. FAA: free amino acids; BCAA: branched-chain amino acids (leucine + isoleucine + valine); Ala: alanine; His: histidine; Ile: isoleucine; Leu: leucine; Val: valine; Lys: lysine; Met: methionine; Thr: threonine; Asn: asparagine; Gly: glycine; Ser: serine; Tyr: tyrosine; Phe: phenylalanine; Trp: tryptophan; Arg: arginine: Glu: glutamate; Gln: glutamine; Asp: aspartate.

Chapter 7

A free amino acid-based diet partially prevents symptoms of cow's milk allergy in mice after oral sensitization with whey

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ABSTRACT

Amino acid-based formulas (AAFs) are used for the dietary management of cow's milk allergy (CMA). Whether AAFs have the potential to prevent the development and/or symptoms of CMA is not known. The present study evaluated the preventive effects of an amino acid (AA)-based diet on allergic sensitization and symptoms of CMA in mice and aimed to provide insight into the underlying mechanisms. C3H/HeOuJ mice were sensitized with whey protein or with phosphate-buffered saline as sham-sensitized control. Starting two weeks before sensitization, mice were fed either a protein-based diet or an AA-based diet with an AA composition based on that of the AAF Neocate, a commercially available AAF prescribed for the dietary management of CMA. Upon allergen challenge, allergic symptoms, mast cell degranulation, whey-specific immunoglobulin levels in serum and $FoxP3^+$ cell counts in jejunum sections were assessed. Compared to mice fed the protein-based diet, AA-fed mice had significantly lower acute allergic skin responses. Moreover, the AA-based diet prevented the whey-induced symptoms of anaphylaxis and drop in body temperature. While the AA-based diet had no effect on levels of serum IgE and mucosal mast cell protease-1 (mMCP-1), AA-fed mice had significantly lower serum IgG2a levels and tended to have lower serum IgG1 levels ($p = 0.076$). Additionally, the AA-based diet prevented the whey-induced decrease in $FoxP3^+$ cells in the jejunum. This study demonstrates that feeding an AA-based diet partially prevents allergic symptoms of CMA in mice. Differences in $FoxP3^+$ cell counts and in the serum levels of IgG2a and IgG1 may suggest enhanced anti-inflammatory and tolerizing capacities in AA-fed mice. These results indicate that next to being effective in the dietary management of CMA, AAFs may also have the potential to inhibit the development of CMA, which warrants further research.

INTRODUCTION

Cow's milk allergy (CMA) is one of the most commonly occurring food allergies in infancy, affecting up to 3% of the children at 1 year of age in developed countries [1, 2]. CMA in infancy represents an increasing global health and economic burden, which is caused not only by an increased prevalence over the last decades, but also by an increased persistence, severity and complexity of the condition [3-6]. Following ingestion of cow's milk, affected children usually present moderate symptoms involving the skin, the gastrointestinal and/or the respiratory tract, but life-threatening systemic anaphylaxis may also occur [7, 8]. In fact, cow's milk is described as one of the most common foods capable of inducing fatal anaphylactic reactions in infancy [9, 10]. In addition to these acute clinical manifestations, CMA in early life can also have long-lasting effects, including delays in growth and development [11, 12], as well as increased risk of developing atopic diseases later in life [13, 14]. Hence, strategies to suppress or prevent the development of CMA are of major importance.

To date, the standard dietary management of CMA in children is allergen avoidance through elimination of cow's milk from the diet [15]. Without appropriate substitution, however, such an elimination diet may lead to nutritional deficiencies and poor growth [16]. A variety of infant formulas have been developed and acknowledged to be suitable substitutions for cow's milk. Extensively hydrolysed formulas (eHFs) are recommended for infants with mild CMA, whereas for infants with severe CMA and for infants who either do not tolerate eHFs or for whom eHFs fail to resolve CMA symptoms, amino acid-based formulas (AAFs) are recommended [17]. Besides being nutritionally adequate [18], AAFs provide relief and a faster recovery from symptoms of CMA, including gastrointestinal (*e.g.* vomiting and diarrhoea) and skin-related symptoms (*e.g.* atopic eczema) [11, 19-21]. Moreover, nutritional intervention with an AAF normalizes the growth of infants with CMA and is overall well-tolerated [22-25]. AAFs are therefore regarded as an effective way of dietary management of CMA.

Whereas numerous studies have investigated AAFs as a dietary management option for CMA, little is known about the potential of AAFs to prevent allergic sensitization and clinical symptoms of CMA. Yet, AAFs have been shown to exert potent immune-modulatory effects both in infants and in *in vitro* immune models [26, 27]. For instance, in infants with non-IgE-mediated CMA, a commercially available AAF prescribed for the dietary management of CMA (*i.e.* Neocate) reduced levels of pro-inflammatory cytokines interleukin-6 (IL-6) and Tumor Necrosis Factor alpha (TNFα), which are both indicated to drive allergic sensitization [26, 28, 29]. This reduction was accompanied by a significant decline in epithelial derived interleukin-33 (IL-33) and in T-cell helper type 2 (TH2)-

associated cytokines IL-4 and IL-13, which are all key contributors to the development of food allergy [30]. Based on these findings, we hypothesized that AAFs can prevent the development of CMA. Therefore, the present study evaluated the preventive effects of an amino acid (AA)-based diet on allergic sensitization and allergic symptoms of CMA, using an extensively validated murine model of orally induced CMA [31].

METHODS

Animals, diets and consumption measurement

Four-week old female, specific pathogen-free C3H/HeOuJ mice which were bred and raised for at least two generations on a cow's milk protein-free diet were purchased from Charles River Laboratories (Maastricht, The Netherlands). All mice were housed in filter-topped makrolon cages (n = 5 per cage) on a 12 h light/dark cycle with unlimited access to food and water at the animal facility of Utrecht University and were acclimatized for 7 days. Animal care and use was performed in strict accordance with the principles of good laboratory animal care as stated by the European Directive 2010/63/EU for the protection of animals used for scientific purposes. All experimental procedures were approved by an independent ethics committee for animal experimentation (DEC Consult, Soest, The Netherlands).

Mice were fed *ad libitum* either a control, semi-purified AIN-93G soy protein-based diet (SSniff Spezialdiaten GmbH, Soest, Germany) [32], or an experimental amino acid (AA)-based diet with an AA composition based on that of the commercially available AAF Neocate (Nutricia) (*Table 1*). Other than the protein content, the diets were identical in all nutrients. Two weeks before the first sensitization, mice were placed on either of the two diets until the end of the study protocol (*Figure 1*). For food consumption measurement, food was weighed per cage with intervals of 3 to 4 days when food was refreshed. The body weight of all mice was measured at study days 1, 2, 3, 4, 5, 6, 8, 13, 20, 27, 34, 41 and 48. Throughout the entire duration of the study, the welfare of the animals was carefully monitored through observation of appearance, body functions (*e.g.* changes in body temperature) and behaviours.

Experimental animal procedures

Upon arrival, 40 mice were randomly assigned to one of the following four groups (n = 10 per group): 1) a sham-sensitized control group fed the protein-based control diet, 2) a sham-sensitized experimental group fed the AA diet, 3) a whey-sensitized control group fed the protein-based control diet and 4) a whey-sensitized experimental group fed the AA diet.

Whey-sensitized mice were sensitized orally via gavage with 20 mg of homogenized whey (WPC60, Milei, Friesland Campina) in 500 μL phosphate-buffered saline (PBS), containing 10 μg cholera toxin (CT) (List Biological Laboratories) as an adjuvant [33]. Sham-sensitized mice received 10 μg CT in 500 μL PBS. Mice were sensitized once a week for 5 consecutive weeks, starting at day 14 (*Figure 1*). One week after the final sensitization, all mice received an intradermal (i.d.) injection of 10 μg homogenized whey in 10 μL PBS in the pinnae of both ears to measure the acute allergic skin reaction as the primary study outcome. Ear thickness was measured in duplicate for each ear before and 1 h after i.d. injection with whey, using a digital micrometer (Mitutoyo). Whey-induced ear swelling was calculated by subtracting the basal ear thickness from the ear thickness measured at 1 h after i.d. injection. Moreover, at 15, 30, 45 and 60 min after the i.d. injection, the body temperature of the mice was measured using temperature transponders (IPTT-300, Biomedical data systems) which were injected subcutaneously 7 days before the sensitization started (*Figure 1*). In addition to the measurement of body temperature, anaphylactic shock severity was scored using a validated 0- to 5-point scoring system (*Table 2*) adapted from Li *et al*. [34]. Animals reaching a shock score of 4 (n = 1), considered a humane endpoint, were euthanized and not considered for further analysis. One day after the measurement of the acute allergic skin reaction, the mice were challenged intragastrically (i.g.) with 100 mg homogenized whey in 500 μL PBS. After 30 minutes, blood samples were taken via orbital extraction under terminal anaesthesia (isoflurane/air), followed by cervical dislocation. Sera were stored at −80°C for immunoglobulins and mouse mast cell protease-1 (mMCP-1) analyses.

Group	n	Statistical comparisons
1. Sham-sensitized mice fed the control diet	10	
2. Sham-sensitized mice fed the AA diet	10	
3. Whey-sensitized mice fed the control diet	10	
4. Whey-sensitized mice fed the AA diet	10	

Figure 1. Schematic representation of the experimental model and the experimental groups. AA: amino acid.

Table 1. The composition of the protein-based control diet and the amino acid-based experimental diet

Components	Control diet (g/kg)	Amino acid diet (g/kg)
	Carbohydrates	
Corn starch	397.5	397.5
Dextrinized corn starch	132.0	132.0
Sucrose	100.0	100.0
	Fiber	
Cellulose	50.0	50.0
	Protein / Amino acids	
Soy protein	200.0	–
Free amino acids	3.0	203.0
L-Alanine	–	8.0
L-Arginine	–	14.2
L-Aspartic acid	–	13.2
L-Cysteine	1.0	5.2
L-Glutamine	–	21.5
L-Glycine	–	12.5
L-Histidine	–	8.1
L-Isoleucine	–	12.5
L-Leucine	–	21.3
L-Lysine	–	14.6
L-Methionine	–	3.4
L-phenylalanine	–	9.6
L-Proline	–	11.2
L-Serine	–	9.3
L-Threonine	–	10.5
L-Tryptophan	–	4.2
L-Tyrosine	–	9.6
L-Valine	–	13.6
L-Carnitine	–	0.1
Taurine	–	0.4
DL-methionine	2.0	–
	Fat	
Soybean oil	70.0	70.0
	Other	
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.014	0.014

Table 2. Scoring system for symptoms of systemic anaphylaxis

Score	Symptoms
0	No symptoms
1	Scratching and rubbing around the nose and head
2	Puffiness around the eyes and mouth, pilar erecti, reduced activity, and/or increased respiratory rate
3	Wheezing, laboured respiration, and cyanosis around the mouth and the tail
4	No activity after prodding or tremor and convulsion
5	Death

Measurement of allergen-specific immunoglobulins and mMCP-1 in serum

Levels of whey-specific IgE, IgG1 and IgG2a were determined by ELISA in serum collected after orbital extraction. Microlon plates (Greiner) were coated with 100 µL whey (20 µg/ml) in carbonate/bicarbonate coating buffer (0.05 M, pH = 9.6; Sigma-Aldrich) for 18 h at 4°C. Subsequently, plates were washed and blocked for 1 h with 2% human serum albumin (HSA) in PBS. Serum samples were incubated for 2 h at room temperature (RT), after which plates were washed and incubated with 1 µg/ml biotin-labelled rat anti-mouse IgE, IgG1 or IgG2a (BD Pharmingen) in PBS for 90 min at RT. Plates were subsequently washed, incubated with 0.5 µg/ml streptavidin-horseradish peroxidase (Sanquin) in PBS for 1 h at RT and developed with tetramethyl benzidine substrate (Pierce, Fisher Scientific). After 10 min, the reaction was stopped with 4 M H_2SO_4 and absorbance was measured at 450 nm with a microplate reader (BioTek, Powerwave HT). The results are expressed as arbitrary units (AU) with pooled sera from whey-alum-immunized mice serving as positive reference to compose a titration curve. Concentrations of mMCP-1 in serum were determined using a commercially available ELISA kit (eBioscience), according to the manufacturer's instructions.

Immunohistochemistry for Forkhead box P3 (FoxP3)

The small intestine of each mouse was dissected, and the jejunum was fixed in 4% formaldehyde in PBS for 24 h at RT, dehydrated and subsequently embedded in paraffin. Sections of 5 µm were cut using a microtome (Leica Microsystems) and stained for intracellular FoxP3 expression. Paraffine sections were dewaxed and boiled for 12 min in 0.01 M sodium citrate buffer (pH = 6.0). Next, sections were washed in demineralized water and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 15 min at RT. Sections were washed, blocked for 90 min with 5% normal rabbit serum (Dako) in PBS with 3% bovine serum albumin (BSA), washed again and incubated overnight at 4°C with rat anti-mouse FoxP3 purified antibody (12.5 µg/ml, 14-5773, eBioscience) or rat IgG2a isotype (2.5 µg/ml, 14-4321, eBioscience) in 3% BSA/PBS as a

control. After washing, slides were incubated with a biotinylated rabbit-anti-rat antibody (5 μg/mL, 312-065-003, Jackson ImmunoResearch) in 3% BSA/PBS and 1% normal mouse serum (Invitrogen) and subsequently incubated with avidin biotin complex (Vectastain Elite ABC Kit, Vector Laboratories) in 3% BSA/PBS for 1 h at RT. The staining was visualized using 0.05% 3,3′-diaminobenzidine tetra-hydrochloride (DAB; Sigma-Aldrich) in Tris buffer (pH = 7.6) for 8 min followed by counterstaining of the sections with haematoxylin (Merck Millipore). Next, sections were dehydrated and covered with Pertex mounting medium (Histolab) and a cover glass. FoxP3-positive ($FoxP3^+$) cells were counted only in completely attached villi and were counted blindly and in duplicate by two independent scientists. Results are expressed as $FoxP3^+$ cells per villus. Graphic images were taken with an Olympus BX50F microscope equipped with a Leica DFC320 digital camera.

Statistical analysis

Results are expressed as means ± the standard error of the mean (SEM). Normal distribution was tested for each readout by D'Agostino & Pearson normality tests. Differences between preselected pairs (n = 4; *Figure 1*) were analysed by means of a one-way analysis of variance (ANOVA) with a post hoc Bonferroni test to correct for multiple comparisons. Levels of mMCP-1 and immunoglobulins in serum were log-transformed to obtain normality. Anaphylactic shock scores were analysed using a Kruskal-Wallis test with a post hoc Dunn's test. Correlations between serum immunoglobulin levels and clinical parameters were assessed by Pearson correlation when data were normally distributed, or otherwise by Spearman Rho correlation. All repeated measurements of the anaphylactic shock score were taken into account simultaneously when testing for correlations, by using the area under the curve (AUC) of this parameter as calculated using the measurements at 0, 15, 30 and 60 min after i.d. challenge. All calculations and statistical analyses were performed using GraphPad Prism Software, version 7. P-values < 0.05 after adjusting for multiple testing were considered statistically significant. Trends were indicated when $p < 0.10$.

RESULTS

The AA diet reduces the allergic skin response and prevents anaphylactic symptoms in response to whey protein

One hour after the i.d. challenge, the ear swelling of whey-sensitized mice fed either the control or the AA diet was significantly higher than that of diet-matched sham-sensitized mice (*Figure 2A*). A significantly lower ear swelling was found in whey-sensitized mice fed the AA diet compared to whey-sensitized mice fed the control diet (*Figure 2A*). In sham-sensitized mice, no difference in ear swelling was observed between the two groups.

The body temperature and anaphylactic symptoms were assessed at 15, 30 and 60 min after the i.d. challenge. At 30 min, whey-sensitized mice fed the control diet had a lower body temperature, an indicator of anaphylactic shock, than diet-matched sham-sensitized mice (*Figure 2B*). In contrast, no significant difference in body temperature was found at any of the time points between whey-sensitized mice fed the AA diet and diet-matched sham-sensitized mice (*Figure 2B*). Anaphylactic shock symptoms were scored as described above (*Table 2*). One of the whey-sensitized mice fed the control diet reached a shock score of 4 and hence was euthanized and excluded from further analyses. At all time-points, the anaphylactic shock score of whey-sensitized mice fed the control diet was significantly higher than that of diet-matched sham-sensitized mice (*Figure 2C-E*). In contrast, anaphylactic shock scores of whey-sensitized mice fed the AA diet remained low and were not significantly different from those of diet-matched sham-sensitized mice at any of the time points (*Figure 2C-E*). Compared to whey-sensitized mice fed the control diet, whey-sensitized mice fed the AA diet had significantly lower anaphylaxis scores at 30 and 60 min after i.d. challenge (*Figure 2D* and *2E*).

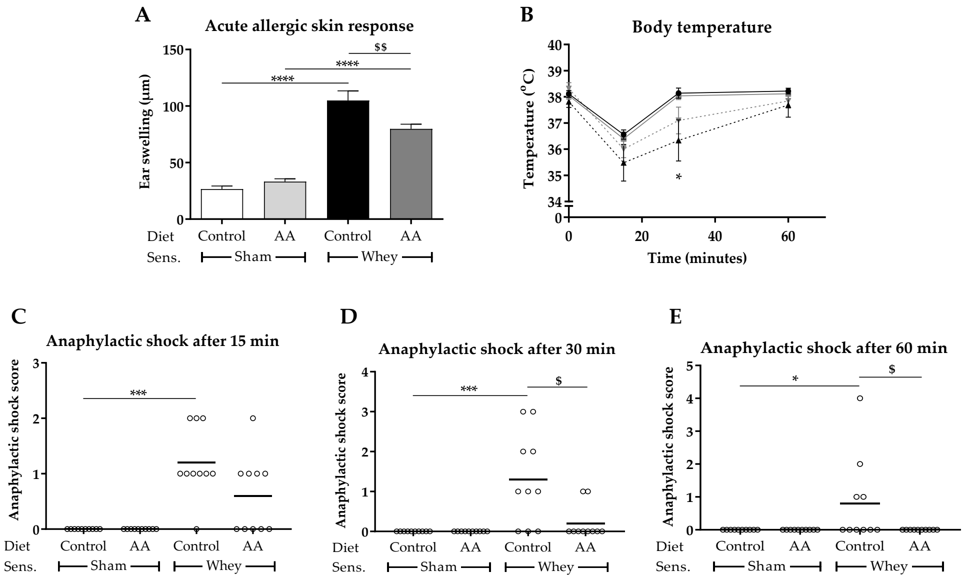

Figure 2. The acute allergic skin response, body temperature and anaphylactic shock severity of the mice. Ear swelling was measured before and 1 h after i.d. challenge with whey **(A)**. Body temperature **(B)** and anaphylactic shock severity **(C-E)** was measured before and 15 min, 30 min and 60 min after i.d. challenge. Groups are as follows: sham-sensitized mice fed the control diet (n = 10); sham-sensitized mice fed the amino acid (AA) diet (n = 10); whey-sensitized mice fed the control diet (n = 9); whey-sensitized mice fed the AA diet (n = 10). Values are expressed as mean ± SEM. Differences between whey-sensitized and sham-sensitized mice are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. Differences between whey-sensitized mice fed the control diet and those fed the AA diet are indicated by $^{\$}p < 0.05$, $^{\$\$}p < 0.01$. Differences are analysed with a one-way ANOVA followed by a Bonferroni post hoc test (**A-B**) or a Kruskal-Wallis test with a post hoc Dunn's test (**C-E**). Sens.: sensitization.

The AA diet lowers serum levels of whey-specific IgG1 and IgG2a, but did not affect serum levels of whey-specific IgE and mMCP-1

Levels of mMCP-1 and whey-specific immunoglobulins were analysed in mouse sera obtained 30 min after the oral challenge. The levels of mMCP-1 and whey-specific IgE, IgG1 and IgG2a were significantly higher in the sera of whey-sensitized mice compared to sham-sensitized mice, irrespective of the diet (*Figure 3A-D*). No differences were observed in mMCP-1 and whey-specific IgE levels between whey-sensitized mice fed the control diet and those fed the AA diet (*Figure 3A* and *3B*). However, whey-sensitized mice fed the AA diet had a tendency towards lower levels of IgG1 ($p = 0.076$; *Figure 3C*) and had significantly lower levels of IgG2a (*Figure 3D*) compared to whey-sensitized mice fed the control diet. With regards to mean differences, serum levels of IgG1 and IgG2a in whey-sensitized mice fed the AA diet were 3.7 and 28.8 times lower, respectively, than those of whey-sensitized mice fed the control diet. No differences were observed in any of the tested sera parameters between sham-sensitized mice fed the AA diet and those fed the control diet (*Figure 3A-D*).

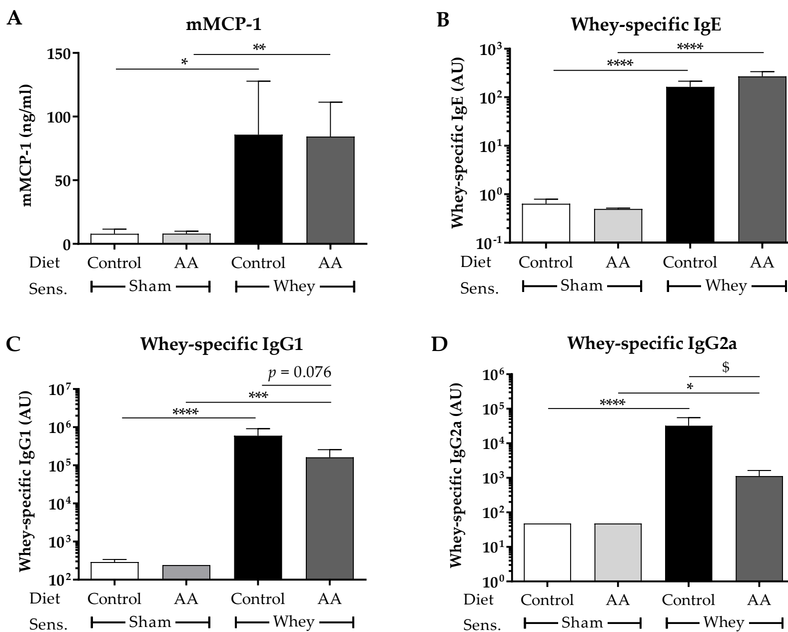

Figure 3. Levels of mMCP-1 and whey-specific immunoglobulins in serum of the mice. Serum of all mice (n = 9-10 per group) was harvested 30 min after the oral challenge. Serum levels are shown for mMCP-1 **(A)**, whey-specific IgE **(B)**, whey-specific IgG1 **(C)** and whey-specific IgG2a **(D)**. Values are expressed as mean ± SEM. Significant differences between whey-sensitized and sham-sensitized mice are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. Differences between whey-sensitized mice fed the control diet and those fed the amino acid (AA) diet are indicated by $^{\$}p < 0.05$. Differences were analysed with a one-way ANOVA followed by a Bonferroni post hoc test for selected groups after log-transformation of the data. AU: Arbitrary Units; Sens.: sensitization.

Whey-specific immunoglobulins correlate with the induction of allergic symptoms but only IgG1 and IgG2a correlate with the severity of symptoms

Validation studies of the murine model for CMA used in the present study showed that the induction of allergic symptoms in this model is partly mediated by allergen-specific IgE. To confirm the validity of the model in the present study, we therefore tested for correlations between allergic symptoms and serum immunoglobulin levels in all mice fed the control diet (n = 19). This indeed revealed strong positive correlations between the acute allergic skin response and serum levels of IgE, as well as IgG1 and IgG2a (*Figure 4A-4C*). Similarly, the AUC of the anaphylactic shock score strongly correlated with serum levels of IgE (r = 0.603, $p = 0.006$), IgG1 (r = 0.946, $p < 0.001$) and IgG2a (r = 0.893, $p < 0.001$).

To evaluate the relation between the severity of allergic symptoms and whey-specific immunoglobulin levels, regression analysis was performed using data from only whey-sensitized mice (n = 19). The severity of the allergic skin response in whey-sensitized mice positively correlated with serum levels of IgG1 (*Figure 4D*) and IgG2a (*Figure 4E*), but not with IgE (*Figure 4F*). Similarly, the AUC of the anaphylactic shock score positively correlated with IgG1 (r = 0.509, $p = 0.026$) and tended to positively correlate with IgG2a (r = 0.413, $p = 0.079$), but not with IgE (r = -0.026, $p = 0.916$).

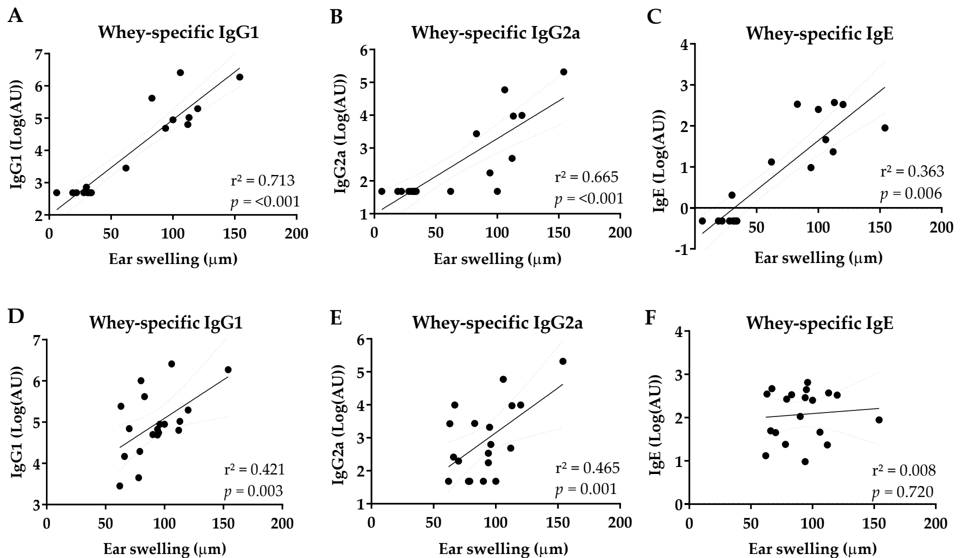

Figure 4. Correlations between immunoglobulin levels in serum and the allergic skin response. Correlations are shown for ear swelling and serum levels of whey-specific IgG1 (**A**), IgG2a (**B**) and IgE (**C**) in all mice fed the control diet (n = 19). Moreover, correlations are shown for ear swelling and serum levels of whey-specific IgG1 (**D**), IgG2a (**E**) and IgE (**F**) in all whey-sensitized mice (n = 19). Immunoglobulin levels were log-transformed prior to the testing of correlations by Pearson correlation. AU: Arbitrary Units.

The AA diet prevents the decrease in FoxP3⁺ cells in the jejunum of whey-sensitized mice

As the state of allergen sensitization has been associated with the depletion of regulatory T (T_{reg}) cells following allergen exposure, jejunum sections were stained for FoxP3, a common marker for T_{reg} cells. Significantly fewer $FoxP3^+$ cells were detected in whey-sensitized mice fed the control diet compared to diet-matched sham-sensitized mice (*Figure 5A*). Whey-sensitized mice fed the AA diet had significantly more $FoxP3^+$ cells than whey-sensitized mice fed the control diet (*Figure 5A*). The AA diet prevented the whey-induced decrease in $FoxP3^+$ cells, as there was no significant difference between whey-sensitized mice fed the AA diet and diet-matched sham-sensitized mice ($p = 0.468$). No significant difference was observed between sham-sensitized mice fed the control diet and those fed the AA diet (*Figure 5A*). In addition, no differences were observed in villus morphology between mice on the different diets (*Figure 5B* and *5C*).

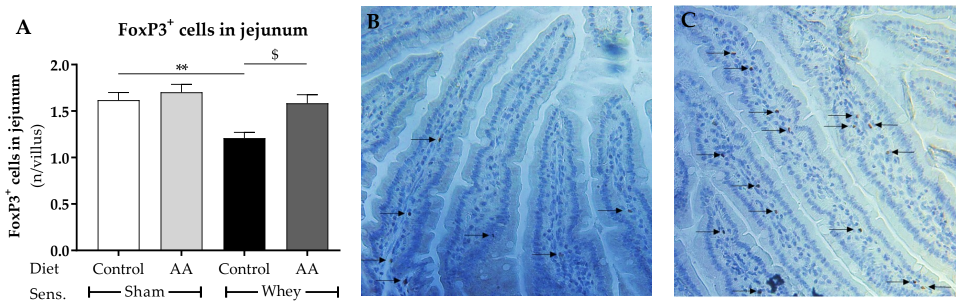

Figure 5. Immunohistochemical staining of FoxP3 in jejunum sections of the mice. Cell counts of $FoxP3^+$ cells in jejunum sections (n = 9-10 per group) expressed as number of positive cells per villus **(A)**. Values are expressed as mean ± SEM. Significant differences between whey-sensitized and sham-sensitized mice are indicated by $^*p < 0.05$ and $^{**}p < 0.01$. Differences between whey-sensitized mice fed the control diet and those fed the amino acid (AA) diet are indicated by $^{\$}p < 0.05$. Differences are analysed by one-way ANOVA followed by a Bonferroni post hoc test. Representative images of the immunohistochemical staining are shown for whey-sensitized mice fed the control diet **(B)** and whey-sensitized mice fed the AA diet **(C)**. Arrows (⟶) indicate positive intracellular staining for FoxP3. Sens.: sensitization.

Body weight and food intake are largely similar for mice fed the AA diet and mice fed the control diet

To evaluate whether the compositional differences between the diets influenced the nutritional status of the mice, body weight and food intake of the mice were monitored throughout the study period. No significant differences were observed in body weight (*Figure 6A*) or food intake (*Figure 6B*) between any of the groups at any of the individual

time points. Also, total food intake during the study was not different between the groups. However, there was a tendency towards an increase in body weight gain over the entire study period in whey-sensitized mice fed the AA diet compared to whey-sensitized mice fed the control diet (p = 0.056; data not shown).

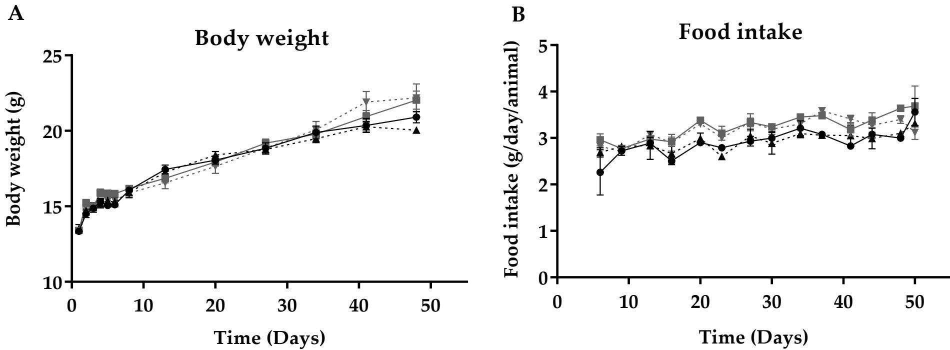

Figure 6. The body weight (A) and food intake (B) of the mice throughout the study period. Groups are as follows: sham-sensitized mice fed the control diet (n = 10); sham-sensitized mice fed the amino acid (AA) diet (n = 10); whey-sensitized mice fed the control diet (n = 9); whey-sensitized mice fed the AA diet (n = 10). Food intake was measured per cage (n = 2 per group). Differences are analysed with a one-way ANOVA followed by a Bonferroni post hoc test (**A**) or a Kruskal-Wallis test with a post hoc Dunn's test (**B**). Values are expressed as mean ± SEM.

DISCUSSION

Amino acid-based formulas (AAFs) are used for the dietary management of cow's milk allergy (CMA). It is well-known that AAFs effectively reduce allergic symptoms and improve growth in children with CMA [11, 19-22, 35]. However, whether AAFs also have potential to prevent allergic sensitization and/or symptoms associated with CMA is not known. In the present study, a murine model of CMA was used to examine the preventive effects of an amino acid-based (AA) diet on allergic sensitization and clinical symptoms. We showed that the AA diet inhibited the allergen-induced acute allergic skin response and effectively protected against anaphylactic symptoms and drops in body temperature. The results further indicated that this beneficial clinical effect could not be explained by a lowered mast-cell degranulation or a diminished production of whey-specific IgE. However, higher $FoxP3^+$ cell counts in the jejunum, combined with lower levels of whey-specific IgG2a and IgG1 in serum may suggest that tolerizing and anti-inflammatory capacities in AA-fed mice are enhanced, compared to whey-sensitized mice fed a protein-based diet.

Studies in humans reported that an increase in allergen-specific IgE, IgG1 and IgG2a is typical for IgE-mediated CMA [36]. The present study similarly showed that the induction of allergy in mice was associated with an increase in serum levels of each of these immunoglobulins. This indicates that the murine model used in this study is a suitable model for IgE-mediated CMA, which is described as one of the most commonly occurring food allergies in infants [37]. In addition to a lower severity of CMA symptoms, we observed that whey-sensitized AA-fed mice had a tendency ($p = 0.056$) towards more weight gain during the study, as compared to whey-sensitized mice fed the control diet. Interestingly, studies in infants with IgE-mediated CMA report similar clinical- and growth-related improvements following dietary management with an AAF [38]. This further indicates that the murine model used in the present study has appreciable overlap with the human condition.

The lower severity of CMA symptoms in whey-sensitized AA-fed mice was not accompanied by a reduction in serum levels of whey-specific IgE and mMCP-1, which are known mediators of allergic symptoms [39, 40]. However, these mice had (a tendency towards) lower serum levels of allergen-specific IgG2a and IgG1, compared to whey-sensitized mice fed the control diet. The lower serum IgG2a and IgG1 levels may be partially responsible for the observed clinical improvements in these mice, as these antibodies can have a mediating role in a variety of allergic symptoms [41-46]. For instance, allergen-specific IgG1 and IgG2a are both capable of inducing systemic anaphylaxis in mice, via distinct pathways from that of IgE [44, 47, 48]. The present study supports this concept, as serum levels of IgG1 and IgG2a positively correlated with the severity of the acute allergic skin response and anaphylaxis in whey-sensitized mice. For IgG1, a positive association with the severity of the acute allergic skin response in CMA mice has also been reported earlier [33].

The finding that serum levels of whey-specific IgE and mMCP-1 were not affected by the AA diet indicates that this diet did not specifically modulate T_H2-type immune responses in this model. This is supported by our observation that both T_H2-type IgG1 as well as T_H1-type IgG2a were lower or tended to be lower in serum of AA-fed mice. The lower serum IgG levels in AA-fed mice coincided with higher numbers of intestinal cells positively stained for FoxP3, which is a marker for regulatory T (T_{reg}) cells [49]. $FoxP3^+$ T_{reg} cells can directly and indirectly inhibit IgG production by B cells [50-52], hence it is plausible that the lower IgG production in AA-fed mice in the current study is mediated by $FoxP3^+$ T_{reg} cells. These T_{reg} cells have also been reported to control the severity of allergen-induced systemic anaphylaxis in both mice and humans [53-55]. Thus, the higher number of $FoxP3^+$ T_{reg} cells we found in AA-fed mice could underlie the lower severity of anaphylactic symptoms in these mice.

FoxP3⁺ T_{reg} cells play vital roles in inducing oral tolerance to food antigens and, more generally, in controlling inflammation [49, 56, 57]. Hence, the higher number of jejunal $FoxP3^+$ cells we observed in AA-fed mice may indicate that these mice have enhanced tolerizing and anti-inflammatory capacities compared to whey-sensitized mice fed the control diet. The observed lower levels of both T_H1- and T_H2-type IgGs in serum of AA-fed mice similarly suggest an anti-inflammatory effect of the AA diet. To confirm this, future studies on the preventive effects of AAFs on CMA should examine systemic and intestinal levels of pro- and anti-inflammatory cytokines, as well as T_H1 and T_H2 cytokines after allergen challenge. The capacity of AAFs to exert anti-inflammatory effects during inflammatory conditions like food allergy has been shown previously. For instance, in a study in CMA infants, intake of an AAF (*i.e.* Neocate) with an AA composition similar to that of the AA diet used in the current study reduced colonic inflammation [26]. This was evidenced by lower baseline levels of pro-inflammatory cytokines TNFα and IL-6 in supernatants of cultured colonic biopsies of allergic infants with the AAF in their diet compared to those without the AAF in their diet. Similarly, *ex vivo* treatment of these biopsies with the AAF reduced the production of pro-inflammatory cytokines [26]. Interestingly, supplementation of only the AA fraction of the aforementioned AAF to LPS-stimulated human peripheral blood mononuclear cells led to similar reductions in pro-inflammatory cytokine and chemokine production [27]. This indicates that the free AAs in the AAF are, at least partially, responsible for the observed anti-inflammatory effects. As free AAs are known to have AA-specific and diverse functions, including immunomodulatory functions [58-60], it is plausible that the specific AA composition of the diet used in the present study contributes to the observed effects.

The AA diet used in the current study contained a relatively high amount of free glycine and glutamine, which are both considered conditionally essential AAs. In terms of millimole AA per kg of feed, glutamine and glycine were the two most abundant AAs in the AA diet, together comprising more than 20% of all AAs present. These two free AAs are known to have a range of immunomodulatory effects. For instance, free glutamine supplementation is shown to decrease the production of pro-inflammatory cytokines while increasing anti-inflammatory cytokines by various immune cells, to improve epithelial barrier function and to protect against inflammation-induced intestinal damage in a variety of *in vitro*, *in vivo* and *ex vivo* immune models [61-65]. In a mouse model of colitis, a glutamine-enriched diet fully prevented the colitis-induced decrease in blood $FoxP3^+$ cells and in FOXP3 expression in the mesenteric lymph nodes [66], similar to the $FoxP3^+$ cell-preserving effects of the AA diet observed in the current study. Next to glutamine, also glycine is known to exhibit profound anti-inflammatory effects, as shown in numerous *in vitro* and *in vivo* immune models [67-69]. Additionally, oral exposure to free glycine prevented the onset of CMA in mice, which was accompanied by a reduction in serum

levels of IgG1 and IgG2a [70]. Based on these findings, it can be speculated that the immunomodulatory and clinical effects of the AA diet observed in the present study are in part mediated by the relatively high content of free glutamine and glycine in the diet.

In addition to the presence of free AAs, also the absence of protein in the AA diet could have contributed to the lower severity of clinical symptoms in AA-fed mice. That is, various studies have suggested that continuous exposure to food proteins in early life is important for the development of a mature cytokine production profile and adequate immune cell populations in the gut-associated lymphoid tissues [71, 72]. An immature mucosal cytokine and immune cell profile may limit the overall ability to respond to antigenic stimulation in the mucosa and thus can potentially lead to a lower severity of allergic symptoms following allergen challenge. We observed, however, that whey-sensitized AA-fed mice produced similar levels of IgE and mMCP-1 in response to antigenic stimulation as whey-sensitized protein-fed mice, suggesting that the AA-fed mice did not have a general impaired ability to respond to the allergen. Nevertheless, further studies on the effects of a protein-free diet on intestinal and immune development are crucial to better understand the applicability of AAFs for the prevention of CMA.

In summary, the present study showed that intake of an AA diet before and during allergic sensitization prevented, at least partially, the development of allergic symptoms in allergen-challenged whey-sensitized mice. The exact mechanism underlying the observed effects has yet to be revealed, however, this study suggests the involvement of $FoxP3^{+}$ T_{reg} cells, IgG2a antibodies and potentially IgG1 antibodies. Although confirmation in humans is critical as findings in mice do not always translate to humans, these data indicate that besides being effective in the dietary management of CMA, AAFs may also have the potential to protect against CMA development. Further investigations are warranted to better understand this protective effect and the underlying mechanisms thereof, ideally in studies that compare the effects of multiple AAFs with varying AA compositions.

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7

Chapter 8

Summarizing discussion

Exclusive breastfeeding in the first months of life supports optimal infant growth and is associated with the protection against various immunological conditions in early life [1]. Because of these benefits, the World Health Organization (WHO) recommends exclusive breastfeeding for the first 6 months of life [2]. For newborns whose mothers are unable to breastfeed, infant milk formulas (IMFs) are available. Compared to breastfed infants, however, infants partially or fully fed an IMF have a different growth pattern and a higher occurrence of specific atopic diseases, most notably cow's milk allergy (CMA), which is the most common food allergy in infancy [1, 3-5]. Bioactive elements present in HM but not in IMFs are thought to play a role in these differences. One of the HM elements that may be relevant in this perspective are free amino acids (FAAs), *i.e.* amino acids (AAs) that in contrast to conjugated AAs are not part of protein or peptides. The FAA fraction in HM has been largely neglected in the field of early life nutrition, yet studies in animals show that specific dietary FAAs can have profound bioactive effects during early life development [6-8]. These effects include the stimulation of intestinal growth and the promotion of immunological mechanisms that are among others relevant for the protection against food allergic sensitization. This urges the need for a better understanding of the composition of FAAs in HM, and for investigations in the potential roles of FAAs in early life growth and immunity.

To this end, this thesis presents detailed analyses of the composition and variability of FAAs in HM in the first 6 months of lactation. To investigate whether FAAs could play a role in early life growth and immunity, correlations were tested between the levels of FAAs in HM and infant growth and immunological conditions in early life. These analyses were also conducted for the total AAs (TAAs; *i.e.* the sum of conjugated AAs and FAAs) in HM to evaluate whether the obtained results were determined by the AA being a FAA. Finally, to investigate whether FAAs could play a role in the prevention of food allergic sensitization, the effects of partial or complete substitution of dietary protein by a selected FAA or a mix of FAAs on the development of CMA in mice were assessed. The current chapter summarizes and discusses the main outcomes of these analyses, and proposes opportunities for future research.

The unique and changing composition of FAAs in human milk

It is well-established that the levels of all conjugated AAs in HM decrease with progressing lactation. This decrease is considered to meet the changing requirements of the growing infant [9]. While ample data exist on temporal changes of conjugated AAs, for FAAs these data are scarce and the few studies that have previously investigated this were restricted to the first 2 to 3 months of lactation [10-14], thereby not covering the recommended period of exclusive breastfeeding. We therefore assessed the temporal changes of FAAs in HM for the

first time in a longitudinal study design that extends up to 6 months of lactation (*Chapter 2*). Contrary to the decreasing levels of all conjugated AAs in the first 3 months of lactation, we found that levels of free glutamine and glutamate prominently increased during this period, along with smaller but significant increases of free serine, threonine and aspartate (*Figure 1A*). Similarly, in another, larger cohort we found that levels of each of these FAAs in HM were higher at 6 months than at 6 weeks of lactation, and that this difference was the largest for glutamine and glutamate (*Chapter 5*). Interestingly, previous studies revealed similar temporal increases of glutamine and glutamate in HM [10-14], and most of these studies have also reported increases for free serine, threonine and aspartate [12-14]. These apparent characteristic changes of FAA levels in HM seem to be specific for the first 3 months of lactation, as we observed that levels of all FAAs remained stable thereafter (*Figure 1A*). The inter-study consistency in the dynamics of FAAs in HM is remarkable considering the large inter-individual variation in the FAA levels in HM (*Chapter 2, 3*). As the different studies have been performed in various geographical locations and ethnic groups, these data collectively indicate that the dynamics of FAAs in HM, at least those of glutamine and glutamate, are globally consistent and thus evolutionary conserved. This strongly suggests that there is a need for an adequate intake of FAAs in early life, and that these needs may change during the infant's first 3 months.

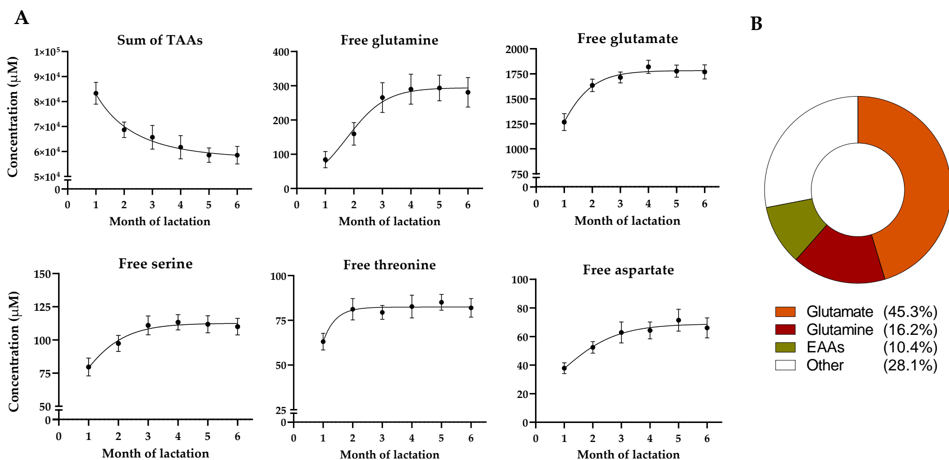

Figure 1. The dynamics and abundance of free amino acids (FAAs) in human milk. (A) The sum of all total amino acids (TAAs) and the levels of free glutamine, glutamate, serine, threonine and aspartate in the first 6 months of lactation (*Chapter 2*). Data are presented as mean ± SEM. **(B)** The abundance of free glutamate, glutamine and the sum of all essential amino acids (EAAs) relative to the sum of all FAAs in milk collected at 6 months of lactation (*Chapter 5*).

We further demonstrated considerable differences between the levels of individual FAAs in the first 6 months of lactation (*Chapter 2*, *5*). Most notable were the high levels of free glutamine and glutamate, which together comprised up to 60% of all FAAs in HM (*Figure 1B*). In contrast, levels of most free essential AAs (EAAs) were relatively low, together accounting for only 10% of the FAAs in HM (*Figure 1B*). Importantly, these differences were also reflected in the relative contribution of FAAs to TAAs; while this contribution was below 2% for all EAAs, for Glx (glutamine + glutamate) this reached up to 18%. This shows that not only the temporal changes but also the composition is largely different between FAAs and conjugated AAs in HM, further indicating a differential need for FAAs and conjugated AAs in early life. Moreover, these data suggest that the need for AAs in the readily available, free form is higher for some of the AAs considered nutritionally non-essential, notably glutamine and glutamate, than for the EAAs.

A potential role for FAAs that increase throughout lactation in the support of early life growth

Given that FAAs are the building blocks for protein synthesis, and that specific dietary FAAs have been associated with growth in young animals [8, 15], it can be hypothesized that an adequate FAA intake in early life is important for infant growth. In line with this, a positive association between levels of free glutamine in HM and infant length at 4 months of age has previously been reported [16], and a recent study revealed that levels of free glutamine, glutamate, threonine and alanine were higher or tended to be higher in HM for infants that had faster weight gain in the first 2 months of life [17]. These findings warranted further investigations in larger cohorts with stricter HM sampling procedures, which is crucial as FAAs are variable not only throughout lactation, but also within a single feed (*Chapter 3*). We therefore investigated the correlations between infant weight and length gain and FAAs in HM, sampled in a strictly controlled manner at 6 weeks and 6 months of lactation, in a large cohort consisting of 671 mother-infant pairs (*Chapter 5*). In line with the aforementioned results of previous studies [16, 17], we found positive correlations between free glutamine and glutamate in 6-week HM and infant weight gain in the first 5 weeks of life, and a tendency for positive correlations between free threonine and glutamine in 6-week HM and infant weight gain and length gain during this period, respectively. An additional positive correlation was found between free serine in 6-week HM and infant weight gain, which has not been reported earlier. Importantly, these correlations were not observed for the TAA variants of these AAs and were also not observed at 6 months of lactation, demonstrating that these correlations are specific for the free form of these AAs and are dependent on the age of the infant.

Consistent with the observed correlations, studies in healthy young animals have shown that dietary supplementation of free glutamine, glutamate and serine increases daily body weight gain [18-22]. For glutamine, this effect has also been demonstrated in low birthweight infants [23, 24]. Combined with the correlational evidence, these findings point towards a role for free glutamine, glutamate, serine and potentially threonine in HM in the support of infant growth, at least in the first several weeks to months of life. Of note is that the levels of each of these FAAs in HM increase during the first 3 months of lactation (*Chapter 2*). It is therefore plausible that this increase relates to a changing need for these FAAs for the maintenance of adequate infant growth. Additional studies with a finer time-spacing between longitudinally collected HM samples are warranted to better understand the timeframe in which these FAAs may be relevant for infant growth.

Of the FAAs that correlated with infant growth, glutamine and glutamate have been most extensively investigated in relation to early life development. In human neonates, the majority of dietary free glutamine and glutamate is taken up by the intestines [25-27]. This, combined with the finding that these FAAs are relatively abundant in HM suggests important functions for these FAAs in the developing human intestines. Extensive studies in young animals have shown that dietary supplementation of these FAAs promotes intestinal growth and maturation, among others through stimulating enterocyte proliferation, supporting villi formation, promoting the synthesis of tight-junction proteins relevant for intestinal structure and integrity, and shaping the intestinal microbiota composition (*Chapter 4*). Granted that intestinal growth and maturation in early life is crucial for optimal food digestion and absorption [28], it is plausible that these FAAs support infant growth via these mechanisms.

Similar to glutamine and glutamate, the FAAs serine and threonine have also been associated with the promotion of intestinal growth and structure in young animals, though evidence is more limited [18, 29-31]. A recent study in infants also indicated a role for free threonine in HM in shaping early life intestinal microbiota colonization, a process which is closely related to early life growth [32, 33]. Thus, it is possible that these FAAs also support infant growth via promotion of intestinal development. This may also explain why none of the four FAAs correlated with infant growth anymore at 6 months of lactation (*Chapter 5*), when several of the most profound developmental changes of the intestines may have already occurred in the infant [34, 35]. Alternatively, an explanation for the correlations being dependent on the age of the infant may lie in the changing capacity of the developing infant digestive system to hydrolyse milk protein and thereby generate FAAs *de novo*. That is, in the first 2-3 months of life this hydrolysing capacity may be limited due to low concentrations and activities of gastric and luminal proteolytic enzymes, while at 6 months of age the concentrations of most of these enzymes are already comparable to those of adults

[36, 37]. Thus, it is plausible that the developing intestines are more dependent on dietary FAAs in the earlier stages of life, and that FAAs in HM may thus be of particular physiological relevance for infant growth during these early stages.

A potential role for specific FAAs in the protection against food allergy development

In addition to being key regulators of cell growth, FAAs are immunologically active components involved in regulating the proliferation, activation and functionality of immune cells [38]. Accordingly, dietary supplementation of FAAs has been associated with profound immunomodulating effects in young animals, both locally in the intestines and systemically [6, 39-41]. These effects include the stimulation of anti-inflammatory and anti-oxidative mechanisms, modulations in the activity of T_H1 and T_H2 cells, and the stimulation of phagocytic capacities, which are all relevant processes in the protection against infections and allergic sensitization in early life (*Chapter 4*). We therefore assessed whether FAAs in HM are associated with the risk for commonly occurring atopic and infectious diseases in infancy (*Chapter 5*). Modified Poisson regression analysis revealed no consistent associations between FAAs and infant wheeze, atopic dermatitis and lower respiratory tract infections, but revealed a negative association between free asparagine in 6-week HM and the risk of food allergy at 1 year of age, and suggested similar potential negative associations for free arginine, citrulline and glutamine in 6-week HM. At 6 months of lactation, these associations were not observed anymore. These preliminary findings may suggest that immunomodulatory effects of FAAs in HM predominantly occur locally in the intestines, and support our previous notion that dietary FAAs may be of particular physiological relevance in the earlier stages of life. The FAAs arginine, glutamine and citrulline are metabolically closely related and have an important precursor relationship in both animals and humans [42]. It is not surprisingly therefore that there is some overlap in the physiological effects described for these FAAs following dietary intake. In young animals, these FAAs are shown to promote the intestinal barrier function and to protect against intestinal damage [43-46], and arginine and glutamine have additionally been implicated in the maintenance of intestinal immune homeostasis through modulating immunity in the gut-associated lymphoid tissues (GALT) [6, 47-49]. These effects could provide a biological explanation for the suggested negative associations of these FAAs with infant food allergy. However, while our preliminary findings support the concept that FAAs in HM, or certain compositions thereof, may be associated with the protection against food allergy, confirmation in future human observational studies is crucial as our cohort included only a small number of food-allergic infants and therefore had limited statistical power.

The FAA glutamine has previously been linked to the protection against food allergy development, though only at the conceptual level [50, 51]. To investigate this potential link experimentally, we evaluated the effects of dietary glutamine supplementation on the development of CMA in young mice (*Chapter 6*). Prior and during allergic sensitization, mice were fed a control diet containing no free glutamine or a diet in which part of the protein content was replaced by either 5.0 g/kg (0.5% w/w) or 20.0 g/kg (2% w/w) free glutamine. The FAA to TAA ratio of Glx in the 0.5% and 2% glutamine diets resembled the lower and higher end of the range of this ratio in mature HM, respectively. We found that mice fed 2% glutamine had a lower severity of allergic symptoms following a dermal allergen challenge than mice fed the control diet. The lower severity of clinical symptoms was accompanied by higher serum and faecal levels of allergen-specific IgA, and a lower ratio of serum allergen-specific IgE to IgA, which are modulations that have previously been related to the development of oral tolerance to cow's milk protein in infants [52-55]. Moreover, the 2% glutamine diet prevented the increase in intestinal levels of branched-chain fatty acids (BCFAs) that is characteristic for food allergy in both mice [56] and human infants [57, 58] and thought to be related to the intestinal inflammatory processes associated with the condition. Similar but less pronounced modulations were observed in mice fed 0.5% glutamine, demonstrating that the effects of glutamine were dose-dependent. Collectively, these data indicate that dietary glutamine supplementation may be an effective strategy to promote clinical tolerance development to food, and further support the hypothesis that an adequate intake of this AA in early life may be important for an optimal protection against food allergy.

To better understand the extent to which dietary glutamine could support the development of clinical tolerance to food, similar investigations but with an oral rather than a dermal allergen challenge to induce allergic symptoms are warranted. Such investigations would more closely resemble the clinical condition in infants, where food allergic symptoms develop following food ingestion, and may also reveal a more potent clinical benefit of glutamine already at lower concentrations. That is, we found that glutamine enhanced allergen-specific faecal IgA – an indicator for intestinal IgA – already at the lower dose (*Chapter 6*). Research indicates a prominent role for intestinal allergen-specific IgA in the suppression of allergic symptoms upon allergen ingestion, through neutralization of the ingested allergens in the intestinal lumen [59, 60]. Thus, suppressive effects of intestinal IgA on the development of allergic symptoms may be best observed following an oral allergen challenge, which in contrast to a dermal challenge enables a direct interaction between the allergen and intestinal IgA. It is therefore possible that the clinical benefits of dietary glutamine supplementation are somewhat underestimated in our murine model for CMA.

Besides enhanced faecal and serum IgA, glutamine-supplemented mice had a higher ratio of T_H1-related IgG2a to T_H2-related IgG1 in serum and a higher frequency of activated T_H1 cells in the mesenteric lymph nodes (MLNs) (*Chapter 6*). Moreover, restimulated MLN cells of these mice produced higher levels of the T_H1-related cytokines IFNγ and IL-2. These immunomodulatory effects are in line with those reported in various other studies in healthy and immune-compromised animals, in which dietary glutamine enhanced systemic and intestinal IgA levels [51, 61, 62] and specifically promoted T_H1-type cytokine production by immune cells of the GALT [63-66]. Interestingly, it has recently been shown that oral glutamine supplementation also enhances faecal IgA levels in human neonates, indicating that dietary glutamine has similar immunomodulating capacities in humans [23]. It is well-known that IgA production and T_H1 immune functions are limited in early life. Evidence points towards the importance of breastfeeding for a timely and adequate maturation of T_H1 immunity and IgA production [67-69]. It is tempting to speculate that free glutamine in HM contributes to these critical processes of infant immune development, which are not only important for the protection against food allergy, but also for the protection against intestinal infections (*Chapter 4*). In line with this concept, oral supplementation of glutamine to low birthweight neonates has been associated with a decreased risk of gastrointestinal infections in the first years of life [70]. It would therefore be interesting to study the levels of free glutamine in HM in relation to infant intestinal infections, which we were not able to investigate in our cohort study (*Chapter 5*) due to limited data on this infant health outcome.

The secretion of free glutamine and glutamate into human milk

The potential of dietary free glutamine to protect against food allergy development (*Chapter 6*) and intestinal infections [70], and the relation between free glutamine and glutamate in HM and infant growth (*Chapter 5*) warrants an understanding of the mechanisms underlying the secretion of these FAAs into HM. The relatively high levels of these FAAs in HM and their unique dynamics throughout lactation suggest that the human mammary gland has developed specialized mechanisms to ensure adequate levels of these FAAs in the milk. HM is the secretory product of mammary epithelial cells (MECs), which can transfer components directly from maternal plasma into milk [71]. For free glutamine and particularly glutamate, however, levels in HM are considerably higher than the levels in plasma of lactating mothers [32, 72, 73]. It is discussed that such differences may be mediated by proteases present in HM, which can hydrolyse protein and thereby release peptides and potentially FAAs into HM [32, 74], though the contribution of proteases to the FAA levels in HM has never been actually investigated. A comparative analysis of the FAAs in human foremilk and hindmilk may give an indication about this potential contribution,

as hindmilk remains in the mammary gland in the presence of native HM proteases for a longer time than foremilk, which has been related to the higher peptide count found in hindmilk [75]. Contrary to the findings related to peptides, we found that most FAAs, including glutamine and glutamate, were slightly lower in hindmilk (*Chapter 3*). This, combined with the finding that the levels and activity of proteases in HM decrease in the first months of lactation [76] while glutamine and glutamate levels increase, suggests that HM proteases do not play a major role in ensuring adequate levels of these FAAs in HM. Rather, these findings suggest a mechanism that involves active synthesis and subsequent secretion of these FAAs by human MECs.

To date, no studies have investigated the synthesis and secretion of free glutamine and glutamate in the human mammary gland. Given the functional and morphological similarities between the human and porcine mammary gland [77], the latter may be an adequate model for such investigations. This is enforced by the finding that, similar to the levels of free glutamine and glutamate in HM, levels of these FAAs in sow milk are high overall and increase with progressing lactation [78, 79]. In sows, free glutamine and glutamate levels in milk can be increased through dietary supplementation of branched-chain AAs (BCAAs, *i.e.* leucine, valine and isoleucine) [80, 81]. *In vitro* studies in porcine MECs similarly showed that the synthesis and secretion of free glutamine and glutamate, and to a lower extent that of asparagine and aspartate, were enhanced by BCAA supplementation [82]. This effect was strongly dependent on the dose of BCAAs and on the activity of the BCAA catabolic enzyme branched-chain aminotransferase (BCAT), suggesting that the secretion of free glutamine and glutamate in sow milk is, at least in part, regulated through mammary gland BCAA catabolism via BCAT. Studies in sows further demonstrated that the mammary gland expresses remarkably high levels of BCAT [82, 83], and that the expression of BCAA transporters in the mammary gland, responsible for BCAA uptake from the circulation, increases with progressing lactation [84]. These findings may provide a biochemical explanation for the high levels of free glutamine and glutamate in milk, and for the observed increases in these levels throughout lactation. Future studies should investigate whether free glutamine and glutamate secretion in HM may be regulated via a similar metabolic mechanism in humans. For this, initial studies could focus on evaluating whether their levels in HM are related to maternal BCAA intakes. Understanding this potential relation may provide novel insights in the dietary AA needs for lactating women to ensure optimal levels of glutamine and glutamate in their breastmilk at all stages of lactation.

The influence of maternal characteristics on the levels of FAAs in human milk

The metabolism of AAs in tissues is known to be influenced by age, body composition and health conditions [85, 86]. Hence, it is of interest to investigate whether the FAA composition in HM is influenced by maternal characteristics. Such investigations may help to understand the large inter-individual variation observed for FAAs in HM (*Chapter 2, 3*), which is important in light of the suggested roles of FAAs in early life growth and immunity. We therefore investigated the correlations between maternal age and anthropometrics and the FAA levels in HM, at 6 weeks and at 6 months of lactation (*Chapter 5*). No consistent correlations were found for maternal age and height, but we observed negative correlations between maternal pre-pregnancy BMI and weight and free glutamine and asparagine in HM, both at 6 weeks and at 6 months of lactation. Though significance was lost after adjusting for putative confounders, these findings are relevant in light of a recent study that reported that levels of free glutamine and asparagine were significantly reduced (by 32% and 49%, respectively) in milk of obese women [87]. In contrast, levels of the BCAAs have previously been reported to be higher in milk of obese women [88]. We similarly found a tendency for a positive correlation between the BCAAs leucine and isoleucine in 6-week and 6-month HM and maternal BMI (*Chapter 5*). As explained earlier, studies in sows indicate that BCAAs are catabolized in the mammary gland by the enzyme BCAT, leading to the synthesis and secretion of among others free glutamine and asparagine. BCAT activity is shown to be negatively affected by obesity, in various tissues both in animals and in human [89, 90]. Whether obesity also affects BCAT activity in the mammary gland is unknown, but this may underlie the higher BCAA levels and lower glutamine and asparagine levels in milk of obese women.

The lower levels of glutamine and asparagine in milk of obese women could lead to a lower intake of these FAAs by their infants. As described earlier, we observed a preliminary negative association between the level of asparagine in HM and the risk for food allergy in infancy (*Chapter 5*), and we found a dose-dependent, protective effect of dietary glutamine against the development of food allergy in mice (*Chapter 6*). Studies in low birthweight infants have also indicated a preventive effect of oral glutamine intake in the neonatal period on the development of atopic dermatitis in the first years of life [70, 91]. It should hence be investigated in future research whether such deviations in milk of obese women could contribute to the apparent association between maternal obesity / BMI during early lactation and infant atopic disease [92-94].

Several studies have indicated that the relation between breastfeeding and infant atopic disease development is modified by maternal allergic status [95-97]. It is argued that

alterations in the HM composition of allergic mothers contribute to this. This is supported by studies showing differences in the concentrations of factors associated with allergy, including IL-4 and IgA, between HM of allergic and non-allergic mothers [98-100]. We found that the level of free glutamine is lower in HM of allergic mothers at 6 months of lactation, compared to HM of non-allergic mothers (*Chapter 5*). It can be speculated that this reduction may have a negative impact on the allergy-protective capacities of the milk. However, we could not distinguish between an active and non-active maternal allergy, and no difference in the free glutamine content was observed at 6 weeks of lactation. Therefore, to better understand the potential implications of this reduction for infants of allergic mothers, longitudinal studies investigating the FAA levels in HM of mothers with an active allergy are warranted.

Potential implications for infant feeding and future research

The results presented in this thesis demonstrate that HM provides the infant not only with conjugated AAs, but also with a unique and changing composition of readily available FAAs. Levels of several FAAs in HM were associated with infant growth and potentially with the risk of food allergy in infancy, and for free glutamine a protective effect against the development of food allergy was also shown experimentally in mice. These findings highlight the importance to continue research on the functional roles of FAAs in infant development. Our data add to the understanding of the complexity and the functionality of the HM composition, and emphasize the importance of breastfeeding for the infant to provide the full scope of benefits that may stem from the diverse and changing composition of FAAs in HM. The associations observed between maternal characteristics and the levels of several FAAs in HM call for further research into factors that could influence the FAA composition in HM. In particular, it is of interest to investigate the relation between maternal diet and the levels of FAAs in HM, and more specifically the effect of maternal BCAA intakes on the levels of free glutamine and glutamate in HM. Understanding which maternal factors influence the composition of FAAs in HM, and by which mechanisms, could provide valuable insights for developing nutritional support for lactating mothers to benefit infant health.

When breastfeeding is not possible, feeding a standard, cow's milk-based IMF is generally considered the best alternative for most infants [101]. Compared to breastfed infants, however, IMF-fed infants have a differential metabolic profile and growth pattern, which has been associated with an increased risk for obesity in childhood and later in life [1, 102-104]. Moreover, IMF-fed infants have a higher risk of developing cow's milk allergy (CMA) and have a slower postnatal physiological decline in intestinal permeability, which renders the infant more susceptible to infections in early life [1, 4, 102, 105]. A general goal within

the field of infant nutrition is to advance the formulation of IMFs to better mimic the physiological advantages of breastfeeding. Currently, most IMFs contain a higher protein content than HM, which is considered to play important roles in some of the differential characteristics between IMF-fed and breastfed infants. Recent advancements in the understanding of the essential AA (EAA) requirements of infants enables a reduction in the protein content, and initial evidence indicates that infants fed a reduced protein IMF indeed have a growth pattern and metabolic profile that better mimics that of breastfed infants [106-109]. A reduced protein IMF may additionally improve the postnatal decline in intestinal permeability, as shown in piglets [110]. While protein levels in current IMFs are high, levels of most FAAs are low, with the level of free glutamate being only 5-10% of that in HM and free glutamine usually being absent [111, 112]. Results of this thesis indicate that the addition of these FAAs to reduced protein IMFs may lead to further improvements in the growth pattern and intestinal permeability of IMF-fed infants, and might provide these infants with a better protection against CMA. In line with this concept, it was recently shown that feeding a reduced protein IMF with added FAAs (*i.e.* glutamine, glutamate, alanine and taurine) to rhesus monkeys better mimicked the growth and metabolic performance of breastfed monkeys than feeding a reduced protein IMF without FAAs [113]. However, as levels of free glutamine and glutamate, as well as that of many other elements in HM change in the first months of lactation, it is desired to ultimately aim for age-based IMFs that are tailored to more accurately meet the changing requirements of the infant.

For infants that cannot be breastfed and also have a familial history of allergy, some clinical studies indicated that exclusively feeding certain partially hydrolysed formulas (pHFs) may decrease the risk of developing CMA, compared to exclusively feeding a standard IMF [114-117]. In pHFs, the cow's milk proteins have been hydrolysed into smaller peptides. Besides having reduced allergenicity, these peptides can induce oral tolerance to cow's milk protein *in vivo* [118-120] and thus may underlie the preventive effect of pHFs observed in said clinical studies. There are, however, also clinical studies that failed to find any benefit of these formulas for the prevention of CMA in infancy [121], which warrants an understanding of the factors that could contribute to these inconsistent findings. In this context, differences in the peptide profiles of the pHFs used in the different studies, which occur due to the varying protein hydrolysation methods applied by different manufacturers, are frequently discussed, as tolerogenic capacities of peptides can be dependent on their length and AA sequence [122, 123]. Besides the varying peptide profiles, however, pHFs also vary largely in their content of FAAs [111], which are released from proteins during the hydrolysis process. We showed that a FAA-based diet, which did not contain any peptides that could induce tolerance, was able to promote clinical tolerance to cow's milk protein in mice (*Chapter 7*). Moreover, as described earlier, we found that dietary glutamine protected against symptoms of CMA in mice in a dose-dependent manner

(*Chapter 6*), and a previous study found similar dose-dependent clinical effects of oral intake of the FAA glycine [124]. These findings indicate that also the varying FAA profiles between pHFs can contribute to the inconsistent preventive efficacies reported. Thus, for pHFs to potentially become useful for the prevention of CMA in atopy-prone infants that cannot be breastfed, it may be important for future research to focus not only on identifying suitable peptide profiles for tolerance induction, but also on identifying suitable FAA profiles.

Overall conclusions and final remarks

To understand how breastfeeding contributes to an optimal infant health and development, extensive research is being conducted in the physiological functions of individual HM components. Until now, FAAs in HM have been largely neglected as compounds that may be of physiological relevance for the infant. In the present thesis it is shown that levels of FAAs in HM change in the first months of lactation in a manner that is AA-specific and different from the changes in levels of conjugated AAs, indicating that there is a need for an adequate FAA intake in early life. The presented results further support the hypothesis that an adequate intake of specific FAAs, notably but not limited to free glutamine, could be important for infant growth and for an optimal protection against food allergies. Overall, our findings provide evidence that FAAs in HM are of physiological relevance to the infant and, perhaps most importantly, call for further investigations on the roles of FAAs in early life development. The importance of understanding these roles is highlighted by the finding that FAA levels in HM may be affected by certain maternal characteristics, such as body mass index and allergy status. To this end, more large-scale longitudinal studies are warranted that investigate the FAAs in HM in relation to infant growth and immunological conditions, notably food allergies. Moreover, additional *in vivo* experiments are required to further elucidate the underlying mechanisms by which dietary FAAs can influence early life growth and immunity. Such studies not only help to better understand the role of breastfeeding in infant development, but could also lead to opportunities for optimizing the functionality of available breastmilk substitutes, which generally contain considerably lower levels of FAAs than HM.

Eventually, as our understanding of the physiological relevance of individual HM components grows, it is desirable for future studies to move towards more holistic approaches by investigating the physiological effects of combinations of HM components, including but not limited to FAAs. After all, this is how bioactive molecules are presented to the infant through breastfeeding.

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Annex

Nederlandse samenvatting
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Nederlandse samenvatting

Moedermelk wordt beschouwd als de meest geschikte voeding voor een zuigeling. Het bevat een breed scala aan componenten waarvan de concentraties zich aanpassen aan de veranderende behoeften van het kind en die tezamen de benodigdheden bieden voor een gezonde ontwikkeling. De Wereldgezondheidsorganisatie (WHO) adviseert om gedurende de eerste zes maanden na de geboorte uitsluitend borstvoeding te geven. De reden hiervoor is dat dit gezondheidsgerelateerde voordelen biedt aan de zuigeling, in vergelijking met het geven van kunstmatige zuigelingenvoeding. Zo is het erkend dat kinderen die uitsluitend zijn gevoed met moedermelk gedurende deze periode optimaal groeien. Dit is van groot belang, omdat groeipatronen in het vroege leven zijn geassocieerd met het risico op het ontwikkelen van bepaalde chronische aandoeningen later in het leven, waaronder obesitas. Naast het waarborgen van een optimale groei ondersteunt moedermelk de ontwikkeling en functionaliteit van het immuunsysteem van de zuigeling. Zo biedt het voeden van moedermelk bescherming tegen infecties en verlaagt het mogelijk het risico op bepaalde allergische aandoeningen in de eerste levensjaren. Een voorbeeld van een dergelijke aandoening is koemelkallergie, een voedselallergie waarbij het immuunsysteem overmatig reageert op eiwitten in koemelk en waarvoor tot op heden nog geen curatieve behandeling beschikbaar is. Er is grote interesse in het identificeren van de componenten in moedermelk die een rol spelen bij de gunstige effecten van borstvoeding op de groei en het immuunsysteem van de zuigeling. Dit verbreedt namelijk niet alleen ons begrip van de wijze waarop borstvoeding een gezonde ontwikkeling van het kind waarborgt, maar biedt ook mogelijkheden voor het optimaliseren van de samenstelling en functionaliteit van kunstmatige zuigelingenvoeding. Dat laatste is belangrijk omdat het exclusief geven van borstvoeding in de eerste zes maanden na de geboorte niet voor elke moeder mogelijk is, om praktische of medische redenen.

Onderzoek toont aan dat componenten in moedermelk die in staat zijn om de ontwikkeling en functies van de darmen te ondersteunen een belangrijke bijdrage kunnen leveren aan zowel de waarborging van optimale groei als de bescherming tegen voedselallergieën. De darmen zijn namelijk niet alleen belangrijk voor de digestie en absorptie van nutriënten die nodig zijn voor de groei, maar bevatten ook de meerderheid van de immuuncellen in het lichaam en spelen een cruciale rol in het reguleren van immuunreacties op allergenen in voedsel. Een van de componenten in moedermelk die mogelijk relevant zouden kunnen zijn in deze context, maar die nog onderbelicht zijn in de literatuur, zijn vrije aminozuren, waarvan meer dan twintig varianten voorkomen in moedermelk. Dit zijn aminozuren die, in tegenstelling tot geconjugeerde aminozuren waar eiwitten uit bestaan, direct beschikbaar zijn voor opname in het lichaam. In preklinische studies is veelvuldig aangetoond dat bepaalde vrije aminozuren in staat zijn om de groei en de ontwikkeling van de darmen te bevorderen, en om de functies van het darmgeassocieerde immuunsysteem te ondersteunen. Desondanks is het nog niet uitvoerig onderzocht of vrije aminozuren in

moedermelk mogelijk een rol spelen bij het waarborgen van een optimale groei van de zuigeling en bij de bescherming tegen voedselallergieën in het vroege leven. In het huidige proefschrift is deze potentiële rol van vrije aminozuren in moedermelk daarom verder onderzocht.

Om meer inzicht te verkrijgen in de mogelijke biologische relevantie van de verschillende vrije aminozuren in moedermelk, zijn in *hoofdstuk 2* de concentraties hiervan gedurende de eerste zes maanden van lactatie gemeten, evenals de totale concentraties van elk aminozuur, bestaande uit de som van de vrije en conjugeerde aminozuren. Terwijl de totale concentraties van elk aminozuur in moedermelk afnam gedurende de eerste drie maanden van lactatie, namen de concentraties van een aantal vrije aminozuren (te weten glutamine, glutamaat, serine, threonine, aspartaat en glycine) toe gedurende deze periode. Deze toename was het grootst voor de aminozuren glutamine en glutamaat, die samen meer dan 60% van de totale hoeveelheid vrije aminozuren in moedermelk vormden. Deze resultaten laten zien dat concentraties van specifieke vrije aminozuren in moedermelk veranderen naarmate de zuigeling ouder wordt, wat suggereert dat er een noodzaak is voor een adequate inname van vrije aminozuren in het vroege leven. De grote hoeveelheid vrije glutamine en glutamaat ten opzichte van de andere vrije aminozuren in de moedermelksamples, samen met de sterke verandering in de concentraties van deze twee aminozuren gedurende lactatie suggereert dat deze aminozuren in het bijzonder mogelijk belangrijke functies hebben in de ontwikkeling van de zuigeling.

In *hoofdstuk 3* is aangetoond dat de concentraties van vrije aminozuren in moedermelk niet alleen veranderen naarmate de zuigeling ouder wordt, maar ook binnen één voeding. De melk die als laatste vrijkomt (achtermelk) bevatte minder vrije aminozuren dan de melk die als eerst vrijkomt (voormelk). De verschillen tussen de twee melkfracties waren het grootst voor vrije glutamine, waarvan de concentratie in achtermelk meer dan 10% lager was dan in voormelk. In de praktijk komt het voor dat er uitsluitend achtermelk wordt gevoed aan vroeggeboren kinderen met een laag geboortegewicht, omdat deze melkfractie meer vet en daarmee meer energie bevat, waardoor deze kinderen sneller kunnen groeien om de achterstand in te halen. Onze resultaten suggereren dat deze handelswijze er ook toe leidt dat de zuigeling een lagere hoeveelheid vrije aminozuren binnenkrijgt. Dit onderstreept het belang van het begrijpen van de functies van vrije aminozuren in moedermelk in de ontwikkeling van de zuigeling. Daarnaast toont deze studie de relevantie aan van het standaardiseren van de melkfractie die wordt verzameld in studies waarin de samenstelling en functies van aminozuren in moedermelk worden onderzocht. Dit helpt in het minimaliseren van de interindividuele variabiliteit in de concentraties van aminozuren in moedermelk, wat robuustere statistische analyses mogelijk maakt.

In *hoofdstuk 4* werd de huidige kennis van de samenstelling en de functies van vrije aminozuren in moedermelk beschreven en bediscussieerd. De mogelijke functies van vrije glutamine en glutamaat in moedermelk werden in het bijzonder uitgelicht, omdat deze aminozuren in relatief grote hoeveelheden aanwezig zijn in moedermelk en toenemen in concentratie naarmate de zuigeling ouder wordt, zoals beschreven in *hoofdstuk 2*. Aan de hand van resultaten uit preklinische studies werd belicht dat deze twee aminozuren een breed scala aan effecten kunnen hebben in de ontwikkelende darm. Zo kunnen glutamine en glutamaat de darmgroei stimuleren, de darmmorfologie bevorderen, de darmbarrière ondersteunen en de kolonisatie van de darmen door micro-organismen moduleren. Daarnaast zijn deze aminozuren onmisbaar voor het optimaal functioneren van immuuncellen en kunnen beide aminozuren de functies van het darmgeassocieerde immuunsysteem beïnvloeden. Zo is onder andere aangetoond dat glutamine en glutamaat in staat zijn om inflammatoire en T helper 2-type (TH2) immuunreacties in de darmen te remmen, die een cruciale rol spelen in de pathogenese en pathofysiologie van allergische aandoeningen, zoals voedselallergie. Gebaseerd op deze preklinische vindingen werd de hypothese gesteld dat glutamine en glutamaat in moedermelk mogelijk een rol spelen in de bescherming tegen allergische aandoeningen, evenals in de ondersteuning van een gezonde groei in het vroege leven.

In *hoofdstuk 5* zijn in een cohort met meer dan 600 moeder-kind paren de concentraties van vrije aminozuren in moedermelk onderzocht in relatie tot zowel de groei van de zuigeling in de eerste levensmaanden, als tot het risico op het ontwikkelen van veelvoorkomende immunologische aandoeningen in de eerste levensjaren. Er werden positieve correlaties gevonden tussen de concentraties van vrije glutamine, glutamaat en serine in moedermelk en de gewichtstoename van de zuigeling in de eerste zes weken van het leven. Voor vrije glutamine werd er tevens een positieve correlatie gevonden met de lengtetoename gedurende deze periode. Tezamen suggereren deze bevindingen dat deze aminozuren in moedermelk een bijdrage leveren aan een gezonde groei in het vroege leven. Er werden geen correlaties gevonden tussen aminozuren in moedermelk en het risico op atopische dermatitis, piepende ademhaling (een symptoom van onder andere astma en luchtweginfecties) en bovenste luchtweginfecties. Wel werden er voor een aantal aminozuren (te weten asparagine, citrulline, arginine en glutamine) negatieve correlaties gevonden tussen de concentraties in moedermelk en het risico op het ontwikkelen van voedselallergie in het eerste levensjaar. Omdat het cohort maar relatief weinig kinderen met voedselallergie bevatte is bevestiging van deze correlaties in grotere cohorten noodzakelijk. Desalniettemin toont deze vinding de relevantie aan van verder onderzoek naar de potentie van vrije aminozuren om de ontwikkeling van voedselallergie tegen te gaan.

In *hoofdstuk 6* werd onderzocht of vrije glutamine de capaciteit heeft om de ontwikkeling van voedselallergie tegen te gaan, gebruikmakend van een gevalideerd muismodel voor koemelkallergie. Er kon worden aangetoond dat muizen die een dieet verrijkt met 2% vrije glutamine kregen, minder ernstige acute allergische symptomen ontwikkelden na toediening van het koemelkallergeen ten opzichte van muizen die een controledieet kregen. De verminderde ernst van allergische symptomen in de glutamine-gevoede muizen ging gepaard met onder andere hogere concentraties van allergeen-specifieke immunoglobuline A (IgA) antilichamen in het serum en de feces, en een verhoogd percentage van geactiveerde T helper 1 (TH1) cellen in de mesenteriale lymfeklieren. Deze modulaties zouden ten grondslag kunnen liggen aan de verminderde ernst van allergische symptomen. Uit eerder onderzoek is namelijk gebleken dat allergeen-specifieke IgA antilichamen en TH1 cellen een remmende werking kunnen hebben op allergische reacties, respectievelijk door allergenen te neutraliseren en door TH2 immuunreacties te remmen. Deze resultaten benadrukken de potentie van vrije glutamine om de functies van het immuunsysteem te moduleren, en ondersteunen de hypothese dat vrije glutamine in moedermelk een bijdrage kan leveren aan de bescherming tegen voedselallergie in de vroege stadia van het leven.

Wanneer voor kinderen met ernstige koemelkallergie geen borstvoeding beschikbaar is, kunnen zij baat hebben bij een speciale kunstmatige zuigelingenvoeding waarbij de eiwitten, die een allergische reactie op kunnen wekken, zijn vervangen door vrije aminozuren. Een dergelijke voeding is effectief in het behandelen van de klachten geassocieerd met koemelkallergie en stimuleert daarnaast de groei van de zuigeling. In *hoofdstuk 7* werd, gebruikmakende van het eerdergenoemde muismodel voor koemelkallergie, aangetoond dat het voeden van een aminozuur-gebaseerd dieet ook effectief is in de preventie van symptomen van koemelkallergie. Opvallend was dat muizen die gevoed waren met het aminozuur-gebaseerde dieet beschermd waren tegen het ontwikkelen van allergeen-geïnduceerde systematische anafylaxie. Deze bescherming ging gepaard met een reductie in de concentraties van antigeen-specifieke IgG antilichamen in het serum en een verhoging van de hoeveelheid Foxp3-positieve cellen in de dunne darm. Deze cellen, ook wel regulatoire T cellen genoemd, hebben een anti-inflammatoire functie en spelen een belangrijke rol bij het induceren van tolerantie tegen allergenen in voedsel. Deze vindingen suggereren dat anti-inflammatoire en tolerantie-inducerende effecten mogelijk ten grondslag liggen aan de beschermende werking van het aminozuur-gebaseerde dieet. Echter, verder onderzoek is nodig om het exacte onderliggende mechanisme te achterhalen.

Samenvattend laat dit proefschrift zien dat de concentraties van vrije aminozuren in moedermelk veranderen naarmate de zuigeling ouder wordt, dat de manier waarop deze concentraties veranderen verschilt per aminozuur en dat deze veranderingen los staan van

de veranderingen in de concentraties van geconjugeerde aminozuren in moedermelk. Dit suggereert dat er een noodzaak is voor een adequate inname van vrije aminozuren in het vroege leven. Verder suggereren de resultaten gepresenteerd in dit proefschrift dat een adequate inname van specifieke vrije aminozuren, in het bijzonder glutamine, gedurende deze periode mogelijk belangrijk kan zijn voor een optimale groei en voor de bescherming tegen voedselallergieën. Deze bevindingen roepen op tot verder onderzoek naar de functies van vrije aminozuren in moedermelk in de ontwikkeling van de borstgevoede zuigeling. Het begrijpen van deze functies kan onder andere mogelijkheden bieden voor het optimaliseren van de samenstelling en functionaliteit van kunstmatige zuigelingenvoeding, dat doorgaans aanzienlijk lagere concentraties van vrije aminozuren bevat dan moedermelk.

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Joris,
December 2021, Utrecht

About the author

Joris van Sadelhoff was born on May 30, 1992 in Zevenaar, the Netherlands. After graduating from high school in 2010, he moved to Nijmegen where he studied Molecular Life Sciences at the Radboud University. In 2013, he received the bachelor's degree, after which he continued with a master in Science, Management and Innovation and a research master in Clinical Biology. During the research master, he did an internship within the Developmental Biology department at the Radboud Institute for Molecular Life Sciences, under supervision of Dr. Jo Zhou and Dr. Willem van den Akker. Here, he investigated the molecular basis of a neurodevelopmental disorder, through differentiating patient-derived induced pluripotent stem cells into glutamatergic neurons. His second research internship was conducted within the Immunology department of Danone Nutricia Research in Utrecht. During this internship, he explored the effects of free amino acids on inflammation-induced muscle breakdown *in vitro*, under supervision of Dr. Jeroen van Bergenhenegouwen and Dr. Anita Hartog. In 2016, he finished both masters with distinction (cum laude), after which he joined the Immunology department of Danone Nutricia Research as a Junior Scientist. Here, he investigated the immunomodulating effects of free amino acids in *in vitro* models for allergic diseases. In 2018, Joris started as a PhD candidate in the Pharmacology division of the Utrecht Institute for Pharmaceutical Sciences. During his PhD, he was trained in immunology by following the Infection and Immunity PhD program at the Utrecht University. In close collaboration with Danone Nutricia Research and under supervision of Prof. Dr. Johan Garssen, Dr. Frank Redegeld, Dr. Astrid Hogenkamp and Dr. Selma Wiertsema, Joris investigated the concentrations of free amino acids in human milk and their potential roles in early life growth and immunity. The results of this research are compiled in this thesis.

List of publications

List of publications

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