

Allergen Shedding in Human Milk from Mothers of Preterm Infants: Proteomic and Peptidomic Feasibility and Pilot Analysis

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Abstract

Rationale:

There is little information regarding the allergen content of milk feeds in the preterm population. Previous studies have evaluated specific proteins/peptides via ELISA, but no studies have performed a broad analysis of the allergenic peptide content and protease activity of milk feeds in this population. Preterm infants spend a critical window of time for immune development in the Newborn Intensive Care Unit (NICU), and may receive fortified donor milk, maternal milk or formula feeds via nasogastric tube or bottle instead of fresh breastmilk via breastfeeding.

Methods:

To evaluate feasibility, we initially performed mass spectrometry on four human milk samples (two term and two preterm) from the Mommy's Milk Human Milk Biorepository (HMB) which included maternal surveys of diet and environmental exposures. We analyzed the results against the University of Nebraska FASTA database and UniProt for a total of 2211 protein sequences. We then further analyzed 5 samples from the Microbiome, Atopy and Prematurity (MAP) pilot study along with formula and human milk fortifier controls and performed not only mass spectrometry, but also peptidomic and protease activity analysis.

Results:

Each HMB sample had between 806 and 1007 proteins, with 37 to 44 non-human proteins/sample encompassing 26 plant and animal species. Bovine proteins were the most numerous; seven unique *Bos taurus* proteins were found in all four samples, and three contained Bos d 5. Cat, dog, mosquito, salmon, and crab were detected in all four samples. All donors ingested fish, shellfish and tree nuts, and all had salmon and crab proteins in their milk samples; two almond proteins were detected in three samples. Aeroallergens, including dust mite (Der f 28, Der f 25) and mold (Cla h 4) were identified in all samples. Two samples contained allergens to latex (Hev b 9) and chicken (Gal d 10). One sample contained several unique proteins, including carrot, two molds (including Pen c 19) and Der f 33-like protein. In the preterm MAP samples, 784 digested non-human proteins were identified, 30 were non-bovine in origin. Proteins from 23 different species including aeroallergens, food, and contact allergens were identified. Protease activity was highest in human milk samples without human milk fortifier and lowest in preterm formula.

Conclusions:

These findings represent the first preterm milk feed mass spectrometry and protease analysis with identification of known allergenic proteins to food, contact and aeroallergens. The varying degree of protein detection may reflect variable individual secretion and augmentation of feeds. This raises questions of whether the composition of milk feeds in the NICU impact the development of atopic disease in the preterm

population and whether the complex interaction between allergens, proteases, and other human milk components can serve to induce sensitization or tolerance to allergens in infants.

Key Messages Regarding Feasibility

1. What uncertainties existed regarding the feasibility?

This was the first time that human milk samples were being run through the mass spectrometer to look for allergen peptides at the University of California San Diego

2. What are the key feasibility findings?

We were able to find several food and environmental allergens in the samples that were run. The samples had good overlapping alignment which meant the results were reliable and there was differential findings compared to formula which would not be expected to have many allergen peptides aside from those associated with cow milk. We also found there was no difference in variety of allergens between human milk from mothers of term and preterm infants.

3. What are the implications of the feasibility findings for the design of the main study?

We were able to subsequently run pilot samples from our Microbiome, Atopy, and Prematurity study through the mass spectrometer and also perform protease studies on these pilot samples. We will now be able to expand our analysis to look at the larger cohort of samples we have collected longitudinally over the first year of life to see how they compare to our feasibility and pilot runs.

Introduction

There has been increasing recognition of the protective role of human milk and early food exposure in the development of atopic conditions such as eczema, food allergy, and asthma.¹⁻⁴ Asthma is the most prevalent chronic disease in children, affecting over 300 million people worldwide⁵ and disproportionately affects preterm infants. In one study, infants born before 37 weeks (moderate to late preterm) were 50% more likely to develop asthma, and infants born before 32 weeks (extremely to very preterm) were three times as likely to develop asthma⁶. While mechanical ventilation and other causes of direct damage to the lung may account for some of this increased risk for the development of asthma in the preterm population, other factors may also play a role. Lower rates of atopy in term infants were associated with exclusive breastfeeding for the first four months of life⁷, lack of antibiotic exposure (either maternal intrapartum or early in infancy),⁸ vaginal delivery,⁹ and furry pets in the home.¹⁰ Preterm infants spend this critical period in the neonatal intensive care unit and some are exposed to antibiotics and c-section delivery but most importantly, almost all experience most of their nutrition via a nasogastric tube or a bottle containing previously frozen maternal or donor breast milk, instead of fresh breastmilk via breastfeeding.

Recommendations regarding the introduction of allergenic foods to infants has shifted from avoiding allergenic foods until 1 year of age to early introduction prior to 6 months of age, as the latter has been shown to be associated with a decreased risk of food allergy during a critical window of the infants immune development.⁴ This approach is thought to reduce the induction of Type 2 inflammation that is responsible for allergic conditions. Given that early exposure to food proteins can alter the development of food allergies

later in life, early nutrition has become an area of interest in studying the pathogenesis of atopic disease.¹¹ Over 70% of peanut reactions occur on the first known exposure, indicating prior sensitization, perhaps transcutaneously, via inhalation, or via human milk.^{12,13} Since human milk is often the primary source of nutrition in infancy, it has been postulated that it could be a source for allergen introduction.¹⁴ Although IgE-mediated reactions to human milk are rare, they do occur, demonstrating its immunogenic nature.^{15 16}

Airborne allergens from house dust mite have also been found in human milk at similar quantities to food allergens.¹⁷ One dust mite protein, Der p1 has demonstrated both Toll-like receptor agonist and protease activities, which could potentially initiate allergic immune responses.¹⁸ In addition to food and environmental allergens, human milk also contains many other bioactive substances, including endogenous proteases and protease inhibitors, immunoglobulins, soluble receptors, cytokines, human milk oligosaccharides (HMOs), fatty acids, and microbes.^{11,19 20–23}

Human milk proteomics studies have utilized different methods for protein identification, including Western blotting, ELISA, and mass spectrometry, which may account for heterogeneity between experiment results.^{24–31} Mass spectrometry has been utilized to determine the protein content in human milk, however, there are few studies that are primarily focused on allergenic proteins.¹¹ Studies utilizing mass spectrometry, which allows for a broader untargeted search for proteins, have identified 1200-1600 total proteins in human milk, the vast majority of non-human proteins derived from cow's milk, with one study also identifying dog, horse, cat, chicken and rice proteins.^{15,32} The degree of protein alteration prior to its appearance in human milk is unknown, and studies are conflicting. Some studies have found that peanut Ara h 1 and 2,¹⁴ ovalbumin,³² and gliadin²⁵ are not degraded in human milk. This contrasts with other studies that identified fragments of β -Lactoglobulin^{15,33} and α -S1 casein.⁵³

Proteases have been identified in human milk and appear to play a significant role in infant digestion, but the interaction between endogenous human milk proteases and non-human proteins such as house dust mite proteases and human milk fortifiers (HMFs) proteases has not been extensively studied.³⁴

The development of tolerance versus sensitization to allergens is complex and depends on the interaction and often multi-directional relationship between many different factors, such as maternal history, milk composition, gut immunology and microbiome and external environment.²¹ Allergen shedding in human milk may be a way to educate the infant's immune system and modulate allergy risk in the infant.³⁵ Given advancements in medicine that have led to increased survival of preterm infants, we aim to examine longer term outcomes such as allergic sensitization or tolerance in this population by examining human milk protein composition and exposure. Due to newer techniques in proteomic and peptidomic analysis, and the paucity of data regarding the ability of HM to induce tolerance or sensitization to allergens, we developed a feasibility pilot study on a subset of milk samples to investigate how nutrition and environmental exposures may impact allergen shedding in human milk in preterm infants.

Methods

Sample Collection

Four human milk samples (2 from mothers of term infants and 2 from mothers of preterm infants) were analyzed from the Mommy’s Milk Human Milk Biorepository (HMB) to evaluate the feasibility of analyzing human milk samples by untargeted mass spectrometry (Table 1a). The Mommy’s Milk HMB was founded at the University of California, San Diego in 2014 with the goal of building a constant but rotating inventory of 3,000 human milk samples available for future research.³⁶ Following informed consent, women provide 50 mL up to a full pump of expressed breast milk (convenience sample). Participants are interviewed about their sociodemographic characteristics, pregnancy history, dietary intake using a standard questionnaire (<https://www.nutritionquest.com>), medication exposure, lifestyle habits, maternal stress, anxiety and depression, breastfeeding behaviors, and signs and symptoms of potential adverse reactions in the offspring. Data on growth of the infant/toddler are captured from medical records, and neurodevelopmental assessments are conducted longitudinally. Sample collections occur at UC San Diego, community sites, or the participant’s home. Human milk samples are stored and shipped on ice within 24 hours of collection to the Mommy’s Milk lab where the sample is aliquoted and stored at -80°C until requested for study analysis.

Table 1
a Maternal Dietary History: Mommy’s Milk Human Milk Repository (HMB) Samples

Sample ID number	Infant’s gestational age	Infant’s gender	Cow milk	Egg	Wheat	Nuts	Fish / Shellfish
R1	36 weeks	Female	no	no	yes	yes	yes
R2	33 weeks	Female	yes	no	yes	yes	yes
R3	39 weeks	Male	yes	yes	yes	yes	yes
R4	40 weeks	Female	no	yes	yes	yes	yes

Table 1
b Maternal Dietary History: Microbiome, Atopy, and Prematurity (MAP) Samples

Sample ID number	Cow’s milk	Egg	Soy	Wheat	Peanut	Tree Nuts	Fish	Shrimp	Shellfish
J7	rarely	daily	never	daily	daily	daily	daily	never	never
J8	weekly	weekly	rarely	weekly	never	never	weekly	weekly	never
J9	daily	daily	rarely	weekly	daily	weekly	rarely	rarely	rarely
J10	weekly	weekly	rarely	weekly	weekly	monthly	monthly	never	never
J11	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
J12	daily	daily	rarely	daily	weekly	weekly	monthly	monthly	never

We also analyzed 5 human milk samples and 1 formula sample (J11) from mothers and preterm infants recruited into the Microbiome, Atopy and Prematurity (MAP) pilot study (*under review*)(Table 1b). The MAP

study population recruited 48 preterm infants, ≤ 34 weeks, and their mothers, from Jacobs Medical Center at UCSD in the Newborn Intensive Care Unit (NICU) and Scripps Memorial La Jolla NICU. At birth, prenatal (maternal antibiotics and diet, pregnancy morbidities, smoking, pet ownership, family history of asthma and other social and demographic information) and perinatal (method of delivery, need for resuscitation) factors/exposures were documented at the time of enrollment. Parents were given a History and Allergy Questionnaire at enrollment which asked about family history of asthma, smoking, allergies, medication, dietary history during pregnancy and postnatally (**Supplemental table 1**). Milk, stool and saliva samples from the study participants were collected weekly and stored immediately at 4°C and transferred to -80°C within 36 hours post collection. For this pilot study, first week milk samples were analyzed from 6 infant/parent couplets. Since the majority of preterm infants require fortification to support their growth and development, half of the milk samples (3/6) analyzed from the MAP study contained human milk or formula based fortifiers. To control for those additives, an additional 2 samples of regular and hydrolyzed HMF (Enfamil human milk fortifier acidified liquid (Mead Johnson)) and one formula sample (Enfamil premature (Mead Johnson)) were analyzed.

This research was performed in accordance with the ethical principles for medical research involving human subjects outlined in the Declaration of Helsinki. The study protocol was approved by the University of California, San Diego's Human Research Protections Program IRB# 181711

Sample preparation for proteomic analysis

Milk samples were thawed on ice prior to preparation for proteomic analysis. Guanidine-HCl was added to 2 μ L of milk sample to achieve a final concentration of 6 M. The samples were boiled for 10 minutes followed by 5 minutes cooling at room temperature. The boiling and cooling cycle was repeated 3 times. The proteins were precipitated with the addition of methanol to a final volume of 90% followed by vortex and centrifugation at 14,000 rpm on a benchtop microfuge for 10 minutes at 25°C. The soluble fraction was removed by flipping the tube onto an absorbent surface and tapping to remove any liquid. The pellet was suspended in 200 μ L of 8 M Urea made in 100 mM Tris pH 8.0. Tris (2-carboxyethyl) phosphine and chloroacetamide were added to final concentrations of 10 mM and 40 mM, respectively and the mixture was vortexed for 5 minutes. 3 volumes of 50 mM Tris pH 8.0 was added to the sample to reduce the final urea concentration to 2 M. Trypsin was added in a 1:50 protein ratio and incubated at 37°C for 12 hours. The solution was then acidified using TFA (0.5% TFA final concentration) and mixed. Samples were desalted using 100 mg C18-StageTips as described by the manufacturer protocol. The peptide concentration of the samples was measured using BCA after resuspension in sample loading buffer and a total of 0.5 μ g was injected for each label free quantification run.

Sample preparation for peptidomic analysis

To remove high molecular weight milk proteins, 100 μ L of human milk was mixed with 900 μ L of methanol and vortexed for 5 seconds. The samples were kept at 25°C for 30 minutes followed by centrifugation at 12,000 rpm for 10 minutes at 25°C. 500 μ L of supernatant was transferred to a fresh tube dried in a vacuum centrifuge. The samples were hydrated in 0.5 mL of 0.5% formic acid and 5% acetonitrile (ACN) solution and desalted using a Sep-PAK C18 1 cc Vac (Waters Corporation, Milford MA) according to the manufacturer's

protocol with the exception that 40% ACN was used to elute peptides. The eluents were dried in speed-vac in preparation for mass spectrometry analysis.

Liquid Chromatography with Tandem Mass Spectrometry (LC-MS-MS)

Trypsin-digested peptides were analyzed by ultra-high pressure liquid chromatography (UPLC) coupled with tandem mass spectroscopy (LC-MS/MS) using nano-spray ionization. The nanospray ionization experiments were performed using a Orbitrap fusion Lumos hybrid mass spectrometer (Thermo) interfaced with nano-scale reversed-phase UPLC (Thermo Dionex UltiMate™ 3000 RSLC nano System) using a 25 cm, 75-micron ID glass capillary packed with 1.7- μ m C18 (130) BEH™ beads (Waters corporation). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–80%) of ACN (Acetonitrile) at a flow rate of 375 μ L/min for 2h. The buffers used to create the ACN gradient were: Buffer A (98% H₂O, 2% ACN, 0.1% formic acid) and Buffer B (100% ACN, 0.1% formic acid). Mass spectrometer parameters are as follows; an MS1 survey scan using the orbitrap detector (mass range (m/z): 400-1500 (using quadrupole isolation), 120,000 resolution setting, spray voltage of 2,200 V, Ion transfer tube temperature of 275°C, AGC target of 400,000, and maximum injection time of 50 ms) was followed by data dependent scans (top speed for most intense ions, with charge state set to only include +2-5 ions, and 5 second exclusion time, while selecting ions with minimal intensities of 50,000 at which the collision event was carried out in the high energy collision cell (HCD Collision Energy of 30%), and the fragment masses were analyzed in the ion trap mass analyzer (With ion trap scan rate of turbo, first mass m/z was 100, AGC Target 5000 and maximum injection time of 35ms). Protein identification and label free quantification was carried out using Peaks Studio 8.5 (Bioinformatics solutions Inc.)

Analysis was performed in two separate runs – the first included the samples from the breast milk repository, and the second included the samples from the MAP study, formula, and fortifier. Database searches were carried out against a reference database that included FASTA protein sequences of known protein allergens from the University of Nebraska (<http://www.allergenonline.org/> (used version 19, published 02/10/19)) that was combined with human proteome UniProt sequences using Peaks 8.5 (Bioinformatics Solutions) search engine. This database included known allergenic peptides and all predicted human proteome. The hits were filtered at 1% false discovery rate (FDR) before being considered for further analysis. All positive peptide sequence results were verified by blasting original sequences against UniProt and NCBI (<https://www.uniprot.org/blast/>) to confirm accuracy. Results where at least one of the two of the databases did not have > 70% identity to the labeled species-specific protein were discarded.

Protease Activity

Samples were diluted 50-fold in 50 mM Tris-HCl, pH 9.0, 150 mM NaCl and assayed with 25 μ M RR-AMC (Santa Cruz Biotechnology, sc-281540) in triplicate wells on a black 384-well plate. The final volume in each well was 30 μ L and the assay was performed at 25°C. Activity was monitored for 2 hours on a BioTek HTX plate reader with excitation of 360 nm and emission of 460 nm. Activity was reported as the change in relative change in fluorescence units per sec (RFU/sec).

HMB samples did not contain fortifier and did not undergo protease analysis. (Summary of milk feed content and analysis performed- Table 2)

Table 2
Milk Sample Composition and type of analysis

Sample ID	Milk Sample Composition	Proteomics	Peptidomics
J7	HM + fortifier	Yes	Yes
J8	HM	Yes	Yes
J9	HM	Yes	Yes
J10	HM + fortifier	Yes	Yes
J11	preterm formula	Yes	Yes
J12	HM	Yes	Yes
R1	HM	Yes	No
R2	HM	Yes	No
R3	HM	Yes	No
R4	HM	Yes	No

HM: human milk; fortifier: Enfamil Human milk fortifier (non-hydrolyzed)

Results

Proteomic Feasibility Study from the Mommy's Milk Human Milk Biorepository samples

To determine the feasibility of detecting allergenic peptides/proteins in human milk, we performed mass spectrometry on four (2 term and 2 preterm) trypsin-digested breast milk samples (R1, R2, R3, R4) and utilized the University of Nebraska FASTA and UniProt databases for a total of 2211 sequences for comparison. Each sample had between 806 and 1007 peptides with 28 to 38 non-human proteins per sample encompassing 23 different plant and animal species (Table 3). We detected peptides from various food, venom/salivary, and airborne sources. There were no appreciable differences between term and preterm samples in terms of total protein content. One sample accounted for over 50% of the non-human peptide variability (R4).

Table 3

List of pertinent bovine peptides and all NH non-bovine peptides stratified by sample proportion in Fortifiers, HBM, and MAP samples

Species	Sample	Proteins	Digested vs Undigested
Bos taurus (cow)	14/14	β -Lg (Bos d 5)	Digested and non-digested, in formula samples only
	13/14	α -S1-casein (Bos d 9), β -casein (Bos d 11), serum albumin (Bos d 6)	-Bos d 9 – 3 isoforms; digested only in the human samples, digested and non-digested in formula samples -Bos d 11 – 5 isoforms; both digested and non-digested in human and formula samples -Bos d 6 – 5 isoforms; all digested in human and formula samples
	12/14	α -lactalbumin (Bos d 4)	4 isoforms were digested in human and formula samples; one isoform digested and non-digested in formula only
	9/14	α -lactalbumin precursor	Digested in human and human samples
	8/14	C3, β -casein A3, ceruloplasmin	-C3 – 2 isoforms; digested in human and formula samples - β -casein A3 – digested and non-digested in human and formula samples -ceruloplasmin – 4 isoforms; digested in human and formula samples
	7/14	Histone H4, antithrombin III	-histone H4 – 19 isoforms, digested in human and formula samples -antithrombin III – digested in human and formula samples
	6/14	Lactadherin, κ -casein (Bos d 12)	-lactadherin – digested in human and formula samples
	5/14	Monocyte CD14	2 isoforms, digested in human and formula samples
	4/14	C4, lactoperoxidase, α -S2-casein (Bos d 10), Ig J	-C4 – digested and non-digested in human samples only -Ig J – digested in human and formula samples
	3/14	C7, PGH2 isomerase, CD 59, LPS binding protein	-C7 – 2 isoforms, digested in human and formula samples

Total number of samples:14 (4 from Mommy's Milk Human Milk Repository, 6 from Microbiome, Atopy, and Prematurity Study, 4 Human Milk Fortifier Samples)

Species	Sample	Proteins	Digested vs Undigested
	2/14	C9, CD 109, C5a	-C9 – 2 isoforms; digested in human and formula samples -C5 – digested in human and formula samples
	1/14	Lipocalin (Bos d 2), CD 5, CD 81, CD 166, T cell surface CD3	-Bos d 2 – digested in formula samples only
Bos mutus (yak)	14/14	β -casein isoform X2	Digested and non-digested in human and formula samples
Felis catus (cat)	13/14	Serum albumin (Fel d 2)	All digested in human and formula samples
Equus caballus (horse)	12/14	Serum albumin (Equ c 3)	All digested in human and formula samples
Canis lupus familiaris (dog)	11/14	Serum albumin (Can f 3)	All digested in human and formula samples
	8/14	Albumin precursor	All digested in human and formula samples
	2/14	Albumin partial, CE1	-CE1: digested in formula samples only
Sus scrofa (pig)	10/14	Serum albumin (Sus s 1), albumin partial	Sus s 1 and albumin partial – all digested in human and formula samples
Dermatophagoides farinae (dust mite)	9/14	Heat shock protein (Der f 28)	All digested in human and formula samples
	5/14	Triosephosphate isomerase (Der f 25)	2 isoforms; digested in human samples only
	1/14	Tubulin α chain	
Aedes aegypti (mosquito)	10/14	Heat shock cognate 70 (Aed a 8)	All digested in human and formula samples
Salmo salar (salmon)	5/14	β -enolase (Sal s 2)	Digested in human samples only
	4/14	Enolase, aldolase A	-Enolase – digested in human samples only
	3/14	Collagen α (Sal s 6.0102)	Digested and non-digested, in formula samples only

Total number of samples:14 (4 from Mommy's Milk Human Milk Repository, 6 from Microbiome, Atopy, and Prematurity Study, 4 Human Milk Fortifier Samples)

Species	Sample	Proteins	Digested vs Undigested
	2/14	Aldolase A fructose bisphosphate	
Hevea brasiliensis (latex)	8/14	Enolase 1 (Hev b 9)	Digested, in human samples only
	2/14	Enolase 2 (Hev b 2)	
Procambarus clarkii (crayfish)	7/14	Triosephosphate isomerase (Pro c 8)	Digested, in human samples only
Penicillium citrinum (fungus)	4/14	Heat shock 70 kDa protein (Pen c 19)	Digested, in human samples only
Blattella germanica (cockroach)	3/14	α -amylase (Bla g 11)	Digested, in human samples only
Gallus gallus (chicken/egg)	5/14	β -enolase	Digested, in human samples only
Scylla paramomosain (crab)	4/14	Triosephosphate isomerase	
Prunus dulcis (almond)	3/14	Pru du 6, Pru du 6.0101	
Oreochromis mossambicus (tilapia)	3/14	Tropomyosin	
Thunnus albacares (tuna)	3/14	α -amylase (Thu a 2.0101)	Digested, in human samples only
Triticum aestivum (wheat)	2/14	HMW glutenin subunit 1By9, Tri a TPIS, triosephosphat-isomerase (Tri a 31)	-HMW glutenin subunit 1By9 – digested, in human samples only -Tri a TPIS – digested, in human samples only -Tri a 31 – digested, in human samples only
Curvularia lunata (mold)	1/14	Enolase	
Daucus carota (carrot)	1/14	Peptidyl-prolyl cis trans isomerase	
Catharanthus roseus (periwinkle)	1/14	Peptidyl-prolyl cis trans isomerase	

Total number of samples:14 (4 from Mommy's Milk Human Milk Repository, 6 from Microbiome, Atopy, and Prematurity Study, 4 Human Milk Fortifier Samples)

Species	Sample	Proteins	Digested vs Undigested
Ambrosia artemisiifolia (ragweed)	1/14	Putative pectate lyase precursor (Amb a 1)	Digested, in human samples only
Total number of samples:14 (4 from Mommy's Milk Human Milk Repository, 6 from Microbiome, Atopy, and Prematurity Study, 4 Human Milk Fortifier Samples)			

In their dietary histories all mothers ingested fish, shellfish, nuts, and wheat. Two out of the four mothers did not consume cow's milk (dairy), although bovine peptides were found in all samples. Two of the four mothers did not ingest egg, yet egg protein was detected in both of those samples. Interestingly, the one mother who drank almond milk was the only one who did not have almond detected.

Proteomic Pilot Study from the MAP samples

After the protocol was validated with the samples from the HMB repository, a subsequent mass spectroscopy run was performed on 5 human milk samples, 1 formula sample, 2 regular milk fortifier samples, and 2 hydrolyzed milk fortifier samples. All the milk samples except for one (J9) were a mix of maternal expressed breast milk or donor breast milk. J9 contained maternal expressed breast milk only.

We performed proteomic and peptideomic analysis on the MAP samples and HMFs. We identified a total of 784 digested non-human proteins, 754 that were bovine in origin and 30 non-bovine in origin. Hydrolyzed HMF samples had significantly fewer proteins (average of 48) compared to non-hydrolyzed HMF (average of 264), human milk (average of 256) and formula (average of 236) (Figure 1).

In total, we identified proteins from 23 different species, including aeroallergens, food and contact allergens (Table 3). For quality control purposes peptide alignment maps were made for two common allergens, beta-lactoglobulin and cat albumin. The maps demonstrated consistent overlap, thereby supporting that these proteins were indeed identified and digested similarly between samples. (**Supplemental Figure 1**)

Bovine peptides were the most numerous of the non-human peptides detected in human milk samples without HMF. Specific allergenic bovine peptides (β -lactoglobulin, α - and β -casein, α -lactalbumin) were in the highest relative quantification in regular fortifier and formula, intermediate in hydrolyzed fortifier and lowest in human milk samples (**Supplemental Figure 2**).

Peptidomics studies of the MAP samples identified peptides that were generally less than 40 amino acids in length (Figure 2). Most non-bovine peptides were digested (28/33) and found in samples with human milk and fortifier which contrasts with only a few (2/28) being found in the formula sample.

Protease activity

Upon analysis of the amino acids at the N- and C- termini of peptides, we discovered that there was a high frequency of proline (P) and glutamine (Q) residues in the MAP samples. This was not surprising because human casein is the major protein in these samples and 17% of residues in this protein are proline while 11% are glutamine. Samples J8-J11 are notable as they have peptides that were cleaved after lysine (K) and

arginine (R) at the N-terminal side of peptides. This is not seen for J7 and J12 (Figure 3). To quantify protease activity in these human milk samples, we assayed the samples with the fluorogenic substrates, Arg-Arg-AMC. We found that the most abundant protease activity was in human milk sample J9, while the formula only sample J11, had the lowest activity (Figure 4).

Discussion

To our knowledge, this is the first combined human milk mass spectrometry and protease analysis using clinically relevant NICU preterm milk samples. While the detection of food peptides in the milk samples is interesting and somewhat expected, the size, breadth, and variety of food and aeroallergens as well as other non-food peptides is fascinating, especially when comparing human milk to cow milk formula. Furthermore, we found differential protease activity between the samples with the highest being in maternal expressed breast milk alone, without fortifier (J9) and the lowest in formula (J11).

The presence of allergen peptides in human milk does not appear to be accidental and may be linked to the development of allergy. In one study, there was an increase in atopy in children who were breastfed by atopic mothers and found to have high HM dust mite (Der p 1) levels; this was not noted in the offspring of mothers without atopic history regardless of Der p 1 level in human milk.¹⁸ In food allergy, maternal cow's milk avoidance was associated with increased cow's milk allergy in offspring, mediated by a lower cow's milk specific IgA and possibly the lack of cow's milk protein exposure.²³ In our analysis, bovine peptides were the most numerous of the non-human peptides detected in the human milk only samples (without formula or fortifier) and specific allergenic bovine peptides (β -lactoglobulin, α - and β -caseins, α -lactalbumin) were found in the highest relative quantification in regular fortifier and formula samples and lowest in the human milk samples.

Multiple other common food allergens have been identified in human breastmilk (HBM) studies. Ovalbumin has been detected in HBM in 8.3%-76% of subjects,^{24,30,31,37} while ovomucoid was identified in 78% of subjects in one study.³⁰ There appeared to be a dose-response phenomenon between maternal egg intake and infant serology, whereby for each additional egg ingested, HBM ovalbumin concentration increased by 25% and infant egg-specific IgG4 increased 22%.³⁷ Egg protein (β -enolase) was found in our analysis although specific ovalbumin and ovomucoid peptides were not identified. Regarding peanut protein, one study of 23 lactating females found that after a 50 g oral peanut load, 48% of female subjects' HM samples contained peanut.¹⁴ Another small study demonstrated peanut allergen (Ara h 6) in HM that was functional and IgE-reactive as evidenced by in vitro assays and the observation that administration to mice lead to partial oral tolerance.³⁸ Peanut protein was not identified in our analysis. With wheat protein, gliadin was detected in 67.5% – 100% breast milk samples in two studies.^{25,39} Multiple different wheat peptides were identified in our samples, but not gliadin.

In the food diaries associated with the maternal milk samples that were not augmented with fortifier or formula, some foods that were reported as consumed did not show up in the samples. Conversely, in other cases, foods that were not reportedly consumed, did show up in the samples. While recall bias and ensuing inaccuracy may partially account for these discrepancies, there is also the issue of timing of food

consumption with respect to appearance in human milk. Moreover, the capability of excreting specific proteins may vary between mothers and further impact the presence of allergenic proteins, which further complicates attempts at correlating dietary ingestion and breast milk peptides.

We know that antigen-presenting cells introduce processed allergens to T-helper lymphocytes and proceed down a TH2 pathway in allergic conditions.⁴⁰ How the allergen is processed, the role of proteases, and the exact conformation of different allergenic proteins in human milk is not known, although the size of the original protein was better elucidated in our study. We demonstrated that many bovine peptides are found digested (original protein size > 40 amino acids) and free (original protein < 40 amino acids), indicating that there are a variety of different parent proteins. These proteins were mostly found shared between the human milk plus fortifier or formula samples. Conversely, human milk samples without fortifier had relatively few digested bovine peptides, supporting that most cow's milk-derived peptides originated from smaller proteins. Interestingly, cow's milk allergy is one of the first to appear in infants' and the majority of those are sensitized to caseins, which may be able to cross the GI border relatively intact as they coagulate in acidic conditions and may be less susceptible to proteolysis.^{32,41} A variety of caseins of different sizes were identified in our formula, fortifier and human milk samples, although the allergenicity of these specific caseins are not definitively established in this current analysis. Additionally, the exact origin of these proteins, although presumably diet-derived, is unknown. As opposed to various sizes, the majority of non-bovine peptides in our human milk samples were digested, thereby originating from peptides over 40 amino acids in length.

Assuming that most of these peptides were generated by proteases, we looked at the amino acid sequence in the protein that ended up being the substrate for cleavage. Sample J9 (pure maternal expressed breast milk) from the MAP study showed the highest protease activity. Previous proteomic studies have shown differences in the presence of proteases and protease inhibitors in HM between allergenic and non-allergenic mothers.⁴² There is evidence that an imbalance between protease and protease inhibitors in HBM could allow for easier penetration of allergens.^{43,44} Specifically, reduced cystatin, a protein inhibitor that has been detected in HM, secreted by epithelial cells has been linked to easier penetration of Der p1 through skin.⁴⁵ Furthermore, protease inhibitors have been detected in the stool of infants who have received HBM indicating that these protease inhibitors may be active in the gastrointestinal tract.⁴⁶ It is thought this complex interplay between allergens, proteases, and protease inhibitors is important in the pathogenesis of atopy, and protease inhibitors are being evaluated as a potential therapeutic agent to treat asthma and other atopic conditions.

There are several limitations to this study. We started with a small batch of samples to assess feasibility in this pilot trial. There is inconsistency between dietary documentation between the two sample groups. We will have a more consistent and larger sample size in our future analysis. Other sample-based limitations include the lack of multiple "pure" samples that contain only maternal expressed breastmilk without fortifier or the use of pooled donor human milk. Theoretically, a subtractive analysis could be considered, with inference of protein content of breast milk via exclusion of proteins found in fortifier, however, this is limited due to the overlap of proteins between fortifier and human milk and the variability between the samples, including differences in protein content between the two samples of the same fortifier. Moreover, since a

large proportion of preterm infants receive supplementation, donor milk, or formula, our results reflect the real world setting in the NICU. Our subject dietary history did not include the temporal relationship of specific food ingestion and sample collection. Thus, secretion kinetics cannot be concluded, and contamination/inadvertent consumption is an issue with the self-reported dietary histories. Closer analysis of maternal diet and timing of consumption may help to determine the kinetics of human milk peptides and the degree of contamination (dietary or via mass spectrometry) that could account for the detection of proteins that are not found in the diet. Database limitations are also possible. We did not manually blast all proteins against NCBI and Uniprot databases, only those which were positively identified, so it is possible that there were false negatives and proteins were not identified due to inaccurate database sequences

Future directions and Conclusions

We have taken a large step forward in identifying what a preterm infant immune system may encounter in their milk feeds, however, it was beyond the scope of this study to determine the origin of the human milk peptides identified. This is an area we plan to investigate in the future. Peptides may be secreted by lactocytes or enter via the bloodstream. It is also not known where proteolytic cleavage occurs, whether it is locally in the breast or in the GI tract/blood, which could be further investigated by paired blood samples in future studies.

The interaction between allergen, protease, and protease inhibitors also warrants further investigation. Identifying which proteases and protease inhibitors are present in our MAP samples would be of great interest, particularly if their presence or absence augments the development of atopic conditions in infants who have been in the NICU. We do plan to follow subjects out to 5 years and look for the development of allergic outcomes in our MAP cohort. The use of formula, fortifiers, and donor milk are important in optimizing the growth and development of preterm infants. However, their use may have unintended long-term consequences, that need further investigation.

In conclusion, the detection of various allergenic peptides and protease activity in our milk samples raises more questions about how modifying feeds in the NICU may impact the development of atopy in preterm infants. Ultimately, whether human milk can serve to induce allergic sensitization or tolerance in an infant is an area of research that needs much further exploration.

Declarations

The data that support the findings of this study are available from the corresponding author, SL, upon reasonable request.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The authors confirm contribution to the paper as follows: study conception and design: KL, MG, AJO, BG, SLL, SL; data collection: KL, DM, SB, KB, CC, KSE, SL, MG, AJO, BG, SLL, SL; analysis and interpretation of results: KL, SL, MG, AJO, BG, SLL, SL; draft manuscript preparation: KL, SB, KB, KSE, MG, AJO, BG, SLL, SL. All authors reviewed the results and approved the final version of the manuscript.

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Figures

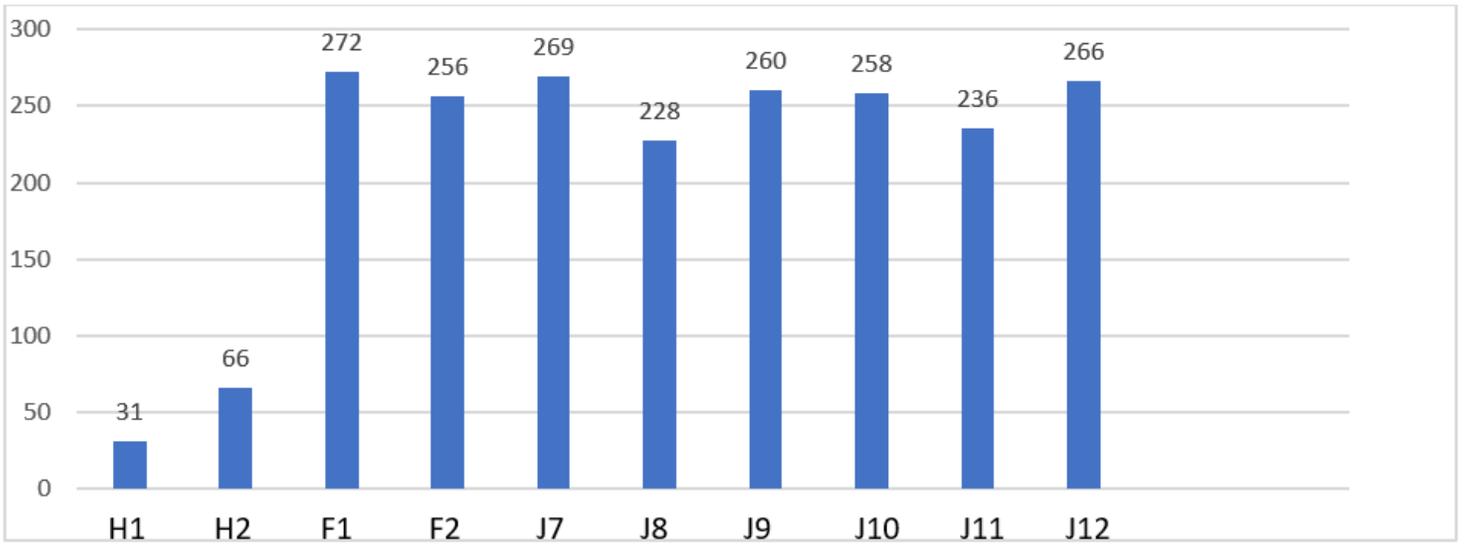


Figure 1

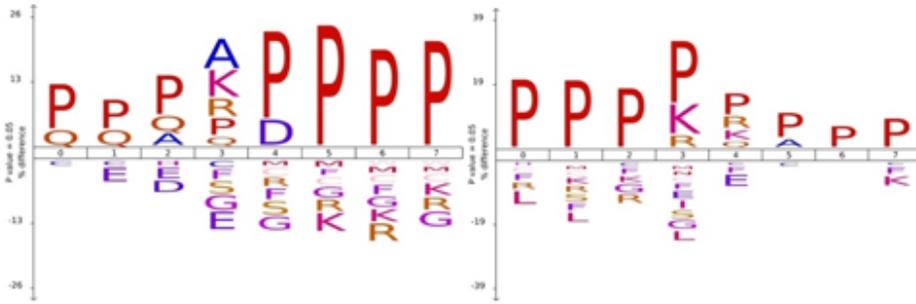
Total Non-Human Proteins in MAP Samples

H- Hydrolyzed Human Milk Fortifier

F- Regular Human Milk Fortifier

J11 Enfamil Preterm Formula

Sample J9



Sample J7

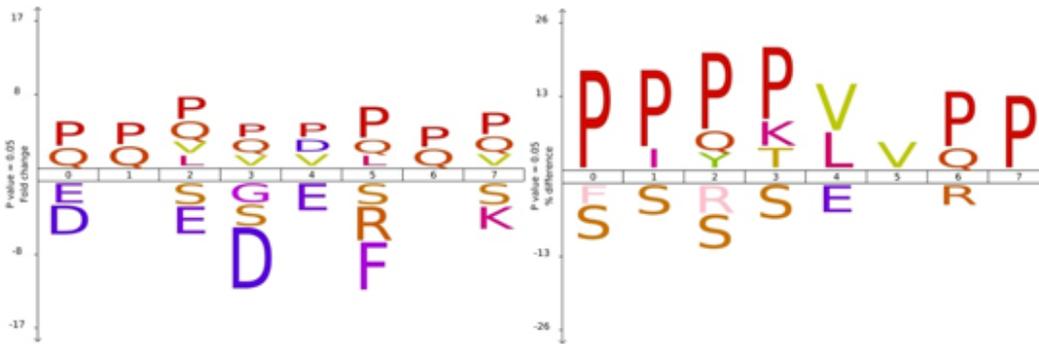


Figure 3

Protease Activity in MAP Samples

Frequency of amino acids at the 4 positions or either side of the cleaved bond (Positions 0 to 7 where cleavage occurs between 3 and 4). The left image on the tab corresponds to the amino acids found on the N-terminal side of the peptides while the right image corresponds to the amino acids found on the C-terminal side of the peptides. Sample J7 is fortified HBM while J9 is unfortified HBM. Amino Acid Codes: Proline-P, Glutamine-Q, Lysine (K), Arginine (R)

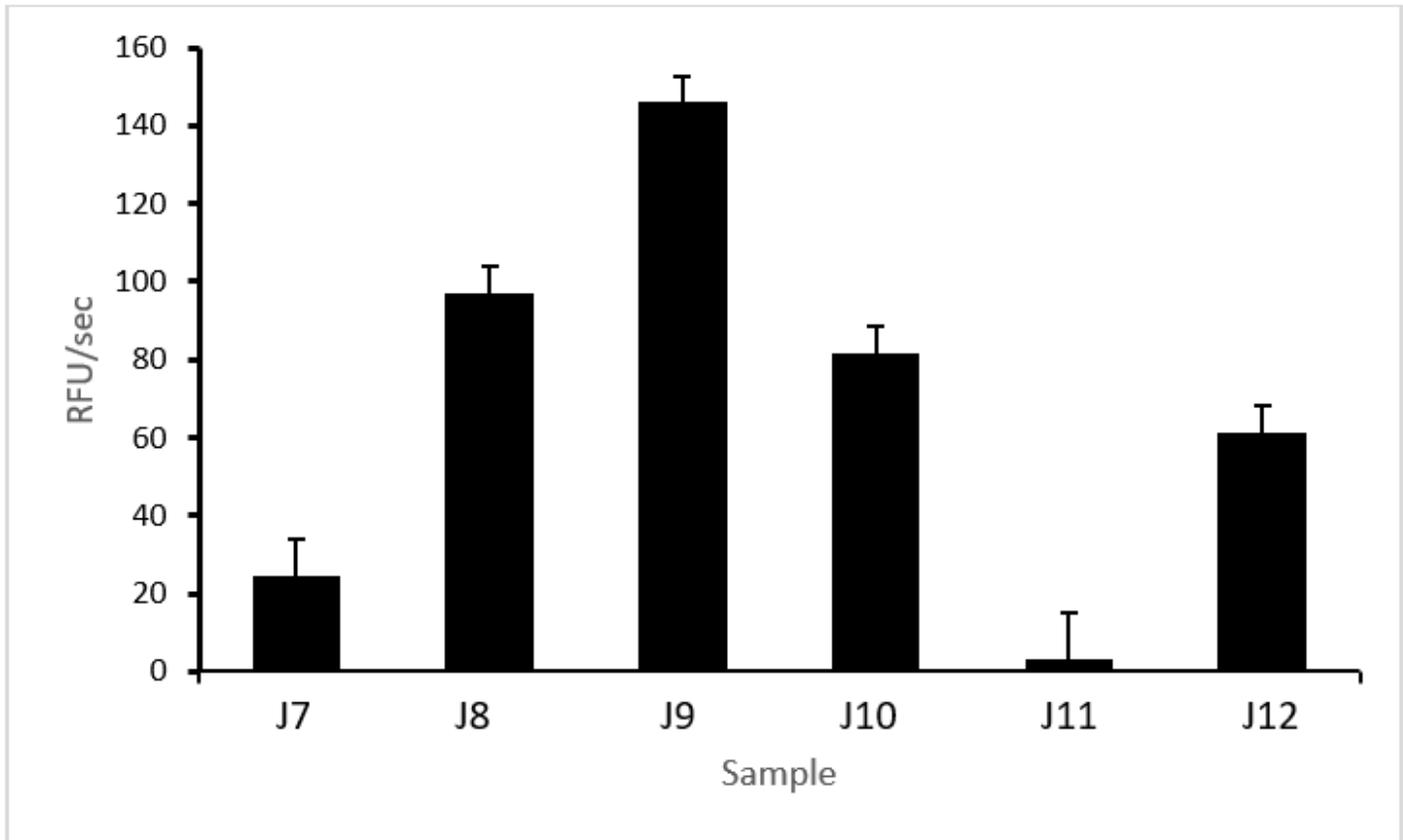


Figure 4

Protease activity in MAP Samples

Proteolytic activity in milk samples using the fluorogenic dipeptide substrates Arg-Arg-AMC.

J9 Maternal Expressed Breast Milk Only. J11 Fortified Formula

Supplementary Files

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