The Journal of Nutrition
Genomics, Proteomics, and Metabolomics

Personalized Profiling Reveals Donor- and Lactation-Specific Trends in the Human Milk Proteome and Peptidome

Jing Zhu,[1,2] Kelly A Dingess,[1,2] Marko Mank,[3] Bernd Stahl,[3,4] and Albert JR Heck[1,2]

[1]Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, University of Utrecht, Utrecht, The Netherlands; [2]Netherlands Proteomics Center, Utrecht, The Netherlands; [3]Danone Nutricia Research, Utrecht, The Netherlands; and [4]Chemical Biology and Drug Discovery, Utrecht Institute for Pharmaceutical Sciences, University of Utrecht, Utrecht, The Netherlands

ABSTRACT

Background: Human milk is the most genuine form of personalized nutrition, whereby its nutritional and bioactive constituents support the changing needs of the growing infant. Personalized proteome profiling strategies may provide insights into maternal–infant relationships. Proteins and endogenous peptides in human milk play an important role as nutrients for growth and have distinct functionality such as immune defense. Comprehensive monitoring of all of the human milk proteinaceous components, including endogenous peptides, is required to fully understand the changing role of the human milk proteome throughout lactation.

Objective: We aimed to investigate the personalized nature of the human milk proteome and peptidome for individual mother–infant dyads.

Methods: Two individual healthy milk donors, aged 29 and 32 y and both of a normal BMI, were longitudinally observed over weeks 1, 2, 3, 4, 6, 8, 10, 12, and 16 postpartum. Milk collection was standardized. Comprehensive variations in the human milk proteinaceous components were assessed using quantitative LC-MS/MS methods.

Results: We longitudinally profiled the concentrations of >1300 milk proteins and 2000 endogenous milk peptides spanning 16 wk of lactation for 2 individual donors. We observed many gradual and alike changes in both donors related to temporal effects, for instance early lactation was marked by high concentrations of proteins and peptides involved in lactose synthesis and immune development. Uniquely, in 1 of the 2 donors, we observed a substantial anomaly in the milk composition, exclusively at week 6, likely indicating a response to inflammation and/or infection.

Conclusions: Here, we provide a resource for characterizing the lactational changes in the human milk proteome, encompassing thousands of proteins and endogenous peptides. Further, we demonstrate the feasibility and benefit of personalized profiling to monitor the influence of milk on the development of the newborn, as well as the health status of each individual mother–infant pair. *J Nutr* 2021;151:826–839.

Keywords: personalized profiling, proteomics, peptidomics, lactation, human milk composition

Introduction

Personalized proteome profiling is steadily gaining momentum, becoming just now feasible through recent technical advances in MS-based proteomics, enhancing sensitivity and throughput. Applying these advancements to human milk allows for the more robust analyses of the molecular components of human milk for individual mother–infant dyads (1). A personalized profiling approach adheres to the current movement for research to improve methodologies for individualized healthcare, taking into account a person's unique characteristics (2). Moreover, along with the widely accepted view that each person has his or her own unique DNA, it is becoming more and more apparent that our proteomes may be just as unique. This idea led to the statement by Leigh Anderson, a prominent pioneer in plasma proteomics, "that in future biomarker analysis, it will become standard practice to interpret each test value against the individual's personalized baseline, instead of against a population distribution, as is currently the case" (3). Most personalized proteome profiling studies to date have focused on plasma or serum, likely due to the fact that blood sample collection is less invasive than tissue biopsy and cerebrospinal fluid collection (4, 5).

We propose that human milk represents an ideal and very important source for personalized proteome and endogenous peptidome profiling, as it provides meaningful insights into the development and health of each mother–infant pair. Unlike

Manuscript received September 9, 2020. Initial review completed November 17, 2020. Revision accepted December 18, 2020.
First published online March 9, 2021; doi: https://doi.org/10.1093/jn/nxaa445.

other sampling of other biofluids, milk sampling is noninvasive, being a part of the normal daily routine for most nursing mothers. Just as blood is a universal reflection of an individual's state or phenotype (4), milk is a reflection of the maternal–infant health status, forming the bridge between 2 interconnected individuals, and is tailor made for each mother–infant pair (6, 7). The act of feeding at the breast propagates enteromammary transfer and mammary gland production of diverse human milk components, such as proteins and endogenous peptides, which deliver maternal immunity to the infant (8). Personalized human milk profiling is of particular interest as newborns are intricately dependent on the immune system of their mother to protect them against a plethora of pathogens and antigens that they encounter for the first time. It is therefore worthwhile to have an in-depth inventory of all proteins and endogenous peptides in human milk, further building upon previous studies (6, 7, 9).

In profiling the composition of human milk, 2 crucial and dynamic dimensions should be taken into account, *1*) change over time and *2*) highly donor-specific aspect. Therefore, we argue that human milk profiling is best performed by taking frequent samples throughout lactation, covering the early period (here the first 16 wk) in which the infant is exclusively breastfed. Thus, instead of a larger cohort of donors and/or pooled samples, we chose to longitudinally monitor 2 individual donors individually. We focused not only on the human milk proteome but additionally profiled the often neglected endogenous peptidome, as several endogenous peptides present in human milk are known to have their own unique functionalities compared to corresponding precursor proteins (10–14).

Supported by the Netherlands Organization for Scientific Research (NWO) funding the large-scale proteomics facility Proteins@Work (project 184.032.201) embedded in the Netherlands Proteomics Centre and the TOP-Punt Grant (718.015.003). Additional support for this research was provided by Danone Nutricia Research. JZ additionally acknowledges support from the Chinese Scholarship Council (CSC).

Author disclosures: MM and BS are employees of Danone Nutricia Research. JZ and KAD were enrolled as PhD students at Utrecht University during this study and received partial financial support from Danone Nutricia Research. All other authors report no conflicts of interest.

JZ and KAD contributed equally.

Present address for JZ: Beijing Institute of Nutritional Resources, Beijing, China. Address correspondence to AJRH (e-mail: a.j.r.heck@uu.nl).

Supplemental Figures 1–9, Supplemental Tables 1 and 2, and Supplemental Methods are available from the ''Supplementary data'' link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.

Abbreviations used: AA, amino acid; AGC, automatic gain control; B3GNT3, N-acetylgalactosaminide β-1,3-N-acetylglucosaminyltransferase 3; B4GALT1, β-1,4-galactosyltransferase; CASB, β-casein; CFB, complement factor B; CID, collision-induced dissociation; CLU, clusterin; DEFA3, neutrophil defensin 3; EThcD, electron-transfer/higher-energy collision dissociation; FCN-1, ficolin-1; FDR, false discovery rate; FUCA1, α-L-fucosidase; FUT3, galactoside 3(4)-L-fucosyltransferase; GLB1, β-galactosidase; HCD, higher-energy collisional dissociation; HEXA, β-hexosaminidase subunit α; HEXB, β-hexosaminidase subunit β; HMO, human milk oligosaccharide; LALBA, α-lactalbumin; LCN2, neutrophil gelatinase-associated lipocalin; LFQ, label-free quantification; MAC, membrane attack complex; MEC, mammary epithelial cell; MFGM, milk fat globular membrane; MHC, major histocompatibility complex; MPO, myeloperoxidase; NME1, nucleoside diphosphate kinase A; PD, proteome discoverer; PGM1, phosphoglucomutase-1; pIgR, polymeric Ig receptor; PLG, plasminogen; PQI, protein quantitation index; pRM, parallel reaction monitoring; PRTC, peptide retention time calibration; RT, retention time; SC, secretory component; SERPINA1, α-1-antitrypsin; SERPINF2, α-2-antiplasmin; SERPINI1, neuroserpin; sIG, secretory Ig; SLC5A1, sodium/glucose cotransporter 1; ST6GAL1, β-galactoside α-2,6-sialyltransferase 1; UGP2, UTP-glucose-1-phosphate uridylyltransferase.

Materials and Methods

Human subjects and milk samples

The observational protein and endogenous peptide profiling of human milk was done with longitudinal samples collected from 2 healthy donors. Maternal and infant demographic information for each donor is provided in **Supplemental Table 1**. Samples were collected according to standardized conditions (15) to avoid variation in the milk composition due to diurnal effects, expression of fore compared with hindmilk, and degradation of proteins by proteases. Donors were instructed to collect milk as an expression of 1 entire breast, by an electric pump, between the hours of 09:00 and 13:00. Pumped milk was transferred into provided 2-mL Eppendorf tubes, with 2-mL syringes (BP Plastipak) containing protease and phosphatase inhibitors. A protease inhibitor (Complete Mini EDTA-free, Sigma-Aldrich) and a phosphatase inhibitor (PhosSTOP, Sigma-Aldrich) were added as one-ninth of the collection volume. Samples were transferred back to the laboratory on dry ice and stored at −80°C until thawed for analysis.

Relevant information regarding the sample collection was recorded by the mothers, including the time and date of collection, left or right breast, and total volume of milk pumped (**Supplemental Table 2**). Written informed consent was obtained prior to sample collection. All samples used were donated to Danone Nutricia Research in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Whole-milk shotgun proteome analysis

The in-solution digestion of whole milk was adapted from previous methods (16). Briefly, whole-milk proteins were exacted, reduced, alkylated, and then digested overnight. The resulting tryptic peptides were separated and analyzed using LC-MS/MS coupling of an Agilent 1290 Infinity HPLC system (Agilent Technologies) to a Q Exactive Plus hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in data-dependent acquisition mode and high-energy collision dissociation (HCD) was used for MS/MS fragmentation. Raw shotgun LC-MS/MS data were searched with Proteome Discoverer (PD) (version 2.2, Thermo Scientific) using the Mascot search engine (version 2.6.1) against a UniProt Swiss-Prot database (17): Homo sapiens (canonical and isoform) (December 2018, 20,417 entries) and filtered with a 1% false discovery rate (FDR). Label-free quantification (LFQ) and retention time (RT) alignment were enabled for shotgun analysis to determine protein intensity. A protein quantitation index (PQI) was calculated using the mean intensity of unique peptides direct proportionality between PQI and protein abundance demonstrated in this method (18), and equations can be seen in **Supplemental Figure 1**. Full details of protein extraction, LC-MS/MS analysis, and PD searches and an explanation of PQI are provided in **Supplemental Methods**.

Whole-milk shotgun peptidome analysis

Methods for whole-milk peptide extraction have been previously described in detail (19, see the liquid–liquid extraction section). Changes to the previously described method were made to scale all reagents to a 100-μL whole-milk starting volume. The resulting endogenous peptides were separated and analyzed using LC-MS/MS coupling in an Agilent 1290 Infinity HPLC system (Agilent Technologies) to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in data-dependent

acquisition mode and triplicate measurements were done in parallel using collision-induced dissociation (CID), HCD, and electron-transfer/higher-energy collision dissociation (EThcD) for MS/MS fragmentation. Raw shotgun LC-MS/MS data were searched with PD (version 2.2, Thermo Scientific) with the same library and search engine settings as described above, with some exceptions. For the nondigested endogenous peptides, no fixed modifications were selected, because samples were not reduced and alkylated. Variable modifications included Met oxidation, phosphorylation (S, T, Y), and N-terminal acetylation. A semitryptic digest was chosen for cleavage specificity, LFQ and RT alignment were not enabled, as they are not compatible with semitryptic searches. All data were filtered by 1% FDR. A modified PQI was set up to determine endogenous peptide concentration (equations can be seen in Supplemental Figure 1). Full details of endogenous peptide extraction, LC-MS/MS analysis, and PD searches and an explanation of PQI are provided in the Supplemental Methods.

Determination of protein and endogenous peptide concentrations

Protein pellets from the endogenous peptide extraction were used for amino acid (AA) hydrolysis to determine total AA content to quantify protein and endogenous peptide. Protein pellets were saved from the endogenous peptide extraction and were washed with ultrapure water to remove excess trichloroacetic acid. Total AA hydrolysis was achieved by addition of 6N hydrochloric acid (Sigma-Aldrich), followed by heating for 20 h at 110°C. Neutralized, washed samples were then analyzed by LC-MS performed on a Dionex Ultimate 3000 autosampler and pump (Thermo Scientific) coupled online to a Q Exactive mass spectrometer (Thermo Scientific). AAs were separated using a Sequant ZIC-pHILIC column (2.1 × 150 mm, 5 μm, guard column 2.1 × 20 mm, 5 μm; Merck). The MS operated in polarity-switching mode with spray voltages of 4.5 kV and −3.5 kV. AAs were identified on the basis of exact mass within 5 ppm and further validated by concordance with retention times of standards. The calibration stock solution of all AAs was prepared as equal parts MEM amino acid solution and MEM EAGLe nonessential amino acid solution, and 200 mM of L-glutamine. Quantification was based on peak area using LCquan software (Thermo Scientific). Full details are provided in the Supplemental Methods.

Targeted PRM LC-MS and data analysis

For PRM analysis, peptides were chosen based on general rules for targeted proteomics (20). For the selected peptides, stable isotope-labeled standards terminated with C-terminal heavy Arg/Lys were purchased from JPT Innovative Peptide Solutions (Supplemental Dataset 2). The tryptic digested peptides with spiked heavy labeled peptides and Pierce™ peptide retention time calibration mixture (PRTC) were analyzed by LC-MS/MS using an UltiMate 3000 HPLC (Thermo Fisher Scientific) coupled online to a Q Exactive High-Field X quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). Summing extracted peak areas of non-interfered fragment ions per each precursor ion were used for calculating ratios of light to heavy in the pairs of each target peptide and its stable isotope standard. Peptide amounts were generated by the ratio of light to heavy multiplying amount of stable isotope-labeled peptide and then further derived to molar concentrations and milligram per milliliter concentrations (Supplemental Dataset 2). Full details are provided in Supplemental Methods.

Statistical analysis

The statistical analysis and all figures were generated with R version 3.4.2, using ggplot2 (version 2.2.1). Statistical tests included Pearson correlations and unsupervised hierarchical clustering. Additionally, *z*-scores were generated for each protein in individual donors across the lactation period.

Data and software availability

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (21) partner repository with the dataset identifier PXD014917 and 10.6019/PXD014917. PRM data have been deposited to Panorama (22) which is a freely available, open-source repository server application for targeted MS assays, via this link: https://panoramaweb.org/i5FXO3.url. Additionally, all plotted proteome and peptidome data have been made available for user interaction at this link: https://milkprofiling.hecklab.com/.

Results

Extending human milk proteome profiling with endogenous peptides

One of the principal goals of this work was to profile the human milk proteome in depth, consisting of both proteins and endogenous peptides, in a personalized manner. The overall workflow is schematically depicted in **Supplemental Figure 3**. In brief, we applied a shotgun proteomics approach (Supplemental Dataset 3) (16) and in parallel a peptidomics approach (19) (Supplemental Dataset 4), analyzing each sample in triplicate for accurate LFQ. We then performed targeted quantitative validation using PRM (Supplemental Dataset 2). The LFQ in triplicate showed high quantitative accuracy with a mean Pearson correlation coefficient of 0.99 for proteins (Supplemental Dataset 5) and 0.97 for endogenous peptides (Supplemental Dataset 6).

The parallel profiling of the endogenous peptidome enlarged the depth of the human milk proteome, both qualitatively and quantitatively. The identified tryptic-digested peptides and endogenous peptides originated cumulatively from 1588 milk proteins, 1528 from proteomics (Supplemental Dataset 3) and 227 from peptidomics (Supplemental Dataset 4) (**Figure 1**A). Thus, analyzing the peptidome added 60 protein identifications to the human milk proteome. Applying stringent filtering criteria, quantitative values for abundances could be monitored for 1320 proteins and 2096 endogenous peptides. In this set of identifications there are still some redundancies, due to the presence of highly polymorphic proteins in milk; notably immunoglobulins and proteins related to the major histocompatibility complex (MHC). We observed that the major quantitative contributors to the proteome and endogenous peptidome were distinctive (Figure 1B). In terms of abundance, 65% of the endogenous peptidome originated from caseins (α-s1-, β-, and κ-), which was 2 times more than the casein abundance observed in the proteome. Osteopontin was the second most abundant precursor for the endogenous peptides, although it only made up 0.2% of the proteome (Figure 1B). In contrast, several other abundant proteins in milk, α-lactalbumin, serum albumin, IgA1, and lysozyme C did not contribute at all to the endogenous peptidome. These distinct characteristics of the endogenous peptidome and proteome hint at their complementary functional roles in human milk.

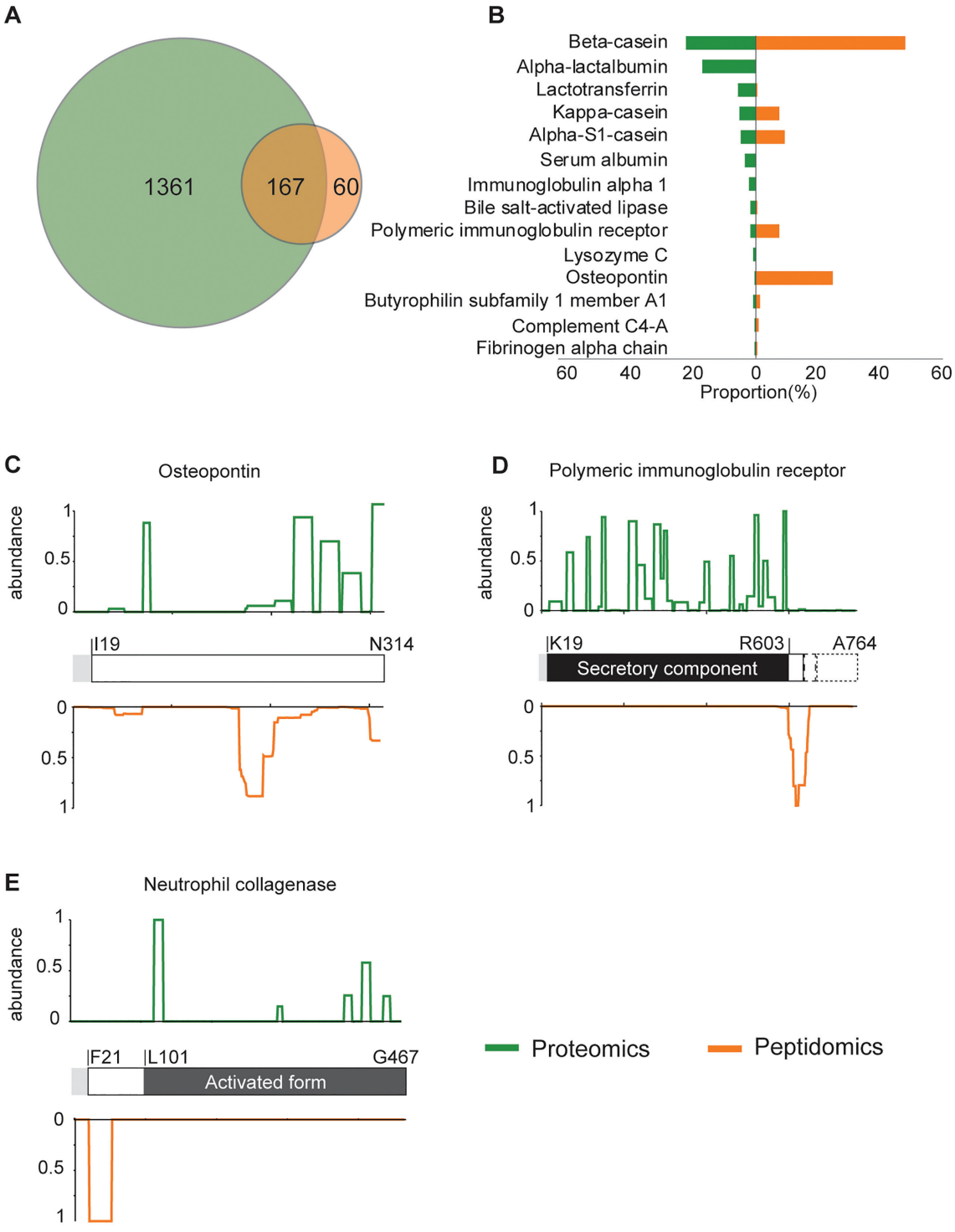

FIGURE 1 Personalized human milk proteome profiling by proteomics and peptidomics. (A) Number and overlap of identified proteins in human milk, for which evidence was detected either by shotgun proteomics (green) or from the analysis of endogenous peptides by peptidomics (orange). This color scheme is used throughout all panels of the figure. (B) Major contributors to the human milk proteome and peptidome. For the proteome data, LFQ was used based on all detected unique tryptic peptides of the protein. For peptidomics, the intensities were summed from all endogenous peptides originating from a precursor protein. While the proteome was dominated by whey proteins, clearly, the caseins were the major contributors to the peptidome, followed by pIgR and osteopontin. (C–E) Endogenous peptides in the peptidome originate from specific parts of the protein sequence differing from the proteome. For 3 illustrative proteins (C) osteopontin, (D) pIgR, and (E) neutrophil collagenase, the sequences are schematically depicted in the middle, whereby the grey box annotates the N-terminal signaling peptides, the black box the protein sequence as present in human milk. The top of each figure depicts the sequence and abundance of detected tryptic peptides of the proteins in the proteomic data, and the bottom depicts the sequence and abundance of detected endogenous peptides originating from the same proteins in the peptidomics data. Clearly, in (D) many tryptic peptides of pIgR are detected in the proteomics data covering its secretory domain, whereas the endogenous peptides observed from pIgR originate exclusively from the transmembrane and cytoplasmic regions that are known to be cleaved off. Similarly, for neutrophil collagenase (E) there is little overlap in sequence covered by the tryptic peptides and the endogenous peptides. LFQ, label free quantification; pIgR, polymeric immunoglobulin receptor.

Intriguingly, the covered sequence for the same protein by shotgun proteomics and endogenous peptidomics revealed selective proteolysis (Figure 1C–E). In the case of the aforementioned osteopontin, the identified peptides by proteomics and peptidomics were found to span the whole protein sequence (Figure 1C). This is in sharp contrast to the polymeric Ig receptor (pIgR), a transmembrane protein primarily responsible for transporting IgA and IgM into milk from mammary epithelial cells (MECs), for which the proteomics and peptidomics data covered almost mutually exclusive regions of the sequence (Figure 1D). The extracellular domain of pIgR is secreted from MECs and the secretory component (SC) is endoproteolytically cleaved. The SC forms tight complexes with secretory IgA (sIgA) or secretory IgM (sIgM) (23). For pIgR all endogenous peptides originated from a small stretch in the sequence, spanning AAs 567–648, where the extracellular component of pIgR is cleaved. In contrast, the peptides detected by shotgun proteomics covered exclusively the secretory component (Figure 1D). Another example is neutrophil collagenase, in which peptides from proteomics data originating exclusively from the activated form and the endogenous peptides all originated from the N-terminus cleaved off upon activation of the precursor (Figure 1E). Our complementary proteomics and peptidomics data revealed several more such processing events. The data described here can be assessed in an online database we created and made publicly available (https://milkprofiling.hecklab.com/). We conclude that the parallel analysis of proteins and endogenous peptides may reveal a deeper physiological overview of the human milk proteome, and thus a more complete view of the biological processes occurring during lactation. Next, we investigated changes occurring in the personalized human milk proteome profiles, focusing on processes regulating glycan synthesis, aiding in infant digestion, and building up components of the immune system.

Personalized characteristics of human milk

To be able to quantify protein abundances, we first determined the total protein concentration in each milk sample using total AA analysis. Protein concentrations were then compared with data reported in a recent meta-analysis (24), from which we concluded that both donors exhibited protein concentrations in line with the metadata and followed the observed general regression trend (**Supplemental Figure 4A**). Although the protein concentrations in the milk samples from our 2 donors adhered those of the reported broader population range, they showed distinctive individual characteristics. One obvious difference was the proportion of whey and casein proteins (Supplemental Figure 4B), where donor 1 (depicted throughout the manuscript in red) had a substantially larger proportion of whey proteins and endogenous peptides compared to the second donor (depicted throughout the manuscript in turquoise), observable in all milk samples, at all time points. Additionally, in the 2 donors the ratio of whey to casein proteins was distinct, albeit still within the reported biological variation of previous studies. It is generally accepted in the field of human milk proteomics that this ratio is reduced roughly from 90:10 in colostrum to 60:40 during mature lactation (25).

We examined the 11 most abundant proteins (making up >70% of the total protein concentration) in the 2 donors over lactation (**Figure 2**A). The ranking of the 11 most abundant proteins was similar in both donors, but the amount of each protein was individual specific. For example, the most abundant whey protein, α-lactalbumin (LALBA), was about 2 times more abundant in donor 1 than donor 2 at the first time point; however, it became nearly equal by week 16 (Supplemental Figure 4C). For several other proteins the concentration profiles displayed individual-specific trends, as illustrated for lactoferrin and serum albumin in Supplemental Figure 4D and E, and for several casein proteins β-, α-s1-, and κ-casein in Supplemental Figure 4F–H.

To extend our analysis beyond these most abundant milk proteins, we performed hierarchical clustering of all quantified milk proteins (Figure 2B) and endogenous milk peptides (Figure 2C). Both for the proteins and endogenous peptides, samples from 1 donor always clustered together, reiterating that a main factor affecting the human milk proteome is the individual donor. The clusters depicted in Figure 2 show several distinctive trends in both proteomics and peptidomics data, which we discuss in more detail below. Of note is the milk sample provided by donor 1 in week 6, which is a clear abnormality, reflected in both the proteome and peptidome. As our primary initial motivation was to monitor "normal" lactational changes in individual donors, we excluded the data of week 6 from both donors in our initial analysis. The observed changes in week 6 for donor 1 will be discussed separately.

Glycan synthesis across lactation

The changes in protein abundances in human milk may have several causes, originating from 4 physiological pathways in the mammary gland. The dominant pathway is the exocytotic pathway (26, 27), followed by the transcytosis pathway (28, 29), and a pathway unique to MECs via the budding of the milk fat globule membrane (MFGM) (28, 30). The remaining fourth pathway, the paracellular pathway, directly transports serum substances and cells and is only active during times of early lactation, involution, or inflammation (31, 32). Via these pathways, the intrinsically generated proteins and endogenous peptides in MECs and those from circulation become present in human milk. Changing concentrations of distinct components in human milk are a reflection of the changes in these pathways, echoing the functional needs of the infant and the biological processes occurring in the lactating mother.

To delineate all the changes observed in the proteome and peptidome, we first focused on the group of proteins involved in glycan synthesis. Human milk is known to be rich in carbohydrates, notably lactose, human milk oligosaccharides (HMOs), and glycoconjugates. Lactose, known to be a key driver of milk production (33), is a disaccharide synthesized primarily in the MEC. We were able to detect nearly all proteins involved in the lactose synthesis pathway (**Figure 3**A). Briefly, lactose synthesis starts with the transport of glucose and galactose from the basement membrane into MEC, continues with the interconversion of UDP-galactose, and ends with the production of lactose by lactose synthase, which is a 1:1 complex of LALBA and β-1,4-galactosyltransferase (B4GALT1) (34). We were able to detect the sodium/glucose cotransporter 1 (SLC5A1), which provides 1 possible way for glucose and galactose to enter the MEC. However, we did not detect the solute carrier family 2 proteins, thought to be the primary route for glucose entry into MEC (35). SLC5A1 has been previously detected in human milk by transcriptomics of human MFGM (36, 37). Our identification of all soluble proteins involved in the metabolism of D-glucose instead of those for D-galactose suggests that the predominant route for UDP-galactose interconversion occurs via D-glucose.

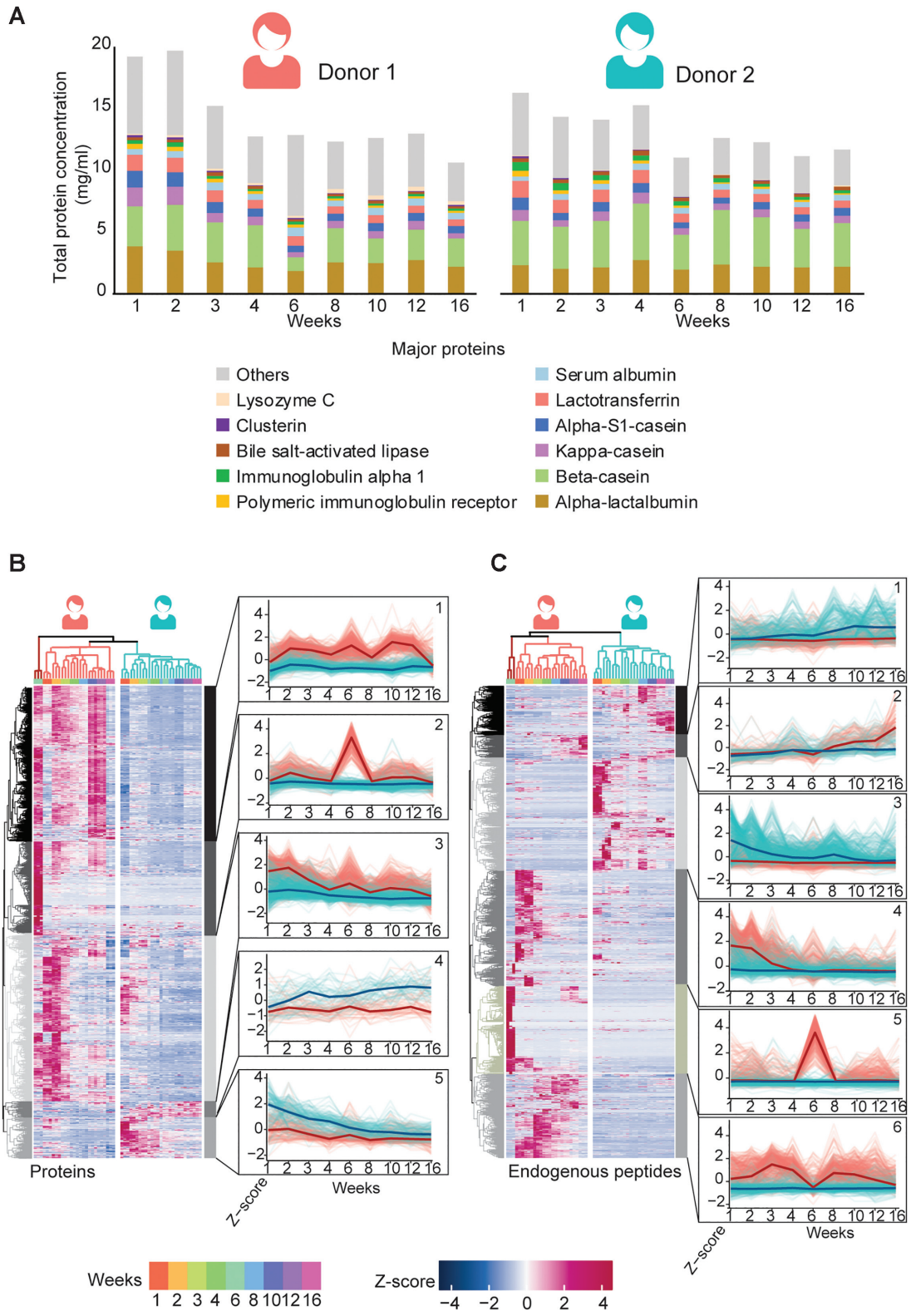

FIGURE 2 Quantitative analysis of concentrations of human milk proteins in 2 individual donors. (A) Concentrations of the most abundant human milk proteins across lactation, from weeks 1 to 16 postpartum, in 2 individual donors. The concentrations of the milk proteins are depicted in stacked bars, using a different color for each major milk protein. (B) Heatmap of the changes in concentration of all human milk proteins across the lactation period. The quantitative proteomics data was clustered in 5 groups, consisting of 435, 262, 464, 44, and 115 proteins, respectively. (C) Heatmap of the changes in concentration of endogenous peptides across the lactation period. These data were clustered in 6 groups, containing 219, 103, 496, 516, 390, and 372 peptides, respectively. The column annotations are colored by weeks. The scale bar for the *z*-score weight is displayed. The insets show *z*-scores for proteins or endogenous peptides in different clusters compared with the lactation time, the median is indicated by the bold line, and all lines are color-coded in red for donor 1 and turquoise for donor 2. The different clusters are discussed in the main text and in Figure 3, but clearly, donor 1 experienced an anomalous event around week 6, as revealed both in the proteomics and peptidomics data.

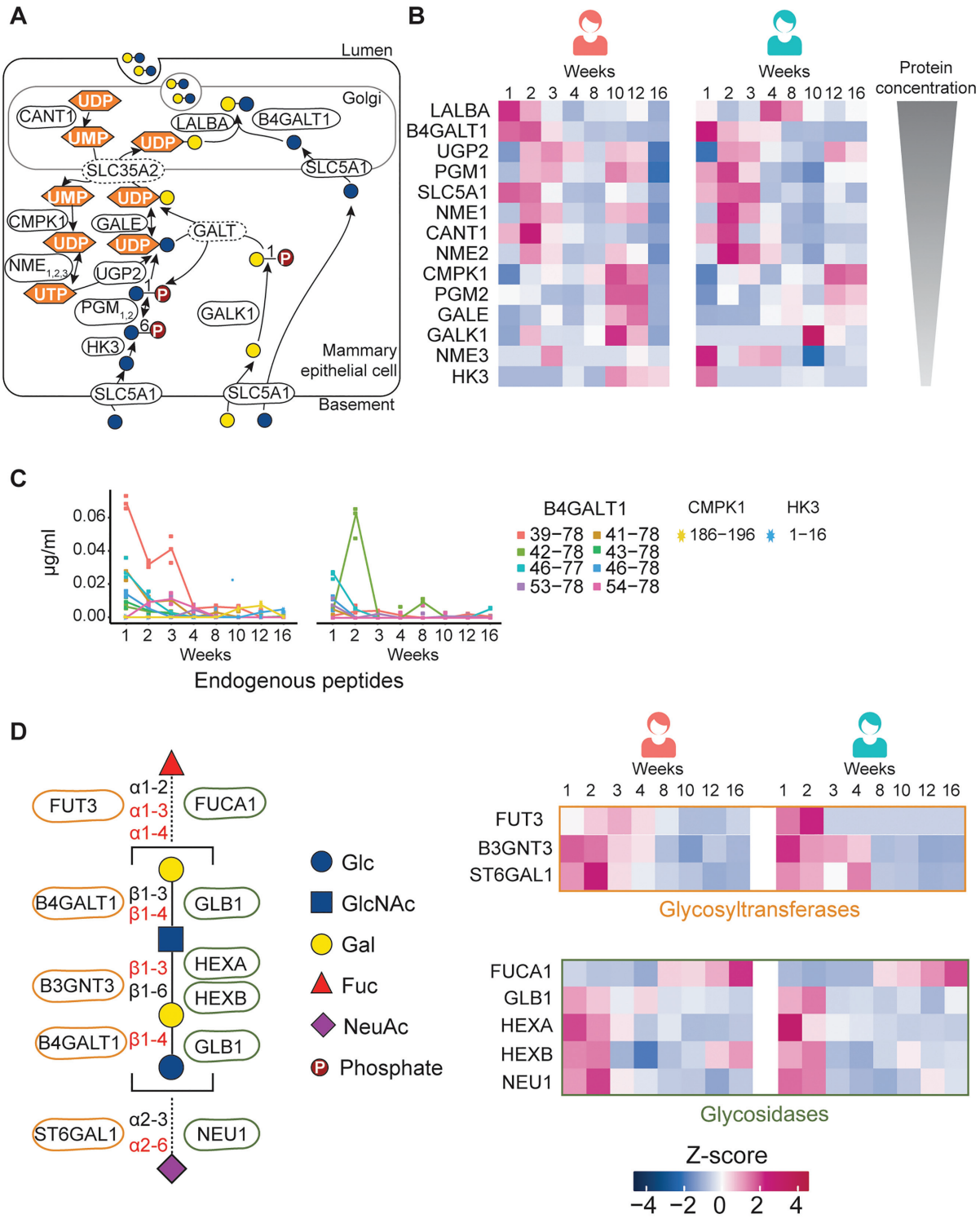

FIGURE 3 Trends in abundance over lactation of proteins involved in lactose synthesis (excluding the week 6 time point) (A) Overview of the proteins involved in lactose synthesis detected in human milk, where all but 2 proteins were identified. The identification status of the proteins are annotated: solid line = identified; dashed line = not identified. (B) Heatmaps depicting the abundance of proteins in human milk involved in lactose synthesis for donors 1 and 2, in descending protein concentration. Two trends can be observed, with the first half of proteins having the highest concentration at early lactation and declining in later lactation, and the second half of proteins having the inverse trend. (C) Declining lactational trends in both donors for selected endogenous peptides derived from B4GALT1, CMPK1, and HK3. Data points indicate the values of each technical replicate; lines are linked by the median of the data points in each week for each donor. (D) Simplified overview of the biosynthesis pathway of human milk oligosaccharides with the observed proteome data overlaid. All possible glycosidic linkages are depicted. The red color indicates the relevant linkages for the identified glycosyltransferases (outlines of the protein are brown) and glycosidases (outlines of the protein are dark green). In the middle, changes in abundance of these glycosyltransferases and glycosidases are depicted. The glycosyltransferases and glycosidases had a higher concentration in early lactation and steadily declined throughout lactation with the exception of FUCA1. The used glycan nomenclature is depicted on the right. The scale bar for *z*-score weight is also displayed. All protein names are represented from their gene name from Uniprot (17). B4GALT1, β-1,4-galactosyltransferase; FUCA1, α-L-fucosidase.

We ordered all proteins involved in lactose synthesis by descending concentration and found that the most abundant proteins in both donors belonged to lactose synthase (Figure 3B). The last step of lactose synthesis is likely limited by B4GALT1 as its co-actor LALBA is the most abundant protein in the human milk proteome, being approximatly100 times more abundant than B4GALT1. Higher concentrations of B4GALT1 were observed at early lactation in both donors (Figure 3B). In the endogenous peptidome, several peptides covering the N-terminus of the lumenal part of B4GALT1 were identified from both donors (Figure 3C). These endogenous peptides were also present at higher concentrations in early lactation. Interestingly, the trends over lactation for B4GALT1 in the proteome and peptidome data seem to complement each other, especially in the period from weeks 1 to 3 for the most abundant endogenous peptide (sequence 39–78 for donor 1 and sequence 42–78 for donor 2). The higher concentration of B4GALT1 in early lactation may suggest a higher rate of lactose synthesis driving milk production.

The proteins critical for the interconversion of UDP-Glucose from D-glucose, including phosphoglucomutase-1 (PGM1), UTP-glucose-1-phosphate uridylyltransferase (UGP2), and nucleoside diphosphate kinase A (NME1), all displayed similar trends (Figure 3B). During the 16-wk observation period, both donors exhibited 2 maxima in abundance for these proteins, 1 at ∼weeks 2–3 and 1 at ∼10 wk. These observations at the protein level are in line with previous transcriptomics studies in which the results suggested that these profiles reflect the regulatory role of these proteins in lactose synthesis (36, 37).

Besides serving as 1 of the main energy resources for infants, lactose is also the primary building block for HMOs, which can be further elongated/branched, fucosylated, and/or sialylated, facilitated by various glycosyltransferases and glycosidases (Figure 3D). We detected several of the glycosyltransferases and glycosidases involved in the biosynthesis or degradation of HMOs. All identified glycosyltransferases, notably galactoside 3(4)-L-fucosyltransferase (FUT3) for fucosylation, β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) for sialylation, N-acetylgalactosaminide β-1,3-N-acetylglucosaminyltransferase 3 (B3GNT3), and the aforementioned B4GALT1 for elongation and branching, exhibited higher concentrations in early lactation. We also detected several glycosidases involved in glycan hydrolysis, such as tissue α-L-fucosidase (FUCA1) for hydrolysis of the fucose from α1–2, α1–3, α1–4, or α1–6-linked fucosyl oligosaccharides (38), sialidase-1 (NEU1) for α2–3 or α2–6 linked sialic acid, and β-galactosidase (GLB1), β-hexosaminidase subunit α (HEXA), and β-hexosaminidase subunit β (HEXB) for hydrolysis of elongated and branched glycans. For both donors all glycosidases displayed the highest concentrations in the first 2 wk, with the exception of FUCA1 (Figure 3D). The increasing trend in concentration during lactation for FUCA1 was similar between the 2 donors and is in agreement with the literature (39). Together, the declining concentration of FUT3 and inclining concentration of FUCA1 may contribute to the reported overall declining concentration of fucosylated HMOs throughout the course of lactation (40, 41).

Unfortunately, it remains difficult to extract precise conclusions on the downstream effects solely based on these trends in concentrations, because glycan synthesis in human milk is complex and involves not only synthesis of HMOs, but also protein glycosylation and the formation of glycosphingolipids. Moreover, enzyme abundance does not necessarily correlate with enzyme activity. Still, the overall emerging picture is that the synthesis of lactose and HMOs in human milk is tightly regulated and reflected by changes in abundance of the principle enzymes involved. Encouragingly, similar trends in enzyme concentrations throughout lactation were observed for both donors (Figure 3A–D) indicating that these changes represent a generic phenomenon.

Aiding Infants' Digestive System by Human Milk

Infants are born with an immature digestion system, producing small amounts of gastric acid and exhibiting low protease activity in comparison to older children and adults (42). Human milk may serve as a compensatory factor for this digestive insufficiency. We looked for evidence supporting this hypothesis in our proteomics and peptidomics data. Indeed, higher concentrations of several proteases and lipases were observed in early lactation. One illustrative example is plasmin, which is 1 of the main proteases in the mammary gland and human milk (12). We identified the inactive form of plasmin, plasminogen (PLG) in our proteome data. Plasmin, the short activated form of PLG, is formed by losing the activation peptides (sequences 20–97). We detected endogenous peptides from PLG (sequence 20–38 and 20–39) in the peptidome spanning 1 of the 2 activation peptides, indicating activation of PLG (**Supplemental Figure 5**). The majority of active plasmin in milk is known to be associated with casein micelles (43), this is supported in our data in which the majority of the endogenous peptides were derived from caseins, especially from β-casein (CASB), and none from the equally abundant LALBA. Previous studies have shown the same peptidome trends (12, 14, 44).

Besides PLG, we could identify several protease inhibitors involved in the PLG system, α-1-antitrypsin (SERPINA1), α-2-antiplasmin (SERPINF2), and neuroserpin (SERPINI1) (Supplemental Figure 5). The overall trends in abundance over lactation for PLG and the 3 protease inhibitors were alike for both donors, following a general decline throughout lactation (Supplemental Figure 5). Interestingly, in both donors SERINA1 had a 10-fold higher concentration than any other protein detected in the PLG system. The overall similar trends of PLG and its inhibitors could be indicative of the systematic balance in human milk. Furthermore, a recent study analyzing the human milk peptidome utilizing gastric samples from preterm infants showed that milk proteases were actively breaking down proteins within the human infant's gastrointestinal tract (45). These declining trends adhere to the accepted theory that proteases and protease inhibitors are less required in human milk at later lactational stages, as the infant matures and is better able to provide self-components necessary for protein digestion.

Immunoglobulins and other immune components in milk

Human milk is known to provide essential immune components to protect the infant and help drive the development of the infant's naïve immune system. Among the critical immune components in human milk are the immunoglobulins (Igs), as they are transported from mother to infant, carrying the personal memory of pathogens faced by the individual mother and providing protection when the adaptive immune system of the infant is still maturing (46).

An issue in immunoglobulin detection and quantification by MS-based shotgun proteomics is the highly polymorphic nature of the hypervariable regions of these molecules. Therefore, we exclusively selected peptides belonging to the constant heavy chains of Igs in the proteomics data to estimate the

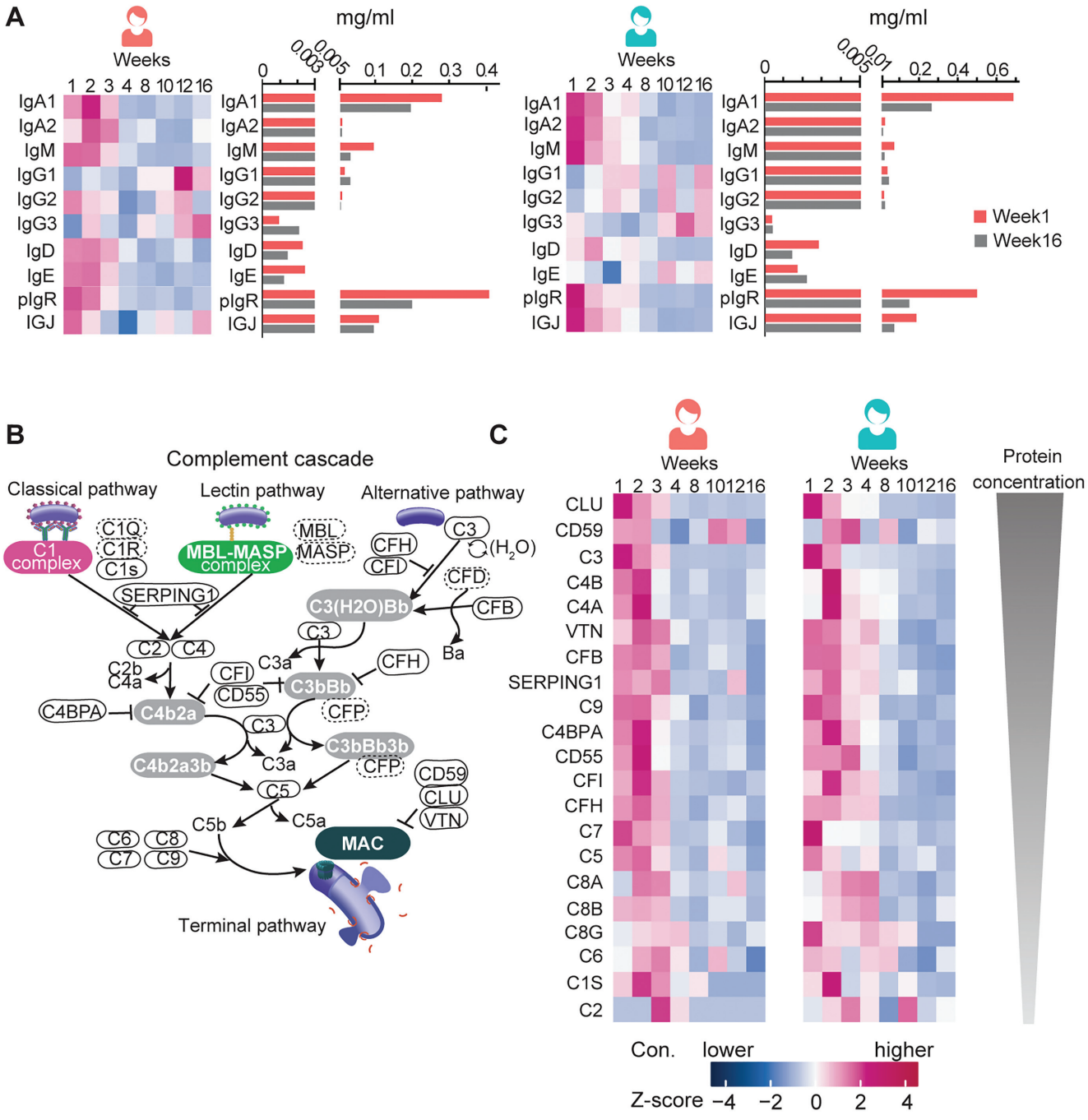

FIGURE 4 Trends in abundance of proteins involved in the immune system (excluding the week 6 time point). (A) Heatmaps depicting the abundance of immunoglobulins in human milk for donors 1 and 2. The bar charts, to the right of the heatmaps, compare the abundance of each selected immunoglobulin in week 1 compared with week 16. In both donors IgAs, IgM, IgD, IGJ, and pIgR decrease in abundance over time, whereas IgGs somewhat increase in abundance. IgE was the only Ig that had an opposite trend between the 2 donors. (B) Overview of the complement cascade with annotated proteins detected in human milk. The involved proteins are depicted in white boxes. The majority of proteins in this cascade were identified in this study, as denoted by the outlines of boxes: solid line = identified; dashed line = not identified. The convertase products formed from various precursor proteins are depicted in grey boxes. (C) Heatmaps depicting proteins from the complement cascade in human milk for donors 1 and 2. Most proteins decreased in abundance in milk as lactation progressed, having the greatest concentration during the first 3 wk. The scale bar for *z*-score weight is displayed. All protein names are represented from their gene name from Uniprot (17).

concentrations. In this manner, we were able to quantify Igs of the different IgAs, IgM, IgGs, IgD, and IgE classes (Figure 4A). IgAs and IgM in human milk are predominantly present in their secretory forms (i.e., sIgA and sIgM), in which the dimeric IgAs or pentameric IgMs form a macromolecular complex with a joining J-chain (IGJ) and a secretory component (SC) derived from pIgR (Figure 1D). Thus, we also quantified the abundance of pIgR and IGJ (Figure 4A). We found that in milk the secretory Igs (sIgA and sIgM) were by far the most dominant Igs, whereby sIgA1 was found to be the most abundant in both donors. It is well known that the main function of sIgA is in the neutralization of pathogens and

toxins without causing inflammation, since IgAs do not activate complement (47, 48). In our data, the high abundance of sIgA is reflected by the equally abundant detection of all its components: IgA1, pIgR, and IGJ. The sIgs showed a declining trend throughout lactation in both donors, with the exception of IgA2 in the first donor, which exhibited more variation (Figure 4A).

IgGs, the dominant Igs in plasma, are relatively much less abundant in human milk than IgA and IgM. Notably, their trend in abundance was opposite to that of the sIgs, being low in concentration at early lactation, and moderately increasing over time. Of all IgGs, IgG1 was found to be the most abundant. IgGs are known to be transferred from the mother to the fetus during pregnancy (49). The production of IgGs in the infant develops after birth (7), and therefore the small but substantial increase in IgGs in milk may provide a means to complement the synchronous decrease of IgG in infants after birth and during early infancy. This gradual switch from a high concentration of sIgAs and sIgM to IgG has been previously reported (7, 9). It has been hypothesized that this Ig switching was due to the functionality of milk transforming from a direct pathogen-killing state through sIgA1 to the promotion of a mother-independent immune response of the infant (7). Further importance of the Igs in human milk was exemplified in that no Igs contributed to the endogenous peptides of the peptidome, suggesting Igs are stable in human milk and are resistant to proteolytic cleavage.

Proteins involved in the complement cascade are also significant immune components in human milk. This cascade consists of 3 activation pathways (i.e., classical, lectin, and alternative) and a terminal pathway leading to the assembly of the membrane attack complex (MAC) (50, 51). As illustrated in Figure 4B, among the 3 activation pathways, proteins of the alternative pathway were found to be the most abundant in human milk. We ordered the proteins in the complement cascade by descending concentrations (Figure 4C). Although the 2 donors displayed some differences, the general trends in protein concentrations were similar. As expected, the majority of proteins involved in activating the complement system had higher concentrations at early lactation, declining at later stages. We also found that most of the proteins involved in inhibiting the complement system, such as clusterin (CLU), vitronectin (VTN), complement decay-accelerating factor (CD55), complement factor I (CFI), and complement factor H (CFH), followed similar trends in abundance as the activators. This may indicate that the complement cascade in human milk not only contributes to the host defense of the infant but also regulates the immunologic balance to minimize inflammatory pathology, especially in early lactation.

Abnormal milk composition at week 6, uniquely in donor 1

Initially, our aim was to characterize changes in the human milk proteome and peptidome in individuals during exclusive breastfeeding; however, due to the sensitive nature of personalized profiling we were able to detect abnormal changes as well. While we observed that the majority of the human milk proteome and peptidome were similar in concentration between the 2 donors throughout lactation, there were notable differences in absolute concentrations of particular proteins and endogenous peptides. By focusing our analysis on individuals, we noticed that at 1 time point (week 6) from donor 1 the milk composition was rather abnormal, as clearly visible from the heatmaps of the proteins and endogenous peptides (Figure 2B and C). The most striking abnormalities were observed in cluster 2 for the proteins and clusters 5 and 6 for the endogenous peptides (Figure 2B and C). Additionally, numerous proteins and peptides were exclusively detected at week 6 for donor 1. This aberrant behavior was quite striking, in particular when taking into consideration that both the proteome and peptidome of week 4 and week 8 samples behaved "normally." This raised the distinctive question: what happened with donor 1 around week 6?

To address this question, we first further describe how drastic these changes were. The proteome atypical cluster 2 contained no less than 262 proteins, accounting for 20% of all identified proteins (**Figure 5**A, Supplemental Dataset 3). The summed amount of these 262 proteins in cluster 2 reached ~3 mg/mL in week 6, which was at least 3 times higher than what was observed at all other time points for donor 1, but also when compared to donor 2 (Figure 5B).

In the peptidome data, we observed 2 atypical clusters in sync with the week 6 abnormality observed in the proteome, annotated as cluster 5 and 6 (Figure 2C, Supplemental Dataset 4). Cluster 5 and cluster 6 contained 390 and 372 endogenous peptides, accounting in abundance for, respectively, 19% and 18% of all quantified endogenous peptides (Figure 5C). The summed amount of these endogenous peptides in cluster 5 was ~200 μg/mL in week 6 and only 20–50 μg/mL in all other weeks, Figure 5D. An opposite trend was observed for the endogenous peptides in cluster 6, where the total peptide concentration was 32 μg/mL in week 6, dropping from 128 μg/mL in week 4 and rising again to 106 μg/mL in week 8. None of these endogenous peptides showed similar fluctuations in donor 2. Strikingly, the quantitative change of these peptides contributed in abundance to 70% of the complete endogenous peptidome at week 6 (Figure 5D).

We next attempted to classify the proteins involved in the detected abnormality using gene annotation and STRING (52) (**Supplemental Figure 6**, Supplemental Dataset 7). The majority of the proteins were found to be involved in regulation of the immune system or related to inflammatory responses, as well as proteins related to cytoskeleton, coagulation, and histones. From the gene annotation, and the relatively short occurrence of the event (exclusively at week 6) we hypothesized that donor 1 experienced a short-term inflammatory event. Several signature proteins originated from neutrophils, suggesting their involvement. The trends in abundance of several neutrophil proteins are depicted in Figure 5E; myeloperoxidase (MPO), neutrophil defensin 3 (DEFA3), neutrophil gelatinase-associated lipocalin (LCN2) (53), and 2 subunits (i.e., S100A8 and S100A9) that form calprotectin, which is well known to be secreted from neutrophils during proinflammatory responses (54). The trend in abundance of another signature protein, ficolin-1 (FCN-1), was further validated by using a targeted PRM assay (**Supplemental Figure 7**).

Abnormal changes at week 6 in donor 1 were also observed for other actors related to innate immunity, including several involved in the complement cascade, acute phase proteins, carrier proteins such as CLU and several apo-lipoproteins. For several of these proteins, confirmation of the anomalous changes observed in week 6 was achieved by targeted PRM assays (**Supplemental Figure 8**). Complement factor B (CFB) displayed an extremely deviating high concentration at week 6 (Figure 5F). CFB can break into 2 parts, Ba (sequence 26–259) and Bb (sequence 260–764), when the alternative pathway is activated (Figure 4B). For CFB we detected several endogenous peptides, all originating from the Ba fragment. The

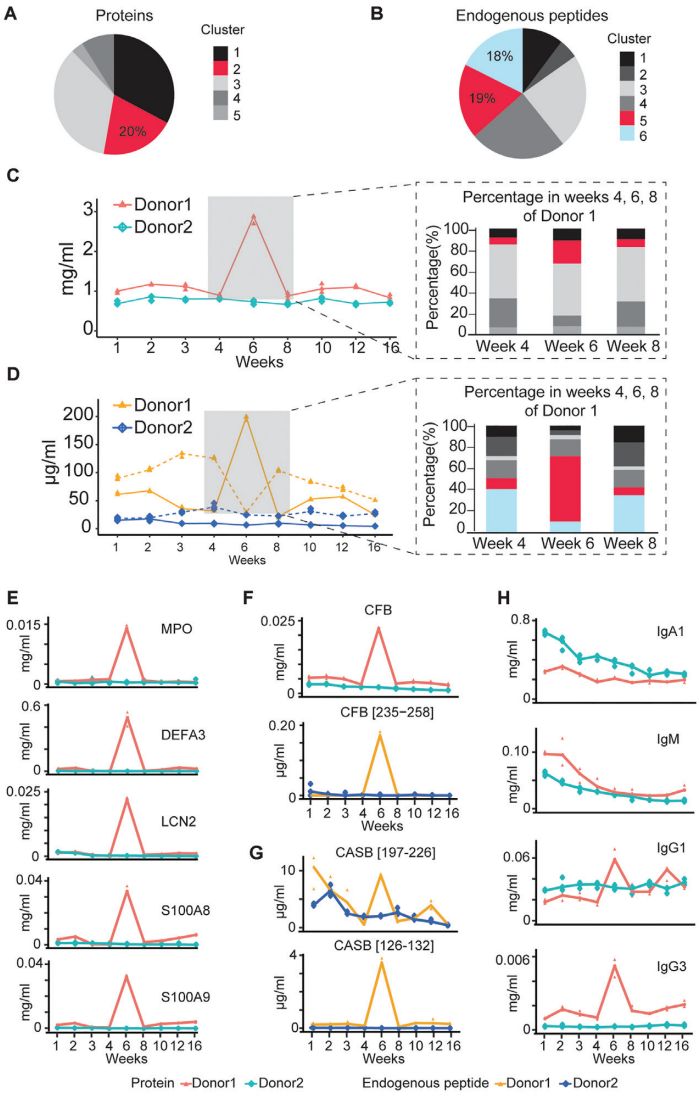

FIGURE 5 Numerous proteins and endogenous peptides display a unique signature in donor 1 around week 6. (A) Pie chart depicting the number of proteins present in each of the clusters from Figure 2B. The aberrant proteins making up cluster 2 are indicated in red and contribute to 20% of the total proteome. (B) Pie chart depicting the number of endogenous peptides present in each of the clusters from Figure 2C. The aberrant peptides making up clusters 5 and 6 are indicated in red and blue and contribute to 19% and 18% of the total peptidome, respectively. (C) Summed contribution to the milk proteome of all proteins from cluster 2 over the lactation time for donor 1 (red) and donor 2 (turquoise). Data points indicate the values of each technical replicate; lines are linked by the median of the data points in each week for each donor. On the right, the contribution of each cluster to the milk proteome is depicted for weeks 4, 6, and 8 as detected for donor 1, with the same color scheme as the pie chart. The percentage of the altered proteins in red is greater in week 6 than weeks 4 or 8, and there is a clear shift in the overall proteome as the distribution of each cluster is different. (D) Summed contribution to the milk peptidome of all endogenous peptides from clusters 5 (solid line) and 6 (dashed line) over the lactation time for donor 1 (yellow) and donor 2 (blue). Data points indicate the values of each technical replicate; lines are linked by the median of the data points in each week for each donor. On the right, the contribution of each cluster to the milk peptidome is depicted for weeks 4, 6, and 8 as detected for donor 1, with the same color scheme as the pie chart. At week 6 there is a clear increase in the percentage of peptides contributing to cluster 5 in relation to weeks 4 and 8. In addition, there is a clear decrease in the peptides contributing to cluster 6 in relation to weeks 4 and 8. All together this results in a total shift in the composition of the human milk peptidome at week 6. (E) Trends across lactation for proteins, which indicate the presence of neutrophils, in donors 1 and 2. Proteins from top to bottom: myeloperoxidase (MPO), neutrophil defensin 3 (DEFA3), neutrophil gelatinase-associated lipocalin (LCN2), protein S100-A8 (S100A8) and protein S100-A9 (S100A9), indicate a clear spike at week 6 specifically in donor 1. Data points indicate the values of each technical replicate; lines are linked by the median of the data points in each week for each donor. (F) Trends across lactation for select proteins and endogenous peptides from the complement cascade, complement factor B (CFB), in donors 1 and 2. The proteins and peptides indicated from the complement cascade show an evident increase at week 6 in donor 1 compared to all other time points. Data points indicate the values of each technical replicate; lines are linked by the median of the data points in each week for each donor. (G) Trends across lactation for endogenous peptides from beta-casein (CASB). Both peptides have antimicrobial or immune modulatory functionality, and differ in trend from their precursor protein across lactation, between donors. Data points indicate the values of each technical replicate; lines are linked by the median of the data points in each week for each donor. (H) Trends across lactation for immunoglobulins A1 (IgA1), M (IgM), G1 (IgG1), and G3 (IgG3), in donors 1 and 2 across lactation. Secretory Igs (sIgA1 and sIgM) showed declining trends across lactation whereas IgG1 and IgG3 showed increasing trends in both donors 1 and 2. The IgGs also showed an increase at week 6, which was not seen in the sIgs. The total concentration of IgGs remained lower than that of sIgs throughout lactation. Data points indicate the values of each technical replicate; lines are linked by the median of the data points in each week for each donor. All protein names are represented from their gene name from Uniprot (17).

most abundant peptide at week 6 (sequence 235–258) was at least 8 times higher in concentration than at all other time points in donor 1, and higher than in donor 2 (Figure 5F). The extreme abundance of CFB at week 6 in donor 1 hints at a critical role in activating the downstream complement cascade in donor 1 at week 6.

Several of the highly abundant milk proteins, such as caseins, lactoferrin, and polymeric Ig receptor, did not display any abnormalities at week 6 in donor 1 (Supplemental Figure 4D–H). In contrast, some endogenous peptides originating from these proteins did show sharp abnormalities. Two of the most notable examples originated from the protein CASB, including the peptide sequence 197–226 (LLNQELLLNPTHQIYPVTQPLAPVHNPISV) and the peptide sequence 126–132 (FDPQIPK) (Figure 5G). Several peptides of CASB have been linked to immune-modulating and antibacterial functions (55). The peptide CASB 197–226 is part of a sequence with reported antimicrobial activities against *Escherichia coli*, *Yersinia enterocolitica*, and *Staphylococcus aureus* (56). Also CASB 126–132 (FDPQIPK) is part of a peptide known to have an immune-modulating function (57). CASB provides thus a key example, showing that regulation of abundance at both the protein and endogenous peptide levels can be quite distinct, thus exemplifying the merit of monitoring the proteome and peptidome levels in parallel for an accurate compositional analysis of body fluids.

The most abundant milk Igs (sIgAs and sIgM) did not display any abnormalities at week 6, at either the proteome or the peptidome level. However, the IgGs, and in particular IgG3 displayed a peak in abundance at week 6, uniquely in donor 1 (Figure 5H and **Supplemental Figure 9**). IgG3 antibodies are particularly effective in the induction of effector functions and are potent proinflammatory antibodies (58). As the main portion of IgGs are transported from serum via neonatal Fc receptors (FcRn) (59), our observations may suggest the inflammation was present in the serum of the mother rather than localized in her breast, although this inflammation may also lead to a differing mammary physiology.

The observed abnormality at week 6 in the proteome and peptidome data, exclusively for donor 1, was not anticipated at the start of our study. Following up on this finding, a maternal self-report revealed that the infant had a skin rash on the cheeks, elbows, and knees at week 6, but was fortunately fully recovered within 1–2 wk. While no absolute conclusion can be made, it is interesting to note the overlap in time of these events. Moreover, the entirety of our data, and especially the anomalous data, provides an endorsement for personalized proteome and peptidome profiling.

Discussion

In the first weeks to months postpartum, the composition of human milk is vital in determining how the child develops. It is the first food source seen by the infant outside of the womb, driving the establishment of overall growth, the immune system, and the gut microbiome. It is therefore highly beneficial to develop and improve methods to monitor the composition of human milk, ideally taking samples from individual donors in a longitudinal manner, as the composition may change over the course of lactation. There is an increasing interest, both in academic research and in the clinic, to move toward personalized approaches, in diagnosis and ultimately in treatment. This paradigm shift, initially limited by lack of biomedical analytical capabilities and extreme costs, is now slowly becoming feasible.

Here, as a proof of concept, we introduce an approach aimed at personalized human milk peptidome and proteome profiling, monitoring the lactational changes over time in 2 distinct donors. Using small amounts of sample, received from consenting donors in a noninvasive manner (i.e., during normal breastfeeding), we reached a depth allowing quantitative analysis from both the proteome and the endogenous peptidome. As in serum profiling, with its abundant albumin and Igs, the dynamic range obtainable in human milk is limited largely due to a few highly abundant casein and whey proteins. Still, we were able to monitor quantitatively around 1300 milk proteins and 2000 endogenous milk peptides in the 2 donors over a 16-wk longitudinal period.

Our initial aim was to monitor how the milk composition changes over a defined lactation period, and we observed many similar changes in the 2 donors, both in the proteome and peptidome. These alike trends confirmed largely known patterns in milk maturation aimed at providing the right components, addressing the nutritional needs of the infant, and aiding the infant's digestive and immune systems. Although trends in abundances of many proteins over the lactation period were similar in the 2 donors, the absolute concentrations still varied substantially. Illustratively, our data for the 2 donors over lactation revealed that donor 1 had an IgA1/IgM ratio ranging between 3 and 8; whereas this ratio range was 11–20 in donor 2 (Figure 5H). Although both donors displayed a similar continuous decrease in abundance of IgA1 over the lactation period, the IgA1 concentration in donor 2 at week 16 was still higher than that of donor 1 at week 1. Our analysis of the human milk proteome provides evidence that the protein and endogenous peptide composition of human milk is highly personalized, justifying that proteomics profiling should best be done against the baseline of the individual, instead of against a pooled or population cohort.

One of the most intriguing and unexpected findings in our study was the striking abnormality observed in the milk composition of 1 sample acquired at 1 time point in 1 donor. Without planning to monitor this phenomenon, the longitudinal highly frequent sampling and personalized profiling enabled us to appreciate this unexpected temporal abnormality. Exclusively, in donor 1 at week 6, hundreds of proteins and endogenous peptides changed dramatically in abundance. The parallel monitoring of endogenous peptides next to the milk proteome turned out to be highly beneficial. The observed changes in the peptidome were in numbers and in amplitude even higher than in the proteome, and hinted at the activation of certain enzymes and other factors involved in an immune response that would have been otherwise missed. The time window of this anomaly was abrupt, occurring only at 1 time point, with only minor evidence leading up to it at week 4 and with little memory effect detected at week 8. Most of these abruptly altered proteins and endogenous peptides originated from serum and immune cells (notably neutrophils), hinting at a short inflammatory response. This response would then result in differing mammary physiology, specifically allowing for serum substances and cells to translocate into the MEC by direct transport via the paracellular pathway. Unfortunately, we could not establish whether this altered composition was due to an inflammatory issue in the mother or child. Other studies have reported incidences of altered milk compositions due to inflammation in human milk (60), bovine milk (61), and active infant infections (62).

Anecdotally, donor 1 sought physician care for the infant's skin rash around week 6. The donor's self-report indicated that no intervention was done and that she continued with normal breastfeeding of the infant. The skin rash resolved on its own shortly thereafter and at the same time, the "abnormality" in the milk composition was resolved. Although we have no evidence for a correlation between these events, it is at least intriguing that they simultaneously occurred in the same time window. From our work, we can also conclude that a mother with no observable clinical symptoms may experience a temporal abnormal milk composition, here likely caused by inflammatory mediators from herself or her infant. This further corroborates the importance of taking a personalized approach in human milk profiling, monitoring the health of both the mother and infant.

Our approach demonstrates that such personalized human milk profiling is now technically achievable, although still quite laborious. With advancements in automation and further improvements in the data analysis pipeline, it should become feasible to do more extensive profiling from a larger set of donors and a longer duration over lactation. Such analysis will shed more light on the molecular composition of the most important source of nutrition during the early life stages of the newborn, and especially display how this food source is dynamically changing due to interactions in the mother infant dyad.

Acknowledgments

We acknowledge the donors for their support in providing the milk samples and to Harm Post (Hecklab, Utrecht University) for help with sample collection and transportation. Additionally we like to thank Gerrit Witte (Danone Nutricia Research) for performing the AA hydrolysis, Esther Zaal (Hecklab, Utrecht University) for performing the AA MS and data analysis, Henk van den Toorn (Hecklab, Utrecht University) for helping to create our online interactive data tool, and our colleagues Vojtech Franc and Kelly E Stecker (Hecklab, Utrecht University) for proofreading the manuscript.

The authors' responsibilities were as follows—JZ, KAD, and AJRH: conceptualized the project; JZ and KAD: performed all experiments; JZ and KAD: were responsible for the data curation and statistical analysis; JZ and KAD: were responsible for visualization; BS and AJRH: provided resources; JZ, KAD, and AJRH: were responsible for the writing; MM and BS: were responsible for editing, and all authors: read and approved the final manuscript.

References

1. Casavale KO, Ahuja JKC, Wu X, Li Y, Quam J, Olson R, Pehrsson P, Allen L, Balentine D, Hanspal M, et al. NIH workshop on human milk composition: summary and visions. Am J Clin Nutr 2019;110(3):769–79.
2. Loos RJF. From nutrigenomics to personalizing diets: are we ready for precision medicine? Am J Clin Nutr 2019;109(1):1–2.
3. Anderson L. Six decades searching for meaning in the proteome. J Proteomics 2014;107:24–30. doi: 10.1016/j.jprot.2014.03.005.
4. Geyer PE, Holdt LM, Teupser D, Mann M. Revisiting biomarker discovery by plasma proteomics. Mol Syst Biol 2017;13(9):942.
5. Anderson L, Razavi M, Skates S, Anderson NG, Pearson TW. Squeezing more value from the analytes we have: personal baselines for multiple analytes in serial DBS. Bioanalysis 2016;8(15):1539–42.
6. Zhang L, de Waard M, Verheijen H, Boeren S, Hageman JA, van Hooijdonk T, Vervoort J, van Goudoever JB, Hettinga K. Changes over lactation in breast milk serum proteins involved in the maturation of immune and digestive system of the infant. J Proteomics 2016;147:40–7. doi: 10.1016/j.jprot.2016.02.005.
7. Gao X, McMahon RJ, Woo JG, Davidson BS, Morrow AL, Zhang Q. Temporal changes in milk proteomes reveal developing milk functions. J Proteome Res 2012;11(7):3897–907.
8. Zhu J, Dingess KA. The functional power of the human milk proteome. Nutrients 2019;11(8). doi: 10.3390/nu11081834.1834
9. Zhang Q, Cundiff JK, Maria SD, McMahon RJ, Woo JG, Davidson BS, Morrow AL. Quantitative analysis of the human milk whey proteome reveals developing milk and mammary-gland functions across the first year of lactation. Proteomes 2013;1(2):128–58.
10. Wada Y, Lonnerdal B. Bioactive peptides derived from human milk proteins–mechanisms of action. J Nutr Biochem 2014;25(5):503–14.
11. Wada Y, Lonnerdal B. Bioactive peptides released from in vitro digestion of human milk with or without pasteurization. Pediatr Res 2015;77(4):546–53.
12. Dallas DC, Guerrero A, Khaldi N, Castillo PA, Martin WF, Smilowitz JT, Bevins CL, Barile D, German JB, Lebrilla CB. Extensive in vivo human milk peptidomics reveals specific proteolysis yielding protective antimicrobial peptides. J Proteome Res 2013;12(5):2295–304.
13. Dallas DC, Guerrero A, Khaldi N, Borghese R, Bhandari A, Underwood MA, Lebrilla CB, German JB, Barile D. A peptidomic analysis of human milk digestion in the infant stomach reveals protein-specific degradation patterns. J Nutr 2014;144(6):815–20.
14. Dallas DC, Weinborn V, de M, Bell JM, Wang M, Parker EA, Guerrero A, Hettinga KA, Lebrilla CB, German JB, Barile D. Comprehensive peptidomic and glycomic evaluation reveals that sweet whey permeate from colostrum is a source of milk protein-derived peptides and oligosaccharides. Food Res Int 2014;63(Pt B):203–9.
15. Geraghty SR, Davidson BS, Warner BB, Sapsford AL, Ballard JL, List BA, Akers R, Morrow AL. The development of a research human milk bank. J Hum Lact 2005;21(1):59–66.
16. Zhu J, Garrigues L, Van den Toorn H, Stahl B, Heck AJR. Discovery and quantification of non-human proteins in human milk. J Proteome Res 2018. doi: 10.1021/acs.jproteome.8b00550.
17. UniProt Consortium. UniProt: a worldwide hub of protein knowledge. Nucleic Acids Res 2019;47(D1):D506–D15.
18. Ahrne E, Molzahn L, Glatter T, Schmidt A. Critical assessment of proteome-wide label-free absolute abundance estimation strategies. Proteomics 2013;13(17):2567–78.
19. Dingess KA, van den Toorn HWP, Mank M, Stahl B, Heck AJR. Toward an efficient workflow for the analysis of the human milk peptidome. Anal Bioanal Chem 2019;411(7):1351–63.
20. Lange V, Picotti P, Domon B, Aebersold R. Selected reaction monitoring for quantitative proteomics: a tutorial. Mol Syst Biol 2008;4:222. doi: 10.1038/msb.2008.61.
21. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ, Inuganti A, Griss J, Mayer G, Eisenacher M, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res 2019;47(D1):D442–D50.
22. Sharma V, Eckels J, Taylor GK, Shulman NJ, Stergachis AB, Joyner SA, Yan P, Whiteaker JR, Halusa GN, Schilling B, et al. Panorama: a targeted proteomics knowledge base. J Proteome Res 2014;13(9):4205–10.
23. Johansen FE, Braathen R, Brandtzaeg P. Role of J chain in secretory immunoglobulin formation. Scand J Immunol 2000;52(3):240–8.
24. Lonnerdal B, Erdmann P, Thakkar SK, Sauser J, Destaillats F. Longitudinal evolution of true protein, amino acids and bioactive proteins in breast milk: a developmental perspective. J Nutr Biochem 2017;41:1–11. doi: 10.1016/j.jnutbio.2016.06.001.
25. Donovan SM. Human milk proteins: composition and physiological significance. Nestle Nutr Inst Workshop Ser 2019;90:93–101. doi: 10.1159/000490298.
26. McManaman JL, Neville MC. Mammary physiology and milk secretion. Adv Drug Deliv Rev 2003;55(5):629–41.
27. Linzell JL, Peaker M. Mechanism of milk secretion. Physiol Rev 1971;51(3):564–97.
28. Wooding FB. The mechanism of secretion of the milk fat globule. J Cell Sci 1971;9(3):805–21.
29. Ollivier-Bousquet M. Transferrin and prolactin transcytosis in the lactating mammary epithelial cell. J Mammary Gland Biol Neoplasia 1998;3(3):303–13.

30. Lee H, Padhi E, Hasegawa Y, Larke J, Parenti M, Wang A, Hernell O, Lonnerdal B, Slupsky C. Compositional dynamics of the milk fat globule and its role in infant development. Front Pediatr 2018;6:313. doi: 10.3389/fped.2018.00313.
31. Verd S, Ginovart G, Calvo J, Ponce-Taylor J, Gaya A. Variation in the protein composition of human milk during extended lactation: a narrative review. Nutrients 2018;10(8). doi: 10.3390/nu10081124.1124
32. Ninkina N, Kukharsky MS, Hewitt MV, Lysikova EA, Skuratovska LN, Deykin AV, Buchman VL. Stem cells in human breast milk. Hum Cell 2019;32(3):223–30.
33. Arthur PG, Kent JC, Hartmann PE. Metabolites of lactose synthesis in milk from women during established lactation. J Pediatr Gastroenterol Nutr 1991;13(3):260–6.
34. Ramakrishnan B, Qasba PK. Crystal structure of lactose synthase reveals a large conformational change in its catalytic component, the beta1,4-galactosyltransferase-I. J Mol Biol 2001;310(1):205–18.
35. Macheda ML, Williams ED, Best JD, Wlodek ME, Rogers S. Expression and localisation of GLUT1 and GLUT12 glucose transporters in the pregnant and lactating rat mammary gland. Cell Tissue Res 2003;311(1):91–7.
36. Lemay DG, Ballard OA, Hughes MA, Morrow AL, Horseman ND, Nommsen-Rivers LA. RNA sequencing of the human milk fat layer transcriptome reveals distinct gene expression profiles at three stages of lactation. PLoS One 2013;8(7):e67531. doi: 10.1371/journal.pone.0067531.
37. Wright EM, Loo DD, Hirayama BA. Biology of human sodium glucose transporters. Physiol Rev 2011;91(2):733–94.
38. DiCioccio RA, Barlow JJ, Matta KL. Substrate specificity and other properties of alpha-L-fucosidase from human serum. J Biol Chem 1982;257(2):714–8.
39. Wiederschain GY, Newburg DS. Human milk fucosyltransferase and α-L-fucosidase activities change during the course of lactation. J Nutr Biochem 1995;6(11):582–7.
40. Samuel TM, Binia A, de Castro CA, Thakkar SK, Billeaud C, Agosti M, Al-Jashi I, Costeira MJ, Marchini G, Martinez-Costa C, et al. Impact of maternal characteristics on human milk oligosaccharide composition over the first 4 months of lactation in a cohort of healthy European mothers. Sci Rep 2019;9(1):11767. doi: 10.1038/s41598-019-48337-4.
41. Thurl S, Munzert M, Boehm G, Matthews C, Stahl B. Systematic review of the concentrations of oligosaccharides in human milk. Nutr Rev 2017;75(11):920–33.
42. Fernandez E, Perez R, Hernandez A, Tejada P, Arteta M, Ramos J. Factors and mechanisms for pharmacokinetic differences between pediatric population and adults. Pharmaceutics 2011;3(1):53–72.
43. Heegaard CW, Larsen LB, Rasmussen LK, Hojberg KE, Petersen TE, Andreasen PA. Plasminogen activation system in human milk. J Pediatr Gastroenterol Nutr 1997;25(2):159–66.
44. Dingess KA, de Waard M, Boeren S, Vervoort J, Lambers TT, van Goudoever JB, Hettinga K. Human milk peptides differentiate between the preterm and term infant and across varying lactational stages. Food Funct 2017;8(10):3769–82.
45. Beverly RL, Underwood MA, Dallas DC. Peptidomics analysis of milk protein-derived peptides released over time in the preterm infant stomach. J Proteome Res 2019;18(3):912–22.
46. Field CJ. The immunological components of human milk and their effect on immune development in infants. J Nutr 2005;135(1):1–4.
47. Cerutti A. The regulation of IgA class switching. Nat Rev Immunol 2008;8(6):421–34.
48. Macpherson AJ, Slack E. The functional interactions of commensal bacteria with intestinal secretory IgA. Curr Opin Gastroenterol 2007;23(6):673–8.
49. Murphy KP. Janeway's immunobiology. 8th ed. London (UK): Garland Science, 2012.
50. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. Cell Res 2010;20(1):34–50.
51. Ogundele M. Role and significance of the complement system in mucosal immunity: particular reference to the human breast milk complement. Immunol Cell Biol 2001;79(1):1–10.
52. Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, Doerks T, Julien P, Roth A, Simonovic M, et al. STRING 8–a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res 2009;37(Database issue):D412–6.
53. Rorvig S, Ostergaard O, Heegaard NH, Borregaard N. Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. J Leukoc Biol 2013;94(4):711–21.
54. Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. J Leukoc Biol 1993;53(2):197–204.
55. Lebrilla CBD, Guerrero A, Dallas DC, German BJ, Khaldi N. Antibacter Pept. United States: The Regents of the University of California(Oakland, CA, US), 2014.
56. Cui X, Li Y, Yang L, You L, Wang X, Shi C, Ji C, Guo X. Peptidome analysis of human milk from women delivering macrosomic fetuses reveals multiple means of protection for infants. Oncotarget 2016;7(39):63514–25.
57. Wada Y, Phinney BS, Weber D, Lönnerdal B. In vivo digestomics of milk proteins in human milk and infant formula using a suckling rat pup model. Peptides 2017;88:18–31. doi: https://doi.org/10.1016/j.peptides.2016.11.012.
58. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. Front Immunol 2014;5:520. doi: 10.3389/fimmu.2014.00520.
59. Jiang X, Hu J, Thirumalai D, Zhang X. Immunoglobulin transporting receptors are potential targets for the immunity enhancement and generation of mammary gland bioreactor. Front Immunol 2016;7:214. doi: 10.3389/fimmu.2016.00214.
60. Wockel A, Abou-Dakn M, Beggel A, Arck P. Inflammatory breast diseases during lactation: health effects on the newborn-a literature review. Mediators Inflamm 2008;2008:1. doi: 10.1155/2008/298760.
61. Zhang L, Boeren S, van Hooijdonk AC, Vervoort JM, Hettinga KA. A proteomic perspective on the changes in milk proteins due to high somatic cell count. J Dairy Sci 2015;98(8):5339–51.
62. Riskin A, Almog M, Peri R, Halasz K, Srugo I, Kessel A. Changes in immunomodulatory constituents of human milk in response to active infection in the nursing infant. Pediatr Res 2012;71(2):220–5.