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Changes over lactation in breast milk serum proteins involved in the maturation of immune and digestive system of the infant

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ABSTRACT

To objective of this study was to better understand the biological functions of breast milk proteins in relation to the growth and development of infants over the first six months of life. Breast milk samples from four individual women collected at seven time points in the first six months after delivery were analyzed by filter aided sample preparation and dimethyl labeling combined with liquid chromatography tandem mass spectrometry. A total of 247 and 200 milk serum proteins were identified and quantified, respectively. The milk serum proteome showed a high similarity (80% overlap) on the qualitative level between women and over lactation. The quantitative changes in milk serum proteins were mainly caused by three groups of proteins, enzymes, and transport and immunity proteins. Of these 21 significantly changed proteins, 30% were transport proteins, such as serum albumin and fatty acid binding protein, which are both involved in transporting nutrients to the infant. The decrease of the enzyme bile salt-activated lipase as well as the immunity proteins immunoglobulins and lactoferrin coincide with the gradual maturation of the digestive and immune system of infants. The human milk serum proteome didn't differ qualitatively but it did quantitatively, both between mothers and as lactation advanced. The changes of the breast milk serum proteome over lactation corresponded with the development of the digestive and immune system of infants.

Biological significance: Breast milk proteins provide nutrition, but also contribute to healthy development of infants. Despite the previously reported large number of identified breast milk proteins and their changes over lactation, less is known on the changes of these proteins in individual mothers. This study is the first to determine the qualitative and quantitative changes of milk proteome over lactation between individual mothers. The results indicate that the differences in the milk proteome between individual mothers are more related to the quantitative level than qualitative level. The correlation between the changes of milk proteins and the gradual maturation of the gastrointestinal tract and immune system in infants, contributes to a better understanding of the biological functions of human milk proteins for the growth and development of infants.

1. Introduction

Breast milk is the best nutrition for infants. It provides essential nutrients and bioactive and immunologic constituents that support optimal growth and development [1,2]. Infants who are exclusively breastfed for at least six months have a lower morbidity from gastrointestinal and allergic diseases, acute otitis media, and respiratory infections [2–4]. Additionally, breast milk reduces the risk of sepsis, necrotizing enterocolitis, and death, especially in very low birth weight infants [5]. Breastfed infants have a higher IQ than formula fed infants, even when corrected for all known confounders [6,7]. These short and long term advantages of breastfeeding may, among other reasons, be related to bioactive proteins in human milk, although other factors such as glycosylation, fatty acid quality and quantity may play a role as well.

Human milk proteins include caseins, milk fat globule membrane (MFGM) proteins, and milk serum proteins. The milk serum proteins are also referred as whey proteins. Milk serum proteins account for

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60% of total proteins [8] and are therefore the dominant group in human milk proteins. In addition to a high proportion of essential amino acids and coenzymes for biosynthesis of lactose [9], milk serum has many bioactive proteins [10]. These bioactive proteins play important roles in regulating the maturation of the immune and digestive system of infants [11]. These proteins have been shown to change over lactation [12–15]. For example, IgA is the dominant immunoglobulin in human milk and is much higher in colostrum compared to mature milk [12–14]. Also, the changes over lactation of α-lactalbumin (LALBA), lactoferrin (LTF), and some other low abundant proteins have previously been shown [1,14]. The understanding of the human milk proteome is still not complete [16], especially with regard to the variation of the milk proteome between individual mothers.

Although breast milk is considered to be the best nutrition for infants, it is unfortunately not always available for due to, for instance, insufficient production or maternal use of medication. The alternative source of nutrition, infant formula, should ideally have similar nutritional value and bioactive functions as breast milk for optimal growth and development of infants [3]. A comprehensive understanding of the human milk proteome may lead to better understanding of the needs of infants, which may contribute to the improvement of infant formula.

The objective of this study is therefore to investigate the changes of the milk proteome from four individual mothers over a six month lactation period by shotgun proteomic techniques.

2. Materials and methods

2.1. Sample collection

Human milk samples were collected from women who gave birth at the obstetric department in VU medical center (VUmc) in Amsterdam. All women who delivered singleton term infants (gestational age 37–42 weeks) were eligible for this study. Women with hemolysis elevated liver enzymes, low platelet syndrome, history of breast surgery, and (gestational) diabetes mellitus were excluded. The institutional medical ethical review board approved the study and written informed consent was obtained from all participants. Participants were asked to donate milk samples during six months after delivery. The samples were collected weekly in the first month, every two weeks in the second and third month, and monthly afterwards. Approximately 5–10 mL were collected in a polypropylene bottle after 1 min of pumping for every sample and stored at −18 °C immediately afterwards.

In total, 28 women were recruited between September 2013 and June 2014, of whom four mothers continued breastfeeding and thus collected milk samples for six months. The other women dropped out from the second month onwards due to several reasons, such as insufficient milk production, mastitis or work related stress. Detailed information of the four included women and their infants is shown in Table 1. Since previous studies on distinct proteins showed a difference especially in early lactation [1,17], samples were assessed weekly in the first month and every two months (at weeks 8, 16, and 24) thereafter.

Table 1
Characteristics of participants and their infants.

Mother	Maternal age	Gestation age	Parity	Delivery mode	Gender of infant	Birth weight (g)
1	30	263	3	Vaginal	Boy	3450
2	33	273	2	C-section	Boy	4064
3	39	270	2	Vaginal	Girl	3348
4	35	288	1	Vaginal	Girl	2705

2.2. Milk serum separation

The separation of milk serum was performed according to a previous study [18–20]. The samples were centrifuged at 1500 ×*g* for 10 min at 10 °C (Beckman coulter Avanti J-26 XP centrifuge, rotor JA-25.15). The milk fat was removed and the obtained supernatant was transferred to the ultracentrifuge tubes followed by ultracentrifugation at 100,000 ×*g* for 90 min at 4 °C (Beckman L-60, rotor 70 Ti). After ultracentrifugation, samples were separated into three phases. The top layer was remaining milk fat, the middle layer was milk serum (with some free soluble caseins), and the bottom layer (pellet) was casein. Milk serum was used for filter aided sample preparation as described below after the measurement of protein content by the BCA protein assay (Fisher Scientific).

2.3. Proteomic techniques

2.3.1. Filter aided sample preparation

Filter aided sample preparation (FASP) was performed as previously described [21]. Milk serum samples (20 μL), including samples of each time point and pooled samples of each included woman, were diluted in 100 mM Tris/HCl pH 8.0 + 4% SDS + 0.1 M Dithiotreitol (SDT-lysis buffer) to get a 1 μg/μL protein solution. Samples were then incubated for 10 min at 95 °C, and centrifuged at 18,407 *g* for 10 min, after cooling down to room temperature. Twenty microliter of each sample were directly added to the middle of 180 μL 0.05 M iodoacetamide/100 mM Tris/HCl pH 8.0 + 8 M urea (UT) in a low binding Eppendorf tube and incubated for 10 min while mildly shaking at room temperature. The sample was transferred to a Pall 3 K omega filter (10–20 kDa cutoff, OD003C34; Pall, Washington, NY, USA) and centrifuged at 15,871 *g* for 30 min. Three repeated centrifugations at 15,871 *g* for 30 min were carried out after adding three times 100 μL UT. After that, 110 μL 0.05 M NH_4HCO_3 in water (ABC) were added to the filter unit and the samples were centrifuged again at 15,871 *g* for 30 min. Then, the filter was transferred to a new low-binding Eppendorf tube. One hundred microliter ABC containing 0.5 μg trypsin were added followed by overnight incubation at room temperature. Finally, the sample was centrifuged at 15,871 *g* for 30 min, and 3.5 μL 10% trifluoroacetic acid (TFA) were added to the filtrate to adjust the pH value of the sample to around 2. These samples were ready for dimethyl labeling.

2.3.2. Dimethyl labeling

The dimethyl labeling was carried out by on-column dimethyl labeling according to [18]. The trypsin digested samples of pooled milk serum from each individual mother collected at the different time points were labeled with light reagent (the mix of CH_2O and cyanoborohydride), whereas trypsin digested milk serum samples of the individual mothers at each time point were labeled with heavy reagent (the mix of CD_2O and cyanoborohydride). Stage tips containing 2 mg Lichroprep C18 (25 um particles) column material (C18 + Stage tip) were made in-house. The C18 + Stage tip column was washed 2 times with 200 μL methanol. The column was conditioned with 100 μL of 1 mL/L formic acid in water (HCOOH) after which samples were loaded on the C18 + Stage tip column. The column was washed with 100 μL 1 mL/L HCOOH, and then slowly flushed with 100 μL labeling reagent (0.2% CH_2O or CD_2O and 30 mM cyanoborohydride in 50 mM phosphate buffer pH 7.5) in about 10 min. The column was washed again with 200 μL 1 mL/L HCOOH. Finally, the labeled peptides were eluted with 50 μL of 70% acetonitrile/30% 1 mL/L HCOOH from the C18 + Stage tip columns. The samples were then dried in a vacuum concentrator (Eppendorf Vacufuge®) at 45 °C for 20 to 30 min until the volume of each sample decreased to 15 μL or less. The pairs of light dimethyl label and heavy dimethyl label were then mixed up and the volume was adjusted to exactly 100 μL by adding 1 mL/L HCOOH. These samples were ready for analysis by LC–MS/MS.

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