Original Article

Comparisons of proteomic profiles of whey protein between donor human milk collected earlier than 3 months and 6 months after delivery

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Background and Objectives: Human milk has nutritional, protective, and developmental advantages for premature infants. However, proteomic information of low abundant protein of donor milk is insufficient. The purpose of this study is to analyze and compare the proteome of low abundant protein of donor milk obtained at different postpartum ages other than the colostrum. **Methods and Study Design:** Donor breast milk from 12 healthy mothers was collected 15 days, 2 months and 6 months after delivery and stored by medically approved methods. The whey milk proteomes were analyzed by mass spectrometry and classified using bioinformatics analysis. **Results:** Human milk obtained 15 days and 2 months after delivery showed more abundant expression of whey proteins related to the generation of precursor metabolites and energy, metabolism, and catalytic activity, compared with milk collected at 3 months. Immune and transport-related proteins were abundant at all time points. Proteins involved in cellular movement, immune cell trafficking, and the carbohydrate metabolism network was more abundant in whey milk collected at 15 day and 2 months using a network analysis. **Conclusions:** We report proteomic information for human donor whey protein. As significant changes were found in whey proteome collected earlier than 2 months and 6 months after delivery, selecting human donor milk earlier than 2 months might be more helpful for early postnatal recipients.

Key Words: bioinformatics, Human donor milk, postnatal age, postpartum age, proteomics

INTRODUCTION

Proteins in human milk have nutritional, protective, and developmental advantages for neonates.[1] Moreover, premature infants fed human milk have lower incidences of necrotizing enterocolitis, retinopathy of prematurity and various infectious diseases.[2,3] However, the rate of exclusively breast fed-premature infants is still relatively low.[4] This is one reason why donor milk from a human milk bank in Korea is now an option for premature infants born in Korea.[5] Donor milk, similar to mother's milk, has a protective effect to necrotizing enterocolitis, compared with premature formula.[6,7] Unfortunately, use of donor milk for premature or sick infants remains unpopular.

In Korea, only small amounts of donor milk are used for infants under intensive care.[5] A lack of detailed information of the availability and safety of donor milk concerning its nutritional and immunological composition is a major reason. As well, most donor milk is not preterm milk and the collection time varies from 1 month to as much as 12 months after delivery.[5]

Recently, a significant difference of proteome in human whey milk was reported between as early as 1 week

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and 3 months postpartum.[8] However, no proteomic information was provided later than these postpartum ages to help select a more appropriate collection time for younger recipient infants. We conducted this study to investigate the proteome of whey protein of donor milk at different postpartum ages from a human milk bank based on molecular function with bioinformatics analysis.

MATERIALS AND METHODS

Milk samples

Human donor milk was obtained from the human milk bank of Kyung Hee University Hospital between August 2011 and January 2012. This study was approved by the Ethics Review committee of the Medical Research Institute, Konkuk University Hospital (KUH-1040023). Inclusion criteria were milk donated from non-smoking healthy mothers, from mothers who were negative in the serologic tests for hepatitis B virus, syphilis and human immunodeficiency virus, and from mothers who followed the nutrition instructions for breast feeding mothers.[9] Human milk was collected from 12 mothers at 15 days, 2 months, and 6 months after delivery. The collected samples were frozen and stored in plastic containers at -20 ℃ after pasteurization. Pasteurization of donor milk was performed at the recommended temperature of 62.5℃ for a period of 30 minutes prior to its use. The milk samples were thawed at 4℃ for microbial tests and then ultracentrifuged at 4℃ (20,000 g for 30 min) so that the cream layer (lipid) at the top could be removed. To deplete the samples of casein, $CaCl_2$ was added to adjust the final calcium concentration to 0.06 M. The pH was adjusted to 4.6 and samples were incubated at room temperature for 1 hr and then ultracentrifuged at 4℃ (20,000 g for 60 min), and the supernatant was obtained. The amount of proteins in 1 ml of human whey milk was measured using the Bradford method.[10]

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) fractionation and in-gel digestion

The samples were fractionated using 12% SDS-PAGE after dilution 1:1 with Laemmli sample buffer. Gels were run using Tris/Glycine/SDS buffer and then stained using Coomassie blue. The gels were cut to separate each sample lane, followed by in-gel tryptic digestion and peptide extraction. Formic acid (1%) was added after the peptide extracts were completely dried.

Protein identification, database searches, and label-free semiquantification

The products were analyzed by nanoliquid chromatography-electrospray ionization-tandem mass spectrometry, using a Synapt nano-ultra-performance liquid chromatography/time-of-flight hybrid tandem mass spectrometer (Waters, USA) according to the manufacturer's instruction. The results of the mass spectrometry were analyzed for label-free quantitative proteomics, using ProteinLynx Global Systemversion 2.4 (Waters), which was set up to search against the human protein data base of the Universal Protein Resource Knowledge Base (UniprotKB, version 2012_06_20) assuming the digestion enzyme was trypsin, with a maximum of 1 missed cleavage allowed. Fragment *b*- and *y*-ion were used in peptide sequencing. The changes of proteome in the postpartum age groups of 15 days, 2 months, and 6 months were determined with the quantification algorithm of ProteinLynx Global System, including the peptides with an accepted identity (greater than 95% protein probability).

Bioinformatics analysis

Gene Ontology (GO) analysis of the biological process, cellular components, and molecular functions of these proteins were performed for the proteins that showed statistically significant quantitative expression changes, using protein annotation through the evolutionary relationship (PANTHER) classification system (http://www.pantherdb.org/) and Ingenuity Pathway Analysis (IPA) software (Ingenuity System Inc, USA) using human genome as a reference. A linear by linear association test and χ^2-test was performed to compare the expressed proteins of three postpartum groups with Bonferroni correction. The signal network and protein function estimation were analyzed using IPA software.

RESULTS

The proteomic analysis revealed significant differences in expressions of several proteins between whey proteins of human milk at 15 days, 2 months, and 6 months after delivery. Proteins showing statistically significant expression changes were categorized on the basis of their functions using GO analysis with the PANTHER and IPA programs. Table 1 shows the relative expression amounts of detected proteins that showed significant changes between milks collected at three postpartum ages. Among these, several functional groups, including cell-to-cell signaling and interaction (n=21), carbohydrate metabolism (n=9), lipid metabolism (n=9), molecular transport (n=16), and small molecule biochemistry (n=15) showed statistically significant expression changes from 15 days to 6 months after delivery (Table 1).

Proteins related with biologic processes

Milk obtained at 6 months contained only a few of the whey proteins in most of the categories. As shown in Table 2 and Figure 1 (A), human milk collected at 15 days and 2 months after delivery had similar expression patterns of proteins related with biological process. Comparison of whey milk proteins from milk collected at 15 days and 2 months revealed no significant differences (data not shown). However, milk at 2 months showed more abundant whey protein expression related to generation of precursor metabolites and energy (p=0.025), and metabolic process (p=0.006) compared with milk obtained at 6 months, in a linear by linear association test.

The most prominent finding was the high expression of metabolic process-associated proteins in milk obtained at 15 days and 2 months (32.8%), but a rapidly decreasing pattern in milk obtained at 6 months (13.8%). Moreover, cellular processes, transport and immune system processes related proteins showed similar patterns. On the contrary, proteins related with cell communication and developmental processes in the whey protein at 6 months had similar expression patterns with milk at 15 days, which were lower than in milk at 2 months. Only the expressions of proteins related to cell communication were

Table 1. Proteins and their functional categories that showed significant changes in whey proteome between human milks collected at different postpartum ages (15 days, 2 months, and 6 months postpartum; $p<0.05$)

Molecular and cellular functions[†]	Protein	Ratio of relative expression amount		
		15 days/15 days	2 month/15 days	6 month/15 days
Cell to cell signaling and interaction	Complement C3	1	0.47	0.18
	Monocyte differentiation antigen CD14	1	1.09	0.36
	Complement C4B	1	0.42	0
	Lactoferrin	1	0	0
	Macrophage mannose receptor 1	1	0	0.32
	Alpha 1 acid glycoprotein 1	1	0.61	0.26
	Plasma protease C1 inhibitor	1	0	0
	Osteopontin;	1	0	4.85
	Kallikrein 6	1	0	0
	Keratin type I cytoskeletal 16	0[§]	0[§]	1[§]
	Macrophage mannose receptor 1	1	0	0
	Polymeric immunoglobulin receptor	1	0.94	0.34
	Serum albumin	1	0	0
	Vitamin D binding protein	1	0	0
	Ig gamma 1 chain C region	0[§]	0[§]	1[§]
	IGK protein	1	9.12	2.01
	IGL protein	1	1.17	4.48
	Ig alpha 1 chain C region	1	0.3	0.41
	Beta 1 4 galactosyl transferase 1	1	0	0
	Keratin type II cytoskeletal 1b	0[‡]	1[‡]	0.74[‡]
	Serpin B5	1	0	0
Carbohydrate metabolism	CD14 molecule	1	1.09	0.36
	Keratin type I cytoskeletal 14	0[§]	0[§]	1§
	Proactivator polypeptide	1	0	0
	Osteopontin	1	0	4.85
	Apolipoprotein A I	1	0.41	0.53
	Xanthine dehydrogenase oxidase	1	0	0
	Bile salt dependent lipase oncofetal isoform Fragment	1	0.68	0.4
	Apolipoprotein A II	1	0.39	0
	Beta 1 4 galactosyl transferase 1	1	0	0
Lipid metabolism	Serum albumin	1	0	0
	Apolipoprotein A I	1	0.41	0.53
	Apolipoprotein A II	1	0.39	0
	CD14 molecule	1	1.09	0.36
	Lactoferrin	1	0	0
	Proactivator polypeptide	1	0	0
	Complement C3	1	0.47	0.18
	Bile salt dependent lipase oncofetal isoform Fragment	1	0.68	0.4
	Serpin peptides inhibitor	1	0.63	0.74
Molecular transport	Serum albumin	1	0	0
	Apolipoprotein A I	1	0.41	0.53
	Apolipoprotein A II	1	0.39	0
	Complement C3	1	0.47	0.18
	Beta casein	1	2.18	0.62
	Vitamin D binding protein	1	0	0
	Hemopexin	0[§]	0[§]	1[§]
	Polymeric immunoglobulin receptor	1	0.94	0.34
	Proactivator polypeptide	1	0	0
	Ras related protein Rab 11A Fragment	1	0	0
	Monocyte differentiation antigen CD14	1	1.09	0.36
	Transthyretin	1	0.7	0.76
	Lactoferrin	1	0	0
	Serpin B5	1	0	0
	Xanthine dehydrogenase oxidase	1	0	0
	Bile salt dependent lipase oncofetal isoform Fragment	1	0.68	0.4

[†]Molecular functional categorization and comparison between three groups (15 days, 2 months, and 6 months postpartum) was determined using Gene Ontology (GO) analysis using protein annotation through protein annotation through evolutionary relationship (PANTHER) classification system and Ingenuity Pathway Analysis (IPA) software (Ingenuity System Inc, USA).

[‡]These proteins were not detected in 15days. The ratios of relative expression amount are 15 days/2month, 2 month/2 month, and 6 month/2 month, respectively.

[§]These proteins were not detected in 15days and 2month. The ratios of relative expression amount are 15 days/6 month, 2 month/6 month, and 6 month/6 month, respectively.

Table 1. Proteins and their functional categories that showed significant changes in whey proteome between human milks collected at different postpartum ages (15 days, 2 months, and 6 months postpartum; $p<0.05$). (cont.)

Molecular and cellular functions[†]	Protein	Ratio of relative expression amount		
		15 days/15 days	2 month/15 days	6 month/15 days
Small molecule Biochemistry	Serum albumin	1	0	0
	Apolipoprotein A I	1	0.41	0.53
	Apolipoprotein A II	1	0.39	0
	Complement C3	1	0.47	0.18
	Monocyte differentiation antigen CD14	1	1.09	0.36
	Lactoferrin	1	0	0
	Serpin peptides inhibitor	1	0.63	0.74
	Transthyretin	1	0.7	0.76
	Proactivator polypeptide	1	0	0
	Bile salt dependent lipase oncofetal isoform Fragment	1	0.68	0.4
	Keratin type I cytoskeletal 14	0[§]	0[§]	1[§]
	Xanthine dehydrogenase oxidase	1	0	0
	Hemopexin	0[§]	0[§]	1[§]
	Osteopontin	1	0	4.85
	Keratin type II cytoskeletal 1b	0[‡]	1[‡]	0.74[‡]

[†]Molecular functional categorization and comparison between three groups (15 days, 2 months, and 6 months postpartum) was determined using Gene Ontology (GO) analysis using protein annotation through protein annotation through evolutionary relationship (PANTHER) classification system and Ingenuity Pathway Analysis (IPA) software (Ingenuity System Inc, USA).
[‡]These proteins were not detected in 15days. The ratios of relative expression amount are 15 days/2month, 2 month/2 month, and 6 month/2 month, respectively.
[§]These proteins were not detected in 15days and 2month. The ratios of relative expression amount are 15 days/6 month, 2 month/6 month, and 6 month/6 month, respectively.

Table 2. Enrichment analysis of expressed proteins categorized into biological process and molecular function using Gene Ontology analysis

Categories	Molecular and cellular function	15 days n (%)	2 months n (%)	6 months n (%)	$p^{\dagger}$
Biological process	Cell communication	4 (3.4)	10 (8.4)	9 (11.3)	0.031
	Cellular process	10 (8.4)	14 (11.8)	13 (16.3)	0.474
	Transport	14 (11.8)	13 (10.9)	12 (15.0)	0.543
	Cellular component organization	4 (3.4)	5 (4.2)	4 (5.0)	0.564
	Apoptosis	2 (1.7)	3 (2.5)	1 (1.3)	0.889
	System process	5 (4.2)	1 (0.8)	4 (5.0)	0.926
	Reproduction	1 (0.8)	1 (0.8)	0 (0)	0.494
	Response to stimulus	8 (6.7)	6 (5.0)	6 (7.5)	0.894
	Homeostatic process	1 (0.8)	0 (0)	0 (0)	0.262
	Developmental process	7 (5.9)	10 (8.4)	7 (8.8)	0.425
	Generation of precursor metabolites and energy	5 (4.2)	1 (0.8)	0 (0)	0.025
	Metabolic process	39 (32.8)	39 (32.8)	11 (13.8)	0.006
	Cell cycle	4 (3.4)	2 (1.7)	0 (0)	0.086
	Immune system process	14 (11.8)	11 (9.2)	10 (12.5)	0.947
	Cell adhesion	1 (0.8)	3 (2.5)	3 (3.8)	0.163
	Total	119 (100)	119 (100)	80 (100)	NA
Molecular function	Transporter activity	5 (7.8)	6 (9.0)	4 (11.1)	0.777
	Translation regulator activity	2 (3.1)	0 (0)	0 (0)	0.112
	Transcription regulator activity	2 (3.1)	5 (7.5)	0 (0)	0.577
	Enzyme regulator activity	6 (9.4)	4 (6.0)	4 (11.1)	0.921
	Catalytic activity	19 (29.7)	17 (22.4)	5 (13.9)	0.044
	Motor activity	1 (1.6)	0 (0)	1 (2.8)	0.824
	Receptor activity	2 (3.1)	3 (4.5)	5 (13.9)	0.083
	Structural molecule activity	6 (9.4)	6 (9.0)	6 (16.7)	0.494
	Ion channel activity	0 (0)	0 (0)	1 (2.8)	0.151
	Binding	21 (32.8)	24 (35.6)	10 (27.7)	0.420
	Total	56 (100)	67 (100)	36 (100)	NA

[†]Tested by linear by linear association test between three postpartum groups.

more abundant in milk obtained at 6 months ($p=0.031$).

Proteins related with molecular function

This category showed similar expression patterns with the biological process category. In the milk obtained at 15 days and 2 months, proteins related to catalytic activity (29.7% and 22.4%, respectively) and binding (32.8% and 35.6%, respectively) were expressed in abundance and

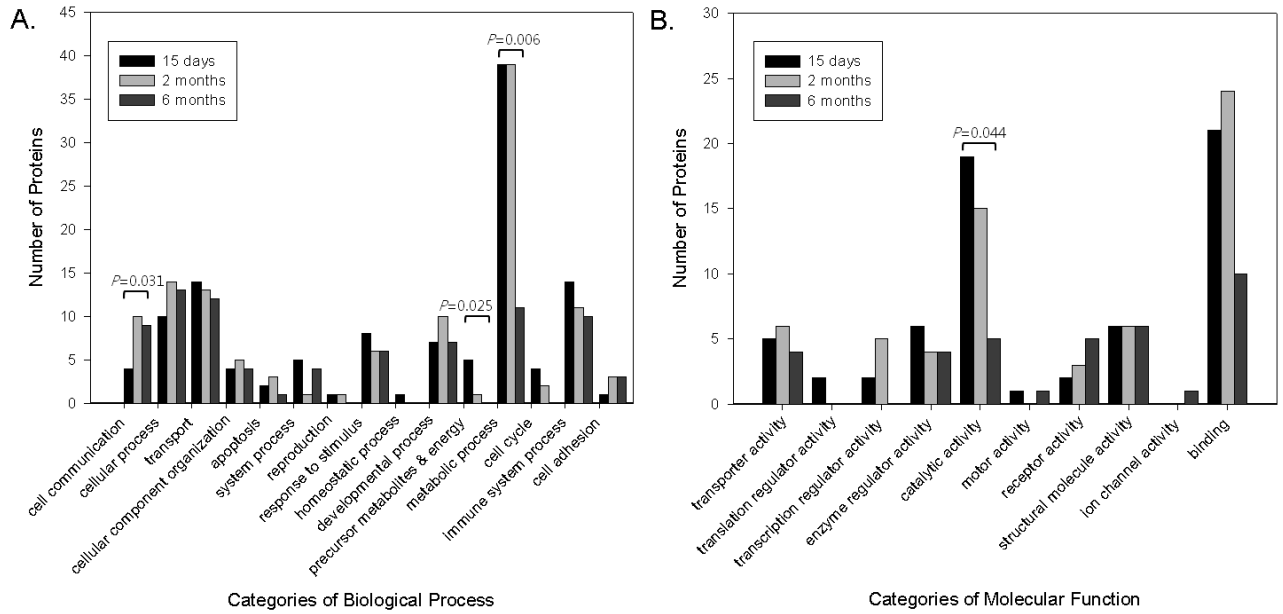

Figure 1. Classification of human milk whey proteome at 15 days, 2 months and 6 months postpartum including proteins related with biological process (A) and molecular function (B) categories.

there were no significant differences between these groups (data not shown). However, the expression of catalytic activity related proteins was decreased at 6 months (p=0.044). In addition, the numbers of binding-related proteins showed rapidly decreasing trends as well (13.9% and 27.7%).

Analysis of proteins associated with pathway network

Proteins with a significant change in whey protein of human milk obtained at 15 days, 2 months and 6 months after delivery were analyzed with IPA bioinformatics software to determine the dominant pathways in each milk group. As shown in Figure 2, cellular movement and the immune cell trafficking network was significantly associated with the proteome changes in human milk collected at 15 days and 6 months. Apolipoprotein, complement components, CD 14, low density lipoprotein, and lactotransferrin were up-regulated in milk obtained at 15 days. On the contrary, hemopexin, IgG (dsDNA-IgG immune complex), keratin, and serpin peptidase inhibitor clade A (SERPIN) were up-regulated in the human milk obtained at 6 months. However, no significant difference was found between milk obtained at 15 days and 2 months (data not shown).

DISCUSSION

In the present study, proteomic information for donor milk is reported. There were remarkable expression differences of whey proteins between the milk collected earlier than 2 months and 6 months after delivery.

Human milk obtained at 15 days and 2 months more abundantly expressed proteins related with generation of precursor metabolites and energy, metabolic processes, and catalytic activity than milk obtained at 6 months. The immune system processes and transport related proteins were highly expressed for all periods. The cellular movement, immune cell trafficking, and carbohydrate metabolism network-related protein groups were more abundant in human milk obtained at 15 days in a network analysis, compared with milk obtained at 6 months, without any significant differences between milk obtained at 15 days and 2 months. These results indicate that the whey proteome of human milk changes markedly between 2 months and 6 months, based on the analysis of this set of samples.

Significant changes of macronutrients between colostrum, transitional human milk, and mature human milk have been reported. In addition, several proteomic studies have focused on specific molecular functions, including protein phosphorylation and glycosylation.[11-14] However, longitudinal proteomic information is lacking. Recently, two proteomic studies on human milk reported a significant difference of proteome between 1 week and 3 months.[8,11] Secretory IgA, IgM, complement system including C3 and SERPINs, extracellular matrix associated proteins, and carbohydrate metabolism compared with 3 month old milk were more abundant in transitional milk (1 week), whereas IgG was more abundant in mature milk (3 months).[8,11] Even though the authors focused on milk of less than 28 days and 3 months postpartum, the reports were consistent with our results. Another recent study involved whey proteomes of 1 week and 1 month milk, but the authors focused mainly on the difference of the compositions between term and preterm milk, and did not provide enough information on the milk composition in different postpartum groups other than colostrum.[15]

To our knowledge, this is the first report on the whey proteomic profiles of term Korean donor milk studied longitudinally up to 6 months, excluding colostrum. Significant changes in whey proteome between earlier than 2 months and 6 months after delivery were found. Selecting donor milk earlier than 6 months might be more helpful for younger recipient infants who have an early postnatal age because the components are suspected to change abruptly at this time. Very recently, Zhang et al reported a seminal data set article of time-dependent changes of the

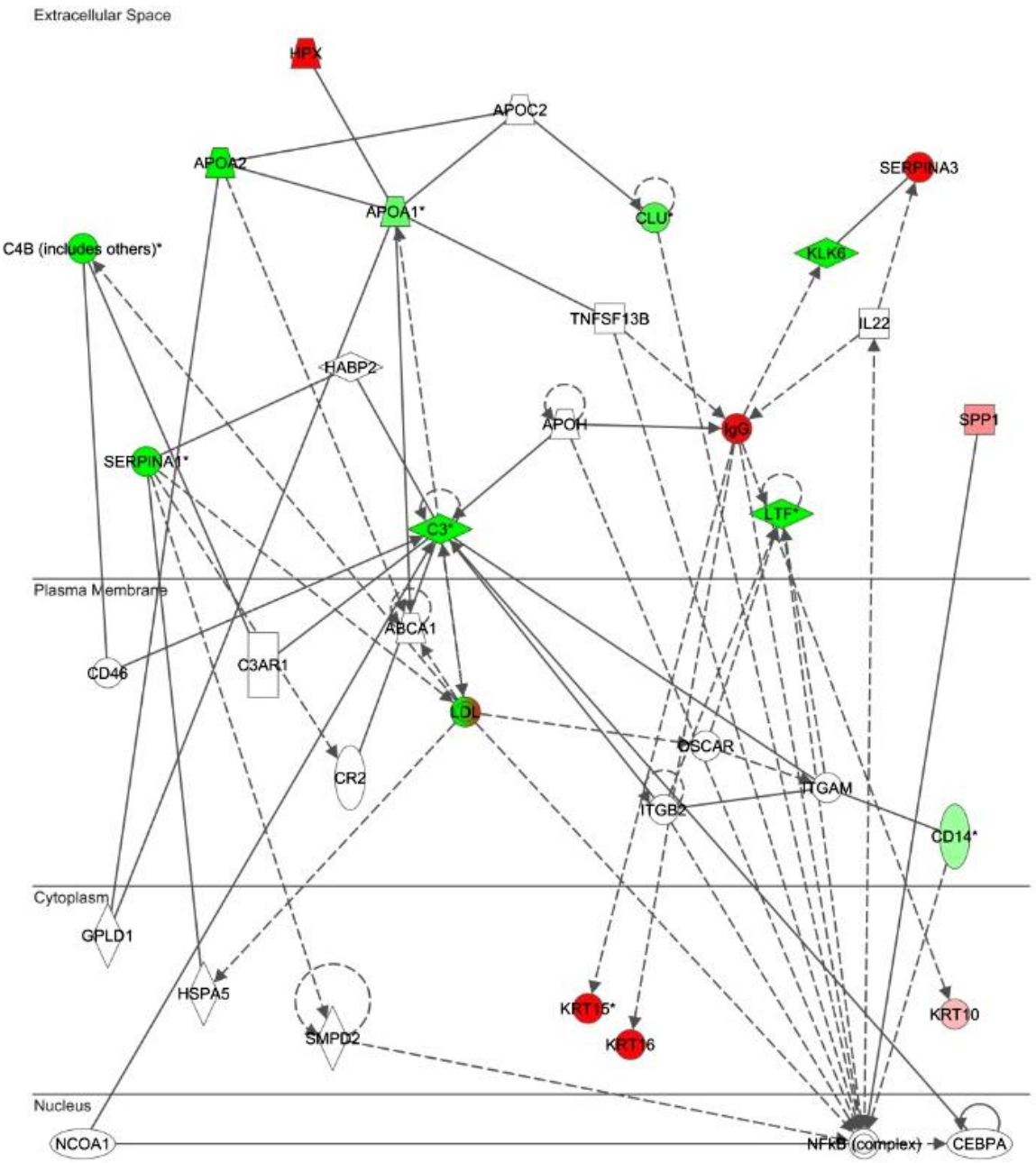

Figure 2. Analysis of cellular movement and immune cell trafficking network.[a]

whey proteome in individual women over a six month lactation period in Dutch people (upcoming issue, published online ahead of print).[16] When comparing the quantitative changes of the whey proteome between Dutch and Korean donors, similar patterns were found although, some variations were observed. This may lead us to have a better understanding about the importance of human milk proteins in the health and development of infants, which can be used in improving infant formula.

There were several limitations in this study. First, the number of samples was not sufficient to emphasize the necessity of selecting earlier donor milk for younger infants. Even though we tried to enroll healthy mothers, small numbers of samples could be a major limitation because the composition of human milk varies between individuals, especially mothers who have a disease, including gestational diabetes or mastitis.[17-21] Second, milk was collected only at 15 days, 2 months, and 6 months postpartum. To provide more reliable evidence of differences between various postpartum age groups, more milk samples need to be collected from more detailed subgroups. Thirdly, we investigated the whey proteome of human milk, not the whole milk proteome. However, abundant proteins in human milk have been well described. Thus, we intended to study less abundant proteins in the current study. Lastly, we do not know the ideal composition of human milk for premature infants. Even though the composition of milk changed abruptly between 2 and 6 months after delivery, it does not indicate that 2 months milk is always ideal milk for younger infants. Clinical trials evaluating the effect of selected early donor milk are warranted, and further studies need to be performed, including more numbers of human milk at more detailed postpartum age groups.

In summary, the whey proteomic information of donor human milk was obtained. As significant changes were found in whey proteome between earlier than 2 months and 6 months after delivery, selecting human donor milk

at earlier than 6 months might be more helpful for younger recipient infants.

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AUTHOR DISCLOSURES

The authors report no declarations of interest.

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