

## The impact of freeze-drying on the glycoproteomic profiles of human milk

Won-Ho Hahn<sup>1</sup>, Seong-Phil Bae<sup>1</sup>, Hookeun Lee<sup>2</sup>, Jong-Moon Park<sup>2</sup>, Suyeon Park<sup>3</sup>,  
Joohyun Lee<sup>4</sup>, and Nam Mi Kang<sup>5</sup>,★

<sup>1</sup>Department of Pediatrics, School of Medicine, Soon Chun Hyang University, Seoul Hospital, Seoul, Korea

<sup>2</sup>College of Pharmacy and Gachon Institute of Pharmaceutical Science, Gachon University, Incheon, Korea

<sup>3</sup>Department of Biostatistics, Soon Chun Hyang Medical Center, Seoul, Korea

<sup>4</sup>Department of Crop Science Konkuk University, Seoul, Korea

<sup>5</sup>Department of Nursing, Konkuk University, Chungju, Korea

(Received September 17, 2019; Revised May 20, 2020; Accepted May 27, 2020)

**Abstract:** Human milk (HM) glycoproteins play important roles protecting infants against various pathogens. Recently, freezing HM is reported to affect some glycoproteins and freeze-drying is suggested as an alternative method. However, the effects of freeze-drying on HM glycoproteins were not evaluated yet. Six fresh HM samples were collected from three healthy mothers at 15 and 60th days of lactation from each mother. Each sample was divided into frozen and freeze-dried subgroups yielding totally 12 samples, and the glycoproteomic analysis was performed by liquid chromatography mass spectrometry. The results were compared between samples of 15 and 60th days of lactation, and before and after the freeze-drying. Totally, 203 glycoproteins were detected. The glycoprotein levels were not different between two groups of 15/60th day of lactation and before/after freeze-drying groups ( $P > 0.050$ ). In addition, significant correlation of glycoprotein levels was found between the different lactation stages ( $r = 0.897$ ,  $P < 0.001$ ) and the status of freeze-drying ( $r = 0.887$ ,  $P < 0.001$ ) in a partial correlation analysis. As no significant change of HM glycoproteins was not found after the freeze-drying, we hope that introducing freeze-drying to HM banks is supported by the present study. This work was supported by the National Research Foundation (NRF) of Korea grant funded by the Korea government (MSIP) (No.2017R1D1A1B03034270; No.2020R1A2C1005082).

**Key words:** freeze-drying, human milk, lyophilization, mass spectrometry, glycoprotein, glycoproteomics

### 1. Introduction

The glycoproteins are principal factors that play the inhibitory roles against the various pathogens.<sup>1,2</sup> Moreover, glycoproteins and oligosaccharides released from milk glycoproteins are well known to serve as

growth substrates for probiotic bacteria including bifidobacteria.<sup>3</sup> Recently, more than 400 glycosylated proteins are identified in HM by mass spectrometry.<sup>4,6</sup> Most well-known HM glycoproteins are lactoferrin, mucins, lactadherin, lactalbumin, bile salt-stimulated lipase, secretory immunoglobulin A,  $\kappa$ -casein, lacto-

★ Corresponding author

Phone : +82-(0)2-450-3033 Fax : +82-(0)43-840-3958

E-mail : nmkang03@kku.ac.kr

This is an open access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

peroxidase, and osteopontin.

Even though HM feeding is known as a gold standard for the optimal nutrition and immunological protection, providing donated HM to sick neonates who cannot be fed their own mother's milk is recommended as the next best option.<sup>7</sup> One of the main processes at HM banks is freezing HM at -70 °C or at -80 °C for preservation safely. However, it has been documented that macronutrients are not largely affected, but some biological or immunologic factors are destroyed by the freezing.<sup>7</sup> Unfortunately, expensive equipment and large spaces for storage are required for deep freezing and it is not available in most of HM banks.<sup>8</sup> Thus, numbers of guidelines for HM banks permit storing at -20 °C if the deep freezer is not available.

However, several concerns were issued on the process. The general freezing was reported as less effective in the aspects of bactericidal and nutrients preserving performances.<sup>8-11</sup> For examples, lactoferrin levels, one of the most well-known glycoproteins in HM were found to be lowered if frozen for 3 months or more.<sup>11</sup> Even though only five days of refrigeration had minimal effects on the lactoferrin levels, 3 months of freezing at -18 to -20 °C decreased by 37 %, and 6 months of freezing at -20 °C decreased by 46 %. Thus, they even concluded that occasionally providing fresh HM to critically ill neonates having frozen milks would be necessary.<sup>11</sup>

Interestingly, the freeze-drying was issued as one of the promising storage methods requiring less resource for longer storage and transport for HM banking in the future. Storing the HM at -80 °C had similar bactericidal activity compared with freeze-drying HM. Moreover, freeze-drying was superior to storage at -20 °C with regard to microbial and bactericidal capacities.<sup>10</sup> Freeze-drying achieved suitable conservation for protecting nutritional properties and oxidative integrity of HM.<sup>12</sup> Moreover, the authors found that HM oligosaccharides were not affected by freeze-drying recently.<sup>13</sup> In addition, it was reported that the freeze-drying of HM was effective for preservation of the nutrients and immunologic factors.<sup>12,14-18</sup>

However, there has been no study on the effects of freeze-drying on the glycoproteins in HM. Thus, this study was conducted to investigate the effects of freeze-drying on the glycoproteomic profiles of HM.

## 2. Methods and Materials

### 2.1. Human Milk samples

Human donor milk was obtained from the mothers who agreed to participate in the present study in April 2016. This study was approved by the ethics review committee of the Medical Research Institute, Konkuk university hospital (KUH-7001355-201712-E-060). Inclusion criteria were milk donated from non-smoking healthy mothers who delivered their baby vaginally with normal in the serologic tests for hepatitis B virus, syphilis and human immunodeficiency virus, and from mothers who followed the nutritional instruction to breast feeding mothers.<sup>19</sup> Totally six fresh HMs were collected from three mothers at 15 and 60th days after delivery. The mean age of mothers was  $30.0 \pm 4.4$  years. Each collected sample was divided into two plastic tubes containing 20 mL of HM; (1) not freeze-dried (liquid) and (2) freeze-dried. Totally 12 samples were sent for the proteomic analysis.

The samples were frozen and stored at -80 °C before and after each treatment. The freeze-drying was performed for 24 hours with 5 ml milk samples at  $\leq -70^\circ\text{C}$  and  $\leq 1.33$  Pa, using Freeze-dryer (Freezone 4.5 Liter Benchtop Freeze dry system<sup>®</sup>, Labconco, Kansas City, MO, USA).

### 2.2. Chemicals and reagents

Concanavalin A (Con A), wheat germ agglutinin (WGA), formic acid, ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ), calcium chloride ( $\text{CaCl}_2$ ), Manganese (II) chloride ( $\text{MnCl}_2$ ), and iodoacetamide (IAA) were purchased from Sigma (St. Louis, MO, USA). Tris (2-carboxyethyl) phosphine (TECP) was obtained from Thermo Fisher Scientific (Rockford, IL, USA). PNGase F was purchased from Roche (Indianapolis, IN, USA). Sequencing-grade modified trypsin was acquired from Promega (Madison, WI, USA).

Acetonitrile (ACN), and high-performance liquid chromatography (HPLC) grade water were purchased from J.T. Baker (Phillipsburg, NJ, USA).

### 2.3. Protein assays

The total protein concentration of all the samples was measured using bicinchoninic acid (BCA) assay with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

### 2.4. Automated digestion of HM proteins

Since casein accounts for most of the HM, it needs to be removed to properly analyze the protein. Therefore, the casein was removed using the depletion prior to digesting the proteins. HM proteins (100 µg each) were each dissolved in 0.1 M Tris-HCl containing 8 M urea at pH 8.5. Prepared samples were transferred on a 96-well filter plate (PALL Corporation, Ann Arbor, MI, USA) and the plate was put on a liquid handling robotic system (Agilent Technologies, Santa Clara, CA, USA) connected to an automated platform controlled by the VWorks software. From here onward, all protein digestion processes such as denaturation, incubation, reduction, alkylation, and tryptic digestion were operated on the liquid handling robotic system and Multiscreen Vacuum Manifold™ (Millipore, Billerica, MA, USA) according to a previously optimized protocol.<sup>20</sup> HM samples were incubated with 100 µL of 5 mM TECP for 30 min at 37 °C at 900 rpm, followed by alkylation with 100 µL of 5 mM IAA for 1 h at 25 °C in the dark at 300 rpm. The proteins were digested overnight with sequencing-grade modified trypsin at an enzyme:protein ratio of 1:50 (w/w) at 37 °C. The trypsin activity was stopped by adding formic acid and the pH was decreased to 2-3. The peptides were dried using a SpeedVac (Bio-Rad, Hercules, CA, USA), and the resultant peptides were used for glycopeptide enrichment.

### 2.5. Automated enrichment of glycopeptide

Enrichment of glycopeptides was performed according to previously optimized procedures on a 96-well filter plate using the liquid handling robotic system connected to the automated platform controlled

by the VWorks software.<sup>21,22</sup> The proteolytic peptides were resuspended in 90 µL of lectin mixture (50 µg ConA, and 50 µg WGA in 90 µL of 2 × binding buffer (2 mM MnCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 1 M NaCl, and 40 mM Tris-HCl, pH 7.6)). The peptide and lectin mixtures were placed on a 96-well filter plate and incubated for 1 h at room temperature for the lectin binding reaction. The lectin captured peptides were washed with 100 µL binding buffer (1 mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.3) of four times. Subsequently, 100 µL of 40 mM NH<sub>4</sub>HCO<sub>3</sub> was added to the filter plate and vacuumed twice for 15 min each. The PNGase F solution (5 unit of PNGase F in 100 µL of 40 mM NH<sub>4</sub>HCO<sub>3</sub>) was added to the filter and incubated for 3 h at 37 °C. Finally, the glycopeptides were eluted by applying vacuum for 15 min and filter plate was washed twice with 100 µL of 40 mM NH<sub>4</sub>HCO<sub>3</sub>. The final eluted glycopeptides were desalted using Sep-Pak Vac 1 mL (50 mg) C18 cartridges, and subsequently dried on a SpeedVac.

### 2.6. Liquid chromatography mass spectrometry (LC-MS/MS) analysis

Before LC-MS/MS analysis, all dried glycopeptides were resuspended in 50 µL of 0.1 % formic acid, and 1 µg of peptide from each sample was injected onto an Acclaim PepMap™ 100 C18 nano-trap column (3 µm, 100 Å, 75 µm × 2 cm) at a flow rate of 2.5 µL/min for 5 min in 0.1 % formic acid. The peptides were separated using an PepMap™ RSLC C18 nano-column (2 µm, 100 Å, 75 µm × 50 cm) at a flow rate of 300 nL/min, and analysis was performed with a Q-Exactive orbitrap hybrid mass spectrometer along with an Easy nano-ESI-LC 1000 system (Thermo Scientific, San Jose, CA, USA). The mobile phase solvents consisted of (A) 0.1 % formic acid and (B) 0.1 % formic acid in 90 % acetonitrile. The gradient was linearly set up as below; holding 4 % of solvent (B) for 14 min, from 4 % to 40 % of solvent (B) for 106 min, from 40 % to 96 % of solvent (B) for 0.1 min, and holding at 96 % of solvent (B) for 10 min, and equilibrating the column at 4 % solvent (B) for 20 min. A data dependent acquisition method was used and the top 10 precursor peaks were selected

and isolated for fragmentation. Ions were scanned at high resolution (70,000 in MS1, 17,500 in MS2 at  $m/z$  400) and the MS scan range (400-2000  $m/z$ ) at both MS1 and MS2 levels. Precursor ions were fragmented with normalized collisional energy (NCE) 27. A dynamic exclusion of 30 s was set to minimize repeated analyses of the same precursor ions.

### 2.7. Peptide identification and protein quantification

The acquired raw files were processed by MaxQuant (version 1.5.8.3) for peptide identification and protein quantification.<sup>23,24</sup> Searches were performed for specific tryptic peptides containing up to two missed cleavage, fixed carbamidomethyl (C) modification and oxidation

(M) & carbamyl (N-term) & deamidation (NQ) set as a variable modification. Filters were set to a protein FDR of 0.01 and a PSM FDR of 0.01. MaxQuant output was visualized using Perseus (version 1.5.8.5).

### 2.8. Statistical and bioinformatics analysis

The mean % of peak intensities of each HM glycoproteins were compared between paired data sets (HMs of 15 and 60th lactation days, and pre- and post-freeze dried milks) were calculated by Wilcoxon signed rank test using SPSS<sup>®</sup> version 18.0 (SPSS Inc., Chicago, IL, USA) to find out the effects of lactation stages and freeze-drying on the protein composition. Partial correlation analysis was performed to calculate correlations between the protein compo-

Table 1. Most abundant 15 glycoproteins detected in the human milk

Accession No. <sup>a)</sup>	Description	# AAs <sup>b)</sup>	MW [kDa] <sup>c)</sup>	calc. pI <sup>d)</sup>
P05814	Beta-casein OS=Homo sapiens GN=CSN2 PE=1 SV=4 - [CASB_HUMAN]	226	25.36576	5.782715
P01876	Immunoglobulin heavy constant alpha 1 OS=Homo sapiens GN=IGHA1 PE=1 SV=2 - [IGHA1_HUMAN]	353	37.63064	6.507324
E7EQB2	Lactotransferrin (Fragment) OS=Homo sapiens GN=LTF PE=1 SV=1 - [E7EQB2_HUMAN]	696	76.57689	8.016113
P07498	Kappa-casein OS=Homo sapiens GN=CSN3 PE=1 SV=3 - [CASK_HUMAN]	182	20.2926	8.924316
P01833	Polymeric immunoglobulin receptor OS=Homo sapiens GN=PIGR PE=1 SV=4 - [PIGR_HUMAN]	764	83.23166	5.744629
P00709	Alpha-lactalbumin OS=Homo sapiens GN=LALBA PE=1 SV=1 - [LALBA_HUMAN]	142	16.21412	4.995605
P47989	Xanthine dehydrogenase/oxidase OS=Homo sapiens GN=XDH PE=1 SV=4 - [XDH_HUMAN]	1333	146.3301	7.664551
A0A0G2JMB2	Immunoglobulin heavy constant alpha 2 (Fragment) OS=Homo sapiens GN=IGHA2 PE=1 SV=1 - [A0A0G2JMB2_HUMAN]	340	36.48505	6.100098
D6RF34	Alpha-S1-casein OS=Homo sapiens GN=CSN1S1 PE=1 SV=1 - [D6RF34_HUMAN]	169	19.87681	5.554199
S4R371	Fatty acid-binding protein, heart (Fragment) OS=Homo sapiens GN=FABP3 PE=1 SV=1 - [S4R371_HUMAN]	132	14.77769	6.800293
P47710	Alpha-S1-casein OS=Homo sapiens GN=CSN1S1 PE=1 SV=1 - [CASA1_HUMAN]	185	21.65758	5.376465
P19835	Bile salt-activated lipase OS=Homo sapiens GN=CEL PE=1 SV=3 - [CEL_HUMAN]	753	79.2717	5.338379
P02768	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 - [ALBU_HUMAN]	609	69.3215	6.277832
E7ER44	Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=1 - [E7ER44_HUMAN]	708	77.91976	8.118652
P02788	Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=6 - [TRFL_HUMAN]	710	78.13192	8.118652

<sup>a)</sup>UniProt Knowledgebase name, <sup>b)</sup>number of amino acid, <sup>c)</sup>molecule weight, <sup>d)</sup>isoelectric point

sitions of HM from lactation periods, and between the samples treated with freeze-drying. Gene ontology analysis was performed for the biological process, molecular functions, and the cellular components were performed for the detected glycoproteins.

### 3. Results

**3.1. Comparisons of the human milks glycoproteins between the different lactation stage groups (15 and 60 postpartum days) and freeze-drying status (pre- and post freeze-drying)**

Totally, 203 different glycoproteins were detected in HM samples. Most abundantly detected 15 glycoproteins were presented in *Table 1*. There was no difference of glycoprotein expression patterns between two lactation periods in the Wilcoxon signed-rank test ( $P > 0.050$ , data not shown). In addition, no significant compositional difference was found between pre- and post freeze-drying groups as well ( $P > 0.050$ , data not shown).

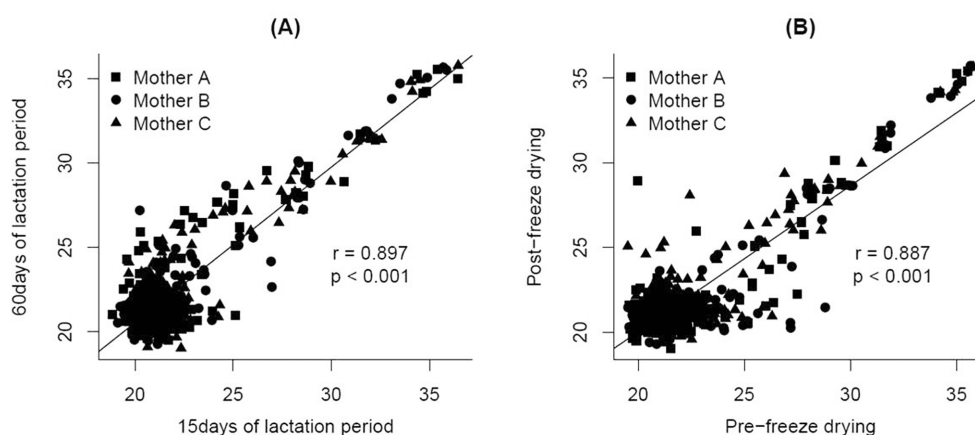
**3.2. Partial correlation analysis of the human milks glycoproteins between the different lactation stage groups (15 and 60<sup>th</sup> lactation day) and freeze-drying status (pre- and post freeze-drying)**

*Fig. 1* demonstrates partial correlation between

normalized label free quantification intensity of glycoproteins of HM samples from each mother. The amount of glycoproteins obtained at 15 and 60<sup>th</sup> lactation day from each mother showed significant correlation ( $r = 0.897$ ,  $P < 0.001$ , *Fig. 1(A)*). Moreover, the amount of glycoproteins of before and after the freeze-drying was significantly correlated with each other ( $r = 0.887$ ,  $P < 0.001$ , *Fig. 1(B)*). The correlation coefficients were high enough to mention that the difference of the lactation phases and freeze-drying status had very small effects on the amount of glycoproteins in HM.

### 3.3. Gene ontology analysis

The detected HM glycoproteins were classified by GO analysis. The enriched gene ontology term of total glycoproteins in the human milk was presented in *Table 2*, and the classification of human milk glycoproteins at 15 and 60 postnatal day (pre-freeze drying), and post-freeze drying was shown in *Fig. 2*. There were small variations, the functional groups of glycoproteins were found to have similar expression patterns between different lactation groups and the different freeze-drying status. In addition, the string analysis was performed. However, only very small numbers of glycoproteins were found to have different expression patterns and no significant pathway network was specified (*Fig. 3*).



*Fig. 1.* Partial correlation analysis of the normalized label free quantitative intensity of human milk glycoproteins between the different lactation stage groups (A), and the before and after the freeze-drying groups (B).

Table 2. Enriched gene ontology term of total glycoproteins in the human milk

#Term ID	Description	Observed gene count	FDR	Matching proteins in the network (labels)
<b>Biological process</b>				
GO:0007589	body fluid secretion	4	2.37E-05	CEL, CSN2, CSN3, XDH
GO:0046903	secretion	7	4.74E-05	ALB, CEL, CSN2, CSN3, LTF, PIGR, XDH
GO:0007595	lactation	3	0.00031	CSN2, CSN3, XDH
GO:0006810	transport	9	0.001	ALB, CEL, CSN1S1, CSN2, CSN3, FABP3, LTF, PIGR, XDH
GO:0002385	mucosal immune response	2	0.0083	LTF, PIGR
GO:2000116	regulation of cysteine-type endopeptidase activity	3	0.0122	CSN2, LTF, XDH
GO:0065008	regulation of biological quality	7	0.0241	ALB, CEL, CSN2, CSN3, FABP3, LTF, XDH
GO:0031640	killing of cells of other organism	2	0.0417	ALB, LTF
GO:0050829	defense response to Gram-negative bacterium	2	0.0417	LALBA, LTF
GO:0051817	modification of morphology or physiology of other organism involved in symbiotic interaction	2	0.0432	ALB, LTF
GO:0050830	defense response to Gram-positive bacterium	2	0.0433	LALBA, LTF
GO:2000117	negative regulation of cysteine-type endopeptidase activity	2	0.0433	CSN2, LTF
<b>Molecular function</b>				
GO:0005576	extracellular region	10	1.09E-07	ALB, CEL, CSN1S1, CSN2, CSN3, FABP3, LALBA, LTF, PIGR, XDH
GO:0005615	extracellular space	7	1.04E-05	ALB, CSN2, CSN3, FABP3, LALBA, LTF, PIGR
GO:0016528	sarcoplasm	2	0.0133	FABP3, XDH
<b>Cellular component</b>				
GO:0005504	fatty acid binding	2	0.0095	ALB, FABP3
GO:0004869	cysteine-type endopeptidase inhibitor activity	2	0.0198	CSN2, LTF

#### 4. Discussion

In the present study, the freeze-drying was found not to influence significantly the glycoproteomic profiles of HM. Thus, the freeze-drying seems to be a promising option to make not only the cost-effect benefits in addition to the better preservation of nutritional and functional components for the HM banking. If the freeze-drying is found as a safe method for the HM banking, there would be advantages in the transporting effectively besides of the better storing. Because the transportation of frozen milks

needs more difficult ways to keep the “cold chains”, the cost for the complex packing and temperature controlling would be reduced significantly.<sup>7,8</sup> In practice, storing pasteurized HM up to 6 months in general freezers and up to 12 months after freeze-drying are documented in the recommendations of Brazilian milk banks. Moreover, transporting freeze-dried milks are recommended to be performed at room temperature.<sup>15,16</sup> Moreover, no significant difference in glycoproteomic profiles was found between the lactation periods of 15 and 60 postpartum days in the present study. The HM samples of 15 and

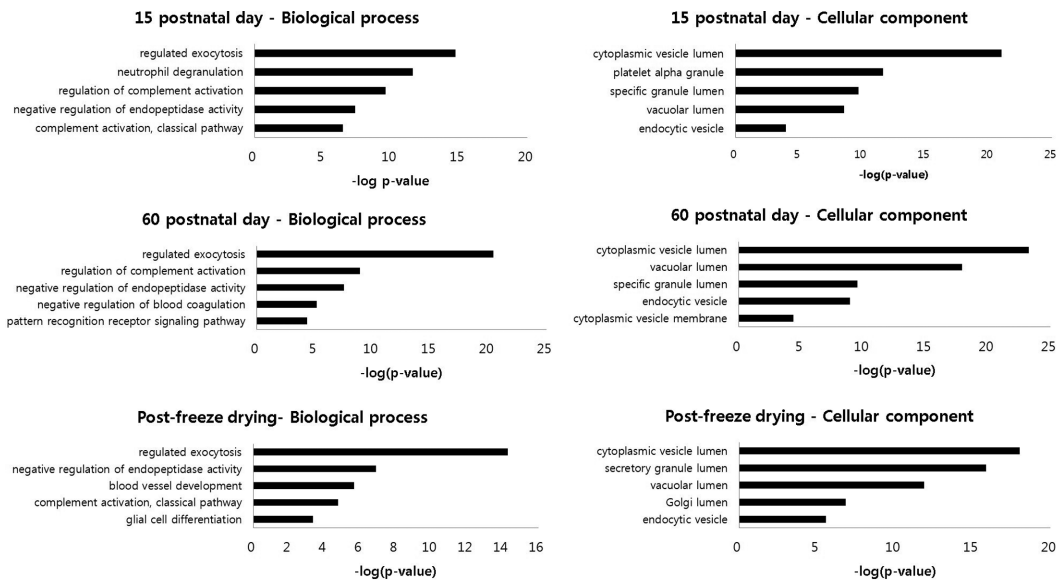


Fig. 2. Classification of human milk glycoproteins at 15 and 60 postnatal day (pre-freeze drying), and post-freeze drying.

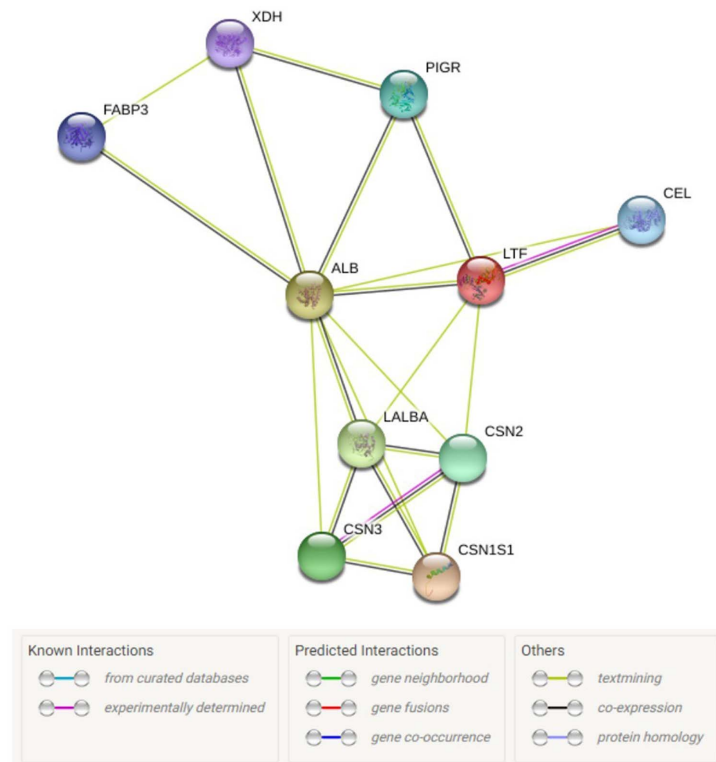


Fig. 3. Analysis of human milk glycoproteins associated with pathway network.

60 days were selected to find the obvious effects of lactation stages on the HM components because the

lactation stages is defined as transitional (> 3-15 days) and mature (>16 days) milk.<sup>25</sup> It was not an expected

result because it was reported that some of glycoproteins were different between early and later milks. Peterson et al. reported that mucin and lactadherin were higher in early milk (<15 d postpartum) than in later milk (15-90 d postpartum) but butyrophilin was not different.<sup>26</sup> However, there has been not enough reports on the glycoprotein expression levels in the different lactation stages and the numbers of enrolled subjects were too small. It needs to be tested in the further studies. However, the pattern of the HM glycoprotein expression is strongly correlated with immunologic responses implying defense mechanisms against various pathogens as demonstrated in *Table 2* and *Fig. 2*. It shows similar patterns with the previously reported proteomic profiles.<sup>6,27</sup>

There was unique aspect in the current study. In order to reduce the error of the investigator's experiments, or to reproduce a large number of samples, an automation system is essential. Thus, the investigators have developed an automated system combining the conventional filter-aided sample preparation (FASP) and filter aided capture and elution (FACE) methods.<sup>21,22</sup> In this experiment, a proteomic automated sample processing system was used to apply human breast milk samples and successfully extracted glycopeptides.

To our knowledge, this is the first report on the effects of freeze-drying on the HM glycoproteomic profiles. However, there are several limitations in this study. First, the number of samples was not sufficient to determine the minimal effects of freeze-drying on the glycoproteomic profiles. However, it was technically difficult to study with large samples numbers considering the analysis method, mass spectrometry. It would be one of the reasons why there was no difference of glycoprotein composition between the milk samples of 15 and 60 days of lactation periods. If larger number of samples, and earlier (<15 d) and later (>60 d) milks were enrolled, there might be a difference in the composition of HM, following the previous study.<sup>26</sup> Second, storage length of sample milks was not enough longer than months. Thus, studies on the preservation length of freeze-dried milks without changes of milk proteomics and/or without growth of infectious agents are

warranted in the future. Third, we could not get the N- and O- glycan data in the current study because we focused on the de-glycosylated peptide present in breast milk. However, N- and O-glycans are known to be important with multibiofunctional health benefits for neonates and are recently reported to vary during lactation.<sup>28</sup> Thus, they are worth to be studied in the further studies to establish the freeze-drying safety on the HM regarding infant's health issues.

In summary, the freeze-drying process was found not to affect the glycoproteomic profiles of HM. In addition, no significant difference between milks obtained at 15 and 60 postpartum days. As significant changes of glycoproteomic profiles were not found after freeze-drying, we hope that the freeze-drying would be supported to play an important role in HM banks in the future. However, studies with larger sample numbers are warranted and the storage length of HM after freeze-drying without changes of glycoproteomic profiles need to be evaluated in the further study.

## Acknowledgements

This work was supported by the National Research Foundation (NRF) of Korea grant funded by the Korea government (MSIP) (No.2017R1D1A1B03034270; No.2020R1A2C1005082).

## References

1. B. Liu and D. S. Newburg, *Breastfeeding. Med.*, **8**, 354-362 (2013).
2. J. A. Peterson, S. Patton and M. Hamosh, *Biol. Neonat.* **74**, 143-162 (1998).
3. S. Karav, A. Le Parc, J. M. Leite Nobrega de Moura Bell, S. A. Frese, N. Kirmiz, D. E. Block, D. Barile and D. A. Mills, *Appl. Environ. Microbiol.* **82**, 3622-3630 (2016).
4. C. E. Molinari, Y. S. Casadio, B. T. Hartmann, A. Livk, S. Bringans, P. G. Arthur and P. E. Hartmann, *J. Proteome. Res.*, **11**, 1696-1714 (2012).
5. X. Gao, R. J. McMahon, J. G. Woo, B. S. Davidson, A. L. Morrow and Q. Zhang, *J. Proteome. Res.*, **11**, 3897-



- 3907 (2012).
6. W. H. Hahn, J. H. Song, J. B. Seo, J. E. Lee, J. S. Lee, S. Song, J. Lee and N. M. Kang, *Asia. Pac. J. Clin. Nutr.*, **27**, 204-210 (2018).
  7. A. DeMarchis, K. Israel-Ballard, K. A. Mansen and C. Engmann, *J. Perinatol.*, **37**, 469-474 (2017).
  8. Italian Association of Human Milk Banks, S. Arslanoglu, E. Bertino, P. Tonetto, G. De Nisi, A. M. Ambruzzi, A. Biasini, C. Profeti, M. R. Spreghini and G. E. Moro, *J. Matern. Fetal. Neonatal. Med.*, **23**, 1-20 (2010).
  9. N. R. Garcia-Lara, D. E. Vieco, J. De la Cruz-Bertolo, D. Lora-Pablos, N. U. Velasco and C. R. Pallas-Alonso, *J. Pediatr. Gastroenterol. Nutr.*, **57**, 377-382 (2013).
  10. J. Salcedo, M. Gormaz, M. C. Lopez-Mendoza, E. Nogarotto and D. Silvestre, *J. Pediatr. Gastroenterol. Nutr.*, **60**, 527-532 (2015).
  11. D. E. Rollo, P. G. Radmacher, R. M. Turcu, S. R. Myers and D. H. Adamkin, *J. Perinatol.*, **34**, 284-286 (2014).
  12. M. V. Cortez and E. A. Soria, *Breastfeeding. Med.*, **11**, 551-554 (2016).
  13. W. H. Hahn, J. Kim, S. Song, S. Park and N. M. Kang, *J. Matern. Fetal. Neonatal. Med.*, **32**, 985-991 (2017).
  14. B. Lozano, A. I. Castellote, R. Montes and M. C. Lopez-Sabater, *Int. J. Food. Sci. Nutr.*, **65**, 703-707 (2014).
  15. D. Gutierrez and J. A. de Almeida, *J. Hum. Lact.*, **14**, 333-335 (1998).
  16. Centre for Clinical Practice at NICE. National Institute for Health and Clinical Excellence: Guidance. *Donor Breast Milk Banks: The Operation of Donor Milk Bank Services*. London: National Institute for Health and Clinical Excellence (UK); 2010.
  17. J. Castro-Albarrán, B. R. Aguilar-Uscanga, F. Calon, I. St-Amour, J. Solís-Pacheco, L. Saucier and C. Ratti, *Dry. Technol.*, **34**, 1801-1809 (2016).
  18. A. Cavazos-Garduño, J. C. Serrano-Niño, J. R. Solís-Pacheco, J. A. Gutierrez-Padilla, O. González-Reynoso, H. S. García and B. R. Aguilar-Uscanga, *J. Food Nutr. Res.*, **4**, 296-302 (2016).
  19. A. K. Leung and R. S. Sauve, *J. Natl. Med. Assoc.*, **97**, 1010-1019 (2005).
  20. J. Jeon, J. Yang, J. M. Park, N. Y. Han, Y. B. Lee and H. Lee, *J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci.*, **1092**, 88-94 (2018).
  21. A. B. Arul, J. M. Park, H. Lee, J. H. Baek, J. Jeon, E. Ji, J. W. Oh and K. P. Kim, *Curr. Proteomics.*, **13**, 55-60 (2016).
  22. A. B. Arul, J. M. Park, H. Lee, N. Y. Han and J. Jeon, *Curr. Proteomics.*, **13**, 48-54 (2016).
  23. J. Cox and M. Mann, *Nat. Biotechnol.*, **26**, 1367-1372 (2008).
  24. J. Cox, M. Y. Hein, C. A. Lubber, I. Paron, N. Nagaraj and M. Mann, *Mol. Cell. Proteomics.*, **13**, 2513-2526 (2014).
  25. J. Zhu and K. A. Dingess, *Nutrients.*, **11**, (2019).
  26. J. A. Peterson, M. Hamosh, C. D. Scallan, R. L. Ceriani, T. R. Henderson, N. R. Mehta, M. Armand and P. Hamosh, *Pediatr. Res.*, **44**, 499-506 (1998).
  27. Y. Liao, R. Alvarado, B. Phinney and B. Lonnerdal, *J. Proteome. Res.*, **10**, 1746-1754 (2011).
  28. Y. Lu, J. Liu, Y. Jia, Y. Yang, Q. Chen, L. Sun, S. Song, L. Huang and Z. Wang, *J. Agric. Food Chem.*, **67**, 10702-10712 (2019).

---

### Authors' Positions

Won-Ho Hahn : Associate Professor  
 Seong-Phil Bae : Assistant Professor  
 Hookeun Lee : Professor  
 Jong-Moon Park : Research Professor

Suyeon Park : Researcher  
 Joohyun Lee : Associate Professor  
 Nam Mi Kang : Professor