Lactation Stage-Related Expression of Sialylated and Fucosylated Glycotopes of Human Milk α -1-Acid Glycoprotein

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Abstract

Background: Because terminal sugars of α -1-acid glycoprotein (AGP) are reported to be involved in anti-inflammatory and immunomodulatory processes, their expressions might have an influence on the proper function of immune system of newborns. Here, relative amounts of sialylated and fucosylated glycotopes on human milk AGP over normal lactation were investigated.

Materials and Methods: AGP concentration and relative amounts of its sialylated and fucosylated glycovariants were analyzed in early colostrum, colostrum, and transitional and mature milk samples of 127 healthy mothers by lectin-AGP enzyme-linked immunosorbent assay using α 2,3- and α 2,6-sialic acid and α 1,2-, α 1,3-, and α 1,6-fucose specific biotinylated *Maackia amurensis*, *Sambucus nigra*, *Ulex europaeus*, *Tetragonolobus purpureus*, and *Lens culinaris* lectins, respectively.

Results: AGP concentration in human milk was about 30 times lower than in plasma of lactating mothers and decreased gradually over lactation. Milk AGP showed significantly higher expression of sialylated and fucosylated glycotopes in comparison with those of plasma AGP. Milk AGP glycovariants containing α 2,6-sialylated and α 1,6- and α 1,2-fucosylated glycotopes showed the highest relative amounts in early colostrums. With progression of lactation, the expressions of glycotopes α 1,2-fucosylated decreased starting from Day 4 and those of α 2,6-sialylated and α 1,6-fucosylated from Day 8 of lactation, whereas the level of α 2,3-sialyl-glycotope was almost constant over 45 days of lactation. In contrast, the expression of α 1,3-linked fucose on AGP was low in colostrums and significantly higher in transitional and mature milk.

Conclusions: The relative amounts of sialylated and fucosylated glycovariants of human hindmilk AGP significantly varied between Days 2 and 45 of normal lactation.

Introduction

HUMAN MILK, APART FROM NUTRIENTS, delivers bioactive molecules that elicit immunomodulatory, antibacterial, and pathogen-binding activities. Most of them are glycoproteins, revealing their biological activity through the mechanism of a sugar-lectin recognition interaction.¹⁻⁴ The changes in proteome of human milk over lactation have been determined in detail^{5,6}; however, the knowledge concerning variations in glycosylation is limited for several abundant milk glycoproteins, such as lactoferrin,^{2,7} bile salt-stimulated lipase,⁸ mannose receptor, tenascin, and xanthine dehydrogenase.² Froehlich et al.² suggested that the dynamic changes of the milk protein glycosylation, bound with periods of lactation,

adjust to meet the needs of the infant and may suggest the roles that the glycan structures could play biologically.

Human α -1-acid glycoprotein (AGP), a positive acute-phase plasma glycoprotein (molecular mass of 40–43 kDa),⁹ because of its high carbohydrate content (40–45% of its total mass), seems to be a good model to study glycosylation changes during lactation. AGP is mainly synthesized by the liver, but local production has also been reported in several other tissues, including human mammary epithelial cells,¹⁰ endothelial cells, alveolar macrophages, monocytes, and polymorphonuclear leukocytes.^{9,11} The concentration of AGP in serum of healthy humans reaches levels of 0.5–1.0 g/L and can increase up to fivefold in acute-phase reactions.¹¹ In nonpathological conditions, AGP is present in bovine

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colostrum after 12 hours ($162 \pm 63.7 \mu\text{g/mL}$) and after the third day is not directly detectable.¹²

The glycan part of human plasma AGP is very heterogeneous and consists of five *N*-linked complex-type oligosaccharides, able to form up to 15–20 glycoforms. The glycans can be bi-, tri-, or tetra-antennary and are terminated by sialic acid and fucose in varying degrees.^{9,13} Alteration of AGP glycosylation is tissue-specific and is reported to be associated with normal and pathological pregnancy,^{14–16} as well as with inflammation, cancer, liver abnormalities, and cardiovascular diseases.⁹ Fetal AGP has a different oligosaccharide pattern compared with that of an adult and carries three *N*- and three *O*-linked oligosaccharide chains and unusual lactosamine structural motifs. Amniotic AGP elicits alterations in glycan part, particularly an increase in branching of *N*-glycans as well as in the degree of fucosylation and sialylation.^{13–15}

The glycans of AGP are reported to influence its biological activities and to modulate cell-to-cell interactions and cellular signaling.^{9,17} During the inflammatory process the sialylated and fucosylated glycotopes of AGP are reported to be involved in anti-inflammatory and immunomodulatory reactions. Van Dijk et al.¹⁷ have suggested that AGP molecules carrying sialyl-Lewis^x glycotopes may interfere with leukocyte–endothelial interactions by binding to E- or P-selectin and possess anti-inflammatory properties. It was also proposed that AGP could act as a nonspecific antimicrobial agent, due to the fact that AGP can inhibit the infection of red blood cells caused by *Plasmodium falciparum*, possibly because of the large amount of sialic acid molecules exposed on the surface of the protein, making AGP a nonspecific competitor for cell surfaces. Moreover, AGP reduces the attachment and phagocytosis of *Mycoplasma pneumoniae* by human alveolar macrophages.^{9,18–20} Ceciliani et al.¹² suggested that, because of AGP immunomodulatory properties, it is conceivable that AGP may contribute to the immunoregulatory molecules expressed in bovine colostrum. Moreover, during inflammation in the bovine mammary gland, AGP has been shown to inhibit several activities of neutrophils,^{12,21} and it induces the expression of several pro- and anti-inflammatory cytokines by monocytes.²² So far, there are no reports concerning fucosylation and sialylation of human milk AGP over lactation.

In our study, we analyzed the alterations in the expression of lectin-reactive sialyl- and fucosyl-glycotopes on human milk and lactating mother's plasma AGP between Days 2 and 45 of normal lactation. The relative amounts of $\alpha 2,3$ - and $\alpha 2,6$ -linked sialic acids and $\alpha 1,2$ -, $\alpha 1,3$ -, and $\alpha 1,6$ -linked fucoses to AGP, extracted from milk and plasma samples by polyclonal anti-AGP antibodies, partly deglycosylated, and coated on enzyme-linked immunosorbent assay (ELISA) plates, were finally determined by a panel of specific lectins *Maackia amurensis* agglutinin (MAA), *Sambucus nigra* agglutinin (SNA), *Ulex europaeus* agglutinin (UEA), *Tetragonolobus purpureus* agglutinin (LTA), and *Lens culinaris* agglutinin (LCA), respectively. The lectin–AGP ELISA did not determine the “true” structure of the human AGP glycans, but allowed observation of the changes of terminal glycotope expressions reflecting their accessibility in vivo for the interaction with endogenous lectins. Such an approach allows determining the reactivity of large numbers of milk and plasma samples with respective lectins without time and cost-consuming isolation procedures.^{15,16}

Materials and Methods

Participants

Samples of milk ($n=127$) were obtained from healthy lactating women receiving regular perinatal care at the 1st and 2nd Departments of Gynecology and Obstetrics at Wrocław Medical University, Wrocław, Poland. Lactating women were recruited for a protocol approved by the Ethics Committee at Wrocław Medical University (number KB-30/2013). Informed consent was obtained from all participants. Participants ranged in age from 21 to 35 years and showed a prepregnancy body mass index between 18 and 25 kg/m². For inclusion in the study, participants had to have a good state of health and normal uncomplicated pregnancy. Women who used tobacco products, illicit drugs, or alcohol or with abnormal lactation (e.g., mastitis) or were pregnant with multiple fetuses were excluded.

Sample collection and preparation

Samples of human milk were collected by a trained nurse from the breast by manual expression at the end of nursing (hindmilk) by complete breast emptying, once per day, at the same time (8:00–10:00 a.m.). There was significant interindividual variation in volume of hindmilk, between 5 mL for colostrum to 100 mL for mature milk. All milk samples were frozen in plastic containers and stored immediately at -20°C until analysis. Skim milk (aqueous phase) was prepared by centrifugation at 3,500 *g* at 4°C for 30 minutes, after which the fat layer and cells were removed. All samples were assayed by the same researcher.

Samples of milk were divided into the following groups: (1) early colostrum (Days 2–3 of lactation; $n=19$), (2) colostrum (Days 4–7 of lactation; $n=53$), (3) transitional milk (Days 8–14 of lactation; $n=37$), and (4) mature milk (Days 15–45 of lactation; $n=18$). Additionally, plasma samples from lactating women on postpartum Day 2 ($n=14$) and healthy volunteers ($n=29$) were included.

Determination of AGP concentration

The concentration of AGP was determined according to the procedure described earlier¹⁴ using goat anti-human AGP antibodies (prepared by Prof. T. Stefaniak from the Department of Veterinary Prevention and Immunology, Wrocław Agriculture Academy, Wrocław, Poland). An AGP preparation, purchased from Sigma (St. Louis, MO), was used as a standard protein. The human serum protein calibrator (Dako, Copenhagen, Denmark) was included in the test to check the precision and accuracy of the method.

Lectin–AGP ELISA for differentiating sialyl and fucosyl glycoforms

Sialyl- and fucosyl- glycotope expression on a constant amount (100 ng) of AGP was determined by lectin–AGP ELISA according to a slightly modified procedure described earlier^{14,15} using specific biotinylated lectins (Vector Laboratories, Inc., Burlingame, CA) with well-described binding preferences.²³ MAA and SNA are known to have binding preferences to sialic acid linked by anomeric glycosidic $\alpha 2,3$ and $\alpha 2,6$ linkages, respectively. UEA, LTA, and LCA are known to have binding preferences to fucose linked by

anomeric glycosidic α 1,2, α 1,3, and α 1,6 linkages, respectively. However, LTA is known to recognize α 1,3 fucose as part of the Lewis^x determinant, but not in the sialyl-Lewis^x structure.²⁴

The experimental details of the lectin-AGP ELISA are as follows: goat anti-human AGP antibodies, oxidized with sodium periodate and desialylated with *Vibrio cholerae* neuraminidase, diluted (2,000 times) in 10 mM Tris-buffered saline containing 1 mM CaCl₂ and 1 mM MgCl₂, pH 8.5, were coupled to a polystyrene microtiter ELISA plate and used to extract AGP from the milk and plasma samples. For the test procedure, 100- μ L of human milk or plasma samples, which were prediluted in 10 mM Tris-buffered saline and 0.05% Tween 20, pH 7.4, to a final AGP concentration of 1 mg/L, were taken. The presence of the sialyl- or fucosyl-glycotopes on AGP was detected by a reaction with specific biotinylated lectins MAA, SNA, UEA, LTA, and LCA, respectively. The AGP-lectin complex was quantified with phosphatase-labeled ExtrAvidin® (Sigma) and then detected by the reaction with disodium 4-nitrophenyl phosphate (Merck, Darmstadt, Germany). The absorbance was measured in a Stat Fax 2100 microplate reader (Awareness Technology Inc., Palm City, FL) at 405 nm with the reference filter at 630 nm. All samples were analyzed in duplicate. Controls were performed to demonstrate the specificity of lectins as well as the absence of detectable endogenous reactive materials. The positive control for MAA, SNA, UEA, LTA, and LCA was a native haptoglobin and an asialo-haptoglobin preparation derived from ovarian cancer fluid.²⁵ The negative control was a human albumin preparation (Sigma) included in the test instead of milk and plasma samples. The background absorbance was low (<0.05 absorbance units [AU]) when Tris-buffered saline was included in the tests instead of (1) lectin, (2) ExtrAvidin-alkaline phosphatase (AP), and (3) milk or plasma samples.

Statistical analysis

The statistical analysis was performed with the STATISTICA version 10.0 software package (StatSoft, Inc., Tulsa, OK). For statistical significance, the Kruskal-Wallis test and the Mann-Whitney U test were used. The results were shown as the mean $\pm$ SD and the median with 25th–75th percentiles. The correlations were estimated according to Spearman. A two-tailed *p* value of <0.05 was considered significant.

Results

AGP concentration in human skim milk

The individual values of AGP concentration ranged from 6 to 51 mg/L in the human skim milk between Days 2 and 45 of lactation, showing a weak negative correlation with days of lactation ($r = -0.32$) (Fig. 1). Its concentration in the skim milk was nearly 30 times lower than in the normal plasma (Table 1). The mean and median values of AGP concentration were the highest in the early colostrum group (mean, 22.9 $\pm$ 11 mg/L; median, 21.2 [16–25.3] mg/L) and then decreased in the transitional milk group (mean, 17.9 $\pm$ 7.3 mg/L; median, 15.7 [13.6–18.7] mg/L), and the lowest value was in the mature milk group (mean, 13.6 $\pm$ 5.1 mg/L; median, 11.9 [9.2–16.2] mg/L). Moreover, the concentration of AGP in transitional milk was significantly higher than in the mature milk group (17.9 $\pm$ 7.3 vs. 13.6 $\pm$ 5.1 mg/L, $p < 0.008$) (Table 1 and Fig. 1).

The mean and median values of AGP in plasma of lactating women (611 $\pm$ 152 mg/L and 549 mg/L, respectively) did not differ significantly from the values of AGP concentration in normal plasma of nonpregnant women (550 $\pm$ 78 mg/L and 550 mg/L, respectively).

Expression of MAA- and SNA-reactive glycotopes

All samples of milk AGP reacted with MAA and SNA, indicating that the AGP glycans were potentially terminated with α 2,3- and α 2,6-linked sialic acid, respectively (Table 1). The expression of SNA-reactive glycotopes on AGP showed negative correlation ($r = -0.45$) with the duration of normal lactation, but no correlation was found with the expression of MAA-reactive glycotopes ($r = -0.1$) (Table 2 and Fig. 1).

The relative reactivity of milk AGP with SNA (specific to α 2,6-sialyl-glycoform) was nearly at the same levels in the early colostrum and colostrum groups (1.2 $\pm$ 0.2 AU and 1.2 $\pm$ 0.3 AU, respectively), but it became significantly lower in the transitional (1 $\pm$ 0.3 AU, $p < 0.003$) and mature milk (0.6 $\pm$ 0.3 AU, $p < 0.004$) groups. The relative reactivity of milk AGP with MAA (specific to α 2,3-sialyl-glycoform) for the early colostrum, colostrum, transitional, and mature milk groups was nearly at a similar level (0.4 $\pm$ 0.2 AU, 0.5 $\pm$ 0.2 AU, 0.5 $\pm$ 0.2 AU, and 0.4 $\pm$ 0.2 AU, respectively).

The relative reactivity of plasma AGP of lactating mothers with SNA (0.4 $\pm$ 0.1 AU) and MAA (0.2 $\pm$ 0.1 AU) was similar to that of the normal plasma group (0.4 $\pm$ 0.1 AU and 0.2 $\pm$ 0.1 AU, respectively); however, it was significantly lower ($p < 0.00001$) compared with the respective values of milk AGP-lectin reactivity (Table 1).

Expression of LCA-, LTA-, and UEA-reactive glycotopes

The milk AGP reactivity with fucose-specific lectins showed a weak positive correlation for LTA (specific to α 1,3-linked fucose; $r = 0.47$) and weak negative correlations for LCA (specific to α 1,6-linked fucose; $r = -0.5$) and UEA (specific to α 1,2-linked fucose; $r = -0.3$) with the day of lactation of healthy mothers (Table 2 and Fig. 1). Moreover, the milk AGP reactivity with fucose-specific lectins showed a weak positive correlation for LCA (specific to α 1,6-linked fucose; $r = 0.24$) and negative correlation for LTA (specific to α 1,3-linked fucose; $r = -0.48$) with the concentration of human skim milk AGP (Table 2).

All samples of milk AGP reacted with LCA, 81% of samples with UEA, and 67% with LTA. The high reactivity of LCA with milk AGP remained nearly at the same level during Days 2–3 and 4–7 of lactation (1.3 $\pm$ 0.4 AU and 1.4 $\pm$ 0.4 AU, respectively) and significantly decreased within 8–14 (1 $\pm$ 0.4 AU; $p < 0.0001$) and 15–45 (0.7 $\pm$ 0.3 AU; $p < 0.009$) days of lactation. The reactivity of UEA with milk AGP showed its highest value during Days 2–3 of lactation (0.8 $\pm$ 0.6 AU), during Days 4–7 days significantly decreased (0.4 $\pm$ 0.3 AU; $p < 0.03$), and remained at an almost unchanged level in the transitional and mature milk groups (0.3 $\pm$ 0.2 and 0.3 $\pm$ 0.1, respectively). In contrast, the reactivity of LTA with milk AGP was low and remained nearly at the same level in the early colostrum and colostrum groups but then significantly increased during Days 8–14 (0.3 $\pm$ 0.2 AU; $p < 0.05$) and 15–45 (0.4 $\pm$ 0.1 AU; $p < 0.009$) of lactation (Table 1).

FIG. 1. (A) The α -1-acid glycoprotein (AGP) concentration and reactivity of skim milk AGP with (B and C) sialic acid- and (D–F) fucose-specific lectins during milk maturation between Days 2 and 45 of normal lactation. The AGP concentration and reactivity of skim milk AGP with lectins were determined by methods given in Materials and Methods. Linear regression is shown with a solid line; the 95% confidence intervals are shown by dotted lines. AU, absorbance units; LCA, *L. culinaris* agglutinin; LTA, *T. purpureus* agglutinin; MAA, *M. amurensis* agglutinin; SNA, *S. nigra* agglutinin; UEA, *U. europaeus* agglutinin.

AGP of plasma samples of lactating mothers as well as normal plasma lacked reactivity with α 1,2-, α 1,3-, and α 1,6-linked fucose-specific lectins (UEA, LTA, and LCA, respectively).

Discussion

Our results show that AGP concentration in human milk of healthy donors and the degree of AGP sialylation and fucosylation declined between Days 2 and 45 of normal lactation. Moreover, milk AGP was more heavily decorated by sialic acid and fucose moieties accessible for the reaction with lectin receptors than plasma AGP of lactating mothers and healthy individuals.

Human milk AGP is about 0.1% of total milk proteins, and its concentration is about 30 times lower than in plasma of lactating mothers and healthy individuals (Table 1). The decrease of AGP and other protein concentrations in human

milk over normal lactation correlates with the total protein concentration decline.^{2,26}

The data of reactivities of specific lectins with AGP (Table 1 and Fig. 1) reflect the relative amounts of sialyl- and fucosylglycotopes accessible for reaction with lectins. However, such an approach did not determine the true amount of sugar or structure but mimics similar type of interaction observed in vivo between exposed glycotope and specific lectin-type receptor. The reactivities of human milk and plasma AGP with the panel of sialic acid-specific (MAA and SNA) and fucose-specific (UEA, LTA, and LCA) lectins, which react specifically with terminally located α 2,3- and α 2,6-linked sialic acid and α 1,2-, α 1,3-, and α 1,6-linked fucose, respectively, show that human milk AGP sialylation and fucosylation patterns are significantly different from those reported for normal human plasma AGP. The observed differences were dependent on AGP origin. The plasma AGP of lactating mothers (Table 1) and normal nonpregnant and pregnant women,^{14,15} which originated from hepatic

TABLE 1. α -1-ACID GLYCOPROTEIN (AGP) CONCENTRATION AND RELATIVE AMOUNTS OF SIALYL- AND FUCOSYL-AGP-GLYCOFORMS IN HUMAN MILK OVER LACTATION

Group (number of samples)	AGP (mg/L)	AGP reactivity (AU) with lectins specific to*				
		Sialic acid		Fucose		
		SNA (α 2,6)	MAA (α 2,3)	LCA (α 1,6)	LTA (α 1,3)	UEA (α 1,2)
Early colostrum (Days 2–3) (n = 19)						(n = 17)
Mean $\pm$ SD	22.9 $\pm$ 11	1.2 $\pm$ 0.2	0.4 $\pm$ 0.2	1.3 $\pm$ 0.4	0.15 $\pm$ 0.17	0.8 $\pm$ 0.6
Median (25 th –75 th percentile)	21.2 (16–25.3)	1.2 (1–1.5)	0.4 (0.3–0.5)	1.4 (1.1–1.6)	0.1 (0–0.2)	0.5 (0.4–1)
Colostrum (Days 4–7) (n = 53)						(n = 45)
Mean $\pm$ SD	18.0 $\pm$ 5.2	1.2 $\pm$ 0.3	0.5 $\pm$ 0.2	1.4 $\pm$ 0.4	0.2 $\pm$ 0.2	0.4 $\pm$ 0.3 ^a
Median (25 th –75 th percentile)	18.2 (14.2–21.7)	1.2 (1–1.3)	0.5 (0.3–0.7)	1.5 (1.1–1.6)	0.2 (0–0.4)	0.3 (0.2–0.5)
p value						p < 0.03
Transitional milk (Days 8–14) (n = 37)						(n = 26)
Mean $\pm$ SD	17.9 $\pm$ 7.3	1 $\pm$ 0.3 ^b	0.5 $\pm$ 0.2	1 $\pm$ 0.4 ^b	0.3 $\pm$ 0.2 ^b	0.3 (0.2–0.3)
Median (25 th –75 th percentile)	15.7 (13.6–18.7)	1 (0.8–1.1)	0.5 (0.3–0.6)	1 (0.7–1.3)	0.3 (0.2–0.4)	0.3 $\pm$ 0.2
p value		p < 0.003		p < 0.0001	p < 0.05	(n = 15)
Mature milk (Days 15–45) (n = 18)						
Mean $\pm$ SD	13.6 $\pm$ 5.1 ^c	0.6 $\pm$ 0.3 ^c	0.4 $\pm$ 0.2	0.7 $\pm$ 0.3 ^c	0.4 $\pm$ 0.1 ^c	0.3 $\pm$ 0.1
Median (25 th –75 th percentile)	11.9 (9.2–16.2)	0.5 (0.4–1)	0.4 (0.3–0.4)	0.7 (0.6–0.9)	0.4 (0.4–0.5)	0.3 (0.2–0.4)
p value	p < 0.008	p < 0.004		p < 0.009	p < 0.009	
Lactating mothers' plasma (n = 14)						
Mean $\pm$ SD	611 $\pm$ 152 ^d	0.4 $\pm$ 0.1 ^d	0.2 $\pm$ 0.1 ^d	0.0 $\pm$ 0.0 ^d	0.0 $\pm$ 0.0 ^d	0.0 $\pm$ 0.0 ^d
Median (25 th –75 th percentile)	549 (450–738)	0.4 (0.3–0.5)	0.2 (0.2–0.3)			
Normal plasma n = 29						
Mean $\pm$ SD	550 $\pm$ 78 ^d	0.4 $\pm$ 0.1 ^d	0.2 $\pm$ 0.1 ^d	0.0 $\pm$ 0.0 ^d	0.0 $\pm$ 0.0 ^d	0.0 $\pm$ 0.0 ^d
Median (25 th –75 th percentile)	550 (488–588)	0.4 (0.4–0.5)	0.2 (0.2–0.3)			

The reactivity of 100 ng of AGP with *M. amurensis* agglutinin (MAA) (specific to α 2,3-linked sialic acid), *S. nigra* agglutinin (SNA) (specific to α 2,6-linked sialic acid), *L. culinaris* agglutinin (LCA) (specific to α 1,6-linked fucose), *T. purpureus* agglutinin (LTA) (specific to α 1,3-linked fucose), and *U. europaeus* agglutinin (UEA) (specific to α 1,2-linked fucose) are expressed as the absolute value of absorbance units (AU) at 405 nm based on the lectin–AGP enzyme-linked immunosorbent assay^{14,15} as described in Materials and Methods.

*The glycosidic linkage of sialic acid and fucose is given in parentheses after the agglutinin.

The Mann–Whitney U and the Kruskal–Wallis tests were used for statistical calculations. A p value of <0.05 was considered significant. Significant differences from the other groups are indicated: ^aearly colostrum (Days 2–3), ^bcolostrum (Days 4–7), ^ctransitional milk (Days 8–14), and ^dmature milk.

TABLE 2. CORRELATION OF α -1-ACID GLYCOPROTEIN (AGP) CONCENTRATION AND RELATIVE AMOUNTS OF SIALYL- AND FUCOSYL-AGP VARIANTS IN HUMAN MILK WITH RESPECT TO MILK MATURATION

	Correlation coefficient (r value)					
	Milk maturation	AGP				
		SNA-reactive	MAA-reactive	LCA-reactive	LTA-reactive	UEA-reactive
AGP concentration	–0.32	0.29	NS	0.24	–0.48	NS
AGP SNA-reactive	–0.45	—	NS	0.65	NS	NS
AGP MAA-reactive	NS		—	NS	NS	NS
AGP LCA-reactive	–0.51			—	–0.38	0.29
AGP LTA-reactive	0.47				—	–0.22
AGP UEA-reactive	–0.30					—

The values of “r” calculated according to the Spearman method correspond to correlation among AGP concentration, relative amounts of human milk sialyl- and fucosyl-AGP glycotopes, and human milk maturation from Day 2 to Day 45 of lactation.

AGP LCA-reactive, AGP glycovariant reactive with *L. culinaris* agglutinin (LCA) specific to α 1,6-linked fucose; AGP LTA-reactive, AGP glycovariant reactive with *T. purpureus* agglutinin (LTA) specific to α 1,3-linked fucose; AGP MAA-reactive, AGP glycovariant reactive with *M. amurensis* agglutinin (MAA) specific to α 2,3-linked sialic acid; AGP SNA-reactive, AGP glycovariant reactive with *S. nigra* agglutinin (SNA) specific to α 2,6-linked sialic acid; AGP UEA-reactive, AGP glycovariant reactive with *U. europaeus* agglutinin (UEA) specific to α 1,2-linked fucose; NS, nonsignificant correlation (p $\geq$ 0.05).

synthesis, were found to be weakly α 2,3- and α 2,6-sialylated, and they did not contain at all or had negligible amounts of α 1,2-, α 1,3-, and α 1,6-linked fucoses. In contrast, the milk AGP derived mainly from local synthesis showed significantly higher expression of α 2,6- than α 2,3-linked sialic acid and was strongly α 1,2-, α 1,3-, and α 1,6-fucosylated. Nwosu et al.²⁷ reported that a high degree of fucosylation was a general feature for human milk glycoconjugates.

The relative amounts of sialyl- and fucosyl-glycotopes of human milk AGP gradually changed during lactation (Table 1 and Fig. 1); nevertheless, they were still higher than in plasma AGP. The sialylation profile (i.e., relatively stable amount of α 2,3-sialylated [MAA-reactive] AGP with simultaneous decreasing of α 2,6-sialylation [SNA-reactive]) was found to be analogous to that described by Wang and Brand-Miller,²⁸ with the decrease of α 2,6-sialylated milk oligosaccharides (HMOs) and relatively stable level of 3' sialyllactose throughout lactation. Also, previous reports by Carlson²⁹ and Landberg et al.⁸ indicated that the level of protein-bound sialic acid, as well as its total amount, in human milk was the highest at the first 2 weeks of lactation and then gradually decreased over lactation.

The α 1,2- (UEA-reactive) and α 1,6- (LCA-reactive) fucosylated glycotopes of milk AGP, which were rather absent in plasma AGP, were highly exposed during the first week of lactation, but their appearance decreased gradually in mature milk. However, the expression of UEA-reactive glycotopes in human milk AGP varied among individuals as a result of the nonsecretor status of mothers who do not express (or express in minute amounts) α 1,2-fucosylglycotopes in their milk or other body secretions.^{30,31} In our randomized study, 19% ($n = 24$) of milk samples did not react with UEA, and we assumed that they were from nonsecretors, so that those samples were excluded from the statistical analysis. The expression of α 1,3-fucosylated glycotopes had quite a different profile than those of other terminally located sugar structures: its relative amount was low in colostrums and significantly higher in transitional and mature milk (Table 1). Fucose α 1,3-linked, as a part of Lewis^x and sialo-Lewis^x antigens, is almost not detected in normal non-inflammatory tissues, but it appears during an acute-phase reaction acting as modulators of selectin-mediated cell adhesion.^{9,17} In early colostrum samples (Table 1) of healthy mothers, α 1,3-linked fucose showed low expression in most cases; however, a high SD of mean value and increasing values after the delivery within and after first week of lactation indicate individual differences in α 1,3-linked fucose expression. That fact was probably linked with pregnancy, delivery, and postpartum hormonal imbalance,³² which may lead to individual response and modulation of synthesis of Lewis^x antigen.

Although according to Froehlich et al.² there is no common pattern for the alterations of particular milk glycoproteins during lactation, the sialylation and fucosylation changes are more or less similar to those of third-trimester amniotic AGP,^{14,15} other milk glycoproteins,^{7,8} and abundantly present HMOs.^{4,30,31} It seems likely that the same set of glycosyltransferases is involved in elongation and branching of glycans of glycoconjugates and HMOs in lactating mammary glands.^{3,4,33} Additionally, hormones, especially at the hormone target organs, such as the mammary gland, can affect the local synthesis³⁴ and glycosylation of glycoproteins. Lactation, being a unique neuroendocrine state, is associated with an

increase in prolactin³⁵ and a decrease in estrogen secretion,³ which are known to regulate expression of different glycosyltransferases involved in O- and N-linked glycan synthesis.³²

Gao et al.⁶ suggested that significant changes between transitional and mature milk reflect a consequence of a transformation in defense mechanisms that occur from newborns to young infants. Thus, human milk might be considered a continuation of amniotic fluid, in view of its protecting role connected with mucosal immune system, because human milk serves as an exogenous source of immunological factors that protect the infant directly and modulate the infant's own immune system.³⁶ The immune system of the newborn is influenced by the maternal immunity transferred via the human milk, which contains a wealth of components, among them glycoconjugates, that provide specific, as well as a nonspecific, defenses against infectious agents^{37,38} and are considered a part of the innate immune system.^{26,39,40} In particular, the fucosylated and sialylated glycotopes, being engaged in many biological recognition processes and signal transduction events based on selectin-mediated cell-cell interactions, are able to modulate reactions in the immune system, manifesting its anti-inflammatory properties.^{9,17,41}

Conclusions

The relative amounts of sialylated and fucosylated AGP glycovariants of human skim milk, similar to HMOs, significantly varied between Days 2 and 45 of normal lactation. However, the results are preliminary; detailed structural and functional studies of human milk AGP glycans will hopefully shed additional light on the possible role of AGP glycovariants.

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Disclosure Statement

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References

1. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. *Annu Rev Nutr* 2005;25:37–58.
2. Froehlich JW, Dodds ED, Barboza M, et al. Glycoprotein expression in human milk during lactation. *J Agric Food Chem* 2010;58:6440–6448.
3. Kobata A. Structures and application of oligosaccharides in human milk. *Proc Jpn Acad Ser B Phys Biol Sci* 2010; 86:731–747.
4. Bode L. Human milk oligosaccharides: Every baby needs a sugar mama. *Glycobiology* 2012;22:1147–1162.
5. D'Alessandro A, Scaloni A, Zolla L. Human milk proteins: An interactomics and updated functional overview. *J Proteome Res* 2010;9:3339–3373.
6. Gao X, McMahon RJ, Woo JG, et al. Temporal changes in milk proteomes reveal developing milk functions. *J Proteome Res* 2012;11:3897–3907.
7. Barboza M, Pinzon J, Wickramasinghe S, et al. Glycosylation of human milk lactoferrin exhibits dynamic changes

- during early lactation enhancing its role in pathogenic bacteria-host interactions. *Mol Cell Proteomics* 2012;11: M111.015248.
8. Landberg E, Huang Y, Strömquist M, et al. Changes in glycosylation of human bile-salt-stimulated lipase during lactation. *Arch Biochem Biophys* 2000;377:246–254.
  9. Ceciliani F, Pocacqua V. The acute phase protein α 1-acid glycoprotein: A model for altered glycosylation during diseases. *Curr Prot Pept Sci* 2007;8:91–108.
  10. Gendler SJ, Dermer GB, Silverman LM, et al. Synthesis of alpha 1-antichymotrypsin and alpha 1-acid glycoprotein by human breast epithelial cells. *Cancer Res* 1982;42:4567–1573.
  11. Fournier T, Medjoubi NN, Porquet D. Alpha 1-acid glycoprotein. *Biochim Biophys Acta* 2000;1482:157–171.
  12. Ceciliani F, Pocacqua V, Provasi E, et al. Identification of the bovine α 1-acid glycoprotein in colostrum and milk. *Vet Res* 2005;36:735–746.
  13. Shiyan SD, Bovin NV. Carbohydrate composition and immunomodulatory activity of different glycoforms of α 1-acid glycoprotein. *Glycoconj J* 1997;14:631–638.
  14. Orczyk-Pawilowicz M, Hirnle L, Kątnik-Prastowska I. Alterations of N-glycan branching and expression of sialic acid on amniotic fluid alpha-1-acid glycoprotein derived from second and third trimesters of normal and prolonged pregnancies. *Clin Chim Acta* 2006;367:86–92.
  15. Orczyk-Pawilowicz M, Hirnle L, Kątnik-Prastowska I. The expression of fucose isoforms of amniotic and plasma alpha-1-acid glycoprotein derived from 2nd and 3rd trimester normal pregnancies. *Clin Biochem* 2009;42:1517–1523.
  16. Orczyk-Pawilowicz M, Hirnle L, Kątnik-Prastowska I. High expression of α 1,2- and α 1,6-linked fucoses on amniotic AGP as a biomarker of fetal postmaturity risk. *J Immunoassay Immunochem* 2011;32:103–113.
  17. Van Dijk W, Brinkman-Van der Linden ECM, Havenaar EC. Glycosylation of alpha₁-acid glycoprotein (orosomucoid) in health and disease: Occurrence, regulation and possible functional implications. *Trends Glycosci Glycotechnol* 1998;10:235–245.
  18. Maeda H, Nishi K, Mori I. Facilitating effects of alpha-1 acid glycoprotein on the passage of erythrocytes through the membrane-filter. *Life Sci* 1980;27:157–161.
  19. Athamna A, Kramer MR, Kahane I. Adherence of *Mycoplasma pneumoniae* to human alveolar macrophages. *FEMS Immunol Med Microbiol* 1996;15:135–141.
  20. Orczyk-Pawilowicz M, Kątnik-Prastowska I. Terminal monosaccharide expression on amniotic glycoproteins as biomarkers of fetus maturity. *Biochem Soc Trans* 2011;39: 344–348.
  21. Burvenich C, Van Merris V, Mehrzad J, et al. Severity of *E. coli* mastitis is mainly determined by cow factors. *Vet Res* 2003;34:521–564.
  22. Tilg H, Vannier E, Vachino G, et al. Antiinflammatory properties of hepatic acute phase proteins: Preferential induction of interleukin 1 (IL-1) receptor antagonist over IL-1 beta synthesis by human peripheral blood mononuclear cells. *J Exp Med* 1993;178:1629–2636.
  23. Wu AM, Lisowska E, Duk M, et al. Lectins as tools in glycoconjugate research. *Glycoconj J* 2009;26:899–913.
  24. Yan L, Wilkins PP, Alvarez-Manilla G, et al. Immobilized *Lotus tetragonolobus* agglutinin binds oligosaccharides containing the Le^x determinant. *Glycoconj J* 1997;14:45–55.
  25. Kątnik I, Jadach J, Krotkiewski H, et al. Investigating the glycosylation of normal and ovarian cancer haptoglobins using digoxigenin-labeled lectins. *Glycosyl Dis* 1994;1: 97–104.
  26. Lönnerdal B. Bioactive proteins in breast milk. *J Paediatr Child Health* 2013;49:1–7.
  27. Nwosu CC, Aldredge DL, Lee H, et al. Comparison of the human and bovine milk N-glycome via high-performance microfluidic chip liquid chromatography and tandem mass spectrometry. *J Proteome Res* 2012;11:2912–2924.
  28. Wang B, Brand-Miller J. The role and potential of sialic acid in human nutrition. *Eur J Clin Nutr* 2003;57:1351–1369.
  29. Carlson SE. N-Acetylneuraminic acid concentrations in human milk oligosaccharides and glycoproteins during lactation. *Am J Clin Nutr* 1985;41:720–726.
  30. Chaturvedi P, Warren CD, Altaye M, et al. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology* 2001;11: 365–372.
  31. De Leoz ML, Gaerlan SC, Strum JS, et al. Lacto-N-tetraose, fucosylation, and secretor status are highly variable in human milk oligosaccharides from women delivering preterm. *J Proteome Res* 2012;11:4662–4672.
  32. Groër M, Davis M, Casey K, et al. Neuroendocrine and immune relationships in postpartum fatigue. *MCN Am J Matern Child Nurs* 2005;30:133–138.
  33. Zivkovic AM, German JB, Lebrilla CB, et al. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. *Proc Natl Acad Sci U S A* 2011;108:4653–4658.
  34. Erbağci AB, Cekmen MB, Balat O, et al. Persistency of high proinflammatory cytokine levels from colostrum to mature milk in preeclampsia. *Clin Biochem* 2005;38: 712–716.
  35. Ostrom KM. A review of the hormone prolactin during lactation. *Prog Food Nutr Sci* 1990;14:1–43.
  36. Goldblum RM, Hilton S. Amniotic fluid and the fetal mucosal immune system. In: Ogra PL, Mestecky J, Lamm ME, et al., eds. *Mucosal Immunology*, 2nd ed. London, Academic Press, 1999:1555–1564.
  37. Garofalo RP, Goldman AS. Cytokines, chemokines, and colony-stimulating factors in human milk: The 1997 update. *Biol Neonate* 1998;74:134–142.
  38. Goldman AS. Evolution of the mammary gland defense system and the ontogeny of the immune system. *J Mammary Gland Biol Neoplasia* 2002;7:277–289.
  39. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. *Annu Rev Nutr* 2005;25:37–58.
  40. Newburg DS. Glycobiology of human milk. *Biochemistry (Moscow)* 2013;78:771–785.
  41. Schauer R. Sialic acids as regulators of molecular and cellular interactions. *Curr Opin Struct Biol* 2009;19:507–514.

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