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Original research article

Alpha 2,3- and alpha 2,6-sialylation of human skim milk glycoproteins during milk maturation

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ABSTRACT

Human milk is a source of glycoconjugates, sialylated forms of which enrich the newborn immature immune system and are crucial for their proper development and well-being. Here, we analyzed the expression of α2,3-/α2,6-sialylated glycotopes on skim milk glycoproteins over lactation. Milk samples were analyzed by lectin-blotting using α2,3- and α2,6- sialic acid specific *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) lectins and sialyl- and asialyl-T antigen specific *Artocarpus integrifolia* (Jacalin) and *Arachis hypogaea* (PNA) lectins. The reactivities of MAA, SNA, Jacalin and PNA with milk glycoproteins showed that they are heavily decorated with α2,3-/α2,6-linked sialic acid and sialyl-T antigen and to a lesser degree with asialyl-T antigen. Despite individual differences of particular glycoproteins, a sharp and significant decline of α2,6-sialylated glycotopes and sialyl-T antigens and a weaker but significant decrease of α2,3-sialylated glycotopes and asialyl-T antigens on milk glycoproteins during milk maturation was observed. The expression of α2,3-/α2,6-sialylated glycotopes, sialyl- and asialyl-T antigens corresponds to milk maturation but differs in relation to the analyzed glycoprotein. Sialylated milk glycoproteins are considered as a part of innate immunity provided to neonates. Further investigations are needed to understand if they may be useful in milk banking to control the biochemical quality of milk.

Introduction

Sialic acid (SA) belongs to a family of nine-carbon atom acids. It can be attached by α2,3- and α2,6-glycosidic linkage to the terminal or subterminal Gal and/or GalNAc moieties of glycoconjugates and can form polysialylated structures in which SAs are linked by α2,8-glycosidic bonds (Kątnik-Prastowska, 2003; Varki and Gagneux, 2012). The presence of sialic acid on the *N*- and *O*-glycans of proteins gives the negative charge, has an impact on conformation stabilization, and protects against recognition and removal by the liver receptor for asialylated glycoproteins (Kątnik-Prastowska, 2003). The expression of α2,3- and α2,6-linked sialic acid is tissue-specific, related to the developmental stages and depends on the pathophysiological status (Katnik-Prastowska, 2003; Orczyk-Pawilowicz et al., 2015b; Vallejo et al., 2000; Wang, 2012; Yasukawa et al., 2005).

The biological role of sialylated glycotopes may vary and is related to the type of linkage, namely α2,3-, α2,6- and α2,8-. Sialic acid can serve as a ligand for sialoadhesin and selectin families and in that way can modulate cell adhesion and communication, and signal transduction events (Kątnik-Prastowska, 2003; Schauer, 2009; Varki and Gagneux, 2012; Wang, 2012). Glycoconjugates of the neonatal mammalian central nervous system, in contrast to adults, are rich in α2,3-sialylated glycotopes, whereas α2,6-sialylated glycotopes are present in small quantities or are absent (Vallejo et al., 2000). Moreover, the increase of α2,3- and/or α2,6-sialylated glycotopes on *N*- and/or *O*-glycans is associated with inflammation, cancerogenesis and metastasis (Fujiwara et al., 2002; Kątnik-Prastowska, 2003; Schauer, 2009; Varki and Gagneux, 2012; Yasukawa et al., 2005).

Abbreviations: asialyl-T antigen, Galβ1,3GalNAc-; HMOs, human milk oligosaccharides; Jacalin, lectin from *Artocarpus integrifolia*; MAA, lectin from *Maackia amurensis*; PNA, lectin from *Arachis hypogaea*; SA, sialic acid, *N*-acetyl-5-neuraminic acid; sialyl-T antigen, sialyl-Galβ1,3GalNAc-; SNA, lectin from *Sambucus nigra*.

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Fucosylated and sialylated glycoconjugates of human milk, can participate as inhibitors in blocking the interactions between lectin receptors of the pathogen and host (infants) epithelial cells (Liu and Newburg, 2013; Royle et al., 2003). The inhibition of pathogen adhesion has been confirmed for sialylated glycans of S-IgA and mucin (S-fimbriated *Escherichia coli*) (Schroten et al., 1998), lactadherin (*Rotavirus*) (Yolken et al., 1992) and κ-casein (*Streptococcus mutans* GS-5) (Vacca-Smith et al., 1994). Moreover, it has been shown that *Enterovirus 71* binds sialylated glycans of human P-selectin glycoprotein-1, in contrast to the preferential binding of α2,6-sialylated glycans by influenza and α2,3-sialylated glycans by Coxsackie virus (Mistry et al., 2011; Yang et al., 2009).

The majority of human milk sialic acid is linked to milk oligosaccharides (HMOs) (80%) (Coppa et al., 1999; Martín-Sosa et al., 2004; Thurl et al., 2010). Moreover, sialic acid can be attached to the glycans of glycoproteins (16%) and glycolipids (0.2%) and can exist as free sialic acid (3%) (Martín-Sosa et al., 2004). However, due to tremendous structural diversity and multivalency of sialylated glycans of glycoproteins, they are considered as a better mechanism of pathogen capture than the relatively short and simple HMO structure (Bernardi et al., 2013). Moreover, alterations of sialylation pattern affect functions as well as anti- and pro-inflammatory properties of some glycoproteins (Shade and Anthony, 2013). The sialylation of human milk glycoconjugates is the highest during the first days of lactation, then significantly decreases, reaching in mature milk 60% of the initial level and is strongly associated with the decrease of sialylated HMOs (Bode, 2012; Martín-Sosa et al., 2004). The sialylated HMOs decrease the occurrence of necrotizing enterocolitis, may act as anti-inflammatory agents and contribute to a lower incidence of inflammatory diseases in breast-fed infants (Bode et al., 2004; Jantscher-Krenn et al., 2012). Moreover, sialyl-Le^x glycotopes of HMOs can serve as ligands for selectins and might inhibit leukocyte rolling and adhesion to the endothelial cells (Bode, 2012; Bode et al., 2004). In an animal model, the sialylated brain glycoconjugates play critical roles in mediating cell-to-cell interactions important for neural development and improving the capability of learning and memory (Wang, 2012).

Human milk *N*-glycome at the third month of lactation contains 57% sialylated and 75% fucosylated structures (Nwosu et al., 2012). So far, the changes in concentration and distribution of sialic acid during human milk maturation have been shown for total sialic acid level as well as for its bound and free forms (Martín-Sosa et al., 2004). The changes in sialylation over lactation have been shown for lactoferrin (Barboza et al., 2012; Froehlich et al., 2010), bile-salt-stimulated-lipase (Landberg et al., 2000), α_1-acid glycoprotein (Orczyk-Pawiłowicz et al., 2014, 2015a) and fibronectin (Orczyk-Pawiłowicz et al., 2015b). However, there are no data concerning overall analysis of sialoproteome in relation to the different types of sialic acid linkages. The application of lectins with well-defined specificity, in contrast to high-performance liquid chromatography and mass spectrometry, allows for obtaining information regarding alterations in expression of α2,3-/α2,6-sialylated glycotopes, which can be related to changes of biological activity (Kątnik-Prastowska, 2003; Varki and Gagneux, 2012). Additionally, lectin based methods offer a more convenient approach to determining protein sialylation pattern as the need of cost consuming procedures for glycan release prior to analysis is eliminated and the time required for sample analysis is substantially reduced (Zhang et al., 2016).

The aim of this study was to investigate the α2,6- and α2,3-sialylation and antigen T expression of human skim milk glycoproteins during milk maturation from the 2nd to 47th days of healthy mother's lactation. The human skim milk glycoproteins were analyzed semi-quantitatively by lectin- blotting using lectins specific to α2,3- (MAA: *Maackia amurensis* lectin) and α2,6-linked (SNA: *Sambucus nigra* lectin) sialic acid (Knibbs et al., 1991; Shibuya et al., 1987) and specific to sialyl-T (Sialyl-Galβ1,3GalNAc-) (Jacalin: *Artocarpus integrifolia* lectin) and asialyl-T (Galβ1,3GalNAc-) (PNA: *Arachis hypogaea* lectin) antigens, respectively (Bourne et al., 2002; Hennigar et al., 1987; Wu et al., 2009).

Materials and methods

Participants

Samples of milk ($n = 43$) were obtained from healthy breastfeeding mothers (aged from 21 to 35 years) receiving regular perinatal care at the 1st Department of Gynecology and Obstetrics at Wroclaw Medical University, Poland. For inclusion in the study, breastfeeding women had to have a good state of health and normal single uncomplicated pregnancy. Women who used tobacco products, illicit drugs, or alcohol or with abnormal lactation were excluded. All mothers who agreed to give their milk for the biochemical analysis were acquainted with the protocol approved by the Ethics Committee at Wroclaw Medical University (KB-30/2013). Informed written consent was obtained from all lactating mothers.

Sample collection and preparation

Samples of human milk from 2 to 47 days of lactation were collected by a trained nurse from the breast by manual expression at the end of nursing (hindmilk) by complete breast emptying. To avoid time-dependent alterations in protein composition between two times of day, namely diurnal and nocturnal (França et al., 2010), milk samples were collected once per day, at the same time (8:00–10:00 a.m.). All milk samples were frozen and stored immediately at -20 °C until analysis. Skim milk was prepared by centrifugation at 3 500 *g* at 4 °C for 35 min, after which the fat layer and cells were removed.

For analysis the samples were collected from mothers who have secretor status with Se+/Le+ phenotype (information obtained from previous studies) (Lis-Kuberka et al., 2015; Orczyk-Pawiłowicz et al., 2015b). To minimize the impact of individual differences among the mothers, the selected milk samples from the same days of lactation were pooled before analysis by mixing an equal volume of an individual skim milk sample. The following groups were formed: (1) colostrum (day 2 of lactation; $n = 2$), (2) colostrum (day 3 of lactation; $n = 5$), (3) colostrum (days 4–5 of lactation; $n = 7$), (4) transitional milk (days 7–8 of lactation; $n = 5$), (5) transitional milk (day 10 of lactation; $n = 4$), (6) transitional milk (days 12–14 of lactation; $n = 5$), (7) mature milk (days 15–17 of lactation; $n = 5$), (8) mature milk (days 30–35 of lactation; $n = 5$) and (9) mature milk (days 39–47 of lactation; $n = 5$).

Methods

Determination of protein concentration

The total protein concentration in human skim milk pooled samples was determined by bicinchoninic methods with the Bicinchoninic Acid Protein Assay Kit (Sigma, St. Louis, MO, USA) and bovine albumin as a standard.

SDS electrophoresis

The skim milk pooled sample containing 30 μg of protein was denaturated at 100 °C for 5 min and loaded to SDS-PAGE in a 7.5% gel, according to Laemmli (1970). After electrophoresis, the separated proteins were transferred onto nitrocellulose membrane according to Towbin et al. (1979).

Lectin-blotting

The reactivities of sialic acid and T antigen-specific lectins with human skim milk glycoprotein bands were analyzed by lectin-blotting using MAA (α2,3-linked sialic acid), SNA (α2,6-linked sialic acid), Jacalin (Sialyl-Galβ1,3GalNAc-) and PNA (Galβ1,3GalNAc-) biotin-labeled lectins (Vector Laboratories Inc., Burlingame, USA) with well-known specificity.

After the SDS-PAGE, the separated milk proteins as well as positive (human haptoglobins from ovarian cancer fluid, glycophorin and asialoglycophorin) (Kątnik et al., 1994; Orczyk-Pawiłowicz et al., 2015b), negative (human albumin) controls, and standards with molecular mass ranging from 250 to 10 kDa (Precision Plus Protein standards, Bio-Rad) were transferred onto nitrocellulose membrane, and then the membrane was blocked with 2% Tween-20 in Tris buffered saline (TBS), pH 7.5 at 32 °C for 1 h. After washing, the membranes were incubated with biotin-labeled lectins: MAA (1 μg/ml), SNA (0.2 μg/ml), Jacalin (0.5 μg/ml), and PNA (5 μg/ml) for 1 h at 32 °C in TBS with 0.1% Tween 20 (TBS-T) containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.5. The formed lectin-glycoprotein complex was detected by the reaction with phosphatase-labeled ExtrAvidin (Sigma, St. Louis, MO, USA) diluted 1:20 000 in TBS-T, pH 7.5 at 32 °C for 1 h. After washing, the colored reaction was developed in a freshly prepared solution of: 5-bromo-4-chloro-3-indolylphosphate (Sigma) and nitroblue tetrazolium chloride (Sigma) in 0.1 M Tris/HCl, pH 9.5, containing 0.05 M $MgCl_2$, 0.1 M NaCl at room temperature for 50 s. The blots with all lectins were carried out twice, and the results are presented as the mean value obtained from two experiments. The variation between two blots (interassay coefficient) is: 2.2% for SNA, 5.7% for MAA, 7.6% for PNA, and 1.8% for Jacalin, respectively.

Densitometric analysis

The intensity of bands corresponded to the lectin reactivity with the particular glycoproteins. The intensity of each line was quantified by densitometric analysis with the myImageAnalysis software (Thermo Scientific, New Hampshire). The relative amount of glycotopes on each glycoprotein band was expressed as a mean of pixels $\times 10^6$ for the individual band obtained from two blots.

Glycoprotein molecular mass determination

To determine the molecular mass of separated human skim milk glycoproteins, the Precision Plus Protein standards for SDS-PAGE (Bio-Rad) were used. Based on molecular mass of standards, a calibration curve of the log of molecular mass (Mm) versus R_f was generated.

Statistical analysis

The statistical analysis was performed with the STATISTICA 10.0 software package (StatSoft, Inc., Tulsa, OK, USA). The correlations

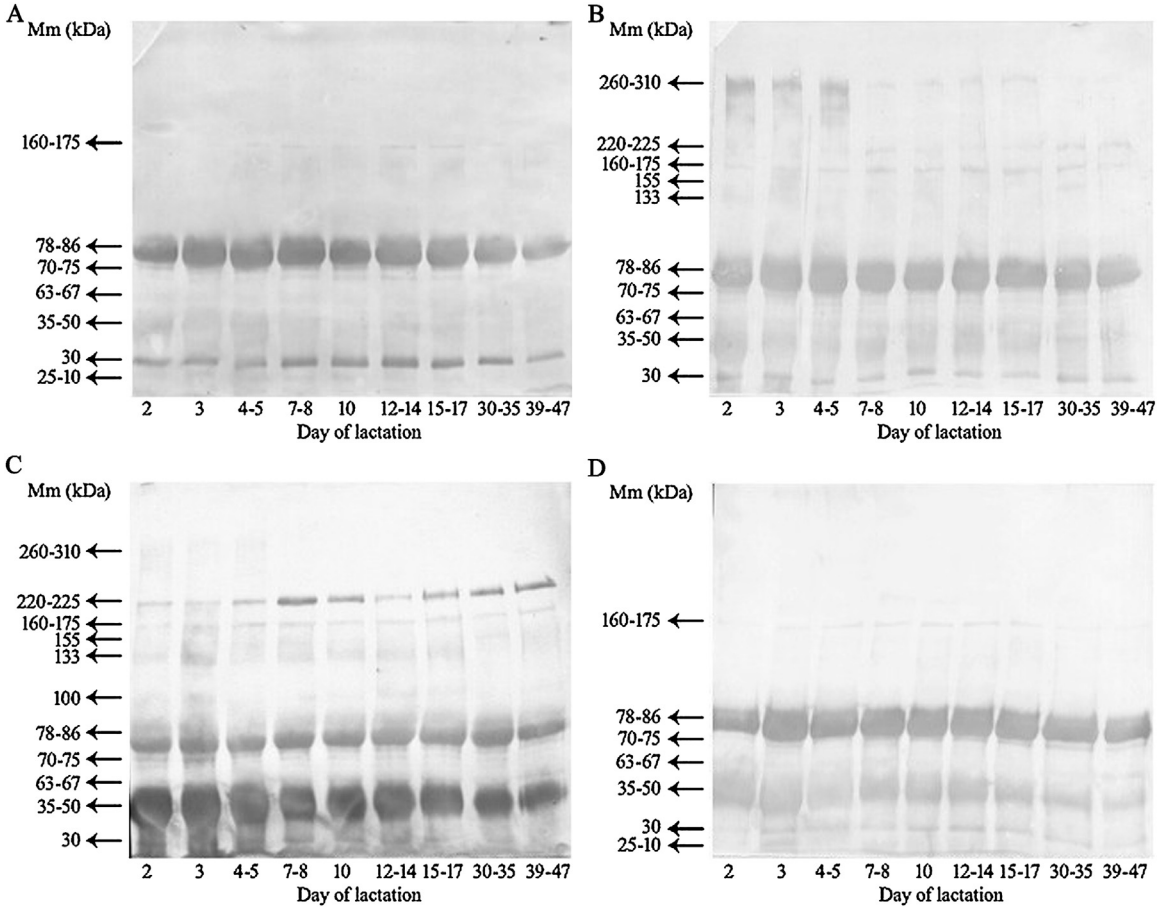

Fig. 1. Representative patterns of human skim milk glycoprotein reactivities with α2,3- and α2,6-sialic acid and sialyl- and asialyl-T antigen specific lectins during lactation. A pooled milk sample containing 30 μg of proteins was loaded per lane of SDS-PAGE. After separation, the glycoproteins were transferred onto nitrocellulose, and the membrane was subjected to the reaction with (a) *Maackia amurensis* (α2,3-linked sialic acid), (b) *Sambucus nigra* (α2,6-linked sialic acid), (c) *Artocarpus integrifolia* (sialyl-T antigen), and (d) *Arachis hypogaea* (asialyl-T antigen) biotinylated lectin (Vector Laboratories Inc. Burlingame. USA), respectively. The formed lectin-glycoprotein complex was detected by the reaction with phosphatase-labeled ExtrAvidin (Sigma. St. Louis. MO. USA). All blots were done in duplicate. For experimental details see Material and Methods.

Table 1
Relative amounts of α2,3- and α2,6-sialylated glycotopes and sialyl- and asialyl-T antigens on human skim milk glycoproteins over lactation.

Mm of protein band [kDa]	Lectin (glycotope recognized)	Relative amounts of the glycotope [pixels × 10^6] revealed by lectin- immunoblotting shown in Fig. 1									r
		Day of lactation									
		2nd	3rd	4th-5th	7th-8th	10th	12th-14th	15th-17th	30th-35th	39th-47th	
260–310	MAA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
	SNA	21.8	12.80	13.00	1.02	1.10	1.15	0.91	0.55	0.00	−0.88
	Jacalin	6.53	4.02	3.59	0.00	0.00	0.00	0.00	0.00	0.00	ND
	PNA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
220–225	MAA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
	SNA	4.49	2.69	2.28	3.63	3.85	2.08	1.28	1.79	2.17	−0.73
	Jacalin	3.45	4.45	3.85	10.10	6.05	3.58	5.07	6.40	8.37	NS
	PNA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
160–175	MAA	8.80	7.53	4.65	8.23	7.93	8.58	10.10	7.27	7.18	NS
	SNA	4.44	3.15	2.51	3.70	3.67	4.11	2.70	2.40	1.59	NS
	Jacalin	2.27	3.75	2.36	2.14	1.71	2.26	3.02	2.18	2.95	NS
	PNA	1.26	1.39	1.49	1.39	1.66	1.36	1.50	0.68	0.86	NS
155	MAA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
	SNA	3.42	3.28	2.57	2.55	3.16	2.76	2.33	2.23	0.00	−0.81
	Jacalin	2.19	3.25	1.99	1.57	1.22	2.32	1.95	2.00	2.90	NS
	PNA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
133	MAA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
	SNA	1.19	1.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
	Jacalin	3.85	8.16	3.20	3.64	3.46	3.68	3.68	0.00	0.00	NS
	PNA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
100	MAA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
	SNA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
	Jacalin	3.42	4.87	2.01	1.95	1.90	2.10	2.83	1.80	1.79	−0.72
	PNA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
78–86	MAA	44.50	48.70	42.00	50.10	41.30	42.50	48.10	40.40	36.90	NS
	SNA	31.90	37.70	35.90	37.90	34.90	40.60	33.80	30.20	22.90	NS
	Jacalin	40.60	44.60	34.10	34.80	32.90	30.60	33.00	39.60	30.30	−0.67
	PNA	26.30	34.70	28.30	32.50	29.10	31.70	31.50	24.10	20.30	NS
70–75	MAA	8.46	7.99	16.40	15.40	14.70	15.00	16.50	12.70	13.40	NS
	SNA	11.80	10.20	8.79	9.64	9.07	9.57	6.82	6.86	2.15	−0.87
	Jacalin	10.10	13.30	8.25	6.37	5.94	7.71	7.06	1.09	5.31	NS
	PNA	2.97	4.75	4.51	5.45	5.96	5.72	6.21	3.81	4.04	NS
63–67	MAA	1.22	12.20	7.88	8.09	9.92	9.88	9.86	8.67	11.00	NS
	SNA	6.46	3.29	4.48	4.74	5.55	5.76	3.93	3.04	1.89	NS
	Jacalin	6.50	6.18	5.67	5.72	5.57	6.36	3.68	7.35	9.62	NS
	PNA	3.65	2.52	2.03	2.43	1.62	2.23	2.06	2.37	1.90	NS
35–50	MAA	47.00	40.20	38.20	34.10	30.30	28.70	27.90	29.00	30.10	−0.85
	SNA	35.30	26.00	25.30	27.50	20.00	25.60	18.60	15.80	7.16	−0.88
	Jacalin	89.50	77.30	71.90	59.30	64.0	61.00	62.10	59.40	48.0	−0.82
	PNA	32.00	32.30	25.00	22.80	24.10	23.00	20.00	16.30	9.99	−0.93
30	MAA	13.90	14.80	13.40	14.20	16.70	12.70	15.40	14.00	12.70	NS
	SNA	9.49	6.57	5.04	6.36	6.32	6.95	6.45	6.14	4.67	NS
	Jacalin	19.20	17.59	10.36	8.56	6.93	6.43	5.07	6.26	4.79	−0.98
	PNA	5.10	8.75	6.94	6.16	9.19	6.67	6.80	5.77	4.66	NS
25−10	MAA	1.65	2.31	3.57	1.75	1.61	1.45	1.16	0.00	0.00	NS
	SNA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
	Jacalin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND
	PNA	1.78	3.26	2.77	2.71	2.71	2.4	2.53	2.56	2.55	NS

For analysis of α2,3- and α2,6- sialylation and sialyl- and asialyl-T antigens of milk glycoproteins, the samples were collected only from mothers who have secretor status with Se+/Le+ phenotype (information obtained from previous studies).
The MAA- (α2,3-linked sialic acid), SNA- (α2,6-linked sialic acid), Jacalin- (sialyl-T antigen) and PNA- (asialyl-T antigen) reactive bands revealed by lectin- immunoblotting and shown in Fig. 1 were analyzed using myImageAnalysis software (ThermoScientific, NewHampshire). The relative amounts of α2,3- and α2,6-sialylated glycotopes and sialyl- and asialyl-T antigens on each glycoprotein band are expressed as the mean number of pixels × 10^6 for the individual band obtained from two independently done blots.
r – correlation coefficient with lactation days.
NS – not significant with a *p*-value equal to or higher than 0.05.
ND – not determined.

were estimated according to Spearman. A p-value lower than 0.05 was regarded as significant.

Results

Sialic acid- and T antigen-specific lectin reactivity with human milk glycoproteins

The lectin-blotting pattern of human skim milk glycoproteins over lactation showed strong reactivities with sialyl-T antigen (Jacalin), α2,6- and α2,3-sialic acid (SNA and MAA) specific lectins, and lower reactivity with asialyl-T antigen (PNA) (Fig. 1). The semi-quantitative differences between the particular milk glycoprotein-lectin reactivities over milk maturation from the 2nd to 47th day of lactation are given in Table 1.

Expression of MAA-reactive bands

The MAA reactivity (Fig. 1a, Table 1) of the 35–50 kDa glycoprotein showed a strong negative correlation with milk maturation ($r = -0.85$), whereas the reactivity of the 160–175 kDa, 78–86 kDa, 70–75 kDa, 63–67 kDa, 30 kDa and 25–10 kDa glycoproteins did not (Table 1). The 260–310 kDa, 220–225 kDa, 155 kDa, 133 kDa, and 100 kDa glycoproteins lacked the MAA-reactive glycotopes during lactation from the 2nd to 47th day.

Expression of SNA-reactive bands

The SNA reactivity (Fig. 1b, Table 1) of the 260–310 kDa ($r = -0.88$), 220–225 kDa ($r = -0.73$), 155 kDa ($r = -0.81$), 70–75 kDa ($r = -0.87$), and 35–50 kDa ($r = -0.88$) glycoprotein bands showed a strong negative correlation with the progression of lactation. In contrast, the relative amount of SNA-reactive glycotopes of 160–175 kDa, 78–86 kDa, 63–67 kDa and 30 kDa glycoproteins did not significantly change over lactation. The 133 kDa, 100 kDa and 25–10 kDa bands were not recognized by SNA, with the exception of low SNA reactivity with the 133 kDa glycoprotein observed for the colostrum samples from the 2nd and 3rd (0.9 and 1.7% of total reactivity, respectively) day of lactation (Table 1).

Expression of Jacalin-reactive bands

The Jacalin reactivity (Fig. 1c, Table 1) of the 100 kDa ($r = -0.72$), 78–86 kDa ($r = -0.67$), 35–50 kDa ($r = -0.82$) and 30 kDa ($r = -0.98$) glycoprotein bands showed a strong negative correlation with milk maturation. In contrast, the relative amount of Jacalin-reactive glycotopes of 220–225 kDa, 160–175 kDa, 155 kDa, 133 kDa, 70–75 kDa, and 63–67 kDa glycoproteins did not. The 25–10 kDa band was not recognized by Jacalin, but for the 260–310 kDa glycoprotein band low Jacalin reactivity was observed for the colostrum samples from the 2nd, 3rd and 4th–5th (3.5, 2.1 and 2.4% of total reactivity, respectively) day of lactation only (Table 1).

Expression of PNA-reactive bands

PNA reacted with 160–175 kDa, 78–86 kDa, 70–75 kDa, 63–67 kDa, 35–50 kDa, 30 kDa, and 25–10 kDa glycoprotein bands of human milk (Fig. 1d, Table 1), but only the 35–50 kDa band showed a strong negative correlation ($r = -0.93$) with lactation progression. In contrast, the 260–310 kDa, 220–225 kDa, 155 kDa, 133 kDa and 100 kDa bands were not recognized by PNA at all (Table 1).

General tendency of sialic acid- and T antigen-specific lectin reactivity with human skim milk glycoproteins over lactation

The general tendency of the α2,3- and α2,6-sialylation as well as sialyl- and asialyl-T antigen expression of human milk glycoproteins over lactation, is presented in Fig. 2. The level of total SNA-reactive glycoproteins from the 2nd to the 39th-47th day gradually and significantly decreased ($r = -0.95$) (Fig. 2a). Similarly, the level of total MAA-reactive glycoproteins decreased ($r = -0.67$) during lactation, although in comparison to SNA-reactive glycoproteins, the correlation coefficient is lower (Fig. 2a). In a similar fashion, the level of total Jacalin- and PNA-reactive glycoproteins significantly decreased ($r = -0.81$ and $r = -0.71$, respectively) (Fig. 2a), but the level of asialyl-T antigen was considerably lower than the level of sialyl-T antigen (Fig. 2a).

Based on the analyses of the sialic acid- as well as asialyl- and sialyl-T antigen specific lectin reactivity of particular milk glycoproteins over lactation, two general types of changes were

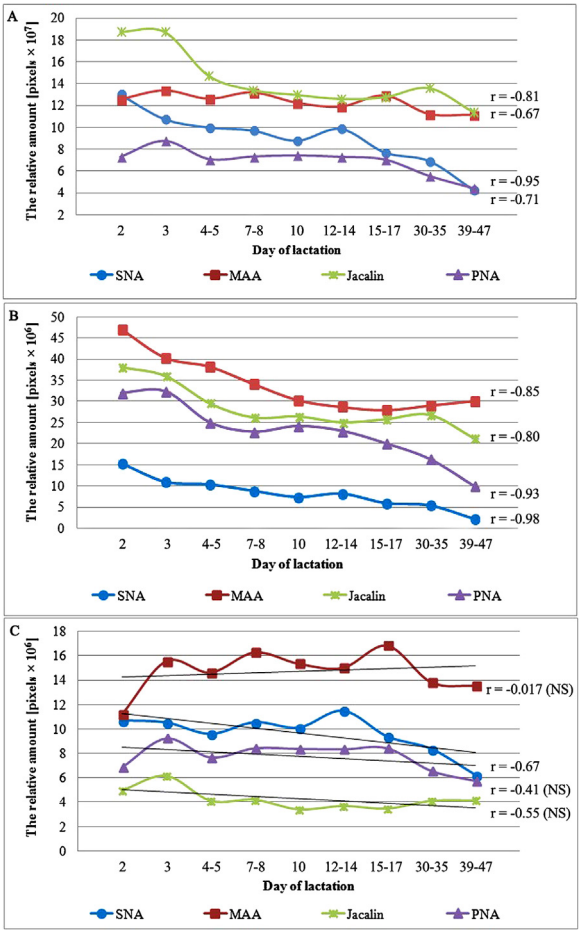

Fig. 2. General pattern of α2,3- and α2,6-sialic acid and sialyl- and asialyl-T antigen specific lectin reactivity of human skim milk glycoproteins over lactation.
The mean value of the relative amounts of the lectin reactive milk glycoproteins was calculated for (a) all glycoprotein bands, (b) those showing a significant correlation and (c) no significant correlation with milk maturation.
For the calculation, the following SDS-PAGE bands (Table 1) were selected in: (a) all bands, (b) reactive with MAA – 35–50 kDa, SNA – 260–310 kDa, 220–225 kDa, 155 kDa, 70–75 kDa, 35–50 kDa, Jacalin – 100 kDa, 78–86 kDa, 35–50 kDa, 30 kDa and PNA – 35–50 kDa; and in (c) reactive with MAA – 160–175 kDa, 78–86 kDa, 70–75 kDa, 63–67 kDa, 30 kDa, 25–10 kDa, SNA – 160–175 kDa, 133 kDa, 78–86 kDa, 63–67 kDa, 30 kDa, Jacalin – 260–310 kDa, 220–225 kDa, 160–175 kDa, 155 kDa, 133 kDa, 70–75 kDa, 63–67 kDa and PNA – 160–175 kDa, 78–86 kDa, 70–75 kDa, 63–67 kDa, 30 kDa, 25–10 kDa. For other details see under Table 1. r = correlation coefficient with lactation days.

selected (Fig. 2b and c). The first one (Fig. 2b) was associated with a significant decrease of MAA, SNA, Jacalin and PNA reactivity with milk glycoproteins. The second type of changes was related to no significant differences/alterations of MAA, SNA, Jacalin and PNA reactivity with milk glycoproteins (Fig. 2c).

α2,3- and α2,6-sialic acid specific lectin reactivity with human milk glycoproteins

The first type of changes, namely decrease of the reactivity, was observed for SNA-reactive 260–310, 220–225, 155, 70–75 and 35–50 kDa bands ($r = -0.98$) (Fig. 2b) and for the MAA-reactive 35–50 kDa band ($r = -0.85$) only. For both SNA- and MAA-reactive glycoproteins, from the beginning of lactation to 15th-17th day a gradual decline was observed. However, from the 30th-35th day a slight increase of MAA-reactive glycoproteins was observed, while for SNA-reactive glycoproteins a further decline was observed (Fig. 2b).

For the second type of changes, MAA-reactive 160–175, 78–86, 70–75, 63–67, 30 and 25–10 kDa bands demonstrated a relatively stable ($r = -0.017$) (Fig. 2c) trend over lactation, although a marked increase of reactivity from day 2 to 3; after that the level stays about the same with some variations for individual days until day 15–17, when the level decreases, was observed. In contrast, SNA-reactive 160–175, 133, 78–86, 63–67 and 30 kDa bands, which did not show a correlation with milk maturation individually, in total, apart from variations for individual days, showed approximately the same level until day 12–14 and after that it drops significantly ($r = -0.67$, $p < 0.05$) (Fig. 2c).

Sialyl-T and asialyl-T antigen specific lectin reactivity with human milk glycoproteins

The first type, namely decrease of the reactivity over lactation, was observed for Jacalin-reactive 100, 78–86, 35–50 and 30 kDa bands ($r = -0.80$) (Fig. 2b) and for the PNA-reactive 35–50 kDa band ($r = -0.93$) only. For both Jacalin- and PNA-reactive glycoproteins, from the beginning of lactation to the 7th-8th day a gradual decline was observed, then from the 8th to 14th day of lactation their level was relatively stable, and next a significant decrease was observed for PNA-reactive glycoproteins only (Fig. 2b).

For the second type of changes, Jacalin-reactive 260–310, 220–225, 160–175, 155, 133, 70–75 and 63–67 kDa bands and PNA-reactive 160–175, 78–86, 70–75, 63–67, 30 and 25–10 kDa bands elicited an insignificant decrease over lactation progression ($r = -0.55$, $p > 0.05$ and $r = -0.41$, $p > 0.05$, respectively) (Fig. 2c), although with slight variation for particular days.

Discussion

The study shows that human skim milk glycoproteins over lactation from the 2nd to 47th day are decorated with α2,3- and α2,6-linked sialic acid and sialyl-T antigen (sialyl-Galβ1,3GalNAc-) and to a lesser degree with asialyl-T antigen (Galβ1,3GalNAc-) (Fig. 1, 2a). During milk maturation, despite individual differences of particular glycoproteins, a sharp and significant decline of α2,6-sialylated glycotopes and sialyl-T antigens and a weaker but significant decrease of α2,3-sialylated glycotopes and asialyl-T antigens on milk glycoproteins was observed.

The α2,6-sialylated glycoproteins analyzed in this study show a gradual decrease as lactation progresses, reaching at days 39–47 about 33% of that observed for colostrum. The significant decrease of α2,6-sialylated glycotopes on human skim milk glycoproteins overlaps with the decrease of α2,6-sialylated HMOs such as sialyllacto-*N*-tetraose c and 6′-sialyllactose (Coppa et al., 1999; ten Bruggencate et al., 2014; Thurl et al., 2010). Alpha-2,3-sialylated glycoproteins also showed a decrease, but their total level was reduced by about 11% only. Among all analyzed α2,3-sialylated glycoproteins, only the 35–50 kDa band, which constitutes 22–37%, showed a strong negative correlation with milk maturation ($r = -0.85$). However, the level of the majority (~63–78%) of α2,3-sialylated glycoproteins (160–175, 78–86, 70–75, 63–67, 30, 25–10 kDa) remained almost constant, like the 3′-sialyllactose level (Coppa et al., 1999).

The relative amounts of sialyl-T (Jacalin-reactive) and asialyl-T (PNA-reactive) antigens of milk glycoproteins are strongly connected with milk maturation, reaching at the 39th–47th day of lactation for both antigens ~61% of the colostrum level. The simultaneous decrease of both forms of T antigen suggests that the decrease of T antigen expression during lactation predominates over the decrease of *O*-glycan sialylation and indirectly indicates that the decrease of sialylation is associated mainly with N-linked glycans. However, the trends are a general tendency observed for overall milk glycoproteins, and for individual glycoproteins deviations can occur. The presence of such glycotopes might be an additional advantage, as another and novel, absent from HMOs, glycotope with potential ability to serve as a decoy in interaction with bacterial adhesins. Similar to *N*-/*O*-glycans of human milk S-IgA (Royle et al., 2003), sialyl- and asialyl-T antigens of skim milk glycoproteins can be considered as an additional element of the innate immune system which can potentially participate in interaction/inhibition of pathogen adhesion to epithelial cells (Bernardi et al., 2013).

Based on the reactivity of lectins with particular milk glycoprotein bands, two types of α2,3- and α2,6-sialylation and sialyl- and asialyl-T antigen expression patterns related to normal lactation are distinguished (Fig. 2b and c). The first comprises the glycoprotein bands which showed a negative correlation with milk maturation (α2,3- (1 band), α2,6-sialic acid (5 bands), sialyl-T antigens (4 bands), and asialyl-T antigens (1 band), which constitute 14%, 50%, 36% and 14% of total glycoprotein bands, respectively) (Fig. 2b). The second type, although variable, did not show significant correlations, with the exception of SNA-reactive bands, which did not significantly correlate with milk maturation individually, but when added together, showed a significant decrease ($r = -0.67$, Fig. 2c). Interestingly, among all SNA- and MAA-reactive glycoprotein bands, one of the most abundant (78–86 kDa) containing lactoferrin and the secretory component of IgA showed a relatively stable level of both α2,3- and α2,6-sialic acids. These glycoproteins are known to play principal roles in innate immunity, and their sialylated glycoforms have antibacterial potential (Barboza et al., 2012; Royle et al., 2003).

A significant decrease in α2,6-sialylation with milk maturation was observed for the 260–310 kDa band, which corresponds to soluble/skim milk mucins (Liu et al., 2012; Parry et al., 2006) and is bound with significant decrease of mucin level over lactation ($r = -0.88$), as was reported previously (Lis-Kuberka et al., 2015). The reactivity pattern of SNA and Jacalin showed that colostral mucins elicited high content of α2,6-sialylated glycotopes as well as sialyl T antigen. In contrast, we did not observe MAA- and PNA-reactivity with the 260–310 kDa band under the test conditions, probably due to the low level of α2,3-sialylated glycotopes and asialyl-T antigens as well as the low level of colostral soluble/skim milk mucins, below 100 mg/l (Hanisch et al., 1990). The α2,6-sialylation of 220–225 and 35–50 kDa bands, which contain fibronectin (FN) and α_1-acid glycoprotein (AGP), respectively, among others, shows a negative correlation with day of lactation and overlaps with milk maturation changes reported previously for FN and AGP based on Lectin-FN/AGP-ELISA (Orczyk-Pawiłowicz et al., 2014, 2015b). However, for α2,3-sialylation of 220–225 and 35–50 kDa bands, the observed trends were

different; namely, there was a significant decrease for the 35–50 kDa band and no detected reactivity for the 220–225 kDa band. Both bands showed presence of sialyl-T antigens, but a significant decrease was observed for the 35–50 kDa band only. Over lactation there was no PNA- reactivity with the 220–225 kDa band. In contrast, PNA- reactivity with the 35–50 kDa band was observed and significantly decreased during lactation. The differences in α2,3-sialylation and asialyl-T antigens on 220–225 and 35–50 kDa bands (Orczyk-Pawiłowicz et al., 2014, 2015a, 2015b) are related to the presence of more than one glycoprotein differentially glycosylated per band, which affected the net result. Moreover, the 35–50 kDa band, except α_1-acid glycoprotein, also contains heavy chains of S-IgA and IgG. Between the 2nd and 5th weeks of lactation the concentration of S-IgA in human milk decreased, while IgG was unchanged, although at the beginning of lactation it was higher (Broadhurst et al., 2015). The calculated ratio of lectin-reactivity to proteins for the 35–50 kDa band changed over lactation from 0.61 to 0.18 for SNA-reactivity, from 1.55 to 1.20 for Jacalin-reactivity, and from 0.55 to 0.25 for PNA-reactivity, however it should be pointed out that the observed trends are the net result of both decreasing protein concentration and decreasing lectin-reactivity and for individual glycoproteins a derogation from the general trend may exist. In contrast, the ratio MAA-reactivity: proteins is almost unchanged (from 0.81 to 0.75), suggesting that the observed trend is related to the decreasing protein level only.

Conclusion

Over the progression of lactation the profile of human skim milk glycoprotein sialylation differed in relation to the type of sialic acid linkage to the glycan of glycoprotein as well as to the analyzed glycoprotein. The changes observed in the α2,6- and α2,3-sialylation of milk glycoproteins overlap with trends for sialylated HMOs during normal lactation and correspond to physiological stages of milk maturation. Human milk is rich in bioactive sialylated glycoproteins which are considered as a part of innate immunity provided to neonates by a mother and can influence the effectiveness of defense mechanisms by inhibition of sialic acid-dependent pathogens adhesion to the mucosa of newborn. Further investigations are needed to understand if sialylated glycoproteins have specific biological activity in human milk. Moreover, the analysis of milk sialylation may be useful in milk banking to control the biochemical quality of milk.

Conflict of interests

The authors declare that they have no conflict of interests.

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