Milk sugars beyond lactose

- Metabolic fate of neutral milk oligosaccharides in infants -

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Justus-Liebig-Universität Giessen

vorgelegt von

Viktoria Dotz
M.Sc. Ernährungswissenschaften,
geb. 25.01.1985 in Ladoschskaja (RUS)

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Erstgutachter: Prof. Dr. Clemens Kunz
Institut für Ernährungswissenschaft, FB09
Justus-Liebig-Universität Giessen
Wilhelmstr. 20, 35392 Gießen

Zweitgutachter: Prof. Dr. Bernhard Spengler
Institut für Anorganische und Analytische Chemie, FB08
Justus-Liebig-Universität Giessen
Schubertstr. 60 Haus 16, 35392 Gießen

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Abstract

Free oligosaccharides in human milk (HMO) are complex carbohydrates structurally based on lactose and present at concentrations of 5–20 g/L. They are considered to be resistant to digestion in the breastfed infant's stomach and small intestine, and to serve as energy source selectively for beneficial microbiota in the bowel. Apart from this prebiotic effect, HMO have been found to exert various biological activities in numerous *in vitro* studies, that indicate not only local functionality in the gut, but also, e.g. immunomodulatory or anti-infective, effects within the body. However, a direct link between structure and function *in vivo* has not yet been provided. This, in turn, is due to the enormous structural diversity of HMO: More than 150 different structures have been characterized to date.

In this work, the metabolic pathways and excretion profiles of HMO were investigated *in vivo* to extend our knowledge on the sites of HMO utilization or modification and thereby to provide hints on the structure-function relationship of HMO in the infant. Therefore, a platform based on solid phase extraction and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for a high throughput-profiling of oligosaccharides from human milk, infants' urine and feces was established, complemented by isomer separation *via* liquid chromatography. It was used to follow the time course and possible metabolites of ^{13}C-enriched and non-enriched oligosaccharides in milk and infant urine from ten mother-infant pairs during 36 hours after the application of ^{13}C-galactose to the mothers. Intestinal absorption and subsequent renal excretion of intact structures, in particular (fucosylated) hexaoses, could be deduced for all infants. However, in some infants, excretion was delayed, pointing at a longer gut passage or systemic circulation of those certain structures. Moreover, structure-specific modifications and utilization in the upper parts of the gut, where absorption is thought to take place, were hypothesized. The overall oligosaccharide profiles in the feces of breastfed infants could be categorized into three groups, i.e. i) high diversity with many HMO-like structures, ii) only one or a few oligosaccharides with rather low signal intensity, and iii) no HMO. The patterns showed some association with the infants' age. However, further research is needed to investigate the underlying causes, e.g. gut maturity or stool frequency.

Novel metabolites were identified in both, infant urine and feces. They could be characterized as acetylated HMO or HMO degradation/modification products, generated by the infants or by their gut microbiota, such as secretor- or Lewis-specific HMO in the feces/urine of infants fed nonsecretor or Lewis-negative milk. Lacto-*N*-tetraose as a major oligosaccharide in milk was significantly reduced especially in fecal samples. Moreover, the secretor-specific structure lacto-*N*-fucopentaose I, which is highly abundant in secretor milk, was not detected in the urine of the infants fed Lewis b secretor milk, which indicates a selective utilization of this specific structure.

Abbreviations

%RSD	relative standard deviation
2-AA	2-aminobenzoic acid
2-AB	2-aminobenzamide
Ac	acetyl residue
ACN	acetonitrile
ATT	6-aza-2-thiothymine
C(G)E	capillary (gel) electrophoresis
CID	collision-induced dissociation
CV	coefficients of variance
ESI	electrospray ionization
fA_{M+1}	fractional abundance of the isotopomer M+1
FL	fucosyllactose
Fuc	fucose(s)
FucT	fucosyltransferases(s)
Gal	galactose
GlcNAc	*N*-acetylglucosamine
GlcNAcT	*N*-acetylglucosaminyltransferase(s)
Hex	hexose
HexNAc	*N*-acetylhexosamine
HILIC	hydrophilic interaction liquid chromatography
HMO	human milk oligosaccharide(s)
HPAEC	high-performance anion-exchange chromatography
IRMS	isotope ratio MS
LC	liquid chromatography
LID	laser-induced dissociation
LIF	laser-induced fluorescence detection
LN(n)H	lacto-*N*-(*neo*)hexaose
LNdFH	lacto-*N*-difucohexaose
LNFP	lacto-*N*-fucopentaose
LNnT	lacto-*N*-*neo*tetraose
LNT	lacto-*N*-tetraose
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
OS	oligosaccharide(s)
PAD	pulsed amperometric detection
PGC	porous graphitized carbon
RBC	red blood cells
RP	reversed-phase
SPE	solid phase extraction
TFA	trifluoroacetic acid
TOF	time-of-flight

Chapter 1

1 Introduction and scope*

Mother's milk is the first and sole natural nutrition in a mammal's life. Exclusive breastfeeding during the first 4–6 months after birth is recommended from the World Health Organisation and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (Agostoni *et al.* 2009; Kramer and Kakuma 2001). This is due to various health benefits of breastfeeding compared to formula-feeding, despite significant improvements of infant formulae since the invention of the 'Soup for infants' by Justus von Liebig in 1865. For a long time, human/mother's milk has been the gold standard for infant nutrition, the composition of which was the reference for milk formulas. However, recently pediatricians regard the performance of the breastfed infant as a more suitable reference measure, i.e. physiological, biochemical and functional parameters, since the adaptation of formula to human milk's macro- and micronutrient composition has still not resolved the large differences in performance between breastfed and formula-fed infants (Hernell 2011). This may partly be due to confounders like psychosocial aspects of breastfeeding, but also to specific 'functional' compounds of human milk, which are low-concentrated or even absent in bovine milk, the common basis for infant formula. Those are, for example, leukocytes, nucleotides, long-chain polyunsaturated fatty acids and glycolipids, glycoproteins, as well as free oligosaccharides (OS) (Hernell 2011; Schack-Nielsen and Michaelsen 2007). The major beneficial effects of breastfeeding include protection against various types of infections during infancy, and as long-term effects a lower prevalence of immune-related diseases, such as type I diabetes and atopic dermatitis, a lower risk of hypertension and obesity, and enhanced cognitive development (Agostoni *et al.* 2009; Verduci *et al.* 2014). Due to their potential anti-infective, prebiotic and immune-modulating properties as found in numerous *in vitro*, animal and some observational human studies, free OS from milk seem to be a key to our understanding of the protective mechanisms of breastfeeding.

1.1 Quantitative and structural aspects of glycans present in infant gut

Glycan is a "generic term for any sugar or assembly of sugars, in free form or attached to another molecule" (Varki *et al.* 2009). The most prominent milk glycan is 4-O-ß-D-galactopyranosyl-D-glucopyranose, commonly known as lactose (Fig. 1-1). It is a major energy source for the breastfed infant and an important osmoregulator during milk secretion from the mammary gland, comprising the most abundant solid fraction in human milk.

In addition, lactose is the starting building block for the formation of free human milk oligosaccharides (HMO) (Grollman *et al.* 1965). HMO are the third major component of human milk, whereas the quantity and structural variety of bovine milk OS is notably lower (Table 1-1).

* Manuscript in preparation combined with Chapter 6

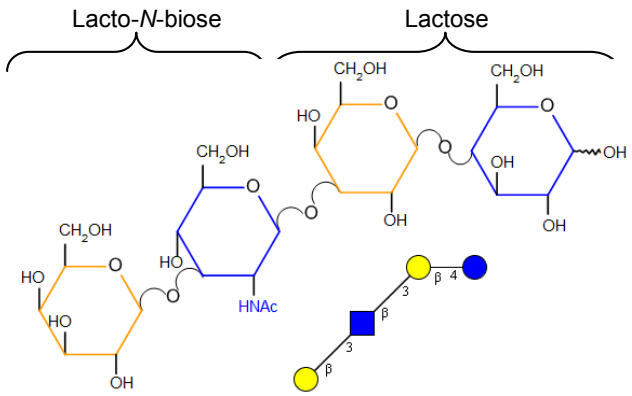

Fig. 1-1 Chemical structure of the milk oligosaccharide lacto-*N*-tetraose with its notation according to the recommendations of the Consortium for Functional Glycomics (drawn using Accelrys Draw 4.2 and GlycoWorkbench 1.1, respectively)

As described in detail in **Chapter 2**, the biosynthesis of complex HMO starts with the enzymatic elongation of the lactose core by iβ1-3-*N*-acetylglucosaminyltransferase. The consecutive action of β1-3- or β1-4-galactosyltransferase results in the major core structure lacto-*N*-tetraose (LNT) (Fig. 1-1), or its *neo*-form (LNnT), respectively. Further elongation and branching, in addition to the peripheral attachment of fucose(s) (Fuc) and/or *N*-acetylneuraminic acid(s) to lactose or the complex cores, results in a unique variety of 100–200 distinct structures, as described for human milk (Urashima *et al.* 2009; Wu *et al.* 2011; Wu *et al.* 2010). However, only a few of the most abundant structures comprise over a half of the total HMO amount, and the biological functions seem to be as multi-faceted as the structural variety. The biological role as investigated so far is presented in more detail in **Chapters 1.3** and **2.3**.

Table 1-1 Macronutrients and functional glycans in human milk

	Human	Bovine	References
Lactose	56–69 g/L [a)]	46–48 g/kg [b)]	(Coppa *et al.* 1993; Jensen *et al.* 2012; Thurl *et al.* 2010)
Lipids	18–34 g/L [a)]	37–61 g/kg [b)]	(Gidrewicz and Fenton 2014; Jensen *et al.* 2012)
- **sialylated glycolipids**	0.001–0.020 g/L	0.004–0.011 g/L	(Georgi *et al.* 2013; Jensen 2002)
- **neutral glycolipids**	5 µM	>27 µM 0.020 g/L	(Newburg and Chaturvedi 1992) (Jensen 2002)
Free oligosaccharides	7.7–20.9 g/L [a)]		(Coppa *et al.* 1993; Thurl *et al.* 2010)
- **neutral**	5.6–10.5 g/L [a)]	0.001–0.003 g/L [a)]	(Kunz *et al.* 1999; Thurl *et al.* 2010;
- **sialylated**	2.2–2.5 g/L [a)]	0.050–1.5 g/L [a)]	Urashima *et al.* 2013)
Glycosaminoglycans	0.41 g/L	0.06 g/L	(Coppa *et al.* 2011a)
Protein	9–20 g/L [a)]	33–45 g/kg	(Gidrewicz and Fenton 2014, Jensen et al. 2012)
- **glycoproteins**	~70% of total protein		(Froehlich *et al.* 2010)

[a)] mean values, varying by lactation period or other factors; [b)] variation range

Free lactose as a product from the action of the enzyme complex lactose synthetase is known to be formed only in the lactating mammary gland (Brew *et al.* 1968). However, various glycosyltransferases are expressed in various tissues and organs and can form HMO-like substructures attached to lipids or proteins in non-lactating individuals as well. Examples are the lacto- and the *neo*lacto-subfamiliy of glycosphingolipids, which are located in cell membranes, especially in secretory tissues (Schnaar *et al.* 2009). Structural examples for some common glycans as also found in human milk are given in .

Glycosphingolipids are the major class of glycolipids detected in human milk, mainly as part of the milk fat globule membrane. The concentration of sialylated glycolipids is substantially lower in bovine milk and the composition differs from human milk (Newburg and Chaturvedi 1992; Pan and Izumi 2000).

However, the major fraction of human milk lipids is not glycosylated and serves as main energy source for the breastfed infant (Table 1-1).

Proteins constitute about 1% of human milk (Table 1-1), with whey proteins and caseins being the most abundant protein fractions, followed by peptones and milk fat globule membrane proteins (Khan *et al.* 2012). The quantitative and qualitative composition of the human milk proteome as well as the glycoproteome can vary significantly over the course of lactation and with other factors (Froehlich *et al.* 2010). A comprehensive review of the total human milk proteome reported on 285 distinct gene products, 106 of which were identified as conserved protein core, also found in the bovine milk proteome (D'Alessandro *et al.* 2010). Glycosylation is a common posttranslational modification in proteins; it occurs in most secreted and membrane-bound proteins, including a major part of human milk proteins. Approximately 70% of the human milk proteins may be glycosylated (Froehlich *et al.* 2010). Oligosaccharides are covalently attached to polypeptide chains by specific glycosyltransferases in one of two possible mechanisms: either to a threonine or serine residue *via* *O*-glycosidic bond or to an asparagine during *N*-glycosylation (Fig. 1-2).

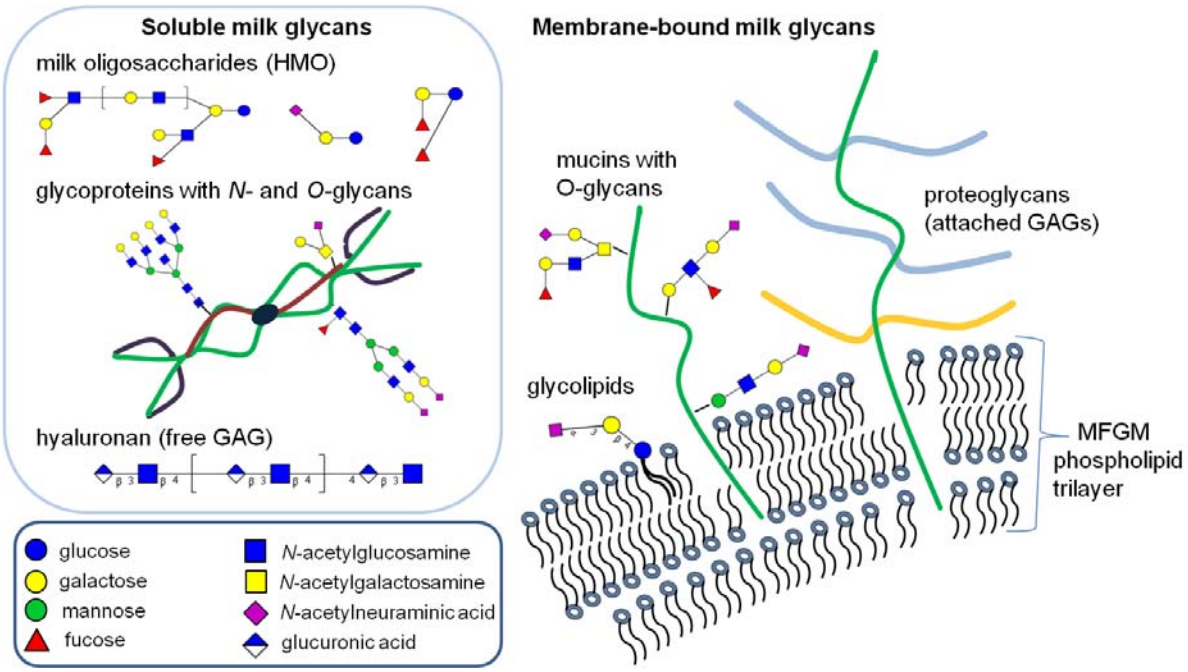

Fig. 1-2 Structural examples of human milk glycans, free (HMO) or attached to lipids/proteins, soluble or embedded in the milk fat globule membrane (MFGM). Secretory IgA and mucin schematics (green line for polypeptide chain), and glycosaminoglycans (GAG) as free hyaluronan or attached to protein (blue and yellow lines for heparin and chondroitin sulphate) are shown. Glycan symbols depicted using GlycoWorkbench (Ceroni *et al.* 2008)

The human milk glycoproteome is therefore highly complex. Hence, the glycans of only few human milk glycoproteins have been characterized so far, e.g. lactoferrin, secretory immunoglobulin A, and bile salt-stimulated lipase (Huang *et al.* 2015; Mechref *et al.* 1999; Smilowitz *et al.* 2013b). Nevertheless, since these glycoproteins are among the most abundant proteins in human milk, their contribution to the total pool of complex glycans ingested by the breastfed infant is appreciable (Froehlich *et al.* 2010). Attempts have also been made to characterize the glycoproteome of milk mucins and milk fat globule membrane proteins (Wilson *et al.* 2008).

The glycan structures bound to proteins and in particular to glycolipids in milk as well as those synthesized endogenously by intestinal epithelial cells have structural similarities (see Fig. 1-2 and (Tailford *et al.* 2015)). That may lead to ambiguous results when analyzing the metabolic fate of exogenous free oligosaccharides, i.e. HMO. However, the presence of such complex HMO-like substructures

released from endogenous or milk glycoconjugates would require specific endoglycosidases, which have not been described to be present in the infant gut to date, as further discussed in **Chapter 4**.

Another glycoconjugate class detected in human milk is that of glycosaminoglycans (mucopolysaccharides). That are anionic heteropolysaccharides containing repeating units of hexosamine and hexuronic acid (Fig. 1-2). Except for hyaluronic acid, glycosaminoglycans are bound to polypeptide chains, forming large proteoglycan macromolecules (Esko *et al.* 2009). The composition and quantities of glycosaminoglycans are significantly different in human and bovine milk and change over lactation, as can be seen in most other milk glycan families as well (Table 1-1).

Monosaccharides have also been detected in human milk, ranging in concentrations from about 0.7 µM for UDP-galactose and 1.5 mM for glucose (Arthur *et al.* 1991), building the precursors used for the biosynthesis of lactose and other milk glycans.

Overall, human milk contains substantial amounts of glycans from all common mammalian glycan classes with substantial structural and quantitative differences compared to bovine milk, which commonly is the basis for formula (Fig. 1-2, Table 1-1). The group of unbound oligosaccharides, i.e. HMO, is of particular interest, since no other body fluid or tissue in humans contains as high concentrations as found in human milk. As also is known for various glycoconjugates exhibiting certain structural motifs, HMO have been linked to defense mechanisms against pathogens, which is crucial in the first months after birth when the naive immune system of an infant faces a vast variety of harmful, but also beneficial microorganisms. Hereby, histo-blood group antigens synthesized by the products of the Secretor (Se) and Lewis (Le) genes seem to play a key role as explained in the following.

1.2 Histo-blood group antigens ABH, Secretor and Lewis

A variety of certain recognition epitopes can be found on glycoconjugates throughout many species. The best-known among them are the ABH (also called ABO) epitopes determining the blood group of an individual (Fig. 1-3). Although various tissues and blood contain ABH antigens on their glycoproteins and glycolipids, the A and B-antigens seem not to play such a significant role in human milk as in blood (Egge 1993; Gustafsson *et al.* 2005). Secretory organs such as salivary or mammary glands express an alpha1,2-fucosyltransferase (FucT) encoded by a gene that is distinct from the alpha1,2-FucT-encoding gene in non-secretory tissues. Therefore, it is referred to as the Se gene, which is active in approx. 80% of Europeans (Grubb 1948). However, even in Se-positive lactating women, who are able to synthesize the precursor for A and B antigens, i.e. H substance, in their secretions, A and B epitopes are barely found on HMO (Rudloff and Kunz 2012). Instead, the products from the activity of the FucT encoded by the Lewis gene, in addition to those synthesized by the Se-gene related FucT (Fig. 1-3), determine the oligosaccharide profile in human milk (see also Fig. 2-1).

Se status and Le blood group were linked to certain HMO structures in human milk already in the 1960's (Grollman and Ginsburg 1967). This resulted in a classification of human milk into four different groups according to the concentrations of HMO structures with Se and/or Lewis epitopes: Se+/Le+ (also called Le^b), Se-/Le+ (Le^a), Se+/Le- (Le^0 Se), or Se-/Le- (Le^0 non-Se) (Erney *et al.* 2000; Kobata 2010; Stahl *et al.* 2001; Thurl *et al.* 2010). The serological incidence among Europeans is approximately 70%, 20%, <10%, and <1%, respectively (Oriol *et al.* 1986; Race and Sanger 1975). However, these numbers are only rough estimations, since the determination of the Le and Se type *via* serotyping appears ambiguous in many cases, especially during changes of physiological conditions, such as cancer, pregnancy, and infancy (Hammar *et al.* 1981; Hirano *et al.* 1987; Lawler and Marshall 1961). This is, in part, due to the fact that the antigen-bearing glycosphingolipids which react with the antibodies in the serotyping test are not expressed in erythrocytes, but are acquired secondarily, mainly from intestinal cells (Marcus and Cass 1969). Moreover, the expression of the enzymes alpha1,2-FucT2

and alpha1,3/4-FucT3, which are the products of the Se and Le genes, respectively, has been found to vary significantly between different tissues and secretions (Orntoft *et al.* 1991; Watkins 1995).

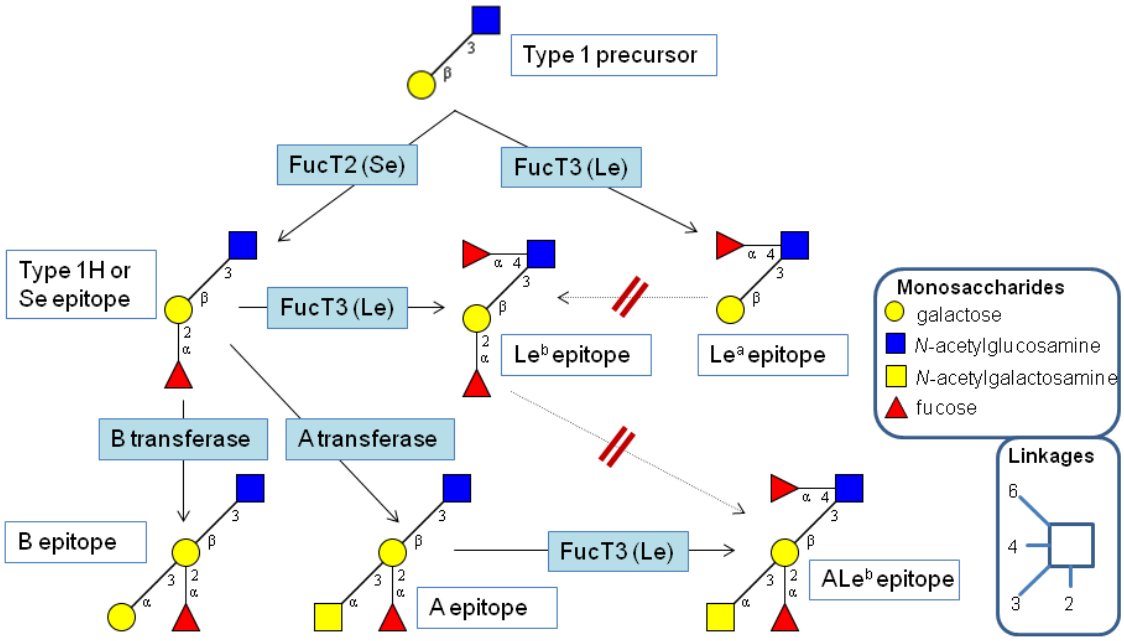

Fig. 1-3 Formation of blood group ABO and Lewis antigens. FucT, fucosyltransferase; Le, Lewis; Se, secretor. Dashed, red-crossed arrows indicate inadmissible reactions. Compiled from (Watkins 1995). Glycan symbols depicted using GlycoWorkbench (Ceroni *et al.* 2008).

Gastrointestinal expression of the various blood group glycosyltransferases has been linked to OS modifications observed in breastfed infants' feces (Albrecht *et al.* 2011a; Albrecht *et al.* 2011b). Therefore, the variety of structures being excreted in a breastfed infant's feces not only depends on the HMO and overall glycomic profile in its mother's milk, but also on the infant's blood group and Se status and age, as was first described by Arne Lundblad's group referring to fecal OS data from in total nine infants (Table 1-2). For example, mother 1 was Se-/Le+, blood group 0, and her infant's feces contained Se-specific OS, although they were not expected to be expressed in milk. This was linked to the infant's Se+ phenotype and a possible expression of alpha1,2-FucT2 in its gastrointestinal tract ((Sabharwal *et al.* 1988a), 1st three rows of Table 1-2). In infant 5, who had the same phenotype as its mother, i.e. Se+/Le+, blood group A, no Se or Le-specific OS were detected in its feces, in spite of their presence in mother's milk. Furthermore, larger A-active OS, i.e. penta- up to heptasaccharides, were detected in feces only. This indicates intense utilization/absorption and modification in the infant's gut ((Sabharwal *et al.* 1991), Table 1-2).

All this resulted in considerations of an alternative concept for the understanding of the role of Le/Se epitopes, less rigid than the classification of the ABH blood groups, which is essential in transfusion, but may possibly obscure crucial aspects of the biological significance of these carbohydrate motifs. In **Chapter 2.5** the inter-individual variability of the HMO pattern according to the anticipated biosynthesis pathways and its possible significance for the breastfed infant are discussed in more detail. Furthermore, **Chapter 2** as a whole shows how modern glycoanalytical methodologies can promote research in that complex field. Not only is the knowledge on the potential biological effects of distinct glycans in breast milk crucial for our understanding of their biological role in the infant (see **Chapters 1.3 and 2.3**), but it is also essential to elucidate the pathways and mechanisms of HMO utilization and metabolization for a better understanding of the processes *in vivo*. Hereby, glyco-analytics capable not only to characterize possible novel metabolites, but also to discriminate them according to their Le/Se specificity, will play a significant role in future HMO research, as demonstrated in **Chapter 4**.

Table 1-2 ABH blood group (BG) and Lewis/secretor-specific oligosaccharides in mother's milk and infant feces according to Lundblad and coworkers

		Se	Le	A	B	Remarks	Reference
1.	**Mother**	o	x	o	o	Milk data not reported	(Sabharwal *et al.* 1988a)
	infant	x	x	o	x	15 weeks pp	
	feces	╬	x	o	o		
2.	**Mother**	x	x	o	o	2-3 weeks pp.	(Sabharwal *et al.* 1991)
	milk	x	x	o	-		
	infant	x	x	x	-		
	feces	x	x	o	-		
3.	**Mother**	x	x	o	o	2-3 weeks pp.	(Sabharwal *et al.* 1991)
	milk	x	x	o	-		
	infant	x	x	x	o		
	feces	x	x	o	-		
4.	**Mother**	x	x	x	o	2-3 weeks pp.	(Sabharwal *et al.* 1991)
	milk	x	x	x	-		
	infant	x	x	o	o		
	feces	x	x	x	-		
5.	**Mother**	x	x	x	o	5 weeks pp.	(Sabharwal *et al.* 1991)
	milk	x	x	x	-	A-tetrasaccharide, but no larger	
	infant	x	x	x	o		
	feces	o	o	x	-	A-tetrasaccharide and larger	
6.	**Mother**	x	x	x	x	2-3 weeks pp.	(Sabharwal *et al.* 1991)
	milk	x	x	x	-		
	infant	x	x	x	o		
	feces	x	x	x	-		
7.	**Mother**	-	-	-	-	BG/milk data not reported	(Sabharwal *et al.* 1984)
	infant	x	-	x	o	4 weeks pp.; Le BG not reported	
	feces	o	o	x	-	no HMO detected	
8.	**Mother**	-	-	-	-	pooled milk	(Sabharwal *et al.* 1988b)
	infant	o	x	x	o	preterm	
	feces	o	x	o	-		
9.	**Mother**	-	-	-	-	pooled milk	(Sabharwal *et al.* 1988b)
	infant	x	x	x	o	preterm	
	feces	o	x	x	-	Various HMO detected; A-active not 3 days pp., but after 8 weeks	

x, presence of BG oligosaccharides and ╬ indicating obvious modification in infant's gut; o, not detected; -, not reported

1.3 Functional aspects of human milk glycans

Beyond the nutritive value of lactose as main carbohydrate source, a prebiotic property is anticipated for all the complex carbohydrate classes, i.e. HMO, glycosaminoglycans, glycolipids and glycoproteins. This is evidenced by studies showing that common infant gut bacterial strains are able to utilize human milk glycans for growth and do possess respective glycosidases, as further discussed in **Chapter 1.4.2**. Moreover, a substantial part of HMO has been found in the feces of breastfed infants, supporting the general opinion of HMO being non-digestible by human enzymes and serving as carbohydrate source to the infant intestinal microbiota (see also **Chapter 1.4**). Other, specific biological effects – with some links to Se status – have been described for the different glycan motifs contained in human milk, as recently reviewed in (Coppa *et al.* 2013; Etzold and Bode 2014; Georgi *et al.* 2013; Kunz *et al.* 2014; Newburg and Grave 2014). These include i) anti-adhesive, anti-microbial, anti-viral, ii) intestinal cell-response modulating, iii) immune-modulating effects, as mainly found *in vitro* in addition to some animal studies and observational studies in humans *in vivo*.

In addition to the lactose's function as osmotic regulator during milk secretion, non-digestible oligosaccharides, including HMO, may regulate the consistence and water content of the bolus during gut passage and prevent constipation in infants (Scholtens *et al.* 2014). Second, glycosylation protects certain milk proteins from proteolysis in stomach and intestine (Davidson and Lonnerdal 1987). Subsequently,

they can exert their specific functions, such as defense against pathogens, modulation of organ development and immune response within the infant's gut. After the absorption of the bioactive (glyco)peptides even systemic effects are possible (Froehlich *et al.* 2010; Georgi *et al.* 2013).

Since the different classes of glycans present in human milk often share specific structural motifs, such as the Se epitope (s. **Chapter 1.2**), *in vitro* biological activity of glycolipids, glycoproteins, glycosaminoglycans and primarily HMO against various pathogens has been shown, e.g. as antimicrobial agents (Herrera-Insua *et al.* 2001; Newburg *et al.* 1995; Ruiz-Palacios *et al.* 2003; Ruvoen-Clouet *et al.* 2006). Several extensive reviews on the functions of HMO were published recently (Bode and Jantscher-Krenn 2012; Castanys-Munoz *et al.* 2013; Kunz *et al.* 2014; ten Bruggencate *et al.* 2014), and a brief overview on Le and/or Se-related effects is given in **Chapter 2.3**. Therefore, only some specific features from very recent publications are summarized in Table 1-3.

Table 1-3 Overview on recent findings on structure-specific effects of human milk oligosaccharides

Functional property	**Structural feature(s)**	**(Model) organism**	**Reference**
Prebiotic	2'-FL, 3-FL, di-FL, 3'-SL, 6'-SL	*In vitro: Bifidobacteria spp. (B.), Bacteroides spp., Lactobacillus spp., Clostridium spp., Enterococcus spp., Streptococcus spp., Staphylococcus spp., Enterobacter spp., E. coli*	(Yu *et al.* 2013)
	Total HMO, LNT, LNnT, 2'-FL, 3-FL, 3'-SL, 6'-SL	*In vitro:* 24 *B. breve* strains isolated from infant feces, *B. longum infantis* ATCC 15697 and *B. animalis lactis* JCM 10602 as positive & negative controls	(Ruiz-Moyano *et al.* 2013)
Cell interaction bacteria-intestinal cell	HMO (vs. lactose as negative control)	*B. longum infantis, B. bifidum* isolated from infants; Caco-2, HT-29 cells	(Chichlowski *et al.* 2012)
Anti-microbial	Total HMO	Enteropathogenic *Escherichia coli (EPEC)* in intestinal cell line and *in vivo* in mice	(Manthey *et al.* 2014)
	2'FL, 6'SL, LNnT	Enterobacteriaceae (do not grow on these HMO)	(Hoeflinger *et al.* 2015)
	Total, neutral, acidic HMO, 3'SL	Uropathogenic *E. coli* in human bladder epithelial cells	(Lin *et al.* 2014)
	Bioengineered 2'-FL and 3-FL	*Campylobacter jejuni*, EPEC*, Salmonella enterica* serovar *fyris*, *Pseudomonas aeruginosa* in human intestinal Caco-2 and respiratory epithelial cell lines	(Weichert *et al.* 2013)
Anti-viral	32 individual HMO (out of 247 tested)	Rotavirus attachment proteins	(Yu *et al.* 2014)
	Total HMO, 3'-SL, 6'-SL, LN*n*T	Rotavirus-infected cell lysates in MA-104 cells and *in situ* in piglets	(Hester *et al.* 2013)
Gut motility	2'-FL and 3-FL	Mouse distal colon segments	(Bienenstock *et al.* 2013)
Immune-modulation	Total HMO, FL-mix, SL-mix, 2'-FL, LN*n*T	Peripheral blood mononuclear cells from piglets (*ex vivo*)	(Comstock *et al.* 2014)
	HMO, 2'FL	Various intestinal epithelial cell lines and mice upon inflammation	(He *et al.* 2016)
Protecting HIV-transmission	Total HMO concentration	HIV-infected, breastfeeding women	(Bode *et al.* 2012)

1.4 HMO digestion and metabolization

1.4.1 Oral and gastrointestinal digestibility and absorption

According to a recent review on glycan degradation in breastfed infants (Dallas *et al.* 2012), a few enzymes present in the human digestive tract may be able to degrade human milk glycans. Those are exoglycosidases, detected in human saliva (β-galactosidase), homogenized gastric mucosa or homogenized brush border tissue. However, most likely they do not play a quantitatively significant role, since i) linkage-specificity is not determined or not matching human milk glycans in several cases, and ii) data from homogenized tissue does not assure their presence on cell surface with possible contact with the substrates (Dallas *et al.* 2012).

These considerations are supported by *in vitro* digestibility studies showing HMO to be resistant to the whole range of conditions and enzymes present in the human oro-gastrointestinal tract, as summarized in Table 1-4. Engfer and colleagues obtained similar results by applying human and porcine intestinal brush border membranes and human duodenal aspirates to digest neutral and acidic HMO fractions or single HMO standards *in vitro* (Engfer *et al.* 2000).

Table 1-4 *In-vitro* digestibility of HMO and related carbohydrates

Substrate [a]	Salivary amylase [b]	HCl pH 2.5 [c]	Pancreatic amylase [c]	BBMV pH 5-7 [d]	PBS [c]
Neutral HMO	0%	0%	0%	2 h: +monosaccharides 4-24 h: +lacto-*N*-triose, Lac, monosaccharides	0%
Acidic HMO	0%	Slightly (+NeuAc, Lac)	0%	<5% (+monosaccharides)	0%
Lactose (Lac)	0%			100%	
Lactulose				12%	

[a] Fractions extracted from human milk or single HMO standards were used; Lac as positive control for lactase from BBMV, lactulose as non-digestible reference carbohydrate; [b] incubated at 37°C for 1 min at pH 7.0 and for 2 h at pH 5.5; [c] incubated at 37°C for 2 h. [d] BBMV, brush border membrane vesicles from porcine small intestine. Compiled from (Gnoth *et al.* 2000).

In-vivo or *ex-vivo* studies on the digestibility of HMO are rare due to practical and ethical reasons. However, a few investigations do exist. For example, a study in suckling neonatal rats showed significant modifications of the oligosaccharide patterns throughout all the segments of the small intestine, when being fed HMO (Jantscher-Krenn *et al.* 2013).

Substantial amounts of HMO were detected many years ago in feces and urine from breastfed infants (Table 1-2, (Chester *et al.* 1981; Rudloff *et al.* 1996)). Therefore, the absorbability of HMO has also been examined previously. LNT and lacto-*N*-fucopentaose (LNFP) I were shown to be transported and retained from the apical to basolateral compartment of intestinal cells *in vitro* (Gnoth *et al.* 2001). Another study found 3% of the apically applied LNT in the basolateral compartment after 90-min incubation (Eiwegger *et al.* 2010).

Indirect evidence for the absorption of various HMO structures *in vivo* by detecting HMO structures in the urine of breastfed infants has been provided (Rudloff *et al.* 1996). Absorption has been determined at 1–3% *via* intrinsic isotopic labeling of HMO and isotope-ratio (IR) MS analysis of the total OS fraction in urine (Rudloff *et al.* 2006; Rudloff *et al.* 2012). Recently, two independent investigations detected higher concentrations of several neutral HMO structures in the blood of breastfed vs. formula-fed infants (Goehring *et al.* 2014; Ruhaak *et al.* 2014). This indicates the uptake of OS ingested *via* moth-

er's milk for the former, but also the presence of endogenous or formula-derived HMO-like structures in the latter, as discussed in **Chapter 4.5**. An origin from sources other than mother's milk can be deduced particularly from the high abundance of sialylated OS in formula-fed infants as reported in (Ruhaak *et al.* 2014). Since complex HMO structures are based on other (core) structures which are also present in human milk in high amounts (**Chapter 2.2**), a discrimination of intact human milk-derived vs. degraded HMO, in practice, can only be achieved *via,* e.g. stable-isotopic, labeling of the OS prior to ingestion *in vivo*, as successfully applied by our group and reported in **Chapter 3**.

1.4.2 HMO metabolism

Due to the assumed non-digestibility of HMO in the upper digestive tract and their possible function as the so-called 'Bifidus factor' (historical review in (Kunz 2012)), i.e. their possible prebiotic properties, HMO metabolization is thought to mainly take place in the infant colon, where concentrations of microbial organisms range from approximately 10^8–10^{10} rRNA gene copies/g as deduced from fecal sample analyses (Palmer *et al.* 2007). Thus, numerous studies on the *in-vitro* metabolization of oligosaccharides and other glycans from human milk by various bacterial species and strains have been performed to date. Major focus is on *Bifidobacteria* and *Bacteroides*, since they represent highly abundant genera especially in newborns and have been shown to grow well on HMO fractions and single compounds as sole carbohydrate source (Marcobal *et al.* 2010; Yu *et al.* 2013). The genome of *Bifidobacterium longum* subsp. *infantis* strains contains gene clusters with capacity to bind, import and cleave milk oligosaccharides, e.g. by intracellular fucosidases and sialidases (LoCascio *et al.* 2010). Further bacterial enzymes putatively involved in HMO degradation are summarized in (Garrido *et al.* 2013; Kitaoka 2012). Other bacterial species are not able to assimilate complex HMO, but secrete extracellular hydrolases prior to the translocation of degraded smaller HMO fragments instead. Therefore, the HMO-utilizing breastfed infant's gut microbiome is thought to be comprised of a complex cooperative mixed-species consortium (Sela and Mills 2010). HMO utilization between different *Bifidobacteria* species and even within the same species, but between different strains, has been found to be highly structure-specific (Ruiz-Moyano *et al.* 2013; Strum *et al.* 2012). Conceivably, the metabolic profile from glycan degradation in the infant's gut *in vivo* should vary immensely due to individual differences in microbiota composition and quantity (Fallani *et al.* 2010; Penders *et al.* 2005). Taken into account the structural diversity of the OS pattern and quantity in human milk, as well as a contribution of endogenous glycans and possible modifications by the infant's gut cells, the variety of OS structures excreted in breastfed infants' feces must be enormous, as indicated in a few profiling studies (Albrecht *et al.* 2011b; De Leoz *et al.* 2013). Furthermore, fecal OS profiles seem to undergo dynamic changes in association with the infants' age, with an increasing number and amount of metabolites occurring from 3–4 months after birth (Albrecht *et al.* 2011a).

However, *in vivo* metabolites data in literature are limited to publications including only few study subjects and detected OS structures (Table 1-2). To obtain more information on the *in-vivo* processes of OS metabolization and modification in the infant gut, two sets of fecal samples from 30 infants in total were analyzed, as reported in **Chapters 4** and **5**. Moreover, possible HMO metabolites after absorption, along with hypotheses on the sites of modification and utilization, are presented in **Chapter 4** by urinary OS data, which is unique so far.

1.5 State-of-the-art HMO analytics

Since human milk is a highly complex mixture of heterogeneous compounds, as presented in **Chapter 2**, the analysis of milk components including HMO is challenging. Therefore, multiple purification steps are often necessary. Traditionally, after delipidation (only milk) and protein precipitation, size exclusion and ion-exchange chromatographic purification were performed prior to analysis by gas, paper, affinity or thin-layer chromatography (Hallgren *et al.* 1977; Kobata 2010; Rudloff *et al.* 1996; Sabharwal *et al.* 1991). The preferred strategies for purification and analysis of milk components by (glyco)proteomic, (glyco)lipidomic and glycomic approaches were briefly reviewed recently (Dallas *et al.* 2013).

Currently used strategies for purification, separation and detection of HMO and related OS as relevant for this work are described in the following. Of note, a brief overview of various methods for HMO analysis published before 2012 is given in (Ruhaak and Lebrilla 2012).

1.5.1 Sample preparation strategies

During the last decade, solid phase extraction (SPE) has widely been used for OS purification, as it allows high-throughput operation and the use of various stationary phases. Ion-exchange resins were traditionally applied for the retention of polar compounds as are glycans. However, they have mainly been replaced by other materials that are easier to handle (Hanai 2003). For example, porous graphitized carbon (PGC) is widely used to purify non-derivatized HMO and related OS (Albrecht *et al.* 2011b; Blank *et al.* 2011; De Leoz *et al.* 2013; Dotz *et al.* 2015), as it is highly selective for glycans (Koizumi 1996). PGC material is highly stable over the whole pH range, and it has a homogenous structure as compared to silica or ion-exchange resins (Hanai 2003). The retention mechanisms are complex and not completely understood (Ruhaak *et al.* 2010b), but are thought to base mainly on hydrophobic and electrostatic interactions (Gu and Lim 1990). Hydrophobicity is higher than in other reversed-phase (RP) materials (Koizumi 1996), enabling an elution of the hydrophilic glycans while hydrophobic compounds are still retained. Hydrophilic interaction liquid chromatography (HILIC) materials are gaining increasing popularity in OS purification, as they are non-expensive and widely applicable also for glycopeptides as well as derivatized glycans (Reiding *et al.* 2014; Ruhaak *et al.* 2010b; Selman *et al.* 2011). However, HILIC SPE has not been applied on HMO or related OS from urine or feces so far.

Various derivatization methods have been developed for different purposes in OS analysis, as reviewed in (Harvey 2011; Ruhaak *et al.* 2010b). In context of HMO, permethylation and reductive amination by fluorescent tags have mainly been applied as derivatization techniques. Permethylation has, among others, the following advantages in MS analysis of OS: i) enhanced signal intensities/sensitivity, ii) preservation of labile sialic acids, iii) simultaneous detection of both neutral and sialylated OS species in the positive ion mode, iv) enhanced cross-ring fragmentation during tandem MS (MS/MS) (Alley *et al.* 2013). Furthermore, increasing the hydrophobicity of OS allows liquid chromatography (LC) separation *via* RP materials, as enabled by permethylation or the attachment of hydrophobic tags such as 2-aminobenzamide (2-AB) (Alley *et al.* 2013; Ruhaak *et al.* 2010b). The latter, 2-AB, has the advantage of allowing for a straightforward quantitative LC-fluorescence OS analysis, which is extensively used in glycomics, especially in HILIC-mode LC (Ruhaak *et al.* 2010b). This has also been demonstrated for animal milk OS profiling (Albrecht *et al.* 2014), for the quantitation of HMO consumption from rat intestines (Jantscher-Krenn *et al.* 2013), and for in-depth structural characterization of novel HMO (Blank *et al.* 2012b). 2-Aminobenzoic acid (2-AA) is another very popular fluorescent tag in OS analytics, since it provides a single negative charge and enhances MS ionization and detection (Ruhaak *et al.* 2010b). Furthermore, 2-aminoacridone has been applied for on-line fluores-

cence-electrospray ionization (ESI)-MS detection of HMO in HILIC-mode separation on an amide HPLC column (Galeotti *et al.* 2012).

However, most approaches applied in HMO research did not imply derivatization techniques to keep sample preparation as simple and fast as possible, especially in the context of high-throughput analysis. In the following, the analytical techniques as used in this work are presented in more detail, followed by a brief overview of other methods found in literature within the field of HMO research.

1.5.2 High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

As mentioned above, fluorescently labeled OS can be isomer-separated and detected *via* HILIC- or RP-HPLC after appropriate derivatization. Alternatively, effective isomer separation and quantification of non-derivatized HMO can be performed in HPAEC-PAD (Dotz *et al.* 2015; Erney *et al.* 2000; Kunz *et al.* 1996; Rudloff *et al.* 2006; Thurl *et al.* 2010). In the hydroxide-containing mobile phase, oxyanions are formed from carbohydrates at pH >12, and thus are separated *via* a sodium acetate gradient on polystyrene-divinylbenzene beads, which act as anion-exchange resin (Rohrer *et al.* 2013). Retention is based on charge and size of the OS molecules. At alkaline conditions oligosaccharides are oxidized on a gold electrode and generate a current, which is registered by the PAD, resulting in sensitivity at picomolar range (Rohrer *et al.* 2013). Identification is done *via* external calibration based on retention time (**Chapter 4**). However, coupling to a mass spectrometer after thorough desalting has also been shown useful in the analysis of OS from infants' urine (Bruggink *et al.* 2010). In the current work data from HPAEC-PAD with the advantage of isomeric separation were combined with the structurally informative and rapid MS analysis *via* MALDI-TOF-MS as shown in **Chapter 4**.

1.5.3 MALDI-TOF-MS

MS approaches are rapidly emerging, also in the field of glycan and HMO analysis. Known as soft-ionization techniques, MALDI- and ESI-MS are widely used for HMO profiling, characterization, and (relative) quantitation. From the obtained *m/z* values monosaccharide composition can be directly calculated (Table 1-5). After fragmentation *via* MS/MS, structural features of the molecules of interest can further be evaluated. This is a major advantage compared to LC alone that relies only on the retention time of the analytes lacking structural information. Nevertheless, co-detection of isobaric structures and ion suppression are major limitations of direct MS, resulting in decreased signal intensities. This occurs to a high extent in direct ESI-MS of analyte mixtures obtained from complex biological matrices, where residual salts and other interfering compounds affect OS ionization and moreover lead to complex mass spectra due to multiple charge states (Zaia 2010). In contrast, MALDI-TOF-MS is the preferred technique for profiling complex samples, especially from large-scale clinical studies, due to its ease-in-use and high-automation and high-throughput potential (Blank *et al.* 2012a; Zaia 2010). The multi-facetted aspects of glycan analysis *via* MALDI-TOF-MS were extensively reviewed recently (Harvey 2015).

Table 1-5 Mass increment values of carbohydrate structures and possible metal ion adducts relevant in HMO analysis

Carbohydrates	Mass increment
Deoxyhexose (e.g. fucose)	146.06
Hexose (e.g. glucose or galactose)	162.05
N-acetylhexosamine (e.g. *N*-acetylglucosamine)	203.08
N-acetylneuraminic acid	291.10
Adduct ions	
Sodium	22.99
Potassium	38.96

The principles of the Ultraflex TOF/TOF instrument from Bruker Daltonics, as used in this work, are shown schematically in Fig. 1-4. The purified oligosaccharide (mixture) is being co-crystallized with the MALDI matrix substance, e.g. 2,5-dihydroxybenzoic acid (DHB), on a sample target plate. Under vacuum conditions the sample-matrix co-crystals are excited by a nitrogen laser emitting at 337 nm (Suckau *et al.* 2003). In the positive ion mode this usually results in desorption and ionization of the OS molecules as singly charged quasimolecular ions after alkali metal adduction. The ions are accelerated applying 25 keV/8 keV in MS or MS/MS mode, respectively, and are subsequently separated according to their *m/z* value during their drift in the field-free TOF region. Through the timed ion selector only the precursor ion with its fragments can pass through if MS/MS mode is active and the *m/z* of the respective precursor has been set. In source 2, precursor and fragment ions are post-accelerated, since their potential energy is 'lifted' by additional 19 keV in LID MS/MS mode (s. **Chapter 1.5.4**). Subsequently, in TOF2 region the ions are separated according to their *m/z*, since the smaller fragments have had lower initial energy, which is proportional to their mass and deduced from the initial 8 keV in the precursor. An ion mirror (reflectron) focuses and redirects the ion-beam and extends the flight length, which results in higher resolution in the dual micro channel plate detector (Suckau *et al.* 2003).

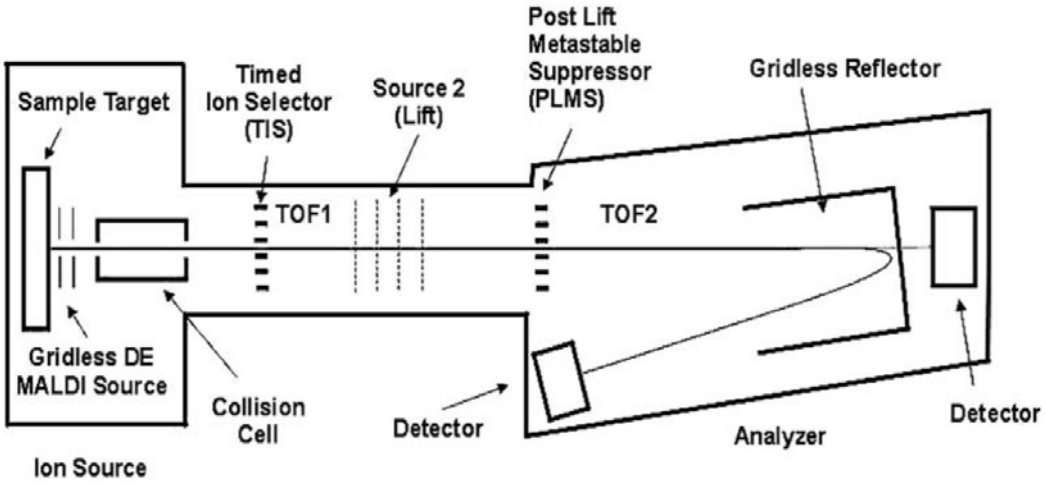

Fig. 1-4 Schematic of the MALDI-LIFT-TOF/TOF mass spectrometer. TOF1 ranges from the MALDI ion source to the LIFT cell, TOF2 from the second accelerator stage in the LIFT cell to the reflector [Reproduced with kind permission from Springer Science+Business Media (Suckau *et al.* 2003)]

MALDI-TOF-MS has not only been used for rapid OS profiling in human milk, infant urine and feces (Dotz *et al.* 2015), **Chapters 4** and **5**, but has also been shown useful for monitoring dynamic urinary excretion of HMO intrinsically labeled by the stable isotope ^{13}C (Dotz *et al.* 2014), s. **Chapter 3**.

1.5.4 Tandem MS

Tandem MS provides the additional value of structural elucidation of the analytes, although the obtained information is often limited in case of oligosaccharides as compared to polypeptides. This is due to the high structural diversity of glycans with variable branching, linkage and modification possibilities, as described for HMO in **Chapter 2**. Therefore, no universal database or software for fragment spectra interpretation of the human glycome is available so far (An and Lebrilla 2011), though attempts have been made towards such tools also in the context of HMO (Totten *et al.* 2014; Wu *et al.* 2011; Wu *et al.* 2010).

In HMO analysis, the following tandem MS methods have recently been applied for structural characterization: collision-induced dissociation (CID) (Albrecht *et al.* 2010; Blank *et al.* 2012b; Ferreira *et al.* 2010; Jovanovic *et al.* 2014; Wu *et al.* 2010), laser-induced dissociation (LID) (Blank *et al.* 2011; Dotz *et al.* 2015), infrared multiphoton dissociation (De Leoz *et al.* 2013; Wu *et al.* 2010), and – very novel for glycan fragmentation studies – electron-transfer dissociation (Han and Costello 2011). Glycosidic bond cleavages, resulting mainly from low-energy CID in ion trap instruments, provide monosaccharide compositional and sequential information. According to the widely used nomenclature from (Domon and Costello 1988), B and C, or Y and Z fragments are generated, containing the reducing or the non-reducing end, respectively. For the determination of branching and linkage patterns, cross-ring cleavages generating A and X fragments, are necessary. Those are more abundant in techniques which allow higher energy transfer (Zaia 2010). However, the specific structure of the OS highly affects dissociation behavior in the respective MS/MS instrument (Alley *et al.* 2013). An unusual example is given in **Chapter 4**, where highly abundant cross-ring fragments of a possible HMO metabolite were obtained by LID in MALDI-TOF/TOF MS/MS analysis.

1.5.5 Other recent analytical techniques for OS originating from milk

In addition to the previously mentioned classic OS analysis methods by fluorescence HPLC and HPAEC-PAD as well as our more recent MALDI-TOF-MS approach, the following recent developments should not remain unmentioned.

LC-ESI-MS is known to be superior to most other techniques for the structural characterization of glycans due to the possibility of separation of structural isomers, e.g. prior to MS/MS for acquiring additional structural information. LC column materials commonly used for OS separation are PGC and HILIC, as already described in **Chapter 1.5.1** for OS purification. PGC has been used for quantification of major OS in human milk (Bao *et al.* 2013), in bacterial fermentation samples *via* triple-quadrupole MS (Yu *et al.* 2013), or in nanoLC-chip-TOF MS for quantification of bacterial HMO consumption *via* deuterated internal OS standards (Strum *et al.* 2012). The latter approach has been extended to infant fecal and urine (De Leoz *et al.* 2013) as well as plasma samples (Ruhaak *et al.* 2014). HILIC-LC-MS has been applied for the quantification of OS from infant plasma and urine *via* triple-quadrupole MS (Goehring *et al.* 2014) and in Orbitrap-MS to quantify the major OS in bovine milk (Liu *et al.* 2014).

A promising alternative to LC-based approaches for OS profiling is capillary (gel) electrophoresis, either in multiplexed high-throughput manner with laser-induced fluorescence detection (CGE-LIF) (Kottler *et al.* 2013), or as CE-LIF with the possibility of additional MS characterization (Albrecht *et al.* 2010). The latter approach has revealed unprecedented insights into the possible metabolization of HMO in the breastfed infant (Albrecht *et al.* 2011a; Albrecht *et al.* 2011b), which have generated hypotheses that among others were intensively studied in this current work (s. **Chapter 1.6**).

Other approaches include off-line PGC-LC-MALDI-TOF-MS (Yu *et al.* 2014), direct ESI-ion trap-MS^n (Ashline *et al.* 2014), and MALDI-quadrupole-TOF-MS (Jovanovic *et al.* 2014). Furthermore, nuclear

magnetic resonance has been applied for metabolic profiling of human milk (Pratico *et al.* 2014), for quantification of major HMO (Smilowitz *et al.* 2013a), to determine Le and Se epitopes from HMO (van Leeuwen *et al.* 2014), or to characterize OS from milk of different primates (Taufik *et al.* 2012). Moreover, for an unambiguous in-depth structural characterization, a combination of the described techniques with more laborious traditional strategies, such as linkage-specific exoglycosidase digestion (Albrecht *et al.* 2014) or GC-MS of permethylated, hydrolyzed, peracetylated HMO still has its place in modern HMO analytics (Blank *et al.* 2012b; Wu *et al.* 2010).

1.6 Project scope and objectives

The examination of the metabolic fate of HMO in the exclusively breastfed infant is the superior objective of this work. This implies two major scientific questions:

1) What individual HMO structures are found in breastfed infants' urine, as evidence for their intestinal absorption and indication for systemic and local effects of certain HMO structures within the infant's organism?
2) Which possible metabolites (next to intact HMO) can be found in particular in infants' urine and feces, describing the metabolic fate of HMO?

The first question is based on the hypothesis that in addition to small, fucosylated and non-fucosylated compounds even large, very complex, multiply fucosylated HMO structures may be absorbed, as was deduced from the detection of such structures in fast atom bombardment-MS after gel filtration of infant urinary OS (Rudloff *et al.* 2012). IR-MS data of urine from infants fed ^{13}C-enriched mother's milk supported this. However, by analyzing the mass isotopomer enrichment of ^{13}C-labeled urinary OS over time in the current project by means of MALDI-TOF as an organic MS method, both, the milk origin and the structural composition of the detected molecular species, can be assured within the same measurement. Therefore, an analytical approach had to be developed allowing for i) reliable determination of OS isotopomer distribution from human milk and infant urine and ii) rapid sample preparation and sensitive analysis, since hundreds of samples with only small analyte amounts (urine) had to be analyzed from the 36 h cohort (**Chapter 3**; Fig. 1-5). To introduce the reader to the challenges and potentials of modern HMO analytics in the context of large-scale clinical studies, **Chapter 2** further gives an overview on the structural and functional features of HMO, with focus on the Le and Se epitopes.

The second question arose from the few data available in literature indicating intense modification and degradation of HMO in the lower gut due to altered OS patterns in infants' feces, as demonstrated by the studies of Lundblad's group (Table 1-2) and more recently in (Albrecht *et al.* 2011a). In addition, Chaturvedi and colleagues claimed that no modifications in the upper intestine seem to take place, as deduced from urinary OS profiles resembling the milk ones (Chaturvedi *et al.* 2001). In order to prove these hypotheses, the methods established for the preparation and analysis of milk and urinary oligosaccharides in the first part of this project had to be adjusted to enable sensitive MS analysis of OS from infant feces (Fig. 1-5). Subsequently, oligosaccharide profiles could be compared, providing indications on the metabolic fate of the oligosaccharides ingested *via* mother's milk. Supportively, the presence of Le and Se-specific OS in milk, urine and feces, by means of an established HPAEC-PAD method, capable to separate isomers, was analyzed. Using MALDI-TOF/TOF-MS the structural composition of possible metabolites as deduced from profile MS measurements was proven (**Chapter 4**; Fig. 1-5).

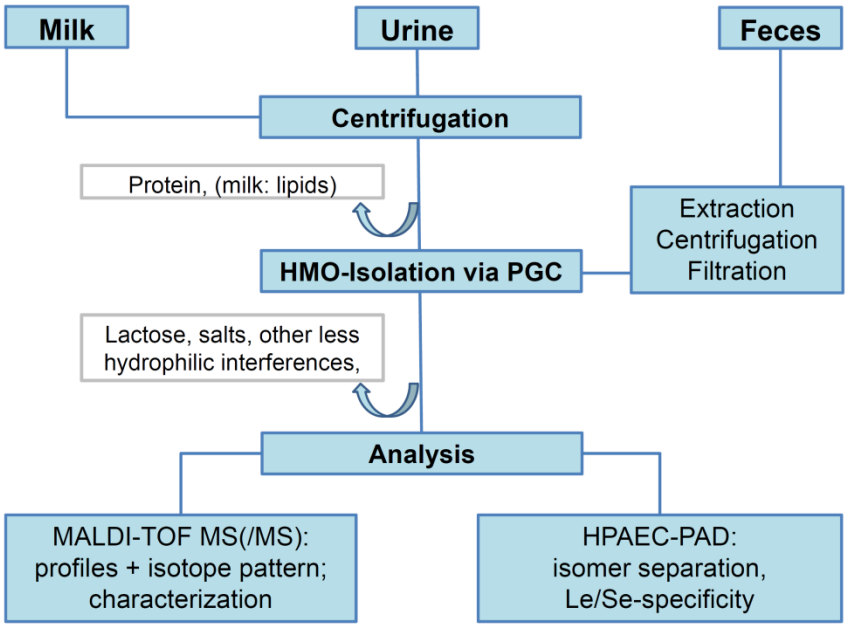

Fig. 1-5 Schematic of the strategy for OS analysis in human milk and infants' urine and feces

Additional fecal samples from another cohort comprised of breastfed as well as formula-fed infants of two different ages were analyzed to strengthen the findings described in **Chapter 4**. Moreover, the MALDI-TOF-MS method has been modified to enable automated, rapid and simple high-throughput OS analysis from infant feces in future investigations (**Chapter 5**).

Chapter 2

AN Advances in Nutrition An International Review Journal

Human Milk Oligosaccharides and Lewis Blood Group: Individual High-Throughput Sample Profiling to Enhance Conclusions From Functional Studies[1,2]

Dennis Blank,[3,5] Viktoria Dotz,[4,5] Rudolf Geyer,[3] Clemens Kunz[4*]

[3]Institute of Biochemistry, Faculty of Medicine and [4]Institute of Nutritional Science, Justus-Liebig-University of Giessen, Giessen, Germany

ABSTRACT

Human milk oligosaccharides (HMO) are discussed to play a crucial role in an infant's development. Lewis blood group epitopes, in particular, seem to remarkably contribute to the beneficial effects of HMO. In this regard, large-scale functional human studies could provide evidence of the variety of results from in vitro investigations, although increasing the amount and complexity of sample and data handling. Therefore, reliable screening approaches are needed. To predict the oligosaccharide pattern in milk, the routine serological Lewis blood group typing of blood samples can be applied due to the close relationship between the biosynthesis of HMO and the Lewis antigens on erythrocytes. However, the actual HMO profile of the individual samples does not necessarily correspond to the serological determinations. This review demonstrates the capabilities of merging the traditional serological Lewis blood group typing with the additional information provided by the comprehensive elucidation of individual HMO patterns by means of state-of-the-art analytics. Deduced from the association of the suggested HMO biosynthesis with the Lewis blood group, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry profiles of oligosaccharides in individual milk samples exemplify the advantages and the limitations of sample assignment to distinct groups. *Adv. Nutr. 3: 440S–449S, 2012.*

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[2] Author disclosures: D. Blank, V. Dotz, R. Geyer, and C. Kunz, no conflicts of interest.

[5] Authors contributed equally to this work.

* To whom correspondence should be addressed. E-mail: Clemens.kunz@uni-giessen.de.

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2 Human milk oligosaccharides and Lewis blood group – Individual high-throughput sample profiling to enhance conclusions from functional studies (Manuscript 1)

2.1 Abstract

Human milk oligosaccharides (HMO) are discussed to play a crucial role in an infant's development. Lewis blood group epitopes, in particular, seem to remarkably contribute to the beneficial effects of HMO. In this regard, large-scale functional human studies could provide evidence of the variety of results from *in vitro* investigations, although increasing the amount and complexity of sample and data handling. Therefore, reliable screening approaches are needed. To predict the oligosaccharide pattern in milk, the routine serological Lewis blood group typing of blood samples can be applied due to the close relationship between the biosynthesis of HMO and the Lewis antigens on erythrocytes. However, the actual HMO profile of the individual samples does not necessarily correspond to the serological determinations. This review demonstrates the capabilities of merging the traditional serological Lewis blood group typing with the additional information provided by the comprehensive elucidation of individual HMO patterns by means of state-of-the-art analytics. Deduced from the association of the suggested HMO biosynthesis with the Lewis blood group, the matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry profiles of oligosaccharides in individual milk samples exemplify the advantages and the limitations of sample assignment to distinct groups.

2.2 Introduction

Free lactose-derived oligosaccharides in human milk are present at concentrations ranging approximately from10 to 20 g/L; hence, several grams of these unique components pass through the breast-fed infant's gut daily (Rudloff *et al.* 2012; Thurl *et al.* 2010; Urashima *et al.* 2012). Various health protective actions have been deduced from *in vitro* investigations, i.e., prebiotic, anti-infective, or immune effects (Bode 2009; Kunz *et al.* 2000), which might partially be associated with the presence of fucosylated oligosaccharide structures. Those are determined by the expression of the Se and Le genes in the mammary gland. Hence, distinct patterns of milk oligosaccharides according to the Le/Se types Le(a+b-) non-Se, Le(a-b+) Se, and Le(a-b-) Se or non-Se genes have been described by several investigators (Erney *et al.* 2001; Kobata 2010; Thurl *et al.* 2010). The prevalence in white individuals averages 22%, 72%, and 6%, respectively (Race and Sanger 1975), whereas 80% of the Europeans secrete ABH substances in saliva and other secretions; thus, they are typed as Se (Grubb 1948; Schiff 1932). Observational studies indicate that certain HMO from secretor are associated with various preventive effects, such as reducing diarrhea and promoting intestinal maturation in preterm neonates (Morrow *et al.* 2011; Newburg *et al.* 2004).

The final evidence of the functionality of the Le-related HMO compounds in humans has to be confirmed by intervention studies with large numbers of participants. Therefore, reliable high-throughput screening methods are needed to determine the oligosaccharide profiles in individual milk samples. Due to high inter- and intra-individual variations in HMO expression, the relationship between the serologically detectable Le blood group and the corresponding oligosaccharide pattern in milk can only be used for a crude milk classification, even though serological detection as a routine method is rapid and highly practicable. Nevertheless, detailed information on the HMO composition in milk samples provides a more accurate indication of the substances actually ingested by the infant. For this purpose, new developments in the field of glycomics have great potential for facilitating the handling of large

sample and data sets from follow-up investigations on the correlation of Le-active components in infants' feeding and their health (Liu *et al.* 2010; Rakus and Mahal 2011; Ruhaak *et al.* 2010a).

In this review, we demonstrate the correlation of the serologically detected Le blood group and the expressed HMO pattern in the milk of the donors and show how this information can be enhanced by recent high-throughput HMO screening methods. On the basis of the suggested biosynthesis of HMO, specific variances in the HMO pattern are taken as examples to highlight the necessity of careful individual milk sample analysis.

2.3 Biosynthesis of Le and Se gene-related oligosaccharides in milk

In the past years, the structures of the major HMO have been thoroughly characterized (Urashima *et al.* 2011; Wu *et al.* 2010). From these data, some structural rules have been deduced and the biosynthetic pathways of the neutral HMO have been proposed (Fig. 2-1). Unfortunately, experimental data on the biosynthesis of HMO are lacking to date.

Because the reducing end of the unbound oligosaccharides from milk consistently contains lactose, which is the major macronutrient in human milk, this disaccharide is assumed to be the initial substrate for HMO synthesis. Lactose is formed in the Golgi apparatus by the action of the lactose synthase complex containing α-lactalbumin and β1–4-galactosyltransferase (Brew and Hill 1975). UDP-activated galactose (Gal) is attached to glucose-I-P with high affinity due to the presence of α-lactalbumin, which is only expressed in the lactating mammary gland of mammals.

We speculate that analogous to the O-glycosylation of proteins in the Golgi of submaxillary and gastrointestinal secreting cells, the glycosyltransferases for HMO synthesis might occur as membrane-bound glycoproteins and process the oligosaccharide sequentially by the addition of a single monosaccharide from sugar nucleotides. Those are synthesized in the cytosol and conveyed to the Golgi lumen *via* specific membrane antiporters, e.g., GLUT1 for monophosphorylated glucose (Brockhausen 1995; McManaman and Neville 2003; Roth 1995).

Thus, elongation (a), branching (b), and fucosylation (c) of lactose and derived structures might be performed by the concerted action of (a) iβ1–3-*N*-acetylglucosaminyltransferase and β1–3- and β1–4-galactosyltransferase for type 1 and type 2 chains, respectively, and (b) Iβ1–6-*N*-acetylglucosaminyltransferase as depicted in Fig. 2-1. Following the suggested rules of HMO synthesis, no further elongation is observed for a terminal type 1 chain (indicated by a no entry sign in Fig. 2-1) (Almeida *et al.* 1997; Kobata 2010; Sasaki *et al.* 1997). The final fucosylation and Le antigen formation is achieved by the consecutive action of α1–2-, α1–3-, or α1–3/4-fucosyltransferases (FucT) (c), as summarized in Fig. 2-2.

The presence of at least 1 functional allele of the Le gene results in the expression of an α1–3/4-FucT (FucTIII), which is able to attach GDP-activated Fuc in the O-4 position to GlcNAc in type 1 (Galβ1–3GlcNAc) chains, resulting in Le epitopes, as shown in Fig. 2-1. The same enzyme forms O-3-Fuc units at the GlcNAc residue of type 2 (Galβ1–4GlcNAc) chains yielding Le^x and, in secretors, Le^y epitopes, however with lower specificity than for type 1 substrates due to steric aspects (Khare *et al.* 1985). The *O*-3-fucosylation of the reducing glucose residue is known to be accomplished by the Le-gene–dependent FucTIII as well (Eppenberger-Castori *et al.* 1989). Furthermore, the formation of Le^x and Le^y epitopes can also be performed by different α1–3-FucTs, i.e., FucTIII–VII and FucTIX (Koda *et al.* 2001).

Although secretory tissues and fluids have predominantly FucTII activity encoded by the Se gene, i.e., milk (Betteridge and Watkins 1985; Shen *et al.* 1968; Watkins 1995), saliva, or stomach tissue (Chester and Watkins 1969), in human serum, both FucTII and the H gene–controlled FucTI are pre-

sent (Le Pendu *et al.* 1985). Both enzymes transfer GDP-activated Fuc in α1–2-position to β-D-galactosides prior to the formation of Le^b and Le^y epitopes (Fig. 2-1).

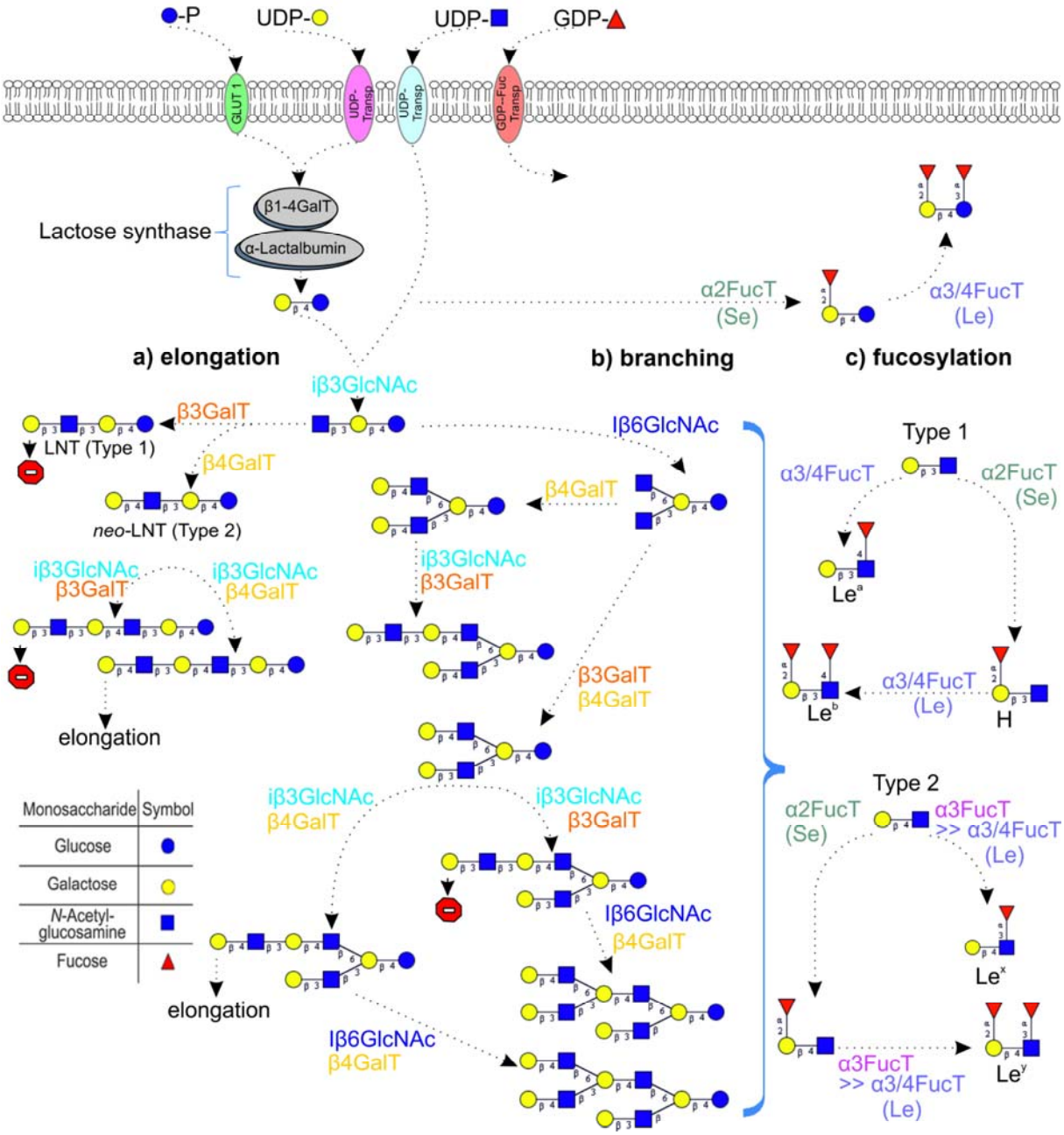


Fig. 2-1 Biosynthesis of neutral complex human milk oligosaccharides (HMO). The assumed biosynthetic pathway starts from the activated monosaccharides and includes the most important enzymes only [*N*-acetylglucosaminyltransferases (GlcNAcT)]: iβ3GlcNAcT attaches *N*-acetylglucosamine (GlcNAc) in the β1–3 position to terminal galactose (Gal), Iβ6GlcNAcT attaches GlcNAc in β1–6 position to terminal Gal. Galactosyltransferases (GalT): β3GalT attaches Gal in the β1–3 position to GlcNAc and β4GalT attaches Gal in the β1–4 position to GlcNAc. Fucosyltransferases (FucT): α2FucT attaches fucose (Fuc) in the α1–2 position to terminal Gal, secretor (Se) enzyme, α3FucT attaches Fuc in the a1–3 position to GlcNAc, α3/4FucT attaches Fuc in the α1–3/4 position to GlcNAc and in the α1–3 position to Glc of the lactose core, Lewis (Le) enzyme. The no entry signs mean that no further elongation takes place. Fucosylation is indicated exemplarily for terminal type 1 and type 2 chains. Glycan structures are depicted according to the recommendations of the Consortium of Functional Glycomics using the GlycoWorkbench software tool (Ceroni *et al.* 2008).

The Le and Se gene-encoded FucTs compete for the substrates so that in secretors Le^a structures are also found in milk and other secretions, but not on erythrocytes or in plasma (Daniels 2002; Watkins 1995).

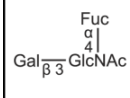
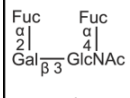
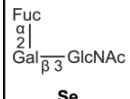
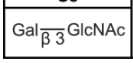
Epitope	Lewis phenotype, prevalence	Active fucosyltransferase(s)	Genotype
 **Le^a**	Le(a+b-), 22%	α1-3/4-FucT (FucTIII)	LeLe, sese or Lele, sese
 **Le^b**	Le(a-b+), 72%	α1-3/4-FucT (FucTIII), α1-2-FucT (FucTII)	LeLe, SeSe or Sese or Lele, SeSe or Sese
 **Se**	Le(a-b-), 6%	α1-2-FucT (FucTII)	lele, SeSe or Sese
		—	lele, sese

Fig. 2-2 The Lewis (Le) and secretor (Se) gene–related glycan epitopes. The Le and Se epitopes, which are characteristic for the Le phenotype in red blood cells and in human milk, are synthesized by the listed fucosyltransferases (FucTs). The Le and Se genes code for the active FucTs in presence of at least 1 functional allele (heterozygous with Lele or Sese, homozygous with LeLe or SeSe). The prevalence of the Le phenotypes is conferred to Europeans (Race and Sanger 1975). Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine.

Individuals with mutations resulting in the nonfunctional FucTs FucTII and FucTIII are usually typed as non- Se and Le negative or Le(a-b-), respectively (Bhende *et al.* 1952; Koda *et al.* 2001), and therefore should not secrete α1–2- and/or α1–4-fucosylated structures into milk.

2.4 Functional aspects of Le blood group–related HMO

Despite the fact that the Le histo-blood group system was discovered more than half a century ago, our knowledge about its biological functions is based mainly on speculations. In contrast, the role of the α1–3-FucTs IV and VII, which synthesize Le^x and Le^y epitopes, seems to be proven because their corresponding genes are highly conserved among mammals and contribute to the formation of selectin ligands (Marionneau *et al.* 2001).

Considering that the Le and Se genes are mainly expressed in secretory tissues, which are in contact with the environment and, therefore, with a large number of various microorganisms, the manifold carbohydrate antigens in secretions and epithelial cells might provide protection against pathogens (Daniels 2002; Marionneau *et al.* 2001). Marionneau *et al.* suggested that providing different cell surface receptors for several pathogens, Le^a antigens accomplish resistance against Le^b-binding pathogens and vice versa (Marionneau *et al.* 2001). Microbial lectins recognize host glycans in the gut, which are presented by mucins and glycolipids, enclosing ABH and Le blood group recognition sites. This promotes colonization, which may have adverse health effects in case of pathogen adhesion (Lane *et al.* 2010). HMO bearing Le epitopes and other recognition sites attach to the pathogens and inhibit their adherence to intestinal cell surfaces. Subsequently, the pathogen-HMO complex can be excreted (Garrido *et al.* 2011; Kunz *et al.* 2003).

Many attempts have been made to investigate the functions of HMO, including Le-specific structures; however, most of these studies were conducted *in vitro*. A brief overview is given in Table 2-1. HMO have antiadhesive properties, possibly resulting in the reduction of infections with *Campylobacter jejuni, Escherichia coli, Vibrio cholerae, Shigella,* and *Salmonella* species or in a decrease in HIV-1 mother-to-child transmission. The bifidogenic effect of HMO, which has been known for decades, might also be assisted by the interaction of bifidobacteria with the Le epitopes because *Bifidobacterium infantis* is able to both use and bind different glycans from human milk and intestinal cells *in vitro*, including Le-specific structures (Garrido *et al.* 2011) (see also other symposium papers).

Because the availability of HMO compounds adequate for interventional clinical trials has been limited to date, *in vivo* functional studies are rare. Nevertheless, data from observational investigations give an insight into the possible associations between the Le or Se phenotypes and diseases. α1–2-fucosylated HMO decreased *Campylobacter jejuni* infections in mice *in vivo* (Ruiz-Palacios *et al.* 2003) and significantly prevented diarrhea in breast-fed infants in a dose-dependent manner (Morrow *et al.* 2004; Newburg *et al.* 2004). Furthermore, low or non-Se status was strongly associated with adverse outcomes in preterm infants, e.g., mortality and necrotizing enterocolitis (Morrow *et al.* 2011), and with Crohn's disease (McGovern *et al.* 2010), giving further indication for the involvement of α1–2-fucosylated structures in the immune-related processes of gut development and health.

Table 2-1 Effects of Lewis and secretor gene–related factors [a)]

	Effect	Factor	Investigated in	Method	Reference
in vitro	Bifidogenic	Lewis a, type 1 H-trisaccharide	*B. longum ssp. infantis*	Genotyping; Glycan array	(Garrido *et al.* 2011)
	Anti-adhesive	HMO, i.a. Lewis epitope bearing	*C. jejuni, E. coli, Vibrio cholerae, Shigella, Salmonella,* HIV-1	Various	Reviewed in (Bode 2009)
	Anti-adhesive vs. *C. jejuni*	α1-2-Fuc-HMO	Carcinoma-derived human epithelial cells	Bacterial adherence assay	(Ruiz-Palacios *et al.* 2003)
ex vivo	Anti-adhesive vs. *C. jejuni*	α1-2-Fuc-Lac, neutral HMO	Fresh human intestinal mucosa	Bacterial adherence assay	(Ruiz-Palacios *et al.* 2003)
in vivo	Colonization with *C. jejuni* reduced	α1-2-Fuc-OS	Pups of transgenic mice	CFU counting after intestinal resection	(Ruiz-Palacios *et al.* 2003)
	Preventive vs. diarrhea from *C. jejuni, Calicivirus*	α1-2-Fuc-HMO	infants	Serological blood group classification; HPLC (HMO quantification)	(Morrow *et al.* 2004)
	Preventive vs. diarrhea from *E. coli*	α1-2-Fuc-HMO	infants	Serological blood group classification; HPLC (HMO quantification)	(Newburg *et al.* 2004)
	Association with mortality, gram negative sepsis and necrotizing enterocolitis	Low or non-secretor status	preterm infants	Genotyping; phenotyping (enzyme immunoassay in saliva)	(Morrow *et al.* 2011)
	Association with Crohn's disease	Non-secretor status	pediatric/adult individuals	Genotyping	(McGovern *et al.* 2010)

[a)] CFU, colony-forming units; Fuc, fucose; HMO, human milk oligosaccharides; Lac, lactose.

Considering the potentially important role of Le and Se epitopes in the infant's digestive tract, it is noteworthy that infants are typed Le(a-b-) in the first months of life, as discussed in the following section. This could be due to a reduced Le and Se antigen expression in the immature gut of neonates because gastrointestinal epithelial cells are suggested to be the main source for Le-specific glycolipids in blood after reabsorption (Hanfland and Graham 1981; Ramsey and Sherman 2000). Nevertheless, strong Le^a activity has been detected consistently in the fecal samples collected after birth (meconium) as well as at the 6-mo follow-up. Le^b reactivity, if present, was complementary to Le^a activity. Even

though the investigation was not representative, it is striking that the feces of the formula-fed neonates exhibited slightly lower Le reactivity than their exclusively breast-fed counterparts (Larson *et al.* 1990).

Because several grams of HMO pass through the breastfed infant's gut daily, they may compensate for the initial lack of Le and Se antigens in the neonate's intestine.

In addition to the lower production of Le and Se antigens in the newborn, there are also observations of decreased Fuc content of fecal glycans in younger infants (Pang *et al.* 1987) and formula-fed infants compared with older and breast-fed infants, respectively (Albrecht *et al.* 2011b). Fucosylated HMO in milk of Se but not in Le^a non-Se decrease steadily in the first 3 mo of lactation (Thurl *et al.* 2010), which might be due to an adaptation of the oligosaccharide composition in milk to the infant's gut maturity. Interestingly, HMO seem to be involved in the infant's intestinal cell maturation (Kuntz *et al.* 2009). In a recent study, the fecal oligosaccharides of mixed-fed infants resembled the breast milk oligosaccharide patterns with few modifications. In contrast, the fecal oligosaccharide profiles from exclusively breast-fed preterm infants were substantially different from those of their mothers' milk, showing an intense metabolism in the digestive tract. Interestingly, the authors reported the additional modification of the HMO with ABH epitopes several weeks postpartum (Albrecht *et al.* 2011a), a phenomenon first described by Lundblad as well (Lundblad 1993). Albrecht et al. explained these time-dependent variations in the infant's individual gastrointestinal adaptation to enteral food (Albrecht *et al.* 2011a).

Le-specific HMO might also contribute to the protective effect of breastfeeding against urinary tract infections (Marild *et al.* 1990) because uropathogenic *E. coli* has been found to attach to glycolipids in non-Se women, presumably causing more urinary tract infections than in Se, in whom the receptor is masked by the additional α1–2-Fuc (Stapleton *et al.* 1992). Some of the Le and Se-specific HMO structures have been detected in the urine of lactating women (Lundblad 1978; Zopf *et al.* 1979) and, more recently, also in the urine of breast-fed infants. The data suggest that the intact compounds reach the circulation after absorption in the infant's gut and might, therefore, display systemic and local effects in the infant (Rudloff *et al.* 2012; Rudloff *et al.* 1996).

2.5 Identification of Le blood group–related compounds

Traditionally, Le blood group determination is performed by serological methods, which can become a challenging task. Cross-reactions of the commonly used antibodies are described as well as the presence of side products leading to misinterpretation of results (Henry *et al.* 1995b). For example, healthy Le (a-b-) and Le(a+b-) individuals can show a slight expression of Le^b epitopes in plasma, which may be due to mutation with incomplete inactivation of FucTIII and FucTII, respectively (Bjork *et al.* 1987; Henry *et al.* 1994; Miller *et al.* 1954). The saliva of Le^a non-Se can also contain Le^b antigens as a result of slight FucTI activity in the salivary glands (Wang *et al.* 1994).

Red blood cells (RBC) do not synthesize Le antigens themselves, but acquire them secondarily from tissues (Marcus and Cass 1969; Sneath and Sneath 1955). Therefore, the serological phenotyping of RBCs does not necessarily describe the Le genotype of a donor because the Le epitope expression may differ in various tissues (Mollicone *et al.* 1994b; Orntoft *et al.* 1991; Yazawa *et al.* 1996). Genetic factors and several conditions such as diseases, infections, transfusions, and bone marrow transplantations can lead to alterations in the Le phenotype, hence, leading to misinterpretation of the blood group determination (Hirano *et al.* 1987; Langkilde *et al.* 1990; Makni *et al.* 1987; Needs *et al.* 1987; Stigendal *et al.* 1984).

In pregnancy, the prevalence of Le(a-b-)-typed women can increase threefold, which is most likely due to an increased attachment of Le-active glycolipids to plasma lipoproteins with a subsequent decrease

in the antigen quantity on erythrocytes (Hammar *et al.* 1981; Yazawa *et al.* 1996). In these individuals, discordant Le phenotypes can be detected on RBC and saliva.

A similar situation can be observed in neonates. Most of them are typed Le(a-b-) by serological detection on RBC, whereas in saliva, Le- and Se-related epitopes are already expressed, according to the genotype of the infant (Lawler and Marshall 1961). Several weeks after birth, Le^a antigen can also be detected on erythrocytes, whereas Le^b antigens are fully present in blood only at the age of 6 y due to delayed activation of the Se gene-controlled FucTII (Jordal 1956).

Because of the discrepancies and the numerous influencing factors of the Le phenotype determination in various tissues and body fluids, the data obtained from RBC phenotyping for Le blood group identification need to be regarded with suspicion (Henry *et al.* 1995a). Nevertheless, as routine method in the clinical sector, it is convenient for screening purposes. For the assignment of milk samples to the distinct Le groups, the serological RBC-based typing of the milk donors remains a valuable tool to roughly estimate the HMO pattern expressed in milk. The link between the Le blood group and the oligosaccharide profile in milk is explained by the correspondence between the biosynthesis of the Le-active glycans present on RBC and the formation of free fucosylated oligosaccharides in the mammary gland (see previously). However, because of the described variations in serological phenotyping as well as individual and lactation time-specific alterations in HMO expression, serology by itself is not sufficient to predict the relative amount of single Le-type oligosaccharides in milk. Detailed information on the expressed oligosaccharide patterns might be a more appropriate basis for future research on HMO, especially for functional clinical studies and investigations on the metabolism and the biological activity of HMO.

HMO monitoring of individual samples and sample mixtures is an important research topic about which excellent studies were conducted in the past. Various methods starting from paper chromatography, HPLC, and HPAEC over MS methods to latest developments in HPLC-chip-MS and CGE-LIF techniques have been applied (Albrecht *et al.* 2010; Blank *et al.* 2011; Kobata *et al.* 1969; Kunz *et al.* 1996; Leo *et al.* 2009; Marino *et al.* 2011; Ninonuevo *et al.* 2008; Thurl *et al.* 1997; Viverge *et al.* 1990; Wu *et al.* 2010; Yang *et al.* 2011). All these powerful methods provide detailed insights into the oligosaccharide pattern of individual milk samples, frequently paired with further information about the relative amount of single isomers. Some of these techniques require sophisticated and time-consuming sample preparation procedures and/or large quantities of sample material, which is a drawback for large sample sets. The combination of a simple, automated, and standardized sample workup procedure combined with standard MALDI-TOF-MS analysis provides the analytical power needed for a high-throughput glycomics approach for HMO profiling (Blank *et al.* 2011).

2.6 Variation of the HMO pattern

Automated MALDI-TOF-MS(/MS) as an HMO screening method enables the Le blood group correlation of a large milk sample set and delivers, at the same time, the distinct HMO pattern of each milk sample with high reliability (Blank *et al.* 2011).

Recent findings in the oligosaccharide pattern of single milk samples show the necessity of individual sample monitoring. The discrepancy described between the serologically detected blood group and the expression of Le antigens in other body fluids and tissues seems to be conferrable to Le-type oligosaccharides in human milk.

From the genetic point of view, a more consistent oligosaccharide pattern might be expected within a distinct Le blood group (Mollicone *et al.* 1994a). However, several studies showed that the proportion of distinct oligosaccharide structures can vary greatly among individual milk samples, depending on

the lactation period and/or Le status (Blank *et al.* 2011; Kobata *et al.* 1969; Kunz *et al.* 2000; Stahl *et al.* 2001; Thurl *et al.* 2010; Viverge *et al.* 1990).

The results of the MALDI-TOF-MS(/MS) screening of 40 milk samples from mothers with serologically determined Le blood group underlined these findings. Fig. 2 3 displays mass spectra from 2 Le(a-b-) and 2 Le(a-b+) donors, which differ markedly in their HMO pattern. The 95% CIs calculated for each signal within an Le blood group are indicated by shaded and open bars. In case of coincidence be¬tween the measured signal intensity and the predicted interval, the bar is shaded; otherwise, the CI is shown by an open bar. Each signal represents a distinct oligosaccharide composition with several structural isomers.

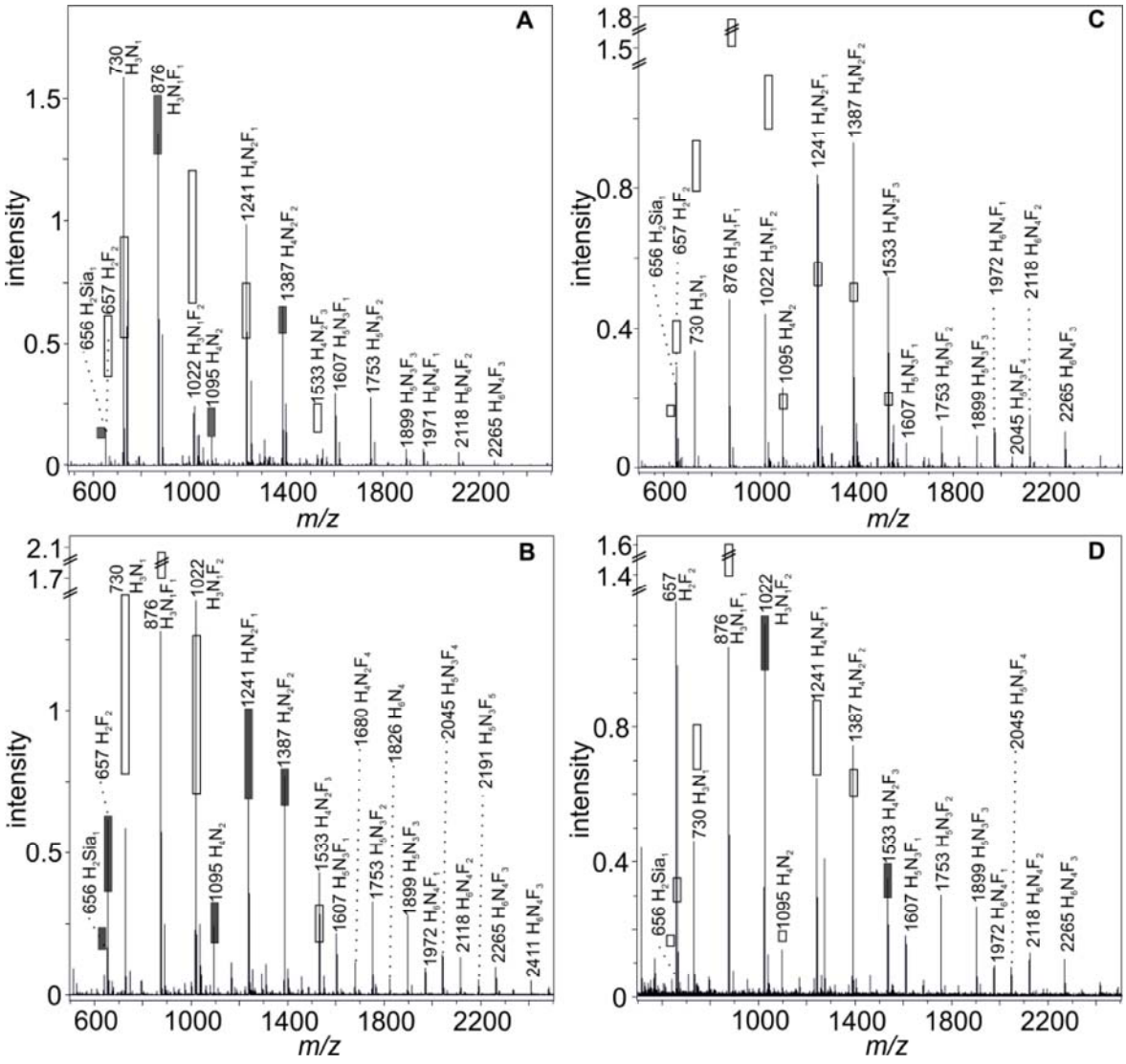


Fig. 2-3 Matrix-assisted laser desorption/ionization time-of-flight MS profile spectra of 4 individual milk samples. Spectra displayed were obtained from 4 women: 2 serologically typed as Le(a-b-) (A,B) and 2 as Le (a-b+) (C,D), respectively. The signals represent sodium adducts. The 95% CI, calculated for each human milk oligosaccharide composition, base on data from 40 individual milk samples. CIs are illustrated by open bars if the measured signal intensity is not in the expected range and by shaded bars if it is. Relative CIs are described in (Blank *et al.* 2011) and are applied to the measured signal intensities. Due to high variance in the signal intensities of the high molecular weight HMO, CIs were only calculated for signals up to *m/z* 1533. Compositions are calculated using GlycoPeakfinder software (Maass *et al.* 2007).

The HMO pattern of a serologically Le(a-b-)-typed woman is depicted in Fig. 2-3A. A strong overexpression of LN(n)T (*m/z* 730), as well as the monofucosylated lacto-*N*-(neo)hexaose (LN(n)H) (*m/z*

1241) was apparent. The multiply fucosylated species, such as difucosyllactose (*m/z* 657), difucosylated LN(n)T (*m/z* 1022) and the trifucosylated LN(n)H (*m/z* 1533) were substantially lower than expected or even absent. All of these 3 structures should contain a remarkable amount of α1–2-Fuc, whereas monofucosylated and difucosylated LN(n)H might contain mainly α1–3/4 fucosylated compounds. This leads to the conclusion that the donor might have an inefficient α1–2-FucT in the mammary gland, which is Se-gene dependent. Two explanations are possible for the anomaly in the depicted mass spectrum: i) this donor might belong to the rare subgroup of Le(a-b-) non-Se and ii) the donor's Le phenotype is actually Le(a+b-) because the HMO profile resembles that of an Le(a+b-) pattern with regard to the calculated CIs (data not shown).

The second Le(a-b-) sample showed the opposite phenomenon (Fig. 2-3B). The nonfucosylated precursor for the complex HMO, LN(n)T (*m/z* 730) and its monofucosylated form redundant (*m/z* 876) were underexpressed, whereas the multiply fucosylated species difucosylated LN(n)T (m/ z 1022) and trifucosylated LN(n)H (*m/z* 1533) were overexpressed. Moreover, unusually intensive signals up to a mass of 2500 Da were detected in high abundance in this specific sample. This suggests that this donor expressed several highly efficient glycosyltransferases as well as FucTs forming a diverse HMO pattern also in the higher mass range, conforming to an Le(a-b+) HMO pattern rather than the expected Le(a-b-)-specific one. Because the HMO spectra shown in Fig. 2-3A and B are atypical Le(a-b-) and resemble those characteristic of Le(a+b-) and Le(a-b+), respectively, the suspicion is raised that the milk HMO profiles may also reflect the partial discordance of the serological RBC- and saliva-based Le phenotyping in pregnant women, as discussed previously.

Fig. 2-3C shows the HMO spectrum of an Le(a-b+) donor expressing an atypical HMO pattern, which cannot be assigned to any Le blood group. All signals from *m/z* 657 to 1022 were expressed in substantially lower intensity than expected, whereas the signals in the mass range from *m/z* 1095 to 1533 were overexpressed. Hence, a shift to high molecular weight HMO can be observed for this particular sample. As already discussed for Fig. 2-3B, also in this example highly efficient glycosyltransferases might be responsible for the observed variation.

Only slight deviations in the Le(a-b+) spectrum are seen in Fig. 2-3D, except for the unexpectedly high proportion of difucosyllactose (*m/z* 657).

The demonstrated variations in the MALDI-TOF-MS profile spectra confirm the conclusion of (Thurl *et al.* 2010) that each lactating woman expresses an individual HMO pattern, even though an assignment of the HMO profiles to the distinct Le blood groups was applicable for the majority of the milk samples in our investigations (see later).

The importance of screening methods to detect the individual oligosaccharide profile is further emphasized by the tandem mass spectrometry analysis of the precursor *m/z* 1022 in a milk sample from a woman typed Le(a+b-) (Fig. 2-4). As a major isomer, an LN(n)T core bearing 1 Fuc at the reducing end and the $Le^{a/x}$ epitope at the nonreducing end is expected in a milk sample from an Le(a+b-) donor. The most intensive fragment signals at *m/z* 730 ($Y_{4\beta}B_{4\alpha}$) and m/ z 876 ($Y_{4\beta}$) result from the dissociation of 1 and 2 Fuc residues, respectively [fragment ions are designated in accordance with the nomenclature of (Domon and Costello 1988)]. In addition, the signals *m/z* 696 ($B_{3\alpha}$), *m/z* 511 ($Y_{2\alpha}$), and *m/z* 365 ($Y_{2\alpha}B_{4\alpha}$) underline the presence of the likeliest precursor structure. Strikingly, an indication for the presence of an $Le^{b/y}$ epitope is given by the signal *m/z* 680 ($B_{2\alpha}$), a difucosylated *N*-acetyllactosamine unit. The serologically detected Le blood group does not explain the presence of an $Le^{b/y}$ epitope because of the lack of the Se gene–dependent FucTII in Le (a+b-) individuals. Nevertheless, the difucosylation of either a terminal type 1 or 2 *N*-acetyllactosamine unit in this milk sample is an indication for α1–2-FucT activity. This finding confirms the unexpected presence of α1–2-fucosyl HMO in 2 serologically typed Le(a+b-) donors by Newburg et al. (Newburg *et al.* 2004). These findings might be ex-

plained by a slight activity of the H gene–controlled FucTI, which has been detected at least in the saliva of Le(a+b-)-typed individuals (Wang *et al.* 1994). The application of the fragmentation analysis of individual oligosaccharides for structural characterization is described for various MS techniques (Albrecht *et al.* 2010; Amano *et al.* 2009; Broberg 2007; Ferreira *et al.* 2010; Ninonuevo *et al.* 2006; Pfenninger *et al.* 2002; Wu *et al.* 2011; Wu *et al.* 2010; Yamagaki *et al.* 2006; Yang *et al.* 2011). Hence, tandem MS analysis can also provide additional structural information for individual HMO compositions in the case of HMO screening.

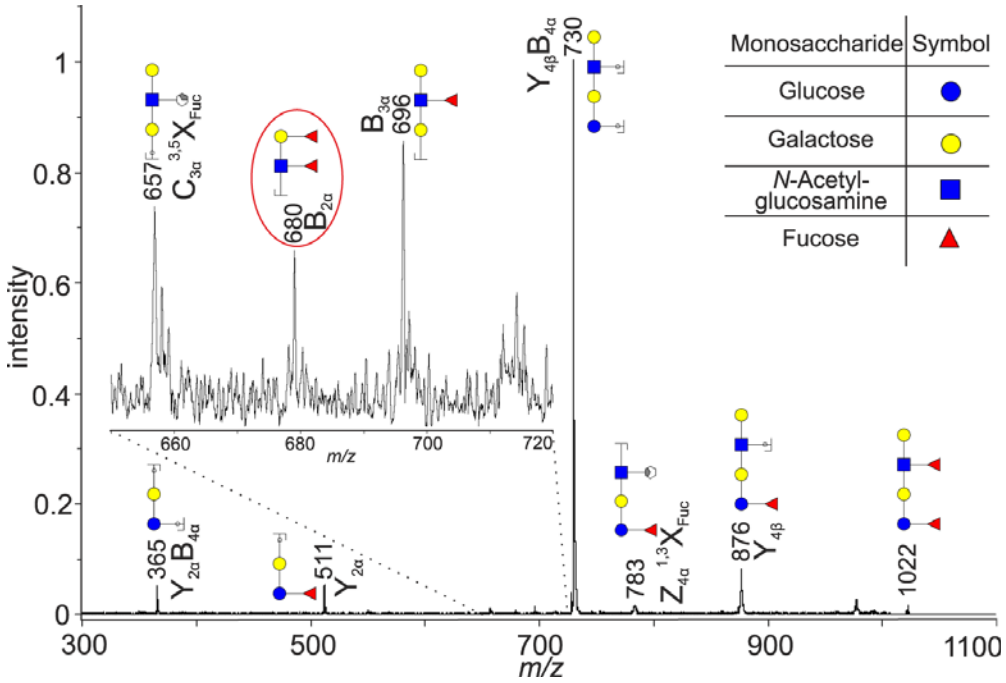


Fig. 2-4 Matrix-assisted laser desorption/ ionization time-of-flight MS/MS analysis of purified human milk oligosaccharides of a Lewis (a+b-) donor. Inset shows range from *m/z* 650 to *m/z* 720 at 50× magnification. The obtained fragment ions were assigned according to the recommendations of the Consortium of Functional Glycomics using GlycoWorkbench (Ceroni *et al.* 2008). Fragment ions are designated in accordance with the nomenclature of (Domon and Costello 1988). In some cases, fragments may be formed by different fragmentation pathways, only 1 of which is illustrated. All fragment ions represent sodium adducts. The unexpected signal is circled in red.

The presented examples demonstrate the drawbacks of serological Le blood group classification and emphasize the need for individual sample mapping. Le phenotyping in human milk by MALDI-TOF-MS(/MS) analysis and subsequent statistical data evaluation provide the opportunity not only to assign the specimens to definite groups, but also to reveal unusual tendencies for each individual milk sample, including its unique HMO profile (Blank *et al.* 2011).

Fig. 2-5 shows the results from a discriminant analysis of 40 individual milk samples measured threefold. Using the new screening approach, 95% of the samples were correctly assigned to the serologically detected Le phenotype in blood by at least 2 of 3 measurements. Specifically, 99% of all serologically Le(a-b+), 100% of the Le(a-b-), and 68% of the Le(a-b-) typed samples were assigned to the previously determined Le blood group. Nevertheless, the partial inhomogeneity of the Le-specific HMO profiles described previously is also evident from the wide distribution of the samples within the Le blood groups. The overlapping area of the Le(a-b+) and Le(a-b-) typed samples, in particular, displays the similarity of the oligosaccharide profiles in several specimens with a different Le phenotype, most likely resulting from their Se gene activity. However, the fact that the majority of the milk samples were matched to the serologically detected Le blood group shows that a classification using both approaches can be useful despite the variations in the expression level of single oligosaccharide composition in

milk. The location of each breakpoint in the coordinate plan therefore provides information about the Le phenotype tendency of a distinct milk sample and at the same time information about its actual oligosaccharide pattern. Consequently, the new MS screening approach provides a fast and material-saving option for individual milk sample mapping with detailed information on the expression level of individual oligosaccharide compositions.

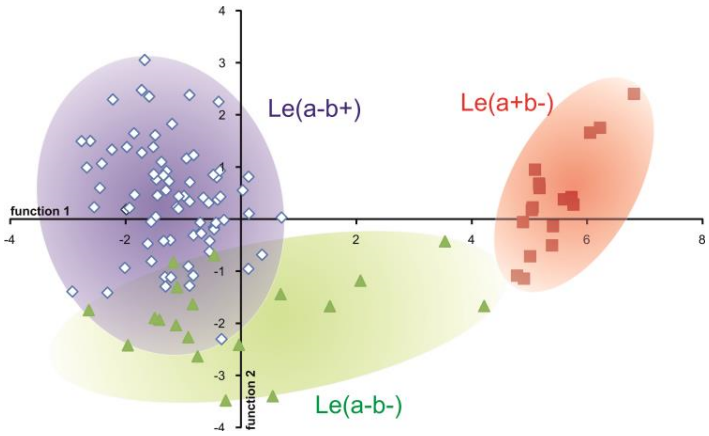


Fig. 2-5 Discriminant analysis. The results obtained for 113 single matrix-assisted laser desorption/ionization time-of-flight MS and redundant MS/MS measurements of 40 milk samples underwent discriminant analysis. Discriminant function 1 is plotted on the x-axis and discriminant function 2 on the y-axis. Open diamonds, red squares, and green triangles represent HMO samples from Le(a-b+), Le(a+b-), and Le(a-b-) donors, respectively. The distribution of each group is indicated by colored shading. Reproduced with kind permission from Springer Science+Business Media (Blank *et al.* 2011), Figure 7.

2.7 Conclusion

Serological Le blood group determination can only offer a first indication of the expressed oligosaccharide pattern in human milk. Modern high-throughput screening methods can support the traditional serological RBC analysis and provide detailed information on the relative abundance for each oligosaccharide composition, thus, enhancing or qualifying the conclusions of functional studies. In particular, the capability of individual glycan epitope recognition and its relationship to observable, biologically relevant effects will be of great benefit. Furthermore, reliable Le phenotype screening, e.g., by the MALDI-TOF-MS approach presented here, can replace serological determination on erythrocytes if no blood sample is available.

The variations between the serologically detected Le blood group and the Le phenotype in other body fluids and tissues, which are frequently reported in literature, were also detectable in human milk using the novel screening approach. Based on the determined individual HMO patterns, some speculations about the activities of certain glycosyltransferases in the milk donors were deduced by taking into consideration the information from the suggested HMO biosynthetic pathway.

The combination of the traditional serological Le blood group detection supported by modern milk screening methods will lead to a solid glycan characterization as the basis for future research on the effects of HMO from native milk.

2.8 Acknowledgments

We thank Professor G. Bein and Professor H. Jomaa (Institute of Immunology and Transfusion Medicine, University Hospital Giessen-Marburg, Germany) for performing the Lewis blood group determination of blood samples as well as Dr. P. Gilbert and his team (St. Josef's Hospital Giessen) for the collection of milk and blood samples. All authors read and approved the final manuscript.

Chapter 3

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^{13}C-labeled oligosaccharides in breastfed infants' urine: Individual-, structure- and time-dependent differences in the excretion

Viktoria Dotz[2], Silvia Rudloff[2,3], Dennis Blank[4,5], Günter Lochnit[4], Rudolf Geyer[4], and Clemens Kunz[1,2]

[2]Institute of Nutritional Sciences, University of Giessen, Wilhelmstrasse 20, 35392 Giessen, Germany; [3]Department of Pediatrics, University of Giessen, Feulgenstrasse 12, 35392 Giessen, Germany; and [4]Faculty of Medicine, Institute of Biochemistry, University of Giessen, Friedrichstrasse 24, 35392 Giessen, Germany

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Human milk oligosaccharides (HMOs) have been paid much attention due to their beneficial effects observed in vitro, e.g., prebiotic, anti-infective and anti-inflammatory properties. However, in vivo investigations with regard to HMO metabolism and functions are rare. The few data available indicate that HMOs are absorbed to a low extent and excreted via urine without noteworthy modifications, whereas the major proportion reaches infant's colon undigested. Via intrinsic ^{13}C-labeling of HMOs during their biosynthesis in the mammary gland of 10 lactating women, we were able to follow the fate of ^{13}C-labeled oligosaccharides (OSs) from their secretion in milk to the excretion in the urine of their breastfed infants. To a certain extent, we could therefore discriminate between original HMOs and non-labeled OSs derived from degradation of HMOs or endogenous glycoconjugates. By means of our novel, rapid, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)-based approach, we found a homogeneous time pattern of isotopomer enrichment in milk among all subjects and between single OS species. In contrast, the time curves from infants' urine varied strongly between individuals and OS species, though the overall MALDI-TOF MS profile resembled those of the mothers' milk. Our data suggest that neutral HMOs might be processed and/or utilized differentially after or upon absorption from the gut, as deduced from their structure-dependent variation in the extent of tracer enrichment and in the retention times in infant's organism. This sheds new light on the role of HMOs within infant's body, beyond the intestine and its microbiota alone.

Keywords: human milk oligosaccharides / in vivo / MALDI-TOF MS / metabolic fate / stable isotope

[1]To whom correspondence should be addressed: Tel: +49-6419939041; Fax: +49-6419939039; e-mail: clemens.kunz@ernaehrung.uni-giessen.de; clemens.kunz@uni-giessen.de
[5]Present address: Boehringer Ingelheim Pharma GmbH & Co. KG, Quality Germany, Birkendorfer Strasse 65, 88397 Biberach an der Riss, Germany.

3 ^{13}C-labeled oligosaccharides in breastfed infants' urine: Individual-, structure- and time-dependent differences in the excretion (Manuscript 2)

3.1 Abstract

Human milk oligosaccharides (HMO) have been paid much attention due to their beneficial effects observed *in vitro*, e.g., prebiotic, anti-infective and anti-inflammatory properties. However, *in vivo* investigations with regard to HMO metabolism and functions are rare. The few data available indicate that HMO are absorbed to a low extent and excreted *via* urine without noteworthy modifications, whereas the major proportion reaches infant's colon undigested. *Via* intrinsic ^{13}C-labeling of HMO during their biosynthesis in the mammary gland of 10 lactating women, we were able to follow the fate of ^{13}C-labeled oligosaccharides (OS) from their secretion in milk to the excretion in the urine of their breastfed infants. To a certain extent, we could therefore discriminate between original HMO and non-labeled OS derived from degradation of HMO or endogenous glycoconjugates. By means of our novel, rapid, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)-based approach, we found a homogeneous time pattern of isotopomer enrichment in milk among all subjects and between single OS species. In contrast, the time curves from infants' urine varied strongly between individuals and OS species, though the overall MALDI-TOF-MS profile resembled those of the mothers' milk. Our data suggest that neutral HMO might be processed and/or utilized differentially after or upon absorption from the gut, as deduced from their structure-dependent variation in the extent of tracer enrichment and in the retention times in infant's organism. This sheds new light on the role of HMO within infant's body, beyond the intestine and its microbiota alone.

3.2 Introduction

It is well known that breastfed infants have various health benefits compared with formula-fed babies, e.g. less-frequent autoimmune diseases and fewer or less severe infections of the respiratory, intestinal and uro-genital tract (Pisacane *et al.* 1992; Schack-Nielsen and Michaelsen 2007). In this regard, HMO are supposed to contribute to these beneficial outcomes through their prebiotic, anti-infective and immunomodulatory effects, as revealed from numerous *in vitro* and few animal and human observational studies (Bode 2012; Kunz *et al.* 2000). Since HMO are products of the action of different glycosyltransferases in the mammary gland and, therefore, evolve large structural variety, it is not surprising that their efficacy and absorption have been found to be dependent on the individual structures (Bode 2012; Gnoth *et al.* 2001), including specific sites of fucosylation (Morrow *et al.* 2011) or sialylation (Bode *et al.* 2004). Lactose as the backbone of HMO can be elongated with GlcNAc and Gal residues to form GlcNAcβ1-3Gal or GlcNAcβ1-4Gal units, the former resulting in the main core structure, LNT, the latter in *neo*-LNT or LNnT. These structures can be further elongated and branched, or also modified by the attachment of α1-2-/α1-3- or α1-4-linked fucose and/or *N*-acetylneuraminic acid, the latter forming acidic HMO (Kobata 2010).

Although OS are the third largest component of human milk with concentrations ranging from 5 to 20 g/L (Coppa *et al.* 2011b; Thurl *et al.* 2010), their metabolism and functions *in vivo* are poorly understood. This is partly due to several practical and analytical limitations. In previous studies with and without the use of stable isotopes, we found a renal excretion of 1–3% of individual HMO in exclusively breastfed infants (Rudloff *et al.* 2012; Rudloff *et al.* 1996). Over the last years, various state-of-the-art methodologies have emerged enabling a sophisticated identification of the majority of the >200 distinct OS structures found in human milk, infant feces and urine. These techniques include chroma-

tography (Chaturvedi *et al.* 2001; Rudloff *et al.* 2012), capillary electrophoresis (Albrecht *et al.* 2011a; Shen *et al.* 2000), mass spectrometry (Klein *et al.* 1998; Kogelberg *et al.* 2004) as well as on-line coupled combinations of those (De Leoz *et al.* 2013; Galeotti *et al.* 2012).

Practical constraints of clinical studies on HMO include their limited availability and affordability for the use in humans (Rudloff and Kunz 2012). Ethical considerations are also impeding the much-needed functional studies, since infants as well as lactating women are particularly vulnerable subpopulations. Consequently, study design and sampling procedures should be of minimal discomfort and risks for mother and child. Therefore, we used intrinsic stable isotope labeling as an established and straightforward, noninvasive technique (Davidsson and Tanumihardjo 2011).

Breastfeeding women ingested ^{13}C-Gal, which was incorporated into HMO during their synthesis in the mammary gland. Subsequently, we were able to track the stable isotope tracer over the course of time including its renal excretion in the breastfed child by measuring ^{13}C-enrichment in whole or fractionated mother's milk and infants' urine by means of IRMS (Rudloff *et al.* 2006; Rudloff *et al.* 2012). Although IRMS is very sensitive in the determination of isotope enrichment, it does not distinguish between single compounds, unless they were isolated prior to analysis in a tedious procedure requiring large sample volumes, in particular, in case of urinary OS. Hence, we have developed a rapid MALDI-TOF-MS-based approach (see also (Blank *et al.* 2011)) for the determination of isotopomer enrichment in nonderivatized, neutral OS from human milk and infants' urine, enabling us to discriminate between the renal excretion of labeled, intact OS originating from milk (HMO) and nonlabeled OS of endogenous origin or resulting from HMO degradation. A dynamic insight into the metabolic fate of individual OS species in lactating women and their breastfed children over time is provided, and thereby indication is given for their functionality within infant's organism and the structures of active compounds.

3.3 Results

Directly before and up to 36 h after an oral application of ^{13}C-Gal to 10 lactating mothers, milk and their infants' urine were collected at each nursing, yielding >200 single milk and urine samples in total (Fig. 3-1).

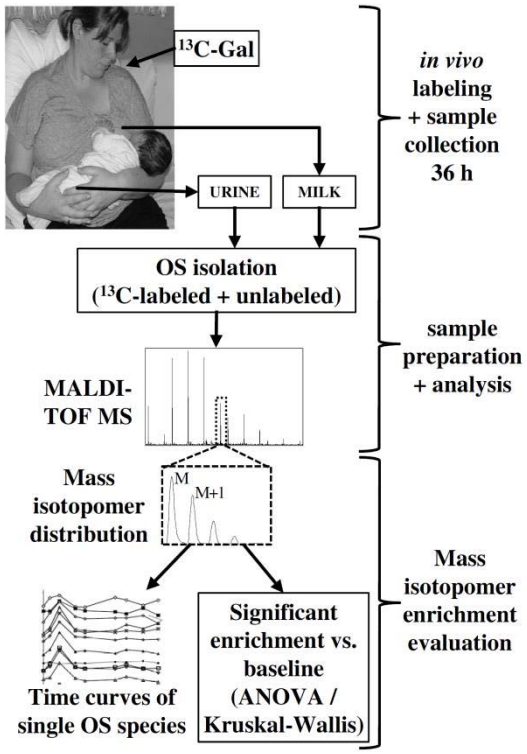


Fig. 3-1 Workflow of the in vivo isotope labeling experiment with mass isotopomer distribution evaluation *via* MALDI-TOF-MS. ^{13}C-Gal was ingested by 10 lactating women and found to be incorporated into milk OS. Labeled and unlabeled OS were, in part, excreted in the urine of their breastfed infants. Enrichment of OS with ^{13}C was followed over time by comparing the fractional abundances of the mass isotopomers at $0 < t < 36$ h with those at baseline.

Some of the single urine samples could not be used for the analyses due to their insufficient volume or to fecal contamination in the diaper, causing discontinuations in the time course during the 36 h examination period. To adjust for the high variation of the urine volume and, therefore, analyte concentration prior to OS extraction, we determined creatinine concentration in each urine sample, since it is commonly used as reference parameter in clinical studies. The concentrations ranged from 30 to 250 mg creatinine/L of infants' urine. A urine volume corresponding to 0.1 mg creatinine was found to be optimal for OS extraction and MS analysis.

After SPE, the OS profile of each individual sample was acquired by MALDI-TOF-MS in positive ion mode (Fig. 3-2). The OS species in milk and urine which could be detected in any of the 36 h samples of an individual are listed in Table 3-1 (page - 34 -). OS profiles showed high interindividual variability, particularly in urine. For example, the molecular species with nominal mass 2044 for the lightest isotopomer $[M+Na]^+$, referring to the glycan composition $Hex_5HexNAc_3Fuc_4$, was only detected in the milk of women 3–9 and the urine of children 5 and 6. The *m/z* values, shown in the present work, represent nominal masses for sodiated glycans as detected in MALDI-TOF-MS. MALDI mass spectra provide mass isotopomer patterns of individual OS structure compositions (Fig. 3-1 and Fig. 3-2). Those were the basis for the determination of fractional abundances, fA_{M+1} in %, in single OS components after tracer administration. For example, LNT and its structural isomer LNnT with $^{12}C_{25}{}^{13}C_1H_{45}{}^{14}N_{16}O_{21}$ or $^{12}C_{26}{}^{1}H_{44}{}^{2}H^{14}N_{16}O_{21}$ or $^{12}C_{26}{}^{1}H_{45}{}^{15}N_{16}O_{21}$ are part of the same isotopomer M+1 with calculated nominal masses of 708, or 731 when detected in MALDI-TOF-MS as sodiated ions.

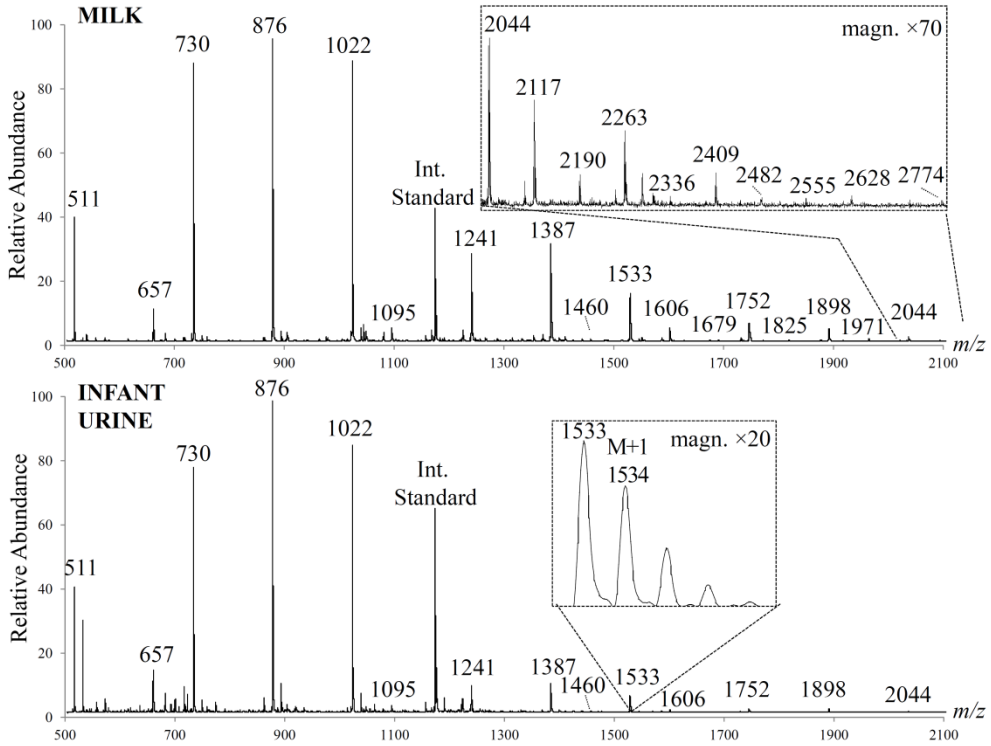


Fig. 3-2 MALDI-TOF-MS profiles of OS in human milk and infants' urine of mother–child pair 6 after ^{13}C-Gal administration. The *m/z* values are given as nominal mass for $[M+Na]^+$ ions; for structural compositions see Table 3-1. Maltoheptaose was used as internal standard (*m/z* 1175).

An internal standard, maltoheptaose with *m/z* 1175 $[M+Na]^+$, was added prior to each MS measurement as control substance with constant isotopomer distribution (Fig. 3-2). The fA_{M+1} value of the samples collected prior to tracer application was regarded as baseline, except for woman 9 and infants 1, 8–10. Since no baseline sample was available from these subjects, theoretical fA_{M+1} values,

as calculated from the chemical formula of the OS and the natural isotope abundances of H, C, N and O, were applied to the statistical tests instead.

3.3.1 Time course of biosynthesis of milk OS over 36 h

For all 10 lactating women, an increase in fA_{M+1} could be observed in many OS species over time after ^{13}C-Gal ingestion. Some variability occurred in terms of the time pattern and the magnitude of tracer enrichment between both, subjects and single OS species. However, the overall time pattern of the fA_{M+1} values in Fig. 3-3 is rather uniform with two or even three maxima within 36 h. Fig. 3-3A shows data averaged from all 10 women, i.e., means of all fA_{M+1} determined in samples collected within a 4-h time interval. The coefficients of variance (CV) of the fractional abundances in larger OS with *m/z* ≥ 1242 were ≤5% at most time points. Those OS species, which were most abundant in milk, i.e. isotopomers at *m/z* 731, 877, 1023, varied by 3−9%. The time pattern of fucosyllactose (*m/z* 512) showed highest inter-individual variations (3.43 ≤ CV ≤ 21.03%). An example of the time course of isotopomer enrichment in HMO of a single donor is depicted in Fig. 3-3C. The isotopomer abundances exhibit a consistently clear double-maximum time pattern in the milk of woman 3, even in case of fucosyllactose at *m/z* 512 (Fig. 3-3C).

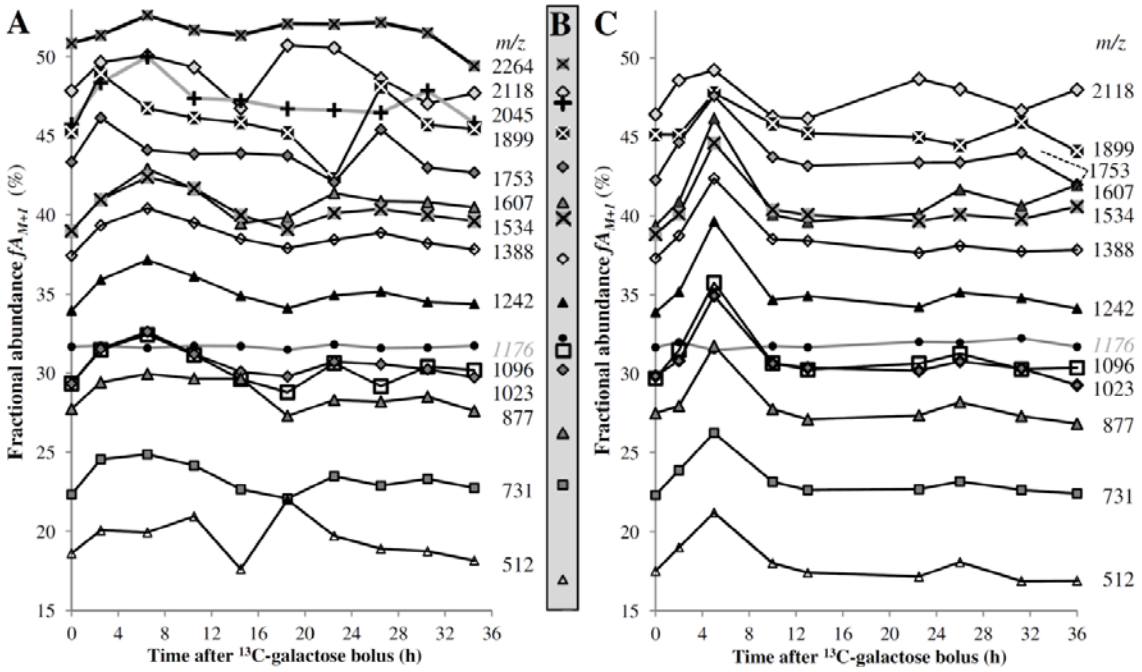
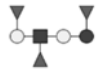
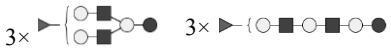
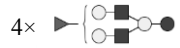
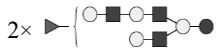
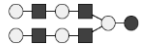
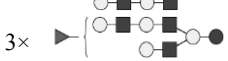
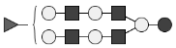
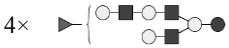
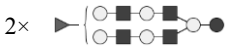
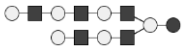
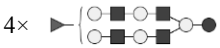
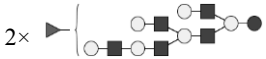
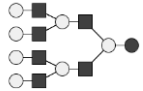
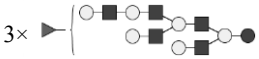
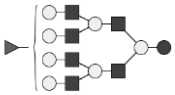
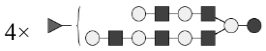


Fig. 3-3 Fractional abundances of the oligosaccharide isotopomer M+1 (fA_{M+1}) in milk during 36 h after oral ^{13}C-Gal intake. Values were calculated from isotopomer abundances of M and M+1 (see Fig. 3-1), as analyzed by MALDI-TOF-MS, and shown as means of the measurements from all women (A), and woman 3 alone with mean values of the triplicate measurements (C). Theoretical fA_{M+1} values were calculated from the chemical formula of the glycan and natural H, C, N, O isotope abundances (B). The *m/z* values of OS are given for $[M+1+Na]^+$ ions. For structures see Table 3-1; *m/z* 1176 refers to the internal standard maltoheptaose.

In order to confirm the significance of the isotopomer enrichment in milk and urine, we evaluated whether the MALDI-TOF-MS fA_{M+1} values of the individual triplicate measurements from each sample at a certain time point differ significantly from baseline (Table 3-2). The individual data were grouped into 4-h intervals after statistical evaluation. The significant enrichment in the first half of the 36 h collection period persisted for ≤12 h in milk, starting directly at ≤4–8 h after tracer ingestion. Enrichment in the later period was more widely distributed over time and was significant in fewer OS species, indicating a third peak enrichment in women 2, 3 and 10. A slightly deviating time pattern was seen in milk samples of subject 5, in which the second maximum was more pronounced than the first (Table 3-2).

Table 3-1 Neutral HMO detected in 10 mother–child pairs by MALDI-TOF-MS over 36 h

m/z	Chemical composition	Structural composition [a]	Detected in [b] milk	urine
511	Hex_2Fuc_1		1-9	1,3-9
657	Hex_2Fuc_2		1,3-9	1,3-9
730	$Hex_3HexNAc_1$		1-10	1-10
876	$Hex_3HexNAc_1Fuc_1$		1-10	1-10
1022	$Hex_3HexNAc_1Fuc_2$		1-10	1-10
1095	$Hex_4HexNAc_2$		1-10	1-10
1168	$Hex_3HexNAc_1Fuc_3$		3-9	6,7,9
1241	$Hex_4HexNAc_2Fuc_1$		1-10	1-10
1387	$Hex_4HexNAc_2Fuc_2$		1-10	1-10
1460	$Hex_5HexNAc_3$		1-10	3,5,6,9, 10
1533	$Hex_4HexNAc_2Fuc_3$		1-10	1,3-10
1606	$Hex_5HexNAc_3Fuc_1$		1-10	1,3,5,6,1 0
1679	$Hex_4HexNAc_2Fuc_4$		3-9	none
1752	$Hex_5HexNAc_3Fuc_2$		1-10	1-3,5-7, 10

m/z	Chemical composition	Structural composition [a]	Detected in [b] milk	urine
1825	$Hex_6HexNAc_4$		1,3,4,6,7,9	none
1898	$Hex_5HexNAc_3Fuc_3$		1-10	1-3,5-7, 10
1971	$Hex_6HexNAc_4Fuc_1$		1-10	none
2044	$Hex_5HexNAc_3Fuc_4$		3-9	5,6
2117	$Hex_6HexNAc_4Fuc_2$		1-10	1
2190	$Hex_7HexNAc_5$		3-8	none
2263	$Hex_6HexNAc_4Fuc_3$		1-10	1
2336	$Hex_7HexNAc_5Fuc_1$		1,3,4	none
2409	$Hex_6HexNAc_4Fuc_4$		2-9	none
2482	$Hex_7HexNAc_5Fuc_2$		2-4,6,7	none
2555	$Hex_8HexNAc_6$		4,6	none
2628	$Hex_7HexNAc_5Fuc_3$		2,4,6	none
2701	$Hex_8HexNAc_6Fuc_1$		4	none
2774	$Hex_7HexNAc_5Fuc_4$		2,4,6	none

The *m/z* values refer to monoisotopic $[M+Na]^+$ ions.

[a] Structural examples for the various isomers, according CGF notation using GlycoWorkbench (Ceroni et al. 2008): Square, *N*-acetyl-D-glucosamine; filled circle, glucose; open circle, Gal; triangle, fucose.

[b] Subject number is indicated if the listed oligosaccharide species was detected in any of the 36 h samples.

Table 3-2 Statistically significant ^{13}C-enrichment of neutral OS in mothers' milk and infants' urine over 36 h

	Net time after ^{13}C-galactose								
	0–4	**4–8**	**8–12**	**12–16**	**16–20**	**20–24**	**24–28**	**28–32**	**32–36**
Milk 1	1023*; 1242; 1388*	1023#; 1242#; 1388#; 1607*; 1899*	512; 1023#; 1096; 1242#; 1388#; 1607	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Urine 1 [a)]	1388	n.s.	–	–	512#; 877#; 1388*	–	–	–	877; 1388
Milk 2	731*; 877*; 1023; 1388#; 1534*; 1753*	731*; 877; 1023;1242;1388#; 1534; 1753#	731*; 877#; 1023; 1242; 1388#; 1534#; 1753*	–	1388*; 1534*	1388*	n.s.	1388	1534
Urine 2	n.s.	n.s.	877#; 1023#	–	n.s.	–	877	877	–
Milk 3	512; 731; 1023; 1096; 1242; 1388	512#; 731#; 877#; 1023#; 1096#; 1242#; 1388#; 1534#; 1607*	n.s.	n.s.	–	n.s.	1023; 1242	n.s.	1534
Urine 3	–	n.s.	n.s.	731; 1023	–	n.s.	731	–	n.s.
Milk 4	877; 1023; 1242#; 1388#; 1534#	1242#; 1388#; 1534#	877; 1388*	n.s.	n.s.	n.s.	1242#; 1388#; 1534*	1388*	n.s.
Urine 4	n.s.	n.s.	731; 877*; 1388#	731	n.s.	–	1388	1023	–
Milk 5	–	1388*	1388*	1388	–	1388#; 1534*	–	1388#; 1534	1388
Urine 5	n.s.	1242*	512; 731*; 1023; 1242#	–	1242*	1242#	1023*	731*; 877*; 1023*; 1242	–
Milk 6	512; 1023#; 1096; 1534#; 1607*;	1023#; 1534#; 1607#; 1899*	731; 1023#; 1534*; 1607	–	n.s.	1023*; 1534; 1899	731; 1023#; 1534*; 1899*	512; 1023*; 1607	1607
Urine 6	1242#; 1534#	512*; 1023; 1534	731*; 1534	–	512*; 1023*; 1534	512#	512*; 1023*; 1242*; 1534#	512; 731*; 877; 1023*; 1242; 1534*	731; 1534
Milk 7 [b)]	n.s.	731#; 877*; 1023; 1242#; 1388; 1534#	731#; 877*; 1023; 1242#; 1388*; 1534*	–	731	731; 877; 1242*; 1534	877; 1242; 1534*	1242	877
Milk 8	n.s.	731#; 877#; 1023#; 1242#; 1388#; 1534; 1607*; 1753*	731#; 877#; 1023#; 1242#; 1388#; 1534*; 1607*; 1753#	731#; 877#; 1023#; 1242#; 1388#; 1534; 1607; 1753*	–	1023; 1242; 1388*	1388	n.s.	n.s.
Urine 8 [a)]	1023	n.s.	877; 1534*	–	–	877; 1023	–	731; 1023	n.s.
Milk 9 [a)]	877*	877#;1023#; 1388*	877#;1023*;1388	877	–	n.s.	n.s.	n.s.	877#
Urine 9 [a)]	–	877	512; 731*; 877*; 1388	512; 731#; 877	–	731*; 877#; 1023; 1388	–	731; 1388*	n.s.
Milk 10	n.s.	731; 877*; 1242	n.s.	731*; 877	–	n.s.	n.s.	731	n.s.
Urine 10 [a)]	n.s.	731	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

The *m/z* of oligosaccharides (OS) are given for $[M+1+Na]^+$ ions, as analyzed by MALDI-TOF-MS, which are significantly enriched when compared with baseline with $P < 0.05$ or with $P < 0.01$ (*), or $P < 0.001$ (#); n.s., not significant; –, no data. For OS structures see Table 3-1.

[a)] Due to a lack of baseline sample in woman 9 and infants 1, 8–10, multiple comparisons were performed on the basis of values calculated from the chemical formula of the glycan and natural H, C, N, O isotope abundances (see explanation in the Materials and methods and Results sections).

[b)] No statistical analysis was performed for urine 7 due to unclear documentation of urine collection time.

3.3.2 Time course of the urinary excretion of OS in breastfed infants

As determined from fA_{M+1} in MALDI-TOF-MS, the excretion of labeled OS in infants' urine was less pronounced than in milk and did not exhibit a uniform time pattern. Nevertheless, when compared with baseline, an increase of the fA_{M+1} values could be seen in many OS structures, with a maximum at 8–12 h after ^{13}C-Gal ingestion (Fig. 3-4). Accordingly, a significant increase of the isotopomer abundances was observed for the period between 4 and 16 h and at 20 h after ^{13}C-Gal ingestion (Table 3-2).

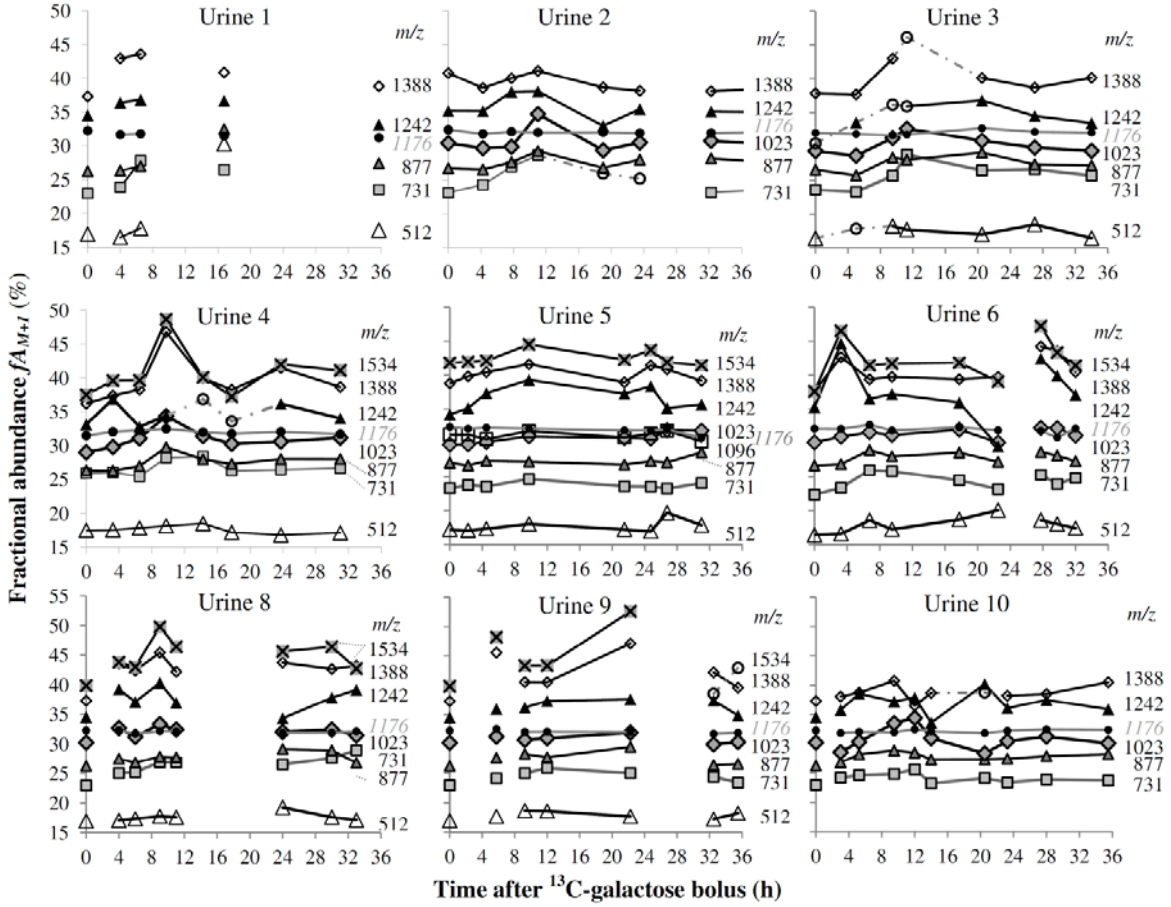


Fig. 3-4 Fractional abundances of the oligosaccharide isotopomers M+1 (fA_{M+1}) in urine samples of nine infants during 36 h after their mothers' oral ^{13}C-Gal intake. Each curve represents the time course of the oligosaccharide with the *m/z* value given for $[M+1+Na]^+$ ions, as analyzed by MALDI-TOF-MS. For structures see Table 3-1; *m/z* 1176 refers to the internal standard maltoheptaose. Discontinuations in the graphs 1, 2, 6, 8–10 are caused by a lack of a urine sample when there was fecal contamination or insufficient volume. For the same reason, baseline values in graphs 1, 8–10 were not measured, but calculated from the chemical formula of the glycan and natural H, C, N, O isotope abundances (see explanation in the Materials and methods and Results sections). Open circle with dashed line: Values with 10–15%RSD within the triplicate measurements. Otherwise the RSD was below 10%.

3.3.3 Method validation parameters

Sample preparation did not show any effects on the fA_{M+1} of the OS isotopomers, as indicated by percent relative standard deviation (%RSD) values not exceeding the variations originating from the MALDI-TOF-MS instrument: The %RSD values from three different preparations of the same infant's urine and mother's milk, with fA_{M+1} values averaged from the analysis of three sample spots each, ranged from 0.85 (*m/z* 731) to 4.72 (*m/z* 1534) and 0.14 (*m/z* 1753) to 5.06 (*m/z* 1023), respectively. The spot-to-spot repeatability (intra-sample) of the fA_{M+1} values ranged from

1.11 (*m/z* 877) to 2.95 (*m/z* 1534) and from 0.66 (*m/z* 1388) to 5.27 (*m/z* 1607) %RSD, as measured from 10 different spots of one infant's urine and mother's milk OS preparation. The variation within the triplicate was monitored for each molecular species in each sample, as the signal intensity of the larger OS, in particular, in the urine was highly subject-dependent. Most %RSD values were within the range measured for repeatability evaluation. However, a few OS species reached values of 10–15% (especially *m/z* 1242, 1534) in some samples. Those data have been marked in the diagrams (Fig. 3-4, Supporting Information Fig. S3-1) and excluded from the statistical tests. The same applies to low-abundant OS species having poor isotopomer patterns. Another uncertainty was the M+1 isotopomer peak of sialyllactose, an acidic OS detectable in the positive ion mode, which overlapped with the M isotopomer of difucosyllactose at *m/z* 657 (see also (Blank *et al.* 2011)). Therefore, we excluded difucosyllactose from further analyses. The resolution of the M+1 OS isotopomers ranged from 6,500 to >12,000.

Structural identity of the OS was confirmed by fragmentation analysis (see also (Blank *et al.* 2011)). Furthermore, MALDI-TOF-MS runs with or without preceding SPE steps with blanks of pure water or dextran hydrolysate, or single HMO in water confirmed the absence of the OS species detected in the real samples, which are listed in Table 3-1. Hence, an experimental cross-contamination of individual HMO samples could be excluded.

The internal standard detected at *m/z* 1176 did not exhibit significant variations in fA_{M+1} over time, as was also confirmed by statistical evaluation (Fig. 3-3 and Fig. 3-4; Table 3-2). As an additional control for instrument-related effects on the measured fA_{M+1} values, we compared them with theoretical fA_{M+1} values calculated from the natural isotopic H, C, N and O abundances and the chemical formula of the OS (see Fig. 3-3B and Fig. 3-4).

3.4 Discussion

Our noninvasive study design comprised sample collection over 36 h with 10 time intervals, resulting in a finer time scale, but also in large numbers of samples with low sample volumes and low analyte concentrations in many cases. Thus, our approach, including automated SPE combined with rapid and sensitive MALDI-TOF-MS analysis (see also (Blank *et al.* 2011)), facilitates comprehensive *in vivo* metabolic studies with stable isotopes in infants, beyond a clinical setting.

To our knowledge, no comparable studies have been done so far to simultaneously trace the fate of >10 distinct glycans over time *via* two organisms, beginning from their biosynthesis in the mother up to their excretion *in vivo* in her infant. Several publications are available on studies applying ^{13}C tracers for elucidating the fate of single hexoses in infants and lactating women (Kalderon *et al.* 1989; Lapidot 1990; Sunehag *et al.* 2002), having their focus on monosaccharides only.

3.4.1 Isotopomer abundances in mother–child pairs over time

Isotopomer enrichment in milk OS, as measured by MALDI-TOF-MS, was observed in all 10 women a few hours after tracer ingestion, followed by a decline at 12–20 h (Fig. 3-3A). This pattern was, except for fucosyllactose, homogeneous in all analyzed OS, which indicates a successive incorporation of the ^{13}C tracer into distinct HMO in the mammary gland after the single-dose application. Our previous IRMS data from whole milk and total neutral or acidic OS fractions showed a similar time pattern (Rudloff *et al.* 2006). As has previously been suggested, the two- and three-maximum patterns over time in total enrichment of milk fractions might be explained by the secondary utilization of the tracer from the carbohydrate storage in other compartments (Rudloff *et al.* 2006), such as glycogen, since the second maximum appears after night fast. Slight

variations in the time pattern of tracer enrichment have been observed in more complex milk OS with *m/z* ≥ 1753, equivalent with difucosylated octaoses and larger structures (Fig. 3-3A and C). Since each molecular species in our data set represents a pool of various structural isomers, this is not surprising due to very likely differences in the kinetics of the biosynthesis of each isomer, which should be more pronounced in complex than in small OS species with less isomers.

The strong inter-individual and inter-structural variation in the tracer enrichment of the urinary OS over time (Fig. 3-4), in particular, if compared with a rather uniform pattern in milk, is an intriguing new finding. Moreover, it is surprising due to the fact that the overall MALDI-TOF-MS profiles from urinary OS resembled those from mother's milk (Fig. 3-2), which is in line with previous publications without stable isotope labeling (Chaturvedi *et al.* 2001; De Leoz *et al.* 2013). Various explanations are possible for this striking deviation. First, despite the normalization to creatinine, the amount of labeled OS in the urine varied, as time points for diaper change were not explicitly regulated. Secondly, gastrointestinal transit time may vary to a high extent, especially in infants (Sievers *et al.* 1993). After 1 h of gastric transit (Vivatvakin *et al.* 2010) HMO are thought to reach the proximal small intestine intact (Gnoth *et al.* 2000). Then, various other individual factors, such as absorption, circulation and the excretion of the OS, may have contributed to the variations in time. This can, in part, explain the delayed increase of isotopomer enrichment in several OS species in the urine of infants 2–5 and 10.

Regarding the metabolic fate of single OS species in each mother–child pair, we partly observed the appearance of the same ^{13}C-enriched OS in infants' urine as in mothers' milk at a similar net time after tracer ingestion (Table 3-2). This indicates the excretion of intact HMO in infants' urine after intestinal absorption, even for large and fucosylated OS, such as the mono-, di- and trifucosylated hexaoses, with *m/z* 1242, 1388 and 1534, respectively. Strikingly, the ^{13}C-enrichment of those OS in infants' urine, but not in milk, seems to be higher than those of the tetraoses, *m/z* 731, 877, 1023, in urine 4, 5, 6, 8 and 9 (Fig. 3-4). One may speculate that the larger OS had been excreted in their intact form, originating from mother's milk, whereas tetraoses may, at least to a certain extent, reflect degradation products from larger HMO, which had lost the ^{13}C tracer. Rudloff et al. reported that LNT was found to comprise the label in both Gal moieties (Rudloff *et al.* 2006). Unfortunately, further examination by means of fragmentation was not possible due to poor resolution of the isotopomers in the fragmentation mode. Furthermore, comparability between the OS species might be limited by accuracy considerations as discussed below.

An unexpected observation was that the excretion of labeled OS in the urine of infants 1 and 9 was still significant at 28–36 h, although the enrichment in milk had been nonsignificant already about 16–20 h before. This finding suggests that OS from milk may stay in infant's organism for several hours after absorption before being excreted *via* urine. Keeping in mind the various potential functions of HMO, including both, intestinal as well as systemic effects (Bode 2012), the question regarding retention time within the gut and child's organism seems crucial for a better understanding of their biological role *in vivo*. In this context, it is noteworthy that we also determined the isotopomer abundances in six fecal samples available from infant 1 between 8 and 32 h after ^{13}C-Gal application to the mother (Supporting Information Fig. S3-1). The time pattern of the fA_{M+1} varied remarkably between OS species. Interestingly, differences over time in the intestinal appearance of the same OS species in HMO-fed rats were recently reported by (Jantscher-Krenn *et al.* 2013). *In vitro* experiments on the transport of HMO by intestinal Caco-2 cells indicated the possibility of re-secretion of some OS structures (Gnoth *et al.* 2001), probably causing variations in their intestinal transit time. As soon as stable isotope-labeled HMO structures are available in future, studies applying singly labeled components to breastfed infants, preferably with collection of feces and urine for >36 h, should provide a more detailed insight into the metabolic fate of dis-

tinct structures. Since larger/smaller HMO may be degraded/modified to structures, which themselves resemble HMO structures again, stable isotope labeling techniques are the method of choice for the elucidation of their structure-specific metabolic fate in the infant.

In addition to the fA_{M+1}, we monitored the values for fA_{M+2}. The overall time patterns were similar to those of M+1. However, because of lower precision and higher variation in the fA_{M+2} of the internal standard, we excluded the M+2 data from our calculations. The resulting uncertainty can be neglected here due to the low levels of precursor enrichment (Hellerstein and Neese 1999).

3.4.2 Methodical considerations

Spot urine samples are known to vary in the concentration of the excreted substances, i.e., OS as well as peptides and other interfering hydrophilic substances (Gowans and Fraser 1987). Consequently, we standardized the urine volume for sample preparation to the creatinine concentration to assure comparability between the urine samples during SPE and MALDI-TOF-MS analysis (see also (Peelen *et al.* 1994). Urinary creatinine excretion is known to be constant throughout the day and is therefore a common parameter for the excretion rate of various urinary analytes (Srivastava *et al.* 2007). In our samples, urinary creatinine concentration and, thus, presumably the analyte concentration varied by a factor of about 10.

The preparative and analytical procedure was validated based on the MALDI-TOF-MS signal intensity and the fractional abundance precision and the resolution of the isotopomers. Supportively, we matched the measured baseline isotopomer abundances to their theoretical value and monitored the fA_{M+1} of the internal standard. In six women, we observed positive deviation of the baseline fA_{M+1} from theoretical values in milk OS at *m/z* 512 and 876 by 8–26 and 6–12%RSD, respectively. This may be due to the chemical addition of hydrogen in the ion source, which is reported to increase with analyte concentration (Hellerstein and Neese 1999). Congruously, these two molecular species count among the most abundant in the MALDI mass spectra from milk. Negative deviation from the expected theoretical values by 8% was observed in three women for the ions at *m/z* 1096, $Hex_4HexNAc_2$, an OS species which was low-abundant in most samples.

3.4.3 Conclusions

MALDI-TOF-MS has been proven as a suitable technique for the determination of OS isotopomer enrichment in human milk and infants' urine after *in vivo* labeling using a ^{13}C tracer. Following this approach, we were able to trace the metabolic fate of carbohydrates from the biosynthesis of individual OS species in the mammary gland to their excretion in the breastfed child over the course of time, and thus to discriminate between original HMO and other nonlabeled OS originating from possible endogenous sources or from HMO degradation. The direct comparison of OS from milk and urine allowed us to detect remarkable differences in the time course and extent of tracer enrichment between single OS species in infants' urine. Former investigations from other groups did not suggest any modifications or notable utilization of HMO after absorption, as nonlabeled urinary OS were analyzed, which reflected the specific profile found in mother's milk. Thus, we provide new indications for the role of HMO within infant's organism, which has possibly been underestimated so far. Consequently, differentiated investigations in infants with single, stable isotope-labeled HMO are still much-needed for a better understanding of the biology of HMO.

3.5 Materials and Methods

^{13}C-Gal (D-Gal; 1-^{13}C, purity >99%) was obtained from Eurisotop (Saint-Aubin Cedex, France). Maltoheptaose (analytical grade), water, acetonitrile (ACN; HPLC grade), NH_4HCO_3 and 6-aza-2-thiothymine (ATT) were purchased from Sigma- Aldrich, Steinheim, Germany. Trifluoroacetic acid (TFA; ULCMS grade) was purchased from LGC Standards,Wesel, Germany

Subjects and study design

Exclusively breastfeeding women (n = 10; 3–6 months postpartum) and their healthy, term infants participated in the study. The consumption of naturally ^{13}C-rich food was restricted. The study was approved by the ethics' committee of the University of Giessen. Written informed consent was obtained from all women. In the morning, mothers orally ingested a single dose Gal bolus consisting of 23 g Gal + 2 or 4 g ^{13}C-Gal, dissolved in 50 mL of drinking water (Rudloff *et al.* 2006; Rudloff *et al.* 2012).

Sample collection

Milk (5–10 mL) had been obtained by manual expression before the Gal bolus was given (baseline value) and at the beginning of each nursing during the following 36 h (Fig. 3-1). Infants' urine was collected in diapers consisting of cellulose, free of other adsorptive material (Procter & Gamble, Frankfurt, Germany). In three infants, adhesive bags were used additionally for urine collection and were changed/emptied before each nursing and immediately frozen at −20°C, as well as the diapers. For urine extraction, diapers uncontaminated with feces were thawed and mechanically pressed. In previous experiments, it was verified that the urine collection *via* diapers and urine bags did not affect HMO analysis (Rudloff *et al.* 2012). The time course of isotopomer enrichment could not be analyzed in infant 7 due to an unclear documentation of urine collection time.

Isolation of OS from human milk and infants' urine

HMO were prepared for extraction as described by (Blank *et al.* 2011). Fifty microliters of milk were centrifuged (14,000 × g) at room temperature for 20 min after the addition of 450 µL pure water. The aqueous phase was transferred into a 96-well plate. SPE with porous graphitic carbon (PGC) cartridges (HyperSep-96 Wells, 25 mg; Thermo Scientific, Bellefonte PA) was performed *via* a Hamilton Microlab Starlet liquid handling system (HamiltonRobotics, Reno, NV). Cartridges were equilibrated with 2 × 250 µL 40% ACN in water (v/v), followed by 2 × 250 µL 20% ACN in water (v/v), both containing 0.1% (v/ v) TFA. Prior to and after sample loading, the cartridges were washed with 5 × 500 µL 0.1% aqueous TFA and fully dried by vacuum. OS were eluted with 400 µL aqueous 40/0.1% ACN/TFA solution and dried overnight in a vacuum centrifuge (Blank *et al.* 2011).

SPE of OS from urine was performed manually using a Chromabond Vacuum manifold (Macherey-Nagel, Düren, Germany) with PGC cartridges (HyperSep Hypercarb 50 mg; Thermo Scientific, Bellefonte PA). After centrifugation (14,000 × g, 20 min, 4°C), 2–4 mL of diluted urine were loaded onto PGC cartridges, which were equilibrated as described for milk in the previous paragraph, but with 2 × 1 mL each. Following the washing with 5 × 1 mL 0.1% aqueous TFA, OS were eluted in two fractions using 2 × 250 µL aqueous 40/0.1% ACN/TFA solution. For normalization of the amount of urinary constituents applied in the extraction procedure, creatinine concentration was determined using a colorimetric kit from R&D Systems (Parameter™, Minneapolis, MN).

OS analysis by MALDI-TOF-MS

OS were re-suspended in HPLC grade water and mixed with maltoheptaose as an isotopically nonenriched internal standard. The matrix, 35 mM ATT in 25 mM aqueous NH_4HCO_3, was pipetted in triplicate onto a MALDI AnchorChip™ 600 target plate (Bruker, Bremen, Germany) and mixed 1:1 with the OS sample. The matrix-sample spots were dried under cold air. Mass spectra were acquired using an Ultraflex I instrument (Bruker, Bremen, Germany), equipped with a nitrogen laser emitting at 337 nm. In the positive ion reflector mode, the voltage was set to 25 kV for acceleration, 26.3 kV for the reflector and 1.715 kV for the detector. Matrix ion deflection was off due to its impact on the detection of ions with *m/z* < 700. OS profiles were acquired in the mass range of *m/z* 360–3000. For calibration, dextran hydrolysate was used. Structural identification was performed by MALDI-TOF/TOF-MS in the laser-induced dissociation mode, as described by (Blank *et al.* 2011). Data acquisition and analysis were done by the flexControl and flexAnalysis 3.0 software (Bruker, Bremen, Germany), respectively. The isotopomer peaks were detected by the Centroid algorithm. Fractional abundances for each OS isotopomer M+1 were calculated as:

$$fA_{M+1}\,(\%) = \frac{I_{M+1} \times 100}{I_M + I_{M+1}}$$

where I_M is the intensity (height) of the mass isotopomer containing ^{1}H, ^{12}C, ^{14}N, ^{16}O and I_{M+1} is the intensity of its first isotopic peak (Fig. 3-1).

The theoretical fA_{M+1} were calculated by the Isotope Pattern Utility (flexAnalysis 3.0, Bruker, Bremen, Germany) based on the chemical formula of the OS and a natural abundance of the ^{13}C-isotope of 1.082%.

Statistical analysis

The fA_{M+1} of triplicate MALDI-MS measurements were processed in SPSS Statistics 20.0 (IBM, Ehningen, Germany) by one-way analysis of variance followed by Dunnett's one-tailed t-test, comparing the means of triplicates for each time point with the baseline sample. In case of unequal group variances– i.e. a significant Levene's test–Kruskal–Wallis H-test, followed by Dunnett's T3 post hoc test were run instead. The threshold for significance was set at $P < 0.05$.

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Conflict of interest

None declared.

3.6 Supporting Information

Supplementary data for this article is available online at http:// glycob.oxfordjournals.org/ and in the following.

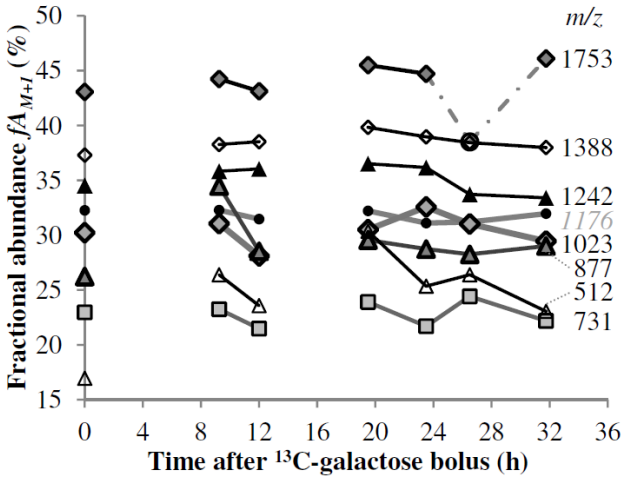

Fig. S3-1 Fractional abundances of the oligosaccharide isotopomers M+1 (fA_{M+1}) in fecal samples of infant 1 during 36 h after the mother's oral ^{13}C-Gal intake. Each curve represents the time course of the oligosaccharide with the *m/z* value given for $[M+1+Na]^+$ ions, as analyzed by MALDI-TOF-MS. For structures see Table 3–1; *m/z 1176* refers to the internal standard maltoheptaose. Baseline values were not measured, but calculated from the chemical formula of the glycan and natural H, C, N, O isotope abundances. Open circle with dashed line: value with 10–15%RSD within the triplicate measurements.

Chapter 4

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RESEARCH ARTICLE

Metabolic fate of neutral human milk oligosaccharides in exclusively breast-fed infants

Viktoria Dotz[1], Silvia Rudloff[1,2], Christina Meyer[1], Günter Lochnit[3] and Clemens Kunz[1]*

[1] Institute of Nutritional Science, University of Giessen, Giessen, Germany
[2] Department of Pediatrics, University of Giessen, Giessen, Germany
[3] Institute of Biochemistry, Faculty of Medicine, University of Giessen, Giessen, Germany

Scope: Various biological effects have been postulated for human milk oligosaccharides (HMO), as deduced from in vitro, animal, and epidemiological studies. Little is known about their metabolic fate in vivo in the breast-fed infant, which is presented here.
Methods and results: Human milk and infant urine and feces were collected from ten mother–child pairs and analyzed by MALDI-TOF MS (/MS), accompanied by high-performance anion-exchange chromatography with pulsed amperometric detection. Previously, we detected intact small and complex HMO in infant urine, which had been absorbed from gut, as verified via intrinsic ^{13}C-labeling. Our current work reveals the presence of novel HMO metabolites in urine and feces of breast-fed infants. The novel metabolites were identified as acetylated HMOs and other HMO-like structures, produced by the infants or by their gut microbiota. The finding of secretor- or Lewis-specific HMO in the feces/urine of infants fed with nonsecretor or Lewis-negative milk suggested a correspondent modification in the infant.
Conclusion: Our study reveals new insights into the metabolism of neutral HMO in exclusively breast-fed infants and provides further indications for multiple factors influencing HMO metabolism and functions that should be considered in future in vivo investigations.

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1 Introduction

Oligosaccharides (OS) are the third most abundant component in human milk with concentrations ranging from 7 to 20 g/L [1]. The quantity and structural variety are unique among mammalian species: Over 150 distinct structures with various structural isomers, which are lacking in bovine milk-based infant formula, have been described so far. Since human milk OS (HMO) are enzymatic products of different glycosyltransferases in the mammary gland and, therefore, evolve high structural variety, it is not surprising that their absorption and metabolism may largely be dependent on individual structures, including specific structural motifs such as fucose (Fuc) and sialic acid [2]. HMO are marginally digested by human intestinal enzymes [3], whereas infant gut microbiota can utilize various HMO structures as carbohydrate source [4–6].

Biosynthesis starts with lactose as the backbone of HMO, which can be elongated with *N*-acetylglucosamine (GlcNAc) and galactose (Gal) residues to form GlcNAc-β1-3Gal or GlcNAc-β1-4Gal units, resulting in the main core structures lacto-*N*-tetraose (LNT) and *neo*-LNT (LN(n)T), respectively.

Correspondence: Professor Clemens Kunz, Institute of Nutritional Science, University of Giessen, Wilhelmstr. 20, 35392 Giessen, Germany
E-mail: clemens.kunz@uni-giessen.de
Fax: +49-6419939049

Abbreviations: **Ac**, acetyl-; **FL**, fucosyllactose; **Fuc**, fucose; **Gal**, galactose; **GlcNAc**, *N*-acetylglucosamine; **Hex**, hexose; **HexNAc**, *N*-acetylhexosamine; **HMO**, human milk oligosaccharide(s); **HPAEC-PAD**, high-performance anion-exchange chromatography with pulsed amperometric detection; **Le**, Lewis; **LNdFH**, lacto-*N*-difucohexaose; **LNFP**, lacto-*N*-fucopentaose; **LNT**, lacto-*N*-tetraose; **LN(n)T**, lacto-*N*-(neo)tetraose; **OS**, oligosaccharide(s); **Se**, secretor

*Current address: Department of Chemistry and Pharmaceutical Sciences, VU University Amsterdam, Amsterdam, The Netherlands

4 Metabolic fate of neutral human milk oligosaccharides in exclusively breastfed infants (Manuscript 3)

4.1 Abstract

Scope: Various biological effects have been postulated for human milk oligosaccharides (HMO), as deduced from *in vitro*, animal, and epidemiological studies. Little is known about their metabolic fate *in vivo* in the breast-fed infant, which is presented here.

Methods and results: Human milk and infant urine and feces were collected from ten mother-child pairs and analyzed by MALDI-TOF-MS (/MS), accompanied by high-performance anion exchange chromatography with pulsed amperometric detection. Previously, we detected intact small and complex HMO in infant urine, which had been absorbed from gut, as verified *via* intrinsic ^{13}C-labeling. Our current work reveals the presence of novel HMO metabolites in urine and feces of breast-fed infants. The novel metabolites were identified as acetylated HMO and other HMO-like structures, produced by the infants or by their gut microbiota. The finding of secretor- or Lewis-specific HMO in the feces/urine of infants fed with nonsecretor or Lewis-negative milk suggested a correspondent modification in the infant. **Conclusion:** Our study reveals new insights into the metabolism of neutral HMO in exclusively breast-fed infants and provides further indications for multiple factors influencing HMO metabolism and functions that should be considered in future *in vivo* investigations.

4.2 Introduction

OS are the third most abundant component in human milk with concentrations ranging from 7 to 20 g/L (Thurl *et al.* 2010). The quantity and structural variety are unique among mammalian species: Over 150 distinct structures with various structural isomers, which are lacking in bovine milk-based infant formula, have been described so far. Since HMO are enzymatic products of different glycosyltransferases in the mammary gland and, therefore, evolve high structural variety, it is not surprising that their absorption and metabolism may largely be dependent on individual structures, including specific structural motifs such as Fuc and sialic acid (Gnoth *et al.* 2001). HMO are marginally digested by human intestinal enzymes (Gnoth *et al.* 2000), whereas infant gut microbiota can utilize various HMO structures as carbohydrate source (Garrido *et al.* 2012; Kitaoka 2012; Marcobal *et al.* 2011).

Biosynthesis starts with lactose as the backbone of HMO, which can be elongated with GlcNAc and Gal residues to form GlcNAc-1-3Gal or GlcNAc-1-4Gal units, resulting in the main core structures LNT and neo-LNT, respectively. These structures can be further elongated and branched, or also modified by the attachment of α1-2-/α1-3- or α1-4-linked Fuc and/or *N*-acetylneuraminic acid, the latter forming acidic HMO (Blank *et al.* 2012a). α-Fuc-(1→4)[Gal-β-(1→3)]-GlcNAc residues are referred to as Le^a epitope, α-Fuc-(1→2)-β-Gal as Se or blood group H epitope. Four different milk groups have been described in women referring to the presence or absence of these two epitopes on HMO resulting from the expression of the respective glycosyltransferases in the mammary gland (Blank *et al.* 2012a; Thurl *et al.* 2010).

Many beneficial effects of breast-feeding might be associated with the presence of specific HMO, such as the lower prevalence of infections of the respiratory, intestinal, and urogenital tract, or of autoimmune diseases due to their postulated prebiotic, anti-infective, and immunomodulatory effects (Bode 2012; Kunz *et al.* 2014; Schack-Nielsen and Michaelsen 2007). However, because of ethical and analytical limitations, most studies have been performed *in vitro* or in animals, giv-

ing only limited insight into the functions and metabolic pathways of HMO in the breast-fed child. Human metabolic studies are usually restricted to the comparison of the OS profile in mother's milk with those in infant urine and feces to keep study design noninvasive in these particularly vulnerable subpopulations, since interventional studies in formula-fed infants are not feasible as long as synthetic HMO are hardly affordable or available for use in humans (Rudloff and Kunz 2012).

Thus, our knowledge of the *in vivo* metabolism of HMO is limited, since studies on the excretion of single OS structures in infants either included only one or two study subject(s) (De Leoz *et al.* 2013; Rudloff *et al.* 2012; Sabharwal *et al.* 1988a; Sabharwal *et al.* 1984; Sabharwal *et al.* 1988b) or detected only few OS structures (Goehring *et al.* 2014; Rudloff *et al.* 1996; Sabharwal *et al.* 1991). Among those, only the publications by Sabharwal et al. described possible modification products, i.e. from blood group A transferase, in infants' feces, whereas other groups reported that the patterns of urinary and fecal OS in breast-fed infants resembled those of their mothers' milk (Chaturvedi *et al.* 2001; Coppa *et al.* 2001). More recently, OS in milk and infants' feces from ten mother–child pairs were investigated by CE with laser-induced fluorescence and ESI-MS, providing information on the possible modifications taking place in the infants' gut (Albrecht *et al.* 2011a). However, data on urinary metabolites providing information on the processes within the infant's organism after the absorption of HMO are lacking.

In previous studies with and without intrinsic stable isotope labeling of HMO, we found a renal excretion of 1–3% of HMO structures in the urine of exclusively breast-fed preterm and full-term infants analyzed by HPAEC-PAD (Rudloff *et al.* 1996) and IRMS or fast atom bombardment-MS (Rudloff *et al.* 2012). A large variety of neutral and acidic OS, and even trifucosylated hexaoses as found in milk were detected. Recently, we reported on the time course of distinct HMO in exclusively breast-fed infants as traced *via* ^{13}C-labeling and measured by MALDI-TOF-MS (Dotz *et al.* 2014). Thereby we revealed time and structure-dependent variations, suggesting significant metabolization and/or the coexcretion of structurally related non-HMO. In the present study, we compare the presence of individual OS structures from mothers' milk with those in the urine and feces of their exclusively breast-fed infants by using a sensitive and rapid MALDI-TOF-MS(/MS) approach, supported by isomer-specific HPAEC-PAD analysis. We also describe possible HMO metabolites, which are reported for the first time for infant urine and infant feces.

4.3 Materials and Methods

Subjects

Ten exclusively breast-feeding women participated in the study with their healthy term infants, aged 2–6 months, and gave their written informed consent. All procedures involving human subjects were approved by the ethics committee of the University of Giessen (permission number 77/00) (Rudloff *et al.* 2012).

Sample collection

Milk (5–10 mL) was obtained by manual expression at the beginning of each nursing during 36 h. Infant urine and feces were collected during the same 36 h in absorber-free diapers (Procter & Gamble, Frankfurt, Germany). In three infants, adhesive bags were used additionally for urine collection and were changed before each nursing and immediately frozen at –20°C, as well as the diapers. Urine diapers were visually examined for any fecal contamination. For urine extraction, diapers were thawed and mechanically pressed (Rudloff *et al.* 2012). Mothers were advised to immediately change the diapers after defecation to avoid urinary contamination.

Sample preparation and analysis

Chemicals were purchased from the following providers: maltoheptaose (analytical grade), water, ACN (HPLC grade), ammonium bicarbonate, ATT, and 2,5- dihydroxybenzoic acid from Sigma-Aldrich (Steinheim, Germany); TFA (ULC-MS grade) from LGC Standards (Wesel, Germany), sodium hydroxide from Baker (Deventer, The Netherlands); anhydrous sodium acetate from Dionex (Idstein, Germany), and water (Rotisolv ULC-MS) from Carl Roth (Karlsruhe, Germany). OS standards for HPAEC-PAD identification were either from Elicityl (Crolles, France), Dextra (Reading, UK), Glycom A/S (Copenhagen, Denmark), or Carbosynth Ltd (Berkshire, UK).

OS extraction

HMO were extracted as previously described (Blank *et al.* 2011). Briefly, milk was centrifuged after the addition of pure water. SPE with porous graphitic carbon cartridges (HyperSep-96Wells, 25 mg; Thermo Scientific, Bellefonte, PA, USA) was performed *via* a Hamilton Microlab Starlet liquid handling system (Hamilton Robotics, Reno, NV, USA). Cartridges were equilibrated with 40% ACN in water v/v, followed by 20% ACN, both containing 0.1% TFA. Prior to and after sample loading, cartridges were washed with 0.1% aqueous TFA and fully dried by vacuum. OS were eluted with 40% ACN containing 0.1% TFA (Blank *et al.* 2011).

Extraction of OS from urine was performed manually using a Chromabond Vacuum manifold (Macherey-Nagel, Düren, Germany) with porous graphitic carbon cartridges (HyperSep Hypercarb 50 mg) (Dotz *et al.* 2014). Briefly, after centrifugation, urine was loaded onto cartridges, which were equilibrated as described above. Following to washing with 0.1% aqueous TFA, OS were eluted in two fractions using aqueous 40/0.1% ACN/TFA solution. For normalization of the amount of urinary constituents applied in the extraction procedure, creatinine concentration was determined using a colorimetric kit from R&D Systems (ParameterTM, Minneapolis, MN, USA).

Fecal samples from diapers were thawed and dissolved in water (15 mg/mL) in duplicates, pasteurized (20 min, 65°C), and gently stirred for 1.5 h at 20°C prior to two-step centrifugation (10 000 × g, 15 min, 4°C) and filtration through a 0.45 µm nylon membrane (Rotilabo, Carl Roth). Extraction was performed automated as described in (Blank *et al.* 2011) for HMO extraction.

OS analysis

After elution, OS were dried overnight in a vacuum centrifuge, and resuspended in pure water. An HPAEC-PAD system, ICS-5000, equipped with a Carbo Pac PA-1 column (250 × 4 mm; 30°C) and a guard column, was operated by Chromeleon 6.80 software (all: Dionex). The gradient parameters at a constant flow rate of 0.5 mL/min were as follows: 100% eluent A, 0.1 M sodium hydroxide in water (0–15 min); 100% eluent B, 0.1 M sodium hydroxide and 0.25 M sodium acetate in water (83–87 min); 100% eluent A (87–107 min). External OS standards were used for identification.

Mass spectra were acquired using an Ultraflex I instrument (Bruker Daltonics, Bremen, Germany), equipped with a nitrogen laser emitting at 337 nm; a dextran hydrolysate was used to calibrate the equipment and the analytical method (Blank *et al.* 2011). Maltoheptaose was added as internal standard to OS extracts to assure comparable concentration in all samples. In triplicate, the OS from 36 h milk and infants' urine and fecal samples each were mixed 1:1 with ATT matrix (35 mM in 25 mM aqueous ammonium bicarbonate) on a MALDI AnchorChip^{TM} 600 target plate (Bruker Daltonics). The matrix-sample spots were dried under cold air. OS profiles were acquired in positive-ion reflectron mode over a mass range of *m/z* 360–3000 (Dotz *et al.* 2014). In addition, 0.25–1 µL of the OS extracts from milk, urine, and feces each were applied in triplicate onto the target plate, dried under cold air, and resuspended with 0.5–1 µL aqueous 32 mM 2,5-dihydroxybenzoic

acid/0.1% TFA matrix solution, which was again dried under cold air. This “reverse thin layer method,” modified from (Nishikaze and Amano 2009), results in higher sensitivity and improved S/N ratio in the low molecular mass range when applied to either standards or sample extracts. Acquisition was performed in the mass range of *m/z* 340–3200, with matrix ion deflection for ions with *m/z* < 300. The OS nature of the detected ions was confirmed by MALDI-TOF/TOF-MS in the laser-induced dissociation mode, as described in (Blank *et al.* 2011). Data acquisition and analysis were performed by flexControl and flexAnalysis 3.0 software (Bruker Daltonics), respectively.

4.4 Results

During the 36-h study, milk and infant urine samples were available from all ten mother–child pairs, whereas fecal samples were only obtainable from six children. Consequently, 98 milk, 73 urine, and 17 fecal samples were analyzed *via* MALDI-TOF-MS, whereas one to two distinct samples of each type from each mother–child pair were analyzed by HPAEC-PAD to obtain Se- and Le-specific HMO profiles after isomer separation.

4.4.1 Overall OS profiles in infant urine and feces compared to milk

Over 70 OS compositions have been detected by MALDI-TOF-MS in the 36-h sample set as listed in Supporting Information Table S4–1. More than 20 OS species thereof have not been described for breast-fed infants so far. Table 4-1 contains mainly those molecular species detected in urine or feces that are different from those in milk, indicating possible HMO metabolites or OS of non-HMO origin.

In Fig. 4-1, MALDI-TOF mass spectra from milk, urine, and fecal OS from two mother–child pairs are shown, with colored structures representing HMO, and unfilled figures for newly found OS species. The urine of infant a2 (left panel) contained a variety of OS species that have not been described previously, while complex HMO were hardly detectable, so that LN(n)T and its monofucosylated form dominated the urinary OS profile. In contrast, urinary OS in infant b2 still showed a structural diversity similar to milk, though the relative intensities of fucosylated OS at *m/z* 511, 657, 1022, and 1387 were lower than those of nonfucosylated OS at *m/z* 730, 1095, 1460 (Fig. 4-1, right panel). Analogously, urinary mono- and difucosylated Hex_2 as well as complex HMO were reduced in infant b5 (Supporting Information Fig. S4-1E). The urinary OS of infants a1 and c showed patterns similar to their mothers’ milk with a comparable diversity of structures, but with some additional non-HMO structures. In infants b1, b3-4, b6-7, Hex_2Fuc_1 increased and was the predominant peak, whereas larger HMO with *m/z* > 1200 decreased as compared to milk (Supporting Information Fig. S4-1A–H).

Various OS species have been detected in feces only, in particular, in samples from infants a2 and b1, e.g. ions at *m/z* 1282.5, 1298.4, 1339.5, or 1542.6. At the same time, complex HMO were hardly detectable in the feces of these two infants (Table 4-1; Fig. 4-1; Supporting Information Fig. S4-1B). The fecal OS profile of infant b2 showed a strong shift toward low- or nonfucosylated HMO (Fig. 4-1), with LN(n)T now being most prominent. In infant b3, LN(n)T was also relatively increased in feces compared to milk, whereas the opposite was the case in infant c, in whom, in addition, a relative increase of larger, more complex fecal HMO was observed (Supporting Information Fig. S4-1C and H). A strong increase in the larger HMO and non-HMO structures at *m/z* 1079 and 1444 was observed in infant a1 (Supporting Information Fig. S4-1A). The presence of LNT has been confirmed in all urine and fecal samples analyzed by HPAEC-PAD, while its isomer LN(n)T was not detectable in the urine of infants b1 and b5 (Table 4-2).

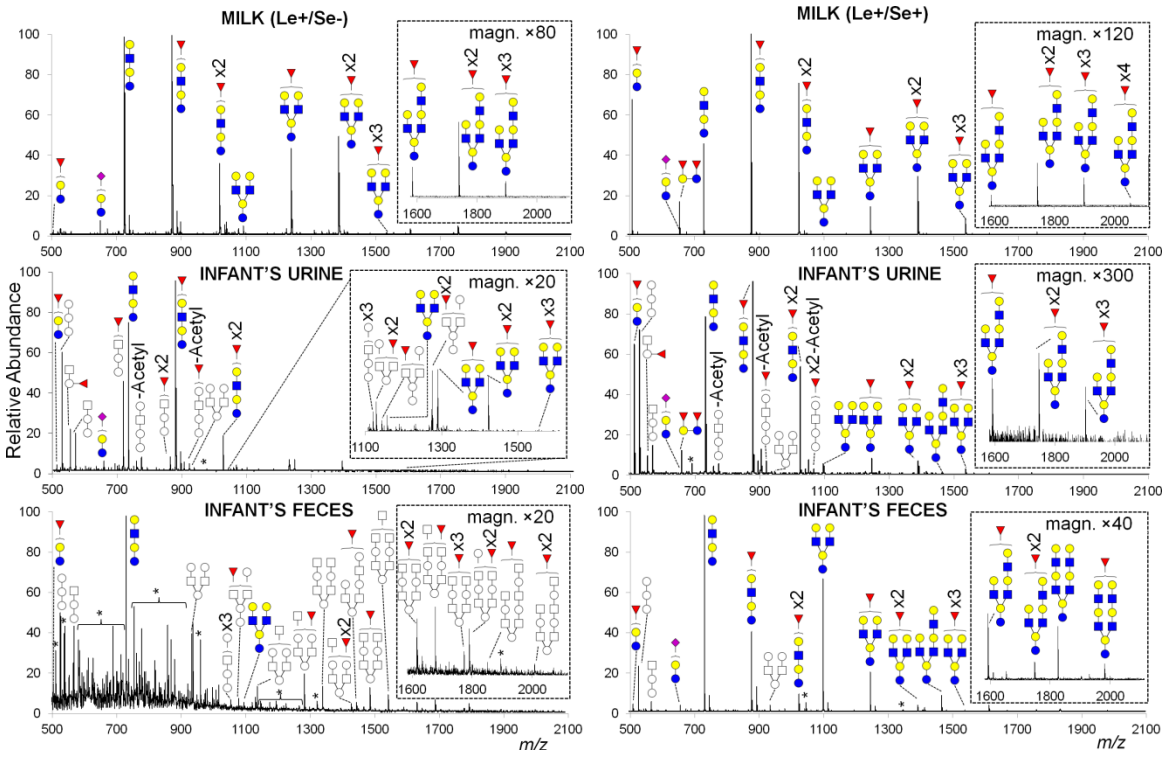

Fig. 4-1 MALDI-TOF-MS profiles of oligosaccharides in mother's milk, infant urine and feces from mother-child pair a2 (left panel) and b2 (right panel), as detected with 2,5-dihydroxybenzoic acid/TFA matrix. Ac, acetyl-; H, hexose; N, *N*-acetylhexosamine; F, fucose; pink diamond, *N*-acetylneuraminic acid; unknown oligosaccharides: open circle, hexose; open square, *N*-acetylhexosamine. Asterisks indicate non-oligosaccharide origin.

The urinary and fecal OS profiles as measured *via* HPAEC-PAD showed many variations as compared to the corresponding milk samples. Besides shifts in the relative intensities of the single HMO peaks, additional peaks, e.g. at t = 27.5 min, were detected in many samples (Fig. 4-2B). However, those could not be further characterized.

4.4.2 Possible modification and degradation products

Mainly in infants' urine, and in very low concentrations also in some milk and fecal samples, we detected acetylated (Ac) OS (Table 4-1). Those were $Ac_1Hex_2Fuc_1$, $Ac_1Hex_3HexNAc_1$, $Ac_1Hex_3HexNAc_1Fuc_{1/2}$, and $Ac_1Hex_4HexNAc_2Fuc_2$ at *m/z* 553, 772, 918, 1064, and 1429, respectively. Accordingly, MALDI-TOF/TOF-MS analyses confirmed the presence of characteristic fragment ions (Fig. 4-3; Supporting Information Table S4-2). Acetylated HMO fragments (*m/z* 772 and 918) as well as isomer-specific glycosidic bond cleavage or cross-ring fragmentation products could be assigned for Ac-Lacto-*N*-difucohexaose (LNdFH) II and I, respectively. The loss of the Ac residue, reflected in the cross-ring fragments of Ac-LNdFH I at *m/z* 816 and 962, suggests its attachment to glucose. We can exclude instrument-related adduction of Ac residues, as no ions at *m/z* corresponding to acetylated maltoheptaose, added as internal standard prior to the MALDI-TOF analysis, were detected. Neither do we expect modifications during sample preparation, as we still detected the respective acetylated structures in infant urine after an alternative extraction procedure *via* gel filtration, avoiding solvents containing acetyl residues. Moreover, we could not detect molecular species corresponding to acetylated OS in urinary samples of an infant from another cohort, exclusively fed with commercial galacto-OS-enriched formula (unpublished data). Consequently, as far as we know, this is the first report on acetylated neutral OS in humans.

Table 4-1 OS from mothers' milk and infants' urine and feces (excerpt)

m/z [M+Na]$^+$	Composition					Occurrence in MALDI-TOF-MS[b]			Reference[c]	
	Ac	H	N	F	MS/MS[a]	milk	urine	feces	urine	feces
553.2	1	2	0	1	yes	none	(a2),b1-2,(b3,b5),b6-7,c	(c)		
569.2	1	3	0	0	yes	(a2,b2)	all (b4)	(a2),b3		
771.3	0	2	2	0	yes	none	a1-2,b1-5,(b6),b7,c	a1,(a2),b1-2		
772.2	1	3	1	0	yes	(a1-2,b1-6,c)	a2,b1-3,b5,(b6),c	a2		
819.3	0	3	0	2	-	none	b4	none		d)
860.3	0	2	1	2	yes	(a1,b1,b3-7,c)	(a1),a2,b1-3,b5-7	a1,(a2),(b3),(c)		e)
917.3	0	2	2	1	yes	none	(a1-2,b3-4,b6-7)	a1,c		
918.3	1	3	1	1	yes	(a1,b1-7)	(a1),a2,(b1),b2-6,(b7),c	none		
933.3	0	3	2	0	yes	(b3-5,b7,c)	(all)	a1-2,b2,c		
1064.4	1	3	1	2	yes	a1, b1-4	a1-2, b1-7	none		
1079.4	0	3	2	1	yes	(all)	(all)	a1,(a2),b1,(b2),c		e)
1136.4	0	3	3	0	-	none	(a1-2,b2,b6)	(a1),a2,b1		
1184.4	0	4	1	2	yes	(b1-2),b3,(b4-7),c	b1,b4,c	b1,c		d)
1225.4	0	3	2	2	yes	(a1-2),(b1-6),b7,(c)	(all)	a1-2,b1,c		e)
1282.5	0	3	3	1	-	none	none	(a1),a2,(b1)		
1298.4	0	4	3	0	-	none	none	(a1),b1		
1339.5	0	3	4	0	-	none	none	(a1),a2,b1,(b3)		
1371.5	0	3	2	3	yes	(a1,b1-b7,c)	(a2),b3,(b5),b6	none		
1403.5	0	5	2	1	yes	(all)	(a1,b2,c)	a1,c		
1429.5	1	4	2	2	yes	(a1,b2,b6-7)	a1-2,(b1-5),b6,(b7),c	none		
1444.5	0	4	3	1	-	none	(a1,c)	a1-2,(b1)		
1542.6	0	3	5	0	-	none	none	(a1),a2,b1		
1631.6	0	3	4	2	-	none	none	a2,(b1)		
1745.6	0	3	6	0	-	none	none	b1		
1793.6	0	4	4	2	-	none	none	a2		

[a] Yes, composition confirmed *via* MS/MS (see Supporting Information Table 2); -, no reliable MS/MS spectra available due to, e.g., low abundance.
[b] Letters a, b, and c were assigned according to the Le- and Se-specific HPAEC-PAD profiles from milk, e.g. Fig. 2.
[c] Reference number is given if one or more compounds with the respective *m/z* value were previously described. [d] (Albrecht *et al.* 2011a); e) (Sabharwal *et al.* 1984)
Brackets indicate low intensity, i.e. S/N ratio <20. Ac, Acetyl; H, Hex; N, HexNAc; F, Fuc.

It is noteworthy that we also analyzed urine samples from women a2 and b1 (Supporting Information Fig. S4-2). Interestingly, the MALDI-TOF-MS profiles of the OS in these women's urine were more similar to those of their infants' urine than to milk, containing higher proportions of $Ac_1Hex_3HexNAc_1$ and other non-HMO.

The majority of the OS structures newly detected in infant urine or feces showed a higher proportion of HexNAc units than in original HMO, many of which were nonfucosylated. Only few structures, e.g. at *m/z* 1184 or 1403, had a lower proportion of HexNAc (Table 4-1 and Supporting Information Table S4-1). *Via* MALDI-TOF/TOF-MS/MS, we confirmed the OS composition of these structures (Supporting Information Table S4-2), and evaluated the site of fucosylation in $Hex_2HexNAc_1Fuc_1$ at *m/z* 714, which revealed Se-specific Hex_1Fuc_1-fragments in the milk of women b2,5,6, and c, and in the feces of infant b2. In contrast, in almost all urine samples, only $HexNAc_1Fuc_1$ fragments were detected (Supporting Information Fig. S4-3).

4.4.3 Le- and Se-specific OS in milk, infants' urine and feces

Table 4-2 gives an overview of the major neutral isomeric OS structures detected in milk and infant urine and feces from ten mother–child pairs *via* HPAEC-PAD. The presence of Le as well as Se-specific structures, such as LNFP II, 2'-fucosyllactose (2'FL), or LNdFH I, in milk of women b1-7, is characteristic for the Le^b phenotype (Fig. 4-2A). Accordingly, the absence of Le-specific OS in milk along with the presence of α1-2-fucosylated structures is typical for Le−/Se+, as detected

in woman c (Fig. 4-2A). Women a1 and a2 were classified as Le[a], since their milk contained α1-4-fucosylated OS, but was lacking Se-specific structures (Fig. 4-2A).

The Se-specific OS 2'FL, di-FL, LNFP I, and LNdFH I were detected not only in the urine and feces from infants fed Se+ milk (subjects b1-7, c), but also in samples from infants a1 and/or a2, though fed non-Se milk (Fig. 4-2B, Table 4-2). The abundance of di-FL in the urine samples available from infant a1 was sufficient to confirm its identity in MALDI-TOF/TOF-MS/MS by the detection of its mono- and defucosylated fragments at *m/z* 511 and 365, respectively (not shown). Le-specific LNFP II was detected in the feces of infant c, fed Le−/Se+ milk, which is lacking Le epitopes.

Table 4-2 Major neutral OS in mothers' milk and infants' urine and feces, detected by HPAEC-PAD

Peak no.	Oligo-saccharide	Structure [a]	RT [b] [min]	Epi-tope	Detected in [c]			*m/z* [e] $[M+Na]^+$
					milk	urine	feces [d]	
1	LNdFH I		13.9	Le, Se	b1-7	a1,b1-3,b5-7	a1,b1,b3	1022.4
2	3-FL		14.2		a1, c	b2,b4,b6-7,c	b1-2	511.2
3	LNFP II		16.6	Le	a1-2,b1-7	a1-2,b1-4,b6	a1-2,b1,c	876.3
4	Di-FL		18.5	Se	1,b1-7	a1,b1-7	a2,b1,b3,c	657.2
5	Lac		20.7		all	all	a1-2,b1-3,c	365.1
6	2'FL		24.5	Se	b1-7, c	a1-2,b1-7,c	a2,b1-3,c	511.2
7	LNFP I		31.1	Se	b1-7, c	a1,c	a1,b3,c	876.3
8	LNnT		34.6		all	a1-2,b2-4,b6-7,c	a1-2,b1-3,c	730.2
9	LNT		40.5		all	all	a1-2,b1-3,c	730.2

[a] Blue circle, glucose; yellow circle, galactose; blue square, *N*-acetylglucosamine, red triangle, fucose; for linkage designation, see Fig. 4-3.
[b] Retention time in HPAEC-PAD.
[c] Letters a, b, and c were assigned according to the Lewis (Le) and secretor (Se)-specific HPAEC-PAD profiles from milk, e.g. Fig. 4-2. **Se**, α-fucosyl-(1→2)-β-galactosyl-; **Le**, α-fucosyl-(1→4)[galactosyl-β-(1→3)]-*N*-acetylglucosaminyl-.
[d] Fecal samples were only available from infants a1-2, b1-3, and c. Infants' samples with Le/Se-specific structures, not matching the Le/Se-specificity of their mother's milk, are labeled gray;
[e] as detected in MALDI-TOF-MS, see Supporting Information Table S4-1.

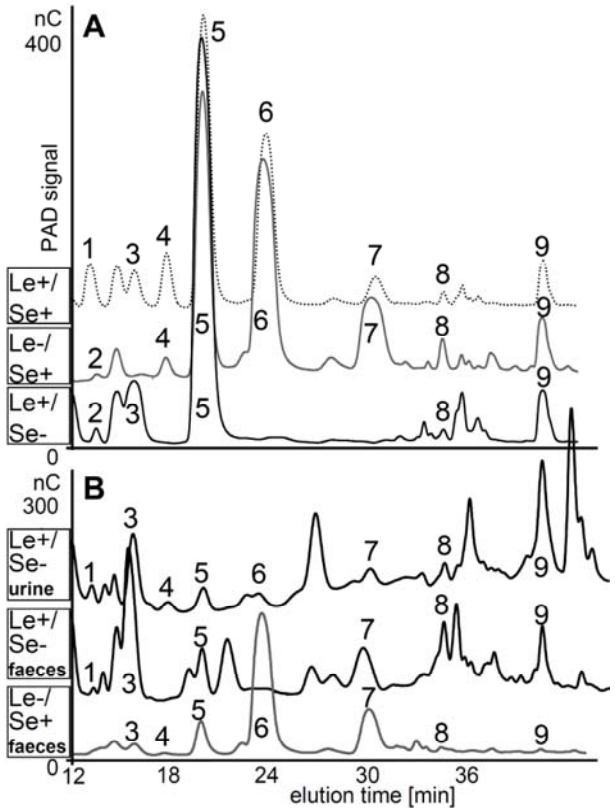

Fig. 4-2 HPAEC-PAD profiles of major OS in (A) milk from women b2 (Le+/Se+), c (Le−/Se+), and a1 (Le+/Se−); (B) urine and feces from infant a1 and feces from infant c. For OS structures see Table 4-2. An excerpt of 12–44 min out of the total run of 107 min is shown.

Se-specific LNFP I could not be detected in any of the analyzed urine samples of the infants fed Le^b milk. The same applied for LNFP I and its Le-specific isomer LNFP II in two of three infants' feces, which were obtainable from Le^b-fed infants (Table 4-2).

4.5 Discussion

Our simplified and rapid method for OS extraction from only 50 µL of milk and 2–4 mL of infant urine and 15 mg of fecal samples (see also (Blank *et al.* 2011; Dotz *et al.* 2014)), as compared to 10- to 60-fold higher sample requirement in (Albrecht *et al.* 2011a; De Leoz *et al.* 2013; Rudloff *et al.* 1996), enabled us to acquire OS profiles from repeated sample collection over 36 h. Thus, intra-individual variation over time could be compensated to some extent. Moreover, our MALDI-TOF-MS technique provides higher resolution than chromatography methods, which have been used conventionally for HMO analysis (Chaturvedi *et al.* 2001; Coppa *et al.* 2001; Rudloff *et al.* 2012; Rudloff *et al.* 1996; Thurl *et al.* 2010).

Urinary OS in breast-fed infants seem not simply to reflect the pattern found in their mothers' milk: The overall MALDI-TOF-MS profiles showed variation in terms of diversity of HMO-related peaks or the relative intensities of fucosylated versus nonfucosylated structures in most infants as compared to their mothers' milk. This finding was also confirmed by our HPAEC-PAD data, showing some shifts between the relative abundances of the single isomeric HMO structures. In the only publication reporting on the urinary versus milk OS profiles, the authors argued the converse (Chaturvedi *et al.* 2001). However, the authors did not show any data on the individual structures detected in urine. In contrast, in a recent publication, some major HMO structures consistently showed better correlations between the levels in mother's milk and infant plasma than infant urine (Goehring *et al.* 2014), supporting our finding of altered OS profiles in breast-fed infants' urine, and furthermore suggesting an enrichment or depletion of certain structures "after" intestinal absorption. In this regard, the absence of Se-specific LNFP I in the urine of all seven infants fed Le^b

milk was most intriguing, considering its predominance in milk of Le^b individuals (Fig. 4-2A, peak 7) with concentrations around 1–2 g/L, making it the second largest component after 2'FL (Thurl *et al.* 2010). Since we were able to detect LNFP I in the urine of infant c as well as LNFP II in most of the Le^b-fed infants, selective intestinal or systemic utilization of LNFP I seems more likely than a reduced absorption of this specific structure only in Le^b-fed infants.

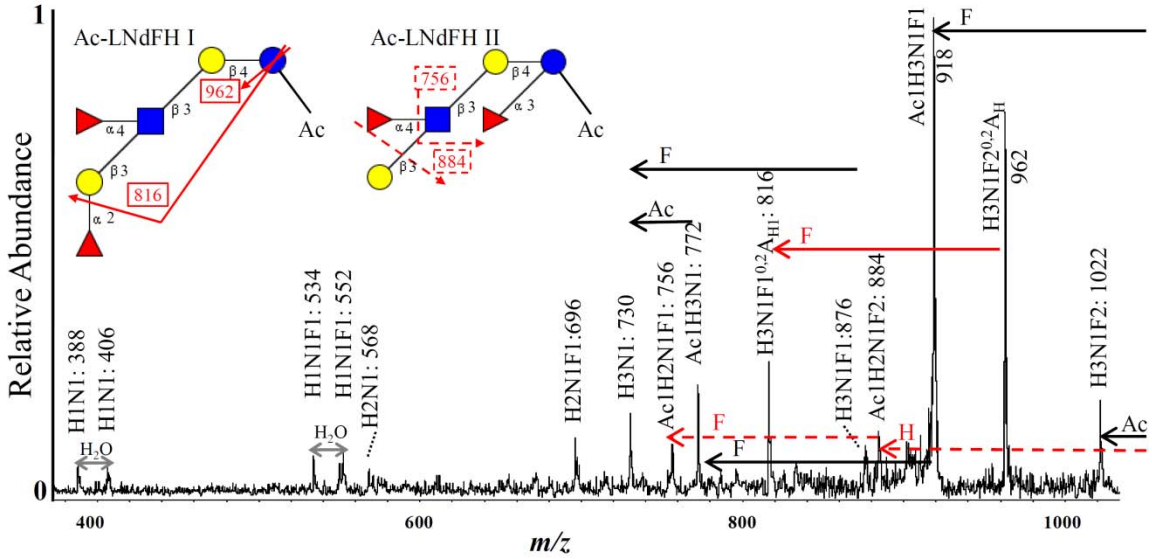

Fig. 4-3 MALDI-TOF/TOF-MS/MS of oligosaccharides at *m/z* 1064 in urine from infant b2. Ac, acetyl; F, fucose; H, hexose; N, *N*-acetylhexosamine. Solid and dashed red lines indicate isomer-specific fragmentation in Ac-lacto-*N*-difucohexaose I and II, respectively; black arrows apply to both isomers.

Our investigation of modification products of HMO revealed a novel type of OS in humans, i.e. acetylated neutral HMO in breast-fed infants' and breast-feeding women's urine. So far, only few studies revealed the significance of *O*-acetylation in OS, limited to acetylated sialic acids, e.g. as found in milk of echidna (Kamerling *et al.* 1982; Oftedal *et al.* 2014). *O*-acetylated sialic acids play an important role in human immunology, bacterial and viral infections, and are linked to type I diabetes, rheumatoid arthritis, and autoimmune disorders (Muthana *et al.* 2012), processes that HMO and/or breast-feeding are also associated with (Bode 2012; Kunz *et al.* 2014; Schack-Nielsen and Michaelsen 2007). A detailed structural characterization and the examination of the biological role of acetylated HMO in future studies is required to enhance our knowledge of the beneficial effects of breast-feeding on mother and child.

As further indication for HMO modification, we detected urinary LNFP I, di-FL, and LNdFH I in infant a1, though fed non-Se milk. Among those, only LNdFH I, which is Le^b-specific, has been reported before in the urine of an infant fed non-Se milk (De Leoz *et al.* 2013). Nevertheless, di-FL has previously been detected in the urine of starved Se-individuals (Lundblad 1978), which can be explained by the activity of α-2-FucT, found in various tissues besides the gastrointestinal tract (Watkins 1995). Recently, a novel sialylated lactosamine structure was detected in formula- and breast-fed infants' plasma, along with unexpectedly high concentrations of its other isomers (Ruhaak *et al.* 2014). Future studies should reveal the origin and biological role of these unexpected compounds in the infant.

With regard to fecal OS, we could roughly identify three groups of infants, as far as possible from only six individuals, i.e. (i) infants a1 and c with MALDI-TOF-MS profiles showing similar or even higher complexity than their mothers' milk, (ii) infants b2 and b3 with decreased relative intensities

of fucosylated HMO, and (iii) infants a2 and b1 with reduced HMO diversity or relative intensities of HMO, respectively, but increased number and intensity of non-HMO. For the first, the overall profiles were comparable with urine. Nevertheless, unexpected HMO structures were detected in the feces of infants a1, a2, and c (Fig. 4-2, Table 4-2). This suggests α-2-FucT activity (Sabharwal *et al.* 1988a), as described above also for urine, and α-4-FucT in the gut of infant c. Potential endogenous or xenobiotic sources of HMO-analogs are also conceivable, since human glycolipids and microbial lipo-OS share some identical or similar substructural features with HMO (Houliston *et al.* 2011). However, the detection of these substructures would require the activity of specific endoglycosidases, which have not been described in humans so far. The fact that we were able to detect LNT and LNnT in many infants' urine and all fecal samples (Table 4-2) is contradictory to the findings of Albrecht (Albrecht *et al.* 2011a). This may be explained by an ongoing degradation of HMO in the fecal slurries, kept overnight at 4°C by Albrecht et al. In contrast, we pasteurized the fecal samples directly after thawing to inactivate any bacterial activity prior to OS extraction.

The urinary $Hex_2HexNAc_1Fuc_1$ at *m/z* 714, bearing a HexNAc-Fuc epitope (Fig. 4-2, Supporting Information Fig. S4-3), may be a degalactosylated or deglucosylated cleavage product from LNFP II or III. Its presence in the urine of both, infants as well as lactating women, indicates intense degradation or modification of the precursors in circulation or body cells. In our recent report on the excretion of intrinsically labeled HMO in the same sample set as presented here, rather poor tracer enrichment of smaller urinary OS at *m/z* < 1200 was determined in infants a2 and b2 (alias infants 10 and 5 in (Dotz *et al.* 2014)). This indicates a coabundance of structurally related OS not originating from mother's milk. Since endogenous sources for such unlabeled HMO-like structures in urine are rather unlikely, as discussed above, a contribution from degradation products after the loss of the ^{13}C-tracer may be an alternative explanation.

The detection of additional OS structures with higher proportion of HexNAc as compared to original HMO, especially the larger ones in the feces of infants a2 and b1 (Table 4-1), suggest intestinal cleavage of the reducing-end glucose along with a modification by HexNAc-transferases, such as blood group A transferase (Albrecht *et al.* 2011a; Sabharwal *et al.* 1988b). In addition, the release of structurally related OS from mucins or glycoproteins by microbial or intestinal enzymes is a probable explanation (Dallas *et al.* 2012; Marcobal *et al.* 2011). A direct contribution of OS from plasma glycoproteins to the urinary profile is unlikely, since the few free neutral OS, which were detected recently in human serum (Iwatsuka *et al.* 2013), did not match the urinary OS compositions of our cohort. Nevertheless, a few HMO-like structures were recently detected in exclusively formula-fed infants' plasma (Goehring *et al.* 2014; Ruhaak *et al.* 2014), suggesting endogenous origin. The formula itself, since based on bovine milk, may also be a possible source.

Analogously, the OS with higher proportion of Hex found in a few infants' urine and fecal samples may be the products of blood group B transferase. However, the formation of Le^b, blood group A, and B-active structures implies the presence of Se/H epitopes (Watkins 1995). Consequently, in infants fed Le^a milk, the consecutive action of two distinct glycosyltransferases has to take place, which is energy-consuming, and therefore indicating to play an important role in the infant's gut and organism. α1,2-Fucosylated HMO were shown to be protective against pathogenic intestinal bacteria not only *in vitro, in vivo* in rats, and *ex vivo*, but also in a prospective epidemiological study (Morrow *et al.* 2004; Ruiz-Palacios *et al.* 2003). Le^a and Le-negative phenotypes were shown to be associated with recurrent urinary tract infections in women and children, respectively (Jantausch *et al.* 1994; Sheinfeld *et al.* 1989). Consequently, the presence or absence of Le and Se epitopes on epithelial surfaces of the intestinal and urinary tract as well as on soluble agents such as OS seems to have an impact on host–pathogen interactions.

Within the infant gastrointestinal tract, microbiota is known to be the key element in HMO metabolization (Dallas *et al.* 2012; Garrido *et al.* 2012; Marcobal *et al.* 2011). In turn, the composition of endogenous (host) as well as exogenous (mother's milk) glycans, especially those bearing the Se epitope, is thought to influence infant gut microbiota composition (Garrido *et al.* 2012; Wacklin *et al.* 2011). In the current work we have provided further indications for an involvement of ABO and Le-related fucosyltransferases in the modification of HMO, not only in the infant's gut, but also after absorption. The similar trend toward less fucosylation in infant b2's urine and feces points to an onset of intense utilization over the entire Fuc-HMO range in the upper segments of the gut, where absorption mainly takes place. In a recent study with rats, a decrease in the relative abundances of 2'FL and LNT were detected already in parts of the small intestine (Jantscher-Krenn *et al.* 2013). Since the impact of salivary, gastric, and small intestinal glycosidases on human milk glycan degradation is estimated to be negligible (Dallas *et al.* 2012; Gnoth *et al.* 2000), the contribution of HMO absorption or utilization by microorganisms in the small intestine might be of greater significance than estimated before. However, an evidence for that can only be provided by studies with applications of single, stable isotope-labeled HMO structures with blood sample collection in infants, which implies some ethical and technical constraints (see also (Ruhaak *et al.* 2014)).

In summary, *via* the combination of simplified OS extraction, rapid MALDI-TOF-MS, and isomer separation by HPAEC, we compared OS profiles from 36-h milk, urine, and fecal samples collected from ten mother–child pairs. Additional MALDI-TOF/TOF-MS/MS analyses revealed novel OS structures in infants' feces and urine, most likely degradation or modification products from microbial and endogenous enzymes in the infants' gut and organism. For the first time, we report on acetylated neutral HMO in infants' urine, which might have other functions and bioactivity as compared to original HMO. Moreover, by means of HPAEC-PAD we found Se- and Le-specific HMO in infant feces and, for the first time, in urine, which were not present in their mothers' milk, and therefore must have been synthesized by the infant. This finding may be relevant for future metabolic and functional studies of HMO, which should consider the Le, ABO blood group, and Se status of the infants as an important cofactor in addition to the infants' age and microbial composition in the gut.

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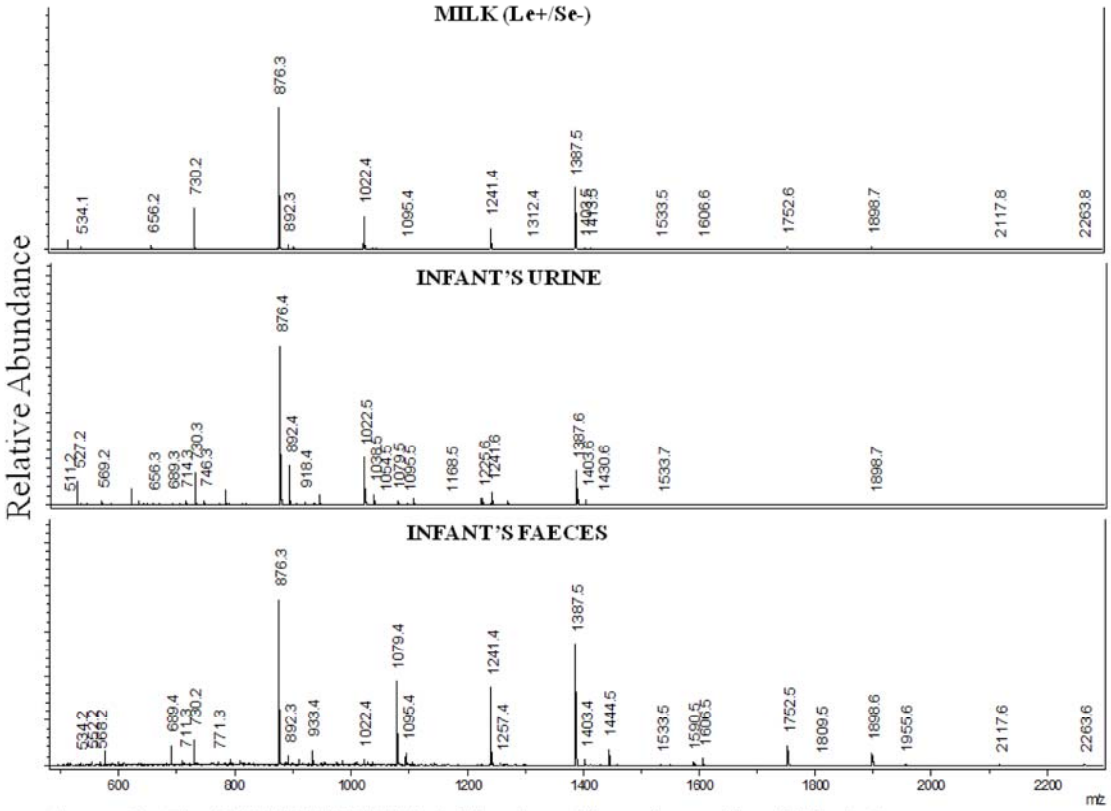
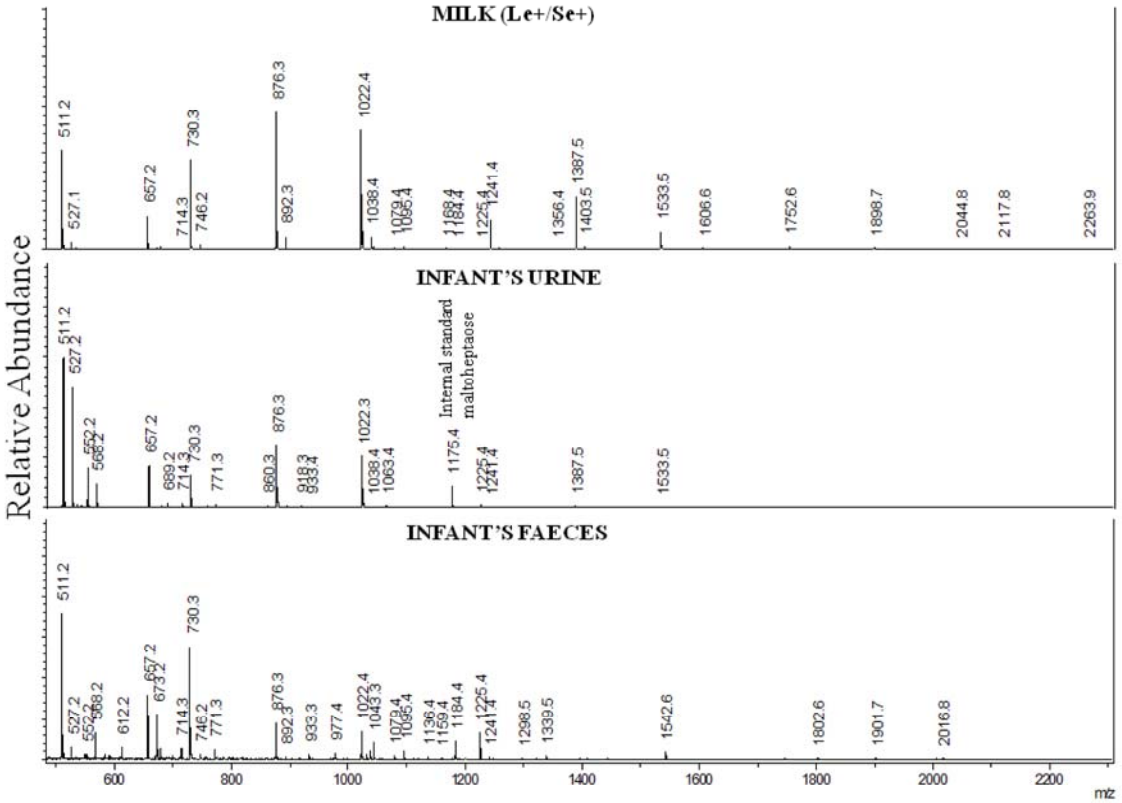
The authors have declared no conflict of interest.

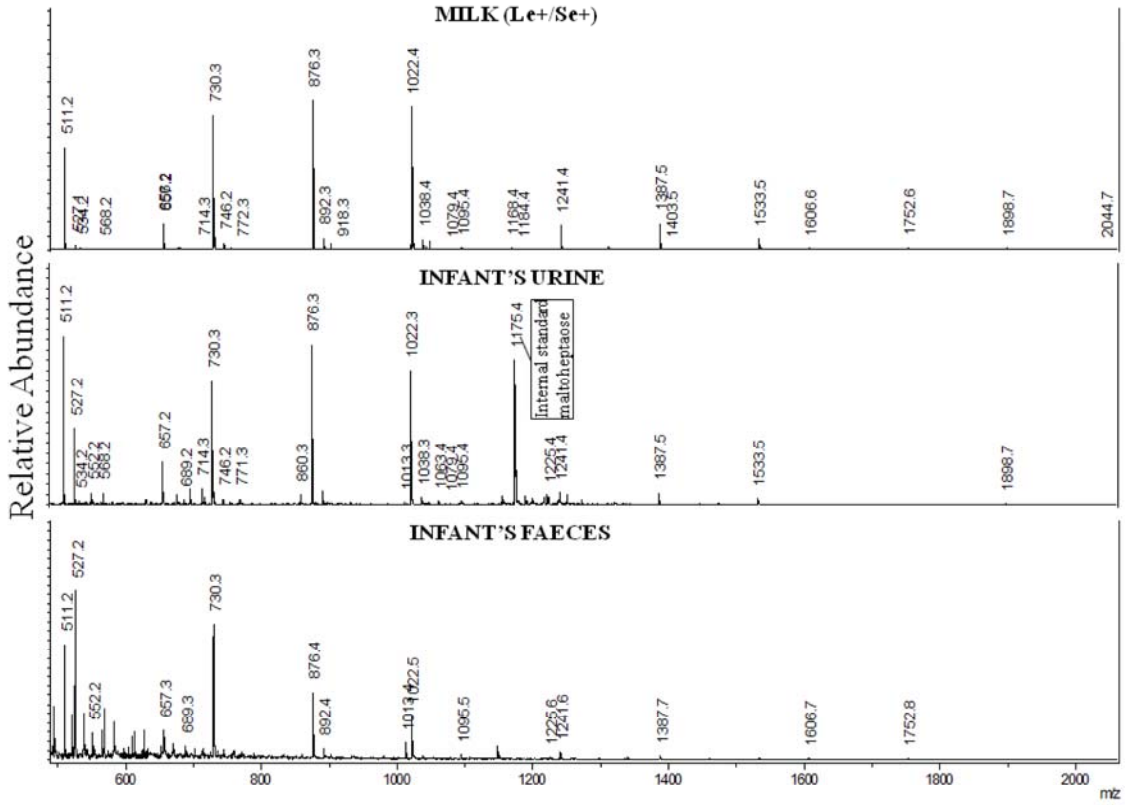
4.6 Supporting Information



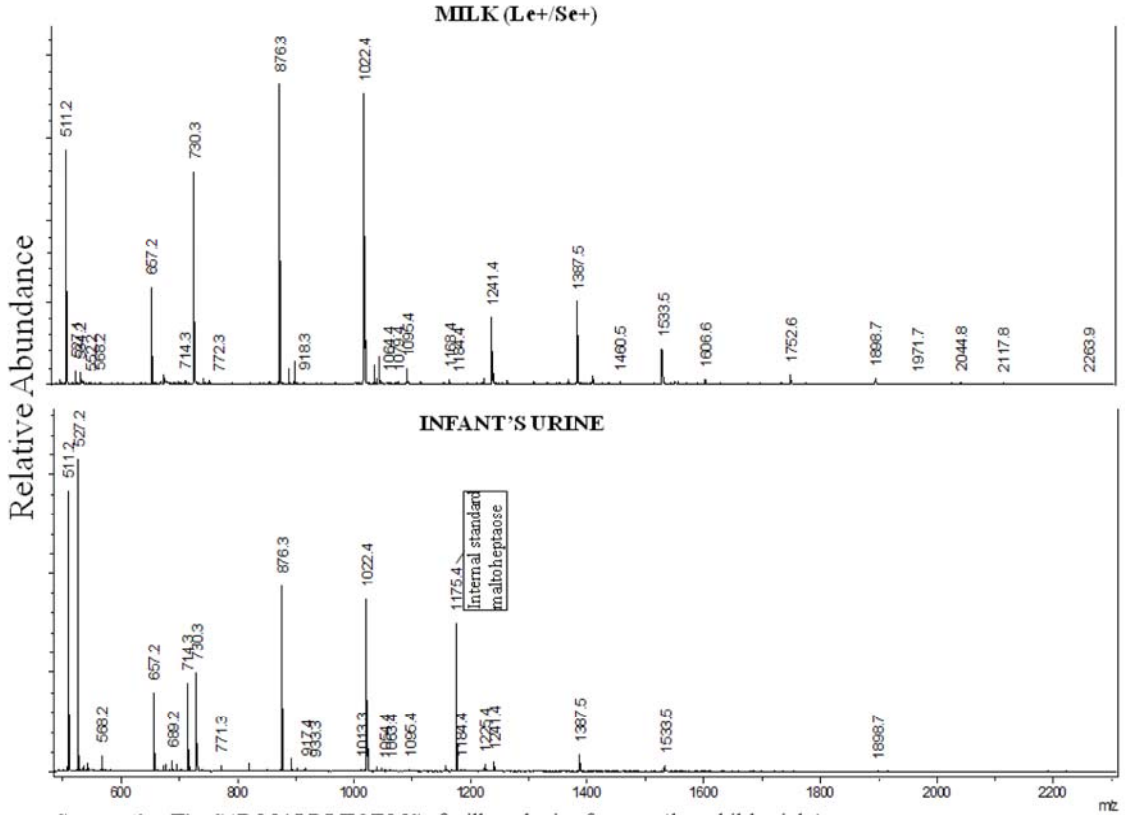
Supporting Fig. S1A MALDI-TOF MS of milk, urine and faeces from mother-child pair a1



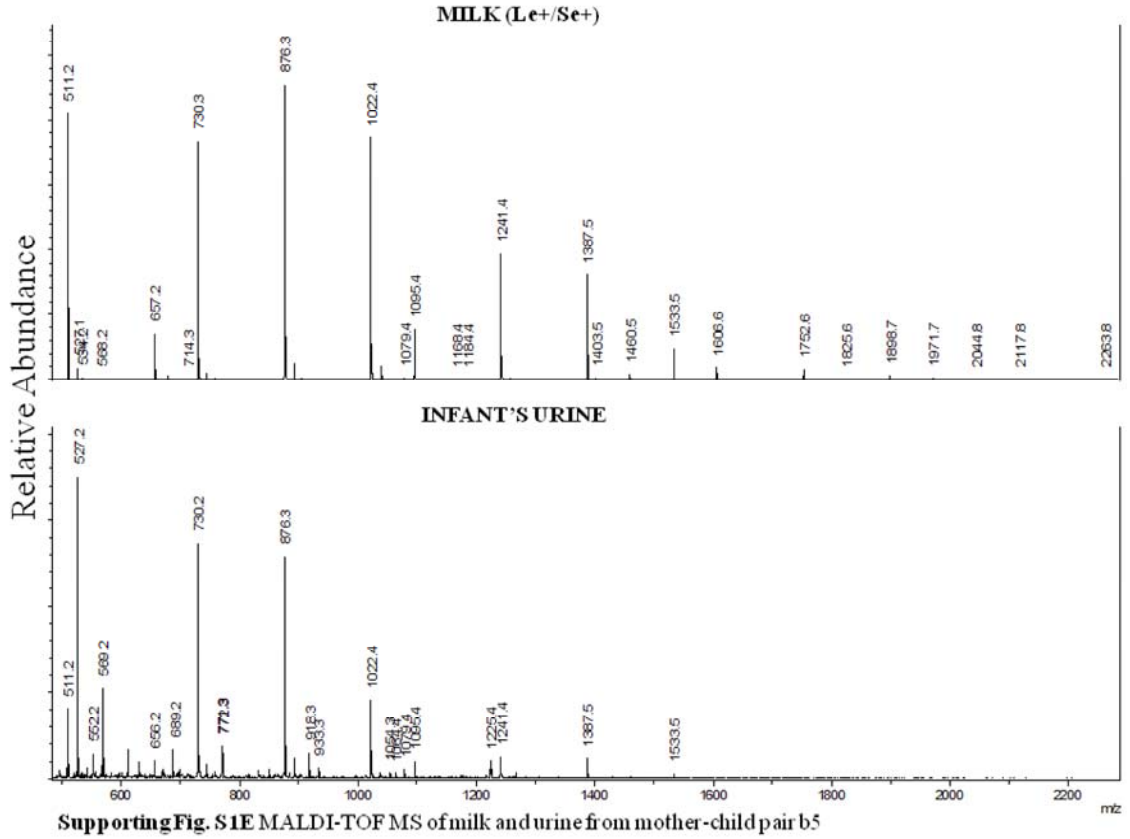
Supporting Fig. S1B MALDI-TOF MS of milk, urine and faeces from mother-child pair b1



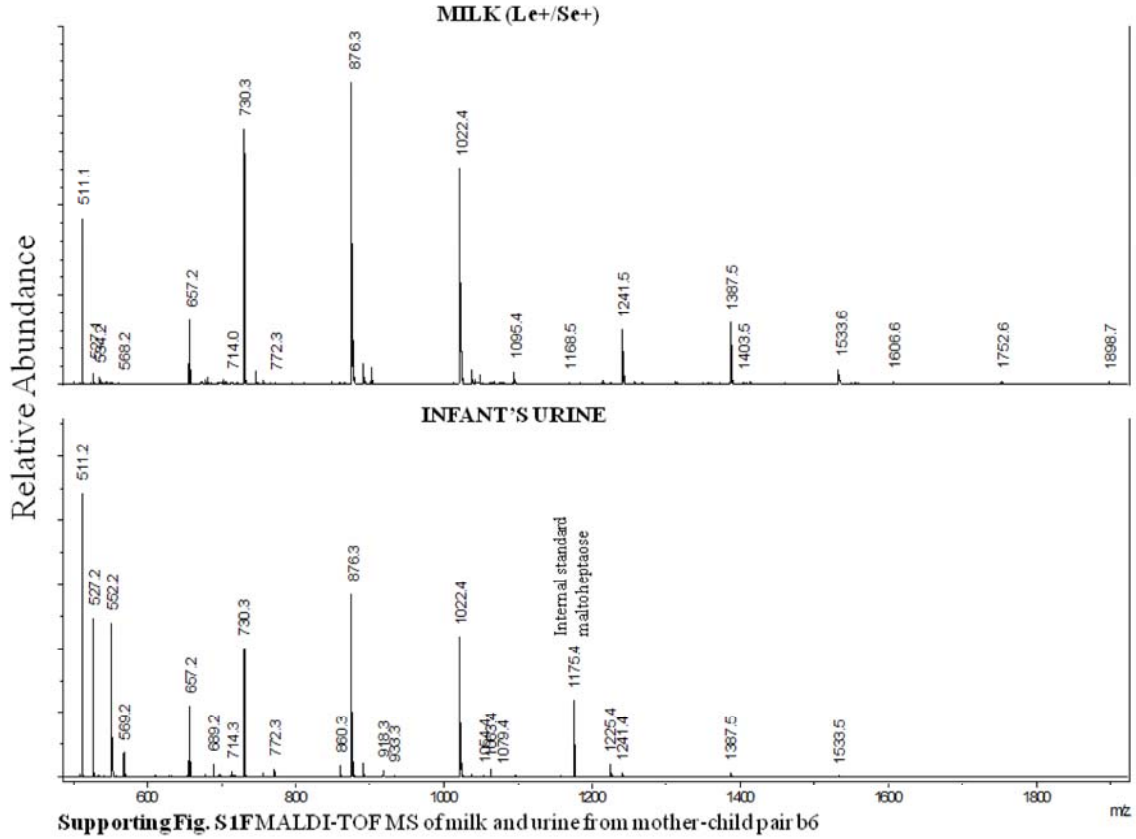
Supporting Fig. S1C MALDI-TOF MS of milk, urine and faeces from mother-child pair b3



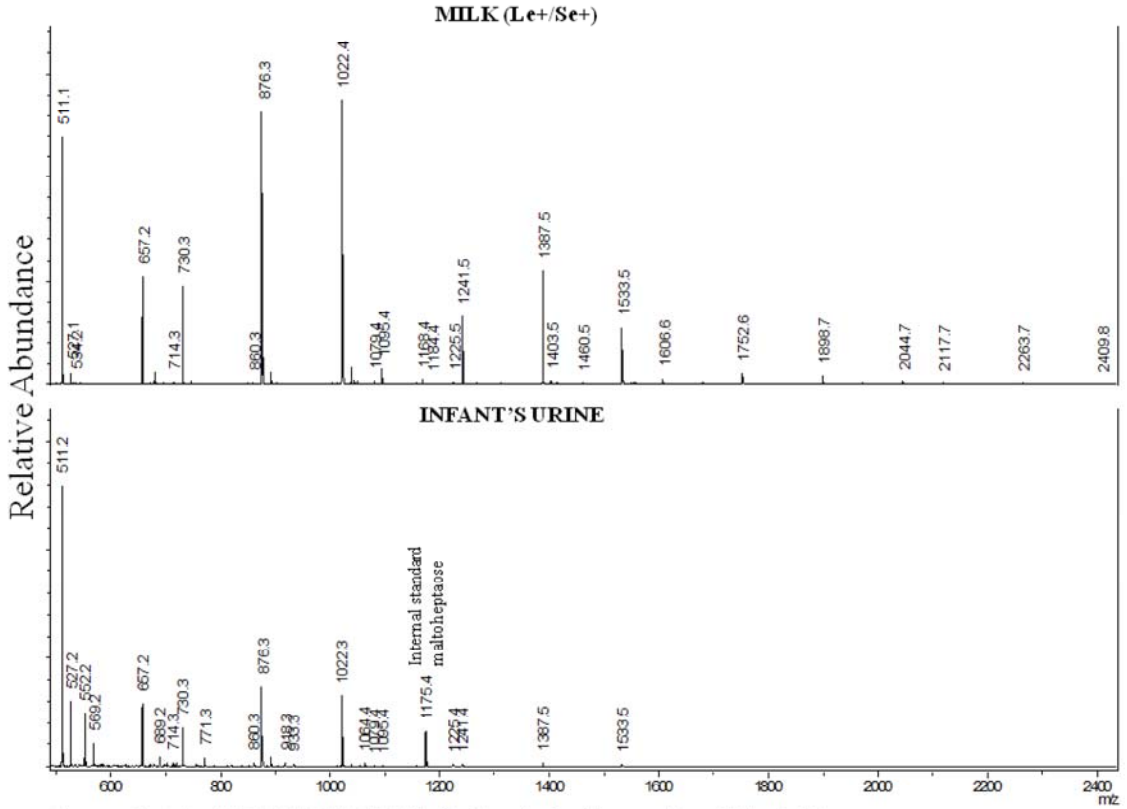
Supporting Fig. S1D MALDI-TOF MS of milk and urine from mother-child pair b4



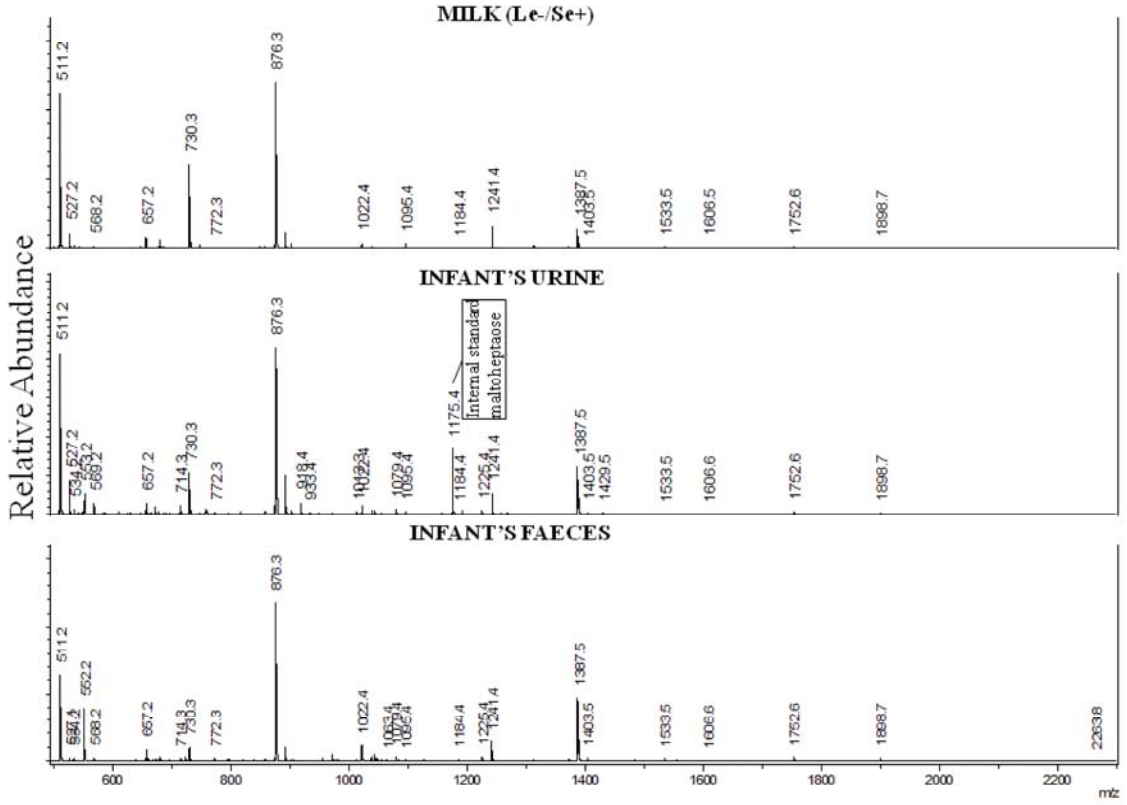
Supporting Fig. S1E MALDI-TOF MS of milk and urine from mother-child pair b5



Supporting Fig. S1F MALDI-TOF MS of milk and urine from mother-child pair b6



Supporting Fig. S1G MALDI-TOF MS of milk and urine from mother-child pair b7



Supporting Fig. S1H MALDI-TOF MS of milk, urine and faeces from mother-child pair c

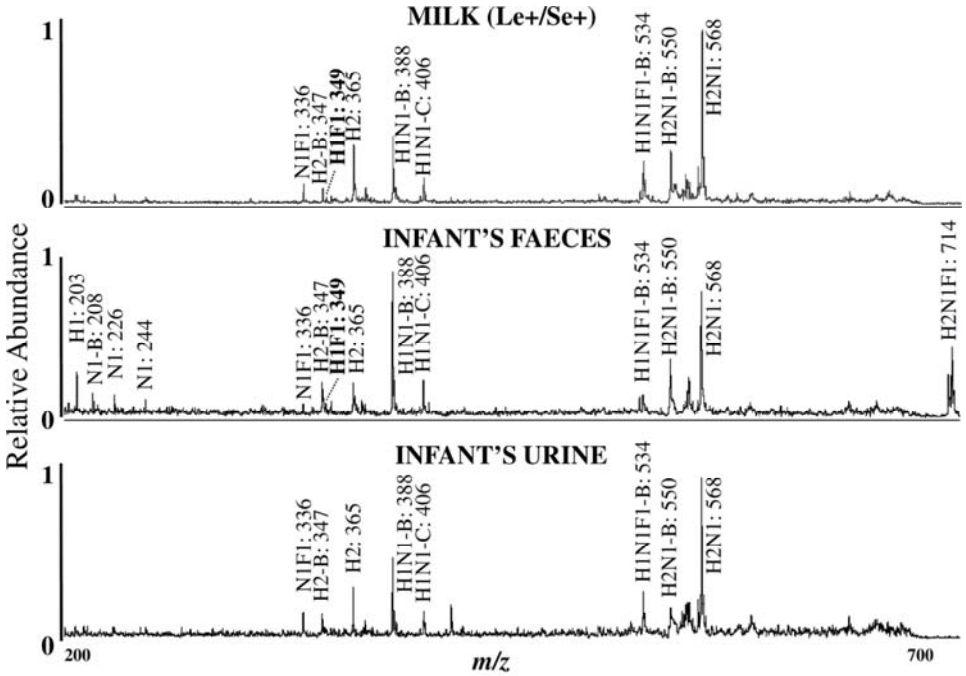
Table S4-1 Oligosaccharides from mothers' milk, infants' urine and feces

m/z $[M+Na]^+$	Composition[a]				Occurrence in MALDI-TOF-MS[b]			Reference[c]	
	Ac	H	N	F	milk	Infant urine	Infant feces	Infant urine	Infant feces
406.1	0	1	1	0	none	a2,(b3),b4,(b6-7)	a1-2,b1-3, c		
511.2	0	2	0	1	all	(a1-2),b1-b7,c	a1,b1-3,c	(Chester *et al.* 1981)	(Sabharwal *et al.* 1988a)
552.2	0	1	1	1	(a1,b3-5,b7)	(a1),a2,b1-3,(b5),b6-7,c	b3,c	(Rudloff *et al.* 2012)	
553.2	1	2	0	1	none	(a2),b1-2,(b3,b5),b6-7,c	(c)		
568.2	0	2	1	0	all	all	all (b3)		
569.2	1	3	0	0	(a2,b2)	all (b4)	(a2),b3		
657.2	0	2	0	2	(a1),b1-7,c	a1,b1-7,c	(a1-2),b1-3,c	(Chester *et al.* 1981)	(Sabharwal *et al.* 1988a)
673.2	0	3	0	1	(b1-6),b7,(c)	(b1-4,b7,c)	(a1),b1-2,c		(Albrecht *et al.* 2011a)
714.2	0	2	1	1	all	all	a1,(b1),b2,(b3),c	(Rudloff *et al.* 2012)	(Sabharwal *et al.* 1991)
730.2	0	3	1	0	all	all	all	(Rudloff *et al.* 2012)	(Sabharwal *et al.* 1988a)
771.3	0	2	2	0	none	a1-2,b1-5,(b6),b7,c	a1,(a2),b1-2		
772.2	1	3	1	0	(a1-2,b1-6,c)	a2,b1-3,b5,(b6),c	a2		
860.3	0	2	1	2	(a1,b1,b3-7,c)	(a1),a2,b1-3,b5-7	a1,(a2),(b3),(c)		(Sabharwal *et al.* 1984)
876.3	0	3	1	1	all	all	a1,b1-3,c	(Rudloff *et al.* 2012)	(Sabharwal *et al.* 1988a)
901.3	0	1	2	2	(a1,a2,b1-3,b5-7,c)	(a2,b1-2,b5-7,c)	none		
917.3	0	2	2	1	none	(a1-2,b3-4,b6-7)	a1,c		
918.3	1	3	1	1	(a1,b1-7)	(a1),a2,(b1),b2-6,(b7),c	none		
933.3	0	3	2	0	(b3-5,b7,c)	(all)	a1-2,b2,c		
1022.4	0	3	1	2	all	(c),a1-2,b1-7	a1,b1-3,c	(Dotz *et al.* 2014)	(Sabharwal *et al.* 1991)
1038.3	0	4	1	1	all	(all)	a1,b1-2,c		(Albrecht *et al.* 2011a)
1047.4	0	1	2	3	(a1,b1,b3-7)	(a1,b1,b3,b5-7)	(a2)		
1054.3	0	5	1	0	(b3)	(all)	(c)		
1063.4	0	2	2	2	none	(all)	c		
1064.4	1	3	1	2	a1,b1,b3-4	(a1,b1-7)	none		
1079.4	0	3	2	1	(all)	(all)	a1,(a2),b1,(b2),c		(Sabharwal *et al.* 1984)
1095.4	0	4	2	0	all	(a1-2,b1),b2,(b3-7,c)	a1,b1-2,(b3),(c)	(Dotz *et al.* 2014)	(Albrecht *et al.* 2011a)
1136.4	0	3	3	0	none	(a1-2,b2,b6)	(a1),a2,b1		
1168.4	0	3	1	3	b1-4,(b5),b6-7	(b1,b3-4,b6)	none	(Dotz *et al.* 2014)	
1184.4	0	4	1	2	(b1-2),b3,(b4-7),c	b1,b4,c	b1,c		(Albrecht *et al.* 2011a)
1225.4	0	3	2	2	(a1-2),(b1-6),b7,(c)	(all)	a1-2,b1,c		(Sabharwal *et al.* 1984)
1241.4	0	4	2	1	all	(a1-2,b1),b2-3,(b4-7,c)	a1,b1-3,c	(Dotz *et al.* 2014)	(Sabharwal *et al.* 1988b)
1267.4	1	3	2	2	(b3,b6)	(all)	none		
1282.5	0	3	3	1	none	none	(a1),a2,(b1)		
1298.4	0	4	3	0	none	none	(a1),b1		
1339.5	0	3	4	0	none	none	(a1),a2,b1,(b3)		
1371.5	0	3	2	3	(a1,b1-b7,c)	(a2),b3,(b5),b6	none		
1387.5	0	4	2	2	all	a1,(a2),b1-b7,c	a1,(b1),b2,(b3),c	(Rudloff *et al.* 2012)	(Sabharwal *et al.* 1988b)
1403.5	0	5	2	1	(all)	(a1,b2,c)	a1,c		
1428.5	0	3	3	2	none	none	(a1-2)		

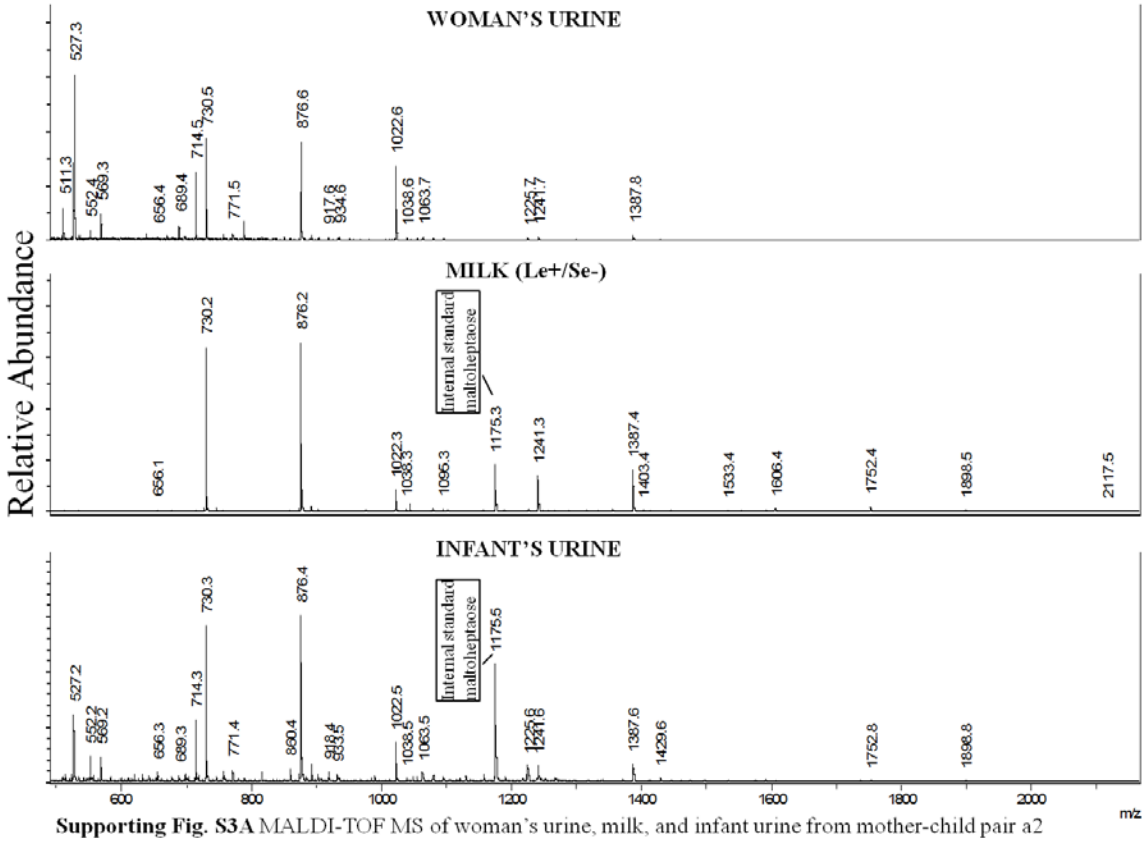
1429.5	1	4	2	2	(a1,b2,b6-7)	a1-2,(b1-5),b6,(b7),c	none		
1444.5	0	4	3	1	none	(a1,c)	a1-2,(b1)		
1460.5	0	5	3	0	all	(a2,b1-3,b5-6)	a1,(b1),b2-3	(Dotz *et al.* 2014)	
1485.7	0	3	4	1	none	none	a2		
1533.5	0	4	2	3	all	(a1-2),b1-7,(c)	a1,b1-3,c	(Rudloff *et al.* 2012)	(Sabharwal *et al.* 1988a)
1542.6	0	3	5	0	none	none	(a1),a2,b1		
1590.6	0	4	3	2	(a1,b1,b3,b5-7)	(b5-6,c)	a1,(a2,b1),c		
1606.6	0	5	3	1	all	(a2,b2-3,b5,c)	a1,b2,(b3),c	(Dotz *et al.* 2014)	(Albrecht *et al.* 2011a)
1631.6	0	3	4	2	none	none	a2,(b1)		
1679.6	0	4	2	4	b1-b7	none	none		
1688.6	0	3	5	1	none	none	a2		
1736.6	0	4	3	3	(b1-2,b5-7)	(a2,b3-6)	none		
1745.6	0	3	6	0	none	none	b1		
1752.6	0	5	3	2	all	(a1-2,b2-5,b7,c)	a1,b2,(b3),c	(Dotz *et al.* 2014)	(Albrecht *et al.* 2011a)
1777.6	0	3	4	3	none	none	a2		
1793.6	0	4	4	2	none	none	a2		
1802.7	0	1	5	4	none	none	b1		
1825.6	0	6	4	0	(b4),b5,(b7)	none	b2,(b3)		
1850.7	0	4	5	1	none	none	(a2)		
1898.7	0	5	3	3	all	(a1-2,b2-5,c)	a1,(b1),b3,c	(Dotz *et al.* 2014)	(Albrecht *et al.* 2011a)
1948.7	0	1	5	5	none	none	b1		
1971.7	0	6	4	1	(a1-2,b1-4),b5,(b6),b7,(c)	none	b2,(b3)		
1996.7	0	4	5	2	none	none	(a2)		
2005.8	0	1	6	4	none	none	(b1)		
2044.7	0	5	3	4	(b2,b3),b4,(b5-6),b7	(b2,b3)	none	(Dotz *et al.* 2014)	(Albrecht *et al.* 2011a)
2117.8	0	6	4	2	a1,(a2),b1,(b2),b3-5,(b6-7,c)	(c)	(a1,b3)	(Dotz *et al.* 2014)	
2190.8	0	7	5	0	(b1-5,b7)	none	none		
2208.8	0	1	7	4	none	none	(b1)		
2263.8	0	6	4	3	a1,(b1),b2-3,(b4-6),b7,(c)	(c)	a1,c	(Dotz *et al.* 2014)	
2336.8	0	7	5	1	(b5,b7,c)	none	none		
2409.9	0	6	4	4	(a1,b1-6),b7	none	none		
2482.9	0	7	5	2	(a1,b3-5,b7)	none	none		
2555.9	0	8	6	0	(b3,b7)	none	none		
2628.9	0	7	5	3	(a1,b3,b7)	none	none		
2702.0	0	8	6	1	(b7)	none	none		
2775.0	0	7	5	4	(a1,b3,b7)	none	none		

[a)] Ac, acetyl-; H, hexose; N, *N*-acetylhexosamine; F, fucose;
[b)] Letters a, b, and c were assigned to the mother-child pairs according to the Lewis and secretor-specific HPAEC-PAD profiles from milk as shown in Fig. 2.
[c)] Reference number is given if one or more compounds with the respective *m/z* value were previously described. Brackets indicate low intensity, i.e. signal-to-noise ratio<20.

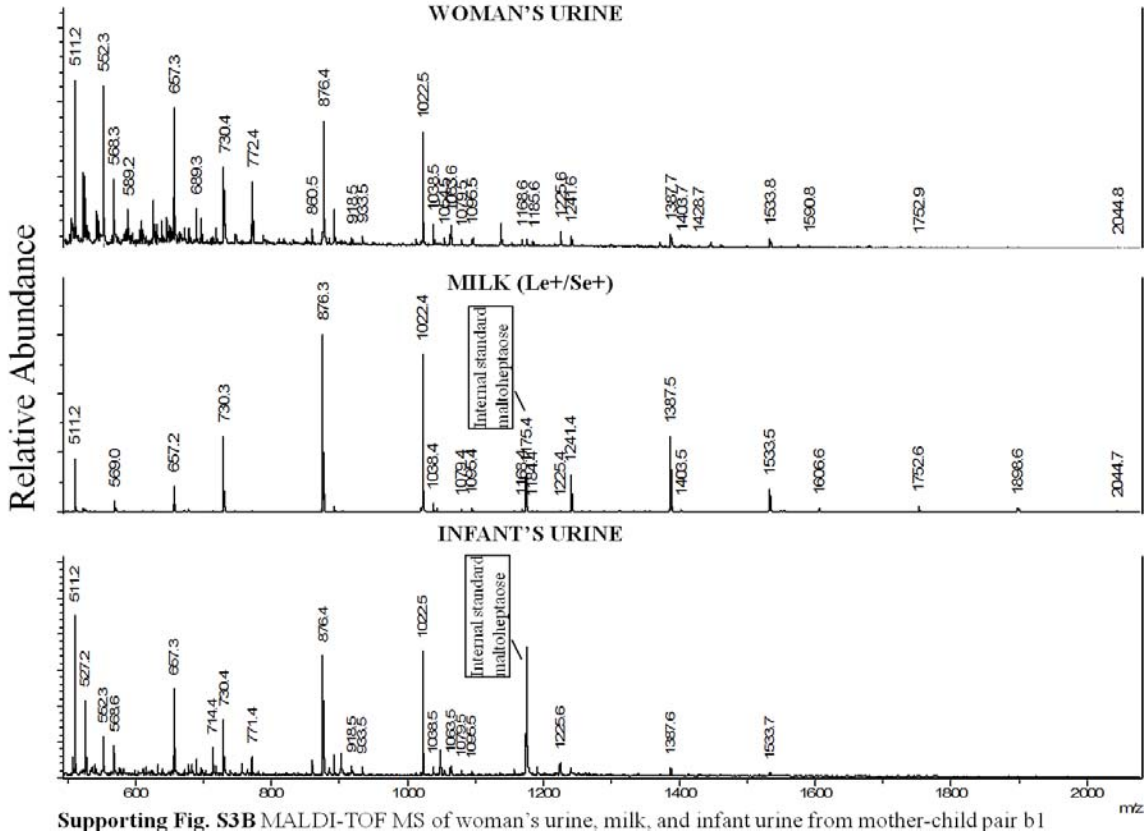
Supporting Information Table S4–2 (extensive Excel file) is available online at the publisher's site.



Supporting Fig. S2. MALDI-TOF/TOF MS/MS of oligosaccharides with m/z 714. Ac, Acetyl; H, hexose, N, *N*-Acetylhexosamine; F, fucose; B/Y or C/Z fragment assignment according to Domon B, Costello CE (1988) Glycoconjugate J 5:397–409.



Supporting Fig. S3A MALDI-TOF MS of woman's urine, milk, and infant urine from mother-child pair a2



Supporting Fig. S3B MALDI-TOF MS of woman's urine, milk, and infant urine from mother-child pair b1

Chapter 5

5 Oligosaccharides in feces of breast-fed and formula-fed infants at different ages (Manuscript 4)*

Viktoria Dotz[1,#], Stefanie Thomanek[1], Günter Lochnit[2], Rüdiger Adam[3], Horst Schroten[3], Clemens Kunz[1]

[1]Institute of Nutritional Science, University of Giessen, Giessen, Germany

[2]Institute of Biochemistry, Faculty of Medicine, University of Giessen, Giessen, Germany

[3]Pediatric Gastroenterology, Department of Pediatrics, University Hospital Mannheim, Mannheim, Germany

Keywords: feces, infant nutrition, human milk oligosaccharides, MALDI-TOF-MS, metabolism, microbiota

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Current address: Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands

5.1 Abstract

Beneficial effects have been proposed for human milk oligosaccharides (HMO), as deduced mainly from in vitro studies. To date, in vivo evidence of the link between certain oligosaccharide structures in milk and their consumption by infant gut microbiota is missing. Although many studies have described HMO patterns in human milk from larger cohorts, data on the excretion of HMO and possible metabolites from the infant gut are still very limited. However, from smaller-scale studies an age-dependency according to infant gut maturation and microbiota adaptation has previously been hypothesized. To further investigate this, we profiled fecal oligosaccharides from term-born infants who were exclusively breast-fed, formula-fed or mixed-fed at the age of six weeks, and from a follow-up of a subgroup at six months of age (INFABIO study). Automated matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) analyses revealed the presence of HMO and metabolites in the feces of most, but not all breast-fed infants at six weeks, with highly varying patterns. Formula-fed infants at six weeks and most of the breast-fed infants at six months did not excrete HMO-like structures in their feces, the latter corresponding to the hypothesis of age-dependency. Together with our previous results that were contradictory to what has been proposed by others, here, we suggest alternative explanations for the described age and feeding-mode dependency of oligosaccharide excretion in infants below six months of age.

5.2 Introduction

OS as a major solid fraction of human milk are thought to have beneficial effects for the breast-fed infant, as is known from various *in vitro* and some animal studies (Bode and Jantscher-Krenn 2012; Kunz *et al.* 2014; Smilowitz *et al.* 2014). Interventional studies in infants are lacking to date due to a scarce availability of single human milk OS (HMO) for human use (Rudloff and Kunz 2012). The high structural diversity of HMO, which notably are lacking in bovine milk-based infant formulas, hampers acquiring a sophisticated knowledge on 'what structures are important'. Metabolic studies might help in this regard by showing which structures are utilized at which sites of the infant´s organism.

HMO were found to be only marginally digested by human intestinal enzymes (Engfer *et al.* 2000; Gnoth *et al.* 2000), while infant gut microbiota can utilize certain HMO as sole carbohydrate source *in vitro* (Marcobal *et al.* 2010; Yu *et al.* 2013). In particular, *Bifidobacteria* and *Bacteroides* are known to consume various HMO in a structure-specific manner *in vitro* (Asakuma *et al.* 2011; Marcobal *et al.* 2011; Satoh *et al.* 2013). This has indirectly been linked to previously reported differences in fecal microbial composition between breastfed and formula-fed infants.

Only very recently, studies trying to prove a direct link between HMO structures and their prebiotic function *in vivo* in infants have been reported. A recent proof-of-concept study indicated an association between the fecal HMO composition and gut microbiota of two breastfed infants over time, though both parameters differed substantially between the two infants (De Leoz *et al.* 2015). Another recent investigation showed a relationship between the fecal microbiome of exclusively breastfed infants (n=16) and OS composition in milk that was ingested by these infants (Wang *et al.* 2015). However, since the variety of both OS and microbiota composition is vast, reliable analytics, optimally in a high-throughput manner for large-scale omic studies, are inevitable for a better understanding of their interaction and relation to health and disease. The importance of integrated metagenomic and metabolomic information on systems biology level in the adult organism has been recognized recently (Aw and Fukuda 2015).

First attempts to profile fecal OS and possible metabolites of HMO *in vivo* in infants by modern omics-suitable methods have been undertaken recently. However, studies either included only one or two study subject(s) (De Leoz *et al.* 2015; De Leoz *et al.* 2013), or showed data from premature infants (Albrecht *et al.* 2011b; De Leoz *et al.* 2013), whose intestinal function may be very different from healthy term infants. Only one publication reported on fecal OS follow-up data from ten term infants, but this time no comparison was drawn with formula-fed babies as negative controls for HMO ingestion (Albrecht *et al.* 2011a).

Previously, we reported on the metabolic fate of HMO in ten exclusively breast-fed infants by comparing MALDI-TOF-MS(/MS) data from mother's milk, infant urine and feces and by tracking *in vivo* ^{13}C-labeled HMO (Dotz *et al.* 2014; Dotz *et al.* 2015). Here we present an automated high-throughput glycomic method, which we applied on fecal samples from in total 24 term infants who were breastfed, formula-fed or mixed-fed. Thus, we take a first step towards an integrated omics approach for a better understanding of the structure-function relationship of HMO in the early childhood.

5.3 Materials and Methods

Subjects

Fecal samples were collected from 24 full-term infants participating in the Diet and Environment longitudinal study of the European project INFABIO at the ages of 6 weeks and 6 months. From two infants, 2 different fecal samples each were obtained at the age of 6 months. The data presented in the current work were obtained from infants recruited from the region of Dusseldorf (Germany) only. Written informed consent was obtained from all mothers. Information on events concerning pregnancy and delivery as well as the feeding method, health history of the baby, and antibiotic treatment of both mother and child, were obtained from the mothers *via* repeated questionnaires (Fallani *et al.* 2010).

Sample collection

Fecal samples were placed in a sterile plastic box and maintained under anaerobic conditions at 4°C using anaerocult A (Merck, Nogent sur Marne, France) for a maximum of 4 hours before processing. After homogenization by mechanical kneading for 3 minutes, aliquots of 1 g (wet weight) were added to 9 mL of anaerobic phosphate-buffered saline and mixed again. Fecal slurries were centrifuged at 5000 x *g* at 20°C for 15 min, and thereafter, the supernatants were filtered through a 0.22 µm membrane. Filtrates were kept frozen at –20°C prior to further processing and analysis.

Sample preparation and analysis

Chemicals were purchased from the following suppliers: maltoheptaose (analytical grade), water, ACN (HPLC grade), and 2,5-dihydroxybenzoic acid from Sigma-Aldrich (Steinheim, Germany), TFA (ULC-MS grade) from LGC Standards (Wesel, Germany).

In duplicates, OS were extracted automated as previously described (Blank *et al.* 2011; Dotz *et al.* 2015), with few modifications. Briefly, SPE on PGC cartridges (HyperSep-96 Wells, 25 mg; Thermo Scientific, Bellefonte PA, USA) was performed using a Hamilton Microlab Starlet liquid handling system (Hamilton Robotics, Reno, NV, USA). Cartridges were equilibrated with 40% ACN and 0.1 % TFA in water (v/v), followed by 20% ACN and 0.1 % TFA, and washed with 0.1% aqueous TFA. Thawed fecal filtrates were loaded onto the cartridges and eluted with 40% ACN containing 0.1% TFA. The eluates were fully dried by vacuum.

OS were re-suspended in pure water and mixed with maltoheptaose, functioning as positive control and as internal standard during MALDI-TOF-MS analysis. Mass spectra were acquired using an Ultraflex I instrument (Bruker Daltonics, Bremen, Germany), equipped with a nitrogen laser emitting at 337 nm; a dextran hydrolysate was used to calibrate the equipment and the analytical method (Blank *et al.* 2011; Dotz *et al.* 2015). Fecal OS extracts were pipetted in duplicates onto a MALDI target plate by the lab robot, and were allowed to dry. Subsequently, samples were re-suspended in 1 µL 2,5-dihydroxybenzoic acid matrix solution (5 mg/mL 25% ACN) by the lab robot, and were allowed to dry.

OS profiles were acquired in positive-ion reflectron mode over a mass range of *m/z* 300–3000. Data acquisition and analysis were performed by flexControl and flexAnalysis 3.0 software (Bruker Daltonics, Bremen, Germany), respectively.

5.4 Results

In total, 24 fecal samples were available from infants who were exclusively breast-fed (B), 7 samples from exclusively formula-fed (F), and 6 from mixed-fed (M) infants (Table 5-1). Samples were not always available at both time points (6 weeks/months) from the same infant. For example, from the six infants who were exclusively formula-fed at 6 weeks, no follow-up sample could be obtained at 6 months of age. In contrast, for infants 13–15 fecal samples were only available at the age of 6 months.

Table 5-1 Cohort sub-groups with sample IDs according to age and feeding mode

Age	breast-fed (B)	mixed-fed (M)	formula-fed (F)
6 weeks	1B – 12B [a)]	16M – 18M	19F – 24F
6 months	1BB – 7BB, 13XB – 15XB	16MM – 18MM	12BF

[a)] Sample IDs: number indicating participant ID, first and second letters indicating feeding mode at 6 weeks and 6 months, respectively; X stands for unknown feeding mode at the age of 6 weeks

Since spectra obtained from fecal samples contained numerous background peaks as well as detector noise up to an *m/z* of 700 (Fig. 5-1, upper panel), a minimum cut-off of *m/z* 700 was set for all further data analysis. This was also observed in pure water blanks that have undergone the same preparation procedure as the fecal specimens (not shown).

5.4.1 Oligosaccharide profiles in infants' feces at six weeks

In total, more than 50 signals matching possible OS compositions have been detected by MALDI-TOF-MS in the sample set at the age of 6 weeks (Supporting Information Table S5–1). Most of these detected species were obtained from samples of exclusively breastfed or mixed-fed infants, and were matching *m/z* values as seen in many milk samples, as exemplified in Fig. 5-1 (lower panel).

Among the 12 exclusively breastfed infants a variety of OS profiles was observed at six weeks. In seven infants many OS species as known from human milk could be detected, i.e. infants 2B, 4B, 7B, 9B–12B (Fig. 5-2 and Table 5-2, HMO group).

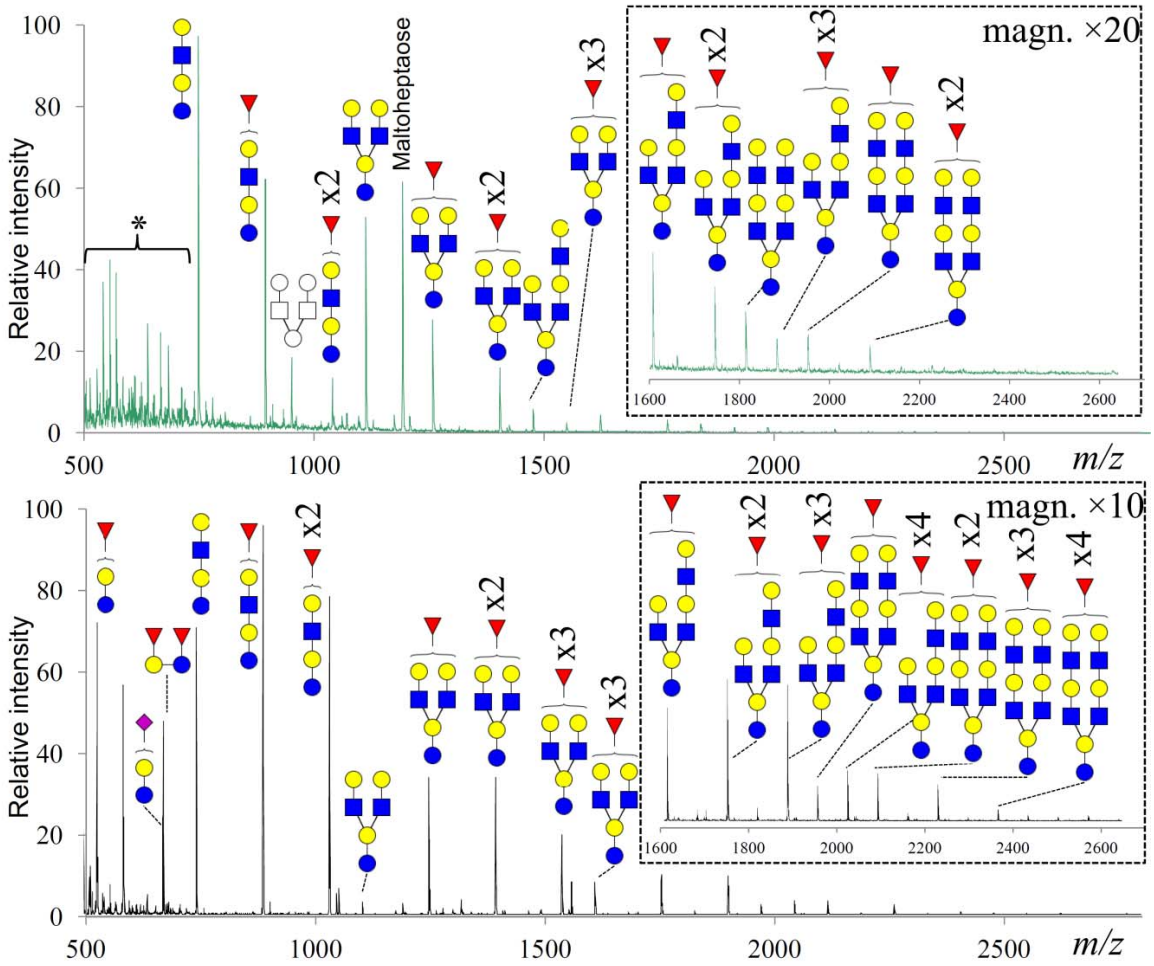

Fig. 5-1 MALDI-TOF mass spectra of a fecal sample from a breastfed infant at 6 weeks (upper panel) and a milk sample (lower panel) from the INFABIO cohort. Suggested structures as known from milk oligosaccharide species: blue circle, glucose; yellow circle, galactose; blue square, *N*-acetylglucosamine; red triangle, fucose; pink diamond, *N*-acetylneuraminic acid; unknown oligosaccharide: open circle, hexose; open square, *N*-acetylhexosamine. Asterisk indicates non-oligosaccharide origin.

Feces of infants 3B, 5B and 8B showed an OS profile clearly distinguishable from milk, where certain OS species predominated and common, especially larger HMO were clearly diminished (Fig. 5-2). For example, in infant 5B hardly any OS > *m/z* 1600 could be detected, whereas difucosylated LN(n)T at *m/z* 1022 was dominating the profile (Supporting Information Fig S5–1). In infants 1B, 3B and 8B non-typical HMO structures, such as $Hex_2HexNAc_1Fuc_1$ at *m/z* 714.2, $Hex_2HexNAc_2$ at 771.3, $Hex_3HexNAc_2Fuc_1$ at *m/z* 1079.4 or $Hex_3HexNAc_2Fuc_2$ at 1225.4, were increased relatively to common HMO signals (Fig. 5-2 and Table 5-2, N>H-2 groups versus ≥ hexaoses group). Furthermore, in child 1B intensities were very low (Supporting Information Table S5-1). No OS species were detected in infant 6B (not shown).

The mixed-fed infant 16M showed an OS profile similar to that of 5B (Supporting Information Fig. S5–1), whereas no OS matching HMO or possible metabolite compositions could be detected in the feces of the other two mixed-fed babies 17M and 18M, neither in the six formula-fed infants 19F–24F (not shown).

Table 5-2 Oligosaccharide groups derived from relative intensities of fecal oligosaccharides

OS group	5B	9B	1B	4B	7B	10B	12B	2B	11B	8B	3B
HMO	100.0	92.9	84.6	94.0	91.9	96.8	93.3	90.3	96.7	30.1	70.9
Fucosylated	95.0	83.3	75.8	66.3	47.9	77.7	88.1	87.9	91.6	50.8	93.7
A-active	2.6	7.0	57.4	2.8	1.3	2.8	5.2	4.7	3.1	38.1	93.3
N>H-2	2.6	7.9	64.0	5.3	7.8	3.9	7.2	9.2	4.1	59.9	95.3
N<H-2	1.3	2.0	0.0	1.3	0.6	1.8	1.8	1.4	3.1	0.0	0.0
≤tetraoses	93.5	75.2	91.2	56.4	51.4	73.5	54.5	58.3	68.9	75.6	70.5
≥hexaoses	5.2	16.9	0.0	38.8	41.5	23.7	40.2	34.1	26.0	5.5	0.9

MALDI-TOF-MS signal intensities of each putative oligosaccharide species (Supporting Information Table S5–1) were divided by the intensity sum of all detected oligosaccharide species from that list; arithmetic means of these relative intensities were calculated from the technical quadruplicates (preparation duplicate × MALDI-TOF-MS spot duplicate), and were summed into groups. OS groups were created as follows: HMO = 714 + 730 + 876 + 1022 + 1038 + 1064 + 1095 + 1168 + 1184 + 1241 + 1387 + 1460 + 1533 + 1606 + 1679 + 1752 + 1825 + 1898 + 1971 + 2044 + 2117 + 2190 + 2263 + 2336 + 2409 + 2482 + 2555 + 2628 + 2701 + 2774 (all m/z detected in human milk except for low-abundant ones, as based on Table S4–1 in **Chapter 4**); Fucosylated: all species containing at least one fucose; A-active: 714 + 860 + 1079 + 1225 (corresponding to masses of A-tetrasaccharide up to A heptasaccharide); N>H-2 or N<H-2: all species containing a higher/lower proportion of HexNAc as compared to HMO core structures ($Hex_2HexNAc_1)_n$; ≤ tetraoses: only species containing up to four core monosaccharides (Hex and/or HexNAc); ≥ hexaoses: only species containing at least six core monosaccharides.

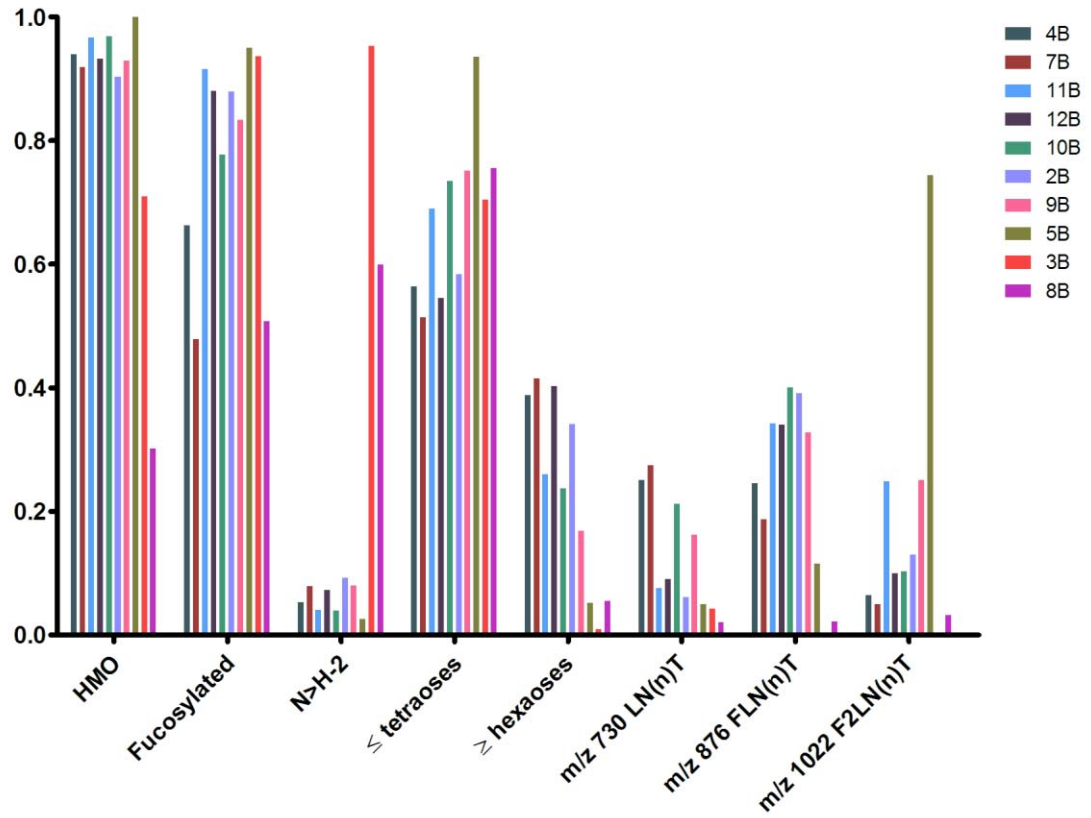

Fig. 5-2 Relative signal intensities of fecal oligosaccharides and oligosaccharide groups from 10 exclusively breastfed infants. For explanation, see Table 5-2.

5.4.2 Oligosaccharides in infants' feces at six months

In 10 out of 14 infants at 6 months of age, no signals matching HMO or potential metabolites could be detected. That were fecal samples from i) exclusively breastfed infants 2BB–4BB, 6BB, 7BB, 15XB, or ii) mixed-fed infants 16MM-18MM, or iii) infant 12BF, who was exclusively formula-fed at the age of six months (not shown). The spectra from the repeatedly obtained samples in

infants 2B and 3B were consistent thereby. In contrast, in three exclusively breastfed infants multiple OS signals were detected by MALDI-TOF-MS. However, OS concentration in the fecal extracts seemed very low, as could be seen in the relatively high signal of the spiked standard maltoheptaose (Fig. 5-3). Furthermore, in the exclusively breast-fed infant 13XB only the OS species $Hex_3HexNAc_3$ at *m/z* 1136 was observed (not shown).

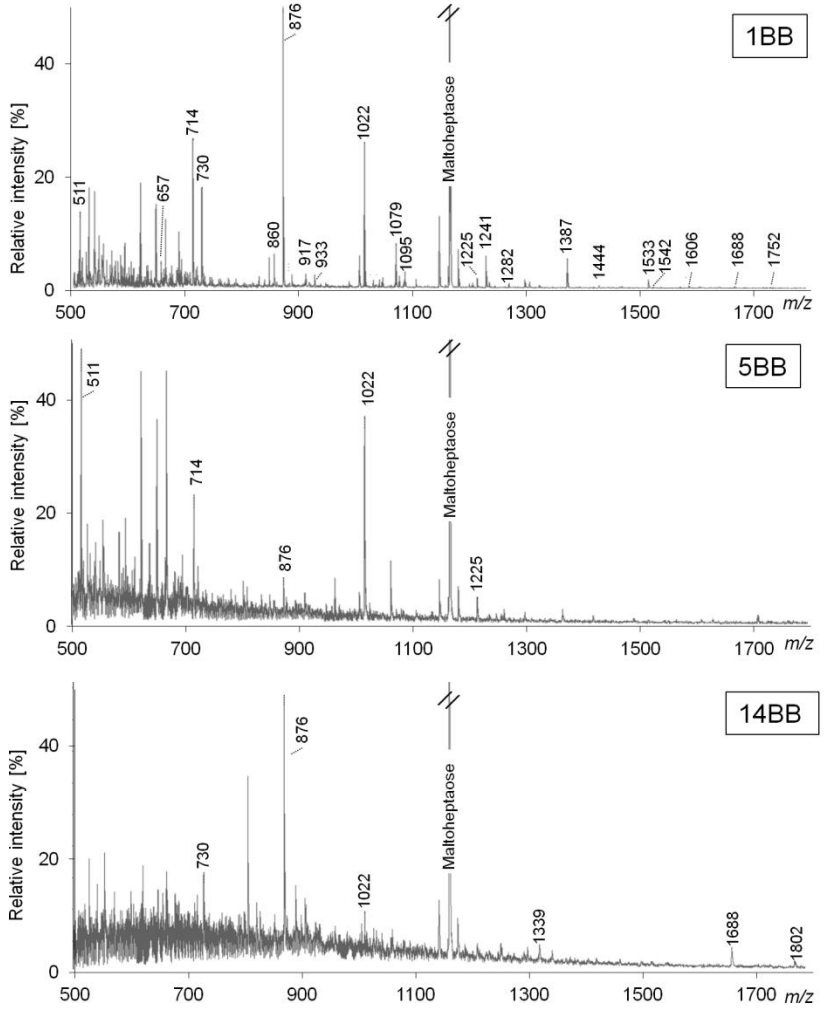

Fig. 5-3 MALDI-TOF mass spectra of fecal oligosaccharides from exclusively breastfed infants at the age of six months. Only molecular species matching HMO or possible metabolites according to Table S4–1 in Chapter 4 Supporting Information were annotated.

5.5 Discussion

Our automated and rapid method for OS extraction from infant feces as previously described (Dotz *et al.* 2015) has successfully been applied on fecal specimens from a sub-cohort of the INFABIO study. In addition, it has been modified to enable reliable automated spotting and measuring. Initially, fecal samples were collected for a characterization of the intestinal microbiome (Fallani *et al.* 2010). The HMO-containing supernatant resulting in course of the extraction of bacterial cells from feces has now been proven suitable for OS analysis by our MALDI-TOF-MS approach.

At the age of six weeks three different groups of exclusively breast-fed full-term infants could be assigned on the basis of their fecal OS profiles: i) high diversity with up to 30 HMO-like structures

in an individual sample, ii) predominance of only one or a few OS with low signal intensities in some cases, and iii) no OS (Table 5-3).

Table 5-3 Oligosaccharide (OS) diversity in feces of exclusively breastfed infants at 6 weeks / months of age

Age	High diversity of OS (>10)	Few OS (5–7)	No OS
6 weeks	2B, 4B, 7B, 9B–12B [a)]	(1B), 3B, 5B and 8B	6B
6 months	1BB	5BB, (14XB)	2BB–4BB, 6BB, 7BB, 13XB, 15XB

[a)] for sample ID definitions and abbreviations, see Table 5-1
Brackets indicate very low intensity as compared to spiking standard maltoheptaose.

The majority of the six weeks old infants were among the first group, but each profile still was unique and some showed very specific features. For example, in the fecal OS profile of infant 7B, which is shown in Fig. 5-1 (upper panel), the proportion of non-fucosylated OS, e.g. at *m/z* 1095 or 1460, was clearly increased compared to the profiles known from milk as analyzed by the same (Fig. 5-1, lower panel) or comparable methods (Blank *et al.* 2011; Dotz *et al.* 2015). Previously, we have reported on the same apparent trend in another infant's fecal and also urinary OS profile (infant b2 in (Dotz *et al.* 2015)). This indicates a higher fucosidase activity in these infants' guts. Intriguingly, we detected a remarkably high relative intensity signal at *m/z* 933 in the same infant. This $Hex_3HexNAc_2$ structure has been detected in other fecal samples in our investigations and also by others (Albrecht *et al.* 2011a; Albrecht *et al.* 2011b), but only in one other case it was found to be as increased (infant a2 in (Dotz *et al.* 2015)).

The profiles in the second group were not very consistent, except for the overall lower abundance of larger OS as compared to the first group. For instance, infant 5B had reduced abundances of larger OS structures and a remarkably high proportion of difucosylated LNT (isomers) at *m/z* 1022, which on the other hand could not be detected in infants 1B and 3B. However, the latter might be a consequence of an anyhow lower presence of this molecular species in milk, as known from Lewis a and some Lewis negative milk samples (Blank *et al.* 2011). This could not be proven here due to a lack of mother's milk samples. The predominance of the signal at *m/z* 1022 was also observed in the mixed-fed infant 16M (Supporting Information Fig. S5–1) and reported by Albrecht and colleagues in one infant (A2) (Albrecht *et al.* 2011b). An intriguing finding was the huge relative abundance of structures with a high proportion of HexNAc to Hex in infants 1B, 3B and 8B (Table 5-2), as we have reported previously in a small cohort (Dotz *et al.* 2015). To a major part, this can be attributed to an attachment of a GalNAc by the action of blood group A enzyme at the intestinal mucosa (Albrecht *et al.* 2011a; Sabharwal *et al.* 1984), as can be seen in the great overlap of the N>H-2 group and the group of presumably A-active OS in Table 5-2.

The absence of OS signals in the feces of exclusively breast-fed infant 6B at six weeks as well as in most fecal samples obtained at the age of six months (7 out of 10), might either reflect a highly effective degradation/consumption in the gut or simply be a result of two major methodical limitations. First, a fecal sample (aliquot) taken only once might be not representative due to the well-known heterogeneity and high variability of the water content/viscosity of fecal samples, especially in infants. Here, we analyzed two different aliquots from the same fecal sample. However, multiple samples were available from only two infants. A second important methodical consideration which has been ignored in fecal OS profiling studies so far is the high variability in frequency of feedings and/or defecation, which is strongly associated with infant age and feeding mode (Tunc *et al.* 2008). In older and in formula-fed infants stool frequency is lower than in younger and

breast-fed infants. This should in turn have a substantial impact on the microbial metabolization efficiency of single OS structures, possibly even more than mere microbiota composition. Marcobal and colleagues have shown that *Bacteroides* species have a sequential degradation pattern for HMO with different preferences after approximately 5 vs. 15 hours of *in vitro* incubation (Marcobal *et al.* 2011). Future studies should take these aspects into consideration by, e.g., documenting stool frequency and collecting fecal specimens repeatedly over a few days to assure representative OS profiling. Having these data available might have helped explaining why we could hardly detect any OS signals in the feces of infant 1 at six weeks, in contrast to the follow-up sample at six months. So far, that finding is contradictory to the reports of Albrecht and colleagues, who claimed a gradual decrease of HMO abundance with age, hypothesizing an association with gut (microbiota) maturity (Albrecht *et al.* 2011a).

Moreover, sample treatment in different laboratories may have contributed to inconsistencies of data on HMO excretion in infant feces. Contrary to our previous (Dotz *et al.* 2015) and our current results, others were not able to detect LNT in any of the fecal samples (Albrecht *et al.* 2011a; Albrecht *et al.* 2011b). This could be due to an ongoing degradation/metabolization of OS during incubation prior to extraction. In contrast, we performed a short incubation in phosphate-saline buffer after thorough homogenization. Previously, a simple 1-hour incubation in water after 20-min pasteurization was also shown sufficient for a reliable fecal OS profiling by MALDI-TOF-MS (**Chapter 4**) (Dotz *et al.* 2015). Thus, our approach for automated extraction, MALDI-plate spotting and TOF-MS measurement presented here clearly features the highest rapidness and throughput among all MS-based methods for the analysis of fecal OS from infants published during the recent years (see (Albrecht *et al.* 2010; De Leoz *et al.* 2015; De Leoz *et al.* 2013) and our comments in (Dotz and Kunz 2013)). It only takes approximately 10–15 hours for the preparation and analysis of up to 96 samples, sample drying included. Consequently, our approach is particularly valuable as it will enable large-scale studies with straightforward analysis of both intestinal microbiome of infants as well as its possible HMO metabolites. Moreover, oligosaccharides from milk samples can also be analyzed by the same approach without major modifications (Blank *et al.* 2011; Dotz *et al.* 2015), allowing for gaining as much of an insight into the structure-function relationship of HMO as is possible from non-invasive studies.

Overall, our findings provide further evidence for the excretion of intact and metabolized OS structures originating from human milk in case of breast-fed infants. Although the excretion patterns of HMO structures and metabolites showed an association with age as suggested previously by others, we have seen exceptions and have proposed alternative hypotheses according to our previously unpublished data. Future large-scale follow-up studies are required to further reveal the factors influencing the high variability and temporal dynamics of infant gut microbiota and HMO composition, providing a crucial basis for our understanding of the unique interaction between these two.

5.6 Supporting Information

Table S5-1. Relative intensities of neutral oligosaccharides in exclusively breast-fed infants' feces, detected by MALDI-TOF-MS at the age of 6 weeks

				Infant ID [a]										
m/z [b]	**H**	**N**	**F**	**5B**	**9B**	**1B**	**4B**	**7B**	**10B**	**12B**	**2B**	**11B**	**8B**	**3B**
714.2	2	1	1	2.6	1.0	48.6	0.1	0.0	1.4	1.4	0.0	1.1	15.4	66.2
730.2	3	1	0	5.0	16.2	24.2	25.1	27.4	21.2	9.0	6.1	7.6	2.0	4.2
771.3	2	2	0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	16.3	0.0
860.3	2	1	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	3.7	0.0
876.3	3	1	1	11.5	32.7	11.8	24.5	18.7	40.0	34.1	39.1	34.3	2.1	0.0
892.3	4	1	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.4	0.0
901.3	1	2	2	0.0	0.0	6.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
917.3	2	2	1	0.0	0.0	0.0	0.2	0.0	0.3	0.0	0.0	0.0	0.0	0.0
933.3	3	2	0	0.0	0.2	0.0	1.6	5.6	0.2	0.5	2.0	0.1	0.0	1.6
1022.4	3	1	2	74.4	25.1	0.0	6.4	5.0	10.3	10.0	13.0	24.8	3.2	0.0
1038.3	4	1	1	1.3	1.8	0.0	0.5	0.2	1.1	0.9	0.8	1.9	0.0	0.0
1063.4	2	2	2	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.1	0.0	0.0
1064.4	3	1	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.4	0.0
1079.4	3	2	1	0.0	3.5	8.8	2.5	1.1	1.3	2.5	3.8	1.0	12.9	27.0
1095.4	4	2	0	0.0	0.3	0.0	5.7	15.6	0.9	2.0	2.6	0.6	0.0	0.5
1136.4	3	3	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	4.4	0.0
1168.4	3	1	3	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.8	0.0	0.0
1184.4	4	1	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.9	0.0	0.0
1225.4	3	2	2	0.0	2.4	0.0	0.1	0.2	0.1	1.3	1.0	0.8	6.0	0.0
1241.4	4	2	1	0.0	3.7	0.0	13.5	9.6	6.1	10.8	9.5	5.6	0.0	0.0
1267.4	3	2	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
1298.4	4	3	0	0.0	0.0	0.0	0.1	0.4	0.0	0.2	0.7	0.0	0.0	0.0
1339.5	3	4	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
1371.5	3	2	3	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
1387.5	4	2	2	3.7	6.9	0.0	9.9	6.1	11.1	13.3	8.5	10.0	0.0	0.0
1403.5	5	2	1	0.0	0.2	0.0	0.8	0.3	0.6	0.9	0.5	0.3	0.0	0.0
1444.5	4	3	1	0.0	0.2	0.0	0.4	0.3	0.1	0.7	1.1	0.2	0.0	0.4
1460.5	5	3	0	0.0	0.0	0.0	0.9	2.0	0.0	0.2	0.6	0.0	0.0	0.0
1533.5	4	2	3	1.5	3.9	0.0	1.0	1.1	0.6	4.3	2.7	4.7	0.0	0.0
1590.6	4	3	2	0.0	0.5	0.0	0.1	0.1	0.3	0.6	0.7	0.2	0.0	0.0
1606.6	5	3	1	0.0	0.1	0.0	2.0	1.7	0.6	1.6	1.7	0.4	0.0	0.0
1679.6	4	2	4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0
1736.6	4	3	3	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0
1752.6	5	3	2	0.0	0.5	0.0	1.8	1.2	1.6	2.6	2.3	1.5	0.0	0.0
1825.6	6	4	0	0.0	0.0	0.0	0.3	0.9	0.0	0.0	0.1	0.0	0.0	0.0
1898.7	5	3	3	0.0	0.5	0.0	0.6	0.6	1.4	1.6	1.4	1.3	0.0	0.0
1971.7	6	4	1	0.0	0.0	0.0	0.7	0.7	0.0	0.1	0.4	0.0	0.0	0.0
2044.7	5	3	4	0.0	0.0	0.0	0.0	0.1	0.0	0.4	0.4	0.5	0.0	0.0
2117.7	6	4	2	0.0	0.0	0.0	0.6	0.4	0.1	0.4	0.5	0.1	0.0	0.0
2190.8	7	5	0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0
2263.8	6	4	3	0.0	0.0	0.0	0.2	0.1	0.1	0.3	0.3	0.2	0.0	0.0
2409.9	6	4	4	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.0
SUM				15,606	49,625	1,819	74,991	74,701	56,108	56,519	53,095	72,516	4,868	11,246
1175.4	7	0	0	66.1	15.6	1454.4	11.2	18.7	17.6	12.7	19.8	19.6	354.6	114.7

[a] Number indicates participant ID; B, breast-fed

[b] MALDI-TOF-MS signal intensities of each putative oligosaccharide species with monoisotopic $[M+Na]^+$ were divided by the absolute intensity sum (**SUM**) of all detected oligosaccharide species from that list; arithmetic means of these relative intensities were calculated from the technical quadruplicates (preparation duplicate × MALDI-TOF-MS spot duplicate). H, number of hexoses; N, number of *N*-acetylhexosamines; F, number of fucoses. *M/z* 1175.4 indicates internal standard maltoheptaose.

Only signals with a relative intensity of ≥0.1 are included.

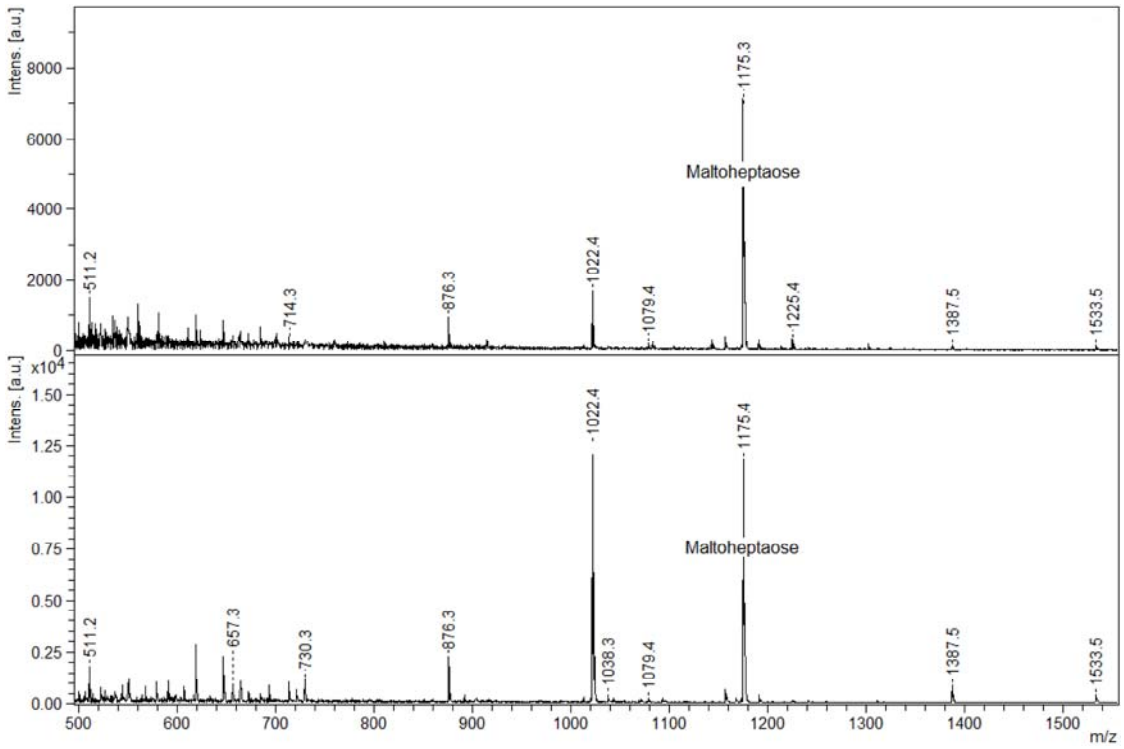

Fig. S5-1 MALDI-TOF mass spectra of oligosaccharides from feces of the mixed-fed infant 16M (top) and the exclusively breast-fed infant 5B (bottom) at the age of six weeks. Maltoheptaose was added prior to analysis as positive control.

Chapter 6

6 General discussion*

Breastfeeding is beneficial to both mother and child, not only during infancy, but also later in life (**Chapter 1**). Partly digestible OS ingested by the infant *via* breast milk are postulated to have various beneficial effects. However, their metabolic fate *in vivo* in healthy term infants had not been investigated in detail prior to the studies presented in this work. In the following, the analytical methodology that had to be developed first as well as data obtained by these methods are discussed.

6.1 HMO analysis by MALDI-TOF-MS and HPAEC-PAD

By using our developed method for profiling OS in MALDI-TOF-MS, approx. 80 distinct molecular species could be detected when taken together milk, infant urine and feces extracts (**Chapter 4**). By adjustments of the method for high-throughput screening of HMO (Blank *et al.* 2011) (**Chapter 2**), in this work we were able to also extract and detect fecal OS using a lab robot for the laborious steps. However, extraction and detection of OS from urine was more challenging due to low and highly varying concentrations of the analytes and higher abundance of interfering hydrophilic compounds. These issues were addressed by normalizing the applied urine volume to the creatinine concentration and by performing manual SPE instead of using the lab robot allowing for more controlled/slower processing at critical steps such as sample application and elution. Nevertheless, OS extraction from feces is not trivial either. As we discussed previously, the common procedure for OS extraction by keeping watery fecal slurries overnight might result in ongoing degradation/metabolization of OS during incubation. This might have occurred in the studies of Albrecht and coworkers (Albrecht *et al.* 2011a; Albrecht *et al.* 2011b), as they were not able to detect LNT in fecal samples in contrast to our results (**Chapters 4 & 5**). Strikingly, De Leoz and coworkers also reported on the presence of LNT in all fecal samples of in total three infants, although they also kept the fecal samples as water slurries overnight (De Leoz *et al.* 2015; De Leoz *et al.* 2013). However, the authors were still applying laborious Folch extraction and overnight incubation for their studies though claiming their method to be rapid and high-throughput. We criticized this in a short letter (Dotz and Kunz 2013). In contrast, we have found a short pasteurization and incubation step to be sufficient for a reliable fecal OS profiling by MALDI-TOF-MS.

The rapidness and sufficient isotopic resolution of our MALDI-TOF-MS approach enabled us to analyze replicates of milk and urine samples collected during 36 hours for tracing ^{13}C-labeled HMO (**Chapter 3**). This approach can easily be applied to future bioavailability and metabolic studies with single stable-isotope labeled OS (**Chapter 6.2**).

The same extracts as used for MALDI-TOF could easily be injected into our established HPAEC-PAD system for the additional evaluation of Le- and Se-specific structures after isomeric separation, since no derivatization is necessary for PAD (Kunz *et al.* 1996). Thereby, we could gain additional information on OS modifications taking place in the infants after ingestion of HMO. However, HPAEC-PAD is not able to characterize novel structures, since identification is only done by

* Manuscript in preparation, combined with Chapter 1

retention time matching. Coupling of HPAEC to MS would have been desirable, but is recognized as very challenging due to the extremely high salt concentration at the HPAEC outlet.

Further limitations of our approach mainly originating from recognized MALDI-TOF-MS-typical problems are discussed in the following. Some of the detected signals may originate from potassium adducts of the same species that is detected at *m/z* lower by 16 Da when sodiated, e.g. $[M+Na]^+$ = 1022 and $[M+K]^+$ = 1038 (see Table S5-1). The addition of lithium acetate, sodium chloride, or potassium chloride to the matrix solution for a suppression of an adduct formation from the other salts as suggested in literature (Pfenninger *et al.* 1999) did not completely suppress the others, but even resulted in lower signal intensities for OS from infant feces or urine (not shown). Using a high-resolution MS, e.g. an FT-capable instrument, would be another alternative, which was not available for our studies, however. In-source fragmentation as a well-known issue in MALDI-TOF-MS analysis (Zaia 2010), may also have contributed to some detection uncertainty. Therefore, care has been taken not to apply too high laser intensity during the measurements. Moreover, due to the well-known problem of sialic acid loss during MALDI-TOF-MS analysis, especially in the positive-ion reflectron mode (Zaia 2010), we were not able to reliably analyze acidic HMO in our samples (**Chapter 4**). LC-MS approaches with softer ES ionization or derivatization of the sialylated species by permethylation or other techniques would enable detection of sialylated OS, however, at a cost of lower throughput, higher sample consumption and substantially higher efforts (**Chapter 1.5.5**). Some of the mass overlaps and uncertainties could be resolved by fragmentation in MALDI-TOF/TOF (**Chapter 4**). This technique was indispensable for the characterization of novel structures, in particular acetylated HMO.

In spite of the aforementioned methodical constraints, our approach for automated extraction, MALDI-plate spotting and TOF MS measurement presented here clearly features the highest rapidness and throughput among all MS-based methods for the analysis of fecal and milk OS published during the recent years (see (Albrecht *et al.* 2010; De Leoz *et al.* 2015; De Leoz *et al.* 2013). Our approach starting from thawed samples until having the mass spectra available takes approximately 10–15 hours for 96 samples if overnight SpeedVac drying after SPE is also counted. The only manual steps are pipetting samples before and after incubation and filtration of each sample *via* syringe filters. However, in principle these steps can also be easily accomplished by a lab robot on well plates. As pointed out in **Chapter 2**, the here presented MALDI-TOF-MS approach, together with other state-of-the-art analytical techniques, will allow for gaining as much of an insight into the structure-function relationship of HMO as is possible from non-invasive studies in the near future.

6.2 Metabolic fate of milk oligosaccharides in infants

Deduced from our findings described in **Chapters 3**, **4**, and **5**, intact, degraded and modified HMO are passing through the entire gastrointestinal tract and are clearly able to be absorbed *in vivo* by the breastfed infant. This has been shown by characterizing various HMO-like structures in infant feces and urine, respectively. The various (classes of) metabolites as detected in our studies, are summarized in Table 6-1. Our finding of acetylated neutral OS in infants' and lactating women's urines is novel. *O*-acetylated sialic acid motifs have been detected on OS in Tasmanian echidna milk (Oftedal *et al.* 2014). In general, *O*-acetylation of sialylated glycans plays an important role in human immunology, bacterial and viral infections, and is linked to type I diabetes, rheumatoid arthritis and autoimmune disorders. However, the role of and the possible metabolization sites/mechanisms leading to the formation of neutral acetylated HMO is to be investigated in the future. We hypothesize that HMO are enzymatically acetylated in the infants' and wom-

en's liver, kidneys', or other body cells excluding gut (Fig. 6-1). A contribution of the intestine to the acetylation of HMO at least in women is unlikely, as it would imply secretion from circulation into the gut with subsequent re-absorption.

Another class of possible urinary OS metabolites is reported in literature from a study in infants having the lysosomal storage disease galactosialidosis (Bruggink *et al.* 2010). That is HMO-like structures with gluconic acid at the reducing end, most likely following a C1-oxidation of the Glc residue. If that modification occurred in our samples, it would have overlapped with potassium adducts of the usual HMO peaks due to the mass difference of +16 Da. In addition, urinary OS bearing myo-inositol instead of Glc at the reducing end have been reported in pregnant Se women (Hallgren *et al.* 1977). Future studies should investigate the metabolic pathways and the role of glycans excreted in the urine of breastfed and formula-fed infants also in the context of disorders in glycan metabolism. That data, together with a more detailed knowledge on glycans in pregnant and lactating women's urines, may boost our understanding of the role of the various glycan structures circulating pre- and postnatally in the mother's and baby's body. Urinary glycans / metabolites furthermore bear great potential in terms of biomarker discovery not only in the context of congenital disorders of glycosylation (Bruggink *et al.* 2010; Peelen *et al.* 1994), but also for monitoring of placental and fetal functionality (Dessi and Fanos 2013; Hallgren *et al.* 1977).

Table 6-1 Summary of possible HMO metabolites as presented in Chapters 4 and 5

Detected OS species	Possible metabolite class	Detected in
+42 Da	acetylation	infants' & lactating women's urine
> proportion of HexNAc compared to original HMO	cleavage of terminal hexoses, attachment of BG A (GalNAc)	infant urine & feces
> proportion of Hex compared to original HMO	attachment of BG B (Gal)	infant urine & feces
additional Le epitope		feces of infant fed Le-negative milk
additional Se epitope		urine and feces of infants fed non-Se milk

Regarding the overall OS profiles, infant urine showed less inter-individual variation than feces, as could be seen in MALDI-TOF-MS and HPAEC-PAD profiles (**Chapter 4**). In general, smaller intact structures were detected and variation in the relative intensities of fucosylated vs. non-fucosylated species was observed, when compared to the respective milk profiles. Certain structures seemed to be strongly depleted in urine, such as the Se-specific LNFP I. Previously, an indication for different intestinal absorption or tubular re-absorption of fucosylated OS between Se and non-Se starved adults was reported (Lundblad 1966). Moreover, our data from intrinsic ^{13}C-labeling and tracing of a variety of HMO until their excretion in infants' urine revealed remarkable differences in the temporal dynamics of HMO absorption and excretion (**Chapter 3**). Fecal profiles in exclusively breastfed infants could be classified as follows: i) high diversity with many HMO-like, also complex structures, ii) predominance of only one or a few OS or OS groups (e.g. fucosylated vs. non-fucosylated), and iii) no OS or only a few with very low signal intensities (**Chapters 4** & **5**).

Finding HMO-like structures only in feces of exclusively and in one partially breastfed, but not exclusively formula-fed infants further points at the role of these compounds within the infant's

gut. Urine samples from formula-fed infants were not available for our studies which would have helped distinguishing OS from endogenous origin and mother's milk to further reveal the relevance of HMO within the infant's organism. However, in a recent publication from another group measurable amounts of lactosyl-OS were found in the urine of both breastfed and formula-fed infants. Intriguingly, much higher total amounts and more complex (fucosylated) structures clearly originating from human milk have been found (only) in breastfed infants' urine (Goehring *et al.* 2014). Importantly, the concentrations and variety of structures was strongly correlated between OS in the infants' urine/plasma and the ingested mother's milk (Goehring *et al.* 2014). Consequently, the contribution of dietary glycans seems more significant than the baseline excretion of, e.g. blood-group and Se-specific, OS as also found in starved adults (Lundblad 1978). This was shown for the urinary excretion of A- and B-trisaccharide (Chester *et al.* 1979) as well as other, e.g. Le- and Se-specific, OS in adults orally given (ga)lactose (Strecker *et al.* 1976). These non-complex dietary saccharides might experience modifications by blood group glycosyltransferases in intestinal cells prior to their uptake in circulation and their renal excretion (Chester *et al.* 1979). Assuming a protective effect of HMO against uropathogenic bacteria (Lin *et al.* 2014), the renal excretion of bioactive HMO in infants or endogenously modified HMO-like sub-structures, also in adults, could possibly benefit individuals at any age, if relevant amounts of active structures are excreted *via* kidneys. Moreover, those OS circulate in the organism before renal excretion so that even systemic effects, analogously to those speculated for infants (**Chapter 1.3**), can be anticipated. Especially the urine of pregnant and lactating women contains a variety (Fig. S4-3) and significant amounts of lactosyl-OS (Hallgren *et al.* 1977), that probably originate from HMO synthesis in the mammary gland as speculated in **Chapter 4** (Supporting Information Fig. S4–2). Future research should investigate the role of certain HMO-like structures in context of their antimicrobial properties not only in the pediatric field, but also as candidates for novel antibiotics in general.

A contribution of HMO-like structures to the OS pool in infant feces and urine from sources other than original HMO is very likely in spite of the fact that we used ^{13}C-labeling strategy. Degradation products from larger HMO which resemble smaller HMO structures (**Chapter 2.2**) and still were carrying the label would not be distinguishable from the same, but intact structures originating from milk. Moreover, ^{13}C-labeled galactose that has been applied to lactating women in our experiment has also been incorporated into proteins or lipids present in their milk (Rudloff *et al.* 2006). Given the possibility of releasing HMO-like structures from glycoconjugates (Fig. 1-2), by yet unknown mechanisms in the infant's gut, this would have generated additional uncertainty in the overall pool of ^{13}C-enriched OS that we have measured (**Chapter 3**). OS released from glycolipids by a yet undescribed endoglycosylceramidase were proposed as possible source of lactosyl-OS in urine (Bruggink *et al.* 2010; Dotz *et al.* 2015; Lundblad 1966). However, already in the 70's it was shown that inducing (ga)lactosuria clearly decreased urinary excretion of OS with reducing-end glucose (Strecker *et al.* 1976). The authors pointed out that transglycosylation of highly abundant, free saccharides in plasma, i.e. normally Glc, is therefore more likely. In pediatrics, future studies with application of single stable-isotope labeled HMO structures to formula-fed infants will enable a clear distinction between endogenous, xenobiotic and milk OS. Nevertheless, this work in total provides a unique additional insight into relevant sites and aspects possibly influencing degradation, metabolization and modifications taking place throughout the entire lifetime of the human milk glycome, starting from biosynthesis until the excretion in infants, as depicted in Fig. 6-1.

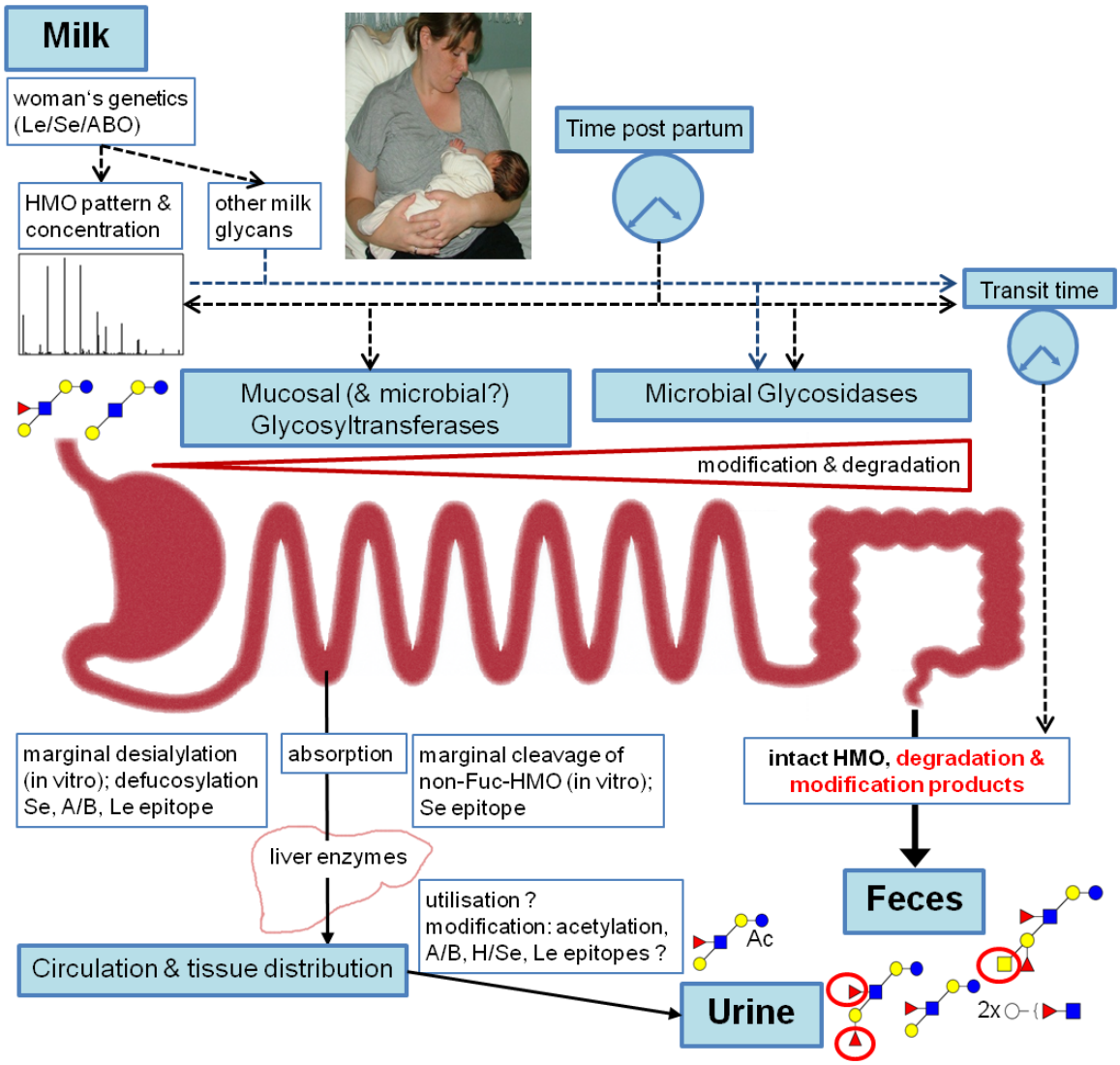

Fig. 6-1 Metabolic fate of human milk oligosaccharides with postulated sites of modification and degradation. Pathways are depicted by solid lines, possible influencing factors by dashed lines.

The main factor determining structural variety and concentration of OS in milk is the Le and Se status of the mother. HMO composition is thought to have an impact on microbiota composition in the infant's gut. Gut microbiota in turn is a major metabolization factor of HMO. However, the infant's age – possibly linked to feeding/stool frequency, gastrointestinal transit time and gut maturation (**Chapter 5**, (Albrecht *et al.* 2011a)) – as well as the infant's Le/Se phenotype and ABO blood group also seem to contribute to the overall pool of OS excreted in feces and urine of breastfed infants. Yet unknown sites of metabolization such as for the here described acetylation are to be explored in the future. Again, isotopically labeled single HMO structures will be of great importance in that context. Their urgent need is demonstrated by our further finding of selective utilization of LNFP I or the intense defucosylation taking place prior to absorption of HMO into circulation (**Chapter 4**). Contrary to what has been postulated before, the latter finding indicates that some metabolization, i.e. fucosidase activity, might already occur in the small intestine, where HMO absorption, but not yet microbial fermentation, is thought mainly to take place. **Chapter 3** shows an overall overlap of the urinary ^{13}C-HMO excretion time profiles with those of HMO secretion in milk, at least regarding larger HMO structures. This supports the hypothesis of an early absorption. An early metabolization of HMO has also been found in rats previously (Jantscher-Krenn *et al.* 2013). However, although animal studies can in general be useful in investigation of

HMO digestion and fermentation, care must be taken when specific structural motifs are analyzed. For example, the small-intestinal mucins of mice are dominated by sialylated OS and the colon mucins by fucosylated structures, which is reversed in humans (Holmen Larsson *et al.* 2013). Mucin glycosylation is expected to have a strong impact on gut colonization (Tailford *et al.* 2015), and thus HMO metabolization, throughout the gastrointestinal tract. The individual gut mucin glycosylation in infants should therefore be kept in mind as one of the factors probably biasing future clinical studies on microbiota-HMO correlation.

Finally, the question 'What structures really matter' remains unresolved. However, the structural diversity found in infants' urine and feces can even exceed that of human milk due to the additional modifications as described in this work. Reducing HMO's functional spectrum to their prebiotic effects seems even more short-sighted when the large variety and amounts of structures found in urine and plasma of breastfed infants as well as pregnant and lactating women is taken into account. Currently, prebiotic OS produced from plants or biotechnologically, such as fructo-OS or galacto-OS are added to commercial formulas (Eiwegger *et al.* 2010). Their structures do not resemble HMO in any regard, i.e. GlcNAc, Fuc, *N*-acetylneuraminic acid are not present in those structures; linkage and branching patterns also differ significantly (Bode 2012). Attempts are being made to extract HMO-like structures from bovine milk (Zivkovic and Barile 2011). However, amounts of fucosylated OS are very low, there is a predominance of type II over type I structures, which is reverse in human milk, and bovine milk contains non-human and therefore potentially allergenic structural motifs (Albrecht *et al.* 2014; Bode 2012). Moreover, it is very likely that the spectrum of OS structures as a whole, in interaction with the different aspects mentioned above and with other glycans present in human milk, may exert its optimal effects.

Therefore, future research should focus on the evaluation of the metabolic pathways of neutral and sialylated glycans, including glycolipids, glycoproteins/peptides as well as free OS, not only in the breastfed infant, but also in its mother. Le/Se and ABO blood group should be determined in both mother and child, using reliable analytics. Moreover, preferably several fecal and urine samples should be collected over a longer time period for improved representativeness, and feeding/stool frequency should be documented. Next to the production of stable-isotope labeled single HMO for human use and as standards for quantitative glycomics, reliable cutting-edge glycoanalytical methodologies with improved data analysis tools will be very much needed in the future to substantially promote our understanding of the unique biological benefits of breastfeeding in the context of glycobiology.

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Zusammenfassung (summary in German)

Humanmilch enthält eine Vielzahl an komplexen Kohlenhydraten, die sich biosynthetisch von dem Milchzucker Laktose ableiten. Ihre Konzentrationen betragen mit ca. 5–20 Gramm pro Liter etwa ein Zehntel des Gehalts von Laktose. Bisher wurden zahlreiche biologische Effekte von Humanmilcholigosacchariden (HMO) anhand von Ergebnissen aus *in vitro* Studien beschrieben. Sie sollen u.a. präbiotisch und antiinfektiös im Darm des gestillten Säuglings wirken. Darüber hinaus könnten sie auch immunmodulierende und andere positive Eigenschaften im Körper haben, sofern die aktiven Substanzen im Dünndarm absorbiert werden. Um den endgültigen Beweis für die Wirksamkeit der einzelnen Substanzen beim Säugling *in vivo* zu erbringen, wären Interventionsstudien mit Einzelsubstanzen notwendig. Diese sind aufgrund mangelnder Verfügbarkeit synthetischer HMO bislang nicht möglich. Daher wurden in der vorliegenden Arbeit der Metabolismus und die möglichen Ausscheidungswege von HMO direkt beim gestillten Säugling untersucht, um so Hinweise auf deren Struktur-Funktionsbeziehungen *in vivo* zu erhalten. Dafür wurde eine analytische Plattform entwickelt, die eine effiziente und zuverlässige Identifizierung von Oligosacchariden aus Muttermilch, Säuglingsurin und –fäzes ermöglicht.

In **Kapitel 1** der vorliegenden Arbeit werden die aktuellen Kenntnisse zum Metabolismus und der Funktion von HMO sowie die gängigsten Methoden der Glykananalytik anhand von Literatur vorgestellt. **Kapitel 2** verdeutlicht die große Relevanz der modernen Glykananalytik für die Erforschung der Struktur-Funktionsbeziehungen von HMO mit Fokus auf spezifische HMO-Strukturen mit Lewis- oder Sekretorepitopen (genetisch determinierte Blutgruppenantigene in Humanmilch).

Kapitel 3 beschreibt die praktischen Studien zum Zeitverlauf der HMO-Ausscheidung im Säuglingsurin 36 Stunden nach Einzelgabe von ^{13}C-Galaktose an die Mütter. Mittels Matrix-unterstützter Laser Desorption/Ionizations-Massenspektrometrie mit Flugzeitanalysator (MALDI-TOF-MS) wurden bei allen zehn Säuglingen intakte, auch sehr komplexe HMO detektiert. Allerdings war die Exkretion bestimmter Strukturen bei manchen Kindern verzögert, was auf eine individuell längere Darmpassage oder Blutzirkulation dieser hinweist. Des Weiteren gab es Hinweise auf eine unerwartet frühe Metabolisierung bestimmter Strukturen in oberen Darmabschnitten.

Die möglichen Metaboliten nach ihrem Abbau oder Modifikation und/oder Aufnahme im Darm wurden bei derselben Probandengruppe mittels MALDI-TOF-MS und Flüssigchromatographie untersucht (**Kapitel 4**). Diese konnten mittels Tandem-MS als acetylierte HMO oder verschiedene HMO-Abbauprodukte charakterisiert werden, zusätzlich zu HMO-Strukturen, die offenbar erst nach der Aufnahme von den Enzymen des Kindes mit Lewis- und/oder Sekretorepitopen versehen worden waren. Einige Hinweise auf strukturspezifische Utilisation im Darm oder Organismus des Säuglings wurden darüber hinaus erlangt, beispielsweise aufgrund stark verminderter Ausscheidung von Lakto-*N*-tetraose im Stuhl oder Lacto-*N*-fukopentaose I im Urin.

Die Oligosaccharidmuster von weiteren 24 Säuglingen wurden ebenfalls mittels MALDI-TOF-MS untersucht (**Kapitel 5**). Dabei wurden drei Untergruppen anhand der HMO-Muster im Stuhl der gestillten Säuglinge im Alter von sechs Wochen beobachtet: i) hohe Diversität an Oligosaccharidstrukturen mit Dominanz von intakten HMO, ii) wenige Oligosaccharide mit eher niedriger Signalintensität und iii) keine HMO oder HMO-Metaboliten. Im Alter von sechs Monaten wurden bei den gestillten Säuglingen keine oder sehr wenige HMO(Metaboliten) detektiert. Des Weiteren konnten bei den formelernährten und bei zwei von drei gemischt ernährten Säuglingen keine Oligosaccharide im Stuhl detektiert werden.

In **Kapitel 6** werden die neuen Erkenntnisse auf der Basis vorhandener Literatur abschließend diskutiert und es werden Perspektiven für künftige Humanstudien über HMO aufgezeigt.

Eidesstattliche Erklärung

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

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Viktoria Dotz

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