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Urinary excretion of *in vivo* ^{13}C-labelled milk oligosaccharides in breastfed infants

Silvia Rudloff[1,2], Gottfried Pohlentz[3], Christian Borsch[2], Michael J. Lentze[4] and Clemens Kunz[2]*

[1]*Department of Pediatrics, Justus-Liebig-University Giessen, Feulgenstrasse 12, D-35392 Giessen, Germany*
[2]*Institute of Nutritional Science, Justus-Liebig-University Giessen, Wilhelmstrasse 20, D-35392 Giessen, Germany*
[3]*Institute for Medical Physics and Biophysics, University of Münster, Robert-Koch-Strasse 31, D-48149 Münster, Germany*
[4]*Department of Pediatrics, University of Bonn, Adenauerallee 119, D-53113 Bonn, Germany*

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Abstract

Recent observations indicate that human milk oligosaccharides (HMO) are involved in a variety of physiological processes in infants. Their metabolic fate, however, is virtually unknown. We investigated metabolic aspects in infants after endogenous ^{13}C-labelling of HMO. An oral bolus of natural and ^{13}C-labelled galactose (Gal; 23 g Gal + 4 g ^{13}C-Gal) was given to ten lactating women. Aliquots of milk at each nursing as well as breath samples from the mothers and urine from their infants were collected over 36 h. The ^{13}C-enrichment of HMO and their renal excretion was determined by isotope ratio-MS; characterisation was achieved by fast atom bombardment-MS. After the Gal bolus was given, an immediate ^{13}C-enrichment in milk and in infants' urine was observed which lasted 36 h. Mass spectrometric analysis of ^{13}C-enriched urinary fractions confirmed the excretion of a variety of neutral and acidic HMO without metabolic modification of their structures. Components with glucose split off at the reducing end were also detectable. Quantitative data regarding the infants' intake of lacto-*N*-tetraose and its monofucosylated derivative lacto-*N*-fucopentaose II ranged from 50 to 160 mg with each suckling, respectively; renal excretion of both components varied between 1 and 3 mg/d. Since the intake of individual HMO by the infants was in the range of several hundred mg per suckling, i.e. several g/d, and some of these components were excreted in mg amounts as intact HMO with the infants' urine, not only local but also systemic effects might be expected.

Key words: Milk oligosaccharides: *In vivo* ^{13}C-labelling: Infants: Urinary excretion

Human milk contains a large variety of complex oligosaccharides in concentrations ranging from 10 to 20 g/l[1,2]. The quantity of these components does not only depend on the lactational stage of the mother but is also affected by the expression of specific glycosyltransferases in the mammary gland[1–3]. Genes encoding for H and Lewis a or x antigens as well as the secretor status determine the presence of α1,2-, α1,3- and/or α1,4-fucosylated core structures of oligosaccharides. In addition, different patterns of sialylation, i.e. the attachment of α2,3- and/or α2,6-linked *N*-acetylneuraminic acid (NeuAc), increase the variability of human milk oligosaccharide (HMO) to a number of about 115 structures characterised so far[4,5]. The biological significance of HMO for the infant, however, has not been proven yet. Since the 1950s, HMO have been thought to be growth-promoting factors for the so-called 'bifidus flora' in the gut of breastfed infants[6]. Recently, the analysis of the genome of *Bifidobacteria* indicated their evolutionary adaptation to preferentially use specific milk components, particularly HMO as their substrate[7,8]. However, the bifidogenic effect of HMO themselves and their direct impact on the intestinal microbiota are difficult to demonstrate *in vivo*. The same applies to other specific *in vitro* functions of HMO, such as their potential to influence inflammatory and infectious processes via inhibition of the attachment of pathogens to epithelial cells or leucocyte endothelial and neutrophil platelet interactions[9–12]. Animal and preclinical studies indicate that oligosaccharides like those in human milk may play an important role in many physiological processes such as influencing cell recognition and cell signalling, cell adhesion, the composition of the microbiota or even affecting neurodevelopment[13–15].

To better understand the underlying mechanisms, more information regarding the metabolic fate of HMO in the infants

Abbreviations: FAB-MS, fast atom bombardment-MS; Gal, galactose; HMO, human milk oligosaccharides; HPAEC-PAD, high pH anion exchange chromatography with pulsed amperometric detection; IR-MS, isotope ratio-MS; LNFP II, lacto-*N*-fucopentaose II; LNT, lacto-*N*-tetraose; NeuAc, *N*-acetylneuraminic acid; NeuAcLac, *N*-acetylneuraminyl-lactose; PDB, Pee Dee Belemnite.

* **Corresponding author:** Professor C. Kunz, fax +49 641 9939049, email clemens.kunz@ernaehrung.uni-giessen.de

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is needed. In a previous study[16], we demonstrated that the application of stable isotopes to lactating mothers leads to the preferential incorporation of ^{13}C-galactose (Gal) into lactose and milk oligosaccharides. The ^{13}C-enrichment of HMO in this pilot study was high enough to investigate the metabolic fate of HMO in the infants. In an earlier study, we showed in one mother and her infant that the combination of fast atom bombardment-MS (FAB-MS) and isotope ratio-MS (IR-MS) is suitable to address specific questions with regard to the metabolism of HMO in humans[17]. In the present study, we report the renal excretion of intact and partly degraded complex oligosaccharides in ten infants after *in vivo* labelling of HMO by administering ^{13}C-Gal to their mothers.

Subjects and methods

Subjects and study design

Exclusively breastfeeding women (n 10; 3–6 months postpartum) participated in the present study. Three mothers had already participated in our previous study[16] and seven were newly recruited. Some dietary restrictions, i.e. the avoidance of naturally ^{13}C-rich foodstuffs (e.g. corn, pineapple, fish), were given. The mothers were asked not to drink any milk for breakfast on the study day and to record their daily food intake during the study period. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committee of the University of Giessen. Written informed consent was obtained from all subjects/patients.

Between 08.00 and 09.30 hours, immediately after breakfast, mothers orally ingested a Gal bolus consisting of 23 g Gal + 4 g ^{13}C-Gal dissolved in about 50 ml of drinking water. The purity of ^{13}C-Gal (D-Gal; 1-^{13}C) from Eurisotop (Saint-Aubin Cedex, France) was determined to be higher than 99 %.

Milk and breath sampling of the women

A milk sample (5–10 ml) was collected immediately before the Gal bolus was given (baseline value) and at the beginning of each nursing during the following 36 h[16]. At the same time, mothers took breath samples and collected their urine in 3–4 h fractions.

Sampling of the infants' urine

For urinary collections, diapers consisting purely of cellulose and free of other absorptive material (Procter & Gamble, Frankfurt, Germany) were used. Urine was collected in adhesive bags which were emptied or replaced before each nursing. Diapers were changed before each nursing and immediately frozen at −20°C. For urine extraction, diapers were thawed and mechanically pressed leading to urine yields of about 70–80 %. In previous experiments, it was verified that the urine collection via diapers and adhesive urine bags did not affect HMO analysis.

Separation of milk fractions and characterisation of milk carbohydrates

The separation of whole milk into fractions with fat, proteins and carbohydrates followed by the removal of lactose was achieved by ultracentrifugation, acetone treatment and gel filtration chromatography of the carbohydrate fraction as described earlier[18]. Then, neutral and acidic HMO were characterised by chromatography and MS (see the following subsections).

High pH anion exchange chromatography with pulsed amperometric detection

High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Carbo Pac PA-1 column (250 × 4·6 mm inner diameter) equipped with a guard column and a Model PAD 2 detector (Dionex, Sunnyvale, CA, USA) were used for characterisation of neutral and acidic HMO at the following conditions: eluent A, 100 mM-NaOH; eluent B, 100 mM-NaOH and 250 mM-sodium acetate. The elution programme started with 3 ml of buffer A followed by a gradient of up to 100 % buffer B in 30 min and a re-equilibration volume of 5 ml buffer A. The flow-rate of 1·0 ml/min was used and 25 μl of 1 or 4 mg/ml solutions were injected. Molar response factors were determined by injecting three to six times equimolar amounts of each oligosaccharide as described previously[18].

Fast atom bombardment-MS

Oligosaccharides (50–100 μg) were dissolved in 500 μl of pyridine–acetic anhydride (1:1, v/v). The mixture was stirred at room temperature overnight. Then the solvent was evaporated under N_2 and the residue was used for mass spectrometric analysis without further purification. The FAB-MS analysis was carried out on a VG-ZAB-T four-sector instrument (Fisons Instruments, Manchester, UK); caesium was used for atom bombardment[16]. The applied acceleration voltage was 8 kV. Thioglycerol–*m*-nitrobenzylalcohol (1:1, v/v) was used as matrix to which 1 μl of a homogeneous sample solution in chloroform–methanol (1:1, v/v) was added. For spectra recording, positive ions (FAB +) were detected on the second photomultiplier point detector. The spectra were run in a mass range from 100 to 3000 atom mass units with a scan rate of 8 s per decay and up to ten scans were accumulated.

Determination of ^{13}C enrichment by isotope ratio-MS

^{13}C enrichment was determined as $\delta\,^{13}C_{PDB}$ measured in duplicate in whole milk, milk carbohydrates, total urine and urinary carbohydrate fractions by IR-MS (Isoprime; Isoprime Limited, formerly: GV Instruments; Manchester, UK) after total combustion at 1020°C (EuroEA, Eurovector, Milan, Italy)[16]. Breath samples were analysed in a BreathMat IRMS Microgas IRMS, Isoprime Limited). ^{13}C elimination rates were then quantified as described in the following subsection.

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^{13}C analysis by isotope ratio-MS

The ^{13}C content of a sample is expressed as delta value ($\delta\,^{13}C_{PDB}$) (‰) which is the relative difference between the ^{13}C:^{12}C ratio of a sample (Sa) and the international Pee Dee Belemnite (PDB) standard with a $(^{13}C{:}^{12}C)_{PDB}$ ratio of 0·0112372,

$$\delta^{13}C_{PDB}\,(‰) = ({}^{13}R_{Sa} - {}^{13}R_{PDB})/({}^{13}R_{PDB}) \times 1000$$

where $^{13}R_{Sa} = (^{13}C/^{12}C)_{Sa}$ is the isotope ratio of the unknown sample and PDB is Pee Dee Belemnite.

Data evaluation was performed with MassLynx inorganic version 4 software (GV Instruments) taking into account the ^{17}O-content of the sample and the blank correction for the carbon of the tin cups[16]. For an easier comparison of the data, the results were expressed as $\Delta\delta\,^{13}C_{PDB}$ (corrected for the ^{13}C:^{12}C ratio at baseline). The accepted standard deviation was $<0{\cdot}2$‰ for total urine and isolated carbohydrates and $<0{\cdot}7$‰ for total milk. The accepted deviation for breath sample analyses was $<0{\cdot}3$‰.

Calculation of the cumulative ^{13}C-enrichment in milk and urine

To determine the ^{13}C-enrichment in milk and urine, total organic carbon (TOC) and ^{13}C-content (at%) for each sample were measured. Then, the excreted amounts of ^{13}C were calculated, taking into account the baseline ^{13}C values and the ^{13}C-bolus given.

Using this ^{13}C-at%$_{sa}$ and the TOC of a sample, the absolute amount of ^{13}C of each sample (Sa) was then calculated as follows:

$$^{13}C_{sa}\,(g) = TOC_{sa}\,(g) \times \frac{^{13}C - at\%_{sa}}{100},$$

where at% is atom%.

Calculation of the cumulative ^{13}C exhalation

The percentage recovery of the ingested ^{13}C amount in breath ($^{13}C_{cum}$) was calculated using the equation shown in the previous subsection. Thereby, the exhaled amount of ^{13}C is defined as

$$^{13}C_{excreted}\,(mol/kg) = \Delta\delta^{13}C_{cum}\,(‰ \times h) \times {}^{13}R_{PDB} \times CO_2 - P\,(mmol/kg \times h),$$

with $\Delta\delta\,^{13}C_{cum}$ being the cumulative ^{13}C exhalation.

Determination of galactose and lactose content

Gal and lactose contents of milk and urine were photometrically determined using a colorimetric kit from Boehringer Mannheim (Mannheim, Germany).

Calculation of ingested milk volumes and respective amounts of human milk oligosaccharide

To determine the amount of milk ingested by the infants, the milk volume was determined by weighing the infants on a digital electronic balance before and after each nursing. Oligosaccharides were quantified by HPAEC-PAD analysis. Peak areas of lacto-*N*-tetraose (LNT) and lacto-*N*-fucopentaose II (LNFP II) were then calculated as percentage of the total amount of oligosaccharides applied, corrected by the molar response of the amperometric detector[18].

Results

^{13}C-enrichment of whole milk and $^{13}CO_2$ exhalation

In all milk samples, there was an immediate increase in ^{13}C-enrichment of whole milk in the first hours after the oral ^{13}C-Gal bolus was given to the mothers followed by a second smaller ^{13}C-peak in six out of ten milk samples from the next morning (Fig. 1). To get an indication of how much Gal was metabolised for energy production, ^{13}C-enrichment of breath was determined by analysing $^{13}CO_2$. Exhalation over 36 h compared to the ^{13}C:^{12}C ratio in whole milk resulted in a similar course although with a much higher enrichment in most samples (Fig. 1).

HPAEC-PAD analysis of the neutral and acidic fractions after Sephadex G25 gel filtration chromatography revealed a rather complex mixture of carbohydrates which were then characterised by FAB-MS. As an example, the composition of the major acidic and neutral HMO is given in Table 1. The molecular weight of these oligosaccharides varied between about 500 and 2500 Da.

Determination of the ^{13}C-enrichment of the infants' urine

The ^{13}C:^{12}C ratio of urine fractions collected over 36 h after the Gal bolus was given to their mothers is shown in Fig. 1. The ^{13}C-enrichment follows about the same pattern as in whole milk. Also, the first ^{13}C-appearance in most urine fractions is delayed as compared to the corresponding milk fraction.

To ensure that the higher ^{13}C:^{12}C ratio is not due to the excretion of free ^{13}C-Gal, we determined the total Gal excretion by enzymatic methods. The data which are exemplified for one infant in Fig. 2 revealed that the excretion of free Gal was only high in the first sample after the Gal bolus was given to the mother, and returned to baseline afterwards. In contrast to the total Gal excretion, the ^{13}C-enrichments in the same urine fractions reached their maximum value 10–11 h (fraction 4) after the Gal bolus was given to the mother.

To characterise ^{13}C-enriched carbohydrates, urine fractions were subjected to Sephadex gel filtration to obtain components of different molecular sizes and to separate monosaccharides and lactose from oligosaccharides. These fractions were further characterised by HPAEC-PAD, IR-MS and FAB-MS. The major components in these fractions are shown in Table 2. Not only small oligosaccharides such as the lactose derivatives FucLac and Fuc_2Lac were detected but also LNT, one of the main core structures in HMO (Table 2). Moreover, the mono-, di- and trifucosylated derivatives of LNT, lacto-*N*-hexaose, lacto-*N*-octaoses and lacto-*N*-decaose could be found. Also, besides neutral components, *N*-acetylneuraminyl-lactose (NeuAcLac), the major acidic component in HMO and the sialylated derivative of LNT,

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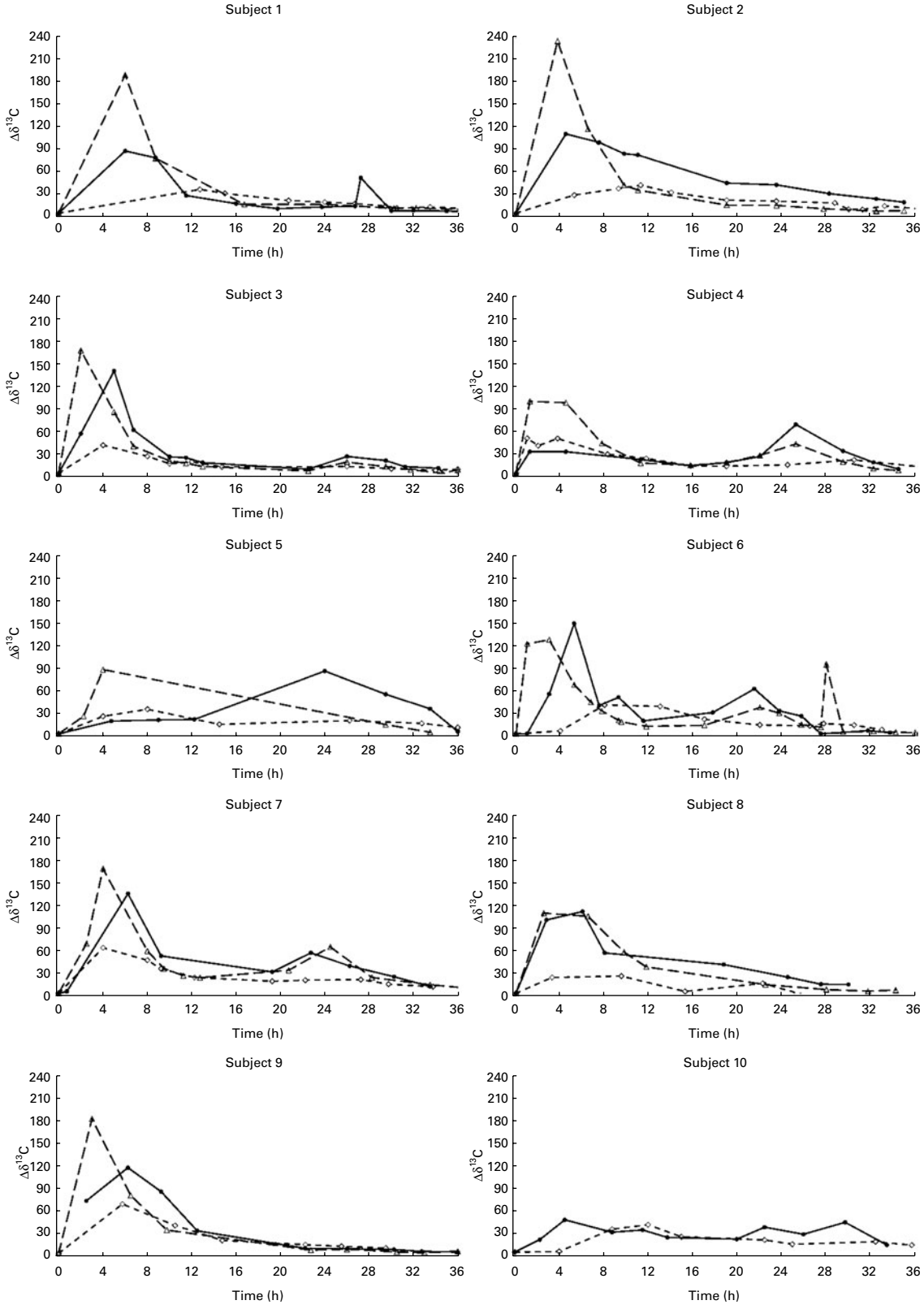

Fig. 1. ^{13}C-enrichment of whole milk (–◆–) and infants' urine (–◇–) and $^{13}CO_2$ exhalation by the mothers (–△–) during the first 36 h after the oral intake of a galactose (Gal) bolus consisting of 4 g ^{13}C-Gal + 23 g Gal by the mothers. The $\delta\,^{13}C_{PDB}$ (‰) values of each sample are corrected over the baseline values of each respective sample obtained at time point 0, immediately before the Gal intake. The bolus was taken after breakfast which varied between 08.00 and 09.30 hours.

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Table 1. Fast atom bombardment-MS of the major neutral and acidic human milk oligosaccharides from one mother in fractions after Sephadex 25 chromatography

Nominal *m/z* values of detected molecular ions			
$[M + H]^+$	$[M + Na]^+$	Oligosaccharide	Composition
Neutral peracetylated oligosaccharides			
	701	Lac	Hex_2
	931	FucLac	$FucHex_2$
1254	1276	LNT	$Hex_3HexNAc$
1484	1506	Fuc_1LNT	$FucHex_3HexNAc$
1714	–	Fuc_2LNT	$Fuc_2Hex_3HexNAc$
1829	–	LNH	$Hex_4HexNAc_2$
2059	2081	Fuc_1LNH	$FucHex_4HexNAc_2$
2289	2311	Fuc_2LNH	$Fuc_2Hex_4HexNAc_2$
2634	2656	Fuc_1LNO	$FucHex_5HexNAc_3$
2864	2886	Fuc_2LNO	$Fuc_2Hex_5HexNAc_3$
3094	3116	Fuc_3LNO	$Fuc_3Hex_5HexNAc_3$
3209	3231	Fuc_1LND	$FucHex_6HexNAc_4$
Acidic peracetylated oligosaccharides			
1036	1058	NeuAcLac (Lacton)	$NeuAcHex_2$
1096	1118	NeuAcLac	$NeuAcHex_2$
1671	–	NeuAcLNT	$NeuAcHex_3HexNAc$
2028	–	$NeuAc_2$LNT (Lacton)	$NeuAc_2Hex_3HexNAc$
2246	–	NeuAcLNH	$NeuAcHex_4HexNAc_2$
2476	–	$NeuAcFuc_1$LNH	$NeuAcFucHex_4HexNAc_2$

Lac, lactose; Hex, hexose; NAc, *N*-acetyl; FucLac, fucosyl-lactose; LNT, lacto-*N*-tetraose; FucLNT, fucosyl-lacto-*N*-tetraose; FucLNH, fucosyl-lacto-*N*-hexaose; NeuAcLac, *N*-acetylneuraminyl-lactose; NeuAcLNT, *N*-acetylneuraminyl-lacto-*N*-tetraose.

N-acetylneuraminyl-lacto-*N*-tetraose, were also present. In addition, fucosyl-lactosamin and fucosyl-lacto-*N*-triose were identified, with glucose split off from the reducing end of the oligosaccharides (Table 2).

Quantification of lacto-N-tetraose and lacto-N-fucopentaose II in milk and in the infants' urine

As the milk volume ingested by an infant per suckling was known, we were able to determine in one infant the total amount of LNT and LNFP II taken up with each suckling

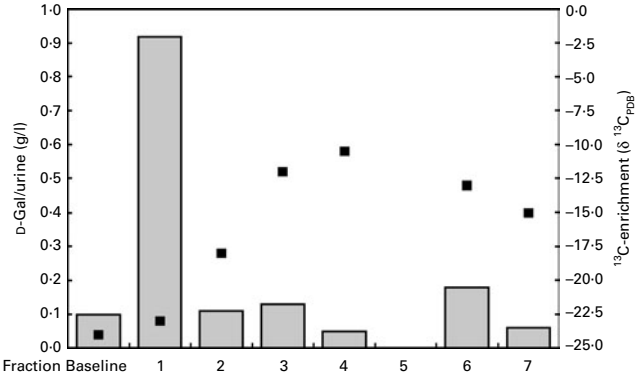

Fig. 2. Excretion of free urinary galactose (□) and ^{13}C-enrichment (■) in fractions of an infant's urine over 24 h.

Table 2. Fast atom bombardment-MS of neutral and acidic oligosaccharides from an infant's urine

Nominal *m/z* values of detected molecular ions			
$[M + H]^+$	$[M + Na]^+$	Oligosaccharide	Composition
Neutral peracetylated oligosaccharides			
679	701	Lac	Hex_2
908	–	FucLacNAc	FucHexHexNAc
909	931	FucLac	$FucHex_2$
1139	–	Fuc_2Lac	Fuc_2Hex_2
1196	1218	Fuc_1LNTri	$FucHex_2HexNAc$
1254	1276	LNT	$Hex_3HexNAc$
1484	1506	Fuc_1LNT	$FucHex_3HexNAc$
1714	1736	Fuc_2LNT	$Fuc_2Hex_3HexNAc$
	1853	Hex_6	
2289	2311	Fuc_2LNH	$Fuc_2Hex_4HexNAc_2$
2519	2541	Fuc_3LNH	$Fuc_3Hex_4HexNAc_2$
Native acidic oligosaccharides			
$[M - H]^-$			
632	–	NeuAcLac	$NeuAcHex_2$
997	–	NeuAcLNT	$NeuAcHex_3HexNAc$
1347	–	$NeuAcFuc_1$ HexNAcLNT	$NeuAcFucHex_3HexNAc_2$

Lac, lactose; Hex, hexose; FucLacNAc, fucosyl-lactosamin; FucLac, fucosyl-lactose; Fuc_1LNTri, fucosyl-lacto-*N*-triose; LNT, lacto-*N*-tetraose; NAc, *N*-acetyl; FucLNT, fucosyl-lacto-*N*-tetraose; LNH, lacto-*N*-hexaose; FucLNH, fucosyl-lacto-*N*-hexaose; FucLNO, fucosyl-lacto-*N*-octaose; FucLND, fucosyl-lacto-*N*-decaose; NeuAcLac, *N*-acetylneuraminyl-lactose; NeuAcLNT, *N*-acetylneuraminyl-lacto-*N*-tetraose; NeuAcFucLNH, *N*-acetylneuraminyl-fucosyl-lacto-*N*-hexaose.

(Table 3). The total intake of both components varies between 46 and 98 mg for LNT and between 63 and 157 mg for LNFP II per suckling. The determination of the same components in the urine fraction allowed us to quantify the total urinary excretion of these HMO (Table 4).

Discussion

There is increasing evidence, supported in recent years by animal studies, that HMO have specific beneficial effects on the infant[14,15]. Concomitantly with these observations, progress in biotechnology today allows to produce at least some of the major milk oligosaccharides to be potentially added to infant formula at reasonable costs. However, to be able to decide which compound should be added, and in which concentrations or combinations, studies are needed regarding absorption, metabolism and functions in the infants. Previously, we have shown that a major part of a ^{13}C-Gal bolus orally given to lactating mothers was immediately transported to the lactating gland and is directly incorporated into HMO[16]. These results have been confirmed in the present study. Thus, using stable isotopes, an *in vivo* ^{13}C-labelling of HMO can be achieved to address important questions with regard to their metabolic fate.

Here, we show that in urine of ten infants, HMO are found in all samples collected within 36 h after a ^{13}C-Gal bolus was given to their mothers. The infants' urinary oligosaccharides can only be derived from milk as the biosynthesis of HMO occurs exclusively in the lactating mammary gland. Also, the excretion of intact HMO in the present study with

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Table 3. Milk concentration and total intake of lacto-*N*-tetraose (LNT) and lacto-*N*-fucopentaose II (LNFP II) per suckling in one infant
(Mean values and standard deviations from five samples of one day)

		Concentration (mg/ml)*					
		LNT		LNFP II		Total intake through milk (mg)	
Suckling	Volume (ml)	Mean	SD	Mean	SD	LNT	LNFP II
1	97·0	0·77	0·01	1·10	0·03	74·73	106·30
2	126·1	0·78	0·01	1·25	0·04	98·22	157·17
3	58·2	0·79	0·00	1·09	0·01	46·20	63·53
4	97·0	0·77	0·09	1·07	0·11	75·08	103·80
5	97·0	0·57	0·00	0·85	0·01	55·56	82·25

term infants at 3–6 months postpartum verifies our earlier data in preterm infants showing very similar data without using stable isotopes[19].

Besides intact HMO, we also detected cleavage products in which not, as expected, the terminal Gal but the glucose moiety on the reducing end had been split off. At the moment, no plausible explanation can be given for such an unusual metabolic degradation step. There is only one congress contribution from Lundblad's group on the renal excretion of oligosaccharides in a few preterm and full-term infants using mass spectrometric methods for analysis[20]. The authors found that small 'typical milk oligosaccharides' were excreted by human milk-fed infants. Most of the analysed urine samples contained 2′- and 3-FucLac as well as Fuc_2Lac. They could not detect these components in the urine of non-breastfed infants. A further comparison with our data regarding complex oligosaccharides such as LNT and fucosylated and/or sialylated derivatives as well as NeuAcLac is not feasible, since Lundblad and co-workers did not report the analysis of such components.

There are two possible explanations for the occurrence of 'modified' oligosaccharides in urine; either they may originate from human milk itself or they are synthesised endogenously from smaller precursors. Although there is no evidence from *in vivo* studies yet, these modifications may derive from bacterial activity within the gut. Also, if such HMO modifications occur within the colon, the underlying mechanism of the succeeding absorptive process needs to be demonstrated as well.

In the present study, we also determined the amount of HMO an exclusively breastfed infant may receive with each suckling. We showed that the intake of individual oligosaccharides by the infant ranges from 50 to 150 mg per suckling. This large amount of oligosaccharides which 'rinse' the whole digestive tract emphasises the potential of anti-adhesive or cellular effects shown for individual carbohydrates in many *in vitro* studies[2,9,11,21–24]. However, to exert systemic effects, HMO must be absorbed and transported in the peripheral blood to specific cells where they might also be metabolised. Although HMO are considered to be indigestible[25], we detected specific components like LNT and LNFP II in the infants' urine indicating intestinal absorption. As the renal excretion was in the range between 1 and 3 mg/d, the actual intestinal absorption must be much higher, and hence, larger amounts of these HMO must have been circulating in the infant's blood. Therefore, in addition to local functions of HMO within the gastrointestinal tract, systemic effects such as the adhesion of leucocytes to endothelial cells or the interaction of platelets with neutrophils may be influenced[12,23]. Recently, an impact of HMO on brain glycoconjugate composition has also been discussed. Carlson & House[26] compared an intra-peritoneal administration to an intra-gastrical application of NeuAc on rat brain composition, and found that both oral and systemic routes resulted in significantly more cerebral and cerebellar glycolipid and glycoprotein NeuAc than did glucose injections. Compared to free NeuAc, orally given NeuAcLac, the major acidic oligosaccharide in human milk, was even more effective on brain composition. These data supported an earlier observation by Witt *et al.*[27] comparing radiolabelled free NeuAc and NeuAcLac; the authors showed a preferential incorporation of ^{14}C-NeuAcLac in rat brain gangliosides. Our data suggest that the absorption of HMO in breastfed infants supports the possibility that sialic acid from HMO is utilised as a substrate for the biosynthesis of components including gangliosides or glycoproteins (e.g. for the brain).

Table 4. Urinary concentration and total excretion of lacto-*N*-tetraose (LNT) and lacto-*N*-fucopentaose II (LNFP II) per suckling in one infant
(Mean values and standard deviations from five samples of one day)

		Concentration (μg/ml)					
		LNT		LNFP II		Total excretion in urine (mg)	
Urine sample	Volume (ml)	Mean	SD	Mean	SD	LNT	LNFP II
1	35·0	13·4	0·2	13·1	0·4	0·47	0·46
2	25·0	21·2	0·5	22·6	1·5	0·53	0·56
3	26·3	11·4	0·2	12·4	0·5	0·30	0·33
4	19·7	7·3	0·1	7·0	0·1	0·14	0·14
5	23·2	11·6	0·3	13·3	0·1	0·27	0·31

In conclusion, we have shown that the application of ^{13}C-labelled HMO alleviates investigations regarding the metabolic fate of HMO in human subjects. The intake of HMO by the infants ranges within several hundred mg per suckling. Some of these components are excreted as intact oligosaccharides or as slightly modified metabolites within the infants' urine. Therefore, HMO have the potential for not only local but also systemic effects. Such effects, however, remain to be demonstrated in future studies. The enormous biotechnological progress in recent years will enable necessary studies *in vivo*.

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