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Exploring human breast milk composition by NMR-based metabolomics

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Breast milk is a complex fluid evolutionarily adapted to satisfy the nutritional requirements of growing infants. In addition, milk biochemical and immunological components protect newborns against infective agents in the new environment. Human milk oligosaccharides, the third most abundant component of breast milk, are believed to modulate the microbiota composition, thus influencing a wide range of physiological processes of the infant. Human milk also contains a number of other bioactive compounds, the functional role of which has not yet been clearly elucidated. In this scenario, NMR-based metabolic profiling can provide a rapid characterisation of breast milk composition, thus allowing a better understanding of its nutritional properties.

Keywords: ^{1}H NMR; HMO; milk metabolome; metabolic profiling

1. Introduction

Human milk is a complex fluid that simultaneously provides nutrients and bioactive components, thus orchestrating infant development. Milk constituents influence several physiological processes, including growth, modulation and maturation of the immune system, protection from toxins and pathogens, cognitive development and the establishment of the intestinal microbiota (Wu & Chen 2009). Human milk oligosaccharides (HMOs) represent the third most abundant components of human milk after lactose and lipids, are minimally digested by the infant and are believed to act as bifidogenic substrates (Sela & Mills 2010; Bode 2012).

The composition of human milk strongly differs among individuals and over the course of lactation (Chaturvedi et al. 2001; Thurl et al. 2010). In particular, the content and the molecular structures of HMOs reflect the expression and activity of specific fucosyltransferases in the lactating mammary gland (Kobata 2010). Human milk also contains a vast array of other bioactive compounds, the functional role of which has not yet been clearly elucidated.

Over the past decade, high-throughput analytical technologies, such as HPLC, mass spectrometry and NMR, allowed the characterisation of hundreds of HMO structures (Martin-Pastor & Bush 2000; Wu et al. 2010) and other classes of nutrients (Sundekilde et al. 2013), including lipids (Kovács et al. 2005).

NMR methods have been shown to allow a rapid, non-destructive identification and quantification of low-molecular weight metabolites in whole cow and sheep milk (Hu et al. 2007; Lamanna et al. 2011). Moreover, recent studies have shown the potential of NMR-based metabolic fingerprinting to investigate human milk composition (Cesare Marincola et al. 2012)

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and to compare the differences in metabolic profiles between human and rhesus milk (O'Sullivan et al. 2012).

In this study, we have applied ^{1}H NMR spectroscopy to disentangle the complex mixture of metabolites present in breast milk hydrosoluble extracts and characterise the human milk metabolome.

2. Results and discussion

The characterisation of the ^{1}H NMR spectrum of milk hydroalcoholic phase is very challenging, due to the presence of a large number of overlapped peaks, especially between 3.5 and 5.50 ppm. ^{1}H NMR spectrum of the polar phase can be divided into three main spectral regions. The aliphatic region, from 0 up to 3.5 ppm, contains amino acids (alanine, isoleucine, lysine, threonine and valine), tricarboxylic acid cycle intermediates (citrate, succinate and α-ketoglutarate), short chain fatty acids (acetate and valerate), lactate, *N*-acetyl moieties, *N*-trimethyl moieties (choline, carnitine and acetylcarnitine) and oligosaccharides (sialic acid and fucosylated derivatives). The sugar region, between 3.5 and 5.5 ppm, includes a series of overlapped signals, due to the presence of simple (glucose, lactose and myoinositol) and complex sugars (HMOs). The most intense signals (δ 5.22, 4.69 and 4.47) were assigned to the anomeric protons of free lactose, superimposed to the peaks of some lactosyl units of HMOs. The aromatic region includes aromatic amino acids (histidine and its methyl derivatives, phenylalanine and tyrosine) and phenolic compounds (4-hydroxyphenylacetic acid).

Two dimensional (2D) homo- and heteronuclear NMR experiments allowed the characterisation of small molecular weight metabolites, especially in the sugar region. In particular, fucosylated oligosaccharides could be discriminated based on the fucose H-1, H-5 and H-6 signals. Details of fucose resonance assignments by TOCSY and HSQC NMR experiments are reported in Supplementary material (Figures S1 and S2). Two dimensional NMR experiments allowed also the identification of sialic acid units (Neu5Ac), which were assigned to the structures of two acidic oligosaccharides: 3′sialyl-lactose and 6′sialyl-lactose, according to a previous study (Osthoff et al. 2008). However, the accurate integration of these resonances was not possible due to the intense signal superimposition.

A total of 43 metabolites were identified, as reported in the Supplementary material (Table S1).

Comparison of breast milk NMR spectra showed three different spectral profiles (or milk groups) characterised by specific patterns of fucosylated oligosaccharide resonances (Figure 1). Milk group 1 ($n = 11$) exhibited all the signals of fucosylated oligosaccharides, while milk group 2 ($n = 7$) lacked α1,2-fucosylated structures (2′FL, 2′ fucosyllactose; LDFT, lactodifucotetraose; LNFPI, lacto-*N*-fucopentaose I; LNDFHI, lacto-*N*-difucohesaose I). In milk group 3 ($n = 2$), α1,2- and α1,3-fucosylated oligosaccharides were found, whereas LNFPII (lacto-*N*-fucopentaose II), LNDFHI and LNDFHII (lacto-*N*-difucohesaose II), with α1,4-fucose residues, were missing. Previous studies have shown that fucosylation pattern of HMOs was correlated to the activity of specific enzymes (Thurl et al. 2010; Totten et al. 2012). In particular, α(1,2)-fucosyltransferase (FucT2) is responsible for a α1-2 linkage of a fucose residue to the Galβ(α1-3)GlcNAc unit of the oligosaccharide chain, while α(1,3/4)-fucosyltransferase (FucT3) transfers a fucose in an α1-4 or/and α1-3 linkage to a sub-terminal *N*-acetylglucosamine residue of the HMO. FucT2 is encoded by the secretor gene (Se), while FucT4 is expressed by the Lewis blood group gene (Le). As a result of the possible expression of these two enzymes, there are four possible mother phenotypes based on HMOs: Se^+/Le^+, Se^-/Le^+, Se^+/Le^- and Se^-/Le^-.

Accordingly, we associated the first milk group with the phenotype denoted as Se^+/Le^+, the second milk group with Se^-/Le^+ phenotype and the last metabotype (milk group 3) to Se^+/Le^- phenotype.

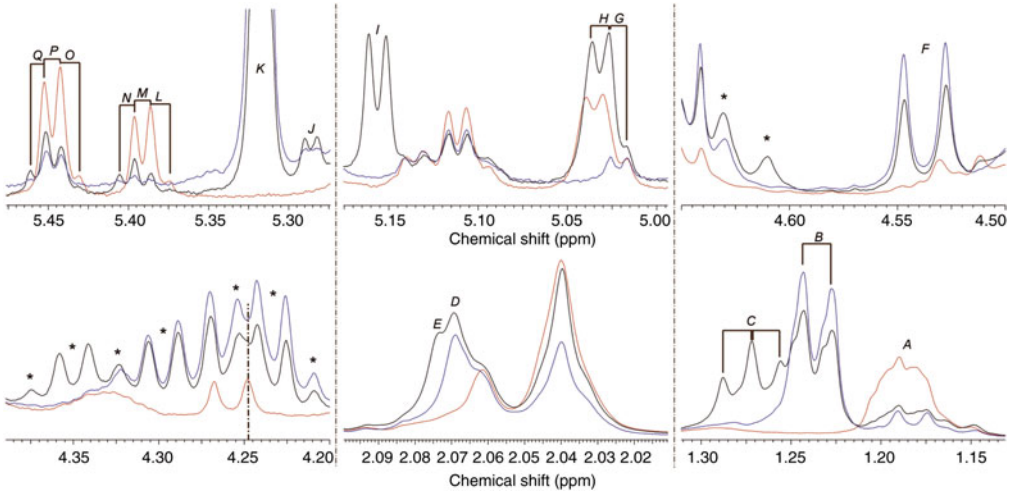


Figure 1. Sections of three ^{1}H NMR spectra of human breast milk, displaying the most evident differences among the three different spectral profiles. Black: milk containing all classes of fucosylated oligosaccharides; red: milk not containing Fuc(α1-2)Gal units; blue: milk not containing α1,4-fucosylated oligosaccharides. Key in Table 1. ∗Overlapped signals of H-5 fucose moieties and H-1 galactose (β1-4).

A summary of the detected fucosylated HMOs with the relative diagnostic resonances for phenotype identification is reported in Table 1.

To test whether other metabolite levels could differ between milk groups 1 and 2, we have applied a Projection to Latent Structures-Discriminant Analysis (PLS-DA) on metabolites shared by these two sample classes. The third milk group was not considered due to a not statistically representative sampling.

The PLS-DA model (Figure 2) showed a clear separation between the two milk groups. The model was described by two latent variables (LVs) that explained 31% and 13% of the total *X*-variance, respectively. R_y^2 was 0.860, while Q^2 was 0.695. α1,3-Fucosylated oligosaccharides, namely 3′FL (3′ fucosyllactose), LNDFHII and derivatives, were the significant discriminating metabolites and resulted two- and fivefold higher in Se^-/Le^+ milk compared with Se^+/Le^+ milk, respectively (Table S2).

The total amount of *N*-acetylglucosamine did not vary significantly between the two phenotypes, although the GlcNAc residues conjugated with α1,3-fucose were more abundant in non-secretor (Se^-) women ($P < 0.001$). The increase in α1,3-fucosylated oligosaccharides observed in Se^-/Le^+ milk was in agreement with previous observations obtained by LC/MS (Thurl et al. 2010), indicating an increase in FucT3 activity in mammary glands of non-secretor mothers. Our results suggest that the oligosaccharide synthesis in Se^-/Le^+ phenotypes is 'balanced' by an increase in FucT3 activity.

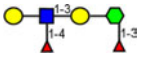
How these variations in HMO composition related to different mother phenotypes could impact on the infant gut microbiota is still a matter of investigation.

3. Experimental

3.1. *Sample collection*

Milk samples were collected from 20 healthy pregnant women, aged between 18 and 45 years old who were recruited from the Department of Obstetrics, Gynecology and Neonatology, at University Hospital of Bari, Italy. Each woman delivered at term (over the 36th week of

Table 1. Structures of fucosylated oligosaccharides detected in the three milk classes, together with the diagnostic chemical shift and the corresponding expressed phenotypes.

Structure	Fucosylated oligosacc.	Group	δ Fucose (ppm)	Group 1 Se^+/Le^+	Group 2 Se^-/Le^+	Group 3 Se^+/Le^-
	3′FL, LNFPV	H-1 Fuc(α1-3)αGlc	5.39(M)	√	√	√
		H-1 Fuc(α1-3)βGlc	5.44(P)			
		H-6 Fuc(α1-3)Glc	1.19(A)			
	LNFPIII and branched	H-1 Fuc(α1-3)GlcNAc′	5.11	√	√	√
		H-1 Fuc(α1-3)GlcNAc″	5.13			
		H-6 Fuc(α1-3)GlcNAc	1.19(A)			
	LNDFHII	H-1 Fuc(α1-4)GlcNAc	5.03(H)	√	√	X
		H-1 Fuc(α1-3)αGlc	5.38(L)			
		H-1 Fuc(α1-3)βGlc	5.43(O)			
		H-6 Fuc(α1-3)Glc	1.14(A)			
	LNDFHI and branched	H-1 Fuc(α1-2)Gal	5.16(I)	√	X	X
		H-1 Fuc(α1-4)GlcNAc	5.03(H)			
		H-6 Fuc(α1-2)Gal	1.29(C)			
		CH_3 GlcNAc(β1-6)	2.08(E)			
	2′FL	H-1 Fuc(α1-2)Gal	5.32(K)	√	X	√
		H-6 Fuc(α1-2)Glc	1.24(B)			
		H-1 Gal(β1-4)	4.53(F)			
	LDFT	H-1 Fuc(α1-2)Gal	5.29(J)	√	X	√
		H-1 Fuc(α1-3)αGlc	5.40(N)			
		H-1 Fuc(α1-3)βGlc	5.46(Q)			
		H-1 Fuc(α1-2)Gal	1.28(C)			
	LNFPI and branched	H-1 Fuc(α1-2)Gal	5.32(K)	√	X	√
		H-3 Fuc(α1-2)Gal	1.24(B)			
		CH_3 GlcNAc(β1-6)	2.06(D)			

Notes: ▲, fucose; ■, *N*-acetylglucosamine; ●, galactose; ⬢, glucose. List of abbreviations: 2′FL, 2′ fucosyllactose; 3′FL, 3′ fucosyllactose; LDFT, lactodifucotetraose; LNFPI–V, lacto-*N*-fucopentaose I–V; LNDFHI–III, lacto-*N*-difucohesaose I–III. Key: √, presence of corresponding resonances; X, absence of corresponding resonances. Letters in brackets, refer to Figure 1.

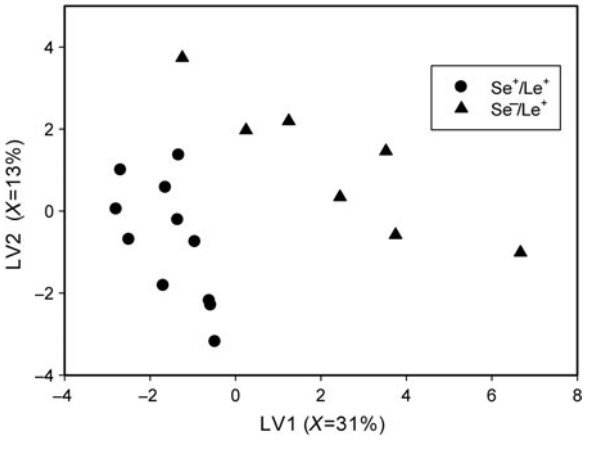


Figure 2. Score plot of PLS-DA for the two milk groups, belonging to secretor (Se^+/Le^+) and non-secretor (Se^-/Le^+) mothers. The model was described by two LVs that explain 31% and 13% of the *X*-variance, respectively.

pregnancy). Exclusion criteria were the use of antibiotics during the study period, diabetes and other chronic illness. A written informed consent was obtained from each subject before enrolment. Breast milk samples were collected 1 month after delivery in sterile plastic tubes, using a manual breast-pump, and immediately frozen at −20°C.

3.2. *Sample preparation*

A total of 600 μL of human milk were vortexed in 3 mL of a methanol–chloroform (2:1) mixture in polypropylene tubes. A total of 400 μL of distilled water and 1 mL of chloroform were sequentially added, then the mixture was vortexed again and kept overnight at 4°C. Polar and organic phases were separated by centrifugation at 11,000*g* at 4°C for 20 min. The extraction procedure was a modification of the method previously set up for tissues, cells and biological fluids (Miccheli et al. 1988, 2006; Ricciolini et al. 1991). The polar phases (top layer) and organic phases (bottom layer) were separately collected, dried under N_2 flux and preserved at −80°C until NMR analysis. The dried polar samples were re-dissolved in 600 μL of D_2O phosphate buffer solution (pH 7.4) containing 2 mM (final concentration) sodium 3-(trimethylsilyl) propionate-2,3-d_4 (TSP) as ^{1}H NMR reference, and transferred to 5 mm NMR glass tubes for analysis.

3.3. *^{1}H NMR spectroscopy*

NMR spectra of polar extracts were acquired at 298 K using a Bruker Avance 400 spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) equipped with a 9.4 T magnet operating at a ^{1}H frequency of 400.13 MHz (see Supplementary material for more details on the acquisition method). Signals assignment was achieved by standard 2D experiments (COSY, TOCSY, HSQC, HMBC and DOSY) on selected samples and confirmed by comparison with the literature (Hounsell 1995; Osthoff et al. 2008; Taufik et al. 2012), web database (Wishart et al. 2013) and in-house database. One-dimensional (1D) NMR spectra were processed and quantified by using ACD Lab 1D NMR Manager ver. 12.0 software (Advanced Chemistry Development, Inc., Toronto, ON, Canada), whereas 2D NMR spectra were processed by using Bruker TopSpin ver.3.1 (Bruker BioSpin GmbH). The acquired NMR spectra were manually phased and baseline was corrected, and referenced to the chemical shift of the TSP methyl resonance at δ 0.00.

Quantification of metabolites was obtained by comparing the integrals of their specific signals with the internal standard TSP integral. Overlapped signals between 1.14 and 1.28 ppm, 2.03 and 2.08 ppm and between 5.35 and 5.45 ppm were measured by peak fitting analysis on the selected multiplets, using the fitting function provided by NMR Manager software. The concentrations are expressed as μmol L^{-1}.

3.4. *Data analysis and statistics*

To evaluate the differences in milk metabolic profiles of Se^+/Le^+ and Se^-/Le^+ mother phenotypes, multivariate and univariate analyses were applied. Multivariate analysis was carried out using Unscrambler 9.8 software (CAMO, Oslo, Norway). Only metabolites common to the two milk groups were considered; data were mean-centred and scaled before analysis. Projection of PLS-DA was applied to maximise the discrimination between different milk groups (Barker & Rayens 2003). Uncertainty test, based on cross validation, Jack-knifing and stability plots, was applied to test the significance of the model (Martens & Martens 2000). Mann–Whitney rank sum test was also applied to assess the differences on the metabolite levels between the two milk groups; a P value < 0.05 was considered significant.

4. Conclusions

In conclusion, the NMR-based metabolic profiling of hydrosoluble milk extracts allowed the characterisation of breast milk metabolome, as well as the identification of three different milk classes on the basis of HMOs. Multivariate analysis of NMR data allowed the characterisation of changes in milk metabolome between Se^+/Le^+ and Se^-/Le^+ mothers.

The proposed approach, integrated with the analysis of the liposoluble extracts, may represent a powerful tool to evaluate the effect of patho-physiological conditions of mothers on milk composition and the influence of dietary interventions on milk metabolome.

Supplementary material

Supplementary material relating to this article is available online, alongside Tables S1 and S2 and Figures S1 and S2.

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