Breast milk: To each his own. From metabolomic study, evidence of personalized nutrition in preterm infants

Breast milk: to each his own. From metabolomic study, the evidence of a personalized nutrition in preterm infants.

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Running title:

Metabolomic study of preterm breast milk for personalized nutrition.

Authors contributions

Dr. S.P. conceptualized and designed the study, drafted the initial manuscript, and approved the final manuscript as submitted.

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Dr. I.Z. contributed to draft the manuscript, reviewed and revised the manuscript, and approved the final version as submitted.

Dr. F.B contributed to data collection and to draft the manuscript, and approved the final manuscript as submitted.

Drs. M.T and A.V. carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted.

Dr. C.B. conceptualized and designed the data collection instruments, contributed to draft the manuscript, and approved the final manuscript as submitted.

Dr. M.C. carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted.

Dr. G.B. conceptualized and designed the study, critically reviewed the manuscript for important intellectual concept, and approved the final manuscript as submitted.

All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved

ABSTRACT

Objective: The composition of milk from mothers delivering prematurely differs from full-term mature milk and changes over time. The aim of this study is to test the hypothesis that changes in milk metabolomic profile from mothers delivering prematurely persist over time when compared with mother delivering at term.

Methods and results: NMR spectroscopy was used to analyze metabolome pattern of human milk samples, collected from 18 mothers. Twelve mothers collected 12 term milk samples (one for each mother), once between 4 to 7 days after delivery. Six mothers delivering prematurely (from 29 to 31 weeks of GA), collected three samples each, once a week after delivery until the 3rd week after birth.

Principal Component Analysis (PCA) showed two distinct metabolites groups, one represented by the 18 preterm milk samples, and the other by term milk samples. Metabolite profiling identified that lactose and oligosaccharides levels were significantly more represented in preterm than in milk term samples.

Conclusions: Preterm milk metabolome pattern undergoes a maturation during the first 3 weeks after birth, but at the end of the third week still does not resemble the term milk pattern. The specific changes in mother milk metabolomic profile according to their offspring, might reflect the different nutritional requirement of each preterm infants. This knowledge is crucial to move from standardized nutritional protocols to a tailored, individualized nutrition in preterm infants.

Keywords

Human milk; Metabolomics; Preterm; NMR spectroscopy; Nutrition

Abbreviations:

GA: Gestational Age

SD: Standard Deviation

NMR: *Nuclear Magnetic Resonance*

PCA: *Principal Component Analysis*

PLS-DA: *Partial Least Square regression Discriminant Analysis*

HMOs: *Human milk oligosaccharides*

INTRODUCTION

Human milk is the best nourishment for the healthy growth and development of infants [1], [2] . Milk is species specific and has a dynamic composition in terms of nutritive components and non-nutritive bio-active factors [3] that makes it uniquely suited for feeding infants [4]. The benefits of breast-feeding on child health are numerous, but, according to European Society for Pediatric Gastroenterology Hepatology And Nutrition (ESPGHAN), the most documented effect is the reduced risk of infectious diarrhea and acute otitis media [2]. Besides these advantages, some studies demonstrate that breastfed infants have better short- and long-term outcomes compared to those of formula-fed infants [5, 6]. Formula-fed infants have different growth patterns, nutritional status and gut microbiota that may be associated with higher risk of obesity, diabetes and cardiovascular diseases later in life. Even with all the current attempts to modify the composition of infant formulas, in order to make them more similar to human milk, huge differences persist between these two kinds of milk [7-9].

Just as in the case of term infants, the first choice of nourishment for preterm newborns should be the mother's own milk, and when this is not available, either at all or in sufficient quantity, donor milk from lactating mothers represents the best choice [10].

Recently we reported that there is a clear difference in the composition between milk collected from mothers delivering extremely preterm (23-25 weeks of gestational age, GA) compared to term one. Furthermore, we found a common temporal variation in the first three weeks of lactation of preterm deliveries. The milks tend to have very similar properties to that collected around the 30th week of post-natal age [8]. These data suggested that, after three weeks of lactation, the milk composition seems to be more homogeneous, as its "maturation" reaches completion.

The present study aims to evaluate if the milk of mother who delivered at mean GA of 30 (more mature milk) has a similar composition compared to that collected from mothers with term delivery. In particular, we aim to assess whether and when the metabolic profiles of milk samples from mothers of premature infants resemble the metabolic profiles of term mature milk, sampling weekly until the third week of post-natal age.

MATERIALS AND METHODS

Using nuclear magnetic resonance (NMR) spectroscopy, we analyzed 30 milk samples, obtained from 18 mothers delivering between 29th and 41st weeks of GA, and collected directly from the milk bank of Santa Maria alle Scotte Hospital in Siena from January 2013 to August 2015. Written informed consent was obtained from all the enrolled mothers.

Mothers were Caucasian, healthy and had a similar home environment. They did not consume alcohol, smoke, or drugs. All mothers had a similar lifestyle and followed a varied and balanced diet. Milk samples were collected from 18 mothers. Twelve mothers collected 12 term milk samples (one for each mother), once between 4 to 7 days after delivery. Six mothers delivering prematurely (from 29 to 31 weeks of GA), collected three samples each, once a week after delivery until the 3rd week after birth. Milk samples were collected from mothers in the morning, before breastfeeding. Then, one ml of each milk sample was processed and stored in the milk bank until completing the recruitment. All milk samples were analyzed in the same time..

(Table 1).

Table 1 - Discriminating features of enrolled mothers and time of samples collection

	Preterm (n = 6)	**Term (n = 12)**
Mean gestational age at the time of delivery (weeks), Standard Deviation	30 ± 1	40 ± 1
Mean mother's age at the time of delivery (years), Standard Deviation	35 ± 5	31 ± 3
Mean time of milk collection (days after birth), Standard Deviation		
1st sample	5 ± 2	6 ± 1
2nd sample	14 ± 2	
3rd sample	21 ± 3	

Butylated hydroxytoluene (BHT) was added to each sample for its antioxidant properties [11] and the samples were frozen and stored at -80°C until the analysis was performed. Before analysis, we extracted and separated the water-soluble metabolites from fat-soluble ones. In each sample of milk, 8 ml of chloroform/methanol/water solution was added (1:1:0.3 respectively). Then, these new samples were placed in glass beaker at 0°C for 15 minutes and, subsequently, centrifuged at a speed of 4000 rpm at 4°C for 20 minutes to separate the methanol/water phase from the chloroform phase. Finally, the methanol/water phase was transferred into small beakers and frozen, ready for the NMR analysis.

For NMR experiments, samples were lyophilized to eliminate water and methanol, then treated with deuterated water and trimethylsilyl-2,2,3,3-tetradeuteropropoic acid 20mM to the calibration of the chemical shift at 0 ppm. 600 µl of sample was then transferred to NMR tubes and the pH was adjusted to 6 ± 0.02 for a first measurement. A second measurement was then carried out at a pH 2.50 ± 0.02, because the chemical shift of ionizable substances is highly pH dependent and, at a pH

2.50, all chemical shift values have a reproducibility of ± 0.01 ppm. NMR measurements were performed with a Bruker Avance DRX 600 MHz using a probe SEI (Selected Enhance Inverse). The spectra were acquired at a constant temperature of 298.0 ± 0.1 K using a 90° pulse. A delay of 10 seconds in the sequence was included to allow the complete relaxation of the spin system. We applied a line broadening function of 0.3 Hz before Fourier transformation. The water signal, still present in the sample, was suppressed by a saturation pulse of the signal itself, with a duration of 2 seconds. 32K data points per scan and the accumulation of 128 transients were used for the acquisition of the spectra (Figures 1 and 2).

Figure 1 - Spectrum representing the metabolomic profiling of human breast milk collected from mothers delivering at term.

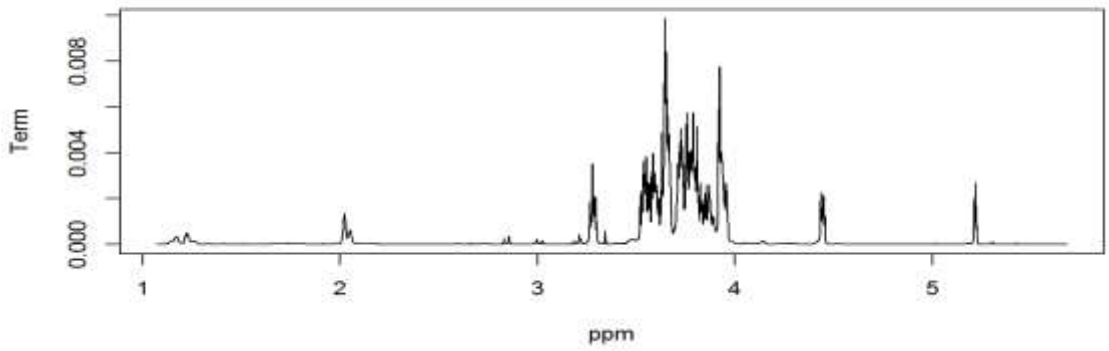
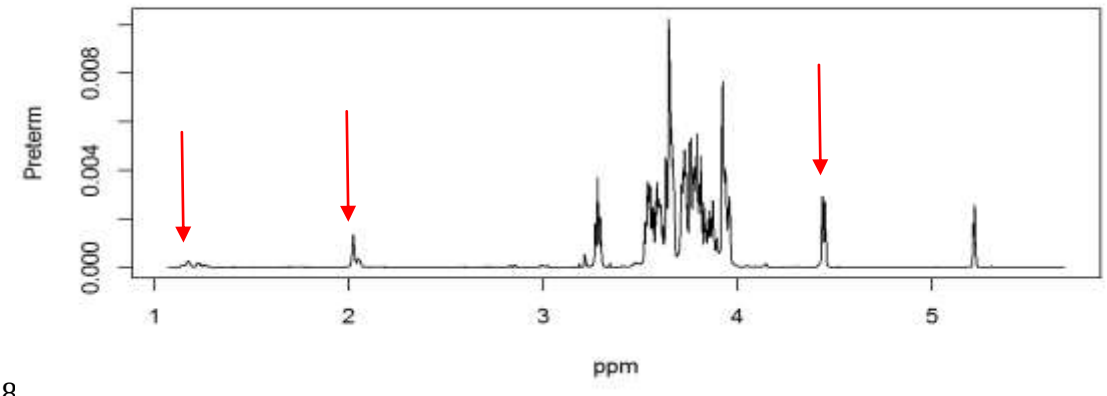


Figure 2 – Spectrum representing the metabolomic profiling of human breast milk collected from mothers delivering prematurely.

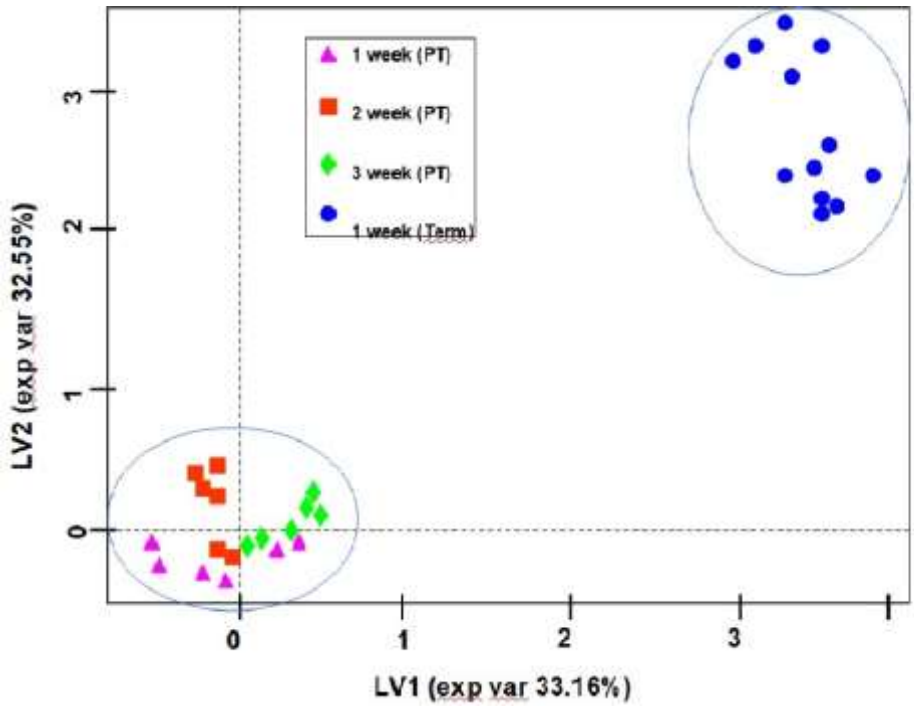


For statistical analyses, we first performed Principal Component Analysis (PCA) with an unsupervised technique, to find trajectories and clustering in our data [12, 13]. Then, Partial Least Square regression Discriminant Analysis (PLS-DA) was performed in order to identify differences in the metabolites profiles [14, 15].

RESULTS

PLS-DA analysis performed on auto-scaled data shows a distinct grouping of milk samples and a significant difference between the metabolic profiles of milk from mothers with preterm delivery and the milk from mothers of term infants, at least until the fourth week (Figure 3).

Figure 3 - Discriminant plot PLS-DA. Two distinct metabolic groups are represented: the bottom left circle denotes preterm human milk samples, collected during the three weeks of lactation after birth; the upper right circle denotes the full term human milk, collected between 4 to 7 days after parturition.



Profiling analysis led to the identification of the metabolite as lactose (4.46 ppm) which is present in higher concentration in preterm milk samples. Furthermore, milk samples from mothers of preterm infants had higher peaks of oligosaccharides, especially those fucosylated, such as fucose at a concentration of 1.23 ppm, N-acetyl-neuraminic acid at 2.06 ppm, and N-acetyl-glucosamine at 2.04 ppm (Figure 4, Table 2).

Figure 4 - Comparison of the spectra representing metabolite profiling identified that lactose (4.46 ppm) and oligosaccharides levels, especially those fucosylated (fucose, 1.23 ppm; N-acetyl-neuraminic acid, 2.06 ppm; N-acetylglucosamine, 2.04 ppm) were significantly higher in preterm milk samples compared to full term milk.

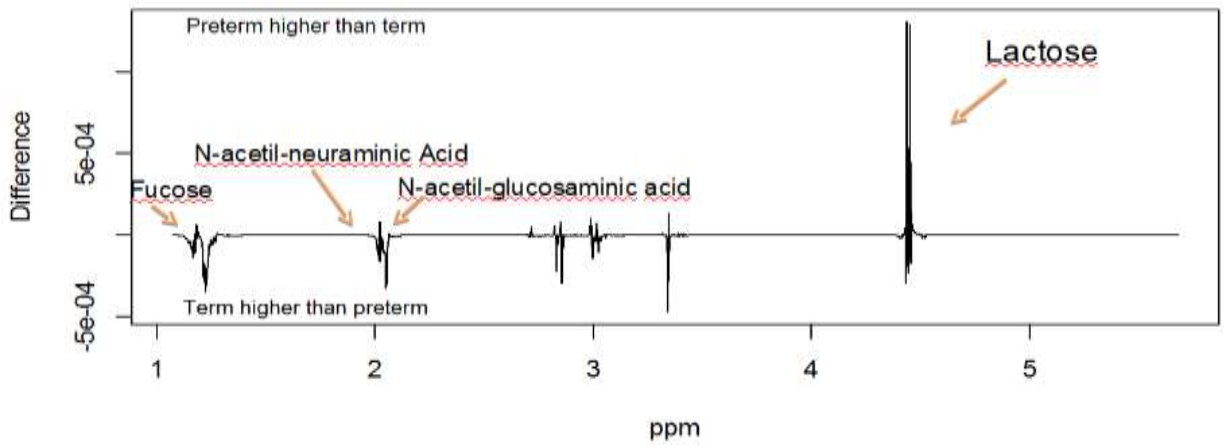


Table 2- NMR identification of metabolites in breast milk

Metabolites	**1H Chemical shift (ppm)**
Lactose	4,46 ppm
Fucose	1,23 ppm
N-acetil-neuraminic acid	2,06 ppm
N-acetil-glucosaminic acid	2,04 ppm

DISCUSSION

Human breast milk does not have a single composition but rather changes over time, from colostrum to transitional milk and finally to mature milk, during the course of lactation [2]. Understanding the composition of human milk therefore provides an important tool for planning infants' feeding, particularly for fragile, high-risk infants as preterm [3]. Metabolomics should be considered as a key technology for this purpose. Measuring in a quantitative manner the complete set of low molecular weight metabolites that are the end products of gene expression, NMR metabolome is able to provide a molecular snapshot of biological fluids as human milk [16]. Using the NMR metabolome approach, we demonstrated that milk from mothers delivering preterm

infants undergoes a sort of “maturation” in the first three weeks of lactation, but, at the end of the 3rd week post-partum, it does not resemble the milk of term infants yet, because of its higher level of lactose and oligosaccharides. The technique that we used intercepts small molecules but not aminoacid. The disaccharide lactose is the principal sugar of human milk and the most common macronutrient, while oligosaccharides constitute the third most common solid component of human milk after lactose and lipids and they have important non-nutritional properties[17, 18]. Synthesized by glycosyltransferases, human milk oligosaccharides range from 3 to 32 sugars in size and differ in composition from those of any other mammal [17], being species-specific. They have lactose at the reducing end and fucose or sialic acid at the non-reducing end [19]. In contrast with bovine milk, most of human milk oligosaccharides are fucosylated, including 1 to 15 fucoses, and their production depends on enzymes encoded by the genes associated with the expression on the Lewis group system [18]. The presence of fucosylated oligosaccharides in human milk depends on a maternal secretory status that is determined by the presence of a alpha 1,2 fucosyltransferase gene (FUT2) and it is identified by NMR spectroscopy by the level of 2-fucosyllactose [20, 21]. Higher levels of this metabolite in preterm milk than term samples were identified by Sundekilde et al. in a metabolomics study. They also found differences between preterm and full-term milk in the level of lactose, citrate and other metabolites [21].

It is now widely accepted that oligosaccharides are non-nutritive bio-active factors whose primary site of activity is the lumen of the gut. Oligosaccharides and their protein conjugates function as soluble “decoy” receptors for pathogens that inhibit the adhesion of pathogens to oligosaccharides receptors expressed on gut surface. Besides, they have been described to exert immunomodulatory effects [22]. In addition, oligosaccharides are prebiotic agents that selectively encourage the growth of commensal bacteria. The advantages for the gastrointestinal tract include dose-dependent protection against necrotizing enterocolitis and late-onset sepsis [23-26]. There is also a trophic effect on gut in terms of more rapid maturation of motility and a more rapid decrease in permeability. This results in lower gastric residual volumes and facilitates early achievement of full

feedings when prebiotics are used together with probiotics [23]. Prebiotics also promote growth of *Bifidobacteria* and decrease growth of pathogens in the gut [27].

It is also possible that an amount of oligosaccharides indirectly affects sites distant from the gut, perhaps through secondary metabolites from fermentation that traverse the bloodstream. In this way, they protect infants from late-onset sepsis and, when eventually excreted into the urine, reduce the risks of urinary tract infections [28]. For these reasons, human milk oligosaccharides content should be looked at as part of the innate immune system by which mothers protect their infants from disease by breastfeeding [18].

Finding that milk collected from mothers of preterm has higher bioactive properties than the milk collected from mothers delivering at term is crucial for a tailored, individualized nutrition.

Although this study is limited by the small number of samples, the data come from a well-defined population, with no variability with respect to location, health and lifestyle. The strenght of the paper is the use of highly sophisticated method to evaluate metabolomic profile of human preterm breast milk such as NMR spectroscopy.

The obtained data suggest that human milk is not only species-specific, but also "gestational age-specific".

In the current clinical practice, if the mother has insufficient milk for preterm infants, milk from donor mothers delivering at term is considered an alternative [29]. Unfortunately, the differences in the metabolic profile of breast milk of women who delivered preterm newborns compared to term might lead to some nutritional deficiencies in preterm newborns affecting their proper growth [30,31]. This paper sheds a light on the need to use a more personalized nutrition in preterm newborns, by adding oligosaccharides to breast milk from mothers delivering at term.

CONCLUSIONS

Our results contribute to understanding the peculiar nutritional needs of breastfed preterm newborns. The high content of oligosaccharides in preterm breast milk, assessed through

metabolomic investigation, provides new insights about dietary modification to attain precise and specific nutritional characteristics of preterm breast milk. From the point of view of precision medicine, in order to promote proper growth, it is desirable that preterm infants receive their own mother's milk, or, at least, a donor human milk from lactating mothers who delivered at the same gestational age.

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Declaration of interest

There were no authors' potential conflicts of interest for this work.

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