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The application of High-Resolution Nuclear Magnetic Resonance (HR NMR) in metabolomic analyses of meconium and stool in newborns. A preliminary pilot study of MABEL project: Metabolomics approach for the assessment of Baby-Mother Enteric Microbiota Legacy

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ABSTRACT

Background: The microbial population living in our intestines plays a key role in several metabolic, nutritional, physiological, and immunological processes. It is known that infant gut microbiota composition has both horizontal transmission delivery and environmental conditions and a vertical one, from mother to child, related to how the infant is fed (breastfed or infant formula). Detailed information on the composition of meconium and feces from newborns may help predict the most prevalent and hazardous conditions affecting pregnancies, mothers, and babies, including pre-term birth, preeclampsia, and gestational diabetes mellitus for example.
Methods: This work aims to demonstrate the feasibility of the whole High-Resolution proton Nuclear Magnetic Resonance (1H HR NMR) procedure in metabolomic analysis in preterm newborns. Thus, multiple samples of meconium and stool were collected from 3 pairs of premature twins and their metabolite profiles were acquired and exploited by combining the NMR technique with univariate and multivariate analysis.
Results: The analysis showed that an impact on the metabolite profile was visible concerning both the sex of the newborns and the couplet of origin. Most of the variation between twin couplets was seen with butyric acid concentration in meconium/stool samples.
Conclusions: Despite the low number of samples, the described NMR procedure showed to be a suitable approach to evaluate the similarities of the molecular profiles of different samples, offering a non-invasive and informative approach to understanding the metabolic and nutritional status of preterm infants. Future metabolomic analysis should be supported by microbiome analysis, such multiomic approach will provide more complex view on development of preterm newborns.

1. Introduction

"All disease begins in the gut", wrote Hippocrates about two thousand years ago. This statement is still mostly true, except for those diseases that are strictly related to genetic modifications. In March 2005 the scientific magazine "Science" focused on the 10-meter-long tube of

Abbreviation: ANOVA, Analysis of Variance; ASCA, ANOVA Simultaneous Component Analysis; BW, Body Weight; CPMG, Carr-Purcell-Meiboom-Gill; CS, Cesarian section; FDR, False Discovery Rate; FID, Free Induction Decay; GA, Gestational Age; HOD, Hydrogen Deuterium Oxide; LOS, late-onset sepsis; MA, male; FM, female, MABEL, Metabolomics Approach for the assessment of Baby-Mother Enteric Microbiota Legacy; ME, meconium; NEC, necrotising enterocolitis; NOESY, Nuclear Overhauser Enhancement Spectroscopy; PCA, principal component analysis; SCFAs, short-chain fatty acids; ST, stools; TSP, sodium (3-trimethylsilyl)-2, 2, 3, 3-tetradeuteriopropionate.

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the gastrointestinal tract, named gut, the mechanisms, and activities of which were still largely unknown. In this special issue, this tube was defined as "the inner tube of life", as it constantly maintains a complex interchange of information with the brain and visceral organs [1]. In fact, since then, many scientific works have begun to describe the importance of the gut's role in crucial biological mechanisms. Later, the relationship of this hidden "metabolic organ" with human well-being, including host metabolism, physiology, nutrition, and immune function gradually emerged. The gut tube harbors a complex microbial population, consisting of at least 100 trillion commensal bacteria divided into approximately 160 species [2]. This population, called microbiota, is responsible for keeping a healthy state as it is involved not only in the digestion and absorption of nutrients but also in the homeostatic maintenance of host immunity [3] and metabolism [4]. Hence, its disruption or alteration is proven to be linked with an enhanced risk of atopic and diarrheal diseases and with disorders such as inflammatory bowel disease and metabolic syndrome, like diabetes and obesity [5–7].

The composition of the microbiota in the gut is highly variable and it can substantially change throughout life [8]. At birth, humans allegedly present a sterile gastrointestinal tract, but over time, starting from weaning, a distinct brew of bacterial species, different from individual to individual, develops in the gut. This mix is partly determined by the individual's genetics and partly by environmental conditions. Nevertheless, several studies confirm the presence of a bacterial core that is constant in each individual and a dynamic core that is characterized by its capacity to rapidly respond to environmental changes [9]. The complexity of the gut population, together with its key role in the appearance or alleviation of human disease, leads to an explosion of interest in studying the bacterial component of the microbiota. This deep interest is shown for example by the emergence of several projects such as The Human Microbiome Project [10] or MetaHit [11]. The goals of these projects are manifold, such as discovering more about the relationship between health and microbiome, characterizing the individual's gut bacterial profile, and developing bioinformatics tools needed to support these goals. Comparatively to the relationship between microbiota and human health (Fig. 1), which has been extensively researched, little is known about vertical microbiota transmission (from mother to newborn) during and after pregnancy and undoubtedly about maternal and perinatal health. In this last case, the capacity for predicting the most prevalent and hazardous conditions affecting pregnancies, mothers, and babies, including pre-term birth, preeclampsia, and gestational diabetes mellitus for example, is still very limited [12]. For this reason, potential technologies and new research methods able to predict these maternal and perinatal conditions are challenging. Metabolomic biomarkers are one of these new promising tools and deserve special attention due to their potentially important role in the prediction or early diagnosis of maternal complications [12].

Considering the renewed interest in these last years on the link between mother-newborn microbiota and given that information on the vertical transmission is still lacking, the overall objective of the MABEL (Metabolomics Approach for the assessment of Baby-Mother Enteric Microbiota Legacy) project will evaluate the possibility of a microbial legacy transmitted from mother to preterm and term newborns during pregnancy and its modification during the early breast or bottle milk feeding period. Of note, metabolome maturation is not as well explored

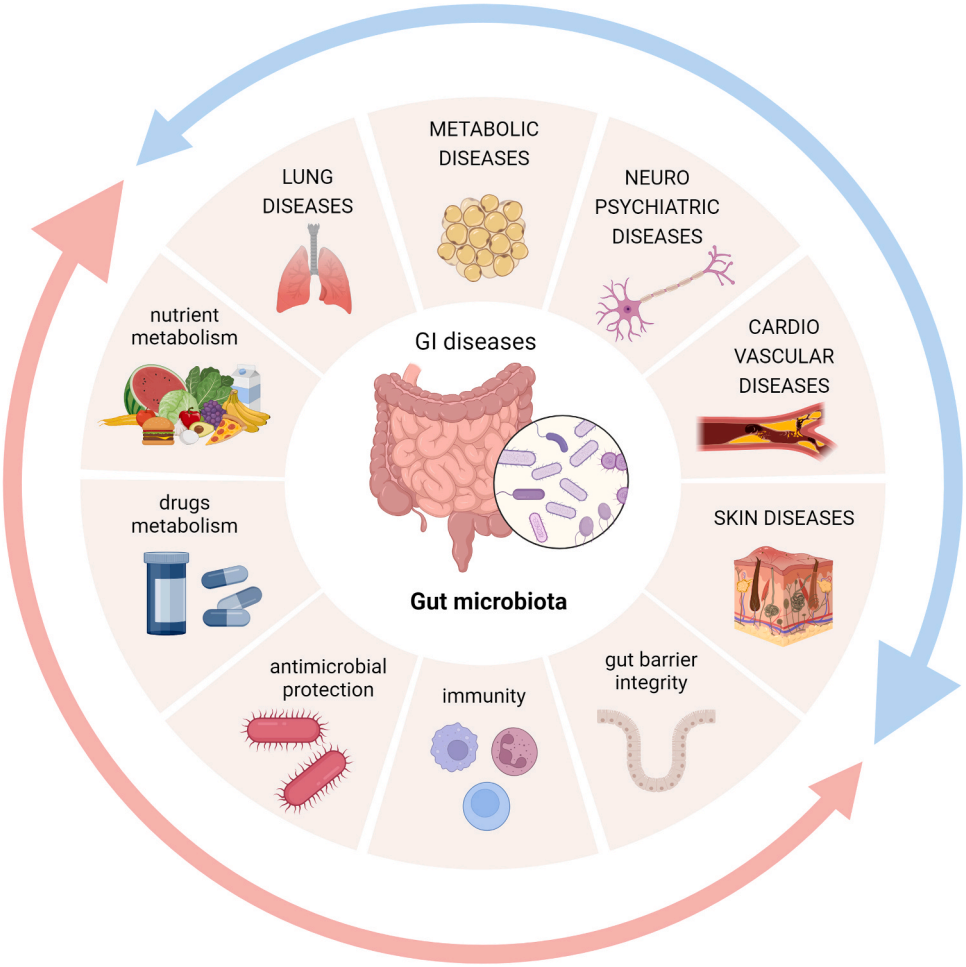

Fig. 1. The mechanisms (Red arrow) through which gut microbiota affect intestinal and extraintestinal health (blue arrow).

as the microbiota [13], especially in premature infants. Moreover, metabolome maturation can play a role in necrotising enterocolitis (NEC) and late-onset sepsis (LOS), which are the most common causes of death in the neonatal period [14]. Through the integration of High Resolution-Nuclear Magnetic Resonance (HR NMR) metabolomic data from stool and meconium samples, MABEL would like to investigate the link between mother and newborn gut microbes. A vertical microbiota transmission can be supposed if both mother and infant share specific microbiome, microbiota, and metabolomic biomarkers. From this point of view, MABEL will enhance the very recent tendency to try to investigate and understand both the vertical microbiota transmission and at a second stage the microbiota modulation upon environmental changes such as mode of delivery and nutritional conditions [15]. Moreover, the quantification of NMR-visible molecules in meconium and preterms should provide considerable information and should help investigators understand the role of stool chemical components in early children's development in normal and pathologic conditions [16]. Thus, the analysis of meconium and stool using NMR enables the early detection of metabolic disorders in preterm infants. By identifying abnormalities in the biochemical composition, healthcare professionals can implement timely interventions to manage and treat potential health complications.

In the present work, a pilot study has been designed to test the whole NMR procedure feasibility in preterm newborns, from the preparation of samples to the spectroscopic analysis. HR NMR spectroscopy was used to detect components of the water-soluble and organic fractions of meconium and stool samples from 6 preterms (3 pairs of twins: Male (MA)/Female (FM), FM/FM, and MA/MA). The study aimed also to assess whether the sensitivity of NMR is enough to measure the substantial differences in the metabolic profile of the samples.

2. Materials and methods

For this study, aqueous extracts of 36 samples from meconium and feces collected at different times, were subjected to HR NMR spectroscopy.

2.1. Meconium and stool collection

Stool samples were collected from 3 pairs of twins born prematurely (Gestational Age (GA): 29.14 ± 1.6 weeks; Body Weight (BW) 1120 ± 257 g) (Table 1). Two pairs of twins were dizygotic (FM/MA and FM/FM), while one pair was monozygotic (MA/MA). All babies were born by cesarean section (CS), and the mothers received a prophylactic antibiotic before surgery. The condition of the children after delivery was assessed as average. From the first day of life, they were fed with breast milk and milk from a human milk bank. After three weeks of life, some of them were fed artificially. Due to early (four children) or late (three children) infection, they received antibiotics.

The analysis included the child's first stool (meconium) and stool samples collected at weekly intervals until the day of discharge from the hospital. Stool samples were collected from diapers using a standardized biological material collection kit (Stool Sample Application System (SAS); Immundiagnostik, Bensheim, Germany). The samples were collected by previously trained hospital staff or by the parents according to an established procedure and stored in a refrigerator (for a maximum of eight hours) before transport. Transport to the laboratory took no longer than 60 min, at 6–8 °C. The stool samples were then frozen at − 20 °C until metagenomic (data not shown) and metabolomic analyses were conducted.

Table 1
Sample summary reporting information on twin couplets: gestational age, sex and body weight at birth.

Couplet	Sex	GA (Weeks + Days)	BW (g)
1	FM	30 + 3	1390
1	FM	30 + 3	1550
2	MA	30 + 3	820
2	FM	30 + 3	1190
3	MA	26 + 4	710
3	MA	26 + 4	1060

2.2. NMR samples preparation

Frozen fecal and meconium samples were thawed at room temperature and shaken before use. They were prepared for HR NMR analysis by adding 1 mL of deionized water (H_2O^{00}) to 80 mg from each sample. The mixture was homogenized by mixing with a vortex for 5 min, followed by centrifugation for 10 min at 14,000 rpm and 4 °C. Subsequently, 540 μL of supernatant was added to 60 μL of 1.5 M deuterated water (D_2O – Eurisotope, Cambridge Isotope Laboratories, Inc, UK) phosphate buffer solution containing 0.1 % trimethylsilylpropanoic acid (TSP – Eurisotope, Cambridge Isotope Laboratories, Inc, UK) and sodium azide (NaN_3 – Sigma Aldrich®, Germany) 2 mM. The homemade buffer was set at pH 7.40 and it was added to the samples to minimize peak shift due to residual pH changes. Before analysis, samples were centrifuged for 10 min again, and then 590 μL was transferred into an HR NMR 5 mm tube [17].

2.3. NMR spectra acquisition

Proton HR NMR (1H HR NMR) spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz. Magnetic field homogeneity was optimized by gradient shimming before acquisition. The temperature was maintained at 298 K and the lock was performed on the D_2O signal. The Hydrogen Deuterium Oxide (HOD) residual signal was suppressed by presaturation, whereas broad signals from slowly tumbling molecules were removed by including a standard 1D experiment using Carr-Purcell-Meiboom-Gill (CPMG) filter to a free induction decay (FID) sequence. The filter was made up of a train of 400 echoes separated by 800 μs, for a total time of 328 ms. Each spectrum was acquired with the following acquisition parameters: number of dummy scans, DS = 16; recycle delay, D1 = 5.00 s; number of loops, TD0 = 64; 90° pulse width = 11.83 μs; number of scans, NS = 4; number of points, TD = 32,768; spectral width, SW = 12.00 ppm; acquisition time, AQ = 2.28 s. The recycle delay was set to 5 s, keeping into consideration the longitudinal relaxation time of the protons under investigation. Before the CPMG experiment, for each sample a one-dimensional Nuclear Overhauser Enhancement Spectroscopy (NOESY) spectrum was recorded with the following acquisition parameters: number of dummy scans, DS = 4; recycle delay, D1 = 5 s; 90° pulse width = 11.830 μs; number of scans, NS = 4; number of points, TD = 32,768; spectral width, SW = 12.00 ppm; acquisition time, AQ = 2.27 s. Water suppression was achieved by irradiation of the water peak during RD and t m [17,18]. The resulting spectra were first optimized and then analyzed with both univariate and multivariate statistical analysis.

2.4. NMR spectra processing

Fourier transformation, phasing, and baseline correction of the 1H HR NMR spectra were performed with Bruker TopSpin (version 3.5 pl 6, Bruker Biospin, Milan, Italy) by using an automatic command apk0.noe that also applies a line broadening of 1 Hz. All spectra were referenced to the TSP resonance at δ 0.00 ppm then the FID files were imported into Matlab (Matlab v R20215b, The Mathworks, Natick, USA). A combined Matlab file, containing all spectra, was then uploaded on SigMa [19].

2.5. NMR data statistical analysis

The regions, with δ below 0.20 ppm or above 10.00 ppm in both meconium and stool water spectra were removed, as they contain only noise. After this, spectra were normalised to unit area and an outlier

spectrum, with broader signals, was removed from the analysis (data not shown). The resulting spectra were processed using SigMa to generate a metabolite table of 35 samples and 63 features, including 23 intervals from 15 known metabolites, 30 unknown, well-resolved signals, and 10 bins. The metabolite table was then analyzed using Matlab (Matlab version R20215b, The Mathworks, Natick, USA) and PLSToolbox (version 9.2, Eigenvector Research, Inc., Manson, WA). First, Principal Components Analysis (PCA) [20] was performed, to identify possible groupings according to the study factors, such as sex, time of sampling, and couplets of twins. ANOVA Simultaneous Component Analysis (ASCA) [21], calculated with 1000 iterations, was also employed to assess the impact of these factors on the metabolite profiles. Finally, one-way ANOVA with False Discovery Rate (FDR) correction was included in the analysis to identify the most significant metabolites involved in sample grouping. Spectral peaks were assigned by comparing their chemical shift and multiplicity with the SigMa database, and literature, and by using Chenomx NMR suite 8.6 software (Edmonton, Canada, http://www.chenomx.com/) [16,21–25].

3. Results

The preliminary results of this study demonstrated the capability of 1 H HR NMR to yield important information on the chemical composition of meconium and stool with a minimum sample preparation [16,26]. Fig. 2 shows the 1 H HR NMR spectrum of the water-soluble fraction of meconium (T0) and stool emitted after 9 weeks of life (T9). Signals falling in the region from 0.50 ppm to 4.40 ppm and from 5.40 ppm to 9.00 ppm were expanded on the vertical scale to visually appreciate signals belonging to minority species. Several well-resolved peaks corresponding to specific metabolites are visible. In Table 2 the assigned metabolites are listed and it has been indicated also if they are present in meconium (ME), stools (ST), or both. The strongest signals are related to the resonance of lactate (1.333 ppm) and acetate (1.923 ppm) and they are present in both meconium and stool. In the latter case, the concentration of both metabolites is much higher. The overlapping of stools' spectra shows differences in the molecular profile. Thus, to further investigate the variabilities among sample classes, unsupervised principal component data analysis was performed. PCA showed that specific sample grouping was observed along the first two principal components (PC1 and PC2), accounting for 32 % and 25 % of the overall variance, according to the sex of the newborns (Fig. 3A) and time of sampling (not shown). Moreover, some clustering trends concerning the couplet of the twin were observable across PC3 and PC5 (8 % and 5 % of the variance, respectively) (Fig. 3B). Low percentages of variation in PCA are typical of metabolomic studies [27], where many different factors can perturbate the metabolome [28,29], so it is expected to observe trends at lower PCs. Loadings from these two scoreplots are shown in Fig. 3C and 3D, indicating the main spectral features driving sex-based or couplet-based clustering. For example, lactic acid is particularly low in samples from the second couplet of twins. As many different factors (such as biological variations) may affect the metabolome hampering the PCA results [29,30], ASCA was then performed to understand the specific impact of the different investigated factors [31]. Results showed that the time-point factor had a larger variation explained, paired with a significant impact on the data matrix (Table 3). Moreover, as also experienced through PCA, sex had a significant impact on the dataset. This proves that already sex-specific metabolite fingerprints are present for each individual from birth, moreover, it shows how the metabolite profiles change significantly even in the first few days of life. The differences in microbiota diversity, called α-diversity, between the genders highlight that male infants began with a low diversity index, whereas female infants have a higher diversity index shortly after birth compared to males during the first 10 days of life. Then, the diversity of the gut microbial community developed over time and appeared similar in males and females during the third 10 days of life [32].

ANOVA was calculated to identify significant features of the study factors. Significant p-values (not FDR, corrected) were found for sex separation and couplet comparison. The most interesting result is that butyrate was capable of distinguishing the first couplet of twins from the two other couplets, being at lower concentrations in the latter two. Moreover, the region containing signals from glycerol, together with two binned regions in the aliphatic part of the spectra were found at higher levels in female samples (Table 4).

4. Discussion

There are a lot of research works confirming the capability of NMR coupled with Metabolomics as a powerful approach for tissue and body fluid analysis due to its relatively high sensitivity and its ability to detect a large number of analytes in the same sample [2,33–36]. In this research work, the main approach used to investigate the metabolome perturbations induced by time points (weeks), gender, couplet, and individual factors is based on the principles of unsupervised latent features discovery, applied to NMR spectra, to get a holistic perspective of the metabolomic phenotype [37]. The NMR data analysis points out how the concentration of lactate and acetate metabolites increases over time as the weeks go by. Their modulation is driven by the maturation of the microbiota [38]; Ximenez and Torres [39] describe how the maturation of microbiota in newborns is significantly affected by diet: breast or formula milk [40]. Human milk favors the growth of *Bifidobacterium* and *Lactobacillus* due to the presence of nutrients and substances with prebiotic functions like oligosaccharides [39]. *Bifidobacterium* has beneficial effects on the development of gut microbiota during early and subsequent infant physiological state and health, thus it results in one of the dominant bacterial genera. Together with *Lactobacillus,* these bacterial genera metabolize maternal milk carbohydrates and as a result, infants have higher concentrations of lactate and acetate [41]. Acetate and lactate play a key role in improving intestinal defense against *Escherichia coli* 0157:H7 (acetate) and help maintain gut barrier function through stimulation of enterocyte proliferation (lactate) [39].

The meconium and feces showed also the presence of several metabolites that cover other molecular families such as amino acids and organic acids [25,42]. In the first case, amino acids are both essential as leucine, isoleucine, phenylalanine, threonine, tryptophan, and valine, and non-essential as asparagine, alanine, glycine, and tyrosine. Concerning the organic acids, a high representation is given by aspartate, formate, glutamate, succinate, and short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate. SCFAs are extremely important as they cover a key role in neuro-immunoendocrine regulation [43]. For this reason, it is important to maintain a certain concentration which depends on both diet (major or minus fiber consumption) and above all microbiota composition [43]. Del Chierico, Vernocchi, et al. [25] point out that low levels of acetate and butyrate are associated with high levels of some Proteobacteria families and low levels of Bifidobacteria and Clostridiaceae. In contrast, high levels of acetogenic metabolism were correlated with increased levels of some *Clostridium* families, which metabolize carbohydrates to acetate and SCFAs. It is well known that the α-diversity [44,45] microbiota community in infants changes during the first days of life and it is different from male to female. This means that the level of certain metabolites may change over time, such as the level of SCFAs [32]. Furthermore, the concentration of metabolites in newborn feces may be also affected by the kind of feeding. In fact, the abundance of human milk oligosaccharide-degrading *Bifidobacterium* species, including *Bifidobacterium longum*, *B. breve*, and *B. bifidum* is positively correlated with the abundance of lactic acid [46].

Among the detected metabolites, butyrate has an important biological role. Butyrate is a 4-carbon SCFA essentially produced in the colon by gut microbes and represents the 15 % ratio of the total SCAFs [47]. Together with acetate (C2), propionate (C3), and valerate (C5), represent a vital group of metabolites that a) influence immune modulation, b) control anabolic processes, c) serve as an energy source for colonocytes, and are precursor metabolites for lipogenesis and gluconeogenesis

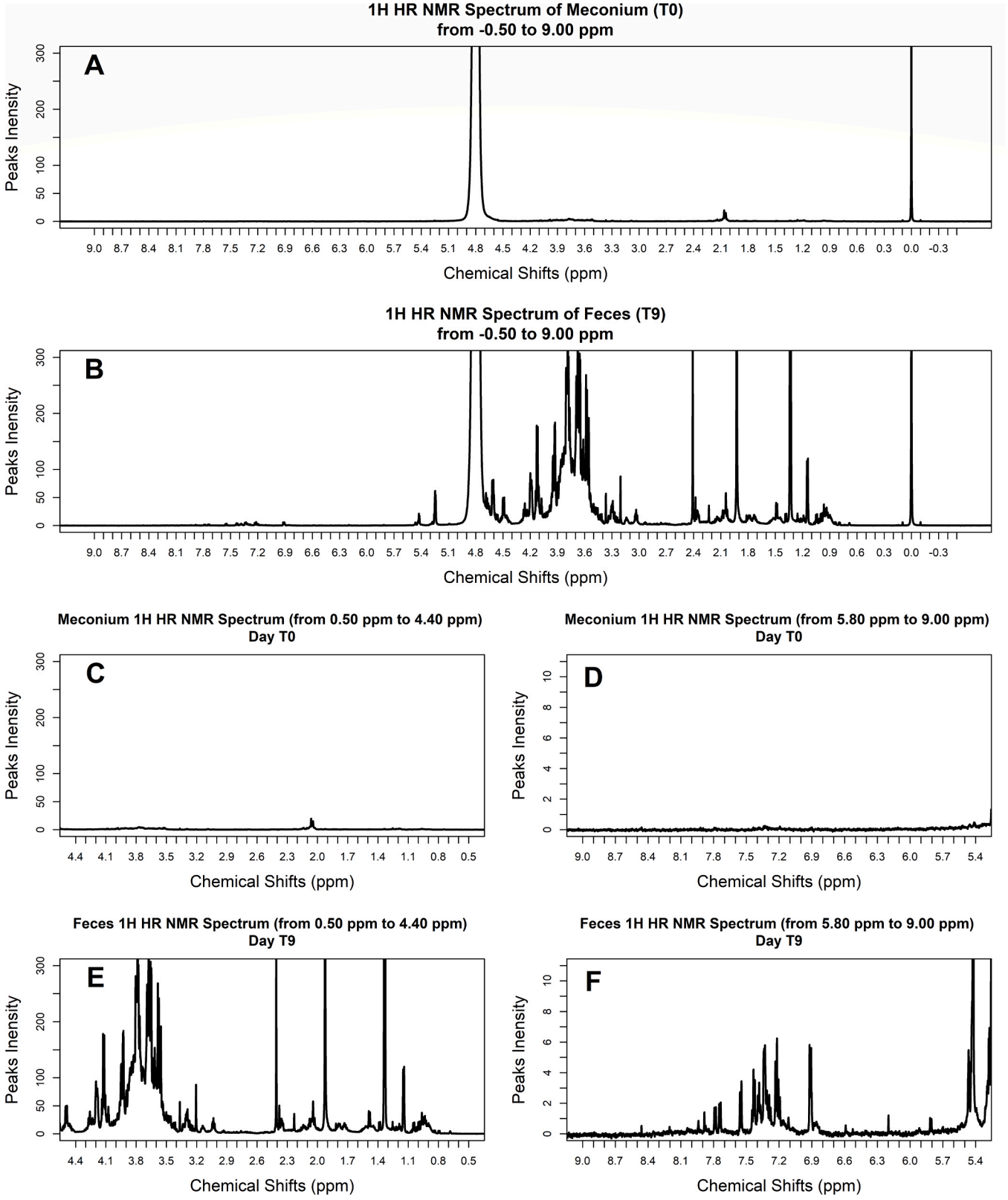

Fig. 2. Regions of the 600 MHz 1 H HR NMR spectrum of both meconium and fecal extracts used in this study. The region from 5.80 ppm to 9.00 ppm was expanded on the vertical scale to better appreciate the presence of small signals of meconium and fecal extracts. A) 1 H HR NMR whole spectrum of meconium extract (from −0.5 ppm to 9.00 ppm). B) 1 H HR NMR whole spectrum of fecal extract at T9, (from −0.5 ppm to 9.00 ppm). C) and D) regions from 0.50 ppm to 4.40 ppm and 5.40 ppm to 9.00 ppm respectively of meconium extract. E) and F) regions from 0.50 ppm to 4.40 ppm and 5.40 ppm to 9.00 ppm respectively of fecal extract.

Table 2
List of 1 H NMR-derived metabolites that differ significantly between meconium (ME) and stools (ST). * Key: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, tt = triplet of triplets.

Metabolites in Meconium (ME) and Stools (ST) NMR Spectrum				
	Metabolites	Stools (ST)/ Meconium (ME)	Multiplicity*	δ (ppm) Chemical - Shifts
1	Acetate	ST and ME	s	1.923
2	Acetone	ST	s	2.205
3	Asparagine	ST	dd/dd	2.839/2.956
4	Aspartate	ST	q/dd	2.686/2.816
5	Alanine	ST and ME	d	1.483
6	Butyrate	ST	t/m/t	0.898/1.561/ 2.161
7	Choline	ST and ME	s	3.205
8	Creatinine	ST	s	3.032
9	Dimethylamine	ST and ME	s	2.715
10	Formate	ST	s	8.459
11	Glycine	ST	s	3.553
12	Glycerol	ST	q/dd/m	3.568/3.611/ 3.783
13	Glutamate	ST	dd	2.371
14	Isoleucine	ST	d	0.993
15	Lactate	ST and ME	d	1.333
16	Leucine	ST	t	0.969
17	Malonate	ST	s	3.134
18	Methanol	ST and ME	s	3.365
19	N, N-dimethylglycine	ST	s	2.919
20	Phenylalanine	ST	d/m/m	7.335/7.380/ 7.433
21	Propionate	ST	t/q	1.059/2.186
22	Succinate	ST	s	2.392
23	Threonine	ME	d/m	1.33/4.265
24	Trimethylamine N-oxide	ST	s	3.253
25	Tryptophan	ST	t/t/s/d	7.205/7.289/ 7.328/7.551
26	Tyrosine	ST	d/d	6.912/7.225
27	Uracile	ST	d/d	5.809/7.550
28	Valine	ST	d/d	0.891/1.044
29	3- 3-Hydroxyisovalerate	ST	s	2.358

[48]. From an intestinal level, butyrate has multiple effects on 1) immune regulation, b) intestinal mobility, c) cell growth and differentiation, d) intestinal barrier function, and e) inflammatory and oxidative status [47]. In addition, a growing number of studies have stressed the role of butyrate in the prevention and inhibition of colorectal cancer [49,50]. In infants, the concentration of this SCFA increased significantly between 3 and 12 months of age, as explained by Nilsen, Madelen Saunders, et al. [48] who detected a four-fold increase in the relative abundance of butyrate between 6 and 12 months of age. The authors observed furthermore that the *Eubacterium rectale* and *Ruminococcus gnavus* networks are positively and negatively correlated to butyrate, respectively.

For what regards glycerol, less is known about its presence in early feces, whilst in adult human feces, significant amounts of glycerol reach the colon microbiota daily through the diet and/or by in situ microbial production. Glycerol is the backbone molecule of all triglycerides. Their digestion and absorption are never complete [51], so a mixture of glycerol, free fatty acids, and undigested glycerides enters the colon. At the same time, glycerides may be hydrolysed into free fatty acids and glycerol by bacterial lipases, enzymes common to a wide range of gut bacteria [51]. Colonic glycerol levels can also be increased by in situ microbial synthesis, release from desquamated epithelial cells, and intestinal clearing of endogenous plasma glycero or release from desquamated epithelial cells [51,52]. Gender differences in fecal glycerol may be caused by different microbiota composition in female and male newborns. Glycerol metabolism is dependent on lactobacilli and enterococci [51], genera belonging to the family Lactobacillaceae and Enterococcaceae whose abundance in newborns may vary according to sex [53]. Moreover, women show a significantly greater increase in systemic glycerol concentrations than men in response to moderate increases in circulating adrenaline and/or norepinephrine. This suggests that women have a greater increase in peripheral lipolysis in response to catecholamines compared to men and that women are more sensitive to the lipolytic effects of catecholamines allowing them to achieve similar or higher levels of lipid mobilisation and utilisation compared to men [54]. Again, the gender differences in microbiota taxonomic profile, diversity, and function were evidenced by Chen, Li, et al. [53]. Interestingly diversity was favoured in preterm females mimicking this gender better outcome under perinatal complications. In addition, their microbiota during the very first month of life was dominated by Enterococcaceae as compared to males in whom the abundance of Streptococcaceae, Lactobacillaceae, Clostridiaceae, Eubacteriacae was evident during this period [55]. The function of the microbiota in the latter was predicted to be predominantly immunological [56]. Though gender differences in the microbiome at the early stage of life need further clarification, it was postulated that sex hormones might mediate the work of action of the immune system thus the difference between boys and girls [57]. It is especially sound as the transient increase in testosterone in newborn boys was evidenced, and found to last weeks after the delivery [58]. Interestingly, this period coincides with the start of the colonization by microbiota.

Due to the pilot nature of the study and the small sample size, it was not possible to assess the impact of nutrition, antibiotic use, or other clinical factors on the metabolome. Such relationships should be taken into account in further studies. Another limitation of the study is the lack of analysis of the microbiota, which may be particularly important for assessing the impact of gender on the metabolome.

5. Conclusion

The results of this study showed the feasibility of the whole NMR procedure in metabolomic analysis in preterm newborns. Moreover, this research work improved and extended previous knowledge regarding the composition of human meconium and established data on the concentration of several metabolites therein such as amino acids and organic acids. This method was also able to demonstrate that the same sibling shares the same metabolome, but it is different from couplet to couplet. There are several metabolites involved in this clustering such as butyrate, which plays a key role in couplet separation, and glycerol, of which the faecal concentration may depend on lactobacilli and enterococci abundance, which can be different in female vs male newborn. Moreover, seems also that sex deeply affects the metabolomics profile of samples, so males (M) and females (F) are separately grouped in the PC space (Fig. 3A). In conclusion, even if these are preliminary results, NMR-based metabolomics reveals itself as a suitable approach to evaluate the similarities of the molecular profile of different samples, this is the reason why this approach can be of help to evaluate correspondences in the metabolomic profile of mother and son. Moreover, the combination of NMR and meconium analysis offers a non-invasive and informative approach to understanding the metabolic and nutritional status of preterm infants. This knowledge can be crucial for tailoring medical interventions, optimizing nutritional support, and ensuring the best possible outcomes for the health and development of these vulnerable newborns. Furthermore, the results highlight the need of pairing metabolomic analysis with microbiome investigation, such multiomic approach will provide more complex view on development of preterm newborns.

Ethics approval and consent to participate

Consent to perform the study in the Department of Neonatal Diseases was obtained from the Bioethics Committee of the Pomeranian Medical

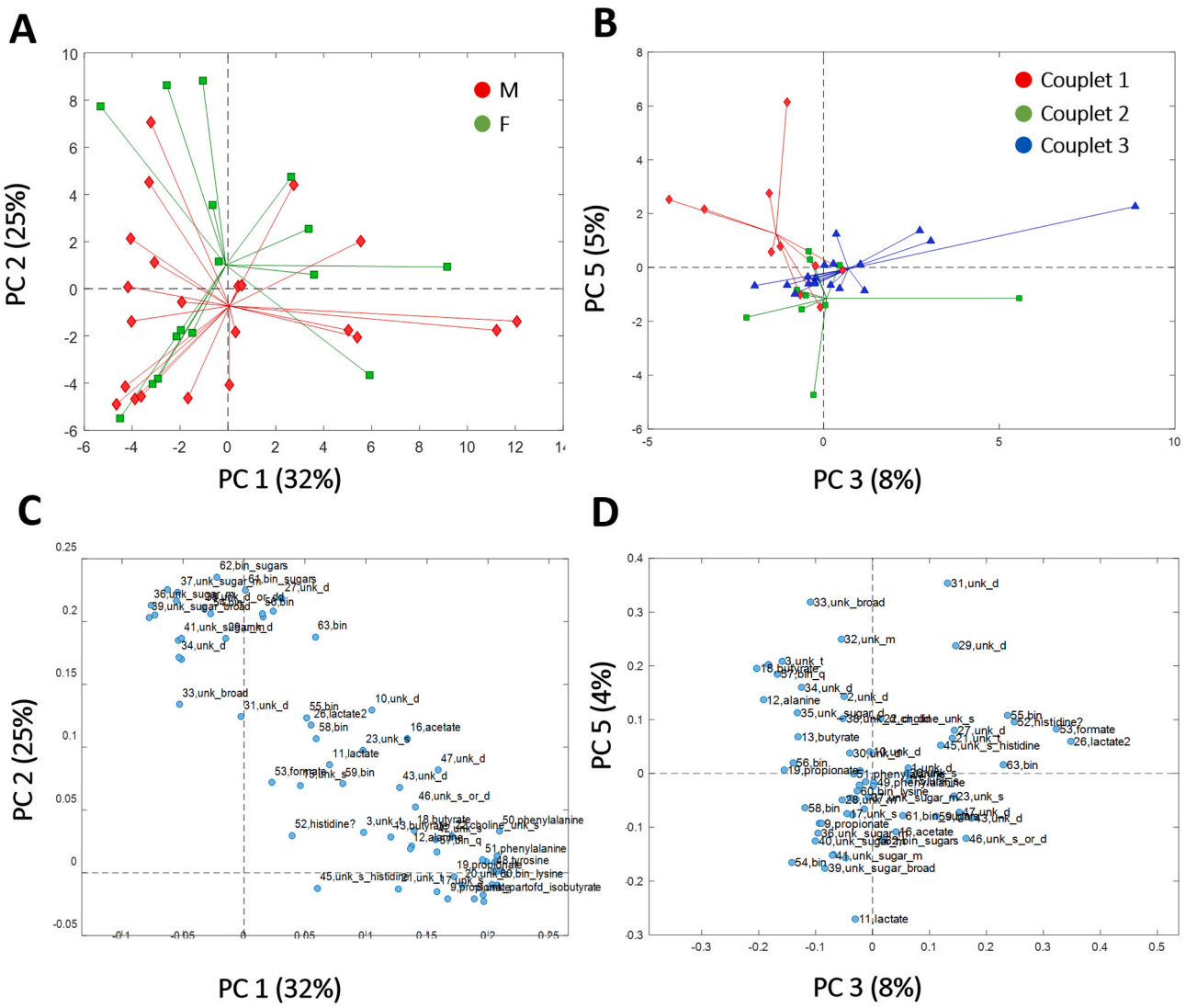

Fig. 3. Principal Components Analysis (PCA) of metabolic profiles from 36 samples of both meconium and stool from 6 CS-delivered newborns. Samples were analysed over the 9 weeks following birth. A) PC1 and PC2 score plot. Samples are clusterized according to the sex, B) PC3 and PC5 score plot. This combination reveals a clusterization based on the different couples C) and D) are the loadings plot of PC1vs PC2 and PC3 vs PC5 respectively.

Table 3
ASCA-based partitioning of the variance derived from the four main effects (sex, couple, week, and individual) using the global dataset (36 urine NMR variables) and calculated with 1000 iterations.

ASCA		
Factor	% Variation	P-value
Sex	4.24	0.014
Couple	5.16	1
Time (Week)	47.25	0.001
Individual	5.16	1

University with Resolution No. KB-0012/55/14 of 30 June 2014. The study followed the Declaration of Helsinki (2013). Written informed consent was obtained from participating patients (parents).

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Table 4
One-way ANOVA with False Discovery Rate (FDR) correction was included in the analysis to identify the most significant metabolites involved in sample grouping.

Males vs Females					
feat n.	feat name	p-value	FC M/F	ppm max	ppm min
24	Glycerol	0.04	0.58	3.59	3.54
58	bin	0.00	0.37	2.10	2.01
59	bin	0.04	0.28	2.43	2.39
Couplet 1 vs 2					
feat n.	feat name	p-value	FC couplet1/2	ppm max	ppm min
4	butyrate	0.01	9.11	0.92	0.88
18	butyrate	0.03	7.17	2.18	2.14
Couplet 1 vs 3					
feat n.	feat name	p-value	FC couplet1/3	ppm max	ppm min
4	butyrate	0.02	3.09	0.92	0.88
18	butyrate	0.04	3.08	1.62	1.50
33	unk_broad	0.02	1.84	5.05	5.01
34	unk_d	0.01	2.04	5.14	5.12
58	bin	0.01	3.12	2.10	2.01
Couplet 2 vs 3					
feat n.	feat name	p-value	FC couplet2/3	ppm max	ppm min
34	unk_d	0.01	2.48	5.14	5.12
58	bin	0.02	2.51	2.10	2.01

CRediT authorship contribution statement

Alessia Trimigno: Writing – review & editing, Visualization, Validation, Methodology, Formal analysis, Data curation. **Beata Łoniewska:** Writing – review & editing, Visualization, Resources. **Igor Łoniewski:** Writing – review & editing, Visualization, Resources. **Gianfranco Picone:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Conceptualization. **Karolina Skonieczna-Żydecka:** Writing – review & editing, Visualization, Resources. **Mariusz Kaczmarczyk:** Writing – review & editing, Visualization, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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