Original article

Exploring the effect of vitamin D and DHA supplementation on the urine metabolome of preterm infants by ^{1}H NMR-based metabolomics

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Abstract

Background and objectives: Vitamin D and docosahexaenoic acid (DHA) insufficiency and deficiency could potentially have a great impact on health outcomes in preterm infant. Due to the importance of early nutrition intervention in this population and given the lack of metabolomic studies concerning the supplementations effect on the metabolome of preterm infants, 44 premature infants were studied, divided in two groups, one receiving only vitamin D (DS) and the second both vitamin D and DHA (D-DHAS) supplementation. Two were the main objectives of the study: 1) to look at changes over time in the urinary metabolic profiles of infants before and over two months of supplementation; 2) to compare the urinary metabolome of the two groups after supplementation.

Methods: ^{1}H NMR-based metabolomics approach was used to analyze urine samples obtained from preterm newborns at three different time points: at the time of hospital discharge and before supplementation (T0), 1 month (T1) and 2 months (T2) after the beginning of supplementation.

Results: A clear temporal dynamics of the urinary metabolic profiles of preterm infants was highlighted by OPLS analysis. Both groups were characterized by growing levels of betaine, *N,N*-dimethylglycine, creatinine, creatine and guanidinoacetate and diminishing levels of myo-inositol and hydroxyproline with increasing postmenstrual age (PMA). Additionally,

for D-DHAS citrate and dimethylamine increased, while lactate decreased over time. OPLS-DA clearly discriminated the two groups after two months of supplementation. Compared to DS, D-DHAS group was characterized by higher levels of betaine, *N,N*-dimethylglycine, creatinine and dimethylamine and lower amounts of lactate and myo-inositol.

Conclusions: Metabolomic analysis of urine from the neonatal period could be a useful tool to understand metabolic processes linked to early nutrition and supplementation. According to our results, vitamin D supplementation exerts in preterm newborns positive effects evaluated with urinary metabolomics. Moreover, it seems that the supplementation with vitamin D and DHA exerts a higher antioxidant and protective action on newborns, and it could also positively affect the body fat composition.

Keywords

Metabolomics, vitamin D, DHA, supplementation, urine, preterm infants.

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How to cite

Introduction

Nutrition in early life could have a strong effect on immediate and lifetime health [1]. Nutritional alterations during this period might influence the metabolic changes occurring *in utero*, during birth and the postnatal weaning period and have an impact on cardiovascular disease risk, bone health and cognitive function in early adulthood [2]. This phenomenon is known as "Early metabolic programming of long-term health and disease" or "Developmental origins of health and disease" [3]. Programming may occur as a result of modification of internal signal due to environmental factors including drugs or diet [4, 5]. Early nutritional intervention is particularly critical for preterm infants to promote growth similar to the intrauterine growth rate and to support functional development [6]. Enhancing the nutritional support of this population of infants is of great importance even after hospital discharge to improve their quality of life [7].

The recent insights into the several functions of vitamin D have aroused interest in the role it can play in decreasing the risk of many chronic illnesses, including cancers, autoimmune diseases, infectious diseases, and cardiovascular disease [8] and there is therefore growing attention in its potential use in health intervention and disease prevention [9]. Developmental processes regulated by vitamin D include lung development, maturation of the immune system and bone formation [10]. As vitamin D is mostly transferred to the fetus during the third trimester, its deficiency is very common in preterm infants [11]. Moreover, since breast milk may not meet the need for vitamin D and the required volume of preterm formulas to achieve sufficient intake of vitamin D is too high, a vitamin D supplementation is recommended [12].

Docosahexaenoic acid (DHA) is an essential fatty acid (FA) particularly important for neurodevelopment and new evidences suggest that the benefits of DHA supplementation extend beyond the brain [13]. As well as for vitamin D, premature infants are at risk of DHA deficiency and its circulating levels directly correlate with health outcomes [14]. Previous studies have demonstrated that premature infants with higher circulating DHA levels presented a lower risk of disease. Caplan et al. have shown that long-chain polyunsaturated fatty acids (PUFA) supplementation, including DHA, reduced the incidence of necrotizing enterocolitis (NEC) and intestinal inflammation in a neonatal rat model [15]. DHA supplementation decreases the severity of retinopathy of prematurity (ROP) in very low birth weight (VLBW) infants [16] and reduces the incidence of bronchopulmonary dysplasia (BPD) [17] as well as improves vision and neurodevelopmental outcomes [18].

Most of supplementation strategies have focused on increasing vitamin D and DHA content in mother's milk or infant formula.

Over the past two decades, nuclear magnetic resonance (NMR) has emerged as one of the principal analytical techniques used in metabolomics [19]. Metabolomics can be defined as an approach based on the systematic study of the complete set

of metabolites (the metabolome) present in a given biological system (fluids, cells, or organisms), which are the end products of gene expression [20]. By detecting and quantifying hundreds of small-molecular substances (< 1,500 Da) in tissues or body fluids, the metabolomics approach explores the genetic-environment-health interaction and provides a 'metabolome snapshot', useful to investigate the metabolic responses of living systems to the disease and external stimuli [21]. Metabolomics appears to be a promising tool in neonatology, especially in premature infants. It could be used for the monitoring of postnatal metabolic maturation, the identification of biomarkers as early predictors of outcome, the diagnosis and monitoring of various diseases and the "tailored" management of paediatric disorders [22].

Recently, Braithwaite et al. have hypothesized that the interaction that occurs between vitamin D and the metabolome is more critical in determining the extent of treatment success in the wider patient population than merely the dose or the form consumed [9].

Activated vitamin D has often been described as a hormone due to its broad effects on various organ systems. In this study, a metabolomics approach based on ^{1}H NMR spectroscopy was applied to explore the dynamic changes of the metabolome of two groups of preterm infants receiving different types of vitamin D and DHA supplementation over a period of two months after hospital discharge.

Methods

Urine samples and study population

Urine samples were obtained from 44 preterm infants born between the 27th and the 34th weeks of gestation with a mean birth weight of 1,538 ± 475 g, admitted to the Neonatal Intensive Care Unit (NICU), University of Cagliari, Italy. Ethical committee approved the study protocol and written informed consent was obtained from the parents before enrolment in the study. Exclusion criteria were congenital malformations.

The patient population was divided into two groups, each one composed by 22 infants, according to the type of supplementation used. The first group received only a vitamin D supplement (DS) (3MilD®, Milte, 400 I.U./day), while the second group received a vitamin D-DHA enriched supplement (D-DHAS) (Ditrevit Forte®, Humana, 400 I.U./day of vitamin D and 20 mg/day of DHA).

Infants' urine was sampled from 0 to 5 months of life at three different time points: at the time of hospital discharge and before supplementation (T0), 1 month (T1) and 2 months (T2) after the beginning of supplementation.

Urine collection was performed using a non-invasive method with a cotton wool ball held into the disposable diaper. Each urine sample (2-3 ml) was aspirated with a syringe, transferred to a sterile 2 ml vials, and then stored at -80°C until the NMR analysis. Once collected, urine samples were immediately transferred to the NMR laboratory and frozen at -80°C.

Metabolomic analysis

Sample preparation

Urine samples were thawed at room temperature before ^{1}H NMR analysis. To avoid any possible bacterial growth during the preparation, an aliquot of 8 μL of a 1% aqueous solution of NaN_3 was added to 800 μl of urine. The samples were then centrifuged at 12,000 g for 10 min at 4°C to remove any solid particles and 630 μL of the supernatant solution was mixed with 70 μL of 1.5 M phosphate buffer solution (pH 7.4) containing TSP (final concentration 1 mM). The mixture was vortexed, and 650 μL was transferred into a 5 mm wide NMR tube.

NMR spectroscopy

^{1}H NMR experiments were performed at 300 K on a Varian UNITY INOVA 500 spectrometer (Agilent Technologies, Inc., Santa Clara, CA), operating at a frequency of 499.83 MHz. One-dimensional (1D) ^{1}H NMR spectra were obtained using a standard pulse sequence (1D NOESY) with presaturation during relaxation and mixing time for water suppression. For each urine spectrum, 128 scans were collected in 64k data points over a spectral width of 6,000 Hz using a recycle time of 3.5 s, and a mixing time of 0.1 s. After Fourier transformation with 0.3 Hz, line broadening spectra were phased and baseline corrected, and the chemical shift scale was set by assigning a value of $\delta = 0.00$ ppm to the signal for the internal standard TSP. After Fourier transformation with 0.3 Hz, line broadening spectra were phased and baseline corrected, and the chemical shift scale was set by assigning a value of $\delta = 0.00$ ppm to the signal for the internal standard TSP.

Spectral processing and statistical analysis

Before statistical analysis, the NMR spectra were processed using MestReNova, version 12.0.1 (Mestrelab Research SL, Santiago de Compostela, Spain) and corrected for misalignments in chemical shift primarily due to pH-dependent signals. Excluding the portions with the residual water (δ 4.6-5.2) and TSP resonances, each spectrum was integrated (binned) using 0.001 ppm integral regions between 9.5 and 0.5 ppm. Bins were normalized to the sum of the total spectral area to compensate for the overall concentration differences. The final data set was automatically reduced to ASCII file and converted into an Excel® file. The NMR data set was imported into SIMCA 14.1 (Umetrics, Umea, Sweden), Pareto-scaled, and analyzed by unsupervised and supervised method: Principal Component Analysis (PCA), Orthogonal Projections to Latent Structures (OPLS) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). Metabolites were identified based on the literature and the Human Metabolome Database (http://www.hmdb.ca/).

PCA was initially applied to reduce the dimensionality of multivariate data while preserving most of the variance within the dataset. Essentially, the purpose of this unsupervised method is to express the main information contained in the initial variables in a lower number of uncorrelated variables (principal components, PCs) which are combinations of the initial measurements, highlighting the variance within the data set and removing redundancies. Graphically, the results of a PCA consist of score plots, giving an indication of any grouping in the data set, and loading plots, indicating which variables are important in reference to the score patterns. Separation of samples in clusters signifies differences between groups as represented by the loadings for those PCs. Generally, the relevant information of PCA analysis are retained by the first two or three principal components (PC1, PC2, and PC3).

OPLS analysis was run to investigate the temporal dynamics of the infant urinary metabolome using the postmenstrual age (PMA) as continuous-response variable, Y. OPLS-DA, a supervised classification technique, was used to discriminate the two groups of preterm infants according to the different type of supplementation (i.e. vitamin D vs vitamin D with DHA). Compared to the classical projection of latent-structure analysis (PLS), both methods reduce model complexity and allow improved interpretation of the spectroscopic variations by removing non-correlated variation in the data. Briefly, they facilitate the separation of the systematic variation in X into two parts, one linearly related to Y (predictive information) and one unrelated to Y (orthogonal information).

The quality of the OPLS and OPLS-DA models is described by the R^2X, R^2Y and Q^2Y parameters. R^2X and R^2Y represent the explained variation of the X and the Y matrices, respectively, while Q^2Y estimates the predictability of the model. The statistical significance of R^2Y and Q^2Y was estimated through a response permutation testing [23]. In this test, the Y matrix is randomly re-ordered (400 times in this study), while the X matrix is kept constant. This means that the Y data remain numerically the same, but their positions are shifted by random shuffling. Each time a new OPLS-DA model is fitted using X and the permuted Y matrix, providing a reference distribution of R^2Y and Q^2Y for random data. An intercept of the Q^2Y regression line below zero and an R^2Y intercept value significantly lower than the corresponding original one are indicative of a valid model. The contribution of variables to the scores pattern was evaluated by analyzing the coefficient (covariance) plots, presented using a back-scaling transformation in order to preserve the original spectral appearance [24]. This allows each data variable to be plotted with a color code which relates to the significance of class discrimination as calculated from the correlation matrix. A correlation coefficient of ± 0.40 was adopted as a cutoff value. In addition, correlations between metabolites and PMA were evaluated using Pearson's correlation test. The strength of correlations was considered weak ($0 < |r| < 0.3$), moderate ($0.3 < |r| < 0.5$) and strong ($0.5 < |r| < 1$) according to the Pearson's coefficient value (r). P-value < 0.05 was considered significant.

Results

Two groups of preterm infants, each comprising 22 subjects, were studied. After hospital discharge, each group underwent a different type of vitamin D supplementation over a period of 2 months: the first group received only vitamin D (DS), while the second group received a supplementation of vitamin D enriched with DHA (D-DHAS). Numerical data related to demographic, perinatal and clinical characteristics are summarized in **Tab. 1**. At the baseline, no significant differences between the

Table 1. Characteristics of the patients enrolled in the study.

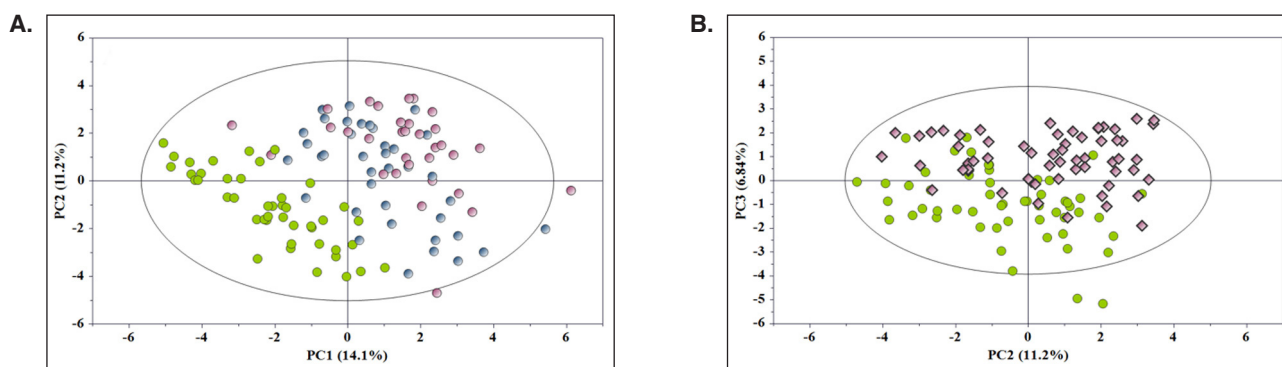|		Supplementation		p-value
		Vitamin D n = 22	Vitamin D + DHA n = 22	
Gestational age, weeks (mean ± SD)		32 ± 2	31 ± 2	0.09
Weight at birth, g (mean ± SD)		1577 ± 567	1503 ± 382	0.58
Male, n (%)		15 (68)	12 (55)	0.43
1-min Apgar index, mean (range)		7 (1-10)	8 (4-9)	0.57
5-min Apgar index, mean (range)		8 (5-10)	8 (6-10)	0.69
Hospital stay, days, mean (range)		33 (4-86)	38 (19-84)	0.15
Type of feeding	Formula milk, n (%)	9 (41)	12 (54)	0.46
	Breast milk, n (%)	1(4)	3 (14)	0.33
	Mixed, n (%)	12 (55)	7 (32)	0.10

two groups were assessed in any of the parameters (Mann-Whitney test).

Overall, 132 samples of urine were collected at three time points and analyzed by ^{1}H NMR spectroscopy. **Fig. 1** shows the scores plot of the PCA model built with all spectra to get a general overview of the data set. The first three principal components jointly explained 32.1% of the total variance. The distribution of objects in the PC1 vs PC2 plot (**Fig. 1A**) evidenced a time-sampling related impact on the urine metabolic profile for all infants. In addition, a separation between the two supplementation groups (i.e. DS and D-DHAS groups) was clearly visible by combining PC2 and PC3 (**Fig. 1B**).

Using PMA as Y variable, an OPLS model was built for each group of infants to further analyze the temporal dynamic of metabolome (**Fig. 2**). Both models presented a good predictability (Q^2Y value of 0.538 and 0.702 for DS and D-DHAS group, respectively). Intercept values for Q^2Y and R^2Y

obtained from the permutation plot (not shown) were -0.400 and 0.463 for the DS group ($p < 0.0001$) and -0.439 and 0.459 for the D-DHAS group ($p < 0.0001$). The metabolites that significantly increased with PMA were betaine, N,N-dimethylglycine (N,N-DMG), creatinine, creatine, guanidinoacetate (GAA) for both groups of infants and citrate and dimethylamine (DMA) only for D-DHAS group. The levels of myo-inositol and hydroxyproline decreased for both groups over postmenstrual time, while lactate diminished only for D-DHAS group. **Tab. 2** shows the Pearson's correlation coefficient between PMA and the most significant metabolites from OPLS models.

Concerning the influence of supplementation on the urinary metabolome, **Fig. 3** shows the score (**Fig. 3A**) and loading (**Fig. 3B**) plots of the OPLS-DA model built for the pairwise comparison of sample groups at T2. Statistical parameters of this model demonstrated its robustness and a good predictive ability ($R^2Y = 0.948$; $Q^2Y = 0.771$;

Figure 1. PCA score plots of ^{1}H NMR preterm urine spectra: **A)** PC1 vs PC2 colored according to the sampling time (T0: green, T1: blue, T2: pink); **B)** PC2 vs PC3 colored in relation to the type of supplementation (Vitamin D: green circle, Vitamin D + DHA: pink diamonds). The ellipse represents the 95% confidence region for Hotelling's T2 statistic in score plots.
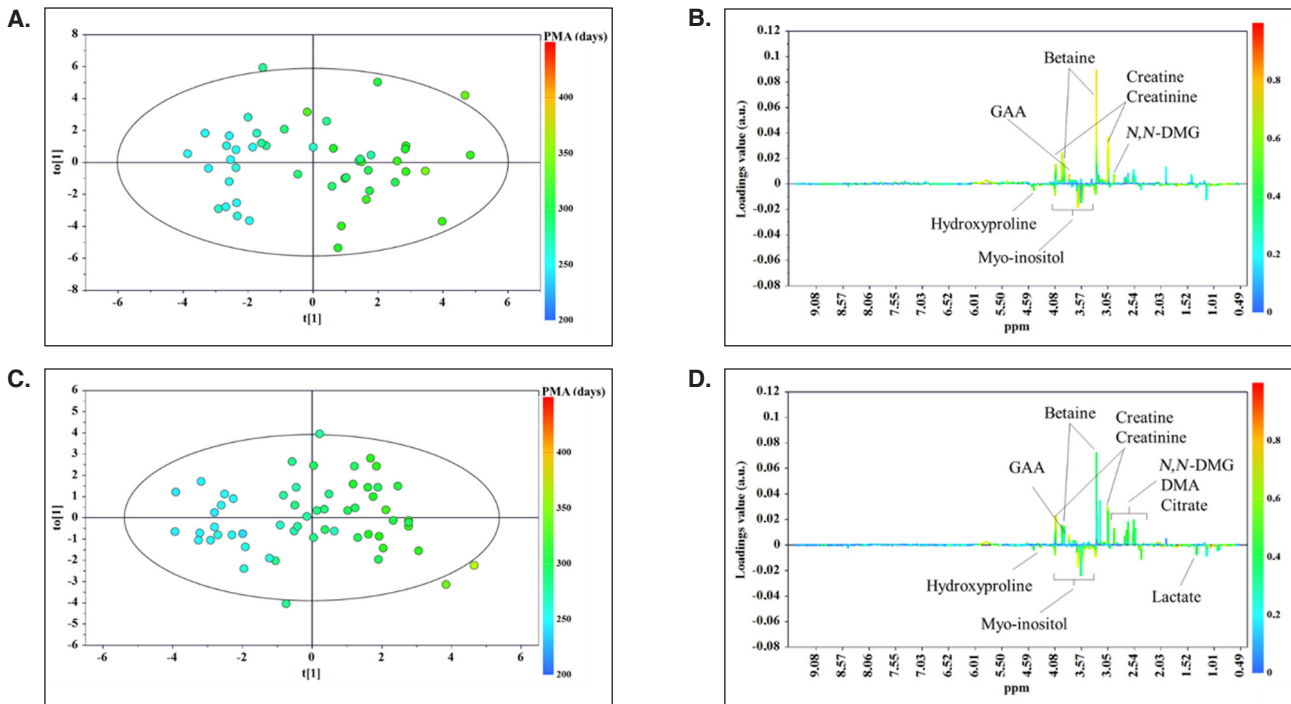
Figure 2. OPLS score (left) and loading (right) plots of **A-B)** DS group ($R^2Y = 0.726$, $Q^2Y = 0.538$, $p = 5.3 \cdot 10^{-7}$); **C-D)** D-DHAS group ($R^2Y = 0.898$, $Q^2Y = 0.702$, $p = 2.3 \cdot 10^{-13}$). Scores are colored according to the postmenstrual age (PMA, days). Only the signals from the major contributing metabolites are labeled (cutoff value: p(corr) ≥ |0.4|).
DS: Vitamin D supplementation group; D-DHAS: Vitamin D and DHA supplementation group.

Table 2. Pearson's coefficients (r) for correlation between postmenstrual age (PMA) and the most significant metabolites from OPLS models.

|		DS			D-DHAS	
		r	p		r	p
Betaine	↑	0.64	$7.7 \cdot 10^{-7}$	↑	0.46	$2.8 \cdot 10^{-4}$
***N,N*-DMG**	↑	0.60	$5.7 \cdot 10^{-6}$	↑	0.59	$7.6 \cdot 10^{-7}$
Citrate	–	0.27	N.S.	↑	0.43	$5.9 \cdot 10^{-4}$
Creatine	↑	0.48	$4.9 \cdot 10^{-4}$	↑	0.47	$1.9 \cdot 10^{-4}$
Creatinine	↑	0.58	$1.3 \cdot 10^{-5}$	↑	0.74	$1.9 \cdot 10^{-11}$
GAA	↑	0.64	$1.2 \cdot 10^{-6}$	↑	0.71	$4.6 \cdot 10^{-10}$
Myo-inositol	↓	-0.53	$9.7 \cdot 10^{-5}$	↓	-0.58	$1.6 \cdot 10^{-6}$
Hydroxyproline	↓	-0.61	$3.6 \cdot 10^{-6}$	↓	-0.53	$1.7 \cdot 10^{-5}$
Lactate	-	-0.23	N.S.	↓	-0.30	0.02
DMA	-	0.29	N.S.	↑	0.52	$2.7 \cdot 10^{-5}$

DS: Vitamin D supplementation group; D-DHAS: Vitamin D and DHA supplementation group; *N,N*-DMG: *N,N*-dimethylglycine; GAA: guanidinoacetate; DMA: dimethylamine. r, Pearson correlation coefficient: weak correlations |0| < r < |0.3|; moderate correlations |0.3| < r < |0.5|; strong correlations: |0.5| < r < |1.0|. N.S. no significant correlations, p > 0.05.
Arrows indicate upward (↑) or downward (↓) temporal variations of the metabolites according to the OPLS analysis.

$R^2Y_{int} = 0.671$; $Q^2Y_{int} = -0.495$; $p = 2.6 \cdot 10^{-8}$). According to the corresponding loadings plot analysis, compared to DS group, D-DHAS group exhibited higher levels of betaine, creatinine, *N,N*-DMG and DMA and lower content of myo-inositol and lactate. These findings are summarized in **Tab. 3**.

Discussion

In the present study, ^{1}H NMR-based metabolomics was used to explore the dynamic variations in the urinary metabolic profiles of two groups of preterm infants underwent a different type of supplementation (DS group: vitamin D;
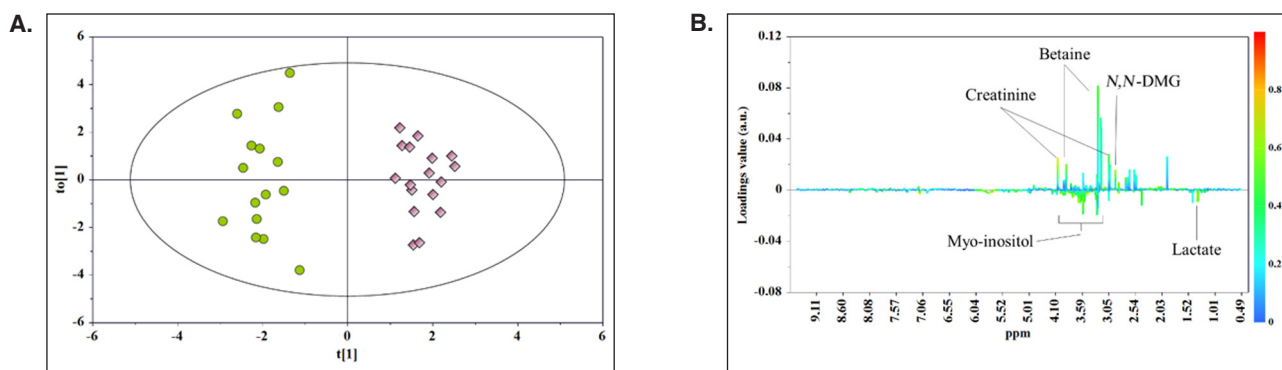
Figure 3. OPLS-DA score (**A**) and loading (**B**) plots for the pairwise comparison between DS (circle) and D-DHAS (diamonds) groups ($R^2Y = 0.948$, $Q^2Y = 0.771$, $p = 2.6 \cdot 10^{-8}$). Only the signals from the major contributing metabolites are labeled (cutoff value: p(corr) ≥ |0.4|).
DS: Vitamin D supplementation group; D-DHAS: Vitamin D and DHA supplementation group.

Table 3. Most significant metabolite variations in urines of D-DHAS group compared to DS group, their function and clinical significance.

Metabolites	D-DHAS	p	Metabolite function	Clinical significance
Betaine	↑	0.001	Donor of methyl groups	Promotes demetilation
N,N-DMG	↑	$2 \cdot 10^{-4}$	Antioxidant	Improves antioxidant action, brain protection
Creatinine	↑	$1.2 \cdot 10^{-5}$	Creatinine excretion	Improves renal function
DMA	↑	0.003	Product of choline metabolism by gut microbiota	Inversely correlates with body fat
Myo-inositol	↓	$8.2 \cdot 10^{-4}$	Second messanger Glucidic homeostasis	Improves metabolic homeostasis and protect from cellular damage
Lactate	↓	0.002	Product of the anaerobic metabolism	Improves aerobic metabolism

DS: Vitamin D supplementation group; D-DHAS: Vitamin D and DHA supplementation group; *N,N*-DMG: *N,N*-dimethylglycine; DMA: dimethylamine.

D-DHAS group: vitamin D and DHA) over a period of 2 months, after hospital discharge. The unsupervised analysis suggested differences in the urinary metabolome of infants related to both the sampling time (T0, T1, T2) and the type of supplementation.

The OPLS analysis revealed similar dynamic modifications for the two classes of infants according to the PMA. In particular, betaine, *N,N*-DMG, creatinine, creatine and GAA increased while myo-inositol and hydroxyproline decreased over time in both groups. Creatinine and creatine have been documented to be age-dependent [25]. The rate of urinary excretion of creatinine, which arises from the conversion of creatine within muscle tissue, has been positively correlated with weight, height and postconceptional age [26]. Moreover, it could be associated to the maturation of infants' renal functions. Creatine plays an essential role in maintaining the energy homeostasis necessary for brain development and function. It is transported through the blood stream and taken up by tissues with high-energy demands,

such as the brain and skeletal muscle. It is worth reminding that, in mammals, daily creatine requirements are achieved through uptake from the diet and a two-step biosynthetic pathway involving L-arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT). A higher concentration of GAA was observed in urine of preterm compared to term-newborn [27]. Thus, since preterm birth might alter the creatine biosynthesis pathway, the growing level of GAA in the patients under investigation could be ascribed to their prematurity. Betaine and *N,N*-DMG are both derived from choline. Betaine is synthesized by choline dehydrogenase and betaine aldehyde dehydrogenase in gut mucosa, liver and kidney [28] and is secreted in large quantities during the neonatal period [29]. It can act as an osmolyte or methyl group donator to homocysteine to form methionine and S-adenosylmethionine. A possible link between the methyl metabolism and oxidative stress in preterm infants was hypothesized by Friesen et al. highlighting the relationship between the choline-betaine-dimethylglycine path-

way and the response to oxidative stress through the provision of glycine for glutathione synthesis [30].

Myo-inositol is a common micronutrient particularly abundant in neural and endocrine tissues. The total body content of inositol is similar to or higher than that of glucose but inositol is mainly intracellular [31]. Due to the immaturity of preterm newborn, a large renal loss of inositol is frequent in the early postnatal period, in which the renal loss is often not compensated by the nutritional intake. In our study, the decrease of this metabolite in urine of both groups of preterm infants indicates a progressive maturation of renal functions with increasing PMA. Hydroxyproline is a collagen turnover compound considered a marker of infant growth [32] and bone reasorption and its urinary excretion was found to be higher in preterm compared with normal term infants [33, 34]. Bone reasorption and formation are complex processes mediated by several biochemical and hormonal factors, including calcium, phosphorous, collagen, PTH, alkaline phosphatase and vitamin D. In the present study the decreasing level of this metabolite over time in both groups of infants could reasonably attributed to a reduction of bone reasorption in favor to bone formation.

Interestingly, a significant temporal increase of citrate and DMA and decrease of lactate were observed only for D-DHAS group. As pointed out by the OPLS-DA, the levels of lactate and DMA after two months of supplementation were also significant different compared to DS group. Additionally, changes in betaine, *N,N*-DMG, creatinine and myo-inositol discriminated the two groups. In particular, myo-inositol and lactate levels were lower in D-DHAS group compared to the DS supplementation, while *N,N*-DMG, betaine, creatinine and DMA were more abundant. These results are summarized in **Tab. 3**. In groups of the same gestational age and birth weight, a lower content of myo-inositol in the urine corresponds to a better wellbeing: in fact, since myo-inositol is mainly intracellular, lower values in urine of myo-inositol are related to lower cellular and organism stress, in particular for the brain [35]. Again, lower values of lactate in urine in the D-DHAS group are related to lower cellular and organism stress, in particular for the brain. *N,N*-DMG is an antioxidant and it protects the neurodevelopment and the neuronal cell function [35]. DMA is a product of choline metabolism by the gut microbiota and from an animal study it is inversely correlated with body fat [36].

This work is not without limitations, including the low number of enrolled patients and the absence of information on mother characteristics (such as tobacco habits). Despite that, the multivariate statistical analysis performed in the present study indicated significant differences between the two types of supplementation, providing robust models. Overall, our preliminary findings suggested higher antioxidant and protective action by supplementation with vitamin D and DHA compared to those with vitamin D, as well as a possible positive influence on the body fat composition. Additional work will be needed to validate these results.

Declaration of interest

The Authors declare that there is no conflict of interest.

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Errata corrige: doses were absent in the first publication of this article and were added in the Methods section on page 3; errors were corrected in two sentences of the Discussion on page 8.