Metabolic Impact of Infant Formulas in Young Infants. An Outlook From the Urine Metabolome

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Abstract

Although breast milk is the ideal food source for newborns during the first six months of life, a high percentage of children receive infant formulas. There is evidence that specific diet habits may influence individual metabolic profile. Therefore, in newborns, such profile can be influenced by the use of infantile formulas given the composition differences that display compared to human milk. Up to now there are no reports in the literature that address this issue. Thus, this work aims to compare the metabolic profile of full-term newborns that were feed with either breast milk (n=32) or infantile formulas (n=21). Metabolic profile was established based on urine analysis through gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (H-NMR). Results evidenced a more gluconeogenic profile in breast fed infants. In addition, infant formula fed infants presented urinary excretion of metabolites derived from specific compounds present in this type of diet that were not observed in breast fed infants. Finally, it was observed that in infant formula fed infants there was excretion of basal levels of metabolites of clinical relevance. This results show the importance of understanding the metabolic impact of diet in newborn population in normal and pathological contexts.

Introduction

Infants constitute a special population that presents several differences compared to older children and adults in terms of metabolism, diet and life style. During the first months of life, infants experience metabolic changes due to several causes: high growth, catabolic and anabolic rates, tissue maturation among others. For these reasons, newborn nutrition must fulfill specific qualitative and quantitative requirements in order to allow an adequate growth but also to promote tissue maturation and prevent disease states[1].

Although breast milk is the ideal food source for newborns, considering its nutritional composition and that it provides digestive enzymes, bacteriostatic components, normal microbiota, growth factors, immunoprotective and bifidogenic factors. Despite of this, during the six months of life, a high percentage of children receive infant formulas in their diet due to different factors that negatively influence breast feeding like psychological distress of the mother, type of delivery, maternal education, marital status, among others[2–6]. In fact, according the United States Food and Drug Administration (FDA), 40, 50 and 75% of babies of 3, 6 and 12 months, respectively, receive infant formula[7]. Similar results have been reported worldwide indicating that between 30–60% of children below six months old receive infant formulas [8–10].

Nutritional composition of infant formulas greatly varies depending on the age of the child. Thus, starter formulas are indicated for newborns and might be used up to 4–6 months of age, while follow-on formulas are used in older infants (from 4–6 months to 1 year of age). In general, infant formulas have higher content of protein, lipids, carbohydrates, vitamins and minerals, which may alter child's metabolism[11].

Up to now some studies have been conducted exploring the effect of infant formula based nutrition on newborn intestinal function[7], immunological system development[11], association to allergies and infectious diseases, and duration of breastfeeding[12,13], however there is limited evidence in the literature about how these formulas influence the newborn metabolism. Recently, Slupsky et.al evaluated the post-prandial metabolic response of newborns to diets consisting of either infant formulas with or without lactose versus breast feeding, observing an increase of plasmatic levels of glucose, insulin and amino acid in infants fed with formulas[11]. Such findings highlight the importance to further study the metabolic impact of those diet induced changes in the levels of nutritional substrates.

Taking the above into account, this work aims to analyze the influence of infant formula consumption in the metabolic profile in children between 0 and 4 months of age. For this, a general analysis of urine metabolome was performed using ^{1}H-NMR in samples from children receiving infant formula versus breast-fed children. Then, using these samples, a further analysis of newborn metabolism was done by determining the organic acid excretion profile using GC-MS since, in human metabolism, organic acids (OA) are intermediate metabolites of different metabolic pathways including metabolism of amino acids, fatty acids and carbohydrates. Results showed general changes in urine metabolome, especially regarding the excretion of carbohydrates and organic acids. Differences observed suggest a marked gluconeogenic profile in breast fed infants. In addition, formula fed infants presented urinary excretion of metabolites derived from specific compounds present in this type of diet that were not observed in breast fed infants. Finally, it was observed that in infant formula fed infants there was excretion of basal levels of metabolites of clinical relevance for the diagnosis of genetic metabolic disturbances like organic acidurias. This results show the importance of understanding the metabolic impact of diet in newborn population in normal and pathological contexts.

Results

A total of 53 urine samples from newborns of both genders were taken upon signature of informed consent by the parents or legal representative (32 samples for BM and 21 samples for IF). In the IF group, infants received a variety of formulas commercially available. Detailed description of the population is presented in Table 1. Chemical analysis of urines samples processed was negative for nitrites, ketone bodies, proteins, bilirubin and glucose. Urinary pH ranged from 5 to 7. Creatinine levels ranged from 2.5 mg/dl to 30mg/dlIn 7 samples dipstick marked positive for leukocytes, in 6 of them it was an isolated finding. In one sample hematuria was also observed, thus this sample was excluded from the study. In addition, two more samples were excluded due to the presence of an abnormal profile that differed from the pattern observed in the other samples.

Table 1. Characteristics of the study population.

Group	*n*			Birth Weigth (g)*	Age (days)*
	Total	Female/Male	VD/CS		
Breast Milk	32	17/15	26/6	3079 (2470-3630)	72 (22-120)
Infant Formula	21	7/14	17/4	3055 (2350-3600)	80 (16-150)

VD: vaginal delivery **CS**: cesarean section. * Reported as mean (min-max).

Qualitative NMR analysis of the urine samples allowed the discrimination of 19 metabolites including organic acids, amino acids (Table 2). Analysis of NMR spectra showed several differences among groups. Thus, in the infant formula group it was observed the increase of a signal at 2.24 ppm identified as Acetone. In addition, a decrease in the signals corresponding to Betain-TMAO (3.27 – 3.91 ppm) and Creatine (3.03-3.939 ppm) was observed compared to the breast-feeding group. Differences were also observed regarding the zone among 3,0 ppm – 4,0 ppm, which presented a higher number of signals in the samples from breast feeding group, corresponding to oligosaccharides and carbohydrates. (Figure 1).

The urinary organic acids profile obtained by GC-MS consisted of around 40 metabolites including short and medium chain organic acids. Around 40% of such metabolites presented statistically significant differences between both populations in addition four metabolites were only observed in the group fed with infant formula (Figure 2. Table S1). Differences were also observed between males and females for 15 metabolites.

We also observed changes in the excretion of some metabolites depending on the age of the subject either decreasing or increasing with age. From those metabolites with decreasing trend the only one that was statistically significant for both populations was lactic acid (Table 2. Figure S1). However, when analyzing independently each population it was observed a significant decreasing trend in two more metabolites in breast fed infants while no differences were detected in the infant formula group (Table 2. Figure S2).

Six metabolites showed a statistically significant increasing trend in both populations. When analyzing independently each population it was observed a significant decreasing trend in two more metabolites in the infant formula group while no differences were detected in breast fed infants (Table 2).

In silico quantitative metabolic analysis revealed that formula-fed infants increased the excretion levels of metabolites related mainly to amino acid metabolism, fatty acids and carbohydrates (Figure 3). Outstandingly, carbohydrate metabolism was enriched mainly due to the high difference observed in the excretion of Sorbitol (D-glucitol) in urine samples from formula-fed infants.

Table 2. Lists the chemical shift values of the chemical function used to identify the metabolites observed in H-NMR spectra

NUMBER	COMPOUND	δ^1H (pH=7.0)
0	Unidentified (found in the area corresponding to lipids).	1.170 (t)
1	3-Aminoisobutyric acid	1.187-1.212 (d)
2	Lactic Acid	1.33-1.347 (d)
3	Alanine	1.475-1.499 (d)
4	Acetic Acid	1.93 (s)
5	N-Acetyl región	1.991-2.027-2.044-2.066-2.09 (s)
6	Acetone	2.24 (s)
7	Succinic Acid	2.412 (s)
8	Cítric Acid	2.509-2.56-2.651-2.70 (d,d)
9	Dimethylamine (DMA)	2.717 (s)
10	Trimethylamine (TMA)	2.931 (s)
11	Creatine	3.03-3.939 (s,s)
12	Creatinine	3.05-4.071 (s,s)
13	Betaine	3.27 (s) – 3.91 (s)
14	Glycine	3.57 (s)
15	Hippuric Acid	3.96-7.52-7.61-7.82(d,t,t,d)
16	Formic Acid	8.44 (s)
17	-N-Phenylacetyl-L-glutamine	7.328-7.391 (t)
18	L-Histidine	7.156 (s)
19	L-Taurine	3.426 (t)

δ1H: Chemical Displacement (ppm), Singlete (s), Doublet (d), Triplet (t).

Discussion

The organic acids excretion profiles and the NMR spectra of normal urine are indicators of the metabolic function in the newborn. The metabolism is affected directly by the alimentation pattern of the individual, for example, it has been observed that food composition, additives and preservers influence the metabolite excretion pattern in urine favoring the excretion of metabolites like D-glucitol, 4-Hydroxy-benzoic acid, oxalic acid, vanillactate, among others[14,15]. In fact, studies performed mainly in adults and

children have demonstrated that specific dietary habits, due to cultural background or age, may influence the organic acid excretion profile[12,15]. Newborn population is exposed to different class of nutrients according to their food, thus, it is important to generate a deeper knowledge of newborn metabolism and the metabolites normally excreted in urine and the impact of diet on them. In general, our results show several differences in the metabolic profiles observed by H^1-NMR and GC-MS analyses between infants receiving infant formula compared to breast fed infants. In fact, metabolic pathway analysis using the obtained results evidenced changes in urinary excretion patterns of metabolites mainly associated to amino acid metabolism, which may be related to the higher protein content of infant formulas. In addition, effects of formula lipidic and carbohydrate components was also observed (Fig. 3).

The study was performed in 50 samples from infants receiving either exclusive breastfeeding or that receive infant formula that fulfill inclusion criteria. In addition, sample quality was further assessed by dipstick chemical analysis of urine samples which was considered normal in most cases. Only one sample was excluded considering the presence of blood and leukocytes. Other 6 samples presented positive results for leukocyte esterase test, the principle of leukocyte detection in dipsticks for urinalysis. Although this test display good sensitivity, specificity and positive predictive value for urinary tract infection diagnosis (around 80%) [16–18], several causes of false positive may results; leukocytes may originate from other structures different from the urinary tract, particularly the female genital tract, and leukocyturia may continue even if an infectious process has been resolved, in addition the presence of bacteria from vaginal fluid may be other source of esterase activity[19,20]. Thus, this observation isolated is not necessarily suggestive of urinary tract infection[18]. Moreover, up to 37% of false positive results has been reported for this test, due to unknown causes[21]. Therefore, considering the absence of other abnormalities in the urinalysis, the absence of any clinical symptomatology associated to an infectious disease and that no abnormalities were observed in the urinary organic acid profile, the samples were included in the analyses.

The urinary organic acids profile obtained by GC-MS differed from previous reports for newborn urine samples due to the additional presence, in both populations, of some metabolites reported as normal in adults including 3-hydroxy-adipate lactone, associated to long fasting period[22]; heptenedioic acid, a dicarboxylic acid derived from odd fatty acid omega and beta oxidation[22]; and glycolic, 4-hydroxy-benzoic and 3-hydroxy-sebacic acids, which are metabolites with dietary origin derived from food additives, flavorings, and fruit or vegetable extracts. The presence of the latter group of metabolites in this population might suggest the transference of artifacts from mother´s diet to the newborn through breast feeding. It is also possible that those metabolites might be constituents of infantile formulas[23].

Some other metabolites were only observed in the population receiving infant formulas like D-glucitol, 4-deoxytetronic and homovanillic acids, which have been associated previously with glucose rich diets and sweeteners that might be present in infant formulas[24]. Furthermore, although not previously associated to infant formulas, lauric acid was also observed in this group. In fact, the presence of this medium chain fatty acid may be related to the high content of this metabolite in such diet[25]. In a similar way, acetone

was also detected in this group by H^1-NMR (Fig. 1). The presence of this ketone might be related to the fact that infant formulas are derived from cow milk, and a transient ketotic state has been previously reported in cows during lactation, this seems to be reinforced by the fact that no other ketone bodies were observed[26].

Other metabolites observed only in the group receiving infant formulas have not been previously associated to dietary habits, but they do have been reported associated to pathological conditions. Such is the case of 2-methyl-3-keto-valeric, 3,4-dihydroxybutiric and 3-hydroxy-3-methylglutaric acids which have been associated to propionic aciduria, deficiency of succinic semialdehyde dehydrogenase, and deficiency of 3-hydroxy-3-methyl-glutaril-CoA dehydrogenase respectively[15,22]. These metabolites were observed in low quantities and their excretion was not simultaneous with other pathological ones. Thus, our results suggest that children receiving infant formulas excrete basal levels of these metabolites. Such findings are important when interpreting urinary profiles for diagnostic purposes in children where any of the above-mentioned conditions is being studied.

Urine metabolic profiles of newborn receiving infant formulas also differed from profiles from the breast-feeding group regarding the excretion of creatine and betaine, showing decreased levels of these metabolites (Fig. 1). Such findings might be related to the decreased content of these metabolites in infant formulas compared to breast milk, as well as reports showing a similar behavior for choline, a betaine precursor[27–29]. In addition, less signals are evident in the area of carbohydrates in the urinary H^1-NMR profile of this group, which may reflect the less oligosaccharide content of infant formulas since they are made with bovine milk[30]. Similar reports have been reported previously by Marincola et al.[31].

In addition to the above mentioned differences, changes in the levels of some metabolites were associated to infants' age (Fig. 2C-D). This results shed some light on metabolic transitions occurring during the first months of life and the influence of diet on such behavior. Thus, in the breast feeding group, a statistically significant decreasing trend was observed for succinic, lactic and 4-hydroxy-phenyl-lactic acids (Fig. 2C-D). The behavior observed for 4-hydroxyphenyllactic acids, metabolites associated to gut bacterial metabolism, might be related to the changes that occur on the gut microbiota during the first year of life[32]. Besides, the high levels of succinic (a tricarboxylic acid – TCA- cycle intermediary) and lactic acids (a gluconeogenic substrate) during the first days of life suggest that there is a high rate of gluconeogenesis from amino acids (Fig. 2C-D). Such rate might be related to an increased energetic requirement in the newborn due to the transition from a constant nutrient supply during fetal life to the nursing cyclic feeding scheme[32,33]. The tendency observed for succinic and lactic acids, coincide with the higher levels of fumaric and 2-cetoglutaric acids observed in breastfed infants (Fig. 2A), and reports of reinforcing the idea that newborns receiving breast milk have a high gluconeogenesis rate to compensate the low glycemic index of this milk, compared to infant formulas, since lactose is the main carbohydrate while, at least in the population analyzed, 78% of the formulas used contained other carbohydrate sources including corn syrup, maltodextrins and sucrose[34,35]. Other authors have found similar decresing trends for Kreb's cycle metabolites and lactic acid[24,36,37]. Moreover, it has been reported that newborn

metabolism highly relies on lipid catabolism during the first days of life. This situation induces an increased flux through the TCA cycle and a decrease in NAD concentrations resulting in stimulation of lactate dehydrogenase activity and a subsequent increase of lactic acid[38–40].

In contrast to the observed in the newborns receiving breast feeding, in the population receiving infant formulas, the only metabolite that presented a decreasing trend was lactic acid (Fig. 2C), suggesting that under this diet newborn metabolism normalizes faster. In fact, the difference in the excretion pattern of other metabolites, like fumaric and succinic acids, between both populations (Fig. 2A, C) may be related with the higher content of glucose rich carbohydrates in infant formulas compared to breast milk, which inhibits gluconeogenesis with the subsequent reduction in the urinary levels of TCA cycle intermediaries.

Summed to the metabolites decreasing with age, several metabolites tend to increase with age, including adipic, 3-methyladipic and 3-methylglutaric acids, as well as the phenylacetylglutamine. The behavior of first two metabolites might suggest an increased lipidic metabolism independently of the diet, moreover, the increasing excretion of 3-methyladipic acid, in both groups, suggest that newborn metabolism uses alternative catabolic pathways for fatty acid oxidation since it is a metabolic intermediary from ω-oxidation of phytanic acid, which is usually metabolized through α-oxidation in adults[22]. Changes observed in 3-methylglutaric acid and phenylacetylglutamine suggest adaptations with age regarding lysine metabolism and gut microbiota activity[15], respectively, and coincide with previous results [22–24].

In the population receiving infant formulas, an increasing trend was also observed for methylsuccinic and 2-methyl-3-hydroxy-butyric acids (Fig. 2F). These results indicate a higher isoleucine catabolism. The trend observed might be explained by the increasing volume of milk that the infants' intake as they grow, and considering that protein content of infant formulas is higher compared to breast milk, there is more amino acids available favoring the use of catabolic pathways[32]. Moreover, our results coincide with the increased amino acid catabolism reported by other authors in infant-fed population[41].

It is important to note that high excretion of 3-methylglutaric, methyl succinic and 2-methyl-3-hydroxy-butyric have also been associated to different organic acidurias (Table 4). Although their presence in normal urine has been previously reported, the description of changes in their levels with age in the population receiving infant formulas is of great importance to better interpret urinary organic acid excretion profiles in newborns when an organic aciduria is been investigated[15,42].

In sum, our results indicate that differences in composition and nutritional contributions between breast milk and infant formulas greatly impact metabolism of neonates (Fig. 4). Moreover, it seems that newborns under breast feeding have an increased gluconeogenic rate using amino acids as important energy substrates. This situation generates high amounts of TCA cycle intermediaries as well as increased levels of other metabolites that tend to decrease as the infant grows. Such behavior was almost abolished when infant formulas are introduced, in fact the higher content of several nutrients lead to increased levels to metabolites not commonly observed in breast fed infants. Our results are in line with previously reported evidence from serum metabolome suggesting energetic metabolism changes

between breast-fed and formula-fed infants41. Although such reports increased ketogenesis and fat oxidation, our results showed changes in gluconeogenesis. Such differences may be related to the metabolomics approach used, the specific metabolic profiles studied as well as the sample used

Table 4
Metabolites observed in the present study that have been associated to organic acidurias.

	METABOLITE	ORGANIC ACIDURIA
NEWBORNS RECEIVING BREAST MILK FEEDING	Heptenedioic acid	Dicarboxylic Aciduria
NEWBORNS RECEIVING INFANT FORMULA FEEDING	2- methyl- 3-ketovaleric Acid	Propionic Aciduria
	3,4 Dihidroxybutyric Acid	Deficiency of succinic Semialdehyde-dehydrogenase 4-Hydroxybutyric Aciduria Intolerance Lactose
	2-methyl-3-Hydroxybutyric Acid	Oxothiolase deficiency
	3-Hydroxy-3-methyl-glutaric Acid	3-Hydroxy-3-methyl glutaric Aciduria
RELATION IN BOTH POPULATION	Methylmalonic Acid	Methylmalonic Aciduria
	Methyl succinic	Malonyl –CoA decarboxylase deficiency
	Glutaric Acid 3 methyl glutaric	Glutaric Acidurias Type I,II and III

by each group, since serum is the ideal sample for detecting fatty acids and ketone bodies in early infancy, since due to the high use rate of ketones in this period, low amounts are observed in urine [43,44]. In contrast, krebs intermediarias as well as other gluconeogenic substrates are detected more efficiently in urine by GC-MS15. Despite of this, both evidences point out that higher carbohydrate content of infant formulas shifts infant metabolism towards using carbohydrates as the main energy source downregulating other catabolic pathways.

The tendencies observed for some metabolites contrast with other studies comparing infants fed with infant formulas and breast milk. Such is the case of the reported by Dessi et al. that found that glucose, galactose and glycine were higher in infant formula population while adipic, aconitic and aminomalonic acids were higher in breast fed infants[40]. However, such data was obtained from population within the first day of life, which was not included in this study, and there are limited studies regarding time evolution of metabolic profile within the first months of life.

Finally, results regarding gender specific changes in the urine metabolome are contradictory with reports of changes in the first days of life as well as in adults, however studies that show no differences have also been reported[39–41, 45,46]. Here we observed that males presented higher levels of metabolites related to Krebs cycle, lactic acid and fatty acids, while those observed increased in females are related to infant formula artifacts (v.g. Lauric, glucitol) and metabolites derived from amino acid metabolism. Although increased fatty acid metabolism was also observed in adults, the behavior of other metabolites differed from that reported in adults and newborns within the first week of life, although it is important to consider differences regarding the population studied, the methodological approach as well as the metabolic profile analyzed in each study[45–48]. These results highlight the necessity to better understand metabolic changes in terms of urinary organic acids with time.

In sum, our results highlight the importance of extensive characterization of the effects of diet on newborn metabolism in order to improve nutritional state and future metabolic implications. Moreover, the information obtained might be useful to give valuable information for improving the design of infantile nutritional substitutes and supplements. In addition, the information obtained extend the knowledge of normal urine excretion pattern of this population, including information regarding some metabolites of diagnostic value in different scenarios. Further studies should aim to widen the metabolic picture here depicted including other group of metabolites not considered here in order to better asses the metabolic impact of dietary supplements in the neonatal period.

Methods

Subjects and Sampling

Population consisted of healthy full term infants below 4 months of age, without familial history of organic aciduria, neonatal deaths or recurrent abortion that have not presented any infectious disease or received any medication in the previous 15 days.

Based on their diet, two groups were established, a control group receiving only breast milk (BM) and the study group consisting of those receiving infant formulas (IF), either alone or alternated with breast milk. The study was approved by the ethical committee from science school of Pontificia Universidad Javeriana, and all methods were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained prior to the sampling from all participant's legal guardians.

A random urine sample was collected after spontaneous urination into urine collection bags and then transferred to urine collecting bottles. Specimens were stored frozen at -20 °C without preservants, until processing up to fifteen days for organic acids and up to six months for ^{1}H-NMR.

^{1} H-NMR Profiles

Sample preparation. Previous creatinine determination, samples were treated according to the method described by Aygen et.al: 180 µL of potassium phosphate buffer 1,5M pH 7.0 is added to 540 µL of urine

sample including 05 Mm of deuterated (3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid or TMSP-d4 as internal standard[49,50].

^{1} H-NMR Spectra acquisition. The urine samples were investigated using 400 MHz Bruker Avance III, (5 mm BBO BB -1H/D Z-GRD Z8248/0031). For each sample, a one-dimensional ^{1}H-NMR spectrum was acquired at 296°K using a NOESYPR1D (1D Nuclear Overhauser Effect Spectroscopy with water pre-saturation) pulse sequence with a relaxation delay of 4 s and a fixed delay of 12,30 µs. Each spectrum was acquired in 64 FIDS (Free induction decays) with an acquisition time of 3.41 s [51].

Data analysis. Phase and baseline corrections were performed manually, using Mestrenova program. Data was normalized according to the internal standard. All spectra were calibrated to internal TSP (0,00). Water and Urea signals were manually removed [52]. Data Alignment was performed using iCOshift toolbox 3.1.1 in the MATLAB® version R2016b software[53]. Finally, assignment of metabolite resonances was performed by comparison with published literature data (HMDB [22], BMRB [54], and the tool Resurrecting and Processing NMR Spectra On-line (NMRDB)[55].

Organic Acids analyses

Chemical analyses and creatinine concentration of each sample were determined before analysis of organic acids. For chemical analysis, a reagent strip (Uriscreen® 10) including testing for pH, proteins, glucose, ketones, occult blood, bilirubin, urobilinogen, nitrite, leukocyte esterase and specific gravity was used. Creatinine was determined using a commercial Jaffé kit according to manufacturer instructions (LabTest Ref. 96–300)[56– 58].

For organic acids extraction from urine samples, a modification of the method reported by Wilten et al. was used [59]. Briefly, 4 g of NaCl were added to 2 mL of urine and acidic pH was adjusted with 6M HCl. As internal standard 100 µL of 0,1M 2-phenylbutyric acid were added. Then a double liquid extraction was performed using first 2 mL of ethyl acetate and then 2 mL of ethyl ether. Organic phase of both extractions was combined and submitted to chemical drying with Na_2SO_4 followed by evaporation with heat and nitrogen atmosphere. Finally, samples were trimethylsilylated using BSTFA and heating at 80 °C for 20 minutes.

Separation and analysis were done by gas-chromatography in a Hewlett-Packard 6870 GC coupled to mass spectrometer Hewlett-Packard 5973.

Data Analysis

Peaks in the chromatogram were identified manually according to the retention time and contrasting the mass spectrum with a human organic acids database. Relative abundance of each metabolite was established by obtaining peak areas that were then normalized against internal standard peak area and mmol of creatinine.

Correlation analyses were performed among the relative abundance of the observed metabolites per samples, the age of the subject and the diet. For this Spearman's rank correlation analysis was performed.

Declarations

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AUTHOR CONTRIBUTIONS

AMCB: Collection, processing, analysis of samples by GC / MS and construction of the manuscript.

SCC: Collection, processing and analysis of samples by GC / MS

NP: Sample Processing, H^+ NMR Sample Analysis, Manuscript Review

MYP: Collection and processing of samples by GC / MS

JGM: Analysis of results and construction of the manuscript

OYEP: Analysis of results and construction of the manuscript

ARL: Statistical analysis of GC / MS results and revision of the manuscript

ETHICS DECLARATIONS - COMPETING INTERESTS

The authors declare no competing interests.

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Tables

Table 3 not available with this version

Figures

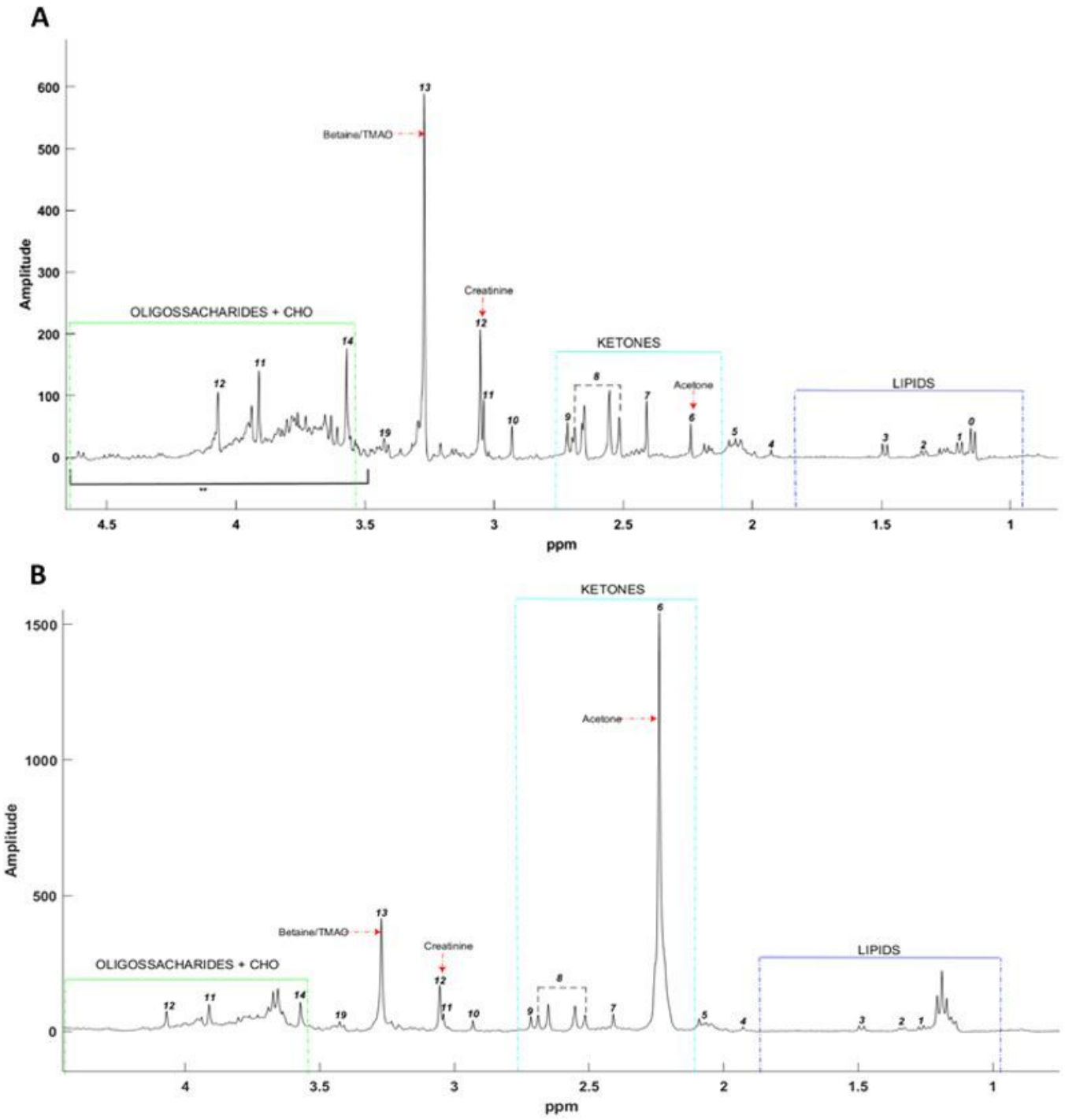

Figure 1

Mean spectra obtained from NMR analyses performed to urine samples from newborns. A. Breast feeding group. B. Infant formula group. The region shown correspond only to a segment of the spectra between 1 and 4 ppm, where the main differences between both populations were found. 0. Not identified triplet. 1. 3-Aminoisobutiric acid. 2. Lactic acid, 3.L-Alanine, 4. Acetic acid, 5. N-acetyl region, 6. Acetone, 7. Succinic acid, 8. Citric acid, 9. DMA, 10. TMA, 11. Creatine, 12. Creatinine, 13. Betaine/ TMAO, 14. L-Glycine, 15. Hippuric acid, 16. Formal acid, 17. Alpha-N-phenylacetyl-L-glutamine, 18. L-Histidine, 19. L-Taurine.

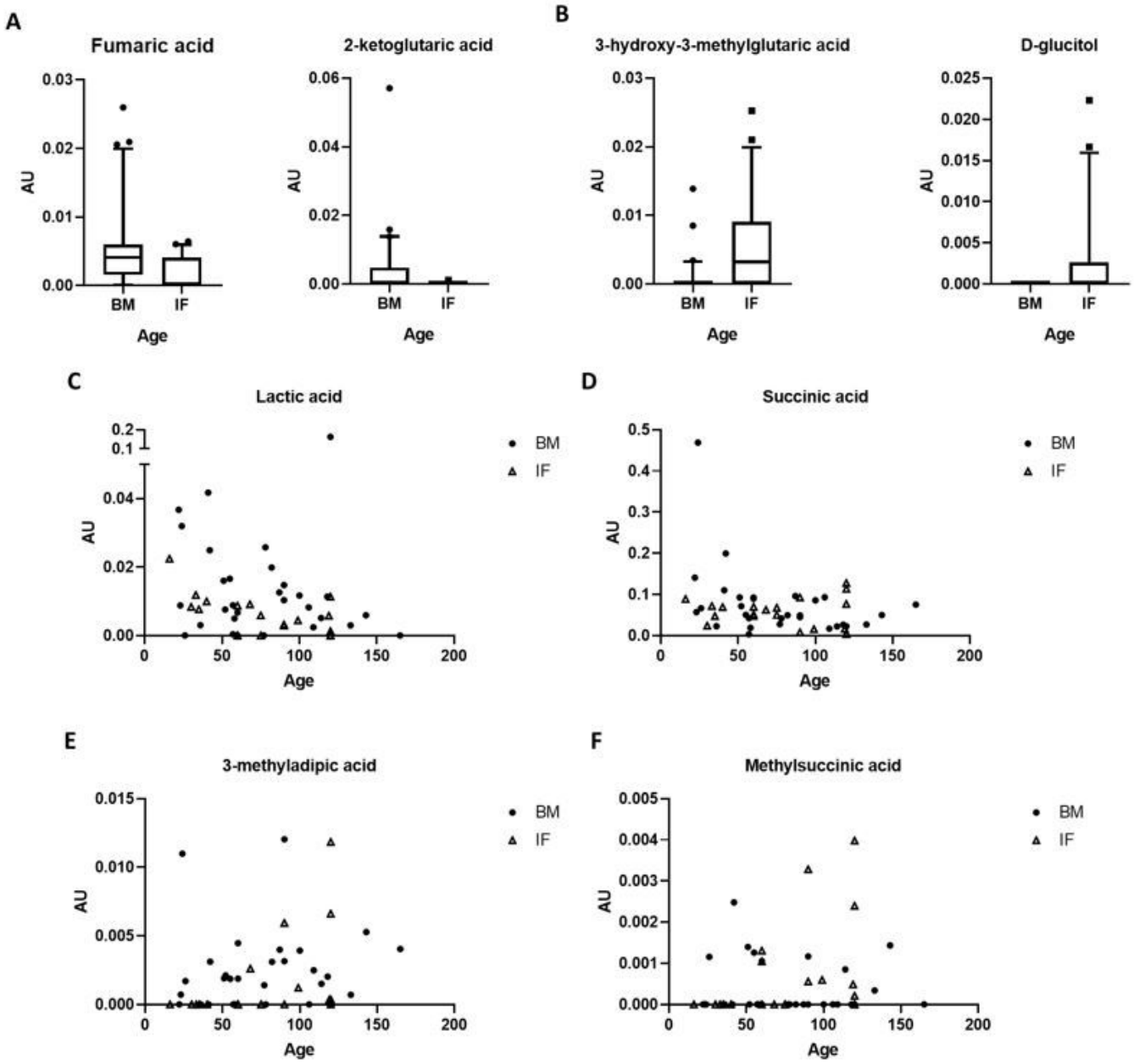

Figure 2

Metabolic signatures according to diet. A. Metabolites increased in population receiving Breast Milk (BM). From the 5 metabolites that showed a statistical significant pattern Fumaric and 2-ketoglutaric acid are shown as representative. B. Metabolites increased in population receiving infant formula (IF). From the 10 metabolites that showed a statistical significant pattern 3-hydroxy-3-methyl glutaric and D-glucitol are shown as representative. C-D. Metabolites that show tendency to decrease with age. C. From the three metabolites that showed a statistical significant tendency in both population Lactic acid is shown as representative. D. From the two metabolites that showed a statistical significant tendency in breast fed population succinic acid is shown as representative. E-F. Metabolites that show tendency to increase with age. E. From the six metabolites that showed a statistically significant tendency in both population 3-methyladipic acid is shown as representative. F. From the two metabolites

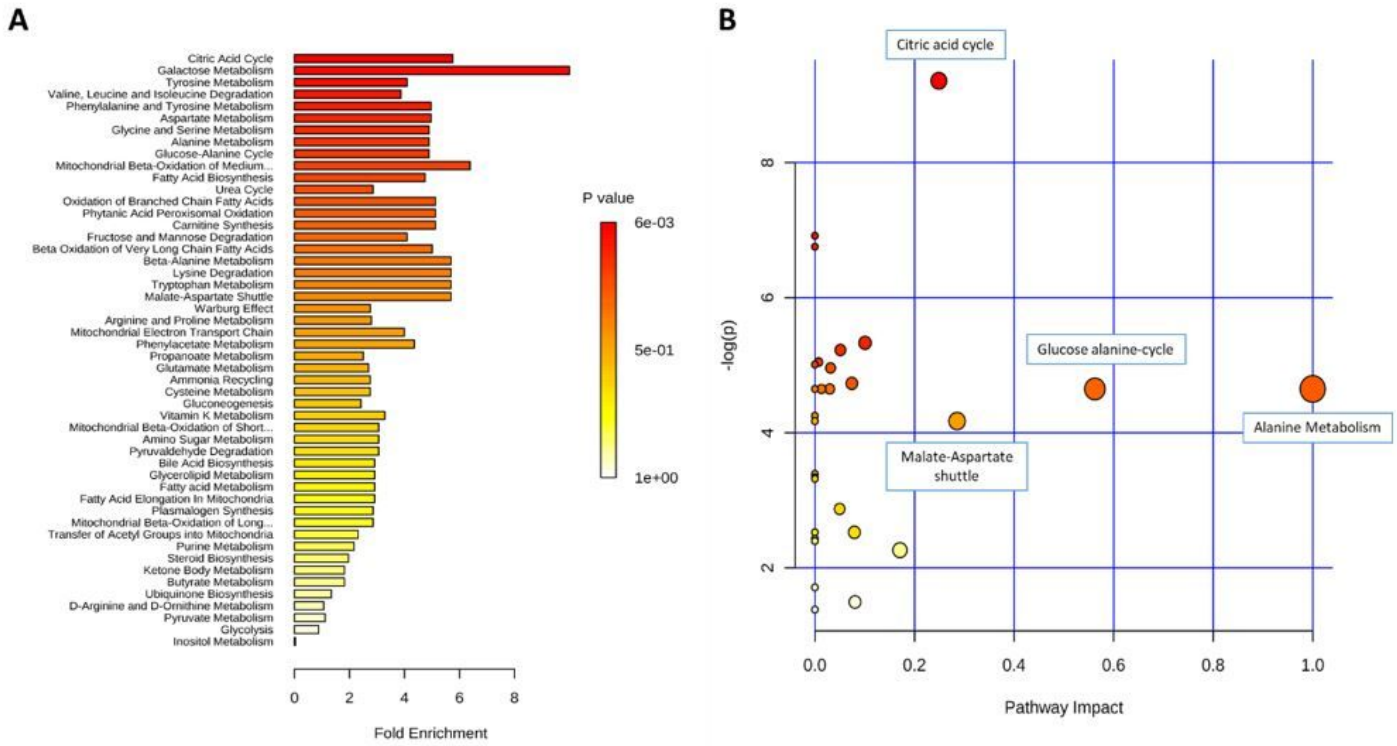

Figure 3

Metabolic enrichment analysis. A. Metabolite enrichment analysis. B. Pathway enrichment analysis. Results for quantitative metabolic analysis performed using MetaboAnalyst 4.0 using Small Molecule Pathway database as source (SMPDB).

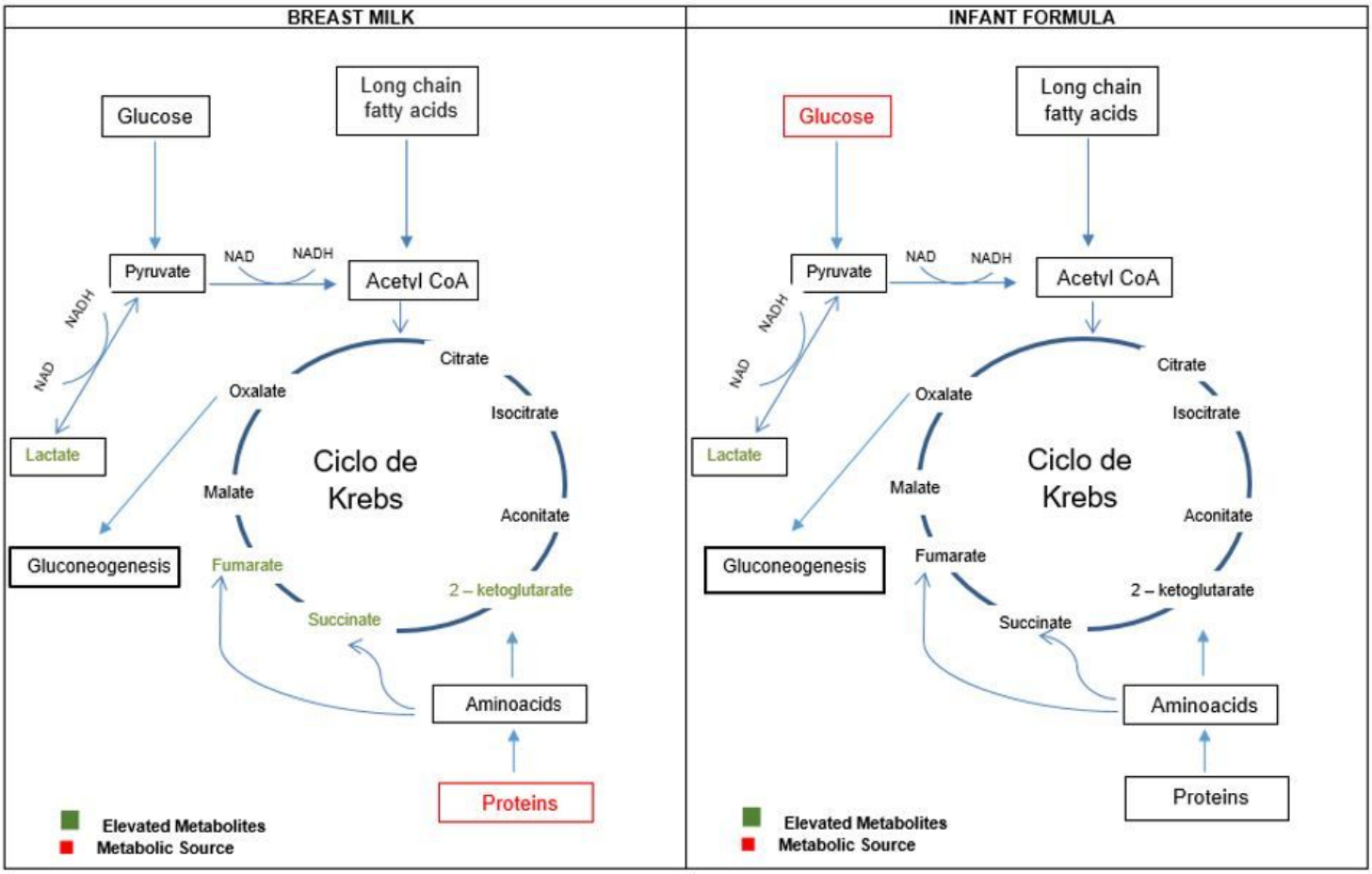

Figure 4

Summary of the metabolic impact of diet in neonates

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