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Research article

Metabolic impact of infant formulas in young infants. An outlook from the urine metabolome

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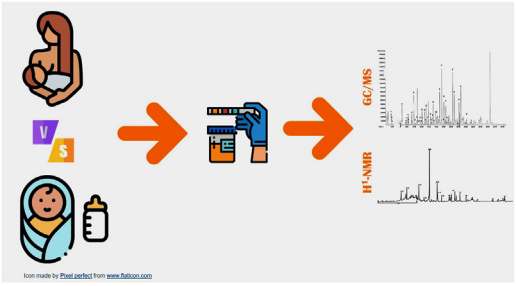
GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: 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.

Objectives: 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). Methods: Metabolic profile was established based on urine analysis through gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (H-NMR).

Results: our results evidenced a more gluconeogenic profile in breast-fed infants characterized by elevation of Kreb's cycle intermediaries like fumaric, succinic and ketoglutaric acids compared to infants receiving infant formula. 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, for instance D-glucitol, and 4-deoxytetronic. Moreover, in infant formula fed infants there was excretion of basal levels of metabolites of clinical relevance like 3-hydroxy-3-methyl-glutaric, 2-methyl-3-keto-valeric and 3,4-dihydroxybutyric.

Conclusion: These results show the importance of understanding the metabolic impact of diet in newborn population in normal and pathological contexts.

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1. 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, transition to a periodic feeding supply, tissue maturation among others [1].

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, by the sixth month 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, 3, 4, 5, 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% to 60% of children below six months old receive infant formulas [8, 9, 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, compared to human breast milk, which may alter child's metabolism [11].

Up to now several 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, duration of breastfeeding [12, 13], and newborn metabolism [11, 14, 15, 16, 17]. In terms of latter, metabolomics approaches are heterogeneous including targeted and non-targeted [15, 16]. Considering that some targeted metabolomic approaches have diagnostic purposes [18], it is important to study the metabolic impact of diet induced changes in the levels of nutritional substrates on specific metabolites. Such is the case of the analysis of urinary organic acid, that is used for diagnostic approach organic acidurias and other inborn errors of metabolism, since organic acids (OA) are intermediate metabolites of different metabolic pathways including metabolism of amino acids, fatty acids and carbohydrates. Therefore, OA analysis represent a powerful metabolomic tool that provides a wide picture of the human intermediate metabolism. In this sense, OA analysis can be informative beyond the diagnostic scenario for understanding human metabolism and the factors influencing it (v.g. age, sex, diet, etc.) in the context of precision medicine [18, 19, 20].

In terms of infant formula impact on OA levels in infants, previous studies have reported changes in dicarboxylic acids, ketone bodies, fatty acids, amino acid derivates and phenolic compounds between formula-fed and breast-fed infants [14, 15, 16, 17]. Such findings have been observed using different samples (urine, plasma, serum, dried blood spots and faeces) and technical approximations including LC-MS, NMR, GC-MS among others [15, 16]. In the diagnostic scenario of OA are evaluated in urine using GC-MS with a protocol that allows the simultaneous evaluation of the above mentioned metabolites [21, 22, 23]. Using this methodology, studies related to the impact of dietary habits on the urinary OA profile in newborns are limited, although there are some reports regarding the normal profile in this population [12, 23, 24, 25].

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 OA excretion profile using GC-MS in samples from children receiving infant formula versus breast-fed children. Additionally, a broader metabolomics analysis was also performed by ^{1}H-NMR. 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.

2. Material and methods

2.1. 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. Subjects recruitment was performed on the basis of an open invitation to participate. Target population consisted of Colombian children living in Bogota city.

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. Samples were collected at participant's place of residence. Samples were then transported refrigerated to the installations of the Institute of Inborn Errors of Metabolism at Pontificia Universidad Javeriana where specimens were stored frozen at -20 °C without preservants until processing. Sample storage was up to fifteen days for organic acids and up to six months for ^{1}H-NMR.

At time of sampling information regarding child heath, exclusion criteria and diet was collected by interview with the mother according an established data sheet including details related to anthropometric measures at birth, diet and exclusion criteria (supplementary table 1). Based on these information, 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.

2.2. ^{1}H-NMR profiles

2.2.1. 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 0.5 mM of deuterated (3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid or TMSP-d4 as internal standard [26, 27].

2.2.2. ^{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 presaturation) pulse sequence with a relaxation delay of 4s 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 [28].

2.2.3. 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 (δ0,00). Water and Urea signals were manually removed [29]. Data Alignment was performed using iCOshift toolbox 3.1.1 in the MATLAB version R2016b software [30]. Finally, assignment of metabolite resonances was performed by comparison with published literature data in HMDB [31],

BMRB [32], and the tool Resurrecting and Processing NMR Spectra On-line (NMRDB) [33].

2.3. 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 kit based on the Jaffé method according to manufacturer instructions (LabTest Ref. 96-300).

For organic acids extraction from urine samples, a modification of the method reported by Witten et al. was used [34]. Briefly, 4g of NaCl and 100μL 6M HCl were added to 2 mL of urine. As internal standard 100μL of 0,1M 2-phenylbutyric acid were added. Then a double liquid extraction was performed using first 2mL of ethyl acetate and then 2mL 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 min.

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

2.3.1. 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, a non-parametrical test, Spearman's rank correlation analysis, was performed since data did not show normal distribution.

3. 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, 33% received also breast milk. Infants within this group received a variety of commercially available formulas. In supplementary Table 2 general nutritional information of such formulas is available and detailed description of the population is presented in supplementary Table 3. 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 30 mg/dL. In seven samples dipstick marked positive for leukocytes, in six 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. One of these samples showed a ketosis suggestive profile (very high excretion of lactic, 3-hydroxibutiric and adipic acid compared to all samples) and the other one presented as a high outlier for more than 40% of the metabolites.

Qualitative NMR analysis of the urine samples allowed the discrimination of 19 metabolites including organic acids, amino acids (Supplementary Table 4). 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 breastfeeding 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 eight metabolites were only observed in the group fed with infant formula (Table 1, Figure 2 a-b, Supplementary figure 1). Univariate analysis also revealed statistically significant sex differences were also observed for 15 metabolites, 10 of them showed higher levels in females and five in male population (Table 1).

We also observed changes in the excretion of eight metabolites depending on the age of the subject. Stearic, palmitic and lactic acids decrease with age (Figure 2c-d, Table 1, Supplementary figure 2); while phenylacetylglutamine, adipic, 3-methyl-glutaric, 4-deoxytetronic and 3-methyl-adipic acids increase with age (Figure 2e-f, Table 1, Supplementary figure 2). When analyzing independently each population some differences were observed compared to the observed behavior on the total population: in infants receiving infant formula the effect of age on stearic and palmitic acids was not evident while an increasing trend was observed for 2-methyl-3-hydroxy-butiric, methylsuccinic and adipic acids (Figure 2 e-f, supplementary Table 5 – Figure S3B). In contrast, breastfeed infants the only two metabolites affected by age were succinic and 4-hydroxyphenyl-lactic acids that display a decreasing trend (supplementary Table 6 - Figure S3A).

4. 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 [35, 36]. 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, 36]. 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 (Table 1, Figure 1). 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 and that coincides with previous studies [14, 17] (Figure 3). In addition, effects of formula lipidic and carbohydrate components were also observed.

The study was performed in 50 samples that were considered normal according to their negative results of dipstick chemical analysis, except for 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%) [37, 38, 39]. This observation isolated is not necessarily suggestive of urinary tract infection since false positive may result from the presence of bacteria from vaginal fluid, leukocyturia may continue even if an infectious process has been resolved, and in up to 37% of the cases the cause is unknown [39, 40, 41, 42]. Taking this into account, and considering the absence of other abnormalities in the urinalysis, clinical symptomatology associated to an infectious disease and abnormalities in the urinary organic acid profile, the samples were included in the study.

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 (Table 1) [23]. These metabolites include 3-hydroxy-adipate

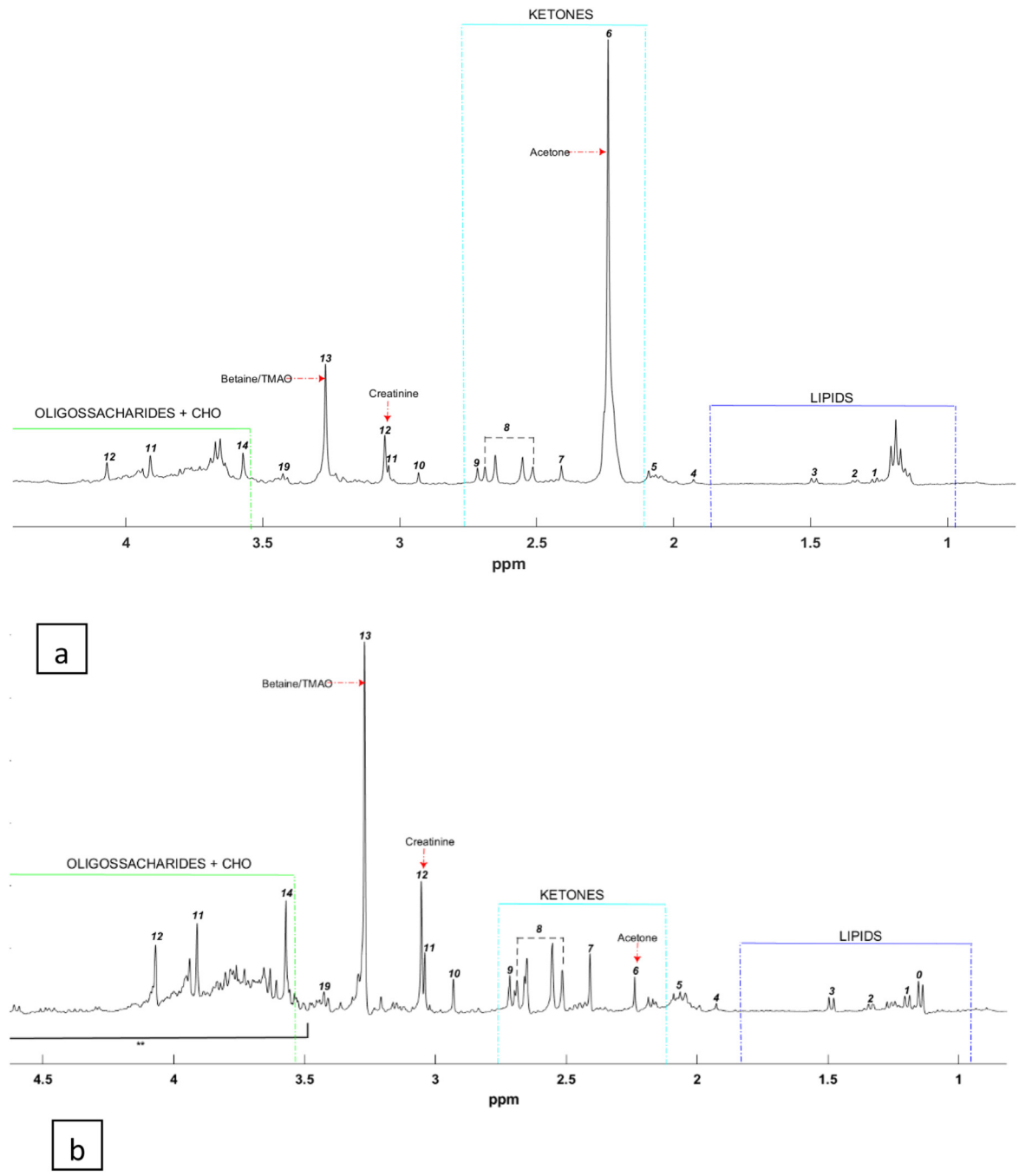

Figure 1. Mean spectra obtained from NMR analyses performed to urine samples from newborns. a. Infant formula group. **b**. Breast feeding group. The region shown correspond only to a segment of the spectra between 1 and 4 ppm, where the main differences between both populations were found. **0.** Not 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.

lactone, associated to long fasting period [31]; heptenedioic acid, a dicarboxylic acid derived from odd fatty acid omega and beta oxidation [31]; 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 breastfeeding. It is also possible that those metabolites might be constituents of infantile formulas [43].

Some other metabolites were only observed in the population receiving infant formulas (Table 1, Figure 2 and Supplementary figure 1) 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 [44]. In a similar way, acetone was also detected in this group by ^{1}H-NMR (Figure 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 [45].

Other metabolites observed only in the group receiving infant formulas have not been previously associated to dietary habits, but they do

Table 1. Organic acids detected in urine of individuals between 0 and 4 months of age receiving or not infant formulas.

Metabolite (Acid)	Retention time (min)	Relative Abundance/(SD) (Breast Milk)	(Infant formula)	P value[a] Diet	P value[a] Age	R	P value[a] Gender
Lactic	3,74	0,0167/(0,0297)	0,0059 (0,0056)	0,038	0,023	-0,32	0,038[M]
2 OH butyric	2,95	0,0003/(0,0009)	0,0005 (0,0014)	0,344	0,051	0,277	0,344
Glicolic*	4,04	0,0013/(0,0029)	0,0053 (0,0120)	0,001	0,708	-0,054	0,001[F]
3 OH isobutiric	5,02	0,0034/(0,0036)	0,0018 (0,0019)	0,024	0,545	-0,088	0,070
Oxalic	5,50	0,0028/(0,0089)	0,0024 (0,0030)	0,613	0,557	0,085	0,613
Pyruvic**	5,53	ND	0,0056 (0,0022)	0,080	0,684	-0,031	0,080
2 methyl 3 OH butyric	5,76	0,0005 (0,0021)	0,0003 (0,0007)	0,205	0,079	0,251	0,233
Methylmalonic	3,95	0,0028 (0,0040)	0,0047 (0,0137)	0,103	0,391	0,124	0,103
3 OH isovaleric	7,96	0,0020 (0,0049)	0,0040 (0,0064)	0,032	0,134	-0,215	0,032[F]
Urea	8,25	0,0190 (0,0211)	0,0125 (0,0143)	0,150	0,914	0,016	0,150
Octanoic**	8,94	ND	0,0065 (0,0018)	0,080	0,990	-0,002	0,080
Ethylmalonic	9,37	0,0047 (0,0051)	0,0046 (0,0024)	0,313	0,719	-0,052	0,313
Phosphoric	9,32	0,0012 (0,0017)	0,0015 (0,0063)	0,948	0,957	0,008	0,948
Succinic	9,95	0,0752 (0,0852)	0,0570 (0,0349)	0,612	0,054	-0,274	0,612
Methylsuccinic	10,24	0,0004 (0,0007)	0,0007 (0,0012)	0,344	0,111	0,228	0,344
Fumaric	10,72	0,0059 (0,0067)	0,0017 (0,0024)	0,011	0,335	-0,139	0,011[M]
4-Deoxytetronic**	11,18	ND	0,0005 (0,0014)	0,003	0,008	0,369	0,003[F]
Phenoxyacetic	11,57	0,0116 (0,0234)	0,0092 (0,0227)	0,921	0,651	-0,066	0,921
Glutaric	11,85	0,0033 (0,0067)	0,0025 (0,0036)	0,966	0,516	0,094	0,966
3 Methylglutaric	11,98	0,0001 (0,0003)	0,0002 (0,0006)	0,163	0,002	0,428	0,163
3 OH Adipate Lactone*	12,15	0,0038 (0,0060)	0,0024 (0,0060)	0,266	0,399	0,122	0,266
3 methylglutaconic	12,21	0,0025 (0,0036)	0,0017 (0,0026)	0,306	0,674	-0,061	0,484
3,4 Dihydroxybutyric**	12,52	ND	0,0017 (0,0023)	0,000	0,816	-0,034	0,000[F]
Citramalic	13,06	0,0013 (0,0030)	0,0057 (0,0002)	0,072	0,536	0,090	0,072
Adipic	13,29	0,0070 (0,0150)	0,0088 (0,0183)	0,858	0,023	0,320	0,858
3 methyl adipic	13,77	0,0024 (0,0029)	0,0015 (0,0031)	0,034	0,013	0,349	0,033[M]
Heptenedioic*	14,46	0,0033[M] (0,0035)	0,0031 (0,0058)	0,172	0,201	0,184	0,172
2 OH glutaric	14,62	0,0022 (0,0035)	0,0009 (0,0014)	0,171	0,203	-0,183	0,171
3 OH 3 Methylglutaric	15,04	0,0009 (0,0030)	0,0055 (0,0070)	0,000	0,652	0,065	0,000[F]
2 Ketoglutaric	15,06	0,0045 (0,0111)	ND	0,012	0,666	0,063	0,012[M]
4 OH Benzoic*	15,08	0,0023 (0,0071)	0,0011 (0,0030)	0,301	0,244	-0,168	0,301
4 OH phenylacetic	15,19	0,0211 (0,0274)	0,0405 (0,0555)	0,147	0,217	0,178	0,147
Lauric**	15,52	ND	0,0019 (0,0043)	0,010	0,499	0,098	0,010[F]
Suberic	16,09	0,0054 (0,0073)	0,0035 (0,0048)	0,271	0,864	0,025	0,271
Aconitic	16,98	0,0377 (0,0392)	0,0243 (0,0315)	0,020	0,989	-0,002	0,020[M]
HVA**	17,01	ND	0,0107 (0,0229)	0,003	0,970	0,005	0,003[F]
Hippuric	17,49	0,0339 (0,0290)	0,0243 (0,0223)	0,162	0,712	-0,054	0,162
2-methyl 3- ketovaleric**	18,01	ND	0,0022 (0,0009)	0,029	0,610	-0,074	0,029[F]
Citric	18,14	0,0755 (0,0425)	0,0632 (0,0422)	0,068	0,954	-0,008	0,068
VMA	18,53	0,0098 (0,0127)	0,0071 (0,0097)	0,128	0,498	-0,098	0,128
4 OH phenylactic	18,91	0,0030 (0,0055)	0,0025 (0,0062)	0,420	0,152	-0,205	0,420
D-Glucitol**	20,06	ND	0,0034 (0,0062)	0,001	0,553	-0,086	0,001[F]
Palmitic	20,53	0,3065 (0,2858)	0,0034 (0,1517)	0,145	0,017	-0,337	0,145
3 OH Sebacic*	20,92	0,0168 (0,0256)	0,0105 (0,0104)	0,577	0,695	-0,057	0,577
4 OH hippuric	22,19	0,0108 (0,0140)	0,0108 (0,0137)	0,828	0,494	-0,099	0,828
Stearic	22,71	0,2071 (0,1927)	0,1341 (0,1011)	0,231	0,010	-0,363	0,231
PAG	24,69	0,0060 (0,0058)	0,0162 (0,0149)	0,033	0,004	0,403	0,033[F]

ND: Not detected.
HVA: Homovanillic Acid.
VMA: Vanilmandelic Acid.
PAG: Phenyl Acetyl Glutamine.
[a] Statistical significance of Spearman's correlation coefficient between age and diet with metabolite levels among the total population.
[M/F] Indicates if higher values are attained in Males ([M]) or Females ([F]). Differences are highlighted only in those that showed statistical significance.
* Metabolites not previously reported in newborn population.
** Metabolites only observed in urine form infant formula group.

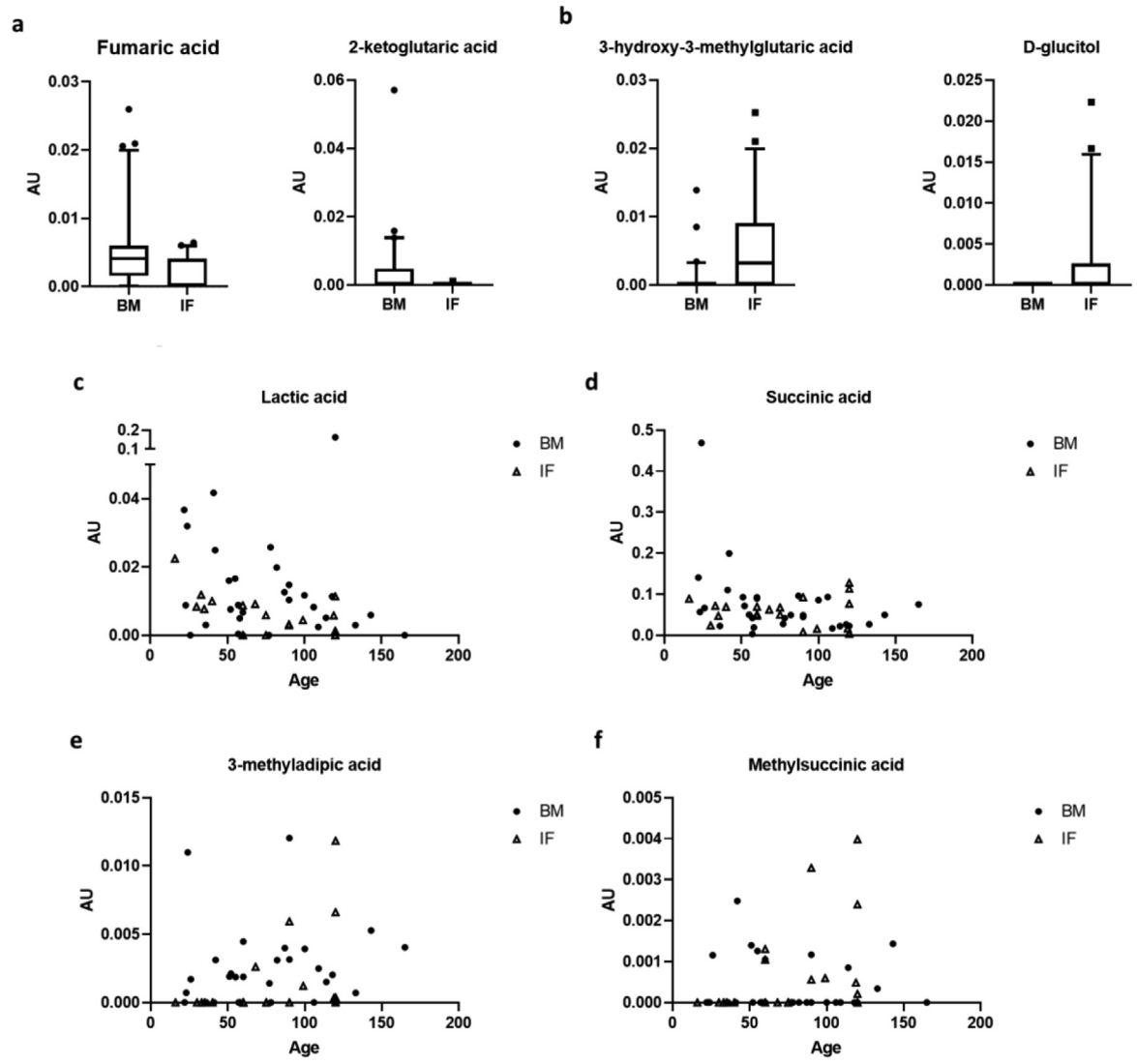

Figure 2. Metabolic signatures according to age and diet. a. Metabolites increased in population receiving Breast Milk (BM). From the 5 metabolites that showed a statistical significant pattern Fumaric ($p = 0.011$) and 2-ketoglutaric acid ($p = 0.012$) are shown as representative. **b.** Metabolites increased in population receiving infant formula (IF). **c-f** Behavior of representative metabolites according to age (expressed in days) and diet. From the 10 metabolites that showed a statistical significant pattern 3-hydroxy-3-methyl glutaric ($p = 0.000$) and D-glucitol ($p = 0.001$) 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 ($p = 0.023$). **d.** From the two metabolites that showed a statistical significant tendency in breast fed population succinic acid ($p = 0.028$) is shown as representative. **e-f.** Metabolites that show tendency to increase with age. **e.** From the five metabolites that showed a statistically significant tendency in both population 3-methyladipic acid ($p = 0.013$) is shown as representative. **f.** From the seven metabolites that showed a statistical significant tendency in population receiving infant formula (IF) methylsuccinic acid ($p = 0.046$) is shown as representative. In all figures Y-axis correspond to arbitrary units (AU) that represent semiquantitative units as defined in methods. Graphical representation of the other metabolites are shown in Figures S1–S3. *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.

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 [31, 36]. These metabolites were observed in low quantities and their excretion was not simultaneous with other pathological one characteristic of such diseases. Our findings are important for 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 (Figure 1). Such findings might be related to the decreased content of these metabolites in infant formulas compared to breast milk, since a similar behavior has been reported for choline, a betaine precursor [46, 47, 48]. 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 [49]. Similar results have been reported previously [17, 50].

In addition to the above-mentioned differences, changes in the levels of some metabolites were associated to infants' age (Figure 2 C–F, Supplementary figure 2). These 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-fed group, a statistically significant

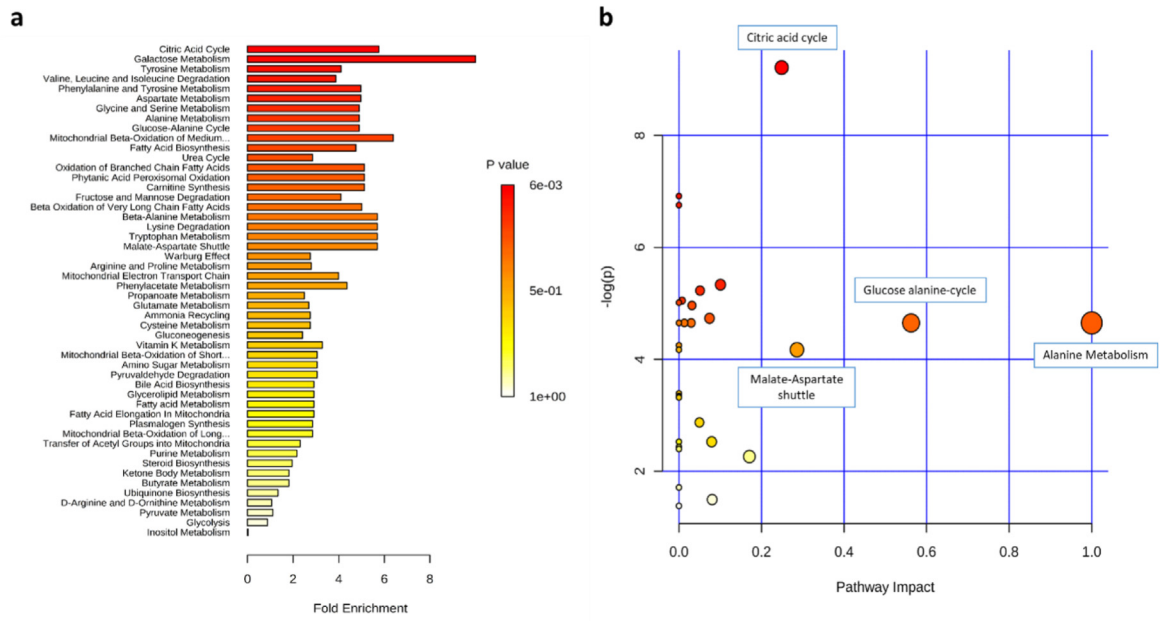

Figure 3. Metabolic enrichment analysis. a. Metabolite enrichment analysis. b. Pathway enrichment analysis. Results for quantitative metabolic analysis of all metabolites evaluated (Table 1) using MetaboAnalyst 4.0 using Small Molecule Pathway database as source (SMPDB).

decreasing trend was observed for succinic, lactic and 4-hydroxy-phenyl-lactic acids (Figure 2 C-D, Supplementary 3A). The behavior observed for 4-hydroxyphenyllactic acid, associated to gut bacterial metabolism, might be related to the changes that occur on the gut microbiota during the first year of life [51]. 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 and reproduces the findings reported by He et al. [14] (Figure 4). 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 [51, 52]. The tendency observed for succinic and lactic acids, coincide with the higher levels of fumaric and 2-cetoglutaric acids observed in breastfed infants (Figure 2A), reinforcing the idea that newborns receiving breast milk have a high gluconeogenesis rate. Other authors have found similar decreasing trends for Kreb's cycle metabolites and lactic acid [24, 53, 54]. 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

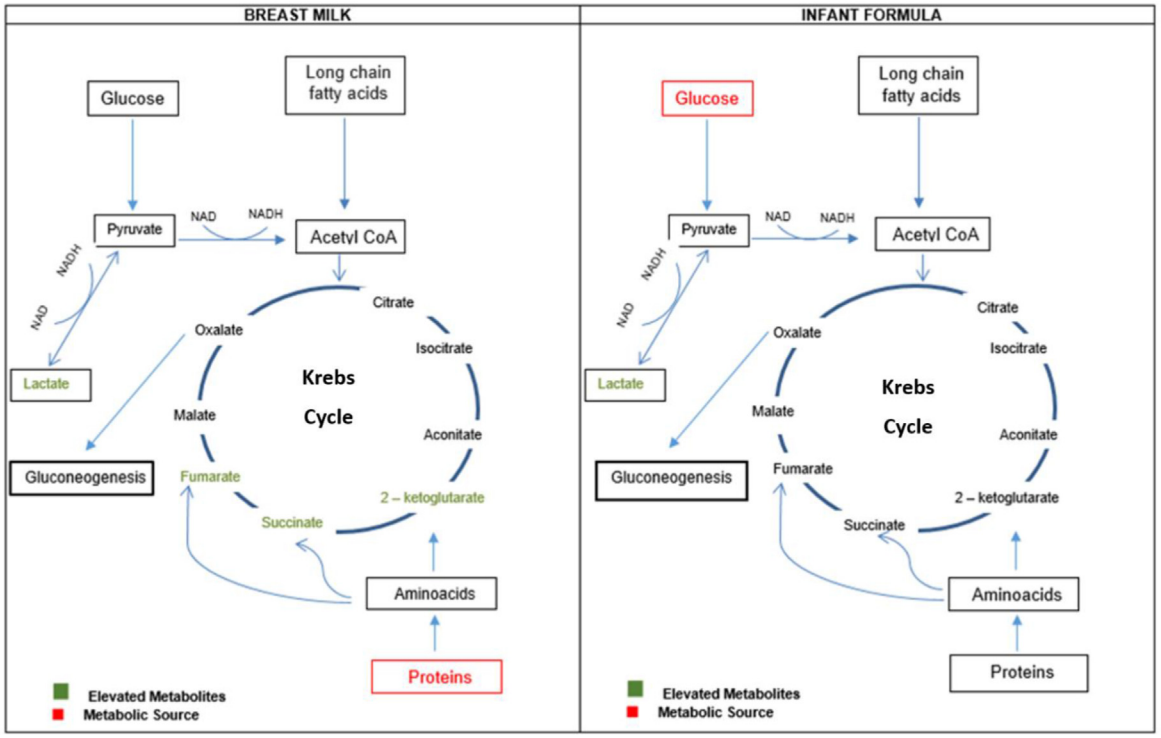

Figure 4. Summary of the metabolic impact of diet in neonates.

dehydrogenase activity and a subsequent increase of lactic acid [55, 56, 57].

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 (Figure 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 (Figure 2 A, 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 (Figure 2E, Supplementary figure 2B). 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 [31]. Changes observed in 3-methylglutaric acid and phenylacetylglutamine suggest adaptations with age regarding lysine metabolism and gut microbiota activity [36], respectively, and coincide with previous results [24, 31, 43].

In the population receiving infant formulas, an increasing trend was also observed for methylsuccinic and 2-methyl-3-hydroxy-butyric acids (Figure 2F, Supplementary figure 3B). 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 [51]. Moreover, our results coincide with the increased amino acid catabolism reported by other authors in infant-fed population [14].

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 2). 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 [36, 58].

Our results are in line with previously reported evidence from other metabolomics approaches suggesting energetic metabolism changes between breast-fed and formula-fed infants [14, 17]. Although some studies report increased ketogenesis and fat oxidation, our results showed mainly changes in gluconeogenesis. Such differences may be related to the metabolomics approach used, the specific metabolic profiles studied as well as the sample used 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 [59, 60]. In contrast, Krebs intermediaries as well as other gluconeogenic substrates are detected more efficiently in urine by GC-MS [61]. 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 (Figure 4).

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 [57]. 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.

Results regarding gender specific changes in the urine or plasma metabolome are controversial with reports of changes in the first months of life as well as in adults, however there are also studies that show no differences [14, 19, 25, 56, 57, 62, 63, 64]. Here we observed (Table 1) that males presented higher levels of metabolites related to Krebs cycle (fumaric, aconitic and 2-ketoglutaric), 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 (v.g. 3-hydroxy-isovaleric, 2-methyl-3-keto-valeric, and 3-hydroxy-3-methyl-glutaric acids). Similar results were observed by Caterino et al 2020 regarding differential behavior of Krebs cycle metabolites among sexes [19] and interestingly data from acylcarnitine profile in newborns (48–72 h of life) that present higher isocalerylcarnitine (C5) in females, which could correlate with the high hydroxy-isovaleric acid observed in our female population [63]. 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 [11, 25, 62, 65]. These results highlight the necessity to better understand influences of gender and age to the urinary organic acids profile which for the case of gender was not further explored in the present study.

In conclusion, this study is a first approximation to the impact on urinary organic acid profile of infant formula nutrition in newborns. Our results suggest that it would be interesting to further analyze the impact of diet considering an intake quantitative analysis, the effect different formulas and changes due to mixed nutrition (infant formula – breast feeding) that were not addressed in the present study. The information obtained extend the knowledge of normal urine excretion pattern of this population, showing that metabolites like Kreb's cycle intermediaries, lactic acid, derivatives from branched-chain amino acids and phenylalanine are highly influenced by diet and age. In addition, some differences were also observed between males and females, as well as diet-dependent excretion of metabolites of diagnostic value like 3-hydroxy-3-methyl-glutaric, 2-methyl-3-keto-valeric and 3,4-dihydroxybutyric. All these information is relevant for better interpretation of organic acid profiles in diagnostic scenarios, as well as a baseline for urinary metabolomics approaches in health and disease.

Table 2. 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 Methyl succinic	Methylmalonic Aciduria Malonyl –CoA decarboxylase deficiency
	Glutaric Acid 3 methyl glutaric	Glutaric Acidurias Type I, II and III

Declarations

Author contribution statement

Angie Marcela Calvo Barbosa, Stefany Casallas Cortes, Martha Yaneth Parra, Ninna Pulido: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Alexander Rodríguez-López: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Johana Guevara-Morales, Olga Yaneth Echeverri: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at The metabolomics and metadata reported in this paper are available via repository of Pontificia Universidad Javeriana: https://repository.javeriana.edu.co/handle/10554/53434.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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