UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

FACULTAD DE MEDICINA

Centro de Investigación en Ciencias de la Salud y Biomedicina (CICSaB)

CARACTERIZACIÓN DEL PERFIL METABOLÓMICO URINARIO EN MUJERES CON DIABETES MELLITUS GESTACIONAL Y SUS RECIÉN NACIDOS A TRAVÉS DEL ABORDAJE MULTIÓMICO

TESIS QUE PRESENTA

M. C. ANA SOFÍA HERRERA VAN OOSTDAM

PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS BIOMÉDICAS BÁSICAS

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Urinary Metabolites Altered during the Third Trimester in Pregnancies Complicated by Gestational Diabetes Mellitus: Relationship with Potential Upcoming Metabolic Disorders

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Abstract: Gestational diabetes mellitus (GDM) is a disorder in pregnancy with highest impact in the future life of both mother and newborn. Increasing incidence, economic impact, and potential for severe GDM-related pregnancy complications are some factors that have motivated the deep study of physiopathology, risk factors for developing GDM, and potential biomarkers for its diagnosis. In the present pilot study, we analyzed the urinary metabolome profile of GDM patients in the 3rd trimester of pregnancy, when GDM is already established and the patients are under dietary and pharmacological control. An untargeted metabolomics method based on liquid chromatography–mass spectrometry analysis was developed to identify differentially expressed metabolites in the GDM group. We identified 14 metabolites that are significantly upregulated in the urine of GDM patients, and, more importantly, we identified those related with the steroid hormone biosynthesis and tryptophan (TRP) metabolism pathways, which are associated with GDM pathophysiology. Thus, these metabolites could be screened as potential prognostic biomarkers of type two diabetes mellitus, coronary artery disease and chronic renal failure in future follow-up studies with GDM patients.

Keywords: gestational diabetes mellitus; metabolomics; urine; tryptophan; mass spectrometry.

1. Introduction

Gestational diabetes mellitus (GDM) is the most common complication of pregnancy worldwide and is defined as hyperglycemia that is recognized for the first time during pregnancy [1]. The disease is usually diagnosed in the second or third trimester of pregnancy in patients with no history of diabetes prior to gestation [2].

In Mexico, the prevalence of GDM ranges from 8.7–17.7%. Women in Mexico are a high-risk group for developing GDM, which increases in incidence in older, overweight and obese women (body mass index > 30 kg/m2), or those with a family history of type two diabetes (T2D) [3,4].

Hyperinsulinism with increased peripheral insulin resistance (IR) is a characteristic of GDM. Hyperglycemia during pregnancy promotes epigenetic changes to the fetus and is associated with increased risk for chronic diseases during adult life [5]. Maternal hyperglycemia results in exaggerated fetal anabolism, growth of fetal adipose tissue, and bone and muscle tissue leading to macrosomia. The newborns are prone to neonatal hypoglycemia, hyperbilirubinemia, hypocalcemia, respiratory distress syndrome, and polycythemia [6].

Women with GDM are, on average, seven times more likely to develop T2D. Approximately 50% of mothers with GDM will develop T2D within ten years—along with cardiovascular morbidity, metabolic syndrome, and renal complications [7,8]. However, the metabolic mechanisms underlying this pathophysiology remain poorly understood. Metabolomics studies have confirmed that highest circulating concentrations of different metabolites in patients with T2D and GDM are associated with IR and pancreatic β-cell dysfunction [9]. A range of untargeted and targeted metabolomics methodologies have been developed to characterize the metabolome. Amino acids and its derivatives—organic acids, lipids, and fatty acids—are some dysregulated metabolites identified in studies conducted principally with maternal serum or plasma [10]. Only a few studies have investigated the excretion profile of maternal urine [11–16]. Urine is very useful for clinical applications because it is available in large quantities, can be collected in a non-invasive manner, and sample treatment is relatively simple. However, the biological interpretation of the urine

metabolome remains challenging due to the effect of physiological factors or hydration status.

While the search for biomarkers capable of predicting GDM early in pregnancy (first and second trimesters) has been the main goal of metabolomics studies conducted in pregnant women, it is also very important to investigate the metabolic alterations in late pregnancy, when GDM has been established. The identification of dysregulated circulating metabolites in GDM patients—metabolites which have also been found to be dysregulated in patients with diabetes, renal failure, cardiovascular complications, and hypertensive disorders—may reflect unmanaged GDM or ineffective response to treatment and dietary control, leading to the aforementioned diseases. Recently, dysregulated tryptophan (TRP) and purine metabolism have been described as the major pathophysiology of GDM [16]. Besides, chronic kidney disease secondary to T2D is also associated with accumulation of toxic TRP metabolites due to both inflammation and impaired kidney function [17]. Dysregulation of TRP-kynurenine (KYN) and KYN-nicotinamide adenine dinucleotide (NAD) metabolic pathways has been postulated as one of the mechanisms of IR [18]. Recently, branched-chain amino acids (BCAAs) and the valine metabolite 3-hydroxybutyrate have shown potential as biomarkers for the transition of GDM to T2D [19]. In conjunction, the study of metabolic pathways dysregulated during the course of GDM could contribute to predicting irreversible metabolic effects in the mother.

In the present study, we analyzed the urinary metabolome of Mexican GDM patients at the 3rd trimester of pregnancy, when GDM is already established and the patients are under dietary and pharmacological control, with the purpose of predicting dysregulated metabolic pathways that could be molecular links associated with negative outcomes after pregnancy.

2. Results

2.1. Clinical and Demographic Characteristics of the Groups Under Study

A total of 35 pregnant women were selected for the study from an initial cohort of 80 patients. 11 women had healthy pregnancies (control group) and 24 patients had GDM (GDM group). The clinical characteristics of the study groups are presented in Table 1. These data belong to the first prenatal control. There were no statistically significant differences between groups for maternal age, pre-gestational body mass index (BMI), and all clinical findings except glucose levels ($p = 0.0029$).

The urea, creatinine, and glucose results measured in the plasma of GDM patients before and after the diagnosis are shown in Figure 1.

2.2. Ultra-Performance Liquid Chromatography (UPLC)-Mass Spectrometry (MS) Urinary Profiles

Typical urinary profiles acquired under positive ion mode are shown in Figure 2.

The methodology's reproducibility was evaluated between quality control (QC) injections, which were run before the samples, and showed good reproducibility for retention time, peak shape, and peak intensity. These were evaluated by direct comparison of overlaid chromatograms (Figure 3A, B) that showed no drifts in retention time, reflecting the stability and reproducibility of the system.

2.3. Metabolites Identification

A total of 4598 features were detected under the conditions employed for the pre-processing of the raw data within UNIFI 1.8.1 (Waters Corp., Milford, MA, USA) and detailed in the Material and Methods section (retention times from 0 to 10 min). Since creatinine normalization had been proven inappropriate for clinical applications [20], in the present study normalization was applied using total ion abundance, scaling the summed abundance of all compound ions in each sample to an equal value.

In the principal component analysis (PCA) score plot, the samples of the control group were only partially separated from the GDM group (variance explained R2Xcum = 60%). PCA was first performed to discover intrinsic treatment-related clusters within the datasets and to identify outliers (Supplementary Figure S1).

Following this, partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were used to improve separation among the groups and to screen for differential metabolites. The OPLS-DA score plots resulted in inter-group separation (Figure 4A). The parameters of the obtained models were satisfied with an acceptable quality of variance explained (R2) and variance predicted (Q2) and are represented in Figure 4B. Differential metabolites were selected based on the separation through the OPLS-DA loadings and variable importance projection (VIP) scores (Figure 4C). VIP represents the extracted variables' ability to discriminate between different treatments. The variables with VIP values greater than 1.5 (VPI > 1.5) were included in the set of metabolites analyzed. The loading plots (S-Plot) identified the metabolites with significant differences in abundance between the study groups (Figure 4D). These variables were further filtered by Mann–Whitney–Wilcoxon test to determine whether a potential biomarker was statistically significant between the two groups. The metabolites with a significant difference after false discovery rate correction ($q < 0.05$) were kept and considered as the potential biomarkers.

The metabolites selected in the OPLS-DA loading S-plot were identified as described in Material and Methods. Identified metabolites with significant changes in expression after false discovery rate (FDR) correction in the GDM group are summarized in Table 2. Moreover, Supplementary Table S1 shows other identified metabolites ($p < 0.05$; $q > 0.05$). The exact measured mass/charge (m/z), mass error (mDa), retention time, and percentage of changes between groups (fold change) are detailed along with the statistical significance of each change. The selected metabolites were identified and classified according to their degree of physicochemical and/or spectral similarity to published data. Mass Spectrometry (MSE) data were manually inspected for the correct identification of major ions.

2.4. Pathway Analysis

The underlying signaling pathways and molecular networks influenced by GDM were explored and visualized by MetPA, a web application for metabolomics analysis. Identified metabolites contributing to the separation of pairwise groups were

imported into MetPA. The “Homo sapiens” library was selected for the database, while hypergeometric test and relative-betweenness centrality were performed for over-representation analysis and pathway topology analysis respectively. Tryptophan metabolism and steroid hormone biosynthesis were classified as important, although other metabolic pathways related to amino acids, lipids, purine, and carbohydrate metabolism were also identified (Figure 5).

When investigated with the same methodology for the relationship of urinary metabolites with diseases, cervical/colon/ovarian cancer, impaired glucose tolerance, diabetes mellitus, coronary artery disease, and chronic renal failure, among others, were predicted (Figure 6).

3. Discussion

In the present pilot study, we analyzed the urinary metabolomics profiles of patients with GDM in the third trimester of pregnancy. At this point, all the patients enrolled in the study were receiving dietary control and/or pharmacological support (insulin or metformin or a combination of both). Since GDM was diagnosed at 24–28 weeks of gestation, the hyperglycemia associated with this condition could have induced persistent metabolic alterations during the pregnancy. The selected participants were matched according to pre-gestational BMI and age, which, although non-significant, were higher on average in the GDM group. In both groups, consistent with epidemiologic statistical reports in the Mexican population [3], overweight and obese women were predominant. As shown in Figure 1, the evolution of the disease was monitored measuring the levels of glucose, creatinine, and urea before and after the GDM diagnosis. The levels of glucose decreased significantly at the 3rd trimester, reflecting a positive effect of treatment and dietary control (three patients received insulin treatment and 21 were treated with metformin 850 mg). However, the significant increase in the levels of serum urea and creatinine in the GDM group after diagnosis means that underlying metabolic disorders continued taking place during the pregnancy. The significantly upregulated metabolites identified in our work by the metabolomics approach belong to the following compound classes: benzopyrans, carboxylic acids and derivatives, glycerolipids, indoles and

derivatives, tetrapyrroles, sphingolipids, and steroid derivatives. Different metabolites within these classes have an impact in the physiopathology of GDM and its complications.

3.1. The Contribution of Identified Metabolites in the Physiopathology of GDM

Since the composition of urine is significantly influenced by diet, measurement of maternal urine can be used to identify a change in dietary pattern. In our study, aspartame (known as trademark canderel), 5-carboxy-alpha-chromanol (related with the Vitamin E metabolism), and cucurbitacin c (present in fruits and cucumber) are related with dietary control in the GDM group. These substances are particularly consumed in Mexican patients under a dietary regimen, as suggested by the Mexican Food System Equivalents [21].

Several compounds classified as steroids and derivatives were also found upregulated in our study: 11-oxo-androsterone-glucuronide, cortolone-3-glucuronide, tetrahydroaldosterone-3-glucuronide, 5-androstene-3b,16b,17a-triol, and 21-deoxycortisol. Some of these metabolites are related to the glucuronidation process—which is used to assist in the excretion of toxic substances, drugs or other substances that cannot be used as an energy source—and have been found altered during GDM [16]. The increase in cortisol cortisol derivative levels during pregnancy is considered as the main cause of the decrease in glucose tolerance. Steroid hormones, which are elevated steadily during pregnancy, are the main hormones that influence β-cell function in early pregnancy and IR, especially in late pregnancy. Estrogen levels change during pregnancy in different states of GDM [22].

Steroid hormones and lipid metabolism are closely related, not only because lipids are precursors of steroid hormones, but also due to the effect on lipid metabolism during pregnancy. In our study, we identified two classes of lipids: sphingolipids and glycerolipids. Diacylglycerols (DGs; belonging to glycerolipids) are intracellular messengers that have been identified as mediators of IR [23]. Regarding sphingolipid metabolism, we detected two species differentially expressed: SM (d18:0/22:0) and Cer (d18:0/23:0). Sphingomyelins are present in animal cell membranes, and the synthesis and degradation of sphingomyelin species produce

signal transduction second messengers that regulate the innate immune response at the feto-maternal interface [24]. Other authors have found pronounced elevations in several species of both saturated and unsaturated sphingomyelins in GDM amniotic fluid [25].

The metabolic breakdown of SM results in ceramides, which are recognized as proinflammatory lipids which are increased in T2D. Ceramide accumulation has demonstrated to be detrimental to pancreatic beta cells and may promote IR, thereby playing a direct role in the pathogenesis of T2D in both the general population and in women with previous GDM [26].

L-tryptophan was found upregulated in our study. Altered levels of TRP have been found in GDM patients [16] as well as in normal pregnancies [9]. TRP is metabolized via TRP-KYN and TRP-methoxyindole pathways.

Regarding the relationship between the identified metabolites and the pharmacological treatment with metformin, in a recent work, Zucker diabetic fatty rats were treated daily for 12 weeks with metformin (200 mg/Kg), which represents a high dose when compared to the maximum human daily dose of 2000 mg/day. In this study, six metabolites were found to have significantly reverted to the normal levels after the therapy, including sphingosine [27]. However, this study was conducted in a model of non-pregnant diabetic rats. A recent report informed about the metabolic profile in women with GDM treated with metformin or insulin [28]. In this study, independently of medication, pregnancy itself had marked influences on amino acid profiles. Metformin treatment of GDM caused a greater increase in serum alanine, isoleucine, and lactate concentrations; this agrees with other previously reports. It was demonstrated that treatment with metformin is associated with increased triglyceride levels and higher atherogenic index of plasma in the third trimester in pregnant women with GDM [29]. Although measures of glucose and C-reactive protein improved with treatment with metformin and insulin, the increase in maternal plasma triglycerides—between randomization to 36 weeks—was greater in women treated with metformin [30,31]. Moreover, previous randomized control

trials of lifestyle advice or metformin in obese or overweight pregnant women have reported little or no effect on standard lipid measurements [32,33].

3.2. Pathway Analysis: Impact on GDM Complications

The metabolic pathways considered as significant (pathway impact > 0.1) in our study were the steroid hormone biosynthesis and TRP metabolism pathways.

In pregnancy, increase in IR occurs due to substantial steroid spectra changes. Major changes in the hypothalamic–pituitary–adrenal/–gonadal axis influence fetal growth and timing of delivery. In the same manner, counter-regulatory hormones—placental growth hormone (GH), glucocorticoid cortisol, and progesterone—progressively increase. It has been reported that gonadal steroids have also been shown to modulate pancreatic function and susceptibility to developing IR and T2D. High levels of androgens are also associated with other serious health consequences, such as high cholesterol, high blood pressure, heart disease, IR, and T2D. Moreover, IR leads to hyperinsulinemia, which is described to induce androgen production and, consequently, hyperandrogenemia directly promotes peripheral IR in women [34]. These mechanisms, when dysregulated, promote the emergence of GDM. Regarding the TRP dysregulation, in a recent study, serum TRP level was found to be significantly higher in T2D and was positively and independently associated with risk of diabetes onset. Patients with higher TRP level tended to present with a higher degree of IR, higher triglycerides, and higher blood pressure [35]. These authors suggest that serum TRP levels increased before IR and T2D, and then depleted gradually along with the progression of T2D. The variation pattern of circulating TRP may represent the compensatory metabolic response to increased oxidative stress related to inflammation as well as the competition with branched-chain amino acids for the same trans-membrane transporter during the development of T2D.

Metabolites of the TRP-kynurenine pathway (i.e., TRP, kynurenine, kynurenic acid, quinolinic acid, 3-hydroxyanthranilic) were also associated with diabetes development in another study [36]. Other authors have reported that downstream bioactive TRP metabolites—kynurenine, kynurenic acid, and quinolinic acid—were

positively and robustly correlated with the severity of kidney disease [18]. The close relationship in the kynurenine pathway between TRP, gamma-interferon, and 2-3-dioxygenase (IDO) as an immuno-modulatory mechanism has since been substantiated [36]. In the specific context of TRP alterations during pregnancy, recent work has also demonstrated similar results: changes in L-TRP in the GDM group were related to an altered serotonin metabolism [37].

TRP is in high demand during pregnancy to meet the increased protein formation for the development of the fetus, and also essential for the production of serotonin in brain, melatonin in the pineal gland, nicotinic acid in liver, etc. This has led to the end for “the tryptophan depletion concept in pregnancy” and its replacement by the “tryptophan utilization concept” [38]. High levels of TRP have also found in pregnancy disorders. A potential role of excessive levels of TRP in preeclampsia has been found, suggesting that high TRP levels can undermine T-cell suppression, resulting in pregnancy complications [39].

The finding of steroid hormones and TRP metabolism dysregulation in our study may be linked with the results obtained in the metabolite set enrichment analysis, where some of the diseases that have been associated with abnormalities in these metabolites are listed (Figure 6). These diseases have been associated with previous GDM history.

Since this study is a pilot and exploratory study, it is limited by a small sample size and the lack of an external validation cohort at the time of the study. The internal cross-validation and univariate methods employed were helpful in validating the OPLS-DA model; this intriguing initial observation, however, requires external validation. Hence, it is imperative that further longitudinal studies be conducted to replicate these results using a larger and more diverse patient cohort.

4. Materials and Methods

4.1. Study Design and Selection of Participants

A cross-sectional study was performed between May and December 2018 to evaluate the metabolomics profile in the 3rd-trimester urine of pregnant women

diagnosed with GDM. The GDM group (n = 24) was composed of patients who were diagnosed with GDM during the second trimester. The control group (n = 11) was constituted by euglycemic women. Patients who developed GDM were matched with women with normal pregnancies based on age and first prenatal body mass index (BMI). The criteria used for the diagnosis of GDM were established in accordance with the parameters established by the American College of Obstetricians and Gynecologists (ACOG) [40]. A routine oral glucose tolerance test (OGTT) was performed at 24–28 weeks' gestation, following the World Health Organization recommendations [41]. Patients enrolled in the study received treatment immediately after the diagnosis and until the delivery. The first-line treatment for pre-gestational and GDM is diet and moderate exercise, which can control up to 70–85% of patients. The first-line pharmacological treatment for gestational diabetes mellitus is insulin, however, in this study, the use of metformin in GDM patients with 20 weeks of gestation or higher was considered as a choice of treatment: (a) when the patient refused the insulin therapy, (b) when the patient was controlled without risk for the binomial, and (c) when the patient stated her agreement with the therapy having signing an informed consent form.

Table 3 summarizes the diagnostic criteria established at Hospital Central. Patients with gestational hypertension, urinary infections, pre-existent T2D, preeclampsia, and chronic renal disease were excluded. Patients were also interviewed and tested for additional comorbidities. Neither cancer nor Polycystic ovary syndrome were reported among the patients. Clinical and demographic data were collected from the medical records for each participant at the first prenatal visit. For GDM patients, levels of glucose, urea, and creatinine were also measured in the 3rd trimester.

4.2. Ethical Considerations

The study was carried out in agreement with the Helsinki Declaration. Signed written informed consent was obtained from all participants prior to interviews and sample collections. The protocol was approved by the Research Ethics Committee of the Hospital Central "Dr. Ignacio Morones Prieto", San Luis Potosi, Mexico, with

Registry: CONBIOETICA-24-CEI-001-201604279. The Registry number of the protocol is 84-17 and it was approved on 19 December 2017.

4.3. Sample Collection and Preparation

Prenatal visits were always scheduled in the morning. Midstream urine samples were collected from each patient at the Hospital Central. The samples were centrifuged at 1200 rpm for 15 min at room temperature (RT) to eliminate cells and/or cellular debris. Then, the urine samples were again centrifuged at 3000 rpm at 4 ◦C, aliquoted and stored at –80 ◦C until use. For metabolomics analysis, urine samples were thawed on ice and vortexed. A 10-microliter aliquot of each sample was pooled to build quality controls (QC) for each group under study. A 100-microliter aliquot of each sample and QCs was diluted with liquid chromatography–mass spectrometry (LC–MS) grade water (1:1 v/v). The mixture was then centrifuged at 14,000 rpm at 4◦C for 15 min. The supernatant was transferred to glass sample vials for UPLC–MS analysis.

4.4. UPLC- MS Method for Metabolomics Analysis

LC–MS grade acetonitrile and water was purchased from JT Baker (Brick Town, NJ, USA). High- purity formic acid (99%) was provided by Thermo Scientific (Rockford, IL, USA).

Samples were analyzed with an ACQUITY UPLC I-Class (Waters Corp., Milford, MA, USA) coupled to a XEVO-G2 XS quadrupole time-of-flight (ToF) mass spectrometer (Waters, Manchester, NH, USA) with an electrospray ionization source. The separation of different metabolites was done in a UPLC Ethylene Bridged Hybrid (BEH) C18 column (2.1 × 100 mm, 1.7 µm) using binary gradient elution of solvents A and B. The mobile phase was A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile. The mobile phase was delivered at a flow rate of 0.5 mL/min, initially with 1% B, followed by a linear gradient to 15% B over 3 min. Then the percentage of B was increased to 50% within 3 min. Over the next 4 min, the gradient was ramped up to 95% B, and the amount of B was then decreased to 1% in 1.1 min. Over 2 min, the percentage of B returned to initial conditions (1%), until the end of

the chromatographic run (13 min). The column temperature was adjusted to 40 ◦C. The injection volume was 5 µL.

Data were acquired in positive electrospray ionization (ESI+) mode with the capillary voltage set to 2.0 kV, the cone voltage to 30 eV and the source temperature to 120 ◦C. The desolvation gas was nitrogen, with a flow rate of 500 L/h and with a temperature of 350 ◦C. Data were acquired from m/z 100 to 2000 in Mass Spectrometry (MSE) mode in which the collision energy was alternated between low energy (6 eV) and high energy (ramped up from 20 to 40 eV).

As a look mass for accurate mass measurements, leucine enkephalin (200 pg/µL in acetonitrile: water (50:50 v/v) + 0.1% formic acid) was infused. For calibration, 0.5 mM sodium formate was used. Five pooled samples (QC) were initially injected to equilibrate the column.

4.5. Data Acquisition and Statistical Analysis

The raw MSE datasets were acquired in continuum mode and processed within UNIFI 1.8.1 (Waters Corp., Milford, USA). The analysis parameters were as follows: retention time of 0–10 min and peak width of 1–30 s. Data within UNIFI 1.8.1 were passed through the apex peak detection and alignment processing algorithms. The intensity of each ion was normalized with respect to the total ion count (TIC) to generate a data matrix that consisted of the retention time, m/z value, and the normalized peak area.

The multivariate data matrix was analyzed by EZinfo software (Waters Corp., Milford, MA, USA) and the univariate analysis was performed with MetaboAnalyst [23]. The data were mean-centered and Pareto-scaled prior to principal component analysis (PCA) and orthogonal projection to latent structures discriminate analysis (OPLS-DA). Potential markers of interest were extracted from the combining VIP plot that was constructed from the loading plots of OPLS-DA. A VIP threshold of 1.5 was considered to select the metabolites.

The nonparametric univariate method, Mann–Whitney–Wilcoxon test was applied to measure the significance of each metabolite, with results adjusted for multiple testing using false discovery rate (FDR) correction, with a mass tolerance of 10 ppm.

Exact molecular mass data (m/z) from significant peaks were used to search the online Human Metabolome Database (HMDB) (http://www.hmdb.ca) for metabolite identities. The identities of key metabolites were confirmed by inspecting the MSE spectra and by comparison of fragmentation pattern with those reported in the HMDB database.

4.6. Pathways Analysis

Metabolite Set Enrichment Analysis and Pathway Analysis were carried out using the pathway analysis module (MetPA) of MetaboAnalyst 3.0. Hypergeometric test and relative betweenness centrality were used for over-representation analysis and pathway topology analysis, respectively.

5. Conclusions

In our pilot study conducted with Mexican women in their the 3rd trimester of pregnancy and previously diagnosed with GDM, we identified 14 metabolites belonging to different classes of compounds which suggest biochemical and metabolic changes orchestrated due to GDM physiopathology. This is, to our knowledge, the first metabolomics study conducted in Mexican women diagnosed with GDM. We found that metabolites from steroid hormone biosynthesis and TRP metabolism pathways could have a significant role in GDM and may be associated with different negative outcomes. The upregulation of these pathways, as a consequence of the oxidative stress and inflammation persistent in GDM, could lead to a higher IR, predisposing to several diabetes-associated complications. These metabolites need to be investigated as potential biomarkers for prognostication in future follow-up studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/20/5/1186/s1, Figure S1: Principal Component Analysis (PCA) scores plots of urine GDM and controls acquired in positive mode.

Table S1: Metabolites differentially expressed ($p < 0.05$) predicted from a multivariate model.

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Figure legends

Figure 1. Levels of (A) glucose, (B) creatinine, and (C) urea measured in plasma of GDM patients before and after GDM diagnosis. Significant differences between the levels in the first prenatal visit (pre-D) and the levels at 3rd trimester (post-D) were found for the three metabolites ($p < 0.05$). Data were analyzed by a paired t-test with the software GraphPad Prism.

Figure 2. Positive ion base peak intensity chromatograms of urine from a healthy control (top) and a GDM patient (bottom) in the third trimester.

Figure 3. Overlaid chromatograms of quality controls (QC) showing good reproducibility for retention time, peak shape, and peak intensity. (A) QCS: overlaid chromatograms of five replicates of QC from the control group. (B) QCD: overlaid chromatograms of five replicates of QC from the GDM group.

Figure 4. (A): Orthogonal partial least-squares discriminant analysis (OPLS-DA) score plots of the urine data set, acquired under positive ion mode. Visual inspection

of the OPLS-DA score plot exhibited tight clusters of samples from each group. (B): Goodness of fit; variance explained R2Y(Cum): 88%, variance predicted Q2(Cum): 59%. (C): Coefficients vs. variable importance in the projection (VIP). The VIP values were also implemented to search for potential biomarkers. Only variables with VIP values higher than 1.5 were highlighted to be important for discrimination. (D): S-plot score plot.

Figure 5. Summary of pathway analysis visualized by MetPA. Steroid hormone biosynthesis and tryptophan metabolism pathways have significant pathway impact (pathway impact > 0.1). The dots represent the pathways that were matched using pathway impact values from pathway topology analysis and p values from pathway enrichment analysis. Colors (varying from yellow to red), means the metabolites are in our data with different levels of significance for enrichment analysis. Other metabolic pathways identified are: Sphyngolipid metabolism, Phenylalanine, Tyrosine and Tryptophan metabolism, Nitrogen metabolism, Nicotinate and nicotinamide metabolism, Glycine, serine and threonine metabolism, Starch and sucrose metabolism, Pentose and glucuronate interconversions, Aminocyl t-RNA biosynthesis, Arginine and proline metabolism, and Purine metabolism.

Figure 6. Metabolite set enrichment analysis for the prediction of disease associated metabolite sets (urine).

Table 1. General clinical characteristics of the pregnant women included in the study.

	GDM (n = 24)	Control (n = 11)	p-Value
Age (years)	31.00 ± 1.401	26.91 ± 1.232	0.0771
Gestational age at sampling (weeks)	32.05 ± 0.9026	34.74 ± 0.6853	0.0672
BMI (Kg/m^2)	30.07 ± 1.268	25.98 ± 1.594	0.0546
Glucose (mg/dL)	92.75 ± 3.174	76.70 ± 1.764	0.0024
SBP (mmHg)	110.4 ± 9.079	108.2 ± 9.816	0.8781
DBP (mmHg)	73.75 ± 9.816	71.82 ± 7.109	0.8161
Creatinine (mg/dL)	0.5262 ± 0.02066	0.5500 ± 0.03643	0.5436
Urea (mg/dL)	13.83 ± 0.9461	16.12 ± 1.734	0.2144
Hb (g/dL)	12.52 ± 0.8055	13.26 ± 1.074	0.1453
Leucocytes ($\times 10^3$)	8.739 ± 1.756	8.000 ± 1.525	0.2339

Data are presented as mean ± standard error of mean (SEM). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure. p-values were determined by independent t-test or Mann–Whitney test. Statistically significant: $p < 0.05$.

Table 2. Differential metabolites dysregulated in GDM urine.

Class	Compound	HMDB	Molecular Formula	Observed *m/z*	RT (min)	*p*-value	FC	Mass Error (mDa)	Adducts
Benzopyrans	**5-carboxy-alpha-chromanol**	HMDB0012798	$C_{19}H_{28}O_4$	338.2322	5.82	2×10^{-4} [a]	4.2	2.0	$NH4^+$
Carboxylic acids and derivatives	**1-Methyl N- -alpha-aspartyl- -phenylalanate (Aspartame)**	HMDB0001894	$C_{14}H_{18}N_2O_5$	295.12879	3.32	4.45×10^{-4} [a]	5.1	0.2	H^+
Glycerolipids	DG (24:0/14:1)	HMDB0007792	$C_{41}H_{78}O_5$	668.6179	9.87	6.36×10^{-4} [a]	3.6	−0.9	NH^{4+}
Indoles and derivatives	L-Tryptophan	HMDB0000929	$C_{11}H_{12}N_2O_2$	205.09703	2.90	3.08×10^{-6} [a]	2.4	−0.5	H^+
Tetrapyrroles and derivatives	L-Urobilinogen	HMDB0004157	$C_{33}H_{48}N_4O_6$	597.3631	5.94	3.75×10^{-3} [a]	3.2	−1.6	H^+
Sphingolipids	Cer (d18:0/23:0)	HMDB0011767	$C_{41}H_{83}NO_3$	638.60751	9.92	5.0×10^{-7} [a]	11	0.1	H^+
	SM (d18:0/22:0)	HMDB0012091	$C_{45}H_{93}N_2O_6P$	789.685	9.62	4.96×10^{-6} [a]	4.2	0.6	H^+
Steroid and steroid derivatives	11-oxo-androsterone-glucuronide	HMDB0010338	$C_{25}H_{36}O_9$	503.2247	5.5	5.44×10^{-4} [a]	2.6	−0.5	Na^+
	cortolone-3-glucuronide	HMDB0010320	$C_{27}H_{42}O_{11}$	543.2789	5.12	5.80×10^{-4} [a]	2.9	−1.1	H^+
	tetrahydroaldosterone-3-glucuronide	HMDB0010357	$C_{27}H_{40}O_{11}$	563.2456	5.09	2.38×10^{-5} [a]	4.6	−0.7	Na^+
	5-androstene-3b,16b,17a-triol	HMDB0000523	$C_{19}H_{30}O_3$	329.21105	5.09	6.10×10^{-5} [a]	4.4	1.9	Na^+
	21-deoxycortisol	HMDB0004030	$C_{21}H_{30}O_4$	347.22121	5.26	2.45×10^{-4} [a]	3.7	−0.6	H^+
	11b, 17a,21-Triydroxypreg-nonolone	HMDB0006760	$C_{21}H_{32}O_5$	365.2317	5.09	2.0×10^{-3} [a]	3.1	−0.4	H^+
	cucurbitacin c	HMDB0034706	$C_{32}H_{48}O$	561.3411	5.92	3.00×10^{-4} [a]	3.3	0.1	H^+

a: significative for false discovery rate (FDR) correction. FC: Fold change. Metabolites in bold are exogenous metabolites.

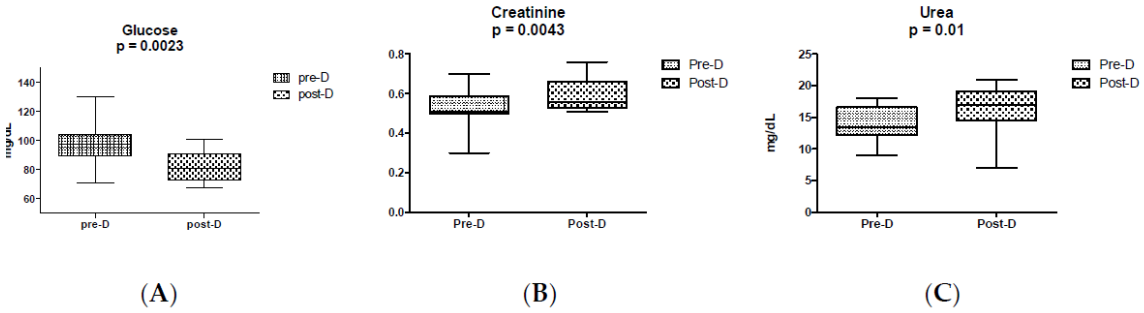
Table 3. Diagnostic criteria for GDM.

Procedure	Glucose Cut Points [a]		
	Time (h)	mg/dL	mmol/L
Step 1: 50 g	Fasting	≥140 mg/dL	7.8
Step 2: 100 g, 3 h OGTT [b]	Fasting	≥95 mg/dL	5.3
	1	≥180 mg/dL	10.0
	2	≥155 mg/dL	8.6
	3	≥140 mg/dL	7.8
75 g, 2 h OGTT [b]	Fasting	≥92 mg/dL	5.1
	1	≥180 mg/dL	10.0
	2	≥153 mg/dL	8.4

a: venous serum or plasma glucose measured at the hospital laboratory. b: Two values meeting or exceeding the cut points are required for diagnosis.

Figures

Figure 1



(A) (B) (C)

Figure 2

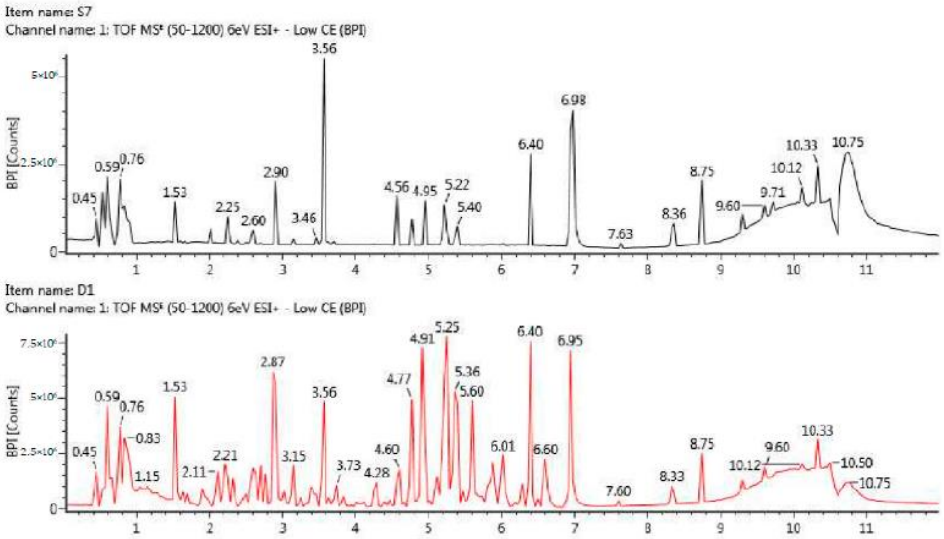
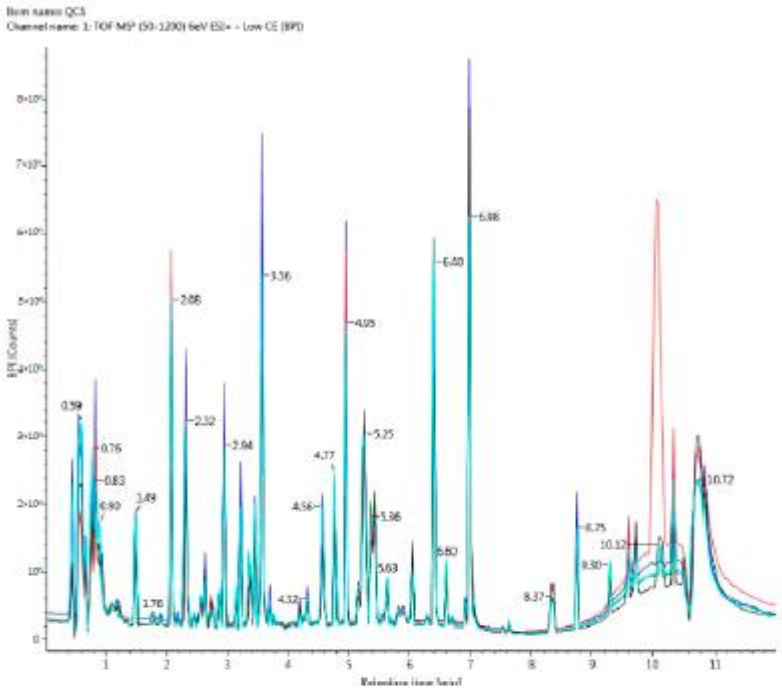
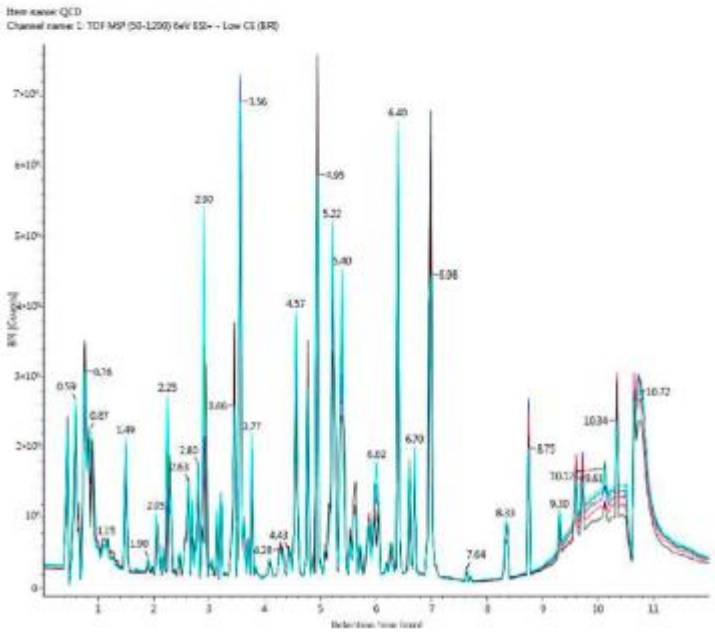


Figure 3

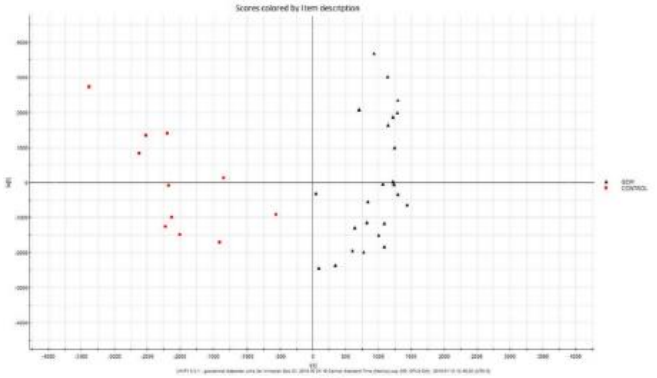


(A)

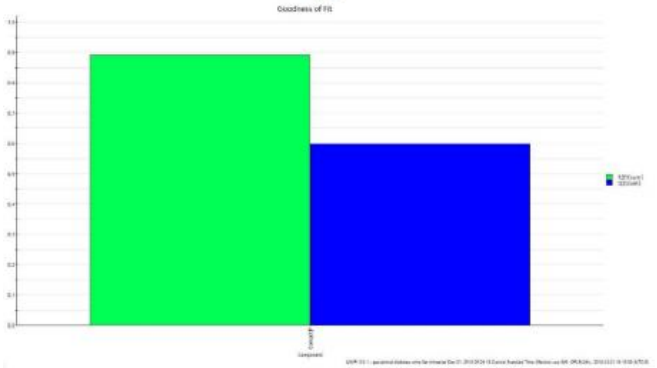


(B)

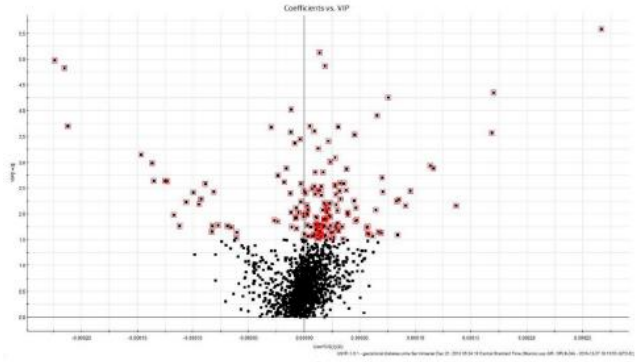
Figure 4


Scores colored by Item description
CONTROL

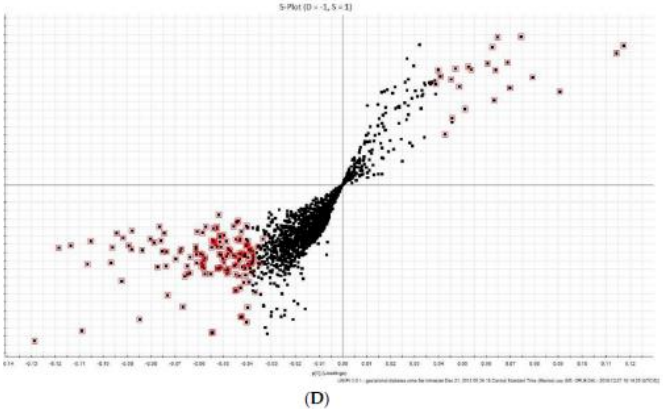
(A)


Goodness of Fit

(B)


Coefficients vs. VIP

(C)


S-Plot (0 = -1, 5 = 1)

(D)

Figure 5

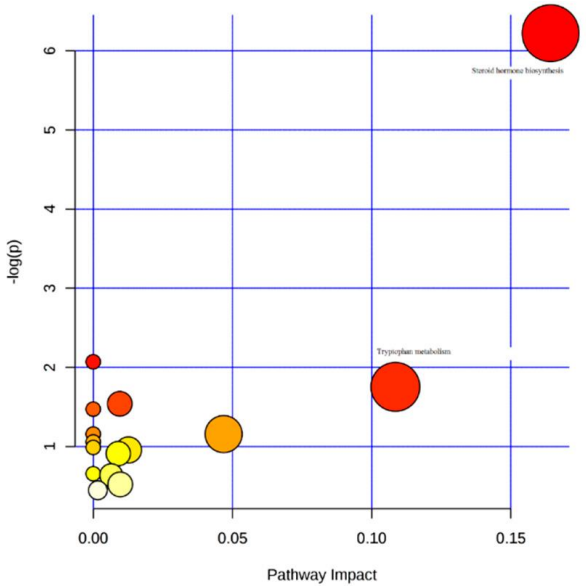
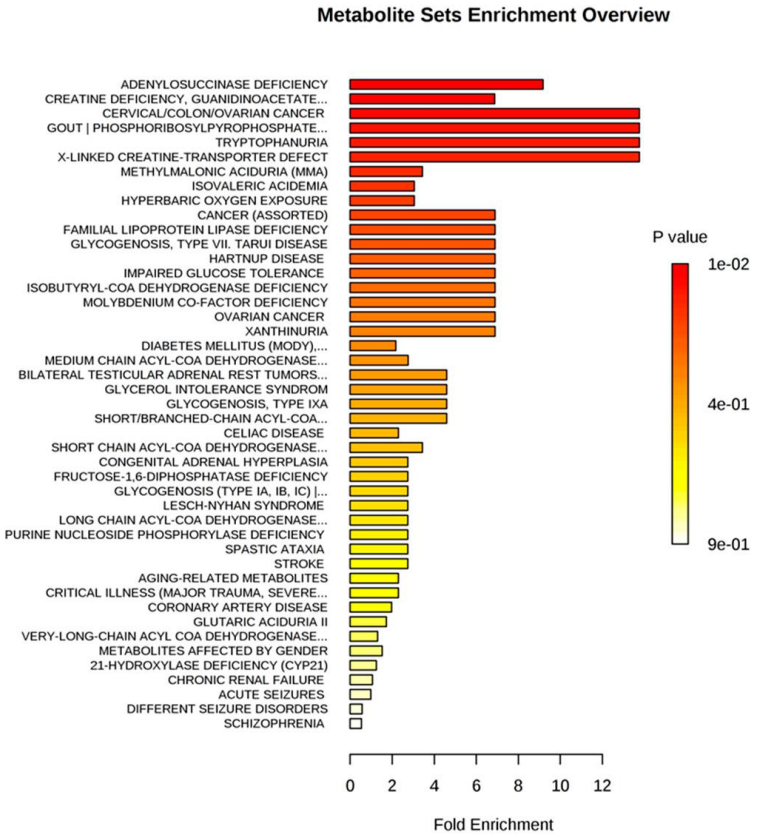


Figure 6



The Urinary Metabolome of Healthy Newborns

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Abstract: The knowledge of normal metabolite values for neonates is key to establishing robust cut-off values to diagnose diseases, to predict the occurrence of new diseases, to monitor a neonate's metabolism, or to assess their general health status. For full term-newborns, many reference biochemical values are available for blood, serum, plasma and cerebrospinal fluid. However, there is a surprising lack of information about normal urine concentration values for a large number of important metabolites in neonates. In the present work, we used targeted tandem mass spectrometry (MS/MS)-based metabolomic assays to identify and quantify 136 metabolites of biomedical interest in the urine from 48 healthy, full-term term neonates, collected in the first 24 h of life. In addition to this experimental study, we performed a literature review (covering the past eight years and over 500 papers) to update the references values in the Human Metabolome Database/Urine Metabolome Database (HMDB/UMDB). Notably, 86 of the experimentally measured urinary metabolites are being reported in neonates/infants for the first time and another 20 metabolites are being reported in human urine for the first time ever. Sex differences were found for 15 metabolites. The literature review allowed us to identify another 78 urinary metabolites with concentration data. As a result, reference concentration values and ranges for 378 neonatal urinary metabolites are now publicly accessible via the HMDB.

Keywords: newborn; metabolites; tandem mass spectrometry; inborn errors of metabolism; reference values.

1. Introduction

According to the 2018 WHO annual report, across the globe, 2.5 million children died in the first month of life. This represents 7000 newborn deaths per day. One third of these deaths occur in the first 24 h of life, while three quarters of infant deaths occur during the first week after birth [1]. Some of the most important causes of neonatal death are preterm birth, intrapartum related events, sepsis/tetanus, infections and congenital abnormalities [2,3].

A neonate is formally defined as a baby that is four weeks old or younger. The neonatal period, a time during which a baby is particularly small and fragile, is characterized by continuous and rapid changes in behavior, physiology and metabolism. During the neonatal stage, a number of important physiological events take place, including the establishment of feeding patterns, remodeling of the immune system, changes to the endocrine system, as well as modifications to the infant's overall metabolism. Usually, the common hospital stay for a newborn is 48 h after a vaginal delivery and 96 h after a caesarean section. However, when medical conditions appear shortly after birth, longer periods of hospitalization are required. It is during these extended stays that multiple hematological, urinary, clinical and biochemical tests are often performed. Therefore, the availability of reference values for hematological, urinary, clinical and biochemical parameters for newborns and neonates is of critical importance.

Indeed, the use of neonatal reference values for biochemical tests (on blood or urine) of selected metabolites have been the basis of newborn screening programs around the world for more than 50 years. While the first tests, introduced by Dr. Robert Guthrie to diagnose inborn errors of metabolism (IEMs), were quite limited and based on simple enzymatic assays [4], over the past 55 years, these biochemical tests have been expanded and improved significantly. Tandem mass (MS/MS) spectrometry is now widely used in the screening of newborns and has greatly expanded the number of metabolite measurements that can be performed [5]. The specificity and sensitivity of tandem mass spectrometry methods may reach values up to 99.99% and 99%, respectively, for IEM diagnoses. With the advent of LC-MS and direct infusion tandem mass spectrometry (DI-MS/MS), an increased number of

less common metabolites and correspondingly rarer IEMs can now be detected with relative ease.

For full term-newborns, infants and children, a modest number of reference biochemical values are available for blood, serum, plasma, urine and cerebrospinal fluid. This information is also available for preterm newborns [6–10]. However, reference values for many common urinary metabolites in newborns are lacking. Indeed, an in-depth literature review, along with detailed comparisons to values reported in the Human Metabolome Database (HMDB) [11] and the human Urine Metabolome Database (UMDB) [12] indicates that only 212 metabolites have reported urinary reference values for newborns and infants (detected and quantified). In contrast, the number of urinary metabolites with adult reference values is >2000 [12]. This suggests that there is a surprising lack of information about normal urine concentration values for a large number of important metabolites in neonates.

In the present work, we used targeted MS/MS assays to identify and quantify 136 metabolites of biomedical interest in the urine from 48 healthy, full-term term neonates collected in the first 24 h of life. The targeted assays that we employed use a combination of liquid chromatography tandem mass spectrometry (LC-MS/MS) and flow injection analysis (FIA-MS/MS). In addition to this experimental study, we also performed a detailed literature review (covering the past eight years and a total of 509 papers) to update the information on neonatal urinary concentration data in the HMDB [11] and the UMDB [12]. Combined with the experimental data reported in this study, the total number of neonatal urinary metabolites with reference concentration data has now grown to 378. By conducting this combined experimental/literature review study, we have now nearly doubled the number of urinary metabolites, with reference concentrations for neonates and infants. All of these metabolites and reference values have now been added to the HMDB [11] and the UMDB [12], and are publicly available at www.hmdb.ca.

2. Results

Table 1 shows the clinical and phenotypic data collected from the 48 singleton, healthy full-term neonates, along with the relevant clinical data from the mothers.

2.1. Concentration Data of 136 Metabolites Measured by Targeted Metabolomics

In the present study, we experimentally determined the concentrations of 136 metabolites, selected due to their known involvement in multiple metabolic pathways associated with metabolic or genetic disorders (such as IEMs), that could manifest in the first 24 h of life. A total of 45 amino acids and biogenic amines, 17 organic acids, 24 glycerophospholipids, 10 sphingomyelins and 40 carnitines and acylcarnitines were detected and quantified in every urine sample. Supplementary Table S1 shows the limit of detection (LOD) for each measured metabolite using our LC-MS/MS approach.

Figure 1a–c show the quantitative results we obtained using our LC-MS/MS method, expressed as mean ± SD for metabolites previously reported in newborns. Additionally, Supplementary Tables S2 and S3 contain the measured concentration data (mean ± SD, raw and normalized to creatinine) for these metabolites. We also report the urinary reference values found in the literature for these compounds, along with values reported in the HMDB [11] and UMDB [12]. When available, the reference value is listed for newborns or neonates. However, when this data is not available, a reference value listed for infants (which is generally assumed to cover babies aged one week to one year) is provided.

Tables 2 and 3 contain the experimental results for metabolites not previously reported in newborn and in human urine, respectively, including the absolute concentration (μM, expressed by mean ± standard deviation (SD)); creatinine-normalized values (μM/mM creatinine) expressed as a mean ± SD; and the 2.5–97.5% percentile range (μM/mM creatinine).

Urinary creatinine (Ucreat) is often used to adjust or normalize urinary analyte concentrations, but we found that Ucreat appeared to be a relatively unreliable reference value in the early newborn period, due to its high variation (ranging from 1000 to 17,000 mM). For this reason, we also provide the absolute concentrations of each metabolite measured in urine.

A total of 86 of these experimentally measured urinary metabolites (64%), along with their concentration ranges, are being reported in neonates/infants for the first time. Of these, 20 metabolites (14% of the compounds identified in this study) are being reported in human urine for the first time ever. Note that the concentration data for some metabolites found through our literature and database searches had more than one reference value, obtained by different laboratories using different methods. This can lead to some minor discrepancies for the numbers reported in these tables.

2.2. Gender-Sex Differences Associated Metabolites

In addition to the combined sex (male + female) results, we also investigated the presence of any sex differences in the measured metabolites. We found that three metabolites (uric acid, butyric acid and octadecadienylcarnitine (C18:2)) were consistently higher ($p < 0.05$) in males than females, while the following 12 metabolites (creatinine, symmetric dimethylarginine, spermine, spermidine, LysoPC a 17:0, LysoPC a 18:1, SM C16:0, SM(OH) C16:1, PC aa 36:0, SM(OH)24:1, PC aa 40:2, and PCaa 40:1) were consistently lower in males than females ($p < 0.05$) (Supplementary Figures S1 and S2). A complete set of tables showing the sex-specific values is included in the Supplementary Material (Supplementary Tables S4–S8). Additionally, the sex-specific values for these urinary metabolites are now available in the HMDB [11] and UMDB [12].

2.3. Impact of Resolution Mode on the Urinary Metabolome of Healthy Newborns

In the present study, 22 newborns were born by vaginal delivery (VD) and 26 newborns were born by caesarian section (CS). Babies born by VD had significantly lower levels of asparagine, lysine and arginine than babies born by CS. Moreover, an increase in glutaconylcarnitine and nonaylcarnitine was also found in VD babies (Supplementary Figure S3).

2.4. Literature Review of the Urinary Metabolome of Healthy Newborns

Finally, we conducted a thorough literature review of other neonatal/infant metabolite concentrations reported over the past eight years, and used this information to

supplement the neonatal urinary data reported in the HMDB and UMDB (www.hmdb.ca). This literature review, which covered an initial set of 509 papers, allowed us to identify another 78 urinary metabolites, with concentration data measured by different platforms (LC-MS/MS, HPLC, 1H-NMR, FIA-MS/MS and GC-MS). Combined with the 212 neonatal urinary metabolites previously reported in the HMDB/UMDB and the 86 neonatal urinary metabolites experimentally detected and reported here, there are now 378 neonatal urinary metabolites that have reported concentration values, and which are publicly accessible via the HMDB or UMDB (Figure 2).

3. Discussion

Over the past decade, our team, as part of the Human Metabolome Project (HMP), has been systematically characterizing human biofluids using a combination of quantitative metabolomic techniques and literature analyses. During that time, we have characterized human (adult) serum [13], cerebrospinal fluid [14], urine [12], saliva [15] and stool samples [16]. Other groups have also analyzed the metabolomes for human breath [17], breast milk [18], bile fluids [19] and hair [20]. We undertook this study to fill in important gaps in our understanding of urinary metabolite concentrations in neonates. While a number of databases and reference textbooks are available that provide reference values for different populations (age, gender, ethnicity) for serum, plasma, CSF and other fluids in newborns [12,13,21–23], there is a paucity of information on urine reference values for newborns and infants. Given that urine samples can be non-invasively collected (via diapers or other simple collection mechanisms) and given that urine provides an invaluable readout of general metabolism, as well as kidney, liver and gut microbiome function, we believe that the development of a reference set of urine metabolites would be highly valuable.

3.1. Comparison of Experimental Values with Reported Reference Values

As described here, we were able to experimentally measure 136 urinary metabolites in 48 full-term healthy neonates. Our combined experimental and literature approach has nearly doubled the total number of metabolites and reference ranges reported

for neonatal/infant urine. In addition to greatly expanding the knowledge of neonatal urine composition, we were also able to quantify 20 metabolites (including acylcarnitines and glycerophospholipds), that had never been reported in human urine previously. Given that several well-known metabolic disorders involve the dysregulation of lipid metabolism and molecules transporting lipids, we believe it is very important to monitor the abundance of these types of compounds in biological fluids. In the specific case of the population selected for this study, several babies were born to mothers who, in some cases, were overweight (see Table 1). The monitoring of lipids during the early stages in life may be useful in preventing the development of future metabolic disorders (such as diabetes or dyslipidemia) in the children of overweight or obese mothers.

In general, the experimental values reported by us are in accordance with previous reference values consulted in the UMDB and the Metagene database (http://metagene.de). We only found notable discrepancies in the concentration values of six compounds: taurine, carnosine, butyric acid, isobutyric acid, 3-hydroxyphenyl-3-hydroxypropionic acid (HPHPA) and indoleacetic acid (Supplementary Table S2). In our study, the interquartile (IQ) range for taurine was 74.64–2841 µmol/mmol creatinine. The reference value reported in the UMDB was 250–910 µmol/mmol creatinine (using NMR) [24]. The abnormal concentration quoted for taurine in the HMDB is 1261 µmol/mmol creatinine, associated with molybdenum cofactor deficiency [25]. However, since all babies included in our study were healthy babies, we must consider other factors contributing to this discrepancy. Relatively few reports on taurine in the perinatal period have been published, but Zaima et al. [26] demonstrated that, in newborns, the urinary taurine concentration was 6222.3 µmol/L on the first day; 1620.1 µmol/L on the third day and on the fifth day 419.3 µmol/L or 1/15 of that of the first day. The daily urinary excretion of taurine was 74.3 micromoles/day on the first day; 79.1 micromoles/day on the third day and 22.7 micromoles/day on the fifth day. So, this suggests that, depending on the day of sampling, the results may be different, with a very large difference between the first and third day after birth. The results obtained in newborns by us correspond to the first 24 h after birth and appear to match well with the data reported by Zaima et

al. Taurine accumulates in the maternal tissue during pregnancy to provide the fetus, via the placenta, with adequate levels and to the newborn via breast milk. Low maternal taurine levels result in low fetal levels [27], but high levels of taurine have been found in maternal plasma in the third trimester among GDM Hispanic women treated with insulin, reflecting altered protein metabolism [28].

We also found that the measured urinary concentration of carnosine was consistently lower (IQ 0.47–35.5 µmol/mmol creatinine) than the value reported in previous literature (IQ 3.05–115.4 µmol/mmol creatinine), which was reportedly measured by the same method (LC-MS/MS) [29]. Carnosine is a dipeptide of the amino acids β-alanine and histidine. It is highly concentrated in muscle and brain tissues. It has been reported that carnosine increases with age as the muscle mass increases in newborns [30]. Because the age of the neonates cited in reference [14] is not known, and given that the urinary samples evaluated in the present work belong to the first 24 h of life, we suspect that the concentration differences in carnosine are likely due to minor age differences in the neonate cohorts being sampled, not unlike those seen with taurine.

In addition to taurine and carnosine, the concentration values of four organic acids (butyric acid, isobutyric acid, 3-hydroxyphenyl-3-hydroxypropionic acid [HPHPA] and indoleacetic acid) are lower than the respective reference values reported for these metabolites (Supplementary Table S2). In the specific case of butyric, isobutyric acid and HPHPA, the literature-derived reference values found by us and listed in Supplementary Table S2 (marked with asterisks) belong to infants (4 weeks to 1 year) and not to neonates (<4 weeks). We suspect that this is the primary reason why our values are significantly lower (by a factor of 5–10) than the reported reference values. It is well known that during the first hours of life, glycogen stores are depleted, and protein catabolism contributes little to energy requirements. This particular metabolic situation seems to be reflected in the reduced urinary excretion of certain organic acids (including butyric and isobutyric acid) compared to older individuals where glycogen stores are fully restored [31]. Furthermore, indoleacetic acid measured in our neonatal cohort were also found to be 10-fold lower than what has been reported in the literature for neonates [32]. Both HPHPA and indoleacetic

acid are known gut microbial metabolites, with HPHPA excretion being associated with gut microbial degradation of dietary phenylalanine or polyphenols [33] and indoleacetic acid being a microbially derived breakdown product of tryptophan [33]. Neonates typically do not have a well-established gut microflora, while older infants do. This difference in intestinal microflora likely contributes to the age-related difference in these two microbially-derived metabolites [34].

3.2. Gender-Sex Differences Associated Metabolites

Through this study, we were also able to identify a number of metabolites that exhibit clear sex-dependent trends, including uric acid, butyric acid and octadecadienylcarnitine (C18:2), which are increased in males, relative to females. In addition, we found that creatinine, symmetric dimethylarginine, spermine, spermidine, LysoPC a 17:0, LysoPC a 18:1, SM C16:0, SM(OH) C16:1, PC aa 36:0, SM(OH)24:1, PC aa 40:2, and PC aa 40:1 are increased in the urine of females, relative to males. The influence of gender in the early newborn metabolome has not been well studied and reliable reference intervals for males and females have not been extensively reported. It is commonly understood that sex differences start in utero [35] and the implementation of sex or gender-dependent strategies in laboratory medicine may help to obtain the correct diagnosis of diseases affecting newborns. Only a handful of studies have been published addressing the effect of gender in the urinary newborn metabolome [30,36,37]. In particular, Diaz et al. [37] reported that allantoin and xanthine levels are higher in the urine of females than in males, which suggests a slightly altered nitrogen metabolism in females, compared to males. In addition, glucose and lactose were also found to be higher in females, but infant females also had lower levels of inositols and other sugars, suggesting that changes in sugar metabolism may be associated with gender in the first days of life [37]. More recently, Caterino et al. [36] analyzed urinary organic acids in healthy Caucasian infants and children (aged 1 month to 36 months), and reported that in the first six months of life, sex differences were more frequent, and the majority of urinary organic acids were higher in males than in females. The authors conclude that sex deeply influences urinary organic acids levels [36]. In our study, we only

found significant sex differences in the level of two organic acids: uric acid and butyric acid (higher in males than in females).

3.3. Impact of Birth Resolution on the Urinary Metabolome of Healthy Newborns

We found significantly lower levels of asparagine, lysine and arginine in the urine of neonates born by VD, than in those born by CS. Moreover, an increase in glutaconylcarnitine and nonaylcarnitine was found in the urine of VD neonates. Early in the postnatal period, major physiological adaptations occur in newborns to cope with stress and extrauterine cold exposure upon exiting the womb. Differences found in relation to delivery mode have been associated with difference in lower gut microflora colonization as well as in alterations of hepatic metabolism [37]. In a recent study, Pierre Martin et al. [38] reported that VD newborns had lower urinary levels of lysine (as we found), as well as lower levels of histidine, relative to CS newborns. It is also important to remember that lysine is involved in carnitine biosynthesis. Indeed, the two main precursors for carnitine biosynthesis are lysine and methionine, which provide the carbon backbone and 4-N-methyl groups of carnitine, respectively. The substrate for carnitine biosynthesis is 6-N-trimethyllysine (TML) [39]. Using up reserves of lysine to produce TML to synthesize carnitine and acylcarnitines would be expected to lead to low levels of lysine. This is consistent with the increased urinary excretion of acylcarnitines and the decreased excretion of lysine in VD newborns found by us. It is also possible that these metabolic differences may arise from the differential nutrition between VD and CS newborns. Neonates from our cohort born by VD were fed with breast milk immediately after delivery. Breast milk is known to be rich in carnitine. The development of ketogenesis in the human neonate is greatly dependent on the exogenous supply of carnitine, because the liver has a limited capacity for de novo carnitine synthesis [40]. Given that VD versus CS metabolic differences were not the primary focus of this study, it is clear that additional mechanistic studies are warranted.

3.4. Urinary Metabolites Associated with IEMs

While most IEM tests are designed for blood or dried blood spots, many health institutions also perform urine tests using targeted assays that employ liquid or gas chromatography coupled with tandem mass spectrometry [41,42]. In a recent study, Kennedy et al. [43] identified (but did not quantify) over 1200 molecules from among 100 clinical urine samples from children (average age of 4.3 years). This study showed clear biochemical signatures for 16 of the 18 IEM diseases tested. This work nicely illustrated the utility of urinary metabolomics for assessing IEMs [43]. Supplementary Table S9 lists the metabolites included in our experimental approach that have been previously identified as urinary markers for different IEMs. A number of IEMs, such as those related to amino acid metabolism; creatinine disorders; fatty acid metabolism and β-oxidation and organic acid disorders; peroxisomal biogenesis and metabolism; aminolevulinic acid dehydratase deficiency; purine and pyrimidine metabolism and urea cycle disorders, can be diagnosed via urinalysis. Urinary metabolic profiling can be used to detect altered levels of intermediate metabolites that result from the incomplete metabolism of amino acids or organic acids.

Carnitines and acylcarnitines are usually measured in plasma to detect IEMs. However, genetic defects in the OCTN2 carnitine transporter can result in a condition known as primary carnitine deficiency. This is associated with a decreased accumulation of intracellular carnitine, higher levels of carnitine in the urine and low levels of carnitine in serum. Because carnitine is transferred from the mother to the child via the placenta, shortly after birth, the levels of carnitine in newborns with carnitine transporter defects could, artefactually, be normal. However, this deficiency could be more easily diagnosed in urine, by detecting an increase in urinary carnitine. Therefore, to properly detect this condition, the additional analysis of urinary organic acids in conjunction with the clinical presentation would allow one to correctly diagnose it [44]. In the study reported by Kennedy et al. [43], they measured urinary concentrations of β-hydroxyisovaleroylcarnitine, α-hydroxyisovalerylcarnitine, tiglylcarnitine, succinylcarnitine, malonylcarnitine, 3-methylglutarylcarnitine and glutarylcarnitine. These authors demonstrated the comparable usefulness of these acylcarnitine biomarkers when determined both in plasma and in urine, for the correct diagnosis of holocarboxylase deficiency and

lysinuric protein intolerance [43]. However, to use urinary acylcarnitines in the diagnosis of IEMs, it is necessary to have reference values for normal concentrations of urinary acylcarnitines. Prior to our work (presented in Table S6), reference concentration values for most neonatal urinary acylcarnitines were not previously available.

In this work, we also provide the urinary concentration values for 24 glycerophospholipids and 10 sphingomyelins. There are at least 40 IEMs with neurological/muscular presentations related to the defects in phospholipid, sphingolipid and long chain fatty acid biosynthesis [45]. Additionally, there are more than 100 IEMs that may lead to primary or secondary defects of complex lipids synthesis and remodeling [46]. Supplementary Table S9 shows the associated IEMs to urinary markers experimentally measured in the present work and the abnormal concentrations reported in literature to diagnose these conditions. We believe that, with the availability of so much more referential data for neonatal urine, it should now be possible to identify a number of other previously undetected or unsuspected IEMs or metabolic conditions in neonates via urinalysis.

3.5. The Importance of Age-Specific Intervals

The availability of quantitatively measured metabolites in biofluids of newborns, particularly in urine, provides extensive and dynamic information that, if followed and controlled over time, can be useful in predicting the “biological age” of infants. Biological age (as opposed to calendar age) is greatly influenced by several factors, such as diet, stress, environment, lifestyle, genetics and disease [47]. Since these factors play important roles in determining the metabolome, urinary metabolomics may provide reliable and sensitive markers to understand the complexity of age-related changes, leading to the identification of novel treatments or strategies for the management of health and disease in early childhood [48]. These age-specific data may also be used for the establishment of inflection points related to metabolic disorders, which is very important in accurate disease diagnosis or prognosis. In this sense, it is important to keep in mind that metabolite concentrations change continuously with growth and age, especially with children and infants. This is often

not fully realized, due to the fact that the majority of clinical studies that have been published are for adults [49–55]. Indeed, to date, there have only been a few comprehensive studies looking at age-specific intervals in children [48,56,57] and newborns [30].

Among the urinary metabolites that are known to vary considerably with age are carnitine, 3-hydroxyisovalerate, creatinine, alanine, and trigonelline. Indeed, these metabolites differ significantly between younger and older groups [54]. Urinary trimethylamine-N-oxide (TMAO) has been found to be higher in infants (aged 1 week–1 year), which may be directly associated to the consumption of milk at this age and/or the corresponding gut microflora found in infants, due to breast feeding [48]. Urinary glycine and glutamine levels have been reported to decline significantly within the first year of life, probably due to their use in supporting the increased growth of skeletal muscle tissue during infancy [48]. Another important metabolite measured in urine that changes with age is creatinine (Ucreat). In particular, it has been noted that creatinine levels increase as children age. However, only a few studies have attempted to measure the values of Ucreat in the first days of life. It has been demonstrated that the mean Ucreat concentration is significantly higher in neonates than in older children [58]. Furthermore, Ucreat is also highly variable, until it begins to stabilize by the first month. Creatinine levels at birth typically do not yet reflect neonatal creatinine clearance, but rather maternal creatinine clearance. Furthermore, because of passive tubular back leak in infants instead of active secretion, creatinine clearance does not yet fully reflect GFR (glomerular filtration rate). Supplementary Table S10 shows a comparison between metabolite concentrations (normalized to creatinine) measured in the present study and in a previous study conducted by our group on healthy adult urine samples [12]. By limiting the comparison to samples analyzed in the same lab using largely the same techniques and workflow (from pre-analytical to post-analytical analysis), we could ensure that any inter-laboratory variation was minimized. From a total of 79 common metabolites, 33 showed a clear, decreasing trend with age, while most others were largely unchanged. In comparing adult urine with newborn urine, we found that a number of amino acids were much higher in newborns than adults, including glycine,

alanine, serine, proline, histidine, lysine, methionine, and most branched-chain amino acids. Amino acids have been previously reported to decrease with age and this phenomenon likely arises from age-dependent changes in cell growth, tissue growth and muscle metabolism [59]. High levels of glycine, serine and proline in newborn urine may be correlated with generally high levels of these amino acids in the newborn body, as high levels of water-soluble metabolites in the urine strongly correlated with levels of water-soluble metabolites in the blood or tissues. These small amino acids are likely needed to support high levels of collagen synthesis in newborns (the most abundant protein in the body). High levels of essential amino acids in newborn urine also reflect their high levels throughout the newborn body. Essential amino acids are obviously needed by newborns to support rapid cell division and cell growth during the first weeks of life [60]. Another group of molecules that showed a decreasing trend with age are biogenic amines. This is in line with previous reports. For example, in children (at five years of age), muscle carnosine levels are initially low but, as children grow, carnosine levels gradually increase before declining and reaching a plateau in adulthood [61]. Dopamine is also observed to be lower in adults than in newborns. This decrease may be explained by the age-related decline in the integrity of the dopamine system, which is seen in most adults [62]. Urinary polyamines, such as putrescine, spermidine and spermine, were also seen to decline with age in our newborn-adult comparison. This result mirrors a previous study that showed that polyamines declined progressively with age [63]. Polyamines are typically elevated in cells that are rapidly dividing (as might be expected for newborns) and can often be seen as metabolic by-products in cancer (in adults).

4. Materials and Methods

4.1. Sample Collection and Research Ethics Approvals

This was a cross-sectional study carried out at the Hospital Central “Dr. Ignacio Morones Prieto”, San Luis Potosi, Mexico, from January 2018 to August 2019. The study was approved by the Research and Ethics Committee, with the registration number 84-17 and folio CONBIOETICA-24-CEI-001-201604279. The protocol

complied with the Declaration of Helsinki. Written informed consent was obtained from the parents of all studied subjects.

At the moment of birth, all newborns were carefully examined by a trained neonatologist. Variables such as weight, sex, Apgar score at 1 min, Apgar score at 5 min, pregnancy resolution, gestational age (Capurro test) and Silverman–Anderson test scores were determined and recorded for each newborn as expediently as possible. After this assessment was complete, one urine sample was collected noninvasively for each newborn. The genitals were cleaned thoroughly, and a sterile bag was placed on the genital area until micturition.

Urine samples contaminated with meconium were discarded. After visual inspection of the urine sample and after at least 1 mL of urine had been excreted by the infant, the sterile bag was removed. The urine sample in the bag was transferred via a micropipette to a sterile 1.5 mL Eppendorf tube. The urine samples were then centrifuged at 3000 rpm to precipitate sediments. Following this, the samples were stored in sterile microtubes at −80 ◦C until further use.

4.2. Chemicals and Internal Standards (ISTD)

Optima™ LC/MS grade formic acid and HPLC grade water were purchased from Fisher Scientific (Ottawa, ON, Canada). Optima™ LC/MS grade ammonium acetate, phenylisothiocyanate (PITC), 3-nitrophenylhydrazine (3-NPH), HPLC grade methanol and HPLC grade acetonitrile (ACN) were also purchased from Sigma-Aldrich (Oakville, ON, Canada). Furthermore, 2H-, 13C-, and 15N-labelled compounds were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA) and from Sigma-Aldrich (Oakville, ON, Canada).

A working internal standard (ISTD) solution mixture in water (for amino acids, biogenic amines, carbohydrates, carnitines and derivatives, phosphatidylcholines and their derivatives) was made by mixing all the prepared isotope-labeled stock solutions together. For organic acids, a working internal standard (ISTD) solution mixture in 75% aqueous methanol was made. All standard solutions were aliquoted and stored at -80 ◦C until further use.

4.3. Metabolite Measurement

4.3.1. Sample Preparation

The urine samples were thawed on ice before analysis. For the analysis of organic acids, 10 µL of an internal standard (ISTD) mixture solution and 10 µL of the samples (three phosphate buffered saline [PBS] blank samples, seven calibration standards, three quality control samples and urine samples) were pipetted directly into the center of corresponding spot in a 96-deep well plate. 30 µL of 75% aqueous methanol was then added to each of the wells, followed by adding 25 µL to each of the following three solutions: 1) 3-nitrophenylhydrazine (250 mM in 50% aqueous methanol), 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (150 mM in methanol) and pyridine (7.5% in 75% aqueous methanol). The whole plate was then shaken at room temperature for 2 h to derivatize the organic acids. After the derivatization reaction, to each plate well, 350 µL of water and 25 µL of butylated hydroxytoluene solution (2 mg/mL in methanol) were added, to dilute and stabilize the final solution. Then, 10 µL was injected into an Agilent 1260 UHPLC-equipped QTRAP 4000 mass spectrometer for LC-MS/MS analysis, using multiple reaction monitoring (MRM) scanning in the negative mode. For the analysis of amino acids, biogenic amines and derivatives, acylcarnitines, lipids and glucose, 10 µL of each sample and 10 µL of the ISTD mixture solution were loaded onto the center of a 96-well filter plate and dried in a stream of nitrogen for 30 min. Subsequently, 50 µL of 5% phenylisothiocyanate (PITC) solution was added to each sample and the whole plate was then incubated at room temperature for 20 min. After incubation, all the samples were again dried under nitrogen for 1.5 h to evaporate excess PITC. The extraction of targeted metabolites was then achieved by adding 300 µL of extraction solvent (5 mM ammonium acetate prepared in methanol). The extracts were obtained by centrifugation into a lower 96-deep well collection plate. To quantify amino acids, biogenic amines and derivatives, extracts were diluted with water in a 1:1 ratio, and 10 µL was injected; to analyze acylcarnitines, lipids and glucose, extracts were diluted five times and 20 µL was injected into an Agilent 1260 UHPLC-equipped QTRAP 4000 mass spectrometer, using multiple reaction monitoring (MRM)

scanning in the positive mode. All data analysis was done using Analyst 1.6.2 (AB SCIEX, Foster, CA, USA) and MultiQuant 3.0.3 (AB SCIEX, Foster, CA, USA).

4.3.2. LC-MS/MS Method

An Agilent reversed-phase Zorbax Eclipse XDB C18 column (3.0 mm × 100 mm, 3.5 µm particle size, 80 Å pore size), with a Phenomenex (Torrance, CA, USA) SecurityGuard C18 pre-column (4.0 mm × 3.0 mm), was used for the LC-MS/MS analysis of organic acids, amino acids, biogenic amines and derivatives.

The LC parameters used for the analysis of amino acids, biogenic amines and their derivatives were as follows: mobile phase A 0.2% (v/v) formic acid in water, and mobile phase B 0.2% (v/v) formic acid in acetonitrile. The gradient profile was as follows: t = 0 min, 0% B; t = 0.5 min, 0% B; t = 5.5 min, 95% B; t = 6.5 min, 95% B; t = 7.0 min, 0% B; and t = 9.5 min, 0% B. The column oven was set at 50 ◦C. The flow rate was 500 µL/min, and the sample injection volume was 10 µL. For the analysis of organic acids, the mobile phases used were A) 0.01% (v/v) formic acid in water, and B) 0.01% (v/v) formic acid in methanol. The gradient profile was as follows: t = 0 min, 30% B;t = 2.0 min, 50% B; t = 12.5 min, 95% B; t = 12.51 min, 100% B; t = 13.5 min, 100% B; t = 13.6 min, 30% B and finally maintained at 30% B for 4.4 min. The column oven was set to 40 ◦C. The flow rate was 300 µL/min, and the sample injection volume was 10 µL.

4.3.3. FIA-MS/MS Method

For the analysis of lipids, acylcarnitines and glucose, the LC autosampler was connected directly to the MS ion source by red PEEK tubing. The mobile phase was prepared by mixing 60 µL of formic acid, 10 mL of water and 290 mL of methanol; and the flow rate was programmed as follows: t = 0 min, 30 µL/min; t = 1.6 min, 30 µL/min; t = 2.4 min; 200 µL/min; t = 2.8 min, 200 µL/min; and t = 3.0 min, 30 µL/min. The sample injection volume was 20 µL.

4.3.4. Quantification

To quantify organic acids, amino acids, biogenic amines and derivatives, an individual seven-point calibration curve was generated for each analyte. The ratios of each analyte's signal intensity to its corresponding isotope-labelled internal standard were plotted against the specific known concentrations, using quadratic regression with a 1/x2 weighting.

Lipids, acylcarnitines and glucose were analyzed semi-quantitatively. Single point calibration of a representative analyte was built, using the same group of compounds that share the same core structure, assuming linear regression through zero. All data analysis was done using Analyst 1.6.2 and MultiQuant 3.0.3.

4.4. Statistical Analysis

GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analyses reported here. Percentiles, mean values and standard deviations (SD) were calculated using standard statistical formulas. Continuous and categorical variables are presented as mean ± SD or median (interquartile range) and number (for percentile) respectively. The Kolmogorov–Smirnov test was used to test the normality of the distribution for continuous variables. A statistical analysis to evaluate gender differences was performed with GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA) and MetaboAnalyst (https://www.metaboanalyst.ca). p-value adjustments for multiple metabolites were carried out by using Benjamini-Hochberg false discovery rate adjustment (FDR < 0.05).

The methodology for determining the reference intervals is based on the recommendations from the International Federation of Clinical Chemistry [64]. All primary reference ranges have been calculated using more than 40 samples, allowing for reliable estimates of the 2.5th and 97.5th centiles. A non-parametric calculation was used to calculate ranges, due to non-normally distributed data.

4.5. Literature Review and Data Entry

The last detailed literature overview conducted by our team on urinary metabolites in newborns was performed in 2012 [12]. A literature search was conducted in

PubMed from November 2019 to February 2020. Our first search filter combined the truncated search term “urin*”. Our second search filter combined the term “newborn” and its synonym “neonat*” with the Boolean operator OR. The third search filter combined the terms “reference value” and “concentration” with the operator OR. Our last search filter used the truncated search term “metaboli*”. We then combined these searches with the Boolean operator AND. Through this preliminary search, we retrieved 509 articles. From this number, 191 were from the last 8 years (the time of the last literature update conducted on the UMDB and HMDB for neonatal reference values). After careful reading through these articles, 34 papers were found to have useful information about metabolite concentration values in newborn urine. From these 34 papers, 78 metabolites with concentration values were obtained and used to update the HMDB/UMDB.

5. Conclusions

The size and chemical diversity of the “measurable” metabolome of healthy neonates appear to be somewhat smaller and simpler than that of children or adults (~300 metabolites vs. ~2000 metabolites). This appears to be due to fundamental differences in neonatal metabolism, as well as differences due to diet, exposures and gut microflora composition. Despite the relative simplicity of neonatal urine in terms of its metabolic diversity, we were surprised by the appearance of several unexpected metabolites. Indeed, 86 of the experimentally measured urinary metabolites had not previously been reported in neonates/infants, and another 20 metabolites are being reported in human urine for the first time ever. Comparisons between neonatal urine and adult urine also show some striking concentration differences for certain metabolites. Much higher levels of essential amino acids, collagen-associated amino acids and acylcarnitines in neonatal urine likely reflect the large pool of these compounds needed to sustain rapid cell growth and cell division in neonates. In addition to the obvious differences between adult and infant urine composition, we also observed clear differences in urinary metabolites between newborn males and females, as well as differences arising due to birth modality (VD vs. CS). Some of our findings reiterated the findings of earlier studies,

while others appear to be quite novel. The clear existence of sex differences in the urine composition of newborns reinforces the need for implementing specific sex-reference values of metabolic markers for female and male neonates. These sex-specific differences may also be relevant in the diagnosis of IEMs. Interestingly, prior to this study, the influence of sex on acylcarnitines, glycerophospholipids, biogenic amines and sphingomyelines urinary levels at birth had not been investigated.

One of the major strengths of our work is the fact that all the urinary samples were collected in the same period of time (the first 24 h of life), which makes the reported measurements more comparable and homogeneous. As a result, we believe that the values reported here should constitute a robust and generally useful set of clinical reference values for the absolute and creatinine-normalized urinary concentration for healthy, full-term neonates. All of these values, along with chemical structures and detailed descriptions of the compounds, are freely available in the UMDB and HMDB (www.hmdb.ca). We hope that such a set of reference values and reference information will be used by clinicians and other health professionals to assist with the diagnosis, prognosis or monitoring of various IEMs and other neonatal health conditions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-1989/10/4/165/s1, Figure S1: Metabolites showing higher urinary concentration levels ($p < 0.05$) in female versus male. Data are shown as Box and Whisker plots. Comparison of continuous variables were performed using the t-test (when data were normally distributed) or Mann-Whitney test (when data were non-normally distributed), Figure S2: Metabolites showing higher urinary concentration levels ($p < 0.05$) in male versus female. Data are shown as Box and Whisker plots. Comparison of continuous variables were performed using the t-test (when data were normally distributed) or Mann-Whitney test (when data were non-normally distributed), Figure S3: Metabolites differentially expressed in vaginal delivery (VD) and Caesarean section (CS) newborns. Data are shown as Box and Whisker plots. Comparison of continuous variables were performed using the t-test (when data were normally distributed) or Mann-Whitney test (when data were non-

normally distributed); Table S1: Limit of detection for measured analytes, Table S2: Metabolites previously reported in newborns and infants, Table S3: Creatinine urinary concentration in newborns. Table S4: Differences in gender (Amino acids and biogenic amines); Table S5: Differences in gender (Organic acids and glucose); Table S6: Differences in gender (Glycerophospholipids); Table S7: Differences in gender (Sphingomyelins); Table S8: Differences in gender (acylcarnitines); Table S9: List of metabolites experimentally measured in the present work that are currently used as primary or secondary markers for Inborn errors of metabolism, Table S10: Comparison between newborn and adult urinary concentration of 79 metabolites measured via LC-MS/MS.

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Figure legends.

Figure 1. Graphical representation of urinary concentrations of (a) amino acids (b) biogenic amines and (c) organic acids, previously reported in newborns by LC-MS/MS. The error bars reflect one standard deviation. (a) Amino acids are represented by their three-letter code. (b) 4-hydroxyproline: Hyp; methionine-sulfoxide: Met(O); acetyl-ornithine: AOR; serotonin: 5-HT; asymmetric dimethylarginine: ADMA; symmetric dimethylarginine: SDMA; total dimethylarginine: TDMA; carnosine: Car; sarcosine: Sar; diacethylspermine: DASpm; betaine: Bet; choline: Cho; alpha-aminoadipic acid: Aad. (c) lactic acid: LA; 3-Hydroxybutyric acid: BHIB; alpha-Ketoglutaric acid: AKG; citric acid: CA; butyric acid: BA; 3-(3-Hydroxyphenyl)-3-hydroxypropanoic acid: HPHPA; succinic acid: SA; fumaric acid: FA; pyruvic acid: PA; hippuric acid: HA; methylmalonic acid: MMA; homovanillic acid: HVA; indoleacetic acid: IAA; Glucose: Glu.

Figure 2. Venn diagram showing the overlap of urine metabolites detected by LC-MS/MS, high performance

Table 1. Clinical data from the newborns and their mothers.

Newborns (*N* = 48)	
Sex Female Male	 17 (35%) 31 (65%)
Gestational age (weeks)	38.2 ± 1.5
Ave. Apgar at 1 min	8 (100%)
Ave. Apgar at 5 min	9 (100%)
Ave. Silverman–Anderson	0 (100%)
Weight (g)	2979 ± 472
Weight (g) males	2992 ± 86.59
Weight (g) females	2882 ± 112.4
Mothers (*N* = 48)	
BMI (pre-gestational)	27.1 ± 4.9
Age (years)	29 ± 7
Resolution Vaginal delivery Caesarean section	 22 (45.8%) 26 (54.2%)
Euglycemic	22 (45.8%)
Gestational Diabetes Mellitus (GDM)	26 (54.2%)

Table 2. Metabolites not previously reported in newborns.

Metabolite	HMDB ID	Mean ± SD (μM)	Mean ± SD (μM/mM Creatinine)	2.5–97.5% Percentile (μM/mM Creatinine)
Histamine	HMDB0000870	0.08 ± 0.03	0.02 ± 0.01	0.01–0.04
Putrescine	HMDB0001414	1.09 ± 1.54	0.31 ± 0.63	0.03–3.42
Methionine sulfoxide	HMDB0002005	7.02 ± 4.70	1.60 ± 0.73	0.60–3.60
N2-Acetylornithine	HMDB0003357	3.28 ± 4.00	0.67 ± 0.75	0.12–3.50
Serotonin	HMDB0000259	0.98 ± 0.67	0.20 ± 0.06	0.10–0.35
DOPA	HMDB0000181	0.20 ± 0.10	0.05 ± 0.03	0.01–0.15
Asymmetric dimethylarginine	HMDB0001539	10.6 ± 6.40	2.31 ± 0.83	0.98–4.90
Symmetric dimethylarginine	HMDB0003334	45.7 ± 30.8	9.80 ± 3.00	5.33–18.1
Spermidine	HMDB0001257	0.25 ± 0.19	0.06 ± 0.05	0.02–0.30
Spermine	HMDB0001256	0.23 ± 0.28	0.06 ± 0.11	0.01–0.62
Diacetylspermine	HMDB0002172	4.64 ± 3.52	1.03 ± 0.59	0.37–3.11
Trimethylamine *N*-oxide	HMDB0000925	59.4 ± 54.0	12.2 ± 10.3	0.30–43.1
p-Hydroxyhippuric acid	HMDB0013678	37.1 ± 24.8	8.03 ± 3.48	4.20–19.6

Table 2. Cont.

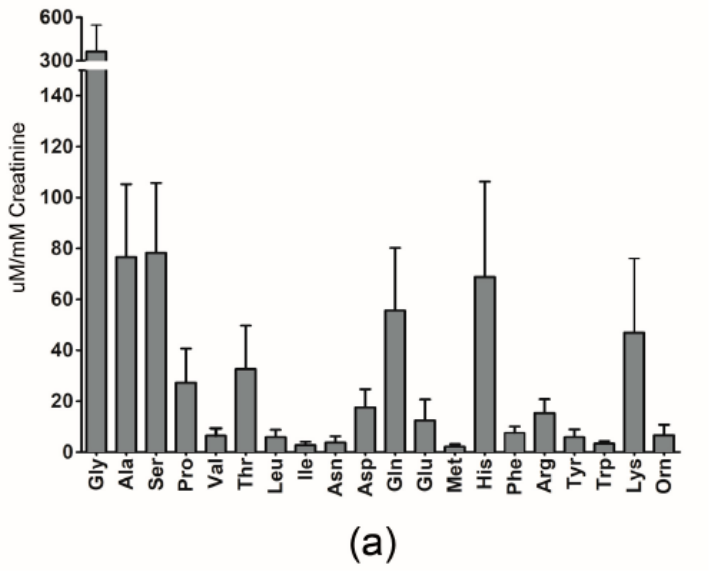
Metabolite	HMDB ID	Mean ± SD (μM)	Mean ± SD (μM/mM Creatinine)	2.5–97.5% Percentile (μM/mM Creatinine)
LysoPC a C16:1	HMDB0010383	0.013 ± 0.011	0.003 ± 0.003	0.0001–0.0150
LysoPC a C16:0	HMDB0010382	0.19 ± 0.20	0.043 ± 0.036	0.007–0.169
LysoPC a C17:0	HMDB0012108	0.018 ± 0.013	0.005 ± 0.001	0.0008–0.0582
LysoPC a C18:2	HMDB0010386	0.03 ± 0.04	0.007 ± 0.007	0.001–0.030
LysoPC a C18:0	HMDB0010384	0.06 ± 0.06	0.015 ± 0.012	0.0008–0.0652
LysoPC a C20:4	HMDB0010395	0.02 ± 0.04	0.005 ± 0.006	0.0–0.03
PC ae C36:0	HMDB0013406	0.02 ± 0.03	0.006 ± 0.013	0.0–0.08
PC aa C36:6	HMDB0008206	0.007 ± 0.006	0.002 ± 0.003	0.0–0.02
PC aa C36:0	HMDB0007886	0.05 ± 0.06	0.013 ± 0.023	0.001–0.130
PC aa C38:6	HMDB0008116	0.08 ± 0.12	0.017 ± 0.020	0.002–0.089
PC aa C38:0	HMDB0007893	0.08 ± 0.04	0.022 ± 0.021	0.005–0.124
PC ae C40:6	HMDB0013422	0.02 ± 0.02	0.005 ± 0.004	0.001–0.021
PC aa C40:6	HMDB0008057	0.04 ± 0.05	0.010 ± 0.013	0.0–0.07
SM(OH) C14:1	HMDB0013462	10.1 ± 0.09	0.02 ± 0.04	0.00–0.25
SM C16:1	HMDB0013464	0.10 ± 0.11	0.02 ± 0.02	0.0–0.11
SM C16:0	HMDB0010168	2.02 ± 2.28	0.50 ± 0.92	0.07–5.31
SM(OH) C16:1	HMDB0013463	0.06 ± 0.08	0.02 ± 0.04	0.0–0.24
SM C18:1	HMDB0012101	0.08 ± 0.10	0.02 ± 0.02	0.0–0.06
SM C18:0	HMDB0012087	0.31 ± 0.37	0.07 ± 0.79	0.01–0.43
SM C20:2	HMDB0013465	0.005 ± 0.005	0.001 ± 0.001	0.0–0.004
SM(OH) C22:2	HMDB0013467	0.03 ± 0.04	0.01 ± 0.01	0.0–0.05
SM(OH) C22:1	HMDB0013466	0.16 ± 0.15	0.04 ± 0.03	0.0–0.15
SM(OH) C24:1	HMDB0013469	0.04 ± 0.05	0.01 ± 0.02	0.0–0.14
Carnitine (C0)	HMDB0000062	8.96 ± 7.07	2.01 ± 1.13	0.79–5.67
L-Acetylcarnitine (C2)	HMDB0000201	4.49 ± 4.32	0.89 ± 0.38	0.38–1.86
Propenoylcarnitine (C3:1)	HMDB0013124	0.08 ± 0.08	0.02 ± 0.01	0.0–0.06
Propionylcarnitine (C3)	HMDB0000824	0.14 ± 0.09	0.03 ± 0.02	0.01–0.07
Butenylcarnitine (C4:1)	HMDB0013126	0.09 ± 0.04	0.02 ± 0.01	0.01–0.04
Butyrylcarnitine (C4)	HMDB0002013	0.53 ± 0.39	0.12 ± 0.07	0.05–0.33
Hydroxypropionyl carnitine (C3OH)	HMDB0013125	0.11 ± 0.05	0.03 ± 0.01	0.01–0.06
Tiglylcarnitine (C5:1)	HMDB0002366	0.34 ± 0.23	0.07 ± 0.03	0.03–0.16
Hydroxybutyryl carnitine (C4OH)	HMDB0002095	0.18 ± 0.10	0.04 ± 0.02	0.01–0.08
Hexenoylcarnitine (C6:1)	HMDB0013161	0.06 ± 0.03	0.020 ± 0.004	0.01–0.02
Hexanoylcarnitine (C6)	HMDB0000756	0.12 ± 0.07	0.03 ± 0.08	0.02–0.05
Hydroxyvalerylcarnitine (C5OH)	HMDB0013132	0.36 ± 0.20	0.08 ± 0.05	0.04–0.29
Glutaconylcarnitine (C5:1DC)	HMDB0013129	0.07 ± 0.04	0.02 ± 0.01	0.0–0.03
Glutarylcarnitine (C5DC)	HMDB0013130	0.20 ± 0.11	0.05 ± 0.02	0.02–0.09
Octanoylcarnitine (C8)	HMDB0000791	0.24 ± 0.19	0.05 ± 0.05	0.02–0.31
Methylglutarylcarnitine (C5MDC)	HMDB0000552	0.27 ± 0.16	0.06 ± 0.02	0.03–0.13
Nonaylcarnitine (C9)	HMDB0013288	0.46 ± 0.38	0.09 ± 0.04	0.03–0.18
Pimelylcarnitine (C7DC)	HMDB0013328	0.21 ± 0.14	0.05 ± 0.02	0.02–0.09
Decenoylcarnitine (C10:1)	HMDB0013205	0.34 ± 0.12	0.09 ± 0.04	0.03–0.20
Decanoylcarnitine (C10)	HMDB0000651	0.29 ± 0.18	0.06 ± 0.02	0.04–0.13

Metabolite	HMDB ID	Mean ± SD (μM)	Mean ± SD (μM/mM Creatinine)	2.5–97.5% Percentile (μM/mM Creatinine)
Dodecenoylcarnitine (C12:1)	HMDB0013326	0.24 ± 0.10	0.06 ± 0.02	0.02–0.14
Dodecanoylcarnitine (C12)	HMDB0002250	0.35 ± 0.27	0.08 ± 0.04	0.03–0.25
Tetradecadienyl carnitine (C14:2)	HMDB0013331	0.05 ± 0.03	0.010 ± 0.003	0.01–0.02
Tetradecenoylcarnitine (C14:1)	HMDB0013329	0.06 ± 0.03	0.01 ± 0.02	0.0–0.1
Tetradecanoylcarnitine (C14)	HMDB0005066	0.11 ± 0.10	0.02 ± 0.02	0.01–0.10
Hydroxytetradecadienylcarnitine (C14:2OH)	HMDB0013332	0.03 ± 0.02	0.007 ± 0.002	0.003–0.014
Hydroxytetradecenoyl carnitine (C14:1OH)	HMDB0013330	0.04 ± 0.02	0.008 ± 0.002	0.004–0.015
Hexadecadienyl carnitine (C16:2)	HMDB0013334	0.02 ± 0.01	0.004 ± 0.002	0.0–0.01
Hexadecanoylcarnitine (C16)	HMDB0000222	0.05 ± 0.03	0.01 ± 0.01	0.01–0.06
Octadecadienylcarnitine (C18:2)	HMDB0006469	0.010 ± 0.003	0.003 ± 0.001	0.0–0.01

Table 3. Metabolites not previously reported in human urine.

Metabolite	HMDB ID	Mean ± SD (μM)	Mean ± SD (μM/mM Creatinine)	2.5–97.5% Percentile (μM/mM Creatinine)
LysoPC a C14:0	HMDB0010379	0.02 ± 0.02	0.006 ± 0.003	0.002–0.012
LysoPC a C18:1	HMDB0002815	0.07 ± 0.09	0.02 ± 0.03	0.0–0.20
LysoPC a C20:3	HMDB0010394	0.014 ± 0.002	0.004 ± 0.003	0.0–0.01
LysoPC a C24:0	HMDB0010405	0.07 ± 0.02	0.02 ± 0.01	0.0–0.05
LysoPC a C26:1	HMDB0029220	0.01 ± 0.01	0.002 ± 0.002	0.0–0.01
LysoPC a C26:0	HMDB0029205	0.01 ± 0.01	0.004 ± 0.005	0.0–0.03
LysoPC a C28:1	HMDB0029221	0.01 ± 0.01	0.002 ± 0.002	0.0–0.01
LysoPC a C28:0	HMDB0029206	0.04 ± 0.01	0.011 ± 0.007	0.0–0.04
PC aa C32:2	HMDB0007874	0.03 ± 0.03	0.01 ± 0.01	0.0–0.03
PC aa C40:2	HMDB0008276	0.01 ± 0.01	0.003 ± 0.008	0.0–0.05
PC aa C40:1	HMDB0007993	0.03 ± 0.04	0.01 ± 0.02	0.0–0.13
Decadienylcarnitine (C10:2)	HMDB0013325	0.14 ± 0.07	0.03 ± 0.01	0.01–0.07
Dodecanedioylcarnitine (C12DC)	HMDB0013327	0.27 ± 0.29	0.05 ± 0.05	0.01–0.29
Hexadecenoylcarnitine (C16:1)	HMDB0006317	0.05 ± 0.02	0.01 ± 0.01	0.0–0.03
Hydroxyhexadecadienylcarnitine (C16:2OH)	HMDB0013335	0.02 ± 0.01	0.004 ± 0.001	0.0–0.01
Hydroxyhexadecenoyl carnitine (C16:1OH)	HMDB0013333	0.04 ± 0.02	0.010 ± 0.004	0.0–0.02
Hydroxyhexadecanoylcarnitine (C16OH)	HMDB0061642	0.04 ± 0.02	0.008 ± 0.003	0.0–0.02
Octadecenoylcarnitine (C18:1)	HMDB0006464	0.02 ± 0.01	0.005 ± 0.004	0.0–0.03
Octadecanoylcarnitine (C18)	HMDB0000848	0.03 ± 0.03	0.01 ± 0.01	0.0–0.07
Hydroxyoctadecenoylcarnitine (C18:1OH)	HMDB0013339	0.02 ± 0.01	0.004 ± 0.002	0.0–0.01

Figure 1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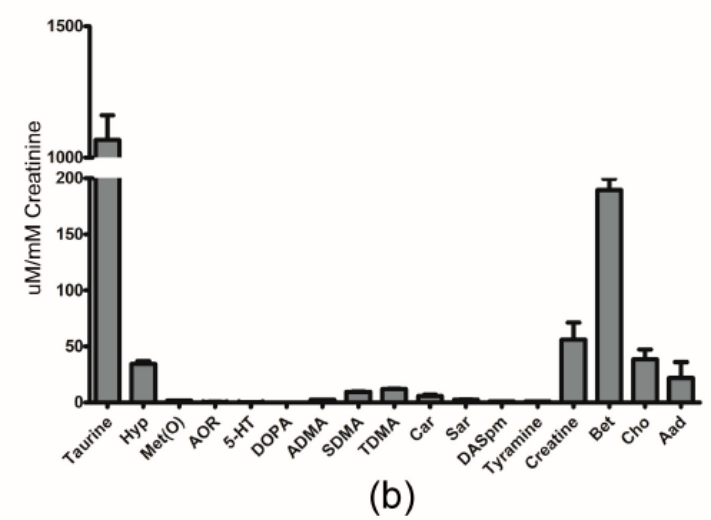
(a)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Sar
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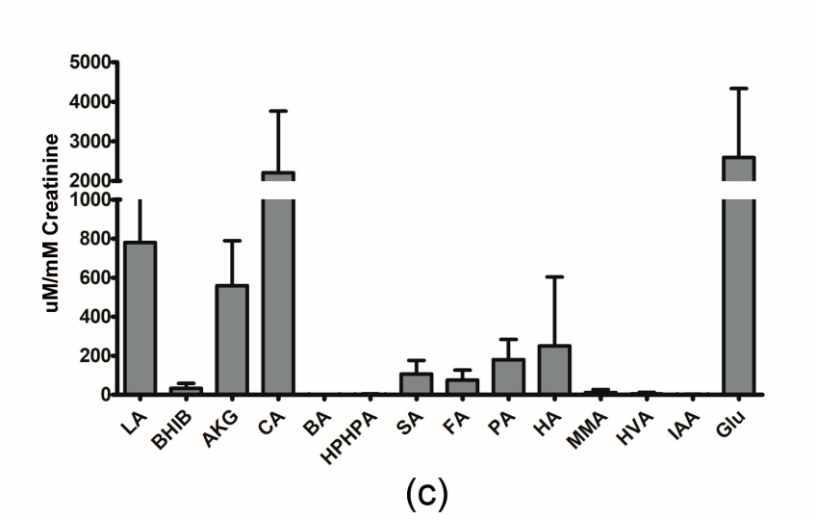
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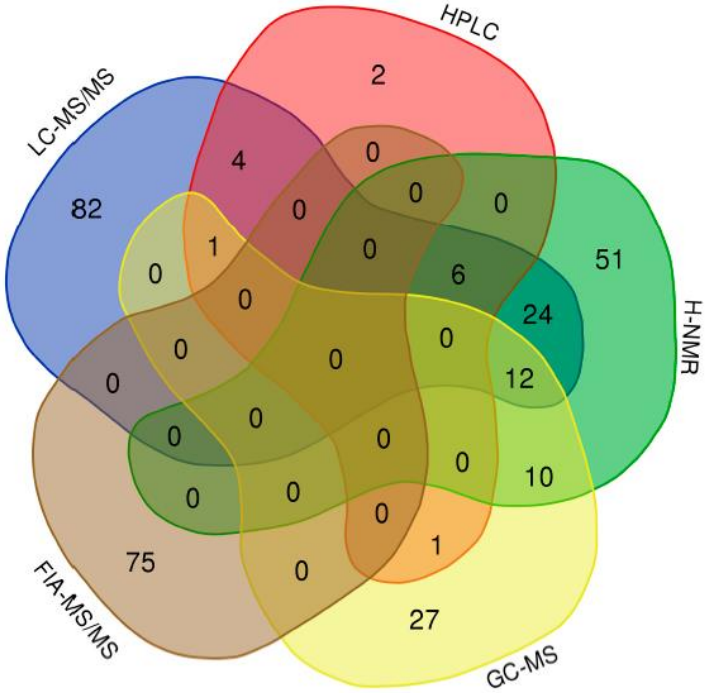
Figure 2



Urinary Metabolomic Profile of Neonates Born to Women with Gestational Diabetes Mellitus

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Abstract: Gestational diabetes mellitus (GDM) is one of the most frequent pregnancy complications with potential adverse outcomes for mothers and newborns. Its effects on the newborn appear during the neonatal period or early childhood. Therefore, an early diagnosis is crucial to prevent the development of chronic diseases later in adult life. In this study, the urinary metabolome of babies born to GDM mothers was characterized. In total, 144 neonatal and maternal (second and third trimesters of pregnancy) urinary samples were analyzed using targeted metabolomics, com- bining liquid chromatographic mass spectrometry (LC-MS/MS) and flow injection analysis mass spectrometry (FIA-MS/MS) techniques. We provide here the neonatal urinary concentration values of 101 metabolites for 26 newborns born to GDM mothers and 22 newborns born to healthy mothers. The univariate analysis of these metabolites revealed statistical differences in 11 metabolites. Multi- variate analyses revealed a differential metabolic profile in newborns of GDM mothers characterized by dysregulation of acylcarnitines, amino acids, and polyamine metabolism. Levels of hexadecenoyl- carnitine (C16:1) and spermine were also higher in newborns of GDM mothers. The maternal urinary metabolome revealed significant differences in butyric, isobutyric, and uric acid in the second and third trimesters of pregnancy. These metabolic alterations point to the impact of GDM in the neonatal period.

Keywords: newborns; metabolomics; gestational diabetes; pregnancy

1. Introduction

Gestational diabetes mellitus (GDM), defined as hyperglycemia during gestation [1], is the most frequent medical complication in pregnancy, affecting 7–10% of all pregnancies worldwide [2]. GDM has been associated with various adverse outcomes both in mothers and newborns. Women with GDM are at higher risk of developing type 2 diabetes (T2D), mainly 3–6 years after delivery, and their offspring is also at higher risk of becoming over- weight and obese [3]. The metabolic abnormalities associated with GDM include increased insulin resistance and β-cell defects, which can occur before conception, especially in populations with a high prevalence of obesity and T2D.

Pregnancy is a complex physiological condition associated with relevant metabolic changes resulting from increased energy and nutrition requirements needed for fetal development [4,5]. However, studies comparing the metabolic profiles of women with GDM and their offspring are still scarce.

There are only a few reports from newborn metabolome addressing the effect of maternal GDM. Most of these studies have been performed in serum from the umbilical cord [6–8]. The first study on this topic reported that the metabolomic profile observed in serum from the umbilical cord of neonates born to GDM women differed between those with and without pregnancy complications, suggesting that a prolonged fetal exposure to hyperglycemia could impact the newborn's metabolome [4]. In cord blood, Lu et al. found that fetal phosphatidylcholine acyl-alkyl C 32:1 and proline showed an independent association with GDM [8]. In a recent work, associations between GDM and higher offspring ketone levels in cord serum at birth were found consistent with maternal ketosis in diabetic pregnancies [9]. Only a study using untargeted metabolomics compared urine and meconium between neonates born to GDM women and healthy controls, finding metabolic differences. However, in this study, no maternal urine or serum samples were available for analyses [5].

In the present work, urine samples were collected from 48 full-term healthy neonates born to GDM mothers and controls. Urine samples were also collected from mothers in the second and third trimesters of pregnancy to investigate whether metabolic alter- ations found in babies could relate to maternal metabolic alterations. All the

samples were analyzed using targeted MS/MS-based metabolomic assays to assess the potential influence of GDM in the early urinary metabolome of newborns. Multivariate analyses revealed a differential metabolic profile in newborns of GDM mothers characterized by the dysregulation of long-chain acylcarnitines, amino acids, short-chain fatty acids, and polyamine metabolism. A dysregulation in the arginine/nitric oxide (Arg/NO) signaling pathway, intestinal dysbiosis, or diet/treatment differences could explain these metabolic alterations. In addition, quantitative values of 101 metabolites measured in newborn urine samples collected within 24 h of birth are presented. Knowing these concentration values is of pivotal importance for clinicians and neonatologists to monitor the impact of GDM in the early stages of life.

2. Results

2.1. Clinical Characteristics of Newborns and Mothers

Comparisons of selected characteristics between mothers and newborns by diabetic status of the mother are shown in Table 1. Newborn gestational age, Apgar scores, and birth weight were similar between those born to GDM and healthy women. Women with GDM were 2.8 years older ($p = 0.05$). The prevalence of overweight was high in both groups (GDM 38.4%, healthy 40.9%; $p = 0.32$). Mean glucose measured at the first prenatal visit was also 7.1 mg/dL higher in women with GDM ($p = 0.03$). From all GDM women, 15 received metformin and one received combined treatment for diabetes control. All women with GDM were diagnosed between weeks 24 and 28 of gestation.

2.2. Urinary Concentration Values of 101 Metabolites of Newborns to Diabetic and Healthy Mothers

From the 136 metabolites analyzed, 101 were absolutely quantified (35 metabolites belonging to glycerophospholipid and sphingomyelin classes were excluded from the sta- tistical analysis as they fell below the limit of detection by the method employed). Table 2 shows the concentration values of the statistically significant metabolites quantified, both for babies born to GDM and healthy mothers. Supplementary Table S1 shows the concen- tration levels for the rest of measured

metabolites. Mann–Whitney U tests revealed that 11 metabolites were statistically different between the study groups (trans-hydroxyproline, glutamic acid, DOPA, spermine, lactic acid, butyric acid, isobutyric acid, C5:1DC, C5DC, C10:2, and C16:1).

2.3. Multivariate Analysis

Figure 1 shows the results from multivariate analyses performed to compare the new- borns of GDM mothers with those from healthy mothers. Supervised PLS-DA (Figure 1A) showed a partial separation with low risk of overfitting ($p = 0.008$ for 2000 permutation test). Figure 1B shows the variable importance in projection for 15 metabolites with a VIP >1.5. C16:1 and spermine were the most discriminant metabolites and remained significant also after FDR adjustment ($q < 0.05$).

When these metabolites were modeled, adjusting by mother's age in years and pre-pregnancy BMI in kg/m2, only spermine remained significant ($p < 0.05$). Such model had a non-significant Hosmer–Lemeshow Chi2 p-value of 0.30, indicating a good fit, and a Nagelkerke R2 of 0.62, indicating that the model explained 62% of the variability of the outcome (i.e., diabetes status of the mother).

The pathway-enrichment analysis revealed that discriminant metabolites were mainly involved in amino acid, carbohydrate, spermine, and spermidine biosynthesis and lipid metabolism-related pathways, as shown in Figure 2.

2.4. Analysis of the Maternal Urinary Metabolome during the Second and Third Trimesters of Pregnancy

To assess the possible relation between the metabolic alterations found in the newborns and those of their mothers, maternal urine samples collected during the second half of the pregnancy were analyzed.

Multivariate analyses did not show a clear separation between healthy and GDM mothers in the second (Figure 3A) or third (Figure 3B) trimesters. VIP analysis (Figure 3C) shows the top 20 metabolites differentially expressed between healthy and GDM mothers in their second and third trimesters. Most of the metabolites altered in GDM newborns were also increased in GDM mothers in the third trimester.

However, only isobutyric, butyric, and uric acid were significantly dysregulated between GDM and healthy mothers, and between trimesters of gestation ($q < 0.05$).

3. Discussion

This study aimed at analyzing the urinary metabolome of babies born to GDM and healthy mothers. To our knowledge, this is the first work reporting quantitative concentra- tion values for 101 metabolites measured in urine within the first 24 h of life of neonates born to GDM mothers. Since there are no previous studies presenting quantitative analyses performed on the urine of newborns of GDM mothers, comparison with these findings was not possible. Despite most urinary metabolite concentrations of all newborns included in the present work being within the normal range, as documented earlier [10], our results showed a differential profile in the urinary levels of some amino acids, polyamines, and carnitines among newborns of GDM mothers.

While we found 11 differential metabolites in the neonatal urine, after the Benjamini Hochberg (BH) procedure, only two metabolites remained as significant. Spermine was increased in the urine of babies born to GDM mothers. The higher levels of spermine in GDM babies could be a result of a dysregulation in the arginine/nitric oxide (Arg/NO) signaling pathway. GDM has been associated with endothelial dysfunction due to a dysregulated endothelial Arg/NO signaling pathway [11]. The activity of this signaling pathway is modulated by D-glucose, adenosine, insulin, and ATP, among other molecules in T2D, GDM, and other diseases coursing with vascular and/or endothelial compromise [12]. An increase in the level of serum arginine in the umbilical artery of women with GDM has been reported, suggesting that GDM upregulates the Arg/NO pathway. This could, in turn, explain the high levels of spermine seen in this and other studies, since the arginine pathway is involved in the polyamine synthesis [11]. Previously, it was found that increased adiposity in children was associated with an increase in the three circulating polyamine levels, including spermine [13]. The authors showed that the spermine level was related to markers of the NO pathway, oxidative stress, inflammation, and leptin [13]. Polyamine metabolism has been involved in adipogenesis, suggesting that increased polyamine levels may be implicated in adipose tissue expandability

during obesity. A close relationship has also been proposed between polyamine levels and the gut microbiota composition. In fact, the gut microbiota area considered as mainly responsible for polyamine levels in the lower part of the intestine from where they are transferred into the bloodstream via the colonic mucosa [14]. Polyamines can also be acquired by breast milk during lactation, although Atiya Ali et al. found that the spermine levels did not differ between breast milk from obese mothers and mothers with normal body weight [15]. The results presented here suggest that spermine could be monitored during the neonatal period and childhood due to its proven relation with metabolic disorders such as obesity.

A significant decrease of urinary C16:1 in babies born to GDM mothers was also observed. In GDM pregnancies, data on carnitines are scarce, but increased carnitine levels in GDM mothers and their offspring have been reported without clear explana- tion [16]. Shokry et al. found that reduced levels of C16:0 in GDM was associated with diminished placental transport of NEFAs and carnitines along with incomplete or reduced FAO, which was reported in insulin resistance and T2D [7]. Several studies have reported an increase in the concentration of long-chain carnitines (C12–C16) in plasma and urine samples of T2D patients, suggesting an incomplete oxidation of long-chain fatty acids, altered activity of the tricarboxylic acid cycle, and an increased flow of fatty acids to the mitochondria, which are molecular mechanisms that contribute to the pathogenesis of insulin resistance [17–19]. Some authors have proposed a similar mechanism for GDM. Lin et al. evaluated the association of plasma acylcarnitine profiles and GDM throughout gestation, reporting that elevated levels of C4, C8:1, and C16:1-OH were associated with an increased risk of GDM [20]. Batchuluun et al. found a specific elevation in the serum of hexanoylcarnitine and octanoylcarnitine among women with GDM and individuals with T2D without alteration in long-chain acylcarnitines [21]. In contrast, Pappa et al. reported for the first time that GDM does not further affect the efficiency of the carnitine system. The authors proposed that the mild effect of GDM on carnitine status could be explained by the concurrent increased gluconeogenesis, a process that does not directly affect carnitine metabolism [16]. The decrease of only one long acylcarnitine in our study is insufficient to suggest one possible molecular

mechanism. Additional studies would be needed to propose a clear explanation for this C16:1 decrease. Although the trend for urinary concentrations of long acylcarnitines was in general to decrease (Supplementary Table S1), no significant differences were found except for C16:1. In line with our results, Sánchez-Pinto et al. compared GDM and no GDM Large for Gestational Age (LGA) new- borns, finding that a history of GDM was associated with lower levels of medium- and long-chain acylcarnitines, although the differences were not significant. From the analysis of the individual acylcarnitines, no remarkable differences were revealed [22].

The urinary metabolome in the second and third trimesters of pregnancy was studied to explore the potential impact of maternal alterations on the newborn urinary metabolome. The study presented here compared urinary metabolites between healthy and GDM women in the second and third trimesters. A distinctive pattern between GDM and healthy pregnant women was not observed. This is in general agreement with what other researchers have reported. For instance, an earlier longitudinal study using a non-targeted approach found that urine metabolites associated with GDM could be detected in the first trimester, but not in the third trimester [23]. In this study, nearly all women diagnosed with GDM were treated with insulin or metformin and dietary/lifestyle interventions to bring their condition under control. This suggests that the urinary metabolic profile of GDM patients could be substantially affected by medical and/or dietary interventions. Similar to that observed here, a large multiethnic study showed that while changes in the maternal urinary profile could be seen during and after pregnancy, no identifiable and reliable biomarkers of GDM were seen [24]. Another study did find alterations in the urinary excretion of some amino acids, but failed to correlate well with the glycemic control of GDM women [25]. Finally, a non-targeted UPLC-MS characterization of second trimester maternal urine and amniotic fluid was unable to find associated changes in the pre-diagnostic GDM group [26]. Overall, the effect of pharmacological treatment (metformin, insulin, or combination of both), diet, and exercise, appears to normalize the metabolic profile of GDM patients to match (or nearly match) that of healthy controls in the second and third trimesters of pregnancy.

Interestingly, levels of butyric and isobutyric acids (SCFAs and BSCFAs, respectively) increased significantly between the second and third trimesters in both healthy and GDM women in this study. This replicates a finding that reported an increase in butanoate metabolism throughout pregnancy in urine samples of GDM women [27] and serum [6].

SCFAs are produced through colonic fermentation of dietary fibers, while BSCFAs are generated from undigested protein reaching the colon and are associated with the fermentation of branched amino acids [28]. In this study, a large proportion (38.5%) of GDM women were overweight (BMI > 25 kg/m2). In overweight women with GDM, a shift in the microbiota composition to higher α-diversity has been observed, along with numerous associations between metabolic and inflammatory patterns and specific bacterial abundance [29]. For instance, in a study with obese Mexican women, with and without metabolic syndrome, firmicutes bacteria were the most abundant gut bacterial phylum [30]. While bacterial presence was not determined in this population, excessive production of SCFA (butyrate) and BSCFA (isobutyrate) was found among GDM women in the second and third trimesters. It has been suggested that there is an obesity-associated gut microbiota that harvests more energy from soluble dietary fiber through fermentation and produces more short-chain fatty acids than lean individuals, influencing host energy metabolism [30]. Butyric and isobutyric acid were also found to be dysregulated in the GDM newborn urinary metabolome, which constitutes a possible connection between maternal and newborn metabolomes influenced by GDM. In addition, intestinal dysbiosis (indirectly reflected by altered levels of butyric and isobutyric acid) can also affect the pool of polyamine metabolism including spermine levels [31].

This study has a number of strengths and weaknesses that need to be highlighted. The application of quantitative, targeted metabolomics to study urinary metabolites of babies born to GDM mothers makes it valuable for clinicians and neonatologists, helping in the screening of the early newborn metabolism. On the other hand, the main limitations relate to the relatively small sample size and the uncontrolled follow-up of dietary regimen in GDM mothers, which may also modulate the maternal metabolome. This led to relatively large standard errors, imprecise estimates, and to

potential selection bias. In spite of the fact that urine is a biofluid with a high content of metabolites and its collection is non- invasive, a number of relevant metabolites for the GDM physiopathology such as lipids had to be excluded because they were below the detection limit for the method employed. Future research is needed to confirm the findings reported here and to address these study limitations.

4. Materials and Methods

4.1. Study Design and Research Ethics Approval

The study was carried out between May 2018 and April 2020 at the Hospital Central "Dr. Ignacio Morones Prieto" from San Luis Potosi, in Mexico. The study design is summarized in Figure 4. The research proposal was revised and approved by the Hospital's Research and Ethics Committee (Registration No. 84-17; CONBIOETICA-24-CEI-001- 201604279). All relevant ethical aspects were carefully considered, and the Helsinki Declaration was followed. Written informed consent was signed by all participant women prior to the interview and to urine sample collection including that of their babies.

4.2. Study Population

Newborns: Forty-eight newborns were examined by a neonatologist (26 newborns of mothers with GDM and 22 of healthy mothers). Collected data included sex, weight, type of delivery, gestational age, APGAR score (i.e., appearance, pulse, grimace, activity, and respiration at minutes 1 and 5), Capurro test, and Silverman–Anderson score (Table 1). The first urine collection from babies born to GDM and healthy women was carried out within the 24 h after birth once the babies' genitals were thoroughly cleaned, placing the samples into sterile bags until micturition. Samples contaminated with meconium were discarded.

Mothers: Forty-eight pregnant women were recruited. The GDM group was composed of 26 patients who were diagnosed with GDM during the second trimester. The control group included 22 euglycemic women. GDM diagnosis was based on a positive oral glucose tolerance test undertaken between weeks 24 and 28 using the diagnostic criteria established by the WHO and the American College of

Obstetricians and Gynecologists. Pregnant women with hypertension or preeclampsia, T2D, urinary infections, chronic renal disease, cancer, or polycystic ovary syndrome were excluded. All GDM patients were given treatment after diagnosis until delivery. In addition to diet and moderate exercise counseling, patients were prescribed metformin, insulin, or a combination of both. Clinical and demographic data were collected from medical records for each participant at the first prenatal visit. A total of 96 urine samples (first-morning urine) from the 48 pregnant women was collected between weeks 24 and 28 (first sample, second trimester), and weeks 30 and 34 (second sample, third trimester) of gestation.

4.3. Metabolite Measurements

A targeted quantitative metabolomics assay called The Metabolomics Innovation Center (TMIC) Prime (TMIC PRIME®) Assay was employed using a combination of direct injection (FIA) MS and reverse-phase high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS). The method was previously described by Zheng et al. [32].

Samples were derivatized prior to MS analysis. Amino acids, biogenic amines and deriva- tives, acylcarnitines, lipids, and glucose were derivatized with phenylisothiocyanate (PITC), while for organic acids, 3-nitrophenylhydrazine, 1-ethyl-3-(3-dimethylaminopropyl) car- bodiimide, and pyridine were added to achieve derivatization.

LC-MS/MS was used for the analysis of amino acids, biogenic amines and deriva-tives, and organic acids. An Agilent reversed-phase Zorbax Eclipse XDB C18 column (3.0 mm 100 mm, 3.5 µm particle size, 80 Å pore size) with a Phenomenex (Torrance, CA, USA) SecurityGuard C18 pre-column (4.0 mm 3.0 mm) was used. The LC and MS parameters are described elsewhere [10]. Seven-point calibration curve was generated for each analyte.

The FIA-MS/MS method was employed for the analysis of lipids, acylcarnitines, and glucose; the LC autosampler was connected directly to the MS ion source by red PEEK tubing. Lipids, acylcarnitines, and glucose were analyzed semi-quantitatively.

4.4. Statistical Analysis

Means and standard deviations (s.d.) or medians with interquartile range (IQR) and percentiles were calculated for continuous data with normal and non-normally distributed data, respectively. Normality of the distributions was assessed by the Kolmogorov–Smirnov test. GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA) was used. Statistical procedures were conducted as described elsewhere for quantitative metabolomics [33]. Univariate analyses were performed using Mann–Whitney rank sum tests and Fisher's exact tests, while principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were conducted using MetaboAnalyst [34]. A 2000- fold permutation test was performed to assess the significance. The differential variables were selected according to three conditions: (1) adjusted $p < 0.05$; (2) fold change between two groups >1.5; and (3) variable importance for the projection (VIP) value obtained from PLS-DA >1.5. Metabolic pathway analysis was carried out using the pathway analysis module of MetaboAnalyst 4.0. The Benjamini and Hochberg method was used to adjust the p values. This method, rather than controlling the false positive rate, controls the false discovery rate (FDR). In the FDR method, p values are ranked in an ascending array and multiplied by m/k where k is the position of a p value in the sorted vector and m is the number of independent tests [35].

5. Conclusions

In this study, an extensive quantitative characterization of the urinary metabolome of newborns to GDM and healthy mothers is presented. Concentration values of 101 metabolites detected in the urine of newborns collected within the first 24 h of life were available for analyses. Spermine and hexadecenoylcarnitine were dysregulated in newborns of GDM mothers. Additionally, urinary maternal samples from the second half of pregnancy were used to assess the influence of GDM in the urinary metabolome of new- borns. GDM mothers in the second and third trimester of gestation had increased urinary levels of isobutyric, butyric acid, and uric acid. Apparently, GDM slightly modulates the maternal metabolome, with altered levels of butyric and isobutyric acid, which are also detected in the urinary metabolome of

newborns. This information is of clinical relevance for neonatologists and pediatricians to monitor the impact of GDM in the early stages of life.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/metabo11110723/s1, Table S1: Concentration values of the metabolites measured for babies born to mothers with GDM and healthy controls.

Author Contributions: Y.L.-H. conceived the experiments; Y.L.-H., A.S.H.-V.O. and J.J.O.-V. conducted the experiments and statistical analysis; Y.L.-H., A.S.H.-V.O. and J.M.-E. analyzed the results; A.S.H.-V.O., J.C.T.-O., V.L.-R. and I.D.R.R. banked the samples and compiled epidemiologic information; A.S.H.-V.O., J.C.T.-O. and V.L.-R. recruited the patients; D.A.H.-V.O. interpreted the data; Y.L.-H., J.M.-E., J.A.L. and M.S.-B. contributed to the study supervision, interpretation of the results, and writing of the manuscript. All authors reviewed and approved the final version of the manuscript and agreed to the published.

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Institutional Review Board Statement: The study was performed in accordance with the Declaration of Helsinki. The study proposal was revised and approved by the Ethics Committee of the Hospital Central “Dr. Ignacio Morones Prieto” from San Luis Potosi, in Mexico (Registration No. 84-17; CONBIOETICA-24-CEI-001-201604279). Informed Consent Statement: Informed consent was obtained from all individuals involved in the study. Written informed consent was signed by all participant women prior to the interview and to the urine sample collection including that of their babies.

Data Availability Statement: The data presented in this study are available in Table S1: Concen- tration values of the metabolites measured for babies born to mothers with GDM and healthy controls.

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Conflicts of Interest: The authors declare no competing interest.

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Figure legends.

Figure 1. Multivariate analysis showing the comparison between urine metabolite data acquired for newborns of GDM (D) and healthy mothers (H). (A) Partial least squares discriminant analysis (2-D PLS-DA) score plots; (B) Variable importance in projection plot. The most discriminating metabolites are shown in descending order of their coefficient scores.

Figure 2. Pathway analysis of the deregulated metabolites in newborns to GDM mothers. The node size is proportional to the enrichment ratio.

Figure 3. Multivariate analysis showing the comparison between urinary metabolite data acquired for GDM and healthy mothers in the second and third trimesters, respectively. (A) Partial least squares discriminant analysis score plots (2-D PLS-DA) of healthy pregnant women with GDM during the second trimester of gestation. (B) 2-D PLS-DA of healthy pregnant women with GDM during the third trimester of gestation. (C) Importance of the variable in the projection graph. The most discriminating metabolites are shown in descending order of their coefficient scores. Second trimester of gestation with GDM (2 d); third trimester of gestation with GDM (3d); healthy second trimester of pregnancy (2h); healthy third trimester of pregnancy (3h).

Figure 4. Study design: Longitudinal study of 48 mother–newborn pairs

Table 1. Comparison of selected characteristics of the pregnant women and their newborns by diabetes status of the mother.

Characteristics	GDM	Healthy	*p*-Value *
Newborns, n (%)	26 (54.1)	22 (45.8)	
Sex, n (%) [b]			
Female	16 (61.5)	4 (18.1)	0.003 **
Male	10 (38.4)	18 (81.8)	
Gestational age (weeks) [a]	38.5 ± 1.3	38.3 ± 1.2	0.5
APGAR score, min 1 [b]	8 (100.0)	8 (100.0)	1.00
APGAR score, min 5 [b]	9 (100.0)	9 (100.0)	1.00
Silverman-Anderson score [b]	0 (100.0)	0 (100.0)	1.00
Weight (g) [a]	3026 ± 399	2943 ± 477	0.4
Delivery, n (%) [b]			
Vaginal	9 (34.6)	13 (59.1)	0.1
C-section	17 (65.4)	9 (40.9)	
Mothers, n (%)	26 (54.1)	22 (45.8)	
Age (years) [a]	28.4 ± 4.7	25.6 ± 2.2	0.05
Pre-BMI (Kg/m^2) [a]	27.87 ± 4.12	25.58 ± 4.22	0.06
Normal weight, n (%) [b]	8 (30.8)	10 (45.5)	0.3
Overweight, n (%) [b]	10 (38.5)	9 (40.9)	0.3
Obese, n (%) [b]	8 (30.8)	3 (13.6)	0.3
Glucose (mg/dL) [a]	86.94 ± 13.3	79.81 ± 8.7	0.03 *
Creatinine (mg/dL) [a]	0.56 ± 0.08	0.57 ± 0.09	0.6
Urea (mg/dL) [a]	14.26 ± 4.0	14.19 ± 3.91	1.0
Hemoglobin (g/dL) [a]	13.02 ± 1.0	12.81 ± 0.75	0.4
Leucocytes ($\times 10^3$) [a]	9.01 ± 2.65	8.34 ± 1.70	0.3
SBP (mm Hg) [a]	113.1 ± 8.7	108.2 ± 9.6	0.07
DBP (mm Hg) [a]	74.23 ± 7.02	72.73 ± 7.67	0.5
Treatment, n (%)			
Metformin	15 (57.7)		
Diet and exercise	10 (38.5)		
Insulin + Metformin	1 (3.8)		

Pre-BMI: prenatal body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure. * p value from Student t-tests were used for normally distributed and Mann–Whitney U tests for non-normally distributed continuous variables, * $p \leq 0.05$, ** $p \leq 0.01$; Chi^2 tests and Fisher's exact tests were used for nominal data. [a] Student's t test (mean ± S.D), [b] Chi square or Fisher's exact tests.

Table 2. Concentration values of statistically significant metabolites measured for babies born to mothers with GDM and healthy controls.

Metabolite	Healthy Newborns	GDM Newborns	*p* Value
	Median (2.5–97.5 IQR) (μM/mM Creatinine)	Median (2.5–97.5 IQR) (μM/mM Creatinine)	
trans-Hydroxyproline	36.0 (8.1–96.65)	26.4 (11.31–60.82)	0.01
Glutamic acid	7.4 (1.88–25.28)	12.4 (3.72–36.52)	0.01
DOPA	0.06 (0.02–0.17)	0.04 (0.01–0.08)	0.04
Spermine	0.03 (0.007–0.09)	0.04 (0.006–0.72)	0.003 *
Lactic acid	85.6 (46.95–797.5)	112.0 (47.14–359.8)	0.04
Butyric acid	0.33 (0.12–0.9)	0.22 (0.06–0.7)	0.02
Isobutyric acid	0.08 (0.03–1.0)	0.05 (0.02–1.0)	0.03
Glutaconylcarnitine (C5:1DC)	0.02 (0.008–0.03)	0.01 (0.005–0.03)	0.009 *
Glutarylcarnitine (C5DC)	0.05 (0.03–0.1)	0.04 (0.02–0.06)	0.006 *
C10:2	0.03 (0.02–0.07)	0.03 (0.014–0.05)	0.01
Hexadecenoylcarnitine (C16:1)	0.012 (0.007–0.03)	0.009 (0.004–0.027)	0.01

$p < 0.05$ was considered statistically significant; * $p < 0.01$.

Figure 1

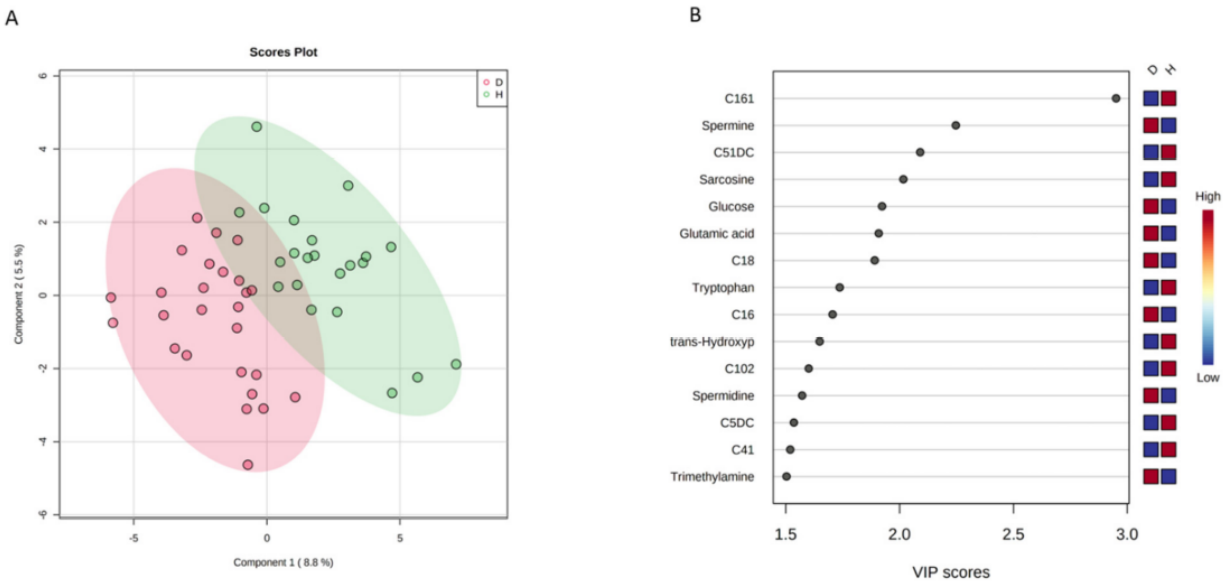


Figure 2

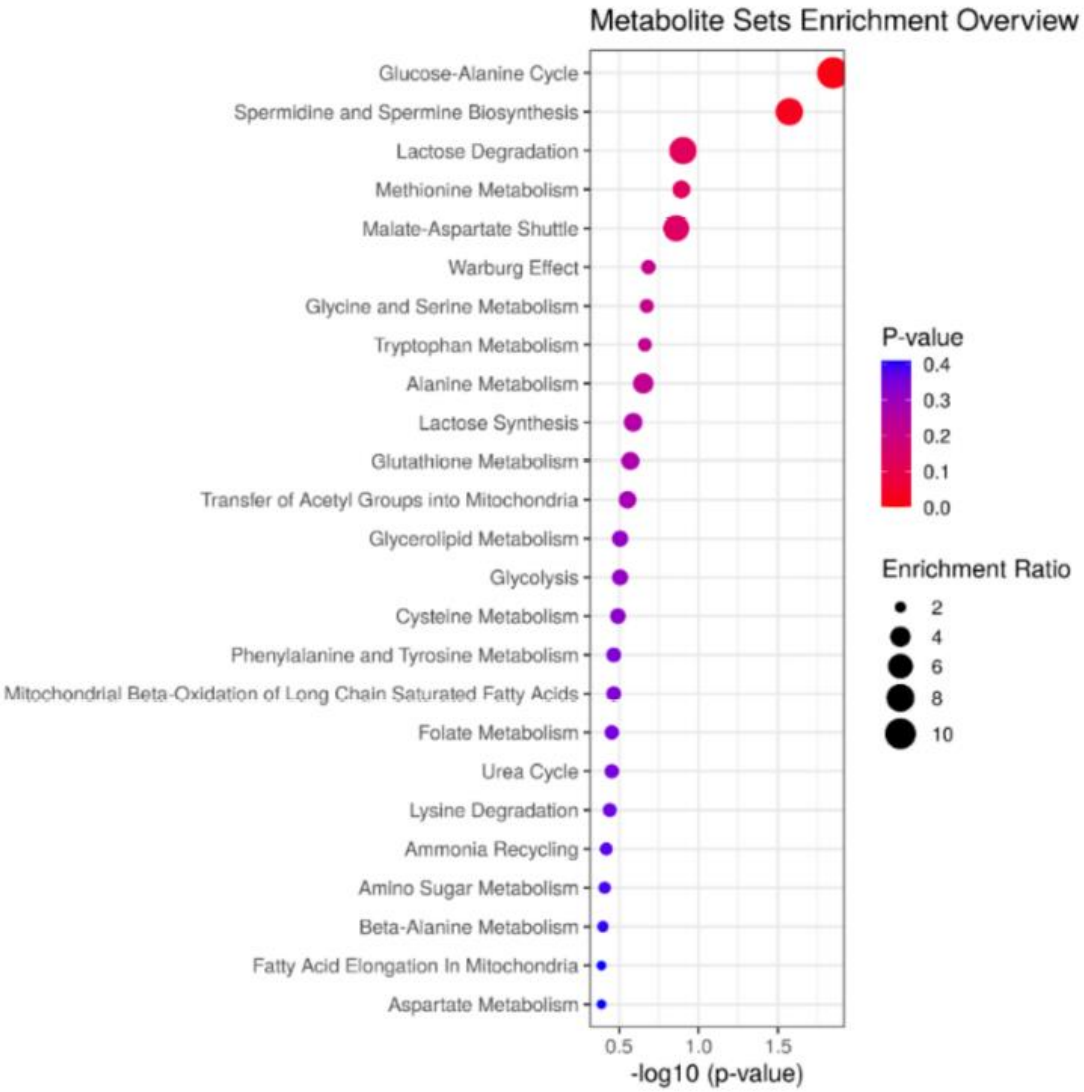

Metabolite Sets Enrichment Overview
Glucose-Alanine Cycle
Spermidine and Spermine Biosynthesis
Lactose Degradation
Methionine Metabolism
Malate-Aspartate Shuttle
Warburg Effect
Glycine and Serine Metabolism
Tryptophan Metabolism
Alanine Metabolism
Lactose Synthesis
Glutathione Metabolism
Transfer of Acetyl Groups into Mitochondria
Glycerolipid Metabolism
Glycolysis
Cysteine Metabolism
Phenylalanine and Tyrosine Metabolism
Mitochondrial Beta-Oxidation of Long Chain Saturated Fatty Acids
Folate Metabolism
Urea Cycle
Lysine Degradation
Ammonia Recycling
Amino Sugar Metabolism
Beta-Alanine Metabolism
Fatty Acid Elongation In Mitochondria
Aspartate Metabolism
0.5
1.0
1.5
-log10 (p-value)
P-value
0.4
0.3
0.2
0.1
0.0
Enrichment Ratio
2
4
6
8
10

Figure 3

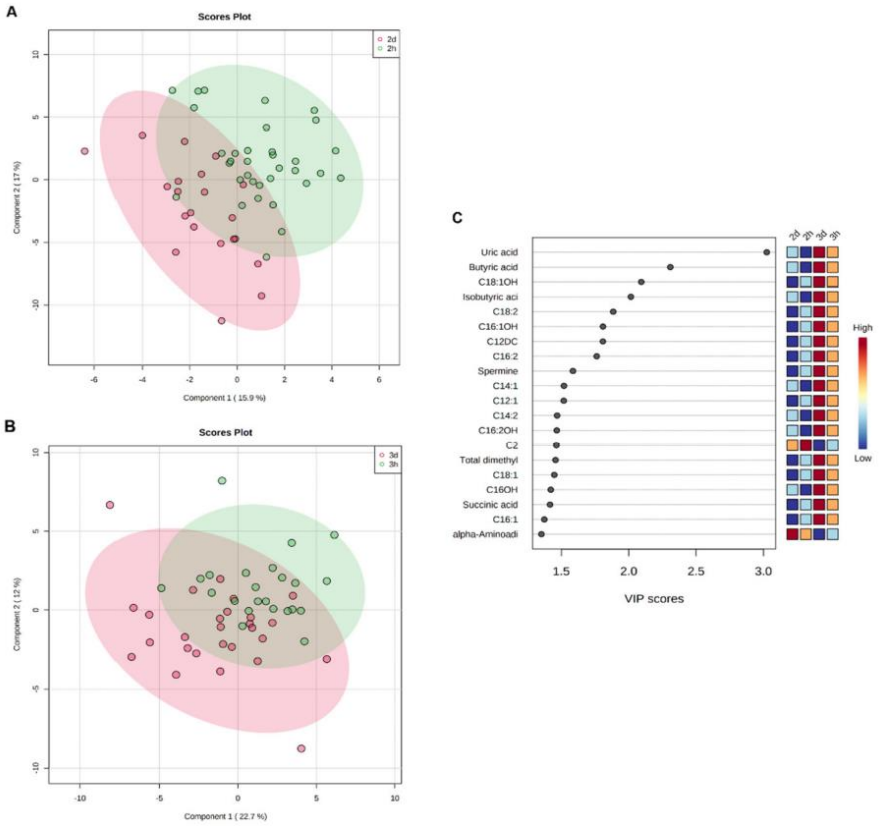
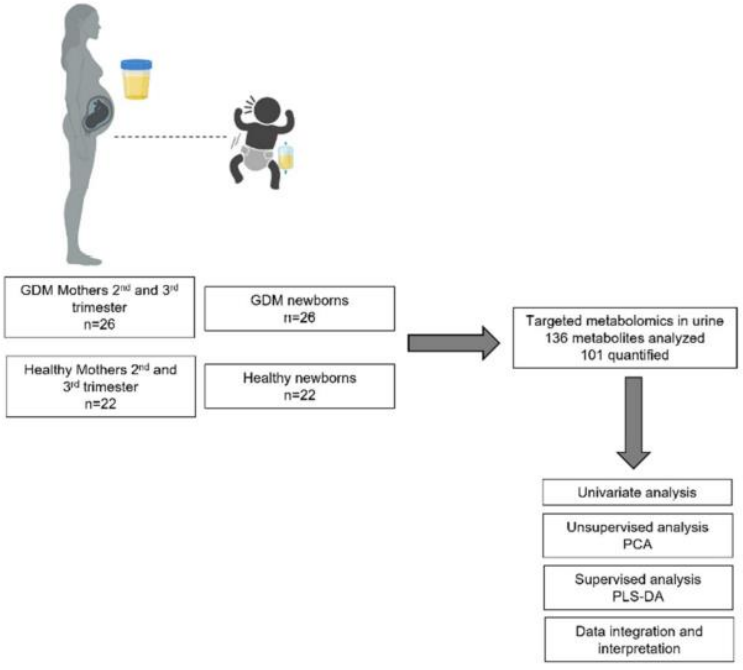


Figure 4



Portada de artículos publicados derivados del trabajo de tesis doctoral

(2019)

International Journal of *Molecular Sciences*

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Article

Urinary Metabolites Altered during the Third Trimester in Pregnancies Complicated by Gestational Diabetes Mellitus: Relationship with Potential Upcoming Metabolic Disorders

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Article

The Urinary Metabolome of Healthy Newborns

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metabolites

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Article

Urinary Metabolomic Profile of Neonates Born to Women with Gestational Diabetes Mellitus

Ana Sofía Herrera-Van Oostdam [1], Mariana Salgado-Bustamante [2], Victoria Lima-Rogel [3], Juan José Oropeza-Valdez [4], Jesús Adrián López [5], Iván Daniel Román Rodríguez [6], Juan Carlos Toro-Ortiz [3], David Alejandro Herrera-Van Oostdam [3], Yamilé López-Hernández [6,7,*] and Joel Monárrez-Espino [8,*]

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Abstract: Gestational diabetes mellitus (GDM) is one of the most frequent pregnancy complications with potential adverse outcomes for mothers and newborns. Its effects on the newborn appear during the neonatal period or early childhood. Therefore, an early diagnosis is crucial to prevent the development of chronic diseases later in adult life. In this study, the urinary metabolome of babies born to GDM mothers was characterized. In total, 144 neonatal and maternal (second and third trimesters of pregnancy) urinary samples were analyzed using targeted metabolomics, combining liquid chromatographic mass spectrometry (LC-MS/MS) and flow injection analysis mass spectrometry (FIA-MS/MS) techniques. We provide here the neonatal urinary concentration values of 101 metabolites for 26 newborns born to GDM mothers and 22 newborns born to healthy mothers. The univariate analysis of these metabolites revealed statistical differences in 11 metabolites. Multivariate analyses revealed a differential metabolic profile in newborns of GDM mothers characterized by dysregulation of acylcarnitines, amino acids, and polyamine metabolism. Levels of hexadecenoylcarnitine (C16:1) and spermine were also higher in newborns of GDM mothers. The maternal urinary metabolome revealed significant differences in butyric, isobutyric, and uric acid in the second and third trimesters of pregnancy. These metabolic alterations point to the impact of GDM in the neonatal period.

Keywords: newborns; metabolomics; gestational diabetes; pregnancy

Portada de artículos publicados durante mis estudios de doctorado en los que participe como co-autora.

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Review

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Biomarkers in Medicine

Placental exosomes viewed from an 'omics' perspective: implications for gestational diabetes biomarkers identification

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Placental exosomes isolated from urine of patients with gestational diabetes exhibit a differential profile expression of microRNAs across gestation

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Article

Clinical Factors Associated with COVID-19 Severity in Mexican Patients: Cross-Sectional Analysis from a Multicentric Hospital Study

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Abstract: (1) Background: Latin America has been harshly hit by SARS-CoV-2, but reporting from this region is still incomplete. This study aimed at identifying and comparing clinical characteristics of patients with COVID-19 at different stages of disease severity. (2) Methods: Cross-sectional multicentric study. Individuals with nasopharyngeal PCR were categorized into four groups: (1) negative, (2) positive, not hospitalized, (3) positive, hospitalized with/without supplementary oxygen, and (4) positive, intubated. Clinical and laboratory data were compared, using group 1 as the reference. Multivariate multinomial logistic regression was used to compare adjusted odds ratios. (3) Results: Nine variables remained in the model, explaining 76% of the variability. Men had increased odds, from 1.90 (95%CI 0.87–4.15) in the comparison of 2 vs. 1, to 3.66 (1.12–11.9) in 4 vs. 1. Diabetes and obesity were strong predictors. For diabetes, the odds for groups 2, 3, and 4 were 1.56 (0.29–8.16), 12.8 (2.50–65.8), and 16.1 (2.87–90.2); for obesity, these were 0.79 (0.31–2.05), 3.38 (1.04–10.9), and 4.10 (1.16–14.4), respectively. Fever, myalgia/arthralgia, cough, dyspnea, and neutrophilia were associated with the more severe COVID-19 group. Anosmia/dysgeusia were more likely to occur in group 2 (25.5; 2.51–259). (4) Conclusion: The results point to relevant differences in clinical and laboratory features of COVID-19 by level of severity that can be used in medical practice.

Keywords: COVID-19; disease severity; Mexico; multivariate analysis; signs and symptoms

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Targeted metabolomics identifies high performing diagnostic and prognostic biomarkers for COVID-19

Yamilé López-Hernández[1,2✉], Joel Monárrez-Espino[3✉], Ana-Sofía Herrera-van Oostdam[4], Julio Enrique Castañeda Delgado[1,5], Lun Zhang[6], Jiamin Zheng[6], Juan José Oropeza Valdez[5], Rupasri Mandal[6], Fátima de Lourdes Ochoa González[5,7], Juan Carlos Borrego Moreno[8], Flor M. Trejo-Medinilla[2,7], Jesús Adrián López[9], José Antonio Enciso Moreno[5] & David S. Wishart[6]

Research exploring the development and outcome of COVID-19 infections has led to the need to find better diagnostic and prognostic biomarkers. This cross-sectional study used targeted metabolomics to identify potential COVID-19 biomarkers that predicted the course of the illness by assessing 110 endogenous plasma metabolites from individuals admitted to a local hospital for diagnosis/treatment. Patients were classified into four groups (≈ 40 each) according to standard polymerase chain reaction (PCR) COVID-19 testing and disease course: PCR−/controls (i.e., non-COVID controls), PCR+/not-hospitalized, PCR+/hospitalized, and PCR+/intubated. Blood samples were collected within 2 days of admission/PCR testing. Metabolite concentration data, demographic data and clinical data were used to propose biomarkers and develop optimal regression models for the diagnosis and prognosis of COVID-19. The area under the receiver operating characteristic curve (AUC; 95% CI) was used to assess each models' predictive value. A panel that included the kynurenine: tryptophan ratio, lysoPC a C26:0, and pyruvic acid discriminated non-COVID controls from PCR+/not-hospitalized (AUC = 0.947; 95% CI 0.931–0.962). A second panel consisting of C10:2, butyric acid, and pyruvic acid distinguished PCR+/not-hospitalized from PCR+/hospitalized and PCR+/intubated (AUC = 0.975; 95% CI 0.968–0.983). Only lysoPC a C28:0 differentiated PCR+/hospitalized from PCR+/intubated patients (AUC = 0.770; 95% CI 0.736–0.803). If additional studies with targeted metabolomics confirm the diagnostic value of these plasma biomarkers, such panels could eventually be of clinical use in medical practice.

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), first identified in China in December 2019, is responsible for the coronavirus disease outbreak (COVID-19)[1]. One year after the pandemic was declared, the infection has caused nearly 2.5 million deaths worldwide[2]. In spite of significant efforts undertaken by government and health authorities to contain the spread, the virus continues to wreak havoc around the world.

Since COVID-19 can lead to multi-organ dysfunction, disease severity is not only the result of pathogen burden[3], but also the consequence of the host's immune response to the infection. It is well known that viruses hijack the host cell machinery for self-replication, as they compete for nutrients and other metabolites to satisfy their bioenergetic and biosynthetic requirements. This metabolic hijacking can lead to an alteration of the host's metabolome[4]. In fact, a number of metabolic pathways have already been found to be consistently altered (glycolysis, fatty acid synthesis, glutaminolysis, pyrimidine metabolism, and tryptophan/kynurenine metabolism) in

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PLOS ONE

RESEARCH ARTICLE

Immunometabolic signatures predict risk of progression to sepsis in COVID-19

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Abstract

Viral sepsis has been proposed as an accurate term to describe all multisystemic dysregulations and clinical findings in severe and critically ill COVID-19 patients. The adoption of this term may help the implementation of more accurate strategies of early diagnosis, prognosis, and in-hospital treatment. We accurately quantified 110 metabolites using targeted metabolomics, and 13 cytokines/chemokines in plasma samples of 121 COVID-19 patients with different levels of severity, and 37 non-COVID-19 individuals. Analyses revealed an integrated host-dependent dysregulation of inflammatory cytokines, neutrophil activation chemokines, glycolysis, mitochondrial metabolism, amino acid metabolism, polyamine synthesis, and lipid metabolism typical of sepsis processes distinctive of a mild disease. Dysregulated metabolites and cytokines/chemokines showed differential correlation patterns in mild and critically ill patients, indicating a crosstalk between metabolism and hyperinflammation. Using multivariate analysis, powerful models for diagnosis and prognosis of COVID-19 induced sepsis were generated, as well as for mortality prediction among septic patients. A metabolite panel made of kynurenine/tryptophan ratio, IL-6, LysoPC a C18:2, and phenylalanine discriminated non-COVID-19 from sepsis patients with an area under the curve (AUC (95%CI)) of 0.991 (0.986–0.995), with sensitivity of 0.978 (0.963–0.992) and specificity of 0.920 (0.890–0.949). The panel that included C10:2, IL-6, NLR, and C5 discriminated mild patients from sepsis patients with an AUC (95%CI) of 0.965 (0.952–0.977), with sensitivity of 0.993(0.984–1.000) and specificity of 0.851 (0.815–0.887). The panel with citric acid, LysoPC a C28:1, neutrophil-lymphocyte ratio (NLR) and kynurenine/tryptophan ratio discriminated severe patients from sepsis patients with an AUC (95%CI) of 0.829

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Rev Invest Clin. 2022;74(6):314-27 ORIGINAL ARTICLE

COVID-19 Outcome Prediction by Integrating Clinical and Metabolic Data using Machine Learning Algorithms

Karen E. Villagrana-Bañuelos[1‡], Valeria Maeda-Gutiérrez[1‡], Vanessa Alcalá-Rmz[1‡], Juan J. Oropeza-Valdez[2], Ana S. Herrera-Van Oostdam[3], Julio E. Castañeda-Delgado[4], Jesús Adrián López[5], Juan C. Borrego Moreno[6], Carlos E. Galván-Tejada[1], Jorge I. Galván-Tejeda[1], Hamurabi Gamboa-Rosales[1], Huizilopoztli Luna-García[1], José M. Celaya-Padilla[1*], and Yamilé López-Hernández[7*]

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diagnostics

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Article

miR-146a, miR-221, and miR-155 are Involved in Inflammatory Immune Response in Severe COVID-19 Patients

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Posgrado en Ciencias Biomédicas Básicas

CBB Ciencias Biomédicas Básicas POSGRADO

Caracterización del perfil metabolómico urinario en mujeres con Diabetes Mellitus Gestacional y sus recién nacidos a través del abordaje multiómico

Examen que presenta:

M en C. Ana Sofía Herrera Van Oostdam

Para obtener el grado de:

Doctora en Ciencias Biomédicas Básicas

UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

Posgrado en Ciencias Biomédicas Básicas

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Asesor externo

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(Unidad Académica de Ciencias Biológicas ,UAZ)

Epidemiología

Diabetes mellitus gestacional (DMG)

Intolerancia a glucosa que se identifica por primera vez durante el embarazo.

Mundial: 2-25%
~ 18 millones de embarazos

En México, la DMG complica 6.9 - 30% de los embarazos

ENSANUT Continua (2020) y ENSANUT COVID-19

Detección de obesidad y sobrepeso en adultos mexicanos: 72.4%

2018, 15.3%
2021, 9.6%

~ 48.6 millones de personas.

Detección de sobrepeso y obesidad en adultos de 20 años o más
2018, 8.4%
2021, 5.1%

Prevalencia de obesidad y sobrepeso en sexo femenino

37.5%
(2012)
(2018)

Díaz-Velázquez (2021); ENSANUT 2018-19: Resultados Nacionales, Instituto Nacional de Salud Pública, 2021; Fröhlich (2021) Molina (2019); Ramírez-Torres (2013)

Factores de riesgo en DMG

Madres
- Obesidad y/o sobrepeso (IMC >25 Kg/m²)
- Edad mayor a 25 años
- Antecedentes de diabetes gestacional
- Antecedentes de enfermedades metabólicas familiares
- Ganancia de 20 Kg de peso en la actual gestación
- Antecedentes de nacimiento de hijos con peso mayor a 4 Kg

Resistencia a la insulina → Hiperglicemia → DGM

Feto
- Hormonas placentarias
- Factores inflamatorios

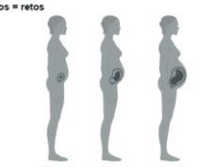
Modificaciones epigenéticas, expresión de proteínas y metabolitos

Diagnóstico y Tratamiento de la diabetes en el embarazo. *Guía Práctica clínica (2016)*; Osman (2022)

Fisiología del embarazo

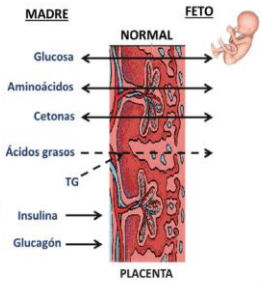
Embarazo = cambios = retos

- Fisiológicos
- Metabólicos
- Hormonales
- Inmunológicos



1° trimestre 2° trimestre 3° trimestre

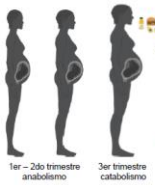
Estrógeno
Progesterona
Prolactina
Hormona del crecimiento placentario
Lactógeno placentario humano



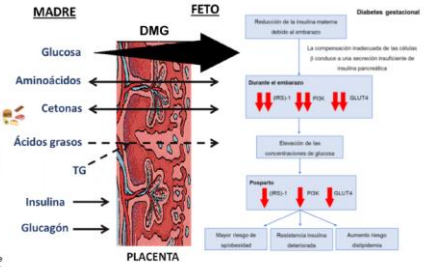
Alteración de la comunicación placentaria materno-fetal en DMG

Embarazo = cambios = retos

- Fisiológicos
- Metabólicos
- Hormonales
- Inmunológicos

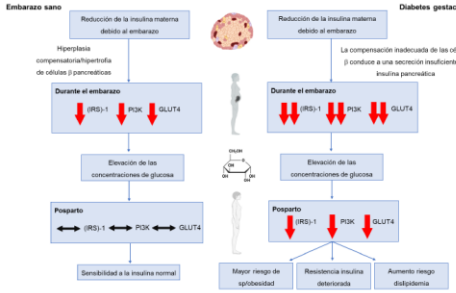


1er – 2do trimestre anabolismo 3er trimestre catabolismo



Fröhlich (2021); Méndez (Modif. Gluckman 2013); Kahn (2020)

Fisiopatología propuesta de la diabetes gestacional



Mousa (2021)

Riesgos maternos y fetales/descendientes asociados con DMG

Materno
- Preeclampsia
- Cesárea
- Complicaciones de parto
- Parto prematuro
- Hemorragia post parto
- DMG recurrente

CTGO

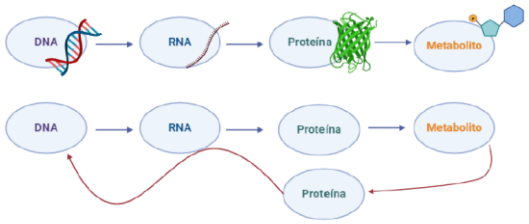
Tiempo	Tamiz 50 gr	Sobrecarga con 100 gr	Sobrecarga 75 gr
Glucosa plasmática en ayuno		>95 mg/dL	>92 mg/dL
Glucosa plasmática 1 hora pos carga	>140 mg/dL	180	180
Glucosa plasmática 2 horas pos carga		155	153
Glucosa plasmática 3 horas pos carga		140	

s tipo 2
la diabetes tipo 2
vascular, nefropatía, retinopatía)
eso/obesidad

Fröhlich (2021); OMS; Kim (2015);Rivero (2014); Reyes (2023)

Enfoque metabolómico

- Estudio de los perfiles globales de metabolitos en una célula, tejido u organismo bajo un conjunto determinado de condiciones.
- Uso de diversos métodos de separación y detección que utilizan equipos de alta potencia, incluida la cromatografía de gases, la cromatografía líquida de alto rendimiento y la espectrometría de masas.



Mousa (2021); Shen (2022) 9

Metabolómica no dirigida

Critical Insight = desafíos

- Analizador de masas de escaneo completo para generar medición de masa para moléculas individuales para permitir cálculos estadísticos seguidos de la adquisición dependiente de datos (DDA) de un subconjunto de muestras para guiar las identificaciones.
- Características de relación masa-carga (m/z) y tiempo de retención (RT) de estructuras provisionales o preliminares.
- Los iones precursores y los iones fragmentados correspondientes se buscan en bases de datos para determinar las asignaciones de metabolitos.

Es importante destacar que:

Las características no siempre son metabolitos

Pueden estar presentes con diferentes valores de m/z.

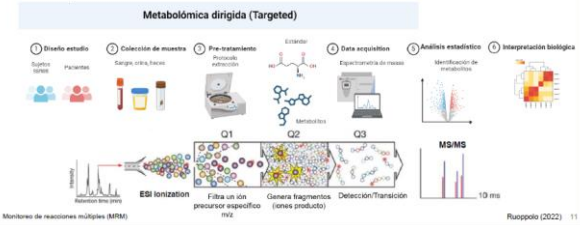
Especies relacionadas:

*isótopos
*pérdidas neutras
*aductos

Schrimpe-Rutledge (2016) 10

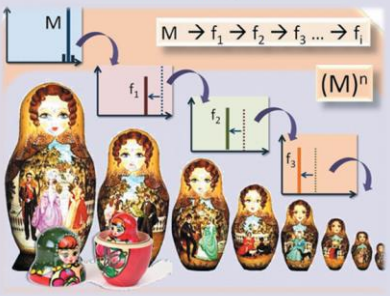
Metabolómica dirigida

- Aplicación analítica de la espectrometría de masas que abarca la medición de grupos definidos de metabolitos caracterizados químicamente.
- Estándares internos añadidos a cada muestra = normalizar las concentraciones de metabolitos en todos los conjuntos y lotes de muestras.
- Emplear el análisis MRM, la identidad exacta de cada metabolito se puede definir.
- Reduciendo el predominio de moléculas de alta abundancia en los análisis.



Ruoppolo (2022) 11

Matrioshka ion-fragmento



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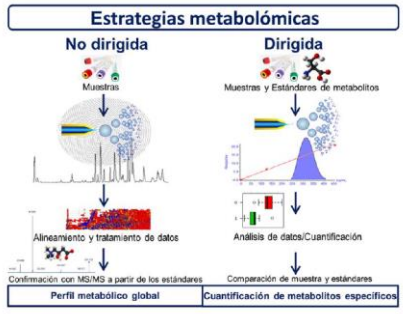
Estrategias metabolómicas



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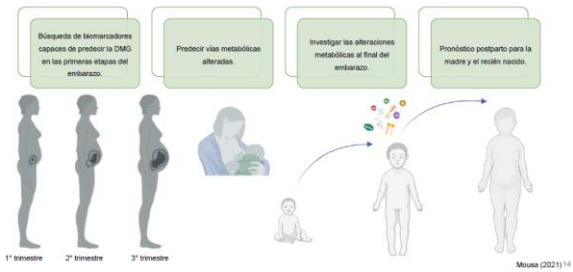
Metaboloma materno-fetal en DMG

Objetivos de los estudios metabolómicos en DMG:



Mousa (2021) 14

Diseño experimental

Servicio de Ginecología y Obstetricia; Neonatología del Hospital Central "Dr. Ignacio Morones Prieto"
Dr. Juan Carlos Toro Ortiz / Dra. Victoria Lima Rogel

Criterios de inclusión	Criterios de no inclusión	Criterios de eliminación
Mujeres +18 años	Antecedente DM1 o DM2	Volumen de muestra insuficiente
< 20 Semanas de Gestación (SDG)	Antecedentes de preeclampsia	Imposibilidad de procesamiento de muestra
Que acepten la participación de su recién nacido	Antecedentes nefropatía	Abandono de estudio

Después de la semana 24 distinguimos dos grupos de embarazadas:

1. Sanas: Niveles de glucosa: 80-100 mg/dl en ayuno; ≤ 140 mg/dl postprandial 1 hora; ≤ 140 mg/dl postprandial 2 horas; ≤ 100 ó 120 mg/dl preprandial, sin cetonurias.

2. DMG: Identificadas en el Servicio de Ginecología con el diagnóstico de DMG: glucemia en ayuno es >110 mg/dl o cuando la glucemia a la hora ≥ 140 mg/dl.

Registro 84-17 (Folio CONBIOETICA-24-CEI-001-201604279)

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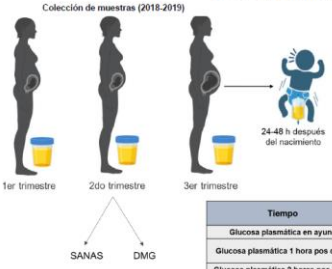
Diseño experimental

Colección de muestras (2018-2019)



Datos clínicos y demográficos se recopilaron de los registros médicos de cada participante en la primera visita prenatal.

Tiempo	Tamiz 50 gr	Sobrecarga con 100 gr	Sobrecarga 75 gr
Glucosa plasmática en ayuno		>95 mg/dL	>92 mg/dL
Glucosa plasmática 1 hora pos carga	>140 mg/dL	180	180
Glucosa plasmática 2 horas pos carga		155	153
Glucosa plasmática 3 horas pos carga		140	

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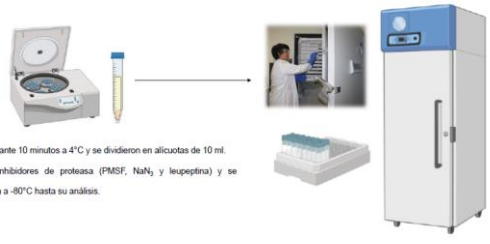
Diseño experimental



- 1200 x g durante 10 minutos a 4°C y se dividieron en alícuotas de 10 ml.
- Cóctel de inhibidores de proteasa (PMSF, NaN_3 y leupeptina) y se almacenaron a -80°C hasta su análisis.

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Diseño experimental general

SANAS **DMG**

1ª Etapa → Perfil metabolómico no dirigido → 3° trimestre

2ª Etapa → Perfil metabolómico dirigido → Recién nacido

3ª Etapa → Perfil metabolómico dirigido → 2° y 3° trimestre, Recién nacido

Ampliar los estudios en matrices que resulten no invasivas mediante el uso de estrategias multiómicas para entender el desarrollo de una patología compleja como DMG.

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Análisis metabolómico no dirigido en orina de mujeres embarazadas de 3er trimestre con DMG

International Journal of Molecular Sciences

Article

Urinary Metabolites Altered during the Third Trimester in Pregnancies Complicated by Gestational Diabetes Mellitus: Relationship with Potential Upcoming Metabolic Disorders

Yamilé López-Hernández [1,*], Ana Sofía Herrera-Van Oostdam [2], Juan Carlos Toro-Ortiz [3], Jesús Adrián López [4], Mariana Salgado-Bustamante [4], Michael Murguı́a [5] and Lourdes Mariela Torres-Torres [3]

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3 Gynecology and Obstetrics Division, Hospital Central "Dr. Ignacio Morones Prieto",7800 San Luis Potosí, Mexico; jcarlotoro@yahoo.com.mx (J.C.T.-O.); mariela.torres161299@gmail.com (L.M.T.-T.)
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Metabolitos urinarios alterados durante el tercer trimestre en embarazos complicados por diabetes mellitus gestacional: relación con posibles trastornos metabólicos futuros

- Estudio piloto
- Perfil ma urinario de paci cientes
- estár lietético y farmacol tabolitos
- Méto ómica no dirigida
- expr cialmente en el gru

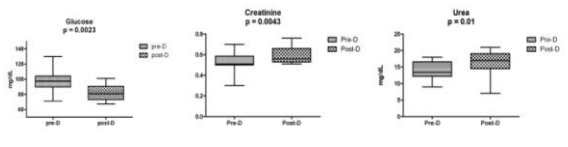
35 mujeres embarazadas de una cohorte inicial de 80 pacientes.

	GDM (n = 24)	Control (n = 11)	p-Value
Age (years)	31.00 ± 1.401	26.91 ± 1.232	0.0771
Gestational age at sampling (weeks)	32.05 ± 0.9026	34.74 ± 0.6853	0.0672
BMI (Kg/m^2)	30.07 ± 1.268	25.98 ± 1.594	0.0546
Glucose (mg/dL)	92.75 ± 3.174	76.70 ± 1.764	0.0024
SBP (mmHg)	110.4 ± 9.079	108.2 ± 9.816	0.8701
DBP (mmHg)	73.75 ± 9.816	71.82 ± 7.109	0.8161
Creatinine (mg/dL)	0.5262 ± 0.02066	0.5500 ± 0.03643	0.5436
Urea (mg/dL)	13.83 ± 0.9461	16.12 ± 1.734	0.2144
Hb (g/dL)	12.52 ± 0.8055	13.26 ± 1.074	0.1453
Leucocytes (×10^3)	8.739 ± 1.756	8.000 ± 1.525	0.2339

media ± error estándar de la media (SEM). BMI: índice de masa corporal; SBP: presión arterial sistólica; DBP: presión arterial diastólica. Los valores de p se determinaron mediante prueba t-Student. Estadísticamente significativo: p < 0.05.

López-Hernández (2019) 20

Niveles de glucosa, creatinina y urea medidos en plasma de pacientes con DMG antes y después del diagnóstico de DMG.



(A) Valores clinicamente sanos < 100 mg/dL

(B) Valores clinicamente sanos 0.5 - 0.8 mg/dL

(C) Valores clinicamente sanos 6 - 43 mg/dL

Se encontró que la glucosa disminuyó significativamente en el tercer trimestre, lo que refleja el efecto positivo del tratamiento y del control dietético en pacientes con resistencia a la insulina tratados con dosis bajas de metformina).

Arias (2018); López-Hernández (2019) 21

Análisis UHPLC-ESI-QTOF-MS

UNIFI 1.8.1 (Waters Corp., Milford, EE. UU.)

Cromatografía de fase reversa

- Fase móvil fue *A*: H_2O con ácido fórmico al 0,1%; *B*
- Columna Waters BEH C18 UPLC híbrida con puente

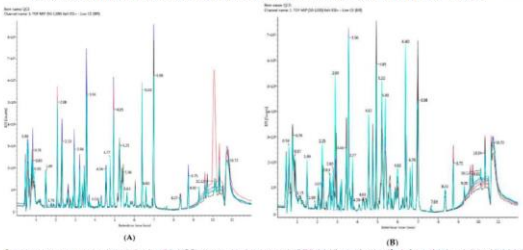
MS: XEVO-G2 XS Q-Tof

- Modo de ionización por electropulverización positiva
- MS^E
- *m / z* 100 - 2000

ACQUITY UPLC I-Class (Waters Corp., Milford, MA, EE. UU.) acoplado a un espectrómetro de masas de tiempo de vuelo (ToF) cuadrupolo XEVO-G2 XS (Waters.)

López-Hernández (2019) 22

Cromatografía líquida de ultra rendimiento (UPLC)-espectrometría de masas (MS) perfiles urinarios



(A) (B)

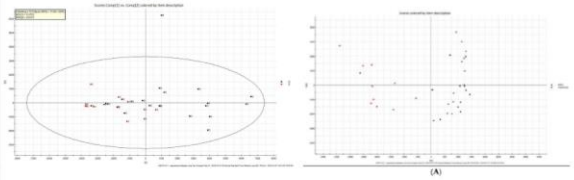
Cromatogramas superpuestos de controles de calidad (QC) que muestran buena reproducibilidad del tiempo de retención, la forma del pico y la intensidad del pico. (A) QCS: cromatogramas superpuestos de cinco réplicas de QC del grupo de control. (B) QCD: cromatogramas superpuestos de cinco réplicas de QC del grupo DMG.

No mostraron variaciones en el tiempo de retención, lo que refleja la estabilidad y reproducibilidad del sistema

López-Hernández (2019) 23

Identificación de metabolitos

Matriz con 4598 variables y 35 muestras



(A)

- La matriz de datos multivariada se analizó mediante el software EZInfo (Waters Corp., Milford, MA, EE. UU.) y el análisis univariado se realizó con MetaboAnalyst.
- Los datos se centraron en la media y análisis multivariado de componentes principales (PCA) y análisis discriminante de estructuras latentes proyección ortogonal (OPLS-DA).
- Los posibles marcadores de interés se extrajeron de la gráfica VIP combinada que se construyó a partir de las gráficas de carga de OPLS-DA. Se consideró un umbral VIP de 1,5 para seleccionar los metabolitos.

López-Hernández (2019) 24

Metabolitos identificados con cambios significativos en la expresión en pacientes con DMG

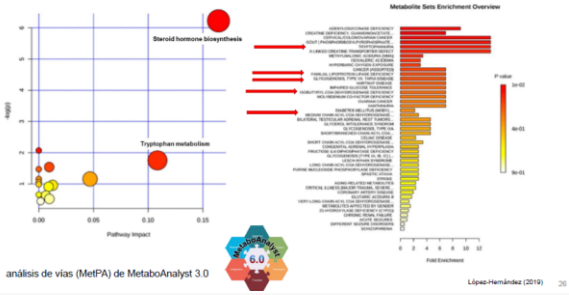
Class	Compound	HMDB	Molecular Formula	Observed m/z	RT (min)	p-value	FC	Mass Error (mDa)	Adducts
Benzopyrans	5-carboxy-alpha-chromanol	HMDB0012798	$C_{12}H_{14}O_4$	238.2312	5.82	2×10^{-6} *	4.2	2.0	M^+
Carboxylic acids and derivatives	3-Methyl N- -alpha-aspartyl- -phenylalanate (aspartame)	HMDB0001894	$C_{14}H_{18}N_2O_5$	295.12879	3.32	4.45×10^{-06} *	5.1	0.2	H^+
Glycerolipids	DG (24:0/14:1)	HMDB0007792	$C_{41}H_{78}O_5$	668.6179	9.87	6.36×10^{-6} *	3.6	-0.9	NH^{4+}
Indoles and derivatives	L-Tryptophan	HMDB0000929	$C_{11}H_{12}N_2O_2$	205.09703	2.90	3.08×10^{-6} *	2.4	-0.5	H^+
Tetrapyrroles and derivatives	L-Urobilinogen	HMDB0004157	$C_{33}H_{44}N_4O_6$	597.3631	5.94	3.75×10^{-6} *	3.2	-1.6	H^+
Sphingolipids	Gr (d18:0/23:0)	HMDB0011767	$C_{47}H_{95}NO_3$	698.68753	9.92	3.0×10^{-7} *	11	0.1	H^+
	SM (d18:0/22:0)	HMDB0012091	$C_{45}H_{93}N_2O_6P$	789.685	9.62	4.96×10^{-6} *	4.2	0.6	H^+
Steroid and steroid derivatives	11-oxo-androsterone-glucuronide	HMDB0010338	$C_{25}H_{38}O_9$	503.2247	5.5	5.44×10^{-6} *	2.6	-0.5	Na^+
	cortolone-3-glucuronide	HMDB0010320	$C_{27}H_{44}O_{11}$	543.2789	5.12	5.80×10^{-6} *	2.9	-1.1	H^+
	tetrahydrodeoxycorsone-3-glucuronide	HMDB0010357	$C_{27}H_{46}O_{11}$	563.2456	5.09	2.38×10^{-5} *	4.6	-0.7	Na^+
	5-androstene-3b,16b,17a-triol	HMDB0000523	$C_{19}H_{30}O_3$	329.21108	5.09	6.18×10^{-5} *	6.4	1.9	Na^+
	21-deoxycortisol	HMDB0004030	$C_{21}H_{30}O_4$	347.22121	5.26	2.45×10^{-5} *	5.7	-0.6	H^+
	11b, 17a,21-Trihydroxypreg-nenolone	HMDB0006760	$C_{21}H_{32}O_5$	365.2317	5.09	2.0×10^{-5} *	3.1	-0.4	H^+
	cucurbitacin c	HMDB0034706	$C_{32}H_{46}O$	561.3411	5.92	3.00×10^{-5} *	3.3	0.1	H^+

a: significative for false discovery rate (FDR) correction. FC: Fold change. Metabolites in bold are exogenous metabolites.

- 5-carboxi-alfa-cromanol (relacionado con el metabolismo de la vitamina E), aspartamo y la cucurbitacina c (presente en frutas y verduras) están relacionados con el control dietético en el grupo con DMG.
- Estas sustancias son consumidas particularmente en pacientes mexicanos bajo un régimen dietético, como lo sugiere el Sistema Alimentario Mexicano Equivalentes (planes de alimentación SMAE)

López-Hernández (2019); Warnen (2018) 25

Contribución de los metabolitos identificados en la fisiopatología de la DMG

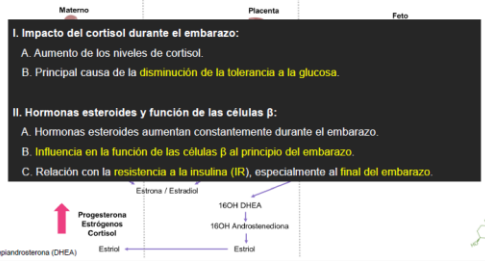


análisis de vías (MetPA) de MetaboAnalyst 3.0

López-Hernández (2019) 26

Las vías metabólicas consideradas significativas en nuestro estudio fueron la vía del metabolismo de esteroides y derivados

La endocrinología del embarazo humano está dominada por múltiples hormonas placentarias que inducen cambios fisiológicos en la madre a favor del embarazo.



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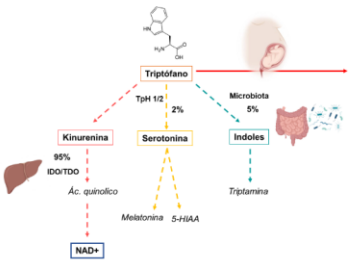
Las vías metabólicas consideradas significativas en nuestro estudio fueron la vía del metabolismo de triptófano (TRP)



Requisitos de triptófano (Trp) durante el embarazo

1.- Necesidad de una mayor síntesis de proteínas por parte de la madre, para el crecimiento y desarrollo del feto.

2.- Ácido quinolínico para la síntesis de NAD+.

3.- Proteger al feto contra el rechazo de los metabolitos KP inmunosupresores.

*citotóxicos/citoprotectoros

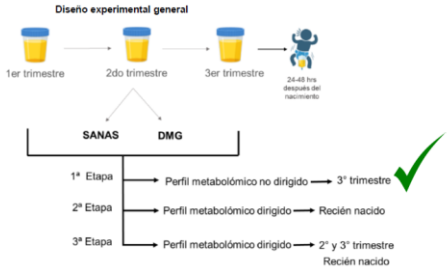
oxidantes/antioxidantes o pro/antiinflamatorias.

DESEQUILIBRIO

Ácido 5-hidroxiindolacético (5-HIAA); nicotinamina adenina dinucleótido (NAD);Indoleamina 2,3-dioxigenasa (IDO);Trp 2,3-dioxigenasa (TDO); triptófano hidroxilasa (TpH)

Badawy (2015);López-Hernández (2019); Miguel (2017) 28

Diseño experimental general



Ampliar los estudios en matrices que resulten no invasivas mediante el uso de estrategias multiómicas para entender el desarrollo de una patología compleja como DMG.

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metabolites MDPI

Article

The Urinary Metabolome of Healthy Newborns

Yamilé López-Hernández [1,*], Juan José Oropeza-Valdez [2], Jorge O. Blanco-Sandate [3], Ana Sofía Herrera-Van Oostdam [4], Jiamin Zheng [5], An Chi Guo [5], Victoria Lima-Rogel [4], Rahmatollah Rajabzadeh [5], Mariana Salgado-Bustamante [4], Jesus Adrian-Lopez [7], C. G. Castillo [3], Emilia Robles Arguelles [7], Joel Monárrez-Espino [8], Rupasri Mandal [5] and David S. Wishart [5,*]

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Metaboloma urinario de recién nacidos sanos

- Durante la etapa neonatal tienen lugar una serie de eventos fisiológicos importantes:
  - Establecimiento de patrones de alimentación
  - Adaptación del sistema inmunológico
  - Cambios en el sistema endocrino
  - Modificaciones en el metabolismo general

Habitualmente, la estancia hospitalaria habitual de un recién nacido es de 48 h tras un parto vaginal y de 96 h tras una cesárea. Sin embargo, cuando las condiciones médicas aparecen poco después del nacimiento, se requieren períodos de hospitalización más prolongados. Es durante estas estancias prolongadas cuando se suelen realizar múltiples pruebas hematológicas, urinarias, clínicas y bioquímicas.

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Metaboloma urinario de recién nacidos sanos

- Para los recién nacidos a término, se encuentran disponibles muchos valores bioquímicos de referencia para *sangre, suero, plasma y líquido cefalorraquídeo*.
- Sin embargo, existe una sorprendente falta de información sobre los valores normales de concentración en **orina** de una gran cantidad de metabolitos importantes en los recién nacidos.
- Una revisión en profundidad de la literatura, junto con comparaciones detalladas con los valores informados en la Base de datos del metaboloma humano (HMDB) y la Base de datos del metaboloma en orina humana (UMDB) indica que solo 212 metabolitos han informado valores de referencia en orina para recién nacidos y lactantes (detectados y cuantificados).

Por lo tanto, la disponibilidad de valores de referencia para parámetros urinarios, clínicos y bioquímicos para recién nacidos es de vital importancia.

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Metaboloma urinario de recién nacidos sanos

Estudio de metabolómica basada en espectrometría de masas en tándem (MS/MS) para identificar y cuantificar 136 metabolitos de interés biomédico en la orina de 48 recién nacidos sanos a término, recolectada en las primeras 24 h de vida.

Newborns (N = 48)	
Sex	
Female	17 (35%)
Male	31 (65%)
Gestational age (weeks)	38.2 ± 1.5
Ave. Apgar at 1 min	8 (100%)
Ave. Apgar at 5 min	9 (100%)
Ave. Silverman-Anderson	0 (100%)
Weight (g)	2979 ± 472
Weight (g) males	2992 ± 86.59
Weight (g) females	2862 ± 112.4
Mothers (N = 48)	
BMI (pre-gestational)	27.1 ± 4.9
Age (years)	29 ± 7
Resolution	
Vaginal delivery	22 (45.8%)
Caesarean section	26 (54.2%)
Euglycemic	22 (45.8%)

examinados cuidadosamente por un neonatólogo

riables para cada recién nacido.

ón, se recogió una muestra de orina de forma no

Se limpiaron minuciosamente los genitales y se

ona genital hasta la micción

Dpto. Ne
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Morones Prieto"
s, Mamás.

López-Hernández (2020) 33

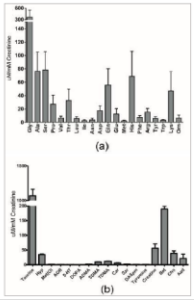
Datos de concentración de 136 metabolitos medidos mediante metabolómica dirigida

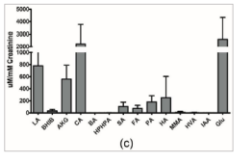
Se detectaron y cuantificaron un total de 45 aminoácidos y aminas biógenas, 17 ácidos orgánicos, 24 glicerofosfolípidos, 10 esfingomielinas y 40 carnitinas y acilcarnitinas.

Límite de detección (LOD) para cada metabolito medido utilizando enfoque LC-MS/MS

López-Hernández (2020) 34

Datos de concentración de 136 metabolitos medidos mediante metabolómica dirigida





Representación gráfica de las concentraciones urinarias de (a) aminoácidos (b) aminas biogénicas y (c) ácidos orgánicos, informadas previamente en recién nacidos mediante LC-MS/MS. media ± SD

López-Hernández (2020) 35

Resultados experimentales de metabolitos no notificados previamente en orina de recién nacidos y orina humana

Metabolitos no reportados previamente en recién nacidos

Metabolite	HMDB ID	Mean ± SD (μM)	Mean ± SD (μM/mM Creatinine)	2.5–97.5% Percentile (μM/mM Creatinine)
Histamine	HMDB0000870	0.03 ± 0.03	0.02 ± 0.01	0.01–0.04
Putrescine	HMDB0001414	1.09 ± 1.54	0.31 ± 0.63	0.03–3.42
Diaceylspermine	HMDB0004174	4.99 ± 5.54	1.83 ± 0.59	0.57–3.11
Trimethylamine *N*-oxide	HMDB0000925	59.4 ± 54.0	12.2 ± 10.3	0.30–43.1
p-Hydroxyhippuric acid	HMDB0013678	37.1 ± 24.8	8.03 ± 3.48	4.20–19.6
LysoPC a C16:1	HMDB0010383	0.013 ± 0.011	0.003 ± 0.003	0.0001–0.0150
LysoPC a C16:0	HMDB0010382	0.19 ± 0.20	0.043 ± 0.036	0.007–0.169
LysoPC a C17:0	HMDB0012108	0.018 ± 0.013	0.005 ± 0.001	0.0008–0.0582

La creatinina urinaria (Ucreat) se utiliza a menudo para ajustar o normalizar las concentraciones de analitos urinarios, pero descubrimos que Ucreat parecía ser un valor de referencia relativamente poco confiable en el período neonatal temprano, debido a su alta variación (que oscila entre 1000 y 17 000 mM).
Por este motivo, también proporcionamos las concentraciones absolutas de cada metabolito medidas en orina

Concentración absoluta (μM, expresada como media ± SD); valores normalizados de creatinina (creatinina μM/mM) expresados como media ± SD; y el rango percentil 2,5–97,5% (creatinina μM/mM)

López-Hernández (2020) 36

Resultados experimentales de metabolitos no notificados previamente en orina de recién nacidos y orina humana

Metabolitos no reportados previamente en orina humana

Metabolite	HMDB ID	Mean ± SD (μM)	Mean ± SD (μM/mM Creatinine)	2.5–97.5% Percentile (μM/mM Creatinine)
LysoPC a C14:0	HMDB00379	0.02 ± 0.02	0.006 ± 0.003	0.002–0.012
Hexadecenoylcarnitine (C16:1)	HMDB0006317	0.05 ± 0.02	0.01 ± 0.01	0.0–0.03
Hydroxyhexadecadienoylcarnitine (C16:2OH)	HMDB0013335	0.02 ± 0.01	0.004 ± 0.001	0.0–0.01
Hydroxyhexadecenoyl-carnitine (C16:1OH)	HMDB0013333	0.04 ± 0.02	0.010 ± 0.004	0.0–0.02

- Un total de 86 de estos metabolitos urinarios medidos experimentalmente (64%), junto con sus rangos de concentración, se informan por primera vez en recién nacidos/lactantes.
- De estos, 20 metabolitos (14 % de los compuestos identificados en este estudio) se encuentran en la orina humana por primera vez.
- Tenga en cuenta que los datos de concentración de algunos metabolitos encontrados a través de nuestra literatura y búsquedas en bases de datos tenían más de un valor de referencia, obtenido por diferentes laboratorios utilizando diferentes métodos. Esto puede generar algunas discrepancias menores en las cifras reportadas en estas tablas.

Concentración absoluta (μM, expresada como media ± SD); valores normalizados de creatinina (creatinina μM/mM) expresados como media ± SD; y el rango percentil 2,5-97,5% (creatinina μM/mM).

López-Hernández (2020) 37

Errores congénitos del metabolismo (IEM)

- En el presente estudio, determinamos experimentalmente las concentraciones de 136 metabolitos, seleccionados debido a su conocida participación en múltiples vías metabólicas asociadas con trastornos metabólicos o genéticos (como los IEM), que podrían manifestarse en las primeras 24 h de vida.
- La especificidad y sensibilidad de los métodos de espectrometría de masas en tándem pueden alcanzar valores de hasta el 99,99% y el 99%, respectivamente, para diagnósticos de IEM.

Lista de metabolitos medidos experimentalmente en el presente trabajo que se utilizan actualmente como marcadores primarios o secundarios de errores innatos del metabolismo.

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Comparación entre las concentraciones de metabolitos (normalizadas a creatinina) medidas en el presente estudio y en un estudio previo realizado por el TMIC en muestras de orina de adultos sanos

De un total de 79 metabolitos comunes, 33 mostraron una tendencia clara y decreciente con la edad, mientras que la mayoría de los demás se mantuvieron prácticamente sin cambios. Al comparar la orina de un adulto con la de un recién nacido, encontramos que una cantidad de aminoácidos era mucho mayor en los recién nacidos que en los adultos, incluidos glicina, alanina, serina, prolina, histidina, lisina, metionina y la mayoría de los aminoácidos de cadena ramificada.

Comparación entre la concentración urinaria de recién nacidos y adultos de 79 metabolitos medidos mediante LC-MS/MS

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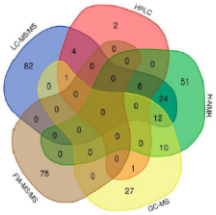
Metaboloma urinario de recién nacidos sanos

Además de este estudio experimental, realizamos una revisión de la literatura (que abarca los últimos ocho años y 509 artículos) para actualizar los valores de referencia en la Base de datos del Metaboloma Humano/Base de datos del metaboloma en orina (HMDB/UMDB).

- Nos permitió identificar otros 78 metabolitos urinarios, con datos de concentración medidos por diferentes plataformas (LC-MS/MS, HPLC, 1H -NMR, FIA-MS/MS y GC -EM).
- Combinados con los 212 metabolitos urinarios neonatales informados previamente en HMDB/UMDB y los 86 metabolitos urinarios neonatales detectados e informados experimentalmente aquí = 378 metabolitos urinarios neonatales que han informado valores de concentración y a los que se puede acceder públicamente a través de HMDB o UMDB



www.hmdb.ca

López-Hernández (2020) 40

Metaboloma urinario de recién nacidos sanos

Metabolitos medidos cuantitativamente en orina, proporciona información que, si se sigue y controla en el tiempo, puede ser útil para predecir la "edad biológica" de los bebés. La edad biológica (a diferencia de la edad calendario) está muy influenciada por varios factores, como la dieta, el estrés, el medio ambiente, el estilo de vida, la genética y las enfermedades.

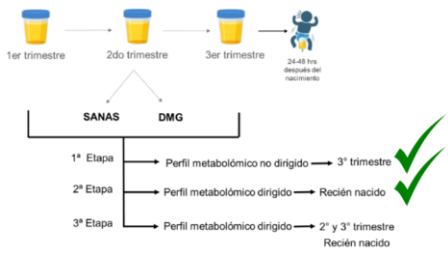
Identificación de nuevos tratamientos o estrategias para el manejo de la salud y la enfermedad en la primera infancia.

Establecer puntos de inflexión relacionados con los trastornos metabólicos, lo cual es muy importante para un diagnóstico o pronóstico preciso de la enfermedad. En este sentido, es importante tener en cuenta que *las concentraciones de metabolitos cambian continuamente con el crecimiento y la edad, especialmente en niños y bebés.*

Hasta la fecha, sólo se han realizado unos pocos estudios exhaustivos que analicen intervalos específicos recién nacidos.

Chiu (2016); Jazwinski (2019); Jove (2017); Scalabre (2017) 41

Diseño experimental general



Ampliar los estudios en matrices que resulten no invasivas mediante el uso de estrategias multiómicas para entender el desarrollo de una patología compleja como DMG.

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Perfil metabolómico urinario de recién nacidos de mujeres con diabetes mellitus gestacional

metabolites

Article

Urinary Metabolomic Profile of Neonates Born to Women with Gestational Diabetes Mellitus

Ana Sofía Herrera-Van Oostdam [1,2], Mariana Salgado-Bustamante [2], Victoria Lima-Rogel [3], Juan José Oropeza-Valdez [4], Jesús Adrián López [5], Iván Daniel Román Rodríguez [6], Juan Carlos Toro-Ortiz [3], David Alejandro Herrera-Van Oostdam [3], Yamilé López-Hernández [6,7,*] and Joel Monárrez-Espino [8,*]

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Citation: Herrera-Van Oostdam, A.S.; Salgado-Bustamante, M.; Lima-Rogel, V.; Oropeza-Valdez, J.J.; López, J.A.; Rodríguez, I.D.R.; Toro-Ortiz, J.C.; Herrera-Van

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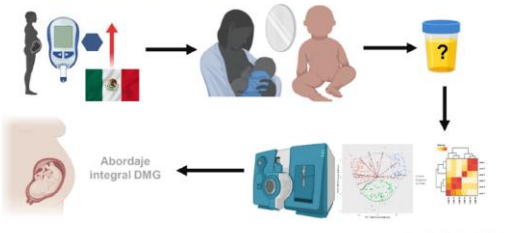
Justificación

La DMG es una de las complicaciones del embarazo más frecuentes con posibles resultados adversos para las madres y los recién nacidos. Sus efectos en el recién nacido aparecen durante el período neonatal o la primera infancia. Por tanto, un diagnóstico oportuno es crucial para prevenir el desarrollo de enfermedades crónicas más adelante en la vida adulta.



Herrera-Van Oostdam (2021) 44

Hipótesis

Existe un perfil metabolómico urinario en mujeres embarazadas con DMG asociado a cambios en el fenotipo metabolómico urinario en sus recién nacidos.

Objetivos

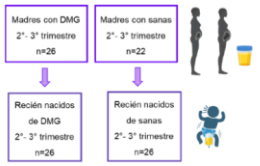
Objetivo principal: Caracterizar el perfil metabolómico urinario de origen de mujeres embarazadas con DMG y evaluar su asociación con el perfil metabolómico urinario fetal para realizar el análisis integral ómico del desarrollo de diabetes gestacional en relación con el binomio materno-fetal.

Objetivos específicos

1.- Análisis dirigido del metaboloma urinario de mujeres embarazadas 2ª y 3ª y sus recién nacidos (binomio).

2.- Integración de datos estadísticos y vías de señalización para el abordaje de DMG.

Características clínicas de las mujeres embarazadas y sus recién nacidos

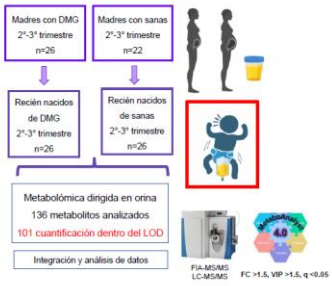


Characteristics	GDM	Healthy	p-Value [a]
Newborns, n (%)	26 (54.1)	22 (45.8)	
Sex, n (%) [b]			0.003 **
Female	16 (61.5)	4 (18.1)	
Male	10 (38.4)	18 (81.8)	
Gestational age (weeks) [a]	38.5 ± 1.3	38.3 ± 1.2	0.5
APGAR score, min 1 [b]	8 (100.0)	8 (100.0)	1.00
APGAR score, min 5 [b]	9 (100.0)	9 (100.0)	1.00
Silverman-Anderson score [b]	0 (100.0)	0 (100.0)	1.00
Weight (g) [a]	3026 ± 399	2945 ± 477	0.6
Delivery, n (%) [b]			0.1
Vaginal	9 (34.6)	13 (59.1)	
C-section	17 (65.4)	9 (40.9)	
Mothers, n (%)	26 (54.1)	22 (45.8)	
Age (years) [a]	38.4 ± 4.7	35.6 ± 3.2	0.05
Pre-BMI (Kg/m²) [a]	27.87 ± 4.12	25.58 ± 4.22	0.06
Normal weight, n (%) [b]	8 (30.8)	10 (45.5)	0.3
Overweight, n (%) [b]	10 (38.5)	9 (40.9)	0.8
Obese, n (%) [b]	8 (30.8)	3 (13.6)	0.3
Glucose (mg/dL) [a]	86.94 ± 13.3	79.81 ± 8.7	0.03 *
Creatinine (mg/dL) [a]	0.56 ± 0.08	0.57 ± 0.09	0.6
Urea (mg/dL) [a]	14.36 ± 4.0	14.19 ± 3.91	1.0

[a] de Student para variables continuas con distribución normal (media ± DE), * p < 0.05, ** p < 0.01; Datos nominales se utilizaron pruebas de Chi2 y pruebas exactas de Fisher, a Prueba t de Student

Herrera-Van Oostdam (2021)

Valores de concentración urinaria de 101 metabolitos de recién nacidos de madres diabéticas y sanas



FC >1.5, VIP >1.5, q <0.05

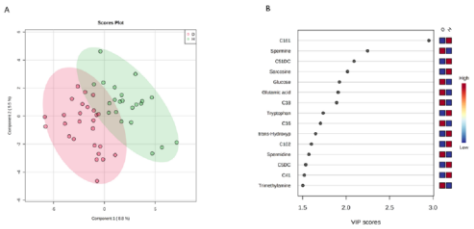
Herrera-Van Oostdam (2021)

Valores de concentración de metabolitos estadísticamente significativos medidos en bebés nacidos de madres con DMG y controles sanos.

Metabolite	Healthy Newborns Median (2.5–97.5 IQR) (μM/mM Creatinine)	GDM Newborns Median (2.5–97.5 IQR) (μM/mM Creatinine)	*p* Value
trans-Hydroxyproline	36.0 (8.1–96.65)	26.4 (11.31–60.82)	0.01
Glutamic acid	7.4 (1.88–25.28)	12.4 (3.72–36.52)	0.01
DOPA	0.06 (0.02–0.17)	0.04 (0.01–0.08)	0.04
Spermine	0.03 (0.007–0.09)	0.04 (0.006–0.72)	0.003 *
Lactic acid	85.6 (46.95–797.5)	112.0 (47.14–359.8)	0.04
Butyric acid	0.33 (0.12–0.9)	0.22 (0.06–0.7)	0.02
Isobutyric acid	0.08 (0.03–1.0)	0.05 (0.02–1.0)	0.03
Glutaconylcarnitine (C5:1DC)	0.02 (0.008–0.03)	0.01 (0.005–0.03)	0.009 *
Glutarylcarnitine (C5DC)	0.05 (0.03–0.1)	0.04 (0.02–0.06)	0.006 *
C10:2	0.03 (0.02–0.07)	0.03 (0.014–0.05)	0.01
Hexadecenoylcarnitine (C16:1)	0.012 (0.007–0.03)	0.009 (0.004–0.027)	0.01

Pruebas U de Mann-Whitney revelaron que 11 metabolitos eran estadísticamente diferentes entre los grupos de estudio (trans-hidroxiprolina, ácido glutámico, DOPA, espermina, ácido láctico, ácido butírico, ácido isobutírico, C5:1DC, C5DC, C10:2 y C16: 1).

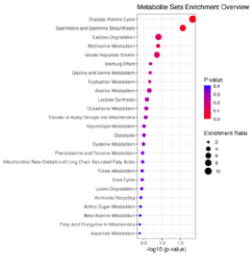
Resultados de los análisis multivariados realizados para comparar los recién nacidos de madres con DMG con los de madres sanas.



(A) Gráficos de puntuación del análisis discriminante de mínimos cuadrados parciales (2-D PLS-DA); (B) Importancia variable en el gráfico de proyección. Los metabolitos diferenciales se muestran en orden descendente de sus puntuaciones de coeficiente.

Herrera-Van Oostdam (2021)

Metabolitos urinarios en recién nacidos están implicados en las vías relacionadas con el metabolismo de aminoácidos, carbohidratos y lípidos

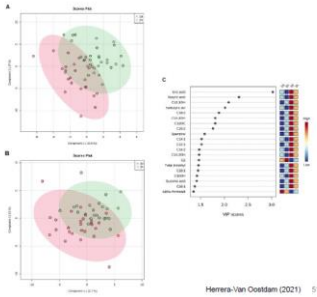


Análisis de la ruta de los metabolitos desregulados en recién nacidos de madres con DMG. El tamaño del nodo es proporcional a la tasa de enriquecimiento.

Herrera-Van Oostdam (2021)

Análisis del metaboloma urinario materno durante el segundo y tercer trimestre del embarazo

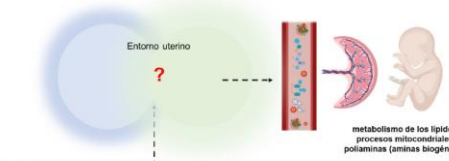
Para evaluar la posible relación entre las alteraciones metabólicas encontradas en los recién nacidos y las de sus madres, se analizaron muestras de orina materna recolectadas durante la segunda mitad del embarazo.



Herrera-Van Oostdam (2021)

Resumen

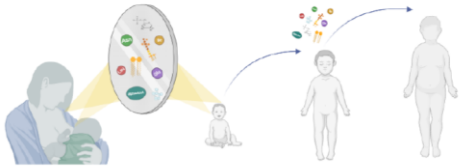
Relación del metaboloma urinario de recién nacidos con madres DMG



- Espermina relacionada con marcadores de estrés oxidativo, inflamación, NOS
- Incrementó de β-oxidación en mitocondrias
- CPT-1 incrementa la producción de citocinas inflamatorias

Codoner-Franch (2011); Hsu (2019); Herrera-Van Oostdam (2021)

¿El perfil metabólico del recién nacido refleja el perfil metabolómico de la madre durante el embarazo?



Conclusiones

Hasta donde sabemos, este es el primer trabajo que informa valores de concentración cuantitativos para 101 metabolitos medidos en orina dentro de las primeras 24 h de vida de recién nacidos de madres con DMG.

Dado que no existen estudios previos que presenten análisis cuantitativos realizados en la orina de recién nacidos de madres con DMG, no fue posible la comparación con estos hallazgos.

A pesar de que la mayoría de las concentraciones de metabolitos urinarios de todos los recién nacidos incluidos en el presente trabajo se encuentran dentro del rango normal, como se documentó anteriormente, nuestros resultados mostraron un perfil diferencial en los niveles urinarios de algunos aminoácidos, poliaminas y carnitinas entre los recién nacidos de madres con DMG.

Conclusiones globales del proyecto metaboloma urinario materno en diferentes etapas del embarazo y sus recién nacidos (2017-2023)

Este fue un proyecto extenso donde se realizaron técnicas de metabolómica no dirigida y dirigida, en orina de mujeres embarazadas sanas y con DMG y su descendencia para analizar vías metabolómicas alteras por este efecto.

Se logró identificar un escaneo completo de metabolitos en orina de mujeres con DMG en el 3° trimestre del embarazo, con principal presencia en la vía del triptófano.

Además, un total de 86 de estos metabolitos urinarios medidos y presentados con sus rangos de concentración, se informan por primera vez en recién nacidos/lactantes y a los que se puede acceder públicamente a través de HMDB o UMDB

Finalmente, se presenta una caracterización cuantitativa extensa del metaboloma urinario de recién nacidos con DMG y madres sanas. Estuvieron disponibles para análisis los valores de concentración de 101 metabolitos detectados en la orina de recién nacidos recolectados dentro de las primeras 24 h de vida. Vías alteradas

Esta información es de relevancia clínica para que los neonatólogos y pediatras monitoreen el impacto de la DMG en las primeras etapas de la vida.

Anexos

PLOS ONE

RESEARCH ARTICLE

Immunometabolic signatures predict risk of progression to sepsis in COVID-19

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"Publish houses of brick, not mansions of straw"

A mi Mamá y Papá, Andre y David

A mis compañerxs el Dr. Juan José Oropeza Valdez, Dra. Sandra Niño Esquivel

Al apoyo del Consejo Nacional de Humanidades, Ciencias y Tecnologías que otorgó la beca con número 862757

"La ciencia no es hecha por fantasmas. Lo hacen humanos reales que trabajan duro todos los días". - Meredith Course
Postdoc en neurociencia, configuró su cámara para lograr una larga exposición mientras trabajaba en la extracción de RNA.
La cámara solo capturó el objeto de alto contraste en movimiento: su bata de laboratorio.