Impact of Gestational Diabetes and Treatment on the Metabolome of Non-invasive Biofluids: Searching for New Biomarkers

Impacto da Diabetes Gestacional e do Tratamento sobre o Metaboloma de Biofluidos Não-invasivos: Procurando Novos Biomarcadores

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Abstract

Introduction: Gestational diabetes *mellitus* may determine pregnancy outcome and impact on offspring health. Knowledge on associated metabolic mechanisms may provide new biomarkers to support diagnosis and treatment protocols. Untargeted metabolomics is effective in biomarker search and, thus, of significant value in this context.
Aims: This work aims to find urinary/salivary metabolic biomarkers of the disease and measure the impact of different treatments on metabolism, to unveil biomarkers for personalized treatment monitoring.
Materials and Methods: Nuclear Magnetic Resonance spectroscopy and multivariate statistical analysis were employed to analyze maternal urine and saliva (n=39) and newborn urine (n=76).
Results: Gestational diabetes impacted significantly on the composition of urine, particularly in the 3rd trimester of pregnancy. Results suggested disruptions in energy metabolism, and gut microflora, pyrimidines and hormonal metabolisms. Saliva composition was less sensitive to disease, however, both biofluids were responsive to treatment type, indicating that metabolic regulation to approach controls is clearer for diet and for insulin/metformin treatments.
Conclusion: Urine metabolome is sensitive to gestational diabetes *mellitus*, providing a valuable source of metabolic biomarkers of the disease and treatment efficacy. Saliva composition is also responsive to treatment, appearing as a potential contributor biofluid for treatment follow-up protocols.

Keywords: gestational diabetes *mellitus* (GDM); metabolomics; urine; saliva; diagnosis; insulin; metformin

> INTRODUCTION

Gestational diabetes *mellitus* (GDM) is an asymptomatic disorder which consists of carbohydrate intolerance with onset or first recognition during pregnancy. (1) The International Diabetes Federation (IDF) estimated that GDM affected 13.4% of all live births in 2021, in females aged 20-49 years old. (2) Given the risks involved with GDM pregnancies and infants (e.g., preeclampsia, macrosomia, neonatal hypoglycaemia and type 2 diabetes

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Resumo

Introdução: A diabetes *mellitus* gestacional pode determinar o desfecho da gravidez e a saúde dos recém-nascidos, e a procura de novos marcadores metabólicos pode apoiar melhorias no diagnóstico e tratamento desta doença. A metabolómica é uma ferramenta eficaz na procura de biomarcadores e, assim, de valor significativo neste contexto.
Objetivos: Pretende-se encontrar biomarcadores metabólicos urinários e salivares da doença e da eficácia de diferentes tratamentos.
Materiais e Métodos: Amostras de saliva e urina maternas (n=39) e de urina de recém-nascido (n=76) foram analisadas por espectroscopia de Ressonância Magnética Nuclear e análise estatística multivariada.
Resultados: A diabetes gestacional afetou significativamente a composição da urina, principalmente no 3º trimestre da gravidez, sendo sugeridos desvios no metabolismo energético, microflora intestinal, e metabolismos das pirimidinas e hormonal. A saliva é menos sensível à doença, no entanto, ambos os biofluidos responderam fortemente ao tipo de tratamento, indicando uma melhor regulação metabólica associada aos tratamentos por dieta e com insulina/metformina.
Conclusão: O metaboloma urinário é sensível à diabetes *mellitus* gestacional, fornecendo uma valiosa fonte de biomarcadores metabólicos da doença e da eficácia do tratamento. A composição da saliva é também responsiva ao tratamento, podendo contribuir com novos marcadores associados a protocolos terapêuticos da GDM.

Palavras-chave: diabetes *mellitus* gestacional; metabolómica; urina; saliva; diagnóstico; insulina; metformina

later in life), [3] there is still a need for early and efficient biomarkers for further refinement of GDM diagnosis and treatment protocols. Metabolomics has been used to search for metabolic markers of GDM and assess the impact of the condition on maternal and fetal metabolisms, using maternal biofluids (blood serum/plasma, urine, breast milk, placenta, hair) and fetal/newborn samples (amniotic fluid, umbilical cord blood, newborn meconium and urine) as reviewed thoroughly in the literature. [4-11] The typical metabolomics workflow (Figure 1) may be applied to biofluids, tissues or cells, subjected to a certain perturbation of interest (e.g. disease). This omic usually employs Mass spectrometry or Nuclear Magnetic Resonance (NMR) spectroscopy as analytical platforms, both producing highly complex records and requiring multivariate statistical analysis (MVA) for meaningful variations in metabolite levels to be detected. Such variations make up a signature that describes the response of the living organism to the disease under study, helping to generate biochemical hypotheses which then require both statistical and biological validation, in order to translate into robust biomarkers, with potential clinical applications.

In the context of GDM research, metabolomic studies have addressed different biofluids, using either NMR or MS-based metabolomics (including lipidomics), mainly aiming at i) searching for early predictive biomarkers, [12-28] ii) defining a GDM metabolic profile which may add to traditional GDM diagnosis, [20,29-35] and iii) follow-up GDM treatment to find metabolic biomarkers of treatment efficacy and pave the way for precision medicine protocols. [33,36-40] In Figure 2 an account of number of publications in this area is shown, as a function of year and biological matrix. It is clear that maternal blood has been the most extensively studied sample type, followed by a consistent interest in urine during pregnancy, for its attractive non-invasive characteristics (which may enable larger, and longitudinal, cohorts to be evaluated). Saliva is another interesting non-invasive biofluid, although to our knowledge, no studies have addressed this biofluid in the context of GDM. On the other hand, much work has been published for urine, although interpretation of complex urine metabolic profiles faces important challenges, namely the impact of confounders (e.g., age, diet, lifestyle, body mass index) and the difficult identification of some metabolites. Despite these, metabolite changes have been found in the urine of pregnant women prior to GDM diagnosis, [14,15,21,26-28,41-43] as well at diagnosis. [29, 33,35,44] Upon diagnosis, reports of maternal urine profiling of a large cohort of GDM women (*n*=823) [29] suggested increased excretion of glucose and citrate with increasing hyperglycemia, whereas other reports unveiled decrease of carnitine in GDM women, and increases in several amino acids and other compounds were also found varying. [29, 33,35,44] The impact of maternal GDM on newborn metabolism through urine [45,46] and meconium [45] has indicated disruptions in acylcarnitines, amino acids, lipid, polyamine and purine metabolisms, partially supported by earlier findings on umbilical cord of GDM newborns. [47] This has been followed by several reports on umbilical cord blood, reporting several metabolic variations in response to GDM. [23,48-54] To our knowledge, few studies of the impact of different GDM treatments on maternal biofluids, [33,36-40] and particularly on maternal urine, [33] have been reported.

The present paper describes a NMR metabolomics study focused on non-invasive biofluids maternal urine

and saliva, to 1) provide a dynamic description of GDM metabolism over pregnancy, to unveil new biomarkers of the disease and 2) measure the impact of different GDM treatments on maternal urinary and salivary compositions, to search for markers of treatment response. A brief note is also added on preliminary results on the impact of GDM on the newborn urinary metabolome.

> MATERIALS AND METHODS

Sampling

Maternal urine and saliva (passive drool method) samples were collected, in the morning at routine medical appointments and diabetes counselling appointments

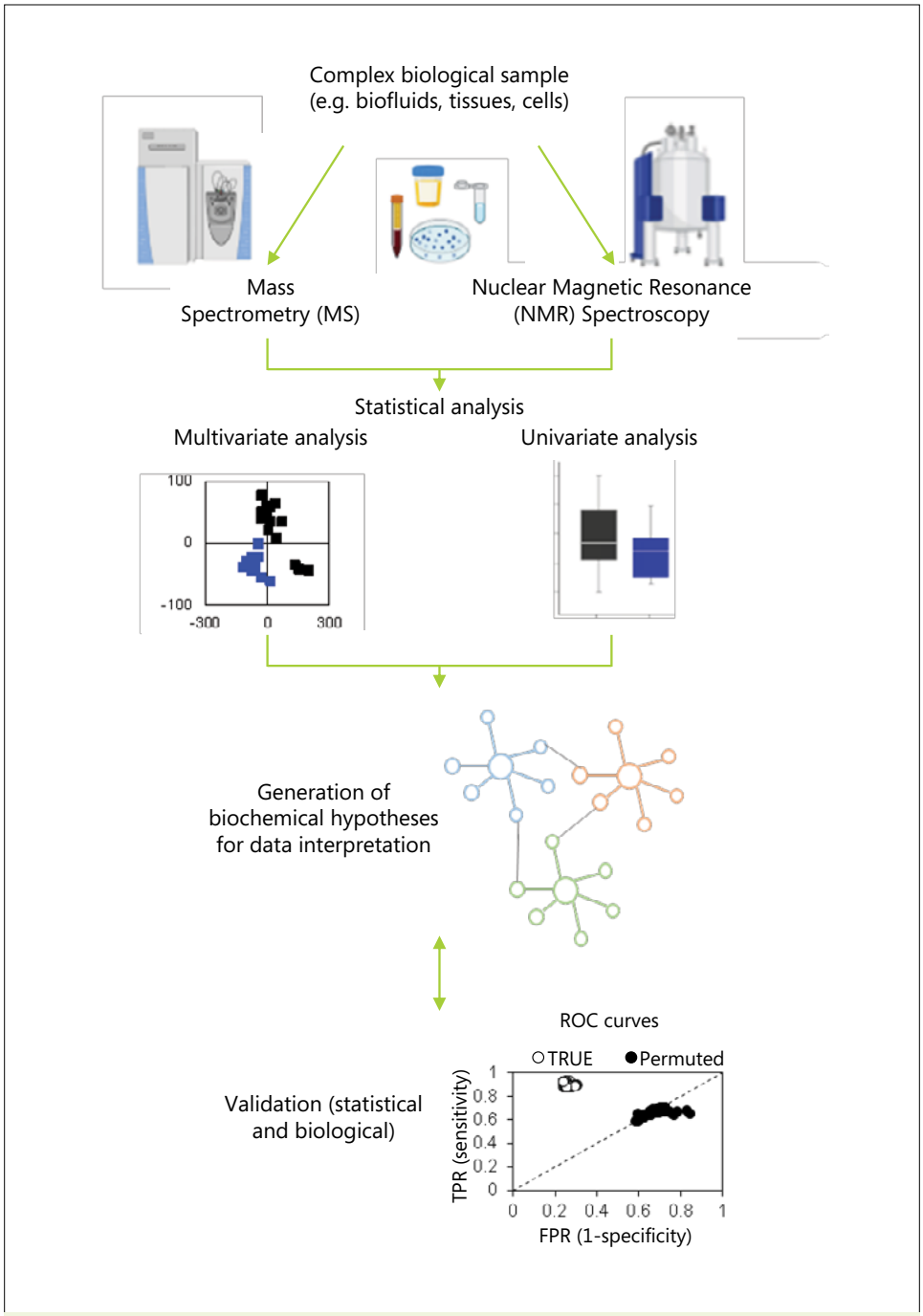

Figure 1 - Schematic representation of a typical metabolomics experimental workflow. NMR, Nuclear Magnetic Resonance. FPR, false positive rate; ROC, receiver operating characteristic curve (for statistical validation); TPR, true positive rate. Figure created with elements available from BioRender.com.

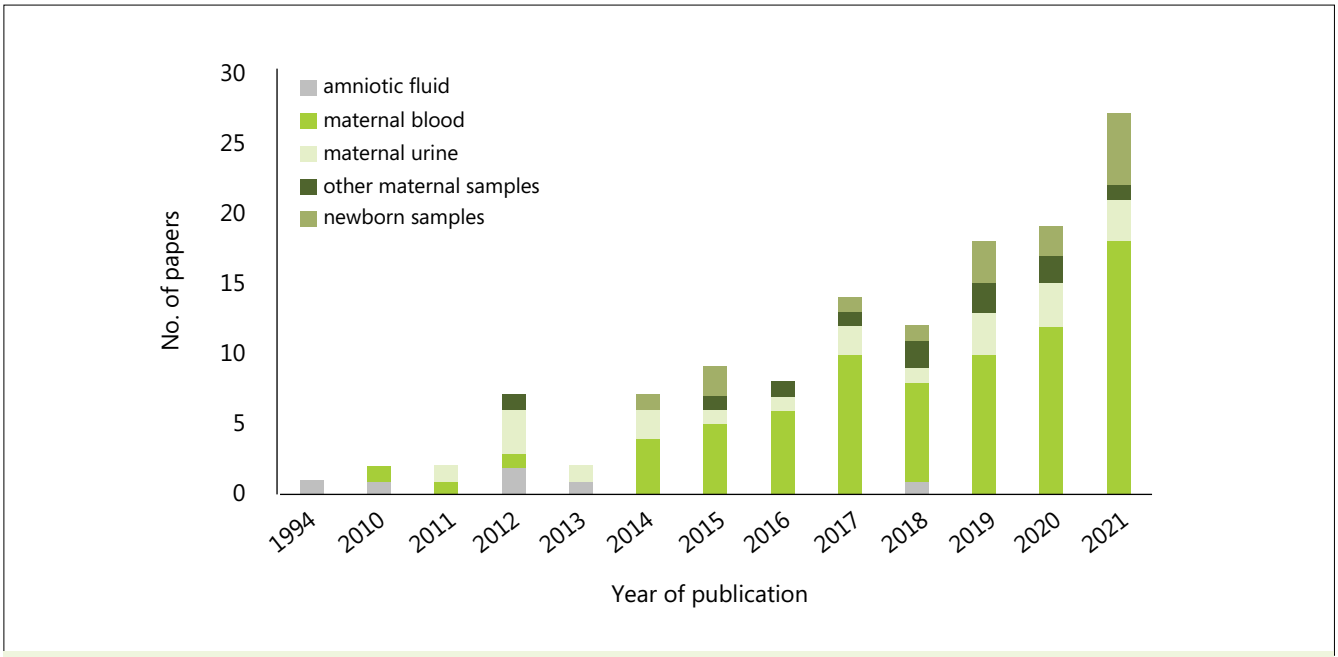

Figure 2 - Number of scientific papers published on GDM metabolomics, as a function of year and colored according to biological matrixes studied. In "Other maternal samples" the following biological matrixes are included: breast milk, feces, exhaled breath condensate (EBC), placenta (tissue) and hair. Newborn samples include umbilical cord blood, urine, meconium and dried blood spots. This information was obtained from consultation of the Web of Science (https://www.webofscience.com/) up to December of 2021.

for women in their 1st, 2nd and 3rd trimesters (1st, 2nd and 3rd T) of pregnancy, at the Maternity Bissaya Barreto (Hospital Center of Coimbra (CHUC) under ethical approval of the CHUC (CHUC-091-17, 25th June 2018) and supported by informed consents from each woman. Table I lists the number of subjects and samples used in this study. GDM diagnosis was based on a first step comprising the measurement of fasting plasma glucose (FPG) at the first prenatal visit (1st T), followed by a second step consisting of the oral glucose tolerance test (OGTT), performed at 24-28 g.w. The guidelines followed for GDM diagnosis may be found elsewhere. [55] Metadata for healthy and treatment type (diet only, insulin, metformin or metformin/insulin combination) for GDM women were obtained from medical records and treated women were followed, with samples collected longitudinally for each woman when possible (Table I). For newborns (46 healthy newborns and 30 babies born

Table I - List of subjects and samples (urine and saliva) for each group under study. Number of samples are shown in brackets, bearing in mind that some women donated more than one sample in some cases. [a] number of subjects and samples used for the comparison between controls and NT-GDM, matching gestational age as far as possible; [b] number of subjects and samples used for the comparison between controls and each GDM treatment. D-T, diet treatment; I-T, insulin treatment; NT-GDM, non-treated GDM; MI-T, metformin/insulin combination treatment; M-T, metformin treatment.

Group	1st T		2nd T		3rd T	
	Urine	Saliva	Urine	Saliva	Urine	Saliva
Controls	13(13)	13(13)	9(9)	9(9)	9(10)[a]/ 12(17)[b]	6(7)[a]/ 9(13)[b]
NT-GDM	3(3)	3(3)	10(10)	8(8)	14(14)	14(14)
D-T	-	-	2(4)	2(4)	7 (9)	6(7)
I-T	-	-	-	-	3(14)	3(14)
M-T	-	-	1(4)	1(3)	5(18)	5(17)
MI-T	-	-	2(5)	2(4)	2(5)	2(3)

from GDM mothers), urine samples were collected as described elsewhere, [56] in the first days of life (days 1-6), under ethical approvals of the Hospital Center of Coimbra: 18/04 and 29/09, and CHUC-091-17, 25th June 2018. Informed consents were obtained from parents/carers for each infant. All samples (maternal saliva and urine, and newborn urine) were stored for up to 3 weeks at -20º C and then transferred to -80 ºC until analysis.

Sample Preparation and NMR Spectroscopy

Maternal and newborn urine samples were thawed at room temperature (RT) and 800 µL (maternal urine) or 600 µL (newborn urine) were centrifuged (4500 g, 25 ºC, 5 min, Sigma 2-16P centrifuge). Sample preparation has been described elsewhere. [33,56] For each urine sample, a standard ^{1}H (proton) NMR spectrum was recorded on a Bruker Avance III HD spectrometer, operating at 500.13 MHz for proton, at 300 K, using acquisition conditions described previously. [33,56] Urine peak identification was carried out with basis on literature, [33,56-58] the Human Metabolome Database (HMDB), [59] and bidimensional (2D) NMR experiments.
Regarding maternal saliva samples, after thawing at RT, each sample was centrifuged (9184 g, 4º C, 1 h). Then, sample preparation and spectra acquisition followed protocol reported previously. [60,61] Saliva peak identification was carried out based on specific literature reports [62-65] and as for urine.

Data Preprocessing and Statistical Analysis

After manual phase and baseline correction, the NMR spectra of urine and saliva were converted into a matrix of rows and columns, corresponding to samples and variables, respectively (Amix 3.9.5, Bruker BioSpin, Rheinstetten, Germany). For urine, spectral regions of water δ 4.50-5.05) and urea (δ 5.60-6.20) were excluded whereas for saliva only that of water was excluded. Spectra alignment was performed to reduce chemical shift variations due to different sample pH, as described elsewhere [66] (Matlab 8.3.0, The MathWorks Inc., Natick, Massachusetts, USA). Spectra were normalized to total spectral area to account for differences in sample concentrations.
Multivariate analysis (MVA) was carried out using SIMCA-P software, version 11.5 (Umetrics, Umeå, Sweden), applying Principal Component Analysis (PCA) and Partial Least Squares – Discriminant Analysis (PLS-DA) to unit variance scaled data. PLS-DA model robustness was evaluated by Q^2 (predictive power) and Monte Carlo cross-validation (MCCV). Classification rates (CR), specificity and sensitivity were calculated and model predictive power was assessed using a ROC curve mapping. PLS-DA models were considered robust when having a predictive power $Q^2 > 0.50$. [67,68] All peaks with no or minimal overlap were integrated in the original spectra (Amix 3.9.5) and normalized to total spectral area. The individual statistical significance of each peak integral was computed by the Wilcoxon rank sum test (based on the assumption that data are non-normally distributed) (R-statistical software). Metabolite variations were expressed as effect size (ES) which accounts for group dispersion. [69]

> RESULTS AND DISCUSSION

Figure 3 shows typical ^{1}H (proton) NMR spectra of maternal urine and saliva (collected in the 1st trimester of pregnancy). In this type of record (or spectra), each metabolite present in the sample contributes with a specific set of peaks depending on its chemical structure. Some examples of metabolites thus detected in urine are shown in Figure 3a, becoming clear that citrate, creatinine, glycine and hippurate predominate in urine samples. The result is a complex record, which reflects tens or hundreds of different metabolites which, together, potentially provide a large wealth of compositional information. Detecting changes in these profiles due to the presence of a disease, such as GDM, compared to controls, is the basis of metabolomic strategies. In addition, a typical spectrum of saliva (Figure 3b) exhibits both narrow and broad peaks, arising from small metabolites and macromolecules (mainly proteins), respectively. The profile of the saliva spectrum is clearly distinct from that of urine, showing lower peak intensity in the higher chemical shift region, and peaks arising mainly from acetate, ethanol, glycine, methanol and propionate.
The use of multivariate analysis methods (both unsupervised methods such as PCA which assess group separation in an unbiased way, and supervised methods such as PLS-DA which maximize group separation and aid interpretation) produces scores scatter plots such as shown for PLS-DA of urine in the three pregnancy trimesters (Figure 4). In this type of plots, each symbol represents one sample (or spectrum) and the statistical robustness of group separation is measured by the value of the Q^2 parameter (predictive power) which, if > 0.5, identifies robust group separation. Inspection of Figure 4 indicates that the graphical separation between urine samples from the diagnosed but non-treated GDM group (NT-GDM, open squares) seems statistically stronger in the

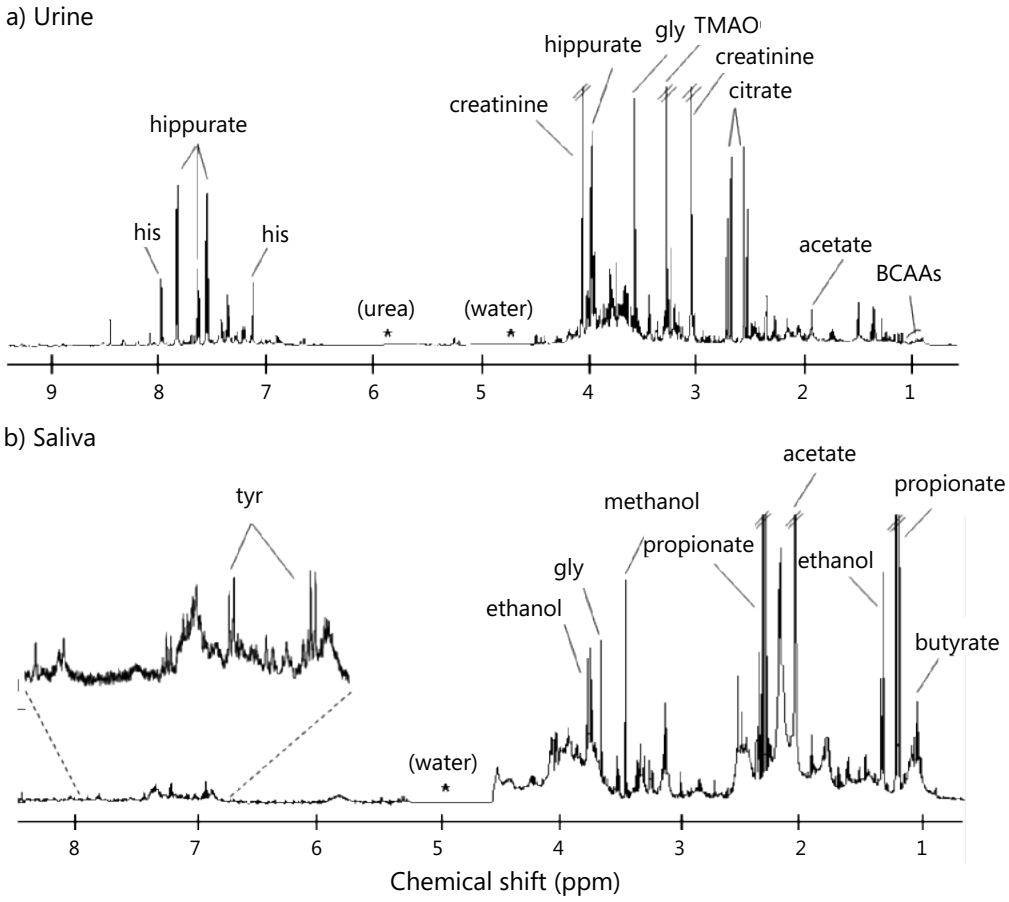

Figure 3 - Examples of ^{1}H NMR spectra of **a)** urine and **b)** saliva collected for a pregnant woman in her 1st trimester of pregnancy. *, spectral regions excluded for urine (water and urea) and saliva (water), due to their random contributions. Abbreviations: 3-letter code used for amino acids; BCAAs, branched chain amino acids; TMAO, trimethylamine-*N*-oxide. Please note that chemical shift scales are distinct between the two spectra.

1st trimester (Figure 4, left; Q^2 0.5, even though with only 3 samples) and 3rd trimester (Figure 4, right; Q^2 0.64).

The next step of the analysis is to identify which metabolites explain the group separation viewed through the scores plots. This is carried out by analysis of the loading plots (not shown) associated to each scores scatter plot and, subsequently by peak integration to quantify metabolite variations. Based on such analysis, the overall metabolite changes and putative biochemical explanations (Figure 5) indicate, firstly, that in the 1st trimester (black symbol following metabolite names) no meaningful changes could be confirmed, most probably due to the low number of samples (n = 3) in the NT-GDM group. In addition, the GDM signature in the 2nd trimester (red symbols after metabolite names, Figure 5) indicate excretion of i) lower levels of galactose, and histidine (changes in glycolysis and tricarboxylic acid (TCA) cycle activities), hippurate (changes in the gut microflora); and ii) higher levels of 5β-pregnane-3α, 20α-diol-3α-glucuronide (P3G, deviations in hormone metabolism), *N*-acetylneuraminic acid (Neu5Ac) and 2-hydroxyisobutyrate (2-HIBA) (possibly related to gut microflora). It is important to note that these changes occur compared to healthy control women in the same trimester of pregnancy, so that they may be interpreted as arising in connection with GDM and not due to pregnancy progression. In the 3rd trimester (green symbols after metabolite names, Figure 5), GDM induces more changes compared to earlier stages. Namely, important changes are noted for metabolites directly or indirectly

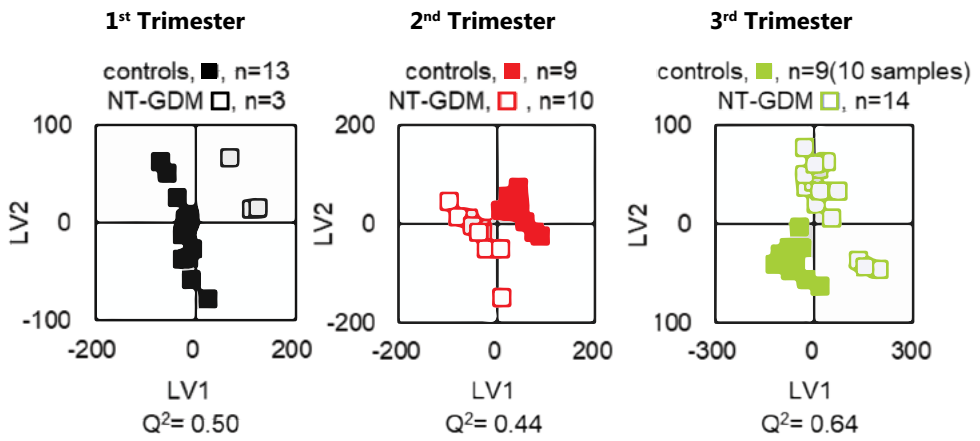

Figure 4 - PLS-DA scores scatter plots obtained for the ^{1}H NMR spectra of maternal urine samples of women with diagnosed and untreated GDM (NT-GDM), compared to healthy pregnant subjects, as a function of pregnancy trimester. LV, latent variable; Q^2, predictive power.

related to energy metabolism, in particular glycolysis and TCA cycle: lower levels of galactose, lactose, 4-deoxythreonic acid (4-DTA), isoleucine, valine (probably required to keep a high energetic metabolism activity), and higher citrate levels, which also may reflect high glycolytic activity (accommodating higher contributions from galactose and lactose). Also in the 3rd trimester, GDM pregnant women excrete lower levels of: i) pseudouridine (change in pyrimidines degradation); ii) hippurate, phenylacetylglutamine (PAG), indoxyl sulphate and *scyllo*-inositol (all four probably reflecting changes in gut microflora); and iii) P3G and progesterone metabolite Pn3G, 3-hydroxyisovalerate (3-HIVA), malonate and estrogens in general, in part confirming the hormonal deviant behavior already suggested in the 2nd trimester of GDM women. These results show, for the first time to our knowledge, that GDM has distinct metabolic signatures depending on the trimester when it affects the pregnancy, with 3rd trimester GDM affecting metabolism more strongly, as viewed by the changes in the excreted metabolome. Interestingly, the NMR metabolomic analysis of urine of babies born from GDM mothers (data not shown), compared to controls offspring, suggested disruptions in gut microflora (observed through changes in hippurate and dimethylamine) and some aspects of energetic metabolism (reflected on distinct levels of alanine and acetone). The impact of GDM on newborn health may shed light into prediction and understanding of GDM long-term effects on children's health, an aspect which justifies further investigation.

A similar study was carried out with saliva samples collected from pregnant controls and GDM women, having shown only a decrease in acetoin in the first trimester and, thus, indicating that the salivary metabolome is significantly less sensitive to the disease than that of urine. We propose that the urinary metabolic changes expressed in Figure 5 may become easily measurable in the urine of pregnant women so that additional information on a potential GDM diagnosis is provided. In addition, if some of these changes are detectable in the urine of control pregnant women, they may be predictive of GDM later in pregnancy.

In relation to the effects of GDM treatment on the excreted metabolome, the PLS-DA scores scatter plot of all groups – controls, diet-treated (D-T), insulin-treated (I-T) and metformin-treated (M-T) (Figure 6a) - indicates that insulin and metformin treatment have different effects on urine composition, whereas the D-T group is positioned close to controls. The treated group positions relatively to the control group (and underlying metabolite differences) may reflect both the severity of the disease (with diet-treated subjects, less severely affected by GDM, approaching controls) and the specific mechanisms of action of the treatment agents. The heatmap in Figure 6b represents the metabolite variations (increases and decreases in red and blue, respectively) of each treated group, compared to healthy pregnancies, with the addition of the insulin/metformin combination treatment (MI-T, right column in Figure 6b). Considering only the identified metabolites (i.e. not

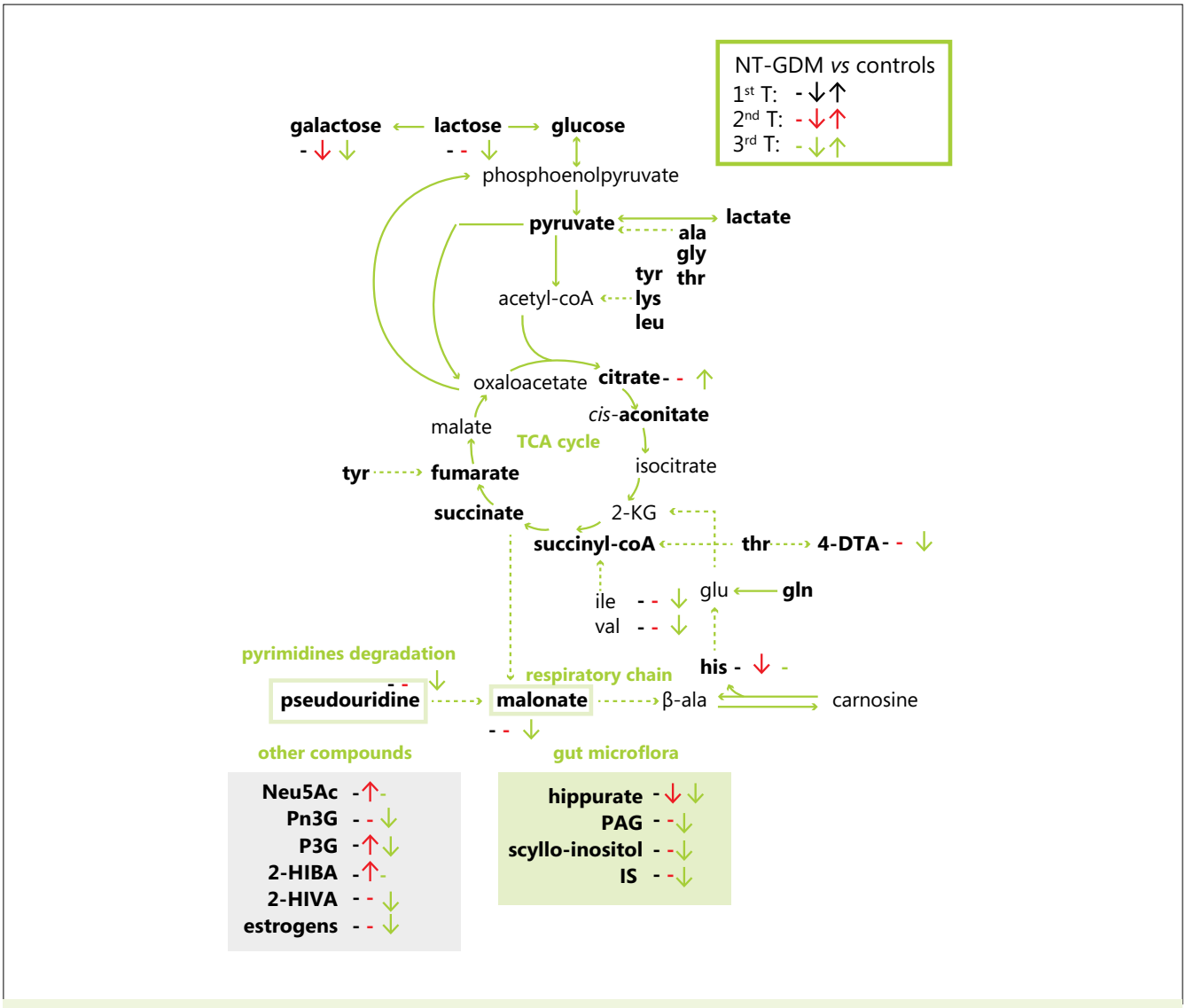

Figure 5 - Putative urine metabolic adaptations detected at the time of GDM diagnosis and before treatment (non-treated GDM: NT-GDM). Altered levels in urinary metabolites are color-coded in black, red and green, for the 1st, 2nd and 3rd trimester, respectively. **Metabolites in bold are those observed by NMR.** Abbreviations: 3-letter code used for amino acids; 2-HIBA, 2-hydroxyisobutyrate; 2-KG, 2-ketoglutarate; 3-HIVA, 3-hydroxyisovalerate; 4-DTA, 4-deoxythreonic acid; IS, indoxyl sulphate; Neu5Ac, *N*-acetylneuraminic acid; P3G, 5β-pregnane-3α,20α-diol-3α-glucuronide; PAG, phenylacetylglutamine; Pn3G, a progesterone metabolite (likely allopregnanolone or corresponding isomers).

the still unassigned resonances shown in the bottom section of the heatmap, which nevertheless are part of the metabolic signatures), the effect of diet treatment regulates most metabolites effectively, as shown by the large number of blank boxes (levels matching those of controls) and the generally weak changes in 2-ketoglutarate (2-KG, a tricarboxylic acid cycle intermediate, reflecting alterations in energy metabolism), malonate (altered fatty acid biosynthesis); and dimethylglycine (DMG) and methylguanidine (amino acid and protein adaptations). It is expected that the return of these four metabolites to control levels would indicate a successful outcome of the treatment. On the other hand, the number of changing metabolites under I-T and M-T is much larger, compared to D-T, with both treatments resulting in significantly distinct signatures. The detailed analysis of these effects is the subject of ongoing work and should provide information on i) the mechanism of action of insulin and metformin, and ii) the extent to which each treatment, and each individual, approach control (healthy) conditions during treatment. This knowledge will enable the personalized follow-up of pregnant wo-

men treated for GDM, through their urinary metabolome, aiding the clinician in the timely definition of individual treatment protocols. Interestingly, when insulin and metformin are combined, the resulting excreted metabolic profile almost completely matches that of controls (except for persisting higher levels of *cis*-aconitate, 4-deoxyerythronic acid (4-DEA), 3-HIVA, gut microflora metabolite 2-HIBA, and amino acid derivatives DMG and creatinine. This suggests that, in this particular cohort, the combined treatment is relatively more suc-

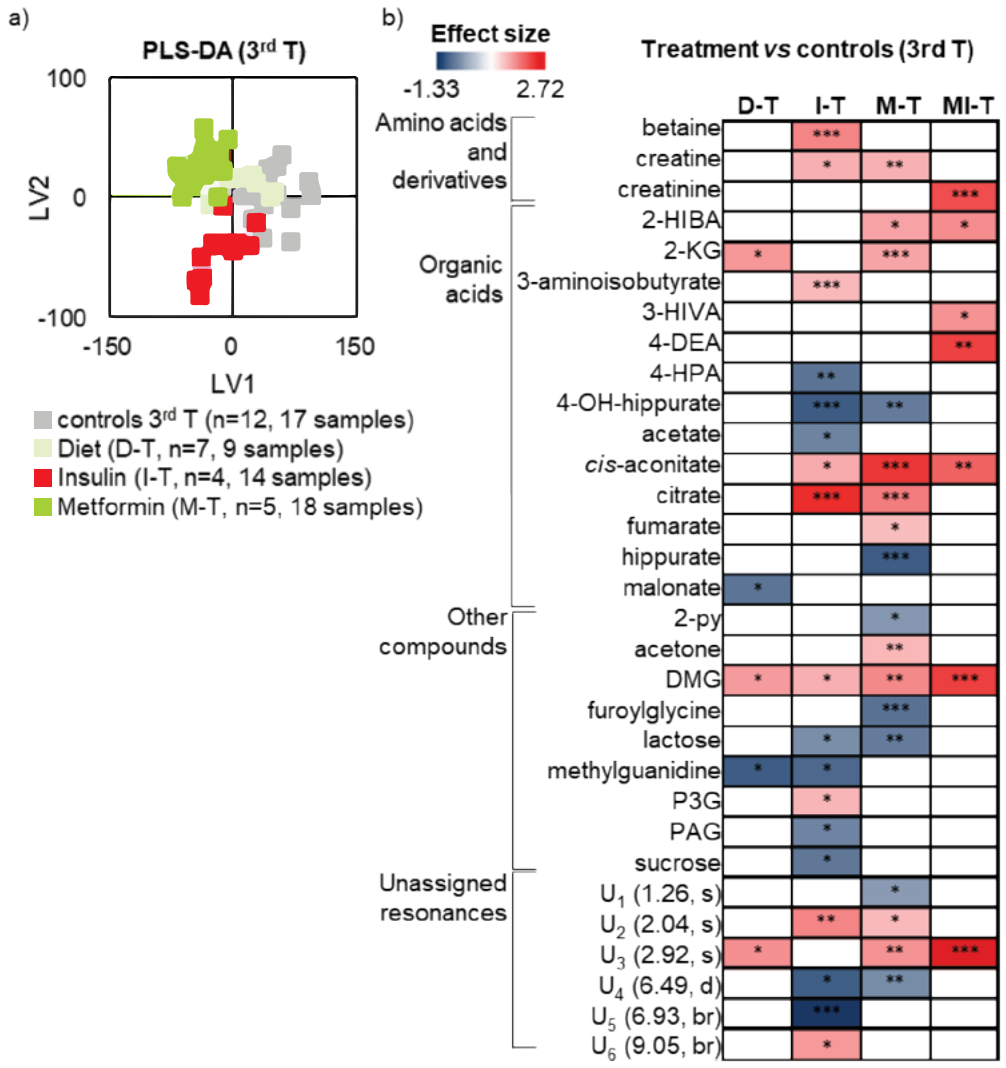

Figure 6 - a) PLS-DA scores scatter plot representing the ^{1}H NMR spectra of urine collected from 3rd T controls (grey squares, n=12, 17 samples), diet-treated subjects (light green squares, n=7, 9 samples), insulin-treated subjects (red squares, n=4, 14 samples) and metformin-treated subjects (green square, n=5, 18 samples; **b)** heatmap representing metabolite variations in maternal urine (expressed as effect size) and shown to increase (red tones) or decrease (blue tones), with statistical relevance (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001). Abbreviations: 2-HIBA, 2-hydroxyisobutyrate; 2-KG, 2-ketoglutarate; 2-py, *N*-methyl-2-pyridone-5-carboxamide; 3-HIVA, 3-hydroxyisovalerate; 4-DEA, 4-deoxyerythronic acid; 4-HPA, 4-hydroxyphenylacetate; 4-OH-hippurate, 4-hydroxyhippurate; DMG, dimethylglycine; D-T, diet-treatment; IS, indoxyl sulphate; I-T, insulin-treatment; MI-T, combination metformin/insulin-treatment; M-T, metformin-treatment; P3G, 5β-pregnane-3α,20α-diol-3α-glucuronide; PAG, phenylacetylglutamine; U^{i}, unassigned resonances ordered by increasing chemical shift (br, broad; d, doublet; s, singlet) .

cessful in approaching the metabolic status of controls, and that the persisting changed metabolites may become valuable indicators of treatment success, in a personalized manner.

In addition, treatment was shown to impact importantly on salivary metabolome too, both in the 2nd and 3rd trimesters of pregnancy (Figure 7 a) and b), respectively). Results shown for a small 2nd trimester cohort, treated either with diet, metformin or insulin/metformin (Figure 7a), again show that diet and the combined treatment

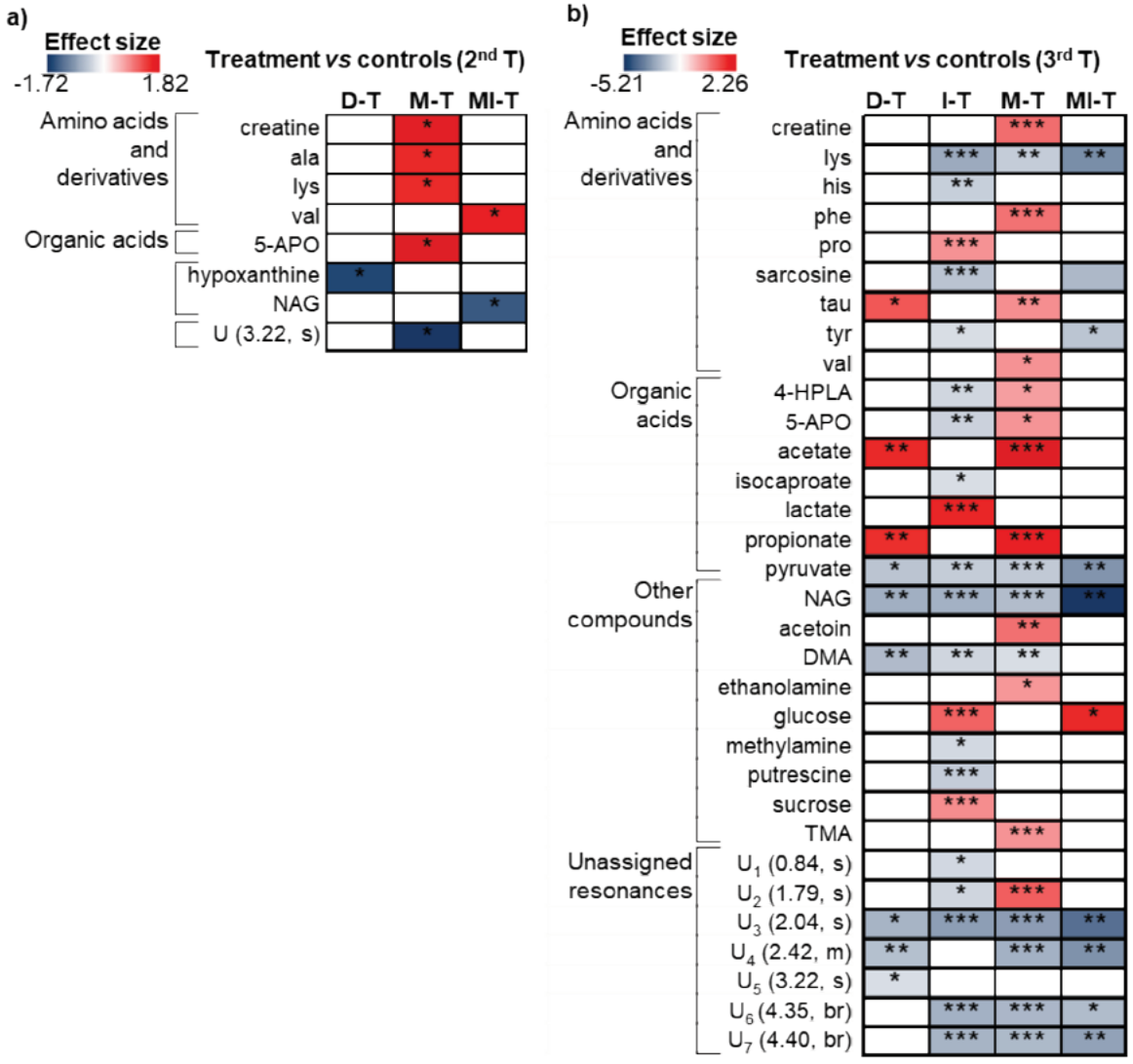

Figure 7 - Heatmap representations of the effects of different treatments in maternal saliva, namely diet (D-T), Insulin (I-T), metformin (M-T) and combination of metformin/insulin (MI-T) on saliva compositions in the **a)** 2nd trimester and in the **b)** 3rd trimester of pregnancy. Metabolite variations are expressed as effect size and shown to increase (red tones) or decrease (blue tones), with statistical relevance (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001. Abbreviations: 3-letter code used for amino acids; 4-HPLA, 4-hydroxyphenyllactate; 5-APO, 5-aminopentanoate; DMA, dimethylamine; NAG, *N*-acetyl group of glycoproteins; TMA, trimethylamine; U_i, unassigned resonances ordered by increasing chemical Shift (br, broad; m, multiplet; s, singlet).

seem more effective to approach healthy conditions (larger number of blank boxes), compared to metformin alone, which seems to disrupt the salivary metabolome to a larger extent. In the 3rd trimester (Figure 7b), the extent of salivary metabolism disruption is significantly more enhanced (larger number of metabolites with altered levels compared to controls), although again diet and the insulin/metformin combined treatment seem to regulate the salivary metabolome more effectively into approaching controls. The above results indicate that, despite the disease not affecting saliva composition, as viewed by NMR metabolomics, different treatments induce distinct salivary metabolic signatures, paving the way to identify, validate and translate to the clinic, both urinary and salivary markers of treatment efficacy (with potential personalized use).

> CONCLUSION

This paper presents a Nuclear Magnetic Resonance metabolomics study of non-invasive maternal biofluids, urine and saliva, with the aims of finding new biomarkers of GDM and of treatment efficacy. As the study considers the disease and treatment effects in different trimesters, one limitation that arises in some cases is the number of available samples, particularly for saliva the collection of which is more lengthy, compared to urine. Despite this, results have clearly shown that the metabolome of urine is sensitive to gestational diabetes *mellitus*, this biofluid providing a valuable source of metabolic biomarkers of the disease and of treatment efficacy. On the other hand, saliva metabolite composition seems to be insensitive to GDM, although it is responsive to treatment. This biofluid appears, therefore, as a potential contributor biofluid for treatment follow-up protocols. <

Department and institution where the work was performed/*Departamento e instituição onde o trabalho foi realizado:*

Sampling was performed at the Maternidade Bissaya Barreto, Centro Hospitalar e Universitário de Coimbra, Portugal, and all sample and data analysis (including all statistical analysis) was performed at CICECO – Aveiro Institute of Materials and Department of Chemistry, at the University of Aveiro, Portugal./A amostragem foi realizada na Maternidade Bissaya Barreto, Centro Hospitalar e Universitário de Coimbra, Portugal, e toda a análise da amostra e dos dados (incluindo toda a análise estatística) foi realizada no CICECO – Instituto de Materiais e Departamento de Química da Universidade de Aveiro, Portugal.

Ethics approval and consent to participate/*Aprovação ética e consentimento para participar:*

This study was approved by the Ethics committee of the Centro Hospitalar e Universitário de Coimbra (references: CHUC-091-17, dated 25 June 2018, 18/04 and 29/09) and by the Portuguese Data Protection Authority (Comissão Nacional de Proteção de Dados) (Authorization no. 1681/ 2018). This study was performed according to the guidelines stated within the Declaration of Helsinki and all participants gave written and signed informed consents./*Este estudo foi aprovado pela Comissão de Ética do Centro Hospitalar e Universitário de Coimbra (referências: CHUC-091-17, de 25 de junho de 2018, 18/04 e 29/09) e pela Autoridade Nacional de Proteção de Dados (Comissão Nacional de Proteção de Dados) (Autorização nº 1681/2018). Este estudo foi realizado de acordo com as diretrizes estabelecidas na Declaração de Helsínquia e todos os participantes deram consentimento informado por escrito e assinado.*

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Conflicts of interests/*Conflitos de interesses:*

The authors individually declare that they have no conflicts of interests./*Os autores declaram a inexistência de conflitos de interesses.*

REFERENCES

1. Creasy RK, Resnik R, Iams JD, editors. Creasy and Resnik's Maternal-fetal medicine: principles and practice. Sixth edition. Philadelphia: Saunders Elsevier; 2009.
2. International Diabetes Federation. IDF Diabetes Atlas. 10th ed. 2021. Available online: https://diabetesatlas.org/atlas/tenth-edition (accessed on 12th May 2022).

3. American Diabetes Association. Standards of medical care in diabetes-2015. Diabetes Care. 2015; 38: S1-93.
4. Dessì A, Marincola FC, Fanos V. Metabolomics and the great obstetrical syndromes —GDM, PET and IUGR. Best Pract Res Clin Obstet Gynaecol. 2014; 29: 156-64.
5. Huynh J, Xiong G, Bentley-Lewis R. A systematic review of metabolite profiling in gestational diabetes mellitus. Diabetologia. 2014; 57: 2453-64.
6. Mao X, Chen X, Chen C, Zhang H, Law KP. Metabolomics in gestational diabetes. Clin Chim Acta. 2017; 475: 116-27.
7. McCabe CF, Perng W. Metabolomics of Diabetes in Pregnancy. Curr Diab Rep. 2017; 17: 57.
8. Chen Q, Francis E, Hu G, Chen L. Metabolomic profiling of women with gestational diabetes mellitus and their offspring: Review of metabolomics studies. J Diabetes Complications. 2018; 32: 512-23.
9. Alesi S, Ghelani D, Rassie K, Mousa A. Metabolomic Biomarkers in Gestational Diabetes Mellitus: A Review of the Evidence. Int J Mol Sci. 2021; 22: 5512.
10. Wang QY, You LH, Xiang LL, Zhu YT, Zeng Y. Current progress in metabolomics of gestational diabetes mellitus. World J Diabetes. 2021; 12: 1164-86.
11. Vladu IM, Clenciu D, Mitrea A, Amzolini A, Micu SE, Crisan AE, et al. Maternal and Fetal Metabolites in Gestational Diabetes Mellitus: A Narrative Review. Metabolites. 2022; 12: 383.
12. Graça G, Duarte IF, Barros AS, Goodfellow BJ, Diaz, SO, Pinto, J. Impact of prenatal disorders on the metabolic profile of second trimester amniotic fluid: a nuclear magnetic resonance metabonomic study. J Proteome Res. 2010; 9: 6016-24.
13. Chen XH, Scholl TO, Leskiw M, Savaille J, Stein TP. Differences in maternal circulating fatty acid composition and dietary fat intake in women with gestational diabetes mellitus or mild gestational hyperglycemia. Diabetes Care. 2010; 33: 2049-54.
14. Graça G, Goodfellow BJ, Barros AS, Diaz S, Duarte, IF, Spagou K, et al. UPLC-MS metabolic profiling of second trimester amniotic fluid and maternal urine and comparison with NMR spectral profiling for the identification of pregnancy disorder biomarkers. Mol Biosyst. 2012; 8: 1243-54.
15. Diaz SO, Barros AS, Goodfellow BJ, Duarte IF, Galhano E, Pita C, et al. Second trimester maternal urine for the diagnosis of trisomy 21 and prediction of poor pregnancy outcomes. J Proteome Res. 2013; 12: 2946-57.
16. de Seymour JV, Conlon CA, Sulek K, Boas SGV, McCowan LME, Kenny LC, et al. Early pregnancy metabolite profiling discovers a potential biomarker for the subsequent development of gestational diabetes mellitus. Acta Diabetol. 2014; 51: 887-90.
17. Bentley-Lewis R, Huynh J, Xiong G, Lee H, Wenger J, Clish C, et al. Metabolomic profiling in the prediction of gestational diabetes mellitus. Diabetologia. 2015; 58, 1329-32.
18. Pinto J, Almeida LM, Martins AS, Duarte D, Barros AS, Galhano E, et al. Prediction of gestational diabetes through NMR metabolomics of maternal blood. J Proteome Res. 2015; 14: 2696-706.
19. Law KP, Han TL, Mao X, Zhang H. Tryptophan and purine metabolites are consistently upregulated in the urinary metabolome of patients diagnosed with gestational diabetes mellitus throughout pregnancy: A longitudinal metabolomics study of Chinese pregnant women part 2. Clin Chim Acta. 2017; 468: 126-39.
20. Law KP, Mao X, Han TL, Zhang H. Unsaturated plasma phospholipids are consistently lower in the patients diagnosed with gestational diabetes mellitus throughout pregnancy: A longitudinal metabolomics study of Chinese pregnant women part 1. Clin Chim Acta. 2017; 465: 53-71.
21. Sakurai K, Eguchi A, Watanabe M, Yamamoto M, Ishikawa K, Mori C. Exploration of predictive metabolic factors for gestational diabetes mellitus in Japanese women using metabolomic analysis. J Diabetes Investig. 2019; 10: 513-20.
22. Mokkala K, Vahlberg T, Pellonperä O, Houttu N, Koivuniemi E, Laitinen K. Distinct Metabolic Profile in Early Pregnancy of Overweight and Obese Women Developing Gestational Diabetes. J Nutr. 2020; 150: 31-7.
23. Walejko JM, Chelliah A, Keller-Wood M, Wasserfall C, Atkinson M, Gregg A, et al. Diabetes Leads to Alterations in Normal Metabolic Transitions of Pregnancy as Revealed by Time--Course Metabolomics. Metabolites. 2020; 10: 350.
24. Tian M, Ma S, You Y, Long S, Zhang J, Guo C, et al. Serum Metabolites as an Indicator of Developing Gestational Diabetes Mellitus Later in the Pregnancy: A Prospective Cohort of a Chinese Population. J Diabetes Res. 2021 Feb 5; 2021: 8885954.
25. Kivelä J, Sormunen-Harju H, Girchenko PV, Huvinen E, Stach--Lempinen B, Kajantie E, et al. Longitudinal Metabolic Profiling of Maternal Obesity, Gestational Diabetes, and Hypertensive Pregnancy Disorders. J Clin Endocrinol Metab. 2021 Oct 21;106(11): e4372-e4388.
26. Koos BJ, Gornbein JA. Early pregnancy metabolites predict gestational diabetes mellitus: implications for fetal programming. Am J Obstet Gynecol. 2021 Feb; 224(2): 215.e1-215.e7.
27. Scott HD, Buchan M, Chadwick C, Field CJ, Letourneau N, Montina T, et al. Metabolic dysfunction in pregnancy: Fingerprinting the maternal metabolome using proton nuclear magnetic resonance spectroscopy. Endocrinol Diabetes Metab. 2020 Nov 18; 4(1): e00201.
28. Piras C, Neri I, Pintus R, Noto A, Petrella E, Monari F, Dessì A, et al. First trimester metabolomics 1H-NMR study of the urinary profile predicts gestational diabetes mellitus development in obese women. J Matern Fetal Neonatal Med. 2021 Sep 16: 1-9.
29. Sachse D, Sletner L, Mørkrid K, Jenum AK, Birkeland KI, Rise F, et al. Metabolic Changes in Urine during and after Pregnancy in a Large, Multiethnic Population-Based Cohort Study of Gestational Diabetes. PLoS One. 2012; 7: e52399.

30. Dudzik D, Zorawski M, Skotnicki M, Zarzycki W, Kozlowska G, Bibik-Malinowska K, et al. Metabolic fingerprint of gestational diabetes mellitus. J Proteom. 2014; 103: 57-71.
31. Scholtens DM, Muehlbauer MJ, Daya NR, Stevens RD, Dyer AR, Lowe LP, et al. Metabolomics reveals broad-scale metabolic perturbations in hyperglycemic mothers during pregnancy. Diabetes Care. 2014; 37: 158-66.
32. Lehmann R, Friedrich T, Krebiehl G, Sonntag D, Häring H-U, Fritsche A, Hennige AM. Metabolic Profiles during an Oral Glucose Tolerance Test in Pregnant Women with and without Gestational Diabetes. Exp Clin Endocrinol Diabetes. 2015; 123: 433-8.
33. Pinto J, Diaz SO, Aguiar, Duarte D, Barros AS, Galhano E, et al. Metabolic profiling of maternal urine can aid clinical management of gestational diabetes mellitus. Metabolomics. 2016; 12: 1-12.
34. Hajduk J, Klupczynska A, Dereziński P, Matysiak J, Kokot P, Nowak D, et al. A Combined Metabolomic and Proteomic Analysis of Gestational Diabetes Mellitus. Int J Mol Sci. 2015; 16: 30034-45.
35. Chen X, de Seymour JV, Han TL, Xia Y, Chen C, Zhang T, et al. Metabolomic biomarkers and novel dietary factors associated with gestational diabetes in China. Metabolomics. 2018 Nov 3; 14(11): 149.
36. Huhtala MS, Tertti K, Pellonperä O, Rönnemaa T. Amino acid profile in women with gestational diabetes mellitus treated with metformin or insulin. Diabetes Res Clin Pract. 2018; 146: 8-17.
37. Huhtala MS, Tertti K, Rönnemaa T. Serum lipids and their association with birth weight in metformin and insulin treated patients with gestational diabetes. Diabetes Res Clin Pract. 2020; 170: 108456.
38. Mokkala K, Vahlberg T, Houttu N, Koivuniemi E, Laitinen K. Distinct Metabolomic Profile Because of Gestational Diabetes and its Treatment Mode in Women with Overweight and Obesity. Obesity. 2020; 28: 1637-44.
39. Liu L, Liu L, Wang J, Zheng Q, Jin B, Sun L. Differentiation of gestational diabetes mellitus by nuclear magnetic resonance--based metabolic plasma analysis. J Biomed Res. 2021; 35: 351-60.
40. White SL, Begum S, Vieira MC, Seed P, Lawlor DL, Sattar N, et al. Metabolic phenotyping by treatment modality in obese women with gestational diabetes suggests diverse pathophysiology: An exploratory study. PLoS One. 2020; 15: e0230658
41. Qiu C, Enquobahrie DA, Frederick IO, Sorensen TK, Fernandez MAL, David RM, et al. Early pregnancy urinary biomarkers of fatty acid and carbohydrate metabolism in pregnancies complicated by gestational diabetes. Diabetes Res Clin Pract. 104: 393-400.
42. Liu X, Wang X, Sun H, Guo Z, Liu X, Yuan T, et al. Urinary metabolic variation analysis during pregnancy and application in Gestational Diabetes Mellitus and spontaneous abortion biomarker discovery. Sci Rep. 2019; 9: 2605
43. Zhao H, Zheng Y, Zhu L, Xiang L, Xu S, Cai Z. Trimester-specific urinary metabolome alterations associated with gestational diabetes mellitus: A study in different pregnancy stages. Chin Chem Lett. 2021; in press.
44. Wang X, Liu H, Li Y, Huang S, Zhang L, Cao C, et al. Altered gut bacterial and metabolic signatures and their interaction in gestational diabetes mellitus. Gut Microbes. 2020; 12: 1-13.
45. Peng S, Zhang J, Liu L, Zhang X, Huang Q, Alamdar A, et al. Newborn Meconium and Urinary Metabolome Response to Maternal Gestational Diabetes Mellitus: A Preliminary Case–Control Study. J Proteome Res. 2015; 14: 1799-809 .
46. van Oostdam ASH, Salgado-Bustamante M, Lima-Rogel V, Oropeza-Valdez JJ, López JA, Rodríguez IDR, et al. Urinary metabolomic profile of neonates born to women with gestational diabetes mellitus. Metabolites. 2021; 11: 723.
47. Dani C, Bresci C, Berti E, Ottanelli S, Mello G, Mecaci F, et al. Metabolomic profile of term infants of gestational diabetic mothers. J Matern-Fetal Neonatal Med. 2014; 27: 537-42.
48. Lowe Jr. WL, Bain JR, Nodzenski M, Reisetter AC, Muehlbauer MJ, Ilkayeva OR, et al. Maternal BMI and Glycemia Impact the Fetal Metabolome. Diabetes Care. 2017; 40: 902-10.
49. Lu Y-P, Reichetzeder C, Prehn C, von Websky K, Slowinski T, Chen Y-P, et al. Fetal Serum Metabolites Are Independently Associated with Gestational Diabetes Mellitus. Cell Physiol Biochem. 2018; 45: 625-38.
50. Shokry E, Marchioro L, Uhl O, Bermúdez MG, García-Santos JA, Segura MT, et al. Impact of maternal BMI and gestational diabetes mellitus on maternal and cord blood metabolome: results from the PREOBE cohort study. Acta Diabetologica. 2019; 56(4): 421-30.
51. Shokry E, Marchioro L, Uhl O, Bermúdez MG, García-Santos JA, et al. Transgenerational cycle of obesity and diabetes: investigating possible metabolic precursors in cord blood from the PREOBE study. Acta Diabetologica 2019; 56: 1073-82.
52. Mansell T, Vlahos A, Collier F, Ponsonby AL, Vuillermin P, Ellul S, et al. The newborn metabolome: associations with gestational diabetes, sex, gestation, birth mode, and birth weight. Pediatr Res. 2021 Sep 15. doi: 10.1038/s41390-021-01672-7. Epub ahead of print. Erratum in: Pediatr Res. 2021 Oct 29.
53. Huhtala MS, Rönnemaa T, Pellonperä O, Tertti K. Cord serum metabolome and birth weight in patients with gestational diabetes treated with metformin, insulin, or diet alone. BMJ Open Diabetes Res Care. 2021; 9: e002022.
54. Ma J, Luo J, He M, Bian X, Li J, Du Y, et al. Umbilical cord blood metabolomics: association with intrauterine hyperglycemia. Pediatr Res. 2021 May 12. doi: 10.1038/s41390-021-01516-4. Epub ahead of print.
55. Almeida MC, Dores J, Ruas L (coordenadores). Consenso "Diabetes Gestacional": Atualização 2017. Rev Port Diabetes. 2017; 12 (1): 24-38.
56. Diaz SO, Pinto J, Barros AS, Morais E, Duarte D, Negrao F, et al.

Newborn Urinary Metabolic Signatures of Prematurity and Other Disorders: A Case Control Study. J Proteome Res. 2016; 15: 311-25.
57. Bouatra S, Aziat F, Mandal R, Guo AC, Wilson MR, Knox C, et al. The human urine metabolome. PLoS One. 2013; 8: e73076.
58. Maitre L, Villanueva CM, Lewis MR, Ibarluzea J, Santa-Marina L, Vrijheid M, et al. Maternal urinary metabolic signatures of fetal growth and associated clinical and environmental factors in the INMA study. BMC Med. 2016; 14: 177.
59. Wishart DS, Guo A, Oler E, Wang F, Anjum A, Peters H, et al. HMDB 5.0: the Human Metabolome Database for 2022. Nucleic Acids Res. 2022; 50: D622-31.
60. Duarte D, Castro B, Pereira JL, Marques JF, Costa AL, Gil AM. Evaluation of Saliva Stability for NMR Metabolomics: Collection and Handling Protocols. Metabolites. 2020; 10: 515.
61. Pereira JL, Duarte D, Carneiro TJ, Ferreira S, Cunha B, Soares D, et al. Saliva NMR metabolomics: Analytical issues in pediatric oral health research. Oral Dis. 2019; 25: 1545-54.
62. Silwood CJ, Lynch E, Claxson AW, Grootveld MC. 1H and (13)C NMR spectroscopic analysis of human saliva. J Dent Res. 2002; 81: 422-7.
63. Takeda I, Stretch C, Barnaby P, Bhatnager K, Rankin K, Fu H, et al. Understanding the human salivary metabolome. NMR Biomed. 2009; 22: 577-84.
64. Dame ZT, Aziat F, Mandal R, Krishnamurthy R, Bouatra S, Bourzouie S, et al. The human saliva metabolome. Metabolomics. 2015; 11: 1864-83.
65. Singh MP, Saxena M, Saimbi CS, Arif JM, Roy R. Metabolic profiling by 1H NMR spectroscopy of saliva shows clear distinction between control and diseased case of periodontitis. Metabolomics. 2017; 13: 137.
66. Veselkov KA, Lindon JC, Ebbels TMD, Crockford D, Volynkin V, Holmes E, et al. Recursive segment-wise peak alignment of biological 1H NMR spectra for improved metabolic biomarker recovery. Anal Chem. 2009; 81: 56-66.
67. Westerhuis JA, Hoefsloot HCJ, Smit S, Vis DJ, Smilde AK, van Velzen EJJ, et al. Assessment of PLSDA cross validation. Metabolomics. 2008; 4: 81-9.
68. Wiklund S, Nilsson D, Eriksson L, Sjostrom M, Wold S, Faber K. A randomization test for PLS component selection. J Chemom. 2007; 21: 427-39.
69. Berben L, Sereika SM; Engberg S. Effect Size Estimation: Methods and Examples. Int J Nurs Stud. 2012; 49: 1039-47.