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ORIGINAL ARTICLE

Urinary metabolomics (GC-MS) reveals that low and high birth weight infants share elevated inositol concentrations at birth

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Abstract

Objective: Metabolomics is a new "omics" platform aimed at high-throughput identification, quantification and characterization of small molecule metabolites. The metabolomics approach has been successfully applied to the classification different physiological states and identification of perturbed biochemical pathways. The purpose of the current investigation is the application of metabolomics to explore biological mechanisms which may lead to the onset of metabolic syndrome in adulthood.

Methods: We evaluated differences in metabolites in the urine collected within 12 hours from 23 infants with IUGR (IntraUterine Growth Restriction), or LGA (Large for Gestational Age), compared to control infants (10 patients defined AGA: Appropriate for Gestational Age). Urinary metabolites were quantified by GC-MS and used to highlight similarities between the two metabolic diseases and identify metabolic markers for their predisposition. Quantified metabolites were analyzed using a multivariate statistics coupled with receiver operator characteristic curve (ROC) analysis of identified biomarkers.

Results: Urinary myo-inositol was the most important discriminant between LGA + IUGR and control infants, and displayed an area under the ROC curve = 1.

Conclusion: We postulate that the increase in plasma and consequently urinary inositol may constitute a marker of altered glucose metabolism during fetal development in both IUGR and LGA newborns.

Keywords

GC-MS, inositol, IUGR, metabolomics, OSC-PLS-DA

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Introduction

Progress in new systems biology methodologies which study metabolic alterations of aetiological processes at the origin of many pathologies are becoming more important. In particular, metabolomics, a new analytical technique defined as the study of the complex system of metabolites, is capable of describing the biochemical phenotype of a biological system [1]. At present, attention is being focused mainly on the formulation of new hypotheses concerning the biological mechanisms that lead to the onset of diabetes and metabolic syndrome in adulthood. Metabolomics has been recently applied to identify several biomarkers (including myo-inositol) for mechanisms which may lead to infantile obesity and the subsequent onset of metabolic diseases [2]. Comprehensive analysis of changes in metabolic profiles during fetal and neonatal life may present an important reference for understanding fundamental biochemical mechanisms, which may lead to consequent metabolic alterations. Previous investigations have demonstrated that fetal malnutrition, whether excessive (overnutrition) or insufficient (hyponutrition), can permanently alter the fetus's metabolic state and increase the risk of chronic diseases later in life. This suggests that neonates with intrauterine growth retardation (IUGR) and large-for-gestational-age (LGA) neonates, despite opposing metabolic characteristics during fetal life, at birth may exhibit a common condition of reduced glucose tolerance which can persist into adulthood, and consequently leads to an increased risk of developing metabolic syndrome related pathologies such as obesity and type 2 diabetes [3]. The current investigation compared urine metabolic profiles of IUGR and LGA neonates to controls to identify metabolic similarities between IUGR and LGA and to identify markers for biochemical alterations during fetal life which may lead to the onset of metabolic syndrome in adulthood.

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Methods

Patient cohort and sample collection

This study was carried out on urine samples of three groups of patients admitted to the Neonatal Intensive Care Unit (NICU) and Puericulture of the University of Cagliari. Ethical committee approved the study protocol and written informed consent was obtained from the parents before enrolment in the study. The first group included 11 IUGR patients (5 males and 6 females) diagnosed with ultrasonography in the prenatal period [4] and with birth weight below the 10th percentile (mean gestational age 37.1). The second group comprised 12 LGA neonates (7 males and 5 females) with birth weight above the 90th percentile (mean gestational age 37.6) while the third group included 10 neonates (5 males and 5 females) with birth weight adequate for gestational age (AGA) (mean gestational age 37.4). The clinical data of each patient were recorded in the hospital registers Urine samples from the three groups were collected within 12 hours from birth (prior to feeding). Each urine sample (2–3 ml) was collected from the patients using a noninvasive method with a ball of cotton, then aspired with a syringe and transferred to a sterile 15 ml Falcon tube. The tubes were then stored at −80 °C until the time of the analysis using the GC-MS technique. An aliquot of 100 μL from each sample formed a urine pooled sample to be analysed with the others as quality control sample.

Sample preparation

One-hundred fifty microliters of urine were transferred in glass vials (2 mL) with PTFE-lined screw caps and evaporated to dryness overnight in an Eppendorf vacuum centrifuge. 30 μL of a 0.24 M solution of methoxylamine hydrochloride in pyridine was added to each vial, samples were vortex mixed and left to react for 17 h at room temperature. Then 30 μL of MSTFA (N-Methyl-N-trimethylsilyl trifuoroacetamide) were added and left to react for 1 h at room temperature. The derivatized samples were diluted with hexane (300 μL) just before GC-MS analysis. The GC-MS analysis was performed on an Agilent 5975C interfaced to the GC 7820 equipped with a DB-5ms column (J & W), injector temperature at 230 °C, detector temperature at 280 °C, helium carrier gas flow rate of 1 ml/min. The GC oven temperature program was 90 °C initial temperature with 1 min hold time and ramping at 10 °C/min to a final temperature of 270 °C with 7 min hold time. 1 μL of the derivatized sample was injected in split (1:20) mode. After a solvent delay of 3 minutes, mass spectra were acquired in full scan mode using 2.28 scans/s with a mass range of 50–700 Amu.

Sample analysis

The chromatogram of the pooled sample was used to build a dedicated library of urinary metabolites: some chromatographic peaks were identified through comparison of retention times and mass spectra with those obtained from authentic samples. Other metabolites were identified using the National Institute of Standards and Technology (NIST08) mass spectral database. In this way, 120 target compounds were identified, thus generating a custom library which was used for later automated analysis of samples by AMDIS (Automated Mass Spectrometry Deconvolution and Identification System, http://chemdata.nist.gov/mass-spc/amdis/) software.

Mathematical model

Partial least squares discriminant analysis (PLS-DA) [5] is a multivariate classification model, which is similar to principal components analysis (PCA) [5], but unlike PCA maximizes the covariance in independent variables (metabolites) and a dependent variable (class labels, e.g. IUGR + LGA, AGA). To capture the maximum variance between clinically defined groups in the first dimension of the PLS-DA model, first latent variable (LV), all information orthogonal to group discrimination needs to be removed from the model. Unfortunately, the plane of separation between class scores in a preliminary PLS-DA model spanned two or more LVs (materials not shown). To simplify model interpretation of which variables had the greatest contribution to the discrimination between clinical classes [6–8], we implemented orthogonal signal correction-PLS (OSC-PLS) using the SIMCAP + 12 Umetrics software [5]. OSC-PLS discriminant analysis (OSC-PLS-DA) was used to maximize the captured variance between samples in the first dimension of the model (LV 1). We generated an OSC-PLS model based on our data and then used the resulting OSC-corrected data as input to generate an OSC-PLS-DA model. Variable loadings for the first LV were compared between PLS-DA and OSC-PLS-DA models. For most variables the magnitude of the model loading did not change greatly, but there were some parameters whose sign for the loading changed; we needed to make sure that the sign of the variable loading accurately reflected each parameter's relative change between classes. In this way we specifically focused on how OSC affects the model's perception of the importance or weights of the variables resulting in significant differences in weights (delta weights) between a pure PLS-DA and an OSC-PLS-DA model. The primary step in this application of OSC filtering to a PLS-DA model is the building of the appropriate Y discrete-values function; the metabolic hypothesis may be tested with the function:

$$y = 0 \ \forall \ samples \ \in AGA$$
$$y = 1 \ \forall \ samples \ \in \ IUGR{-}LGA$$

The Y column is added to the X matrix of the metabolite concentration and can be used to remove the orthogonal components in the X variance matrix.

Biomarker validation

Receiver Operating Characteristic (ROC) curves are generally considered the method of choice for evaluating the performance of potential biomarkers. Some authors have developed a web-based tool designed to assist researchers in performing common ROC-based analyses on metabolomic data also using a multivariate approach [9]. The module provides a well-established approach based on the Partial Least Squares – Discriminant Analysis (PLS-DA) for classification and feature selection. Monte Carlo cross validation (MCCV) with multiple iterations was employed to compute ROC curves and

calculate confidence intervals of the AUCs. The purpose of this module is to create and identify robust predictive models using multiple biomarkers. The authors integrated feature selection and classification procedures for the PLS-DA algorithm. The procedures were repeated several times to identify the best model as well as the most stable features. The input for ROCCET web tools is the data table containing absolute or relative abundance values of compound concentrations. The file should be a data table with samples in rows and features (metabolites) in columns; the phenotype labels must follow immediately after the sample IDs. We uploaded the OSC-X matrix filtered by means of the Y 2-values classification function (classes for AGA versus IUGR-LGA). Data were logarithmically transformed and Pareto scaled and then the ROC analysis with the PLS-DA algorithm was performed. The parameter for classifier performances, the area under the ROC curve, was unitary due to the OSC filtration action. A validation of the OSC-correction approach was conducted in R [10] using the R package DeviumWeb (https://github.com/dgrapov/DeviumWeb). DeviumWeb was used to generate an OSC-PLS-DA model according to previously published methods [11]. The sample scores and variable importance rank were in close agreement between the two approaches.

Results

Our GC-MS analysis of the urine samples showed significant differences among AGA, LGA, and IUGR groups. A comparison between the chromatograms of urine samples collected from AGAs, LGAs and IUGRs are shown in Figure 1.

As can be noted, quantitative and qualitative differences are present among the groups. Subsequently, to elucidate the association between the most important variables, a multivariate statistical approach was performed. The spectra of the urine samples were aligned and the values of the relative concentrations were placed in a numerical matrix with a size of 33×111. An explorative PCA model was built for all three groups together and then for each group of subjects (PCA class). Furthermore, to explore an innovative metabolic hypothesis we later considered performing the analysis within two groups: (1) LGA + IUGR, (2) AGA. The comparison between these groups aimed to explore the common dysfunctional pathways of LGA + IUGR, if they were present. However, with an unsupervised approach the separation among the three groups was not clear (data not shown). For this reason, a method to extract the part of the covariant metabolome using these two groups LGA + IUGR and AGA was adopted. A new function with discrete arbitrary values was created and the samples belonging to the LGA + IUGR class had 1 value whilst the AGA class 0. The binary Y variable created was subsequently used as the single Y function for the OSC filter application to remove the non-covariant part within the metabolic change of interest. After OSC transformation of data, a PLS-DA model was built and a clear separation between the groups (Figure 2) was found with this OSC-PLS-DA model. The OSC-PLS-DA model generated had a high goodness of fit and predictability as indicated by an R2Y value of 0.979 and a Q2 value of 0.903 respectively with a CV-ANOVA p value of $1.8\times$ 10-9. The analysis of the corresponding loading plot revealed variables of importance for the clustering, thus allowing identification of metabolites responsible for the variance observed. The most important variable that significantly contributed to the separation between the LGA + IUGR/AGA groups was myo-inositol which was higher in the LGA + IUGR group compared to AGA group. In addition, the metabolites: urea, glycerol, glucose, citric acid and uric acid were also important for clinical class discrimination. A multivariate analysis of the OSC data was also performed by means of the web tool MetaboAnalyst [9] with the ROCCET module used to calculate the ROC curves for evaluation of the model classification power in terms of sensibility and sensitivity (www.metaboanalyst.ca). The ROC curve for myo-inositol showed an area under the curve almost equal to 1 [Figure 3 left]. The following Figure 3 (right) shows the box plot of the OSC-transformed concentrations of myo-inositol OSC.

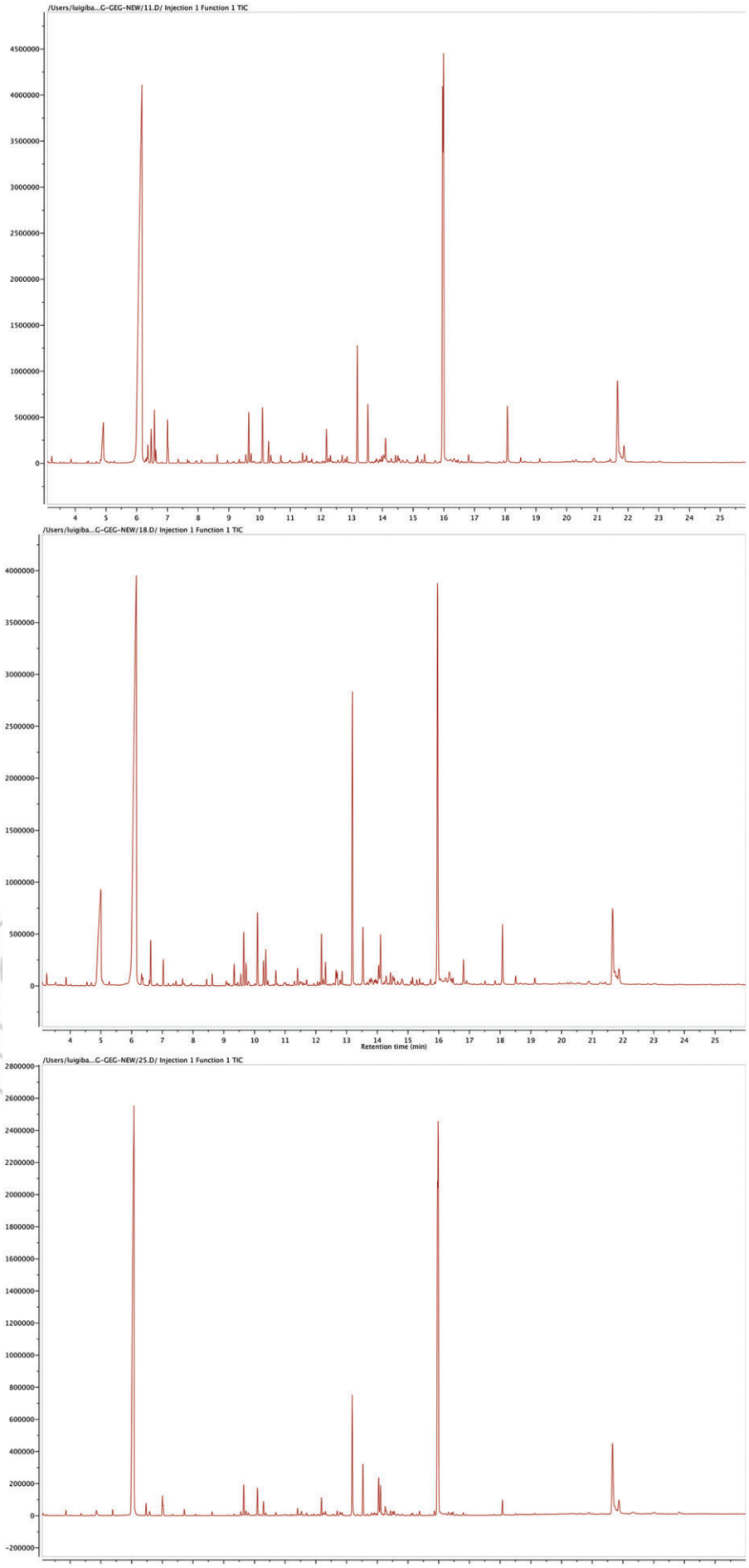
Data were processed also with the R software reproducing results found with the SIMCAP12 (Figure 4).

Discussion

To date, little is known about the overall metabolic state of IUGR and LGA neonates. In clinical practice, only a limited number of metabolites are normally measured in neonates' biological liquids by means of conventional analytical methods. In recent years, metabolomics has assumed an important clinical role since it is capable of making a simultaneous qualitative and quantitative assessment of a consistent number of metabolites in biological fluids so as to supply a description of the present biochemical status of an organism. Some studies in the literature correlate the behavior of single substances such as inositol with intrauterine growth retardation and suggest their possible role as markers in such a pathology [12–14]. Inositol is a carbocyclic polyol (the most important form in nature is myo-inositol) which plays a fundamental role as the structural base of the second messengers in eukaryotic cells, including the inositol phosphates such as phosphatidylinositol and phosphatidylinositol phosphate (PIP) [15]. It is an important metabolite for different cell functions, among which cell growth and survival, the development and functioning of peripheral nerves, osteogenesis and reproduction. Many are the hormones and neurotransmitters that trigger the production of inositol following interaction with their receptors: classic examples include insulin, serotonin, noradrenalin, histamine and angiotensin. Certain organs such as the brain, the liver and the pancreas have inositol concentrations up to 28 times higher than those found in plasma; this emphasizes the importance of this metabolite at the tissue and cellular levels. In this study, the application of metabolomics made it possible to identify molecules responsible for the differences between the diverse metabolic profiles, among which myo-inositol, whose urine content increased in IUGR and LGA neonates compared to controls. Different inositol isomers were found to possess insulin-mimetic properties and were efficient in reducing post-prandial glucose levels in the blood. Anomalies in the inositol metabolism were also associated with insulin resistance and long-term microvascular

COLOR Online / B&W in Print

Figure 1. GC/MS chromatograms of urine samples from LGA (top), AGA (middle), and IUGR (bottom) subjects.



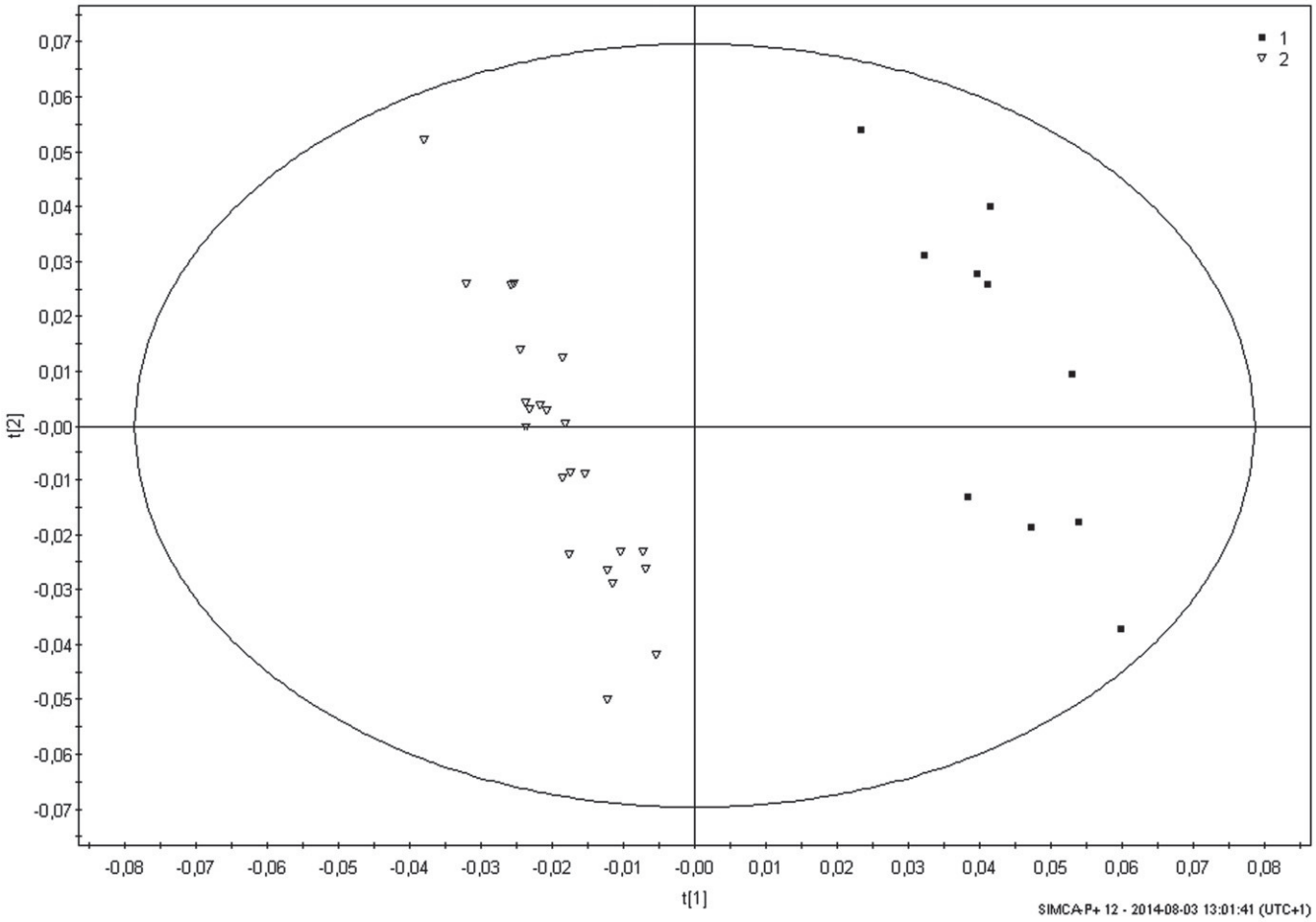


Figure 2. Score Plot of the OSC-PLS-DA model: 1 = black box AGA samples; 2 = Open Diamond LGA + IUGR samples.

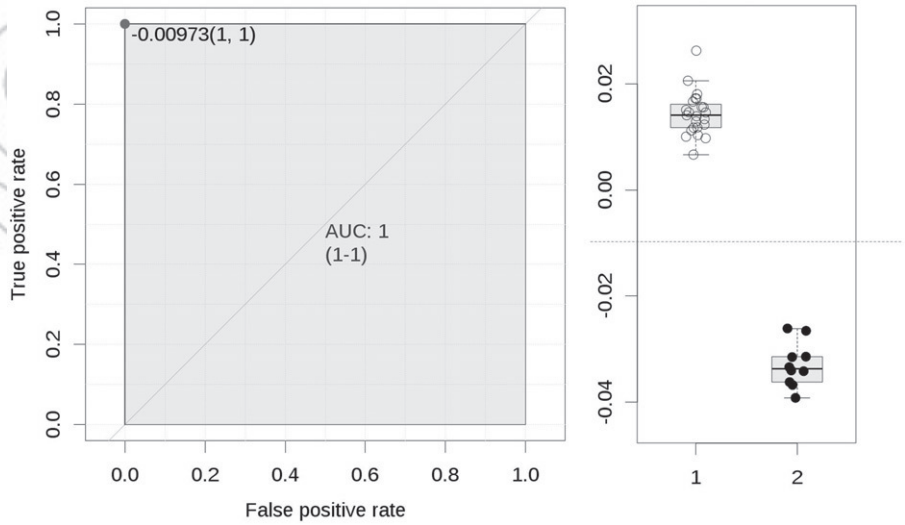


Figure 3. Left: ROC curve for the Inositol OSC feature as biomarker; right: boxplot for the OSC-concentration values for the two classes 1 = AGA and 2 LGA + IUGR.

complications of diabetes, thus stressing a role of inositol or its derivatives in the glucose metabolism [16]. In human subjects with type 2 diabetes and experimental models (rhesus monkeys, rats) there is an intracellular depletion of myo-inositol with an increase in its secretion and at the same time a decrease in the amount of D-chiro-inositol in the urine [17]. This kind of urinary excretion leads to a decrease in the urinary [D-chiro-inositol]/[myo-inositol] ratio. The same anomalous inositol behavior is to be seen in insulin-sensitive tissues (liver, muscle, fat and kidneys) of human and animal diabetic subjects [18]. Some researchers have found a nonlinear, U-shaped association between birth weight and type 2 diabetes which leads to an increased risk both for neonates with high birth weight and those with low birth

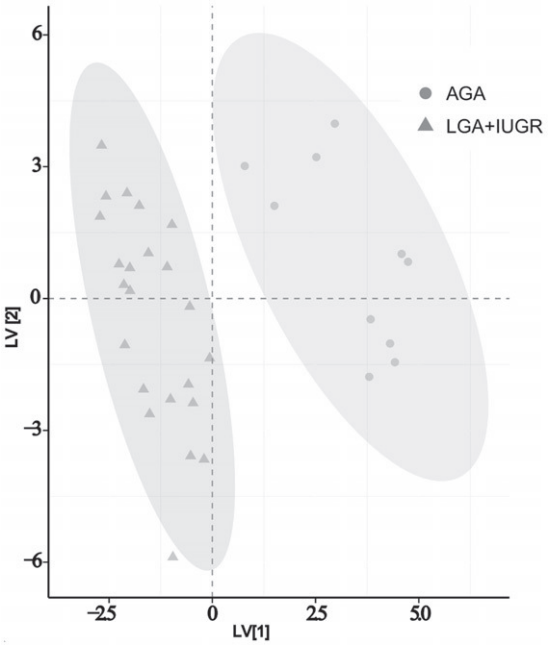


Figure 4. OSC-PLS-DA model calculated in R [10], which reproduced the previous results.

weight for their gestational ages [19]. This confirms what has been observed in cardiovascular [20] and kidney [21] pathologies. All this may explain the altered metabolic pattern found in IUGR and LGA neonates compared to controls and especially the increase in urinary myo-inositol that accumulates in the two pathologies. In this work, patients' urine samples were collected within 12 hours from birth when they had not yet been fed. The contribution of nutritive substances to the fetus is a key factor in the regulation of fetal growth. Many studies in the literature argue that environmental factors acting on the fetus (such as the kind of nutrition) may influence its prenatal development, thus determining structural and functional alterations that appear to be irreversible and continue in the course of postnatal life, leading to an increased risk of developing dysmetabolic diseases in adulthood. Several studies on animal models have confirmed that exposure to a hyper- and hypoglycemic environment in the uterus leads to a reduced glucose tolerance at birth which continues into adulthood, independently of subjects' genetic predisposition [22,23]. It is known that insulin plays a role in favoring lipid and protein synthesis as well as cell growth and inositol is known to be one of its important secondary messengers [24]. Up to now, works have been published to suggest that the glucose mechanism altered during fetal development in IUGRs may be marked by the increase in extracellular myo-inositol and some of these studies employed metabolomics in analyzing patients' samples [25,26]. This is the first work described in the literature that analyzed by means of metabolomics the urine metabolic profiles in IUGR and LGA neonates, comparing them to controls for the purpose of defining the metabolic patterns associated with such pathologies. The urinary increase in inositol (likely related to the high plasmatic concentrations of inositol), often associated with glucose intolerance and insulin resistance in adults, may also be considered valid markers of an altered glucose metabolism during fetal development both in IUGRs and LGAs. It is thus to be hoped that future progress in metabolomics will soon make it possible to arrive at a more effective personalized nutritional therapy for the prevention of chronic adult pathologies in these patients.

Declarations of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper. One of the authors, Dmitry Grapov, is supported by a NIH Grant: NIH 1 U24 DK097154.

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