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Abstract:
Introduction: Metabolomics identifies phenotypical groups with specific metabolic profiles, being increasingly applied to several pregnancy conditions. This is the first preliminary study analyzing placental metabolomics in normal weight (NW) and obese (OB) pregnancies.
Methods: Twenty NW (18.5 ≤ BMI< 25 kg/m2) and eighteen OB (BMI≥ 30 kg/m2) pregnancies were studied. Placental biopsies were collected at elective caesarean section. Metabolites extraction method was optimized for hydrophilic and lipophilic phases, then analyzed with GC-MS. Univariate and PLS-DA multivariate analysis were applied.
Results: Univariate analysis showed increased uracil levels while multivariate PLS-DA analysis revealed lower levels of LC-PUFA derivatives in the lipophilic phase and several metabolites with significantly different levels in the hydrophilic phase of OB vs NW.
Discussion: Placental metabolome analysis of obese pregnancies showed differences in metabolites involved in antioxidant defenses, nucleotide production, NOX signaling, as well as lipid synthesis and energy production, supporting a shift towards higher placental metabolism. OB placentas also showed a specific fatty acids profile suggesting a disruption of LC-PUFA biomagnification. This study can lay the foundation to further metabolomic placental characterization in maternal obesity. Metabolic signatures in obese placentas may reflect changes occurring in the intrauterine metabolic environment, which may affect the development of adult diseases.

TITLE PAGE

1. TITLE:

Preliminary Metabolomics Analysis of Placenta in Maternal Obesity

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ABSTRACT

Introduction: Metabolomics identifies phenotypical groups with specific metabolic profiles, being increasingly applied to several pregnancy conditions. This is the first preliminary study analyzing placental metabolomics in normal weight (NW) and obese (OB) pregnancies.

Methods: Twenty NW (18.5 ≤BMI< 25 kg/m2) and eighteen OB (BMI≥ 30 kg/m2) pregnancies were studied. Placental biopsies were collected at elective caesarean section. Metabolites extraction method was optimized for hydrophilic and lipophilic phases, then analyzed with GC-MS. Univariate and PLS-DA multivariate analysis were applied.

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KEYWORDS

Metabolomics, Placenta, GC-MS, Pregnancy, Obesity

ABBREVIATIONS

AMDIS: Automated Mass spectral Deconvolution and Identification System

ANOVA: ANalysis Of VAriance

BF3: boron trifluoride

BMI: Body Mass Index

CV: Cross Validation

DHA: DocosaHexaenoic Acid

DNA: DeoxyriboNucleic Acid

FDR: False Discovery Rate

FIGO: International Federation of Obstetrics and Gynecology

GC-MS: Gas Chromatography-Mass Spectrometry

GDM: Gestational Diabetes Mellitus

GMD: Golm Metabolome Database

GWG: Gestational Weight Gain
HSD: Honest Significant Difference
IGF-1: Insuline-like Growth Factor-1
LC-MS: Liquid Chromatograph Mass Spectrometers**LC-PUFA:** Long Chain-Polyunsaturated Fatty Acids
MS: Mass Spectrometry
MSTFA: N-Methyl-Ntrimethylsilyltrifluoroacetamide
mTOR: mammalian Target Of Rapamycin
NAD: Nicotinamide Adenine Dinucleotide
NADPH: Nicotinamide Adenine Dinucleotide Phosphate
NIST08: National Institute of Standards and Technology mass spectral database
NMR: Nuclear Magnetic Resonance
OGTT: Oral Glucose Tolerance Test
PBS: Phosphate Buffered Saline
PLS-DA: Partial Least Square-Discriminant Analysis
RT: Room Temperature

HIGHLIGHTS

- Maternal pregestational BMI determines different placental metabolite concentration
- Obese placentas lipophilic profile supports LC-PUFA biomagnification disruption
- The hydrophilic profile suggests a shift towards higher obese placental metabolism

MAIN TEXT

INTRODUCTION

Obesity is spreading worldwide with almost epidemic proportions, representing a risk factor for adverse pregnancy outcomes and offspring's complications **[1-2]**. Maternal obesity is characterized by calorie imbalance and incorrect dietary intake and has been associated with a lipotoxic placental environment, defined by decreased regulators of angiogenesis and increased markers of inflammation and oxidative stress **[3]**. This adverse intrauterine environment may directly affect placental function and metabolism **[4,5]**. Similarly to what occurs with maternal diabetes, increased maternal Body Mass Index (BMI), together with fetal sex, is associated with decreased placental efficiency and histopathologic findings typical of hypoxia and inflammation **[3-6]**.

Metabolomics applies a holistic approach to study the whole metabolite content of cells, tissues or bio-fluids. Metabolomic analysis has recently found applications in several pregnancy-related conditions **[7-13]** allowing for the recognition of different phenotypical groups due to their characteristic metabolic profile. Most of these works reported metabolomic analysis of bio-fluids such as blood, urine or amniotic fluid **[7-13]**.

To the best of our knowledge, there are only few metabolomic studies on placenta tissue extracts using Mass Spectrometry (MS) **[14-20]** or Nuclear Magnetic Resonance (NMR) spectroscopy **[21-23]**. Placental metabolome changes in relation to maternal obesity were only investigated in rats following different diets **[20]**.

The aim of this preliminary study is to examine key placental metabolites associated with maternal obesity. Obese patients were also evaluated according to gestational diabetes. Hydrophilic and lipophilic metabolites were studied through GC-MS (Gas Chromatography-Mass Spectrometry) platform, followed by multivariate statistic protocols.

METHODS

1.Population

The protocol of the study was approved by the Ethical Committee of the Sacco Hospital (Milan) and all women signed a written informed consent. Only singleton spontaneous pregnancies, with maternal age between 18 and 40 years and of Caucasian ethnicity were included in the study. Exclusion criteria were maternal preexisting diseases, fetal and maternal infections,

alcohol or drugs abuse, fetal malformations or chromosomal disorders, BMI< 18.5 or BMI between 25-30. Pregnant women were allocated into two different groups based on their pre-gestational BMI according to the Institute of Medicine (IOM) guidelines **[24]**:

- **Normal weight** (NW) (18.5 ≤BMI< 25 Kg/m^2), n= 20
- **Obese** (OB) (BMI≥ 30 Kg/m^2), n= 18

Obese patients were given specific nutritional advice and recommendations on weight gain in pregnancy. Eight obese women had a diagnosis of Gestational Diabetes Mellitus (GDM) [OB/GDM(+)] based on an Oral Glucose Tolerance Test (OGTT, 75 g), according to FIGO guidelines **[25]**. OB/GDM(+) were constantly checked for glycaemia and were given lifestyle and dietary indications for glycemic control. None of the studied women needed insulin therapy.

Maternal medical history, demographic, anthropometric, and obstetric data, as well as neonatal outcome data were recorded at recruitment and after delivery. Maternal gestational weight gain (GWG) was recorded.

2.Sample Collection

Placentas from elective caesarean section were measured recording placental weight, area and thickness as previously described **[5]**. Placental tissue was collected from a not-impaired part of the placental disc, after discarding the maternal decidua layer, washed in PBS, then cut into small pieces and immediately frozen in liquid nitrogen. The tissue was then transferred into a -80°C freezer. Samples were sent to the University of Cagliari to be analyzed by the GC-MS platform.

3.Sample Preparation

The extraction method was optimized from literature methods **[17-18, 26-27]**. A piece of placental tissue of about 100 mg was rapidly weighed, put in a glass mortar on ice with 1.9 mL of chloroform/methanol/water (1.4/1.4/1, 700/700/500 μl) and homogenized with a Potter-Elvehjem homogenizer for 2 minutes. The mixture was kept at 4°C for 15 min, then centrifuged at 14000 rpm for 10 min at 4°C. The upper (hydrophilic) and lower (lipophilic) phases were separated: the hydrophilic was dried in a vacuum concentrator (Eppendorf Concentrator Plus) overnight; the lipophilic in a glass vacuum desiccator under fume-hood for 2 h. The volume of extraction solution was normalized to 100 mg of tissue, with 1000 μL of the upper phase and

600 µL of the lower being dried down for 100 mg of tissue **[17]**. The dried fractions were stored at -80°C until analysis.

Hydrophilic Phase

30 µL of a solution of methoxylamine hydrochloride in pyridine (0.24 M) were added to each sample, then vortex mixed and left for 17 h at room temperature (RT). 30 µL of MSTFA were added and left for 1 h at RT. Just before GC-MS analysis, samples were diluted with a hexane solution (600 µL) of tetracosane (0.006 mg/mL) as internal standard, then analyzed using a Agilent 5977B interfaced to the GC 7890B 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 for 1 min, ramped by 10°C/min to 270°C with 7 min hold time. The sample (1 µL) was injected in split (1:10) mode. After a solvent delay of 3 min, mass spectra were acquired in full scan mode using 2.28 scans/s with a mass range of 50–700 Amu.

Lipophilic Phase

150 µL of chloroform:methanol (1:1) and 100 µL of 14% BF_3 in methanol were added to each vial, samples were vortex mixed and left for 90 min at 80ºC into an heating block. Once cooled, 600 µL hexane and 300 µL water were added, samples were vortex mixed and centrifuged for 2 min at 1400 rpm. The organic layer (upper) was transferred into glass vials and dried in a vacuum concentrator. Samples were then reconstituted with 400 µL hexane and injected to be analyzed with Agilent 5977B interfaced to the GC 7890B 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 initial column temperature was 60ºC for 2 minutes, ramped by 15ºC/min to 150ºC, and then by 4°C/min to 230ºC, hold for 20 min. The sample (1 µL) was injected in split (1:10) mode. After a solvent delay of 4 min, mass spectra were acquired in full scan mode using 2.28 scans/s with a mass range of 50–700 Amu.

4.Data Analysis

Each acquired chromatogram was analyzed with the free software AMDIS [http://chemdata.nist.gov/mass-spc/amdis] using an in-house made library comprising 222 metabolites. Some metabolites were identified using NIST08 and the GMD [http://gmd.mpimp-golm.mpg.de/] the metabolite was considered positively identified with a match factor ≥70%. For lower values the metabolite was labelled as ‘unknown’. The AMDIS analysis of the

hydrophilic phase analysis produced a matrix containing 78 metabolites: 55 accurately identified, 1 unknown compound matching an equally unknown of GMD group, and 22 unknown molecules recurring in every sample. The lipophilic phase produced a matrix containing 22 metabolites: 17 accurately identified compounds and 5 unknown molecules recurring in every sample. The obtained data matrices were successively subjected to statistical analysis.

5.Statistical Analysis

Sample size was adequate to assure the minimum precision requested for a pilot study **[28]**.

Clinical characteristics and data displayed a normal distribution (Kolmogorov-Smirnov Test), and were thus compared between groups with parametric statistics (t-test). Correction to t-test was applied when the equality of variances assumption was violated (Levene's test). Differences were considered statistically significant when $p \leq 0.05$.

Univariate analysis (ANOVA, ANalysis Of VAriance, and t-test) and multivariate models based on Partial Least Square-Discriminant Analysis (PLS-DA) were performed in MetaboAnalyst 3.0 [http://www.metaboanalyst.ca/] that allows to perform both analyses in the same session **[29-31]**. Raw data matrix was submitted to missing value estimation replacing all the missing values with the half of the minimum positive values in the original data, normalization by sum, Log transformation, and auto scaling and then used for both univariate and multivariate analyses. PLS-DA models were submitted to Cross Validation (CV) for the evaluation of statistical parameters (correlation coefficient- R^2, CV coefficient Q^2). This allowed to determine the optimal number of components for the model description. The permutation test was then applied to each model to investigate its predictive ability using the prediction accuracy test to set a permutation number (n=100 and $p < 0.01$).

RESULTS

Characteristics of the Population

Maternal and delivery characteristics of the two study groups are reported in **Table 1**. No significant differences were observed in fetal and placental data at delivery between OB and NW. Moreover we found no significant differences between OB/GDM(+) and OB/GDM(-) except for maternal basal glycaemia (81.9 ± 7.0 vs 98.0 ± 7.9 mg/dL respectively, $p \leq 0.01$) and placental weight (466.4 ± 65.7 vs 561.1 ± 67.5 g respectively, $p \leq 0.01$) [data not shown].

Hydrophilic Phase

ANALYSIS IN OBESE vs NORMAL WEIGHT

Univariate analysis (t-test) revealed a significantly increased concentration of the metabolite uracil (*p-value = 0.0005*, False Discovery Rate FDR= 0.034). Multivariate PLS-DA analysis was then performed: this model showed good statistical significance (accuracy= 0.77 R^2 = 0.79 Q^2 = 0.48. CV method: 10-fold CV, performance measure $p < 0.01$) [**Figure 1A**]. In obese placentas higher levels of nucleobases (uracil, hypoxanthine and a not clearly identified purine derivative), glucose-6-phosphate, 3-phoshoglycerate, glycerol, nicotinamide and the amino acids tyrosine, isoleucine, phenylalanine, leucine and serine were found. On the other side lower amounts of the amino acids lysine, taurine, aspartic acid and glutamine, along with the nucleosides inosine and guanosine, an inositol isomer and gluconic acid were detected [**Figure 1B**].

DIFFERENCES IN RELATION TO GDM

We then explored the possible role of GDM in the hydrophilic metabolites profile of obese pregnant women. Univariate data analysis (ANOVA with Tukey's HSD, Honest Significant Difference, post-hoc test) of the three classes OB/GDM(+), OB/GDM(-) and NW, gave no significant results. Multivariate data analysis (PLS-DA model) of the three classes OB/GDM(+), OB/GDM(-) and NW, gave accuracy= 0.61 R^2 = 0.83 Q^2 = 0.55. CV method: 10-fold CV, performance measure $p < 0.01$ [**Figure S1A and S1B**].

Lipophilic Phase

ANALYSIS IN OBESE vs NORMAL WEIGHT

Univariate analysis (t-test) revealed no significant differences in metabolite concentration. Multivariate PLS-DA analysis was then performed [**Figure 2A**]. This model showed good statistical significance: accuracy= 0.86 R^2 = 0.62 Q^2 = 0.33; [CV] method: 10-fold CV, performance measure $p < 0.01$. The most interesting metabolites were represented by palmitic acid, showing higher levels in obese subjects, and DHA (DocosaHexaenoic Acid), arachidonic and stearic acid, presenting lower levels [**Figure 2B**].

DIFFERENCES IN RELATION TO GDM

We then explored the possible role of GDM in the lipophilic metabolites profile of obese pregnant women.

Univariate data analysis (ANOVA with Tukey's HSD post-hoc test) of the three classes OB/GDM(+), OB/GDM(-) and NW, gave no significant result.

Similarly, multivariate data analysis (PLS-DA model) of the three classes OB/GDM(+), OB/GDM(-) and NW, gave no significant result ($p = 0.20$).

DISCUSSION

To our knowledge, this is the first study providing preliminary data on a broad range of metabolites in obese placentas delivered by elective caesarean section, thus avoiding molecular alterations due to labor. We applied metabolomics to investigate possible placental metabolic differences that can be relevant in two extreme maternal groups of the BMI scale: obese and normal weight. Of note, this study involved pregnant women with a well characterized clinical condition, undergoing regular prenatal checks in a dedicated clinic, and provided with nutritional and life style advice. This resulted in non-complicated maternal and fetal outcomes, and normal birthweights. Nonetheless, important differences were observed in the placental metabolites. These results may carefully be considered as potential markers for development of adult diseases.

Hydrophilic Phase

GC-MS analysis of the hydrophilic phase revealed altered amounts of several metabolites in obese placentas.

In our study we found significant changes in several amino acids, with OB showing increased levels of tyrosine, isoleucine, phenylalanine, leucine and serine and lower amounts of lysine, taurine, aspartic acid and glutamine. Amino acids are accumulated within the placenta by active transport systems located on the microvillus membrane **[36, 37]**. Taurine is an aminosulfonic metabolite with lower levels in obese or diabetic subjects **[38]**. Indeed, decreased taurine transporter activity has been previously reported in placental villous explants of obese pregnancies **[39]**. With the exception of taurine, maternal obesity has been associated with enhanced placental transporters, mTOR, and IGF-1 signaling pathways activity **[40]**. We have also previously reported higher umbilical amino acid concentrations in pregnancies with gestational diabetes **[41]**. However, in this study obese subjects showed higher values of tyrosine, isoleucine, phenylalanine, leucine and serine but decreased levels of lysine, aspartic

acid and glutamine. Disrupted placental metabolism may account for most of the above mentioned amino acids alterations in obese women. Specifically, we recently observed increased mitochondrial DNA content in placentas of obese women **[42]** potentially leading to decreased fetal oxygen availability and thus altered metabolism **[43-44]**. Moreover, changes in metabolic pathways between the placenta and the fetal liver for glutamine and glutamate have been proposed **[37]**, possibly due to fetal liver overgrowth **[40]**. Furthermore, similar alterations in amino acid levels were previously reported for hyperglycemic mothers **[45]**.

Many of the amino acid that we found altered in obese placentas, together with other impaired metabolites (such as glycerol, uracile, hypoxanthine, a purine derivative, nicotinamide, glucose-6-P, 3-phosphoglycerate, guanosine and inosine) are involved in metabolic pathways supporting nucleotide production, antioxidant defenses and lipid synthesis. Serine has a complex metabolism, being involved in several pathways. Among them, serine involvement in the folate cycle, can contribute to mitochondrial NADPH production **[46]**. Nicotinamide, an important component of NAD^+-NADH co-enzymatic factor, is involved in mitochondrial energy production and in several redox transformations **[47]**. Interestingly, impaired global gene profiling related to mitochondrial dysfunction and altered energy production was reported in obese placentas and maternal blood **[48-49]**. Glycerol is a key intermediate in lipid and energy metabolism through triglycerides, relevant for energy storage. Its increase may be related to enhanced placental fatty acid availability and uptake from the maternal circulation of obese women **[32]**.

Finally, inositol and gluconic acid lower amounts suggest a potentially reduced carbohydrate metabolism leading to enhanced insulin sensitivity **[50]**.

Lipophilic Phase

PLS-DA analysis showed interesting differences in placentas from obese women compared to controls. Interestingly, significantly lower levels of LC-PUFA (Long Chain-Polyunsaturated Fatty Acids) derivatives, arachidonic acid and DHA, were observed. The saturated palmitic acid was instead significantly increased. Changes in the maternal lipid profile have been reported in obesity, with increased triglycerides and decreased levels of high density lipoproteins **[32]**. Moreover, placental expression of fatty acid binding proteins is stimulated in obesity **[33]** together with higher lipid accumulation. Our results confirm and expand these observations indicating that obese women, independently of diabetes, show disruption of the physiologic LC-PUFA biomagnification, leading to decreased availability of arachidonic acid and DHA for

the fetus. This can increase the risk of adverse fetal outcomes and of developing a number of chronic diseases (e.g. metabolic and cardiovascular diseases) throughout postnatal life **[34-35]**.

Limitations

This study shows preliminary data in a limited population sample size.

As such, the aim of this work was to identify metabolic perturbations in our samples of OB and NW placentas. Thus, here we report preliminary results and no definitive conclusion can be drawn from our data. However, this study provides interesting driving information for future metabolic pathways analysis, with a larger sample size.

Obese pregnant women were carefully selected and matched to control pregnancies with similar characteristics except for BMI. For this reason, these results cannot be extended to the general obese population, since their favorable pregnancy outcomes were likely modulated by optimal prenatal care in these women.

This study applied the same analytical platform (GC-MS) to both hydrophilic and lipophilic phase. LC-MS instruments are the most used for lipid profiling studies, allowing for the detection of a very large number of metabolites, covering all lipid classes. Nevertheless, the easier identification of metabolites provided by GC-MS, allowed to get interesting information, although not exhaustive, for this preliminary study.

Another potential limitation is that, due to the small amount of tissue available, no pool sample was prepared.

Conclusions

Placental metabolome analysis of obese pregnancies suggested changes in metabolites' concentrations associated with obesity, specifically higher glycerol levels, with a similar trend for a number of fatty acids. Differences were also found for some amino acids and metabolites involved in nucleotide production, antioxidant defenses and lipid synthesis, suggesting a generalized shift towards higher placental metabolism.

This study can lay the foundation to further metabolomic placental characterization in the context of obesity. This can represent an additional missing link combining the two separated biological compartments of maternal blood and amniotic fluid, already investigated in other studies **[6,7,9,11].**

Moreover, future works based on these preliminary data will help identifying a specific placental phenotype of obese pregnancies, even in the setting of optimal prenatal care and

pregnancy outcomes. These data are not unexpected and lead to two major conclusions: **1)** the need to optimize maternal BMI before conception and **2)** the presence of specific metabolic signatures that may reflect underlying changes in the intrauterine metabolic environment.

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Author Contributions

C.M., G.M.A. and C.N. enrolled patients and performed sample collection and classification. C.F., A.N. and E.P.L. contributed to sample preparation, and GC-MS analysis. A.N. and L.B performed chemometric analysis on the collected data. C.F., F.P., A.N. and I.C. were responsible for the writing of the manuscript. R.P. contributed to the references section. V.F. and I.C provided a critical revision of the manuscript. V.F., V.S., A.D. and L.B. were the project supervisors.

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TABLE 1

		NW n=20	OB n=18
Maternal Data	Maternal Age [yrs]	33.7 ± 5.7	33.9 ± 5.2
	Maternal Pre-Pregnancy BMI [kg/m^2]	21.5 ± 1.6	36.4 ± 4.8 ***
	Maternal GWG [kg]	11.2 ± 3.6	8.8 ± 4.0
	Maternal Basal Glycemia [mg/dL]	78.6 ± 6.9	89.3 ± 11.0 *
Delivery Data	Gestational Age [wks]	39.1 ± 0.2	39.1 ± 0.3
	Fetal Weight [g]	3420.0 ± 401.1	3390.3 ± 461.6
	Placental Weight [g]	479.0 ± 80.8	508.6 ± 80.7
	Placental Efficiency	7.34 ± 1.28	6.76 ± 1.01
	Placental Area [cm^2]	287.0 ± 77.5	247.51 ± 59.1
	Placental Thickness [cm]	1.78 ± 0.56	2.19 ± 0.60

Table 1: Maternal and delivery data (fetal and placental parameters). Data are presented as average ± SD. Student's t-test: OB vs NW: *p≤ 0.05, ***p≤ 0.001.

OB group: OB/GDM(-) and OB/GDM(+) grouped together. BMI: Body Mass Index; GDM: Gestational Diabetes Mellitus; GWG: Gestational Weight Gain; Placental Efficiency: fetal/placental weight ratio.

FIGURES LEGEND

Figure 1: **A)** 2D scores plot showing PLS-DA discrimination between hydrophilic phases from placentas of OB vs NW and **B)** the corresponding VIP score plot.

Figure 2: A) 2D scores plot showing PLS-DA discrimination between lipophilic phases from placentas of OB vs NW and **B)** the corresponding VIP score plot.

SUPPLEMENTARY DATA FIGURES LEGEND

Figure S1: A) 2D scores plot showing PLS-DA discrimination between hydrophilic phases from placentas of NW, OB/GDM(-), OB/GDM(+): accuracy= 0.61 R^2 = 0.83 Q^2 = 0.55; cross validation [CV] method: 10-fold CV, performance measure: prediction accuracy during training $p<0.01$; **B)** the corresponding VIP score plot.

Figure 1
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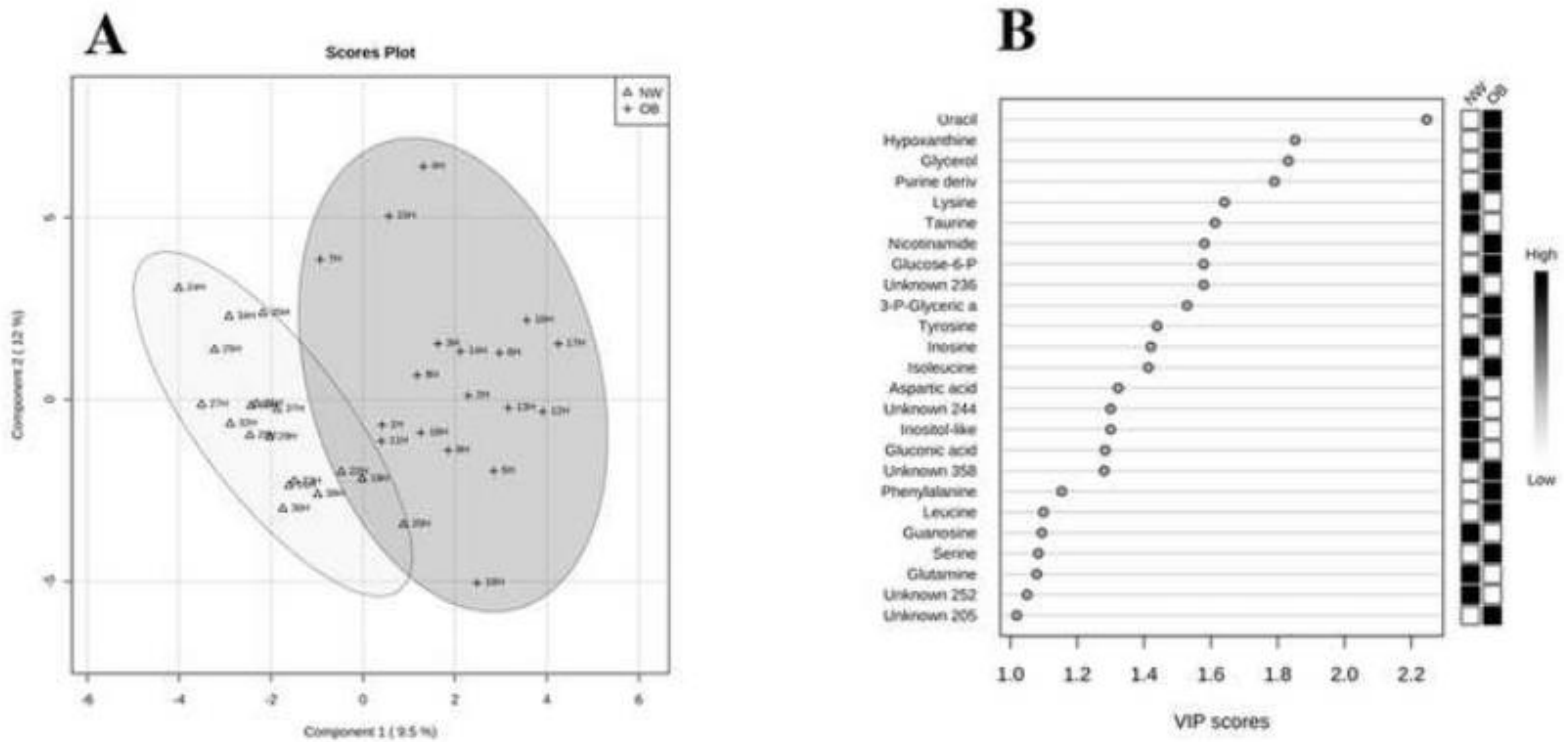

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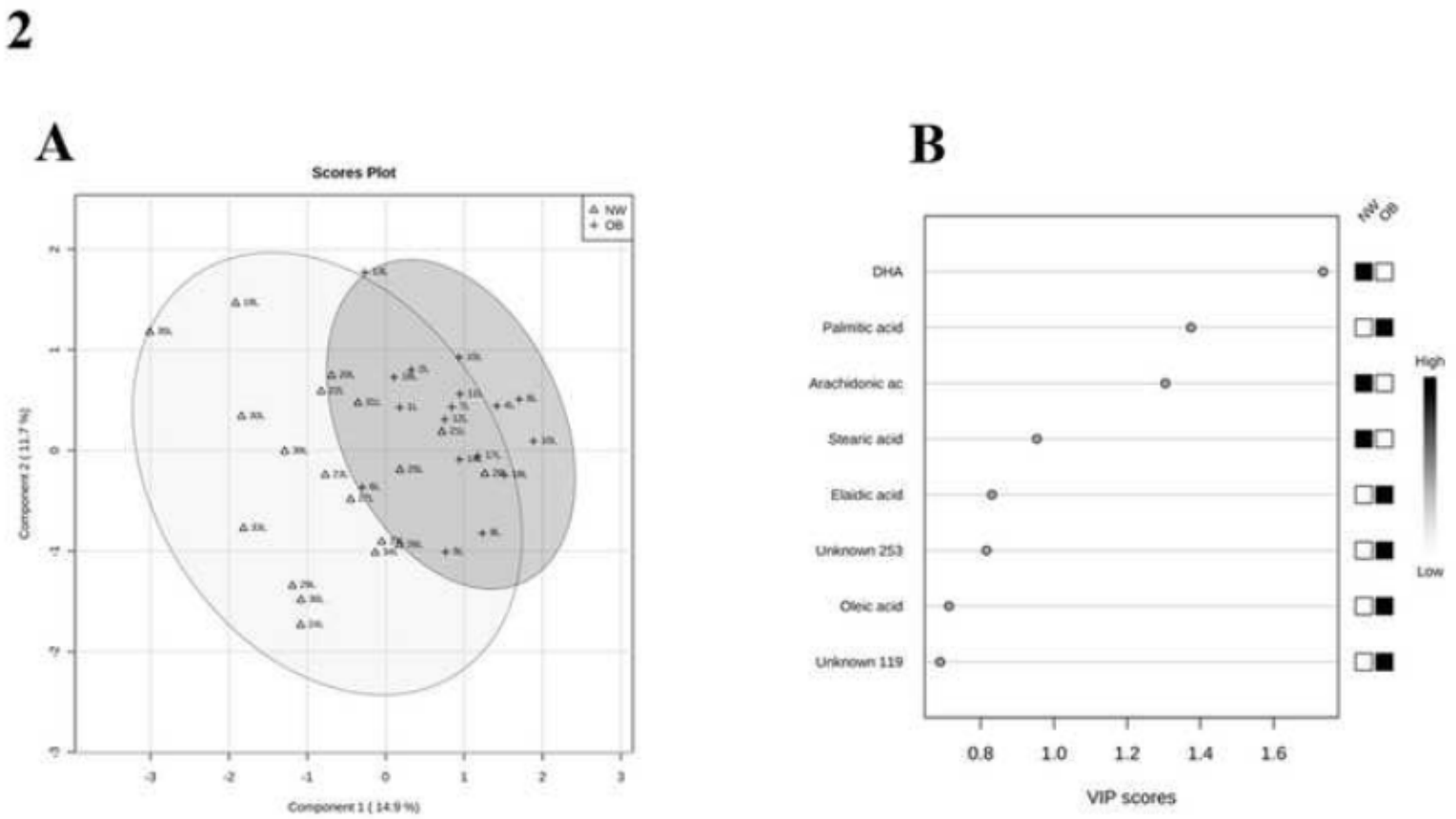

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