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Biomarkers of Morbid Obesity and Prediabetes by Metabolomic Profiling of Human Discordant Phenotypes

Sara Tulipani [a,b,*], Magali Palau-Rodriguez [a], Antonio Miñarro Alonso [c], Fernando Cardona [b,f], Anna Marco-Ramell [a], Bozo Zonja [d], Miren Lopez de Alda [d], Araceli Muñoz-Garach [b,f], Alejandro Sanchez-Pla [c,e], Francisco J Tinahones [b,f], Cristina Andres-Lacueva [a,*]

[a] Biomarkers & Nutrimetabolomic Lab, Nutrition & Food Science Dept, XaRTA, INSA, Campus Torribera, Pharmacy and Food Science Faculty, University of Barcelona, 08028, Spain
[b] Biomedical Research Institute [IBIMA], Service of Endocrinology and Nutrition, Malaga Hospital Complex [Virgen de la Victoria], Campus de Teatinos s/n, Malaga, Spain
[c] Statistics Department, Biology Faculty, University of Barcelona, 08028, Spain
[d] Water and Soil Quality Research Group, Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research [IDAEA-CSIC], Barcelona, Spain
[e] Statistics and Bioinformatics Unit Vall d'Hebron Institut de Recerca [VHIR], 08035, Spain
[f] CIBER Fisiopatología de la Obesidad y Nutrición [CIBERobn], Instituto de Salud Carlos III [ISCIII], Madrid, Spain

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ABSTRACT

Metabolomic studies aimed to dissect the connection between the development of type 2 diabetes and obesity are still scarce. In the present study, fasting serum from sixty-four adult individuals classified into four sex-matched groups by their BMI [non-obese versus morbid obese] and the increased risk of developing diabetes [prediabetic insulin resistant state versus non-prediabetic non-insulin resistant] was analyzed by LC- and FIA-ESI-MS/MS–driven metabolomic approaches.

Altered levels of [lyso]glycerophospholipids was the most specific metabolic trait associated to morbid obesity, particularly lysophosphatidylcholines acylated with margaric, oleic and linoleic acids [lysoPC C17:0: $R = -0.56$, $p = 0.0003$; lysoPC C18:1: $R = -0.61$, $p = 0.0001$; lysoPC C18:2 $R = -0.64$, $p < 0.0001$]. Several amino acids were biomarkers of risk of diabetes onset associated to obesity. For instance, glutamate significantly associated with fasting insulin [$R = 0.5$, $p = 0.0019$] and HOMA-IR [$R = 0.46$, $p = 0.0072$], while glycine showed negative associations [fasting insulin: $R = -0.51$, $p = 0.0017$; HOMA-IR: $R = -0.49$, $p = 0.0033$], and the branched chain amino acid valine associated to prediabetes and insulin resistance in a BMI-independent manner [fasting insulin: $R = 0.37$, $p = 0.0479$; HOMA-IR: $R = 0.37$, $p = 0.0468$]. Minority sphingolipids including specific [dihydro]ceramides and sphingomyelins also associated with the prediabetic insulin resistant state, hence deserving attention as potential targets for early diagnosis or therapeutic intervention.

1. Introduction

Metabolomics [1] is opening avenues to the discovery of biomarkers associated with insulin resistance and type 2 diabetes (T2D) [2–5]. Most of the human large-scale population-based studies carried out so far, however, mirrored the strong epidemiologic relationship between obesity and the impairment of glycemic control, and no emphasis was given to dissect the connection between obesity and diabetes or on the impact of the degree of adiposity in differentiating diabetic and nondiabetic individuals [6–10]. Hence, the identified metabolites of diabetes often coincide with obesity markers and not enable to corroborate the actual contribution of obesity in their predictive capacity.

Moreover, since the establishment of T2D generally occurs in a later phase of the natural history of obesity [11], the identification of biomarkers of early diabetes onset prior to its clinical diagnosis is crucial to define the first metabolic derangements associated with incipient glycemic control impairment, and ultimately promote prediction, early diagnosis and intervention of the disease at earlier stages [12].

Even so, evidence indicates that individuals' risk of developing diabetes may not uniformly depend on their body size [13,14]. Obese population subsets who maintain blood sugar control parameters within the normal range do exist, even at evolved stages of obesity (Body

Abbreviations: HbA1c, glycated hemoglobin; Cer, ceramide; CHOL, total cholesterol; DLDA, diagonal discriminant analysis; FG, fasting glucose; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, Homeostatic Model Assessment; LDA, linear discriminant analysis; LDL-C, low-density lipoprotein cholesterol; n.s., not significant; PC, phosphatidylcholine; PE, phosphatidyletanolammine; PLSDA, Partial least squares projection to latent structures-discriminant analysis; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; QDA, quadratic discriminant analysis; SCDA, nearest shrunken centroid classification; SD, standard deviation; SM, sphingomyelin.

* Corresponding authors at: Biomarkers & Nutrimetabolomic Lab., Nutrition and Food Science Department, XaRTA, INSA, Campus Torribera, Pharmacy and Food Science Faculty, University of Barcelona, Av. Joan XXIII s/n, 08028, Barcelona, Spain.
E-mail addresses: sara.tulipani@ub.edu (S. Tulipani), candres@ub.edu (C. Andres-Lacueva).

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Mass Index, BMI ≥ 40) [15], as well as T2D occur among adult lean individuals [16]. Although the clinical relevance of these subgroups remains debated [17], the study of discordant metabolic phenotypes for obesity and diabetes provides a unique and poorly unexploited opportunity to examine the interrelations between adipose tissue expansion and the gradual development of T2D and its sequelae [disease risk assessment]. However, the studies focused on them are still very scarce, small-scaled [18–20] or not focused on humans [21].

In the present study, we propose that the metabolic profiling of human concordant and discordant phenotypes for obesity and prediabetes/insulin resistance would define the metabolic alterations associated to adipose tissue expansion from those related to the incipient failure in the glucose homeostasis, and help to dissect the connection between the two diseases.

Univariate statistics was first applied to highlight any significant metabolic variation among the phenotypic groups in study. Age-adjusted regression analysis was used to assess the statistical significance of the relations of individual metabolites with the clinical traits of morbid obesity and prediabetes/insulin resistance, and the significant associations were visualized into organic metabolic networks. Finally, the diagnostic power of the most discriminant metabolites in correctly classifying the obese and prediabetic/insulin resistance phenotypes was evaluated.

2. Material and Methods

2.1. Subjects and Study Design

Sixty-four adult individuals (19 men and 45 women) were recruited at the Virgen de la Victoria University Hospital and Carlos Haya Hospital (Málaga, Spain). Overall exclusion criteria were acute or chronic infection, a history of cancer, a history of alcohol abuse or drug dependence, and all type of antidiabetic, corticosteroid, or antibiotic drug treatments. Other treatments including anti-inflammatory, antihypertensive and anti-cholesterolemic agents were recorded, but not restricted. The following measures were used for the clinical characterization of the subjects in study: a) anthropometric markers, measured by trained personnel using standardized techniques: body weight (kg), BMI (calculated as weight in kg/height^2 in m^2), waist circumference (cm), hip circumference (cm) and waist-hip index; b) markers of glucose regulation: plasma concentrations of fasting glucose (FG, mmol/L), fasting insulin (μU/mL), calculated Homeostatic Model Assessment (HOMA-IR index, arbitrary unit), glycated hemoglobin (HbA1c) concentration (%, mmol/mol), when available; c) blood pressure markers: diastolic and systolic blood pressure (mm Hg); d) blood lipid markers (mmol/L): total cholesterol, low-density lipoproteins and high-density lipoproteins cholesterol, and triglycerides.

The individuals were then classified into four sex-matched phenotypic groups according to their BMI (non-obese if: BMI = 18,5–26,9 kg/m^2; morbidly obese if: BMI > 40 kg/m^2) and to the risk of developing type two diabetes based on fasting plasma glucose concentrations and insulin resistance (non-prediabetic/non-insulin resistant state if: FG < 100 mg/dL and HOMA-IR < 2.5; prediabetic/insulin resistant state if: 100 ≤ FG < 126 mg/dL and HOMA-IR > 3.4).

The cut-off of HOMA-IR for identifying insulin resistant individuals was obtained experimentally by dividing the entire initial cohort into quartiles, and revealed to be higher than that generally accepted as the clinical definition of insulin resistance (>2.60), in line with previous reports [13]. The study protocol was approved by the local Ethics and Research Committees (Hospital Universitario Virgen de la Victoria, Málaga) and all participants provided written informed consent.

2.2. Serum metabolomic profiling

Fasting morning serum was stored at −80 °C until analysis. Metabolomic measurements were performed through two different platforms. A TSQ Vantage™ triple quadrupole mass spectrometer with ESI-II Ion Source (Thermo Scientific) equipped with a binary HPLC system was used for the in-house running of the AbsoluteIDQ p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria), through a standardized protocol as described by manufacturer. Data acquisition was carried out using liquid chromatography tandem mass spectrometry (LC-MS/MS, 5 μL injection volume, ESI+, Thermo Scientific Hypersil GOLD 3.0 μm 2.1 × 100 mm HPLC column), and flow injection analysis tandem mass spectrometry (FIA-MS/MS, 10 μL injection volume, ESI+ and ESI-) techniques. The remaining lipid metabolites were quantitatively analyzed via a high-throughput flow injection ESI-MS/MS screening method by Biocrates AG service (Innsbruck, Austria) through a validated protocol.

Serum samples were analyzed in a randomized batch format, to avoid run-order effects. Quality control samples including three reference plasma spiked with increasing concentrations of the targeted metabolites (QC1, QC2, QC3) and zero samples (10 mM phosphate buffer with internal standards) were analyzed every 20 injections, throughout the whole run, to control the stability and performance of the system and evaluate the quality of the acquired data. Quantifications were achieved by multiple reaction monitoring, by reference to multipoint calibration curves and/or in combination with the use of stable isotope-labelled and other internal standards, to compensate for matrix effects, as previously described [22]. Data evaluation and quantitative data analysis was performed with Met*IDQ*™ software (Biocrates Life Sciences AG) enabling isotopic correction and basic statistical analysis. Validated analytical methods were applied, in conformance with FDA Guidelines (U.S. Department of Health and Human Services 2001), as described by the manufacturer (UM-P180-THERMO-3).

2.3. Statistical analysis

Statistical analyses were performed in the R environment (R version 3.1.2). After excluding those metabolic measures below the limits of detection in >25% subjects in any of the phenotypic groups, and with high analytical variance in the QC2 replicates (CV > 25%), 246 successful metabolites remained for further analysis (Supplementary Table 1). Metabolite levels were log-transformed and Pareto scaled, missing values were imputed using nearest neighbor averaging (k = 10) and the potential effects of age and drug intake on the metabolomics data was removed by the application of a feature selector on each dependent variable, according to the Akaike Information Criterion [23].

Univariate statistics was first applied to highlight any significant variation among all the four phenotypic groups in study, and between the morbid obese and prediabetic/insulin resistance phenotypes (ANOVA and HSD Tukey contrasts for pairwise mean comparisons, $p = 0.05$, $q = 0.05$).

Age-adjusted regression analysis was used to assess the statistical significance of the relations of individual metabolites with the clinical traits of obesity (BMI) and prediabetes/insulin resistance (fasting glucose concentrations, HOMA-IR). The significant metabolite-metabolite and metabolite-clinical correlations were visualized into an organic metabolic network (Cytoscape 3.3.0), where nodes represent metabolites while edges configure any positive or negative significant relation among them. Significance (adjusted p-value <0.05) and correlation degree cut-offs were set (adjusted Spearman's partial correlation coefficients > |0.35|) similarly to previous studies [24].

Finally, we evaluated the capacity to correctly classify the subjects in their phenotypic groups by using their metabolic profiling, without the help of clinical predictors, and compared the diagnostic power of the metabolic profiling with that of the clinical measures available. To do that, the most robust metabolic markers were first selected by features selection techniques, so to generate a consensus list of successful metabolic classifiers, and their diagnostic power was evaluated by applying