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

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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.

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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

linear and non-linear classification techniques (Supplementary material).

3. Results

Clinical baseline characteristics of the study subjects are shown in Table 1. Female participants were prevalent, but no gender-dependent differences were detected among groups (Chi-squared test, $p = 0.324$). Table 2 summarizes the serum concentrations of the metabolites which significantly differed among the phenotypic groups. Although the current lack of established reference values for most of the metabolic species analyzed (i.e. lipid molecules), the concentration range (nM to μM) was in line with previous quantifications [25]. On the basis of their partial correlations, the measured metabolites allowed to depict a metabolic network (Fig. 1). Metabolites clearly clustered based on their biochemical classes and pathways membership, and phospholipids made the biggest cluster in the network, followed by amino acids and biogenic amines, ceramides and acylcarnitines sub-networks.

The associations of obesity and glycemic impairment with specific metabolites of the serum metabolic network are shown in Fig. 2. The strongest clinical-metabolite associations were observed between obesity markers and individual lyso- and glycerophospholipid species. More specifically, the levels of three lysophosphatidylcholines (lysoPC) showed very strong inverse relations with BMI (lysoPC C17:0: $R = -0.56$, $p = 0.0003$; lysoPC C18:1: $R = -0.61$, $p = 0.0001$; lysoPC C18:2 $R = -0.64$, $p < 0.0001$), as well as with body weight, waist and hip circumference. Similar but less significant correlations were also observed between obesity markers and serum phospholipids, especially diacyl- and alkyl acyl species with long-chain polyunsaturated fatty acids (PUFA).

The circulating levels of glutamate and glycine levels associated weakly with adiposity markers but strongly with insulin resistance, suggesting to be in the cross-talk between the two pathologies. Glutamate levels particularly showed positive associations with fasting insulin ($R = 0.5$, $p = 0.0019$) and HOMA-IR index ($R = 0.46$, $p = 0.0072$), while glycine concentrations negatively associated with the same parameters (fasting insulin: $R = -0.51$, $p = 0.0017$; HOMA-IR: $R = -0.49$, $p = 0.0033$) (Supplementary Fig. 1). A positive association between the levels of the branched-chain amino acid (BCAA) valine and the degree of insulin resistance was also observed (fasting insulin: $R = 0.37$, $p = 0.0479$; HOMA-IR: $R = 0.37$, $p = 0.0468$), independently from the BMI (Supplementary Fig. 1). Finally, the prediabetic and insulin resistant state confirmed modest but positive correlations with circulating nonpolar sphingolipids including several specific (dihydro)ceramides (increase of ceramide d18:1/C18:0 and dehydroceramides d18:0/C18:0 and d18:0/C22:0) and sphingomyelins (increase of sphingomyelin C18:0).

Metabolic versus clinical predictors. Both choline and ethanolamine-containing lysolipids acylated with margaric acid (C17:0) oleic acid (C18:1) and linoleic acid (C18:2) were the best classifiers for morbid obesity, together with diacyl and acyl alkyl phosphocholines with very long-chain fatty acids (Supplementary Fig. 2). The amino acid valine confirmed to be within the selective markers of prediabetes, together with sphingomyelins C18:0 and C18:1. In contrast, alterations in the circulating levels of the amino acid glycine and different ceramide species were selected as metabolic classifiers of both conditions (e.g. hydroxyceramide C17:0, dihydroceramides C20:0, C22:0 and 24:1). The robustness of the top-ranked metabolic markers in correctly classifying the individuals on the basis of the obese and prediabetic phenotypes was poor in respect to the use of clinical predictors (53 to 56% error in predicting classification), (Supplementary Table 2) reasonably due to the difficulty in clearly defining the metabolic profile of an incipient glycemic impairment. When considering obesity and prediabetes for separate, in turn, prediction capacity improved notably, especially for the morbid obesity phenotype Table 3.

4. Discussion

The use of organic metabolic networks based on age-adjusted regression analysis was helpful in identifying significant associations of individual metabolites with prediabetes or insulin resistance and morbid obesity.

4.1. Early metabolic markers associated to increased risk of diabetes development

4.1.1. Variation in the amino acid profile

Although the objective difficulty in defining the metabolic signature of an incipient glycemic impairment, compared to the characterization of an evolved state of obesity, altered levels of specific amino acids were detected in prediabetic patients, compared to non-prediabetic individuals, so to be proposed as suitable early predictors of increased risk for diabetes.

Glutamate and glycine were the most significantly altered amino acids associated to the prediabetic phenotype (i.e. rise of glutamate *versus* progressive decline of glycine compared with the matched control group), followed by the BCAA valine. Their circulating levels also associated with adiposity markers [namely BMI, body weight and waist

Table 1
Basal anthropometric and clinical characteristics of the study population according to phenotype membership.

Phenotype										
		Non-obese non-prediabetic [4 M; 15F]		Morbidly obese non-prediabetic [2 M; 10F]		Non-obese prediabetic [4 M; 8F]		Morbidly obese prediabetic [9 M; 12F]	ANOVA*	Tukey Contrasts* Obese vs Non-obese	Tukey Contrasts* Prediabetic vs Non-prediabetic
Age [years]	19	47 ± 15	12	43.67 ± 11.30	12	53.67 ± 14.13	21	43.14 ± 8.91	n.s.†	n.s.	n.s.
Weight [kg]	19	64.79 ± 8.90	12	125.77 ± 15.28	12	65.33 ± 6.58	21	147.04 ± 30.41	< 0.0001	< 0.0001	0.011
BMI [kg/m^2]	19	24.13 ± 1.82	12	45.78 ± 4.67	12	24.87 ± 1.75	21	52.67 ± 10.20	< 0.0001	< 0.0001	0.011
Waist circumference [cm]	19	82.37 ± 8.81	12	125.09 ± 12.82	12	90.58 ± 7.97	17	138.82 ± 14.96	< 0.0001	< 0.0001	0.007
Hip circumference [cm]	19	93.84 ± 9.97	12	139.54 ± 15.56	12	99 ± 5.29	16	146.56 ± 15.56	< 0.0001	< 0.0001	0.046
Fasting glucose [mmol/L]	19	90.42 ± 7.79	12	89.75 ± 5.58	12	111.33 ± 11.15	21	113.95 ± 12.62	< 0.0001	n.s.	<0.00001
Insulin [μU/mL]	19	5.47 ± 2.27	12	7.92 ± 2.36	12	14.87 ± 7.29	21	23.89 ± 8.15	< 0.0001	<0.001	<0.00001
HOMA IR	19	1.22 ± 0.52	12	1.76 ± 0.55	12	4.02 ± 1.82	21	6.77 ± 2.58	< 0.0001	<0.001	<0.00001
Systolic pressure [mm Hg]	18	114.06 ± 14.65	12	141.62 ± 18.11	12	126.25 ± 20.25	15	133.6 ± 16.79	0.026	0.022	n.s.
Diastolic pressure [mm Hg]	18	68.83 ± 11.15	12	88.12 ± 9.37	12	78.33 ± 11.31	15	81 ± 8.25	0.01	0.018	0.046
CHOL [mmol/L]	19	177.63 ± 23.76	12	191.5 ± 46.38	12	232.58 ± 39.81	21	198.90 ± 35.74	0.002	n.s.	0.01
C-HDL [mmol/L]	19	56.89 ± 10.42	12	52.75 ± 15.52	12	52.08 ± 17.59	20	41.5 ± 10.50	0.009	0.018	0.011
C-LDL [mmol/L]	19	103.29 ± 23.21	12	98.04 ± 51.85	12	148.53 ± 41.17	19	128.58 ± 29.84	0.002	n.s.	0.001
TAG [mmol/L]	19	80.68 ± 36.46	12	115.25 ± 107.87	12	190.75 ± 106.09	21	149.14 ± 44.65	0.002	n.s.	0.001

Data are presented as mean values and standard desviation.*, adj. p values; † n.s., not significant; CHOL, total cholesterol; LDL-C, low-density lipoproteins cholesterol; HDL-C, high-density lipoproteins cholesterol; TAG, triglycerides.

Table 2
List of serum concentrations and statistical significance of discriminant metabolites among the four phenotipic groups.

	Phenotype										
	n	Non-obese non-prediabetic [4 M; 15F]	n	Morbidly obese non-prediabetic [2 M; 10F]	n	Non-obese prediabetic [4 M; 8F]	n	Morbidly obese prediabetic [9 M; 12F]	ANOVA*	Tukey Contrasts* Obese vs Non-obese	PreT2D vs Non-preT2D
Amino acids [μM]											
Glutamate	17	41.62 ± 17.77	12	56.60 ± 20.73	11	57.78 ± 23.53	18	112.44 ± 77.59	0.0012	0.0038	0.0252
Glycine	17	272.86 ± 70.78	12	202.30 ± 47.16	11	223.31 ± 74.47	18	179.69 ± 30.24	0.0007	< 0.001	0.0429
(Lyso)Phosphatidylcholines [μM]											
lysoPC a C16:0	19	67.88 ± 12.19	12	61.32 ± 17.53	12	85.10 ± 18.34	21	65.39 ± 15.11	0.016	0.0309	n.s.†
lysoPC a C17:0	19	1.16 ± 0.33	12	0.80 ± 0.24	12	1.27 ± 0.25	21	0.83 ± 0.35	0.0007	< 0.0001	n.s.
lysoPC a C18:0	19	18.52 ± 3.52	12	16.58 ± 5.20	12	25.54 ± 5.82	21	18.03 ± 6.02	0.0114	0.0288	n.s.
lysoPC a C18:1	19	15.72 ± 4.01	12	11.36 ± 3.46	12	17.97 ± 4.85	21	10.19 ± 2.54	< 0.0001	< 0.0001	n.s.
lysoPC a C18:2	19	22.77 ± 8.66	12	14.01 ± 4.95	12	23.52 ± 5.03	21	13.16 ± 3.39	< 0.0001	< 0.0001	n.s.
lysoPE a 18:1	18	337.51 ± 128.53	12	265.56 ± 107.40	12	423.20 ± 208.09	20	219.33 ± 55.46	0.0054	< 0.001	n.s.
lysoPE a 18:2	18	443.08 ± 178.20	12	304.74 ± 105.08	12	511.49 ± 221.46	20	300.64 ± 109.29	0.0071	< 0.001	n.s.
lysoPE a 18:0	18	288.05 ± 71.73	12	255.41 ± 107.43	12	330.61 ± 110.56	20	247.44 ± 79.79	n.s.	0.041	n.s.
lysoPE e 18:0	18	9.17 ± 3.68	12	6.40 ± 3.11	12	8.71 ± 3.27	20	5.82 ± 1.79	0.0204	0.0023	n.s.
PC aa 38:6	19	83.39 ± 27.46	12	72.65 ± 26.20	12	96.91 ± 27.08		71.41 ± 23.34	n.s.	0.0494	n.s.
PC ae 34:0	19	1.00 ± 0.24	12	0.78 ± 0.22	12	0.93 ± 0.24	21	0.83 ± 0.25	n.s.	0.0288	n.s.
PC ae C34:1	19	8.07 ± 2.10	12	6.56 ± 1.77	12	7.39 ± 0.98	21	6.39 ± 1.44	n.s.	0.0093	n.s.
PC ae C34:2	19	10.35 ± 2.29	12	7.60 ± 2.38	12	9.81 ± 2.03	21	7.03 ± 2.10	0.0012	< 0.0001	n.s.
PC ae C34:3	19	7.09 ± 2.14	12	5.10 ± 1.51	12	6.34 ± 1.73	21	4.47 ± 1.50	0.0023	< 0.001	n.s.
PC ae C36:2	19	12.35 ± 3.13	12	9.27 ± 2.43	12	12.36 ± 1.67	21	9.11 ± 2.79	0.0044	< 0.001	n.s.
PC ae C36:3	19	8.64 ± 2.11	12	6.36 ± 1.92	12	8.33 ± 1.58	21	5.90 ± 2.00	0.0022	< 0.001	n.s.
PC ae C38:0	19	2.01 ± 0.70	12	1.56 ± 0.40	12	2.50 ± 0.75	21	2.06 ± 0.77	0.0451	n.s.	n.s.
PC ae C38:5	19	19.77 ± 4.48	12	17.07 ± 3.47	12	21.04 ± 4.92	21	16.27 ± 5.37	0.0465	0.0085	n.s.
PC ae C38:6	19	7.97 ± 2.37	12	6.42 ± 1.49	12	8.77 ± 2.03	21	6.30 ± 2.28	0.0162	0.0024	n.s.
PC ae C40:1	19	1.03 ± 0.23	12	0.78 ± 0.23	12	1.19 ± 0.29	21	0.98 ± 0.42	n.s.	0.0309	n.s.
PC ae C40:6	19	4.74 ± 1.46	12	3.46 ± 0.89	12	4.27 ± 0.74	21	3.50 ± 0.91	0.008	< 0.001	n.s.
Phosphatidylethanolamines [nM]											
PE aa 28:5	18	11.90 ± 5.11	12	9.01 ± 7.87	12	8.83 ± 3.31	20	7.28 ± 4.65	0.0465	0.0145	n.s.
PE aa 36:0	18	329.95 ± 158.80	12	261.52 ± 77.35	12	368.27 ± 114.42	20	232.36 ± 78.19	0.0381	0.0103	n.s.
PE aa 38:0	18	546.13 ± 269.07	12	405.42 ± 124.93	12	620.00 ± 215.05	20	336.56 ± 135.35	0.0071	0.0017	n.s.
PE aa 38:1	18	252.75 ± 83.19	12	205.02 ± 48.42	12	289.73 ± 82.17	20	187.67 ± 59.46	0.0127	0.0028	n.s.
PE aa 40:2	18	21.74 ± 10.09	12	15.72 ± 4.49	12	20.17 ± 5.58	20	15.19 ± 3.53	0.0211	0.0024	n.s.
PE aa 40:3	18	30.72 ± 14.56	12	23.73 ± 7.31	12	29.67 ± 9.45	20	19.75 ± 5.58	0.016	0.0029	n.s.
PE ae 34:1	18	126.47 ± 53.58	12	115.97 ± 51.90	12	170.08 ± 66.98	20	96.90 ± 23.06	0.0399	0.0308	n.s.
PE ae 34:2	18	113.92 ± 46.85	12	95.58 ± 36.47	12	154.24 ± 64.21	20	78.59 ± 26.42	0.016	0.0085	n.s.
PE ae 34:3	18	104.75 ± 41.50	12	79.98 ± 42.04	12	118.15 ± 37.73	20	69.85 ± 30.22	0.0414	0.0072	n.s.
PE ae 36:2	18	219.01 ± 88.96	12	198.69 ± 79.07	12	274.53 ± 93.71	20	166.83 ± 54.78	0.0451	0.0221	n.s.
PE ae 36:3	18	346.62 ± 138.18	12	274.69 ± 115.13	12	429.47 ± 160.44	20	228.20 ± 86.26	0.0127	0.0028	n.s.
PE ae 38:2	18	51.07 ± 15.75	12	43.69 ± 11.75	12	53.50 ± 13.86	20	40.81 ± 9.23	n.s.	0.0145	n.s.
PE ae 38:3	18	65.84 ± 25.81	12	53.83 ± 15.53	12	72.72 ± 22.32	20	48.70 ± 15.39	0.0465	0.0107	n.s.
PE ae 38:6	18	873.95 ± 354.25	12	761.72 ± 248.20	12	1186.31 ± 447.20	20	649.16 ± 211.28	0.0118	0.0106	n.s.
PE ae 40:3	18	37.03 ± 10.79	12	29.06 ± 8.03	12	34.52 ± 8.40	20	27.54 ± 7.58	n.s.	0.0083	n.s.
PE ae 40:5	18	200.20 ± 83.81	12	187.99 ± 57.58	12	244.56 ± 81.13	20	160.49 ± 48.36	n.s.	0.0499	n.s.
PE ae 40:6	18	533.53 ± 231.67	12	457.86 ± 138.96	12	631.93 ± 204.40	20	388.93 ± 116.89	0.0204	0.0085	n.s.
PS aa 38:4	18	31.09 ± 12.47	12	55.59 ± 39.85	12	31.64 ± 21.90	20	46.06 ± 27.46	n.s.	0.0221	n.s.
Sphingolipids [nM]											
N_C11_1_Cer	18	0.34 ± 0.24	12	0.62 ± 0.49	12	0.30 ± 0.13	20	0.54 ± 0.47	n.s.	0.0137	n.s.
N_C17_0_[OH] Cer	18	5.84 ± 2.45	12	8.24 ± 5.79	12	7.18 ± 3.40	20	11.34 ± 6.69	0.0399	0.0145	n.s.
N_C18_0_Cer	18	62.36 ± 24.54	12	74.98 ± 30.35	12	94.19 ± 35.43	20	88.77 ± 27.86	0.0414	n.s.	0.0429
N_C18_0_Cer2H	18	14.60 ± 6.49	12	26.86 ± 16.02	12	20.82 ± 7.07	20	34.69 ± 17.71	0.0007	< 0.001	0.0429
N_C18_1_Cer	18	7.33 ± 2.75	12	6.16 ± 3.56	12	7.02 ± 1.62	20	4.74 ± 1.38	0.0212	0.001	n.s.
N_C20_0_[OH] Cer	18	10.09 ± 6.99	12	18.87 ± 12.94	12	10.19 ± 5.21	20	17.94 ± 7.87	0.0089	< 0.001	n.s.
N_C20_0_Cer2H	18	13.53 ± 6.68	12	18.87 ± 7.50	12	16.48 ± 4.96	20	22.64 ± 7.53	0.0129	0.0079	n.s.
N_C22_0_Cer2H	18	68.75 ± 34.65	12	91.21 ± 30.16	12	89.99 ± 25.39	20	119.95 ± 36.37	0.0044	0.0072	0.0429
N_C23_0_Cer2H	18	43.33 ± 20.76	12	62.95 ± 21.30	12	60.38 ± 19.33	20	67.41 ± 21.05	0.0257	0.0308	n.s.
N_C24_0_Cer2H	18	95.08 ± 50.35	12	130.02 ± 49.12	12	119.51 ± 40.81	20	152.96 ± 55.50	0.0393	0.0202	n.s.
N_C24_1_Cer2H	18	47.95 ± 18.73	12	72.87 ± 27.17	12	62.41 ± 14.81	20	83.10 ± 35.28	0.0199	0.0106	n.s.
N_C25_0_Cer	18	118.96 ± 42.34	12	104.53 ± 33.21	12	129.36 ± 39.86	20	90.02 ± 28.75	n.s.	0.0284	n.s.
N_C26_0_Cer	18	21.98 ± 5.69	12	17.81 ± 5.85	12	20.18 ± 5.43	20	16.52 ± 5.16	n.s.	0.0141	n.s.
SM C18:0	19	23.54 ± 4.85	12	30.39 ± 9.63	12	35.38 ± 9.67	21	35.38 ± 12.14	0.007	n.s.	0.0252

Data are presented as mean values and standard desviation.*, adj. p values; † n.s., not significant. PC, phosphatidylcholine; PE, phosphatidyletanolammine; PS, phosphatidylserine; Cer, ceramide; SM, sphingomyelins.

circumference], but in a modest extent. In morbidly obese subjects, for instance, a 2-fold increase in the serum levels of glutamate was particularly observed, compared to non-prediabetic obese controls, suggesting alterations in the glutamate metabolism as a selective metabolic marker of an early onset of diabetes in subjects with high BMI. By its conversion to α-ketoglutarate, a precursor of glutamine, higher concentrations of glutamate might provide an alternative energy source to either glucose via glycolysis or fatty acids via β-oxidation [26], thus possibly playing a compensatory role against glucose and lipid metabolism impairment. Hence reciprocal associations of glutamine and glutamate circulating levels with glycemic impairment might reflect the role of glutamate as a substrate of the tricarboxylic acid cycle. In line with these speculations,

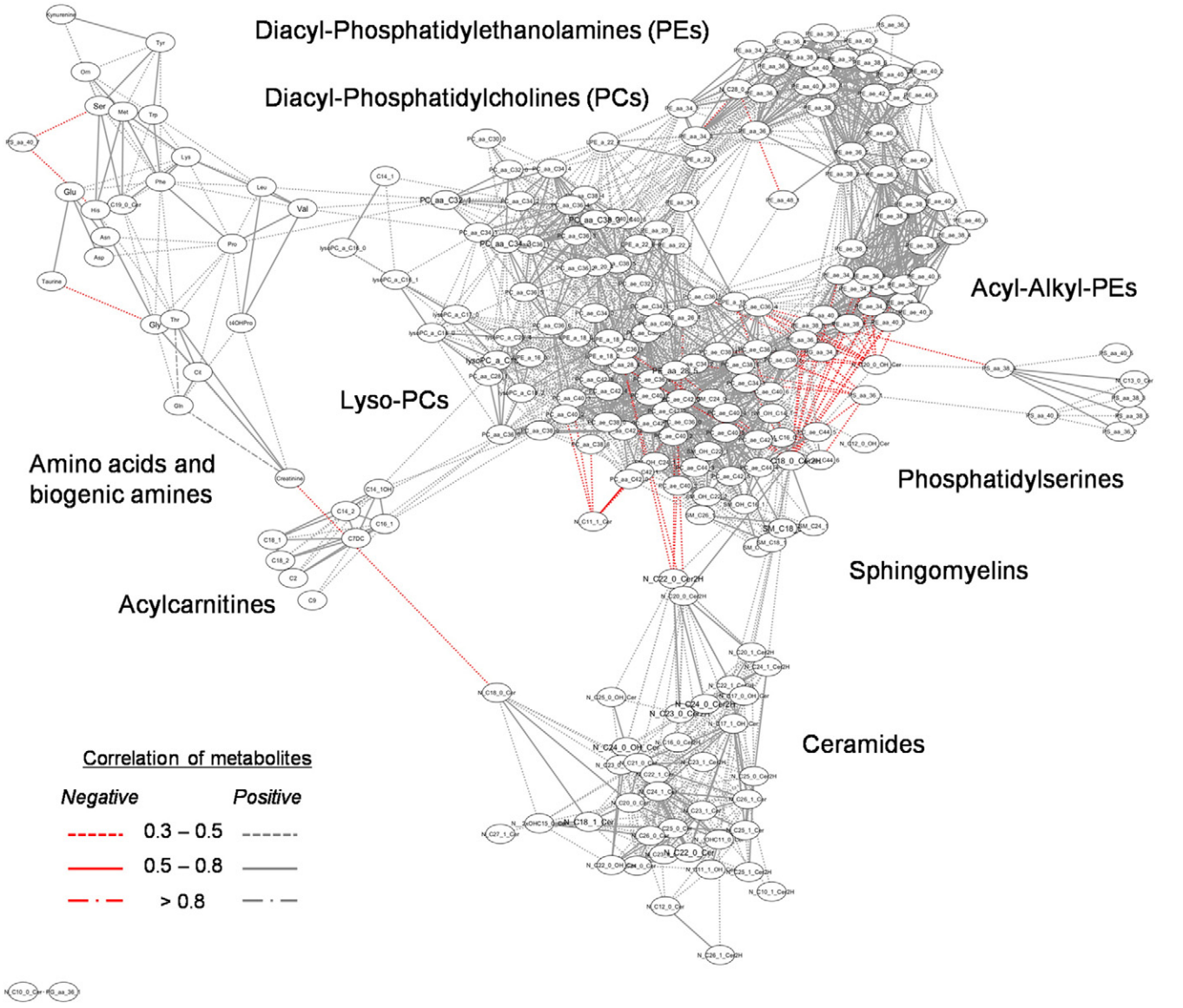

Fig. 1. Serum metabolic network representing the significant correlation (edges) between metabolites (nodes). Adjusted for the other metabolites. Black line represents positive correlation while red line negative correlation. The line format (dotted, solid) indicates the degree of correlation.

in our study glutamine levels decreased progressively across the morbid obese, prediabetic and morbid prediabetic/obese individuals, although differences did not reached the statistical significance.

A strong correlation between insulin resistance and the fasting glutamate has been described in large population-based studies [27], and decreased levels of glycine have been proposed as an early predictor of incident dysglicaemia and insulin resistance in high-risk nondiabetic subjects in follow-up studies [8,9]. Although any causative relations between altered levels of glutamate or glycine and metabolic impairment have been proved so far [28], the circulating concentrations of both metabolites have been shown to drastically reverse to the normal concentration range after gastric bypass surgery or behavioural weight loss and to predict the concomitant improvement of glycemic control [29,30], thus reinforcing the possible mechanistic relation with the beneficial metabolic adaptations associated to weight loss.

It is noteworthy that a low-grade inflammatory state is considered as one of the fundamental mechanisms in the progression of obesity-related diseases [31]. Interestingly, inflammation has been also proposed as an intriguing intersection between the metabolism of the amino acids significantly altered in our study and the development of prediabetes. For instance, in vivo studies have suggested that glycine might suppress the production of pro-inflammatory cytokines (i.e. TNF-α and IL-6), increase adiponectin secretion through the activation of PPAR-γ, and prevent insulin resistance and associated inflammatory diseases [32]. The effects of inflammatory cytokines on glutamate metabolism are also under investigation. In the scenario, the progressive alteration of glutamate and glycine levels from the lean to the 'healthy' morbid obese up to the morbid prediabetic obese phenotype, observed in our study, may confirm a link between the metabolism of these amino acids and a lower inflammatory state.

Finally, in our study the association of BCAA valine with insulin resistance was BMI-independent, and do not confirm a primary association between altered BCAA levels and obesity. The implication of an impaired BCAA metabolism in the development and interconnection of obesity and diabetes is currently a prominent topic of discussion [33]. In line with our findings, elevated blood concentrations of BCAA and their derivatives has been observed as an early manifestation of insulin resistance and diabetes [reviewed in [34]]. A significant correlation between plasma valine concentration and HOMA index has been also demonstrated in subjects spanning normal glucose tolerance, impaired

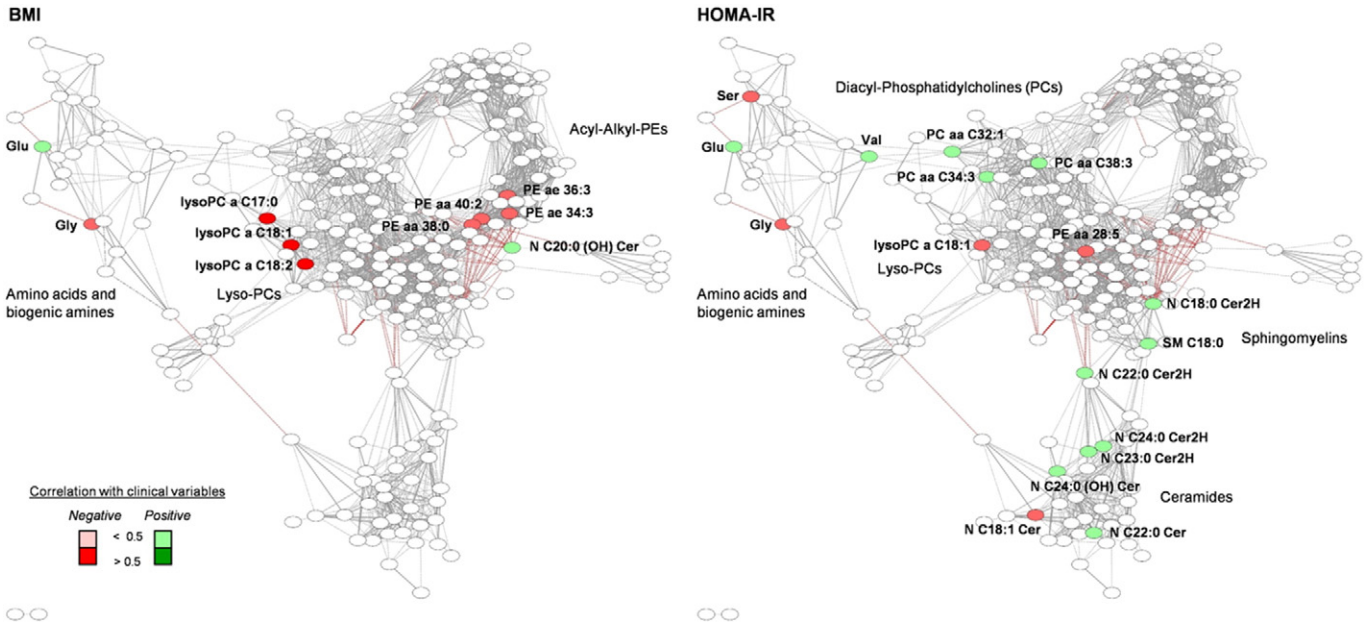

Fig. 2. Association between BMI (A) and glycemic status (B) and individual metabolites within the serum metabolic network of the study cohort. Green color indicates positive correlation while red negative correlation; color intensity indicates the degree of correlation.

glucose tolerance, and diabetes [35], and similar results were obtained adjusting plasma BCAA levels for BMI [2,36] or waist circumference [37]. However, several experimental studies also suggest that increased circulating BCAA would specifically mirror obesity-dependent diabetic states, possibly related to altered adipose tissue BCAA catabolism [18, 38–40]. Although attempts to reconcile these disparate perspectives have been already proposed [41], more investigations are required to reach a definitive overview.

4.1.2. Increase of circulating sphingolipids

A substantive literature has accumulated implicating sphingolipids, especially enhanced ceramide generation, as mediators of diabetes and insulin resistance progression [42–44]. Besides confirming ceramides as an attractive therapeutic target for obesity-associated insulin resistance, our study specifically focused the attention on individual sphingolipid species significantly associated with the prediabetic phenotype, including sphingomyelin species with saturated acyl chains [i.e. sphingomyelin C18:0], ceramide d18:1/C18:0 and dihydroceramides d18:0/C18:0 and d18:0/C22:0. These last observations particularly sustain the concept that dihydroceramides are not merely inert precursors of ceramides, and would confirm a link between the accumulation of dihydroceramides and the changes in the dihydroceramide/ceramide ratio with the impairment of adipose tissue expansion and adipocyte function, through the alteration of membrane-associated processes [45]. Our findings would be

Table 3
Diagnostic power of clinical versus metabolic measures in classifying the subjects according to their BMI and/or prediabetic state.

	Prediction of Obesity						**Prediction of Prediabetes**					
	Clinical classifiers			**Metabolic classifiers**			**Clinical classifiers**			**Metabolic classifiers**		
	misclass.	**brier score**	**P [mean]†**	**misclass.**	**brier score**	**P [mean]**	**misclass.**	**brier score**	**P [mean]**	**misclass.**	**brier score**	**P [mean]**
	[all subjects, obese [n = 33] versus non-obese [n = 31]]						**[all subjects, prediabetic [*n* = 33] versus non-prediabetic [*n* = 31]]**					
DLDA	0.02	0.02	0.98	**0.22**	0.41	0.78	0.08	0.15	0.91	**0.39**	0.73	0.61
LDA	0.01	0.02	0.98	**0.20**	0.30	0.76	0.04	0.07	0.96	**0.40**	0.57	0.58
QDA	0.03	0.05	0.97	**0.26**	0.38	0.72	0.04	0.07	0.96	**0.40**	0.62	0.56
PLSDA	0.02	0.03	0.96	**0.17**	0.28	0.82	0.07	0.12	0.92	**0.42**	0.65	0.57
SCDA	0.02	0.04	0.94	**0.20**	0.37	0.79	0.09	0.15	0.89	**0.39**	0.67	0.59
	[healthy only, obese [n = 12] versus lean [n = 19]]						**[lean only, pre-T2D [*n* = 12] versus healthy [*n* = 19]]**					
DLDA	0.00	0.00	1.00	**0.21**	0.41	0.79	0.06	0.09	0.95	**0.23**	0.44	0.77
LDA	0.01	0.01	0.99	**0.37**	0.65	0.62	0.08	0.15	0.92	**0.35**	0.61	0.64
QDA	0.03	0.05	0.97	**0.37**	0.63	0.63	0.09	0.18	0.90	**0.42**	0.71	0.58
PLSDA	0.04	0.06	0.96	**0.19**	0.34	0.77	0.08	0.13	0.92	**0.30**	0.48	0.66
SCDA	0.00	0.01	0.98	**0.23**	0.41	0.76	0.05	0.07	0.94	**0.26**	0.46	0.73
	[pre-T2D only, obese [n = 21] versus lean [*n* = 12]]						**[obese only, pre-T2D [*n* = 21] versus healthy [n = 12]]**					
DLDA	0.03	0.04	0.98	**0.22**	0.43	0.78	0.06	0.12	0.94	**0.50**	0.96	0.50
LDA	0.05	0.07	0.94	**0.19**	0.31	0.79	0.06	0.10	0.94	**0.52**	0.87	0.48
QDA	0.09	0.17	0.91	**0.20**	0.35	0.79	0.06	0.11	0.94	**0.48**	0.84	0.51
PLSDA	0.06	0.07	0.94	**0.23**	0.35	0.73	0.10	0.19	0.87	**0.50**	0.84	0.49
SCDA	0.03	0.04	0.95	**0.22**	0.42	0.77	0.06	0.12	0.91	**0.41**	0.57	0.52

DLDA,diagonal discriminant analysis; LDA, linear discriminant analysis; QDA, quadratic discriminant analysis; PLSDA, Partial least squares projection to latent structures-discriminant analysis; SCDA, nearest shrunken centroid classification. †The classification performance was determined by common performance metrics including the misclassification rate [indicating the % of error in predicting classification], proper scoring rules [i.e. the Brier Score measuring the accuracy of probabilistic predictions [MSE loss]], and the average probability of correct classificatio [P].

also in line with an increased expression of the CerS1, the most abundant (dihydro)ceramide synthase isoform in skeletal muscle and specifically involved in the synthesis of C18:0 ceramides [44], recently described in mice fed a high-fat diet and associated with alterations in ceramide levels and glucose tolerance [46].

4.2. Morbid obese markers

4.2.1. Drop of glycerophospholipids

Recent large-scale metabolomic studies indicated several choline-containing [lyso]lipids, including lysoPC C18:2, as potential biomarkers of diabetes [7], and lysoPC C18:2 and glycine were confirmed to be predictive markers of diabetes in a second large-scale population-based (KORA) cohort [9]. In these works, however, no emphasis was given to the different degree of adiposity observed between diabetic and non-diabetic individuals (i.e. cases of diabetes often having higher BMI and waist circumference compared to the non-cases), thus not enabling to corroborate the actual contribution of obesity in the predictivity of these metabolic markers. In contrast, in our study, a significant drop of lyso- and glycerophospholipids clearly characterized the morbidly obese phenotype, independently from the glycemic state of the individuals. This would suggest that alterations of the (lyso)lipid metabolism would associate with adipose tissue expansion but not play a pivotal early role in the early onset on glycemic impairment, as also recently suggested [47]. The levels of three lysolipids, namely lysophosphocholines acylated with margaric acid (lysoPC C17:0) oleic acid (lysoPC C18:1) and linoleic acid (lysoPC C18:2), were particularly reduced in morbid obesity. These metabolic intermediates are enzymatically produced during the de−/re-acylation cycles that control the overall lipid species composition, and are considered a readout of β-oxidation. Despite their relatively short half-life, circulating lysoPC C18:1 and C18:2 have been previously described as independent correlates of glucose intolerance and insulin resistance in nondiabetic subjects, besides as putative lipid-signalling molecules [8,48].

In addition to lysolipids, in our study as in previous research, the vast majority of the diacyl glycerophospholipids which markedly decreased in serum of morbidly obese individuals were plasmalogens, namely phospholipids in which one of the two carbon atoms on glycerol is bonded to an alkyl chain via an ether linkage, as opposed to the usual ester linkage. In the compresence of severe obesity and impaired glycemic control, plasmalogens concentrations dropped even more (Table 2). On overall, significant plasmalogens consisted in long-chain and very long-chain PUFA-containing phosphatidylcholines and phosphatidylethanolamines, thus probably mirroring enhanced fatty acid desaturation and elongation activities. A correlation between desaturase enzyme activities and obesity has been also found in several cases [49] and partly explained as a mechanism for modulating packing and degree of order in the membrane phospholipid bilayer. Lipidomic studies on twins discordant for body size (lean vs obese) recently suggested that individuals in the early stage of obesity had increased proportions of very long-chain PUFA-containing phospholipids in their adipose tissue (despite their lower dietary intake of PUFA compared to the lean twins) and a proportional diminishment of phospholipids containing shorter and more saturated fatty acids, regulated by Elovl6 [49]. With adipose cell expansion, more phospholipids have to be incorporated into the cellular membranes. Increasing PUFA content, decreasing plasmalogen concentration and using choline instead of ethanolamine-containing headgroup are known compensatory mechanisms of cell membranes to maintain fluidity, permeability to small molecules at the price, however, of increasing their vulnerability to inflammation. Although focused on the blood compartment and apparently conflicting, our data are consistent with the findings recently obtained at the adipose tissue level, since a down-regulation of plasmalogens in serum of obese twins was previously documented [50]. Certainly, an in-depth analysis of the adipose tissue membrane composition at different stages of obesity and metabolic impairment will be highly hoped to verify the hypothesis. Furthermore, it should be verified whether the circulating glycerophospholipid pool may mirror accumulation and structural functioning in adipose tissue.

5. Conclusions

Our targeted metabolomics approach gave a granular metabolic footprint of morbid obesity and prediabetes/insulin resistance. The alteration in the (lyso)phospholipid metabolism was the most specific trait associated to morbid obesity, particularly mirrored by the circulating levels of lysoPC C17:0, C18:1 and C18:2. Results also indicate glutamate and glycine as biomarkers of early diabetes onset associated to obesity, while the association of valine with glycemic impairment was BMI-independent, hence a primary association between altered branched-chain amino acids levels and obesity was not confirmed. In addition, minority sphingolipids including specific (dihydro)ceramides and sphingomyelins also associated with the prediabetic state, hence deserving attention as potential targets for early diagnosis or therapeutic intervention.

The degree of redundancy in the fatty acyl composition observed across the altered lipid species should deserve attention in future studies (e.g. acylation with non-essential C18:0, C18:1, and essential C18:2n-6 fatty acids was the most common alteration associated to morbid obesity) since suggesting a specific association between their dysfunctional metabolism and the extreme adipose tissue expansion. So far, the mechanistic explanation is not so intuitive.

Certainly, the interpretation of our data needs to be assessed within the context of the limitations of the present work. For instance, it is well recognized that insulin resistance develops on a continuum, thus the use of cutting points of fasting glucose and insulin sensitivity to differentiate phenotypes at high versus low insulin sensitivity could be questionable. As well as, the spectrum of insulin sensitivity in the study cohort was not based on load testing such as the hyperinsulinemic euglycemic clamp and oral glucose tolerance test. Nevertheless, for this reason we experimentally calculated the HOMA-IR cut-off for identifying insulin resistant individuals, and set it at a higher value than usually accepted. Since the lack of significance among phenotypic categories should be interpreted in the context of sample size/statistical power, future research will require larger studies to confirm the predictively of the detected biomarkers in the case of subclinical glycemic impairment in apparently insulin sensitive and glucose tolerant obese subjects. Finally, the authors support large-scale studies to replicate and validate the results, as well as future studies focused on the study of pathways involved.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2016.10.005.

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