UNIVERSITAT DE BARCELONA

Aplicación de la Metabolómica para el estudio de la elevada resistencia a la insulina, dependiente e independiente de la obesidad

Estudio de fenotipos discordantes y efectos metabólicos de la pérdida de peso

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FACULTAD DE FARMACIA Y CIENCIAS DE LA ALIMENTACION

DEPARTAMENTO DE NUTRICION, CIENCIAS DE LA ALIMENTACION Y GASTRONOMIA

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ABREVIACIONES

1,5-AG	1,5-anhydroglucitol
3PG	3-phosphoglycerate
AACE	Asociación Americana de Endocrinólogos Clínicos
ADA	Asociación Americana de Diabetes (del inglés, *American Diabetes Association*)
ADIOL-DS	*Androstenediol disulfate*
AHEAD	*Action for Health in Diabetes*
ALANDINO	Alimentación, Actividad Física, Desarrollo Infantil y Obesidad
AMP	*Adenosine 5'-monophosphate*
ANOVA	Análsisi de la varianza (del inglés, *Analysis of Variance*)
AUC	Área Bajo la Curva (del inglés, *area under the curve*)
BCAA	Amino ácidos de cadena ramificada (del inglés, *Branched chain amino acids*)
CFCA	Cuestionario de frecuencia de consumo de alimentos
CHOL	Colesterol
COSI	*Childhood Obesity Surveillance Initiative*
DBP	Presión diastólica (del inglés, *diastolic blood pressure*)
DG	Diglicéridos (del inglés, *diglycerids*)
DietMed	Dieta Mediterránea
DS	Cruce duodenal
DT2	Diabetes tipo 2
EGIR	Grupo Europeo para el Estudio de la Resistencia a la Insulina
ESI	Ionización en electroesprai (del inglés, *electrospray ionization*)
FDR	*False discovery rate*
FM	Masa grasa (del inglés, *fat mass*)
FXR	*Farnesoid X receptor*
GCA-S	*Glycocholenate sulfate*
GGT	Gamma glutamil transpeptidasa
GIP	Glucosa-dependiente *insulinotropic polypeptid*
GLP-1	*Glucagon-like peptide 1*
GM	Gastrectomía en manga
GOT	Glutamato-piruvato transaminasa
GPC o PC	Fosfatidilcolina
GPE o PE	Fosfatidiletanolamina
GPI o PI	Fosfatidilinositol
GPS o PS	Fosfatidilserina

HbA1c	Hemoglobina glicosilada (del inglés, *glycated haemoglobin A1*)
HCA	Análisis de clustering jerárquico (del inglés, *hierarchical clustering analysis*)
HDL o c-HDL	Lipoproteina de elevada densidad (del inglés, *high-density lipoprotein cholesterol*)
HILIC	*Hydrophylic interaction liquid cromatography*
Hip	*Hip circumference*
HMDB	*Human Metabolome Database*
HOMA-IR	*Insulin resistance calculated by homeostatic model assessment*
HPLA	3-(4-hidroxifenil)lactato
HWL	*High weight loss group*
IDF	Federación Internacional de Diabetes
IMC	Índice de Masa Corporal
IR	Resistencia a la Insulina
KNN	K N*earest Neighbours*
LAGB	Bandas gástricas ajustables de laparoscopia
LASSO	*Least absolute shrinkage and selection operator*
LC	Cromatografía líquida (del inglés, *liquid chromatography*)
LDL o c-LDL	Lipoproteina de baja densidad (del inglés, *low-density lipoprotein cholesterol*)
LG	*Loss Group*
LM	Masa magra (del inglés, *loss mass*)
LOD	Límite de detección (del inglés, *limit of detection*)
LOQ	Límite de cuantificación (del inglés, *limit of quantification*)
LWL	*Low weight loss group*
MCA	Análsisi múltiple de correspondencia (del inglés, *multiple correspondence analysis*)
MedDiet	Mediterranenan Diet
MFA	Análisis de factores múltiples (del inglés, *multiple factor analysis*)
MHO	Obesidad metabólicamente sana (del inglés, m*etabolically healthy obesity*)
MRM	Reacción múltiple monitorizada (del inglés, *multiple reaction monitoring*)
MS/MS	Espectrometría de masas en tandem (del inglés, *tandem mass spectrometry*)
NAA	N-acetilaspartato
NCEP:ATPIII	Programa Nacional de Educación en Colesterol: Tercer Panel de Expertos
OEA	Oleoyl ethanolamide
OECD	Organización para la Cooperación y el Desarrollo Económicos
OGTT	Test de la tolerancia de la glucosa oral (del inglés, *oral glucose tolerance test*)

OMS	*Organización Mundial de la Salud*
OSC	Correción Ortogonal (del inglés, *Orthogonal Signal Correction*)
PA	Presión Arterial
PC	Componente principal (del inglés, *principal component*)
PCA	Análisis de componentes principales (del inglés *principal component analysis*)
PEA	Etanolamide (del inglés, *palmitoyl ethanolamide*)
PLA2	Fosfolipasa (del inglés, p*hospholipases A2*)
PLS-DA	Análisis Discriminante por Mínimos Cuadrados Parciales (del inglés, *Partial Least Squares Discriminant Analysis*)
PREDIMED	Prevención con Dieta Mediterránea
QC	Control de calidad (del inglés, *quality control*)
RAPA	*Rapid Assessment of Physical Activity questionnaire*
rdCV	Doble validación cruzada repetida (del inglés, r*epeated double cross validation*)
RF	Análisis de bosques aleatorios (del inglés, *Random Forest*)
ROC	*Receiver Operating Characteristic*
RP	Fase reversa (del inglés, *reverse phase*)
RSD	Desviación estándar relativa (del inglés, *relative standard desviation*)
RT	Tiempo de retención (del inglés, *retention time*)
RYGB	*Bypass* gástrico en Y de Roux
SBP	Presión sistólica (del inglés, *systolic blood pressure*)
SM	Esfingomielina (del inglés, *sphingomyelin*)
SMet	Síndrome metabólico
SOS	*Swedish Obese Subjects*
SPE	Extracción de fase sólida (del inglés, *solid-phase extraction*)
sPLS	*Sparse Partial Least Squares*
TCA	Cicle tricarboxílico (del inglés, t*ricarboxylic acid cycl*)
TG	Triglicéridos (del inglés, *triglycerides*)
TGR5	*G protein-coupled bile acid receptor 1*
UPLC	*Ultra-performance liquid chromatography*
USDA	*U.S. Department of Agriculture*
VAT	Tejido adiposo visceral (del inglés, *visceral adipose tissue*)
VIP	Proyección de la variable (del inglés, *variable importance for projection*)
Waist	Circumferéncia de la cintura (del inglés, *waist circumference*)
LPLATs	Lisofosfolípido aciltransferasa
GC	Cromatografía de gases (del inglés, *gas chromatography*)

RESUMEN

La obesidad es la 'epidemia' del siglo XXI según la Organización Mundial de la Salud. La obesidad es un desafío de salud debido a su relación epidemiológica con el aumento de riesgo de desarrollar enfermedades metabólicas, como la diabetes tipo 2 (DT2) o el síndrome metabólico. Sin embargo, los mecanismos implicados en la relación entre obesidad y DT2 no están completamente identificados.

El objetivo principal de esta tesis doctoral consiste en la identificación de marcadores metabolómicos de prediabetes/resistencia a insulina, asociados o no a la presencia de obesidad en edad adulta. Este objetivo se ha abordado desde dos perspectivas complementarias: i) utilizando un modelo de fenotipos concordantes/discordantes de obesidad y prediabetes/resistencia a la insulina y ii) estudiando los efectos metabólicos asociados a la pérdida de peso.

En la primera, se realizaron tres estudios metabolómicos observacionales transversales, dos en muestras suero y uno en tejido adiposo visceral (VAT). Los individuos se clasificaron según su Indice de Masa Corporal (IMC), sin obesidad (IMC = 18.5 -26.9 kg/m^2) o con obesidad mórbida (IMC > 40 kg/m^2) y según el riesgo de desarrollar DT2, individuos con baja resistencia a la insulina (HOMA-IR <2.5 y glucosa en ayunas <100 mg/dL) o elevada resistencia a la insulina (glucosa en ayunas de 100-125 mg/dL o HOMA-IR >3.4).

En la segunda perspectiva, se realizó dos estudios metabolómicos prospectivos, en muestras de suero antes y 1, 3 y 6 meses después de la cirugía bariátrica Los sujetos presentaban obesidad mórbida (IMC > 40 kg/m^2), con o sin presencia de factores de riesgo de síndrome metabólico. También se realizó un estudio metabolómico de intervención, en muestras de plasma, antes y después de 12 meses de una intervención con cambios en el estilo de vida, dieta y ejercicio. Los participantes incluidos en este estudio eran adultos con obesidad (IMC=30-40 kg/m^2) y sin síndrome metabólico.

Los tres estudios del modelo de fenotipos concordantes/discordantes revelaron alteraciones en el metabolismo de los lípidos y de los amino ácidos en los pacientes con obesidad y elevada resistencia a la insulina. Estos cambios reflejan parte de los cambios en la composición y la funcionalidad del tejido adiposo visceral en el desarrollo de la obesidad. Con el fin de caracterizar un biomarcador de obesidad con sensibilidad a la insulina, sensible y específico en VAT, se creó un modelo multimetabolito utilizando el glicerofosfatidilinositol (18:0), el glicerofosfatidiletanolamina (18:2), el glicerofosfatidilserina (18:0), el glicerofosfatidilcolina (18:0/18:1), (18:2/18:2) y (18:2/18:3). En suero se creó un biomarcador utilizando 15 diacilglicéridos, el ácido adrénico y el ácido úrico que permitió diferenciar aquellos sujetos con elevada resistencia a la insulina entre toda la población.

Los estudios sobre los efectos metabólicos de la pérdida de peso permitieron observar una modulación del perfil de los amino ácidos y lípidos (diacilglicéridos, acilcarnitinas, ácidos grasos y fosfolípidos) que se asociaba a mejoras metabólicas, con un acercamiento al cuadro metabólico de los sujetos delgados. Productos de la microbiota intestinal del metabolismo de los amino ácidos y de los ácidos biliares mostraron tener un papel clave en los mecanismos implicados a la resistencia a la insulina. Aunque la magnitud de los cambios después de la cirugía bariátrica han sido superiores, al de una intervención con cambios en el estilo de vida a largo plazo, ambas estrategias de pérdida de peso comparten perfiles metabólicos relacionados al metabolismo de los lípidos y a la degradación de los amino ácidos por la microbiota intestinal.

Los efectos de la cirugía bariátrica y los cambios en el estilo de vida, combinando dieta y actividad física, sobre la salud metabólica abren nuevas perspectivas y nuevas hipótesis en el entendimiento de la obesidad y sus enfermedades asociadas. Las especies lipídicas, especialmente el estudio del grado de redundancia en la composición de las cadenas de ácidos grasos de los diacilglicéridos, fosfolípidos y esfingolípidos, así como, el estudio de la degradación de los amino ácidos por la microbiota intestinal podrían abrir nuevas vías de investigación en el campo de la obesidad.

ABSTRACT

Obesity is the 'epidemic' of the twenty-first century, according to the World Health Organization. Obesity is a challenge to health due to its epidemiologic relationship with an increased risk of developing metabolic diseases, such as type 2 diabetes (T2D) and metabolic syndrome. However, the mechanisms involved in the connection between obesity and T2D have not been fully identified.

The main objective of this doctoral thesis consists in the identification of metabolomic markers of prediabetes/insulin resistance associated or not with the presence of obesity in adulthood. This objective has been tackled from two complementary perspectives: i) using a model of concordant/discordant phenotypes of obesity and prediabetes/insulin resistance; and ii) studying the metabolic effects associated with strategies of weight loss.

In the first perspective, three metabolomics observational transversal studies were carried out, two on serum samples and one on visceral adipose tissue (VAT). Subjects were classified according to their body mass index (BMI), either as non-obese (BMI=18.5–26.9 kg/m^2) or obese (BMI>40 kg/m^2), and according to their risk of developing T2D, with low insulin resistance (fasting glucose<100 mg/dL and HOMA-IR<2.5) or high insulin resistance (fasting glucose between 100 and 125 mg/dL or HOMA-IR>3.4).

In the second perspective, two prospective metabolomics studies were carried out on serum samples before and 1, 3 and 6 months after bariatric surgery. Subjects presented morbid obesity (BMI>40 kg/m^2) with or without the presence of other metabolic syndrome risk factors. In addition, a metabolomic analysis was carried out in an interventional study on samples of plasma before and after 12 months of lifestyle intervention, based on a hypocaloric Mediterranean diet (MedDiet) and physical activity. The participants included in this study were adults with obesity (BMI=30–40 kg/m2) and without metabolic syndrome.

The three studies of the concordant/discordant phenotype model revealed alterations in the lipid and amino acid metabolism in patients with obesity and high insulin resistance. These changes revealed alterations in the composition and functionality of VAT. With the aim of identifying characterized, sensible and specific VAT biomarkers of obesity and insulin sensitivity, a multi-metabolite model was built with glycerophosphatidylinositol (18:0), glycerophosphatidylethanolamine (18:2), glycerophosphatidylserine (18:0), and glycerophosphatidylcholine (18:0/18:1), (18:2/18:2) and (18:2/18:3). In serum, a biomarker was built with 15 diglycerides, adrenic acid and uric acid, enabling those subjects with high insulin resistance among the entire population to be distinguished.

The studies on the metabolic effects of weight loss allowed a modulation of the profile of amino acids and lipids (diglycerides, acylcaritines, fatty acids and phospholipids) associated with metabolic improvement. The microbial metabolism of amino acids and bile acids may be the key points of metabolic rearrangement after surgery. Although the magnitude of changes after bariatric surgery was greater than that of an intervention with changes in long-term lifestyle, both weight loss strategies share metabolic profiles related to lipid metabolism and degradation of amino acids by the intestinal microbiota.

The effects of bariatric surgery and changes in lifestyle, combining diet and physical activity, on metabolic health open new perspectives and new hypotheses in the understanding of obesity and its associated diseases. Lipid species, and particularly the study of the degree of redundancy in the composition of fatty acids of diglycerides, phospholipids and sphingolipids, as well as the study of the degradation of amino acids through gut microbiota, could open new pathways for research.

1. ANTECEDENTES BIBLIOGRÁFICOS

1.1 La problemática de la obesidad y la diabetes a nivel mundial

Según la Organización Mundial de la Salud (OMS):

La obesidad es una enfermedad no transmisible y crónica, definida como una acumulación anormal o excesiva de grasa que puede perjudicar la salud de la persona. A nivel general la obesidad es diagnosticada con el índice de masa corporal (IMC), que tiene en cuenta el peso y la altura de la persona. En adultos el sobrepeso se define cuando el IMC es superior o igual a 25 y obesidad cuando es superior o igual a 30.

También se han definido subclases de obesidad según la severidad. Se considera obesidad de clase I cuando el IMC está entre 30-34.9, clase II u obesidad severa cuando el IMC es superior o igual a 35, obesidad mórbida cuando el IMC es superior o igual a 40.

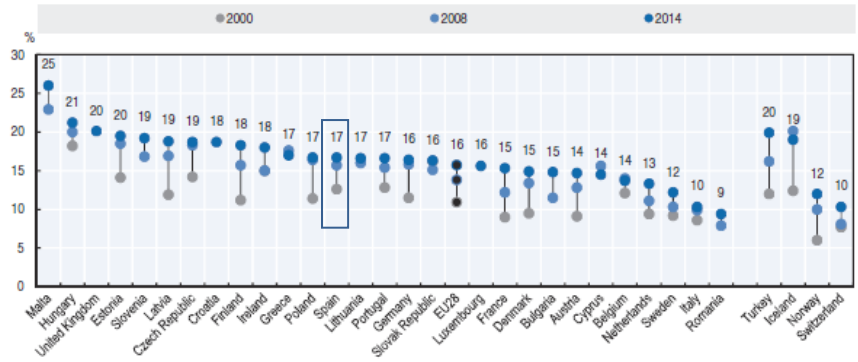
1.1.1 La obesidad en cifras

En todo el mundo, 650 millones de adultos, 340 millones de adolescentes y 41 millones de niños (<5 años de edad) presentan obesidad (IMC ≥ 30), según la Organización Mundial de la Salud (OMS)[1]. La obesidad es la epidemia mundial del siglo XXI, la llamada *globesidad*.

España es el segundo país europeo con más tasa de sobrepeso (IMC ≥ 25). En 2016, el 74% de los hombres españoles adultos y el 61% de las mujeres españolas adultas presentaban sobrepeso[2]. Del mismo modelo, la tasa de obesidad española también estaba por encima de la mediana europea, con un 17% en adultos (IMC ≥ 30)[3] (**Figura 1**) y se poryecta que para el 2030 las tasas de obesidad aumentarán.

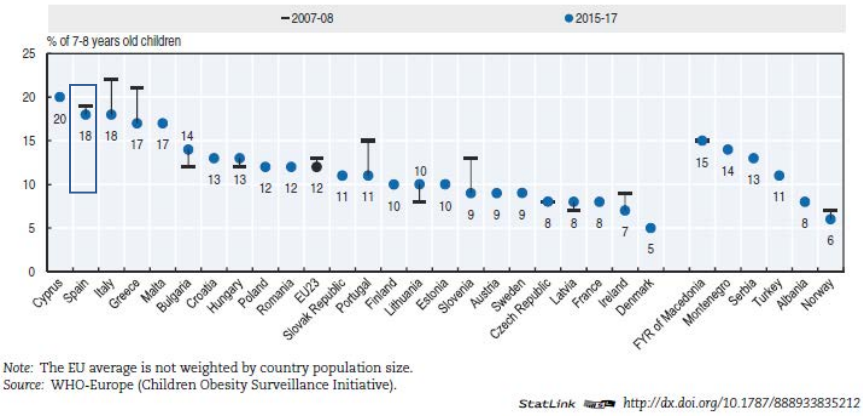
La prevalencia del sobrepeso y de la obesidad en niños en Europa ha aumentado a ritmo alarmante. Según el estudio *COSI*, del inglés *Childhood Obesity Surveillance Initiative*, España se sitúa entre los primeros países europeos con obesidad en niños y adolescentes[3] (**Figura 2**). Así, el estudio *Alandino* y la Sociedad Española de la Obesidad, determinaron que el 18% de los niños españoles de entre 6 y 9 años presentaba obesidad en 2011[4].

Figura 1. Obesidad en adultos, 2000-2014



Obesidad autodiagnosticada. Fuente: Eurostat (EHIS 2008 and 2014) complemented with OECD Health Statistics 2018 for 2000 data and data for non-EU countries, *https://doi.org/10.1787/health-data-en*. Extraída de OECD/EU (2018), Health at a Glance: Europe 2018: State of Health in the EU Cycle[3]

Figura 2. Obesidad en niños entre 7-8 años de edad, 2007-2008 y 2015- 2017



Fuente: extraído de OECD/EU (2018), Health at a Glance: Europe 2018: State of Health in the EU Cycle[3]

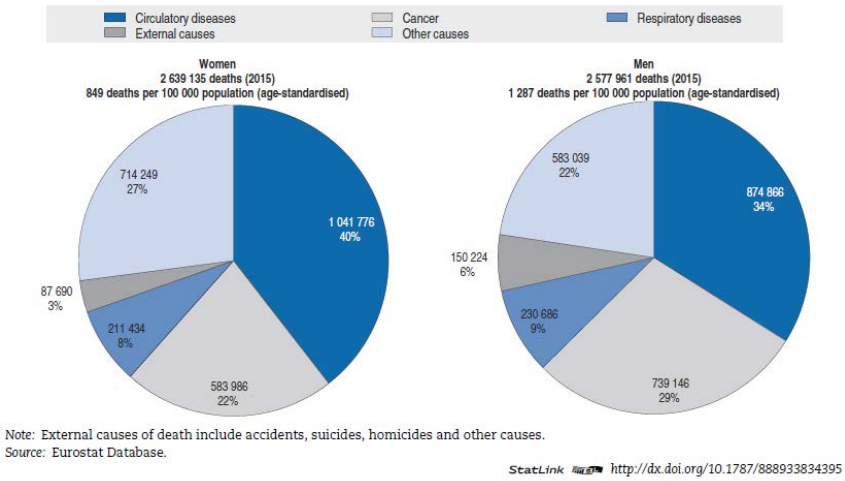
El estudio ALANDINO (Alimentación, Actividad Física, Desarrollo Infantil y Obesidad) forma parte del estudio COSI, del inglés Childhood Obesity Surveillance Initiative, que agrupa 23 países europeos para el estudio de la obesidad. ALANDINO estudió la prevalencia del sobrepeso y la obesidad en niños españoles (6-9 años), entre octubre de 2010 y mayo de 2011, y examinó los diferentes criterios de corte en su cálculo (Pérez-Farinós et al. 2013).

La obesidad representa un gasto económico directo e indirecto para la sanidad pública, ligado al absentismo laboral, a la baja productividad y a la utilización de los

recursos sanitarios y tratamientos asociados[5]. Se estima que la obesidad es responsable del 2-8% de los costes sanitarios en Europa[6].

Las cifras son preocupantes, pero la obesidad va más allá de los números. La obesidad plantea una enorme carga para la salud y el bienestar de las personas. Diversos estudios ya han evidenciado que la obesidad en la niñez está asociada con una mayor probabilidad de muerte prematura y de incapacidad, atribuyéndosele un papel importante en el aumento de riesgo de desarrollar distintos tipos de cánceres y enfermedades metabólicas[7,8]. Según la OMS, la obesidad y el sobrepeso son el principal factor de riesgo de enfermedades cardiovasculares, principal causa de muerte en personas mayores (>65 años) en Europa (**Figura 3**).

Figura 3. Principales causas de muerte en Europa, 2015



Fuente: extraído de OECD/EU (2018), Health at a Glance: Europe 2018: State of Health in the EU Cycle[3]

1.1.2 Factores de riesgo del sobrepeso y la obesidad

La obesidad es una enfermedad multifactorial que va más allá de un desequilibrio entre las calorías consumidas y las calorías gastadas. Se suman factores socioeconómico y sociocultural que crean un entorno *obesogénico*, entre los que destacan los patrones alimentarios, el consumo de tabaco, el sedentarismo y el consumo de alcohol (**Tabla 1**). También existen factores heredables y no

modificables como los factores genéticos, la historia familiar y la raza/etnia, que condicionan la prevención o el desarrollo de sobrepeso u obesidad.

Tabla 1. Factores de riesgo del sobrepeso y la obesidad

Individuales	Socioeconómicos	Ambientales
- Ingesta energética por encima de las necesidades energéticas - Elección de alimentos ricos en calorías y pobres en nutrientes - Actividad física baja - Sedentarismo - Falta o exceso de sueño - Factores genéticos - Ciertas enfermedades - Condiciones psicológicas (ej. depresión, estrés) - Medicamentos específicos	- Bajos niveles de educación - Pobreza	- Falta de acceso a los recursos de actividad física/barrios con pocas zonas peatonales - Áreas geográficas con poco o ningún acceso a alimentos saludables, como productos frescos/comestibles - Virus - Microbiota - Sustancias químicas que alteran el sistema endocrino

Fuente: adaptado de la Organización Mundial de la Salud Fact sheet 311 y Hruby and Hu et al. Pharmacoenomics 2016

De hecho, la principal laguna en el conocimiento de la obesidad no se debe a la cantidad de factores de riesgo, ni a su impacto individual en el desarrollo de enfermedades metabólicas, sino por el efecto de la convergencia de todos ellos.

En los últimos años, la relación entre microbiota intestinal y el desarrollo de enfermedades ha tomado gran relevancia. La microbiota intestinal se ha propuesto como punto confluyente de factores de riesgo de la obesidad, aunque su implicación en el desarrollo de la obesidad y enfermedades relacionadas (por causalidad o por consecuencia) aún está en debate. Como punto clave de esta investigación, este vínculo se desarrollará en el apartado 1.2.

De la misma manera, la dieta es un factor de riesgo ampliamente estudiado[9–11]. En la presente tesis doctoral se ha estudiado el cambio en el patrón alimentario y el incremento de actividad física como tratamientos de la obesidad para la pérdida de peso, detallado en el apartado 1.1.1.2.

1.1.3 Obesidad, una puerta de entrada de enfermedades metabólicas

Gema Frühbeck & Volkan Yumuk describieron la obesidad como una puerta de entrada de enfermedades (*A Gateway Disease*)[6]. Según la OMS, un elevado IMS es un importante factor de riesgo de enfermedades no transmisibles. La obesidad podría ser responsable de al menos el 80% de enfermedades cardiovasculares, diabetes, problemas musculo-esqueléticos y el 40% de cánceres podrían prevenirse a través de la dieta[2].

La prevalencia global de diabetes tipo 2 (diabetes mellitus, DT2) muestra comportamientos similares a la obesidad. 422 millones de personas fueron diagnosticadas de DT2, 2014[2], de los cuales se calcula que el 80-90% presentarán complicaciones asociadas (25% retinopatías, 2% ceguera, 40% nefropatías)[6]. La obesidad es responsable del 80% de los casos de DT2, lo que ha dado lugar al término, *diabesidad* o DT2 dependiente de obesidad[12]. Las mujeres con IMC en el rango 23-25 presentan un riesgo 4 veces mayor de desarrollar DT2 que aquellas con IMC <20. En aquellas con un IMC del 24-25 el riesgo de sufrir DT2 se multiplica por 5, mientras que este probabilidad es 93.2 veces mayor en mujeres con IMC >35[13].

Según la Asociación Americana de Diabetes (ADA del inglés, *American Diabetes Association*), la DT2 es definida como "*la pérdida progresiva de secreción de insulina por parte de las células beta del páncreas en el fondo de la resistencia a la insulina*"[14]. El cribado de diabetes se basa en la detección de los niveles de glucosa plasmática en ayunas ≥126 mg/dL (7.0 mmol/L), ≥ 200 mg/dL (11.1 mmol/L) después de 2 horas de recibir una carga oral de 75-g de glucosa (prueba oral de tolerancia a la glucosa) o presentar niveles ≥ 6.5% según la prueba de hemoglobina

glicosilada (HbA1c) (cuando hay ausencia de hiperglucemia)[15]. Las mayores consecuencias de la DT2 son la presencia de complicaciones severas y la reducción de la esperanza de vida en 8-10 años[15]. Además, la DT2 es una enfermedad difícil de tratar, cara de controlar y de diagnosticar.

Aunque la obesidad se conoce como el principal factor de riesgo de DT2, hay otros factores como la raza/etnia, el género, la edad, la genética y el estilo de vida que también tienen una gran influencia[15].

1.1.4 Salud metabólica o obesidad metabólicamente *sana*

En 1975, Haller and Hanefeld describieron el término de síndrome metabólico (SMet) como *la combinación de factores de riesgo subyacentes que cuando ocurren a la vez terminan en consecuencias adversas, como el desarrollo de DT2, enfermedades cardiovasculares y el incremento en el riesgo de mortalidad en 1.5*. Según la definición, el síndrome metabólico se asocia con la obesidad abdominal, el incremento de la glucosa plasmática, el incremento de triglicéridos (TG), la reducción del colesterol de baja densidad (HDL) y la hipertensión. Los criterios de diagnóstico de la SMet se han desarrollado a partir de la agrupación de factores de riesgo de las enfermedades cardiovasculares fuertemente interrelacionados entre ellos. Diferentes guías clínicas han propuesto definiciones y criterios de diagnóstico del SMet para su rápida identificación, los cuales se resumen en la **Tabla 2**. No obstante, los criterios varian y los individuos pueden ser diagnosticado de SMet con uno o dos definiciones pero no con otras[16].

En la presente tesis doctoral se ha utilizado la definición de SMet del "Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III, ATPIII)"[17] que considera el SMet como una constelación de factores de riesgo mayores y factores de riesgo del hábito de vida sin enfatizar en ningún factor de riesgo. Los criterios para su diagnóstico se encuentran recogidos en la **Tabla 2.**

Tabla 2. Criterios fijados para el diagnóstico de síndrome metabólico según el número de asociaciones influentes

NCEP:ATPIII	OMS	EGIR	AACE	IDF
Al menos tres de los siguientes criterios	Elevados niveles de insulina	Elevadas concentraciones de insulina en ayuna-resistencia a la insulina	Alteración de la tolerancia a la glucosa	Obesidad central = cintura (etnia y género)
+	+	+	+	+
	Dos de los siguientes:	*Dos de los siguientes:*	*Dos de los siguientes:*	*Dos de los siguientes:*
1. Cintura >102 cm (hombres) >88 cm (mujeres)	**1.** Obesidad abdominal Cintura>37''. IMC >30 kg/m^2	**1.** Cintura ≥94 cm (hombres) ≥80 cm (mujeres)	**1.** TG ≥150 mg/dL HDL <40 mg/dL (hombres) <50 mg/dL (mujeres)	**1.** TG ≥150 mg/dL HDL <40 mg/dL (hombres) <50 mg/dL (mujeres)
2. TG >150 mg/dL HDL <40 mg/dL (hombres) <50 mg/dL (mujeres)	**2.** TG >150 mg/dL HDL <35mg/dL (hombres) <39mg/dL (mujeres)	**2.** TG >2 mmol/L HDL <1 mg/dL		
3. PA ≥130/85 mmHg	**3.** PA ≥140/90 mmHg	**3.** PA ≥140/90 mmHg o medicamentos para la hipertensión	**2.** PA ≥130/85 mmHg	**2.** PA ≥130/85 mmHg
4. Glucosa plasmática en ayunas ≥110 mg/dL	**4.** Microalbúmina >30 mg/g	**4.** Glucosa en ayunas ≥6.1 mmol/L		**3.** Glucosa plasmática en ayunas ≥5.6 mmol/L o DT2

Criterios de diagnóstico del Síndrome Metabólico según el número de asociaciones influentes.
AACE, Asociación Americana de Endocrinólogos Clínicos; DT2, Diabetes Tipo 2; Cintura, circunferencia de la cintura; EGIR, Grupo Europeo para el Estudio de la Resistencia a la Insulina; IDF, Federación Internacional de Diabetes; IMC, Índice de Masa Corporal; HDL, lipoproteína de alta densidad; NCEP:ATPIII, Programa Nacional de Educación en Colesterol: Tercer Panel de Expertos-EE.UU; OMS, Organización Mundial de la Salud; PA, presión arterial; TG, triglicéridos. Fuente: adaptada de S. O'Neill et al.[18]

Ciertos estudios han sugerido que bajo ciertas condiciones, la obesidad podría tener un efecto protector de enfermedades, con una relación inversa entre IMC y mortalidad (revisado en Oreopoulos et al.[19]): la *paradoja de la obesidad*. Bajo el concepto "más grasa más enfermedades metabólicas", se ha descrito que alrededor de un 30% de los sujetos presentan efectos cardioprotectores, a pesar de tener elevados los niveles de adiposidad. Estos individuos se han definido como sujetos con niveles favorables de sensibilidad a la insulina, inflamación lipídica, enzimas hepáticas y perfil inmunitario[20]. Por otro lado, adultos delgados también pueden presentar resistencia a la insulina/DT2[21].

El estudio de los fenotipos discordantes de obesidad y resistencia a la insulina/DT2 aporta una única e inexplorada oportunidad para estudiar la conexión entre la expanxión del tejido adiposo y el desarrollo gradual de DT2 y enfermedades asociadas.

Caracterizar los mecanismos protectores asociados a la obesidad metabólicamente *sana* se ha considerado de gran relevancia desde un punto de vista fisiológico, terapéutico y clínico[22]. La aparición de un fenotipo *sano* con obesidad podría conducir a un cambio en el paradigma del tratamiento de esta enfermedad. Consecuentemente, mejorar los factores de riesgo cardiometabólicos asociados a la obesidad podría ser un objetivo más importante a abordar que la pérdida de peso en sí. De esta manera, se podría postergar el tratamiento de pérdida de peso en aquel subgrupo de pacientes con obesidad metabólicamente *sana*.

Sin embargo, la línea entre ser o no ser metabólicamente *sano* puede ser muy estrecha. Recientemente se ha propuesto que, después de aproximadamente cinco años, los sujetos metabólicamente *sanos* terminan desarrollando alguna complicación metabólica, por lo que la salud metabólica en sujetos con obesidad podría ser un estado transitorio[23], y la obesidad, ser una enfermedad independientemente del estado metabólico del sujeto[24].

El concepto de obesidad metabólicamente *sana* o *enferma* utilizado en los estudios de la presente tesis doctoral se basa en los criterios de la NCEP-ATPIII.

1.2 La metabolómica y el estudio de perfiles metabólicos. Una nueva visión para el estudio de la obesidad

El *National Institute of Health* de EE.UU. definió la Metabolómica como la

> *La metabolómica proporciona una lectura directa de la actividad bioquímica de una célula, de un organismo o de un sistema biológico mediante la cuantificación y/o identificación de compuestos de bajo peso molecular (<1 kDa), los llamados* ***metabolitos****, que se encuentran en un sistema biológico, como sangre, orina y tejidos entre otros, derivados de determinadas condiciones fisiológicas y ambientales a las que uno o más organismos están expuestos (****metaboloma****) (Nicholson et al. 1999).*

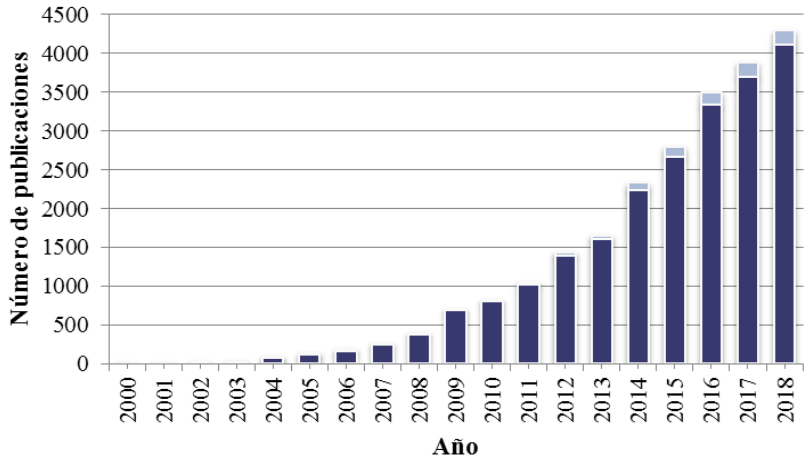
tecnología clave para el descubrimiento de fármacos dentro de su "plan de trabajo para la investigación médica en el siglo XXI", bajo el lema "Nuevos Caminos para los Descubrimientos". Según Nature Opinion, la metabolómica se encuentra entre los cincos sectores tecnológicos más pioneros de la década actual ("The Tomorrow's Giants"), y el *Massachusetts Institute of Technology* Review la destaca como la tecnología emergente que transformará la investigación biológica[25].

A pesar de ser la más joven de las "-ómicas", la metabolómica cuenta con una serie

> *La Human Metabolome Database (HMDB) 4.0 es una plataforma online gratuita que contiene información detallada de las pequeñas moléculas que se encuentran en el cuerpo humano. La HMDB proporciona información de tipo química, clínica y molecular/bioquímica. Cada metabolito está vinculado a diferentes bases de datos, así como a bases de datos de rutas biológicas (Wishart et al. 2018).*

de aplicaciones en el campo de la investigación clínica[26–31], lo cual se refleja en el crecimiento exponencial del número de publicaciones en los últimos años (**Figura 4**).

Figura 4. Número de publicaciones por año en el campo de la metabolómica



Número de publicaciones por año publicadas en PubMed con la búsqueda "Metabolomics" (en azúl marino) y "Metabolomics & Obesity" (en azúl celeste)

La metabolómica se considera una tecnología emergente para: i) el descubrimiento de nuevos biomarcadores relacionados con el diagnóstico precoz o el pronóstico de enfermedades agudas y crónicas, o la estratificación de la población por grupos de riesgo (identificación de nuevos biomarcadores de naturaleza endógena); ii) una mejor comprensión de los mecanismos etiopatogénicos que subyacen a la enfermedad, de importancia crucial en la evaluación de riesgo y en la identificación de nuevas dianas terapéuticas (identificación de mecanismos patogénicos desconocidos); y iii) una mayor comprensión y evaluación de la toxicidad/eficacia de intervenciones terapéuticas en fase de desarrollo, aumentando la capacidad de predecir la variación fenotípica en respuesta a distintos tratamientos farmacológicos[32] (respuesta a tratamientos) y cirugía (fenotipado metabólica intra-operatória, del inglés *surgicalmetabolomics*)[33]. El fenotipado a nivel molecular mediante el "fenotipado metabólico" o "metabotipado" de muestras de tejidos o biofluidos (en inglés, *metabolic profiling* o *metabotyping*) refleja los cambios constantes del metaboloma humano y sus interacciones con el ambiente, la dieta, la microbiota y los genes, definiendo así el fenotipo del organismo (o *metabotipos*). Este hecho permite la estratificación de los pacientes a tiempo real y así mejorar el

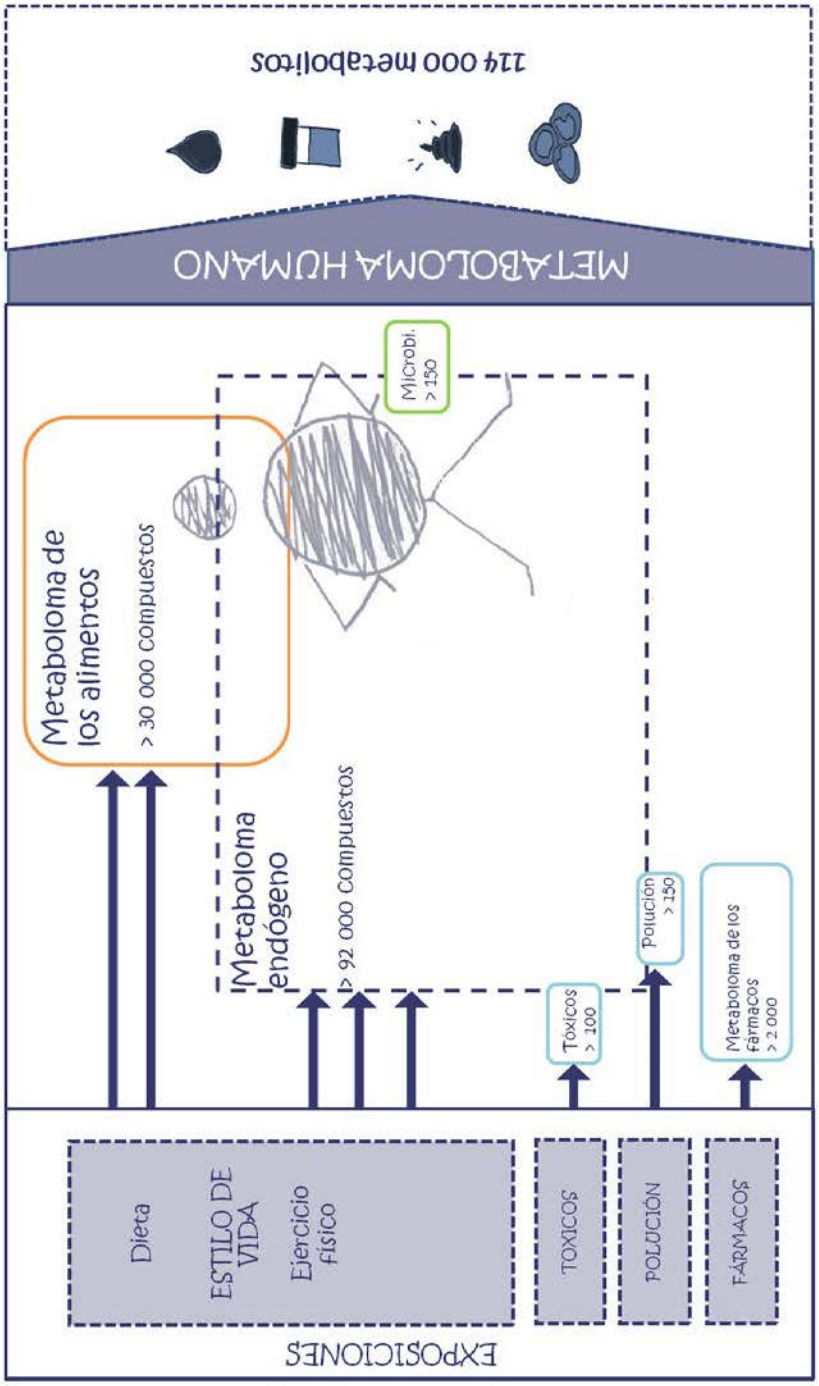
diagnóstico diferencial, la respuesta terapéutica y la predicción de los resultados a largo plazo de una terapia[34].

Actualmente se han caracterizado e identificado alrededor de 114.100 pequeñas moléculas en el cuerpo humano (datos del Human Metabolome DataBase, 2018)[35]. Estos compuestos son el reflejo de las interacciones e interrelaciones de la genómica, transcriptómica y proteómica, proporcionando así un perfil integral del estado biológico de la persona. Según Scalbert et al.[36], el metaboloma humano incluye la integración de: i) el metaboloma endógeno (del inglés, *endogenous metabolome*), que incluye compuestos del metabolismo celular; ii) el metaboloma de alimentos (del inglés, *food metabolome*), formado por los compuestos derivados de los alimentos después de la digestión y el posterior metabolismo en los tejidos y la microbiota; iii) metaboloma de los fármacos (del inglés, *drug metabolome*), y iv) el metaboloma derivado del ambiente y los químicos (del inglés, *exposome*) (**Figura 5**).

El carácter dinámico y susceptible del metaboloma humano ofrece una oportunidad única para el estudio de enfermedades endocrinas, especialmente de las "enfermedades metabólicas" como la obesidad y la DT2 que impactan fuertemente sobre el metabolismo[37].

En los últimos años los métodos analíticos utilizados en metabolómica han avanzado en sensibilidad y reproducibilidad, amplia cobertura de metabolitos y alto rendimiento de número de muestras, hecho que se refleja en el incremento del número de metabolitos caracterizados y de publicaciones de metabolómica en el campo de la salud humana y la investigación biomédica, así como aplicaciones en cultivos y análisis de calidad de alimentos.

Figura 5. El Metaboloma Humano



Fuente: Adaptado de Scalbert et al.[36] y actualizado con datos del Human Metabolome Database 4.0 (http://www.hmdb.ca/), publicado en Wishart et al.[35]

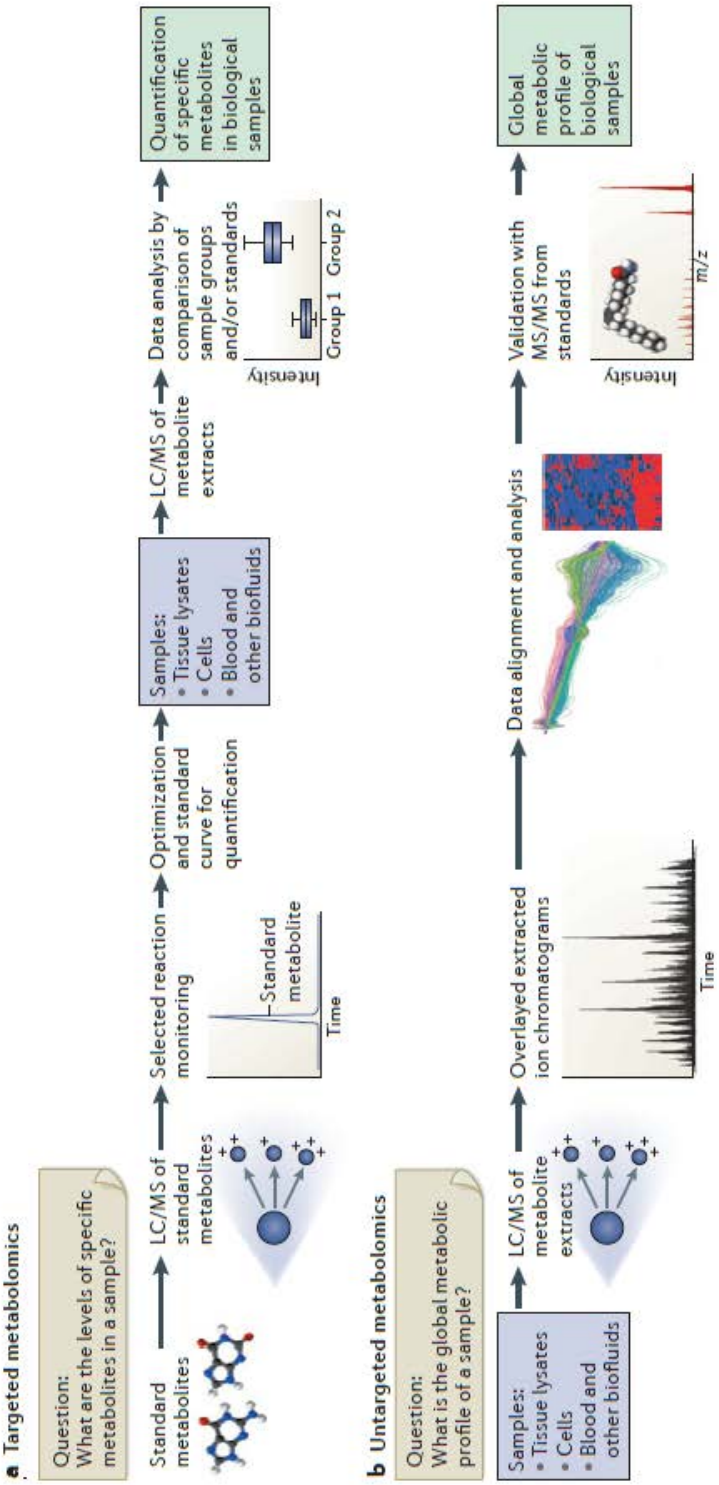
Las técnicas analíticas más comúnmente empleadas en metabolómica son la espectrometría de masas, acoplada o no a cromatografía y la resonancia magnética nuclear. Las dos técnicas analíticas permiten la caracterización de diferentes clases de metabolitos incluyendo lípidos, amino ácidos, azúcares, aminas biógenas y ácidos orgánicos entre otros. Dependiendo de la naturaleza química de los metabolitos que se quieran identificar, como el peso molecular, polaridad y concentraciones, se opta por el uso de una técnica u otra.

El flujo de trabajo aplicado en los estudios metabolómicos se compone de cinco pasos principales: i) la recolección y preparación de la muestra biológica; ii) adquisición y pre-procesamiento de los datos; iii) análisis de datos; iv) identificación de metabolitos e v) interpretación biológica.

Dentro de este esquema general se describen dos enfoques metabolómicos principales. El primero consiste en un **análisis dirigido** o cuantitativo, en el cual se estudia un grupo de metabolitos específicos, predefinidos *a priori*, especialmente guiados por una hipótesis inicial (del inglés, *hypothesis-driven approach*). Alternativamente, el segundo enfoque consiste en un **análisis no-dirigido**, libre de hipótesis iniciales (del inglés, *hypothesis-free approach*), el cual permite tener un enfoque global para obtener una instantánea general y semicuantitativa del metaboloma de una muestra biológica, incluyendo aquellos compuestos que son desconocidos o poco caracterizados, sin focalizar el estudio en la identificación de un grupo concreto de metabolitos y únicamente limitado por la plataforma analítica utilizada, el solvente, la columna y la fuente de ionización. De esta forma, los resultados de esta aproximación permiten la generación de nuevas hipótesis de estudio e ir más allá en la investigación (**Figura 6**).

La espectrometría de masas es la plataforma analítica más potente para la adquisición de perfiles metabolómicos como huellas metabólomicas en matrices biológicas complejas. En el apartado de Metodología se desarrollará en detalle las plataformas y pasos seguidos en cada estudio.

Figura 6. Fujo de trabajo de la metabolómica dirigida y no-dirigida por LC-MS



Fuente: extraído de Patti et al.[38]

1.2.1 La microbiota intestinal en el desarrollo de la obesidad y enfermedades relacionadas

La microbiota intestinal humana alberga trillones de microorganismos de diferentes dominios: Archaea, Bacteria y Eukarya. La mayoría residen en el colon, donde la célula bacteriana se puede encontrar entre 109-1012 UFC/mL y con más de 1000 especies diferentes. El 90% de las especies bacterianas en el intestino adulto pertenecen a dos filos: Firmicultes (Gram positivas) y Bacteroidetes (Gram-negativas). La microbiota intestinal da al huésped numerosas funciones vitales, incluyendo la habilidad de digerir compuestos de la dieta, sintetizar vitaminas y regular el sistema inmunitario (Tremaroli et al. 2008).

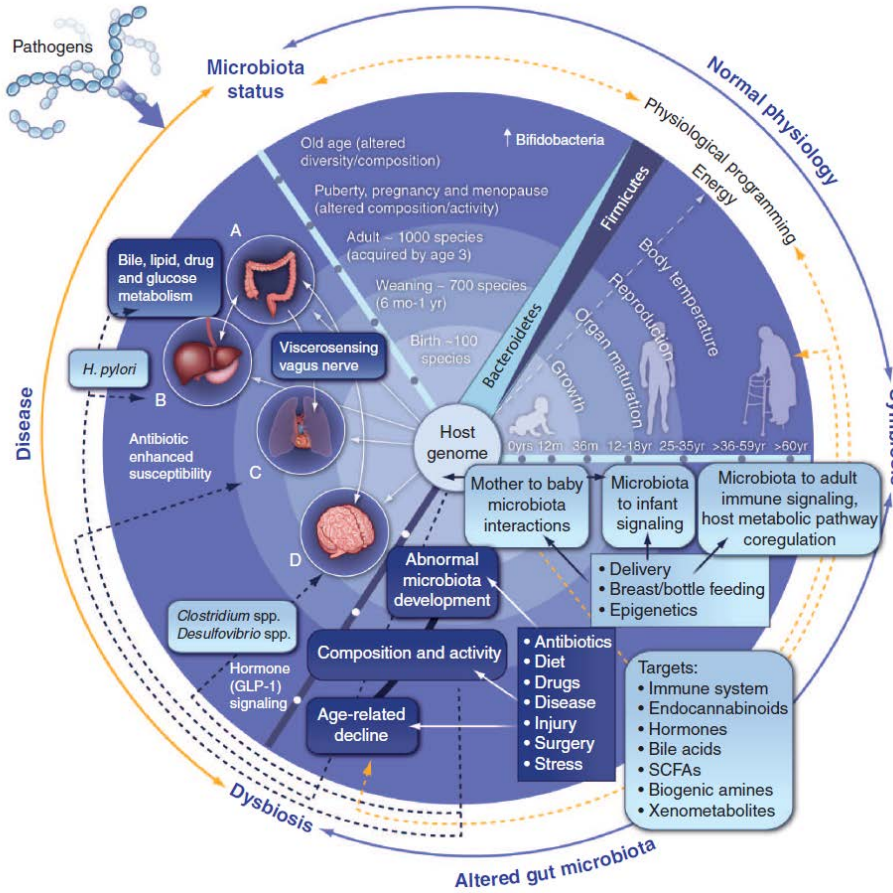
Actualmente disponemos de la metagenómica, cultivo-independiente que permite estudiar las estructuras y funciones de las comunidades microbianas y sus interacciones con el hábitat que ocupan, es un campo emergente que nos ha permitido aproximarnos en el conocimiento de las bacterias residentes en las distintas microbiotas (Tremaroli et al. 2008).

En los últimos años se ha propuesto la microbiota intestinal como un elemento imprescindible para el entendimiento de los mecanismos implicados en la obesidad y sus comorbidilidades. Su emergente auge en la comunidad científica viene dado porqué la microbiota intestinal podría ser la centralita de todos los factores de riesgo de la obesidad; capaz de dar instrucciones a nuestro cerebro y modificar el metabolismo[39], el llamado en inglés *the gut-brain-liver axis*[40]. No obstante, es tal su complejidad que se requieren de estudios complementarios para poder comprender toda su capacidad.

La composición de la microbiota se desarrolla con el huésped ya desde su nacimiento y está estrechamente vinculada con él[41]. Aunque cada individuo posee una microbiota característica, existe un conjunto de colonizadores intestinales comunes entre individuos, especialmente entre miembros de una misma familia[42]. Los factores ambientales (dieta, polución, medicamentos, etc.) modifican la microbiota intestinal, favoreciendo la actividad de colonias bacterianas que pueden impulsar la actividad inductora de enfermedad (*disbiosis*) o una actividad protectora

de enfermedad (*probiosis*). El balance entre la actividad bacteriana fortalecedora de las defensas y el efecto proinflamatorio patológico, fue descrito en 1908 por el premio Nobel *lauréate* Elie Metchnikoff[43] (**Figura 7**).

Figura 7. Influencia de la microbiota intestinal en la salud humana al largo del tiempo.



Fuente: extraído de Nicholson et al.[44]

El vínculo entre microbiota intestinal y el metabolismo del huésped y su comunicación con el tejido adiposo podría influenciar en el desarrollo de alteraciones metabólicas asociadas tanto a la obesidad como a los transtornos del control glucémico. Un creciente número de estudios científicos, tanto en modelos animales como en humanos, han evidenciado el papel de la microbiota intestinal en el metabolismo humano afectando el balance energético, el metabolismo de la

glucosa y riesgo de desarrollar resistencia a la insulina y DT2 y en el estado de inflamación de bajo grado, asociado a la obesidad y enfermedades metabólicas[45]. Bäckhed *et al*. reportó que ratones libres de gérmenes (del inglés, *germ-free*) tenían un 42% menos de grasa corporal que los ratones con microbiota convencional. Cuando los ratones libres de gérmenes se convencionalizaban —se les trasplantaba microbiota de ratones adultos— incrementaban un 60% su peso y desarrollaban resistencia a la insulina al cabo de 2 semanas del trasplante aunque se les redujera el consumo de alimentos[46]. Ley *et al.* observó que ratones genéticamente obesos (*ob/ob*) presentaban una reducción del 50% de Bacteroidetes comparado con ratones hermanos delgados (-/-)[47]. Con una dieta controlada, la proporción de Bacteroidetes disminuía y aumentaba la proporción de Firmicutes en ratones obesos respecto a ratones delgados[42]. Este fenómeno también se observó en humanos[48] .

En ratones se ha observado que la microbiota estimula el metabolismo lipídico del tejido adiposo marrón[49]. Asimismo, cuando los ratones se alimentan con una dieta rica en grasa, su permeabilidad intestinal se ve incrementada y disminuye la expresión de genes que codifican las proteínas implicadas en las uniones estrechas encargadas de mantener la homeostasis de la permeabilidad intestinal[50]. Se ha identificado que los lipolisacáridos de las membranas de las bacterias Gram-negativo son un factor en el desarrollo de la inflamación. El cambio de la microbiota intestinal por el consumo de una dieta rica en grasa incrementaría la permeabildad, facilitando la absorción de los lipolisacáridos del intestino a la sangre, provocando la *endotoxemia metabólica*, desencadenante de la inflamación[50,51].

Sin embargo, todavía no está aclarada la direccionalidad de la relación entre dysbiosis intestinal y desarrollo de patología metabólicas como la obesidad y la diabetes [52]

Diferentes herramientas se han propuesto para entender el impacto de la microbiota en el mantenimiento de la salud y el desarrollo de la DT2. La metabolómica representa una herramienta con un enorme potencial para dilucidar el efecto de la microbiota en la salud. Por este motivo, el primer trabajo de esta tesis doctoral consiste en una revisión bibliográfica de los estudios en humanos que cuantifican o

identifican, mediante una aproximación de metabolómica dirigida o no-dirigida, metabolitos derivados de la interacción microbiota-huésped y asociados con una alteración del metabolismo de la glucosa y/o de la obesidad.

A continuación se adjunta el artículo y el material suplementario de la revisión:

Revisión 1. *Magali Palau-Rodriguez*, Sara Tulipani*, Maria Isabel Queipo-Ortuño, Mireia Urpi-Sarda, Francisco J. Tinahones y Cristina Andres-Lacueva. Metabolomic insights into the intricate gut microbial–host interaction in the development of obesity and type 2 diabetes. Frontiers in Microbiology 2015 6:1–12. doi: 10.3389/fmicb.2015.01151*

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Metabolomic insights into the intricate gut microbial–host interaction in the development of obesity and type 2 diabetes

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Gut microbiota has recently been proposed as a crucial environmental factor in the development of metabolic diseases such as obesity and type 2 diabetes, mainly due to its contribution in the modulation of several processes including host energy metabolism, gut epithelial permeability, gut peptide hormone secretion, and host inflammatory state. Since the symbiotic interaction between the gut microbiota and the host is essentially reflected in specific metabolic signatures, much expectation is placed on the application of metabolomic approaches to unveil the key mechanisms linking the gut microbiota composition and activity with disease development. The present review aims to summarize the gut microbial–host co-metabolites identified so far by targeted and untargeted metabolomic studies in humans, in association with impaired glucose homeostasis and/or obesity. An alteration of the co-metabolism of bile acids, branched fatty acids, choline, vitamins (i.e., niacin), purines, and phenolic compounds has been associated so far with the obese or diabese phenotype, in respect to healthy controls. Furthermore, anti-diabetic treatments such as metformin and sulfonylurea have been observed to modulate the gut microbiota or at least their metabolic profiles, thereby potentially affecting insulin resistance through indirect mechanisms still unknown. Despite the scarcity of the metabolomic studies currently available on the microbial–host crosstalk, the data-driven results largely confirmed findings independently obtained from *in vitro* and animal model studies, putting forward the mechanisms underlying the implication of a dysfunctional gut microbiota in the development of metabolic disorders.

Keywords: metabolomics, gut microbiota, obesity, type 2 diabetes, co-metabolism

Abbreviations: ^{1}H-NMR, proton nuclear magnetic resonance; BA, bile acids; IGT, impaired glucose tolerance; FMO3, flavin monooxygenase 3; FXR, Farnesoid X Receptor; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; NAFLD, non-alcoholic fatty liver disease; OGTT, oral glucose tolerance test; T2D, type 2 diabetes; TGR-5, G-protein coupled receptor; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide.

GUT MICROBIOTA AND *DIABESITY*: ROLE IN ENERGY HARVEST, GUT BARRIER INTEGRITY, ENDOCRINE MODULATION, AND METABOLIC INFLAMMATION

Obesity is a complex,multifactorial disease characterized by an excessive accumulation of fat due to an imbalance between energy intake and expenditure. The linear rise in the prevalence of T2D throughout the normal, overweight and obese ranges is so high that the relative risks of diabetes are 40 times higher when BMI increases above 35 kg/m^2 (Hu et al., 2001; Mokdad et al., 2003; Poirier et al., 2006; World Health Organization [WHO], 2013). The public concern over the obesity epidemic mostly lies in the intimate connection between obesity and T2D (so-called *diabesity*; Astrup and Finer, 2000) and makes the elucidation of mechanisms underlying the co-occurrence of the two diseases a central focus of current biomedical research.

Recently, consideration has started to be given to the gastrointestinal tract as a key point in the development and progression of complex metabolic diseases, since it represents the milieu where interactions between exogenous (i.e., diet, microbiome) and endogenous (i.e., genetic) factors predisposed to disease and the body's defenses (physical barrier, immune system response) actually take place. Increasing evidence indicates in particular the impact of changes in the composition of the human gut microbiota on host metabolism and a variety of diseases (Bäckhed et al., 2005; Moreno-Indias et al., 2014; Shoaie et al., 2015).

Firmicutes (Gram-positive), Bacteroidetes (Gram-negative) and Actinobacteria (Gram-positive) represent over 90% of the phyla and dominate the gut microbiota (DiBaise et al., 2008), but a relevant change in their relative proportion has been described in obesity and T2D. A favorable prevalence of Firmicutes bacteria toward healthy subjects has been observed in both animal models of obesity (Ley et al., 2005) and human obesity (Ley et al., 2006; Turnbaugh and Gordon, 2009), also reviewed in (Turnbaugh and Gordon, 2009; Sanz et al., 2013; Moreno-Indias et al., 2014), although with some discrepancies among data (Schwiertz et al., 2010). Although the potential impact of specific species on host metabolism has already been elucidated, most of the data so far available have reported observed changes at the phylum level. Furthermore, the physiological contribution of Firmicutes in the development of the obese phenotype is still being debated. In turn, some studies have observed a positive correlation between ratios of Bacteroidetes to Firmicutes and plasma glucose concentration, but not with BMI, although this was expected (Larsen et al., 2010).

Different mechanisms have been proposed in the attempt to understand the impact of microbiota both in maintaining metabolic health and in the development of obesity and T2D. Essentially, the intestinal microbial variability has been hypothesized as an important factor in four different processes, namely: (i) the modulation of energy homeostasis by regulating the energy harvest from diet, fat storage, lipogenesis, and fatty acid oxidation (host energy metabolism; Tilg et al., 2009; Musso et al., 2010); (ii) the modulation of the gut barrier integrity by regulating the epithelial permeability, the intestinal motility and the transport of digestion products such as short-chain fatty acids, which are an energy source for colonocytes (Samuel et al., 2008); (iii) the regulation of gastrointestinal peptide hormone secretion, by suppressing the secretion of the lipoprotein lipase inhibitor (fasting-induced adipose factor), determining the release of fatty acids from circulating triglycerides and lipoproteins in muscle and adipose tissue and promoting fat mass accumulation (Bäckhed et al., 2007); and (iv) the modulation of the host inflammatory state by contributing to the systemic increase of lipopolysaccharide, which impairs insulin sensitivity (metabolic endotoxemia; reviewed in Bäckhed et al., 2007; Cani et al., 2007, 2012; Sun et al., 2010; Vrieze et al., 2010; Shen et al., 2013). Evidence of the role of gut microbiota in the preservation of metabolic health also comes from the effect of prebiotics, such as non-digestible carbohydrates, namely non-digestible ingredients that are fermented by specific beneficial bacterial strains, selectively promote the growth and/or activity (release of end-products of bacterial fermentation) of the gastrointestinal microbiota, affecting favorably the host health (Gibson et al., 2010). The intake of prebiotics has in fact been described to act on host endocrine secretion, improve gut barrier integrity by increasing the release of glucagon-like peptide-2 (Cani et al., 2012; Dewulf et al., 2013), stimulate postprandial release of peptides involved in energy homeostasis and/or pancreatic functions such as the anorexigenic glucagon-like peptide-1 and peptide YY, and the decrease of orexigenic peptides such as ghrelin in plasma which in turn modulates food intake (regulators of appetite) and energy expenditure across the entire gastrointestinal tract (Piche et al., 2003; Delzenne and Cani, 2011; reviewed in Vrieze et al., 2010). Furthermore, evidence suggests that the modulation of the host metabolic health by prebiotics intake can be mediated to specific fermentation products (i.e., short-chain fatty acids, predominantly acetate, propionate and butyrate) produced by cross-feeding between *Eubacterium rectale* and *Bifidobacterium thetaiotaomicron* (Venema, 2010); *Propionibacterium* sp. and *Bacteroides* sp. (Hosseini et al., 2011); *Faecalibacterium prausnitzii* and *Roseburia intestinalis/Eubacterium rectale* (Duncan et al., 2004; Venema, 2010) respectively.

THE METABOLOMIC APPROACH

Due to the species specificity of several enzymatic machineries, the gut microbial composition and activity are likely to be characterized by the profile of small metabolites produced in the intestinal lumen, eventually absorbed through the intestinal barrier and further biotransformed by the host. Consequently, the complexity of microbial–host exchanges may be reflected in the specific chemical signature of host circulating biofluids (Nicholson et al., 2012). Metabolomics has recently attracted attention as the most suitable *-omics* technology for investigating complex, polygenic and multifactorial diseases with a strong metabolic etiology, such as obesity and T2D as well as the crosstalk of distinct predisposing factors in disease development

and progression (Faber et al., 2007; Llorach et al., 2012; Du et al., 2013; Kurland et al., 2013). Aimed at the comprehensive analysis of the low- molecular- weight compounds contained in a biological system –by definition, metabolites comprise a plethora of primary or secondary derivatives of the intermediate metabolism (molecular weight below 900 and 2000 Dalton, depending on sources; Beckonert et al., 2010; Psychogios et al., 2011; Hadacek, 2015) metabolomics represents a powerful tool for exploring the crosstalk between the microbial and host metabolism in a more exhaustive fashion.

The workflow applied in metabolomic studies is broadly categorized into five main steps: (1) sample collection, (2) sample preparation, (3) data acquisition, (4) data analysis, and (5) biological interpretation of the results obtained (Llorach et al., 2012). The analytical techniques most commonly used for the characterization of the metabolome of a biological sample are MS and ^{1}H-NMR. Both technologies have their advantages and disadvantages. ^{1}H-NMR implies a non-destructive, non-selective, cost-effective, and relatively sensitive analysis while, compared to ^{1}H-NMR, MS mainly offers potential advantages in terms of sensitivity and, if coupled to different separation techniques such as LC or GC, it provides a means of detecting a broader and complementary range of biomarkers (Faber et al., 2007). LC coupled to electrospray ionization MS is becoming the method of choice for the acquisition of profiling metabolites in complex biological samples (Scalbert et al., 2009) through both targeted (i.e., triple quadrupole-driven) and non-targeted (e.g., quadrupole time-of-flight-, linear trap quadrupole orbitrap-driven) approaches.

The present review aims to summarize the gut microbial–host cometabolites identified so far in humans in relation to obesity and/or T2D by targeted and untargeted metabolomic studies. Since the potential impact of some specific species in host metabolism has already been elucidated, an attempt to associate bacterial producers of the co-metabolites with the metabolic alterations related to the obese, diabetic, or diabese phenotype was also made. A critical view of the current limitations and future directions of metabolomics will accompany the discussion.

MATERIALS AND METHODS

Search Strategy

The following keywords were searched for in the PubMed and Web of Science electronic databases: (Metabolom* [TW] or co-metabol* [TW] or host-gut metabo* [TW] or nuclear magnetic resonance [TW] or MS [TW] or magnetic resonance spectroscopy [TW]) AND (OBES* [TW] OR DIABET* [TW] OR DIABES* [TW]) AND (gut micro* [TW]). Species (human), language (English), and publication date restrictions (2000 to date, last search on November 27th, 2014) were imposed, but there were none for gender, age or ethnicity. Relevant references cited in the selected articles were additionally reviewed. Targeted and untargeted metabolomic approaches driven by ^{1}H-NMR or MS techniques were both included in the selection. Low-molecular-weight (<1000 Da) metabolites significantly up- or downregulated in overweight and obese subjects with/without impaired glycemic control, with respect to controls (i.e., lean, healthy subjects), were the primary outcomes of interest of the review.

RESULTS AND DISCUSSION

Characteristics of the Studies and Metabolic Variations

Only eight human studies successfully met the eligibility criteria for inclusion in the review (details in the Supplementary Material File). As summarized in **Table 1**, seven observational and one interventional study have so far applied a metabolomic approach and specifically identified changes in products of the gut microbial–host co-metabolism in overweight to obese individuals (BMI > 25 kg/m^2) and/or several degrees of impaired glycemic control (ranging from IGT up to T2D) compared to control individuals. Other comorbidities were not described (i.e., hypertension, renal or liver dysfunction).

Overall, the study subjects, designs and objectives were quite heterogeneous despite the small number of retrieved studies (Supplementary Material File), thereby complicating an otherwise integrated and consistent picture of the metabolomic changes observed.

Urine (Salek et al., 2007; Calvani et al., 2010; Zhao et al., 2010; Huo et al., 2015), fasting serum (Huo et al., 2009; Zhang et al., 2009; Suhre et al., 2010) and plasma (Zhao et al., 2010; Campbell et al., 2014) were the biological samples used in these studies. A data-driven untargeted approach was chosen in the majority of the studies (Salek et al., 2007; Huo et al., 2009; Zhang et al., 2009; Calvani et al., 2010; Zhao et al., 2010; Campbell et al., 2014) while two of them provided quantitative information about known targeted metabolites (Suhre et al., 2010; Huo et al., 2015). The metabolic changes observed in these studies and the related interpretations are summarized in **Table 2**.

Co-metabolism of Bile Acids

Two of the metabolomic studies described in this review highlighted a change in the circulating pool of BA in obese patients with insulin resistance or T2D, compared with BMI-matched healthy individuals (Suhre et al., 2010; Zhao et al., 2010). Alterations involved both human-derived (hepatic) structures (primary BA) and gut microbial-produced derivatives (secondary BA).

To the best of our knowledge, it is currently accepted that the bacterial enzymes involved in the biotransformation from primary to secondary BA are not shared across the whole microbial community, although they have been described so far in genera belonging to the four major phyla Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria (Labbé et al., 2014). Furthermore, according to Jones et al. (2014) Actinobacteria and Firmicutes clones would be the only ones able to degrade all conjugated BA, with Bacteroidetes species being limited to tauro- conjugation activities.

After their production in the liver and the eventual glyco- and tauro-conjugation (*N*-acyl amidation with glycine or taurine

TABLE 1 | Human metabolomic studies showing gut microbial–host co-metabolites significantly altered in obese and/or T2D diagnosed patients, respect to controls.

Observational Studies

Disease	Participants[1]	Medication[2]	Approach (analytical technique)	Specimen	Changes respect to the CT group	Reference
Obesity + pre-T2D	Group 1 = 15 (0F) morbidly OB with IR	No	Non-targeted	Spot urine, fasting	↓Hippuric acid, *N*- methylnicotinate ↑ 2-hydroxyisobutyrate	Calvani et al., 2010
	Group 2 (CT) = 10 (0F) healthy NW (with NGT)	No	(^{1}H-NMR)			
Obesity + T2D (treated vs. not)	Group 1 = 15 (8F) OW with treated T2D	Metformin (15)	Non-targeted	Serum, fasting	↑Trimethylamine-*N*-oxide	
	Group 2 (CT) = 20 (10F) OB with untreated T2D	No	(^{1}H-NMR)			
Obesity + T2D (treated vs. not)	Group 1 = 20 (11F) OB with treated T2D	Glyburide (10), glimepiride (6), Gliclazide (4)	Targeted	Spot urine, fasting	↓Hippuric acid (untreated T2D) ↑hippuric acid (with anti-T2D drugs)	Huo et al., 2015
	Group 2 = 20 (11F) OB with untreated T2D	No	(UPLC-MS)			
	Group 3 (CT) = 20 (10F) healthy OB (with NGT)	No				
Obesity + T2D	Group 1 = 30 (13F) OW to OB with untreated T2D	No	Non-targeted	Spot urine, fasting	↓Hippuric acid, *N*- methylnicotinate, ↓*N,N*-dimethylglycine, *N,N* dimethylamine	Salek et al., 2007
	Group 2 (CT) = 12 (4F) healthy NW to OW (with NGT)	No	(^{1}H-NMR)			
Obesity + T2D	Group 1 = 40 (0F) OB with T2D	No (7), antidiabetic medication	Targeted	Serum, fasting	↓Cholate ↑deoxicholate ↓Gamma muricholate	Suhre et al., 2010
	Group 2 (CT) = 60 (0F) healthy OW (with NGT)		(UPLC-MS/MS)			
(pre-)T2D	Group 1 = 74 (42F) NW with T2D	No (48), metformin, acarbose, glipizide or repaglinide as a monotherapy (26)	Non-targeted	Serum, fasting	↓Choline (vs. NGT and vs. IGT)	Zhang et al., 2009
	Group 2 = 77 (44F) NW with IGT	No	(^{1}H-NMR)			
	Group 3 (CT) = 80 (46F) healthy NW (with NGT)	No				
Obesity + pre-T2D	Group 1 = 12 (?F) OB with IGT	No	Non-targeted	Plasma, fasting	↑Glycochenodeoxycholic acid	Zhao et al., 2010
	Group 2 (CT) = 39 (?F) healthy OB (with NGT)		(UPLC-qToF-MS)	Spot urine, fasting	↓Hippuric acid, 3-hydroxyhippuric acid, methyluric acid, methylxanthine	

Single-arm intervention study (weight-loss plan with calorie restriction and exercise)

Disease	Participants	Duration and associated clinical outcomes	Approach (analytical technique)	Specimen	Changes from baseline	Reference
Obesity + pre-T2D	Group = 15 (15F) OB with IR (only 12 up to the end)	0, 14–17 weeks ↓BMI, body fat, V02, fasting	Non-targeted (GC-ToF-MS)	Plasma, fasting and 30, 60, 90, 120 min after OGTT	↑Tricarballylic acid (fasting and after OGTT)	Campbell et al., 2014

[1] *'healthy' stands for NGT subjects. Abbreviations: F, number of females in studies; CT, control; BMI, Body Mass Index; OW, overweight; OB, obese; NW, normal weight; NGT, normal glucose tolerance; IGT, impaired glucose tolerance; IR, insulin resistance; T2D, type 2 diabetes; pre-T2D, prediabetes;* ^{1}*H NMR, proton nuclear magnetic resonance; UPLC-qTOF-MS, reversed-phase ultra performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; GC-TOF-MS, gas chromatography/time-of-flight mass spectrometry.*

[2] *Medication during the study.*

TABLE 2 | Summary of the most significant gut microbial and host co-metabolites identified in the selected studies.

Class	Metabolite	Disease	Change[1]	Anti-T2D drugs effect	Sample	Interpretation	Reference	*Related bacteria (phyla)*
Bile acids (primary)	γ-muricholate (hyocholate)	Obesity + T2D	↓		Blood fluids	Bile acids are proposed as new metabolic integrators of whole body energy homeostasis that influence glucose and lipid metabolism. Subjects with diabetes exhibit alterations in the composition of the bile acid pool and their related biosynthetic pathway. A higher rate of conversion of primary to secondary bile acids by the gut microbiota has been implicated in the observed variation.	Suhre et al., 2010	Firmicutes[3]
	cholate	Obesity + T2D	↓		Blood fluids		Suhre et al., 2010	
	glycochenodeoxycholate	Obesity + pre-T2D	↑		Blood fluids		Zhao et al., 2010	
Bile acids (secondary)	Deoxycholate	Obesity + T2D	↑		Blood fluids		Suhre et al., 2010	
Vitamin metabolites	Choline	(pre-)T2D	↓↓[2]		Blood fluids	In the absence of anti-T2D treatment, alteration of choline metabolism (increased degradation) noticed in T2D patients may result from: (a) an altered demand, possibly by altered lipoprotein turnover/biosynthesis, (b) an altered gut microbiotal activity associated with T2D development, or (c) an osmotic compensation for raised blood glucose concentrations. Low levels of choline would also associate to the prevalence of OB/T2D complications, namely nonalcoholic fatty liver. When associated with metformin, may indicate a possible two-way relationship between the anti-T2D treatment and the gut microbiota. The intestinal bacteria composition would influence glucose metabolism and the mechanisms of action of metformin, and the drug would regulate back the gut microbial function.	Zhang et al., 2009	Firmicutes, Proteobacteria and Actinobacteria[4]
	TMAO	Obesity + T2D		↑	Blood fluids		Huo et al., 2009	
	DMA, DMG	Obesity + T2D	↑		Urine		Salek et al., 2007	
	N-methylnicotinate (trigonelline)	Obesity + (pre-)T2D	↓		Urine	Gut microbial class-specific product of the metabolism of niacin, which is an essential vitamin involved in major physiological functions such as coenzyme in tissue respiration, carbohydrate and lipid metabolism. Trigonelline regenerates glutathione stores that are depleted by oxidative stress in obesity. Moreover, low levels of trigonelline could suggest perturbation in nucleotide metabolism during T2D.	Salek et al., 2007; Calvani et al., 2010	NA

(Continued)

TABLE 2 | Continued

Class	Metabolite	Disease	Change[1]	Anti-T2D drugs effect	Sample	Interpretation	Reference	*Related bacteria (phyla)*
Organic acids and derivates	2-hydroxyisobutyric acid	Obesity + pre-T2D	↑		Urine	Since their production is species specific at the colonic level, changes in their level may reflect (a) significant shifts in the subjects' gut microbe ecology or functional differences in the microbiome metabolic activity between OB with IR and healthy lean individuals and (b) changes in the host metabolism/uptake of gut-derived metabolites, possibly related to a variation in the intestinal mucosa permeability after weight-loss plan with calorie restriction and exercise.	Calvani et al., 2010	Firmicutes[5]
	tricarballylic acid	Obesity + pre-T2D	↑		Blood fluids		Campbell et al., 2014	
Phytochemical and purine metabolites	hippuric acid	Obesity + (pre-)T2D	↓↓↓↓	↑	Urine	Changes in the production of hippurate and derivatives are generally connected to diet and gut microbial activities with the human metabolic phenotype and the blood pressure of individuals. They could indicate a relevant role of the gut microbiota in the pathogenesis of the pre-T2D state and could be related to age progression and gender effects on metabolism in T2D. The reversion of these changes by sulfonylurea treatment would confirm a beneficial effect of anti-T2D drugs on gut microbiota metabolism, besides glucose homeostasis. Higher concentrations in obese humans could reflect the known role of gut microbiota in energy metabolism and immune function of the host.	Salek et al., 2007; Calvani et al., 2010; Zhao et al., 2010; Huo et al., 2015	Firmicutes[5]
	3-Hippuric acid hydroxyhippuric acid				Urine		Zhao et al., 2010	
	Methyluric acid	Obesity + pre-T2D	↓		Urine	Gut microbiota-associated metabolite biomarkers, related to IGT. Accumulating evidence indicates that the gut microbiota is instrumental in the energy metabolism and immune function of the host.	Zhao et al., 2010	NA
	Methylxanthine	Obesity + pre-T2D	↓		Urine		Zhao et al., 2010	

OB, obesity; T2D, type 2 diabetes; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; SU, sulfonylureas; M, metformin hydrochloride; TMAO, trimethylamine-N-oxide; NMN, N-methylnicotinate; DMG, N, N dimethylglycine; DMA, dimethylamine. [1]*In respect to healthy controls.* [2]*Also in T2D vs. IGT but not IGT vs. NGT.* [3]*From: Begley et al., 2005; Holm et al., 2006; Fükiya et al., 2009; Labbé et al., 2014.* [4]*From: Claesun and Balskus, 2012.* [5]*From: Li et al., 2008.*

substituents), primary BA are secreted in the small intestine through the bilis (Ridlon et al., 2006; Hofmann and Hagey, 2008; Swann et al., 2011), where they are first subjected to deconjugation by a bacterial bile salt hydrolase enzyme produced by species of the four phyla, such as *Clostridium, Bacteroides, Lactobacillus, Bifidobacterium,* and *Enterococcus* (Begley et al., 2005). In the ileum, more than 95% of these BA undergo enterohepatic recycling (Swann et al., 2011; Kootte et al., 2012; Nicholson et al., 2012), are absorbed from the intestine and returned to the liver (Ridlon et al., 2006). The remaining 5% escape the enterohepatic circulation and reach the large bowel where the bioconversion into secondary BA is completed, especially by Firmicutes phyla *(Eubacterium* sp. and *Clostridium* sp.; Midtvedt, 1974; Nguyen and Bouscarel, 2008; Swann et al., 2011).

A decrease of primary BA and an increase of secondary BA was observed in the fasting serum of overweight patients with T2D, compared to healthy subjects (Suhre et al., 2010). The authors hence concluded that differences in the gut microbiota of diabetic patients may lead to higher rates of conversion from primary to secondary BA. In turn, Zhao et al. (2010) only observed an increase of glycochenodeoxycholic acid (primary BA) in the plasma of prediabetic individuals, while no changes were noticed in urine. Despite the only partial overlapping of the results, both studies suggested that overweight and obesity may not be the predominant factor implied in the metabolomic changes observed, and thus in linking impaired glucose homeostasis to alterations in BA pool composition. As argued in those studies, the variation of the BA pool in biofluids may depend on different factors, namely a change in the prevalence or activation of the gut microbial species implied in BA bioconversion or an altered reabsorption of BA through the gut mucosa, in turn produced by the disease itself, by dietary or other external changes (i.e., induced by bariatric surgery), or by a combination of these. In any case, the results indicated a probable implication of the modulation of BA biosynthetic pathways in the relationship between gut microbiota and insulin resistance (**Figure 1**).

These findings are in line with independent studies that recently associated changes in BA turnover with *diabesity*. In turn, a reduction of the bacterial enzymatic activities involved in the conversion of primary into secondary BA was observed in diabetic patients compared to healthy controls, and linked to Firmicutes phyla (Labbé et al., 2014). A very similar pattern was also reported in obese patients with diagnosed metabolic syndrome, treated with antibiotic agents (vancomycin and amoxicillin) and associated with both a decreased prevalence of the Firmicutes population and a reduction of peripheral insulin sensitivity (Vrieze et al., 2014). Taken together, these data suggest a possible link between BA and metabolic health (Lefebvre et al., 2009). In line with these findings, BA have recently been proposed as metabolic integrators of whole-body energy homeostasis implicated in the regulation of various metabolic pathways, including their own synthesis and enterohepatic circulation, triglyceride, glucose, and energy homeostasis, by acting as signaling molecules through receptor-dependent and -independent pathways. The role of a dysregulation of this

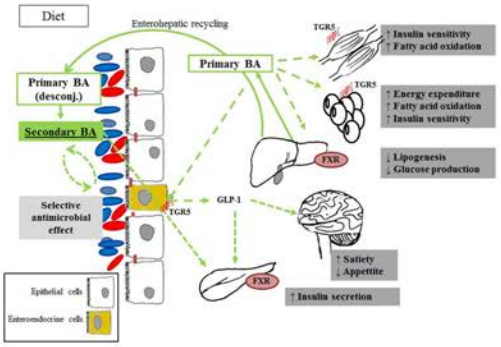

FIGURE 1 | Products of bile acids co-metabolism associated with obesity and diabetes via metabolomic studies. After the ingestion of food, conjugated BA are secreted into the intestinal lumen, are subjected to deconjugation by the intestinal bacteria, and converted to secondary BA. The main effect of secondary BA is mediated by the FXR activation, which in turn act increasing insulin secretion and sensitivity in the pancreas. Primary BA is also involved in glucose metabolism by TGR-5 activation and glucagon-like peptide-1 (GLP-1) release. Host endogenous processes are in solid lines, while microbial production and metabolite actions are in dotted lines.

BA-mediated metabolic control in the pathogenesis of T2D and co-morbidities, such as its attractiveness as a therapeutic target, is now beginning to be elucidated (reviewed in Prawitt et al., 2011). Their action on energy metabolism regulation would occur via both the activation of the nuclear receptor FXR and FXR-independent pathways, such as through the membrane receptor TGR5 expressed in several tissues including gall bladder, ileum, colon, and brown and white adipose tissue.

It is worth noting that preliminary evidence has shown that not all BA activate equally, and the microbial-derived production of secondary BA could be an important mechanism in the regulation of signaling pathways involved in the development of *diabesity* (Nguyen and Bouscarel, 2008). When gut microbial-derived secondary BA are bound to TGR5, the receptor is internalized and a series of adenylate cyclase-dependent signaling is triggered by activating distinct pathways involved in glucose and lipid energy metabolism (Kawamata et al., 2003; Thomas et al., 2008). In tissues, such as brown adipose tissue and muscle, this would lead to an increased mitochondrial activity and oxidative phosphorylation, which has been linked to an insulin sensitization both in genetic and diet-induced models of *diabesity* (Watanabe et al., 2006). In enteroendocrine L-cells (Kawamata et al., 2003), in turn, these signaling pathways would enhance glucose metabolism by stimulating the production of glucagon like peptide, thereby promoting insulin secretion. Finally, recent studies have also shown that BA secretion improves insulin secretion, insulin sensitivity and whole-body glucose homeostasis (reviewed in Thomas et al., 2008), improving liver and pancreatic function in obese mice (Thomas et al., 2009; Tremaroli and Bäckhed, 2012).

Co-metabolism of Vitamins

Products of the gut microbiota-driven metabolic pathway of vitamins such as choline and niacin have been associated with obesity and diabetes.

Choline Metabolism

Although humans may produce choline endogenously (*de novo* hepatic synthesis), dietary intake (e.g., from egg yolk, liver, muscle meats, fish, nuts, legumes) is necessary to meet the demand for body health maintenance (Blusztajn, 1998; Zeisel, 2000). Once dietary choline reaches the intestine, anaerobic intestinal microorganisms, mainly of Firmicutes and Proteobacteria phyla (Romano et al., 2015) catalyze its conversion to TMA, which may be further degraded to several methylamines by the gut microbiota (sym-xenobiotic pathway; Harris et al., 2012), or is absorbed and oxidized to TMAO by the hepatic FMO3 enzyme. Choline may also be converted into betaine and further products (e.g., dimethylglycine) by mammalian mitochondrial pathways in the liver and kidney (Lever and Slow, 2010) (**Figure 2**). The bacterial gene clusters responsible for anaerobic choline degradation started to be identified only recently. Bacterial species belonging to Firmicutes, Actinobacteria and Proteobacteria phyla have been revealed as possessing the necessary enzymatic activities, while Bacteroidetes would be apparently deprived (Craciun and Balskus, 2012). However, the complete diversity of species that contribute to TMAO production in humans still remains unknown (Romano et al., 2015).

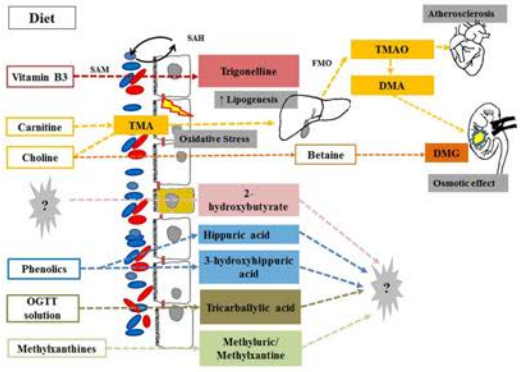

FIGURE 2 | Products of vitamin, phytochemical and purine co-metabolism associated with obesity and diabetes via metabolomics studies. Intestinal microorganisms catalyze the conversion of dietary choline and carnitine into TMA with a direct effect on the intestinal mucose (increased oxidative stress) and is the substrate for the hepatic production of TMAO (associated with cardiovascular disease risk) and TMA. Choline may escape microbial degradation and convert into betaine and further products [i.e., dimethylglycine (DMG)] by mammalian mitochondrial pathways in the liver and kidney where they have an detrimental osmotic effects. For most of the host microbial co-metabolites associated with the diabese phenotype, the eventual role in glucose and lipid metabolism remains unknown. OGTT, Oral glucose tolerance test; SAH and SAM, *S*-adenosylmethionine to *S*-adenosylhomocysteine (methionine cycle).

Recent studies have shown that circulating levels of choline and TMAO are related to cardiovascular disease risk (Dumas et al., 2006; Micha et al., 2010; Wang et al., 2011; Koeth et al., 2013; Warrier et al., 2015), and the gut microbiota-driven TMA-FMO3-TMAO pathway has been particularly recognized as a key regulator of lipid metabolism and inflammation. Increased levels of TMAO have been observed in a leptin-deficient murine model of obesity and T2D (Gipson et al., 2008; Won et al., 2013) and revealed a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice (Dumas et al., 2006). The systemic perturbations of key metabolites of choline have also been related to the progression of T2D, suggesting that in the early stages of diabetes an attenuated conversion of choline into dimethylglycine may occur, which can be observed by the increased levels of TMAO and TMA, with a reversion of this behavior at a later stage of the disease (Guan et al., 2013).

Messana et al. (1998) published the first study linking TMAO and T2D in humans. Using a ^{1}H-NMR approach, increased levels of TMAO and dimethylamine were observed in the urine of diabetic individuals compared to a group of healthy individuals, and were present in high concentrations even in diabetics with good metabolic control (i.e., absence of glycosuria and glycohemoglobin). In the last decade, little progress has been made on the mechanisms that would directly or indirectly involve TMAO in the development of diabetes. Nevertheless, the potential role of an altered composition of the microbiota and its ability to metabolize choline in glucose homeostasis and disease development has become increasingly relevant (Dumas et al., 2006).

To the best of our knowledge, three further metabolomic studies observed a change in choline metabolism, which was associated with impaired glycemic control in humans, within a wide range of BMI (Salek et al., 2007; Huo et al., 2009; Zhang et al., 2009; **Table 1**). In all of them, fasting biosamples were analyzed, thereby avoiding fluctuations in choline concentrations due to dietary intake. Salek et al. (2007) carried out a ^{1}H-NMR-driven metabolomic comparison of urinary changes linked to T2D both in animals (obese mice and rats with autosomal recessive defects in the leptin receptor gene – *db/db* and Zucker *fa/fa*, respectively), and individuals who were overweight to obsese (BMI = 25–40) compared to healthy lean controls. An increased excretion of a product of choline biotransformation, namely *N,N*-dimethylglycine and *N,N*-dimethylamine, distinguished the urinary metabolome of T2D in all species in the study (Salek et al., 2007), suggesting a possible increase in the choline turnover. The authors assumed that the *diabese* phenotype may have a major demand for choline, possibly due to an altered biosynthesis of lipoproteins, an altered metabolism of methylamines – which would play an important osmoregulatory role by degrading dietary choline – or to an altered intestinal microbiota composition (Salek et al., 2007). Although there is a scarcity of data in this regard, Firmicutes and Proteobacteria seem to be the most implicated phyla in the conversion of choline to TMAO (Romano et al., 2015). For this reason, the decline of choline circulating levels and increase of choline subproducts such as TMAO and DMA in obese subjects would be in line

with the increase of the Firmicutes phylum associated with obese phenotype (Ley et al., 2006).

The role of BMI in the observed association was partly downsized in a second study (Zhang et al., 2009). By applying a similar untargeted and ^{1}HNMR-driven approach, in fact, Zhang et al. (2009) demonstrated a decrease in the serum levels of choline in normal-weight subjects with T2D (BMI = 25.9 ± 9.0) compared to non-diabetic lean individuals (normal or IGT); however, no changes in the downstream products of choline metabolism were detected. Aside from increased insulin resistance, the decrease in serum choline is linked to a specific shift in the gut microbial community in the diabetic patients (relative increase in Firmicutes) and to an increase in the prevalence of T2D complications, as NAFLD (Zhang et al., 2009). The role of the microbial community hosted by *diabese* subjects in altering choline metabolism was also tested by assessing the effects of antidiabetic medication (Huo et al., 2009). As shown in **Table 1**, Huo et al. (2015) observed increased serum levels of TMAO in overweight diabetic subjects receiving metformin treatment *versus* untreated *diabese* controls, which may indicate an intestinal bacterial regulation function of metformin. It has already been suggested that antidiabetic treatments have a beneficial effect on gut microbiota metabolism (Huo et al., 2015), although the exact mechanisms are still unclear (Moreno-Navarrete et al., 2012). The authors suggested a link between the deregulation of choline metabolism in T2D and a rupture of the intestinal barrier by oxidative stress (Wei et al., 2008). In any case, a possible two-way relationship between anti-T2D treatment and gut microbiota has been hypothesized.

Niacin Metabolism

Alterations in the niacin (vitamin B3) metabolism have also been observed in association with obesity and T2D, and due to the overlapping in the choline/niacin catabolic pathways (i.e., via betaine and glycine metabolism), may also reflect a dysregulation in choline metabolism (Huang et al., 2012). Through a LC–MS-driven metabolomic approach, an altered urinary excretion of nicotinuric acid (*N*-nicotinoyl-glycine) was recently proposed as a potential marker of metabolic syndrome diagnostic traits and of cardiometabolic risk (Huang et al., 2012). Similarly, an association between the presence of trigonelline (betaine nicotinate) and obese and diabetic phenotypes has been proposed. Despite having a possible exogenous (dietary) origin, trigonelline is mostly biosynthesized by the gut microbiota during the conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine (methionine cycle). By applying a ^{1}H-NMR-based metabolomics approach, Salek et al. (2007) found lower levels of trigonelline in the urine of diabetic (*db/db*) mice and humans, associated with a change in energy and tryptophan metabolism. Further animal (Salek et al., 2007; Won et al., 2013) and human studies (Calvani et al., 2010) confirmed a decline of trigonelline in obesity and diabetes, some authors suggesting that oxidative stress possibly has a role (i.e., via glutathione store depletion) in the observed relationship (Calvani et al., 2010). Trigonelline is known to be involved in major physiological functions including lipid and carbohydrate metabolism.

Co-metabolism of Organic Acids and Derivates

Calvani et al. (2010) identified high levels of 2-hydroxyisobutyrate in the urine of obese people, and the change was associated with a reduced bacterial diversity in 'obese' gut microbiota possibly involved in nutrient and energy harvest (**Tables 1** and **2**). In particular, 2-hydroxyisobutyrate is a product of the microbial degradation of dietary proteins that escape digestion in the upper gastrointestinal tract, and its production has been associated with the presence of specific microbial members such as *Faecalibacterium prausnitzii* (Li et al., 2008), butyrate-producer species with anti-inflammatory effect and to be in low levels in obese and *diabese* individuals compared to healthy subjects (Qin et al., 2012). In addition, Campbell et al. (2014) observed that obese subjects involved in a dietary weight loss program had higher levels of tricarballylic acid after an OGTT compared with the fasting concentrations. Interestingly, tricarballylic acid is a product of gut microbial metabolism of food-derived trans-aconitate, described as an additive contained in the OGTT solution. Once again, the authors suggested a two-way relationship between the obese and gut microbial phenotype (tricarballylic acid production would in turn increase the metabolic activity of specific gut microbes associated with its production).

Co-metabolism of Phytochemicals and Purines

Hippuric acid and 3-hydroxyhippuric acid are two normal urinary components mainly derived from the degradation of plant (poly)phenols and aromatic amino acids (i.e., phenylalanine and tryptophan) by a range of gut microbes, recently found to belong to *Clostridium* sp. (Li et al., 2008). The resulting benzoic acid is then absorbed, subjected to glycine conjugation reaction (by mitochondrial glycine *N*-acyltransferase) and finally excreted in urine (Huo et al., 2015). Decreased levels of hippuric acid (Salek et al., 2007; Calvani et al., 2010; Zhao et al., 2010) and 3-hydroxyhippuric acid in urine have been related to IGT and obesity (Zhao et al., 2010) in both animal and human studies. In turn, the downregulation was reduced in T2D patients after the treatment with sulfonylurea, suggesting the drug potentially has a protector effect on gut microbiota metabolism (Huo et al., 2015). However, a strict dietary assessment is mandatory to dismiss any diet-dependent variation among groups, due to the wide range of phenolic compounds leading to these last-step metabolites following microbial and human biotransformations (lack of specificity; Salek et al., 2007). Moreover, the reasons for their putative associations with obesity and T2D are unknown. Salek et al. (2007) suggested that hippurate could be related to age progression and gender effects on metabolism in T2D, but these suppositions need to be further investigated.

Zhao et al. (2010) observed that subjects with IGT had a reduced excretion of methyluric acid and methylxanthine, which are products of the microbial metabolism of methylxanthines contained for instance in coffee and tea. The authors tentatively interpreted the observed changes as the result of an altered gut

microbiota in the presence of insulin resistance, although their putative role in glucose metabolism is still unknown.

CONCLUSION

Current public health burdens such as obesity and T2D are complex, polygenic, multifactorial diseases with a strong metabolic etiology. Gut microbiota have recently been proposed as a crucial environmental factor in their development, but the metabolic complexity of the symbiotic interaction between the host individual and its microbial community, as well as the impact of this crosstalk between body weight changes and glucose homeostasis, are still unclear.

However, our review highlighted how few metabolomic studies have been specifically conducted so far to identify the role of the gut microbiota in the development and progression of obesity and T2D, at least in humans.

Despite the scarcity, heterogeneity and intrinsic limitations of the metabolomic studies conducted so far aimed at identifying the role of the gut microbiota in the development and progression of obesity and T2D (i.e., wide range of BMI and/or glycemic status evaluated, important sources of variability not considered including ethnic, gender effects, and dietary assessments), the results obtained by these data-driven metabolomic approaches are in line with findings independently obtained from *in vitro* or animal model studies. Products of the microbial/host metabolism of BA, vitamins (choline, niacin), branched fatty acids, purines and phenolic compounds have been described as being altered in (pre-)diabetic subjects, with or without increased BMI, compared with healthy controls. Moreover, few articles show a clear relation between metabolites and their bacterial producers in terms of the complexity of the gut microbiota and the cross-feeding mechanisms that would have combined bacterial effects in the colon ecosystem.

More efforts should be directed in the future toward expanding our knowledge of the metabolic interactions of the host and the gut microbiota, particularly through a strict evaluation of the lifestyle factors (i.e., diet) strongly involved in the modulation of this crosstalk.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01151

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Material Suplementario

REVISION 1

frontiers

Supplementary Material

Metabolomic insights into the intricate gut microbial–host interaction in the development of obesity and type 2 diabetes

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1 Supplementary Data

1.1 Descriptive Results

The search in PubMed and Web of Science databases provided 75 and 162 publications respectively. After adjusting for duplicates, 170 remained, while the first-step selection based on a review of titles, abstracts and keywords excluded 121 publications.

The subjects in study were all adults (18–79 years old) but from different ethnic groups, including Han Chineses (Zhang et al., 2009), Caucasians (Campbell et al., 2014), black people (Campbell et al., 2014), Asians (Campbell et al., 2014), Hispanics (Campbell et al., 2014) and individuals of unknown ethnicity (Zhao et al., 2010; Calvani et al., 2010; Huo et al., 2009; Salek et al., 2007; Huo et al., 2014).

The objectives and study designs were quite heterogeneous, and ranged from exploring metabolomic difference between subjects with different metabolic status (Suhre et al., 2010; Zhao et al., 2010; Zhang et al., 2009; Calvani et al., 2010; Salek et al., 2007; Campbell et al., 2014) to assessing the effects of antidiabetic drugs on the gut microbial ecology and metabolic activity (Huo et al., 2009, 2014). Except for one case (Zhang et al., 2009), the reviewed studies were focused on the co-presence of both metabolic diseases (*diabesity*), and one prospective study described the gut microbial-related metabolomic changes following a diet and exercise weight-loss program in obese and insulin resistant females, thus showing a possible association with reduction in BMI (Campbell et al., 2014).

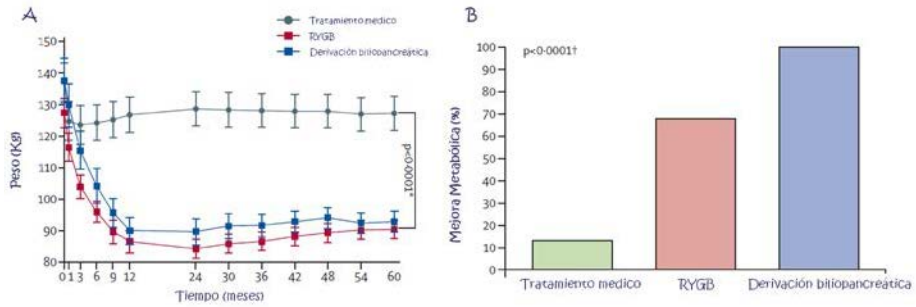
1.2.2 Estrategias de reducción de peso encaminadas a mejoras metabólicas

Estudios clínicos han demostrado que en sujetos con sobrepeso y obesidad una reducción de peso del 5% ya se observan mejoras sobre la salud y la calidad de vida, disminuye el riesgo de desarrollar DT2 en un 30%-60% y reduce en un 2% la glucosa en ayunas en pacientes con obesidad y DT2[53]. La Asociación Americana de Endocrinólogos Clínicos (AAEC) subraya la necesidad de priorizar la mejora de las complicaciones asociadas a la obesidad como punto terapéutico primario sobre la disminución preestablecida del peso *per se*[54]. En 2014, en el Consenso de Obesidad entre AAEC y el Colegio Americano de Endocrinología se acentuó la necesidad de ir más allá del diagnóstico de la obesidad y de potenciar la mejora de la salud y la calidad de vida del paciente[55].

Aún así no está claro si los beneficios metabólicos de la reducción de peso dependen de la pérdida de peso en sí o de otros mecanismos implicados independientes del peso. Los estudios demuestran que la remisión de la resistencia a la insulina y DT2 podría ocasionarse incluso antes de una pérdida de peso significativa[56]. De esta forma, se vuelve a evidenciar un factor desconocido ligado a la resistencia a la insulina y DT2 independiente de la obesidad.

A día de hoy se diferencian dos grandes enfoques para la pérdida de peso y mejoras metabólicas: intervenciones comportamentales a través de las mejoras en el estilo de vida (dieta y ejercicio) y cirugía bariátrica (**Figura 8**)[54].

Figura 8. Cambios en el peso y mejoras metabólicas al largo de 5 años de seguimiento de una intervención de pérdida de peso por tratamiento médico, *bypass* gástrico en Y de Roux y derivación biliopancreática



A) Mediana (Desviación estándar) de los cambios de peso. B) Proporción de pacientes que alcanzan mejoras metabólicas (glicemia, presión arterial y control lipidémico)
Fuente: adaptado de Mingrone *et al.* [57]

1.1.1.1 Intervención por cirugía bariátrica

La cirugía bariátrica es el tratamiento más efectivo y duradero de la obesidad y la DT2[57]. En 1987, el estudio Swedish Obese Subjects (SOS) fue el primer estudio a largo plazo que proporcionó información acerca de los efectos de la cirugía bariátrica sobre la incidencia de los factores de riesgo cardiovasculares y la mortalidad total[58].

El estudio Swedish Obese Subjects (SOS) es un estudio de intervención prospectivo, no-randomizado y controlado que examina los efectos de la cirugía bariátrica sobre la mortalidad y las enfermedades asociadas a la obesidad (Núm: NCT01479452). El estudio empezó en 1987 y tuvo un seguimiento de 4047 sujetos en los dos años y de 1703 sujetos en los diez años con, reclutando pacientes de 480 centros de salud primaria de Suecia. Este estudio lo componen dos brazos paralelos, la cirugía bariátrica vs el tratamiento convencional de la obesidad (https://clinicaltrials.gov/ct2/show/NCT01479452).

La cirugía bariátrica es el tratamiento de elección en pacientes con IMC≥ 40 kg/m^2 con o sin otras complicaciones médicas[54]. A pesar de los riesgos de la cirugía, como potenciales deficiencias nutricionales, aumento de peso en algunos pacientes y la necesidad de la monitorización médica, en 2017 Rubio *et al*. describió la cirugía

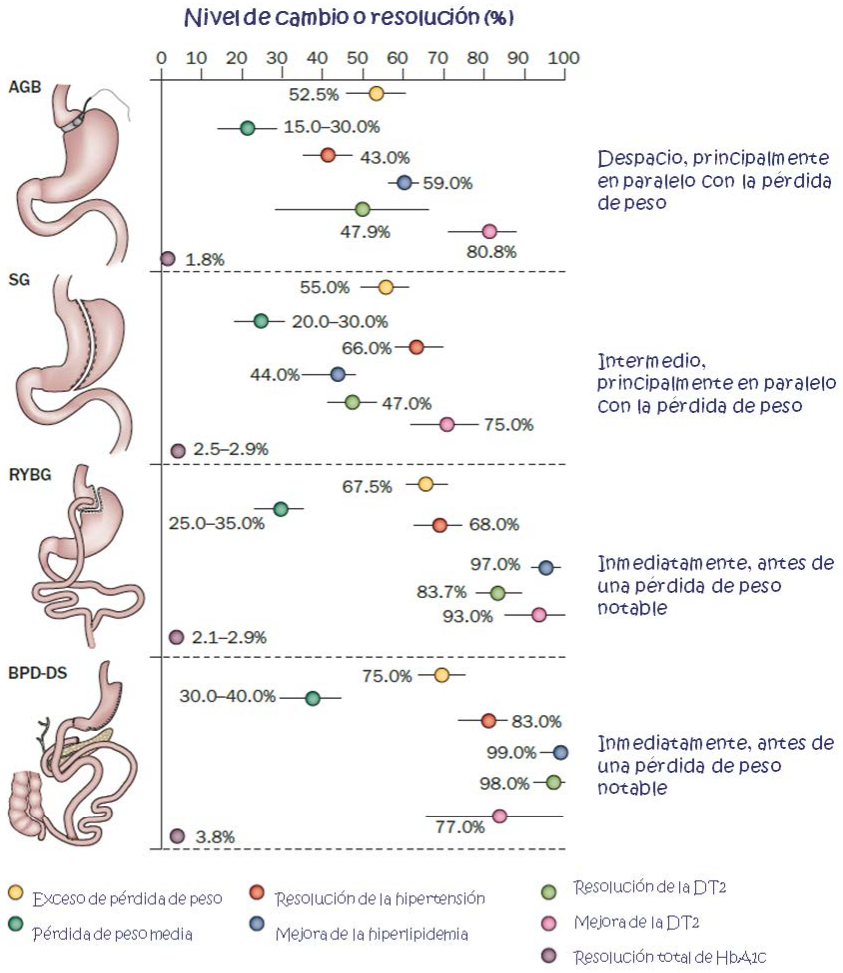
bariátrica como tratamiento para la mejora metabólica y no sólo para la pérdida de peso, dando lugar así el término de **cirugía metabólica**[59]. Actualmente las guías clínicas, como la guía de la AACE ya recomiendan la cirugía bariátrica en pacientes con un IMC ≥ 35 kg/m^2 y con la presencia de una o más complicaciones relacionadas con la obesidad, e incluso en pacientes con IMC ≥ 30 kg/m^2 y complicaciones aunque en este último caso no hay suficientes estudios[54,60].

La cirugía bariátrica se pueden categorizar según el mecanismo de acción, técnicas restrictivas, técnicas malabsortivas y técnicas que combinan los dos mecanismos. Las técnicas restrictivas limitan el tamaño del estómago y por lo tanto reduce la toma de alimentos, incluyendo la gastrectomía en manga (GM) y las bandas gástricas ajustables de laparoscopia (LAGB). Las técnicas malabsorptivas reducen la capacidad de absorción de los nutrientes debido a un intestino pequeño, como el cruce duodenal (DS). Hay procedimientos que tienen efectos restrictivos y malabsortivos como *bypass* gástrico en Y de Roux (RYGB)[61]. En todos ellos se observa una mejora de los niveles glicémicos y una reducción de la necesidad de medicación. Incluso la mayoría de pacientes alcanzan la remisión de diabetes (**Figura 9**). En general, hay estudios que han apuntado que a los 3-10 años de la intervención hay un incremento de los niveles de glucosa en sangre y la necesitad de restituir la medicación de aplicación a pacientes con diabetes.

Sorprendentemente, la mejora o la remisión de la DT2 después de la RYGB ocurre rápidamente al cabo de pocos días de la operación, e incluso antes de que el paciente se haya dado de alta en el centro hospitalario tras la cirugía (**Figura 9**). Este hecho ha conducido a cuestionar los mecanismos implicados en el desarrollo de la DT2 dependientes e independientes de la adiposidad del individuo y destacando posibles mecanismos de fases tempranas o de corto plazo y otros de fases tardías o de largo plazo.

En tres grandes grupos, los mecanismos implicados en las mejoras de la DT2 asociados a la cirugía bariátrica son: cambios en las hormonas intestinales, implicación de los ácidos biliares y/o el perfil del microbioma[62].

Figura 9. Resumen de los efectos de los principales tipos de cirugía bariátrica sobre la pérdida de peso (exceso y media de pérdida de peso) y sus principales comorbilidades (hipertensión, hiperlipidemia y diabetes tipo 2) a los 3-5 años.



Valores expresados como media de la eficacia y 95% de intervalo de confianza. La columna de la derecha indica el patrón temporal de los efectos de la diabetes tipo 2. Abreviaciones: AGB del inglés, adjustable gastric banding; BPD-DS del inglés, biliopancreatic diversion with or without duodenal switch; RYGB del inglés, Roux-en-Y gastric bypass; SG del inglés, sleeve gastrectomy. Fuente: traducido de Frühbeck et al.[63].

A nivel hormonal, se ha observado que los beneficios de la cirugía bariátrica se ven reflejados a corto plazo, especialmente por el incremento de las incretinas (glucagon-like peptide 1 (GLP-1) y la glucosa-dependiente *insulinotropic polypeptid* (GIP)). Estas hormonas tienen efectos insulinotrópicos ─estimulan o afectan a la producción de insulina. Además, GLP-1 estimula la toma de glucosa periférica,

retrasa el vaciamiento gástrico, disminuye la secreción de glucagón, disminuye el apetito y consecuentemente la toma de alimentos. Los niveles de GLP-1 post-cirugía podrían alargarse hasta los 12 y 15 meses. La mejora de la sensibilidad de insulina a nivel periférica a los 3 a 12 meses se ha asociado a una disminución de la grasa[62].

A pesar de las contradicciones entre estudios la mayoría parece apuntar que los ácidos biliares resultan alterados después de la cirugía bariátrica. Incluso pacientes diabéticos presentaron mayores niveles de ácidos biliares en ayunas que aquellos sin diabetes. Los ácidos biliares son activadores de receptores involucrados en el metabolismo de la energía y de la glucosa como el TGR5 y FXR respectivamente[64].

Como se ha comentado anteriormente, la microbiota intestinal es un factor clave asociado con la obesidad y la diabetes. Después de la cirugía bariátrica la composición y diversidad de la microbiota intestinal se ve afectada[65], de este modo, el ratio *Firmicutes*/*Bacteroidetes* disminuye, asociándose con cambios funcionales como alteraciones del flujo biliar, flujo de nutrientes, niveles hormonales, así como restricción gástrica, modulación del estado inflamatorio por cambios en la permeabilidad intestinal[66]. Estos cambios podrían ser responsables de los beneficios de la cirugía bariátrica a largo plazo.

Otros autores han relacionado los cambios a corto plazo con la restricción calórica *per se* asociadas a los primeros meses de la intervención con una disminución drástica de las >3000 kcal/día a las 300 kcal/día[67,68], aunque otros autores lo han desmentido[69].

1.1.1.2 Intervención por cambio de estilo de vida: dieta y ejercicio físico

Diferentes estudios clínicos aleatorizados, como el Programa de Prevención de Diabetes (del inglés *Diabetes Prevention Program*)[70], el Look AHEAD (acrónimo del inglés, *Action for Health in Diabetes*)[71], el estudio fínese de Prevención de Diabetes (del inglés, *Finnish Diabetes Prevention Study*)[72] y el estudio Da Quing[73], han demostrado la eficacia de cambios de estilo de vida para la prevención de la DT2.

Cambios de estilo de vida ligados a una reducción de las calorías ingeridas (entre 500-1000 calorías/día) han demostrado una disminución dede la glucosa en ayunas y la resistencia a la insulina[74–76]. La OMS enfatiza la necesidad de cambiar las políticas sanitarias, de urbanismo y de la industria alimentaria para dar soporte al acceso a un estilo de vida saludable combinando el consumo de un **patrón alimentario saludable** y ejercicio físico. A nivel general la OMS recomienda[77]:

- Limitar el consumo de energía procedente de grasas y azúcares.
- Incrementar el consumo de verduras y vegetales, así como de legumbres, cereales integrales y nueces.
- Mantener una actividad física regular (60 minutos por día en niños y 150 min/semana en adultos).

La Dieta Mediterránea como intervención dietética saludable para la pérdida de peso

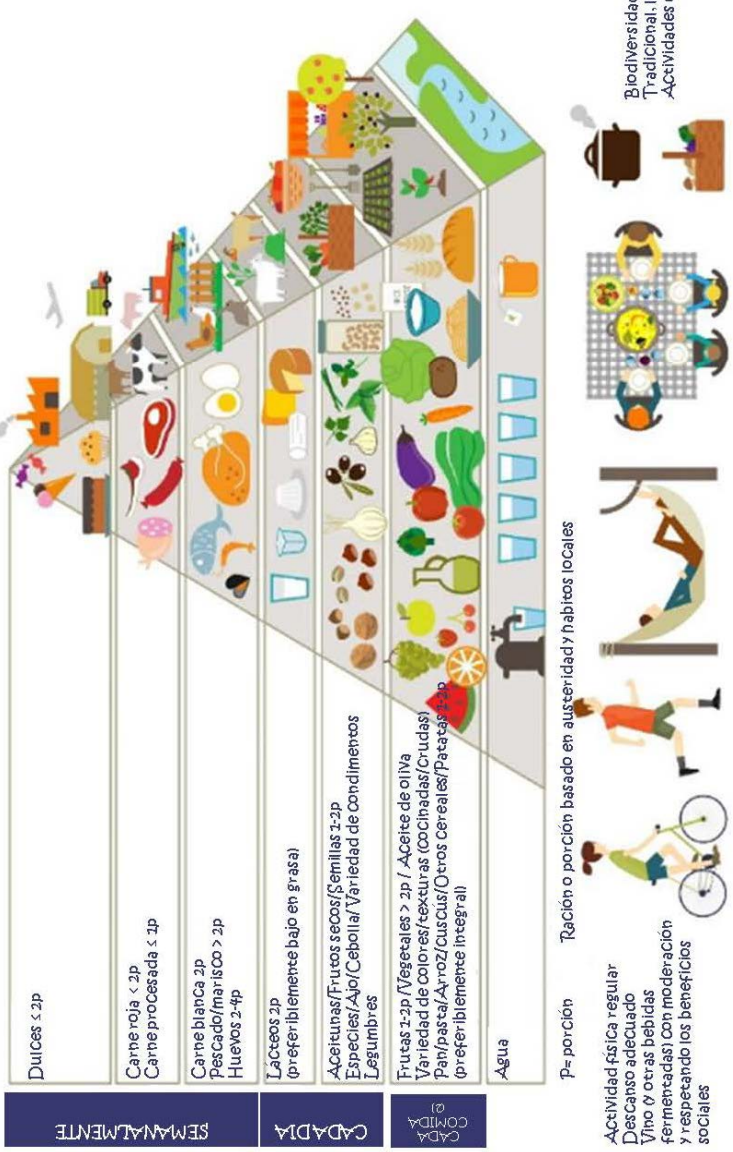
La Dieta Mediterránea (DietMed) se ha postulado como un patrón alimentario saludable con la evidencia científica más elevada en este campo de aplicación. La DietMed es un patrón de estilo de vida de los países del Mediterráneo, que contiene variaciones culturales específicas de cada país. La DietMed tradicional se caracteriza por incluir: i) el aceite de oliva virgen extra como principal fuente de grasa, verduras (incluyendo las de hoja verde), frutas, cereales integrales, legumbres y frutos secos; ii) consumo moderado de pescado y aves de corral; iii) bajo consumo de productos lácteos, carnes rojas, productos cárnicos procesados y dulces; iv) consumo moderado de alcohol entre las comidas.

Además, incluye cambios cualitativos ligados a la forma de comer, entre ellos: i) moderación, tamaño de las porciones; ii) sociabilización; iii) la práctica regular de ejercicio físico; iv) descanso, durante la noche y durante el día en forma de siesta; v) consumo de alimentos estacionales, alimentos frescos y mínimamente procesados y vi) consumo de productos tradicionales, locales y respetuosos con el medio ambiente[78] (**Figura 10**).

Recientemente se ha propuesto el concepto de DietMed 4.0, la DietMed con cuatro beneficios sostenibles: i) mayores beneficios para la salud y la nutrición; ii) bajos impactos ambientales y riqueza en biodiversidad; iii) valores alimentarios socioculturales altos y iv) rentabilidad económica local positiva[79].

La sinergia de compuestos bioactivos que contiene una DietMed son los principales causantes de los efectos beneficiosos sobre la salud metabólica[80] y una disminución en la incidencia de DT2[81]. Además, la DietMed, con elevada proporción de grasa mono-insaturada comparada con una dieta baja en grasa como recomendaba años anteriores la ADA[82], proporciona un mejor control glicémico y un retraso en la necesidad de fármacos anti-diabéticos en pacientes diagnosticados *de novo*[83]. Adicionalmente se ha demostrado un papel protector en el desarrollo de síndrome metabólico.

Figura 10. La nueva pirámide de la Dieta Mediterránea 4.0



Fuente: traducido del *International Foundation of Mediterranean Diet* [84]

En las guías dietéticas de la *U.S. Department of Health and Human Services and U.S. Department of Agriculture (USDA*), *2015-2020*[85] la DietMed forma parte de los tres patrones alimentarios saludables. El aceite de oliva o las legumbres, elementos claves de la DietMed también figuran en las guías de la USDA. Las guías americanas han sido implementadas con el concepto del "MyPlate" (**Figura 11**), un símbolo que sirve como recordatorio de consumo de patrones alimentarios saludables mediante la selección de alimentos saludables entre grupos de alimentos y bebidas, alineados con las recomendaciones de la USDA[85]. Según la USDA y el informe consenso de la ADA y la Asociación Europea para el estudio de la diabetes, la DietMed es un patrón alimentario recomendado en pacientes con DT2[86].

Figura 11. Implementación de las guías *Guías Dietética*s estadounidenses mediante el concepto "MyPlate"

Fuente: extraído de U.S. Department of Health and Human Services and U.S. Department of Agriculture. *2015–2020 Dietary Guidelines for Americans*. 8th Edition. December 2015. Disponible a: http://health.gov/dietaryguidelines/2015/guidelines/.[85]

Existen numerosos estudios que evidencian el efecto de la DietMed en el control de peso en individuos con obesidad[83]. Incluso, una elevada adherencia a la DietMed se ha asociado con un mantenimiento, pérdida de peso y reducciones en la circunferencia, así como una reducción de la grasa abdominal[87]. Estos beneficios se han visto acentuados cuando la DietMed incluye restricciones calóricas y/o la práctica regular de actividad física[86] .

No obstante, al cabo de un año de intervención por cambio de estilo de vida, más del 75 % de los pacientes ganan alrededor de 1-2 kg/año[88]. Esto ha evidenciado la dificultad de cambiar hábitos a largo término, influenciados fuertemente por el entorno en que vivimos[89].

Para identificar los efectos comunes y diferenciales de los dos tratamientos para la pérdida de peso, cambio de estilo de vida y cirugía bariátrica, en la presente tesis doctoral se realizó una actualización del estado del arte. Este trabajo se presenta en formato de revisión bibliográfica, dónde se revisan estudios de metabolómica dirigida y no-dirigida en humanos que identifican metabolitos asociados con la intervención de pérdida de peso por cirugía y por cambio en el estilo de vida en sujetos con obesidad.

A continuación se adjunta la revisión y el material suplementario de:

Revisión 2. *Sara Tulipani, Jules Griffin, Magali Palau-Rodriguez, Ximena Mora-Cubillos, Rosa M. Bernal-Lopez, Francisco J. Tinahones, Barbara E. Corkey y CristinaAndres-Lacueva. Metabolomics-guided insights on bariatric surgery versus behavioral interventions for weight loss. Obesity 2016 24:2451–2466. doi: 10.1002/oby.21686*

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Review
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Metabolomics-Guided Insights on Bariatric Surgery Versus Behavioral Interventions for Weight Loss

Sara Tulipani[1,2], Jules Griffin[3,4], Magali Palau-Rodriguez[1], Ximena Mora-Cubillos[1], Rosa M. Bernal-Lopez[5,6], Francisco J. Tinahones[2,6], Barbara E. Corkey[7], and Cristina Andres-Lacueva[1]

Objective: To review the metabolomic studies carried out so far to identify metabolic markers associated with surgical and dietary treatments for weight loss in subjects with obesity.
Methods: The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed.
Results: Thirty-two studies successfully met the eligibility criteria. The metabolic adaptations shared by surgical and dietary interventions mirrored a state of starvation ketoacidosis (increase of circulating ketone bodies), an increase of acylcarnitines and fatty acid β-oxidation, a decrease of specific amino acids including branched-chain amino acids (BCAA) and (lyso)glycerophospholipids previously associated with obesity, and adipose tissue expansion. The metabolic footprint of bariatric procedures was specifically characterized by an increase of bile acid circulating pools and a decrease of ceramide levels, a greater perioperative decline in BCAA, and the rise of circulating serine and glycine, mirroring glycemic control and inflammation improvement. In one study, 3-hydroxybutyrate was particularly identified as an early metabolic marker of long-term prognosis after surgery and proposed to increase current prognostic modalities and contribute to personalized treatment.
Conclusions: Metabolomics helped in deciphering the metabolic response to weight loss treatments. Moving from association to causation is the next challenge to move to a further level of clinical application.

Obesity (2016) **24**, 2451-2466. doi:10.1002/oby.21686

Introduction

Despite evidence to support their utility, lifestyle-based strategies for weight loss and treatment of obesity (i.e., based on diet and physical activity) have met so far with little success in the long term in terms of permanent weight loss (1). Bariatric surgery is the only current treatment for obesity leading to sustained weight loss (2) and to improvements in glucose regulation, up to a complete remission of type 2 diabetes (T2D) in the short and long follow-up (3-6). Consistent with the causative role of several organs being involved in metabolic homeostasis and both the development of T2D and obesity (e.g., including pancreatic islets, liver, fat cells, but also brain, gut, vasculature, muscle) (7-9), it is accepted that metabolic surgery (the remodeling of metabolism following weight loss) would act on a global rather than local scale through the restoration

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Author contributions: S.T. and C.A.-L. designed the research; M.P.-R., X.M-C., and S.T. conducted the studies' eligibility assessment, the data extraction, and the table generation; S.T., M.P.-R., J.G., B.E.C., F.J.T., and C.A.-L. generated the discussion on the extracted data; S.T. wrote the paper; J.G., B.E.C., F.J.T., and C.A.-L. reviewed/edited the manuscript and proportionate a critical cut to the work though their scientific perspectives; S.T. and C.A.-L. are the guarantors of the study and have the primary responsibility for final content. All authors read and approved the final manuscript.
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of physiological pathways in a wide range of tissues and organs (10). However, a definitive explanation of its systemic effects is still lacking. Evidence also demonstrates that the remission of insulin resistance (IR) and T2D takes place even before significant weight loss has been achieved (within days to weeks after surgery) and does not necessarily associate either with the overall weight loss in the longer term or the surgical technique employed, confirming that other mechanisms beyond weight loss should explain both the rapid and sustained impact of the surgery on the metabolic improvements detected in individuals (11-13). Furthermore, the overall weight loss achieved by the different procedures did not differ in the longer term and was not significantly associated with T2D outcome; thus other mechanisms beyond weight loss should explain the rapid and sustained impact of malabsorptive techniques on IR and glycemic control (14).

Understanding the early and prolonged metabolic adaptations to both dietary and bariatric weight loss procedures may allow us to gain insights into shared and exclusive mechanisms of action of weight loss, help to dissect heterogeneity in response, identify individuals who would more likely benefit from moderate weight loss, predict prognosis, and ultimately guide personalized treatment (15-21) (as reviewed in Ref. 22).

In this context, metabolomics—namely the comprehensive study of ideally all endogenous and exogenous metabolites contained in a biological system at a given moment—is currently considered as the most appropriate *omics* technology to investigate complex, polygenic, and multifactorial diseases with a strong multisystemic metabolic nature (23) such as obesity and T2D (22,24-27).

The primary objective of this article was to review the metabolomic studies carried out so far to identify metabolic markers of response to surgical and dietary treatments for weight loss, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Methods

Data sources

Two electronic databases (PubMed and Web of Science) were searched for keywords (details in Online Supporting Information). Species (human), language (English), and publication date restrictions (from 2000 to date, last search on 4th December 2015) were imposed. Relevant references identified from pertinent articles were additionally reviewed.

Study selection

Types of interventions, study designs, and participants. Prospective intervention trials carried out to study the metabolic response to surgical and/or dietary treatments for weight loss in adult subjects with overweight or obesity (males and females, BMI > 25 kg/m^2) were included. Randomization was expected only for parallel-arm lifestyle intervention trials (surgical procedures being applied according to clinical decision).

Analytical approach. Targeted and untargeted metabolomic approaches driven by mass spectrometry (MS) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) techniques were both included in the selection.

Types of outcome measures. Low-molecular-weight (<1,000 Da) metabolites significantly up- or downregulated following surgical or behavioral weight loss interventions, with respect to baseline and to the eventual parallel intervention, were the primary outcome measures of interest of the review. In the case of single-arm (quasi) trials, the post-intervention changes with respect to baseline were of primary interest, while baseline differences with respect to controls (i.e., lean healthy subjects) were not considered since they were not universally available. Weight loss was the cutoff clinical outcome required for inclusion in the review, except for acute postsurgery studies. In the case of parallel-arm studies, only those metabolites that differed systematically post-intervention (within-group variation during follow-up with respect to baseline) and between interventions (between-group variation) were considered as potential biomarkers of a given intervention, to minimize potential confounders. In the case of multi-time point follow-up studies, only metabolites that significantly differed from baseline at any follow-up point were considered in the summary tables. Additional clinical outcome measures such as known descriptors of glycemic control [i.e., fasting glucose, glucose tolerance, IR—HOMA (homeostasis model assessment), HbAc1b] were reported, when available.

Data extraction

Eligibility assessment was carried out independently by two authors using predefined data fields in an unblinded standardized manner. A third author conducted an independent review of the extracted articles, and cases of disagreements were resolved by majority consensus.

Results

Of the 304 studies initially retrieved, 32 successfully met the eligibility criteria for inclusion in the review (Figure 1).

Study designs

As summarized in Tables 1 and 2, studies ranged from parallel-arm trials comparing over time the effects of surgical versus dietary interventions or of different treatments of the same category (surgical only and behavioral only) up to the analysis of single-arm anti-obesity interventions in respect to baseline. Only 2 of the 32 selected studies included an independent cohort in the study design to replicate/validate the obtained findings (28,29).

Participants

The majority of the studies consisted of small-scale trials (n = 6-71 participants). Only one large-scale metabolomic study (n = 500) has been carried out so far to investigate the effect of a combined behavioral intervention (diet and exercise) on subjects with obesity (validation cohort of the WLM trial) (29). The participation of women was generally higher, or even exclusive (29-33), but gender-dependent variation in the metabolic response to treatment was only discussed in three studies (29,34,35).

Interventions

Gastric bypass (Roux-en-Y, RYGB) was the most widely investigated among surgery procedures (28,29,33,36-53), followed by sleeve gastrectomy (30,40,48,53,54), adjustable gastric banding (36,39), and biliopancreatic diversion with duodenal switch (BD-DS) (41,49). Behavioral weight loss strategies mainly consisted of

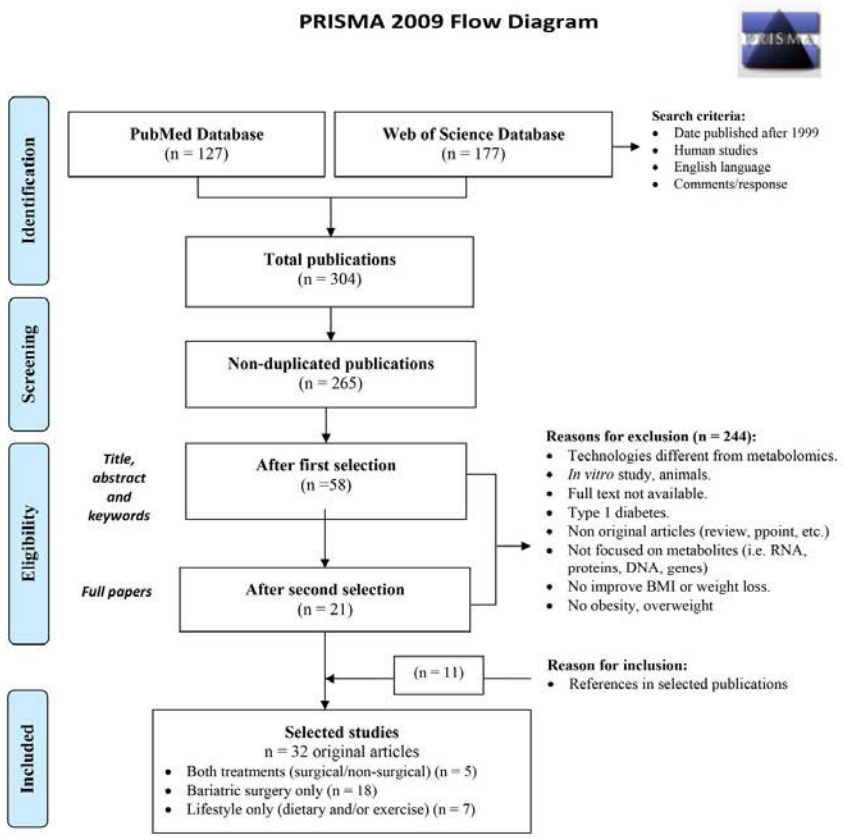

Figure 1 Schematic overview of the search strategy for this review. [Color figure can be viewed at wileyonlinelibrary.com.]

randomized controlled dietary intervention trials, and only three studies also included progressively increasing physical activity in a lifestyle treatment plan (29,32,55). Except for sporadic exceptions (56), hypocaloric diets were the most common interventions, *albeit* with a variable degree of caloric restriction (i.e., −600 to −1,200 kcal/day) (30,31,35,54,55), personalization (i.e., −15% of daily energy requirements) (57), and modification of the macronutrient proportion (i.e., low- and very-low-carbohydrate diets, high vs. low protein intake). In a few cases, weight loss was achieved by adopting a meal replacement diet and through the distribution of specific dietary items to all subjects during weekly visits (28,58).

Follow-up and bio-specimens

The duration of the interventions, as well as the types of specimens and the collection frequency varied among studies, being a relevant source of heterogeneity. Three bariatric studies investigated acute postsurgical effects (≤7 days postoperatively) (40,42,48) as these studies focused on the cause of T2D remission rather than the effect. The majority of the works focused on mid-term (≤6 months) metabolic adaptations, while long-term effects (~1 year) have rarely been described to date (n = 4) (41,47,52,53).

Fasting blood serum and plasma collected in a fasted state were the most frequently accessed biological matrices, followed by urine (spot or 24 hr). Finally, the metabolomic profiling of dysfunctional tissues has been rare to date (55), probably due to invasiveness issues of taking biopsies.

Clinical outcomes

The outcomes of parallel-arm and single-arm designed studies are separately summarized in Supporting Information Tables S1 and S2.

TABLE 1 Summary of included prospective, parallel-arm metabolomic studies evaluating the efficacy of surgical and/or behavioral antiobesity interventions in adult subjects

Interventions	Analytical technique	Specimen	Time points	Participants	Matched groups/variables	Ref.
Bariatric surgery vs. dietary intervention						
RYGB vs. CR/MR (NYONRC cohort)	Targeted (Biocrates p150IDQ assay)	Plasma, fasting and post-insulin infusion	0, at 10 kg weight loss (RYGB = ~1m; CR =~2m)	RYGB = 10 (7F) mOB with T2D CR = 10 (2F) mOB with T2D	Matched for age, weight, BMI, T2D duration, glycemic control (HbA1c)	28
RYGB vs. CR/MR (NYONRC cohort)	Targeted (LC-ESI-QqQ-MS/MS)	Plasma, fasting	0, at 10 kg weight loss (RYGB = ~1m; CR = ~2m)	RYGB = 12 (12F) mOB with T2D CR = 10 (10F) mOB with T2D	Matched for age, gender, weight, BMI, T2D duration, glycemic control (HbA1c)	29
RYGB vs. CR	Targeted (LC-ESI-QqQ-MS/MS)	Plasma, fasting and post-mixed meal tolerance test	−10 d, 2 w	RYGB = 10 (7F) mOB with T2D CR = 10 (2F) mOB with T2D	Matched for age, BMI, T2D medications, insulin sensitivity, FG, fasting insulin	37
SG vs. CR/LowCHO	Targeted (Biocrates p150IDQ assay)	Serum, fasting	0, 6 m	SG = 14 (9F) OB, 8% with incident T2D CR/LowCHO = 12 (6F) healthy OB	Apparently unmatched groups for BMI, T2D, health status	54
SG vs. CR	Targeted (LC-ESI-QTRAP-MS/MS)	Feces	−1 d, 3 m, 6 m	SG = 5 (5F) healthy mOB CR = 5 (5F) healthy mOB	Matched groups for BMI, weight loss, age, sex, non-T2D, Bacteroides-enterotype	30
Bariatric surgery interventions						
Malabsorptive vs. restrictive	Targeted (HPLC-?)	Serum, fasting	0, 1 m, 3 m	MS = 19 (15F) mOB with GT (n = 3), IGT (n = 12), or T2D (n = 4) RS = 15 (3F) mOB with GT (n = 5), IGT (n = 5), or T2D (n = 5)	Matching strategy: nd	38
RYGB vs. AGB	Targeted (?-MS/MS)	Plasma, fasting and post-glucose/insulin infusion	0, at 20% weight loss (RYGB = 16 ± 2 w; AGB = 22 ± 7 w)	RYGB = 10 (9F), OB, non-T2D AGB = 10 (9F) OB, non-T2D	Matched for age, gender, BMI, weight loss, health status	39
RYGB vs. AGB	Targeted (LC-ESI-QqQ-MS/MS)	Plasma, fasting	0, 4 d, 6 w	RYGB = 12 (7F) morbidly, metabolic status: nd AGB = 6 (4F) mOB, metabolic status: nd	Matching strategy: nd	36

TABLE 1. (continued).

Interventions	Analytical technique	Specimen	Time points	Participants	Matched groups/variables	Ref.
RYGB vs. SG	Untargeted (GC-ESI-MS)	Plasma, fasting	−3 d, 3 d	RYGB = 8 (8F) mOB with T2D SG = 7 (6F) mOB with T2D	Matched for age, BMI, metformin therapy, caloric intake, glycemic control	40
RYGB (proximal vs. distal) vs. SG	Untargeted (^{1}H-NMR)	Serum, fasting	0, 3 m, 6 m, 9 m, 1 y	Proximal RYGB = 23 (7F) mOB, metabolic status: nd Distal RYGB = 40 (7F) mOB, metabolic status: nd SG = 8 (7F) mOB, metabolic status: nd	Unmatched for degree of obesity, comorbidities, eating behaviors, patient preferences	53
RYGB vs. DS	Targeted (LC-API-QqQ-MS/MS)	Serum, fasting	0, 6 w, 6 m, 1 y	RYGB = 31 (23F) severely OB with T2D (n = 6) DS = 29 (19F) severely OB with T2D (n = 6)	Matching strategy: nd	41
Prognostic markers of post-op glycemic control						
RYGB vs. DJB	Untargeted (^{1}H-NMR)	Plasma, fasting (?)	7 **d**	Improved = 10 (7F) OB with T2D Non-improved = 12 (6F) OB with T2D	Unmatched for age, gender, BMI, T2D duration, surgical procedure, since assigned after the follow-up	42
RYGB	Targeted (UPLC-ESI and GCxGC-ToF-MS)	Plasma, fasting	0, 4 **d**, 42 d	Improved = 7 (3F) OB with T2D Non-improved = 7 (7F) OB with med-T2D (n = 2 insulin therapy)	Missing	50
RYGB	Targeted (UPLC-ESI-QqQ-MS/MS)	Serum, fasting	0, 1 y	Improved = 26 (13F) OB with T2D Non-improved = 12 (7F) OB with T2D	At baseline, matched for BMI, SBP, DBP, waist, TAG, HDL, LDL, HOMA	52
Behavioral interventions						
CR/low-fat vs. EX	Targeted (LC-ESI-QqQ-MS/MS)	Skeletal muscle biopsies	0, 16 w	CR = 8 (5F) OW to OB, all with IGT EX = 8 (4F) OW to OB with IGT (n = 4) or NGT (n = 4)	Matched for age, gender, BMI, glycemic control	55
Low-fat + mixed nuts (30 g/d) vs. low-fat	Untargeted (LC-ESI-qToF-MS)	Urine, 24 h	0, 12 w	Nuts = 22 (9F) OB with MetS (IFG n = 17) Low-fat = 20 (10F) OB with MetS (IFG n = 13)	Matched for age, BMI, MetS components, baseline energy/ nutrient intake	58

TABLE 1. (continued).

Interventions	Analytical technique	Specimen	Time points	Participants	Matched groups/variables	Ref.
CR vs. CR diet rich in BCAA (20 g/d gelatin vs. whey protein supplementation)	Untargeted (GC-ToF-MS)	Plasma, fasting	0, 8 w	CR rich in BCAA = 16 (16F) OB with MetS (non-T2D) CR (control) = 11 (11F) OB with MetS (non-T2D)	Matched for age, gender, BMI, MetS.	31
Markers of responsiveness to caloric restriction						
CR + adequate/low dairy servings	Untargeted (GC-ToF-MS)	Plasma, fasting	0, 12 w	Low responders = 22 healthy OW to OB (7F) High responders = 22 healthy OW to OB (7F)	At baseline, matched for BMI and health status, unmatched for sedentary time (min/d)	35

T2D or IGT (n = 3), HT, (n = 10), hyperlipidemia (n = 11), obstructive sleep apnea (n = 6).
T2D (n = 3), HT, (n = 9), hyperlipidemia (n = 8), obstructive sleep apnea (n = 3).
T2D/med-T2D (n = 4), HT, (all), hyperlipidemia (n = 4).
AGB, laparoscopic adjustable gastric banding; BCAA, branched-chain amino acids; BMI, body mass index; CR, calorie-restricted; CHO, carbohydrate; d, days; DBP, diastolic blood pressure; DJB, duodenal jejunal bypass surgery; DS, duodenal switch; EX, aerobic exercise training; F, female; FG, fasting glucose; GC, gas chromatography; GT, glucose tolerance; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment; ^{1}H-NMR, proton nuclear magnetic resonance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; LC, liquid chromatography; LC-ESI-QqQ-MS/MS, liquid chromatography-electrospray-triple quatrupole-tandem mass spectrometry; LDL, low-density lipoprotein cholesterol; m, months; med-T2D, on type 2 diabetes medication; MetS, metabolic syndrome; mOB, people with morbid obesity; MS, mass spectrometry; MR, meal replacement; n, number of subjects involved; nd, not available; NGT, normal glucose tolerance; OB, people with obesity; OW, people with overweight; RYGB, Roux-en-Y gastric bypass; SBP, systolic blood pressure; SG, laparoscopic sleeve gastrectomy; TAG, triglycerides; T2D, type 2 diabetes; UPLC-QTOF-MS, ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry; w, week; y, year.

TABLE 2 Summary of included prospective, single-arm (quasi-study) metabolomic studies evaluating the efficacy of surgical or dietary antiobesity interventions in adult subjects

Interventions	Analytical technique	Specimen	Time points	Participants	Control group (baseline only)	Ref.
Bariatric surgery interventions						
RYGB	Targeted (GC-, LC-, SPE-LC-QqQ-MS/MS)	Serum, fasting	0, 3 m, 6 m	14 (14 F) mOB, non-T2D (n = 9) or med-T2D (n = 5)	Missing (pooled technical reference samples from age- and gender-matched)	33
RYGB	Targeted (LC-ESI-QqQ-MS/MS)	Plasma, fasting	0, 3 m, 6 m	13 (10 F) mOB with ≥1 OB comorbidity[a]	Missing	43
RYGB	Targeted (LC-ESI-QqQ-MS/MS)	Plasma, fasting	0, 3 m, 6 m	10 (9 F) mOB with ≥1 OB comorbidity[b]	Missing	44
RYGB	Targeted (lipids) (LC-ESI-QExactive-MS)	Plasma, fasting (?)	0, 3 m	5 (4 F) mOB with ≥1 OB comorbidity[c]	Missing	45
RYGB	Targeted (LC-ESI-LTQ-FTMS)	Plasma, fasting and postprandial	−4 w, 1 w, 4 w, 40 w	5 (1 F) OB, metabolic status: nd	8 (4 F) healthy, lean, age-, gender-, but not diet-matched	46
RYGB	Targeted (LC-ESI-QqQ-MS/MS)	Serum, fasting and post-oral glucose tolerance test	0, 1 y	30 (27 F) mOB or OB with T2D	Missing	47
RYGB	Untargeted (^{1}H-NMR) and (GC-MS)	Plasma, fasting	0, 1 y	10 (?F) OB with T2D	Missing	51
RYGB/SG	Untargeted (^{1}H-NMR)	Spot urine, non-fasting	0, 3–9 d	50 (?F) mOB, metabolic status: nd (RYGB n = 11); SG n = 39)	50 healthy, age- and gender- matched, lean	48
RYGB/BD	Untargeted (^{1}H-NMR)	Spot urine, fasting	0, 1 m, 3 m	2 (0 F) mOB with IR[a]	15 (0 F) mOB with IR 10 age-matched lean	49
Combined behavioral interventions						
CR + EX	Targeted (LC-ESI-QqQ-MS/MS)	Plasma, fasting	0, 6 m (>4 kg weight loss)	500 (63% F) OW to OB, non-med-T2D taking medications for hypertension and/or dyslipidemia	Missing	29

TABLE 2. (continued).

Interventions	Analytical technique	Specimen	Time points	Participants	Control group (baseline only)	Ref.
CR + EX + vitamin/mineral	Untargeted (GC-ESI-ToF-MS)	Plasma, fasting and post-oral glucose tolerance test	0, 14 to 17 w	12 (12 F) sedentary OB with IR	Missing	32
Dietary interventions (unchanged physical activity)						
Isocaloric diet + BSP (4.5 g/meal)	Untargeted (LC-ESI-qToF-MS)	Serum, fasting	0, 12 w	34 (19 F) healthy OW to OB	Placebo group (nd)	56
CR/LowCHO + vitamin/mineral	Untargeted (LC- and GC-ESI-qToF-MS)	Serum, fasting	0, 4 w, 8 w	45 (20 F) "healthy" OB	30 (17F) healthy, lean, gender-matched, unmatched for age, insulin, HOMA, FG	34
Personalized CR	Untargeted (?) (GC-MS)	Serum, fasting	0, 8 w	22 (15 F) old "healthy" OW to OB (non-T2D)	Missing	57
CR (12 w) + WM (21 w)	Targeted, lipidomic (GC-MS, LC-ESI-qToF-MS)	Serum, fasting	0, 33 w	9 (5 F) OW to OB with IFG or IGT and >2 MetS criteria	10 (6F) OW to OB, age-, gender, BMI-, IFG-, and IGT-matched	86

[a]T2D or IGT (n = 3), HT (n = 10), hypertriglyceridemia (n = 11), obstructive sleep apnea (n = 6).
[b]T2D (n = 3), HT (n = 9), hypertriglyceridemia (n = 8), obstructive sleep apnea (n = 3).
[c]T2D/med-T2D (n = 4), HT (all), hypertriglyceridemia (n = 4).
BD, biliopancreatic diversion; BMI, body mass index; BSP, black soybean peptides; CHO, carbohydrate; CR, calorie-restricted; CHO, carbohydrate; d, days; EX, aerobic exercise training; F, female; FG, fasting glucose; GC, gas chromatography; HOMA, homeostasis model assessment; ^{1}H-NMR, proton nuclear magnetic resonance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; IR, insulin resistance; LC, liquid chromatography; LC-ESI-QqQ-MS/MS, liquid chromatography–electrospray-triple quadrupole-tandem mass spectrometry; m, months; med-T2D, on type 2 diabetes medication; MetS, metabolic syndrome; mOB, people with morbid obesity; MS, mass spectrometry; n, number of subjects involved; nd, not available; OB, people with obesity; OW, people with overweight; RYGB, Roux-en-Y gastric bypass; SG, laparoscopic sleeve gastrectomy; SPE-LC-QqQ-MS/MS, solid phase extraction-liquid chromatography-triple quadrupole-tandem mass spectrometry T2D,type 2 diabetes; w, week; y, year.

Besides BMI, measures of glycemic control were the most accessible criteria for defining the metabolic phenotypes in a study at baseline (Tables 1 and 2). Significant improvements in glycemic control (i.e., reduction of circulating HbA1c concentrations, fasting/postprandial insulin, fasting/postprandial HOMA) and a more favorable plasma lipid profile (i.e., increased high-density lipoprotein cholesterol) were commonly observed, confirming the metabolic benefits of weight loss.

Metabolomic approaches

Mass spectrometry combined with different metabolite chromatographic separation (i.e., ultra-performance liquid chromatography, high-performance liquid chromatography, and gas chromatography) was the most commonly used analytical technique, followed by ^{1}H-NMR-based approaches or by the integration of multi-platform analyses (54). An array of targeted and untargeted metabolomic approaches was applied to date, but targeted analysis predominated.

The two distinct approaches are complementary and, if both are not used, the choice between them should depend on the aim of each study. Untargeted metabolomics is the most comprehensive analysis of all the measurable analytes in a sample, including chemical unknowns. Consequently, among its intrinsic limitations, it provides wider opportunities for novel metabolite species, pathway, and target discovery (59). In turn, targeted metabolomics is reliant on *a priori* selection of a subset of biochemically characterized and interpreted metabolites, which often belong to selective established pathways (reduced coverage). Although the targeted approach allows quantitative results, reduces the likelihood of analytical artifacts, and eases up the troubles related to big data analysis and interpretation, the clear definition of the species measured hinders the discovery of novel metabolic perturbations (hypothesis-driven approach).

Discussion

Bariatric versus dietary approaches: shared metabolic adaptations

To our knowledge, only five parallel-arm metabolomic studies have directly compared so far the body systemic response with dietary and surgical treatments for weight loss (28-30,37,54), and groups were well matched only in two of them (for age, body weight, BMI, diabetes duration and management, and also extent of body weight lost during treatment, in kg or % loss) (28,29). From a clinical viewpoint, in all cases the rate of weight loss and reduction of fat mass were faster after the surgery than the dietary changes, together with a more robust impact on glucose homeostasis improvement (28). However, the amount of weight loss was neither solely nor primarily correlated with improvement in IR. As summarized in Table 3, the metabolic adaptations shared by surgical and dietary interventions involved a decrease of several amino acids including branched-chain amino acids (BCAAs), together with an increase of total circulating ketone bodies and acylcarnitines. In contrast, bariatric surgery was associated with a greater decline in circulating BCAAs leucine, isoleucine, and valine than after an equivalent weight loss induced by diet (28), and postsurgical changes were reported soon perioperatively. The decrease of BCAAs and their related metabolites correlated with weight loss after RYGB only.

When including results from single-arm studies in the comparison, the depicted differential metabolic adaptations were confirmed (Supporting Information Table S3). Considering lipid metabolites, both surgical and dietary approaches promoted an overall decline of circulating (lyso)phospholipid species. Despite large-scale metabolomic studies such as those focused on the EPIC cohort recently indicating several choline-containing phospholipids as potential biomarkers of T2D (26,27), the heterogeneous response of this wide lipid class to both treatments hampered the generation of mechanistic explanations for phospholipids. Bariatric procedures were most associated with a significant decline of circulating ceramides (33,43,44), while dietary weight loss programs mainly reported the decrease of long- and very-long-chain fatty acids with variable degrees of saturation, both in the free and esterified form (diacylglycerols and triglycerides) (34,45,55,57).

Restoration of protein metabolism

BCAA. Following both types of weight loss programs, the circulating BCAAs were described as decreasing significantly, associated with improvement in IR and, more importantly, predicted metabolic benefits independently from the amount of weight lost. Although obesity-associated hyperaminoacidemia and the rise in circulating BCAAs are the most widely described shift in protein metabolism associated with obesity and IR, in both adults and childhood (60-62), the mechanism(s) which may underlie an imbalance of protein turnover are numerous and remain unclear.

Overall, the decrease in circulating plasma BCAAs observed following the different weight loss strategies may result from (i) a decrease in protein intake (increased AA catabolism), (ii) a decrease in protein catabolism, observed to be secondary to increased insulin sensitivity, indicative of metabolic amelioration, (iii) an attenuation of tissue-specific alterations in the BCAA metabolism and a promoted amino acid uptake and tissue utilization (increased tissue BCAA catabolism), or (iv) a combination of these factors. Although BCAAs are found in dietary proteins and, therefore, diet remains a potential contributor to the modulation of their peripheral levels, previous studies have suggested that dietary intake accounts for only a small portion of blood BCAA and for their change during weight loss (29,63). Furthermore, the more drastic decrease in circulating AAs detected following surgery should not depend on a decrease in protein intake as both surgical and dietary treatments actually shared a certain degree of caloric restriction, and the decrease in non-proteic amino acids such as ornithine and cystathionine would confirm these suppositions (Supporting Information Table S3). Consequently, other factors clearly influence the observed variations in BCAAs.

Reversion of tissue-specific alterations in BCAA metabolism could contribute, at least in part, to the decline of plasma BCAAs following weight loss treatments. This hypothesis makes sense since the surgery was accompanied by a consistent significant decline of specific products of incomplete mitochondrial oxidation of BCAAs, namely short-chain acylcarnitines C3 and C5 (28,39,64). Furthermore, the activity of key BCAA catabolic enzymes is known to be altered in obesity, in a tissue-specific fashion with alterations in liver and adipose tissue but not in muscle, and these enzymatic changes contribute to the rise in plasma BCAAs (65). In turn, a strong correlation between the expression of BCAA catabolic genes, IR, and T2D has also been demonstrated in humans, and evidence exists of the reversion of the enzymatic alterations in adipose tissue

TABLE 3 Shared and unique metabolic variations observed in parallel-arm bariatric surgery versus dietary intervention studies[a]

Metabolite	Variation[b]		Class/function summary	Ref.
	Post-bariatric surgery	Post-dietary treatment		
3-hydroxybutyrate	↑↑	↑↑	Ketone body	28,37
Total ketones	↑↑	↑↑	Ketone bodies, molar sum	28,37
Phenylalanine	↓↓↓	↓	Glucogenic and ketogenic AAA	28,29,37
Tyrosine	↓↓	↓	Glucogenic and ketogenic AAA	28,29
Isoleucine	↓↓	↓	Glucogenic and ketogenic BAAA	28,29
Leucine	↓↓	↓	Exclusively ketogenic BCAA	28,29
Valine	↓↓↓	-	Glucogenic BCAA	28,37
Alanine	↓↓↓	↓↓	Glucogenic non-essential AA	28,29,37
Asparagine/aspartate	-	↓	AA ratio	28
Glutamate	↑	-	Glucogenic AA	54
Histidine	↓↓	-	Glucogenic AA	28,37
Proline	↓	-	Glucogenic AA	37
Serine	↑	-	Glucogenic AA	37
Ornithine	↓↓	↓	Others, non-proteic AA	28,29
totBCAAs	↓↓	-	Molar sum	28,37
totAAAs	↓	-	Molar sum	37
totAAs	↓↓	-	Molar sum (proteic AAs)	28,37
C2	↑↑	↑↑	Short-chain ACs	28,37
C3 + C5	↓↓	-↓	Short-chain ACs (BCAAs catabolism)	28,37
totACs	↑↑	↑↑	Molar sum	28,37
C4-OH	↑	↑	Short-chain ACs	28
Ci4-D/C4-DC	↓	-	Short-chain ACs (BCAAs catabolism)	28
C16, C18:1	↑	↑	Long-chain (even-) ACs	28
C18:2	↑↓	↑-	Long-chain (even-) ACs	28,54
C16-OH/C14-DC, C18-OH/C16-DC, C20-OH/C18-DC	-	↓	Long-chain (even-) ACs	28
C22	-	↑	Long-chain (even-) ACs	28
C6 - C12, total	-	↑	Medium-chain (even-) ACs, sum	37
C14 - C22, total	↑	↑	Long-chain (even-) ACs, sum	37
Bile acids, conjugated	↑	-	Bile acids, microbial modification	30
NEFA, total	↑↑	↑-	Fatty acids, molar sum	30,37
PC aa C42:0	↓	-	Glycerophospholipids with long-	54
PC aa 32:0, PC aa 32:1, PC aa 40:5	-	↓	Glycerophospholipids with long-	54

[a]All changes have been investigated in plasma/serum samples, collected in the fasted state.
[b]↑ and ↓ respectively indicate a significant post-intervention increase or decrease in relative metabolite concentration, in respect to basal levels.
AAs, amino acids; ACs, acylcarnitines; BCAAs, branched-chain amino acids; C2, acetyl carnitine; C3, propionyl carnitine; C4/Ci4, butyryl carnitine/isobutyryl carnitine; C5, isovaleryl carnitine/3-methylbutyryl carnitine/2-methylbutyryl carnitine; C4-OH, 3-hydroxy-butyryl carnitine/β-hydroxy-butyryl carnitine; Ci4-DC/C4-DC, methylmalonyl carnitine/succinyl carnitine; C6, hexanoyl carnitine; C16, palmitoyl carnitine; C16-OH/C14-DC, 3-hydroxy-hexadecanoyl carnitine/tetradecanedioyl carnitine; C18-OH/C16-DC; C18:2, linoleoylcarnitine; C20-OH/C18-DC, 3-hydroxy-eicosanoyl carnitine/octadecanedioyl carnitine; C22, behenoyl carnitine/docosanoyl carnitine; NEFA, nonesterified fatty acids; PC, phosphatidylcholines; totBCAAs, molar sum of branched-chain amino acids (Val + Leu/Ile); totAAAs, molar sum of aromatic amino acids (Phe + Tyr); totAAs, molar sum of all amino acids measured.

BCAA metabolism by bariatric surgery-induced weight loss in subjects with obesity (66).

Other amino acids. Metabolic surgery was associated with a more drastic decrease in the total amino acid pools than that detected in dietary weight loss programs, particularly driven by the significant decline of the aromatic amino acids phenylalanine and tyrosine (AAAs), as well as ornithine, proline, and histidine (Table 3) (28,37). Subjects with obesity had approximately twofold higher serum phenylalanine concentration than control subjects, which has been proposed as a noninvasive marker of liver dysfunction in obesity, related to liver steatosis. Consequently, AAAs have been proposed as additional markers of metabolic improvement directly or indirectly involved in postoperative adaptations (67).

Surprisingly, the decline of circulating alanine was even more frequently described than the decrease in BCAAs, especially as a

response to surgical procedures. Although the mechanistic explanation is still unknown, plasma alanine levels may decrease in association with reduced dietary intake (i.e., through a low-protein diet) or vitamin deficiency (i.e., alanine metabolism is highly dependent upon enzymes having vitamin B6 as a cofactor) or even a decrease in fasting glucose available (68). Alanine is in fact an important substrate and regulator for glucose metabolism, playing a key role in glucose-alanine cycle between tissues and liver. In muscle and other tissues that degrade amino acids for fuel, alanine is produced during BCAA metabolism and most commonly via reductive amination of pyruvate, a product of muscle glycolysis. The alanine formed then is passed into the blood and transported to the liver, where a reverse of the alanine aminotransferase reaction takes place, and pyruvate regenerated forms glucose through gluconeogenesis, which then returns to muscle. According to this latter scenario, decreased circulating levels of alanine could also indirectly reflect a depletion of pyruvate available in muscle for conversion (increased local oxidation) in respect to what would be expected in the "well fed" state, and this has been associated with blood sugar levels in both diabetes and hypoglycemia.

Finally, despite the overall decreasing aminoacidemia normally associated with obesity, circulating levels of other amino acids such as glycine have been shown to significantly increase after weight loss treatments, and especially following bariatric techniques (Supporting Information Table S3). Low fasting levels of glycine have been observed in individuals with impaired glucose tolerance and proposed as an early marker for IR (27). The rise in glycine may be associated with the positive metabolic effects of weight loss treatments, since it is involved in many different biochemical mechanisms including the regulation of plasma cholesterol and triglyceride levels as well as the restoration of antioxidant glutathione synthesis and reduction of tissue and oxidant damage (69). However, results are still contradictory, as are any eventual causative associations.

Exclusive metabolic shifts after surgery

Five parallel-arm metabolomic studies have focused so far on differentiating the metabolic response to malabsorptive [RYGB and duodenal jejunal bypass surgery] versus restrictive surgeries (laparoscopic sleeve gastrectomy and laparoscopic adjustable gastric banding), and only two of them clearly defined the group-matching strategy (38,39). As shown in Table 4, the main bariatric-specific metabolic response included a significant increase in circulating bile acids (BAs). A decrease in BCAAs valine, leucine, and isoleucine and other glycogenic amino acids (i.e., proline, histidine), the increase of serine and glycine, and a heterogeneous variation in the circulating acylcarnitines were also observed. The very similar metabolic signatures obtained in response to diversionary and non-diversionary surgeries suggest that the metabolic effects are downstream of the primary effects of the surgery despite the different approaches. This was consistent with clinical outcomes. Although a trend of greater improvement in glycemic control was associated with malabsorptive procedures (i.e., significant reduction of fasting glucose and fasting insulin and increase in plasma FGF19 levels), inter-study variation was apparent and hence hampered the definition of clear-cut differences among bariatric techniques.

Restoration of BA metabolism

Altered BA metabolism has been long observed in obesity and T2D, with a significant decline in the circulating BA pool, especially postprandially (70). In contrast, an increase in circulating BAs in humans has been observed following surgical treatment for obesity, including perioperative (<7 days) and both in the fasted (13,38,47) and postprandial state (46). Accordingly, BA secretion has been associated with improvement in insulin secretion, insulin sensitivity, and whole-body glucose homeostasis, as well as improving liver and pancreatic function in animal models of obesity (reviewed in Ref. 71). Malabsorptive techniques were expected to have a greater impact on BA metabolism, due to the anatomical adaptive changes associated with the surgery, including bile delivery to the terminal ileum, decreased enterohepatic BA circulation followed by increased conversion of cholesterol to BAs (13). Nevertheless, the currently available metabolomic studies give insights for a restoration of BA synthesis also sustained by restrictive techniques (30,38). Nevertheless, the high variability in the study designs and the variable change in the BA pool and composition over time made it difficult to pick the nuances of BA changes after dinstict bariatric procedures.

Far from simply being dietary fat emulsifiers and the primary route governing cholesterol homeostasis, BAs have been recognized over the past decade as nutrient-responsive hormones that modulate various metabolic pathways through cell surface and nuclear receptors, including their own synthesis and enterohepatic circulation, but also triglyceride, glucose, and energy homeostasis (reviewed in Ref. 72). According to the most recent hypothesis, changes in bile flow after the surgery may have a direct role in IR amelioration via (i) increased satiety gut hormone responses (i.e., enhancing glucagon like peptide-1 response by L-cell stimulation), leading to reduced food intake and weight loss, (ii) inhibition of gluconeogenesis in a farnesoid X receptor-dependent and -independent manner, or (iii) insulin signaling promotion and glycogen synthase activation, thus aiding insulin-dependent control of glucose metabolism in the liver.

Moreover, preliminary evidence has shown that not all BAs act equally. For instance, Simonen et al. (47) postulated that altered conjugation of BAs after surgery is the actual mediator of metabolic consequences, independent of changes in total serum BAs, suggesting the role of gut microbiota, a key regulator of BA conjugation and secondary BA formation, in the metabolic adaptations observed postsurgery (73). In line with this hypothesis, a significant increase in the bile salt glycine/taurine conjugation ratio was also observed postoperatively (46).

Early prognostic markers of metabolic flexibility: Ketone bodies

Compared with behavioral antiobesity strategies, bariatric surgery is known to trigger very early adaptations. Hence, it is theoretically possible to identify early prognostic markers of the long-term metabolic response, particularly desirable to help clinicians in deciding whether drug therapy is necessary shortly after surgery and ultimately contributing to personalized treatment for obesity.

A NMR-based metabolomic analysis was specifically applied to differentiate the response to metabolic surgery according to diabetic improvement (42). The analysis showed that the metabolite profile of two groups ("improved" vs. "non-improved" diabetic individuals with obesity) differentiated at an early postoperative stage (7 days) leading to an accurate prognosis prediction of long-term glycemic

TABLE 4 Shared and unique metabolic variations observed in parallel-arm malabsorptive versus restrictive surgery studies[a]

Metabolite	Class/function summary	Variation[b]		Ref.
		Malabsorptive surgery	Restrictive surgery	
Bile acids, 1ary	Bile acids, hepatic	-↑	↑↑[a]	38
Bile acids, 2ary	Bile acids, microbial-derived	↑-	↑↑[b]	38
Bile acids, total	Molar sum	↑↑	↑-	36, 38
Alanine	Glucogenic AA	↓↓	↓↓	39, 40
Proline	Glucogenic AA	↓↓	↓-	39, 40
Histidine	Glucogenic AAA	↓↓	↓-	39, 40
Phenylalanine	Glucogenic and ketogenic AAA	↓↓	↓↓	39, 53
Tryptophan	Glucogenic and ketogenic AAA	↓	↓	39
Tyrosine	Glucogenic and ketogenic AAA	↓↓	↓-	39, 53
Isoleucine	Glucogenic and ketogenic BCAA	↓↓	↓↓	39, 53
Leucine	Exclusively ketogenic BCAA	↓↓	↓↓[b]	39, 53
3-methyl-2-oxo-pentanoic acid	BCAA metabolite	-	↑	39
Valine	Glucogenic and ketogenic BCAA	↓	↓	53
Methionine	Glucogenic SAA	↓	↓	39
Ornithine	Others, non-proteic AA	↓	↓	39
Glutamine/glutamate	AA ratio	↓	↓	39
totAAs	Molar sum	↓	↓	39
Serine	Glucogenic AA	↑	↑	39
Glycine	Glucogenic AA	↑↑	↑↑[b,c]	39, 53
C2	Short-chain ACs	↑	-	39
C3	Short-chain ACs (BCAAs catabolism)	↓	↓	39
C5, C5:1, C5OHC3DC	Short-chain ACs (BCAAs catabolism)	↓	↓	39
C3 + C5	Short-chain ACs (BCAAs catabolism)	↓	↓	39
C4-OH	Short-chain ACs	↑	-	39
C4/Ci4	Short-chain ACs	↓	↓	39
C8:1, C8:1OH/C6:1DC, C10:1, C10:2, C10:3	Medium-chain ACs	↓	↓	39
C14OH/C12DC	Long-chain (even-) ACs	↓	↓	39
C16:1OH/C14:1-DC	Long-chain (even-) ACs	-	↓	39
C18:1DC, C18:2OH	Long-chain (even-) ACs	↑	↑	39
totACs	Molar sum	↑	-	39
C10:0	SFAs, medium-chain	↓	-	40
Acetoacetate	Ketone bodies	↑[b]	↑[b]	53
3-hydroxybutyrate	Ketone bodies	↑[b]	↑[b]	53
2-hydroxy(iso)butyrate	Xeno-metabolite, microbial-derived	-	↑	40
Citrate	Energy, Kreb's intermediate	↓↑[b]	-↑[b]	40, 53
Pyruvate	Energy, Kreb's intermediate	↓	↓	53
Propanol	Alcohol, microbial-derived	↓	-	53
Isopropanol	Alcohol, microbial-derived	↓	↓[b,c]	53
Methanol	Alcohol, microbial-derived	↓	-	53
TMAO	Amines metabolism, microbial-derived	↑	-	53
Dimethyl sulfone	Microbial-derived	↑	↑	53

[a]Increase observed only 1 m following the surgery.
[b]Increase observed only 3 m following the surgery.
[c]Increase observed only 6 m following the surgery.
AA, amino acids; AAAs, aromatic amino acids; ACs, acylcarnitines; BCAAs, branched-chain amino acids; C2, acetyl carnitine; C3, propionyl carnitine; C4/Ci4, butyryl carnitine/isobutyryl carnitine; C5, isovaleryl carnitine/3-methylbutyryl carnitine/2-methylbutyryl carnitine; C4-OH, 3-hydroxy-butyryl carnitine/β-hydroxy-butyryl carnitine; C6, hexanoyl carnitine; C8, octanoyl carnitine; C10:3, decatrienoyl carnitine; C16, palmitoyl carnitine; C16-OH/C14-DC, 3-hydroxy-hexadecanoyl carnitine/tetradecanedioyl carnitine; TMAO, trimethylamine N-oxide; totAAs, molar sum of all amino acids measured.

control after surgery (defined through HbA1c values at 3 months). Circulating 3-hydroxybutyrate (3-HB), higher in the improved group, was the most relevant early metabolic feature to positively correlate with a better glycemic control subsequently, together with lower glucose and lipid (low-density lipoprotein, very-low-density lipoprotein) concentrations. The [3-HB]/[glucose] ratio was observed to augment current prognostic modalities and proposed to help clinicians in deciding whether drug therapy is necessary shortly after surgery, ultimately contributing to personalized treatment.

Although starvation-associated ketoacidosis has been proposed as an explanation for the observed changes (increased fatty acid oxidation during perioperative fasting or caloric restriction) (74), this does not explain the selective increase in 3-HB in subjects with a better glycemic prognosis in the long term, since both the improved and non-improved groups underwent the surgical stress or fasting immediately after surgery. A direct effect of 3-HB and ketone bodies on insulin sensitivity has been already shown (75); thus its rise in the early days after bariatric surgery might directly mediate glycemic control enhancement. As 3-HB is formed primarily when the energy source for the peripheral organs is shifted to lipid from glucose often during longer-term fasting, these alterations in metabolism at an early-stage postoperation may be a key factor in the success of longer-term postoperative glucose control. Increases in 3-HB may also reflect a change in the mitochondrial redox state of the liver (from a more oxidative state toward a more reduced state), which would be induced by the surgery only in patients with better responses in terms of their metabolic phenotypes (metabolic flexibility). The rise of circulating metabolites known to alter the liver redox state, including branched-chain ketoacids, BCAAs, and free fatty acids, recently associated with obesity and T2D (75), would support this last hypothesis, in keeping with the recent concept of redox as a master regulator of metabolism (76-78).

Restoration of lipid metabolism

From traditional clinical lipid measures, it is widely appreciated that dyslipidemia and abnormal lipid metabolism are characteristics of obesity, especially in association with abnormal glucose metabolism, i.e., impaired fasting glucose, impaired glucose tolerance, and T2D, while weight loss improves the blood lipid profile. Although lipidomics is now considered a self-standing "omics" technology, studies focused on the comprehensive analysis of lipid diversity were included in this review, due to the overlapping between the metabolomic and lipidomic approach and the strong impact of obesity and weight loss on all traditional lipid homeostasis measures (79,80).

Decline of proinflammatory ceramides. A significant decrease in plasma ceramides was observed in diabetic subjects with obesity after RYGB, in conjunction with significant improvements in cardiovascular risk factors and insulin sensitivity (43). This study was the first to provide *in vivo* evidence following surgically induced weight loss. Subsequently, Graessler et al. (45) found a significant positive correlation between the RYGB-induced decrease of ceramide metabolites and the postoperative levels of HbA1c, together with triglycerides, total cholesterol, and low-density lipoprotein cholesterol. An increasing number of cell systems and animal and human studies have demonstrated a link between increased circulating ceramides and accumulation (as a major component of ectopic fat) and *diabesity*, mediated by inflammatory mechanisms (81). These sphingolipids are generated in response to a variety of mediators, including proinflammatory cytokines, oxidative stress, and increased levels of free fatty acids, and would contribute to the state of IR by facilitating inflammatory signaling pathways (i.e., inhibition of insulin action and subsequent glucose uptake through inactivation of Akt pathway and induction of inflammation through activation of the tumor necrosis factor-α axis) (81,82).

Furthermore, it is noteworthy that ceramides differing in the sphingoid base and the fatty acid chain length and saturation are formed in different cell compartments or membranes, by a variety of different mechanisms, at different times, and potentially with distinct biological functions (e.g., dihydroceramides). In turn, the effect of bariatric surgery was most pronounced on specific ceramide species, such as the long-chain C24:0, already associated with the *diabese* phenotype (83).

In light of recent evidence regarding the role of muscle sphingolipid content in IR (i.e., ceramide enriched with nervonic acid) (56), Mutch et al. (46) speculated that an increase of ceramides in the bloodstream may reflect their mobilization from non-adipose tissues, such as the muscle (26). In confirmation of this hypothesis, elevated intramyocellular lipid deposition was observed in IR and T2D individuals with obesity, while a significant decrease in intramyocellular lipid associated with both reduced circulating sphingolipid molecules and with improved insulin action after RYGB surgery. However, conflicting results have been published (84).

Fatty acid metabolism. Obesity and IR/T2D have been positively associated with decreased fatty acid oxidation, accumulation in the body both in the free and esterified form (lipotoxicity), and to impaired fatty acid elongation and desaturation (higher proportions of saturated species and lower proportions of longer-chain n-6 and n-3 polyunsaturated fatty acid) (85). In contrast, during dietary-based weight loss a significant reduction of circulating short- and medium-chain SFAs has been described, generally coupled with an improvement in insulin sensitivity (57,86). Blood levels of MUFAs and ω-6/ω-3 polyunsaturated fatty acids also decreased significantly in the majority of the studies (Supporting Information Table S3). Interestingly, Perez-Cornago et al. also found that individuals with higher circulating concentrations of palmitoleic acid (C16:1) at baseline experienced a lower reduction in percentage body fat, thus suggesting a certain individual predisposition to responsiveness to the dietary treatment (57). However, any potential causative effect remains controversial (87).

Less-explored metabolic adaptations: Xeno-metabolism and gut microflora

Finally, metabolic adaptations to both weight loss strategies also included changes in less explored pathways (Supporting Information Table S3), such as in purine/pyrimidine metabolism, xeno, and gut microbial metabolism. Changes in xeno-metabolites including phytochemical derivatives have been observed following both dietary and surgical treatments (e.g., *p*-cresol sulfate) or only postoperatively (i.e., 2-hydroxy(iso)butyric acid) as a consequence of the physical restructuring of the gastrointestinal tract following surgery which involves bypassing much of the proximal small intestine (duodenum and jejunum are the predominant absorption sites for phytochemicals) (33,34,49). Although some of them have been already recognized as early biomarkers of IR and glucose intolerance (88), discrepant results have been collected so far.

Since the xeno-metabolome is strongly influenced by the intricate relationship between the host metabolism, the diet, and the gut microbiota composition and activity (24,89), starvation, alterations in vitamin status (i.e., biotin, niacin), and changes in the gut microflora following both types of procedures for weight are all possibly implicated. The gut microbiota have been particularly associated with the development of obesity and its comorbidities, mainly due to their contribution in the modulation of several processes including host energy metabolism, gut epithelial permeability, gut peptide hormone secretion, and host inflammatory state. The symbiotic interaction between the gut microbiota and the host is essentially reflected in specific metabolic signatures, and the application of metabolomics has already allowed new insights on the mechanisms linking the gut microbiota composition and activity with disease development (24). Since the microbiota composition may change rapidly in response to both dietary factors and bariatric procedures (49), the effects of a pronounced alteration of gut microbial ecology postoperatively is a particularly hot topic of research currently.

Pitfalls and limitations

The interpretation of the insights accumulated so far needs to be assessed within the context of the limitations of the reported studies. Some criticisms are relevant to technical limitations of current metabolomic studies in general (e.g., results variability depending on the analytical approaches and platforms employed, the bio-sample selection, preparation, and storage), while others depend on the poorly characterized or highly variable designs of the studies published so far.

From the study design viewpoint, the small scale of the studies and the presence of single-arm designed or parallel-arm studies with poor group-matching criteria were the most common issues hampering biomarker discovery and data exploitation, followed by the common lack of a validation cohort for internal data replication. The limited accessibility of relevant information was another relevant issue (e.g., lack of medication and dietary intake assessment, poor definition of the anthropometric and clinical characteristics of the subjects). Since it is well known that not all subjects with obesity are at the same increased cardiometabolic risk, the metabolic response and effectiveness of a surgical or dietary intervention to treat obesity clearly depend on the metabolic health status of the subjects at baseline (90). Unfortunately, this clinical fact is not reflected in the metabolomic studies carried out to date. Among the reviewed works, in fact, virtually no attempt was observed to stratify individuals with obesity based on their metabolic health phenotype at baseline (i.e., "metabolically healthy" vs. "unhealthy/at risk" phenotypes with obesity), otherwise necessary for personalized follow-up (91). Also the definition of adiposity was heterogeneous and the assessment of body fat percentage, a recognized predictive marker of cardiometabolic risk and responsiveness to dietary treatments (92), was considered only sporadically. Limited data on the variation of inflammatory mediators following weight loss were also available in the reviewed studies. This prevented the search for associations between the metabolic response to the treatments and the improvement of the proinflammatory state, recognized as a potential mechanism linking adipose tissue expansion and cardiometabolic risk. Similarly, the modest length of the follow-up ($\leq$1 year) did not allow us to draw conclusions about the longer-term effects of metabolic surgery, as well as the maintenance phase of any dietary intervention.

Similarly, it is noteworthy that most of the metabolomic studies have focused on examining changes in the overnight-fasted state, although it is trivial that eventual improvement in the metabolic flexibility would manifest more robustly under dynamic challenged conditions, such as an oral glucose tolerance test, a meal or mixed meal tolerance test, or glucose/insulin infusion.

Conclusion

Moving from association to causation is the next challenge for metabolomics to deepen the link between weight loss treatments and reduction of cardiometabolic risk, and so move biomarker discovery to the next level of clinical effectiveness. To achieve this goal, extrinsic and intrinsic limitations should be faced in the near future, including suboptimal study designs and the prevalent application of targeted approaches (hypothesis-driven and not hypothesis-generating).

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Material Suplementario

REVISION 2

Supporting Information (SI) File

Metabolomics-Guided Insights on Bariatric Surgery versus Behavioral Interventions for Weight Loss

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Abbreviations (for Tables)

Lifestyle-based interventions: CR, calorie-restricted; MR, meal replacement; VLCD, very low carbohydrate diet; CHO, carbohydrate; BSP, black soybean peptides; OWP diet, diet high in oat and wheat bread and potato; RP diet, diet high in rye bread and pasta; ITF, inulin-type fructans; EX, aerobic exercise training, WM, weight management.

Surgical treatments: RYGB, Roux-en-Y Gastric Bypass; AGB, Laparoscopic Adjustable Gastric Banding; DS, Duodenal switch; BD-DS, biliopancreatic diversion with duodenal switch; SG/DJB, laparoscopic sleeve gastrectomy with duodenal jejunal bypass surgery; MS, malabsorptive surgeries; RS, restrictive surgeries.

Anthropometric and clinical parameters: NW, normal weight; OW, overweight; OB, obese; mOB, morbidly obese; T2D, type 2 diabetes; med-T2D, medicated T2D; FG, fasting glucose; NGT, normal fasting glucose (≤ 5.6 mmol/l); IFG, impaired fasting glucose (5.6–7.0 mmol/l according to ADA criteria for pre-diabetes); IGT, impaired gucose tolerance (2-hour OGTT plasma glucose concentration 7.8–11.0 mmol/l); IR, insulin resistance (defined as one or more of the following: (a) IFG or OGT as for the ADA criteria, and/or (b) using target Quantitative Insulin Sensitivity Check Index (QUICKI) score, HOMA or logHOMA cut-offs); SBP, systolic blood pressure; DBP, diastolic blood pressure; MetS, metabolic syndrome; FFM, fat-free mass.

Nomenclature of metabolites/metabolite classes. Amino acids (AA): Ala, Alanine; Gly, glycine; Glm, glutamine; Glt, glutamate; Pro, Proline; Met, Methionine; His, Histidine; Val, Valine; Leu/Ile, Leucine/Isoleucine; Phe, phenilalanine; Tyr, Tyrosine; Arg, Arginine; Aspr, asparagine; Asp, aspartic acid; The, theanine; totBCAAs, molar sum of branched-chain amino acids (Val + Leu/Ile); totAAAs, Molar sum of aromatic amino acids (Phe + Tyr); totAAs, molar sum of all amino acids measured; tCys, total cysteine; tHcy, total homocysteine; Tau, taurine; SAAs, sulphur amino acids (Met, Cys, non-protein AA: Hcy, Tau)

Acylcarnitines (ACs): C2, acetyl carnitine; C3, propionyl carnitine; C4/Ci4, butyryl carnitine/Isobutyryl carnitine; C5, isovaleryl carnitine/3-methylbutyryl carnitine/2-methylbutyryl carnitine; C4-OH, 3-Hydroxy-butyryl carnitine/β-hydroxy butyryl carnitine; Ci4-DC/C4-DC, Methylmalonyl carnitine/ Succinyl carnitine; C6, hexanoyl carnitine; C8, octanoyl carnitine; C10:3, Decatrienoyl carnitine; C16, Palmitoyl carnitine; C16-OH/C14-DC, 3-Hydroxy-hexadecanoyl carnitine/Tetradecanedioyl carnitine; C18-OH/C16-DC; C18:2, Linoleoylcarnitine; C20-OH/C18-DC, 3-Hydroxy-eicosanoyl carnitine/ Octadecanedioyl carnitine; C22, Behenoyl carnitine/Docosanoyl carnitine; 3-OH-Hexanyoylcarnitine (C6-OH); Octenoylcarnitine (C8:1); Decenoylcarnitine (C10:1); Decanoylcarnitine (C10); Glutarylcarnitine (C5-DC); Dodecenoylcarnitine (C12:1); Dodecanoylcarnitine (C12); 3-OH-Dodecanoylcarnitine (C12-OH); Tetradecadienoylcarti nine (C14:2); Tetradecenoylcarnitine (C14:1); Tetradecanoylcarnitine (C14); 3-OH-Tetradecenoylcarnitine (C14:1-OH); 3-OH-Tetradecanoylcarnitine (C14-OH); Hexadecenoylcarnitine (C16:1);Hexadecanoylcarnitine (C16); 3-OH-Hexadecenoylcarnitine(C16:1OH); 3-OH-Hexadecanoylcarnitine (C16-OH); Linoleylcarnitine (C18:2); Oleylcarnitine (C18:1); Stearoylcarnitine (C18); 3-OH-Linoleylcarnitine (C18:2-OH); 3-OH-Oleylcarnitine (C18:1-OH). *Lipids*: TAG, triglycerides; DAG, Diacylglycerols; NEFA, non-esterified fatty acids; CholE, cholesterol esters, GPL, glycorephospholipids; (Lyso)PC, (lyso-)phosphatidylcholines; PE, phosphatidylethanolamines; PEO, PE ethers, PI, phosphatidylinositols; IMTG, Intramyocellular triacylglycerol. Sphingolipids: Cer, ceramides; dhCer, dihydroceramides; SM, sphingomielins.

Bile acids (BAs): LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; 7-KCLA, 7-ketolithocholic acid; HCA, hyocholic acid; GUDCA, glycoursodeoxycholic acid; GHCA, glycohyocholic acid; GDCA, glycodeoxycholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; THCA, taurohyocholic acid.

Materials and Methods: Search Strategy

Data Sources. Two electronic databases (PubMed and Web of Science) were searched for keywords according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines.

Search terms in Pubmed were 1) ("Metabolome"[Mesh] OR "Metabolomics"[Mesh] OR "Mass Spectrometry"[Mesh] OR "Nuclear Magnetic Resonance, Biomolecular"[Mesh]) AND ("Bariatric Surgery"[Mesh] OR "Bariatrics "[Mesh]); 2) ("Metabolome"[Mesh] OR "Metabolomics"[Mesh] OR "Mass Spectrometry"[Mesh] OR "Nuclear Magnetic Resonance, Biomolecular"[Mesh]) AND ("Obesity" [Mesh] OR "Diabetes Mellitus, Type 2"[Mesh] OR "Metabolic Syndrome X"[Mesh]) AND ("Diet"[Mesh] OR "Food"[Mesh]). Search terms in Web of Science: 1) (metabolom* OR metabonom*) AND (bariatric surgery* OR gastric bypass* OR gastric banding* OR gastroplasty* OR biliopancreatic diversion* OR duodenal switch* OR laparoscopy*); 2) (Metabolome OR Metabolomics OR Mass Spectrometry OR Nuclear Magnetic Resonance) AND (Obesity OR Diabetes Mellitus, Type 2 OR Metabolic Syndrome X) AND (Diet OR Food) AND ("Human*" NOT "Rat*" NOT "MICE" NOT "RNA" NOT "Gen*").

Species (human), language (English) and publication date restrictions (2000 to date) were imposed. Relevant references identified from pertinent articles were additionally reviewed.

Study Selection.

Types of interventions, study designs and participants. Prospective intervention trials carried out to study the metabolic response to surgical and/or dietary treatments for weight loss in overweight to morbidly obese adult subjects (males and females, BMI > 25 kg/m^2) were included. Randomization was expected only for parallel-arm lifestyle intervention trials (surgical procedures being applied according to clinical decision).

Analytical approach. Targeted and untargeted metabolomic approaches driven by mass spectrometry (MS) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) techniques were both included in the selection.

Types of outcome measures. Low-molecular weight (<1000 Da) metabolites significantly up- or downregulated following surgical or behavioral weight loss interventions, with respect to

baseline and to the parallel intervention, when present, were the primary outcome measures of interest of the review. In the case of single-arm (quasi) trials, the post-intervention changes with respect to baseline were of primary interest, while baseline differences with respect to controls (i.e. lean healthy subjects) were not considered since not universally available. Weight loss was the cut-off clinical outcome required for inclusion in the review, except for acute post-surgery studies. Only those metabolites that differed systematically post-intervention (within-group variation during follow-up with respect to baseline) and between interventions (between-group variation) were considered potential biomarkers of a given intervention. In the case of multi-time point follow-up studies, only metabolites that significantly differed from baseline at any follow-up point were considered in the summary tables. Additional clinical outcome measures such as known descriptors of glyceamic control (i.e. fasting glucose, glucose tolerance, IR – HOMA, HbAc1b) were reported when available.

Data Extraction

Eligibility assessment was carried out independently by two authors using predefined data fields in an unblinded standardized manner. An independent review of the extracted articles was conducted by a third author, and cases of disagreements were resolved by majority consensus.

Supplemental Tables

Table S1. Summary of the main clinical outcomes and the significant metabolites identified during the anti-obesity interventions (parallel-arms design).

Interventions	Clinical outcomes (fasted state only)	Metabolic changes, fasting state		
		SHARED between treatments[a]	UNIQUE for a treatments[b]	Ref.
Bariatric Surgery vs Dietary Intervention				
RYGB vs CR/MR	SHARED:↓ BMI, FG, HOMA, IS, pro-insulin, leptin RYGB only: ↑ rate of weight loss; ↓ CRP; ↑ ALT; CR only ↑ ghrelin	ACs: ↑ short-chain C2, C4-OH, long-chain C16, C18:1, C18:2, totACs; FAs and products: ↑ total ketones, 3β-hydroxybutyrate	RYGB only = AAs: ↓ Ala, Val, Leu/Ile, Phe, Tyr, His, Orn, totAAs totBCAAs; short-/medium-chain ACs :↓ C4-D/C4-DC, C3 + C5, C10:3 CR only = AAs: ↓ Aspr/Asp; long-chain ACs: ↓ C16-OH/C14-DC, C18-OH/C16-DC, C20-OH/C18-DC; ↑ C22	Laferrère B, et al. 2011
RYGB vs CR/MR	SHARED: ↓ BMI, FG, fasting insulin and proinsulin, HOMA; RYGB only: ↑ rate of weight loss, ↓ fasting proinsulin/insulin; CR only: ↓ fasting glucagon	AAs: ↓ Leu, Ileu, Phe, Tyr, Ala, Orn	nd	Shah et al. 2012
RYGB vs CR	SHARED: ↓ BMI , FG (ns); ↑ IS, fasting hormone levels (glucagon, leptin, ghrelin, C-peptide, GIP, GLP-1, amylin, TNF-α and FGF21; >>RYGB, ns); RYGB only:↓ Fasting insulin	AAs: ↓ Ala; ACs: ↑ C2, tot long-chain ACs (C14-C22), totACs; ↓ C3 + C5; FAs and products: ↑ NEFAs, total ketones, 3β-hydroxybutyrate	RYGB only = AAs: ↑ Ser; ↓ Pro, His, Val, Phe, totBCAAs, totAAAs, totAAs CR only = medium-chain ACs: ↑C6 - C12	Khoo C, et al. 2013
SG vs CR/LowCHO	SHARED: ↓ BMI, HbAc1, FPI, TAG, CRP; ↑ HDL-Chol RYGB only: ↓ FG	nd	SG only = AAs: ↑ Gln; long-chain ACs: ↓ C18:2; GPLipids: ↓ PC aa C42:0 CR only = GPLipids: ↓ PC aa 32:0, PC aa 32:1, PC aa 40:5	Oberbach et al. 2011
SG vs CR	SHARED: ↓ BMI, waist, glucose, TAG; ↑ relative weight loss SG only: ↓ SBP; CR only: ↓ DBP, tot/LDL-Chol, ↑ HDL-Chol	nd	SG only = ↑ conjugated BAs (GDCA, TDCA, TCDCA), ↑ totNEFA	Damms et al. 2015
Bariatric surgery Interventions				
Malabsorptive vs Restrictive	SHARED: ↓ BMI, HbAc1, TAG, leptin; ↑ Adiponectin, GLP-1; MS only: ↓ FG, fasting insulin, HOMA; RS only: ↑ GIP	↑ total BAs (at 1m, 3m), 1ary BAs (at 3m), 2ary BAs (at 1m)	RS only: ↑1ary BAs (at 1 m), 2ary BAs (at 3 m)	Nakatani et al. 2009
RYGB vs AGB	SHARED: ↓ BMI, fat mass, lean mass (!), FG, fasting insulin, intrahepatic TAG	AAs: ↓ Ala, Pro, BCAAs, AAAs, Met, His, Gln/Glt, Orn, totAAs; ↑ Gly, Ser, short-chain ACs: ↓C3, C4Ci4, C5, C5:1, C5OHC3DC, medium-chain ACs: ↓ C8:1, C8:1OH/C6:1DC, C10:1, C10:2, C10:3, long-chain ACs: ↓ C14OH/C12DC; ↑ C18:1DC, C18:2OH	RYGB only = short-chain ACs: ↑ C2, C4OH, ↑ totACs AGB only = long-chain ACs: ↓ C16:1OH/C14:1DC	Magkos et al. 2013
RYGB vs AGB	SHARED: nd RYGP only = ↑ plasma FGF19	nd	RYGB only = ↑ total BAs (after 6 w)	Pournaras et al. 2012
RYGB vs SG	SHARED: ↓ HOMA-IR (ns) RYGB only = ↓ FG, (proteomic analysis: ↓ 7 proteins, including Fetuin-A and Retinol binding protein 4 previously linked to IR)	AAs: ↓ Ala	RYGB only = AA: ↓ Pro, His, SFAs: ↓ C10:0; Krebs intermediate: : ↓ citric acid SG only = AAs & catabolites: ↑ 3-methyl-2-oxo-pentanoic, 2-hydroxybutyrate	Jüllig et al., 2014

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proximal RYGB vs distal RYGB vs SG	SHARED: ↓ BMI, weight, fat mass, lean body mass ↑ LDL (at 1 y)	SHARED: : ↑ Gly (at 3, 6 m) ↓ Ile (at 3 m, 1 y) ↓ Leu (at 3 m), Val (at 3 m and 1 y), Phe (3 m, 9 m and 1 y); Carboxylic acid anions: ↑ acetoacetate, citrate, 3-HB (at 3 m), ↓ pyruvate (at 1 y); Others: ↑ dimethyl sulfone (at 3 m, 9 m, 1 y), ↓ Isopropanol (at 3 m, 6 m)	proximal RYGB only = AAs: ↓ His (at 6 m), Leu (at 1 y), Methanol (at 3 m)	↓ Gralka et al. 2015
	RYGB only (proximal and distal) = ↓ lipoproteins, ↓ VLDL (at 6 m, 9 m, 1 y)	RYGB only (proximal and distal) = AAs: ↑ Gly (at 9 and 1 y), ↓ Ile (at 6 m), Tyr, Val (at 6 m-9 m), Leu (at 6 m), Phe (at 6 m); Carboxylic acid anions: ↑ 3-HB (at 6 m), ↓ Isopropanol (at 9 m, 1 y), methanol (at 9 m , 1 y), ↓ Propanol ↑ TMAO. proximal RYGB and SG only = ↑ Gln (at 9 m, 1 y)	distal RYGB only = AAs: ↑ Arg(at 6 m- 1 y), Gln (at 3 m), His (1 y), ↓ Leu (at 9 m); ketone bodies: ↑ acetoacetate (at 6 m); Others: ↓ Creatinine (at 1 y), ↓ Methanol (at 6 m)	
	distal RYGB only = ↑ LDL (at 9 m), ↓ VLDL (at 3 m)	distal RYGB and SG only = ↑ Gln (at 3 m), ↓ Ile (at 9 m); Carboxylic acid anions: ↓ pyruvate (at 6-9 m); Others: ↑ dimethyl sulfone (at 6 m)	SG only = AAs: ↑ Gly (at 3-6 m), ↑ Gln (at 6 m); Krebs intermediate: ↑ citric acid (at 6m)	
RYGB vs DS	SHARED: ↓ BMI (>> for DS, ns), creatinine, GGT RYGP only = ↑ vitamin B12	AAs:↓ Met, Glt, Cystathionine	DS only = AAs: ↑ tHcy, ↓ tCys	Aasheim et al. 2011 British
Prognostic Markers of Post-op Glycemic Control				
RYGB vs DJB	Improved group only: ↓ HbA1c Non-improved only = ↑ FG, LDL-Chol, VLDL	nd	Improved only = ↑ 3-hydroxybutyrate (3-HB), [3-HB]/[glucose] ratio	Kwon et al. 2014
RYGB	SHARED: ↓ BMI, fasting glucose, Hb1Ac	AAs & derivatives: ↓ BCAAs, tyrosine, alanine; ↑ methionine, L-proline; FAs: short and medium-chain: ↓ 2-ethyl-3-hydroxypropionic acid, benzeneacetic acid, 4-methyl-2-oxovaleric acid; long-chain FAs: ↓ C16:0, C18:0; C18:1; SLipids: ↑SM (d18:1/28:0, d18:1/23:3; d18:1/23:1;d18:1/21:0); ↓TAG with long -chain FAs (48:0, 50:1, 55:1, 57:3), ↑TAG (58:9, 58:8, 55:9, 55:8, 53:8, 53:7, 53:3); GPLipids:↓ PC (40:6), ↑LysoPE (18:0); ↑PE (36:3) lower levels in improved subjects); CHO metabolites: ↓ glucose, erythritol, d-galactose, aconitate.	Improved only = ↑TAG (56:2), LysoPE (18:0); ↓ SM (d18:1/25:2), PE (38:3 (only at 42 d), 36:5e, 40:8e), PC (32:0, 32:5, 34:3, 36:5, 40:3e, 40:5e), LysoPC (18:3, 20:3).	Arora et al. 2015
RYGB	SHARED: ↓ BMI, fasting glucose, TAGs, Hb1Ac, HOMA, SBP, Waist, oral hipoglycemic agents, insuline therapy Non-improved only = ↓ tot-Chol Improved group only: ↓LDL-Chol, HDL-Chol. C-peptide	nd	Improved only = BAs (fasting) ↓ CDCA, 7-KLCA;↑ HCA, GHCA, TDCA, THCA Non-improved only = BAs (fasting) ↓ GUDCA.	Yu et al. 2015

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Behavioral Interventions				
CR/Low-Fat vs EX	SHARED:↓ BMI, fat mass	DAG: ↓ total DAG, DAG with long-chain S/MUFA (C14:0/16:0, C14:0/18:0, C14:0/18:1, C16:0/18:0, C16:0/18:1, C16:1/18:1, C18:0/18:1, DI-C14:0, DI-C16:0, DI-C18:1)	DAG: ↓ DAG with long-chain S/MUFA C16:1/18:0, DI-C16:1, DI-C18:0	Dubé et al. 2011
	CR/LowFat only: ↓ FG, fasting insulin, IMCL content; EX only: ↑ FFM, IMCL content	SLipids: ↓ Cer C14:0, Cer C20:0, Cer C24:0, Cer C24:1, ↓ Dihydro-Cer	SLipids: ↓ total Cer, Cer C18:0, ↓ Sphingosine	
CR vs CR diet rich in BCAA	SHARED: at baseline, BCAA positively correlated with HOMA; post-intervention: ↓ BMI, body weight, waist circumference, fasting insulin, total TG, loss of association between BCAA levels and HOMA	nd	CR rich in BCAA only= ↓ trans-4-hydroxyproline, cystine, cysteine, 4-hydroxyproline	Piccolo et al. 2015 (A)
Low-Fat + mixed nuts (30g/day) vs Low-Fat	SHARED: ↓ BMI, body weight, fat mass, waist circumference, SBP, DBP Nuts only = ↓ fasting insulin, HOMA, IL-6; Low-Fat only = ↓ LDL-chol	nd	n Nuts only = PUFA metabolites: ↑10-hydroxy-decene-4,6-diynoic acid sulfate, ↑tridecadienoic/tridecynoic acid glucuronide; ↑dodecanedioic acid; biogenic amines metabolites: ↑N-acetylserotonin sulfate, ↑ hydroxyindolacetic acid	Tulpani et al 2011
Markers of Responsiveness to Caloric Restriction				
CR + adequate/low dairy servings	High-responders only = ↓ BMI, intra-abdominal adipose tissue, gynoid fat, android fat, total fat, weight, hematocrit, leptin concentrations (the change is greater in HR) HR only = ↑ physical activity		High-responders only = at baseline, ↑ stearic, oleic and palmitic, myo-inositol; ↓ 2-ketoisocaproic acids; post-intervention = ↑citric acid, 3-hydroxybutanoic acid	Piccolo et al. 2015 (B)

Abbreviations listed in the Supporting Information File. Nd: not available

Table S2. Summary of the main clinical outcomes and the significant metabolites identified during the anti-obesity interventions (single-arm design).

Interventions	Clinical outcomes (fasted state only)	Metabolic changes, fasting state (post- vs pre-intervention)[a]	Ref.
Bariatric surgery Interventions			
RYGB	↓ BMI, fat mass, lean mass (!), resting energy expenditure, HDL-Chol, TAG, leptin, total caloric intake; (insulin-treated excluded): ↓FG, insulin, HOMA^	AAs & derivatives: ↓ Ala, Leu, Ileu, Val, Tyr, Threo, Lys, kinurenic acid, (tau, aspr at 1 m only, glt at 6 m only) ↑DAG with long-chain MUFA/PUFA (C18:1, C18:2), GPLipids: ↑ PC with long-chain S/MU/PUFA (C18:2, C20:4), (C18:0, C22:6) (C18:0, C18:2); SLipid: ↓ Cer (d18:1/C24:0), erytro-C16-sphingosine, long-chain FAs: ↓ SFAs C18:0, C22:0, ↓ PUFAs ω-6 C18:3); Fatty alcohols :↓C15, ; ↑Xeno-metabolites: ↑ p-Cresol sulfate, ↓ creatine, campesterol	Mutch et al. 2009
RYGB	↓ BMI, FG, fasting insulin, TAG, totChol, LDL-Chol, tot/HDLChol, free FAs, TNF-a; ↑ IS. Resolution/Attenuation of all comorbidities at 6 m	SLipids: ↓ totCer, Cer C14:0, C16:0, C20:0, C24:0	Huang et al. 2011
RYGB	↓ BMI, fasting insulin, HOMA, LDL-Chol, free Fas; ↓ totChol (3 m), ApoB100 and ApoB100/ApoA1 (6 m). Resolution/Attenuation of all comorbidities at 6 m	SLipids: ↓ Cer C16:0, C18:0 (6 m post-op)	Heneghan et al. 2013
RYGB	↓ BMI, totChol, LDL-Chol, TGC	↓TAG with very long-chain MU/PUFA (46 - 54C), ↓ CholEs with medium-chain S/MU/PUFAs (14 - 20C); GPLipids: ↓ LysoPCs with medium-chain S/MU/PUFAs (14 - 20C); ↓ PCs with long-chain S/MU/PUFAs (30 - 40C), ↓ PC ethers with long-chain MU/PUFAs (34:2, 36:3, 36:4), ↓ PEs with long-chain U/PUFAs (34 - 36C), ↓ PE ethers with long-chain PUFAs (34 - 40C), ↓ PI with long-chain MU/PUFAs (34 - 38); SLipids: ↓ Cer with very long-chain MU/PUFA (40 - 42C), ↓ SM with long-chain MUFAs (32 - 42C)	Graessler et al. 2013
RYGB	↓ BMI	BAs (postprandial only): ↑ Gly- and Tau- conjugated	Ahmad et al. 2013
RYGB	↓ BMI, FG, HOMA, ALT, energy expenditure, respiratory quotient, glucose oxidation	BAs (fasting): ↑ totBAs, 2ary: DCA, GLCA, ↓Tau-conjugated (taurocholic acid), Tau/Gly conjugation ratio	Simonen et al. 2012
RYGB	↓ BMI, fat mass, % body fat, fasting glucose, HOMA, LDL-Chol, TAG; ↑ HDL-Chol.	AAs: ↓ Leu, Ileu, Val, Thre, ↑ glutamine/glutamate; ↓ lactate, glucose; ↑ acetate, methylamine, dimethylamine, O-acetyl-carnitine, FAs: ↑ SFA (14-18C, 22C) ; ↓PUFAs (C18:3n3, C18:2n6, C20:5n3, C20:4n3, C20:3n6,	Lopes, et al 2015
RYGB/BD	↓ BMI, Chol, TAG; ↑IS	Xeno-metabolites: ↑ hippuric acid (>> after BD), trigonelline, xanthine; ↓ 2-hydroxyisobutyrate	Calvani, R et al. 2010
RYGB/SG	nd	AAs & derivatives: ↑ Gly; 3-hydroxybutyrate, 2-hydroxyisobutyrate; Xeno-metabolites: ↓ hippuric acid, trigonelline	Friedrich et al. 2012
Combined Behavioural Interventions			
CR + EX	↓ BMI, ↓ HOMA	AAs: ↓ BCAAs (Val, Leu/Ileu), AAAs (Phe, Tyr)	Shah SH, et al. 2012
CR + EX + vitamin/mineral	↓ BMI, VO2peak, MaxPow, FG, fasting insulin, Matsuda index, QUICKI	AAs & derivatives: ↓ Glt, Cys, Arg/Orn; ↑ Ser, Gly, N-methyl-Ala; CHO metabolites: ↓ glucose, insulin, α-ketoglutaric, pyruvic acid, lactic acid, ↑ malic acid; Purine/pyrimidine metabolites: ↓ uridine, uric acid; Xeno-metabolites: ↓ Υ-tocopherol, ↑ HMFA (fasted state only)^	Campbell et al. 2014

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Dietary Interventions (unchanged physical activity)			
Isocaloric diet + BSP (4.5g/meal)	↓ BMI, fat mass, TAG (ns); ↑ HDL-Chol	AAs & derivatives: ↓ L-Pro, Leu/Ileu, Tyr, Trp, phenylpyruvic acid, ↓ 2-phenylGly, ↑ betaine; ACs: ↓ C8; GPLipids: ↓ LysoPCs with long-chain SFAs PC16:0, C18:0, ↑ LysoPCs with long-chain MU/PUFAs C18:1, C18:2, C20:1, and C20:4 ^^	Kim et al. 2013
CR/LowCho + vitamin/mineral	↓ fasting & 2h postprandial insulin, fasting & 2h postprandial HOMA	AAs & derivatives: ↓ Ala, Glt, Pro, Orn, ↑ The, urea; CHO metabolites: ↑ ribose, mannose, ↓ succinic acid, threitol; long-chain FAs: ↓ C16:1, C18:0; Xeno-metabolites: ↑ p-cresol; Other: ↓ pyridine	Gu et al 2013
Personalized CR	↓ BMI, waist circumference, fat mass, FG, DBP, tot-Chol, LDL-Chol. ↓ lean mass and HDL-Chol. Other: ↑ Δ5-desaturase activity	AAs: ↓ Ileu; SFAs: ↓ total, C14:0, C16:0, C18:0, C24:0; MUFAs: ↓ total, C18:1, C20:1; ω-6 PUFAs: ↓ total, C18:2n-6, C20:4n-6, C20:3n-6, C20:2n6; ω-3 PUFAs: ↓ total, C22:6n-3	Perez-Cornago et al. 2014
CR (12w) + WM (21w)	SHARED: ↓ FG, ↑ IS; CR only = ↓ BMI	↓TAG with long-chain SFA/MUFA: i.e 16:0/14:0/14:1; GPLipids: ↓ PC with long-chain SFA/MUFA: i.e 18:0/20:4	Schwab et al 2008

Abbreviations are listed in the Supporting Information File. Nd: not available . ^ Metabolites detected in the fasted state only, from a combined PLS model using fasted/post-OGTT metabolite variances (Campbell et al. 2014). ^^ Selection of metabolites with VIP > 2 (Kim et al. 2013)

Table S3. Shared and exclusive metabolic variations observed after surgical versus behavioral interventions^.

	Class	Variation[a] Post-bariatric surgery	Post-dietary treatment	Ref.
ketone bodies	3-hydroxybutyrate	↑↑↑↑↑	↑	Laferrère B, et al. 2011; Khoo C, et al. 2013; ^Kwon et al. 2014;Friedrich et al. 2012; Gralka, et al. 2015
	Total ketones	↑↑	↑	Laferrère B, et al. 2011, Khoo C, et al. 2013
Bile acids	1ary	↑		Nakatani H, et al. 2009
	CDCA	↓‡		Yu et al. 2015
	HCA	↑‡		Yu et al. 2015
	2ary	↑↑		Nakatani H, et al. 2009; Simonen M, et al. 2012
	7-KLCA	↓‡		Yu et al. 2015
	Gly-conjugated	↑		Ahmad NN, et al. 2013
	GDCA	↑		Dummas et al. 2015
	GHCA	↑‡		Yu, et al. 2015
	GUDCA	↓II,III		Yu, et al. 2015
	Tau-conjugated	↑↓		Ahmad NN, et al. 2013; Simonen M, et al. 2012
	TDCA, TCDCA, THCA	↑↑‡		Dummas et al. 2015; Yu, et al. 2015
	Tau/Gly conjugation ratio	↓		Simonen M, et al. 2012
	total BAs	↑↑↑		Nakatani H, et al. 2009; Pournaras DJ, et al. 2012; Simonen M, et al. 2012
Amino acids and catabolites	Alanine	↓↓↓↓↓↓	↓↓↓	Laferrère B, et al. 2011; Shah et al. 2012; Khoo C, et al. 2013; Magkos F, et al. 2013; Jüllig et al., 2014; Mutch D, et al. 2009; Gu et al 2013 ; Arora, et al. 2015
	N-methyl-Ala		↑	Campbell et al. 2014
	Valine	↓↓↓↓	↓	Laferrère B, et al. 2011; Khoo C, et al. 2013; Mutch D, et al. 2009; Shah SH, et al. 2012; Gralka, et al. 2015
	2-hydroxy(iso)butyric acid	↑↓		Friedrich et al. 2012; Calvani, R et al. 2010
	Isoleucine	↓↓↓↓↓	↓↓↓↓	Laferrère B, et al. 2011; Shah et al. 2012; Mutch D, et al. 2009; Shah SH, et al. 2012; Kim et al. 2013; Perez-Cornago et al. 2014; Gralka et al. 2015; Lopes et al. 2015
	4-methyl-2-oxovaleric acid	↓		Arora, et al. 2015
	Leucine	↓↓↓↓	↓↓↓	Laferrère B, et al. 2011; Shah et al. 2012; Mutch D, et al. 2009; Shah SH, et al. 2012; Kim et al. 2013; Lopes et al. 2015
	2-ethyl-3-hydroxypropionic acid	↓		Arora, et al. 2015
	Phenylalanine	↓↓↓↓	↓↓	Laferrère B, et al. 2011; Shah et al. 2012; Khoo C, et al. 2013; Shah SH, et al. 2012; Gralka, et al. 2015
	benzenacetic acid	↓		Arora, et al. 2015
	Tyrosine	↓↓↓↓	↓↓↓	Laferrère B, et al. 2011; Shah et al. 2012; Mutch D, et al. 2009; Shah SH, et al. 2012; Kim et al. 2013; Arora, et al. 2015
	Tryptophan		↓	Kim et al. 2013
	Kinurenic acid	↓		Mutch D, et al. 2009
	Proline	↓↓↓	↓↓	Khoo C, et al. 2013; Magkos F, et al. 2013; Jüllig et al, 2014; Kim et al. 2013; Gu et al 2013;
	L-proline	↑		Arora, et al. 2015
	Histidine	↓↓↓↓		Laferrère B, et al. 2011; Khoo C, et al. 2013; Magkos F, et al. 2013; Jüllig et al., 2014
	Ornithine	↓↓↓	↓↓	Laferrère B, et al. 2011; Shah et al. 2012; Magkos F, et al. 2013; Gu et al 2013
	Serine	↑↑	↑	Khoo C, et al. 2013; Magkos F, et al. 2013; Campbell et al. 2014
	Glycine	↑↑↑	↑	Magkos F, et al. 2013; Friedrich et al. 2012; Campbell et al. 2014; Gralka, et al. 2015
	2-Phenylglycine		↓	Kim et al. 2013
	Glutamate	↑↓	↓↓	Oberbach A, et al. 2011; Aasheim et al. 2011; Campbell et al. 2014; Gu et al 2013
	Glutamine	↑↑		Oberbach A, et al. 2011; Lopes, et al. 2015
	Lysine	↓		Mutch D, et al. 2009
	Threonine	↓	↑	Mutch D, et al. 2009; Gu et al 2013
	Cysteine		↓	Campbell et al. 2014
	Methionine	↓↓↑		Magkos F, et al. 2013; Aasheim et al. 2011; Arora, et al. 2015
	Cystathionine	↓		Aasheim et al. 2011
	Threonine	↓	↑	Mutch D, et al. 2009; Gu et al 2013
	Glutamine/Glutamate	↓		Magkos F, et al. 2013
	Asparagine/Aspartate		↓	Laferrère B, et al. 2011
	Arginine/Ornithine		↓	Campbell et al. 2014
	totAAAs	↓↓	↓	Khoo C, et al. 2013, Magkos F, et al. 2013; Shah SH, et al. 2012
	totBCAAs	↓↓↓↓	↓	Laferrère B, et al. 2011; Khoo C, et al. 2013; Magkos F, et al. 2013; Arora, et al. 2015; Shah SH, et al. 2012
	totAAs	↓↓↓		Laferrère B, et al. 2011; Khoo C, et al. 2013; Magkos F, et al. 2013

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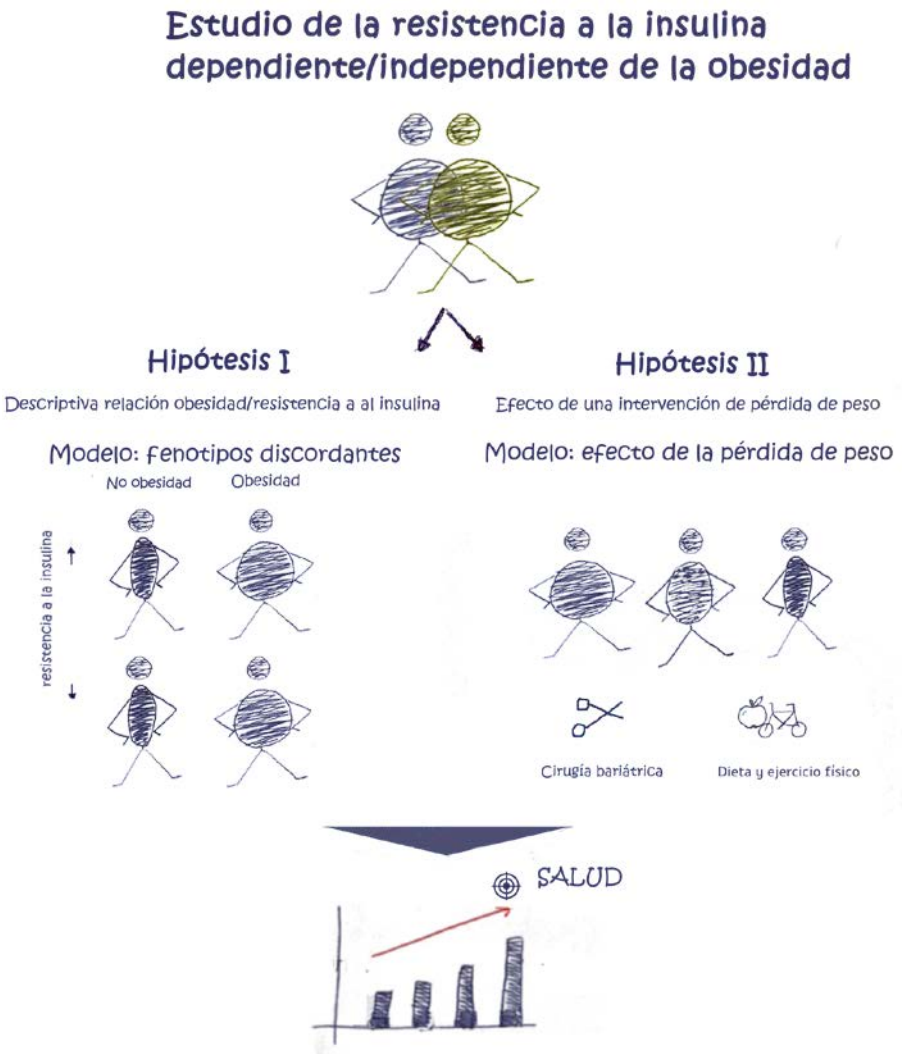
Acylcarnitines	Short-chain (C3, C5)	↓↓↓	↓	Laferrère B, et al. 2011; Khoo C, et al. 2013; Magkos F, et al. 2013
	Short-chain (C2, C4)	↑↓↑↓↑	↑	Laferrère B, et al. 2011; Khoo C, et al. 2013; Magkos F, et al. 2013
	Medium-chain (even-)(C6 - C12)	↓↓↑	↑↓	Laferrère B, et al. 2011; Khoo C, et al. 2013; Magkos F, et al. 2013; Kim et al. 2013
	Long-chain (even-) (≥ C14)	↑↑↓↓↑	↑	Laferrère B, et al. 2011; Khoo C, et al. 2013; Oberbach A, et al. 2011; Magkos F, et al. 2013
	totACs	↑↑	↑	Khoo C, et al. 2013; Magkos F, et al. 2013
CHO metabolites and Organic acids	Malic acid		↑	Campbell et al. 2014
	α-ketoglutaric		↓	Campbell et al. 2014
	Pyruvic acid/pyruvate	↓	↓	Campbell et al. 2014; Gralka, et al. 2015
	Phenylpyruvic acid		↓	Kim et al. 2013
	Lactic acid		↓	Campbell et al. 2014
	Succinic acid		↓	Gu et al 2013
	Citric acid	↓	↑	Jüllig et al., 2014; Piccolo, et al. 2015 (B)
	Glucose, insulin	↓	↓	Arora, et al. 2015; Campbell et al. 2014
	Erythritol	↓		Arora, et al. 2015
	D-galactose	↓		Arora, et al. 2015
	Threitol		↓	Gu et al 2013
	Ribose, mannose		↑	Gu et al 2013
Sphingolipids	Cer, total	↓↓↓	↓	Dube et al. 2011; Mutch D, et al. 2009; Huang H, et al. 2011; Heneghan HM, et al. 2013
	Cer with very long-chain MU/PUFA (40 - 42C)	↓		Graessler J, et al. 2013
	SM with long-chain MUFAs (32 - 42C)	↓↓		Graessler J, et al. 2013; Arora, et al. 2015
	Sphingosine, erytro-C16-sphingosine	↓	↓	Dube et al. 2011; Mutch D, et al. 2009
Fatty acids	SFAs, medium-chain	↓		Jüllig et al., 2014
	SFAs, long-chain (i.e C14:0, C16:0, C18:0, C24:0)	↓	↓	Arora, et al. 2015; Perez-Comago et al. 2014;
	SFAs, total		↓	Perez-Comago et al. 2014;
	S/MUFAs, long-chain C16:1, C18:0, C22:0, C18:1, C20:1	↓↑	↓↓	Mutch D, et al. 2009; Arora, et al. 2015; Gu et al 2013; Perez-Cornago et al. 2014
	MUFAs, total		↓	Perez-Comago et al. 2014;
	PUFAs ω-6, long-chain (i.e C18:3, C18:2n-6, C20:4n-6, C20:3n-6, C20:2n6)	↓	↓	Mutch D, et al. 2009; Perez-Cornago et al. 2014
	PUFAs ω-6, total		↓	Perez-Comago et al. 2014;
	PUFAs ω-3, long-chain (i.e C22:6n-3)		↓	Perez-Comago et al. 2014;
	PUFAs ω-3, total		↓	Perez-Comago et al. 2014;
	NEFAs, total	↑	↑	Khoo C, et al. 2013
Glycerophospholipids	LysoPCs with medium-chain S/MU/PUFAs (14 - 20C)	↓		Graessler J, et al. 2013
	LysoPCs with long-chain SFAs PC16:0, C18:0		↓	Kim et al. 2013
	LysoPCs with long-chain MU/PUFAs C18:1, C18:2, C20:1, and C20:4 ^^		↑	Kim et al. 2013
	LysoPE with long-chain SFAs C18:0	↑		Arora, et al. 2015
	PC with long-chain SFA/MUFA (i.e 18:0/20:4)		↓	Schwab et al 2008
	PCs with very long-chain S/MU/PUFAs (30 - 40C) PC aa C42:0	↓↓↓		Oberbach A, et al. 2011; Graessler J, et al. 2013; Arora, et al. 2015
	PC aa 32:0, PC aa 32:1, PC aa 40:5		↓	Oberbach A, et al. 2011
	PC with long-chain S/MU/PUFA (C18:2, C20:4), (C18:0, C22:6) (C18:0, C18:2)	↑		Mutch D, et al. 2009
	PC ethers with long-chain MU/PUFAs (34:2, 36:3, 36:4)	↓		Graessler J, et al. 2013
	Others (i.e. PE, PS, PI)	↓		Graessler J, et al. 2013
	PE ethers with long-chain PUFAs (34 - 40C)	↓↑		Graessler J, et al. 2013; Arora et al. 2015
	PI with long-chain MU/PUFAs (34 - 38)	↓		Graessler J, et al. 2013

(continued on the following page)

DAG	DAG with long-chain S/MUFA (C14:0/16:0, C14:0/18:0, C14:0/18:1, C16:0/18:0, C16:0/18:1, C16:1/18:1, C18:0/18:1, DI-C14:0, DI-C16:0, DI-C18:1)		↓	Dube et al. 2011
	DAG with long-chain MUFA/PUFA (C18:1, C18:2)	↑		Mutch D, et al. 2009
	DAG with long-chain C16:1/18:0, DI-C16:1, DI-C18:0		↓	Dube et al. 2011
	total DAG		↓	Dube et al. 2011
TAG	TAG with long-chain SFA/MUFA (i.e 16:0/14:0/14:1)		↓	Schwab et al 2008
	TAG with very long-chain MU/PUFA (46 - 58C)	↓↓↑		Graessler J, et al. 2013; Arora, et al. 2015
Other	CholEs with medium-chain S/MU/PUFAs (14 - 20C)	↓		Graessler J, et al. 2013
Purine/Pirimidine deriv.	Xanthine	↑	↑	Calvani, R et al. 2010; Gu et al 2013
	Uridine		↓	Campbell et al. 2014
	Uric acid		↓	Campbell et al. 2014
	Pyridine		↓	Gu et al 2013
Biogenic amines metabolites	N-acetylserotonin sulfate		↑	Tulipani et al JPR 2011
	Hydroxyindolacetic acid		↑	Tulipani et al JPR 2011
Xeno-metabolites (diet- including adulterants, gut microbe-derived)	Methanol/isopropanol/propanol	↓		Gralka et al. 2015
	Hippuric acid	↓↑		Friedrich et al. 2012, Calvani, R et al. 2010
	Trigonelline	↓↑		Friedrich et al. 2012, Calvani, R et al. 2010
	2-hydroxy(iso)butyric acid	↑↓		Friedrich et al. 2012, Calvani, R et al. 2010
	10-hydroxy-decene-4,6-diynoic acid sulfate, tridecadienoic/tridecynoic acid glucuronide, dodecanedioic acid		↑	Tulipani et al JPR 2011
	p-Cresol (sulfate)	↑	↑	Mutch D, et al. 2009; Gu et al 2013
	creatine	↓		Mutch D, et al. 2009
	campesterol	↓		Mutch D, et al. 2009
	ϒ-tocopherol		↓	Campbell et al. 2014
	Dimethyl sulfone	↑		Gralka et al. 2015
	TMAO, dymetilamine	↑↑		Gralka et al. 2015; Lopes et al. 2015
	Betaine		↑	Kim et al. 2013

Abbreviations are listed in the Supporting Information File. ^For bariatric surgery, only metabolites shared by the compared intervention or up- down- regulated at least by RYGB are represented, due to the huge majority of studies centered on the latter surgical technique. ŀOnly in T2D improved, Ĥ Only in T2D non improved; ĦBetween improved vs non-improved T2D.

2. HIPOTESIS Y OBJETIVOS


Estudio de la resistencia a la insulina dependiente/independiente de la obesidad
Hipótesis I
Descriptiva relación obesidad/resistencia a al insulina
Modelo: fenotipos discordantes
No obesidad
Obesidad
resistencia a la insulina
Hipótesis II
Efecto de una intervención de pérdida de peso
Modelo: efecto de la pérdida de peso
Cirugía bariátrica
Dieta y ejercicio físico
SALUD

2.1 Hipótesis de trabajo

Hipótesis 1: *El estudio metabolómico de los fenotipos discordantes obesidad/resistencia a la insulina, diabetes aportará nuevos biomarcadores de riesgo de diabetes que nos permitan profundizar en los mecanismos de acción independientes y asociados a la obesidad e identificar potenciales dianas terapéuticas.*

Hipótesis 2: *La cirugía bariátrica y los cambios en el estilo de vida basados en dieta y actividad física encaminados a la pérdida de peso en sujetos con obesidad, producen cambios metabólicos únicos y compartidos que pueden aportar información sobre los mecanismos implicados en el desarrollo de la resistencia a la insulina, diabetes asociada a la obesidad.*

2.2 Objetivos generales y específicos

El objetivo principal de la presente tesis doctoral es identificar perfiles metabolómicos asociados a elevada resistencia a la insulina dependientes e independientes de la obesidad mediante el modelo de fenotipos discordantes y de los efectos metabólicos asociados a una intervención de pérdida de peso.

Para la consecución de este objetivo se han planteado los siguientes objetivos específicos:

1. Caracterizar los perfiles metabólicos séricos y del tejido adiposo visceral de sujetos con elevada resistencia a la insulina y obesidad y sus rutas metabólicas asociadas al estudio de fenotipos concordantes y discordantes de obesidad y resistencia a la insulina.
2. Identificar biomarcadores, específicos y sensibles en la clasificación de sujetos con elevada resistencia a la insulina o sensibilidad a la insulina en suero y tejido adiposo.
3. Caracterizar los perfiles metabólicos plasmáticos asociados a las mejoras metabólicas a largo plazo que subyace la pérdida de peso por cambios en el estilo de vida mediante dieta Mediterránea y actividad física.

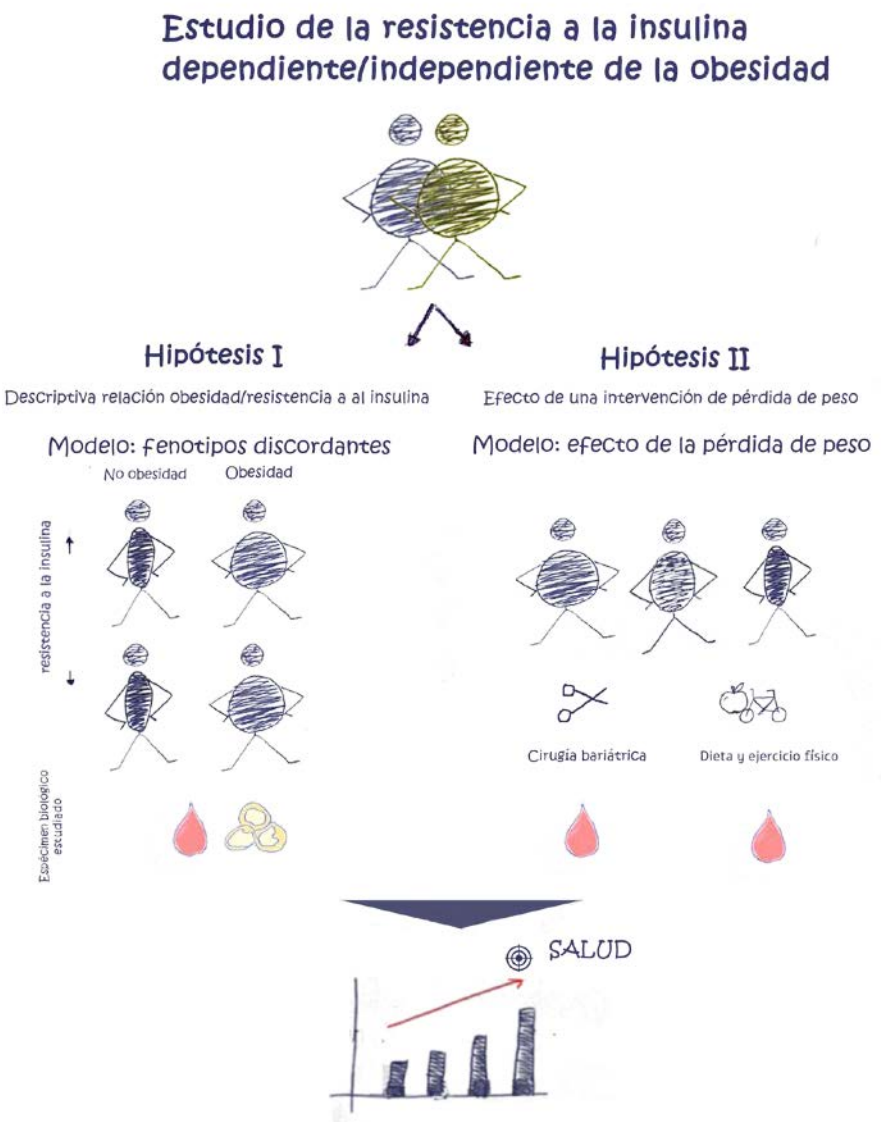
4. Caracterizar los perfiles metabólicos séricos que subyacen las adaptaciones metabólicas post-cirugía bariátrica asociadas a las mejoras metabólicas.
5. Definir perfiles metabólicos séricos de las respuestas a la cirugía bariátrica dependiendo del estado metabólico basal del paciente con obesidad mórbida, metabólicamente *sano* o *enfermo*.

3. METODOLOGIA

3.1 Diseño de estudio

En el siguiente apartado se presenta el diseño de estudio y las características de los participantes de los trabajos de esta tesis doctoral (**Figura 12**).

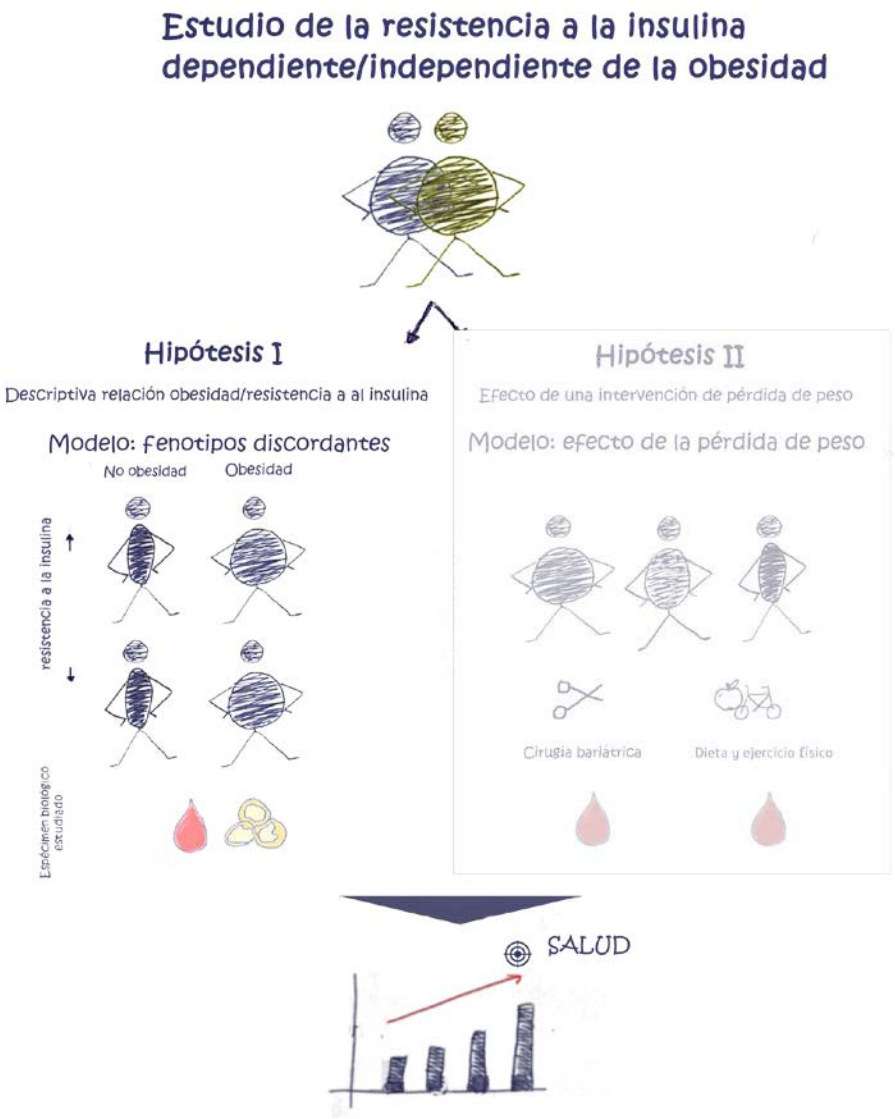
Figura 12. Resumen de los estudios desarrollados



3.1.1 Efecto de la obesidad dependiente e independiente del desarrollo de resistencia a la insulina (HIPOTESIS I)

3.1.1.1 Diseño de estudio de fenotipos discordantes

Figura 13. Modelo de fenotipos discordantes



Diseño del estudio y participantes

El modelo de fenotipos discordantes lo configuran tres análsis metabolómicos realizados sobre un estudio observacional transversal diseñados para identificar la huella metabólica concordante y discordante de la obesidad y la resistencia a la insulina (**Figura 13**). Para esto los análisis metabolómicos se realizaron en 64 muestras de suero y 71 de tejido adiposo visceral.

Los individuos se clasificaron según su IMC en individuos normopeso (IMC = 18.5 -26.9 kg/m^2) o con obesidad mórbida (IMC > 40 kg/m^2) y según el riesgo de desarrollar diabetes tipos 2 en individuos con baja resistencia a la insulina (HOMA-IR <2.5 y glucosa en ayunas <100 mg/dL) o elevada resistencia a la insulina (glucosa en ayunas de 100-125 mg/dL o HOMA-IR >3.4). El punto de corte del HOMA-IR se obtuvo experimentalmente dividiendo toda la cohorte entre cuartiles. Este valor fue más elevado que el de la definición clínica de resistencia a la insulina (>2.6), en línea con otros estudios[90].

Los criterios de exclusión de los participantes fueron la toma de antidiabéticos, corticoides o antibióticos, individuos con abuso a alcohol o fármacos, la presencia de una infección crónica o aguda, enfermedades como la diabetes, hipertensión, enfermedades cardiovasculares, enfermedades psiquiátricas severas, enfermedades sistémicas severas (cáncer, demencia, fallo orgánico).

Variables clínicas y antropométricas

Las variables clínicas que se determinaron en los participantes de este estudio fueron:

- Variables antropométricas, obesidad y hormonas relacionadas
  - Peso/IMC, ratio peso/altura (kg/m^2).
  - Circunferencia de la cintura, medida en cm.

- Circunferencia de la cadera, medida en cm.

- Hormona de la leptina y de la adiponectina. Se analizaron mediante un kit enzimático immunoabsorbete (Diagnostic System Laboratories, Webster, Texas, y DRG Diagnostics GmbH, Marburg, Alemania, respectivamente).

- Variables reguladoras de la glucosa

  - Hemoglobina glicosilada (HbA1c del inglés, *glycated hemoglobin A1c*) .

  - Concentraciones de la glucosa en ayunas (del inglés, *fasing glucose*). Se analizó con el método de *hexokinasa* (equipo, Advia 2400®, Siemens, Alemania).

  - Concentraciones de la insulina en ayunas. Se cuantificó con radioinmunoensayo (BioSource International, Camarillo, Canada).

  - HOMA-IR (del inglés, *Homeostatic Model Assessment resistence insulin index*), calculado a partir de la ecuación:

    HOMA-IR índice = insulina en ayunas × glucosa en ayunas/22.5

- Variables de presión arterial. Medidas con un esfigmomanómetro automático validado (OMRON M4-I) con el paciente en posición asentada.

  - Presión sistólica y diastólica (SBP, DBP del inglés, *systolic* y *diastolic blood pressure*).

- Variables lipídicas. Medidas con el equipo comercial Dimension® AutoAnalyzer (Dade Behring, Deerfield, Ilinois) utilizando el método enzimático (Laboratorios Randox, Crumlin, Country Antrim, Inglaterra).

  - Colesterol total (CHOL del inglés, *total cholesterol*).

  - Colesterol de alta densidad (c-HDL del inglés, *high-density lipoprotein cholesterol*).

  - Colesterol de baja densidad (c-LDL del inglés, *low-density lipoprotein*). Se calculó con la ecuación de Friedwald.

  - Colesterol de muy baja densidad (VLDL del inglés, *very low density lipoprotein*).

  - Triglicéridos (TG del inglés, *triglicerids*).

- Variables indicadoras de la función enzimática del hígado

  - Gamma Glutamil Transpeptidasa (GGT del inglés, *gamma glutamyl transpeptidasa*).

  - Aspartato aminotransferasa o glutamato-oxalacetato transaminasa (GOT del inglés, a*spartate transaminase*).

  - Alanina Aminotransferasa o glutamato-piruvato transaminasa (GPT del inglés, alanine transaminase).

- Variables indicadoras de la función renal, mediante métodos estandarizados (Laboratorios Randox, Antrium, Inglaterra).

  - Ácido úrico, creatinina y urea.

- Variables de inflamación

  - Proteína C- reactiva (CRP del inglés, *C-reactive protein*). Se cuantificó con el kit enzimático immunoabsorbente (BLK Diagnostics (Badalona, España)).

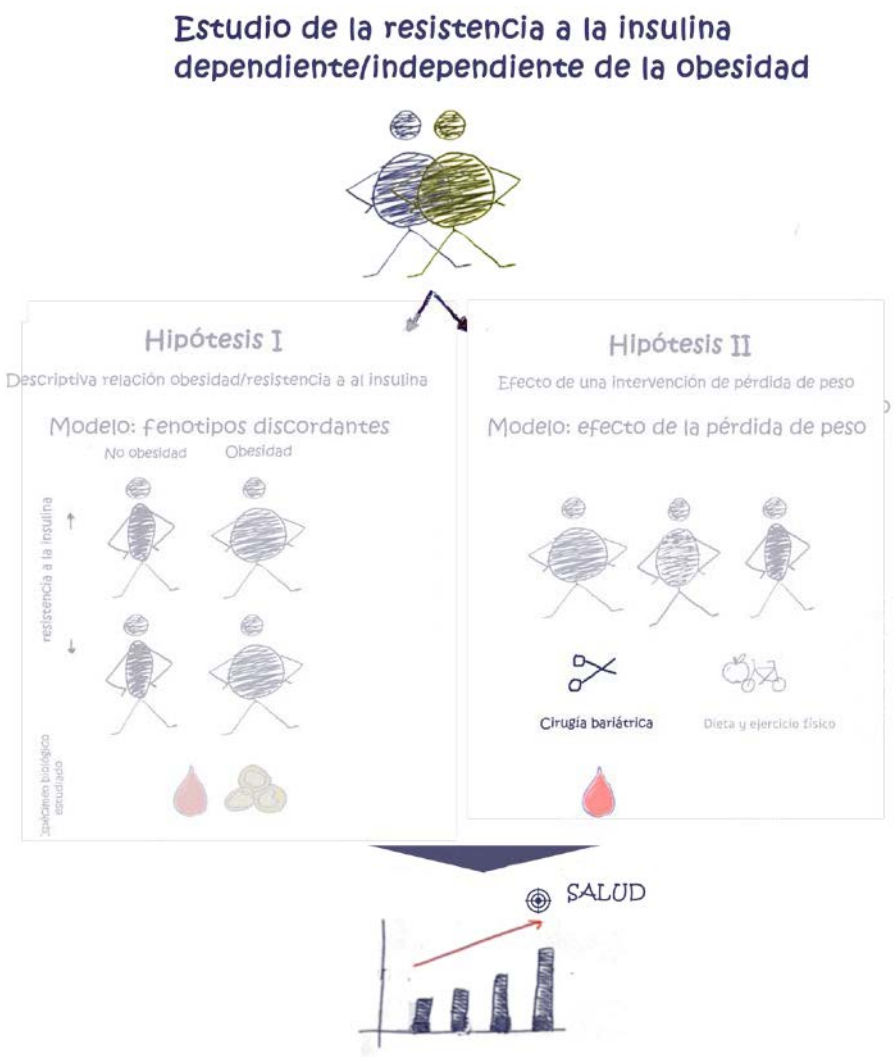
Las muestras eran procedentes del Biobanco del Hospital Clínico Virgen de la Victoria y del Hospital Carlos Haya (Málaga). El protocolo de investigación fue aprobado por el Comité Ético y de Investigación Local (Hospital Universitario

Virgen de la Victoria, Málaga, España) en concordancia con la Declaración de Helsinki y todos los participantes daron su consentimiento escrito en participar en el estudio.

3.1.2 Efecto de la pérdida de peso en la salud metabólica (HIPOTESIS II)

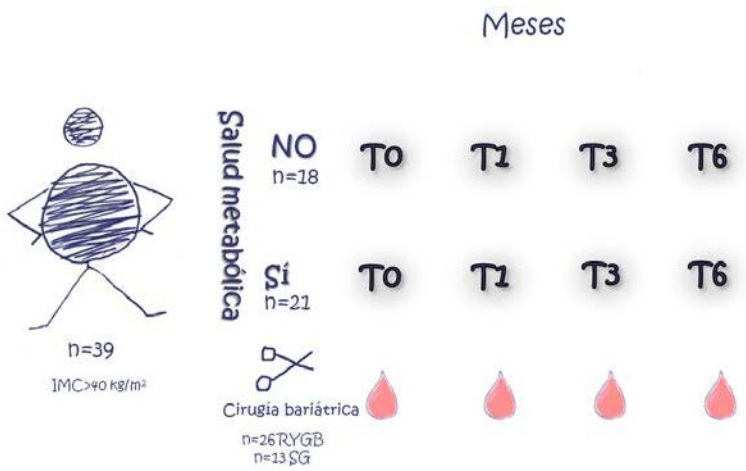
3.1.2.1 Modelo de la cirugía bariátrica

Figura 14. Estudio de los efectos de la cirugía bariátrica



Diseño del estudio y participantes

Figura 15. Diseño de estudio de la cirugía bariátrica



Para estudiar los cambios después de la cirugía bariátrica se realizaron dos análisis metabolómicos complementários en muestras de suero (**Figura 14**). El diseño de estudio fue prospectivo de 39 sujetos de entre 19-59 años de edad con obesidad mórbida (IMC > 40 kg/m^2), con más de 10 años de historia de obesidad y elegibles para cirugía bariátrica.

Los pacientes fueron sometidos a cirugía RYGB o GM y se realizaron los análisis de metabolómica en muestras de suero antes de la cirugía 1, 3 y 6 meses después (**Figura 15**).

Inicialmente todos los participantes fueron agrupados según su estado metabólico, de acuerdo con la definición del ATPIII, considerando los pacientes metabólicamente *enfermos* si presentaba >2 criterios de síndrome metabólica y metabólicamente *sanos* si presentaban ≤2 criterios.

Los criterios de exclusión de los participantes fueron la toma de antidiabéticos, corticoides o antibióticos, individuos con abuso a alcohol o fármacos, la presencia de una infección crónica o aguda, enfermedades como la diabetes, hipertensión,

enfermedades cardiovasculares, enfermedades psiquiátricas severas, enfermedades sistémicas severas (cáncer, demencia, fallo orgánico).

Las muestras de suero pertenecen al Biobanco del Hospital Clínico Virgen de la Victoria y del Hospital Carlos Haya (Málaga). El protocolo de investigación fue aprobado por el Comité Ético y de Investigación Local (Hospital Universitario Virgen de la Victoria, Málaga, España) en concordancia con la Declaración de Helsinki y todos los participantes daron su consentimiento escrito en participar en el estudio.

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- Variables antropométricas, obesidad y hormonas relacionadas
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  - Circunferencia de la cintura, medida en cm.
  - Circunferencia de la cadera, medida en cm.
  - Hormona de la leptina y de la adiponectina. Se analizaron mediante un kit enzimático immunoabsorbete (Diagnostic System Laboratories, Webster, Texas, y DRG Diagnostics GmbH, Marburg, Alemania, respectivamente).
- Variables reguladoras de la glucosa
  - Hemoglobina glicosilada (HbA1c del inglés, *glycated hemoglobin A1c*) .

- Concentraciones de la glucosa en ayunas (del inglés, *fasing glucose*). Se analizó con el método de *hexokinasa* (equipo, Advia 2400®, Siemens, Alemania).

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- HOMA-IR (del inglés, *Homeostatic Model Assessment resistence insulin index*), calculado a partir de la ecuación:

  HOMA-IR índice = insulina en ayunas × glucosa en ayunas/22.5

- Variables de presión arterial. Medidas con un esfigmomanómetro automático validado (OMRON M4-I) con el paciente en posición asentada.

  - Presión sistólica y diastólica (SBP, DBP del inglés, *systolic* y *diastolic blood pressure*).

- Variables lipídicas. Medidas con el equipo comercial Dimension® AutoAnalyzer (Dade Behring, Deerfield, Ilinois) utilizando el método enzimático (Laboratorios Randox, Crumlin, Country Antrim, Inglaterra).

  - Colesterol total (CHOL del inglés, *total cholesterol*).

  - Colesterol de alta densidad (c-HDL del inglés, *high-density lipoprotein cholesterol*).

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  - Alanina Aminotransferasa o glutamato-piruvato transaminasa (GPT del inglés, alanine transaminase).

- Variables indicadoras de la función renal, mediante métodos estandarizados (Laboratorios Randox, Antrium, Inglaterra).

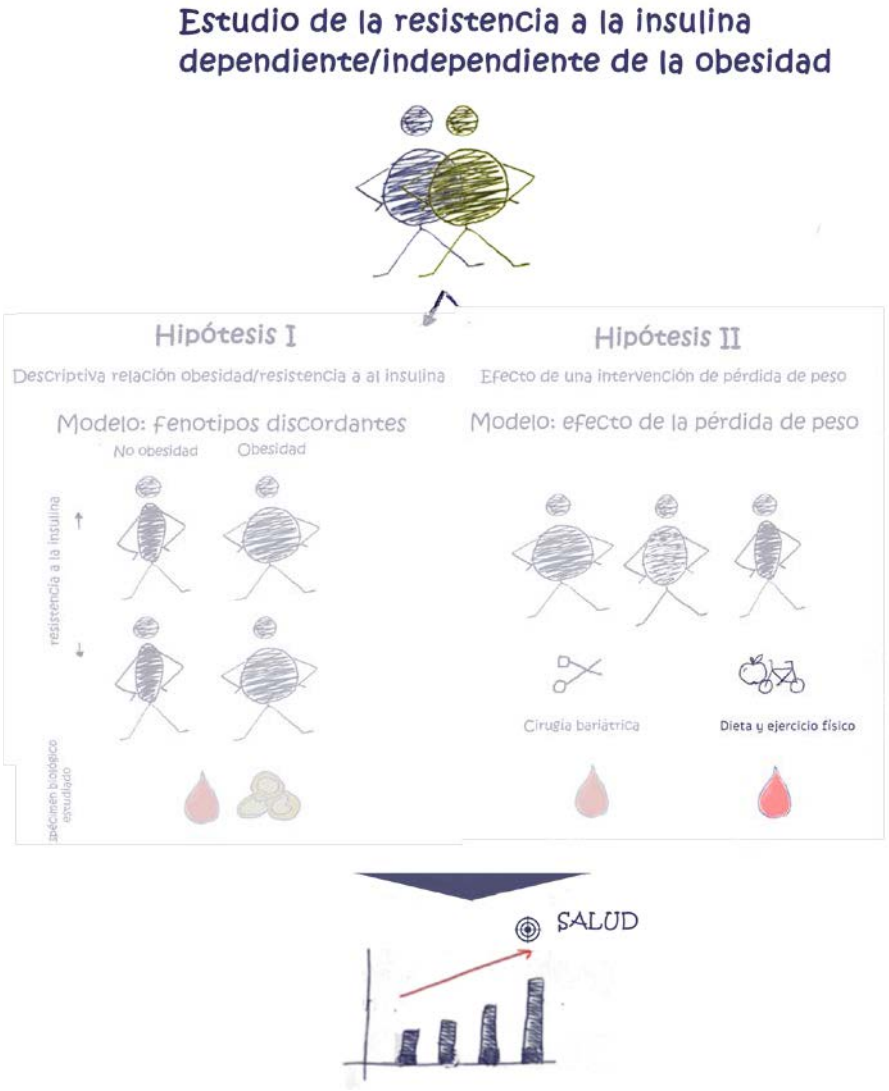
  - Ácido úrico, creatinina y urea.

- Variables de inflamación

  - Proteína C- reactiva (CRP del inglés, *C-reactive protein*). Se cuantificó con el kit enzimático immunoabsorbente (BLK Diagnostics (Badalona, España)).

3.1.2.2 Modelo de intervención en el estilo de vida

Figura 16. Efecto de una intervención con Dieta Mediterránea y ejercicio



Diseño del estudio y participantes

El estudio de intervención se encuentra en el marco del estudio clínico registrado con el número ISRCTN88315555 (http://www.isrctn.com/ISRCTN88315555). Este estudio tiene el objetivo de investigar el efecto de la pérdida de peso, mediante una DietMed hipocalórica y ejercicio físico, en pacientes adultos metabólicamente sanos en la prevención del desarrollo de diabetes y enfermedades cardiovasculares (**Figura 16**).

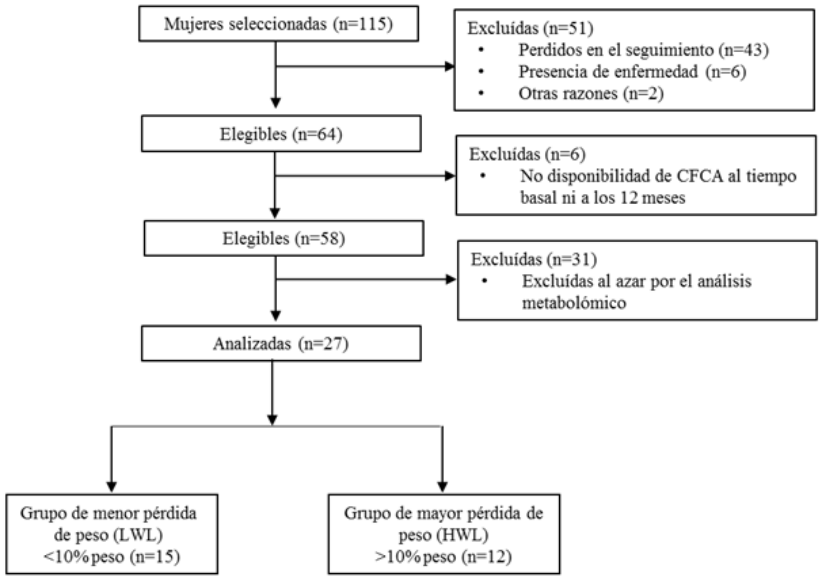
El estudio involucró cuatro centros de primaria de Málaga del servicio de Salud de Andalucía (España) coordinados por la Dra. María Rosa Bernal-López. El protocolo de investigación fue aprobado por el Comité Ético de la Investigación Biomédica de Andalucía en concordancia con la Declaración de Helsinki con consentimiento de los participantes.

La intervención fue un programa basado en una DietMed hipocalórica y la realización de actividad física de manera regular. La Dieta Mediterránica incluyó la ingesta de aceite de oliva virgen extra como principal fuente de grasa visible y la ingesta de vegetales (≥2 porciones/día), frutas (≥3 porciones/día), legumbres (≥3 porciones/semana) y pescado (≥3 veces a la semana), reduciendo el consumo de carne roja y disminuyendo los productos lácteos, bebidas con azúcar y dulces. La dieta se basaba en una reducción de 600 kcal menos/día distribuidas en: 35-40 % grasas (8-10% grasas saturadas), 40-45 % carbohidratos y 20% proteínas. Los participantes se les recomendó la práctica de ejercicio físico alrededor de 150 minutos a la semana durante los 2 años de duración del estudio.

Los participantes recibieron 9 visitas médicas y 4 visitas con la enfermería cada año, las cuales se extendieron en aquellos pacientes que presentaban patologías asociadas. Los controles incluían recomendaciones dietéticas, de higiene general y de valoración clínica. Los participantes fueron seguidos a los 3, 6, 12, 18 y 24 meses.

En el trabajo de metabolómica se realizó en 27 sujetos en plasma con EDTA a tiempo basal y a los 12 meses de la intervención. La selección de las mujeres para el análisis metabolómico fue de acuerdo con los criterios expuestos en la **Figura 17**.

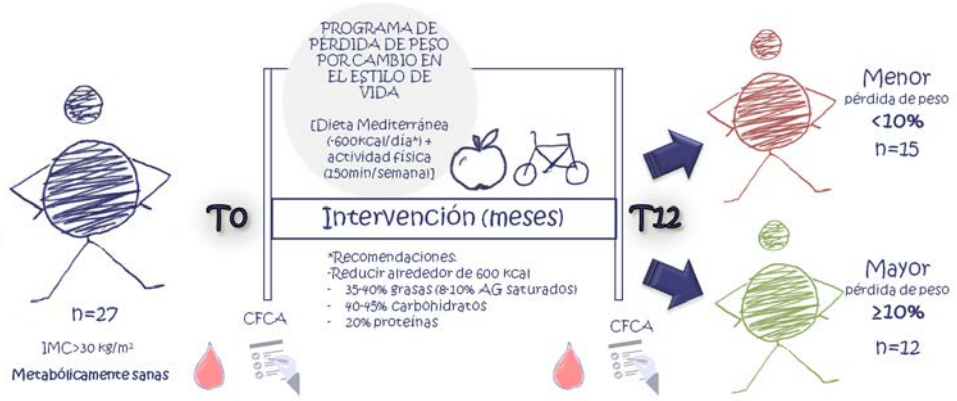
Figura 17. Diagrama de flujo de los participantes incluidos en el estudio



Los participantes incluidos en este estudio eran adultos con obesidad metabólicamente sana (IMC 30-45 kg/m^2). Definidos como metabólicamente sanos según la definición del ATPIII.

Los criterios de exclusión de los participantes fueron la presencia de enfermedades como la diabetes o alteración de la tolerancia a la glucosa, hipertensión, enfermedades cardiovasculares, enfermedades psiquiátricas severas, enfermedades sistémicas severas (cáncer, demencia, fallo orgánico), embarazo en algún momento del estudio, individuos con inmovilización, individuos con abuso al alcohol o fármacos, pacientes que iniciaron un programa de actividad física o empezaron una dieta en los últimos tres meses o perdieron ≥5kg en los últimos 6 meses sin causa conocida (**Figura 18**).

Figura 18. Diseño de estudio del estudio de intervención



Variables clínicas y antropométricas

Las variables clínicas que se han determinado en los participantes de este estudio son:

- Variables de Antropométricas y de obesidad
  - Peso/IMC, ratio peso/altura (kg/m^2)
  - Circunferencia de la cintura, medida en cm
  - Circunferencia de la cadera, medida en cm
  - Masa magra (LM del inglés, *lean mass*)
  - Masa grasa *(*FM del inglés, *fat mass*)
- Variables reguladoras de la glucosa
  - Test de la tolerancia de la glucosa oral (OGTT del inglés *oral glucose tolerance test*), detectado a las 2h con la toma de 75-g de glucosa.

- Hemoglobina glicosilada (HbA1c del inglés, *glycated hemoglobin A1c*).

- Concentraciones de la glucosa en ayunas (del inglés, *fasing glucose*), analizado con el método de *hexokinasa* (equipo, Advia 2400®, Siemens, Alemania).

- Concentraciones de la insulina en ayunas.

- HOMA-IR (del inglés, *Homeostatic Model Assessment resistence insulin index*), calculado a partir de la ecuación:

  HOMA-IR índice = insulina en ayunas × glucosa en ayunas/22.5

- Variables de presión arterial. Medidas con un esfigmomanómetro automático validado (OMRON M4-I) con el paciente en posición asentada.

  - Presión sistólica y diastólica (SBP, DBP del inglés, *systolic* y *diastolic blood pressure*).

- Variables lipídicas. Medidas con el equipo comercial Dimension® AutoAnalyzer (Dade Behring, Deerfield, Ilinois) utilizando el método enzimático (Laboratorios Randox, Crumlin, Country Antrim, Inglaterra).

  - Colesterol total (CHOL del inglés, *total cholesterol*).

  - Colesterol de alta densidad (c-HDL del inglés, *high-density lipoprotein cholesterol*).

  - Colesterol de baja densidad (c-LDL del inglés, *low-density lipoprotein*). Se calculó con la ecuación de Friedwald.

  - Colesterol de muy baja densidad (VLDL del inglés, *very low density lipoprotein*).

– Triglicéridos (TG del inglés, *triglicerids*).

Cuestionarios

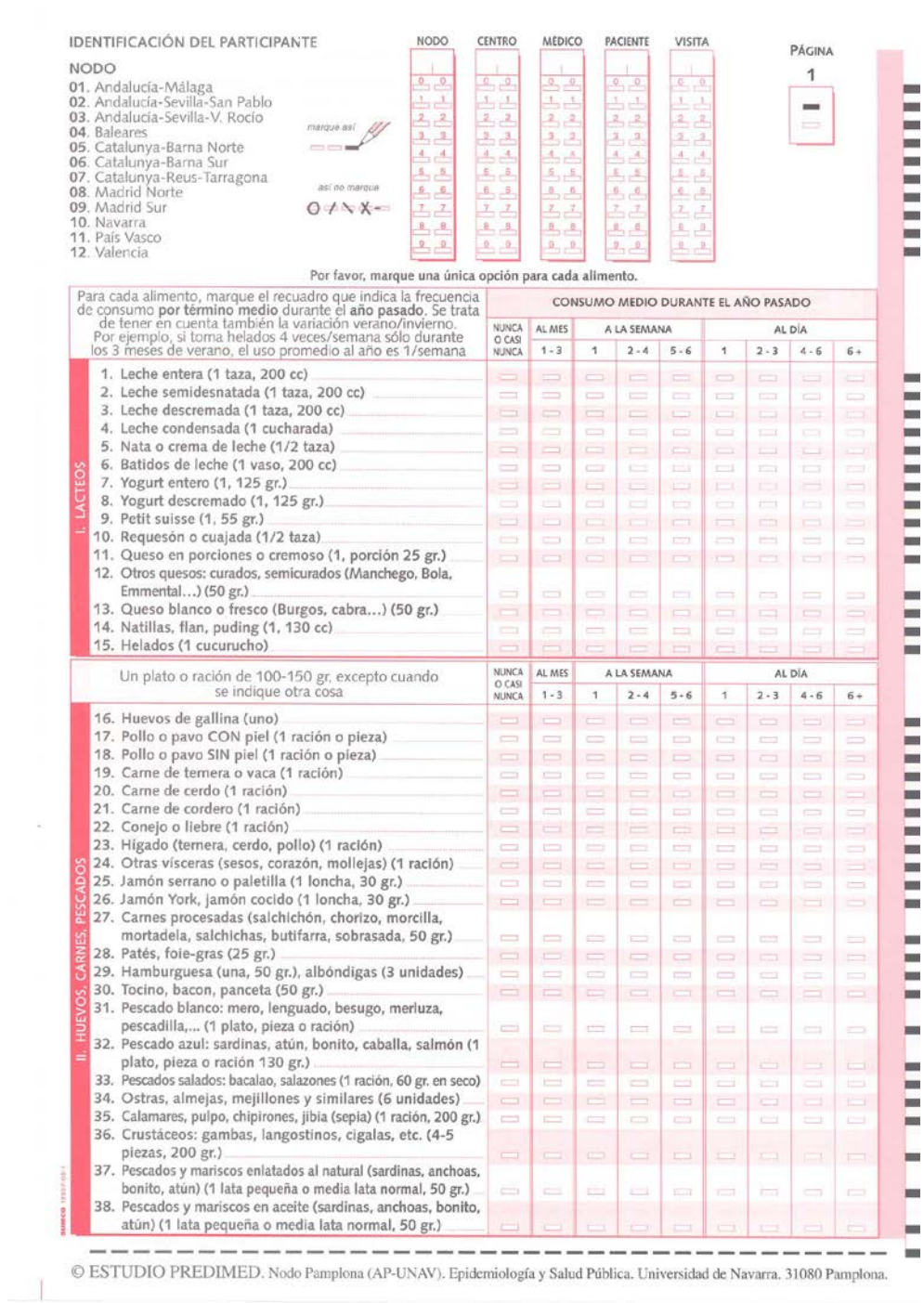
Evaluación de la ingesta dietética

La evaluación de la ingesta dietética se realizó mediante el cuestionario de frecuencia de consumo de alimentos (CFCA).

El CFCA es el cuestionario semicuantitiativo más usado para la estimación de una ingesta habitual de nutrientes específicos o para proporcionar estimaciones de la ingesta a largo plazo (generalmente 6 meses a 1 año) [91,92]. Los CFCA son utilizados en estudios epidemiológicos debido a su bajo coste y su fácil administración, tanto por el entrevistador como por el auto-administrado. Los resultados de los CFCA incluyen tamaños de porciones, alimentos específicos utilizados que proporcionan datos de nutrientes dentro de una categoría de alimentos y recetas usadas para estos alimentos. Los participantes responden sobre la frecuencia de consumo de una lista de alimentos previamente especificada y expresada en varias categorías como “una vez por día o 1-2 veces por semana" y así sucesivamente. Adicionalmente existen preguntas sobre el tamaño de porción de los alimentos y métodos de preparación.

El CFCA utilizado en este estudio fue un cuestionario validado semicuantitativo de 137-items[93] (**Figura 19**). El contenido nutricional se valoró a partir de las tablas españolas de composición de alimentos [94,95].

Figura 19. Cuestionario de frecuencia de consumo de alimentos



IDENTIFICACIÓN DEL PARTICIPANTE

NODO
01. Andalucía-Málaga
02. Andalucía-Sevilla-San Pablo
03. Andalucía-Sevilla-V. Rocío
04. Baleares
05. Catalunya-Barna Norte
06. Catalunya-Barna Sur
07. Catalunya-Reus-Tarragona
08. Madrid Norte
09. Madrid Sur
10. Navarra
11. País Vasco
12. Valencia

marque así

así no marque

NODO | CENTRO | MÉDICO | PACIENTE | VISITA

PÁGINA 1

Por favor, marque una única opción para cada alimento.

Para cada alimento, marque el recuadro que indica la frecuencia de consumo **por término medio** durante el **año pasado**. Se trata de tener en cuenta también la variación verano/invierno. Por ejemplo, si toma helados 4 veces/semana sólo durante los 3 meses de verano, el uso promedio al año es 1/semana

CONSUMO MEDIO DURANTE EL AÑO PASADO

	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1-3	1	2-4	5-6	1	2-3	4-6	6+
I. LÁCTEOS									
1. Leche entera (1 taza, 200 cc)									
2. Leche semidesnatada (1 taza, 200 cc)									
3. Leche descremada (1 taza, 200 cc)									
4. Leche condensada (1 cucharada)									
5. Nata o crema de leche (1/2 taza)									
6. Batidos de leche (1 vaso, 200 cc)									
7. Yogurt entero (1, 125 gr.)									
8. Yogurt descremado (1, 125 gr.)									
9. Petit suisse (1, 55 gr.)									
10. Requesón o cuajada (1/2 taza)									
11. Queso en porciones o cremoso (1, porción 25 gr.)									
12. Otros quesos: curados, semicurados (Manchego, Bola, Emmental...) (50 gr.)									
13. Queso blanco o fresco (Burgos, cabra...) (50 gr.)									
14. Natillas, flan, puding (1, 130 cc)									
15. Helados (1 cucurucho)									

Un plato o ración de 100-150 gr. excepto cuando se indique otra cosa	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1-3	1	2-4	5-6	1	2-3	4-6	6+
II. HUEVOS, CARNES, PESCADOS									
16. Huevos de gallina (uno)									
17. Pollo o pavo CON piel (1 ración o pieza)									
18. Pollo o pavo SIN piel (1 ración o pieza)									
19. Carne de ternera o vaca (1 ración)									
20. Carne de cerdo (1 ración)									
21. Carne de cordero (1 ración)									
22. Conejo o liebre (1 ración)									
23. Hígado (ternera, cerdo, pollo) (1 ración)									
24. Otras vísceras (sesos, corazón, mollejas) (1 ración)									
25. Jamón serrano o paletilla (1 loncha, 30 gr.)									
26. Jamón York, jamón cocido (1 loncha, 30 gr.)									
27. Carnes procesadas (salchichón, chorizo, morcilla, mortadela, salchichas, butifarra, sobrasada, 50 gr.)									
28. Patés, foie-gras (25 gr.)									
29. Hamburguesa (una, 50 gr.), albóndigas (3 unidades)									
30. Tocino, bacon, panceta (50 gr.)									
31. Pescado blanco: mero, lenguado, besugo, merluza, pescadilla,... (1 plato, pieza o ración)									
32. Pescado azul: sardinas, atún, bonito, caballa, salmón (1 plato, pieza o ración 130 gr.)									
33. Pescados salados: bacalao, salazones (1 ración, 60 gr. en seco)									
34. Ostras, almejas, mejillones y similares (6 unidades)									
35. Calamares, pulpo, chipirones, jibia (sepia) (1 ración, 200 gr.)									
36. Crustáceos: gambas, langostinos, cigalas, etc. (4-5 piezas, 200 gr.)									
37. Pescados y mariscos enlatados al natural (sardinas, anchoas, bonito, atún) (1 lata pequeña o media lata normal, 50 gr.)									
38. Pescados y mariscos en aceite (sardinas, anchoas, bonito, atún) (1 lata pequeña o media lata normal, 50 gr.)									

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Por favor, marque una única opción para cada alimento.

	Un plato o ración de 200 grs, excepto cuando se indique	CONSUMO MEDIO DURANTE EL AÑO PASADO								
		NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
			1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
III. VERDURAS Y HORTALIZAS	39. Acelgas, espinacas									
	40. Col, coliflor, brócoles									
	41. Lechuga, endivias, escarola (100 gr.)									
	42. Tomate crudo (1, 150 gr)									
	43. Zanahoria, calabaza (100 gr.)									
	44. Judías verdes									
	45. Berenjenas, calabacines, pepinos									
	46. Pimientos (150 gr.)									
	47. Espárragos									
	48. Gazpacho andaluz (1 vaso, 200 gr.)									
	49. Otras verduras (alcachofa, puerro, cardo, apio)									
	50. Cebolla (media unidad, 50 gr.)									
	51. Ajo (1 diente)									
	52. Perejil, tomillo, laurel, orégano, etc. (una pizca)									
	53. Patatas fritas comerciales (1 bolsa, 50 gr.)									
	54. Patatas fritas caseras (1 ración, 150 gr.)									
	55. Patatas asadas o cocidas									
	56. Setas, níscalos, champiñones									

	Una pieza o ración	CONSUMO MEDIO DURANTE EL AÑO PASADO								
		NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
			1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
IV. FRUTAS	57. Naranja (una), pomelo (una), o mandarinas (dos)									
	58. Plátano (uno)									
	59. Manzana o pera (una)									
	60. Fresas/fresones (6 unidades, 1 plato postre)									
	61. Cerezas, picotas, ciruelas (1 plato de postre)									
	62. Melocotón, albaricoque, nectarina (una)									
	63. Sandía (1 tajada, 200-250 gr.)									
	64. Melón (1 tajada, 200-250 gr.)									
	65. Kiwi (1 unidad, 100 gr.)									
	66. Uvas (un racimo, 1 plato postre)									
	67. Aceitunas (10 unidades)									
	68. Frutas en almíbar o en su jugo (2 unidades)									
	69. Dátiles, higos secos, uvas-pasas, ciruelas-pasas (150 gr.)									
	70. Almendras, cacahuetes, avellanas, pistachos, piñones (30 gr.)									
	71. Nueces (30 gr.)									

	72. ¿Cuántos días a la semana toma fruta como postre?	0	1	2	3	4	5	6	7

	Un plato o ración (150 gr.)	CONSUMO MEDIO DURANTE EL AÑO PASADO								
		NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
			1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
V. LEGUMBRES Y CEREALES	73. Lentejas (1 plato, 150 gr. cocidas)									
	74. Alubias (pintas, blancas o negras) (1 plato, 150 gr. cocidas)									
	75. Garbanzos (1 plato, 150 gr. cocidos)									
	76. Guisantes, habas (1 plato, 150 gr. cocidas)									
	77. Pan blanco, pan de molde (3 rodajas, 75 gr.)									
	78. Pan negro o integral (3 rodajas, 75 gr.)									
	79. Cereales desayuno (30 gr.)									
	80. Cereales integrales: muesli, copos avena, all-bran (30 gr.)									
	81. Arroz blanco (60 gr. en crudo)									
	82. Pasta: fideos, macarrones, espaguetis, otras (60 gr. en crudo)									
	83. Pizza (1 ración, 200 gr.)									

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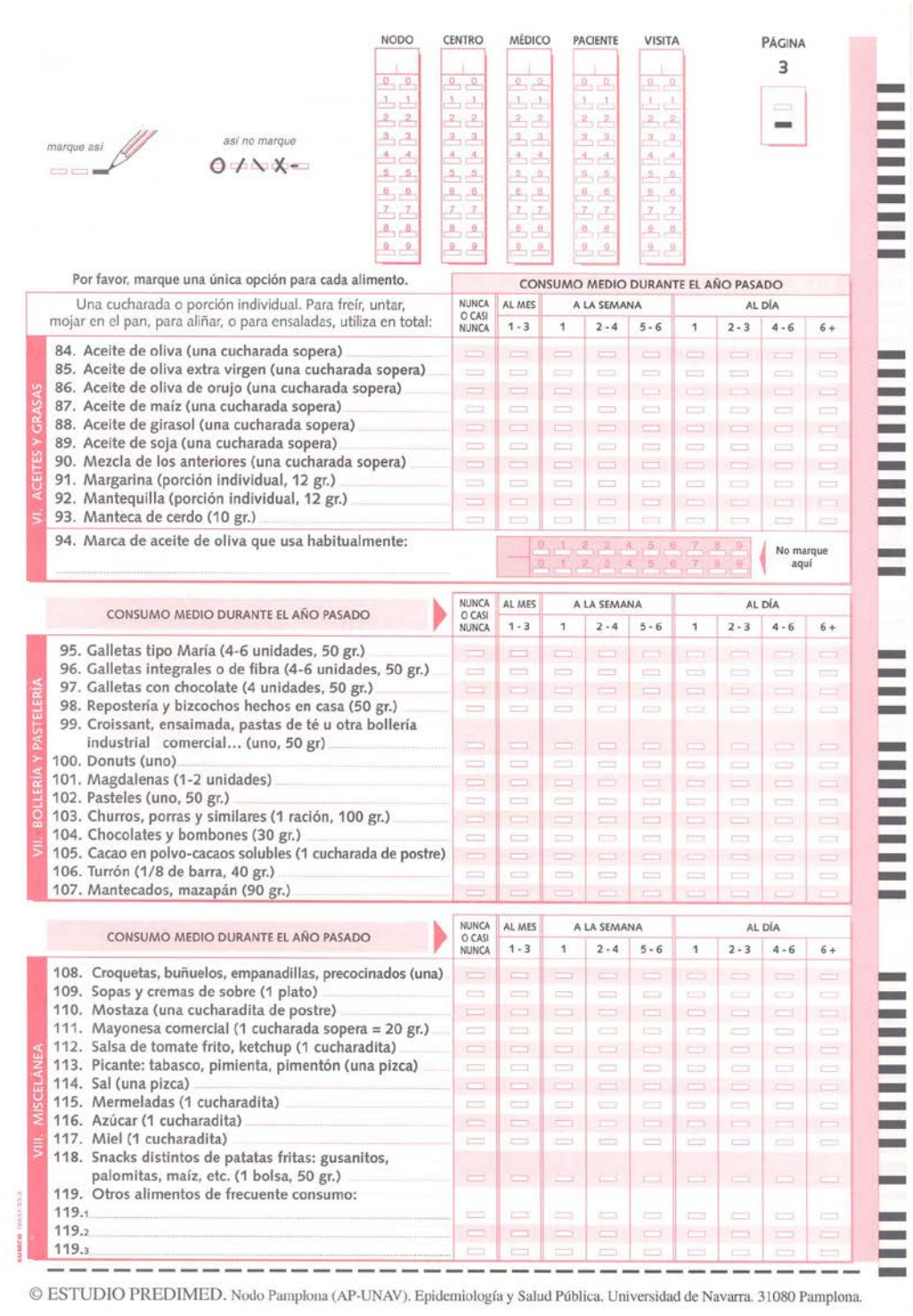

NODO | CENTRO | MÉDICO | PACIENTE | VISITA

PÁGINA 3

marque así

así no marque

Por favor, marque una única opción para cada alimento.

CONSUMO MEDIO DURANTE EL AÑO PASADO

Una cucharada o porción individual. Para freír, untar, mojar en el pan, para aliñar, o para ensaladas, utiliza en total:	NUNCA O CASI NUNCA	AL MES 1-3	A LA SEMANA 1	A LA SEMANA 2-4	A LA SEMANA 5-6	AL DÍA 1	AL DÍA 2-3	AL DÍA 4-6	AL DÍA 6+
VI. ACEITES Y GRASAS									
84. Aceite de oliva (una cucharada sopera)									
85. Aceite de oliva extra virgen (una cucharada sopera)									
86. Aceite de oliva de orujo (una cucharada sopera)									
87. Aceite de maíz (una cucharada sopera)									
88. Aceite de girasol (una cucharada sopera)									
89. Aceite de soja (una cucharada sopera)									
90. Mezcla de los anteriores (una cucharada sopera)									
91. Margarina (porción individual, 12 gr.)									
92. Mantequilla (porción individual, 12 gr.)									
93. Manteca de cerdo (10 gr.)									
94. Marca de aceite de oliva que usa habitualmente:									

No marque aquí

CONSUMO MEDIO DURANTE EL AÑO PASADO

	NUNCA O CASI NUNCA	AL MES 1-3	A LA SEMANA 1	A LA SEMANA 2-4	A LA SEMANA 5-6	AL DÍA 1	AL DÍA 2-3	AL DÍA 4-6	AL DÍA 6+
VII. BOLLERÍA Y PASTELERÍA									
95. Galletas tipo María (4-6 unidades, 50 gr.)									
96. Galletas integrales o de fibra (4-6 unidades, 50 gr.)									
97. Galletas con chocolate (4 unidades, 50 gr.)									
98. Repostería y bizcochos hechos en casa (50 gr.)									
99. Croissant, ensaimada, pastas de té u otra bollería industrial comercial... (uno, 50 gr)									
100. Donuts (uno)									
101. Magdalenas (1-2 unidades)									
102. Pasteles (uno, 50 gr.)									
103. Churros, porras y similares (1 ración, 100 gr.)									
104. Chocolates y bombones (30 gr.)									
105. Cacao en polvo-cacaos solubles (1 cucharada de postre)									
106. Turrón (1/8 de barra, 40 gr.)									
107. Mantecados, mazapán (90 gr.)									

CONSUMO MEDIO DURANTE EL AÑO PASADO

	NUNCA O CASI NUNCA	AL MES 1-3	A LA SEMANA 1	A LA SEMANA 2-4	A LA SEMANA 5-6	AL DÍA 1	AL DÍA 2-3	AL DÍA 4-6	AL DÍA 6+
VIII. MISCELÁNEA									
108. Croquetas, buñuelos, empanadillas, precocinados (una)									
109. Sopas y cremas de sobre (1 plato)									
110. Mostaza (una cucharadita de postre)									
111. Mayonesa comercial (1 cucharada sopera = 20 gr.)									
112. Salsa de tomate frito, ketchup (1 cucharadita)									
113. Picante: tabasco, pimienta, pimentón (una pizca)									
114. Sal (una pizca)									
115. Mermeladas (1 cucharadita)									
116. Azúcar (1 cucharadita)									
117. Miel (1 cucharadita)									
118. Snacks distintos de patatas fritas: gusanitos, palomitas, maíz, etc. (1 bolsa, 50 gr.)									
119. Otros alimentos de frecuente consumo:									
119.1									
119.2									
119.3									

Página 4

Por favor, marque una única opción para cada alimento.

IX. BEBIDAS

	CONSUMO MEDIO DURANTE EL AÑO PASADO								
	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6+
120. Bebidas carbonatadas con azúcar: bebidas con cola, limonadas, tónicas, etc. (1 botellín, 200 cc)									
121. Bebidas carbonatadas bajas en calorías, bebidas light (1 botellín, 200 cc)									
122. Zumo de naranja natural (1 vaso, 200 cc)									
123. Zumos naturales de otras frutas (1 vaso, 200 cc)									
124. Zumos de frutas en botella o enlatados (200 cc)									
125. Café descafeinado (1 taza, 50 cc)									
126. Café (1 taza, 50 cc)									
127. Té (1 taza, 50 cc)									
128. Mosto (100 cc)									
129. Vaso de vino rosado (100 cc)									
130. Vaso de vino moscatel (50 cc)									
131. Vaso de vino tinto joven, del año (100 cc)									
132. Vaso de vino tinto añejo (100 cc)									
133. Vaso de vino blanco (100 cc)									
134. Vaso de cava (100 cc)									
135. Cerveza (1 jarra, 330 cc)									
136. Licores, anís o anisetes... (1 copa, 50 cc)									
137. Destilados: whisky, vodka, ginebra, coñac (1 copa, 50 cc)									

138. ¿A que edad empezó a beber alcohol (vino, cerveza o licores), incluyendo el que toma con las comidas con regularidad (más de siete "bebidas" a la semana)?

Edad (años)

Decena
Unidad

139. ¿Cuántos años ha bebido alcohol con regularidad (más de siete "bebidas" a la semana)?

Años

Decena
Unidad

119. Otros alimentos de frecuente consumo

119.1 (No marque aquí)

119.2 (No marque aquí)

119.3 (No marque aquí)

Si durante el año pasado tomó vitaminas y/o minerales (incluyendo calcio) o productos dietéticos especiales (salvado, aceite de onagra, leche con ácidos grasos omega-3, flavonoides, etc.), por favor indique la marca y la frecuencia con que los tomó:

Marcas de los suplementos de vitaminas o minerales o de los productos dietéticos	CONSUMO MEDIO DURANTE EL AÑO PASADO								
	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6+
140.									
140.1									
140.2									

140 (No marque aquí)

140.1 (No marque aquí)

140.2 (No marque aquí)

Muchas gracias por su colaboración

La adherencia a la Dieta Mediterránea fue medida usando el cuestionario validado de 14-ítems del estudio de PREDIMED [96]. Este cuestionario está compuesto por 12 preguntas acerca la frecuencia del consumo de alimentos y otras 2 preguntas sobre los hábitos de la ingesta de alimentos considerados de la dieta Mediterránea española. Cada respuesta se le asigna una puntuación de 0 o 1, con una puntuación total que va de los 0 a 14. Una puntuación menor a 7 puntos se considera que hay una baja adherencia a la Dieta Mediterránea, por encima de 10, buena adherencia a la Dieta Mediterránea (**Tabla 3**).

Tabla 3. Cuestionario para la evaluación de la adherencia a la dieta Mediterránea

PREGUNTA	VALORACIÓN	Puntos
1. ¿Usa el aceite de oliva como principal grasa para cocinar?	Sí = 1 punto	
2. ¿Cuánto aceite de oliva consume en total al día? (incluyendo el usado para freír, comidas fuera de casa, ensaladas, etc…)	2 ó mas cucharadas = 1 punto	
3. ¿Cuántas raciones de verduras u hortalizas consume al día ? (1 ración = 200 g. Las guarniciones o acompañamientos = ½ ración)	2 ó más (al menos 1 de ellas en ensalada o crudas) = 1 punto	
4. ¿Cuántas piezas de fruta (incluyendo zumo natural) consume al día?	3 ó más = 1 punto	
5. ¿Cuántas raciones de carnes rojas, hamburguesas, salchichas o embutidos consume al día ? (1 ración = 100-150 g)	Menos de 1 = 1 punto	
6. ¿Cuántas raciones de mantequilla, margarina o nata consume al día? (porción individual = 12 g)	Menos de 1 = 1 punto	
7. ¿Cuántas bebidas carbonatadas y/o azucaradas consume al día? (refrescos, colas, tónicas, bitter)	Menos de 1 = 1 punto	
8. ¿Bebe vino? ¿Cuánto consume a la semana?	3 ó más vasos = 1 punto	
9. ¿Cuántas raciones de legumbres consume a la semana ? (1 plato o ración = 150 g)	3 ó más = 1 punto	
10. ¿Cuántas raciones de pescado/mariscos consume a la semana? (1 plato, pieza o ración = 100-150 g de pescado ó 4-5 piezas ó 200 g de marisco)	3 ó más = 1 punto	
11. ¿Cuántas veces consume repostería comercial a la semana? (no casera, como: galletas, flanes, dulces, bollería, pasteles)	Menos de 3 = 1 punto	
12. ¿Cuántas veces consume frutos secos a la semana	1 ó más = 1 punto	

(1 ración = 30 g) ?	
13. ¿Consume preferentemente carne de pollo, pavo o conejo en vez de ternera, cerdo, hamburguesas o salchichas ? (carne de pollo, pavo o conejo: 1 pieza o ración de 100-150 g)	Sí = 1 punto
14. ¿Cuántas veces a la semana consume los vegetales cocinados, la pasta, arroz u otros platos aderezados con salsa de tomate, ajo, cebolla o puerro elaborada a fuego lento con aceite de oliva ? (sofrito)	2 ó más = 1 punto

Fuente: Adaptado de Trichopoulou A et al. [11]

Evaluación del ejercicio físico

El ejercicio físico se evaluó utilizando el cuestionario RAPA del inglés *Rapid Assessment of Physical Activity questionnaire* [97]. Este cuestionario consta de 9 preguntas de sí o no divididas en dos grupos, el RAPA 1 formdo por 7 preguntas para determinar el nivel de actividad aeróbica que realiza el participante y el RAPA 2 formado por 2 preguntas para determinar si se trabaja la fuerza y la flexibilidad. Según las respuestas del RAPA se clasificaron los participantes en sedentarios, con actividad física moderada y cono elevada actividad física, utilizando los puntos de corte descritos en el cuestionario (**Figura 20**).

Figura 20. Cuestionario RAPA para adultos: *Rapid Assessment of Physical Activity*

¿Cuál es su nivel de actividad física?

Valoración del nivel y la intensidad de la actividad física.

Versión adaptada culturalmente y retrotraducida por:
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Escola d'Infermeria La Fe – Valencia – España.
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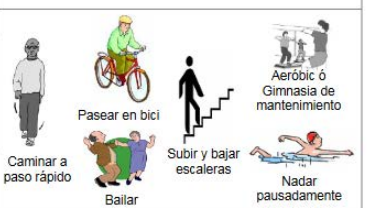
Valoración rápida de la actividad física

Las **actividades físicas** son actividades en las que usted se mueve y aumenta el ritmo del corazón, por encima del ritmo en reposo; y que se realizan por placer, por trabajo o para desplazarse.

Las preguntas siguientes se refieren a la cantidad e intensidad de su actividad física habitual. La intensidad de la actividad se refiere a la cantidad de energía que usted emplea en esas actividades.

Ejemplos de diferentes niveles de intensidad de la actividad física

Actividades ligeras Su corazón late un poco más rápido de lo normal. Se puede hablar y cantar.	 Pasear lentamente · Cuidar o jugar con niños · Barrer o trabajo suave en el jardín
Actividades moderadas Su corazón late bastante más rápido de lo normal. Se puede hablar, pero no cantar.	 Caminar a paso rápido · Pasear en bici · Bailar · Subir y bajar escaleras · Aeróbic ó Gimnasia de mantenimiento · Nadar pausadamente
Actividades intensas El ritmo de su corazón aumenta muchísimo. Se puede hablar o el habla es interrumpida por respiraciones profundas	 Escaladora · Tenis, Frontenis, Padle · Correr o footing · Ciclismo en cuestas

¿Cuál es su nivel de actividad física? *(Marque una respuesta en cada línea)*

			¿Le describe de manera adecuada?
RAPA 1	1	Casi nunca hago actividades físicas.	Sí ☐ No ☐
	2	Hago alguna actividad física **ligera** o **moderada**, pero no todas las semanas.	Sí ☐ No ☐
	3	Todas las semanas hago alguna actividad física **ligera**.	Sí ☐ No ☐
	4	Hago hasta 30 minutos de actividades físicas **moderadas** al menos 5 días a la semana.	Sí ☐ No ☐
	6	Hago 30 minutos o más al día de actividades físicas **moderadas**, 5 ó más días a la semana.	Sí ☐ No ☐
	5	Hago hasta 20 minutos de actividades físicas **intensas** al menos 3 días a la semana.	Sí ☐ No ☐
	7	Hago 20 minutos o más al día de actividades físicas **intensas**, 3 ó más días a la semana.	Sí ☐ No ☐
RAPA 2 (3= ambos 1 y 2)	1	Hago actividades para aumentar la **fuerza muscular**, como levantamiento de pesas o ejercicios de entrenamiento una o más veces a la semana.	Sí ☐ No ☐
	2	Hago actividades para mejorar la **flexibilidad**, como estiramientos o yoga, una o más veces a la semana.	Sí ☐ No ☐

Número de identificación :

Fecha :

Instrucciones para la puntuación

RAPA 1:Aeróbico

Para obtener su calificación y sintetizar por categoría su nivel de actividad física, elija a continuación las respuestas que sean afirmativas en su caso particular. Si el número seleccionado es menor a 6, esto significa que su nivel de actividad física está por debajo de lo recomendable. Del número 6 hacia arriba se encuentra dentro de lo recomendable.

Se considera como: Sedentario:

1. Casi nunca hago actividades físicas..

Se considera como: Poco activo:

2. Hago alguna actividad física ligera o moderada, pero no todas las semanas.
3. Todas las semanas hago alguna actividad física ligera.

Se considera como: Moderadamente activo

4. Hago hasta 30 minutos de actividades físicas moderadas al menos 5 días a la semana.
5. Hago hasta 20 minutos de actividades físicas intensas al menos 3 días a la semana.

Se considera como : Activo

6. Hago 30 minutos o más al día de actividades físicas moderadas, 5 ó más días a la semana.
7. Hago 20 minutos o más al día de actividades físicas intensas, 3 ó más días a la semana.

RAPA 2: Fuerza y flexibilidad

Hago actividades para aumentar la fuerza muscular, como levantamiento de pesas o ejercicios de entrenamiento una más veces a la semana. (1)

Hago actividades para mejorar la flexibilidad, como estiramientos o yoga, una más veces a la semana. (2)

Hago los dos (3)

No hago ninguno (0)

Fuente: Topolski et al.[98]

3.2 Estudio de perfiles metabólicos

Para responder las hipótesis de estudio se realizaron aproximaciones metabolómicas complementarias. La máxima cobertura del perfil metabolómico se da a partir del trabajo multidisciplinar indispensable de químicos, biólogos, estadísticos e informáticos para llegar a unos resultados óptimos. Así poder ofrecer un significativo avance holístico en el estudioi de la biología de sistemas.

Con una hipótesis y un diseño experimental a priori, distinguimos: i) la recolección y preparación de la muestra biológica; ii) adquisición y pre-procesamiento de los datos; iii) análisis de datos; iv) identificación de metabolitos y v) interpretación biológica.

Como se ha explicado en los antecedentes bibliográficos la utilización de una aproximación metabolómica dirigida o no-dirigida nos permite obtener conocimiento complementario de la muestra biológica de estudio. En esta tesis doctoral se han utilizado ambas aproximaciones. En la **Tabla 4** se resumen las principales características de cada aproximación y su elección dependerá del objetivo a alcanzar.

Tabla 4. Resumen de las características de las aproximaciones metabolómicas

Aproximación dirigida	Aproximación no-dirigida
- *Hypothesis-driven approach, grupo de metabolitos específicos*	- *Hypothesis-free approach*
- Cuantitativa, validación de biomarcadores	- Exploratorio, descubrimiento de biomarcadores
	- Idealmente muestras no-procesadas
- Tratamiento pre-cromatográfico	
	- Masa exacta
- Eleva sensibilidad	

3.2.1 Recolección y preparación de la muestra

La recolección y la preparación de las muestras son los pasos iniciales para tomar la muestra, almacenarla y extraer los compuestos de la matriz biológica para mejorar su sensibilidad a la hora de analizarla y ponerlos en un formato compatible con la técnica analítica utilizada[99].

Para garantizar que los metabolitos identificados en la muestra sean exactos a los del momento de recolección la muestra se almacena a -80ºC hasta sus análisis[100]. De esta manera se paran las reacciones bioquímicas que ocurren en la matriz biológica de estudio.

3.2.1.1 Análisis metabolómico dirigido

La preparación de la muestra en el análisis dirigido se ha seguido el procedimiento establecido por el fabricante (Biocrates Life Science AG, Innsbruck, Austria) y se incluyeron los compuestos comerciales (estándares) que posteriormente se cuantificaran y sus correspondientes controles de calidad tal y como se muestra en la **Figura 21**.

Figura 21. Análisis metabolómico dirigido. Distribución de las muestras y los controles de calidad en la placa de inyección.

3.2.1.2 Análisis metabolómico semidirigido

Las muestras se prepararon según las características del sistema analítico Metabolon (Metabolon Inc, Durham, North Carolina USA)[101], mediante una precipitación de proteínas con metanol y agitación vigorosa.

3.2.1.3 Análisis metabolómico no-dirigido

Las muestras que se analizaron por metabolómica no-dirigida fueron expuestas a una primer fase de deproteinización por un solvente acídico (acetonitrilo al 1% de ácido fórmico) seguido de una extracción de fase sólida (SPE del inglés, *solid-phase extraction*) para la precipitación de fosfolípidos, descrito en Tulipani et al.[102].

Las placas se prepararon con los controles de calidad y los estándares internos y externos correspondientes, como se describe en la **Figura 22**. Los controles de calidad son: agua (QC1), *pool* de compuestos (QC2) descritos en [102,103], muestras repetidas del análisis (QC3), solvente de extracción (QC4), suero de referencia o comercial (QC5) y se reinyectaran muestras biológicas para controlar efecto placa de extracción (no necesario si solo se realiza en una sola placa) (QC6).

Figura 22. Análisis metabolómico no-dirigido. Distribución de las muestras y los controles de calidad en la placa de inyección.

3.2.2 Adquisición de datos y pre-procesado

La espectrometría de masas es la plataforma analítica que se empleó en todos los estudios de la presente tesis doctoral. A continuación se detallan las características de cada aproximación.

3.2.2.1 Análisis metabolómico dirigido

La adquisición de los datos se realizó con LC-MS/MS y FIA-MS/MS con un QTRAP 6500 con fuente de ionización en *electrospray* positiva y negativa, siguiendo las especificaciones del comercial. Las muestras fueron analizadas al azar y la cuantificación se logró con monitoreo de reacciones múltiples (MRM del inglés, *multiple reaction monitoring*) a partir de reglas de calibración de puntos múltiplos y con la combinación de estándares enriquecidos isotópicamente y estándares internos para compensar el efecto matriz descrito en[104]. La evaluación de los datos y la obtención de una matriz cruda para su posterior análisis estadístico con los compuestos cuantificados se realizó con el programa MetIDQ™ (Biocrates Life Sciences AG) permitiendo correcciones isotópicas. Los métodos aplicados siguen metodologías validadas y siguen las guías del Food Administration (U.S. Department of Health and Human Services 2001), como describe el fabricante (UM-P18-THERMO-3).

3.2.2.2 Análisis metabolómico semidirigido

La adquisición de datos en el análisis metabolómico semidirigido se realizó con el sistema analítico de Metabolon (Metabolon Inc., Durham, North Carolina, USA) con LC-MS acoplado a Q-Exactive MS/MS con fuente de ionización en *electrospray* positiva y negativa en cromatografía líquida inversa de ultra rendimiento (UPLC del inglés, *ultra-performance liquid chromatography*) y en *electrospray* negativa en cromatografía líquida de interacción hidrofílica [101]. La variabilidad instrumental fue determinada calculando la desviación estándar relativa de los estándares internos que se añadieron a cada una de las muestras antes de la inyección en el MS y mediante el cálculo de la desviación estándar relativa por todos los compuestos

endógenos de los controles de calidad contenidos en muestras de plasma humana ya conocidos.

3.2.2.3 Análisis metabolómico no-dirigido

La adquisición de los datos se realizó con LC-MS con tripleTOF 6600 híbrido cuadrupolo-TOF (AB Sciex, Framingham, MA) con fuente de ionización en *electrospray* positiva y negativa con las condiciones cromatográficas descritas en el protocolo del grupo publicado en Tulipani et al.[102,103]. Las muestras fueron analizadas al azar y cada 15 inyecciones se analizaron los controles de calidad, analíticos y de extracción ya explicados anteriormente.
La evaluación de los datos y la obtención de una matriz cruda para su posterior análisis estadístico se realizaron con el programa MarkerView 1.3.0.1 (AB Sciex, Toronto, Canadà). El programa permite la extracción, detección y el alineamiento de los picos. En cada publicación se especifican los parámetros óptimos utilizados. Con este proceso se obtuvo una tabla de iones que contenían la masa exacta, el tiempo de retención (RT del inglés, *retention time*) y la intensidad de cada ión en cada una de las muestras analizadas.

3.2.3 Análisis estadísticos

Tanto en la metabolómica dirigida como no-dirigida se pueden utilizar distintas técnicas estadísticas para discriminar los metabolitos del estudio o los iones discriminantes entre los grupos de estudio respectivamente.

Pre-procesado y limpieza de los datos

Entre la generación de los datos por la plataforma analítica y la interpretación biológica de los resultados, es necesario la limpieza de los datos, su preparación para ser analizados, y desarrollar el/los análisis estadísticos adecuados para responder la respuesta de estudio. Una vez los datos se han limpiado es importante pre-tratarlos antes de analizarlos[105].

El pre-tratamiento de los datos se realizó con el objetivo de preparar los datos para que los análisis estadísticos posteriores pudieran focalizarse en la información biológica relevante.

Inicialmente, se exploraron los datos y su normalidad, es decir si los datos seguían una distribución normal o de Gauss. Esto se realizó a partir de los gráficos Q-Q (de cuantil), la dispersión de los residuos del modelo y la visualización de los resultados a partir del diagrama de caja y bigotes (del inglés, *box-plot*) y el gráfico de interacción.

En estas etapas se identificaron variables con un número importante de 0. Se ha descrito que entre un 10-40% de los datos de metabolómica contienen valores 0 [106]. Estos valores son los llamados valores perdidos (del inglés, *missing values*). Los valores perdidos pueden ser producidos por distintas razones que tendrán que tenerse en cuenta para tratarlos correctamente[107]. Las razones de un valor perdido pueden ser: i) la variable no es presente en la muestra; ii) la variable es presente en la muestra pero se ha perdido en el análisis analítico; iii) variable es presente en la muestra pero está debajo del límite de detección del equipo (LOD, del inglés *limit of detection*)[107].

Para eliminar parte de los valores perdidos primero se filtró la matriz y se eliminaron todas aquellas variables que no aportaban información. Principalmente se eliminan las variables que: i) todos los sujetos presentan un valor inferior al LOD; ii) se detectaban en muy pocas muestras y 3) son prácticamente constantes. Para eliminar estas variables se utilizó la regla del 80%[108], es decir, se eliminaron todas las variables que al menos uno de los grupos de estudio no tenían más del 80% de los valores. En el caso de algunos estudios se consideró bajarlo al 75%.

Con los valores perdidos que quedaron se realizó una imputación. La imputación pretende remplazar el valor 0 o dato perdido por otro valor y es importante para estudios posteriores especialmente para análisis multivariantes. La técnica que se utilizó fue la técnica de imputación por K vecinos más cercanos (KNN del ingés, N*earest Neighbours*) con un K=10. Este método remplaza los valores perdidos de

una determinada variable de un indiviudo, por la media de los K vecinos más próximos a dicho individuo.

En el caso del estudio[109], al tratarse de un análisis cuantitativo los valores por <LOD o <LOQ se han reemplazado por LOD/√2 y LOQ (del inglés *limit of quantification*) /√2 respectivamente, utilizando del valor teórico del LOD y LOQ de cada variable y proporcionada en el protocolo[104]

Los distintos metabolitos (variables) a analizar presentan diferencias en las concentraciones. Estas diferencias no son proporcionales a la importancia biológica de los niveles de los metabolitos. No obstante, los análisis multivariantes no son capaces de hacer esta distinción. Para solucionar esto se recomienda seguir unos pasos adicionales en el pre-tratamiento de los datos. En los trabajos presentados se escaló y se log-transformó los datos. El método de escalado divide cada variable por un factor, el cual es diferente por cada variable y así ajustar las diferencias entre ellas[105]. Se utilizó el método del auto-escalado (del inglés, *auto-scaling*) en los análisis metabolómicos dirigidos y el método de escalado por *Pareto* en las aproximaciones no dirigidas.

El método de auto-escalado compara los metabolitos en base a su correlación, todos los metabolitos son igual de importantes[105]. Siendo $\tilde{x}$ los datos después de diferentes pre-tratamientos, $\overline{x_i}$ la media y la S_i la desviación estándar.

$$\tilde{x}_{ij} = \frac{x_{ij} - \overline{x}_i}{s_i}$$

El escalado por *Pareto* reduce la importancia relativa de los valores largos pero mantiene la estructura de los datos parcialmente intacta.

$$\tilde{x}_{ij} = \frac{x_{ij} - \overline{x}_i}{\sqrt{s_i}}$$

Análisis estadísticos

3.2.3.1 Análisis univariantes

Los resultados se expresaron en media y desviación estándar y en Palau-Rodriguez et al. (*enviada*) se expresaron en mediana y rango intercuartil (primer y tercer cuartil) debido a la falta de normalidad de los datos. En el caso de las variables metabolómicas en Palau-Rodriguez et al. (*enviada*) los resultados se graficaron utilizando *Box-plots*.

Para las variables dicotómicas, categóricas (o cualitativas) se presentaron el número de participantes y/o los porcentajes (tanto por ciento).

Para la comparación de las medias a tiempo basal o comparación de las medias de los incrementos entre diferentes tiempos se utilizó un *t* test o un análisis de la varianza (ANOVA, del inglés *Analysis of Variance*) cuando se compararon más de dos grupos. En caso de no normalidad la significación se determinó a través de tests de permutaciones.

Para estudiar la evolución de los pacientes a lo largo del tiempo en los estudios prospectivos y o estudiar la interacción entre el factor grupo y tiempo (salud/no salud x tiempo o tipo de cirugía x tiempo) y entre grupos (obesidad x resistencia a la insulina) se utilizó un modelo lineal mixto. Una interacción significativa nos indica un comportamiento diferente de los diversos grupos a lo largo del tiempo.

Para la comparación de las variables cualitativas o categóricas se utilizó el test exacto de Fisher, basado en la distribución hipergeométrica[110].

Tanto en el análisis de ANOVA como los modelos lineales mixtos se incluyeron las variables edad, género y medicamentos ya que se han visto que pueden influenciar el metaboloma humano[111,112]. En los análisis pertinentes también se incluyó el tipo de cirugía[113].

En todos los análisis se han considerado diferencias significativas cuando el p valor era inferior a 0.05.

Para las variables metabolómicas el p valor fue corregido para evitar falsos positivos asociados al testar múltiples hipótesis a través del procedimiento de Benjamin-Hochberg [114].

Correlación

El análisis de correlación se utilizó para identificar si dos variables cuantitativas estaban asociadas. Este análisis permite establecer un grado de asociación mediante el coeficiente de correlación de Pearson (cuando las variables son normales) y el coeficiente de Spearman (cuando las variables son no normales) entre -1 a 1, siendo 0 el valor nulo, la no correlación de las variables y la dirección de 0 a -1 sería una relación inversa y de 0 a 1 una relación directa.

3.2.3.2 Análisis multivariante

Como el nombre indica el análisis multivariante es un conjunto de herramientas estadísticas para analizar observaciones (o muestras de estudios) descritas por múltiples variables. En el caso de los datos de metabolómica los análisis multivariantes resultan ser una herramienta imprescindible, tanto en las aproximaciones dirigidas como no dirigidas. Estas técnicas pretender analizar los datos teniendo en cuenta que cada variable no es una entidad independiente y que puede existir interacción entre ellas. Las técnicas multivariantes que se utilizaron en esta tesis doctoral se clasifican en dos grandes grupos: técnicas **supervisadas**, conocida como técnicas predictivas o dirigidas y técnicas **no supervisadas**, conocidas como técnicas descriptivas o no-dirigidas.

Análisis no supervisados

Uno de los objetivos de los análisis no supervisado es descubrir la estructura inherente de los datos, sin conocimiento a priori de los posibles grupos presentes. Son habitualmente utilizados con fines exploratorios y de reducción de la dimensión de los datos.

a. *Análisis factorial y por componentes principales*

Análisis de componentes principales (PCA, del inglés principal component analysis) *y análsisi múltiple de correspondencia (MCA, del inglés multiple correspondence analysis*)

El PCA es una técnica no supervisada comúnmente utilizada para la reducción de la dimensionalidad, la visualización y la exploración de los datos cuantitativos. El objetivo de este análisis es reducir el número de variables al menor número posible, perdiendo la menor cantidad de información posible de los datos. El PCA crea unas variables "artificiales", llamadas componentes principales (PC, del inglés *principal component*). Los PCs son una combinación lineal de las variables originales, independientes entre sí y que explican una proporción de la variabilidad de los datos que puede cuantificarse a través de los autovalores o valores propios (del inglés, *eigenvalues*)). De esta forma seleccionando las PCs con mayor variabilidad explicada puede describirse el modelo con un número razonablemente reducido de varibles si el porcentaje de variabilidad acumulado explicado es significativo. Cuando las variables de estudio son variables cualitativas se utiliza el análisis múltiple de correspondencia (MCA, del inglés *multiple correspondence analysis*).

El PCA se utilizó en todos los trabajos con fines exploratorios, para identificar valores atípicos, ya sean por las características de los pacientes o para valorar la calidad de los datos analíticos. Los metabotipos creados en Palau-Rodríguez et al.[109] se han visualizado mediante PCA.

Análisis de factores múltiples (MFA, del inglés multiple factor analysis)

En determinadas ocasiones las observaciones o muestras de estudio están descritas por distintos grupos de variables, datos de naturaleza distinta y/o con distinto número de variables en cada grupo. Por ejemplo, en estudios longitudinales como Palau-Rodriguez et al.[109,115], distintos grupo de datos corresponden a los distintos puntos temporales; o la combinación de datos clínicos y metabolómicos utilizados en la publicación presentan naturaleza (variables metabolómicas y variables clínicas) y cantidad de datos diferentes (más variables metabolómicas que clínicas)[115]; también se podrían utilizar para combinar datos de resonancia magnética nuclear con

datos de MS[116]. En estos casos la utilización de un PCA podría ser errónea ya que daríamos el mismo peso a las variables con la mayor varianza y no cuales explican mejor el modelo. Para solventar este problema el análisis de factores múltiples (MFA, del inglés *multiple factor analysis*) permite combinar distintos grupos de datos independientemente de la cantidad y la naturaleza de los datos (sean cuantitativas o cualitativas) [117]. Originalmente el MFA se desarrolló para valorar vinos (observaciones) juzgados por diferentes expertos (subgrupo de datos), combinando las opiniones de todos los expertos sin que las opiniones de ningún experto tuviese más influencia encima de los otros[118].

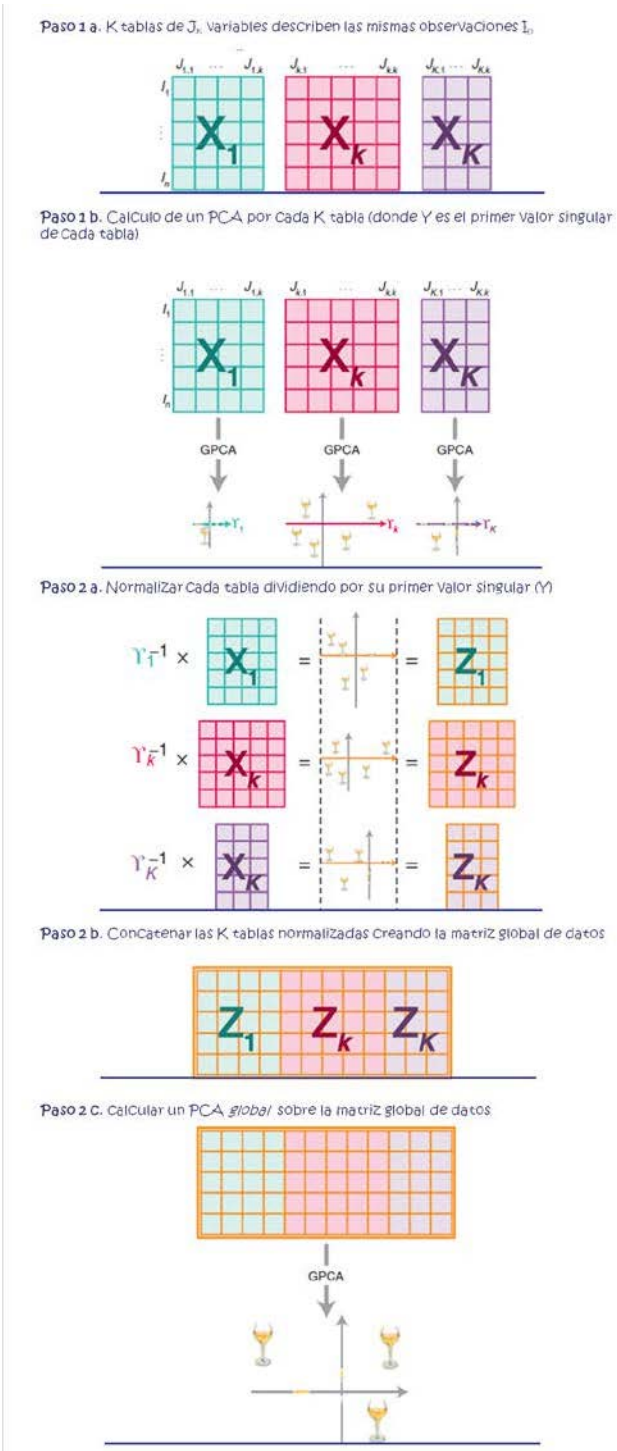
En nuestro conocimiento es la primera vez que se ha utilizado este análisis en estudios de metabolómica longitudinales. Por esto merece brevemente explicar su procedimiento.

El MFA consiste en dos pasos simultáneos: 1) se desarrolla un PCA para cada subgrupo de variables (K) si son cuantitativas o un MCA si las variables son cualitativas. Seguidamente cada subgrupo es normalizado el *primer valor singular* (Y) de cada sugrupo ─que es la raíz cuadrada de cada *eigenvalue*─ para que el PC1 de cada tabla sea igual a 1 y por lo tanto que no hay un grupo de variables que domine por encima de los otros; 2) se concatenan todos los datos normalizados anteriores creando la *matriz global de datos* y se calcula un PCA *global* (GPCA) sobre esta matriz dando un número de puntuación a las observaciones y una carga cada variable (**Figura 23**).

En las publicaciones Palau-Rodriguez et al.[109,115] se utilizó el MFA para explorar la contribución de cada variable, independientemente del tiempo en los cambios post cirugía y la contribución de las variables clínicas y metabolómicas para explicar diferencias entre comportamientos post-cirugía según si los pacientes eran metabólicamente *sanos* o *enfermos* al inicio de la operación. Para la interpretación de los resultados se utilizó: i) el gráfico de grupos de variables, los cuales informan de la dispersión de los subgrupos de variables sobre los dos primeros PCs; ii) el mapa del factor individual, que representa las observaciones con los valores parciales de cada subgrupo; iii) el circulo de correlaciones de las variable, que

representa la correlación de cada variable con los PCs (ejes), mediante el gráfico de un vector sobre una circunferencia que pasa por cada valor 0 de los ejes. El vector puede ir de -1 a 1. Cuando la distancia del vector es cercana a 1 mayor efecto tienen la variable sobre el componente y cuando más cercana a 0 menos influencia tiene la variable sobre el componente y iv) el gráfico de los ejes parciales de cada grupo en los dos primeros PCs. El PCA, MCA y MFA se realizaron mediante el paquete de R *FactoMineR* versión 1.36.

Figura 23. Pasos del MFA



Fuente: adaptado de Abdi H et al. [176]. Utilizando como observaciones diferentes vinos valorados por diferentes expertos (X_k utilizando criterios diferentes)

Análisis de conglomerados o clustering

Dentro de los análisis no supervisados también se encuentran los análisis de conglomerados (del inglés, *clustering analysis*).

Este concepto viene dado con la necesidad de clasificar las observaciones a partir de características parecidas. En la biología, la clasificación de los organismos ha sido una preocupación desde el principio de la investigación. Aristotelos ya clasificó las especies en reinos hasta la aparición del concepto de taxonomía desarrollado por Adanson (1727-1806). En un punto la clasificación puede parecer un método simple de organizar grandes conjuntos de datos y entender la información de forma más fácil y eficiente. Hoy en día, conjuntamente con otras técnicas multivariantes son impresindibles para resumir grandes base de datos, crecientes en distintos campos como son el marketing o la ciencia, llamándolas técnicas de extracción de datos (del inglés, *data mining*) [119].

Varios métodos se han desarrollado para el mismo propósito, clasificar las observaciones de forma objetiva y estable, mediante la creación de *clusters* homogéneos (con cohesión interna) y separados (con aislamiento externo) (**Figura 24**). Aunque no hay una aproximación estándar, la elección de un u otro método dependerá de los datos a analizar [120]. Entre ellos se destacan los métodos de *K-means*, aproximaciones basadas en la densidad, jerárquicos, etc. descritos en [120].

Análisis de clustering: K-means

En Palau-Rodríguez et al.[109] el método de *K-means* ha sido el utilizado para determinar metabotipos de respuesta a la cirugía bariátrica. Por esto, será el que se explique a continuación.

Figura 24. *Clusters* con cohesión interna y/o aislamiento externo

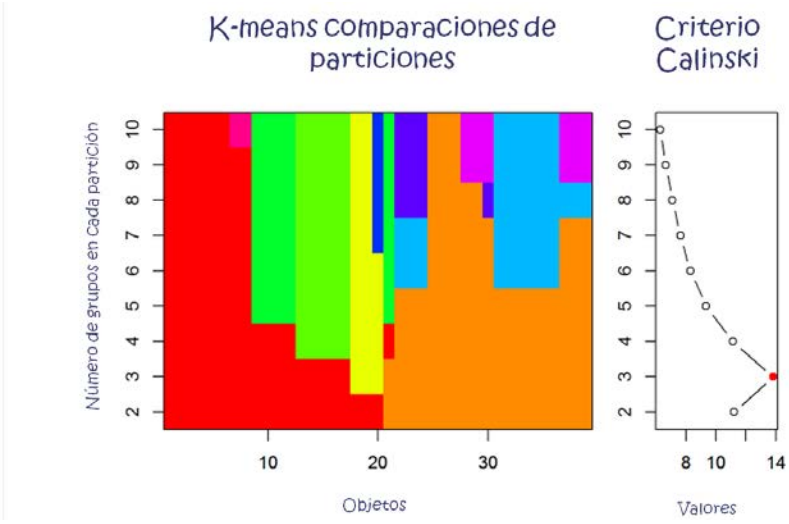
Fuente: extraído del libro Cluster Analysis de Everitt et al. [119] y este con permisos de CRC Press from Gordon, 1980

El método k-means parte de un valor k de grupos deseados y funciona a través de un proceso iterativo en el que se van reasignando los individuos al grupo con la media más cercana y recalculando las medias hasta que el proceso ya no produce cambios en la asignación de los individuos.

Antes de realizar el análisis, la mayoría de algoritmos requieren la identificación del número de grupos que se puede componer los datos de estudio (del inglés, *clusters*). Este paso no es sencillo y diferentes procedimientos se pueden seguir. En el trabajo se ha utilizado el índice Calinski-Haranbasz (1974), el cual en 1985, Milligand and Cooper sugerió como uno de los métodos más efectivos en la determinación de grupos [121].

El índice de Calinski-Harabasz garantiza la compacidad y el aislamiento del grupo a través de la suma mínima de distancia entre las observaciones del grupo y su centro ficticio (centroide) y la distancia máxima entre los grupos, en base al promedio entre y dentro de la suma de cuadrados de los grupos creados para diferentes valores de k en el método, K-means (**Figura 25**).

Figura 25. Gráficos en cascada para la selección del número de grupos óptimos



El gráfico de la izquierda cada color representa un grupo con el número de observaciones (o objetos) en el eje de la X y el número de grupos el eje de la Y. El gráfico de la derecha representa el número óptimo de grupos (punto rojo) a partir del número de criterios (calinski) para determinar la mejor partición. Fuente: Generado con los datos de la publicación Palau-Rodríguez et al. [109] a partir del paquete de R *Vegan* 2.3.5

Para evaluar que los grupos se han creado correctamente y que no se han creado por cuestión del azar se utiliza un sistema de validación interna y externa. La validación interna juzga la calidad de los grupos en base de propiedades estadísticas del método de agrupamiento en sí mismo [122], el mismo índice de Calinski-Harabasz resolvería este paso y sólo sería necesario complementarlo con una validación externa. La validación externa se realizaría identificando los grupos en otro conjunto de datos.

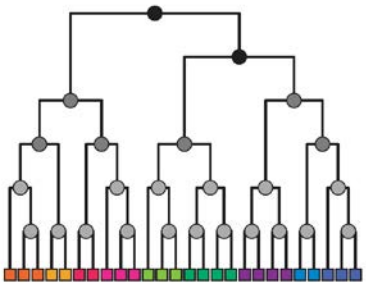
Se utilizó el paquete de R *Vegan* versión 2.3.5 para realizar el análisis de K-means que incorpora el método de creación de los *clusters*.

Análisis de clustering: jerárquico (HCA, del inglés hierarchical clustering analysis)

En la Publicación Palau-Rodriguez et al. (*enviada*) y en la Marco-Ramell et al.[123] se utilizó otro método de análisis de conglomerados llamado análisis jerárquico (HCA, del inglés *hierarchical clustering analysis*) como su mismo nombre indica, es un método que va más allá de agrupar, se basa en la organización de los datos en forma jerárquica, es decir, los datos se dividen en grupos los cuales se subdividen en otros grupos de grupos y así sucesivamente en múltiples escalas[124]. En la mayoría de casos los grupos que se identifican corresponden en unidades conocidas, como nichos ecológicos, redes bioquímicas o grupos químicos. Esta técnica es muy útil cuando se trabaja con muchos datos. Habitualmente las estructuras jerárquicas se representan en árbol o dendograma donde los objetos similares están conectados por vértices y su posición en el diagrama dependerá del nivel de similitud o no con los otros objetos **(Figura 26**). Hay distintas maneras de determinar la distancia entre los objetos. En estos trabajos como medida de distancia se ha utilizado, la distancia Euclídea y como algoritmo, el algoritmo de Ward. En las publicaciones de metabolómica no-dirigidas[115,125] se utilizó este método de *clustering* para tener una idea de cómo pueden estar agrupados los iones facilitando la identificación de metabolitos, de esta manera, cada *cluster* de iones podría corresponder a fragmentos de una misma molécula, moléculas de la misma familia química o relacionadas metabólicamente.

Para este método se utilizó el paquete de R *dendextend* versión 1.5.2.

Figura 26. Representación de un dendograma



Fuente: extraído de: Clauset et al. [124]

Análisis supervisados

Los métodos supervisados parten sobre conocimiento previo de los datos, es decir los individuos están etiquetados en grupos o existe una variable respuesta conocida. Los métodos supervisados tienen el objetivo de encontrar una relación o una estructura en los datos que permitan predecir datos nuevos, ya sea el grupo de pertenencia o el valor de la variable respuesta. Por esto, se habla de datos de entrenamiento (del inglés, *training sets*), que acostumbra a ser parte de los datos, dónde se pretende configurar un modelo que explique la relación entre los datos y se valida en la parte restante de los datos o en un grupo de estudio diferente que son los llamados datos de validación.

Cuando hay pocos datos o si los datos no son uniformes se recomienda utilizar un modelo poco complejo para evitar que el modelo se sobreajuste (del inglés, *overfit*) a los datos, es decir queremos evitar que el modelo se ajuste tan bien a los datos de entrenamiento que sea difícil generalizar en otros datos. Si hay muchos datos se necesitarán modelos más complejos, que permitan mayor variabilidad, para aprender la estructura de todos los datos.

En grandes rasgos, los métodos supervisados utilizados en la presente tesis doctoral se clasificarían en dos: métodos de clasificación, cuando el propósito es predecir la *etiqueta* de los datos o métodos de regresión cuando se predice una variable numérica.

a. Métodos de Regresión

Regresión LASSO (del ingles, least absolute shrinkage and selection operator) *y curvas ROC (del ingles, Receiver Operating Characteristic*)

En la publicación Palau-Rodriguez et al. (*enviada*) y en la publicación Marco-Ramell et al.[125] se utilizó la regresión logística LASSO del inglés *Least Absolute Shrinkage and Selection Operator* junto con las curvas ROC del inglés *Receiver Operating Characteristic* para valorar la especificidad y sensibilidad de los modelos multimetabolito.

La regresión LASSO es una técnica de reducción y selección de variables que puede ser implementada en diferentes modelos de regresión, por ejemplo en regresión logística. La regresión logística se utiliza para conocer qué factores (variables cuantitativas o categóricas) influyen a una variable dependiente binaria (variable cualitativa o categórica dicotómica). Por ejemplo presentar resistencia a la insulina o no. El objetivo del LASSO es obtener un subconjunto de predictores que minimiza el error de predicción para una variable de respuesta cuantitativa. El LASSO hace esto imponiendo una restricción en los parámetros del modelo que causa que los coeficientes de regresión para algunas variables se reduzcan a cero. Estas variables con valor 0 se excluyen del modelo. Las variables con coeficientes de regresión distintos de cero están fuertemente asociadas con la variable de respuesta. La selección de parámetros para el método LASSO se hace por validación cruzada dejando uno fuera (del inglés, *leave-one-out*) y por lo tanto el método de validación que utiliza no tiene que subdividir los datos entre grupo de entrenamiento y grupo de prueba y así su aplicación con conjunto de datos con muchas variables y pocas muestras puede ser más adecuado que otras técnicas[126].

Para la regresión LASSO se utilizó el paquete de R *glmnet* versión 2.0-10.

El coeficiente-lambda fue utilizado por seleccionar las variables más predictivas del modelo y crear los distintos modelos de biomarcadores multimetabolitos:

Biomarcador multimetabolito= λ_1 x metabolito$_1$ + λ_2 x metabolito$_2$ + … + λ_n x metabolito$_n$

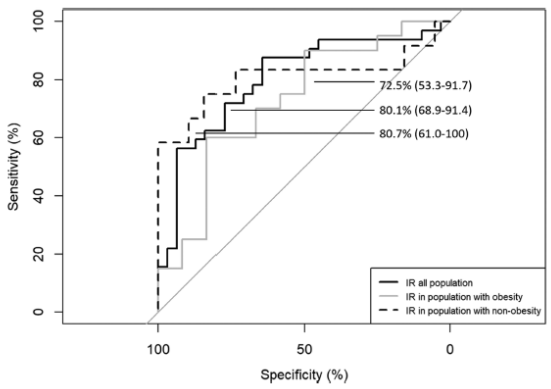
El potencial de los distintos modelos multimetabolitos para ser buenos biomarcadores y los metabolitos seleccionados en la regresión LASSO como potenciales biomarcadores se evaluaron a partir de las curvas ROC (del inglés, *receiving operating characteristics*). Las curvas ROC permiten evaluar clasificadores binarios (insulino resistente si o no) con un resultado continuo o discreto. El área bajo la curva (AUC, del inglés *area under the curve*) de la curva ROC mide el rendimiento de un clasificador a partir del cálculo de la sensibilidad (proporicón de observaciones positivas clasificadas correctamente, es decir clasificar los casos correctamente) y la especificidad (proporción de observaciones negativas correctamente clasificadas, es decir de clasificar los controles correctamente)[127]. Se representa en forma de curva siendo el eje Y la sensibilidad y el eje X la especificidad.

Sensibilidad = Positivos verdaderos / (Positivos verdaderos + falsos negativos)

Especificidad = Negativos verdaderos (Negativos verdaderos + falsos positivos)

En el caso que los potenciales biomarcadores permitan la clasificación correcta de todas las muestras, la AUC tiene un valor de 1 (100%). Al comparar distintos modelos, cuando mejor es la especificidad y sensibilidad, mayor es la AUC, mejor es el clasificador. Si la proporción de falsos positivos es igual a la de falsos negativos la curva ROC es una diagonal (**Figura 27**).

Figura 27. Curva ROC de distintos modelos para identificar la elevada Resistencia a la insulin.



Fuente: extraído de Marco-Ramell et al.[125]

Para las curvas ROC se utilizó el paquete de R *pROC* versión 1.11.0.

b. Métodos de clasificación

Análsis discriminantes: Análisis Discriminante por Mínimos Cuadrados Parciales (PLS-DA, del inglés Partial Least Squares Discriminant Analysis).

Los métodos de discriminación permiten clasificar nuevos individuos dentro de grupos previamente definidos. Además, permiten reducir la dimensionalidad de los datos a partir de la selección de variables (o características) o introducir las variables "artificiales" que resuman la mayoría de la información que nos permite la separación de los grupos (PC). Uno de los métodos más utilizados es el análisis de PLS-DA, del inglés *Partial Least Squares Discriminant Analysis*, una variante de la regresión PLS donde la variable respuesta es el grupo al que pertenecen los individuos y que es muy utilizada para propósitos de discriminación/clasificación. El PLS-DA utiliza la rotación de los componentes del PCA para obtener la máxima separación de clases

Una variación del PLS es el sPLS, del inglés *Sparse Partial Least Squares* que impone el principio de simplicidad (del inglés, *sparsity*) en la regresión PLS, dando lugar a una reducción de las dimensiones y selección de las variables de forma simultánea. Este método es una buena solución cuando la muestra es más pequeña que el número total de variables; y las covariables están muy correlacionadas, pudiendo afrontar respuestas univariantes y multivariantes. La variante para realizar clasificación es el sPLS-DA (Sparse PLS discriminant analysis).

En sPLS-DA se utilizan dos parámetros de ajuste: 1) el "eta", que representa el parámetro de la simplicidad y 2) el "K" que representa el número de componentes latentes ocultos (PCs). "Eta" puede tener un valor entre 0 y 1 y "K" puede tener un valor de 1 al número de variables que contiene los datos. El valor óptimo de "eta" y "K" se determina a partir de obtener el mínimo error de clasificación de las observaciones (del inglés, *misclassification error rate*). El error de clasificación se obtiene por un procedimiento de validación cruzada comparando cada observación con la observación obtenida del modelo sPLS-DA. Este procedimiento coge un conjunto de observaciones al azar que serán el grupo de entrenamiento

(aproximadamente 75% de las muestras) y se crea el modelo y se valida en el grupo de validación, formado por las observaciones restantes (aproximadamente el 25% de las muestras). Este procedimiento se repite varias veces hasta que todas las muestras han entrado alguna vez en el grupo de validación. Este método contribuye a evitar el sobre-ajustamiento del modelo a los datos.

Para el sPLS se utilizó el paquete de R *spls* versión 3.3.3

En la Publicación Marco-Ramell et al.[125] en lugar de utilizar un sPLS-DA se utilizó un OSC-PLS-DA para intensificar la separación de los grupos. El OSC-PLS-DA utiliza como base de clasificación el PLS-DA con un filtro de corrección ortogonal de las señales (OSC, del inglés *Orthogonal Signal Correction*) el cual consiste en eliminar la información de las variables que no están relacionadas (o correlacionadas) con la clase o grupo de estudio[128].

Para validar el modelo además de una validación cruzada se utilizó el test de permutaciones. La prueba de permutación quiere demostrar que el modelo creado permite la clasificación de las muestras y que esta clasificación es mejor que si las muestras fueran escogidas al azar[129]. En esta prueba las *etiquetas* de grupo son permutadas, son asignadas al azar a diferentes individuos. Con las etiquetas "permutadas" en cada objeto se calcula la clasificación del modelo. De esta manera con las etiquetas permutadas el nuevo modelo no tendría que predecir correctamente las clases. Como los grupos están formados al azar, se asume que no hay diferencias existentes entre ellos. El procedimiento del test de permutaciones mide la probabilidad de que la precisión observada en la clasificación real se obtenga por casualidad. El p-valor representa la fracción de los conjuntos de datos aleatorios en los que el clasificador se comportó igual o mejor que en los datos originales.

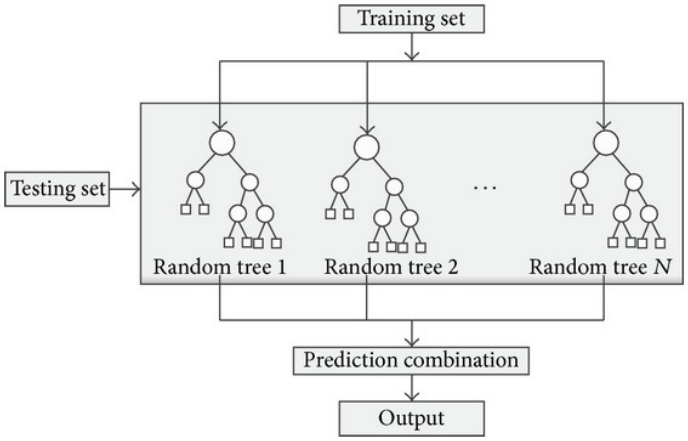
A diferencia del *sparse* el filtro OSC no nos selecciona las variables. En estos casos las variables de interés se seleccionaron a partir del valor VIP, del inglés *variable importance for projection* o el p(corr) (correlación entre las variable y el valor p o peso de metabolito). El valor VIP es una medida que explica la importancia de una variable al modelo PLS. Valores más altos indican una mayor contribución de esta variable, es decir, más importante es la variable para el modelo. Aunque no hay un

punto de corte que permita dividir entre variables más importantes de las menos importantes, en la mayoría de estudios metabolómicos se utiliza un valor VIP superior a 1. Por lo tanto todas aquellas variables con un valor VIP superior a 1 es considerada variable discriminante[130]. En la publicación Marco-Ramell et al. se utilizó un valor de VIP >2 para reducir la posibilidad de obtener falsos positivos y el modelo fue validado por el método de validación cruzada y el test de permutaciones explicado anteriormente.

Análisis de bosques aleatorios (RF, del inglés Random Forest)

El análisis de bosques aleatorios (RF, del inglés *Random Forest*) es un método de clasificación de la familia de clasificación por árboles [131]. Este método empieza por la partición de los datos originales en diferentes subgrupos por remplazamiento (del inglés, *bootstrapping*), es decir, de forma aleatoria. Este paso permite elegir un subgrupo de datos que serán los datos de entrenamiento (corresponden al 63.2% de las muestras) y otros grupos de datos que serán los grupos de prueba (del inglés, *test sets*) (corresponden al 36.8% restante de las muestras) (Etapa 1). Los datos de entrenamiento se utilizarán para construir los árboles mientras que los grupos de prueba se utilizarán para estimar la exactitud de clasificación[132]. A partir de los datos de entrenamiento se construye el árbol de decisión. Cada árbol empieza con un nodo con el conjunto de entrenamiento y este de forma no correlacionada forma árboles de decisión formando subgrupos de datos de entrenamiento y estos sucesivamente serán partidos por otros subgrupos hasta poder asignar una *etiqueta* a la muestra (Etapa 2). En cada punto de división del árbol se seleccionan pequeños subgrupos de variables al azar que permiten mejorar la clasificación de las observaciones. A partir de estas variables se va optimizando los árboles dentro del bosque y se van creando tantos árboles como grupos de entrenamiento se hayan formado hasta tener los nodos-respuestas que sería la clasificación de cada muestra. Para clasificar los datos de prueba se aplica cada muestra a cada uno de los árboles del bosque. Cada árbol produce una clasificación y la clasificación finalmente atribuida por el bosque es la que suma el mayor número de "votos" sobre los árboles del bosque. El algoritmo genera una probabilidad de distribución del bosque aleatorio (**Figura 28)**.

Figura 28. Esquema general del proceso de Random Forest



Fuente: obtenido de Zhang et al.[132]

En este caso se utilizó un RF con validación cruzada doble y repetida para disminuir la probabilidad estadística de sobre-ajustado [133] y un test de permutaciones (n=1000). Se consideró que el modelo de clasificación era correcto cuando p valor del test de permutación era <0.05). Este algoritmo fue desarrollado *in-house* por el grupo del Dr. Rikard Landberg utilizando la base del Random Forest del paquete de R *randomForest* versión 4.6-10 y englobado en el paquete *MUVR*.

Para seleccionar un u otro método supervisado se han tenido en cuenta las ventajas e inconvenientes que aportan cada uno resumidos en (**Tabla 5**).

Tabla 5. Comparación del PLS-DA y el RF según características generales

	PLS-DA	RF
Lidiar con los valores perdidos	●	●
Robustez de los valores atípicos (outliers)	●	●
Potencia de predictibilidad	●	●
Habilidad de clasificar las variables	●	●
Interpretabilidad y visualización de los resultados	●	●
Resistencia al sobre-entrenamiento	●	●
Reducción de las dimensiones	●	●
Resistencia al sobre-ajustado	●	●
Selección de los parámetros	✓	✓
Pre-procesado de los datos	✓	✗

● **Bueno** ● Justo ● **Malo** ✓ Sí ✗ **No**
Fuente: adaptado de Glomski et al. [132]

3.2.4 Identificación de marcadores

En la metabolómica dirigida la identidad de los metabolitos analizados es conocida a priori, ya que se emplean estándares comerciales para su cuantificación, por lo que la etapa de identificación no es necesaria en estos casos.

En el caso de la metabolómica no-dirigida a partir de los iones discriminantes obtenidos en los procedimientos anteriores se seguirá un proceso de múltiples pasos para poder identificar los metabolitos[134]. Para identificar el ión se compara con las características de compuestos estándares incluidos en librarías online, como en librerías formadas con compuestos propios del grupo. En nuestro caso además tenemos datos de la fragmentación de las moléculas por MS/MS. Esta información se puede adicionar en la búsqueda y aproximarnos a la identificación del compuesto. A continuación se detallan los pasos.

Cada ión detectado por LC-MS es caracterizado por un tiempo de retención, una relación masa-carga (m/z) y una intensidad. Los iones puede ser un metabolito, un fragmente de un metabolito que se ha fragmentado en la fuente de ionización o un contaminante. En la mayoría de casos los iones discriminantes entre grupos pueden ser entre centenares a miles, haciendo el proceso de identificación más o menos tedioso. Para facilitar el proceso de identificación primero se realiza un análisis de agrupamiento jerárquico. Este procedimiento agrupa los iones, formando *clusters*, ya sea porque podrían corresponder a fragmentos de una misma molécula, moléculas de la misma familia química o moléculas relacionadas biológicamente. Si los fragmentos son de la misma molécula estos se verán agrupados en un mismo *cluster* y con el mismo RT, ya que la fragmentación se produce después de la cromatografía y el RT viene condicionado por las características cromatográficas. También nos podríamos encontrar que distintos *clusters* podrían presentar el mismo RT, esto podría ser debido a que hay compuestos que co-eluyen en la separación cromatográfica.

Una vez tenemos los iones agrupados en *clusters* sigue la etapa de anotación de los iones. En esta etapa se identifica si cada ión es un ión molecular, es decir, es un ión

que ya representa toda la molécula, un isótopo del mismo (e.g. Carbono[13]), un fragmento de la molécula o un adducto (e.g. sales o aductos con el solvente). Esto se puede predecir calculando la diferencia de m/z entre cada uno de los iones del *cluster* y comprobando las anotaciones con la información de las librerías online, librerías de MS/MS o calculadores de aductos como la http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator.

Aún así, hay un número de iones que no podemos predecir su anotación y se utilizan programas de predicción de la fragmentación MS/MS como MetFrag (http://c-ruttkies.github.io/MetFrag/). Una fragmentación *in silico* permite la predicción de los puntos de fragmentación de una molécula a partir de las condiciones analíticas determinadas y mediante la información que dispone la plataforma online.

Una vez se hayan anotado todos los fragmentos se procede con la identificación de las moléculas teniendo en cuenta aquellos *clusters* que uno de los iones es el ión molecular, el ión de la molécula principal. Hay distintas moléculas que pueden ser el mismo ión molecular ya que tienen el mismo valor de m/z. Los iones que son fragmentos de esta molécula, el RT y su comparación con un patrón comercial o herramientas computacionales *in silico* pueden ayudar en la identificación final.

Las bases de datos que consultamos para la identificación de los metabolitos fueron el HMDB (http://www.hmdb.ca/)[35], Metilin (http://metlin.scripps.edu)[135], MassBank (https://massbank.eu/MassBank/)[136] y LipidMaps (https://www.lipidmaps.org/)[137].

Una vez identificada la molécula se les asigna un nivel de identificación según el grado la certeza de la identificación. Estos niveles fueron consensuados por la Iniciativa de Estándares Metabolómica (del inglés *Metabolomics Standards Initiative*)[134]. El nivel I se les asigna a los metabolitos identificados que su patrón de fragmentación y el RT coincide con el patrón de fragmentación del compuesto comercial fragmentado con las mismas condiciones analíticas. Por lo tanto, asignar un compuesto a nivel I puede depender de la disponibilidad del correspondiente compuesto y de su coste. El nivel II de identificación se le asigna a los compuestos que su fragmentación coincide con la fragmentación de las bases de datos o la

bibliografía científica. El nivel III se les asigna a los compuestos según las características de la clase química. El nivel IV se les asigna a los compuestos que no se han conseguido identificar, también llamados compuestos *unknown*.

En el caso de la metabolómica semidirigida se permite la obtención de una semicuantificación de los metabolitos ya que se compara los iones discriminantes con librerías de compuestos comerciales purificados y analizados por la misma plataforma analítica. Como en el caso anterior, los iones discriminantes se comparan con el RT, la masa exacta y el espectro de MS/MS del compuesto comercial.

3.2.5 Interpretación

La última fase es la interpretación biológica de los hallazgos. Para facilitar esta etapa, existen muchas herramientas bioinformáticas gratuitas, intuitivas y completas. A partir de los metabolitos identificados discriminantes o significativos de nuestro estudio podemos realizar distintos análisis: i) mapear los metabolitos identificados/cuantificados en sus correspondientes rutas metabólica y obtener las rangos de rutas implicadas (del inglés, *over-representation analysis*); ii) facilitar la visualizando de los datos por ejemplo, creando redes de metabolitos relacionados (del inglés, *network analysis*) o iii) identificar las rutas metabólicas más alteradas y aquellas más importantes según la posición que ocupa el metabolito en la ruta (del inglés, *topology pathway analysis*).

Por todo lo mencionado se realizó una revisión bibliográfica de todas las herramientas que hay en el mercado.

A continuación se adjunta la revisión y el material suplementario de:

Revisión 3. *Anna Marco-Ramell, Magalí Palau-Rodriguez, Ania Alay, Sara Tulipani, Mireia Urpi-Sarda, Alex Sanchez-Pla, Cristina Andres-Lacueva. Evaluation and comparison of bioinformatic tools for the enrichment analysis of metabolomics data. BMC Bioinformatics 2018 19. doi:10.1186/s12859-017-2006-0.*

En esta revisión se han analizado las características y posibilidades de herramientas para análisis de enriquecimiento en datos *ómicos*. Para esto se ha utilizado datos de

metabolómica de repositorios públicos y la creación de datos simulados para testar las similitudes y diferencias entre plataformas y su potencial para identificar rutas metabólicas en datos previamente enriquecidos con metabolitos involucrados en rutas particulares.

Durante el transcurso del proceso de publicación de la revisión salió ChemRICH (http://chemrich.fiehnlab.ucdavis.edu/). ChemRICH es una plataforma que se ha utilizado en los trabajos Palau-Rodriguez et al.[109,115] y Marco-Ramell et al.[125]. A diferencia de todas las herramientas mencionadas en la revisión esta plataforma realiza un análisis de enriquecimiento basándose en la similitud química de las especies de estudio[138]. ChemRICH utiliza las similitudes estructurales y las ontologías químicas para agrupar los metabolitos conocidos en categorías. Esta aproximación compara las similitudes químicas utilizando la base de datos Medical Subject Heading y los coeficientes de similitud química Tanimoto para agrupar los metabolitos sin solapamiento de las moléculas. El análisis de enriquecimiento se realiza mediante el test estadístico Kolmogorov-Smirnov.

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Evaluation and comparison of bioinformatic tools for the enrichment analysis of metabolomics data

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Abstract

Background: Bioinformatic tools for the enrichment of 'omics' datasets facilitate interpretation and understanding of data. To date few are suitable for metabolomics datasets. The main objective of this work is to give a critical overview, for the first time, of the performance of these tools. To that aim, datasets from metabolomic repositories were selected and enriched data were created. Both types of data were analysed with these tools and outputs were thoroughly examined.

Results: An exploratory multivariate analysis of the most used tools for the enrichment of metabolite sets, based on a non-metric multidimensional scaling (NMDS) of Jaccard's distances, was performed and mirrored their diversity. Codes (identifiers) of the metabolites of the datasets were searched in different metabolite databases (HMDB, KEGG, PubChem, ChEBI, BioCyc/HumanCyc, LipidMAPS, ChemSpider, METLIN and Recon2). The databases that presented more identifiers of the metabolites of the dataset were PubChem, followed by METLIN and ChEBI. However, these databases had duplicated entries and might present false positives. The performance of over-representation analysis (ORA) tools, including BioCyc/HumanCyc, ConsensusPathDB, IMPaLA, MBRole, MetaboAnalyst, Metabox, MetExplore, MPEA, PathVisio and Reactome and the mapping tool KEGGREST, was examined. Results were mostly consistent among tools and between real and enriched data despite the variability of the tools. Nevertheless, a few controversial results such as differences in the total number of metabolites were also found. Disease-based enrichment analyses were also assessed, but they were not found to be accurate probably due to the fact that metabolite disease sets are not up-to-date and the difficulty of predicting diseases from a list of metabolites.

Conclusions: We have extensively reviewed the state-of-the-art of the available range of tools for metabolomic datasets, the completeness of metabolite databases, the performance of ORA methods and disease-based analyses. Despite the variability of the tools, they provided consistent results independent of their analytic approach. However, more work on the completeness of metabolite and pathway databases is required, which strongly affects the accuracy of enrichment analyses. Improvements will be translated into more accurate and global insights of the metabolome.

Keywords: Bioinformatic tools, Database, Enrichment, HumanCyc, KEGG, Metabolite, Metabolomics, Over-representation analysis, Pathway, Reactome

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Background

Enrichment techniques for 'omics' data are key tools for understanding complex biological systems. These tools reduce the complexity of the data, improve interpretation and understanding of biological systems, and help to generate hypotheses. Although the number of tools for 'omics' is rapidly growing, suitable tools for metabolomics are still scarce. Most of the available tools for metabolomics data have been previously developed for other 'omics' technologies. These tools have been described in detail elsewhere [1–6].

Enrichment tools denote any analytic technique that benefits from molecular pathway or network information to gain insight into a biological system [4]. The most widely used methodology for performing such analysis is termed functional enrichment or over-representation analysis (ORA) [7]. This analysis looks for keywords or descriptors of the set of molecules of interest (e.g. those over-expressed) with respect to a background reference set (e.g. the whole genome/transcriptome/proteome/metabolome or the set of molecules detected by the technology employed) [1]. Classical enrichment analyses employ Fisher's exact test, but many other enrichment methods have derived from it, e.g. hypergeometric, Kolmogorov–Smirnov or Wilcoxon statistical tests [6, 7].

To the best of our knowledge studies evaluating the performance of enrichment tools for metabolite sets do not exist yet. The aim of the present work will be to dissect, for the first time, these techniques. First of all, we have carried out an exploratory multivariate analysis of the state-of-the-art of bioinformatic tools for metabolomics sets to visualize their diversity. Then, we have examined the completeness of metabolite databases, the performance of ORA methods and accuracy of disease-based analyses. For these purposes, we have used datasets from metabolomic repositories, whose results have been already published in peer-reviewed journals. In addition, we have enriched selected metabolic pathways and then compared the outputs of these tools when using real datasets or enriched data. Thus the present study provides a global insight of the current status of bioinformatic tools for the analysis and interpretation of metabolite sets from metabolomic studies.

Methods

Datasets

The list of metabolites used in this work refers to five datasets from metabolomics studies in humans, already published in peer-reviewed journals, whose raw data, study information and the list of identified metabolites are available in MetabolomeXchange [8], an online portal of metabolomics repositories including MetaboLights [9], Metabolomics Workbench [10] and Metabolomic Repository Bordeaux. A brief summary of the datasets is shown in Table 1. These datasets correspond to the following publications: 1) Lanza et al. [11]; 2) Fiehn et al. [12]; 3) Kaluarachchi et al. [13]; 4) Hart et al. [14]; and 5) Zhu et al. [15]. P- and adjusted p-values were obtained from the original papers [11–15] and only metabolites with an adjusted p-value < 0.05 were used for tools comparison. The list of metabolites is shown as (Additional file 1: Table S1).

Search of metabolite identifiers

Bioinformatic tools for enrichment analysis require the metabolite name or code (identifier) from a metabolite database. Although Kyoto Encyclopaedia of Genes and Genomes (KEGG Compound) identifiers [16] are the most commonly used in metabolomics [3, 17], some tools prefer other database identifiers such as PubChem [18], BioCyc/HumanCyc (hereinafter only referred as HumanCyc) [19] or Chemical Entities of Biological Interest (ChEBI) [20].

We analysed the current completeness of the following metabolite libraries: Human Metabolome Database (HMDB) [21], KEGG, PubChem, HumanCyc, ChEBI, ChemSpider [22], the metabolic reconstruction Recon2 [23], METLIN [24] and Lipid Metabolites and Pathways Strategy (LipidMAPS) [25].

The list of significant metabolites from [11–15] was used to assess the completeness of these nine databases. The identification of metabolites had been carried out by original authors in all the datasets, and in some cases KEGG and HMDB identifiers were already provided by authors. Since the HMDB website provides links to other metabolite databases, we started the search of the

Table 1 Main characteristics of the datasets used, extracted from the repository MetabolomeXchange

Dataset	Repository reference	Condition of study	Metabolomic platform	Significant metabolites in publication	Total metabolites analysed by authors	Reference
1	ST000091	Type 1 diabetes	LC(RP)-MS	8	44	[11]
2	ST000383	Type 2 diabetes and obesity	GC-MS	27	106	[12]
3	MTBLS364	Smokers	NMR, LC(HILIC-/RP)-MS	81	–	[13]
4	MTBLS424	Breast cancer	NMR	22	25	[14]
5	ST000284	Colorectal cancer	LC(RP)-MS	42	113	[15]

Abbreviations: GC gas chromatography, *HILIC* hydrophilic interaction liquid chromatography, *LC* liquid chromatography, *MS* mass spectrometry, *NMR* nuclear magnetic resonance, *RP* reverse phase

Table 2 Summary of the tools used to assess the performance of over-representation (ORA) methods and their main characteristics (July 2017). Tools and databases are sorted alphabetically

Tool name	Tool version	Database used	Database version	Test used in this work	Platform	Input code	Website
ConsensusPathDB	32	HumanCyc KEGG Reactome	19.1 (06/2015) 80.0 (10/2016) 59 (12/2016)	Fisher's exact test Fisher's exact test Fisher's exact test	Online	HumanCyc KEGG Reactome	http://cpdb.molgen.mpg.de/
HumanCyc	21.0	HumanCyc	21.0 (12/2016)	Fisher's exact test	Online	Name	https://humancyc.org/
IMPaLA	10	HumanCyc KEGG Reactome	NA NA NA	Fisher's exact test Fisher's exact test Fisher's exact test	Online	HumanCyc KEGG Reactome	http://impala.molgen.mpg.de/
IPA®	NA	IPA® disease	NA	Fisher's exact test, Z-score	Java-based software	KEGG	
KEGGREST	1.17.0	KEGG	NA	–	R	KEGG	https://bioconductor.org/packages/release/bioc/html/KEGGREST.html
MBRole	2.0	HMDB disease HumanCyc KEGG	3.5 (01/2013) 17.1 (06/2013) 54.1 (05/2010)	Hypergeometric test Hypergeometric test Hypergeometric test	Online	HumanCyc KEGG	http://csbg.cnb.csic.es/mbrole2/
MetaboAnalyst	3.0	SMPDB disease KEGG	NA NA	Fisher's exact test, hypergeometric test Fisher's exact test, hypergeometric test	Online	KEGG	http://www.metaboanalyst.ca/
Metabox	NA	KEGG	NA	Hypergeometric test	R	PubChem	https://github.com/kwanjeeraw/metabox
MetaCore™	NA	MeSH and OMIM disease	NA	–	Online	PubChem	https://portal.genego.com/
MetExplore	2.11.2	HumanCyc KEGG	18.0 (02/2014) 74.0 (04/2015)	Fisher's exact test Fisher's exact test	Online	HumanCyc KEGG	http://metexplore.toulouse.inra.fr/metexplore2/
MPEA	(2010)	KEGG	(2010)	Hypergeometric test	Online	KEGG	http://ekhidna.biocenter.helsinki.fi/poxo/mpea
PathVisio	3.2.4	Reactome	54 (10/2015)	Z-score	Java-based software	KEGG	https://www.pathvisio.org/
Reactome	61	Reactome	61 (06/2017)	Fisher's exact test	Online	KEGG	http://reactome.org/

Abbreviations: *NA* not available

Results

Evaluation of the state-of-the-art of bioinformatic tools

Figure 1 displays a similarity plot of the most commonly used bioinformatic tools. Tools were distributed all along the two dimensions revealing their diversity. The first dimension mainly separated tools that: 1) perform ORA and are non-open source, 2) perform ORA and are open-source, and 3) are a metabolite database. On the other hand, the second dimension mainly separated tools that: 1) perform metabolite identification, 2) perform ORA and are not programmable, and 3) perform ORA and are programmable. MetScape and MetaMapp, which only carry out data visualization, were distant in the plot.

Evaluation of the completeness of metabolite databases

Metabolites of the five datasets were used to assess the completeness of the metabolite and pathway databases. Almost all the metabolites presented PubChem (97%), ChEBI (91%), METLIN (91%), KEGG (88%), ChemSpider (87%) and HMDB (86%) identifiers, and the 97% of the lipid subset had LipidMAPS identifiers. In some cases, KEGG, HumanCyc and Recon2 provided chemical class identifiers instead of a single identifier to certain metabolites, especially to the lipid subset (Additional file 1: Table S1 and Additional file 4: Table S4).

Evaluation of over-representation methods

In general, ORA methods yielded consistent results using both real and enriched data in all the range of tools tested (Tables 3 and 4). Also similar results were obtained in paired analyses/tools such as MetaboAnalyst hypergeometric test - Fisher's exact test, MBRole full - *Homo sapiens* database, MPEA top down - bottom up

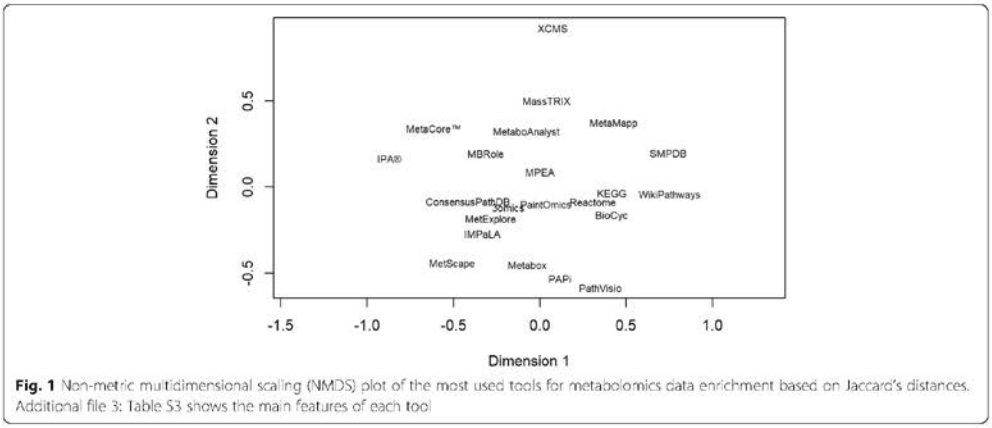

Fig. 1 Non-metric multidimensional scaling (NMDS) plot of the most used tools for metabolomics data enrichment based on Jaccard's distances. Additional file 3: Table S3 shows the main features of each tool

analyses, or ConsensusPathDB - IMPaLA tools, as expected (Tables 3 and 4).

Minor differences in the total number of metabolites/pathway and number hits/pathway were found. For instance, MPEA (Table 3) and MBRole (Table 4) presented a higher number of metabolites/pathway than the other tools. Other divergences were also observed, e.g. MPEA provided higher adjusted *p*-values values (nearly 1 in all the cases) than other tools, or not all the tools mapped the same metabolites of the dataset onto the queried pathways (not shown).

Evaluation of disease-based libraries

The significant metabolites of the five datasets were used to analyse the accuracy of the SMPDB, HMDB, IPA®, MeSH and OMIM disease-based libraries. Outputs revealed that the diseases queried (diabetes type 1 and 2, obesity, respiratory alterations and breast and colorectal cancer) were not successfully identified by these tools, as they appeared in a low position in the list of potential diseases and most of the times they presented a $p > 0.05$ (Table 5).

Discussion

Interpretation of metabolomic data is much less straightforward than that with genomic and proteomic datasets [36]. In the present work we have described the diversity of bioinformatic tools for metabolite sets and have evaluated their performance by exploring three features: the completeness of metabolite databases, ORA approaches and disease-based analyses. To that end, we have used five metabolite sets of blood biomarkers of different diseases obtained from LC-MS, GC-MS and NMR metabolomics approaches. This approach allowed minimizing the possible bias introduced by a given metabolomic platform and thus working with a wide range of metabolites.

Metabolomics is a developing field, thus bioinformatic tools designed to perform enrichment of metabolomics datasets are being developed and released by various groups using diverse statistical tests [3]. Our exploratory multivariate analysis mirrors the high diversity of the currently available tools for the analysis of metabolite sets.

To date about 30,000 endogenous metabolites have already been identified, but this number is rapidly increasing due to advances in high-throughput technologies [21]. Current metabolite databases do not have the full potential to quickly absorb these advances in the description of the endogenous metabolome yet, as not a single metabolite database used in this work covered the full list of significant metabolites of the five datasets. Among all the metabolites databases, PubChem was the one that covered more metabolites from the datasets. However, PubChem is a crowded compound database and presents duplicated metabolite entries, which might produce a larger number of false positives than searching against the KEGG database [49]. To address the low metabolite coverage of metabolite databases, some of them such as KEGG and HumanCyc assign chemical class identifiers to certain types of compounds, especially lipids such as phosphatidylcholines, sphingomyelins or triglycerides. For instance, KEGG coded phosphatidylcholines and sphingomyelins as 'C00157' and 'C00550', respectively, and HumanCyc as 'PHOSPHATIDYLCHOLINE' and 'Sphyngomyelin (class)'.

Missing, ambiguous or redundant entries have been commonly found in public repositories [50]. Indeed metabolites with more than one PubChem, HMDB or ChEBI identifiers were found in this work, which reduce enrichment analyses' accuracy. Several on-going initiatives on identifiers standardization such as BridgeDB and the Chemical Translation Service are trying to

Table 3 Evaluation of over-representation analysis (ORA) outputs of bioinformatic tools employing KEGG pathways. Real (from dataset ST000284) and enriched data were used. The number of total metabolites in the pathway, the number of hits, the ranking of the pathway among all the KEGG pathways (according to their significance), the *p*-value and the adjusted *p*-value were calculated by the tools

Tool	Data	Rank	Total metab.	Hits	*P*-value	Adjusted *p*-value
Alanine, aspartate and glutamate metabolism						
ConsensusPathDB	Real	2	28	8	3.77E-11	9.99E-10
	Enriched	2	28	7	3.76E-13	7.32E-12
IMPaLA	Real	2	28	8	3.77E-11	7.91E-09
	Enriched	2	28	7	3.76E-13	3.00E-10
KEGGREST	Real	NA	28	7	NA	NA
	Enriched	NA	28	7	NA	NA
MBRole (full database)	Real	2	24	8	3.47E-12	2.07E-10
	Enriched	1	24	7	7.23E-14	5.86E-12
MBRole (*Homo sapiens*)	Real	1	24	8	2.31E-11	1.50E-09
	Enriched	1	24	7	3.85E-13	2.00E-11
MetaboAnalyst (Fisher)	Real	1	24	7	3.91E-06	6.74E-05
	Enriched	1	24	7	6.21E-12	4.97E-10
MetaboAnalyst (hyper.)	Real	1	24	7	3.91E-06	6.74E-05
	Enriched	1	24	7	6.21E-12	4.97E-10
Metabox	Real	2	32	8	3.60E-11	5.22E-10
	Enriched	2	32	7	1.34E-13	1.27E-12
MetExplore	Real	3	NA	8	1.03E-08	4.32E-07
	Enriched	2	NA	7	4.42E-10	1.33E-08
MPEA (top down analysis)	Real	1	24	8	4.41E-11	0.660
	Enriched	1	24	7	5.01E-13	0.440
MPEA (bottom up analysis)	Real	1	24	8	1.01E-11	0.170
	Enriched	1	24	7	1.08E-12	1.00
Aminoacyl-tRNA biosynthesis						
ConsensusPathDB	Real	4	52	8	7.89E-10	1.05E-07
	Enriched	9	52	5	2.58E-07	1.12E-06
IMPaLA	Real	4	52	8	7.89E-09	8.74E-07
	Enriched	9	52	5	2.58E-07	1.13E-05
KEGGREST	Real	NA	52	8	NA	NA
	Enriched	NA	52	5	NA	NA
MBRole (full database)	Real	5	75	8	6.07E-08	1.20E-06
	Enriched	12	75	5	1.23E-06	8.30E-06
MBRole (*Homo sapiens*)	Real	5	75	8	3.75E-07	4.87E-06
	Enriched	6	75	5	3.95E-06	3.42E-05
MetaboAnalyst (Fisher)	Real	3	75	8	1.40E-05	3.75E-04
	Enriched	7	75	5	2.72E-05	3.11E-04
MetaboAnalyst (hyper.)	Real	3	75	8	1.40E-05	3.75E-04
	Enriched	7	75	5	2.72E-05	3.11E-04
Metabox	Real	4	56	8	4.28E-09	3.10E-08
	Enriched	4	56	5	8.69E-08	2.15E-07
MetExplore	Real	5	NA	8	1.55E-06	1.69E-06
	Enriched	7	NA	5	1.51E-05	4.52E-04

Table 3 Evaluation of over-representation analysis (ORA) outputs of bioinformatic tools employing KEGG pathways. Real (from dataset ST000284) and enriched data were used. The number of total metabolites in the pathway, the number of hits, the ranking of the pathway among all the KEGG pathways (according to their significance), the *p*-value and the adjusted *p*-value were calculated by the tools *(Continued)*

Tool	Data	Rank	Total metab.	Hits	*P*-value	Adjusted *p*-value
MPEA (top down analysis)	Real	3	53	8	5.32E-09	1.00
	Enriched	7	53	5	1.42E-06	1.00
MPEA (Bottom up analysis)	Real	5	53	8	7.12E-08	1.00
	Enriched	4	53	5	6.57E-08	1.00
Arginine and proline metabolism						
ConsensusPathDB	Real	9	76	7	2.79E-06	1.64E-05
	Enriched	4	76	6	3.94E-08	3.85E-07
IMPaLA	Real	9	76	7	2.79E-09	8.74E-07
	Enriched	4	76	6	3.94E-08	2.18E-06
KEGGREST	Real	NA	77	7	NA	NA
	Enriched	NA	77	6	NA	NA
MBRole (full database)	Real	3	82	10	2.59E-10	8.55E-09
	Enriched	2	82	8	9.30E-12	3.77E-10
MBRole (*Homo sapiens*)	Real	2	82	10	2.58E-09	8.38E-08
	Enriched	2	82	8	6.21E-11	1.61E-09
MetaboAnalyst (Fisher)	Real	2	77	9	6.69E-06	6.74E-05
	Enriched	2	77	8	8.61E-10	3.45E-08
MetaboAnalyst (hyper.)	Real	2	77	9	6.69E-06	6.74E-05
	Enriched	2	77	8	8.61E-10	3.45E-08
Metabox	Real	9	84	7	1.92E-05	6.18E-05
	Enriched	4	84	6	1.25E-08	5.96E-08
MetExplore	Real	2	NA	10	4.03E-08	1.69E-06
	Enriched	1	NA	8	3.34E-10	1.00E-08
MPEA (top down analysis)	Real	4	90	10	1.40E-08	1.00
	Enriched	2	90	7	1.09E-10	1.00
MPEA (bottom up analysis)	Real	2	90	10	2.24E-09	1.00
	Enriched	2	90	8	1.69E-10	1.00

NA means that information was not provided by the tool. Abbreviations: *Fisher* Fisher's exact test, *hyper* hypergeometric test, *NA* not available

overcome redundancy [50–52]. Some tools such as MetaboAnalyst, ConsensusPathDB or PathVisio embrace these initiatives and accept different types of identifiers, which are then transformed into an internal identifier prior to the enrichment analysis [51]. However, this approach also presents different pitfalls. For instance, these tools usually transform the input code into KEGG identifiers, and thus certain types of metabolites such as lipids lose their uniqueness and become a chemical class KEGG identifier. Consequently bioinformatic tools analyse these lipids as a single entity, thereby losing the diversity of these metabolites.

KEGG and HumanCyc are the most used pathway libraries in metabolomics [3, 17] and Reactome is widely used in other 'omics' studies [53]. Thus we have evaluated and compared outputs of ORA methods that employ these pathway libraries. Some limitations prior to ORA analysis were found. For instance, despite the fact that almost all the metabolites of ST000284 dataset had a KEGG code, not all of them were mapped in a KEGG pathway. However, these compounds (e.g. 5-hydroxytryptophan and salicylurate) were mapped in other pathway databases such as Reactome, Wikipathways and SMPDB (not shown). In addition, the KEGG code for glutamic acid (C00025) was not recognized by MetaboAnalyst and the alternative suggested by the tool corresponded to the compound amphetamine (C07514).

The number of total metabolites and hits per pathway varied according to the tool used and those tools that employ the newer database versions (Table 2) presented the higher number of metabolites, as expected.

Table 4 Evaluation of over-representation analysis (ORA) outputs of bioinformatic tools employing Reactome and HumanCyc pathways. Real (from dataset ST000284) and enriched data were used. The number of total metabolites in the pathway, the number of hits, the ranking of the pathway among all the Reactome or HumanCyc pathways (according to their significance), the *p*-value and the adjusted *p*-value were calculated by the tools

Tool	Data	Rank	Total metab.	Hits	*P*-value	Adjusted *p*-value
Reactome						
Metabolism of amino acids and derivates						
ConsensusPathDB	Real	3	272	18	8.46E-14	4.55E-12
	Enriched	1	272	12	7.67E-15	5.75E-13
IMPaLA	Real	3	272	18	8.46E-14	4.21E-11
	Enriched	1	272	12	7.67E-15	1.02E-11
PathVisio	Real	NA	NA	NA	NA	NA
	Enriched	NA	NA	NA	NA	NA
Reactome	Real	9	283	18	1.03E-04	3.81E-03
	Enriched	1	283	12	8.18E-08	1.00E-05
HumanCyc						
tRNA charging						
ConsensusPathDB	Real	2	24	8	9.14E-12	1.92E-10
	Enriched	2	24	5	4.40E-09	1.30E-07
HumanCyc	Real	8	24	8	2.57E-05	0.002
	Enriched	18	24	5	7.90E-05	4.25E-03
IMPaLA	Real	2	24	8	9.14E-12	2.28E-09
	Enriched	2	24	5	4.40E-09	3.51E-07
MBRole (full database)	Real	4	64	8	8.38E-09	1.14E-06
	Enriched	47	64	4	3.58E-07	7.97E-06
MBRole (*Homo sapiens*)	Real	5	64	8	1.26E-04	2.44E-03
	Enriched	11	64	5	1.58E-04	1.52E-03
MetExplore	Real	1	NA	8	7.11E-07	7.75E-05
	Enriched	6	NA	5	4.45E-05	3.60E-04

NA means that information was not provided by the tool. Abbreviations: *NA* not available

Surprisingly, KEGGREST, a R package that provides an updated client interface to the KEGGREST server, did not provide the highest number of total metabolites among the tested KEGG pathways. Despite regular updates to some pathway databases, such as KEGG [16] or Reactome [42], being carried out, most of the tools evaluated do not use up-to-date database versions (Table 2) [54]. Wadi et al. performed an elegant review on the impact of outdated annotations on pathway enrichment analysis, which revealed that many software tools use functional information not updated for years, thereby strongly affecting the quality of the analyses [54].

We can conclude that current ORA methods, despite their differences, provide consistent, robust and reproducible results regardless of their analytic approach (statistical test, *p*-value adjustment or pathway database used), despite the limitations and small differences found between outputs. The most discordant result was obtained with MPEA, probably due to the fact that it employs a different method to handle many-to-many relationships that may occur between the query compounds and metabolite annotations [38].

Although we cannot recommend one tool over the others, we suggest choosing those tools that employ updated metabolite/pathway databases in order to obtain more complete results. Nevertheless, we also consider that the enrichment analysis must not be restricted to a single database or tool. The combined use of libraries such as KEGG, Reactome, HumanCyc or WikiPathways will increase the metabolome coverage and the statistical power of the enrichment analysis.

Disease-based enrichment analysis did not yield accurate results. Although we only used serum/plasma biomarkers, results with other types of biological samples would have been similar. On one hand, metabolite disease sets are not up-to-date. For instance, MetaboAnalyst and MBRole (SMPDB and HMDB disease databases, respectively) base their searches of literature dated

Table 5 Disease-based enrichment analyses of the five datasets performed with MetaboAnalyst (SMPDB disease database), MBRole (HMDB disease database) and IPA® (in-house disease database) and MetaCore (based on MeSH and OMIM annotations). When the exact disease/condition of study was not obtained, a similar disease was selected

Dataset	Disease input	Disease output	Rank	Input number metabolites	Hits output	*P*-value	Adjusted *p*-value
MetaboAnalyst							
ST000091	Type 1 diabetes mellitus	Diabetes mellitus MODY	20	8	2	3.40E-02	5.84E-01
ST000383	Type 2 diabetes mellitus	Diabetes mellitus MODY	4	27	4	8.60E-03	6.69E-01
	Obesity	Obesity	31	27	1	9.07E-02	8.83E-01
MTBLS364	Smokers	–	–	81	–	–	–
MTBLS424	Breast cancer	Mammary tumour	30	22	2	4.08E-03	4.68E-02
ST000284	Colorectal cancer	Cervical/colon/ovarian cancer	46	42	1	8.47E-02	5.30E-01
MBRole							
ST000091	Type 1 diabetes mellitus	–	–	8	–	–	–
ST000383	Type 2 diabetes mellitus	Type 2 diabetes mellitus	8	27	3	1.16E-02	5.48E-02
	Obesity	Obesity	28	27	1	1.08E-01	1.48E-01
MTBLS364	Smokers	Lung Cancer	16	81	31	3.02E-02	9.25E-02
MTBLS424	Breast cancer	Lung Cancer	7	22	6	1.27E-04	1.09E-03
ST000284	Colorectal cancer	Colorectal cancer	44	42	1	5.19E-02	1.14E-01
IPA®							
ST000091	Type 1 diabetes mellitus	–	–	8	–	–	–
ST000383	Type 2 diabetes mellitus	Insulin resistance	21	27	3	6.10E-05	NA
	Obesity	Adipogenesis of fat	264	27	1	1.54E-02	NA
MTBLS364	Smokers	Cough	490	81	11	4.33E-02	NA
MTBLS424	Breast cancer	Gastric cancer	2	22	9	5.03E-11	NA
ST000284	Colorectal cancer	Colorectal cancer	3	42	11	2.31E-08	NA
MetaCore™							
ST000091	Type 1 diabetes mellitus	Type 1 diabetes mellitus	NA	8	0	NA	NA
ST000383	Type 2 diabetes mellitus	Type 2 diabetes mellitus	NA	27	7	NA	NA
	Obesity	Obesity	NA	27	1	NA	NA
MTBLS364	Smokers	Respiratory disorders	NA	81	1	NA	NA
MTBLS424	Breast cancer	Breast neoplasms	NA	22	0	NA	NA
ST000284	Colorectal cancer	Colorectal neoplasms	NA	42	13	NA	NA

Abbreviations: *NA* not available

between 1975 and 2008, as stated in the outputs of these tools. Since 2008, advances in high-throughput techniques have remarkably improved metabolomics analyses and, consequently, more knowledge about these diseases is available. As previously discussed, the use of not updated annotation sets strongly affect the quality of the analyses [54]. On the other hand, metabolites can overlap between unrelated physiopathological events since similar metabolic processes are altered [55]. This fact could complicate the development and accuracy of background sets for disease-based enrichment analysis.

Although extensive work in developing bioinformatic tools for metabolite sets has been carried out in recent years, more effort in improving metabolite/pathway databases and tools is still needed. On one hand, metabolite databases have to rapidly absorb new information from unstoppable advances in high-throughput technologies. On the other hand, enrichment methods should include a wider range of metabolite identifiers (e.g. LipidMAPS, ChemSpider or METLIN) and metabolite pathway databases in order to increase the metabolome coverage. For instance, the LipidMAPS Structure Database contains about 30,000 human endogenous lipids and 12,000 plant lipids, but also databases based on lipid metabolism and signalling pathways, MS/MS spectra and protein-related data [25, 56]. ChemSpider is a general chemical database and offers access to information for almost 25 million experimentally determined structures of natural and synthetic compounds [22]. However, similarly to PubChem, ChemSpider may lead

to a high number of false positives [57]. The METLIN database includes nearly 1,000,000 molecules, ranging from lipids, steroids, plant & bacteria metabolites, small peptides, carbohydrates, exogenous drugs/metabolites and central carbon metabolites, and more than 200,000 MS/MS spectra [24]. Including these information sources in current bioinformatic tools would also involve more effort in the improvement of metabolite identifiers converters. Therefore, there is still a long way ahead to achieve complete metabolite and pathway databases and thus accurate enrichment analyses of metabolite sets.

Conclusions

We have extensively reviewed, for the first time, the state-of-the-art of bioinformatic tools for the enrichment of metabolite sets from metabolomics studies, visualized their diversity, and examined their performance. The redundancy of identifiers, the use of chemical class identifiers and the incompleteness of metabolite databases and disease metabolite sets limit the extent of the analyses and reduce their accuracy. In general, ORA tools provided consistent results among tools revealing that these analyses are robust and reproducible regardless of their analytic approach. However, more work in the completeness of metabolite/pathway databases is required to get more accurate and global insights of the metabolome.

Additional files

Additional file 1: Table S1. Full list of significant metabolites of the five datasets used in the present study (adjusted *P*-value < 0.05). Dataset A refers to dataset ST000091, B to ST000383, C to MTBLS364, D to MTBLS424 and E to ST000284. (XLSX 28 kb)

Additional file 2: Table S2. Enriched data and their main metabolite identifiers for ORA analysis. (XLSX 9 kb)

Additional file 3: Table S3. List of features of the tools analysed by multiple correspondence analysis. Abbreviations: N, no; Y, yes. (XLSX 12 kb)

Additional file 4: Table S4. Number of metabolites with identifiers of the following metabolite databases. Metabolite databases are sorted by the number of identifiers found. *LipidMAPS identifiers were only searched in lipids ($n = 67$), while the rest of identifiers were considered in all the metabolites of the datasets ($n = 147$). (DOCX 16 kb)

Abbreviations

ChEBI: Chemical entities of biological interest; FDR: False discovery rate; HMDB: Human metabolome database; KEGG: Kyoto Encyclopaedia of genes and genomes; LipidMAPS: Lipid metabolites and pathways strategy; NMDS: Non-metric multidimensional scaling; ORA: Over-representation analysis; SMPDB: Small molecule pathway database

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Availability of data and materials

All data generated or analysed during the current study are included in this published article and its supplementary information files. Authors state that data are available for further studies.

Declarations

This manuscript has not been published elsewhere previously and is not being considered by another publication.
All the authors are aware and agree to the content of the paper and their being listed as authors of the manuscript.

Authors' contributions

The authors' contributions to the manuscript were as follows: AMR, ASP and CAL designed the experiments; AMR and MPR selected the bioinformatic tools; ST and MUS tools provided the metabolite datasets; AB enriched the data and contributed to the statistical analyses; AMR and MPR conducted the analysis of the data; AMR wrote the manuscript; AMR, ASP and CAL contributed to the discussion of the manuscript; all the authors reviewed the manuscript. CAL has the primary responsibility for the final content. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Material Suplementario

REVISION 3

Table S1. Full list of significant metabolites of the five datasets used in the present study (P-adjusted<0.05 or p<0.05 if p-adjusted was not provided).

Dataset A refers to dataset ST000091, B to ST000383, C to MTBLS364, D to MTBLS424 and E to ST000284 PC: PHOSPHATIDYLCHOLINE

Table S2. Enriched data and their main metabolite identifiers for ORA analysis

Table S3. List of features of the most commonly used tools for metabolomics data enrichment. Abbreviations: N, no; Y, yes.

Table S1. Full list of significant metabolites of the five datasets used in the present study (P-adjusted<0.05 or p<0.05 if p-adjusted was not provided). PC: PHOSPHATIDYLCHOLINE; ChemS: ChemSpider; Met: Metline

Dataset A refers to dataset ST000091, B to ST000383, C to MTBLS364, D to MTBLS424 and E to ST000284.

Metabolite	Data	ID in metabolite databases								
		HMDB	KEGG	PubChem	ChEBI	ChemS	HumanCyc	Recon2	Lipid MAPS	MET
Amino acids and related compounds										
Acetylglycine, N-	E	HMDB00532	-	10972	61887	10507	-	-	-	-
Alanine, L-	D	HMDB00161	C00041	5950	16977	5735	L-ALPHA-ALANINE	M_ala_L_c	-	11
Aminoadipic acid, 2- (α-)	A, E	HMDB00510	C00956	469	37024	456	CPD-468	M_adpac_c	-	3271
Aminobutyric acid, α-	C	HMDB00452	C02356	80283	35619	72524	2-Aminobutyrate	M_C02356_c	-	35945
Aminobutyric acid, γ-	E	HMDB00112	C00334	119	16865	116	4-AMINO-BUTYRATE	M_4abut_l	-	279
Aminohippuric acid	C	HMDB01867	D06890	2148	104011	2063	-	-	-	3927
Aminoisobutyric acid, β-	A	HMDB02166	C03284	439434	33094	388543	CPD-466	M_3aib_c	-	45884
Arginine, L-	C, E	HMDB00517	C00062	6322	1646	6082	ARG	M_arg_L_c	-	13
Argininosuccinic acid, L-	C	HMDB00052	C03406	16950	15682	16059	L-ARGININO-SUCCINATE	M_argsuc_c	-	389
Argininosuccinic anhydride, L-	C	-	-	-	-	-	-	-	-	-
Aspartic acid, L-	E	HMDB00191	C00049	5960	17053	5745	L-ASPARTATE	M_asp_L_c	-	15
Cystathionine, L-	E	HMDB00099	C02291	439258	17482	388392	L-CYSTATHIONINE	M_cyst_L_c	-	39
Cystine, L-	B	HMDB00192	C00491	67678	16283	60997	CYSTINE	M_Lcystin_c	-	17
Dimethylglycine	E	HMDB00092	C01026	673	17724	653	DIMETHYL-GLYCINE	M_dmgly_c		277
Glutamic acid, L-	A, C, D, E	HMDB00148	C00025	33032	16015	30572	GLT	M_glu_L_c	-	19

Glutamine, L-	D, E	HMDB00641	C00064	5961	18050	5746	GLN	M_gln_L_c	-	18
Glutathione	C	HMDB00125	C00051	124886	16856	111188	GLUTATHIONE	M_gthrd_c	-	44
Glycine	B, D	HMDB00123	C00037	750	15428	730	GLY	M_gly_c	-	20
Guanidinoacetic acid	C	HMDB00128	C00581	763	16344	743	GUANIDOACETIC_ACID	M_gudac_c	-	9
Histidine, L-	B, D, E	HMDB00177	C00135	6274	15971	6038	HIS	M_his_L_c	-	21
Homogentisic acid	E	HMDB00130	C00544	780	44747	759	HOMOGENTISATE	M_hgentis_c	-	331
Hydroxyproline, 4-	E	HMDB00725	C01157	5810	18240	5605	4-HYDROXY-L-PROLINE	-	-	58354
Isoleucine, L-	A, D	HMDB00172	C00407	6306	17191	6067	ILE	M_ile_L_c	-	23
Kynurenine, L-	C	HMDB00684	C00328	161166	16946	141580	L-KYNURENINE	M_4hpro_LT_c	-	72
Leucine, L-	A, B, D	HMDB00687	C00123	6106	15603	5880	LEU	M_leu_L_c	-	24
Lysine, L-	B, C, E	HMDB00182	C00047	5962	18019	5747	LYS	M_lys_L_c	-	25
Methionine, L-	D, E	HMDB00696	C00073	6137	16643	5907	MET	-	-	26
Ornithine, L-	A	HMDB00214	C00077	6262	15729	6026	L-ORNITHINE	M_orn_L_c	-	27
Proline, L-	C, D, E	HMDB00162	C00148	145742	17203	128566	PRO	M_pro_L_c	-	29
Phenylalanine, L-	A, C, D	HMDB00159	C00079	6140	17295	5910	PHE	M_phe_L_c	-	28
Phenylpyruvic acid	E	HMDB00205	C00166	997	30851	972	PHENYL-PYRUVATE	-	-	328
Serine, L-	C	HMDB00187	C00065	5951	17115	5736	SER	M_ser_L_c	-	30
Trimethyllysine	C	HMDB01325	C03793	440120	17311	389120	N6N6N6-TRIMETHYL-L-LYSINE	M_tmlys_c	-	44794
Tyrosine, L-	D	HMDB00158	C00082	6057	17895	5833	TYR	M_tyr_L_c	-	34
Valine, L-	A	HMDB00883	C00183	6287	16414	6050	VAL	M_val_L_c	-	35
Organic acids										

Acetic acid	D	HMDB00042	C00033	176	15366	171	ACET	M_ac_c	-	3206
Benzoic acid	B	HMDB01870	C00180	243	30746	238	BENZOATE	M_bz_c	-	1297
Carboxylic acid	C	-	C00060	-	33575	-	Carboxylates (class)	-	-	-
Citric acid	C	HMDB00094	C00158	311	30769	305	CIT	M_cit_c	-	124
Formic acid	D	HMDB00142	C00058	284	30751	278	FORMATE	M_for_c	-	3202
Fumaric acid	E	HMDB00134	C00122	444972	18012	10197150	FUM	M_fum_c	-	3242
Gluconic acid	B	HMDB00625	C00257	10690	33198	10240	GLUCONATE	M_glcn_c	-	345
Hippuric acid	E	HMDB00714	C01586	464	18089	451	CPD-425	-	-	1301
Ketoisocaproic acid, 2- (α-)	B	HMDB00695	C00233	70	48430	69	2K-4CH3-PENTANOATE	-	-	121
Kynurenic acid	E	HMDB00715	C01717	3845	18344	3712	KYNURENATE	M_kynate_c	-	5683
Lactic acid	D, E	HMDB00190	C00186	107689	422	96860	L-LACTATE	M_lac_L_c	-	116
Leucic acid	E	HMDB00624	C03264	439960	55534	388986	-	-	-	5597
Maleic acid	E	HMDB00176	C01384	444266	18300	392248	-	-	-	4198
Methylsuccinic acid	E	HMDB01844	C08645	10349	29003	9922	DIETHYL-2R3R-2-METHYL-3-HYDROXYSUCCIN	-	-	3710
Oxalic Acid	E	HMDB02329	C00209	971	16995	946	OXALATE	M_oxa_c	-	113
Oxoglutaric acid, 2-	E	HMDB00208	C00026	51	30915	50	2-KETOGLUTARATE	M_akg_c	-	119
Phosphoenolpyruvic acid	E	HMDB00263	C00074	1005	44897	980	PHOSPHO-ENOL-PYRUVATE	M_pep_c	-	152
Pyruvic acid	E	HMDB00243	C00022	1060	32816	1031	PYRUVATE	M_pyr_c	LMFA01060077	117
Uric acid	E	HMDB00289	C00366	1175	17775	1142	URATE	M_urate_c	-	88
Carbohydrates										
Anhydrogalactose, 3,6-	B	-	C06474	16069996	27861	17229511	-	-	-	66407

Erythrose, D-	E	HMDB02649	C01796	439574	23956	388659	-	-		65654
Fructose, D-	B	HMDB00660	C02336	439709	15824	388775	FRU	M_fru_c	-	135
Glucose, D-	B, D, E	HMDB00122	C00031	5793	4167	5589	GLUC	M_glc_D_c	-	133
Glucuronic acid, D-	B	HMDB00127	C00191	444791	4178	392615	GLUCURONATE	M_glcur_c	-	161
Inulobiose	B	HMDB29898	C03323	439552	16751	388643	-	-	-	65636
Xylose, D-	B	HMDB00098	C00181	135191	53455	119104	XYLOSE	-	-	314
Lipids										
(25R)-3alpha,7alpha-dihydroxy-5beta-cholestan-27-oyl taurine	C	-	-	42608426	-	-	-	-	LMST05040008	57994
1-(6-[3]-ladderane-hexanyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphocholine	C	-	-	44256603	-	-	-	-	LMGP01040090	46679
Androsterone sulfate	C	HMDB02759	-	159663	83037	140383	-	M_CE6031_c	-	3559
Arachidonic acid	B	HMDB01043	C00219	444899	15843	392692	ARACHIDONIC_ACID	M_arachd_c	LMFA01030001	193
Cer(d18:1/24:0)	C	HMDB04956	C00195	5283571	17761	4446684	CERAMIDE	-	LMSP02010012	83712
Cer(d18:1/24:1(15Z))	C	HMDB04953	C00195	5283568	17761	4446681	CERAMIDE	-	LMSP02010009	83709
FMC-5(d18:1/22:0)	C	-	-	52931245	-	-	-	-	LMSP05010034	83799
GalCer(d18:1/24:0)	C	-	-	44260152	75186	4955274	A-GALACTOSYLCERAMIDE	M_galside_hs_c	LMSP0501AC05	83800
GlcCer(d14:1/22:1)	C	-	-	70699248	75186	-	A-GALACTOSYLCERAMIDE	M_galside_hs_c	LMSP0501AA67	-
GlcCer(d18:1/23:0)	C	-	-	52931253	75186	34449082	A-GALACTOSYLCERAMIDE	M_galside_hs_c	LMSP0501AA32	83807
Glycerol-3-phosphate	B	HMDB00126	C00093	439162	15978	388308	GLYCEROL-3P	M_glyc3p_c	-	5161

Glycochenodeoxycholic acid	E	HMDB00637	C05466	12544	36274	17215984	GLYCOCHENODEOXYCHOLIC_ACID	M_dgchol_c	LMST0503000	203
Glycocholic acid	E	HMDB00138	C01921	10140	29746	24747221	GLYCOCHOLIC_ACID	M_gchola_c	LMST05030001	202
Heptadecanoic acid	B	HMDB02259	-	10465	32365	10033	-	M_hpdca_c	LMFA01010017	4206
Hydroxybutanoic acid, 2- (α-)	B	HMDB00008	C05984	11266	50612	10792	CPD-3564	M_2hb_c	LMFA01050455	35690
Hydroxybutanoic acid, 3- (β-)	B, D, E	HMDB00357	C01089	441	20067	428	CPD-335	M_bhb_c	LMFA01050463	125
Linoleic acid	E	HMDB00673	C01595	5280450	17351	4444105	LINOLEIC_ACID	M_lnlc_c	LMFA01030120	191
Linolenic acid, α-	E	HMDB01388	C06427	5280934	27432	4444437	LINOLENIC_ACID	M_lnlnca_c	LMFA01030152	192
LysoPC(15:0)	C	HMDB10381	C04230	24779458	580912	24694854	PC	M_lpchol_hs_c	LMGP01050016	61691
LysoPC(16:0)	C	HMDB10382	C04230	460602	64563	405287	PC	M_lpchol_hs_c	LMGP01050018	61692
LysoPC(18:0)	C	HMDB10384	C04230	497299	91313	435389	CPD-8345	M_lpchol_hs_c	LMGP01050026	61694
LysoPC(18:2)	C	HMDB10386	C04230	11005824	91309	9181014	CPD-8347	M_lpchol_hs_c	LMGP01050035	61696
LysoPC(20:0)	C	HMDB10390	C04230	24779473	67058	21403128	PC	M_lpchol_hs_c	LMGP01050045	61700
LysoPC(20:1)	C	HMDB10391	C04230	52924051	67057	24766530	PC	M_lpchol_hs_c	LMGP01050131	61701
LysoPC(20:4)	C	HMDB10396	C04230	24779476	91310	21403155	PC	M_lpchol_hs_c	LMGP01050140	61705
LysoPC(22:6)	C	HMDB10404	C04230	10415542	64567	8590975	PC	M_lpchol_hs_c	LMGP01050056	61713
LysoPC(P-16:0)	C	HMDB10407	C04230	10917802	73841	9093054	PC	M_lpchol_hs_c	LMGP01070006	61716
LysoPC(P-18:0)	C	HMDB13122	C04230	24779527	88779	24823061	PC	M_lpchol_hs_c	LMGP01070009	-
LysoPE(22:6)	C	HMDB11526	C00350	52925132	72734	24769403	Lysophosphatidylethanolamines (class)	-	LMGP02050013	62310
Oleic acid	B	HMDB00207	C00712	445639	16196	393217	OLEATE-CPD	M_ocdcea_l	LMFA01030002	190
Palmitic acid	B	HMDB00220	C00249	985	15756	960	PALMITATE	M_hdca_c	LMFA01010001	187

Palmitoleic acid	B	HMDB03229	C08362	445638	28716	393216	CPD-9245	M_hdcea_x	LMFA01030056	188
PC(15:1/20:4)	C	HMDB07949	C00157	52922330	64482	24766624	PC	M_pchol_hs_c	LMGP01011454	-
PC(16:0/18:1)	C	HMDB07972	C00157	5497103	89013	4593686	PC	M_pchol_hs_c	LMGP01010005	59421
PC(16:0/18:2(9Z,12Z))	C	HMDB07973	C00157	5287971	89823	4450224	PC	M_pchol_hs_c	LMGP01010591	59422
PC(17:1/20:4)	C	-	C00157	52922565	64482	-	PC	M_pchol_hs_c	LMGP01011543	-
PC(18:0/16:0)	C	HMDB08034	C00157	18631368	64482	18601329	PC	M_pchol_hs_c	LMGP01010742	59482
PC(18:0/22:6)	C	HMDB08057	C00157	24778876	84829	24766721	PC	M_pchol_hs_c	LMGP01010821	59504
PC(18:1(9Z)/0:0)	C	HMDB02815	C00157	16081932	28610	17240641	PC	M_pchol_hs_c	LMGP01050032	40298
PC(18:2/18:1)	C	HMDB08137	C00157	52922727	89597	24766800	PC	M_pchol_hs_c	LMGP01011624	59583
PC(20:4/22:6)	C	HMDB08452	C00157	52923327	64482	24767113	PC	M_pchol_hs_c	LMGP01011925	59931
PC(O-16:0/18:1)	C	HMDB13412	C04317	53481707	75268	-	PC	M_pchol_hs_c	LMGP01020003	40067
PC(O-16:0/22:6)	C	HMDB13409	C04317	16759366	1077065	24958571	PC	M_pchol_hs_c	LMGP01020064	40092
PC(O-18:0/0:0)	C	HMDB11149	C04317	2733532	560225	2015318	PC	M_pchol_hs_c	LMGP01060014	40390
PC(O-18:1(11Z)/0:0)	C	-	C04317	21672239	64482	-	PC	M_pchol_hs_c	LMGP01060034	76582
PC(O-20:0/18:2(9Z,12Z))	C	HMDB08270	C04317	24779263	64482	24766932	PC	M_pchol_hs_c	LMGP01020228	76459
PC(P-18:0/16:0)	C	HMDB11239	C00157	53480705	84826	24767514	PC	M_pchol_hs_c	LMGP01030052	62065
PE(18:0/20:2)	C	HMDB09000	C00350	9546824	90467	7825774	L-1-PHOSPHATIDYL-ETHANOLAMINE	M_pe_hs_c	LMGP02010124	40526
PE-Cer(15:2/24:0)	C	-	-	70699029	-	-	-		LMSP03020046	-
PG(20:0/22:0)	C	-	-	52927210	-	-	-	M_pglyc_hs_c	LMGP04010947	79772
SM(d18:1/14:0)	C	HMDB12097	C00550	11433862	17636	960873	Sphingomyelins (class)	-	LMSP03010028	53976

SM(d18:1/16:0)	C	HMDB10169	C00550	5283590	6304	4446703	Sphingomyelins (class)	-	LMSP03010003	41586
SM(d18:1/18:1)	C	HMDB12101	C00550	6443882	17636	494783	Sphingomyelins (class)	-	LMSP03010029	-
SM(d18:1/22:0)	C	HMDB12103	C00550	44260125	17636	24846873	Sphingomyelins (class)	-	LMSP03010006	41589
SM(d18:1/23:0)	C	HMDB12105	C00550	46891684	17636	-	Sphingomyelins (class)	-	LMSP03010078	83778
SM(d18:1/24:0)	C	HMDB11697	C00550	5283595	106884	4446708	Sphingomyelins (class)	-	LMSP03010008	41591
SM(d18:1/24:1)	C	HMDB12107	C00550	44260126	17636	24846874	Sphingomyelins (class)	-	LMSP03010007	-
SM(d18:2/14:0)	C	-	C00550	1143386	-	9608732	Sphingomyelins (class)	-	LMSP03010034	83736
SM(d18:2/15:0)	C	-	C00550	52931135	-	-	Sphingomyelins (class)	-	LMSP03010036	83738
SM(d18:2/21:0)	C	-	C00550	52931187	-	-	Sphingomyelins (class)	-	LMSP03010064	83764
SM(d18:2/22:0)	C	HMDB12091	C00550	44260132	-	24846880	Sphingomyelins (class)	-	LMSP03010092	83792
SM(d18:2/24:0)	C	HMDB12094	C00550	44260134	-	24846882	Sphingomyelins (class)	-	LMSP03010081	83781
SM(d18:2/24:1)	C	HMDB12095	C00550	44260133	-	24846881	Sphingomyelins (class)	-	LMSP03010080	83780
Stearic acid	B	HMDB00827	C01530	5281	28842	5091	STEARIC_ACID	M_ocda_c	LMFA01010018	189
TG(12:0/18:2(9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z))	C	-	C00422	56937282	17855		Triacylglycerols (class)	M_tag_hs_c	LMGL03013492	99347
TG(14:0/18:2(9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z))	C	HMDB42537	C00422	56938181	17855	-	Triacylglycerols (class)	M_tag_hs_c	LMGL03014392	100247
TG(15:0/18:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))	C	HMDB43275	C00422	56938939	17855	-	Triacylglycerols (class)	M_tag_hs_c	LMGL03015157	101005
Others										
13-methyl-4,4-bisnor-8,11,13-podocarpatrien-3-one	C	-	-	-	25212	-	-	-	-	-
Adenylosuccinic acid	E	HMDB00536	C03794	440122	15919	389122	ADENYLOSUCC	-	-	3551
Allantoin	E	HMDB00462	C01551	204	15676	199	R--ALLANTOIN	M_alltn_c	-	89
Benzylalcohol	B	HMDB03119	C005567	244	17987	13860335	BENZYL-ALCOHOL	-	-	-

Bilirubin	C	HMDB00054	C00486	5280352	1699	4444055	BILIRUBIN	M_bilirub_c	-	81
Carnitine	C	HMDB00062	C00318	2724480	11060	2006614	CARNITINE	M_crn_r	-	63461
Choline	D	HMDB00097	C00114	305	15354	299	CHOLINE	M_chol_c	-	56
Creatine	D	HMDB00064	C00300	586	16919	566	CREATINE	M_creat_c	-	7
Creatinine	D, E	HMDB00562	C00791	588	16737	568	CREATININE	M_crtn_c	-	8
Deoxyerythritol, 2-	B	-	-	18302	88063	556326	CPD-16716	-	-	-
Deoxyuridine, 2-	E	HMDB00012	C00526	13712	16450	13118	DEOXYURIDINE	M_duri_c	-	91
Ethanolamine	B	HMDB00149	C00189	700	16000	13835336	ETHANOL-AMINE	-	-	3207
Glyceraldehyde	E	HMDB01051	C02154	751	5445	731	GLYCERALD	M_glyald_c	-	4176
Hypoxanthine	C	HMDB00157	C00262	790	17368	768	HYPOXANTHINE	M_hxan_c	-	83
Methoxyquinoline, 6-	C	-	-	14860	72822	122469	-	-	-	34519
Methyladenosine, 1-	E	HMDB03331	C02494	27476	16020	17216227	1-METHYLADENOSINE	-	-	6888
Tricosanamide	C	HMDB00950	-	52931115	-	24765757		-	LMSP02010021	5896
Uridine	B, E	HMDB00296	C00299	6029	16704	5807	URIDINE	M_uri_c	-	90
Vaccenyl carnitine	C	HMDB06351	-	53477830	89742	-		M_ocdececrn_c	-	58401

Table S2. Enriched data and their main metabolite identifiers for ORA analysis

Enriched data

Name	KEGG	HumanCyc	PubChem
Alpha-Ketoglutaric Acid	C00026	2-KETOGLUTARATE	5961
Creatine	C00300	CREATINE	1060
Creatinine	C00791	CREATININE	33032
Fumarate	C00122	FUM	6274
Gamma-Aminobutyrate	C00334	4-AMINO-BUTYRATE	6057
Glutamic acid	C00025	GLT	6140
Glutamine	C00064	GLN	51
Guanidinoacetate	C00581	GUANIDOACETIC_ACID	444972
Histidine	C00135	HIS	970
Oxaloacetate	C00036	OXALACETIC_ACID	586
Phenylalanine	C00079	PHE	763
Pyruvate	C00022	PYR	588
Tyrosine	C00082	TYR	119

Table S3. List of features of the most commonly used tools for metabolomics data enrichment. Abbreviations: N, no; Y, yes.

Tool	ORA	Integration with other 'omics'	Other enrichments	Pathway visualization	Network visualization	Other visualization	Uses KEGG pathways	Uses BioCyc pathways	Uses Reactome pathways	Uses Wikipathway pathways
3omics	Y	Y	N	N	Y	N	Y	Y	N	N
BioCyc	Y	N	N	Y	Y	N	N	Y	N	N
ConsensusPathDB	Y	Y	N	N	N	Y	Y	Y	Y	Y
IMPaLA	Y	Y	N	N	N	N	Y	Y	Y	Y
IPA®	Y	Y	Y	N	Y	Y	N	N	N	N
KEGG	N	Y	N	Y	N	N	Y	N	N	N
MassTRIX	N	N	Y	N	N	N	Y	N	N	N
MBRole	Y	N	Y	N	N	N	Y	Y	N	N
MetaboAnalyst	Y	N	Y	Y	N	Y	Y	N	N	N
Metabox	Y	Y	N	N	Y	N	Y	N	N	N
MetaCore™	Y	Y	Y	N	Y	Y	N	N	N	N
MetaMapp	N	N	N	N	Y	N	N	N	N	N
MetExplore	Y	Y	N	N	Y	N	Y	Y	N	N
MetScape	N	Y	N	N	Y	N	Y	N	N	N
MPEA	Y	N	N	N	N	N	Y	N	N	N
PaintOmics	Y	Y	N	Y	N	N	Y	N	N	N
PAPi	Y	N	N	N	N	N	Y	N	N	N
PathVisio	Y	Y	N	Y	N	N	N	N	Y	Y
Reactome	Y	Y	Y	Y	Y	N	N	N	Y	N
SMPDB	N	N	N	Y	N	N	N	N	N	N
WikiPathways	N	N	N	Y	N	N	N	N	N	Y
XCMS	N	N	Y	N	N	Y	N	N	N	N

Table S4. Number of metabolites with identifiers of the following metabolite databases. Metabolite databases are sorted by the number of identifiers found. *LipidMAPS identifiers were only searched in lipids (n=67), while the rest of identifiers were considered in all the metabolites of the datasets (n=147).

	Total	PubChem	METLIN	ChEBI	KEGG	ChemSpider	HumanCyc	HMDB	Recon2	LipidMAPS*
Metabolites with identifiers										-
Amino acids and related compounds	35	34	33	34	33	34	32	34	29	-
Organic acids	19	18	18	19	19	18	17	18	13	-
Carbohydrates	7	7	7	7	7	7	4	6	3	-
Lipids	67	67	60	56	57	52	60	53	47	65
Other compounds	19	18	16	18	14	17	15	16	12	-
Total	**147 (100%)**	**144 (98%)**	**134 (91%)**	**134 (91%)**	**130 (88%)**	**129 (87%)**	**128 (87%)**	**127 (86%)**	**104 (71%)**	**65 (97%)**
Unique identifiers										
Amino acids and related compounds	35	34	33	34	33	34	32	34	29	-
Organic acids	19	18	18	19	19	18	17	18	13	-
Carbohydrates	7	7	7	7	7	7	4	6	3	-
Lipids	67	67	60	42	20	52	21	53	20	65
Other compounds	19	18	16	18	14	17	15	16	12	-
Total	**147 (100%)**	**144 (98%)**	**134 (91%)**	**120 (81%)**	**93 (63%)**	**129 (87%)**	**89 (61%)**	**127 (86%)**	**76 (52%)**	**65 (97%)**

4. RESULTADOS

PUBLICACION 1

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

Sara Tulipani, Magali Palau-Rodriguez, Antonio Miñarro, Francisco Cardona, Anna Marco-Ramell, Borja Zonja, Miren Lopez de Alda, Araceli Muñoz-Garach, Alex Sanchez-Pla, Francisco J Tinahones, Cristina Andres-Lacueva

Clinica Chimica Acta

Factor de Impacto: 2.80 Q1 (7/30) MEDICAL LABORATORY TECHNOLOGY (Indexado en Web of Science)

Los resultados de esta publicación fueron presentados en formato de:

- Comunicación póster: XIII Congreso Nacional Sociedad Española de Estudios de la Obesidad, 15-17 Marzo 2017, Sevilla, España, "Aplicación de la Metabolómica al estudio de fenotipos discordantes Obesidad/Diabetes. Perfiles metabólicos hacia la prevención y el tratamiento clínico"
- Comunicación póster: NUGOweek: MECHANISMS OF A LONG-LIFE HEALTH. 7-9 Set 2015 Barcelona, "Aplicación de la Metabolómica al estudio de fenotipos discordantes Obesidad/Diabetes. Perfiles metabólicos hacia la prevención y el tratamiento clínico"

RESUMEN PUBLICACION 1

Objetivos: El objetivo de esta publicación fue diseccionar la conexión obesidad y resistencia a la insulina mediante el estudio metabolómico de fenotipos discordantes obesidad y resistencia a la insulina.

Metodología: Se aplicó una aproximación dirigida, con las plataformas analíticas LC- y FIA-ESI-MS/MS para cuantificar 188 metabolitos (acilcarnitinas, (liso)fosfatidilcolinas, esfingomielinas, amino acidos, aminas biógenas y hexosas) en muestras de suero de sujetos con sensibilidad a la insulina y resistencia a la insulina con obesidad mórbida (n=12 y n=21 respectivamente) y sensibilidad a la insulina y resistencia a la insulina en sujetos con normopeso (n=19 y n=12, respectivamente).

Resultados: Los sujetos con obesidad severa se caracterizaron por tener alterados los niveles de (liso-)glicerofosfolípidos, específicamente aquellas moléculas aciladas con ácido margárico, oleico y linoleico. Varios amino acidos fueron biomarcadores de riesgo de DT2 asociados a obesidad. Entre ellos, el glutamato fue asociado significativamente con la insulina en ayunas mientras que la glicina mostraba asociaciones negativas. La valina, amino ácido de cadena ramificada se asoció con un estado de resistencia a la insulina y pre-DT2, independientemente del IMC. Esfingolípidos minoritarios como las ceramidas y las esfingomielinas, fueron también asociadas con la resistencia a la insulina. Las especies lipídicas, con cadenas 18:0, 18:1 y 18:2n-6 se asociaron con la obesidad mórbida.

Conclusiones: Este estudio identifica potenciales biomarcadores que podrían ayudar en la predicción de la diabetes en un estado más temprano de la enfermedad. Más estudios a grande escala son necesarios para validar estos resultados.

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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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lass Index, BMI ≥ 40) [15], as well as T2D occur among adult lean individuals [16]. Although the clinical relevance of these subgroups remains ebated [17], the study of discordant metabolic phenotypes for obesity nd diabetes provides a unique and poorly unexploited opportunity to camine the interrelations between adipose tissue expansion and the adual development of T2D and its sequelae [disease risk assessment]. owever, the studies focused on them are still very scarce, small-scaled 8–20] or not focused on humans [21].

In the present study, we propose that the metabolic profiling of uman 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 the glucose homeostasis, and help to dissect the connection between ie two diseases.

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

Material and Methods

1. Subjects and Study Design

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

The individuals were then classified into four sex-matched phenopic groups according to their BMI (non-obese if: BMI = 18,5–26,9 g/m^2; morbidly obese if: BMI > 40 kg/m^2) and to the risk of developing pe two diabetes based on fasting plasma glucose concentrations and insulin resistance (non-prediabetic/non-insulin resistant state if: FG < 00 mg/dL and HOMA-IR < 2.5; prediabetic/insulin resistant state if: 00 ≤ FG < 126 mg/dL and HOMA-IR > 3.4).

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

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	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.

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t of serum concentrations and statistical significance of discriminant metabolites among the four phenotipic groups.

	Phenotype								ANOVA*	Tukey Contrasts*	
	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]		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

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

rcumference], but in a modest extent. In morbidly obese subjects, for istance, a 2-fold increase in the serum levels of glutamate was particularly observed, compared to non-prediabetic obese controls, suggesting terations in the glutamate metabolism as a selective metabolic marker 'an early onset of diabetes in subjects with high BMI. By its conversion 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,

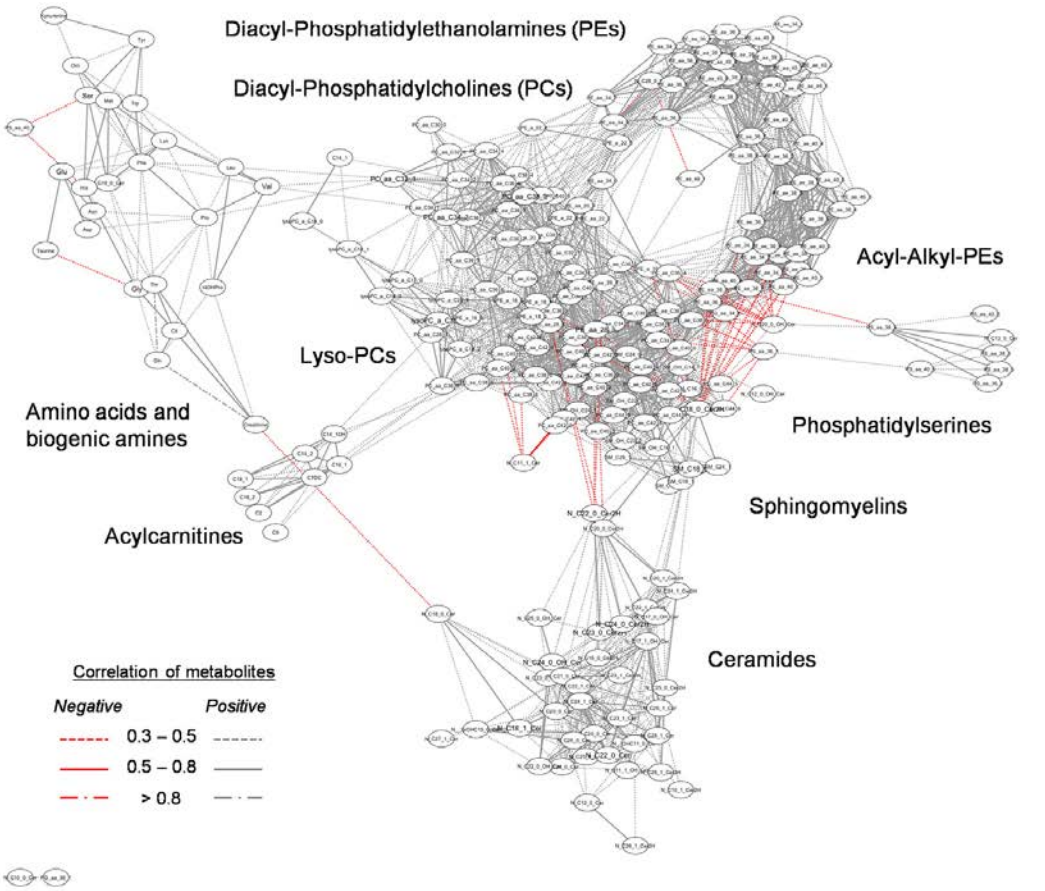


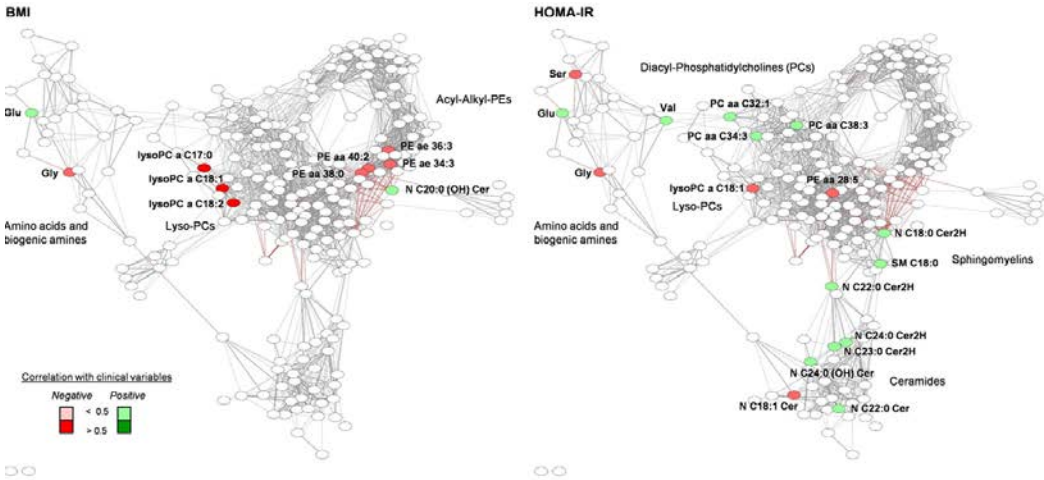
Fig. 1. Serum metabolic network representing the significant correlation (edges) between metabolites (nodes). Adjusted for the other metabolites. Black line represents positive correlation while red line negative correlation. The line format (dotted, solid) indicates the degree of correlation.

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



g. 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 rrelation while red negative correlation; color intensity indicates the degree of correlation.

ucose tolerance, and diabetes [35], and similar results were obtained ljusting plasma BCAA levels for BMI [2,36] or waist circumference 7]. However, several experimental studies also suggest that increased rculating BCAA would specifically mirror obesity-dependent diabetic ates, possibly related to altered adipose tissue BCAA catabolism [18, 3–40]. Although attempts to reconcile these disparate perspectives ave been already proposed [41], more investigations are required to ach a definitive overview.

1.2. Increase of circulating sphingolipids

A substantive literature has accumulated implicating sphingolipids, pecially 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

ble 3
agnostic 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

.DA,diagonal discriminant analysis; LDA, linear discriminant analysis; QDA, quadratic discriminant analysis; PLSDA, Partial least squares projection to latent structures-discriminant analis; SCDA, nearest shrunken centroid classification. †The classification performance was determined by common performance metrics including the misclassification rate [indicating the % 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 assificatio [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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Material Suplementario

PUBLICACION 1

Statistical analysis

Data Preprocessing. Statistical analyses were performed in the R environment (R version 3.1.2) [139]. To ensure valid and robust measurements, those metabolites with values below the LOD in more than 25% subjects in any of the phenotypic groups, and with high analytical variance in the QC2 replicates (CV > 25%) were excluded from further analysis. Missing values of the retained metabolites were imputed using k-nearest neighbors averaging (k=10) (R, *impute* package), and the resulting dataset was normalized. The eventual effects of age and drug intake (anti-inflammatory, antihypertensive and anticholesterolemic agents) on the clinical and metabolomic datasets were investigated by the application of a feature selector on each dependent variable, according to the Akaike Information Criterion. For each clinical and metabolic variable, a model explaining the variability across the subjects by means of the age and the individuals or simultaneous drug intake was obtained, and the model residuals were used as new dataset for further analysis.

UVA and MVA. The metabolites significantly differentiating the four phenotypic groups were assessed by univariate statistics (ANOVA test). To control for the false discovery rate (FDR) associated with multiple hypothesis testing, the p=0.05 significance criterion was adjusted to allow a maximum 5% probability (q= 0.05) of false positive detection [114]. In case of global significance, pairwise mean comparisons were carried out by HSD Tukey contrasts.

The selection of the metabolites making the largest contribution to the classification was first carried out by four different supervised techniques (R, CMA package): a) f-test (F test belonging to the linear hypothesis that the mean is the same for all classes), b) Kruskal test (multi-class generalization of the Wilcoxon rank sum test

and the nonparametric pendant to the F test), c) limma test (‘moderated t’ statistic for the two-class case and ’moderated F’ statistic for the multiclass case) [140], and d) random forest variable importance analysis. Each method ranked the metabolic markers from the most relevant to the less relevant by using its proper 'filter' criteria, and independent cross-validations were used to validate the selected sets of potential metabolic classifiers. The whole data set was randomly split into five equal-size subgroups (20% of samples each): four of them were used as learning set, to develop optimized submodels, whereas the remaining one as test (validation) set, to estimate the degree of model overfitting (n = 10 iterations). Variable selection was carried out in the whole population in study (obesity markers and prediabetes markers in all the subjects, irrespectively on the compresence of the second pathology) and within the phenotypic subgroups of individuals, separately (obesity markers in healthy subjects, obesity markers in prediabetic subjects, prediabetic markers in obese subjects, prediabetic markers in lean subjects).

The capacity of the selected metabolic classifiers in discriminating the four phenotypic groups in study was then evaluated by applying both linear and non-linear classification techniques (R, CMA package), including: a) diagonal discriminant analysis (DDA), b) linear discriminant analysis (LDA), c) quadratic discriminant analysis (QDA), d) Partial least squares projection to latent structures-discriminant analysis (PLS-DA) [141], and e) nearest shrunken centroid classification (SCDA). 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 classification).

The degree of correlation among metabolic markers and standard measures of adiposity and blood sugar control) were also determined based on the Spearman’s rank-order correlation coefficients.

Supplementary Tables

Supplementary Table 1. Summary of metabolites (semi)quantified in blood serum by ESI/MS/MS". Metabolites are grouped in to classes based on metabolic function or structural similarities.

			Healthy_Ln		Healthy_Ob		Pre-T2D_Ln		Pre-T2D_Ob	
Metabolic class (*)	**Short Name**	**Data Type**	**n**	**Concentration (nM, mean ± SD)**	**n**	**Concentration (nM, mean ± SD)**	**n**	**Concentration (nM, mean ± SD)**	**n**	**Concentration (nM, mean ± SD)**
Acylcarnitines	C0	Quanti	19	31.77 ± 7.13	12	34.12 ± 8.73	12	39.30 ± 7.43	21	36.55 ± 6.82
	C2	Quanti	19	6.57 ± 2.22	12	9.15 ± 4.63	12	7.26 ± 1.44	21	6.65 ± 2.45
	C7DC	Semi-quanti	19	0.02 ± 0.01	12	0.02 ± 0.01	12	0.02 ± 0.01	21	0.02 ± 0.01
	C9	Semi-quanti	19	0.03 ± 0.01	12	0.02 ± 0.02	12	0.03 ± 0.01	21	0.02 ± 0.01
	C10:2	Semi-quanti	19	0.02 ± 0.01	12	0.02 ± 0.01	12	0.02 ± 0.01	21	0.02 ± 0.01
	C14:1	Semi-quanti	19	0.11 ± 0.03	12	0.11 ± 0.06	12	0.12 ± 0.02	21	0.11 ± 0.02
	C14:1OH	Semi-quanti	19	0.01 ± 0.01	12	0.01 ± 0.01	12	0.02 ± 0.01	21	0.01 ± 0.00
	C14:2	Semi-quanti	19	0.04 ± 0.02	12	0.04 ± 0.02	12	0.04 ± 0.01	21	0.04 ± 0.01
	C16:1	Semi-quanti	19	0.03 ± 0.01	12	0.03 ± 0.01	12	0.03 ± 0.01	21	0.03 ± 0.01
	C18:1	Semi-quanti	19	0.12 ± 0.04	12	0.13 ± 0.03	12	0.13 ± 0.03	21	0.13 ± 0.04
	C18:2	Semi-quanti	19	0.05 ± 0.01	12	0.05 ± 0.01	12	0.05 ± 0.01	21	0.05 ± 0.01
Amino Acids	Ala	Quanti	17	285.05 ± 76.34	12	254.44 ± 94.30	11	294.56 ± 103.71	18	361.75 ± 87.79
	Asn	Quanti	17	43.50 ± 13.67	12	34.29 ± 11.64	11	29.70 ± 10.60	18	35.59 ± 15.59
	Asp	Quanti	17	23.00 ± 5.21	12	22.68 ± 4.65	11	21.52 ± 8.55	18	21.62 ± 10.07
	Cit	Quanti	17	26.94 ± 9.61	12	20.16 ± 9.51	11	22.40 ± 8.79	18	20.83 ± 7.92
	Gln	Quanti	17	546.59 ± 104.08	12	497.05 ± 113.50	11	499.28 ± 85.89	18	482.49 ± 140.31
	Glu	Quanti	17	41.62 ± 17.77	12	56.60 ± 20.73	11	57.78 ± 23.53	18	112.44 ± 77.59
	Gly	Quanti	17	272.86 ± 70.78	12	202.30 ± 47.16	11	223.31 ± 74.47	18	179.69 ± 30.24
	His	Quanti	17	78.78 ± 12.28	12	74.01 ± 14.66	11	80.43 ± 13.32	18	75.69 ± 7.91
	Ile	Quanti	17	39.38 ± 8.90	12	47.77 ± 35.43	11	45.07 ± 12.96	18	49.70 ± 13.47
	Leu	Quanti	17	96.28 ± 17.87	12	105.62 ± 45.40	11	104.66 ± 23.43	18	113.47 ± 27.10
	Lys	Quanti	17	196.56 ± 36.59	12	204.38 ± 48.23	11	220.20 ± 38.47	18	214.91 ± 41.80
	Met	Quanti	17	27.08 ± 6.99	12	26.29 ± 6.03	11	28.78 ± 6.28	18	22.38 ± 7.49
	Orn	Quanti	17	85.59 ± 26.56	12	105.78 ± 37.14	11	100.05 ± 17.62	18	91.61 ± 24.64
	Phe	Quanti	17	53.79 ± 10.14	12	51.47 ± 8.19	11	52.04 ± 8.59	18	59.35 ± 15.04
	Pro	Quanti	17	120.56 ± 43.36	12	107.53 ± 32.67	11	117.03 ± 33.99	18	138.10 ± 38.06
	Thr	Quanti	17	131.20 ± 24.57	12	108.98 ± 37.88	11	103.26 ± 29.83	18	120.09 ± 30.39
	Trp	Quanti	17	47.25 ± 13.12	12	42.49 ± 8.25	11	58.20 ± 15.53	18	46.89 ± 8.57
	Tyr	Quanti	17	96.41 ± 34.52	12	109.94 ± 31.70	11	129.94 ± 30.57	18	114.35 ± 36.69
	Val	Quanti	17	122.92 ± 34.62	12	128.98 ± 66.78	11	128.12 ± 32.24	18	152.97 ± 37.90
Amino Acids, ratio	Asn_to_Asp_Ratio	Ratio	17	1.96 ± 0.74	12	1.54 ± 0.60	11	1.60 ± 1.11	16	1.59 ± 0.65
	Gln_to_Glu_Ratio	Ratio	17	15.37 ± 7.40	12	9.97 ± 4.40	11	9.67 ± 3.17	18	6.33 ± 3.64
	Glu_to_C0_Ratio	Ratio	19	1.25 ± 0.78	12	1.77 ± 0.73	12	1.38 ± 0.80	21	2.63 ± 2.18
	Glu_to_Lys_Ratio	Ratio	17	0.21 ± 0.09	12	0.30 ± 0.14	11	0.26 ± 0.10	18	0.52 ± 0.34
	Glu_to_Orn_Ratio	Ratio	17	0.51 ± 0.20	12	0.70 ± 0.65	11	0.57 ± 0.17	18	1.24 ± 0.70
	Glu_to_Ser_Ratio	Quanti	17	0.29 ± 0.12	12	0.43 ± 0.23	11	0.43 ± 0.15	18	0.89 ± 0.53
	Tyr_to_tOHPro_Rati	Ratio	17	14.12 ± 6.95	12	17.20 ± 6.75	11	18.67 ± 8.70	18	18.42 ± 12.56
	Glu_to_tOHPro_Ratio	Ratio	17	6.14 ± 3.54	12	9.21 ± 4.78	11	8.11 ± 4.60	18	16.70 ± 11.98
Amino Acids, sum	tot_AA	Quanti	19	2293.26 ± 869.34	12	2401.02 ± 454.93	12	2316.73 ± 830.2	21	2237.18 ± 976.28
	totAAA	Quanti	19	247.16 ± 102.41	12	277.91 ± 49.47	12	293.89 ± 107.1(	21	253.95 ± 113.34
	totBCAA	Quanti	19	231.36 ± 98.92	12	282.36 ± 145.64	12	254.70 ± 102.62	21	270.98 ± 133.38
Biogenic Amines	Taurine	Quanti	17	57.09 ± 21.86	12	71.76 ± 38.37	11	45.08 ± 12.54	18	70.48 ± 31.67
	ADMA	Quanti	17	0.30 ± 0.13	12	0.33 ± 0.14	11	0.26 ± 0.17	18	0.36 ± 0.16
	Creatinine	Quanti	17	69.24 ± 19.30	12	65.27 ± 23.33	11	64.82 ± 18.77	18	67.54 ± 21.67
	Kynurenine	Quanti	17	2.11 ± 0.52	12	2.45 ± 0.59	11	2.40 ± 0.42	18	2.49 ± 0.71
	t4-OH-Pro	Quanti	17	7.94 ± 3.12	12	7.06 ± 2.59	11	8.16 ± 3.11	18	8.00 ± 3.78
Lysophosphatidylcholines	lysoPC a C16:0	Semi-quanti	19	67.88 ± 12.19	12	61.32 ± 17.53	12	85.10 ± 18.34	21	65.39 ± 15.11
	lysoPC a C16:1	Semi-quanti	19	1.67 ± 0.58	12	1.61 ± 0.48	12	2.25 ± 0.82	21	1.80 ± 0.52
	lysoPC a C17:0	Semi-quanti	19	1.16 ± 0.33	12	0.80 ± 0.24	12	1.27 ± 0.25	21	0.83 ± 0.35
	lysoPC a C18:0	Semi-quanti	19	18.52 ± 3.52	12	16.58 ± 5.20	12	25.54 ± 5.82	21	18.03 ± 6.02
	lysoPC a C18:1	Semi-quanti	19	15.72 ± 4.01	12	11.36 ± 3.46	12	17.97 ± 4.85	21	10.19 ± 2.54
	lysoPC a C18:2	Semi-quanti	19	22.77 ± 8.66	12	14.01 ± 4.95	12	23.52 ± 5.03	21	13.16 ± 3.39
	lysoPC a C20:4	Semi-quanti	19	3.63 ± 0.90	12	3.13 ± 0.93	12	4.29 ± 1.27	21	3.45 ± 1.09
	lysoPC a C24:0	Semi-quanti	19	0.26 ± 0.10	12	0.21 ± 0.10	12	0.25 ± 0.15	21	0.29 ± 0.16
	lysoPC a C28:1	Semi-quanti	19	0.20 ± 0.09	12	0.21 ± 0.10	12	0.25 ± 0.12	21	0.26 ± 0.19

(continued on the following page)

Phosphatidylcholines	PC aa C28:1	Semi-quanti	19	1.73 ± 0.40	12	1.97 ± 0.45	12	2.02 ± 0.35	21	1.95 ± 0.50
	PC aa C30:0	Semi-quanti	19	2.43 ± 0.66	12	2.76 ± 1.00	12	3.04 ± 1.17	21	2.94 ± 0.95
	PC aa C32:0	Semi-quanti	19	9.91 ± 2.05	12	9.69 ± 2.08	12	10.45 ± 1.55	21	10.57 ± 2.07
	PC aa C32:1	Semi-quanti	19	7.24 ± 3.40	12	7.85 ± 3.43	12	10.25 ± 6.98	21	10.86 ± 4.90
	PC aa C34:1	Semi-quanti	19	174.91 ± 53.66	12	158.29 ± 48.94	12	201.04 ± 40.35	21	164.29 ± 47.36
	PC aa C34:2	Semi-quanti	19	379.55 ± 102.85	12	344.39 ± 75.23	12	448.17 ± 76.95	21	356.40 ± 83.69
	PC aa C34:3	Semi-quanti	19	7.53 ± 2.58	12	6.73 ± 1.98	12	9.93 ± 2.94	21	8.82 ± 2.92
	PC aa C34:4	Semi-quanti	19	0.95 ± 0.39	12	0.98 ± 0.51	12	1.25 ± 0.58	21	1.11 ± 0.57
	PC aa C36:0	Semi-quanti	19	2.71 ± 0.86	12	2.10 ± 0.68	12	2.89 ± 0.71	21	2.49 ± 1.23
	PC aa C36:1	Semi-quanti	19	43.12 ± 14.10	12	36.08 ± 10.30	12	47.67 ± 8.70	21	40.79 ± 14.41
	PC aa C36:2	Semi-quanti	19	248.60 ± 70.84	12	208.06 ± 50.22	12	279.78 ± 44.27	21	219.35 ± 60.60
	PC aa C36:3	Semi-quanti	19	136.77 ± 48.19	12	122.17 ± 32.25	12	160.44 ± 32.75	21	132.17 ± 41.18
	PC aa C36:4	Semi-quanti	19	150.20 ± 40.43	12	169.80 ± 47.83	12	190.71 ± 42.16	21	178.73 ± 53.32
	PC aa C36:5	Semi-quanti	19	11.07 ± 7.35	12	10.12 ± 4.48	12	16.17 ± 9.37	21	11.80 ± 4.47
	PC aa C36:6	Semi-quanti	19	0.59 ± 0.26	12	0.46 ± 0.24	12	0.73 ± 0.39	21	0.62 ± 0.31
	PC aa C38:0	Semi-quanti	19	3.96 ± 1.32	12	2.95 ± 0.89	12	4.12 ± 1.06	21	2.98 ± 0.98
	PC aa C38:3	Semi-quanti	19	44.73 ± 14.24	12	45.38 ± 13.24	12	58.57 ± 14.98	21	55.63 ± 17.87
	PC aa C38:4	Semi-quanti	19	96.19 ± 26.61	12	107.70 ± 29.37	12	121.02 ± 26.16	21	116.36 ± 37.37
	PC aa C38:5	Semi-quanti	19	38.50 ± 11.23	12	36.58 ± 10.79	12	45.79 ± 10.19	21	36.88 ± 11.45
	PC aa C38:6	Semi-quanti	19	83.39 ± 27.46	12	72.65 ± 26.20	12	96.91 ± 27.08	21	71.41 ± 23.34
	PC aa C40:1	Semi-quanti	19	0.19 ± 0.20	12	0.03 ± 0.11	12	0.09 ± 0.16	21	0.17 ± 0.29
	PC aa C40:2	Semi-quanti	19	0.32 ± 0.08	12	0.25 ± 0.06	12	0.30 ± 0.05	21	0.48 ± 0.61
	PC aa C40:3	Semi-quanti	19	0.56 ± 0.12	12	0.49 ± 0.08	12	0.63 ± 0.13	21	0.79 ± 0.84
	PC aa C40:4	Semi-quanti	19	2.92 ± 0.85	12	3.10 ± 1.03	12	3.38 ± 0.89	21	3.74 ± 1.44
	PC aa C40:5	Semi-quanti	19	6.62 ± 2.06	12	6.41 ± 2.00	12	7.77 ± 2.22	21	7.48 ± 2.84
	PC aa C40:6	Semi-quanti	19	27.13 ± 11.81	12	24.34 ± 8.46	12	32.29 ± 9.36	21	26.18 ± 9.27
	PC aa C42:0	Semi-quanti	19	0.63 ± 0.12	12	0.49 ± 0.12	12	0.56 ± 0.17	21	0.56 ± 0.19
	PC aa C42:1	Semi-quanti	19	0.37 ± 0.08	12	0.29 ± 0.08	12	0.32 ± 0.07	21	0.33 ± 0.13
	PC aa C42:2	Semi-quanti	19	0.33 ± 0.11	12	0.24 ± 0.08	12	0.30 ± 0.08	21	0.29 ± 0.19
	PC aa C42:4	Semi-quanti	19	0.19 ± 0.03	12	0.17 ± 0.02	12	0.18 ± 0.03	21	0.28 ± 0.27
	PC aa C42:5	Semi-quanti	19	0.24 ± 0.05	12	0.23 ± 0.05	12	0.29 ± 0.09	21	0.29 ± 0.16
	PC ae C32:1	Semi-quanti	19	2.10 ± 0.54	12	1.72 ± 0.47	12	1.77 ± 0.32	21	1.90 ± 0.44
	PC ae C34:0	Semi-quanti	19	1.00 ± 0.24	12	0.78 ± 0.22	12	0.93 ± 0.24	21	0.83 ± 0.25
	PC ae C34:1	Semi-quanti	19	8.07 ± 2.10	12	6.56 ± 1.77	12	7.39 ± 0.98	21	6.39 ± 1.44
	PC ae C34:2	Semi-quanti	19	10.35 ± 2.29	12	7.60 ± 2.38	12	9.81 ± 2.03	21	7.03 ± 2.10
	PC ae C34:3	Semi-quanti	19	7.09 ± 2.14	12	5.10 ± 1.51	12	6.34 ± 1.73	21	4.47 ± 1.50
	PC ae C36:0	Semi-quanti	19	0.88 ± 0.26	12	0.78 ± 0.15	12	0.89 ± 0.20	21	0.74 ± 0.21
	PC ae C36:1	Semi-quanti	19	6.83 ± 1.80	12	5.40 ± 1.67	12	6.96 ± 0.78	21	7.20 ± 4.75
	PC ae C36:2	Semi-quanti	19	12.35 ± 3.13	12	9.27 ± 2.43	12	12.36 ± 1.67	21	9.11 ± 2.79
	PC ae C36:3	Semi-quanti	19	8.64 ± 2.11	12	6.36 ± 1.92	12	8.33 ± 1.58	21	5.90 ± 2.00
	PC ae C36:4	Semi-quanti	19	16.77 ± 4.61	12	15.08 ± 3.68	12	18.99 ± 6.32	21	15.28 ± 6.00
	PC ae C36:5	Semi-quanti	19	10.72 ± 3.09	12	9.49 ± 1.92	12	12.02 ± 2.28	21	9.67 ± 3.63
	PC ae C38:0	Semi-quanti	19	2.01 ± 0.70	12	1.56 ± 0.40	12	2.50 ± 0.75	21	2.06 ± 0.77
	PC ae C38:3	Semi-quanti	19	3.86 ± 0.77	12	3.76 ± 1.07	12	4.80 ± 1.01	21	5.23 ± 4.48
	PC ae C38:4	Semi-quanti	19	11.41 ± 2.11	12	10.54 ± 1.87	12	11.79 ± 1.63	21	11.06 ± 3.40
	PC ae C38:5	Semi-quanti	19	19.77 ± 4.48	12	17.07 ± 3.47	12	21.04 ± 4.92	21	16.27 ± 5.37
	PC ae C38:6	Semi-quanti	19	7.97 ± 2.37	12	6.42 ± 1.49	12	8.77 ± 2.03	21	6.30 ± 2.28
	PC ae C40:1	Semi-quanti	19	1.03 ± 0.23	12	0.78 ± 0.23	12	1.19 ± 0.29	21	0.98 ± 0.42
	PC ae C40:2	Semi-quanti	19	1.52 ± 0.43	12	1.50 ± 0.33	12	1.84 ± 0.33	21	1.99 ± 1.27
	PC ae C40:3	Semi-quanti	19	1.38 ± 0.25	12	1.46 ± 0.33	12	1.82 ± 0.49	21	2.61 ± 3.16
	PC ae C40:4	Semi-quanti	19	2.30 ± 0.36	12	2.09 ± 0.34	12	2.36 ± 0.33	21	2.73 ± 1.78
	PC ae C40:5	Semi-quanti	19	3.37 ± 0.79	12	2.72 ± 0.61	12	3.01 ± 0.48	21	3.36 ± 1.51
	PC ae C40:6	Semi-quanti	19	4.74 ± 1.46	12	3.46 ± 0.89	12	4.27 ± 0.74	21	3.50 ± 0.91
	PC ae C42:1	Semi-quanti	19	0.38 ± 0.10	12	0.33 ± 0.08	12	0.38 ± 0.08	21	0.51 ± 0.48
	PC ae C42:2	Semi-quanti	19	0.61 ± 0.16	12	0.49 ± 0.15	12	0.63 ± 0.10	21	0.62 ± 0.37
	PC ae C42:3	Semi-quanti	19	0.88 ± 0.22	12	0.69 ± 0.20	12	0.87 ± 0.19	21	0.80 ± 0.38
	PC ae C42:4	Semi-quanti	19	0.84 ± 0.20	12	0.69 ± 0.23	12	0.75 ± 0.18	21	0.81 ± 0.31
	PC ae C42:5	Semi-quanti	19	1.94 ± 0.31	12	1.68 ± 0.28	12	1.83 ± 0.47	21	1.93 ± 0.55
	PC ae C44:3	Semi-quanti	19	0.13 ± 0.05	12	0.10 ± 0.03	12	0.12 ± 0.04	21	0.16 ± 0.13
	PC ae C44:4	Semi-quanti	19	0.40 ± 0.13	12	0.30 ± 0.06	12	0.33 ± 0.07	21	0.36 ± 0.13
	PC ae C44:5	Semi-quanti	19	1.67 ± 0.44	12	1.45 ± 0.34	12	1.59 ± 0.64	21	1.52 ± 0.53
	PC ae C44:6	Semi-quanti	19	1.17 ± 0.30	12	1.00 ± 0.30	12	1.03 ± 0.36	21	1.03 ± 0.35
Lysophosphatidylethanolamines	LysoPE a 16:0	Quanti	18	201.18 ± 66.64	12	157.53 ± 56.96	12	195.01 ± 74.85	20	151.91 ± 42.32
	LysoPE a 18:0	Quanti	18	288.05 ± 71.73	12	255.41 ± 107.43	12	330.61 ± 110.56	20	247.44 ± 79.79
	LysoPE a 18:1	Quanti	18	337.51 ± 128.53	12	265.56 ± 107.40	12	423.20 ± 208.05	20	219.33 ± 55.46
	LysoPE a 18:2	Quanti	18	443.08 ± 178.20	12	304.74 ± 105.08	12	511.49 ± 221.46	20	300.64 ± 109.29
	LysoPE a 20_4	Quanti	18	140.13 ± 41.38	12	135.74 ± 56.07	12	179.07 ± 64.39	20	132.49 ± 38.22
	LysoPE a 22:6	Quanti	18	172.32 ± 59.85	12	142.89 ± 55.78	12	206.48 ± 93.53	20	150.25 ± 39.10
	LysoPE a 22:4	Quanti	18	9.02 ± 3.29	12	10.21 ± 3.48	12	12.14 ± 5.33	20	12.55 ± 6.17
	LysoPE a 22:5	Quanti	18	22.39 ± 8.95	12	23.69 ± 12.74	12	29.96 ± 16.27	20	27.20 ± 11.79
	LysoPE e 18:0	Quanti	18	9.17 ± 3.68	12	6.40 ± 3.11	12	8.71 ± 3.27	20	5.82 ± 1.79

Phosphatidylethanolamines †	PE aa 20:0	Quanti	18	5.19 ± 1.45	12	4.97 ± 1.81	12	6.33 ± 1.77	20	5.69 ± 3.06
	PE aa 22:2	Quanti	18	9.74 ± 3.86	12	8.70 ± 3.43	12	10.38 ± 4.36	20	8.08 ± 2.74
	PE aa 26:4	Quanti	18	52.15 ± 22.64	12	50.12 ± 38.94	12	48.46 ± 13.32	20	46.27 ± 31.84
	PE aa 28:4	Quanti	18	12.65 ± 5.20	12	12.12 ± 8.43	12	12.32 ± 4.63	20	11.92 ± 6.98
	PE aa 28:5	Quanti	18	11.90 ± 5.11	12	9.01 ± 7.87	12	8.83 ± 3.31	20	7.28 ± 4.65
	PE aa 34:0	Quanti	18	27.02 ± 7.13	12	30.98 ± 13.02	12	30.82 ± 16.22	20	24.78 ± 10.33
	PE aa 34:1	Quanti	18	600.94 ± 502.59	12	869.58 ± 510.67	12	338.81 ± 519.63	20	794.44 ± 404.19
	PE aa 34:2	Quanti	18	1383.65 ± 859.61	12	1669.30 ± 753.52	12	742.67 ± 1043.(	20	1643.90 ± 811.07
	PE aa 34:3	Quanti	18	56.90 ± 28.59	12	66.19 ± 36.54	12	84.66 ± 52.64	20	70.32 ± 31.29
	PE aa 36:0	Quanti	18	329.95 ± 158.80	12	261.52 ± 77.35	12	368.27 ± 114.4	20	232.36 ± 78.19
	PE aa 36:1	Quanti	18	621.36 ± 435.11	12	770.99 ± 392.31	12	972.19 ± 554.4	20	728.69 ± 331.30
	PE aa 36:2	Quanti	18	3138.77 ± 1892.79	12	3523.08 ± 1697.99	12	470.51 ± 2378.(	20	1533.89 ± 1791.6
	PE aa 36:3	Quanti	18	1241.52 ± 971.27	12	1169.42 ± 525.56	12	504.34 ± 1004.5	20	1028.03 ± 570.15
	PE aa 36:4	Quanti	18	1855.01 ± 980.25	12	2454.56 ± 1338.05	12	264.74 ± 1250.3	20	2227.01 ± 1047.1(
	PE aa 36:5	Quanti	18	147.76 ± 71.59	12	154.93 ± 71.61	12	195.86 ± 101.4(	20	144.35 ± 60.43
	PE aa 38:0	Quanti	18	546.13 ± 269.07	12	405.42 ± 124.93	12	520.00 ± 215.0	20	336.56 ± 135.35
	PE aa 38:1	Quanti	18	252.75 ± 83.19	12	205.02 ± 48.42	12	289.73 ± 82.17	20	187.67 ± 59.46
	PE aa 38:2	Quanti	18	167.43 ± 46.27	12	162.47 ± 28.02	12	184.02 ± 49.61	20	151.57 ± 33.17
	PE aa 38:3	Quanti	18	550.72 ± 407.20	12	632.29 ± 412.31	12	729.05 ± 354.4	12	729.05 ± 354.49
	PE aa 38:4	Quanti	18	4487.33 ± 2268.89	12	6333.49 ± 3953.23	12	932.23 ± 2905.(	20	4786.00 ± 2676.6
	PE aa 38:5	Quanti	18	1246.87 ± 830.12	12	1396.61 ± 735.98	12	420.18 ± 732.8	20	1223.11 ± 637.62
	PE aa 38:6	Quanti	18	3173.39 ± 1591.33	12	3459.26 ± 1727.91	12	539.26 ± 2173.(	20	3520.58 ± 2154.4
	PE aa 38:7	Quanti	18	86.83 ± 32.02	12	82.00 ± 29.41	12	92.16 ± 39.28	20	87.52 ± 32.49
	PE aa 40:2	Quanti	18	21.74 ± 10.09	12	15.72 ± 4.49	12	20.17 ± 5.58	20	15.19 ± 3.53
	PE aa 40:3	Quanti	18	30.72 ± 14.56	12	23.73 ± 7.31	12	29.67 ± 9.45	20	19.75 ± 5.58
	PE aa 40:4	Quanti	18	111.06 ± 75.98	12	151.98 ± 115.84	12	156.66 ± 103.4	20	175.25 ± 108.34
	PE aa 40:5	Quanti	18	350.44 ± 294.23	12	455.23 ± 354.45	12	473.51 ± 310.31	20	511.49 ± 316.24
	PE aa 40:6	Quanti	18	1928.46 ± 1047.90	12	2356.19 ± 1433.25	12	759.03 ± 1750.1	20	2681.79 ± 1490.9
	PE aa 40:7	Quanti	18	455.87 ± 219.26	12	421.43 ± 180.78	12	502.27 ± 262.7(	20	419.36 ± 253.79
	PE aa 48:1	Quanti	18	6.38 ± 2.60	12	8.53 ± 4.04	12	6.53 ± 2.21	20	7.99 ± 5.95
	PE ae 34:1	Quanti	18	126.47 ± 53.58	12	115.97 ± 51.90	12	170.08 ± 66.98	20	96.90 ± 23.06
	PE ae 34:2	Quanti	18	113.92 ± 46.85	12	95.58 ± 36.47	12	154.24 ± 64.21	20	78.59 ± 26.42
	PE ae 34:3	Quanti	18	104.75 ± 41.50	12	79.98 ± 42.04	12	118.15 ± 37.73	20	69.85 ± 30.22
	PE ae 36:1	Quanti	18	81.84 ± 37.47	12	82.47 ± 32.54	12	104.47 ± 42.50	20	70.25 ± 22.32
	PE ae 36:2	Quanti	18	219.01 ± 88.96	12	198.69 ± 79.07	12	274.53 ± 93.71	20	166.83 ± 54.78
	PE ae 36:3	Quanti	18	346.62 ± 138.18	12	274.69 ± 115.13	12	429.47 ± 160.4	20	228.20 ± 86.26
	PE ae 36:4	Quanti	18	555.87 ± 311.26	12	538.90 ± 300.46	12	933.50 ± 588.5	20	431.34 ± 171.70
	PE ae 36:5	Quanti	18	357.47 ± 180.63	12	332.59 ± 163.60	12	474.38 ± 173.31	20	288.38 ± 135.55
	PE ae 38:1	Quanti	18	48.52 ± 14.83	12	43.84 ± 9.96	12	51.91 ± 10.84	20	42.48 ± 9.01
	PE ae 38:2	Quanti	18	51.07 ± 15.75	12	43.69 ± 11.75	12	53.50 ± 13.86	20	40.81 ± 9.23
	PE ae 38:3	Quanti	18	65.84 ± 25.81	12	53.83 ± 15.53	12	72.72 ± 22.32	20	48.70 ± 15.39
	PE ae 38:4	Quanti	18	481.07 ± 206.45	12	497.83 ± 224.19	12	363.48 ± 332.8	20	432.78 ± 186.54
	PE ae 38:5	Quanti	18	1016.21 ± 416.27	12	1001.45 ± 389.80	12	573.49 ± 731.3	20	842.40 ± 320.37
	PE ae 38:6	Quanti	18	873.95 ± 354.25	12	761.72 ± 248.20	12	186.31 ± 447.2	20	649.16 ± 211.28
	PE ae 40:1	Quanti	18	47.54 ± 12.91	12	45.70 ± 11.44	12	51.08 ± 20.41	20	44.74 ± 17.29
	PE ae 40:2	Quanti	18	69.16 ± 27.32	12	67.51 ± 21.42	12	67.00 ± 22.29	20	65.23 ± 30.61
	PE ae 40:3	Quanti	18	37.03 ± 10.79	12	29.06 ± 8.03	12	34.52 ± 8.40	20	27.54 ± 7.58
	PE ae 40:4	Quanti	18	74.68 ± 19.55	12	75.06 ± 22.79	12	82.91 ± 24.66	20	66.83 ± 21.73
	PE ae 40:5	Quanti	18	200.20 ± 83.81	12	187.99 ± 57.58	12	244.56 ± 81.13	20	160.49 ± 48.36
	PE ae 40:6	Quanti	18	533.53 ± 231.67	12	457.86 ± 138.96	12	531.93 ± 204.4(	20	388.93 ± 116.89
	PE ae 42:1	Quanti	18	12.65 ± 6.00	12	10.49 ± 3.05	12	12.84 ± 4.34	20	11.78 ± 5.07
	PE ae 42:2	Quanti	18	35.57 ± 13.11	12	37.15 ± 12.23	12	44.04 ± 19.58	20	39.65 ± 17.27
	PE ae 46:5	Quanti	18	20.30 ± 12.50	12	22.59 ± 12.70	12	17.56 ± 4.97	20	20.15 ± 16.87
	PE ae 46:6	Quanti	18	57.73 ± 21.89	12	60.18 ± 38.59	12	54.75 ± 11.44	20	55.51 ± 43.16
Phosphatidylserires †	PS aa 36:1	Quanti	18	27.65 ± 13.76	12	44.38 ± 32.34	12	38.99 ± 22.59	20	39.56 ± 26.55
	PS aa 36:2	Quanti	18	8.03 ± 4.30	12	9.42 ± 5.33	12	11.67 ± 5.72	20	9.18 ± 5.65
	PS aa 38:3	Quanti	18	8.74 ± 4.53	12	8.49 ± 6.16	12	7.96 ± 4.64	20	9.15 ± 6.01
	PS aa 38:4	Quanti	18	31.09 ± 12.47	12	55.59 ± 39.85	12	31.64 ± 21.90	20	46.06 ± 27.46
	PS aa 38:5	Quanti	18	2.26 ± 2.53	12	2.22 ± 2.45	12	2.10 ± 1.73	20	2.64 ± 1.98
Phosphatidylglycerols †	PG aa 34:1	Quanti	18	61.36 ± 42.63	12	57.73 ± 30.47	12	54.90 ± 40.11	20	68.52 ± 30.14
	PG aa 36:1	Quanti	18	58.47 ± 28.74	12	76.80 ± 38.75	12	69.32 ± 20.11	20	77.79 ± 38.71
Phosphatidylserires †	PS aa 40:5	Quanti	18	4.56 ± 3.69	12	5.13 ± 3.19	12	4.47 ± 3.24	20	4.32 ± 3.17
	PS aa 40:6	Quanti	18	12.14 ± 5.78	12	13.46 ± 7.08	12	12.81 ± 7.55	20	12.41 ± 7.63
	PS aa 40:7	Quanti	18	2.82 ± 1.82	12	3.35 ± 1.24	12	3.35 ± 2.33	20	2.69 ± 2.06
	PS ae 36:1	Quanti	18	2.72 ± 2.28	12	3.56 ± 3.10	12	2.45 ± 1.64	20	2.73 ± 2.11
	PS ae 36:2	Quanti	18	4.86 ± 3.86	12	3.23 ± 2.82	12	4.15 ± 1.92	20	3.92 ± 2.96

(continued on the following page)

Ceramides †	N__2xOHC15_0_Cer	Quanti	18	8.38 ± 3.75	12	8.61 ± 3.48	12	10.73 ± 5.26	20	8.51 ± 3.51
	N__OHC11_0_Cer	Quanti	18	4.27 ± 1.33	12	3.41 ± 1.38	12	4.84 ± 1.66	20	4.44 ± 1.31
	N_C10_0_Cer	Quanti	18	26.94 ± 9.66	12	24.18 ± 11.56	12	28.66 ± 12.39	20	23.73 ± 6.10
	N_C10_1_Cer2H	Quanti	18	0.32 ± 0.27	12	0.24 ± 0.19	12	0.49 ± 0.28	20	0.31 ± 0.29
	N_C11_0_(OH) Cer	Quanti	18	3.31 ± 1.77	12	2.70 ± 1.63	12	3.90 ± 2.72	20	3.73 ± 1.33
	N_C11_1_(OH) Cer	Quanti	18	171.40 ± 36.65	12	151.82 ± 56.87	12	188.01 ± 47.20	20	156.25 ± 31.63
	N_C11_1_Cer	Quanti	18	0.34 ± 0.24	12	0.62 ± 0.49	12	0.30 ± 0.13	20	0.54 ± 0.47
	N_C12_0_(OH) Cer	Quanti	18	12.11 ± 8.06	12	11.72 ± 6.51	12	13.58 ± 8.16	20	9.51 ± 6.98
	N_C12_0_Cer	Quanti	18	511.09 ± 91.30	12	474.18 ± 185.32	12	582.11 ± 135.85	20	480.13 ± 111.50
	N_C13_0_Cer	Quanti	18	0.55 ± 0.48	12	0.72 ± 0.48	12	0.57 ± 0.31	20	0.74 ± 0.33
	N_C14_0_Cer	Quanti	18	5.55 ± 1.54	12	5.14 ± 3.09	12	5.19 ± 1.86	20	5.41 ± 1.77
	N_C15_0_Cer	Quanti	18	0.50 ± 0.49	12	0.56 ± 0.33	12	0.47 ± 0.34	20	0.50 ± 0.41
	N_C16_0_(OH) Cer2	Quanti	18	1.89 ± 1.13	12	2.24 ± 1.47	12	2.64 ± 1.13	20	2.01 ± 0.94
	N_C16_0_Cer	Quanti	18	189.13 ± 43.34	12	178.46 ± 29.26	12	208.87 ± 50.77	20	173.42 ± 40.92
	N_C16_0_Cer2H	Quanti	18	24.10 ± 8.76	12	27.78 ± 11.32	12	28.12 ± 7.73	20	26.57 ± 5.56
	N_C17_0_(OH) Cer	Quanti	18	5.84 ± 2.45	12	8.24 ± 5.79	12	7.18 ± 3.40	20	11.34 ± 6.69
	N_C17_0_Cer	Quanti	18	3.45 ± 1.59	12	3.15 ± 1.44	12	3.54 ± 1.76	20	3.38 ± 1.68
	N_C17_1_(OH) Cer	Quanti	18	39.06 ± 15.27	12	41.74 ± 19.24	12	51.28 ± 15.90	20	48.12 ± 16.10
	N_C18_0_Cer	Quanti	18	62.36 ± 24.54	12	74.98 ± 30.35	12	94.19 ± 35.43	20	88.77 ± 27.86
	N_C18_0_Cer2H	Quanti	18	14.60 ± 6.49	12	26.86 ± 16.02	12	20.82 ± 7.07	20	34.69 ± 17.71
	N_C18_1_Cer	Quanti	18	7.33 ± 2.75	12	6.16 ± 3.56	12	7.02 ± 1.62	20	4.74 ± 1.38
	N_C19_0_Cer	Quanti	18	1.69 ± 1.42	12	1.62 ± 0.62	12	1.87 ± 1.21	20	1.65 ± 0.65
	N_C20_0_(OH) Cer	Quanti	18	10.09 ± 6.99	12	18.87 ± 12.94	12	10.19 ± 5.21	20	17.94 ± 7.87
	N_C20_0_Cer	Quanti	18	87.04 ± 21.79	12	82.81 ± 16.51	12	114.66 ± 38.80	20	91.45 ± 23.41
	N_C20_0_Cer2H	Quanti	18	13.53 ± 6.68	12	18.87 ± 7.50	12	16.48 ± 4.96	20	22.64 ± 7.53
	N_C20_1_Cer2H	Quanti	18	0.74 ± 0.40	12	0.88 ± 0.57	12	0.88 ± 0.84	20	0.88 ± 0.67
	N_C21_0_Cer	Quanti	18	13.43 ± 5.18	12	13.20 ± 3.31	12	17.83 ± 5.89	20	14.64 ± 5.21
	N_C22_0_(OH) Cer	Quanti	18	12.80 ± 9.50	12	17.49 ± 8.66	12	17.18 ± 6.97	20	20.21 ± 12.36
	N_C22_0_Cer	Quanti	18	601.37 ± 149.38	12	546.63 ± 143.11	12	733.13 ± 169.85	20	637.62 ± 184.24
	N_C22_0_Cer2H	Quanti	18	68.75 ± 34.65	12	91.21 ± 30.16	12	89.99 ± 25.39	20	119.95 ± 36.37
	N_C22_1_Cer	Quanti	18	11.43 ± 5.10	12	11.72 ± 3.87	12	15.52 ± 6.75	20	12.10 ± 4.05
	N_C22_1_Cer2H	Quanti	18	2.65 ± 0.81	12	3.80 ± 1.71	12	2.93 ± 0.77	20	4.01 ± 1.64
	N_C23_0_(OH) Cer	Quanti	18	14.66 ± 8.78	12	22.18 ± 9.09	12	21.73 ± 11.42	20	20.63 ± 10.25
	N_C23_0_Cer	Quanti	18	484.93 ± 134.42	12	472.52 ± 121.56	12	525.84 ± 142.45	20	487.03 ± 130.56
	N_C23_0_Cer2H	Quanti	18	43.33 ± 20.76	12	62.95 ± 21.30	12	60.38 ± 19.33	20	67.41 ± 21.05
	N_C23_1_Cer	Quanti	18	8.17 ± 2.61	12	7.38 ± 3.25	12	10.00 ± 5.42	20	8.50 ± 3.34
	N_C23_1_Cer2H	Quanti	18	2.34 ± 1.08	12	2.73 ± 1.11	12	2.86 ± 1.29	20	2.94 ± 0.88
	N_C24_0_(OH) Cer	Quanti	18	48.53 ± 36.92	12	67.76 ± 26.34	12	66.03 ± 24.75	20	74.43 ± 37.09
	N_C24_0_Cer	Quanti	18	2021.68 ± 579.05	12	1755.90 ± 480.27	12	2213.55 ± 433.5	20	1873.29 ± 552.82
	N_C24_0_Cer2H	Quanti	18	95.08 ± 50.35	12	130.02 ± 49.12	12	119.51 ± 40.81	20	152.96 ± 55.50
	N_C24_1_Cer	Quanti	18	748.49 ± 294.24	12	713.13 ± 188.12	12	899.41 ± 266.62	20	726.29 ± 219.15
	N_C24_1_Cer2H	Quanti	18	47.95 ± 18.73	12	72.87 ± 27.17	12	62.41 ± 14.81	20	83.10 ± 35.28
	N_C25_0_(OH) Cer	Quanti	18	2.74 ± 1.76	12	4.29 ± 2.74	12	4.47 ± 2.55	20	3.25 ± 1.93
	N_C25_0_Cer	Quanti	18	118.96 ± 42.34	12	104.53 ± 33.21	12	129.36 ± 39.86	20	90.02 ± 28.75
	N_C25_0_Cer2H	Quanti	18	12.26 ± 4.80	12	12.78 ± 4.59	12	13.47 ± 5.45	20	12.23 ± 4.22
	N_C25_1_Cer	Quanti	18	23.49 ± 7.85	12	23.01 ± 7.18	12	27.34 ± 6.83	20	22.22 ± 6.55
	N_C25_1_Cer2H	Quanti	18	3.41 ± 1.39	12	3.38 ± 1.68	12	4.31 ± 1.96	20	3.93 ± 1.86
	N_C26_0_Cer	Quanti	18	21.98 ± 5.69	12	17.81 ± 5.85	12	20.18 ± 5.43	20	16.52 ± 5.16
	N_C26_1_Cer	Quanti	18	10.06 ± 6.70	12	8.20 ± 3.99	12	12.55 ± 3.73	20	9.28 ± 4.21
	N_C26_1_Cer2H	Quanti	18	0.45 ± 0.36	12	0.52 ± 0.36	12	0.45 ± 0.44	20	0.51 ± 0.42
	N_C27_0_Cer	Quanti	18	1.20 ± 0.81	12	1.20 ± 0.66	12	0.99 ± 0.99	20	1.02 ± 0.72
	N_C27_1_Cer	Quanti	18	1.43 ± 1.27	12	1.14 ± 0.91	12	1.31 ± 0.84	20	1.27 ± 0.88
	N_C28_0_Cer	Quanti	18	2.40 ± 1.22	12	2.08 ± 1.13	12	2.03 ± 0.84	20	2.34 ± 0.96
Sphingomielins	SM (OH) C14:1	Semi-quanti	19	4.20 ± 0.98	12	4.20 ± 1.13	12	4.91 ± 1.22	21	4.30 ± 1.42
	SM (OH) C16:1	Semi-quanti	19	2.91 ± 0.70	12	2.87 ± 0.59	12	3.54 ± 0.93	21	3.06 ± 0.89
	SM (OH) C22:1	Semi-quanti	19	11.18 ± 3.35	12	11.32 ± 2.95	12	14.60 ± 3.29	21	13.17 ± 5.47
	SM (OH) C22:2	Semi-quanti	19	10.68 ± 2.69	12	11.62 ± 3.39	12	14.35 ± 5.17	21	11.54 ± 5.96
	SM (OH) C24:1	Semi-quanti	19	2.16 ± 0.51	12	2.08 ± 0.57	12	3.04 ± 0.69	21	2.50 ± 1.19
	SM C16:0	Semi-quanti	19	107.81 ± 19.26	12	105.65 ± 20.94	12	118.30 ± 27.96	21	103.96 ± 20.84
	SM C16:1	Semi-quanti	19	14.21 ± 3.43	12	17.36 ± 3.84	12	17.60 ± 5.78	21	16.15 ± 3.46
	SM C18:0	Semi-quanti	19	23.54 ± 4.85	12	30.39 ± 9.63	12	35.38 ± 9.67	21	35.38 ± 12.14
	SM C18:1	Semi-quanti	19	12.73 ± 3.30	12	15.51 ± 3.82	12	17.34 ± 5.45	21	15.85 ± 4.00
	SM C24:0	Semi-quanti	19	23.21 ± 6.91	12	24.57 ± 7.47	12	29.74 ± 6.08	21	26.31 ± 7.07
	SM C24:1	Semi-quanti	19	58.83 ± 13.23	12	61.68 ± 15.18	12	74.04 ± 25.83	21	58.94 ± 15.54
	SM C26:1	Semi-quanti	19	0.34 ± 0.08	12	0.33 ± 0.08	12	0.42 ± 0.11	21	0.61 ± 0.81

*Raw data are presented (no imputed, pre-normalized); values are given as mean ± standard deviation. † nM concentration range.

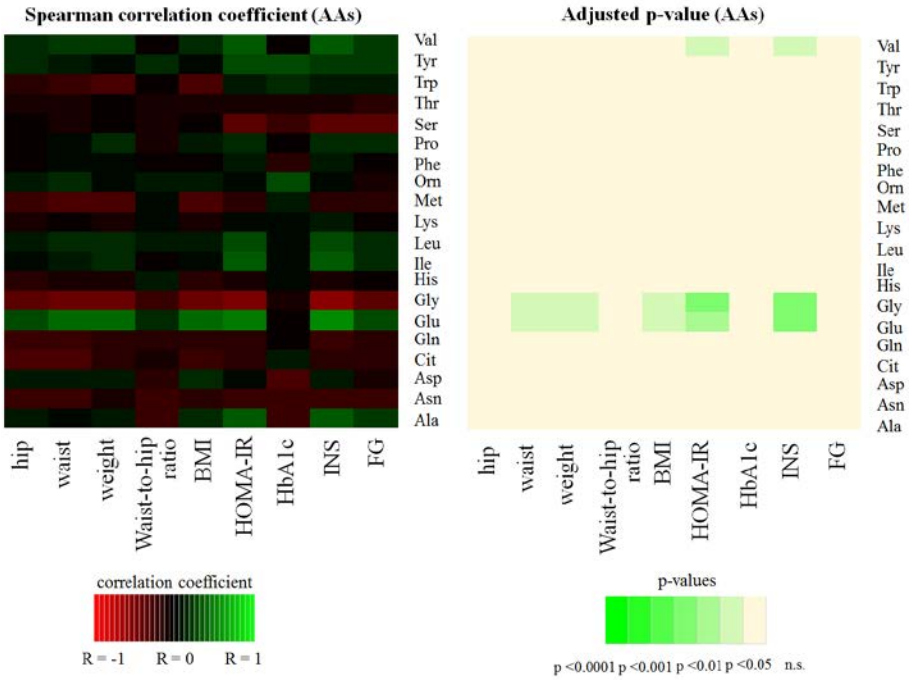
Supplementary Table 2. Diagnostic power of clinical versus metabolic measures in classifying the subjects according to their BMI and/or prediabetic state*.

Prediction of Obesity AND prediabetes (four phenotypic groups)

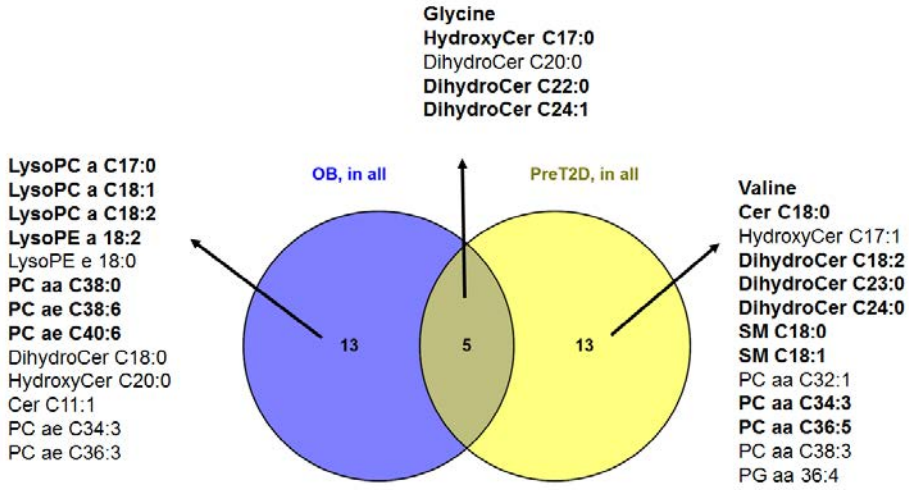
	Clinical			Metabolic		
	misclassification	brier score	P (mean)†	misclassification	brier score	P (mean)
DLDA	0.07	0.11	0.93	**0.55**	1.05	0.45
LDA	0.06	0.09	0.93	**0.53**	0.76	0.44
QDA	0.12	0.23	0.87	**0.56**	0.88	0.41
PLSDA	0.09	0.15	0.89	**0.55**	0.86	0.42
SCDA	0.07	0.12	0.90	**0.54**	0.95	0.44

* The diagnostic power of the selected metabolic markers is compared with that obtained using all the anthropometric and biochemical measures available (clinical predictors), listed in Table 1. † average probability.

Supplementary Figure 1. Correlation analysis between circulating amino acids and clinical markers of morbid obesity and prediabetes. Spearman correlation coefficients (r) and p values were generated from age-adjusted regression analysis of the association between each metabolite and clinical traits of adiposity and blood sugar control.



Supplementary Figure 2. Venn diagram showing the top metabolic classifiers exclusive of the obese or prediabetic phenotypes, and those disrupted in both conditions. Only metabolites selected by at least 50% of the variable selection techniques employed are listed; bold characters indicate 100% agreement among the selection techniques. All individuals were used for the classification purposes.



PUBLICACION 2

Untargeted Profiling of Concordant/Discordant Phenotypes of High Insulin Resistance and Obesity to Predict the Risk of Developing Diabetes

Anna Marco-Ramell, Sara Tulipani; Magali Palau-Rodriguez, Raul Gonzalez-Dominguez, Antonio Miñarro, Olga Jauregui, Alex Sanchez-Pla, Manuel Macias-Gonzalez, Fernando Cardona, Francisco J Tinahones, Cristina Andrés-Lacueva

Journal of Proteome Research

Factor de Impacto: 4.24 Q1 (14/78) BIOCHEMICAL RESEARCH METHODS (Indexado en Web of Science)

Los resultados de esta publicación fueron presentados en formato de:

- Comunicación póster: XIV Congreso Nacional Sociedad Española de Estudios de la Obesidad, 14-16 Marzo 2018, Lleida, España, "Estudio de nuevos biomarcadores mediante metabolómica no-dirigida LC-MS para la predicción del riesgo de diabetes en sujetos con obesidad y elevada resistencia a la insulina".

RESUMEN PUBLICACION 2

Objetivos: Los objetivos de este estudio fueron:

1) Explorar los perfiles metabólicos de elevada resistencia a la insulina y obesidad.

2) Identificar diferencias entre fenotipos concordantes/discordantes de elevada resistencia a la insulina y obesidad.

3) Definir modelos predictivos del riesgo de desarrollar diabetes.

Metodología: Se aplicó un flujo metabolómico no-dirigido utilizando LC-ESI-TripleTOF-MS en muestras de suero de sujetos con sensibilidad a la insulina y resistencia a la insulina con obesidad (n=12 y n=21 respectivamente) y sensibilidad a la insulina y resistencia a la insulina en sujetos con normo-peso (n=19 y n=12, respectivamente).

Resultados: En este estudio se modeló un nuevo marcador de resistencia a la insulina, compuesto por diacilgicéridos (DG), el ácido úrico y el ácido adrénico, con una capacidad discriminatoria del 84.0 % (74.3-93.7). Los metabotipos asociados a la obesidad y la resistencia a la insulina, se caracterizaron por la presencia de distintos marcadores inflamatorios, incluyendo derivados del ácido araquidónico, leucotrienos y ketocolesteterol. Además, también se encontraron niveles elevados de DG y hidroxicorticosterona en los sujetos con insulinorresistencia, mientras que el ácido araquidónico, palmitoleico y glicocólico, así como varios glutamil-peptidos, fueron identificados únicamente en sujetos con obesidad. Los análisis de rutas metabólicas desvelaron una mayor alteración del metabolismo de los glicerolípidos y fosfolípidos en los sujetos con insulinorresistencia, y del metabolismo de los ácidos araquidónico, linolénico y linoleico en sujetos con obesidad.

Conclusiones:

Los resultados de este estudio proponen un potencial biomarcador de resistencia a la insulina que podría ayudar a la detección de la DT2 en un estado más temprano de la enfermedad. Es necesaria la validación de estos resultados en otra cohorte y seguir estos pacientes hasta el desarrollo o no de la enfermedad.

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Untargeted Profiling of Concordant/Discordant Phenotypes of High Insulin Resistance and Obesity To Predict the Risk of Developing Diabetes

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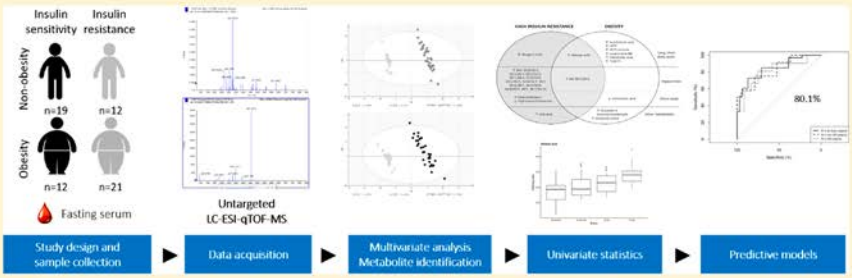
S *Supporting Information*



ABSTRACT: This study explores the metabolic profiles of concordant/discordant phenotypes of high insulin resistance (IR) and obesity. Through untargeted metabolomics (LC-ESI-QTOF-MS), we analyzed the fasting serum of subjects with high IR and/or obesity (n = 64). An partial least-squares discriminant analysis with orthogonal signal correction followed by univariate statistics and enrichment analysis allowed exploration of these metabolic profiles. A multivariate regression method (LASSO) was used for variable selection and a predictive biomarker model to identify subjects with high IR regardless of obesity was built. Adrenic acid and a dyglyceride (DG) were shared by high IR and obesity. Uric and margaric acids, 14 DGs, ketocholesterol, and hydroxycorticosterone were unique to high IR, while arachidonic, hydroxyeicosatetraenoic (HETE), palmitoleic, triHETE, and glycocholic acids, HETE lactone, leukotriene B4, and two glutamyl-peptides to obesity. DGs and adrenic acid differed in concordant/discordant phenotypes, thereby revealing protective mechanisms against high IR also in obesity. A biomarker model formed by DGs, uric and adrenic acids presented a high predictive power to identify subjects with high IR [AUC 80.1% (68.9−91.4)]. These findings could become relevant for diabetes risk detection and unveil new potential targets in therapeutic treatments of IR, diabetes, and obesity. An independent validated cohort is needed to confirm these results.

KEYWORDS: *adrenic acid, diglycerides, insulin resistance, metabolic profiles, metabolomics, obesity, observational study, predictive model, ROC curves, uric acid*

INTRODUCTION

Metabolic disorders such as insulin resistance (IR) and obesity are major health problems. IR plays an important pathophysiological role in the development of diabetes and metabolic syndrome. Obesity is also usually accompanied by other metabolic comorbidities such as IR, diabetes, and cardiovascular complications.[1,2] Nevertheless, not all the subjects with obesity

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develop IR or diabetes, and individuals with IR are not always overweight. Subjects with obesity can be insulin-sensitive (IS) and have normal blood pressure and lipid profiles, whereas normal weight individuals can present IR and β-cell impairment.[3,4] The inclusion of discordant phenotypes in research studies has shed light on new insights into the metabolic processes uniquely related to obesity or diabetes, and therefore dug more deeply into the interrelation between obesity and the development of diabetes.[5]

Metabolomics is the high-throughput technology that explore the global metabolic state (metabolome) of an individual by analyzing the low-molecular-weight compounds (metabolites) within a biological sample.[6] Over the past decade, metabolomics has been used to identify predictive and prognostic biomarkers and to monitor the efficacy of treatments.[7,8] Moreover, metabolomics has also been employed to uncover the molecular processes involved in pathophysiological states and to describe individual metabolic phenotypes (metabotypes), which can be exploited in personalized medicine and public healthcare.[9]

Untargeted metabolomics is a promising tool for elucidating novel mechanisms and finding disease biomarkers. It measures hundreds of metabolites and can detect previously unpredicted metabolic perturbations associated with a certain disease.[6] Few untargeted metabolomic studies have explored the metabolic profiles of diabetes and obesity, and very few of high IR regardless of obesity. The comprehensive analysis of the metabolome of subjects with high IR could be key in discovering a new gold standard to predict the progression of IR and the risk of developing diabetes.

The aims of this work are three-fold: (1) to explore the metabolic profiles of high IR and obesity; (2) to identify differences between concordant/discordant phenotypes of high IR and obesity; and (3) to define a predictive model for the risk of developing of diabetes. To these ends, we have carried out an untargeted metabolomic approach on fasting serum of human concordant/discordant phenotypes of high IR and obesity, followed by multivariate and univariate statistics, and an enrichment analysis. Finally we have built different predictive models of combined serum markers to identify subjects with high IR through a multivariate logistic regression and assessed their performance with ROC curves.

MATERIALS AND METHODS

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 (Malaga, Spain). A detailed description of the study design and inclusion/exclusion criteria has been previously reported.[5]

Individuals were classified according to (1) the risk of developing diabetes type 2, based on fasting plasma glucose (FG) and the Homeostatic Model Assessment-Insulin Resistance index (HOMA-IR), in low IR or IS if FG < 100 mg/dL and HOMA-IR < 2.5, or high IR if 100 ≤ FG < 126 mg/dL and HOMA-IR > 3.4; and (2) body mass index (BMI), in non-obesity if 18.5 < BMI ≤ 26.9 kg/m^2 or subjects with obesity if BMI > 40 kg/m^2. The FG cutoff was defined by the American Diabetes Association,[10] and the HOMA-IR cutoff was obtained experimentally.[5] Subsequently, four sex-matched phenotypic groups were obtained as follows: subjects with (1) IS and non-obesity (control group, n = 19); (2) IS and obesity (n = 12); (3) high IR and non-obesity (n = 12); and (4) high IR and obesity (n = 21).

The protocol was approved by the local Ethics and Research Committees (Hospital Universitario Virgen de la Victoria, Malaga) and all participants provided written informed consent.

Anthropometric and Biochemical Parameters

The following anthropometric and biochemical parameters were measured, as previously described:[5] (1) adiposity markers (body weight (kg), BMI (kg/m^2), waist and hip circumference (cm) and waist-hip ratio); (2) IR markers (FG (mmol/L), fasting insulin (μU/mL), HOMA-IR index); (3) blood pressure (diastolic and systolic blood pressure (mm Hg)); and (4) lipid markers (total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, and triglycerides (TG), mmol/L).

Reagents

Acetylcholine, acetyl-d3-L-carnitine hydrochloride, acetyl-L-carnitine, adrenic acid, L-carnitine, L-citrulline, dodecanoic acid, (−)-epicatechin, gallic acid, glycochenodeoxycholic acid, glycocholic acid, glycocholic acid-(glycyl-1−13C) monohydrate, α-hydroxyisobutyric acid, indole-3-acetic-2,2-d2 acid, L-isoleucine, 7-ketocholesterol, L-leucine, leukotriene B4, margaric acid, palmitic acid, L-phenylalanine, stearic acid, syringic acid, L-tryptophan, uric acid, and L-valine were purchased from Sigma-Aldrich (St. Louis, MO). 4-hydroxyhippuric acid was purchased from PhytoLab GmbH and Co KG (Vestenbergsgreuth, Germany), naringenin from Extrasynthèse (Genay, France), and arachidonic acid from Cymit Quimica (Barcelona, Spain).

UHPLC−MS-grade methanol, acetone, formic acid, and HPLC-grade acetonitrile were purchased from Scharlau Chemie S.A. (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

Quality Controls and Standards

An aqueous mix of metabolite standards (quality control, QC) and internal/external standards was prepared, as previously described,[11] to monitor instrumental stability. Water was used as QC1. A mix of standards (QC2) containing acetylcholine, acetyl-d3-L-carnitine hydrochloride, acetyl-L-carnitine, L-carnitine, L-citrulline, dodecanoic acid, (−)-epicatechin, gallic acid, glycochenodeoxycholic acid, glycocholic acid-(glycyl-1−13C) monohydrate, α-hydroxyisobutyric acid, indole-3-acetic-2,2-d2 acid, L-isoleucine, L-leucine, palmitic acid, L-phenylalanine, stearic acid, syringic acid, L-tryptophan and L-valine, spiked in Milli-Q water and plasma, was prepared (5 ppm final concentration). Finally, a 10% of the samples, randomly selected, were reanalyzed to assess differences between replicates (QC3).

Aqueous solutions of isotopically labeled and unlabeled compounds were also prepared and used during sample extraction. A mixture of glycocholic acid-(glycyl-1−13C) monohydrate and 1-O-stearoyl-*sn*-glycero-3-phosphocholine (25 ppm final concentration) was used as internal standard, and a mixture of indole-3-acetic-2,2-d2 acid and acetyl-d3-L-carnitine hydrochloride (25 ppm final concentration) as external standard.

Adrenic acid, arachidonic acid, glycocholic acid, 7-ketocholesterol, leukotriene B4, margaric acid, palmitoleic acid, and uric acid (50 ppb ppm final concentration) were spiked in Milli-Q

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water and plasma to confirm the identity of annotated metabolites.

Sample Treatment and Data Acquisition

Fasting serum samples (50 μL) were subjected to in-plate hybrid extraction, previously optimized by Tulipani et al. Samples were first deproteinized by acidic solvent precipitation (acetonitrile in 1% formic acid), followed by phospholipid solid phase extraction (SPE)-mediated removal.[12]

A TripleTOF 6600 hybrid quadrupole-TOF mass spectrometer (AB Sciex, Framingham, MA) with Turbo Spray IonDrive source coupled to a Shimadzu Nexera X2 series HPLC system (Kyoto, Japan) (Atlantis T3 RP column 50 × 2.1 mm^2, 5 μm (Waters, Milford, MA)) was used. A linear gradient elution was used ([A] Milli-Q water 0.1% HCOOH (v/v) and [B] methanol (v/v)), at a constant flow rate of 600 μL min^{-1} as follows (time, min; B, %): (0, 1), (4, 20), (6, 95), (7.5, 95), (8, 1), (12, 1). Data acquisition was performed by liquid chromatography−mass spectrometry (LC−MS) from 70 to 850 m/z with positive and negative electrospray ionization (ESI + and ESI−). The sample injections order was randomized to avoid bias. QC samples were analyzed throughout the run every 15 injections to provide measurements of the stability and performance of the system and evaluate the quality of the data.[12,13] Calibration was carried out with calibration solutions for AB Sciex TripleTOF systems (AB Sciex) in ESI+ and ESI− modes.

The mass spectrometry data have been deposited to the MetaboLights repository[14] (https://www.ebi.ac.uk/metabolights/) with the data set identifier MTBLS668.

Data Preprocessing

LC−MS data were preprocessed with MarkerView 1.3.0.1 (AB Sciex) (Tables S1 and S2). Raw data contained 3000 mass features, including redundant mass signals (isotopes, adducts, in-source fragments, etc.). The data sets were filtered out to remove variables that did not appear in more than 25% of any of the groups.[11] The final data sets presented 2607 (ESI+) and 2318 (ESI−) mass features. ESI+ and ESI− data sets were analyzed separately.

Multivariate Statistical Analysis

Partial least-squares discriminant analysis with orthogonal signal correction (OSC-PLS-DA) was used to examine between-group differences in LC−MS data (SIMCA-P+ 13.0 software, Umetrics, Umeå, Sweden). Data were log-transformed and Pareto scaled,[15,16] and an OSC filter was applied to remove the variability not associated with the diseases. Comparisons were performed by comparing the control group (IS and non-obesity, n = 19) with the high IR group (subjects with high IR (non-obesity + obesity), n = 33) or the obesity group (subjects with obesity (IS + high IR), n = 33). The robustness of the models was evaluated through the R^2X (cum), R^2Y (cum), and Q^2 (cum) parameters, cross-validation and permutation tests (n = 200) (Table S3). As a final quality test, the data set was randomly split into ten equal-size subsamples, nine of which were used as a training set while the remaining was used as a validation set. This process was repeated ten times (Table S4). Mass features explaining group separation were selected according to their variable importance for projection (VIP) values (cutoff ≥ 2).

Annotation of Metabolites

A cluster analysis, based on Pearson correlation and Ward's distance method,[17] was used to determine eventual clusters of mass features from the same metabolite (PermutMatrix 1.9.3). MetaNetter, a plugin for Cytoscape (v.2.8.0), was used to define adducts and fragments within the cluster.[18] The annotation of metabolites was carried out by comparing MS and MS/MS experimental data with in-house (MAIT[19]) and online databases including HMDB, METLIN, LipidMAPS, MassBank and MetFrag (±5 mDa mass error tolerance).

The fragmentation of [M+H]+ and [M+Na]+ ions enabled the characterization of fatty acids contained in the glycerolipid structure. The fatty acid composition of diglycerides (DG) was annotated based on characteristic daughter ions in the m/z range 200−400 Da, generated through the release of fatty acids from the glycerol backbone.[20]

Metabolite identity confirmation was carried out by matching peak chromatographic and MS responses (extracted ion chromatogram, product ion scan) to those of commercial reference standards, when available, spiked in Milli-Q water and plasma (50 ppb), on a QStar Elite system (AB Sciex). The analytical parameters were the same as described above.

Univariate Statistical Analysis

Univariate analysis was performed in R to describe differences in clinical and metabolic parameters. Clinical parameters were first log-transformed prior to the analysis. Statistics on metabolic parameters were performed on the raw matrix. Prior to the analyses, data were log-normalized and Pareto scaled. A type III ANOVA for unbalanced groups was performed to assess the effects of obesity and high IR on clinical variables. Fisher's exact test was used to evaluate differences in gender distribution across the groups.[21] A Student's t test was used to confirm that the metabolites with a VIP ≥ 2 differed between groups, and to identify differences between concordant and discordant phenotypes of each metabolic disorder. All p-values were corrected by false discovery rate (FDR) to reduce the probability of false positives.[22] Gender, age and drug consumption were considered as confounders in all the analyses. Only those metabolites with adjusted p-value ≤ 0.05 were considered significant.

Enrichment Analysis

ChemRICH (http://chemrich.fiehnlab.ucdavis.edu/) was used to perform an enrichment analysis of the metabolites that presented VIP ≥ 2 and adjusted p-value ≤ 0.05. ChemRICH utilizes structure similarity and chemical ontologies to map all known metabolites and name metabolic modules. The ChemRICH statistical approach compares chemical similarities using the Medial Subject Headings database and Tanimoto chemical similarity coefficients to cluster metabolites into non-overlapping chemical groups. Enrichment statistical analysis uses a background-independent database test, Kolmogorov−Smirnov-test, using the created clusters.[23]

Predictive Models of Combined Serum Markers

Variable selection was performed with all the metabolites that met both criteria, VIP ≥ 2 and adjusted p-value ≤ 0.05, for high IR to select those compounds that better separate subjects with IS or high IR. A new metabolic variable, total diglycerides (tDG), was created with the arithmetic mean of all DGs.

Variable selection was conducted with the least absolute shrinkage and selection operator (LASSO) logistic regression using a leave-one-out cross-validation.[24] Prior to the analysis, data were log-normalized and Pareto scaled, and adjusted by gender, age, and drug consumption. The lambda-coefficient was

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Table 1. Anthropometric and Biochemical Parameters of the Population of Study

	Mean ± standard deviation				P-value						
	Non-OB IS	Non-OB IR	OB IS	OB IR	IR	Obesity	IR x OB	IS: non-OB vs OB	IR: non-OB vs OB	Non-OB: IS vs IR	OB: IS vs IR
Gender	4M, 15F	4M, 8F	2M, 10F	9M, 12F	n.s.	n.s.	n.s.	n.s	n.s.	n.s.	n.s.
Age (years)	47 ± 15	53.67 ± 14.13	43.67 ± 11.30	43.14 ± 8.91	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Weight (kg)	64.79 ± 8.90	65.33 ± 6.58	125.77 ± 15.28	147.04 ± 30.41	n.s.	5.02E-23	n.s.	1.87E-13	3.53E-09	n.s.	n.s.
BMI (kg/m^2)	24.13 ± 1.82	24.87 ± 1.75	45.78 ± 4.67	52.67 ± 10.20	0.031	1.35E-24	n.s.	2.31E-15	3.39E-09	n.s.	n.s.
Waist circumference (cm)	82.37 ± 8.81	90.58 ± 7.97	125.09 ± 12.82	138.82 ± 14.96	0.021	3.64E-20	n.s.	5.32E-10	3.39E-08	n.s.	n.s.
Hip circumference (cm)	93.84 ± 9.97	99 ± 5.29	139.54 ± 15.56	146.56 ± 15.56	n.s.	5.60E-16	n.s.	6.44E-08	1.68E-07	n.s.	n.s.
Fasting glucose (mmol/L)	90.42 ± 7.79	111.33 ± 11.15	89.75 ± 5.58	113.95 ± 12.62	4.33E-11	n.s.	n.s.	n.s.	n.s.	1.58E-04	2.57E-06
Fasting insulin (μU/mL)	5.47 ± 2.27	14.87 ± 7.29	7.92 ± 2.36	23.89 ± 8.15	2.59E-10	n.s.	n.s.	n.s.	0.005	7.53E-04	8.81E-08
HOMA-IR (index)	1.22 ± 0.52	4.02 ± 1.82	1.76 ± 0.55	6.77 ± 2.58	1.08E-12	0.001	n.s.	n.s.	0.006	1.03E-04	1.99E-08
Systolic pressure (mm Hg)	114 ± 15	126 ± 20	142 ± 18	134 ± 17	n.s	0.001	n.s.	0.010	n.s.	n.s.	n.s.
Diastolic pressure (mm Hg)	69 ± 11	78 ± 11	88 ± 9	81 ± 8	n.s.	6.37E-04	n.s.	0.018	n.s.	n.s.	n.s.
Total cholesterol (mmol/L)	177.63 ± 23.76	232.58 ± 39.81	191.5 ± 46.38	198.90 ± 35.74	0.008	0.001	n.s.	n.s.	0.038	0.002	n.s.
HDL-cholesterol (mmol/L)	56.89 ± 10.42	52.08 ± 17.59	52.75 ± 15.52	41.5 ± 10.50	0.042	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
LDL-cholesterol (mmol/L)	103.29 ± 23.21	148.53 ± 41.17	98.04 ± 51.85	128.58 ± 29.84	0.003	n.s.	n.s.	n.s.	n.s.	0.003	n.s.
Triglycerides (mmol/L)	80.68 ± 36.46	190.75 ± 106.09	115.25 ± 107.87	149.14 ± 44.65	3.21E-04	n.s.	n.s.	n.s.	n.s.	0.002	n.s.

[a]Data are presented as mean ± standard deviation. *P*-values are based on lineal models with gender, age, and drugs as confounders. Gender distribution was explored by Fisher's exact test. *P*-values were adjusted by false discovery rate (FDR). Abbreviations: F, female; IR, insulin resistance; IS, insulin sensitivity; M, male; OB, obesity; n.s., not significant ($p > 0.05$).

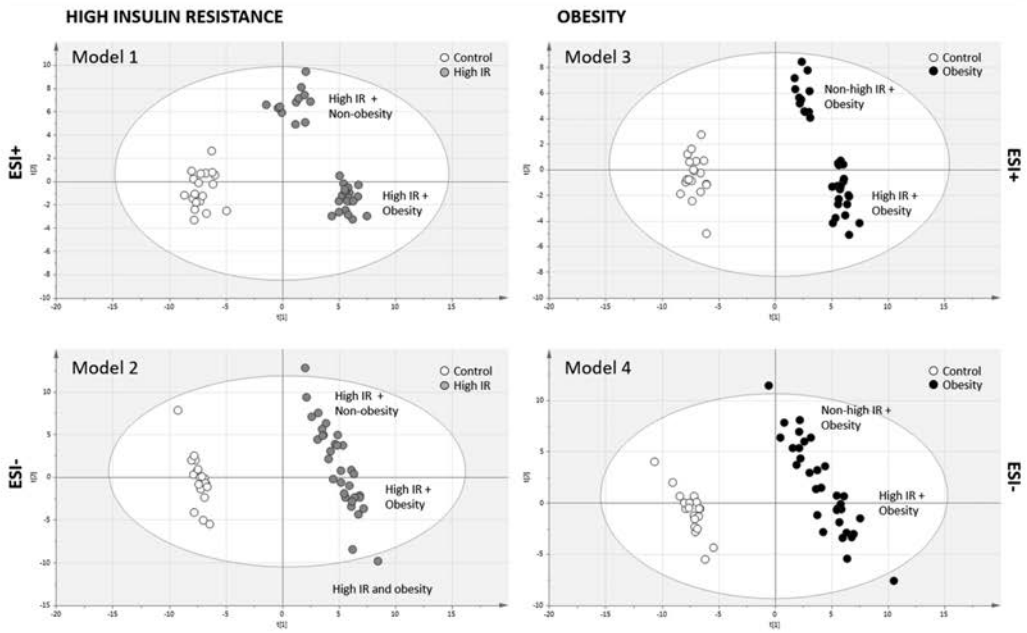

Figure 1. OSC-PLS-DA score plots. The discriminant models separated the control group (individuals with both IS and non-obesity) from patients with high IR (models 1 and 2) or subjects with obesity (models 3 and 4) in both ionization modes. White circles refer to the control group (non-obese IS), gray circles to high IR, and black circles to obesity. Abbreviations: ESI, electrospray ionization; IR, insulin resistance; IS, insulin sensitivity; OSC-PLS-DA, orthogonal signal correction partial least-squares discriminant analysis.

used to choose the most predictive metabolites, and these were employed to build a new parameter, the multimetabolite biomarker model, as follows:

$$\text{Multimetabolite biomarker model} = \lambda_1 \times \text{metabolite}_1 + \lambda_s \times \text{metabolite}_2 + \ldots + \lambda_n \times \text{metabolite}_n$$

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Table 2. Annotated Metabolites Associated with High Insulin Resistance and Obesity[a]

Cluster[a]	Ion mode[b]	RT (min)	Detected mass[c] (m/z)	Error (mDa)	Assignation	Potential marker	Fold change[d]		P-value[e]		Level of evidence[f]
							High IR	Obesity	High IR	Obesity	
Organic acids											
1	ESI+	0.80	169.0351	0.5	[M+H]+	Uric acid	1.48	-	0.002	-	1
	ESI-	0.78	167.0209	0.2	[M-H]-						
Fatty acids											
2	ESI+	5.78	365.2063	0.0	[M+2Na-H]+	Hydroxyeicosatetraenoic acid (HETE)	4.54	4.89	n.s.	0.005	2
			343.2237	0.7	[M+Na]+						
	ESI-	5.73	523.1856	-4.5	3x[M+CHOONa]-						
			388.2153	3.3	13C[M+CHOONa]-						
			387.2136	1.7	[M+CHOONa]-						
			320.2283	2.9	13C[M-H]-						
			319.2270	0.9	[M-H]-						
			301.2166	0.2	[M-H2O-H]-						
3	ESI-	6.68	269.2471	1.5	[M-H]-	Margaric acid	1.51	-	0.045	-	1
4	ESI+	6.76	377.2423	0.4	[M+2Na-H]+	Adrenic acid	1.58	1.64	0.001	3.34E-04	1
			355.2621	-1.4	[M+Na]+						
	ESI-	6.72	399.2495	2.2	[M+CHOONa]-						
			332.2660	1.6	13C[M-H]-						
			331.2630	1.2	[M-H]-						
5	ESI+	5.77	325.2139	-0.1	[M+Na]+	Hydroxyeicosatetraenoic acid (HETE) lactone	4.98	61.43	n.s.	0.012	2
			303.2317	0.1	[M+H]+						
6	ESI-	5.35	404.2109	2.6	13C[M+CHOONa]-	Leukotriene B4 (diHETE)	63.58	66.28	n.s.	0.018	1
			403.2083	1.9	[M+CHOONa]-						
			335.2217	1.1	[M-H]-						
7	ESI+	6.29	277.2083	5.5	[M+Na]+	Palmitoleic acid	-	1.59	-	2.73E-05	1
			255.2311	0.7	[M+H]+						
			238.2243	0.3	13C[M-H-H2O]+						
			237.2208	1.0	[M-H2O+H]+						
			219.2103	1.5	[M-2H2O+H]+						
	ESI-	6.24	253.2176	-0.3	[M-H]-						
8	ESI+	6.42	328.2322	0.6	13C[M+Na]+	Arachidonic acid	-	1.58	-	0.001	1
			327.2287	0.7	[M+Na]+						
	ESI-	6.36	304.2352	1.1	13C[M-H]-						
			303.2325	0.4	[M-H]-						
9	ESI+	5.25	375.2147	-0.5	[M+Na]+	Trihydroxyeicosatetraenoic acid (triHETE)	-	6.02	-	0.009	2
Diglycerides											
10	ESI+	8.55	617.5023	0.3 or 2.7	2x13C[M+Na/H]+	Diglyceride 34:2/36:5	2.43	2.21	3.97E-06	0.022	2
			616.4992	0.1 or 2.5	13C[M+Na/H]+						
			615.4956	0.3 or 2.7	[M+Na/H]+						
11	ESI+	8.27	615.4864	0.6 or 3.0	2x13C[M+Na/H]+	Diglyceride 34:3/36:6	2.44	2.21	1.29E-05	n.s.	2
			614.4852	-1.6 or 0.8	13C[M+Na/H]+						
			613.4820	0.4 or -1.2	[M+Na/H]+						
12	ESI+	8.01	586.4528	0.5 or 1.9	13C[M+Na/H]+	Diglyceride 32:3/34:6	2.12	1.75	0.012	n.s.	2
			585.4499	-1.0 or 1.5	[M+Na/H]+						
13	ESI+	8.17	612.4683	-0.4 or 2.1	13C[M+Na/H]+	Diglyceride 34:4/36:7	2.79	2.37	4.15E-05	n.s.	2
			611.4648	-0.2 or 2.2	[M+Na/H]+						
14	ESI+	8.05	635.4653	-0.7 or 1.7	[M+Na/H]+	Diglyceride 36:6/38:9	2.83	-	4.75E-04	-	2
15	ESI+	8.01	609.4484	0.5	[M+Na]+	Diglyceride 34:5	2.64	2.25	3.69E-04	n.s.	2
16	ESI+	8.92	645.5354	-0.9	2x13C[M+H]+	Diglyceride 38:5	2.03	-	4.95E-05	-	2
			644.5326	-0.4	13C[M+H]+						
			643.5292	-2.0	[M+H]+						
17	ESI+	8.19	588.4690	-1.1 or 1.4	13C[M+Na/H]+	Diglyceride 32:2/34:5	2.21	-	3.42E-04	-	2
			587.4655	-0.9 or 1.5	[M+Na/H]+						
18	ESI+	8.18	662.4834	0.2 or 0.2	13C[M+Na/H]+	Diglyceride 38:7/40:10	2.09	-	0.004	-	2
			661.4814	-1.2 or 1.3	[M+Na/H]+						
19	ESI+	8.44	590.4844	-0.8 or 1.6	13C[M+Na/H]+	Diglyceride 32:1/34:4	2.04	-	1.50E-04	-	2
			589.4814	-1.2 or 1.3	[M+Na/H]+						
20	ESI+	8.79	668.5306	-0.1 or 2.4	13C[M+Na/H]+	Diglyceride 38:4/40:7	1.83	-	0.001	-	2
			667.5285	-1.3 or 1.1	[M+Na/H]+						
21	ESI+	8.41	641.5020	0.6 or 3.0	2x13C[M+Na/H]+	Diglyceride 36:4/38:7	1.83	-	4.97E-04	-	2
			640.4991	0.2 or 2.6	13C[M+Na/H]+						
			639.4962	-0.3 or 2.1	[M+Na/H]+						
22	ESI+	7.91	559.4338	-0.5 or 1.9	[M+Na/H]+	Diglyceride 30:2/32:5	1.58	-	0.009	-	2
23	ESI+	8.36	563.4634	1.2 or 3.6	[M+Na/H]+	Diglyceride 30:0/32:3	1.95	-	0.008	-	2
24	ESI+	8.10	562.4533	-1.0 or 2.4	13C[M+Na/H]+	Diglyceride 30:1/32:4	2.03	-	0.015	-	
			561.4488	0.1 or 2.6	[M+Na/H]+						
Other lipids											
25	ESI+	7.03	424.3265	0.2	13C[M+Na]+	Ketocholesterol, 7-	5.97	5.29	0.002	n.s.	1
			423.3235	-0.2	[M+Na]+						
			402.3444	0.4	13C[M+H]+						
			401.3413	0.1	[M+H]+						

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Table 2. continued

Cluster[a]	Ion mode[b]	RT (min)	Detected mass[c] (m/z)	Error (mDa)	Assignation	Potential marker	Fold change[d]		P-value[e]		Level of evidence[f]
							High IR	Obesity	High IR	Obesity	
26	ESI+	4.82	363.2163	0.3	[M+H]+	Hydroxycorticosterone	0.62	-	0.003	-	2
27	ESI-	5.25	446.2893	1.3	[M-H2O-H]-	Glycocholic acid	-	0.51	-	1.90E-04	1
Dipeptides											
28	ESI+	3.91	284.0794	-2.5	[M+K]+	Glutamyl-Valine	-	61.43	-	0.038	2
			268.1055	-2.5	[M+Na]+						
			247.1265	-2.1	13C[M+H]+						
			246.1236	-2.6	[M+H]+						
			228.1127	-1.7	[M-H2O+H]+						
29	ESI+	6.77	217.1211	-2.9	[M+H]+	γ-Glutamyl-γ-aminobutyraldehyde	-	3.06	-	0.012	2

[a]Metabolites are sorted by their VIP value in the high IR state. Abbreviations: ESI, electrospray ionization; IR, insulin resistance; n.s., not significant (adjusted *p*-value >0.05). The symbol "/" means ambiguity in metabolite annotation. [b]Clusters are listed according to decreasing VIP value. All the mass features met the criteria VIP ≥ 2. [c]Type of ionization. [d]Data obtained by LC-ESI-qTOF-MS. [e]Fold-change of metabolites in the high IR and obesity groups with respect to the control group. [f]Calculated with a Student's *t* test and adjusted by false discovery rate (FDR). Data were log-normalized, Pareto scaled and then adjusted by gender, age and drug consumption. [g]According to the Metabolomics Standards Initiative.[25]

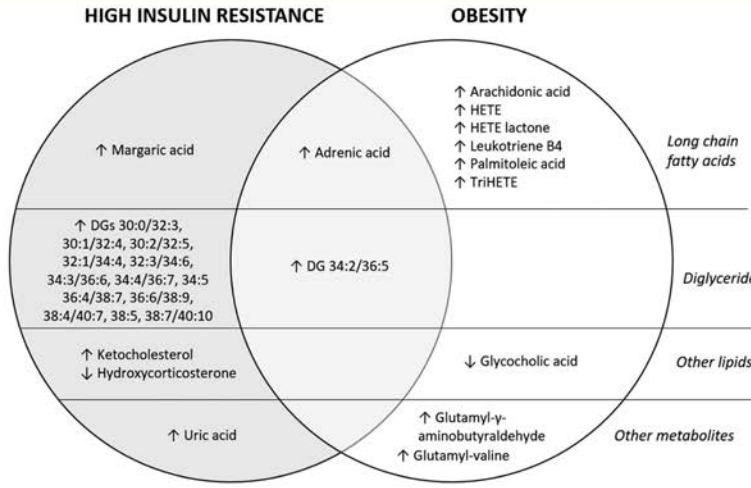

Figure 2. Venn diagram of the metabolic profiles of subjects with high IR and/or obesity. This diagram shows similarities and divergences between the metabolic status of high IR and obesity with respect to subjects with IS and non-obesity. Only metabolites that met the criteria VIP ≥ 2 and adjusted *p*-value ≤0.05 are shown. The symbol "/" means ambiguity in metabolite annotation.

The LASSO regression method was performed in R with the *glmnet* package.

ROC Curves

The global performance of this multimetabolite biomarker model was evaluated through receiver operating characteristic (ROC) curves. The area under the curve (AUC) value, confidence intervals (CIs 95%), sensitivity, and specificity were calculated in R with the *pROC* package.

RESULTS

Anthropometric and Biochemical Parameters

Individuals with high IR presented altered FG, fasting insulin, HOMA-IR index, and lipid metabolism indicators (total cholesterol, HDL, and LDL cholesterol and TG). Subjects with obesity had higher adiposity markers, systolic and diastolic pressure, and total cholesterol than individuals without obesity. No changes were observed in the interaction between high IR and obesity for any of the variables (Table 1).

Differences between concordant and discordant phenotypes of high IR were mainly due to adiposity markers. Subjects with concordant and discordant phenotypes of obesity also presented metabolic differences including FG, fasting insulin, HOMA-IR index, and lipid metabolism (Table 1).

LC–MS Data Quality

Neither carryover nor apparent clustering due to the batch injection order were noticed (Figure S1). The run-to-run repeatability of the QCs across the whole data set met the quality criteria (retention time shift ≤ 0.05 min, mass accuracy deviation <3 mDa and peak area CV < 25%)[11] (Table S1). The generation of the OSC filters removed six and five components (eigenvalue >2), maintaining the 54% and 76% non-orthogonal variation in the original ESI+ and ESI− data sets, respectively.

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The OSC-PLS-DA resulted in four robust models that discriminate metabolic differences among control individuals and subjects with high IR or obesity (Figure 1, Table S3). The PLS score plot showed that the control group and the high IR or obesity groups clearly separated in the first component. The plot also suggested that concordant and discordant phenotypes of each disorder (high IR-obesity vs high IR-non-obesity, and IS-non-obesity vs IS-obesity, respectively) might be metabolically different as they were slightly separated in the second component (Figure 1). A total of 193 (ESI+) and 169 (ESI−) mass features were selected (VIP value ≥ 2) for further metabolite identification(Figure S2).

Metabolic Profiles of High IR and Obesity

A total of 29 metabolites (VIP ≥ 2) were annotated from their m/z value and/or fragmentation pattern, and the identity of eight of them was confirmed with metabolite standards (Table 2). The majority of the metabolites were lipids. We were not able to discern between a molecular ion or sodium adduct in DGs since both species presented a small mass difference with the theoretical mass (<3 mDa). Thus, we provided both annotations.

A Student's t test confirmed that two out of these compounds were shared by both metabolic statuses, 18 were only found in high IR and nine in obesity. Adrenic acid and a DG (34:2/36:5) were common between high IR and obesity, which were higher than in the control group. Metabolomics also revealed that the high IR group presented more DGs, margaric acid, ketocholesterol, and uric acid, and lower levels of hydroxycorticosterone. On the other hand, alterations in lipid metabolism were also found in obesity. For instance, the obesity group showed higher levels of arachidonic acid, HETE, HETE lactone, leukotriene B4, palmitoleic acid and tryhydroxyeicosatetraenoic acid (triHETE), and the dipeptides γ-glutamyl-γ-aminobutyraldehide and glutamyl-valine than the control groups, and lower levels of the bile acid glycocholic acid (Figure 2).

An enrichment analysis was performed with ChemRICH to identify which chemical class was more enriched in each metabolic disorder. ChemRICH revealed that the most enriched chemical class in high IR was DGs (adjusted p-value = 2.2×10^{-20}), while HETEs and unsaturated fatty acids were in obesity (adjusted p-values = 1.7×10^{-05} and 6.0×10^{-04}, respectively) (Table 3). Therefore, we will mainly focus the discussion of the results in these chemical classes.

Table 3. Enrichment Analysis with ChemRICH[a]

Cluster name	Cluster size	p-value	FDR	Altered metabolites
High IR				
Diglycerides	15	2.2E-20	2.2E-20	15
Obesity				
HETEs	3	5.8E-06	1.7E-05	3
Unsaturated fatty acids	4	4.0E-04	6.0E-04	4

[a]ChemRICH utilizes structure similarity and chemical ontologies to map all known metabolites and name metabolic modules. P-values were calculated by applying the Kolmogorov-Smirnov test. Only the metabolites that presented VIP ≥ 2 and adjusted p-value ≤ 0.05 were used.

Metabolic Differences between Concordant/Discordant Phenotypic Groups

Comparisons between phenotypic groups confirmed that the main differences between groups were due to DG and polyunsaturated fatty acid (PUFA) levels, revealing that the degree of dyslipidemia and pro-inflammatory markers could differentiate subjects of distinct phenotypic groups (Figure 3). Among all the PUFAs, adrenic acid was the only metabolite able to distinguish subjects with IS from those with high IR, and individuals with obesity from those without obesity (Table S5).

Predictive Models of Combined Serum Markers

A combined multimetabolite biomarker model to identify individuals with high IR was formed with the arithmetic mean of DGs (tDG), uric acid, and adrenic acid. This model presented a high predictive power. Specifically, the AUC (95% CI) for the multimetabolite biomarker model was 80.1% (68.9−91.4) when analyzing all the population of the study, 72.5% (53.3−91.7) for the subjects with obesity, and 80.7% (61.0−100) for individuals without obesity (Figure 4). Sensitivity and specificity rates were between 70 and 90%. In the case of subjects with obesity, predictive values were slightly lower (Table 4). This predictive model presented better performance than the combination of other lipid markers such as cholesterol or TG between them and/or with uric acid and adrenic acid (Table S6).

■ DISCUSSION

The untargeted profiling of the serum of concordant/discordant phenotypes of high IR and/or obesity allowed exploring the metabolic profiles of these two metabolic statuses and describing their similarities and divergences. In addition, it allowed defining a multimetabolite biomarker model to detect high IR regardless of obesity, which might predict the risk developing diabetes. Large disturbances in lipid metabolism were observed in all the metabolic disorders.

Metabolic Profile of High IR

DGs were the most enriched chemical class in subjects with high IR. This group also presented differences in TG levels, whose levels highly correlate with DG levels (Pearson's correlation coefficient: r = 0.90). However, TG species could not be detected in metabolomic profiles because of their very low polarity, which provokes that most TGs remain adsorbed into the protein precipitate during serum extraction. Furthermore, these neutral lipids are not readily ionized in ESI, unless some modifier is added to mobile phases (e.g., ammonium salts).

Despite the adipocytokines-induced inflammation is the prevailing hypothesis of IR progression, the hypothesis of DG-mediated IR is becoming increasingly important.[26,27] In line with this hypothesis, we observed higher levels of DGs in subjects with high IR regardless of obesity. An accumulation of DGs leads to a cascade of events such as the activation of isoforms of protein kinase C that inhibit sensibility to insulin of insulin responsive tissues, the reduction of fatty acid β-oxidation in the mitochondria, thereby limiting energy production, and lipodystrophy in tissues due to the redistribution of fat.[26,27]

Adrenic acid was the only PUFA whose levels were altered in subjects with high IR, suggesting a certain degree of a pro-inflammatory response. Adrenic acid is a ω-6 PUFA. This class of lipids act as inflammatory mediators by acting as ligands for immune receptors and trigger a perpetual low-grade inflamma-

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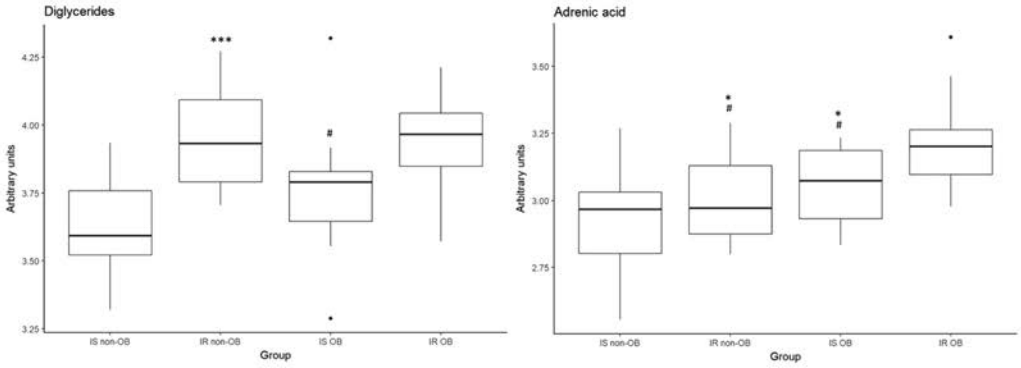

Figure 3. Box plots of the most representative metabolite changes in concordant/discordant phenotypes of high IR and obesity (Table S5). Significances (p-values) are shown with asterisks when compared with the control group as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; or with hash keys when compared with the group of subjects with high IR and obesity as follows: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. Abbreviations: IR, insulin resistance; IS, insulin sensitivity; OB, obesity.

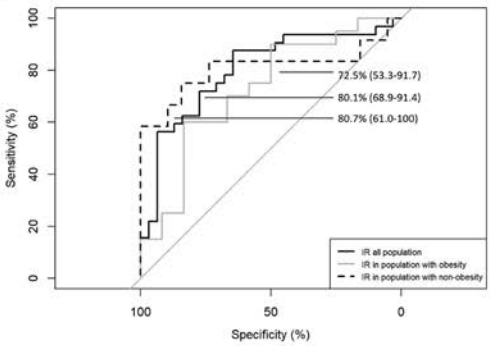

Figure 4. ROC curve parameters of a predictive biomarker model to identify high IR, regardless of obesity. The biomarker model was formed by the arithmetic mean of the 15 DGs annotated (tDG), adrenic acid, and uric acid.

Table 4. ROC Curve Parameters of Prediction Biomarker Model To Identify Subjects with High IR[a]

Prediction	Sensitivity (%)	Specificity (%)	AUC (95% CI)
High IR in all study population	71.9	77.4	80.1% (68.9-91.4)
High IR in population with obesity	60.0	83.3	72.5% (53.3-91.7)
High IR in population without obesity	75.0	84.2	80.7% (61.0-100%)

[a]Biomarker model was formed by the arithmetic mean of the 15 DGs annotated (tDG), adrenic acid and uric acid. Abbreviations: AUC, area under the curve; CI, confidence interval; IR, high insulin resistance.

tion. This low-grade inflammation leads to a cascade of events including inflammatory cell activation, adipocyte growth and dysfunction, oxidative stress and altered signaling.[28,29]

Uric acid, a product of the metabolic breakdown of purine nucleotides, was also higher in subjects with high IR. It is normally excreted by the urine but high concentrations of uric acid in blood are associated with oxidative stress, inflammation and alterations in carbohydrate and lipid metabolism. For instance, hyperuricemia promotes endothelial cell damage and dysfunction, decreases endothelial nitric oxide availability, which limits insulin action, increases reactive oxygen species, and blocks adiponectin synthesis. In addition, hyperuricemia alters gluconeogenesis, fatty acid oxidation, and induces the production of pro-inflammatory mediators. Serum uric acid has been proposed as a risk marker in IR, cardiovascular disease, metabolic syndrome and renal failure, among others.[30,31]

The precursor of aldosterone, hydroxycorticosterone, was lower in subjects with high IR. Hypoaldosteronism has been associated with adrenal insufficiency and diabetic nephropathy.[32] Results from the cohort Framingham Heart Study described a lineal relationship between the glycaemic index and the risk for renal alterations, even before the onset of diabetes.[33] Therefore, alterations in uric acid and hydroxycorticosterone might reflect that subjects with high IR may be prone to develop renal alterations.

Furthermore, higher levels of 7-ketocholesterol might also confirm oxidative processes in high IR. 7-ketocholesterol, also known as 5-cholesten-3β-ol-7-one, is a sterol derived from the oxidation of cholesterol and it has been proposed as a robust biomarker of oxidized LDL particles in a range metabolic disorders.[34] Energy misbalance, hyperglycaemia, and hyperlipidaemia can lead to increase the production of free radicals, which might damage cellular structures and alter metabolic processes.[35,36]

Metabolic Profile of Obesity

Dyslipidemia was also observed in obesity. For instance, the blood levels of free fatty acids (FFA) such as palmitoleic acid and ω-6 PUFAs were higher in the obesity group than in the control group. In physiological conditions, blood FFA levels are tightly regulated. However, in obesity and other metabolic disorders, FFA increase in plasma due to the stress of the adipose tissue, which releases more FFA than in normal conditions.[37]

The enrichment analysis with ChemRICH revealed that HETEs and unsaturated fatty acids were the most enriched chemical classes in subjects with obesity. For instance, adrenic acid, arachidonic acid, HETE, HETE lactone, leukotriene B4 (diHETE), and triHETE levels were found to be higher in the obesity group. These metabolites belong to the ω-6 PUFAs class and, as already commented, they are lipid mediators that trigger a perpetual low-grade inflammation. Arachidonic acid is

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considered the primary source of pro-inflammatory lipid mediators and it is rapidly converted into potent inflammatory mediators such as prostaglandins, thromboxanes, leukotrienes, lipoxins and HETEs, and derivatives, which lead to cascade of events, as described hereinbefore.[28,29] Therefore, the fact that we found more ω-6 PUFAs differentially expressed in obesity than in high IR with respect to the control group (Table 2), and their levels were higher in concordant than in discordant phenotypes (Figure 3, Table S5), suggests that the inflammatory processes in high IR might be at a lower extent than in obesity.

Inflammation and oxidative stress are tightly interconnected processes. For instance, inflammatory cells produce free radicals during the immune response.[35,36] Although 7-ketocholesterol was not altered in obesity, two glutamyl peptides, namely glutamyl-γ-aminobutyraldehyde and glutamyl-valine, levels were higher in obesity. Glutamyl dipeptides, formed by glutamate and another amino acid, are byproducts of glutathione synthesis and their levels are an indirect evidence of glutathione synthesis and amino acid availability.[38] γ-aminobutyraldehyde is the direct precursor of γ-aminobutyric acid (GABA). Both GABA and glutamate stimulate food intake and body weight gain.[39] Valine has also been associated with obesity as branched-chain amino acids (BCAAs) fuel adipocytes.[40] Glutamate and BCAA levels also correlated with anthropometric adiposity markers in a previous study, probably as an alternative energy source to compensate glucose and lipid metabolism impairment.[5] Therefore, higher levels of these dipeptides in obesity might mirror oxidative stress, the stimulation of appetite, body weight gain, and the use of alternative energy sources in the group with obesity.

Bile acids are involved in the absorption of dietary fat and fat-soluble vitamins and modulate cholesterol level, but also regulate energy homeostasis and can act as signaling molecules and inhibit obesity. We found lower levels of glycocholic acid, a primary bile acid conjugated with glycine, in obesity. Thus, alterations in this bile acid might reflect body weight, lipid and carbohydrate metabolism alterations in obesity.[41] In addition, this decrease of primary bile acids might alter the release of glucagon-like peptide-1 (GLP-1), thus modifying satiety and appetite of individuals with obesity.[42] This observation agrees with the higher levels of the dipeptide formed by glutamate and the direct precursor of GABA. Increases in conjugated bile acids have been found in patients with obesity after undergoing bariatric surgery.[43]

Differences between Concordant/Discordant Phenotypes of High IR and Obesity

The main differences between the four phenotypic groups were DGs and PUFA levels. The highest levels of these metabolites were found in subjects with both high IR and obesity, while the lowest levels in individuals with both IS and non-obesity. In addition, this study also revealed that the metabolic profile of subjects with only one metabolic disorder, high IR or obesity, had lower levels of DGs, free fatty acids and pro-inflammatory markers than individuals presenting both disorders. These results might unveil that obesity itself also implies the existence of protective mechanisms against high IR. In line with this observation, differences in pro-inflammatory markers in subjects with obesity and IS or IR have been already described. This observation is also known as the "obese healthy paradox".[44,45]

Among all the metabolites identified as potential markers of discordant phenotypes of high IR and obesity (Table S5), adrenic acid is particularly interesting since it is the only compound whose levels allowed differentiating the four phenotypical groups. Adrenic acid (C22:4 n-6) is a minor ω-6 PUFA in blood, it derives from the elongation of arachidonic acid in the liver and its production increases in inflammation.[46] However, little literature about its role in healthy conditions is known. Further research on this particular lipid could provide more insights about differences between concordant/discordant phenotypes in metabolic disorders.

Multimetabolite Biomarker Model To Predict Risk of Developing Diabetes

IR sets in before disease markers appear and it might remain undiagnosed for a long period, thereby increasing the risk of developing other metabolic alterations. Therefore, there is a need to detect IR rapidly and to monitor its progression to diabetes. Although current markers have a high predictive power, they also present some limitations.[1] Current markers of high IR such as FG, fasting insulin or HOMA-IR presented a high predictive power (not shown, AUC ≈ 95%). It may be because subjects were grouped according their FG levels and HOMA-IR index. However, they may be late markers since when insulin deficiency manifests as hyperglycaemia, considerable pancreatic β-cell insufficiency has already occurred.[47] Thus, the third aim of this work was to identify new markers of high IR. We selected those metabolites that presented a VIP ≥ 2 and adjusted p-value <0.05 and the most predictive metabolites for high IR were chosen. The combination of DGs, uric acid and adrenic acid provided a good predictive model of high IR (AUC 80.1%).

This multimetabolite biomarker model could be a comprehensive indicator of metabolic alterations before β-cell impairment occurs, as it mirrors IR in insulin-responsive tissues, lipotoxicity and certain degree of inflammation (DG), oxidative stress and alterations in carbohydrate and lipid metabolism (uric acid),[30,31] and proinflammatory processes (adrenic acid).[46] Further research with larger cohorts and longitudinal studies should be conducted to validate this model as an early marker of diabetes.

Strengths and Limitations

Although this study is an observational study, the high potential of untargeted metabolomics has provided a snapshot of the metabolome of subjects with high IR and/or obesity at a given time. Thus, we have explored in depth the metabolic profiles of these two metabolic disorders, described their similarities and divergences, formulated hypotheses about discordant phenotypes and mechanistic insights, and defined a predictive model for the risk of developing diabetes. Despite the low number of subjects enrolled in the study and the fact that some individuals were grouped in both high IR and obesity groups, results were robust and in line with previously reported. Complementary metabolomics studies are necessary to provide a comprehensive overview of the metabolome of these metabolic disorders. The authors support large-scale and follow-up studies to replicate and validate the results.

■ CONCLUSION

Through an untargeted metabolomic-driven approach, we have explored the metabolic profiles of concordant and discordant phenotypes of subjects high IR and/or obesity. Large alterations in lipid metabolism, oxidative stress, and inflamma-

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tion were unveiled. In addition, these results allowed to build a multimetabolite biomarker model to predict high IR regardless of obesity that includes the measurement of DGs, uric acid, and adrenic acid. It might be also employed to predict the risk of developing diabetes; however, they need to be externally validated. These findings provide new insights in the research of metabolic diseases and unveil new potential targets in therapeutic treatments of diabetes and obesity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00855.

Principal component analysis score plot of quality controls and serum samples; plot correlating ions with VIP ≥ 2 in high IR and obesity groups in both ionization modes; variation in retention time, peak area, peak height, and detection mass in quality controls and internal and external standard samples; preprocessing parameters in MarkerView; summary of parameters for assessing OSC-PLS-DA predictive ability; summary of parameters to validate OSC-PLS-DA predictive ability; statistical significance of metabolites between phenotypic groups; ROC curve parameters of multimetabolite biomarkers to build predictive biomarker models for high IR (PDF)

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Notes

The authors declare no competing financial interest.

The mass spectrometry data have been deposited to the MetaboLights repository[14] (https://www.ebi.ac.uk/metabolights/) with the data set identifier MTBLS668.

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■ ABBREVIATIONS

AUC, area under the curve; BCAA, branched-chain amino acids; BMI, body mass index; CI, confidence interval; DG, diglyceride; diHETE, dihydroxyeicosatetraenoic acid; ESI, electrospray ionization; FDR, false discovery rate; FFA, free fatty acids; FG, fasting glucose; GABA, γ-aminobutyric acid; HDL, high-density lipoprotein; HETE, hydroxyeicosatetraenoic acid; HOMA-IR, homeostatic model assessment−insulin resistance; IR, insulin resistance; LASSO, least absolute shrinkage and selection operator; LC−MS, liquid chromatography mass spectrometry; LDL, low-density lipoprotein; OSC-PLS-DA, orthogonal signal correction partial least-squares discriminant analysis; PUFA, polyunsaturated fatty acids; QC, quality control; ROC, receiver operating characteristic; tDG, total diglycerides; TG, triglyceride; triHETE, trihydroxyeicosatetraenoic acid; VIP, variable importance in projection

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Material Suplementario

PUBLICACION 2

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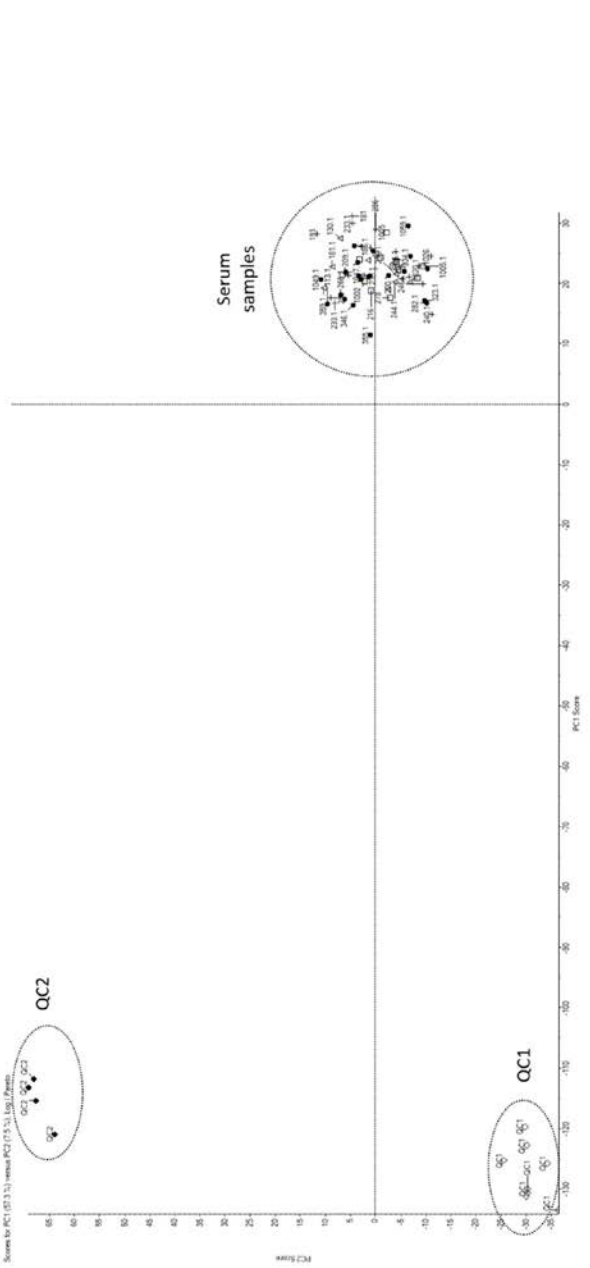

Figure S1. Principal componcnt analysis score plot of quality controls (QC) (water (QC1), QC (QC2)) and scrum samples.

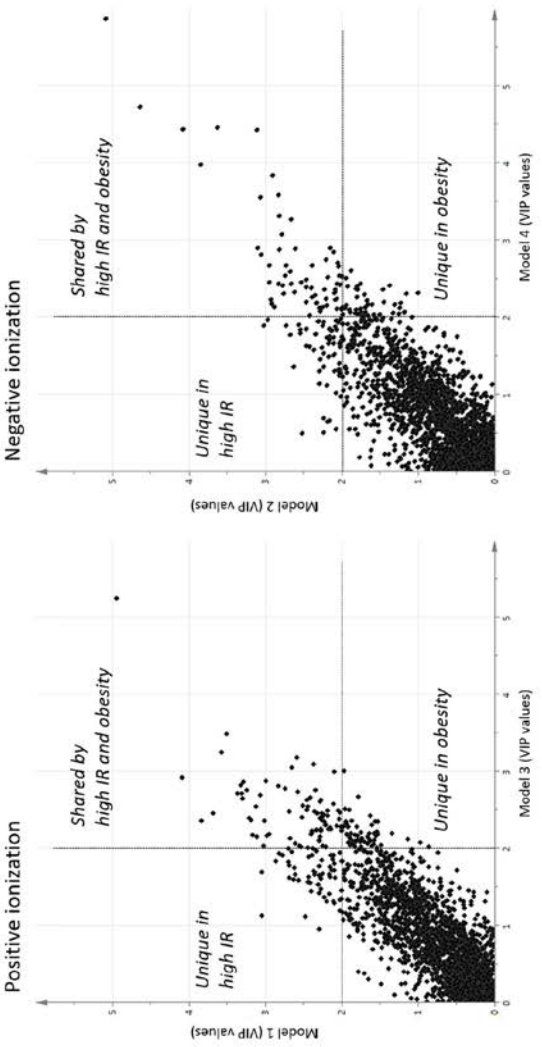

Figure S2. Plot correlating ions with VIP ≥2 in the high IR and obesity group when compared with the control group in both ionization modes. It provides a general overview of the shared and unique mass features between both metabolic states.

Table S1. Variation in retention time, peak area, peak height and detection mass in aqueous QC2 and internal and external standard samples injected in a randomized order throughout the analysis.

Analyte name	Detection mass (m/z)		Retention time (min)		Intensity (peak area)		
	Mean	Error (mDa)	Mean	Shift	Mean	SD	CV (%)
Quality controls							
Positive mode							
L-Carnitine	162.11248	0.016	0.29	0.00	89345	1911	2.14
L-Citrulline	176.10262	-0.345	0.32	0.00	7030	302	4.29
Acetylcholine	146.11733	-0.218	0.37	0.00	38065	737	1.94
L-Valine	118.08564	-0.574	0.43	0.01	1728	73	4.23
Acetyl-L-carnitine	204.12295	-0.072	0.48	0.00	100153	1751	1.75
L-Isoleucine	132.10168	-0.217	0.90	0.01	12214	312	2.55
L-Leucine	132.10168	-0.217	0.90	0.01	12214	312	2.55
L-Phenylalanine	166.08609	-0.150	2.24	0.01	29244	822	2.81
4-Hydroxyhippuric acid	196.06017	-0.251	3.51	0.02	1907	170	8.89
L-Tryptophan	205.09715	0.006	3.56	0.02	25259	715	2.83
Epicatechin	291.08678	0.478	3.98	0.01	32939	1564	4.75
Naringerin	273.07618	0.440	4.73	0.01	72801	3182	4.37
Glycochenodeoxycholic acid	450.32069	-0.708	5.51	0.02	2659	75	2.82
Negative mode							
α-Hydroxyisobutyric acid	103.03985	0.011	1.14	0.00	4768	140	2.93
Gallic acid	169.01396	0.018	1.48	0.00	29801	747	2.51
L-Phenylalanine	164.07143	0.058	2.22	0.00	10701	305	2.85
4-Hydroxyhippuric acid	194.04570	0.062	3.47	0.02	22802	795	3.49
L-Tryptophan	203.08228	0.059	3.52	0.02	25414	588	2.31
Syringic acid	197.04535	0.054	4.02	0.01	21780	559	2.57
Dodecanedioic acid	229.14441	0.034	5.07	0.01	136553	3132	2.29
Glycochenodeoxycholic acid	448.30609	0.034	5.47	0.01	169075	4899	2.90
Palmitic acid	255.23251	0.090	6.55	0.02	2287	101	4.41
Stearic acid	283.26391	0.086	6.98	0.01	1942	154	7.91
Internal and external standards							
Positive mode							
Glycocholic acid-(glycyl-1-13C) monohydrate)	467.3192	-0.512	5.33	0.01	703	137	19.54
Indole-3-acetic-2,2-d2 acid	178.0831	-0.106	4.39	0.01	134126	8857	6.60
Acetyl-d3-L-carnitine hydrochloride	207.1429	1.066	0.40	0.00	247919	52651	21.24
Negative mode							
Glycocholic acid-(glycyl-1-13C) monohydrate)	465.30447	-0.642	5.30	0.02	449798	63308	14.07
Indole-3-acetic-2,2-d2 acid	176.06839	-0.218	4.35	0.03	30174	5004	19.59

Table S2. Preprocessing parameters in MarkerView[a]

Parameter	Description	Value ESI+	Value ESI-
Peak finding	Subtraction offset (scans)	15	15
	Subtraction multiplication factor	1.5	1.5
	Minimum spectral peak width (ppm)	1	1
	Minimum retention time peak width (scans)	3	3
	Noise threshold	5	5
Peak alignment	Retention time tolerance (min)	0.18	0.08
	Mass tolerance (Da)	0.02	0.02
	Intensity threshold	5	5
	Maximum number of peaks	3000	3000

[a] MarkerViewTM 1.3.0.1 software (AB Sciex, Toronto, Ontario, Canada).

Table S3. Summary of parameters for assessing the OSC-PLS-DA predictive ability.

Model	Number of sample classes	Ionization	Number[a)]	$R^2X(cum)$[b)]	$R^2Y(cum)$[b)]	$Q^2(cum)$[b)]	R intercept[c)]	Q intercept[c)]	p-value[d)]
M1	2 (control vs HIR)	POS	1	0.0633	0.890	0.697	0.577	-0.170	2.69E-15
M2	2 (control vs HIR)	NEG	1	0.0633	0.890	0.697	0.665	-0.165	3.69E-13
M3	2 (control vs MO)	POS	1	0.0664	0.952	0.812	0.580	-0.182	1.08E-20
M4	2 (control vs MO)	NEG	1	0.0664	0.952	0.812	0.637	-0.167	3.93E-18

a) Number of components selected.

b) R^2X (cum) and R^2Y (cum) are the cumulative modelled variation in X and Y matrix, respectively, and Q^2 (cum) is the cumulative predicted variation in Y matrix.

c) Obtained after a permutation test (N=200).

d) Value from ANOVA based on the cross-validated predictive residuals. The models were considered significant value with $P<0.001$.

Table S4. Summary of parameters for validate the OSC-PLS-DA predictive ability.

Training set (90%) of models	No.[a)]	$R^2X(cum)$[b,c]	$R^2Y(cum)$[b,c]	$Q^2(cum)$[b,c]	*p*-value	Total correctly classified individuals (%)	Correctly classified individuals from the control group (%)	Correctly classified individuals from the disease group (%)	Fisher probability
M1	1	0.0757 ± 0.0180	0.877 ± 0.011	0.734 ± 0.027	8.11E-13	99.81	100	100	6.24E-14
M2	1	0.0690 ± 0.0042	0.948 ± 0.008	0.795 ± 0.018	6.02E-15	100	100	100	2.10E-14
M3	1	0.0850 ± 0.0019	0.931 ± 0.012	0.841 ± 0.039	1.52E-16	100	100	100	1.30E-14
M4	1	0.0661 ± 0.0030	0.893 ± 0.008	0.688 ± 0.022	3.23E-11	99.80	100	99.69	5.99E-14

a) Number of components selected.
b) R^2X (cum) and R^2Y (cum) are the cumulative modeled variation in X and Y matrix, respectively, and Q^2 (cum) is the cumulative predicted variation in Y matrix
c) Values of R^2X (cum), R^2Y (cum) and Q^2 (cum) corresponding to Mean and Standard Deviation

Table S5. Statistical significance of metabolites between phenotypic groups. P-values and adjusted p-values (by false discovery rate, FDR) are shown. The low number of metabolites and subjects per group highly penalize significances. Abbreviations: aminobut., aminobutyraldehyde; DG, diglycerides; HETE, hydroxyeicosatetraenoic acid; IR, insulin resistance; IS, insulin sensitivity; OB, obese; TriHETE, trihydroxyeicosatetraenoic acid.

	IS: non-OB vs OB		**IR: non-OB vs OB**		**OB: IS vs IR**		**Non-OB: IS vs IR**		**Non-OB IS vs OB IR**	
	p-value	FDR	p-value	FDR	p-value	FDR	p-value	FDR	p-value	FDR
Diglycerides										
DG 30:0 or 32:3	0.420	0.504	0.475	0.528	0.022	0.074	0.035	0.052	0.001	0.001
DG 30:1 or 32:4	0.031	0.066	0.126	0.250	0.339	0.466	0.006	0.013	0.001	0.001
DG 30:2 or 32:5	0.047	0.083	0.105	0.250	0.357	0.466	0.010	0.018	0.001	0.002
DG 32:1 or 34:4	0.017	0.040	0.435	0.518	0.011	0.067	0.001	0.004	1.02E-07	1.11E-06
DG 32:2 or 34:5	0.296	0.423	0.351	0.438	0.015	0.071	0.004	0.011	2.78E-05	6.95E-05
DG 32:3 or 32:6	0.108	0.181	0.021	0.104	0.540	0.616	0.025	0.039	0.022	0.024
DG 34:2 or 36:5	0.047	0.083	0.991	0.991	0.009	0.066	2.36E-04	0.002	3.13E-08	9.39E-07
DG 34:3 or 34:6	0.352	0.459	0.663	0.710	0.007	0.066	0.001	0.004	7.78E-07	3.40E-06
DG 34:4 or 34:7	0.627	0.681	0.176	0.265	0.019	0.072	0.001	0.004	2.04E-05	5.57E-05
DG 34:5 or 34:8	0.321	0.438	0.296	0.395	0.060	0.149	0.006	0.013	7.73E-05	1.66E-04
DG 36:4 or 36:7	0.867	0.897	0.303	0.395	0.065	0.151	0.001	0.004	0.001	0.001
DG 36:6 or 36:9	0.924	0.924	0.007	0.078	0.086	0.172	4.01E-04	0.003	3.06E-04	0.001
DG 38:4 or 40:7	0.483	0.558	0.157	0.250	0.135	0.239	0.001	0.004	0.001	0.002
DG 38:5 or 40:8	0.047	0.083	0.156	0.250	0.083	0.172	2.29E-04	0.002	1.46E-05	4.39E-05
DG 38:7 or 40:10	0.636	0.681	0.127	0.250	0.157	0.261	0.009	0.018	0.001	0.002

DG average	0.118	0.186	0.108	0.250	0.027	0.081	1.62E-04	0.002	3.39E-07	2.55E-06
Fatty acids										
Adrenic acid	0.005	0.017	0.030	0.121	0.006	0.066	0.013	0.022	1.11E-07	1.11E-06
Arachidonic acid	0.002	0.013	0.158	0.250	0.439	0.549	0.060	0.085	1.97E-04	3.69E-04
HETE	0.001	0.010	0.010	0.078	0.324	0.466	0.218	0.284	8.34E-05	1.67E-04
HETE lactone	0.006	0.017	0.776	0.803	0.347	0.466	0.748	0.802	0.082	0.087
Leukotriene B4	0.001	0.010	0.100	0.250	0.732	0.757	0.231	0.289	0.019	0.022
Margaric acid	0.368	0.460	0.045	0.150	0.554	0.616	0.012	0.020	0.145	0.145
Palmitoleic acid	0.004	0.017	0.013	0.078	0.031	0.083	0.243	0.292	7.93E-07	3.40E-06
TriHETE	3.80E-04	0.006	0.013	0.078	0.917	0.917	0.857	0.887	6.17E-05	1.42E-04
Other lipids										
Glycocholic acid	3.03E-06	9.09E-05	0.152	0.250	0.489	0.587	0.935	0.935	0.008	0.010
Hydroxycorticosterone	0.014	0.034	0.109	0.250	0.016	0.071	0.003	0.007	5.79E-07	3.40E-06
Ketocholesterol	0.009	0.026	0.032	0.121	0.007	0.066	0.092	0.126	4.99E-06	1.87E-05
Other metabolites										
Glutamyl-γ-aminobut.	0.005	0.017	0.449	0.518	0.655	0.701	0.360	0.415	0.084	0.087
Glutamyl-Valine	0.178	0.267	0.006	0.078	0.113	0.212	0.520	0.578	0.018	0.021
Uric acid	0.005	0.017	0.289	0.395	0.329	0.466	0.001	0.004	8.16E-06	2.72E-05

TableS6. ROC curve parameters of multimetabolite biomarkers to build a predictive models to identify subjects with high IR. A new parameter, totalDG (tDG), was calculated as the arithmetic mean of the fifteen DGs identified. Abbreviations:AUC, area under the curve;CI,confidence interval; IR,high insulin resistance; tDG,totalDG; TG,tri glycerides.

Models	Sensitivity(%)	Specificity(%)	AUC(95%CI)
Lipids			
tDG	59.4	83.9	72.2%(59.3-85.1)
TG	84.4	64.5	76.2%(64.1-88.4)
Cholesterol	59.4	74.2	65.5%(51.5-79.5)
tDG+TG	78.1	71.0	77.6%(65.8-89.4)
tDG+cholesterol	68.8	77.4	76.3%(64.1-88.5)
TG+cholesterol	87.5	64.5	77.0%(65.0-89.0)
tDG+TG+cholesterol	67.7	78.1	77.4%(65.7-89.2)
Lipids+metabolites			
tDG+adrenicacid	67.7	78.1	77.1%(65.2-89.1)
TG+adrenicacid	81.3	77.4	79.8%(68.4-91.3)
Cholesterol+adrenicacid	62.5	80.6	73.6%(61.6-86.2)
tDG+uricacid	68.8	80.7	73.9%(61.3-86.5)
TG+uricacid	75.0	71.0	76.7%(64.7-88.7)
Cholesterol+uricacid	62.5	64.5	66.6%(53.0-80.3)
tDG+adrenicacid+uricacid	**71.9**	**77.4**	**80.1%(68.9-91.4)**
TG+adrenicacid+uricacid	81.3	74.2	78.7%(67.2-90.3)
Cholesterol+adrenicacid+uricacid	62.5	87.1	75.6%(63.5-87.7)

PUBLICACION 3

Visceral adipose tissue phospholipid signature of insulin sensitivity and obesity. Metabolic fingerprints of insulin resistance dependent and independent of obesity

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Enviada

- Los resultados fueron estudio de patentabilidad por la Fundació Bosch y Gimpera-Universidad de Barcelona. Al final se decidió publicarlos.

RESUMEN PUBLICACION 3

Objetivos: El objetivo de este trabajo fue el estudio de perfiles metabólicos de fenotipos discordantes obesidad y elevada resistencia a la insulina en muestras tejido adiposo visceral (VAT). Se diferencian los siguientes objetivos:

1) Explorar el metaboloma de VAT en sujetos con elevada y baja resistencia la insulina con y sin obesidad y las rutas biológicas asociadas.

2) Identificar diferencias metabólicas entre los metabotipos.

3) Desarrollar un biomarcador de obesidad metabólicamente sana.

Metodología: Se aplicó un flujo metabolómico semicuantitativo UPLC-Q-Exactive-MS/MS en muestras de VAT de sujetos con sensibilidad a la insulina y resistencia a la insulina con obesidad (n=11 y n=25 respectivamente) y sensibilidad a la insulina y resistencia a la insulina en sujetos con normo-peso (n=25 y n=10, respectivamente). Un análisis de bosques aleatorios (en inglés, *Random Forest*), con doble validación cruzada repetida, fue utilizado para identificar los metabolitos discriminantes entre los dos grupos de estudios. El modelo multimetabolito fue creado mediante una regresión tipo LASSO (acrónimo del inglés, *least absolute shrinkage selection operator logistic*) y su rendimiento se evaluó mediante las áreas debajo la curva (AUC, acrónimo del inglés *area under the curve*) de las curvas ROC (acrónimo del inglés *receiver operating characteristic curves*).

Resultados: El metaboloma adiposo de la obesidad fue caracterizado por variaciones en el metabolismo de los amino ácidos, carbohidratos, lípidos y nucleótidos, entre otros. El modelo de bosques aleatorios clasificó correctamente un 85% de los sujetos con obesidad y con un p-valor <0.05. Cuando la obesidad coexistía con sensibilidad a la insulina, se observó una alteración del metabolismo de fosfolípidos con ácidos grasos de 18 átomos de carbonos. Un modelo multimetabolito fue creado con el glicerofosfatidilinositol (18:0), el glicerofosfatidiletanolamina (18:2), el glicerofosfatidilserina (18:0), el

glicerofosfatidilcolina (18:0/18:1), (18:2/18:2) y (18:2/18:3). Este biomarcador presentaba una elevada capacidad predictiva para identificar metabotipos de obesidad con sensibilidad a la insulina en una población con obesidad [AUC 96.7% (91.9-100)] y en una población general (sujetos con y sin obesidad) [AUC 87.6% (79.0-96.2)] con una sensibilidad y especificidad del 88.0% y 100% y 90.9% y 76.7%, respectivamente. La capacidad discriminativa del modelo, entre sujetos normopeso con sensibilidad a la insulina y la población con obesidad y entre la población general fue modesta [AUC 64.8% (44.19-85.4)] y [AUC 49.9% (36.1-63.6)] respectivamente y con una sensibilidad y especificidad del 70.0% y 68.0% y 52.0 y 58.7% en cada caso.

Conclusión: En este estudio se ha identificado signaturas metabólicas comunas y únicas de resistencia a la insulina, asociada y no asociada a la obesidad. Se ha proporcionado un modelo específico y sensible de fosfolípidos con ácidos grasos de 18 átomos de carbono para distinguir sujetos con obesidad y sensibilidad a la insulina, de los sujetos con obesidad y elevada resistencia a la insulina. La validación de estos hallazgos en muestras biológicas, obtenidas por métodos menos invasivos es necesario por su uso en la práctica clínica.

Visceral adipose tissue phospholipid signature of insulin sensitivity and obesity. Metabolic fingerprints of insulin resistance dependent and independent of obesity

Short running title: Visceral adipose tissue metabolomic fingerprints

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ABSTRACT

Objective: To define a metabolic signature in visceral adipose tissue (VAT) of insulin resistance (IR) dependent and independent of obesity with metabolomics.

Research Design and Methods: An untargeted UPLC-Q-Exactive metabolomic approach was performed on the VAT of insulin-sensitive and insulin-resistant subjects with obesity (N=11 and N=25, respectively) and insulin-sensitive and insulin-resistant subjects with non-obesity (N=25 and N=10, respectively). A multi-metabolite model was created with the least absolute shrinkage selection operator logistic regression for metabolic selection and the area under the curve (AUC) of the receiver operating characteristic curves for the evaluation of its global performance.

Results: The VAT metabolome in obesity was characterized by variations in the metabolism of amino acids, carbohydrates, lipids and nucleotides, among others, whereas in combination with high IR, it affected the metabolism of 18 carbon fatty acyl-containing phospholipid species. A multi-metabolite model created by glycerophosphatidylinositol (18:0), glycerophosphatidylethanolamine (18:2), glycerophosphatidylserine (18:0), glycerophosphatidylcholine (18:0/18:1), (18:2/18:2) and (18:2/18:3) presented a high predictive performance to identify the metabotype of 'insulin sensitivity (IS) obesity' among those people with obesity [AUC 96.7% (91.9-100)] and among all the study population [AUC 87.6% (79.0-96.2)].

Conclusions: We showed a unique and shared metabolic signature of IR dependent and independent of obesity. We provided a specific and sensitive model of C18-fatty acyl containing phospholipid metabolites to distinguish subjects with the 'IS obesity' metabotype from those with 'high IR obesity'. The validation of these findings in a more accessible sample such as blood is required for its use in clinical practice.

Obesity has become one of the most important global public health challenges as its prevalence is dramatically increasing. Obesity is usually accompanied by other comorbidities such as type 2 diabetes, insulin resistance (IR) and cardiovascular complications. However, the co-occurrence of obesity and IR hinders the study of the physiopathology associated with obesity or IR *per se*.

Adipose tissue is a metabolically dynamic organ. It is the primary site of storage for excess energy but it is also an endocrine organ that synthesizes a number of biologically active compounds which regulate metabolic homeostasis. Obesity is defined as the excessive growth of adipose tissue depots. A large individual variation in the size, expandability (1) and functionality (2) of the adipose tissue depots leads to a chronic state of 'low-grade' inflammation associated with the diverse risk of developing obesity-related comorbidities (3). In addition, changes in the secretion of these active compounds including leptin, adiponectin and pro-inflammatory molecules may also influence the risk of developing metabolic alterations (1,4). The existence of discordant phenotypes, subjects with obesity with high insulin sensitivity and non-obesity with high insulin resistance, suggest that rather than the amount of fat, the composition of adipose tissue may have a pivotal role in the study of the development of insulin resistance dependent and independent of obesity. However, due to the nature of this sample and the difficulty of obtaining large amounts, few studies analyzed the composition of VAT, especially in those subjects with non-obesity.

Visceral adipose tissue (VAT) is considered to be functionally and metabolically different from other types of adipose tissue, such as subcutaneous adipose tissue (SAT). VAT is more metabolically active than SAT and is characterized as an active endocrine organ with complex roles beyond energy storage (5). Alterations in VAT are closely linked to cardiometabolic abnormalities (6). The global analysis of the metabolic status of the VAT of individuals with obesity and high insulin sensitivity (IS) or high IR by a metabolomic driven approach will allow the profiling of these metabolic phenotypes (metabotypes) and the discovery of potential markers of metabolic healthy obesity (MHO).

The current study was designed to: 1) explore the metabolome of the VAT of subjects with high and low IR with and without obesity and the pathways associated; 2) identify metabolomic differences between metabotypes; and 3) develop a potential biomarker of MHO and high IR in subjects with obesity.

To fulfil these aims, we have performed an untargeted metabolomic driven approach on the VAT of human concordant and discordant phenotypes of obesity and high IR. Data have been analysed by univariate and multivariate statistical analysis, pathway enrichment analysis, regression analysis for variable selection and receiver operating characteristic (ROC) curves. The comprehensive analysis of the metabolome of concordant and discordant phenotypes of obesity and high IR may open the door to new diagnostic, prognostic, therapeutic and lifestyle strategies for the management of MHO and consequently prevent the progression of obesity related comorbidities.

RESEARCH DESIGN AND METHODS

Subjects and study design

Seventy-one adults (27 men and 44 women) were recruited at the Virgen de la Victoria University Hospital (Málaga, Spain). A detailed description of the study design and inclusion/exclusion criteria has previously been reported (7). Briefly, patients were excluded if they had an acute or chronic disease including type 2 diabetes and/or were on anti-hyperglycemic agents, insulin or any drugs that could alter the lipid profile.

Individuals were classified according to their body mass index (BMI), in subjects with non-obesity (BMI = 18.5–26.9 kg/m2) or subjects with morbid obesity (BMI > 40 kg/m^2), and by their risk of developing type 2 diabetes, based on fasting plasma glucose (FG) concentrations and Homeostatic Model Assessment-IR (HOMA-IR) as follows: low IR or IS state (FG < 100 mg/dL and HOMA-IR < 2.5) or high IR state (FG levels 100-125 mg/dL or HOMA-IR > 3.4). The HOMA-IR cut-off was experimentally obtained by dividing the entire initial cohort into quartiles as described previously (7). Individuals were classified into four sex matched phenotypic groups as follows: 1) subjects with low IR or IS and non-obesity, the so

called control group (N = 25); 2) subjects with IS and obesity (N = 11); 3) subjects with high IR and non-obesity (N = 10); and 4) subjects with high IR and obesity (N = 25) as described previously (7).

Anthropometric values and parameters were measured by means of standardized techniques as previously described (7,8). Biopsies of visceral adipose tissue (VAT) were obtained by laparoscopic surgery and frozen at -80ºC until assayed. The protocol was approved by the local Ethics and Research Committee (Hospital Universitario Virgen de la Victoria, Málaga, Spain) and all participants provided written informed consent.

Metabolomic profiling

Sample preparation and analytical metabolomic analysis were conducted at Metabolon Inc. (Durham, NC). Tissue extracts were prepared with the automated MicroLab STAR® system (Hamilton, Bonaduz, Switzerland) with methanol as previously described (9).

Two aliquots of tissue extracts were used for RP-UPLC-MS/MS with positive ion mode electrospray ionization (ESI+), in acidic conditions for hydrophilic and hydrophobic compounds, respectively, and one aliquot with negative ionization (ESI-) in basic conditions. The fourth aliquot was analysed by HILIC/UPLC-MS/MS with negative ion mode ESI. Samples were analysed on an ACQUITY UPLC system (Waters, Milford, MA), coupled with a Q-Exactive mass spectrometer and Orbitrap mass analyser (both from Thermo Scientific, Waltham, MA). The UPLC system was equipped with a UPLC C18 BEH (2.1x100 mm, 1.7 µm) or UPLC BEH Amide (2.1x150 mm, 1.7 µm) columns (Waters). The Q-Exactive system was interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap operated at 35,000 mass resolutions. The MS analysis covered 70-1000 m/z and alternated between MS and data-dependent MSn scans using dynamic exclusion. Further information has been detailed previously (9).

Statistical analysis

Data analysis was carried out in R (v.3.4). First, the dataset was filtered to remove ion compounds that had more than 80% missing values in all the groups. Then, data were logarithmically transformed and pareto scaled and the potential effects of gender, age and drug consumption were corrected. In addition, for multivariate analysis, data were also first imputed with the k-nearest neighbours algorithm (k=5) (10).

Univariate analysis was used to describe clinical and metabolic parameters. An ANOVA for unbalanced groups was performed to assess the effects of obesity and high IR and to compare between groups on clinical and metabolic parameters. All the *p* values were also corrected for multiple testing by the false discovery rate and only the metabolites with adjusted *p* values <0.05 were considered as significant.

The Random Forest (RF) modelling within an in-house developed repeated double cross validation (rdCV) was used to find the most discriminative metabolites between groups (11). The rdCV minimises statistical overfitting, improves modelling accuracy and reduces misclassifications. Further information on this procedure is detailed in (12,13). The following parameters were set: number of repetitions=20, metabolites in the outer loop=5, varRatio=0.8 and number of permutations=200. The validity of the model was assessed through the misclassification rate (<20%) and the fitness of randomly permuted classifications (*p* value<0.05).

Models of classification of 'IS obesity' metabotype

Variable selection was performed with the least absolute shrinkage and selection operator (LASSO) logistic regression using a leave-one-out cross-validation with metabolites with a *p* value <0.05 and adjusted *p* value <0.25. The LASSO method is a multivariate regression model that adds a penalization term to those metabolites that do not contribute to the model. Thus, the most predictive variables are selected into the model, while the coefficients of the remaining variables are shrunk to zero (14). The coefficients of the most predictive metabolites were used to create a new parameter, the multimetabolite biomarker. The global performance of the

multimetabolite biomarker model and its components was evaluated by receiver operating characteristic (ROC) curves: area under the curve (AUC) value, confidence intervals (CIs 95%), sensitivity and specificity. The performance of the multimetabolite biomarker was also assessed in the non-obesity population. The LASSO regression and ROC curves were performed with *glmnet* and *pROC* packages respectively.

Enrichment and correlation analysis

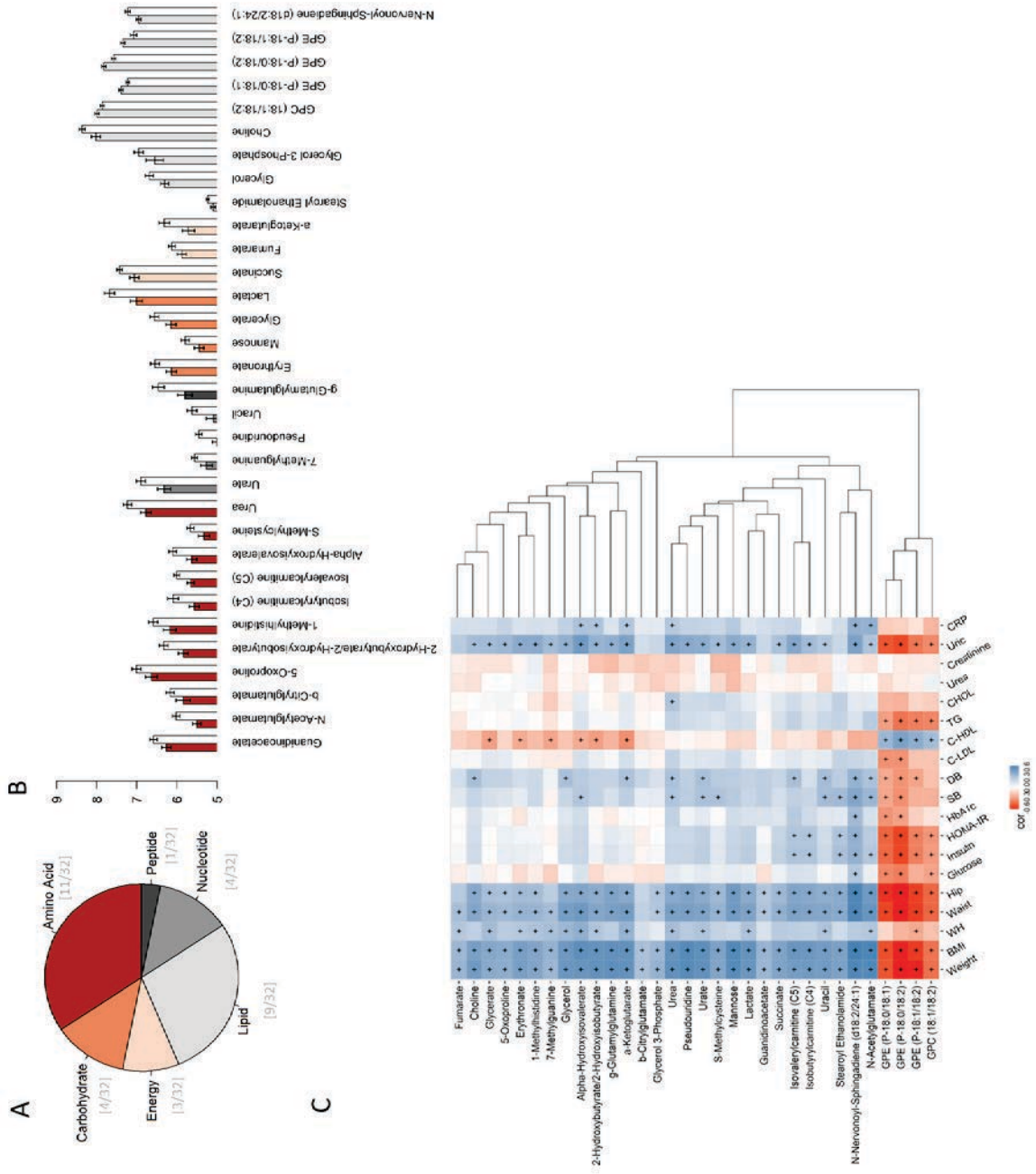
Enrichment analysis was performed by hypergeometric test. Spearman correlation coefficients were calculated to estimate the associations among the selected metabolites and clinical variables and they were represented as a hierarchical clustered correlation matrix with average distance. The *Hmisc* and *ggplot* packages were used for the analysis of correlations and the creation of a heatmap, respectively.

RESULTS

Clinical parameters

Subjects with obesity presented increased adiposity indicators, such as weight, BMI and waist and hip circumferences, as well as higher levels of liver damage markers and uric acid, and lower levels of cholesterol associated with HDL particles compared to individuals with normal weight. Individuals with high IR had elevated concentrations of IR indicators, including fasting glucose and insulin and HOMA-IR, increased levels of triglycerides and cholesterol associated to LDL particles and uric acid. No significant changes were observed in the interaction of obesity x high IR. As expected, concordant and discordant phenotypes of obesity (IS obesity versus high IR obesity) presented differences in IR markers, whereas phenotypes of high IR (high IR non-obesity versus high IR obesity) did not in adiposity and liver damage indicators (Table 1).

Figure 1. Selected metabolites from the comparison between subjects with obesity versus subjects with non-obesity by Random Forest. A) Summary of the chemical classes of the selected metabolites. B) Mean and standard error of the logarithmic transformation of the levels of the discriminant metabolites in the subjects with obesity (white bars) and those with normal weight (coloured and sorted according to the class of the metabolites). C) Hierarchical clustered spearman correlation matrix of the selected metabolites by the Random Forest analysis of subjects with obesity and non-obesity. Adjusted *p* values with significant threshold set at <0.05 are marked with +. Positive correlations are coloured in blue and negative correlations in red.


A
Amino Acid [11/32]
Peptide [1/32]
Nucleotide [4/32]
Lipid [9/32]
Energy [3/32]
Carbohydrate [4/32]
B
Guanidinoacetate
N-Acetylglutamate
b-Citrylglutamate
5-Oxoproline
2-Hydroxybutyrate/2-Hydroxyisobutyrate
1-Methylhistidine
Isobutyrylcarnitine (C4)
Isovalerylcarnitine (C5)
Alpha-Hydroxyisovalerate
S-Methylcysteine
Urea
Urate
7-Methylguanine
Pseudouridine
Uracil
g-Glutamylglutamine
Erythronate
Mannose
Glycerate
Lactate
Succinate
Fumarate
a-Ketoglutarate
Stearoyl Ethanolamide
Glycerol
Glycerol 3-Phosphate
Choline
GPC (18:1/18:2)
GPE (P-18:0/18:1)
GPE (P-18:0/18:2)
GPE (P-18:1/18:2)
N-Nervonoyl-Sphingadiene (d18:2/24:1)
9
8
7
6
5
C
Fumarate
Choline
Glycerate
5-Oxoproline
Erythronate
1-Methylhistidine
7-Methylguanine
Glycerol
Alpha-Hydroxyisovalerate
2-Hydroxybutyrate/2-Hydroxyisobutyrate
g-Glutamylglutamine
a-Ketoglutarate
b-Citrylglutamate
Glycerol 3-Phosphate
Urea
Pseudouridine
Urate
S-Methylcysteine
Mannose
Lactate
Guanidinoacetate
Succinate
Isovalerylcarnitine (C5)
Isobutyrylcarnitine (C4)
Uracil
Stearoyl Ethanolamide
N-Nervonoyl-Sphingadiene (d18:2/24:1)
N-Acetylglutamate
GPE (P-18:0/18:1)
GPE (P-18:0/18:2)
GPE (P-18:1/18:2)
GPC (18:1/18:2)
Weight
BMI
WH
Waist
Hip
Glucose
Insulin
HOMA-IR
HbA1c
SB
DB
C-LDL
C-HDL
TG
CHOL
Urea
Creatinine
Uric
CRP
cor
0.6 0.3 0 -0.3 -0.6

The VAT metabolome in obesity

A total of 422 different metabolites were identified among all the groups of the study. Univariate statistics revealed a variation of 118 metabolites in the VAT metabolome of subjects with obesity independent of high IR (adjusted *p* value of the interaction obesity x high IR >0.05) (Supplemental Table S1). Changes of these metabolites reflected changes in six metabolic pathways: 1) leucine, isoleucine and valine metabolism; 2) TCA cycle; 3) glutathione metabolism; 4) glycolysis, gluconeogenesis and pyruvate metabolism; 5) glycerolipid metabolism; and 6) glycine, serine and threonine metabolism (Supplemental Table S2).

The Random Forest model selected 32 of these metabolites that distinguished between subjects with obesity from those with normal weight (Figure 1A-B, Supplemental Table S3) with a 15 % of misclassification and *p* value<0.001. Among these metabolites, the ten most discriminative metabolites were urate, lactate, N-acetylglutamate, urea, 2-hydroxy(iso)butyrate, succinate, two plasmalogens glycerophosphaethanolamine (GPE), α-hydroxyisovalerate and γ-glutamylglutamine.

The correlation analysis showed negative correlations of glycerophosphatidylcholines and plasmalogens GPE with clinical variables except HDL particles. Positive correlations were observed between all the other 28 metabolites and weight related parameters. In addition, N-nervonoyl-sphingadiene (d18:2/24:1), N-acetylglutamate and steroyl ethanolamide and isobutyrylcarnitine and isovalerylcarnitine (carnitines C4 and C5 respectively) were also positively correlated with glycaemic parameters. Blood pressure correlated positively with choline, glycerol, α-hydroxyisovalerate, α-ketoglutarate, urea, urate, S-methylcysteine, carinitine C5, uracil, stearoyl ethanolamine, N-nervonoyl-sphingadiene (d18:2/24:1) and N-acetylglutamate. HDL particles were also negatively correlated with glycerate, erythronate, 7-methylguanine, α-hydroxyisovalerate, 2-hydroxyisobutyrate and α-ketoglutarate (Figure 1C).

The VAT metabolome in high IR

Subjects with high IR did not show differences in VAT metabolome when compared with subjects with IS. Neither did the effect of high IR in the metabolome depend on the obesity variable, interaction obesity x high IR (adjusted *p* value>0.05), except for plasmalogen GPE (P-18:0/18:2) higher levels were observed in subjects with IS, *p* value=0.001 (adjusted *p* value=0.077). The RF analysis could also not successfully classify subjects with IS from those with high IR (data not shown).

The VAT metabolome of discordant phenotypes: non-obesity with high IR and obesity with IS

The metabolites that distinguished between high IR phenotypes (subjects with high IR, non-obesity versus those with obesity) were the same metabolites observed in the comparison of non-obesity versus obesity, independent of high IR (Supplemental Table S1). No differences were observed between non-obesity phenotypes (subjects with non-obesity, with high IR versus those with IS).

The metabolome between 'IS obesity' and 'high IR obesity' only presented differences in the levels of the metabolite lysolipid GPE (18:2). Specifically, the levels of this lipid species were lower in the 'high IR obesity' metabotype. When we set the adjusted *p* value to 0.25 for descriptive purposes, other phospholipids containing fatty acyl groups of 18 carbons (C18) were also found to be in lower levels in the 'high IR obesity' metabotype. These phospholipids were the lysolipids GPE (18:1), glycerophosphatidylinositol (GPI) (18:0), glycerophosphatidylserine (GPS) (18:0), the GPE (18:0/18:1), GPC (18:0/18:2), GPC (18:2/18:2), GPC (18:2/18:3), GPC (18:2/20:4n6), GPE (18:0/18:2) and the plasmalogen GPE (P-18:0/18:2) (Figure 2A).

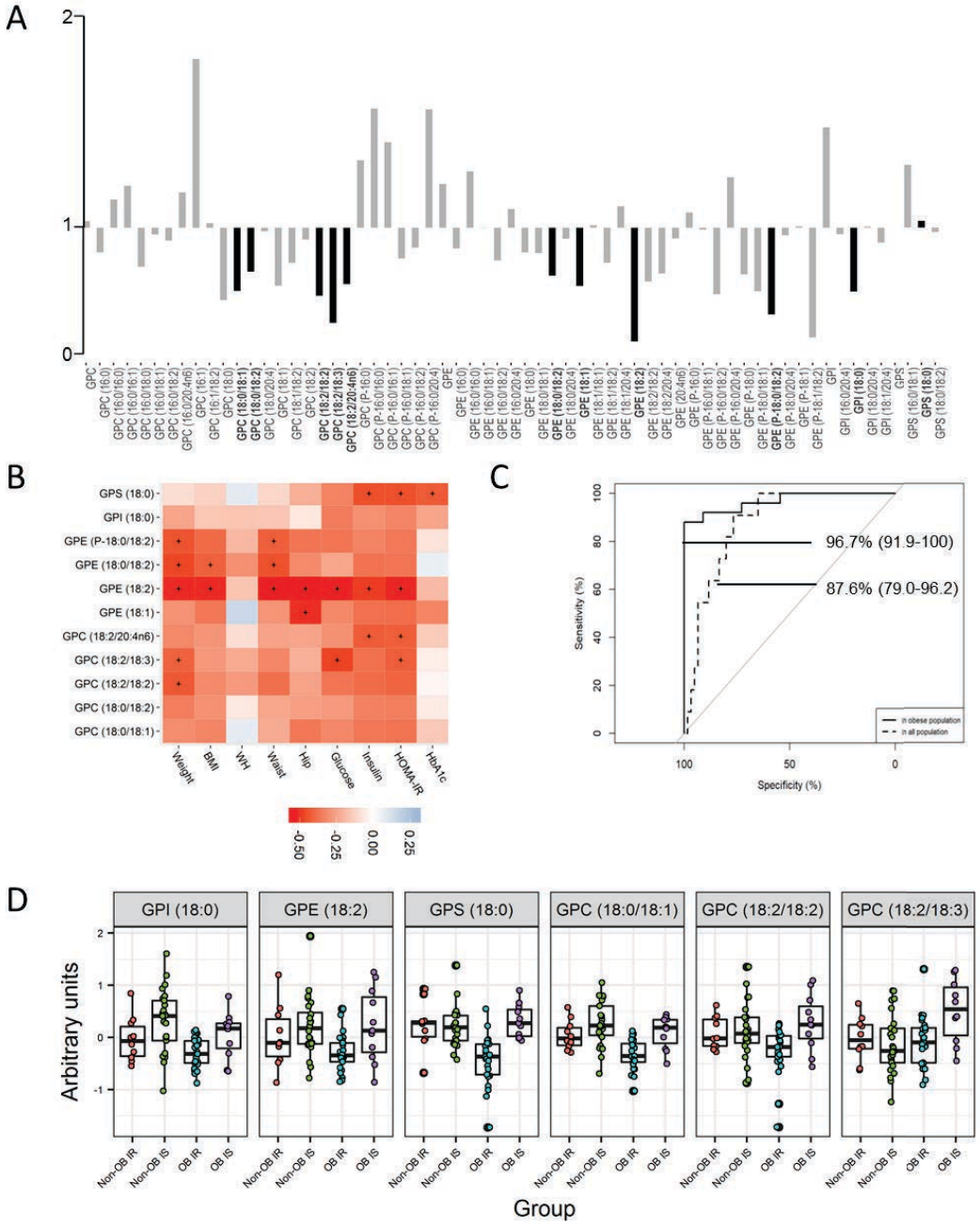
Figure 2. The VAT metabolome of the discordant phenotype of obesity: A) Fold changes in the levels of lipid species between subjects with obesity and high insulin resistance and those with obesity and insulin sensitivity. Lipids significantly different between groups were marked in a dark colour and with bold text (adjusted *p* value<0.25). B) Spearman correlation matrix of the selected lipids with clinical variables. Adjusted *p* values with a threshold set at <0.05 are marked with +. Positive correlations are coloured in blue and negative correlations in red. C) ROC

curves (AUC%, CI 95%) of the multimetabolite biomarker model to identify the 'IS obesity' metabotype among the population with obesity (IS and high IR), or all the subjects of the study (normal weight and obesity and/or IS or high IR). The model was formed by GPE 18:2, GPI 18:0, GPS 18:0, GPC aa 36:1, GPC aa 36:4 and GPC aa 36:5, selected by the LASSO method. D) Boxplot of the levels of the individual metabolites of the multimetabolite biomarker after logarithmic transformation and pareto scaled. Abbreviations: AUC, area under the curve; CI, confidence interval; GPC, glycerophosphatidylcholine; GPE, glycerophosphatidylethanolamine; GPI, glycerophosphatidylinositol; PS, glycerophosphatidylserine.

Figure 2B shows that these species negatively correlated with weight parameters. It is noteworthy that correlations between GPE (18:2) and weight, waist and hip circumferences and fasting glucose, fasting insulin and HOMA-IR were statistically significant.

The model of classification of the 'IS obesity' metabotype was achieved with regression analysis based on the LASSO method. This method was used to select those metabolites that better explained the differences between the ‘IS obesity' and ‘high IR obesity’ metabotype. GPE (18:2) presented a high ability to detect the ‘IS obesity’ metabotype when only analysing the subset with subjects with obesity [AUC 89.1% (78.8-99.4)] and all the population of the study, comprised by non-obesity and subjects with obesity [AUC 71.8% (56.5-87.2)] (Table 2, Figure 2C). However, when this lysolipid was combined with the C18-containing phospholipids GPI (18:0), GPS (18:0), GPC (18:0/18:1), GPC (18:2/18:2) and GPC (18:2/18:3) (Figure 2D), the discriminative ability of this metabolite panel increased, yielding values of AUC 96.7% (91.9-100) in the subset of subjects with obesity and AUC 87.6% (79.0-96.2) in all the study population (Table 2, Figure 2C).

The discriminative ability of the combined multimetabolite model between subjects with ‘IS non-obesity’ in non-obesity population and in all the study population was AUC 64.8% (44.19-85.4) and 49.9% (36.1-63.6) respectively and a sensitivity and specificity of 70.0% and 68.0 % and 52.0% and 58.7 % in each case.


A
B
C
D
96.7% (91.9-100)
87.6% (79.0-96.2)
Sensitivity (%)
Specificity (%)
n obese population
n all population
Arbitrary units
Group
Non-OB IR
Non-OB IS
OB IR
OB IS
GPI (18:0)
GPE (18:2)
GPS (18:0)
GPC (18:0/18:1)
GPC (18:2/18:2)
GPC (18:2/18:3)

CONCLUSIONS

We provided a comprehensive VAT metabolic profiling of concordant/discordant phenotypes of obesity and high IR to deepen the understanding of the intricate relationship between obesity and high IR. To that aim, we identified a VAT multimetabolite panel specific and sensitive for discriminating subjects with IS obesity from those with high IR obesity and also from the overall population. This panel presented a modest ability to discriminate those subjects with normal weight and IS in non-obesity population and in the overall population.

Firstly, in line with previous findings, the VAT metabolome of patients with obesity extensively differentiated from patients with normal to overweight. The pathway analysis revealed changes in the branched chain amino acids (BCAA) in obesity. Alterations in the leucine, isoleucine and valine metabolism were mirrored by changes in the carnitines C4 and C5, originated from BCAA metabolism (15) and α-hydroxyisovalerate. The levels of BCAA tend to be elevated in subjects with obesity, and raised levels of circulating BCAA are associated with forthcoming high IR or type 2 diabetes (16). In subjects with obesity, the impairment of the mitochondrial metabolism may cause increased levels of C4 and C5 by lowering fatty acid oxidation (17). In turn, mitochondrial dysfunction has been associated with high IR (18). However, the interaction high IR x obesity was not found to be statistically significant in this study.

The lipidomic profiling of multiple populations and clinical cohorts has identified that decreased levels of plasmalogens are associated with obesity, as well as with pre-diabetes and diabetes (19). Our results showed that those patients with obesity presented lower levels of phospholipids, specifically GPC and GPE plasmalogens, than those with normal weight, as previously observed (20). Plasmalogens are powerful antioxidants and their upregulation may reduce oxidative stress, improve the lipid dysregulation that accompanies obesity, suppress inflammatory responses and ameliorate the high IR associated with metabolic diseases.

Comparisons between groups suggested that high IR might also modify the metabolism of phospholipids in subjects with obesity, but not in patients with

normal weight. Specifically, GPE (18:2) and other phospholipid species also containing C18-fatty acyl groups including GPE (18:1), GPI (18:0), GPS (18:0), GPC (18:0/18:1), GPC (18:2/18:2) and GPC (18:2/18:3) were lower in the 'high IR obesity' metabotype. In addition, GPE (18:0/18:2) also presented lower levels in subjects with high IR than with IS, regardless of the adiposity. Phospholipids are formed by fatty acyl groups attached to their *sn*1 and *sn*2 of the glycerol backbone (21). They are first formed in the *de novo* pathway from glycerol-3-phosphate and then matured in the remodelling pathway. In this second step, the concerted action of phospholipases A2 (PLA2) and phospholipid acyltransferases (AT) establish the asymmetry and high diversity of phospholipids (22). The phospholipid species GPC and GPE are the major constituents of the plasma membrane, whereas GPI and GPS are less abundant in the cell (23). Lysolipids proceed from the cleavage of an acyl chain of phospholipids by the enzyme PLA2. They play a structural role and act as lipid mediators involved in cell signalling (24).

Changes in arachidonyl-containing lipid species seemed to be less intense in our study than previously reported (21,25), as their levels were not significantly altered in obesity or high IR. Thus, we hypothesize that the occurrence of high IR in subjects with obesity promotes a higher pro-inflammatory state, thus accentuating a greater decrease of C18-containing lipid species. On the other hand, inflammation and oxidative stress are closely interconnected processes (26). The free radicals produced during the inflammatory reaction can also damage phospholipids, especially plasmalogens, thereby reducing their levels (27). How this lipid remodelling affects the plasma membrane is controversial. While some report that the biophysical properties of the membrane remain unaltered (25), others suggest changes in membrane potential and permeability (28) and altered receptor signalling (24). Obesity and related comorbidities lead to expansion, differentiation and remodelling of adipocytes (4). Pietiläinen et al. studied the adipocyte remodelling in monozygotic twin pairs discordant for BMI. The authors observed that the twin with obesity had higher proportions of palmitoleic (C16:1) and arachidonic (C20:4) acids in their adipose tissue and lower levels of saturated fatty acid, linoleic (C18:2) and α-linoleic (C18:3) acids (25). Other studies showed that arachidonyl-containing

species increased during adipocyte differentiation, whereas linolenic-containing lipids decreased due to the enhanced activity of the enzyme AT (22). Phospholipids with arachidonyl groups positively correlated with BMI and increased risk of metabolic syndrome (22). Engelman et al. performed a study on the erythrocyte plasma membrane in dyslipidemia. They also reported that under pathological conditions the enzyme PLA2 is overexpressed and transforms fatty acyl groups linked to GPE and GPC into arachidonyl-containing GPE and GPC (21). Arachidonyl acyl chains are further converted into pro-inflammatory metabolites such as prostaglandins and eicosanoids, and promote a pro-inflammatory response via PPARγ receptors (21,22,25). Pietiläinen et al. also suggested that this pro-inflammatory environment caused by arachidonyl groups makes the adipocytes more vulnerable and prone to inflammatory responses and oxidation (25).

These C18-fatty acyl containing phospholipids presented a high discriminative ability to distinguish the ‘IS obesity’ metabotype from the ‘high IR obesity’ one, but also identify it among all the study population, including subjects with non-obesity. Specifically, GPE (18:2) was the metabolite that presented higher AUC and sensitivity and specificity rates. In addition, we carried out a regression analysis to create a multimetabolite biomarker model to enhance the prediction accuracy of the ‘IS obesity’ metabotype. The resulting model consisted of the combination of GPE (18:2) with other 18 carbon-containing phospholipids GPE (18:1), GPI (18:0), GPS (18:0), GPC (18:0/18:1), GPC (18:2/18:2) and GPC (18:2/18:3). This model presented a very high discriminative ability to identify the ‘IS obesity’ metabotype from ‘high IR obesity’, and distinguish it from all the metabotypes of the study. However, the scarce ability to discriminate subjects with ‘IS non-obesity’ and a missing alternative biomarker to identify this group within all the population, may suggest a different metabolic connection of IR in subjects with obesity than in those subjects with non-obesity.

To our knowledge, there are no studies reporting a model based on levels of metabolites to discriminate subjects with obesity and IS. We propose for the first time a panel of metabolites consisting of phospholipids to identify accurately the ‘IS

obesity' metabotype and potentially MHO subjects. This model might also allow clinicians to monitor the progression from IS to high IR in the population with obesity. Nevertheless, to that aim further research needs to be done in a greater and independent cohort to validate this biomarker model in adipose tissue and identify it in a more accessible biological sample such as blood samples. In addition, it will also be necessary to explore the progression of these lipid species in the pathological state, i.e. obesity with one or more comorbidities, in order to define normality intervals and a disease cut-off.

In conclusion, our study showed that obesity is associated with a huge alteration in the composition of VAT. Subjects with IS and obesity present a modification of the metabolism of phospholipids containing C18-fatty acyl groups. This lipid remodelling might promote pro-inflammatory responses in VAT, which would be enhanced when the patient presents both conditions at the same time. The particular combination of GPE (18:2), GPI (18:0), GPS (18:0), GPC (18:0/18:1), GPC (18:2/18:2) and GPC (18:2/18:3) configure a sensitive and specific biomarker to distinguish subjects with 'IS obesity' metabotype from those with 'high IR obesity'. These findings suggest a useful biomarker for 'IS obesity' in obesity and in the overall population.

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Author contributions

S.T., F.J.-T and C.A.-L. designed the research; M.P.-R. and A.M.R. conducted the research; M.P.-R., A.M.-R, A.M. and A.S.-P., performed the statistical analysis; M.P.R. and A.M.-R. performed the data visualization; M.P.R., A.M.-R., M.M. and P.C.-A. wrote the paper; All the authors contributed to the discussion and/or reviewed/edited the manuscript. All authors read and approved the final manuscript. C.A.L is the guarantor of this work and, as such, had full access to the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflict of interest statement

No potential conflicts of interest relevant to this article were reported

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TABLES

Table 1. Anthropometric and clinical parameters of the subjects of the study*.

							Adjusted *p* value			
	Non-OB IS	**Non-OB IR**	**OB IS**	**OB IR**	**OB**	**IR**	**OB x IR**	**OB IS vs OB IR**	**Non-OB IR vs OB IR**	**Non-OB IS vs non-OB IR**
Gender	M=7, F=18	M=3, F=7	M=3, F=8	M=14, F=11	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Age [years]	47.56 ± 13.66	54.90 ± 13.62	40.36 ± 10.64	42.84 ± 8.99	0.004	n.s.	n.s.	n.s.	0.003	n.s.
Weight [kg]	64.56 ± 8.64	66.10 ± 5.63	125.97 ± 15.00	154.80 ± 31.20	5.92E-24	0.040	n.s.	n.s.	1.08E-08	n.s.
BMI [kg/cm^2]	23.75 ± 2.12	25.30 ± 1.64	46.14 ± 4.58	53.63 ± 9.05	5.38E-26	0.009	n.s.	n.s.	1.08E-08	n.s.
Waist [cm]	81.88 ± 7.93	92.70 ± 3.65	128.00 ± 16.18	146.62 ± 21.73	1.69E-18	0.004	n.s.	n.s.	2.34E-06	0.038
Hip [cm]	94.88 ± 8.26	101.30 ± 3.66	142.40 ± 11.07	151.25 ± 17.80	1.78E-18	0.031	n.s.	n.s.	2.78E-06	n.s.
Waist/Hip [ratio]	0.81 ± 0.04	0.90 ± 0.67	0.87 ± 0.11	0.97 ± 0.11	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fasting glucose [mmol/L]	89.96 ± 7.59	115.50 ± 14.93	88.82 ± 4.58	115.68 ± 11.63	n.s.	2.67E-13	n.s.	1.15E-07	n.s.	4.34E-05
Fasting insulin [μU/mL]	5.33 ± 2.04	12.36 ± 4.04	8.19 ± 2.16	24.81 ± 13.25	0.003	1.03E-10	n.s.	2.89E-06	n.s.	9.13E-04
HbA1c [%]	5.32 ± 0.40	6.14 ± 0.61	5.63 ± 0.10	5.69 ± 0.47	n.s.	n.s.	n.s.	n.s.	n.s.	0.002

HbA1c [mmol/mol]	34.59 ± 4.36	43.59 ± 9.24	37.96 ± 1.05	38.68 ±5.11	n.s.	n.s.	n.s.	n.s.	n.s.	0.002
HOMA-IR [index]	1.18 ± 0.47	3.46 ± 0.99	1.80 ± 0.51	7.10 ± 3.81	0.004	5.75E-14	n.s.	1.15E-07	n.s.	4.34E-05
Systolic pressure [mm Hg]	115.42 ± 13.39	133.70 ± 27.61	131.50 ± 22.55	135.61 ± 18.38	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Diastolic pressure [mm Hg]	71.88 ± 11.54	79.20 ± 8.61	82.00 ± 10.73	84.33 ± 13.43	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Total cholesterol [mmol/L]	179.20 ± 25.26	241.60 ± 42.61	190.18 ± 50.15	201.76 ± 34.53	n.s.	0.003	n.s.	n.s.	n.s.	0.004
HDL [mmol/L]	55.80 ± 10.59	55.60 ± 17.61	45.73 ± 15.43	39.92 ± 12.95	0.022	n.s.	n.s.	n.s.	n.s.	n.s.
LDL [mmol/L]	105.74 ± 24.40	153.04 ± 41.62	107.51 ± 45.71	134.50 ± 28.57	n.s.	8.28E-04	n.s.	n.s.	n.s.	0.018
Triglycerides [mmol/L]	82.36 ± 39.52	166.00 ± 78.62	121.64 ± 112.87	144.47 ± 48.28	n.s.	0.003	n.s.	n.s.	n.s.	0.010
C-reactive protein [mg/L]	2.98 ± 0.77	5.95 ± 2.62	13.80 ± 12.51	8.87 ± 6.24	n.s.	n.s.	n.s.	n.s.	n.s.	0.010
GOT [U/L]	14.60 ± 6.24	14.60 ± 9.62	21.91 ± 8.87	24.64 ± 11.84	1.96E-04	n.s.	n.s.	n.s.	0.018	n.s.
GPT [U/L]	27.48 ± 9.29	32.50 ± 14.62	38.91 ± 16.49	54.20 ± 19.26	2.32E-04	n.s.	n.s.	n.s.	0.018	n.s.
GGT [U/L]	23.64 ± 14.86	37.35 ± 47.62	26.27 ± 17.86	53.88 ± 50.74	0.006	n.s.	n.s.	n.s.	0.003	n.s.
Uric acid [mmol/L]	3.74 ± 0.84	4.76 ± 1.62	5.04 ± 0.78	6.49 ± 1.46	6.01E-05	0.003	n.s.	n.s.	n.s.	0.045
Creatinine [mmol/L]	0.77 ± 0.20	0.77 ± 0.62	0.74 ± 0.17	0.84 ± 0.18	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Urea [mg/dL]	30.05 ± 7.95	35.00 ± 7.62	27.90 ± 7.82	32.68 ± 12.13	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

*Values are shown as Mean ± SD unless otherwise indicated. *P* values were determined ANOVA model and t-test after log-transformed variables where appropriated and corrected for multiple testing by the false discovery rate

Abbreviations: BMI, body mass index; c- LDL, low-density lipoproteins cholesterol; HDL, high-density lipoproteins cholesterol; GOT, aspartate transaminase; GPT, alanine transaminase; GGT, γ-glutamyl transferase; F, female; HbA1c, glycated haemoglobin A1c; HOMA-IR, insulin resistance calculated by homeostatic model assessment; IR, high insulin resistance; IS, insulin sensitivity; M, male; n.s., not significant; OB, obesity; SD, standard deviation.

Table 2. ROC curve parameters of the combined multimetabolite biomarker model to detect subjects with obesity and IS, and of the individual metabolites that are part of this model. Their predictive power was assessed in all the obese subset and in all the study population including individuals with non-obesity.

Detection of OB IS	Sensitivity (%)	Specificity (%)	AUC (95% CI)
Only in population with obesity			
Combined multimetabolite model	88.0	100	96.7 (91.9-100)
GPE 18:2	76.0	90.9	89.1 (78.8-99.4)
GPI 18:0	76.0	81.8	77.5 (56.7-98.2)
GPS 18:0	84.0	72.7	78.8 (61.1-96.8)
GPC aa 18:0/18:1 (GPC aa 36:1)	88.0	63.6	79.3 (61.0-97.6)
GPC aa 18:2/18:2 (GPC aa 36:4)	72.0	72.7	69.5 (48.7-90.2)
GPC aa 18:2/18:3 (GPC aa 36:5)	72.0	72.7	71.6 (50.0-93.3)
In all the study population			
Combined multimetabolite model	90.9	76.7	87.6 (79.0-96.2)
GPE 18:2	63.6	66.7	71.8 (56.5-87.2)
GPI 18:0	81.8	66.7	70.2 (50.3-90.0)
GPS 18:0	72.7	81.7	77.9 (61.9-93.9)
GPC aa 18:0/18:1 (GPC aa 36:1)	63.6	66.7	61.4 (43.3-79.5)
GPC aa 18:2/18:2 (GPC aa 36:4)	72.7	55.0	53.0 (35.1-70.9)
GPC aa 18:2/18:3 (GPC aa 36:5)	72.7	53.3	60.3 (39.1-81.5)

Metabolites are sorted alphabetically. Abbreviations: aa, diacyl; AUC, area under the curve; CI, confidence interval; GPC, glycerophosphatidylcholine; GPE, glycerophosphatidylethanolamine; GPI, glycerophosphatidylinositol; GPS, glycerophosphatidylserine.

Material Suplementario

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Table S1. Statistical significance of discriminative metabolites according to the patient group in obesity, high IR, interaction of both metabolic statuses and discordant phenotypes of both. *P* values were corrected for multiple testing by the false discovery rate (adjusted *p* value) are. Metabolites are sorted alphabetically, according to their metabolic pathway. Abbreviations: IR, insulin resistance; IS, insulin sensitivity; OB, obesity; n.s., not significant. *Compounds identified with a very high level of confidence, but not validated with a standard; # *p* value <0.01 (raw *p* value).

Metabolites	Metabolic pathway	Adjusted *p* value					
		OB	HIR	OB x H IR	OB, IS vs IR	HIR, Non-OB vs OB	Non-OB, IS vs IR
Amino acids							
N-Acetylalanine	Alanine and Aspartate Metabolism	0.036	n.s.	n.s.	n.s.	n.s.	n.s.
N-Acetylasparagine	Alanine and Aspartate Metabolism	0.045	n.s.	n.s.	n.s.	n.s.	n.s.
Creatinine	Creatine Metabolism	0.001	n.s.	n.s.	n.s.	n.s.	n.s.
Guanidinoacetate	Creatine Metabolism	0.001	n.s.	n.s.	n.s.	n.s	n.s.
Beta-Citrylglutamate	Glutamate Metabolism	0.014	n.s.	n.s.	n.s.	0.034	n.s.
Glutamine	Glutamate Metabolism	0.013	n.s.	n.s.	n.s.	n.s.	n.s.
N-Acetylglutamate	Glutamate Metabolism	1.92E-06	n.s.	n.s.	n.s.	0.003	n.s.
N-Acetylglutamine	Glutamate Metabolism	0.002	n.s.	n.s.	n.s.	0.018	n.s.
2-Hydroxybutyrate/2-Hydroxyisobutyrate	Glutathione Metabolism	2.34E-05	n.s.	n.s.	n.s.	0.015	n.s.
5-Oxoproline	Glutathione Metabolism	0.007	n.s.	n.s.	n.s.	n.s.	n.s.
Cysteine-Glutathione Disulfide	Glutathione Metabolism	7.79E-04	n.s.	n.s.	n.s.	0.039	n.s.
Glutathione, Reduced (GSH)	Glutathione Metabolism	0.017	n.s.	n.s.	n.s.	n.s.	n.s.
Ophthalmate	Glutathione Metabolism	0.004	n.s.	n.s.	n.s.	n.s.	n.s.
Betaine	Glycine, Serine and Threonine Metabolism	9.52E-04	n.s.	n.s.	n.s.	n.s.	n.s.
Dimethylglycine	Glycine, Serine and Threonine Metabolism	0.006	n.s.	n.s.	n.s.	n.s.	n.s.
Glycine	Glycine, Serine and Threonine Metabolism	0.037	n.s.	n.s.	n.s.	n.s.	n.s.
N-Acetylglycine	Glycine, Serine and Threonine Metabolism	0.008	n.s.	n.s.	n.s.	n.s	n.s.
N-Acetylthreonine	Glycine, Serine and Threonine Metabolism	0.002	n.s.	n.s.	n.s.	n.s	n.s.
1-Methylhistidine	Histidine Metabolism	7.79E-04	n.s.	n.s.	n.s.	0.018	n.s.
1-Methylimidazoleacetate	Histidine Metabolism	0.002	n.s.	n.s.	n.s.	n.s.	n.s.
Histidine	Histidine Metabolism	0.010	n.s.	n.s.	n.s.	n.s.	n.s.
Imidazole Lactate	Histidine Metabolism	0.004	n.s.	n.s.	n.s.	n.s.	n.s.
2-Methylbutyrylcarnitine (C5)	Leucine, Isoleucine and Valine Metabolism	0.001	n.s.	n.s.	n.s.	0.045	n.s.
3-Methyl-2-Oxobutyrate	Leucine, Isoleucine and Valine Metabolism	0.026	n.s.	n.s.	n.s.	n.s	n.s.
3-Methyl-2-Oxovalerate	Leucine, Isoleucine and Valine Metabolism	0.023	n.s.	n.s.	n.s.	n.s.	n.s.
4-Methyl-2-Oxopentanoate	Leucine, Isoleucine and Valine Metabolism	0.019	n.s.	n.s.	n.s.	n.s.	n.s.
Alpha-Hydroxyisovalerate	Leucine, Isoleucine and Valine Metabolism	2.74E-04	n.s.	n.s.	n.s.	0.018	n.s.
Beta-Hydroxyisovalerate	Leucine, Isoleucine and Valine Metabolism	0.005	n.s.	n.s.	n.s.	n.s	n.s.
Isobutyrylcarnitine (C4)	Leucine, Isoleucine and Valine Metabolism	3.04E-04	n.s.	n.s.	n.s.	0.048	n.s.
Isoleucine	Leucine, Isoleucine and Valine Metabolism	0.034	n.s.	n.s.	n.s.	n.s.	n.s.
Isovalerylcarnitine (C5)	Leucine, Isoleucine and Valine Metabolism	1.31E-04	n.s.	n.s.	n.s.	n.s	n.s.
2-Aminoadipate	Lysine Metabolism	0.021	n.s.	n.s.	n.s.	n.s	n.s.
Pipecolate	Lysine Metabolism	0.019	n.s.	n.s.	n.s.	n.s.	n.s.
Cysteine	Methionine, Cysteine, SAM and Taurine Metabolism	0.002	n.s.	n.s.	n.s.	0.015	n.s.

S-Adenosylhomocysteine (SAH)	Methionine, Cysteine, SAM and Taurine Metabolism	0.037	n.s.	n.s.	n.s.	n.s.	n.s.
S-Methylcysteine	Methionine, Cysteine, SAM and Taurine Metabolism	0.002	n.s.	n.s.	n.s.	n.s	n.s.
Taurine	Methionine, Cysteine, SAM and Taurine Metabolism	0.004	n.s.	n.s.	n.s.	n.s	n.s.
Kynurenine	Tryptophan Metabolism	0.003	n.s.	n.s.	n.s.	n.s.	n.s.
Tryptophan Betaine	Tryptophan Metabolism	0.004	n.s.	n.s.	n.s.	n.s.	n.s.
Citrulline	Urea cycle, Arginine and Proline Metabolism	0.034	n.s.	n.s.	n.s.	n.s.	n.s.
Ornithine	Urea cycle, Arginine and Proline Metabolism	0.010	n.s.	n.s.	n.s.	n.s.	n.s.
Proline	Urea cycle, Arginine and Proline Metabolism	0.038	n.s.	n.s.	n.s.	n.s.	n.s.
Trans-4-Hydroxyproline	Urea cycle, Arginine and Proline Metabolism	0.045	n.s.	n.s.	n.s.	n.s.	n.s.
Urea	Urea cycle, Arginine and Proline Metabolism	5.18E-05	n.s.	n.s.	n.s.	0.018	n.s.
<u>Carbohydrates</u>							
Erythronate*	Aminosugar Metabolism	0.001	n.s.	n.s.	n.s.	n.s	n.s.
N-Acetylglucosamine/N-Acetylgalactosamine	Aminosugar Metabolism	0.011	n.s.	n.s.	n.s.	n.s.	n.s.
N-Acetylglucosaminylasparagine	Aminosugar Metabolism	0.045	n.s.	n.s.	n.s.	n.s.	n.s.
Mannose	Fructose, Mannose and Galactose Metabolism	3.94E-04	n.s.	n.s.	n.s.	0.018	n.s.
1,5-Anhydroglucitol (1,5-AG)	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.003	n.s.	n.s.	n.s.	n.s.	n.s.
3-Phosphoglycerate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.003	n.s.	n.s.	n.s.	0.038	n.s.
Glucose	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.027	n.s.	n.s.	n.s.	n.s.	n.s.
Glycerate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.001	n.s.	n.s.	n.s.	0.045	n.s.
Lactate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.57E-05	n.s.	n.s.	n.s.	0.005	n.s.
Phosphoenolpyruvate (PEP)	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.018	n.s.	n.s.	n.s.	n.s	n.s.
Cofactors and vitamins							
Threonate	Ascorbate and Aldarate Metabolism	0.001	n.s.	n.s.	n.s.	0.043	n.s.
Heme	Hemoglobin and Porphyrin Metabolism	0.010	n.s.	n.s.	n.s.	0.007	n.s.
N1-Methyl-2-Pyridone-5-Carboxamide	Nicotinate and Nicotinamide Metabolism	0.026	n.s.	n.s.	n.s.	n.s.	n.s.
Alpha-Tocopherol	Tocopherol Metabolism	0.002	n.s.	n.s.	n.s.	n.s.	n.s.
Gamma-Tocopherol/Beta-Tocopherol	Tocopherol Metabolism	0.045	n.s.	n.s.	n.s.	n.s.	n.s.
Threonate	Ascorbate and Aldarate Metabolism	0.001	n.s.	n.s.	n.s.	n.s.	n.s.
<u>Energy</u>							
Alpha-Ketoglutarate	TCA Cycle	1.31E-04	n.s.	n.s.	n.s.	0.016	n.s.
Fumarate	TCA Cycle	0.013	n.s.	n.s.	n.s.	n.s.	n.s.
Malate	TCA Cycle	0.011	n.s.	n.s.	n.s.	n.s.	n.s.
Succinate	TCA Cycle	1.31E-04	n.s.	n.s.	n.s.	0.016	n.s.
Succinylcarnitine (C4-DC)	TCA Cycle	0.045	n.s.	n.s.	n.s.	n.s.	n.s.
Lipids							

Deoxycarnitine	Carnitine Metabolism	0.038	n.s.	n.s.	n.s.	n.s.	n.s.
Ceramide (d18:1/14:0, d16:1/16:0)*	Ceramides	0.021	n.s.	n.s.	n.s.	n.s	n.s.
Stearoyl Ethanolamide	Endocannabinoid	0.004	n.s.	n.s.	n.s.	n.s.	n.s.
Propionylcarnitine (C3)	Fatty Acid Metabolism (also BCAA Metabolism)	0.026	n.s.	n.s.	n.s.	n.s.	n.s.
3-Hydroxybutyrylcarnitine (1)	Fatty Acid Metabolism(Acyl Carnitine)	0.013	n.s.	n.s.	n.s.	n.s.	n.s.
Acetylcarnitine (C2)	Fatty Acid Metabolism(Acyl Carnitine)	0.010	n.s.	n.s.	n.s.	n.s	n.s.
Linoleoylcarnitine (C18:2)*	Fatty Acid Metabolism(Acyl Carnitine)	0.038	n.s.	n.s.	n.s.	n.s.	n.s.
Myristoleoylcarnitine (C14:1)*	Fatty Acid Metabolism(Acyl Carnitine)	n.s.	n.s.	n.s.	n.s.	0.046	n.s.
Oleoylcarnitine (C18:1)	Fatty Acid Metabolism(Acyl Carnitine)	0.009	n.s.	n.s.	n.s.	n.s	n.s.
Palmitoleoylcarnitine (C16:1)*	Fatty Acid Metabolism(Acyl Carnitine)	n.s.	n.s.	n.s.	n.s.	0.044	n.s.
Palmitoylcarnitine (C16)	Fatty Acid Metabolism(Acyl Carnitine)	0.002	n.s.	n.s.	n.s.	0.041	n.s.
2-Hydroxyglutarate	Fatty Acid, Dicarboxylate	3.52E-04	n.s.	n.s.	n.s.	0.016	n.s.
Glycerol	Glycerolipid Metabolism	6.75E-04	n.s.	n.s.	n.s.	0.015	n.s.
Glycerol 3-Phosphate	Glycerolipid Metabolism	0.003	n.s.	n.s.	n.s.	n.s.	n.s.
Glycerophosphoglycerol	Glycerolipid Metabolism	0.032	n.s.	n.s.	n.s.	n.s.	n.s.
3-Hydroxybutyrate (BHBA)	Ketone Bodies	0.024	n.s.	n.s.	n.s.	n.s.	n.s.
1-Linoleoyl-GPE (18:2)*	Lysolipid	0.035	n.s.	n.s.	0.017	0.026	n.s.
1-Oleoyl-GPE (18:1)	Lysolipid	n.s.	n.s.	n.s.	n.s.#	n.s.	n.s.
1-Stearoyl-GPI (18:0)	Lysolipid	n.s.	n.s.	n.s.	n.s.#	n.s.	n.s.
1-Stearoyl-GPS (18:0)	Lysolipid	n.s.	n.s.	n.s.	n.s.#	n.s.	n.s.
1-(1-Enyl-Oleoyl)-2-Linoleoyl-GPE (P-18:1/18:2)*	Lysoplasmalogen	2.74E-04	n.s.	n.s.	n.s.	0.005	n.s.
1,2-Dilinoleoyl-GPC (18:2/18:2)	Phospholipid Metabolism	n.s.	n.s.	n.s.	n.s.#	0.045	n.s.
1-Linoleoyl-2-Arachidonoyl-GPC (18:2/20:4n6)*	Phospholipid Metabolism	n.s.	n.s.	n.s.	n.s	0.045	n.s.
1-Linoleoyl-2-Linolenoyl-GPC (18:2/18:3)*	Phospholipid Metabolism	n.s.	n.s.	n.s.	n.s.#	0.043	n.s.
1-Oleoyl-2-Linoleoyl-GPC (18:1/18:2)*	Phospholipid Metabolism	0.019	n.s.	n.s.	n.s.	n.s	n.s.
1-Stearoyl-2-Linoleoyl-GPC (18:0/18:2)	Phospholipid Metabolism	n.s.	n.s.	n.s.	n.s.#	n.s.	n.s.
1-Stearoyl-2-Linoleoyl-GPE (18:0/18:2)	Phospholipid Metabolism	n.s.	n.s.	n.s.	n.s.#	n.s.	n.s.
1-Stearoyl-2-Oleoyl-GPC (18:0/18:1)	Phospholipid Metabolism	0.036	n.s.	n.s.	n.s.#	n.s	n.s.
Choline	Phospholipid Metabolism	9.60E-04	n.s.	n.s.	n.s.	0.045	n.s.
Glycerophosphoserine*	Phospholipid Metabolism	0.014	n.s.	n.s.	n.s.	n.s.	n.s.
1-(1-Enyl-Palmitoyl)-2-Linoleoyl-GPE (P-16:0/18:2)*	Plasmalogen	0.030	n.s.	n.s.	n.s.	n.s	n.s.
1-(1-Enyl-Palmitoyl)-2-Palmitoleoyl-GPC (P-16:0/16:1)*	Plasmalogen	0.026	n.s.	n.s.	n.s.	n.s.	n.s.
1-(1-Enyl-Stearoyl)-2-Linoleoyl-GPE (P-18:0/18:2)*	Plasmalogen	3.52E-04	n.s.#	n.s.	n.s.#	0.045	n.s.

1-(1-Enyl-Stearoyl)-2-Oleoyl-GPE (P-18:0/18:1)	Plasmalogen	0.005	n.s.	n.s.	n.s.	n.s	n.s.
Arachidonate (20:4n6)	Polyunsaturated Fatty Acid (n3 and n6)	0.008	n.s.	n.s.	n.s.	n.s.	n.s.
Docosahexaenoate (DHA, 22:6n3)	Polyunsaturated Fatty Acid (n3 and n6)	0.013	n.s.	n.s.	n.s.	n.s.	n.s.
Docosapentaenoate (DPA, 22:5n3)	Polyunsaturated Fatty Acid (n3 and n6)	0.008	n.s.	n.s.	n.s.	0.016	n.s.
Docosapentaenoate (DPA, 22:5n6)	Polyunsaturated Fatty Acid (n3 and n6)	n.s.	n.s.	n.s.	n.s.	0.003	n.s.
Glycosyl-N-Palmitoyl-Sphingosine (d18:1/16:0)	Sphingolipid Metabolism	0.034	n.s.	n.s.	n.s.	0.047	n.s.
Lactosyl-N-Nervonoyl-Sphingosine (d18:1/24:1)*	Sphingolipid Metabolism	0.043	n.s.	n.s.	n.s.	n.s.	n.s.
N-Nervonoyl-Hexadecasphingosine (d16:1/24:1)*	Sphingolipid Metabolism	0.020	n.s.	n.s.	n.s.	n.s	n.s.
N-Nervonoyl-Sphingadiene (d18:2/24:1)*	Sphingolipid Metabolism	1.21E-04	n.s.	n.s.	n.s.	0.045	n.s.
N-Palmitoyl-Sphingadienine (d18:2/16:0)*	Sphingolipid Metabolism	7.08E-04	n.s.	n.s.	n.s.	0.045	n.s.
N-Stearoyl-Sphingadienine (d18:2/18:0)*	Sphingolipid Metabolism	0.001	n.s.	n.s.	n.s.	n.s	n.s.
Sphingomyelin (d17:2/16:0, d18:2/15:0)*	Sphingolipid Metabolism	0.036	n.s.	n.s.	n.s.	n.s.	n.s.
Sphingomyelin (d18:1/18:1, d18:2/18:0)	Sphingolipid Metabolism	0.004	n.s.	n.s.	n.s.	n.s	n.s.
Sphingomyelin (d18:1/20:1, d18:2/20:0)*	Sphingolipid Metabolism	0.038	n.s.	n.s.	n.s.	n.s.	n.s.
Sphingomyelin (d18:1/22:2, d18:2/22:1, d16:1/24:2)*	Sphingolipid Metabolism	0.002	n.s.	n.s.	n.s.	0.043	n.s.
Sphingomyelin (d18:2/14:0, d18:1/14:1)*	Sphingolipid Metabolism	0.044	n.s.	n.s.	n.s.	n.s.	n.s.
Sphingomyelin (d18:2/16:0, d18:1/16:1)*	Sphingolipid Metabolism	0.025	n.s.	n.s.	n.s.	n.s	n.s.
Sphingomyelin (d18:2/24:1, d18:1/24:2)*	Sphingolipid Metabolism	0.026	n.s.	n.s.	n.s.	n.s.	n.s.
Sphingomyelin (d18:2/24:2)*	Sphingolipid Metabolism	0.001	n.s.	n.s.	n.s.	n.s.	n.s.
4-Cholesten-3-One	Sterol	0.011	n.s.	n.s.	n.s.	n.s.	n.s.
Nucleotides							
Hypoxanthine	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.024	n.s.	n.s.	n.s.	n.s.	n.s.
Urate	Purine Metabolism, (Hypo)Xanthine/Inosine containing	3.24E-05	n.s.	n.s.	n.s.	0.018	n.s.
Xanthine	Purine Metabolism, (Hypo)Xanthine/Inosine containing	9.52E-04	n.s.	n.s.	n.s.	n.s.	n.s.
7-Methylguanine	Purine Metabolism, Guanine containing	0.004	n.s.	n.s.	n.s.	0.003	n.s.
3-Ureidopropionate	Pyrimidine Metabolism, Uracil containing	0.003	n.s.	n.s.	n.s.	0.039	n.s.
Pseudouridine	Pyrimidine Metabolism, Uracil containing	2.34E-05	n.s.	n.s.	n.s.	0.003	n.s.
Uracil	Pyrimidine Metabolism, Uracil containing	1.24E-04	n.s.	n.s.	n.s.	0.005	n.s.
Peptides							
Glycylleucine	Dipeptide	0.038	n.s.	n.s.	n.s.	n.s.	n.s.
Phenylalanylglycine	Dipeptide	6.75E-04	n.s.	n.s.	n.s.	0.003	n.s.
Gamma-Glutamylglutamine	Gamma-glutamyl Amino Acid	8.62E-05	n.s.	n.s.	n.s.	0.018	n.s.
Gamma-Glutamylthreonine	Gamma-glutamyl Amino Acid	3.94E-04	n.s.	n.s.	n.s.	0.004	n.s.

Table S2. Enrichment analysis of the metabolic pathways altered in obesity. Enrichment and p-value mirror the magnitude of the alteration of these pathways. Metabolic pathways are sorted according to the p-value of the alteration.

Metabolic pathway	Significant	Detected	Ratio S/D[a]	Ratio All S/D[b]	Enrichment	p-value[c]
OBESITY						
Leucine, isoleucine and valine metabolism	9	12	0.75	0.28	2.68	0.001
Glutathione metabolism	5	6	0.83	0.28	2.98	0.007
TCA cycle	5	6	0.83	0.28	2.98	0.007
Glycolysis, gluconeogenesis and pyruvate metabolism	6	9	0.67	0.28	2.38	0.017
Glycerolipid metabolism	3	3	1.00	0.28	3.58	0.021
Glycine, serine and threonine metabolism	5	8	0.63	0.28	2.24	0.042

a) Ratio significant metabolites / detected metabolites in a particular metabolic pathway
b) Ratio all significant metabolites (118) / all detected metabolite (422)
c) Calculated with hypergeometric test

Table S3. The most discriminative metabolites in VAT in obesity obtained from multivariate RF analysis. The smaller the VIP value is, the more discriminant the metabolite is. Metabolites are sorted according to their VIP value. Abbreviations: aa, acyl-acyl; ae, acyl-alkyl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; VIP, variable importance in projection.

VIP	Name	Metabolic pathway	Chemical class
11.47	Urate	Purine Metabolism, (Hypo)Xanthine/Inosine containing	Nucleotide
12.45	Lactate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	Carbohydrate
16.50	N-Acetylglutamate	Glutamate Metabolism	Amino acid
18.47	Urea	Urea cycle, Arginine and Proline Metabolism	Amino acid
25.02	2-Hydroxybutyrate/2-Hydroxyisobutyrate	Glutathione Metabolism	Amino acid
25.37	Succinate	TCA Cycle	Energy
27.49	1-(1-Enyl-Stearoyl)-2-Oleoyl-GPE (GPE ae 18:0/18:1,GPE ae 36:1)	Plasmalogen	Lipid
27.91	1-(1-Enyl-Stearoyl)-2-Linoleoyl-GPE (GPE ae 18:0/18:2, GPE ae 36:2)	Plasmalogen	Lipid
35.45	Alpha-Hydroxyisovalerate	Leucine, Isoleucine and Valine Metabolism	Amino acid
36.17	Gamma-Glutamylglutamine	Gamma-glutamyl Amino acid	Peptide
38.09	Pseudouridine	Pyrimidine Metabolism, Uracil containing	Nucleotide
39.63	N-Nervonoyl-Sphingadiene (d18:2/24:1)*	Sphingolipid Metabolism	Lipid
40.93	Uracil	Pyrimidine Metabolism, Uracil containing	Nucleotide
42.88	Erythronate*	Aminosugar Metabolism	Carbohydrate
49.20	1-(1-Enyl-Oleoyl)-2-Linoleoyl-GPE* (GPE ae 18:1/18:2, GPE ae 36:3)	Lysoplasmalogen	Lipid
49.95	Glycerol	Glycerolipid Metabolism	Lipid
52.96	Isobutyrylcarnitine (C4)	Leucine, Isoleucine and Valine Metabolism	Amino acid
53.49	1-Oleoyl-2-Linoleoyl-PC* (GPC aa 18:1/18:2, GPC aa 36:3)	Phospholipid Metabolism	Lipid
62.09	Choline	Phospholipid Metabolism	Lipid
66.45	7-Methylguanine	Purine Metabolism, Guanine containing	Nucleotide
67.48	Fumarate	TCA Cycle	Energy
67.71	Stearoyl Ethanolamide	Endocannabinoid	Lipid
73.92	S-Methylcysteine	Methionine, Cysteine, SAM and Taurine Metabolism	Amino acid
74.33	Alpha-Ketoglutarate	TCA Cycle	Energy
76.67	Glycerate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	Carbohydrate

77.67	Glycerol 3-Phosphate	Glycerolipid Metabolism	Lipid
79.10	Isovalerylcarnitine (C5)	Leucine, Isoleucine and Valine Metabolism	Amino acid
79.78	Beta-Citrylglutamate	Glutamate Metabolism	Amino acid
81.65	5-Oxoproline	Glutathione Metabolism	Amino acid
82.29	Mannose	Fructose, Mannose and Galactose Metabolism	Carbohydrate
82.61	Guanidinoacetate	Creatine Metabolism	Amino acid
82.64	1-Methylhistidine	Histidine Metabolism	Amino acid

PUBLICACION 4

Effects of a long-term lifestyle intervention on metabolically healthy women with obesity: Metabolite profiles according to weight loss response

Magali Palau-Rodriguez, Mar Garcia-Aloy, Antonio Miñarro, M. Rosa Bernal-Lopez, Carl Brunius, Ricardo Gómez-Huelgas, Rikard Landberg, Francisco J Tinahones, Cristina Andres Lacueva

Clinical Nutrition

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Los resultados de esta publicación fueron presentados en formato de:

- Comunicación oral: FoodBall Meeting, Berna, Suiza, Enero 24-26 de 2018, "Impact of weight-loss lifestyle intervention on plasma metabolome of metabolically healthy women with obesity".
- Comunicación oral: Asociación Catalana de Diabetes, Tarragona, España, Abril 6-7 de 2018, "Estudi dels perfils metabòlics ("metabotypes") responedors a l'efecte d'una intervenció amb dieta hipocalòrica i activitat física en la pèrdua de pes i modulació del risc d'aparició de complicacions associades a la diabetis en voluntaris obesos metabòlicament sans".

RESUMEN PUBLICACION 4

Objetivos: El objetivo de esta publicación fue investigar como el perfil metabólico plasmático es afectado por la pérdida de peso en una intervención de estilo de vida basada en la modificación de la dieta por un patrón de dieta Mediterránea hipocalórica y el incremento de actividad física en mujeres con obesidad metabólicamente *sana*.

Metodología: Se aplicó un flujo metabolómico semicuantitativo con UPLC-Q-Exactive-MS/MS en muestras de plasma de 27 mujeres con obesidad metabólicamente *sana*, antes y después de 12 meses de una intervención de estilo de vida basada en dieta Mediterránea hipocalórica y el incremento de actividad física. Las pacientes fueron divididas en dos grupos, según el peso perdido: < 10% (grupo de menor pérdida de peso (LWL del inglés *low weight loss group*) y 10% (grupo de mayor pérdida de peso (HWL del inglés *high weight loss group*)). Un análisis de bosques aleatorios (en inglés, *Random Forest*), con doble validación cruzada repetida, fue utilizado para identificar los metabolitos discriminantes entre los dos grupos de estudios y dentro de un mismo grupo. Estudios de rutas biológicas y análisis de correlaciones fueron utilizados para ayudar en la interpretación biológica de los datos.

Resultados: Trece metabolitos discriminaron entre los dos grupos LWL y HWL. Incluyendo el 1,5-anhidroglucitol, el carotendiol, el 3-(4-hidroxifenil)lactato (HPLA), el N-acetilaspartato (NAA) y diferentes especies lipídicas (2 esfingolípidos, 1 plasmalógeno, fosfolípido, 1 ácido biliar secundario, 2 esteroides y 2 acilcarnitinas de cadena larga). El modelo de bosques aleatorios clasificó los individuos un 88.9% correctamente con y con un p-valor <0.05. Los niveles de 1,5-anhydroglucitol, HPLA y esfingomielinas fueron asociados positivamente con las variables de peso mientras que el NAA y el plasmalógeno correlacionaban negativamente. Cambios en los niveles de HPLA y acilcarnitinas fueron observados en el HWL y se correlacionaron con la glucosa en ayunas y cambios en los niveles

del plasmalógeno correlacionó negativamente con la resistencia a la insulina. Los niveles de colesterol fueron asociados positivamente con los cambios de HPLA, de los esfingolípidos y del 1,5 anhidroglucitol.

Conclusiones: Elevada pérdida de peso después de seguir un patrón de dieta Mediterránea hipocalórica e incrementar el ejercicio físico durante 12 meses es asociado con cambios en el metaboloma plasmático en mujeres metabólicamente *sanas*. Estos hallazgos son asociados con cambios en las variables bioquímicas y antropométricas y podrían sugerir una mejora en el riesgo cardiometabólico en aquellos pacientes que pierden más peso. Es necesario realizar más estudios para investigar si la respuesta de los sujetos con obesidad metabólicamente sana es diferente de la respuesta de la intervención en pacientes con obesidad metabólicamente enferma.

Effects of a long-term lifestyle intervention on metabolically healthy women with obesity: Metabolite profiles according to weight loss response

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Abbreviations:

1,5-AG, 1,5-anhydroglucitol; 16OH-DHEA-S, 16a-hydroxy DHEA 3-sulfate; 3PG, 3-phosphoglycerate; ADIOL-DS (1), androstenediol (3beta,17beta) disulfate (1); ADIOL-DS (2), androstenediol (3beta,17beta) disulfate (2); aHICA, alpha-hydroxyisocaproate; AMP, adenosine 5'-monophosphate; BMI, body mass index; carnitine C24, lignoceroylcarnite; carnitine C26, cerotoylcarnitine; carnitine C3, propionylcarnitine; C-glyTrp, C-glycosyltryptophan; CHOL, cholesterol; cys-gly, oxidized, cysteine-glycine, oxidized; DAG (18:2/18:2) , linoleoyl-linoleoyl-glycerol (18:2/18:2); DBP, diastolic blood pressure; ESI, electrospray ionization; FA, formic acid; FDR, false discovery rate; FM, fat mass; GCA-S, glycocholenate sulfate; glycosyl-ceramide (d18:1/16:0), glycosyl-N-palmitoyl-sphingosine (d18:1/16:0); GPC (18:1/18:2), 1-oleoyl-2-linoleoyl-GPC (18:1/18:2); GPC (P-16:0/18:1), 1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1); GPI (18:0), 1-stearoyl-GPI (18:0); GPI (20:4), 1-arachidonoyl-GPI (20:4); HbA1c, glycated haemoglobin A1; HDL, high-density lipoprotein cholesterol; HILIC, hydrophylic interaction liquid chromatography; Hip, hip circumference; HOMA-IR, insulin resistance calculated by homeostatic model assessment; HPLA, 3-(4-hydroxyphenyl)lactate; HWL, high

weight loss group; Insulin, fasting insulin; LDL, low-density lipoprotein cholesterol; LM, lean mass; LWL, low weight loss group; MedDiet, Mediterranenan diet; MG (18:2), 1-linoleoylglycerol (18:2); MHO, metabolically healthy obesity; MS/MS, tandem mass spectrometry; NAA, N-acetylaspartate; non-HDL, non-high-density lipoprotein cholesterol; OEA, oleoyl ethanolamide; OGTT, oral glucose tolerance test; PEA, palmitoyl ethanolamide; PFPA, perfluoropentanoic acid; PLA, phenyllactate; rd-CV, repeated double cross-validation; RF, Random forest; RP, reverse phase; RSD, relative standard deviation; SBP, systolic blood pressure; SM, sphingomyelin; TG, triglycerides; UPLC, ultra-performance liquid chromatography; Waist, waist circumference

Abstract

Background & Aims

The benefits of weight loss in subjects with metabolically healthy obesity (MHO) are still a matter of controversy. We aimed to identify metabolic fingerprints and their associated pathways that discriminate women with MHO with high or low weight loss response after a lifestyle intervention, based on a hypocaloric Mediterranean diet (MedDiet) and physical activity.

Methods

A UPLC-Q-Exactive-MS/MS metabolomics workflow was applied to plasma samples from 27 women with MHO before and after 12 months of a hypocaloric weight loss intervention with a MedDiet and increased physical activity. The subjects were stratified into two age-matched groups according to weight loss: <10% (low weight loss group, LWL) and >10% (high weight loss group, HWL). Random forest analysis was performed to identify metabolites discriminating between the LWL and the HWL as well as within-status effects. Modulated pathways and associations between metabolites and anthropometric and biochemical variables were also investigated.

Results

Thirteen metabolites discriminated between the LWL and the HWL, including 1,5-anhydroglucitol, carotenediol, 3-(4-hydroxyphenyl)lactic acid, N-acetylaspartate and several lipid species (steroids, a plasmalogen, sphingomyelins, a bile acid and long-chain acylcarnitines). 1,5-anhydroglucitol, 3-(4-hydroxyphenyl)lactic acid and sphingomyelins were positively associated with weight variables whereas N-acetylaspartate and the plasmalogen correlated negatively with them. Changes in very long-chain acylcarnitines and hydroxyphenyllactic levels were observed in the HWL and positively correlated with fasting glucose, and changes in levels of the plasmalogen negatively correlated with insulin resistance. Additionally, the

cholesterol profile was positively associated with changes in acid hydroxyphenyllactic, sphingolipids and 1,5-AG.

Conclusions

Higher weight loss after a hypocaloric MedDiet and increased physical activity for 12 months is associated with changes in the plasma metabolome in women with MHO. These findings are associated with changes in biochemical variables and may suggest an improvement of the cardiometabolic risk profile in those patients that lose greater weight. Further studies are needed to investigate whether the response of those subjects with MHO to this intervention differs from those with unhealthy obesity.

Keywords: metabolomics; metabolically healthy obese; LC-MS; Mediterranean diet; lifestyle intervention; obesity

Introduction

Obesity comprises a variety of different metabolic profiles that diversify the risk of developing metabolic alterations that lead to diseases such as type 2 diabetes [1]. However, while obesity is usually associated with high cardiometabolic risk, it has been suggested that metabolically healthy obesity (MHO) has a different risk profile [2]. Subjects with MHO, despite having an excess of adipose tissue, present a propitious metabolic profile distinguished by higher insulin sensitivity, normal blood pressure, lower inflammatory parameters, lower visceral fat and more normal circulating lipid profiles than those with metabolically "unhealthy" obesity [1]. This may protect them from developing metabolic complications normally associated with obesity [3].

Men and women with obesity are advised or put on treatment to lose weight for better metabolic health [4]. However, it is as yet unclear whether subjects with an MHO phenotype will benefit from weight loss since they show a better cardiometabolic risk profile [1]. On the other hand, some studies have argued that the MHO condition may be a transient state towards a higher metabolic risk state. Therefore, it is important to investigate the effect of weight loss on cardiometabolic health intermediates in the MHO phenotype.

Randomized controlled trials based on a Mediterranean diet (MedDiet) [5] and physical activity [6] have shown their beneficial effects on metabolic health per se. Moreover, when the two are combined even greater benefits have been demonstrated [7,8]. However, the impact of a lifestyle weight loss treatment on the MHO phenotype is poorly understood.

Metabolomics is a powerful high-throughput approach employed to define metabolic profiles through the comprehensive measurement of small molecule metabolites in a biological sample. The metabolome reflects the interaction of the exposome (i.e. the diet, gut microbiota and environmental agents to which an individual is exposed) with the gene cascade. Metabolomics can be used to identify biomarkers of prediction, progression or pathogenesis of conditions and diseases, as

well as providing new clues regarding the mechanisms involved in metabolic deregulation [9,10]. Metabolomics may thus have advantages over other *omics* techniques in the study of diseases with a major metabolic component.

In the present study, we aimed to investigate how the plasma metabolite profiles would be affected according to weight loss response to a long-term lifestyle intervention based on a hypocaloric MedDiet and increased physical activity in women with metabolically healthy obesity. This could provide insights into affected metabolic pathways and potential consequences for cardiometabolic health.

Material and Methods

Subjects and study design

Metabolically healthy women with obesity (BMI ≥ 30 kg/m^2) aged 35–55 years were recruited by their family doctors between June 2013 and April 2014 from four primary health-care centres in the Malaga district of the Andalusian Health Service (Spain) [11]. A participant was considered to be metabolically healthy if they fulfilled ≤1 of the following criteria: elevated fasting plasma glucose (≥100 mg/dL); elevated blood pressure (≥135/85 mmHg or use of blood pressure-lowering agents); elevated triglycerides (≥150 mg/dL or treatment with lipid-lowering medication); or decreased HDL cholesterol (<50 mg/dL). Exclusion criteria were: presence of diabetes or impaired glucose tolerance (≥100 mg/dL); pregnancy or planning to become pregnant during the study; cardiovascular disease; presence of any severe systemic disease such as advanced organ failure, cancer or dementia; immobilized individuals; alcohol or drug abuse; having participated in a weight loss programme in the past three months; or having lost ≥5 kg of body weight in the last six months.

Participants were enrolled into a lifestyle weight-loss intervention with a hypocaloric MedDiet and a recommendation of physical activity for 12 months. The hypocaloric diet was based on a reduction of about 600 kcal in the energy intake with a calorie distribution as follows: 35–40% fats (8–10% saturated fatty acids), 40–45% carbohydrates and 20% protein. Additionally, participants were recommended to practise daily exercise, which involved walking on average for 150

minutes every week throughout the study. The Rapid Assessment of Physical Activity questionnaire was used to determine the activity of the participants [12]. The dietary and physical intervention involved individual appointments with a nutritionist every week during the first two months, followed by monthly visits during the next four months and then once every three months up to 12 months. The study was conducted in accordance with the Declaration of Helsinki, all protocols were approved by the institutional ethical committee (Comité Coordinador de Ética de la Investigación Biomédica de Andalucía) and all participants provided written informed consent. The clinical trial was registered at the ISRCTN registry (https://www.isrctn.com/ISRCTN88315555).

Clinical measurements were taken at baseline and after 12 months of intervention by trained health-care workers, and included anthropometry (weight, height, waist and hip circumference, and body composition), blood pressure and the collection of fasting blood samples. Biochemical analyses were performed in the laboratory of the reference hospital and conducted using routine methods.

Energy and nutrient intakes were determined using a previously validated semi-quantitative 137-item food frequency questionnaire [13] and Spanish food composition tables [14,15]. Adherence to the MedDiet was measured using the 14-item screener from the PREDIMED study [16].

For the present study, participants were classified in two groups according to the percentage of weight loss after 12 months of intervention: <10% (low weight loss group, LWL) and >10% (high weight loss group, HWL).

Metabolomics analysis

All samples were kept at -80 ºC until analysis using the Metabolon analytical system (Metabolon Inc., Durham, North Carolina, USA) [17]. Briefly, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills Geno/Grinder 2000). One aliquot of the resulting supernatant was analysed using an approach based on hydrophilic interaction liquid chromatography -ultra-performance liquid chromatography (UPLC, Waters ACQUITY) coupled to a

Thermo Scientific Q-Exactive tandem mass spectrometer (MS/MS) using negative ion mode electrospray ionization (ESI-). Three aliquots were analysed by reverse phase (RP)-UPLC-ESI-MS/MS, two of them using positive ion mode electrospray ionization and the other using ESI-. The UPLC system was equipped with a UPLC C18 BEH (2.1x100 mm, 1.7 µm) or UPLC BEH Amide (2.1x150 mm, 1.7 µm) column (Waters). The Q-Exactive system was interfaced with a heated ESI source and an Orbitrap mass analyser operating at a 35,000 mass resolution and covering 70–1000 m/z was used. The MS analysis alternated between MS and data-dependent MS^n scans using dynamic exclusion. Instrument variability was determined by calculating the relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the MS. In parallel, overall process variability was determined by calculating the RSD for all endogenous metabolites present in all of the quality control samples created from a large pool of human plasma that were analysed at the beginning and at the end of the experimental run and evenly throughout the run. The median RSD of the analytical platform instrumentation was 3%, whereas the median RSD overall process variability was 6%. These values reflected acceptable levels of variability for both instrument and overall process variability.

Peaks were quantified using the area under the curve and metabolites were identified by comparing them to library entries of purified standards, according to retention time, accurate mass and MS/MS spectral data [18].

Statistical analysis

All the statistical analyses and graphics were computed in R (version 3.3.3), unless otherwise specified. General characteristics of study participants, as well as anthropometric, clinical and dietary data, were examined through univariate statistical analyses. Fisher's exact test was used to compare categorical variables. For quantitative variables, data were analysed using a non-parametric permutation test (n=1000) of a mixed (within and between groups) factorial design using the *ez* package [19] to assess, respectively: i) between-group differences at baseline; ii) within-group differences between before and after the intervention; and iii) between-

group differences in the changes during the intervention. Quantitative data are expressed as median (interquartile range), whereas qualitative data are expressed as number of individuals (percentage).

For metabolomics data, a multi-step process was carried out. Firstly, metabolites not found in at least 80% of the samples in either of the classes were removed (considering the time point and the weight loss group for the definition of classes). Missing values were imputed with the k-nearest neighbours method (k=5) [20]. Data were scaled to set the median equal to 1 and log-transformed. Finally, the differences in metabolites between baseline and 12 months after the intervention period were calculated. Random forest analysis with repeated double cross-validation (RF-rdCV) was used to select metabolites that discriminated between the LWL and the HWL during the intervention process, as well as those metabolites that discriminated between baseline and the 12-month intervention within each group [21,22]. Briefly, RF-rdCV was performed using a procedure developed in-house [23]: the double cross-validation separated the cross-validation into an outer "testing" loop (n=8 CV segments) and inner "tuning" (or validation) loop (n=7 CV segments) to reduce bias from overfitting models [21,22]. The rdCV was repeated 30 times for between-group analysis and 20 times for within-group analysis and misclassification was used for the fitness of the model tuning. Metabolite selection was performed within the inner loop by iteratively turning over successively fewer features, keeping in the subsequent inner loop iterations the 80% most informative metabolites. The validity of the models was assessed using two-tailed permutation tests (n=1000). Additionally, the *p* values of within- and between-group differences in the changes in metabolites selected by each RF-rdCV model were also calculated through intra- and inter-group permutation tests (n=1000) using the above-mentioned *ez* package [19].

Finally, Spearman correlation coefficients were calculated to estimate the associations among the selected metabolites and with clinical variables. Metabolite-clinical correlations were represented as a heat map and metabolite-metabolite-clinical correlations as a network. These *p* values were adjusted by false discovery

rate (FDR) multiple testing, based on the Benjamini-Hochberg procedure, with the significant threshold set at p<0.1 for the adjusted *p* values [24]. The *Hmisc* and *ggplot* packages were used for the analysis of correlation and the creation of the heat map, respectively. The correlation network was performed using Cytoscape 3.3.0.

Results

A total of 115 women with MHO were enrolled in the study. Of these, 43 dropped out during the intervention, six were excluded due to the presence of an illness, two for personal reasons and six for not having completed the food frequency questionnaires at both baseline and 12 months. Finally, 27 women were randomly selected for metabolomics analysis, of whom 15 (55.6%) and 12 (44.4%) lost <10% and >10% of their body weight, respectively (Figure S1). Table 1 presents the characteristics of these participants. In brief, the participants of both groups were of similar ages, and there were similar proportions of menopause state, a high level of studies and smokers among them.

Table 2 shows changes in clinical variables between the two groups. No baseline differences were observed in any of these variables between the groups. After 12 months, anthropometric and body composition parameters had improved in the HWL but remained unchanged in the LWL. The HWL also presented decreases in the oral glucose tolerance test (OGTT) and in glucose levels, but no changes in HbA1c, HOMA-IR or fasting insulin, whereas these three parameters increased in the LWL. Systolic blood pressure, as well as total, LDL and non-HDL cholesterol, also decreased in the HWL but did not do so in the LWL. These differences were also significant between the groups for most of the mentioned variables.

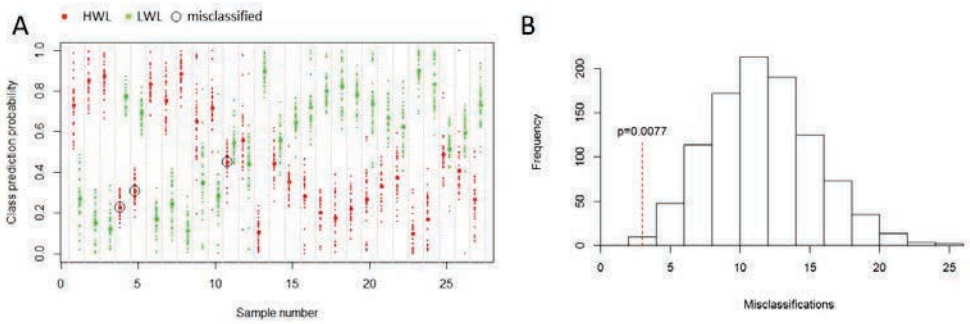
At the beginning of the study the reported energy intake of the HWL was higher than that of the LWL (Table 3). Both groups showed decreases in energy intake through the study, but the women in the HWL presented significantly larger reductions, together with decreases in fat consumption, especially saturated and monounsaturated, and increases in protein intake. In general, the subjects in the HWL had a higher level of adherence to the treatment and also more of them

followed the recommendations for physical activity in the HWL than in the LWL during the programme (Table 3).

Impact of weight loss on plasma metabolomic profile

Accurate classification predictions were obtained both between and within groups using the random forest classification scheme (Figures 1, S2 and S3). While in between-group analysis, 24 out of 27 (88.9%) individuals were correctly classified, in within-HWL analysis, 11 out of 12 (91.7%) were correctly classified, and in within-LWL analysis, all subjects were correctly classified (*p* values of permutation test <0.05).

Figure 1. Results from repeated double cross-validated random forest analysis (rdCV-RF) to classify between weight loss groups: (A) Predictive classification of subjects according to weight loss group (misclassified individuals are highlighted with a circle); (B) Histograms for permutation tests (n=1000) of the rdCV-FR classification of subjects according to weight loss group.

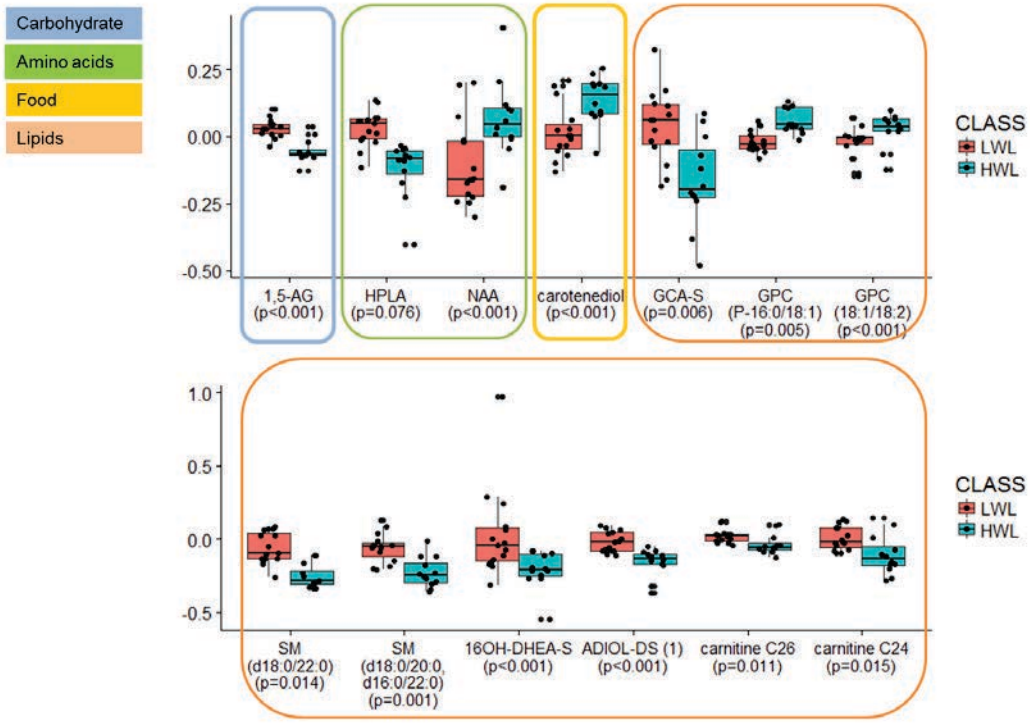


Thirteen metabolites were identified as determinants of the classification between weight loss groups, i.e. differences in 1,5-anhydroglucitol (1,5-AG), 3-(4-hydroxyphenyl)lactate (HPLA), N-acetylaspartate (NAA), the exogenous compound carotenediol, and nine lipids: 1 bile acid, 1 plasmalogen, 1 phospholipid, 2 sphingolipids, 2 steroids and 2 acylcarnitines (Figure 2).

Figure 2. Differences between weight loss groups in metabolites selected by repeated double-cross validated random forest model.

p values were obtained by permutation test (n=1000) of the differences of the changes between groups.

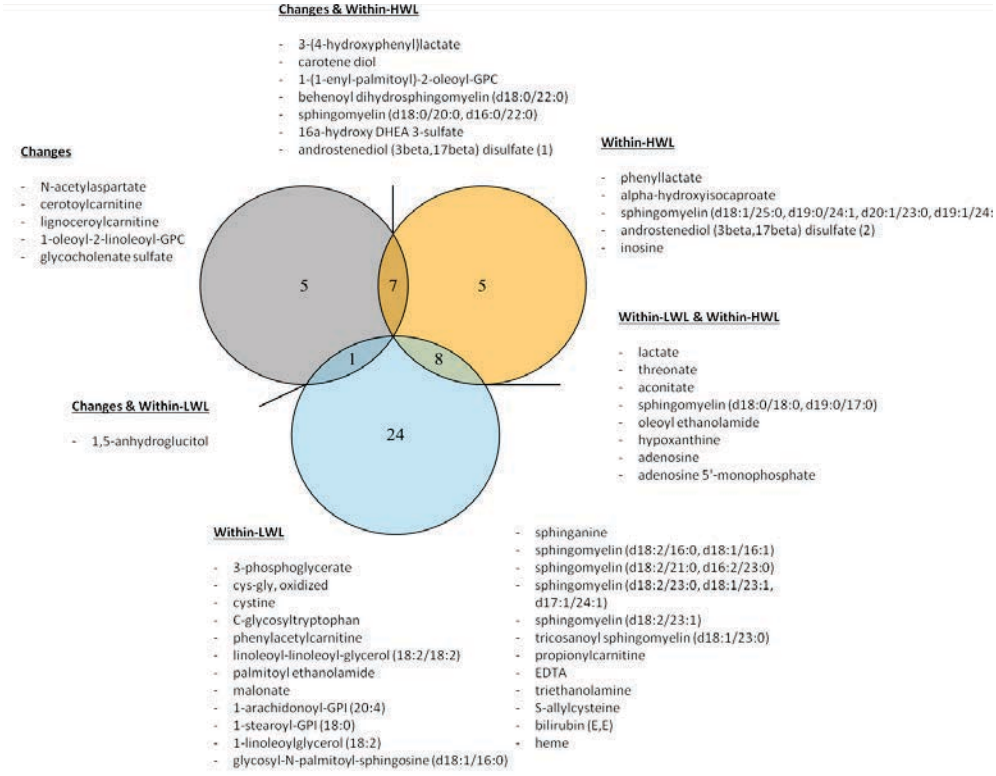
1,5-AG, 1,5-anhydroglucitol; 16OH-DHEA-S, 16α-hydroxydehydroepiandrosterone 3-sulfate; ADIOL-DS (1), androstenediol (3β,17β) disulfate; carnitine C24, lignoceroylcarnitine; carnitine C26, cerotoylcarnitine; GCA-S, glycocholenate sulfate; GPC, glycerophosphocholine; HPLA, 3-(4-hydroxyphenyl)lactate; NAA, N-acetylaspartate; SM, sphingomyelin.



Twenty and 33 metabolites, respectively, were selected from within-HWL and within-LWL analyses. 1,5-AG was also selected from within-LWL analysis, with higher levels at the 12-month intervention than at baseline. Seven metabolites from the between-group analysis were also selected from the within-HWL analysis (Figure 3 and Figure S4). Among these metabolites were the plasmalogen 1-(1-enyl-palmitoyl)-2-oleoyl-sn-glycero-3-phosphocholine (P-16:0/18:1) (GPC (P-

16:0/18:1)) and the exogenous compound carotenediol, which increased during the within-HWL intervention, and significantly more so than in the LWL (Figure S5). The levels of HPLA and some lipids (two steroids and two sphingolipids) decreased after the intervention in the HWL and more so in the HWL than in the LWL.

Figure 3. Venn Diagram of metabolites discriminating between LWL and HWL, within-HWL and within-LWL, selected by repeated double-cross validated random forest modelling.



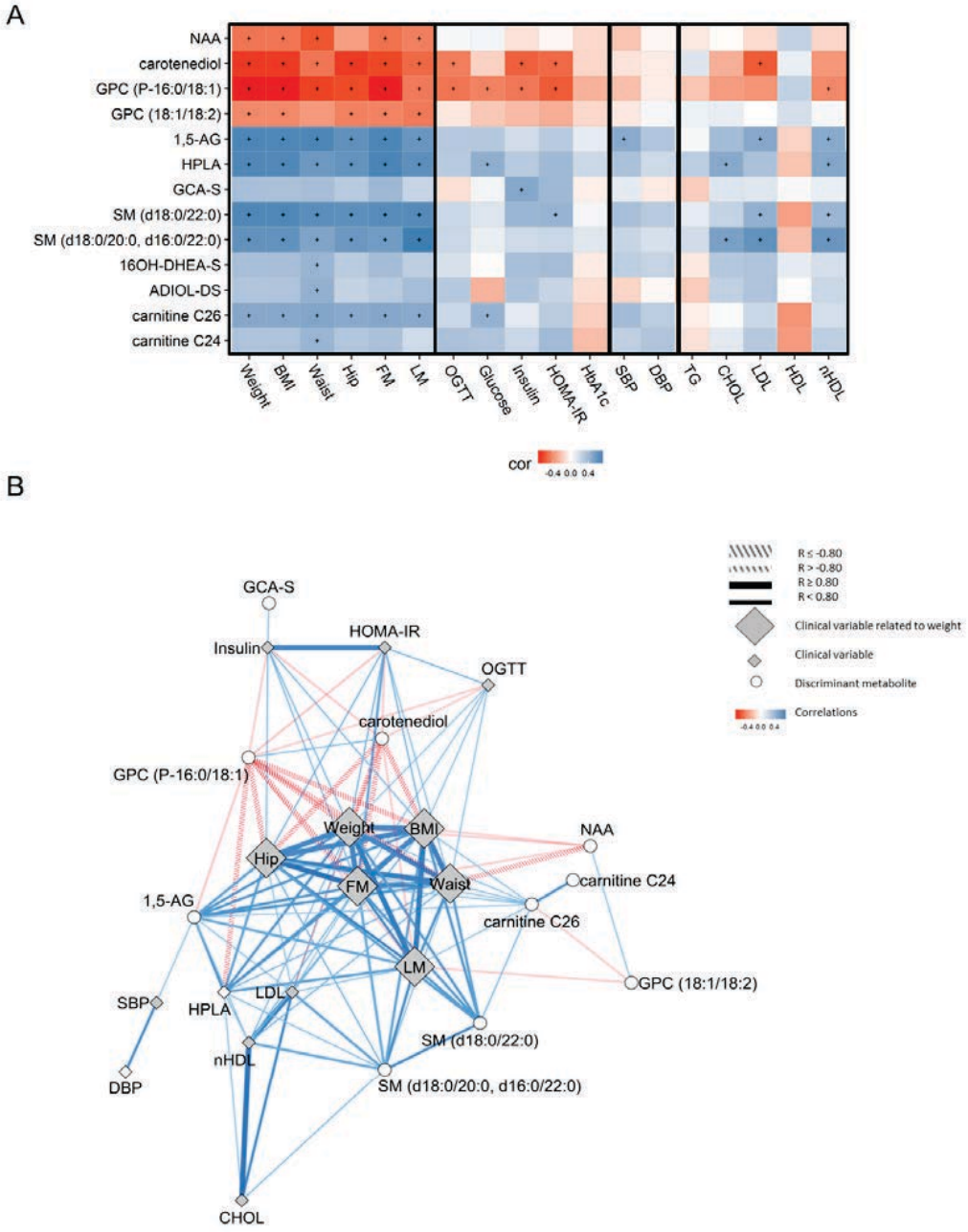
Correlation and pathway analysis

Metabolites that were identified as discriminating between weight loss groups were correlated with changes in clinical variables (Figure 4-A), and their intercorrelations were mapped in an organic metabolic network (Figure 4-B). Positive correlations were presented between changes in the levels of 1,5-AG, HPLA, SMs and carnitine C26 and weight variables, whereas changes in the levels of NAA, carotenediol, GPC (P-16:0/18:1) and GPC (18:1/18:2) correlated negatively with them. Furthermore, GPC (P-16:0/18:1) and carotenediol also correlated inversely with glycaemic variables. In addition, several metabolites, including 1,5-AG, both SMs and HPLA, correlated positively with lipid biochemistry. Correlations between the selected metabolites in the within-group analyses and clinical variables are presented in Figure S6.

Figure 4. Correlations of changes between metabolites and clinical parameters during the intervention program: (A) Metabolite-clinical correlations; (B) Metabolite-metabolite-clinical significant correlations.

Associations determined by Spearman correlations adjusting p values by FDR, with significant threshold set at $p<0.1$. Negative correlations are coloured in red and positive correlations are colored in blue.

1,5-AG ,1,5-anhydroglucitol; 16-OH-DHEA-S, 16a-hydroxy DHEA 3-sulfate; ADIOL- DS, androstenediol (3beta,17beta) disulfate (1); BMI, body mass index; carnitine C26, cerotoylcarnitine (C26); carnitine C24, lignoceroylcarnitine (C24); CHOL, total cholesterol; DBP, diastolic blood pressure; FM, fat mass; GCA-S, glycocholenate sulfate; GPC (18:1/18:2), 1-oleoyl-2-linoleoyl-GPC (18:1/18:2); GPC (P-16:0/18:1), 1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1); HbA1c, glycated hemoglobin A1c; HDL, high-density lipoproteins cholesterol; Hip, hip circumference; HOMA-IR, insulin resistance calculated by homeostatic model assessment; HPLA, 3-(4-hydroxyphenyl)lactate; LDL, low-density lipoproteins cholesterol; LM, lean mass; NAA, N-acetylaspartate; OGTT, oral glucose tolerance test; non-HDL, non-high-density lipoproteins cholesterol; SBP, systolic blood pressure; SM sphingomyelin; TG, triglycerides; Waist, waist circumference


A
NAA
carotenediol
GPC (P-16:0/18:1)
GPC (18:1/18:2)
1,5-AG
HPLA
GCA-S
SM (d18:0/22:0)
SM (d18:0/20:0, d16:0/22:0)
16OH-DHEA-S
ADIOL-DS
carnitine C26
carnitine C24
Weight
BMI
Waist
Hip
FM
LM
OGTT
Glucose
Insulin
HOMA-IR
HbA1c
SBP
DBP
TG
CHOL
LDL
HDL
nHDL
cor
-0.4 0.0 0.4
B
R ≤ -0.80
R > -0.80
R ≥ 0.80
R < 0.80
Clinical variable related to weight
Clinical variable
Discriminant metabolite
Correlations
GCA-S
HOMA-IR
Insulin
OGTT
carotenediol
GPC (P-16:0/18:1)
Weight
BMI
NAA
Hip
FM
Waist
carnitine C24
1,5-AG
carnitine C26
LM
GPC (18:1/18:2)
SBP
HPLA
LDL
SM (d18:0/22:0)
nHDL
DBP
SM (d18:0/20:0, d16:0/22:0)
CHOL

Finally, and in order to identify the most important metabolic pathways involved in these changes, pathway analyses were performed taking into account the discriminant metabolites selected from between-group analysis (Figure 2) and from within-HWL (Figure S4) and within-LWL (Figure S5) analyses.

No specific pathways were statistically significant using the metabolites selected as discriminant in the between-group RF model (Figure S7). Statistically significant pathways altered in the HWL and in the LWL are shown in Figure S8.

Discussion

Although several studies report the metabolic benefits of weight loss in subjects with obesity [7,25], the benefits of a lifestyle intervention for subjects with MHO are not clear. The present study demonstrated differences in the modulation of the plasma metabolome, stratified by weight loss, after a lifestyle weight loss programme based on a hypocaloric MedDiet and physical activity in metabolically healthy women with obesity.

This study shows greater differences in carotenediol levels in the HWL after the intervention and these levels were also observed to increase in this group of women. Carotenediols are vitamin A precursors found mainly in vegetables and fruit-rich diets such as the MedDiet. An increase in their levels could reflect a higher intake of such foods, which is also reflected in their greater adherence to the Mediterranean pattern [26].

1,5-AG discriminated between the HWL and the LWL and was observed to increase in women with lower weight loss. Similar results were observed in a 6-month intervention based on the New Nordic Diet, which is rich in vegetables, whole grains, nuts and seafood products [27,28]. This metabolite has in fact been proposed as a biomarker of short-term glycaemic control and for screening undetected type 2 diabetes in saliva [29]. In line with our results, Lipsky et al. (2016) also observed a higher association of 1,5-AG with BMI and adiposity indicators [30]. Small

differences in the HWL likely reflect improved glycaemic control as a result of a more successful intervention as measured by weight loss.

Lipid metabolism has been extensively studied in obesity [31–34]. This study showed that lipid metabolism was altered, particularly in steroids, glycerophosphatidylcholines and sphingolipid metabolism.

The steroids pathway was regulated differently in the two groups. Although androgen steroid sulfates decreased in the LWL, a higher decline was observed in the HWL. Similar behaviour was observed by Ernst et al. (2013) when weight was lost after bariatric surgery [35]. 16a-hydroxy DHEA 3-sulfate (16OH-DHEA-S) and androstenediol (3beta,17beta) disulfate (ADIOL-DS) seem to be the major players in these changes. DHEA and ADIOL are interconverting molecules through the action of 17-hydroxysteroid dehydrogenase [36]. However, while several studies have attributed an anti-obesity role to DHEA-S [37], others have observed an inverse association between DHEA-S and the leptin hormone and satiety [38]. In addition, DHEA-S could play a role in the regulation of energetic balance in a fasting state or caloric restriction [39]. Steroid sulfation and desulfation are fundamental pathways for endocrine balance, specifically for fat mass distribution and glucose metabolism [40] regulated by sulfotransferases and sulfatase enzymes, respectively. These results could reflect an effect of modulation of the endocrine metabolism, especially in women of the HWL.

SM (d18:0/22:0) and SM (d18/0/20:0, d16:0/22:0) were chosen by the multivariate model to discriminate between the HWL and the LWL. In addition, the overall sphingolipid profile decreased in both groups. Sphingolipids are the most prevalent class of lipid found in circulating LDL and activate inflammatory pathways [41]. Higher levels of sphingolipids are associated with obesity and related co-morbidities [31]. We observed a general decrease in these lipid species, which is in line with results reported after a lifestyle intervention in adolescents [42]. These two were selected and correlated with LDL, nHDL and CHOL. Thus, a downregulation of the sphingolipid pathway could indicate a better LDL profile and consequently potentially a reduction in the risk of developing cardiometabolic diseases.

The plasmalogen GPC (P-16/18:0) discriminated between the HWL and the LWL and correlated negatively with adiposity variables. Plasmalogens act as an endogenous antioxidant produced by peroxisome. The production of plasmalogens is explained as a compensatory mechanism to protect the organism against higher oxidative stress such as in the development of metabolic syndrome [43]. Thus, an increase in GPC (P-16/18:0) levels in the HWL may indicate major protection of this group in the face of obesity complications.

Strikingly, our study also reflects changes in lipid metabolism through changes in very long-chain acylcarnitines. We pointed out lower changes in the HWL than in the LWL. However, little is known about the role of very long-chain acylcarnitines in obesity and associated co-morbidities. Zhang et al. (2014) found higher concentrations of the carnitine C24, but not C26, in newly diagnosed type 2 diabetes subjects, and even in those with pre-diabetes, than in subjects with normal glucose tolerance [44]. Interestingly, higher levels of the C26 carnitine were detected in patients with a peroxisomal biogenesis disorder and it has been proposed as a biomarker in neurodegenerative disorders [45]. However, the implications of our findings are still uncertain.

We also observed that NAA, a marker of neuronal density [46] in the central nervous system, discriminated between the HWL and the LWL. This is in line with previous research, which showed that subjects with overweight and diabetes presented lower levels of NAA in the hippocampus [47] and that NAA in the cortex was positively correlated with physical fitness in elderly adults [48].

Finally, the significant decrease in the HWL of HPLA and PLA, lactobacillus breakdown products of phenylalanine and tyrosine, respectively [49], could reflect either a decreased protein intake or a possible modulation of gut microbiota from the intervention. Subjects with obesity present higher levels of phenylalanine, tyrosine and leucine, among other amino acids [50,51]. In addition, higher levels of microbial product of HPLA were found in children with obesity [47]. HPLA has been proposed as a potential biomarker of a higher percentage of lean mass in young and healthy adults, though with an unknown mechanism [52]. Furthermore, positive

correlations between changes in HPLA and weight loss, dyslipidaemia parameters and OGTT and fasting glucose may suggest a possible global metabolic improvement in those subjects that benefited more from of lifestyle intervention.

A major limitation of this work, inherent to the study design, is that findings cannot be conclusively attributed to weight loss per se, a better adherence to a MedDiet and/or physical activity due to confounding. In addition, at the beginning of the study, the HWL had a greater energy intake than the LWL. In addition to this, the sample size was small and a validated cohort and prospective study is needed to corroborate our results. Moreover, our results are gender dependent and therefore we cannot extrapolate our findings to the general population. However, this limitation also contributed to a strength of this study: the fact that all participants were middle-aged women from a single metabolic phenotype reduced other sources of variability. Moreover, our findings have been obtained using a robust multivariate modelling procedure to acquire the most relevant biomarkers of high weight loss. These results show the potential of metabolomics for metabolic profiling and the identification of potential biomarkers in the onset of diseases.

Overall, our results reveal that weight loss after a lifestyle intervention is associated with the modulation of lipid metabolism, sulfation activation and microbiota metabolism likely associated with a metabolic protective effect. Therefore, this study reinforces the idea that a healthy lifestyle, increased physical activity and weight loss lead to an improved metabolic health status in women with obesity, irrespectively of their initial metabolic state. Further studies are needed to investigate whether the response of those subjects with MHO to this intervention differs from that of those with unhealthier obesity.

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Statement of Authorship

MR.B.-L, R.G.-H., F.J.-T and C.A.-L. conceptualization; M.P.-R., M.G.-A., A.M., C.B. and R.L. data curation; M.P.-R., M.G.-A. formal analysis; C.A.-L. Funding acquisition; M.P.-R., M.G.-A. investigation; M.P.-R., M.G.-A. methodology; M.P.-R., M.G.-A., C.A.-L. project administration; MR.B.-L, R.G.-H., F.J.-T and C.A.-L. resources; A.M., C.B., R.L., C.A.-L. supervision; MR.B.-L, R.G.-H., F.J.-T and C.A.-L. validation; M.P.-R., M.G.-A. visualization; M.P.-R., M.G.-A. writing original draft; M.P.-R., M.G.-A., A.M., C.B., R.L. MR.B.-L, R.G.-H., F.J.-T and C.A.-L. writing review & editing. *C.A.L is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.* All authors read and approved the final manuscript.

Conflict of Interest Statement

The authors declare no competing interests.

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Table

Table 1. Characteristics of the study participants

Variable	All	LWL	HWL	p[a]
n	27	15	12	-
Age, median (Q1,Q3)	45.0 (42.0,48.0)	45.0 (42.0,46.5)	47.0 (40.7,49.5)	0.520
Menopause, n (%)	6 (22.2)	3 (20.0)	3 (25.0)	1.000
High level of studies, n (%)[b]	21 (77.8)	12 (80.0)	9 (75.0)	1.000
Smokers , n (%)	9 (33.3)	4 (26.7)	5 (41.7)	0.448

Table 2. Anthropometric and clinical variables at baseline and after 12 months of intervention according to weight loss group[a]

	LWL (<10% weight loss)			HWL (>10% weight loss)			Treatment effect		
	Baseline	12 months	p[1]	Baseline	12 months	p[1]	Differences LWL	Differences HWL	p[2]
Weight, kg	95.3 (82.9,98.2)	93.3 (83.1,98.8)	0.694	85.4 (80.4,105.1)	71.8 (65.9,82.9)	0.002	-0.2 (-3.5,2.6)	-15.0 (-17.4,-12.1)	<0.001
BMI, kg/m2	36.0 (33.6,37.8)	35.6 (32.7,37.9)	0.722	34.7 (31.8,38.1)	28.3 (26.8,33.2)	0.002	0.0 (-1.5,1.1)	-5.8 (-6.7,-4.7)	<0.001
Waist circumference, cm	111.0 (105.5,118.5)	114.5 (106.5,117.8)	0.441	114.3 (109.1,126.3)	97.0 (94.5,107.5)	<0.001	-0.5 (-2.5,5.0)	-13.8 (-18.1,-9.9)	<0.001
Hip circumference, cm	123.0 (111.3,126.5)	118.0 (111.3,122.0)	0.142	121.5 (118.9,128.0)	111.5 (105.5,114.6)	<0.001	-1.0 (-4.3,0.5)	-11.3 (-14.3,-6.8)	<0.001
Fat mass, %	40.3 (33.6,46.0)	42.6 (31.9,44.4)	0.824	36.9 (34.6,45.4)	25.9 (21.4,33.7)	<0.001	1.1 (-2.3,2.1)	-10.2 (-13.4,-8.6)	<0.001
Lean mass, %	51.7 (47.7,53.7)	50.8 (48.4,52.6)	0.265	49.8 (46.6,55.6)	44.6 (44.1,50.9)	0.002	-0.6 (-1.6,0.7)	-4.0 (-4.9,-3.0)	<0.001
OGTT	100.0 (83.0,112.0)	89.0 (71.0,104.0)	0.665	100.0 (91.8,109.0)	62.5 (57.5,85.5)	0.018	-8.0 (-14.5,0.5)	-27.0 (-42.3,-5.5)	0.029
Glycaemia, LWL/dL	90.0 (86.0,92.5)	85.0 (79.5,93.5)	0.064	88.5 (82.0,93.3)	77.5 (72.0,81.5)	0.005	-3.0 (-7.5,0.0)	-8.5 (-13.0,-5.5)	0.076
Fasting insulin, uU/mL	9.7 (8.7,14.7)	15.2 (11.6,19.1)	<0.001	9.0 (8.4,9.8)	8.7 (8.3,9.7)	0.854	5.0 (1.2,7.0)	0.0 (-0.7,0.5)	0.390
HOMA-IR index	2.1 (1.9,3.2)	3.2 (2.5,3.9)	0.004	2.0 (1.8,2.1)	1.7 (1.5,2.2)	0.990	0.7 (0.1,1.4)	-0.1 (-0.5,0.0)	0.249
HbA1c %	5.4 (5.2,5.5)	5.4 (5.2,5.7)	0.043	5.4 (5.2,5.6)	5.4 (5.3,5.6)	0.833	0.1 (0.0,0.2)	0.0 (-0.1,0.1)	0.156
SBP, mmHg	105 (101,117)	112 (105,122)	0.823	114 (109,124)	108 (100,114)	0.041	4.0 (-4.5,12.5)	-6.0 (-13.3,1.3)	0.176
DBP, mmHg	71 (69,79)	75 (68,83)	0.351	75 (67,87)	74 (67,76)	0.404	2.0 (-4.8,6.8)	0.0 (-11.3,5.5)	0.183
TG, mg/mL	92.0 (67.5,95.0)	79.0 (60.0,114.5)	0.593	85.5 (57.3,106.0)	76.0 (65.3,96.3)	0.474	-1.0 (-17.0,20.5)	0.0 (-26.3,10.3)	0.420
CHOL, mg/mL	184.0 (176.0,207.0)	187.0 (170.0,200.0)	0.182	196.0 (163.8,211.5)	172.0 (159.5,178.3)	0.013	-3.0 (-11.0,3.5)	-12.5 (-29.8,-5.0)	0.039
LDL, mg/mL	124.2 (101.6,135.2)	118.6 (106.2,124.7)	0.117	123.3 (97.0,131.0)	98.4 (88.0,106.3)	0.007	-9.2 (-14.0,4.9)	-18.2 (-23.1,-6.2)	0.031
HDL, mg/mL	51.0 (45.0,56.5)	48.0 (41.5,58.5)	0.628	55.0 (50.0,62.0)	54.5 (52.0,65.5)	0.572	-1.0 (-5.5,2.0)	2.5 (0.0,6.0)	0.456
non-HDL, mg/mL	136.0 (123.0,148.5)	136.0 (119.0,146.0)	0.218	141.5 (112.5,149.0)	115.0 (101.0,126.3)	0.009	-1.0 (-14.0,4.0)	-17.0 (-30.0,-10.3)	0.012

[a]Data are presented as median (interquartile range). There were no statistically significant between-group differences at baseline (*p* values obtained by permutation test, n=1000).
[1]*p* values obtained by permutation test (n=1000) for within-group differences.
[2]*p* values obtained by permutation test (n=1000) for between-group differences of the changes during the intervention.
BMI, body mass index; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin A1c; HDL, high-density lipoproteins cholesterol; HOMA-IR, insulin resistance calculated by homeostatic model assessment; HWL, high weight loss group; LDL, low-density lipoproteins cholesterol; LWL, low weight loss group; OGTT, oral glucose tolerance test; SBP, systolic blood pressure; CHOL, total cholesterol; TG, triglycerides.

Table 3. Baseline energy, nutrient intake and adherence assessment of the Mediterranean diet and 12-week changes according to weight loss group[a]

	LWL (<10% weight loss)			HWL (>10% weight loss)			Treatment effect		
	Baseline	12 months	p[1]	Baseline	12 months	p[1]	Differences LWL	Differences HWL	p[2]
Energy (kcal/day)	2179.2 (1895.6,2465.1)	1705.9 (1432.1,1972.0)	0.013	2691.4 (2304.7,2855.5)*	1655.4 (1378.3,1754.8)	0.002	-341.6 (-670.6,-73.1)	-1014.6 (-1534.4,-607.4)	0.022
Carbohydrate (%)	38.1 (32.4,40.4)	38.0 (33.4,39.9)	0.848	37.6 (34.6,41.4)	39.4 (35.3,42.7)	0.255	-0.5 (-2.9,1.9)	3.4 (-0.7,5.7)	0.497
Protein (%)	21.4 (17.6,23.7)	23.6 (20.2,25.0)	0.152	17.5 (16.4,21.5)	27.5 (25.6,30.3)	<0.001	2.1 (-0.3,3.5)	9.2 (6.4,10.6)	<0.001
Total fat (%)	42.0 (38.4,44.6)	41.5 (37.5,42.5)	0.338	42.2 (37.9,45.6)	33.0 (29.8,35.3)	0.003	-1.1 (-5.7,1.4)	-12.0 (-13.9,-5.4)	0.006
Saturated (%)	11.7 (9.9,13.4)	10.9 (10.0,11.5)	0.060	12.6 (10.8,14.2)	7.9 (6.6,10.0)	<0.001	-0.8 (-2.7,0.4)	-3.5 (-5.1,-2.4)	0.018
Monounsaturated (%)	19.0 (15.1,21.9)	19.3 (15.9,20.7)	0.892	19.5 (16.0,20.6)	15.1 (10.6,17.2)	0.014	0.6 (-1.4,1.7)	-5.8 (-7.1,-0.9)	0.029
Polyunsaturated (%)	7.0 (6.0,8.2)	6.9 (6.0,7.4)	0.385	6.5 (5.4,8.4)	5.8 (5.1,6.5)	0.252	-0.2 (-1.6,0.2)	-0.2 (-3.3,0.6)	0.826
Cholesterol (g/day)	406.2 (369.2,456.4)	308.2 (280.0,373.6)	0.014	439.3 (363.1,473.3)	375.2 (290.4,404.0)	0.003	-78.7 (-118.2,20.1)	-78.8 (-115.9,-41.9)	0.379
Ethanol (g/day)	1.2 (0.0,3.4)	0.4 (0.0,2.1)	0.059	1.6 (0.5,4.2)	1.0 (0.0,2.5)	0.075	-0.1 (-1.2,0.0)	-0.7 (-2.6,0.2)	0.109
Fiber (g/day)	24.4 (20.7,30.5)	20.9 (18.2,27.1)	0.283	31.9 (25.0,34.7)	28.5 (23.7,31.5)	0.763	-4.6 (-8.2, 2.5)	-2.5 (-7.1,5.7)	0.606
MedDiet score	9 (7,10)	9 (7,9.5)	0.878	7.50 (6.75,8.25)	12 (12,12)	0.002	0 (-1,1)	5 (3,5.2)	<0.001
Physically Active[b]	3 (20.0%)	4 (26.7%)	1.00	1 (8.3%)	10 (83.3%)	0.008	13.3% (6.7%)	75.0% (0.0%)	0.004

[a]Data are presented as median (interquartile range) for quantitative variables, or number of subjects (%) for categorical variables.
[b]Women physically active were considered if they reported to perform at least 150 minutes of moderate physical activity per week or 60 minutes of intense physical activity per week, measured using Rapid Assessment of Physical Activity questionnaire. Treatment effect on physical activity is presented as: % of subjects that increased physical activity as recommended (% of subjects that decreased physical activity as sedentary).
[1]*p* values were obtained by permutation test (n=1000) within-groups for quantitative variables or McNemar's test for categorical variables.
[2]*p* values were obtained by permutation test (n=1000) between-group for quantitative variables or repeated measures logistic regression (interaction term) for categorical variables.
**p* values of statistical differences between groups at baseline, obtained by permutation test for quantitative variables or Fisher test for categorical variables: *** p<0.001, ** p<0.01, * p<0.05

HWL, high weight loss group; LWL, low weight loss group

Material Suplementario

PUBLICACION 4

Figure S1. Flow diagram of the participants and sample selection of the study

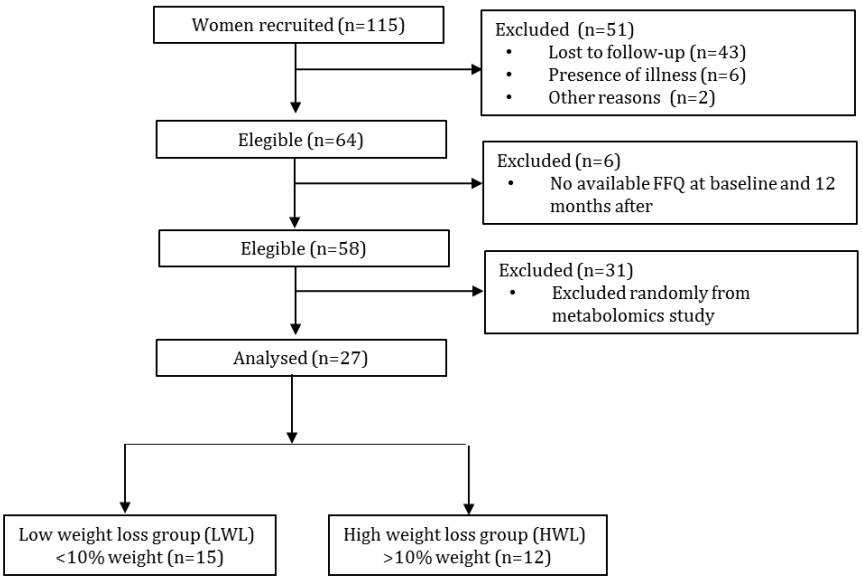
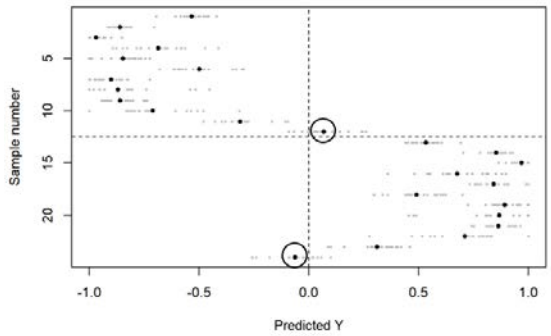


Figure S2. Results from random forest analysis within high weight loss group (HWL): (A) Predictive classification of subjects according to time point (misclassified individuals are highlighted with a circle, and are calculated as those in the quadrants of the right-left diagonal, being the upper and lower half of the graph each other's opposites from the same individual); (B) Histograms for permutation test (n=1000) of the repeated double-cross validated random forest model for classification of subjects according to time point within HWL.

A



B

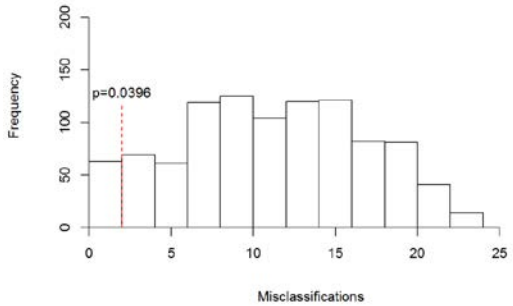


Figure S3. Results from random forest analysis within low weight loss group (LWL): (A) Predictive classification of subjects according to time point (misclassified individuals are highlighted with a circle, and are calculated as those in the quadrants of the right-left diagonal, being the upper and lower half of the graph each other's opposites from the same individual); (B) Histograms for permutation tests (n=1000) of the repeated double-cross validated random forest model for classification of subjects according to time point within LWL.

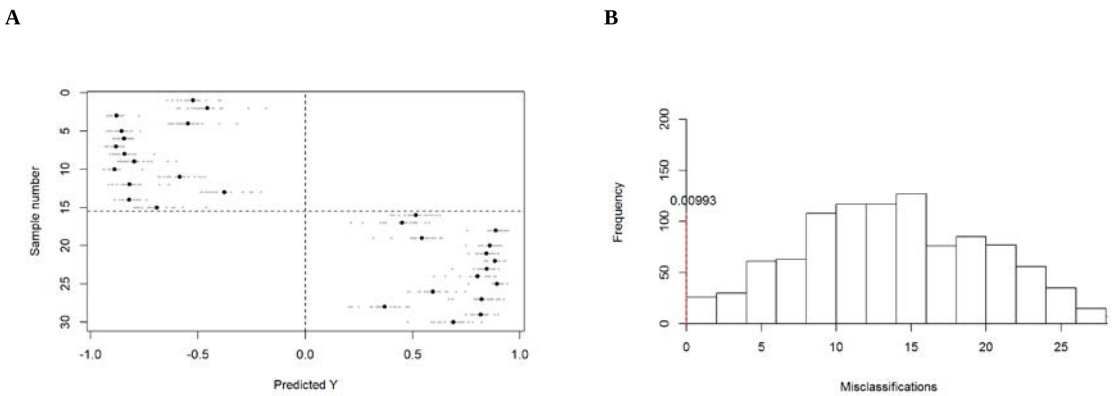
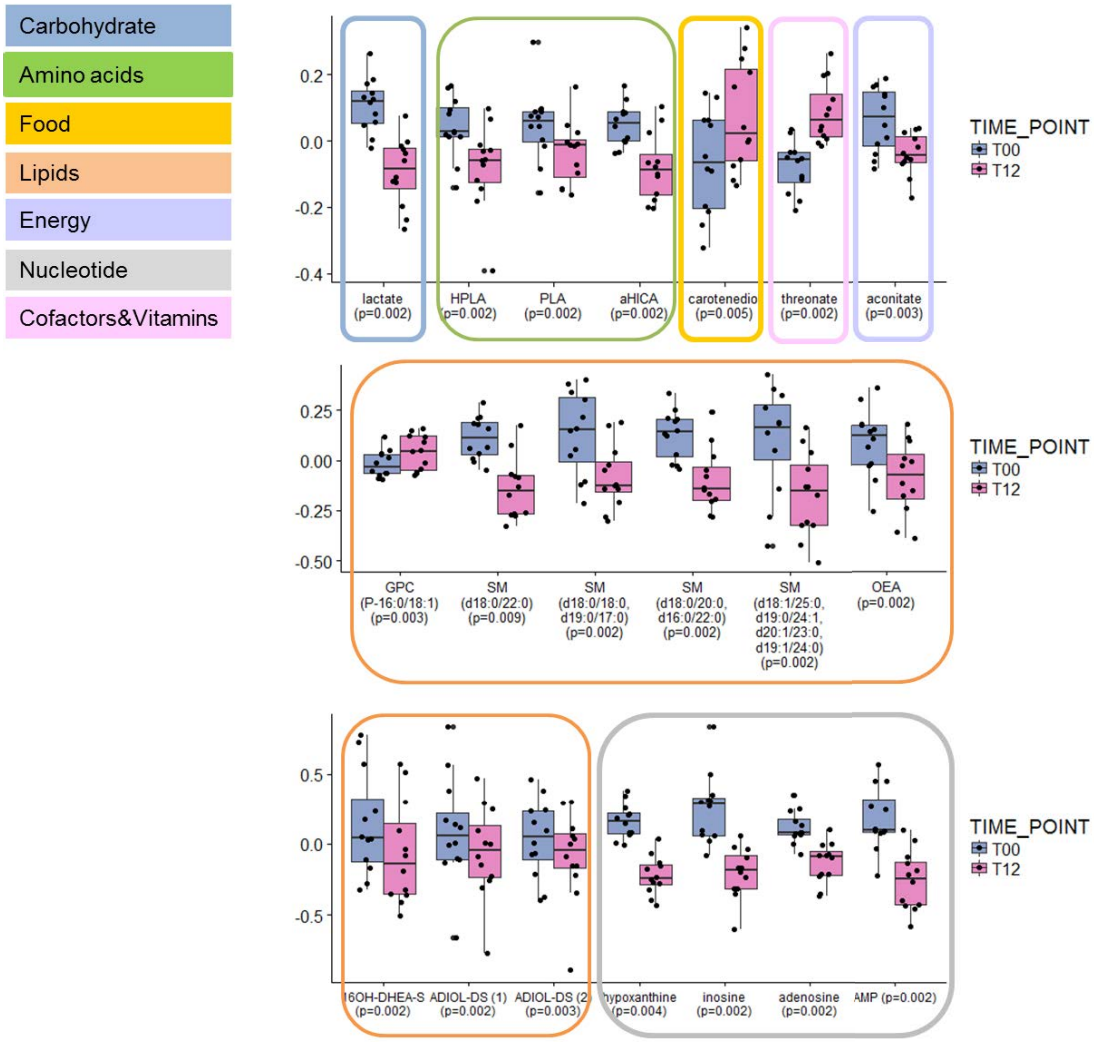


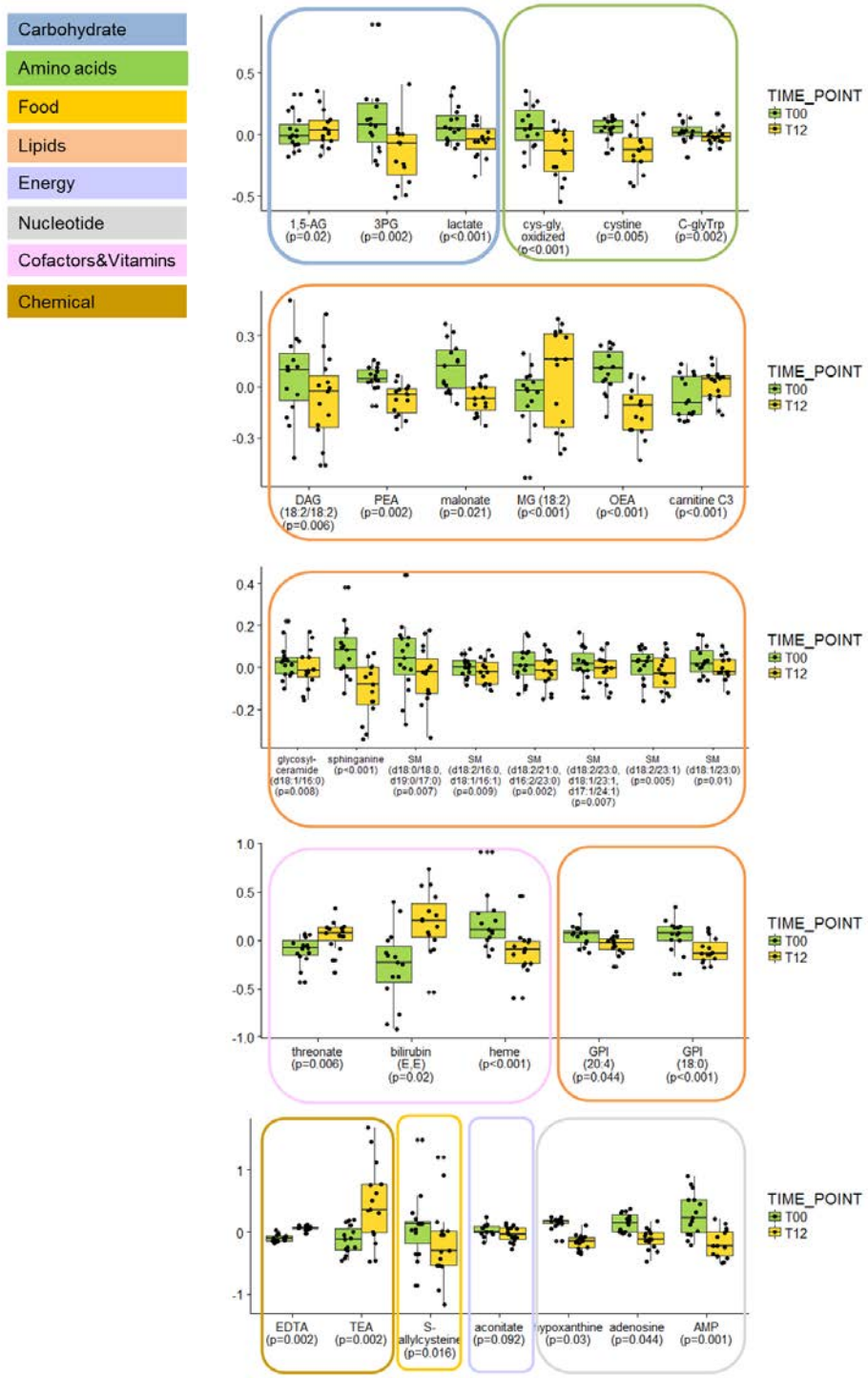
Figure S4. Changes within high weight loss group of metabolites selected by the repeated double-cross validated random forest model.*



*p-values were obtained by permutation test (n=1000) of the changes within loss group.

16OH-DHEA-S, 16α-hydroxydehydroepiandrosterone 3-sulfate; ADIOL-DS, androstenediol (3β,17β) disulfate; αHICA, α-hydroxyisocaproate ; AMP, adenosine 5'-monophosphate; GPC (P-16:0/18:1), 1-(1-enyl-palmitoyl)-2-oleoyl-glycerophosphocholine (P-16:0/18:1); HPLA, 3-(4-hydroxyphenyl)lactate; OEA, oleoylethanolamide; PLA, phenyllactate ; SM, sphingomyelin; SM (d18:0/22:0), behenoyl dihydrosphingomyelin (d18:0/22:0).

Figure S5 Changes within low weight loss group of metabolites selected by the repeated double-cross validated random forest model.*

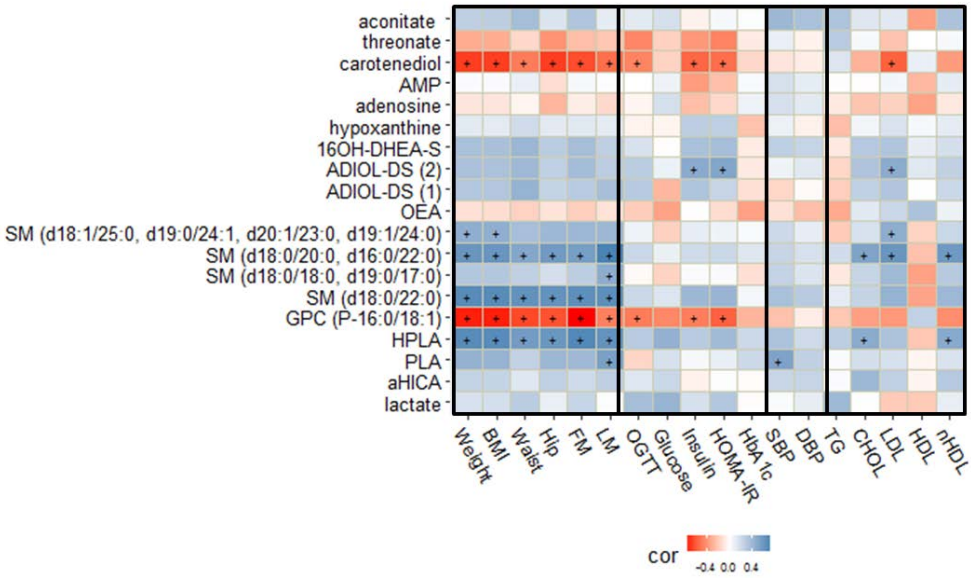


*p values were obtained by permutation test (n=1000) of the changes within maintained group.

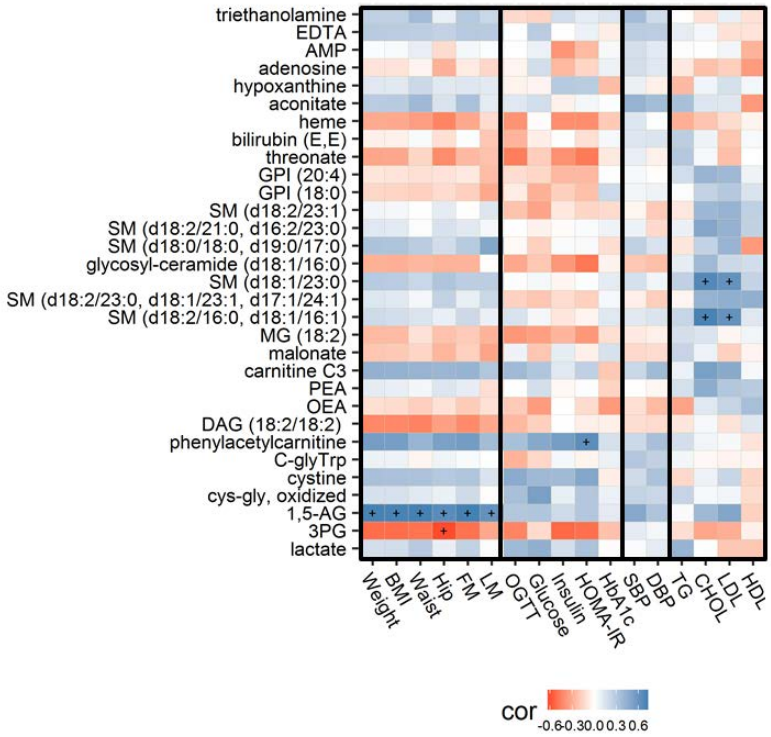
1,5-AG, 1,5-anhydroglucitol; 3PG, 3-phosphoglycerate; ADIOL-S, androstenediol (3α, 17α) monsulfate; AMP, adenosine 5'-monophosphate ; carnitine (C3), propionylcarnitine; C-glyTrp, C-glycosyltryptophan; cys-gly, cys-glycine, oxidized; DG, linoleoyl-linoleoyl-glycerol; EA-S, epiandrosterone sulfate; EDTA, ethylenediaminetetra-acetate; glycosyl ceramide, glycosyl-N-palmitoyl-sphingosine; GPI, stearoyl-glycosylphosphatidylinositol; MG (18:2), 1-linoleoylglycerol (18:2); OEA, oleoylethanolamide; PEA, palmitoyl ethanolamide; SM, sphingomyelin; SM (d18:1/23:0), tricosanoyl sphingomyelin (d18:1/23:0); TEA, triethanolamine;

Figure S6. Correlations of changes between metabolites and clinical parameters during the intervention program. (A) Metabolites selected by the repeated double-cross validated random forest model within high weight loss group and (B) Metabolites selected by the repeated double-cross validated random forest model within low weight loss group *

A

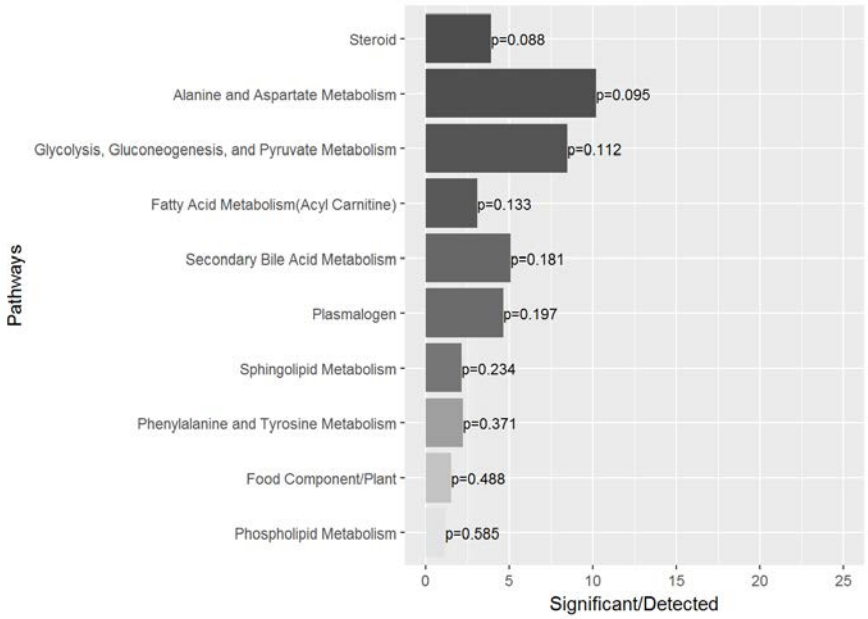


B



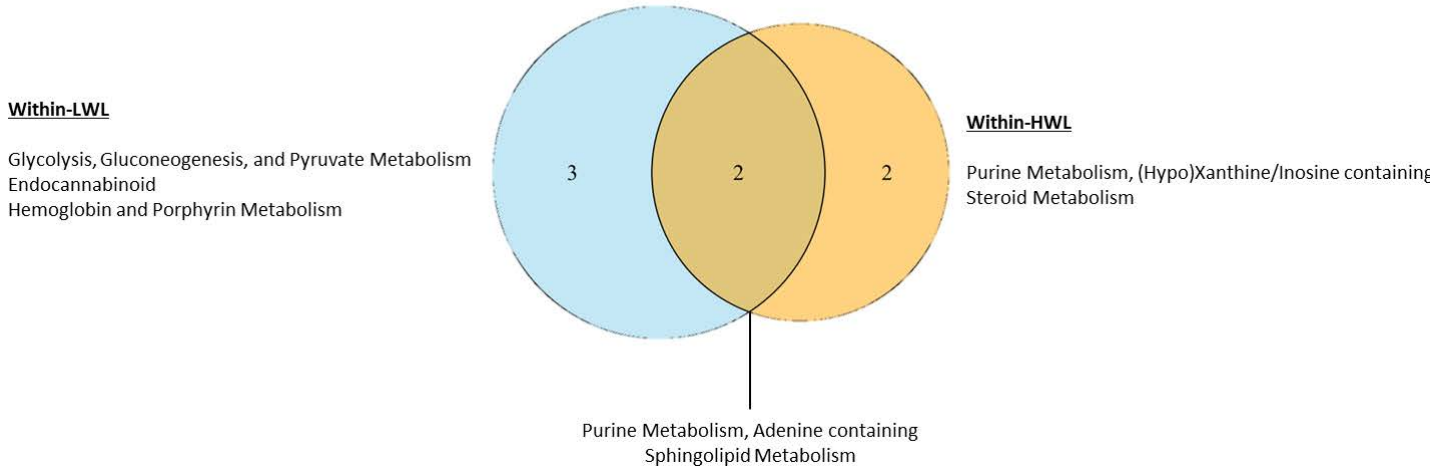
*Associations determined by Spearman correlations adjusting p values by FDR, with significant threshold set at $p<0.1$.
BMI, body mass index; CHOL, total cholesterol; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin A1c; HDL, high-density lipoproteins cholesterol; HOMA-IR, insulin resistance calculated by homeostatic model assessment; LDL, low-density lipoproteins cholesterol; OGTT, oral glucose tolerance test; SBP, systolic blood pressure; TG, triglycerides.

Figure S7. Pathway analysis of discriminant metabolites between groups*



*Pathway analysis was performed through hypergeometric test.

Figure 8. Venn diagram of the statistically significant pathways altered in high weight loss group (HWL) and low weight loss group (LWL) *



*Pathway analysis performed through hypergeometric test taking into account the significant metabolites of within-HWL and within-LWL analysis.

PUBLICACION 5

Metabotypes of response to bariatric surgery independent of the magnitude of weight loss

Magali Palau-Rodriguez, Sara Tulipani, Anna Marco-Ramell, Antonio Miñarro, Olga Jáuregui, Alex Sanchez-Pla, Bruno Ramos-Molina, Francisco J Tinahones, Cristina Andres Lacueva

PLOS ONE

Factor de Impacto: 2.81 Q1 (15/64) MULTIDISCIPLINARY SCIENCES (Indexado en Web of Science)

Los resultados de esta publicación fueron presentados en formato de:

- Comunicación oral: BioMed PhD day, Barcelona, España, 7 Diciembre 2017, "Globesity is a fact.Personalized weight loss intervention through metabolomics".
- Comunicación oral: XIII Congreso Nacional Sociedad Española de Estudios de la Obesidad, 15-17 Marzo 2017, Sevilla, España, "Estudio metabolómico del efecto de la cirugía bariàtrica en individuos obesos mórbidos metabólicamente sanos frente obesos mórbidos metabólicamente enfermos".

RESUMEN PUBLICACION 5

Objetivos: El objetivo de este trabajo fue la identificación de perfiles metabólicos asociados a la respuesta a la cirugía bariátrica y determinanr adaptaciones metabólicas a corto-plazo. Para esto se establecieron tres subobjetivos:

1) Discriminar signaturas metabólicas de respuesta a la cirugía bariátrica asociadas a la condición metabólica basal del paciente, metabólicamente *enfermo* o metabólicamente *sano*.

2) Identificar metabotipos de respuesta a la cirugía bariátrica según la evolución metabólica del paciente.

3) Asociar estos cambios con diferentes grados de mejoras metabólicas según los parámetros antropométricos y clínicos.

Metodología: Se aplicó un flujo metabolómico cuantitativo mediante una aproximación LC- y FIA-ESI-MS/MS para cuantificar 188 metabolitos (acilcarnitinas, (liso)fosfatidilcolinas, esfingomielinas, amino acidos, aminas biogenas y hexosas) en 39 muestras de suero de sujetos con obesidad severa, antes y después de un, tres y seis meses de la cirugía bariátrica. Para el estudio de las tendencias metabólicas post-cirugía se utilizó el análisis de factores múltiples (del inglés, *multiple factor analsis*) y el análisis discrimínate de mínimos cuadrados parciales con filtro *sparce* (del inglés, *sparce partial least squares regression* (sPLS-DA). Para la identificación de metabotipos se utilizó el método de grupos K-means y un análisis lineal mixto para estudiar la evolución del los metabolitos a lo largo del tiempo.

Resultados: La condición metabólica basal de los pacientes no mostró influenciar el metaboloma sérico post-cirugía de los metabolitos cuantificados. Se identificaron cambios temporales del metaboloma después de la cirugía bariátrica, independientemente del estado metabólico basal del individuo. Cambios en los amino ácidos de cadena ramificada (isoleucina, leucina y valina) se observaron ya en

el primer mes post-cirugía. En el periodo de uno a tres meses correlacionaron cambios en los niveles de la carnitina, la fenilalanina, la arginina, la ornitina y varios fosfolípidos acil-etil. Por primera vez, se identificaron dos grupos de pacientes que se diferenciaban en el intervalo de tiempo de seis meses- basal por sus variables metabólicas (metabotipos). Clínicamente, estos metabotipos perdieron peso de manera semejante y presentaban diferentes grados de mejora en su condición de resistencia a la insulina, niveles de colesterol, niveles de lipoproteína de baja densidad y niveles de ácido úrico.

Conclusiones: Este estudio abre nuevas perspectivas en el estudio de la obesidad y abre nuevas hipótesis en los cambios metabólicos asociados a la cirugía.

PLOS ONE

RESEARCH ARTICLE

Metabotypes of response to bariatric surgery independent of the magnitude of weight loss

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Abstract

Objective

Bariatric surgery is considered the most efficient treatment for morbid obesity and its related diseases. However, its role as a metabolic modifier is not well understood. We aimed to determine biosignatures of response to bariatric surgery and elucidate short-term metabolic adaptations.

Methods

We used a LC- and FIA-ESI-MS/MS approach to quantify acylcarnitines, (lyso)phosphatidylcholines, sphingomyelins, amino acids, biogenic amines and hexoses in serum samples of subjects with morbid obesity (n = 39) before and 1, 3 and 6 months after bariatric surgery. K-means cluster analysis allowed to distinguish metabotypes of response to bariatric surgery.

Results

For the first time, global metabolic changes following bariatric surgery independent of the baseline health status of the subjects have been revealed. We identify two metabolic phenotypes (metabotypes) at the interval 6 months-baseline after surgery, which presented differences in the levels of compounds of urea metabolism, gluconeogenic precursors and (lyso) phospholipid particles. Clinically, metabotypes were different in terms of the degree of improvement in insulin resistance, cholesterol, low-density lipoproteins and uric acid independent of the magnitude of weight loss.

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Competing interests: The authors have declared that no competing interests exist.

Conclusions

This study opens new perspectives and new hypotheses on the metabolic benefits of bariatric surgery and understanding of the biology of obesity and its associated diseases.

Introduction

Over the last few decades bariatric surgery has been used as a powerful "disease modifier" for the treatment of morbid obesity, not merely as a strategy for weight loss [1]. To date, bariatric surgery is the most successful treatment for weight loss, metabolic control and effective prevention, remission or delay of type 2 diabetes progression [2].

The metabolic co-morbidities of obesity such as diabetes improve after bariatric surgery even before weight loss occurs. However, why this happens is still unclear. Previous investigations focused on metabolic patterns in a specific view such as according to the type of surgery received [3] or the remission state of the subject before the intervention [4]. Few studies have been carried out to explore signatures after bariatric surgery and their association with the heterogeneity of metabolic improvement [5]. Therefore, unveiling different metabolic fingerprints following bariatric surgery in the earliest stages might help to elucidate the physiological pathways of insulin resistence and the onset of the related co-morbidities.

Metabolomics has emerged in biomedical investigation as a tool to uncover obesity-associated metabolic diseases and could give insights into the prognosis of weight loss intervention through the study of the metabolome. The study of the metabolome through high-throughput mass spectrometry provide a comprehensive overview of the metabolic processes in a given moment and define metabolic phenotypes (metabotypes) of responses to a treatment or an intervention [6].

Hence, the aims of the present study are to: 1) discriminate metabolic signatures of response to bariatric surgery associated with the baseline condition of the patient, "metabolically unhealthy" or "metabolically healthy"; 2) elucidate metabotypes of response to bariatric surgery according to their metabolic evolution; and 3) associate these changes with different degrees of metabolic improvement according to anthropometric and clinical parameters. The baseline condition of the patients dissipate after the surgery. We have defined two metabotypes of response independently of the gender, age or the amount of weight loss but dependent of insulin resistance, cholesterol and uric acid levels. Despite further studies are needed, our results open new hypothesis in the study of obesity and provide a comphensive view of the metabolic changes after the surgery.

Materials and methods

Serum samples from 39 patients with morbid obesity (body mass index (BMI) > 40 kg/m^2) were collected before and 1 (T1), 3 (T3) and 6 (T6) months after bariatric surgery at the Virgen de la Victoria University Hospital (Malaga, Spain). The availability of the samples after the surgery were 38 (T1), 34 (T3), 27 (T6) respectively. Twenty-six patients underwent a Roux-en-Y gastric bypass (RYGB) procedure and 13 patients sleeve gastrectomy (SG). All the patients were adults (19–59 years old), comprising 27 females and 12 males with $>$ 10-year history of obesity. The inclusion and exclusion criteria are detailed in [7]. Briefly, the exclusion criteria were the intake of antidiabetic, corticosteroid or antibiotic drug treatment; the presence of acute or chronic infection, a history of alcohol abuse or drug dependence, a history of cancer.

Other treatment including anti-inflammatory, antihypertensive and anti-cholesterolemic agents were recorded.

Initially, subjects were stratified according to their degree of metabolic syndrome, as defined by the Adult Treatment Panel III criteria [8]: metabolically healthy (MH) subjects with ≤ 2 criteria (n = 21) and metabolically unhealthy (MU) subjects with ≥ 3 criteria (n = 18).

Anthropometric and clinical parameters

Anthropometric and clinical parameters were measured using standardized techniques [9,10]. In this work we report: a) anthropometric markers: body weight, BMI, waist circumference, hip circumference and waist-hip ratio; b) markers of glucose regulation: glycated haemoglobin A1c (HbA1c); plasma fasting glucose and insulin, Homeostatic Model Assessment (HOMA-IR score = fasting insulin x fasting glucose/22.5 AU; c) blood pressure markers: diastolic and systolic blood pressure; d) blood lipid markers: total cholesterol (CHOL), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL), and triglycerides (TG); e) hormones: leptin and adiponectin; f) kidney impairment/disease markers: urea, uric acid and creatinine; g) other standard biochemical markers including liver enzymes, C-reactive protein (CRP).

The protocol was approved by the local Ethics and Research Committee (Hospital Universitario Virgen de la Victoria, Málaga) and all participants provided written informed consent.

Metabolomic analysis

Amino acids and biogenic amines were acquired by liquid chromatography (10 μl injection volume) and acylcarnitines, glycerophospholipids, sphingolipids and total hexoses by flow injection analysis (20 μl injection volume), both coupled with tandem mass spectrometry in positive and negative electrospray ionization modes (S1 Table). All the analyses were performed on a QTRAP 6500 System (AB Sciex, Framingham, MA).

To avoid run-order effects, serum samples were analysed in a randomized batch format. Quality controls were analysed throughout the whole run to control the stability and performance of the system and evaluate the quality of the acquired data [11]. Metabolites were quantified 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 [12].

Isotopic correction was performed with the software Met*IDQ*™ (Biocrates Life Sciences AG). The limits of detection (LOD) and lower (LLOQ) and upper limits of quantification were experimentally determined.

Data pre-processing

Metabolite measures below the LOD or LLOQ in more than 25% of subjects at any time point and/or with high analytical variances (CV>25% in the quality control) were excluded from further analyses (S1 Table).

Values below the LOD and LLOQ were imputed using theoretical LOD and LLOQ values ($LOD/\sqrt{2}$ and $LLOQ/\sqrt{2}$) within every metabolite level across all the samples. Data were log-transformed. The missing values were dealt with using the K-means nearest neighbour imputation method (K = 10). Finally, data were subjected to auto-scaling.

Statistical analysis

T-test for independent samples was used to compare anthropometric/clinical and metabolic variables between patient groups in the preoperative state (baseline). Fisher's exact tests were performed to explore the distribution of males and females. Multiple factor analysis (MFA) was used to explore how the metabolic variables evolved over time, taking into account the three following temporal increments: T1–T0, T3–T1 and T6–T3. Further information about MFA is available as Supporting Information and in [13]. Principal component analysis (PCA) was performed to provide single snapshots of each increment of time in respect to the baseline (T6–T0, T3–T0 and T1–T0). Global metabolic post-surgery changes were evaluated by MFA through the projection of each subject on the individual factor map and each metabolite in the correlation circle, represented as vectors.

Linear mixed models were performed to analyse: 1) the track of single metabolites and anthropometric/clinical variables after the intervention; 2) the changes of these variables at each pre- and post-surgery time point; 3) the influence of the type of surgery; and 4) the baseline health status throughout the evolution of the patient after bariatric surgery.

In order to associate the different patterns of response observed on the MFA's individual factor map, metabolic clusters were determined at T6–T0 with K-means cluster analysis (Supporting Information). The most responsible variables for cluster separation were the most correlated with the PCA (correlations $r > 0.75$ or < -0.75 with the PC1). The application of sparse partial least squares discriminant analysis (sPLS-DA) confirmed them. The sparsity (*eta*) and number of hiding components (*K*) were chosen using a 10-fold cross-validation procedure and the predictability of the model was calculated on a leave-one-out basis. Linear mixed models also confirmed the discriminant metabolites and anthropometric/clinical differences when the *P* value of the interaction *time* x *cluster* was statistically different ($p<0.05$).

Pearson correlation coefficients were calculated to estimate the linear correlation among the discriminant metabolic variables and anthropometric/clinical variables in each metabolic cluster. Metabolite-clinical correlations were represented as a network and metabolite-metabolite correlations as a heatmap.

Enrichment analysis was performed using the bioinformatics tool ChemRich (Chemical Similarity Enrichment Analysis for Metabolomics) for each group of the study and with all the metabolites identified. The ChemRich statistical approach compares chemical similarities using the Medial Subject Headings database and Tanimoto chemical similarity coefficients to cluster metabolites into non-overlapping chemical groups. *P* values are obtained by employing the Kolmogorov-Smirnov test using the created clusters [14].

Since a potential effect on metabolite profiles has been described [15,16]: *gender*, *age*, *drug intake and type of surgery* were used as covariables in the univariate analysis, and only *gender* and *age* in the un-supervised multivariate analysis (K-means and sPLS-DA analysis) to minimize the influence in the formation of the clusters.

All the *P* values reported in this study were adjusted by false discovery rate multiple testing (5%), based on the Benjamin-Hochberg procedure [17]. When linear mixed models were used, the evaluation of the models was performed using the Akaike information criterion [18]. All the statistical analyses and graphics were computed in R3.3.1 except the correlation network, which was performed using Cytoscape 3.3.0.

Results

As shown in S1 Table we quantified 94 lipid metabolites (lysophosphatidylcholines (lysoPCs), acyl-alkyl phosphatidylcholines (PC ae), diacyl phosphatidylcholines (PC aa), sphingomyelins (SMs)) and 46 polar metabolites (acylcarnitines, amino acids and biogenic amines).

General metabolomic patterns over time

We analysed all the quantified metabolites to distinguish a metabolic temporal pattern through MFA analysis. The two first principal components explained the 44.9% of the variability (S1A Fig). The MFA score plot separated the time intervals T3–T1 from T6–T3 and T1–T0 in the PC1 and PC2, but only the PC2 allowed the separation of T6–T3 from T1–T0 (S1B and S1C Fig). Fig 1 shows the most correlated metabolites for each increment of time and principal component ($r > |0.7|$).

Temporal trends according to baseline status: Metabolically healthy (MH) versus metabolically unhealthy (MU) subjects

The individual factor map revealed different metabolic temporal profiles, but they were independent of the baseline health status of the subjects, defined by the Adult Treatment Panel III.

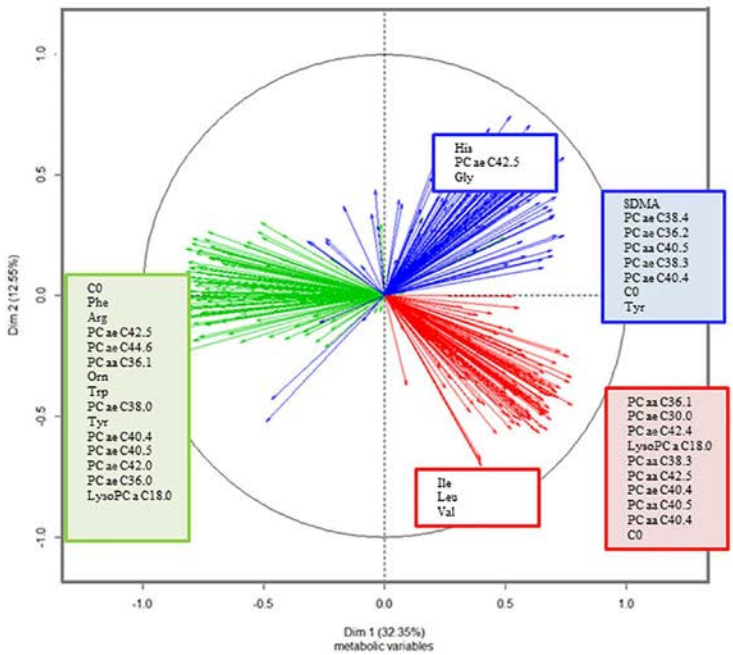

Fig 1. Correlation circle of metabolites in each increment of time in the first and second principal components: In red, metabolites correlated with T1–T0, in green, metabolites correlated with T3–T1, and in blue, metabolites correlated with T6–T3. Up to the most correlated 15 metabolites (correlation higher than 0.7 or smaller than -0.7) to each PC are shown. Metabolites correlated with the first dimension are written in the filled squares and those metabolites correlated with the second dimension are written in the white squares. Variables were adjusted by age and gender. The profiles of PC aa and PC ae and carnitine were also linked to T6–T3 in PC1. Carnitine negatively correlated with PC1 in the increment T3–T1 ($r = -0.88$, $p<0.001$). Changes in lysoPC a C18:0 inversely correlated with T1–T0 in PC1 ($r = 0.77$, $p<0.001$) and changes in T3–T1 in PC1 ($r = -0.82$, $p<0.05$). The increment T3–T1 correlated with a large profile of amino acids and certain PC aa and PC ae in PC1 ($r<-0.85$, $p<0.05$). Changes in tyrosine and PC ae C42:5 were kept in T6–T3 ($r = 0.71$ with PC2, $p<0.01$). Histidine and glycine correlated with the last time period ($r>0.70$ with PC2, $p<0.001$). Hence, T1–T0 was explained by changes in branched-chain amino acids (BCAAs, isoleucine, leucine and valine) ($r<-0.7$ with PC2, $p<0.001$) and PC ae and PC aa ($r>0.7$ with PC1, $p<0.001$).

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S2 Table shows the characteristics of each group at baseline. Clearly, at the baseline MU subjects presented higher levels of fasting glucose, HOMA-IR, VLDL and TG than MH. Metabolically, the MU group also had higher levels of alanine, glutamate, acylcarnitines C10:0 and C10:1, lysoPC a C16:0, C16:1 and C24:0 (S3 Table) than MH. However, the two groups presented a similar response to the bariatric surgery since no anthropometric/clinical or metabolic variables differed between groups ($p>0.05$ for *time* x *health*) (S4 and S5 Tables). A PCA corroborated these results (S2 Fig).

Temporal trends according to K-means cluster analysis

Identification of temporal metabolomic metabotypes. We performed a K-means cluster analysis with metabolite measurements in the time period T6–T0. The optimal number of clusters chosen according to the Calinski-Harabasz index (Supporting information) was two (cluster 1 and cluster 2). PC1 was the main contributor to separating the two clusters (43.2% of the total variability) (Fig 2A). Fifty-one metabolites correlated with PC1 ($r>0.75$ or <-0.75) (Table 1). Linear mixed models and a sPLS-DA, with an $eta = 0.7$, $K = 1$ and goodness of classification of 97.4%, confirmed these results.

In general, cluster 2 presented lower levels in all the selected metabolites at 6 months than at baseline, whereas cluster 1 had stable or even slightly higher levels than at baseline (S6 Table). In addition, the temporal pattern of cluster 1 was significantly different from cluster 2 for these metabolites. While the metabolite profiles of cluster 2 had a similar trend, specifically a sudden decrease followed by a slight recovery that quickly faded away, cluster 1 presented smoother metabolite profiles (S6 Table).

Baseline characterization of the two metabolic clusters. At baseline, clusters showed similar anthropometric/clinical parameters and were equally gender distributed (Table 2). Alanine, histidine, lysoPC a C16:0, C18:1, C20:3, C20:4 and C24:0, PC aa C28:1 and PC ae C30:0 were higher in cluster 2 (S7 Table).

Anthropometric/clinical changes over time. Global changes after the surgery in anthropometric/clinical parameters were also reported, independently of the clusters (Table 3). The two clusters were matched by the magnitude of weight loss (~35%) at 6 months versus baseline, but cluster 2 showed a greater decrease in the levels of fasting insulin, HOMA-IR, CHOL, LDL and uric acid, while cluster 1 showed a greater decrease of urea levels (Table 3).

Correlations between metabolic changes and anthropometric/clinical changes. To identify potential links between anthropometric/clinical and metabolic variables, and to elucidate metabolic differences between clusters, the next step was to correlate anthropometric/clinical variables with metabolites in T6–T0 for each cluster.

Fig 2B presents the correlation of cluster 1 between changes in clinical variables and discriminant metabolites of the clusters (Table 1). Those metabolites that were not the most discriminant between clusters but also correlated were also included in the network. Cluster 2 did not present statistical significance in these metabolites. S3A and S3B Fig correlate discriminant metabolites in cluster 1 and cluster 2 respectively.

The most metabolite classes in each metabotype are shown in S4A and S4B Fig. Both phenotypes present major changes in unsaturated PC ae and PC aa and SMs. The impact of changes in aromatic amino acids and BCAAs were also significant in cluster 1 whereas in cluster 2, saturated and unsaturated lysoPC and the amino acids and BCAAs did not reach statistical significance. Most of the metabolites that compose these groups of compounds decreased in cluster 2, but increased in cluster 1, except aromatic amino acids and the BCAAs, that decreased in both clusters. Data are shown in S8 Table.

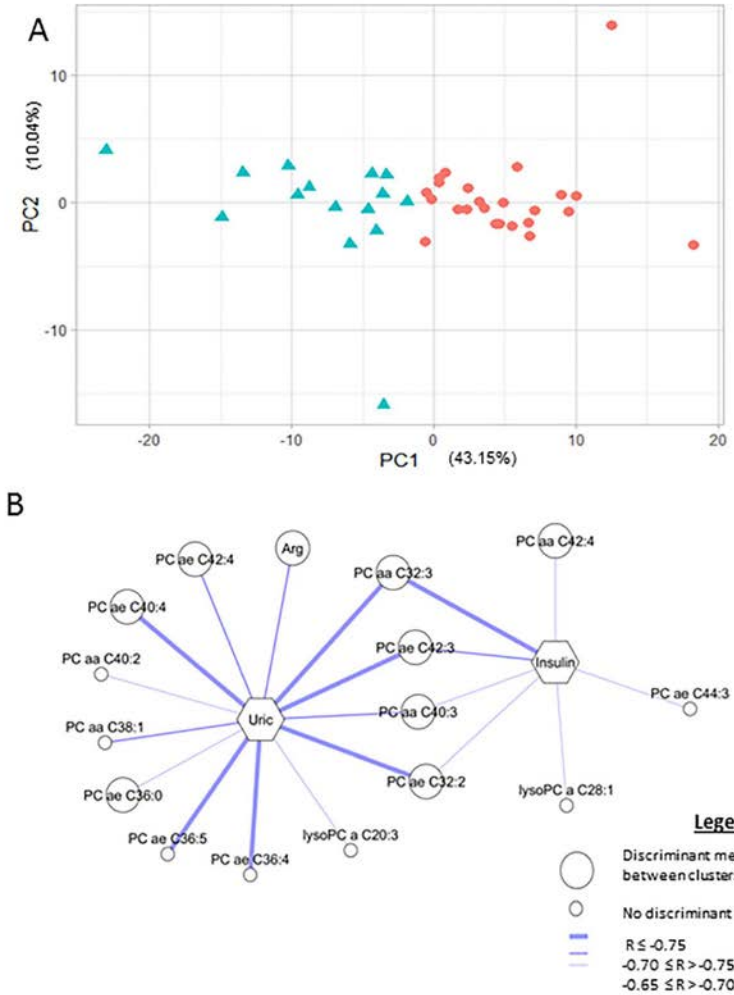

Fig 2. A. Scatter plot of cluster 1 (red dots) and cluster 2 (blue triangles) derived from K-means cluster analysis in the first (PC1) and second (PC2) principal components. B. Network of the correlations at T6–T0 of phenotype 1 between changes in uric acid (Uric) and fasting insulin (Insulin) and metabolites is represented. Only correlations with statistical significance and corrected by false-discovery rate are drawn. The most discriminant metabolites between clusters (correlation with PCA $r > 0.75$ or $r < -0.75$) are represented by a big circle, and other metabolites by a small circle. Correlations $r \leq -0.75$ are represented by a thick line, correlations $-0.70 \leq r > -0.75$ are represented by a medium line and $-0.65 \leq r > -0.70$ by a thin line. Arg: arginine, PC aa: diacyl phosphatidylcholines, PC ae: acyl-alkyl phosphatidylcholines, and lysoPC: lysophosphatidylcholines.

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Table 1. The most discriminating metabolites for K-means at 6-month increment versus baseline (sorted by family and alphabetical order).

Metabolites[a]	Cluster 1[b]	Cluster 2[b]	PC1 correlation[c]	*P*- Time[d]	*P*- Time X Cluster[d]	*P*- 6m X Cluster[d]
Alanine	0.45	-0.72	0.77	<0.05	<0.05	<0.05
Arginine	0.44	-0.71	0.77	<0.001	<0.05	<0.05
Histidine	0.38	-0.61	0.77	<0.001	<0.05	<0.05
Tyrosine	0.50	-0.81	0.78	<0.001	<0.05	<0.001
SDMA	0.51	-0.82	0.81	0.47	<0.05	<0.05
Carnitine	0.50	-0.80	0.81	<0.001	<0.05	<0.001
LysoPC a C16:0	0.46	-0.74	0.80	0.26	<0.001	<0.001
LysoPC a C18:0	0.51	-0.82	0.81	<0.001	<0.001	<0.001
LysoPC a C18:1	0.47	-0.76	0.79	<0.05	<0.05	<0.001
PC aa C28:1	0.55	-0.89	0.85	<0.001	<0.001	<0.001
PC aa C32:0	0.43	-0.69	0.81	0.24	<0.001	<0.001
PC aa C32:3	0.48	-0.76	0.81	0.09	<0.05	<0.001
PC aa C34:1	0.44	-0.71	0.82	0.82	<0.001	<0.001
PC aa C34:2	0.49	-0.78	0.88	<0.05	<0.001	<0.001
PC aa C36:0	0.55	-0.88	0.81	<0.001	<0.001	<0.001
PC aa C36:1	0.51	-0.81	0.82	<0.001	<0.001	<0.001
PC aa C36:2	0.48	-0.77	0.83	<0.001	<0.001	<0.001
PC aa C36:3	0.52	-0.82	0.82	<0.001	<0.001	<0.001
PC aa C36:4	0.38	-0.61	0.80	0.19	<0.05	<0.001
PC aa C38:0	0.58	-0.94	0.83	<0.05	<0.001	<0.001
PC aa C38:4	0.41	-0.65	0.79	<0.05	<0.001	<0.001
PC aa C38:5	0.40	-0.65	0.80	0.20	<0.05	<0.001
PC aa C38:6	0.51	-0.82	0.78	<0.05	<0.001	<0.001
PC aa C40:3	0.50	-0.81	0.79	<0.05	<0.001	<0.001
PC aa C40:4	0.55	-0.89	0.88	<0.001	<0.001	<0.001
PC aa C42:0	0.52	-0.84	0.75	0.48	<0.05	<0.001
PC aa C42:4	0.47	-0.75	0.76	<0.05	<0.001	<0.001
PC aa C42:5	0.47	-0.76	0.82	<0.05	<0.001	<0.001
PC ae C32:2	0.51	-0.81	0.89	<0.05	<0.05	<0.001
PC ae C34:0	0.56	-0.90	0.79	<0.05	<0.001	<0.001
PC ae C34:1	0.52	-0.83	0.90	<0.05	<0.001	<0.001
PC ae C36:0	0.55	-0.88	0.90	0.20	<0.001	<0.001
PC ae C36:1	0.53	-0.85	0.81	<0.001	<0.001	<0.001
PC ae C36:2	0.52	-0.84	0.81	<0.001	<0.001	<0.001
PC ae C38:0	0.52	-0.83	0.86	<0.001	<0.05	<0.001
PC ae C38:6	0.46	-0.74	0.77	<0.001	<0.05	<0.001
PC ae C40:4	0.55	-0.88	0.92	<0.001	<0.001	<0.001
PC ae C40:5	0.50	-0.80	0.86	0.74	<0.05	<0.001
PC ae C40:6	0.53	-0.85	0.85	0.34	<0.05	<0.001
PC ae C42:3	0.53	-0.85	0.83	<0.001	<0.001	<0.001
PC ae C42:4	0.49	-0.78	0.88	<0.001	<0.001	<0.001
PC ae C42:5	0.52	-0.84	0.89	0.31	<0.05	<0.001
PC ae C44:4	0.45	-0.71	0.78	<0.001	<0.05	<0.001
PC ae C44:5	0.47	-0.75	0.84	0.19	<0.05	<0.001
PC ae C44:6	0.49	-0.78	0.79	<0.05	<0.05	<0.001
SM (OH) C14:1	0.51	-0.81	0.87	0.23	<0.001	<0.001
SM (OH) C16:1	0.47	-0.75	0.78	0.28	<0.05	<0.001

(*Continued*)

Table 1. (Continued)

Metabolites[a]	Cluster 1[b]	Cluster 2[b]	PC1 correlation[c]	*P*- Time[d]	*P*- Time X Cluster[d]	*P*- 6m X Cluster[d]
SM C16:0	0.49	-0.79	0.87	0.52	<0.001	<0.001
SM C16:1	0.41	-0.66	0.80	0.30	<0.05	<0.001
SM C24:0	0.54	-0.86	0.76	<0.001	<0.001	<0.001
SM C24:1	0.46	-0.74	0.82	0.45	<0.05	<0.001

[a] Metabolites with R>0.75 or R<-0.75 with the first component.

[b]Cluster 1 (Cl-1) and 2 (Cl-2) were derived from K-means cluster analysis of 140 different biomarkers. Values are changes 6 months respect baseline of variables after being adjusted by age and gender.

[c] Correlation coefficient with the first principal component (43.15%). sPLSDA confirmed the results except His, PC.aa.C36.4, PC.aa.C38.4, PC.aa.C38.5.

[d] *P* value derived from linear mixed model after log-transformed variables: TIME, effect of time over time (baseline, 1, 3 and 6 months after surgery), TIMExCLUSTER, interaction of both variables and 6mXCLUSTER, interaction of group at 6 months vs baseline. The model was adjusted by age, gender, drug intake and type of surgery and corrected for multiple testing by false discovery rate. Total n 39 patients, separate in Cluster 1 (Cl-1, n = 24) and Cluster 2 (Cl-2, n = 15) according K-means clustering at increment 6 month vs baseline. Numbers of serum samples 1, 3 and 6 months after surgery were as follows: Cl-1: 23, 21, 16 and Cl-2 15, 13, 11.

Abbreviations: LysoPC, lysophosphatidylcholine; PC aa, diacyl phosphatidylcholine; PC ae, acyl-alkyl phosphatidylcholine; SM, sphingomyelin; SMDA, symmetric dimethylarginine

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Discussion

No clinical or quantified metabolite confirmed the assumption that the "metabolically unhealthy" and "metabolically healthy" phenotypes respond differently over time to bariatric surgery. Therefore, we believe that the impact of bariatric surgery on the metabolic status is so intense that any initial metabolic differences are nullified.

Our results highlight shared and exclusive profiles of the response to surgery among the subjects throughout 6 months of follow-up, regardless of the baseline metabolic state, gender and age of the subjects and the surgical procedure received.

Overall, the morbidly obese traits almost vanished 6 months after surgery and the metabolic status of the subjects improved, thereby confirming previous reports [19]. For the first time, changes in metabolites at the increment T6–T0 indicated two potentially different metabolic phenotypes (metabotypes) of short-term adaptation to bariatric surgery, hereinafter metabotype 1 (cluster 1) and metabotype 2 (cluster 2). Although clusters were clinically similar at baseline, remarkable differences in the post-surgery progression were observed. Metabotype 2 presented a greater degree of improvement in fasting insulin, HOMA-IR index, total cholesterol, LDL and uric acid 6 months after surgery, whereas metabotype 1 showed lower levels of urea, suggesting over- and down-expression of specific metabolic pathways in each phenotype.

Amino acid remodelling after bariatric surgery

Shared trends after surgery between metabotypes. In line with other works, an increase of branched-chain amino acids (BCAAs, isoleucine, leucine and valine) and aromatic amino acids has been described in obesity and they have been proposed as biomarkers for screening the risk of developing diabetes [20]. This study reports that bariatric surgery remodels amino acid metabolism as early as in the first month post-surgery. An increase in the catabolic enzymes of the BCAAs metabolism in subcutaneous and visceral fat depots after bypass procedure could explain this phenomenon [21]. Recently, it has found that after the intervention, microbial functions involved in pathways of production of these amino acids are more similar to lean subjects [22].

Our study also mirrored changes in urea-nitric oxide (NO) metabolism, specially by changes in ornithine, arginine and symmetric dimethylarginine levels. A recent study pointed

Table 2. Subjects' demographic, anthropometric, biochemical and clinical characteristics before bariatric surgery[a].

	Cluster 1 (n = 24)	Cluster 2 (n = 15)	*P*
Type of surgery, RYGB (nSG)	13 (11)	13 (2)	0.05*
Gender, nF(nM) for RYGB, SG	10 (3), 6 (5)	10 (3), 1(1)	0.73*
Age, y	40.88 ± 9.51	40.67 ± 10.79	0.97
Fasting glucose, mmol/l	107.83 ± 38.05	104.33 ± 19.32	0.80
Fasting insulin, μU/ml	17.17 ± 9.66	18.29 ± 9.14	0.80
HOMA-IR, AU	4.86 ± 3.55	4.94 ± 3.12	0.97
HBA1c, %	5.58 ± 0.21	5.63 ± 0.45	0.84
HBA1c, mmol/mol	37.43 ± 2.30	38.00 ± 4.88	0.84
Weight, kg	136.88 ± 29.82	145.07 ± 22.80	0.42
BMI, kg/m^2	49.45 ± 9.11	52.23 ± 6.99	0.36
Waist-hip ratio	0.96 ± 0.20	0.89 ± 0.14	0.31
Waist circumference, cm	134.67 ± 21.57	137.29 ± 20.93	0.80
Hip circumference, cm	142.19 ± 21.50	154.43 ± 13.76	0.13
Diastolic pressure, mmHg	83 ± 15	83 ± 12	0.90
Systolic pressure, mmHg	135 ± 20	131 ± 11	0.71
LDL, mg/dl	122.46 ± 34.75	138.13 ± 38.11	0.29
HDL, mg/dl	52.42 ± 20.46	45.79 ± 13.77	0.31
VLDL, mg/dl	25.83 ± 12.48	25.77 ± 10.85	0.99
TG, mg/dl	129.17 ± 62.39	128.87 ± 54.23	0.99
CHOL, mg/dl	202.96 ± 43.95	212.53 ± 44.03	0.60
CRP, mg/l	10.50 ± 8.33	9.66 ± 8.90	0.86
Leptin, mg/ml	78.36 ± 35.73	78.29 ± 36.50	1.00
Adiponectin, ng/ml	7.48 ± 3.75	7.95 ± 4.61	0.84
GOT, U/l	26.75 ± 17.65	19.33 ± 7.04	0.16
GPT, U/l	44.83 ± 28.35	44.07 ± 13.62	0.95
GGT, U/l	32.55 ± 17.78	29.73 ± 17.96	0.74
Uric acid, mmol/l	5.45 ± 1.00	5.59 ± 1.66	0.84
Creatinine, mmol/l	0.80 ± 0.17	0.78 ± 0.11	0.75
Urea, mg/dl	31.29 ± 9.60	31.53 ± 10.43	0.97
Medication use n (%)			
Lipid-lowering drugs	1 (4.16)	0 (0%)	1.00
Antihypertensive drugs	7 (29.16)	2 (13.33)	0.27
Antiinflamatory drugs	3 (12.50)	2 (13.33)	1.00

[a]Values are shown as Mean ± SD otherwise it is indicate. *P* values were determined by independent *t*-test after log-transformated variables

* *P* values were determined by fisher's exact test

Abbreviations: AU, arbitrary units; BMI, body mass index; CHOL, total cholesterol; CRP, c-reactive protein; GGT, gamma glutamyl transferase; GOT, aspartate transaminase; GPT, alanine transaminase; HbA1c, glycated haemoglobin A1c; HDL, high-density lipoproteins; HOMA-IR, insulin resitance calculated by homeostatic model assessment; LDL, low-density lipoproteins; RYGP, roux-en-Y gastric bypass; SG, sleeve gastrectomy; TG, triglycerides; VLDL, very low-density lipoprotein.

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a relationship between arginine and its methylated products (asymmetric and symmetric dimethylarginine) and adverse cardiovascular events and all-cause mortality [23]. We also found that these metabolites change differently according to the phenotype of the subject.

In accordance with others [24], we observed an increase in the glycine levels after surgery. Low levels of glycine have been detected in patients with a high risk of developing diabetes, caused by an increase in glutathione consumption [25], therefore this may be an indicator of glycaemic control.

Table 3. Anthropometric and clinical characteristics before bariatric surgery and 1, 3 and 6 months after surgery[a].

	Before surgery	After surgery, months			*P*-Time[b]	*P*-Time X Cluster[b]
		1	3	6		
Fasting glucose, μU/mL	106,49 ± 31,89	89.17 ± 11.25‡	83.76 ± 12.09‖	84.31 ± 11.06§	<0.001	0.44
Cl-1	107,83 ± 38,05	90.73 ± 8.03	83.00 ± 13.19	86.00 ± 11.09		
Cl-2	104,33 ± 19,32	86.71 ± 15.03	85.07 ± 10.27	81.85 ± 10.98		
Fasting insulin, μU/mL	17,60 ± 9,36	13.62 ± 8.25	9.94 ± 3.82§	8.95 ± 3.78§	<0.001	<0.05
Cl-1	17,17 ± 9,66	15.71 ± 9.35	10.57 ± 4.23	10.03 ± 3.92		
Cl-2	18,29 ± 9,14	10.33 ± 4.80#	8.83 ± 2.75	7.45 ± 3.12¶		
HOMA-IR, AU	4,89 ± 3,35	3.06 ± 1.99	2.05 ± 0.86‖	1.89 ± 0.86‖	<0.001	<0.05
Cl-1	4,86 ± 3,55	3.56 ± 2.21	2.15 ± 0.96	2.14 ± 0.87		
Cl-2	4,94 ± 3,12	2.28 ± 1.32¶	1.87 ± 0.65	1.55 ± 0.76		
HBA1c, %	5,59 ± 0,29	5.15 ± 0.26§	5.21 ± 0.29‖	5.20 ± 0.23§	<0.001	0.20
Cl-1	5,58 ± 0,21	5.27 ± 0.24	5.23 ± 0.23	5.16 ± 0.17		
Cl-2	5,63 ± 0,45	5.00 ± 0.20	5.19 ± 0.38	5.23 ± 0.28		
HBA1c, mmol/mol	37,60 ± 3,16	32.73 ± 2.81§	33.46 ± 3.22‖	33.32 ± 2.51§	<0.001	0.18
Cl-1	37,43 ± 2,30	34.10 ± 2.66	33.68 ± 2.56	32.88 ± 1.83		
Cl-2	38,00 ± 4,88	31.13 ± 2.19	33.16 ± 4.11	33.63 ± 3.01		
Weight, kg	140,03 ± 27,31	124.50 ± 23.49‖	113.99 ± 22.31‖	100.41 ± 19.53‖	<0.001	0.90
Cl-1	136,88 ± 29,82	123.49 ± 26.21	112.68 ± 24.25	99.59 ± 23.25		
Cl-2	145,07 ± 22,80	126.16 ± 19.00	116.25 ± 19.16	101.61 ± 13.11		
BMI, kg/m2	50,52 ± 8,37	45.09 ± 7.19‖	41.28 ± 6.80‖	36.42 ± 6.14‖	<0.001	0.87
Cl-1	49,45 ± 9,11	44.45 ± 7.55	40.55 ± 6.78	36.23 ± 7.09		
Cl-2	52,23 ± 6,99	46.14 ± 6.69	42.53 ± 6.91	36.69 ± 4.66		
Waist-hip ratio, ratio	0,93 ± 0,18	0.87 ± 0.11	0.90 ± 0.21	0.85 ± 0.07	0.27	0.07
Cl-1	0,96 ± 0,20	0.89 ± 0.11	0.86 ± 0.08	0.86 ± 0.07		
Cl-2	0,89 ± 0,14	0.85 ± 0.11	0.97 ± 0.33¶	0.85 ± 0.07		
Waist circumference, cm	135,71 ± 21,05	123.92 ± 16.06‖	116.54 ± 13.94‖	108.71 ± 13.87‖	<0.001	0.80
Cl-1	134,67 ± 21,57	124.30 ± 18.43	116.00 ± 15.33	107.65 ± 15.18		
Cl-2	137,29 ± 20,93	123.29 ± 11.80	117.54 ± 11.42	110.35 ± 11.98		
Hip circumference, cm	147,09 ± 19,53	141.65 ± 14.82	131.84 ± 18.98	125.58 ± 13.60‡	<0.001	0.07
Cl-1	142,19 ± 21,50	140.13 ± 16.43	133.25 ± 15.48	125.45 ± 16.13		
Cl-2	154,43 ± 13,76	144.14 ± 11.84	129.23 ± 24.70¶	125.77 ± 9.02		
Diastolic pressure, mmHg	83,58 ± 14,08	76.47 ± 13.08	78.31 ± 12.46	76.71 ± 9.75	<0.05	0.38
Cl-1	83,95 ± 15,16	76.38 ± 12.46	81.57 ± 11.74	78.05 ± 8.65		
Cl-2	83,00 ± 12,79	76.62 ± 14.55	73.43 ± 12.30	74.58 ± 11.35		
Systolic pressure, mmHg	133,55 ± 17,31	124.41 ± 18.85‡	127.46 ± 16.83	126.26 ± 16.54	0.11	0.19
Cl-1	134,68 ± 20,44	120.86 ± 21.06	129.29 ± 20.13	126.63 ± 19.70		
Cl-2	131,75 ± 11,30	130.15 ± 13.42	124.71 ± 10.19	125.67 ± 10.53		
TG, mg/dL	129,05 ± 58,65	121.89 ± 38.22	109.82 ± 38.63	95.56 ± 38.43	<0.01	0.58
Cl-1	129,17 ± 62,39	127.68 ± 43.08	115.04 ± 42.83	102.00 ± 45.13		
Cl-2	128,87 ± 54,23	112.79 ± 28.06	100.86 ± 29.44	86.15 ± 24.47		
CHOL, mg/dL	206,64 ± 43,66	163.75 ± 36.92‖	170.08 ± 37.09§	169.72 ± 45.16‡	<0.001	<0.05
Cl-1	202,96 ± 43,95	170.14 ± 35.42	177.88 ± 35.38	180.58 ± 46.90		
Cl-2	212,53 ± 44,03	153.71 ± 38.29¶	156.71 ± 37.36	153.85 ± 38.85¶		
LDL, mg/dL	128,23 ± 36,32	101.50 ± 29.79	106.35 ± 29.90	106.07 ± 36.70	<0.001	<0.05
Cl-1	122,46 ± 34,75	107.03 ± 27.61	112.66 ± 29.14	113.68 ± 37.35		
Cl-2	138,13 ± 38,11	92.98 ± 32.10¶	95.97 ± 29.18¶	94.65 ± 34.01¶		
HDL, mg/dL	49,97 ± 18,36	38.34 ± 10.16‖	44.11 ± 10.89	46.44 ± 13.26	<0.001	0.63

(*Continued*)

Table 3. (Continued)

	Before surgery	After surgery, months			P-Time[b]	P-Time X Cluster[b]
		1	3	6		
Cl-1	52,42 ± 20,46	40.05 ± 11.88	44.71 ± 11.83	47.89 ± 15.17		
Cl-2	45,79 ± 13,77	35.79 ± 6.41	43.07 ± 9.39	44.31 ± 10.04		
VLDL, mg/dL	25,81 ± 11,73	23.90 ± 7.77	20.50 ± 8.05	18.88 ± 8.30	<0.01	0.44
Cl-1	25,83 ± 12,48	25.74 ± 8.72	22.11 ± 9.24	21.18 ± 9.81		
Cl-2	25,77 ± 10,85	20.68 ± 4.57	17.64 ± 4.49	15.80 ± 4.60		
PCR, mg/l	10,13 ± 8,41	6.69 ± 5.05	5.55 ± 5.46‡	2.55 ± 2.79‖	<0.001	0.81
Cl-1	10,50 ± 8,33	6.86 ± 4.70	4.78 ± 2.99	2.42 ± 1.93		
Cl-2	9,66 ± 8,90	6.50 ± 5.70	6.57 ± 7.76	2.69 ± 3.71		
Leptin, mcg/ml	78,33 ± 35,38	41.15 ± 26.40‖	32.84 ± 16.28‖	23.83 ± 10.28‖	<0.001	0.80
Cl-1	78,36 ± 35,73	45.07 ± 33.81	34.21 ± 18.73	23.40 ± 12.67		
Cl-2	78,29 ± 36,50	36.44 ± 13.58	31.16 ± 13.61	24.36 ± 7.04		
Adiponeptin, mcg/ml	7,70 ± 4,11	9.66 ± 4.16	10.98 ± 7.16	12.76 ± 7.71§	<0.001	0.88
Cl-1	7,48 ± 3,75	9.15 ± 3.46	9.94 ± 4.64	12.15 ± 5.42		
Cl-2	7,95 ± 4,61	10.34 ± 5.08	12.41 ± 9.85	13.68 ± 10.66		
GOT, IU/L	23,90 ± 14,84	34.23 ± 17.18‡	27.61 ± 18.79	21.47 ± 9.32	<0.001	0.27
Cl-1	26,75 ± 17,65	36.33 ± 20.05	27.29 ± 22.19	22.11 ± 10.85		
Cl-2	19,33 ± 7,04	31.07 ± 11.67	28.14 ± 11.51	20.54 ± 6.80		
GPT, U/L	44,54 ± 23,56	61.60 ± 28.91§	45.95 ± 25.98	36.22 ± 13.44	<0.001	0.87
Cl-1	44,83 ± 28,35	60.24 ± 25.87	44.21 ± 28.52	35.79 ± 14.73		
Cl-2	44,07 ± 13,62	63.64 ± 33.89	48.93 ± 21.60	36.85 ± 11.86		
GGT, U/L	31,41 ± 17,66	32.31 ± 15.44	30.89 ± 55.01	19.03 ± 9.89‡	<0.001	0.45
Cl-1	32,55 ± 17,78	36.19 ± 12.87	37.67 ± 67.94	21.28 ± 7.50		
Cl-2	29,73 ± 17,96	26.50 ± 17.54	19.29 ± 14.77	15.92 ± 12.12		
Uric, mmol/L	5,50 ± 1,25	6.53 ± 2.52	5.02 ± 1.23	4.46 ± 1.16	<0.001	<0.01
Cl-1	5,45 ± 1,00	6.50 ± 2.94	5.30 ± 1.23	4.82 ± 1.09		
Cl-2	5,59 ± 1,66	6.57 ± 1.75	4.53 ± 1.10	3.95 ± 1.08¶		
Creatinine, mmol/L	0,79 ± 0,15	0.77 ± 0.20	0.67 ± 0.17‖	0.72 ± 0.15	<0.001	0.15
Cl-1	0,80 ± 0,17	0.78 ± 0.23	0.65 ± 0.20	0.75 ± 0.17		
Cl-2	0,78 ± 0,11	0.75 ± 0.16	0.69 ± 0.12	0.68 ± 0.11		
Urea, mg/dL	31,38 ± 9,79	21.97 ± 8.09§	23.61 ± 8.28§	25.17 ± 7.96‡	<0.001	<0.05
Cl-1	31,29 ± 9,60	23.09 ± 6.05	22.24 ± 8.23	24.35 ± 8.93		
Cl-2	31,53 ± 10,43	20.21 ± 10.56	25.86 ± 8.13	26.33 ± 6.56		

[a] Values are shown as Mean ± SD. Total n 39 patients, separate in Cluster 1 (Cl-1, n = 24) and Cluster 2 (Cl-2, n = 15) according K-means clustering at increment 6 month vs baseline. Numbers of serum samples 1, 3 and 6 months after surgery were as follows: Cl-1: 23, 21, 16 and Cl-2 15, 13, 11.

[b] *P* values represent changes over time (*P*-time) and time x group interaction (*P* -time x group) derived from linear mixed model after log-transformed variables and corrected for multiple testing by the false discovery rate. Taking into account the co-founders: age, gender drug intake and type of surgery.

‡ represents $p<0.05$,

§ represents $p<0.01$ and,

‖ represents $p<0.000$ at 1 month, 3 months or 6 months after surgery vs baseline.

¶ $p<0.05$,

# $p<0.01$ and,

** $p<0.0001$ represents change over time differently between Cl-1 and Cl-2.

AU, arbitrary units; BMI, body mass index; CHOL, total cholesterol; CRP, c-reactive protein; GGT, gamma glutamyl transferase; GOT, aspartate transaminase; GPT, alanine transaminase; HbA1c, glycated haemoglobin A1c; HDL, high-density lipoproteins; HOMA-IR, insulin resitance calculated by homeostatic model assessment; LDL, low-density lipoproteins; TG, triglycerides; VLDL, very low-density lipoprotein.

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Distinctive hallmarks after surgery between metabotypes. Our results show an important association between the increase of insulin sensitivity and the decrease of uric acid levels. Hyperuricemia has been described as a causal factor of insulin resistance due to gluconeogenesis disruption [26]. Paradoxically, the identification of individuals with hyperuricemia and a balanced glycaemia homeostasis indicates a pluri-mechanistic connection between both conditions [27].

Uric acid stimulates hepatic gluconeogenesis [28]. After surgery, uric acid led to the down-regulation of the gluconeogenesis in metabotype 1, mirrored by the restoration of the amino acids metabolism via the glucose-alanine cycle, also observed by others [29]. Metabotype 2 had lower levels of alanine and glycogenic precursors. Hence, metabotype 2 may fail to compensate for the peripheral glucose demand after surgery, resulting in a greater hypoglycaemic state, also indicated in [30]. Previous studies prove that the reduction of glycogenic substrates is independent of the magnitude of weight loss [31].

Metabotype 1 presented higher levels of compounds of the glucose-alanine and urea cycles at 6 months post-surgery. However, the low levels of urea and the negative correlation between arginine and uric acid in metabotype 1 suggest that bariatric surgery inhibited the production of urea-NO and arginine accumulation [32]. Jia et al. showed that beta-cells treated with uric acid triggered an inflammatory response and the overproduction of NO [33]. We observed that after the surgery this would reverse, uric acid decreased and methylated products of arginine metabolism increased after bariatric surgery in metabotype 1.

These observations also emphasize that bariatric surgery engages multiple mechanisms, independent and dependent of NO [23].

Lipid metabolism remodelling after bariatric surgery

For the first time, changes in the PCs profile have been related to the improvement of insulin resistance and uricemia after bariatric surgery, independently of the degree of weight loss. In fact, a recent study has described the protective role of phospholipids in pancreatic islets [34]. However, the interplay between PCs and uric acid after bariatric surgery and a possible connection with insulin resistance has not been reported yet.

Although previous reports did not identify an association between uric acid and PCs, experimentally have been demonstrated that uric acid produces oxidative stress in mitochondria, inhibits the aconitase enzyme in the tricarboxylic acid cycle and impairs the beta-oxidation of fatty acids [35].

The high levels of acetyl-CoA reflect this low capacity of the tricarboxylic acid cycle for beta-oxidation of long-chain fatty acids and an increase in carbohydrate catabolism [36]. The carnitine metabolism disruption is within the crosstalk between the alteration of the glucose and lipid metabolism. It is worth noting that the levels of carnitines C2, C14:1 and C18:1 immediately increased 1 month post-surgery. Thus they mirror acute changes in the metabolism after the surgery rather than carnitine synthesis or dietary origin, as fasting serum was used to reduce the dietary effect, and the levels of lysine and methionine—precursors of carnitine synthesis—remained stable after the surgery.

Large-scale metabolomics studies have demonstrated the association of obesity, high insulin resistance and dyslipidaemia with specific lipids such as SMs and diacyl-PCs [37]. However, few studies have dissected their individual contribution to adipose tissue expansion and/or insulin resistance. Our findings demonstrate beneficial effects of bariatric surgery on metabolic health by the restoration of the sphingolipid-phospholipid metabolism, through the improvement of the lipoprotein profile. (Lyso)PCs have been considered a pathognomonic characteristic of subjects with obesity and high insulin resistance [38]. LysoPCs are produced

by the hydrolysis of PCs during the oxidation of LDL [39] or by the action of lecithin-cholesterol acyltransferase. Therefore, the decreased levels of lysoPCs after surgery observed in metabotype 2 could be explained by a reduction of these biochemical processes [40]. Controversially, other authors stated that there was a decrease in lysoPC levels in insulin resistance subjects with non-alcoholic fatty liver [41], reflecting the role of lysoPCs as lipid-signalling molecules in the homeostasis of glucose [42]. Contrary to expected, PCs and lysoPCs presented similar trends in both phenotypes, suggesting that other mechanisms rather than the over-/downregulation of lysoPCs production from PCs could be modified after surgery.

Oxidized SMs enhance the susceptibility of HDL to aggregation and accumulation in the arterial walls [43] and reduce their clearance. A recent study reported that weight loss after a hypocaloric-diet intervention was associated with a decrease of HDL-SM, together with an improvement of the metabolic status [43]. The different post-surgery metabolic profiles of phenotypes 1 and 2 suggest that the benefits from the bariatric surgery are phenotypically dependent.

Taking all these results together, this study reveals that individuals that experienced a greater improvement of insulin resistance and cardiovascular factors after bariatric surgery had lower levels of gluconeogenic precursors, metabolites of the urea metabolism associated with NO mechanisms, and changes in the microcomponents of lipoproteins.

This short longitudinal design of the study allowed us to explore the earliest changes after surgery, but not changes due to adaptation. Although the MFA showed different post-surgery trends, the low number of patients in the study limited the amount of clusters and members in each one. The groups, formed by K-means cluster analysis,were unbalanced in terms of type of surgery. K-means is an unsupervised method and we could not address this point in the analysis. Consequently, despite considering type of surgery as a co-founder, we cannot affirm that the differences observed along the time are independent of the type of surgery. Several statistical approaches corroborated the results, notwithstanding, an independent cohort and prospective study is needed to confirm them. This work presents an innovative metabolomics and statistical approach to study the metabolic adaptations of patients after bariatric surgery. We have expanded the quantification of the metabolites-metabolic pathways, providing a general perspective and a dissection of the multiple responses to bariatric surgery.

Conclusions

Our data allowed the identification of metabotypes that are more likely to benefit from bariatric surgery, independently of the baseline status of the patients. The complexity of the metabolome requires complementary analytical methodologies in order to expand its coverage for a better understanding of the biological processes. Moreover, other factors such as global lifestyle or even a genetic component might be able to explain these metabotypes. Finally, to go beyond the analysis of individual mechanisms to the study of systems biology from an integral view, further investigation is needed.

Supporting information

S1 File. Multiple factor analysis and cluster analysis.
(DOC)

S1 Fig. Multifactor analysis graphical outputs. A. Eigenvalues of the different principal components (PCx) of the analysis. B. Plot for groups of variables versus the two first principal components. C. Partial axes of each group in the first two PCx. The increment 1 month—baseline is highly correlated with the first dimension and negatively correlated with the second

dimension. The increment 3 months—1 month is negatively correlated with the first dimension. The increment 6 months—3 months is positively explained by the first component and second component.
(TIF)

S2 Fig. Scatter plot of the metabolically healthy obese (red dots) and metabolically unhealthy (blue triangles) individuals in the following increments: A: T1–T0, B: T3–T0 and C: T6–T0.
(TIF)

S3 Fig. Metabolic correlation matrix of the discriminant metabolites of the K-means cluster analysis at the increment T6-T0 A) correlations between metabolites in phenotype 1.B) correlations between metabolites in phenotype 2. Only those correlations with $p<0.05$ are shown.
(TIF)

S4 Fig. Bubble plot of the most impacted metabolite clusters in metabotype 1 (A) and 2 (B) respectivetly. Chemical enrichment statistics was calculated by applying the Kolmogorov-Smirnov test on the metabolites at the increment of time T6-T0. Clusters are generated by chemical similarity and ontology mapping. Cluster colors give the proportion of increased or decreased compounds (red = increased, blue = decreased). *P* values were corrected for multiple testing by false discovery rate and only those clusters with $p<0.05$ are shown.
(TIF)

S1 Table. Summary of the metabolites (semi-)quantified in blood serum by MS/MS and those excluded. Metabolites are grouped into classes based on their metabolic function or structural similarities. According to manufacturer guidelines the detected MRM signal for lipid measurements is a sum of several isobaric/isomeric lipids. "For example: the signal of PC aa C36:6 can arise from at least 15 different lipid species that have different fatty acid composition (e.g. PC 16:1/20:5 versus PC 18:4/18:2), various positioning of fatty acids sn-1/sn-2 (e.g. PC 18:4/18:2 versus PC 18:2/18:4) and different double bond positions and stereochemistry in those fatty acid chains (e.g. PC(18:4(6Z,9Z,12Z,15Z)/18:2(9Z,12Z)) versus PC (18:4 (9E,11E,13E,15E)/18:2(9Z,12Z)))". QC-CV>25%: high analytical variances in the quality control replicates (coeficient of variation >25%); LOD: limit of detection; LLOQ: limit of quantification.
(XLS)

S2 Table. Anthropometric, biochemical and clinical characteristics of metabolically healthy (MH) and unhealthy (MU) individuals before the intervention[1]. [1] Values are shown as Mean ± SD. *P* values were determined by independent t-test after log-transformated the variables * *P* values were determined by fisher's exact test. AU, arbitrary units; BMI, body mass index; BP, blood pressure; CHOL, total cholesterol; C- LDL, low-density lipoproteins cholesterol; C-HDL, high-density lipoproteins cholesterol; CRP, C-reactive protein; DBP, diastolic blood pressure; GOT, aspartate transaminase; GPT, alanine transaminase; GGT, gamma glutamyl transferase; HbA1c, glycated haemoglobin A1c; HOMA-IR, insulin resitance calculated by homeostatic model assessment;; RYGP, Roux-en-Y gastric bypass SBP, systolic blood pressure; SG, sleeve gastrectomyTG, triglycerides. Cardiometabolic risk factors Adult Treatment Panel III criteria): Waist circumference >102 cm for male and >88 for female; TG over 150 mg/dl; HDL ≤40 for male and ≤50 for female; BP, SBP>130mmHg or DBP>85mmHg; fasting glucose over 110 mmol/ml.
(XLS)

S3 Table. Concentrations of metabolites in metabolically healthy (MH) and unhealthy (MU) individuals before the intervention[1]. [1]Values are shown as Mean ± SD (μM) [2] *P* values derived from t-test after log-transformed the variables, p-adjusted are *P* values corrected for multiple testing by the false discovery rate aa, acyl-acyl; ae, acyl-alkyl; LPC a, lysophosphatidylcholines; Cx:y, where x is the number of carbons in the fatty acid side chain; y is the number of double bonds in the fatty acid side chain; DC, decarboxyl; M, methyl; OH, hydroxyl; PC, phophatidylcholine; SM, sphingomyelin.
(XLS)

S4 Table. Anthropometric, biochemical and clinical characteristics of metabolically healthy (MH) and unhealthy (MU) individuals over time[1]. [1]Values are shown as Mean ± SD. Total n 39 patients, separate in metabollicaly health (MH, n = 21) and metabollicaly abnormal (MU, n = 18). [2]*P* values represent changes over time (p-time) and time x group interaction (p-time x group) derived from linear mixed model after log-transformate the variables and corrected for multiple testing by false discovery rate. Taking into acount the co-founders: age, gender, drug intake and type of surgery. *, **,*** represents $p<0.05$, $p<0.01$ and $p<0.0001$ respectivetly at 1 month, 3 months or 6 months after surgery vs baseline estimated in linear mixed-effects models, corrected for multiple testing by the false discovery rate. #, $p<0.05$; ##, $p<0.01$ and ###, $p<0.0001$ represents change over time differently between MH and MU. AU, arbitrary units; BMI, body mass index; CHOL, total cholesterol; C- LDL, low-density lipoproteins cholesterol; C-HDL, high-density lipoproteins cholesterol; CRP, c-reactive protein; GOT, Aspartate transaminase; GPT, Alanine transaminase; GGT, Gamma glutamyl transferase; HbA1c, glycated haemoglobin A1c; HOMA-IR, insulin resitance calculated by homeostatic model assessment; TG, triglycerides.
(XLS)

S5 Table. Concentration of metabolites of metabolically healthy (MH) and unhealthy (MU) individuals after surgery[1]. [1]Values are shown as Mean ± SD. Total n 39 patients, separate in metabollicaly health (MH, n = 21) and metabollicaly abnormal (MU, n = 18).[2] *P* values represent changes over time (p-time) and time x group interaction (p-time x group) derived from linear mixed model after log-transformed variables and corrected for multiple testing by false discovery rate. Taking into account the co-founders: age, gender, drug intake and type of surgery. #, $p<0.05$; ##, $p<0.01$ and ###, $p<0.0001$ represents change over time differently between MH and MU. aa, acyl-acyl; ae, acyl-alkyl; LPC a, lysophosphatidylcholines; Cx:y, where x is the number of carbons in the fatty acid side chain; y is the number of double bonds in the fatty acid side chain; DC, decarboxyl; M, methyl; OH, hydroxyl; PC, phophatidylcholine; SM, sphingomyelin.
(XLS)

S6 Table. Changes in metabolite concentrations of both clusters after surgery. [1]Values are shown as Mean ± SD. Total n 39 patients, separate in Cluster 1 (Cl-1, n = 24) and Cluster 2 (Cl-2, n = 15) according K-means clustering at increment 6 month vs baseline. [2] *P* values represent changes over time (p-time) and time x group interaction (p-time x group) derived from linear mixed model after log-transformed variables and corrected for multiple testing by the false discovery rate. Taking into account the co-founders: age, gender, drug intake and type of surgery. #, $p<0.05$; ##, $p<0.01$ and ###, $p<0.0001$ represents change over time differently between Cl-1 and Cl-2.
(XLS)

S7 Table. Concentrations of metabolites in both clusters before the intervention. [1]Values are shown as Mean ± SD [2]*P* derived from t-test after log-transformed variables and corrected

for multiple testing by false discovery rate.
(XLS)

S8 Table. Raw data with clinical and metabolomic variables in each period of time.
(CSV)

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Material Suplementario

PUBLICACION 5

Supporting information

Metabotypes of response to bariatric surgery independent of the magnitude of weight loss

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Supplementary Methods

Multiple factor analysis

Multiple factor analysis or 'multiple factorial analyses' (MFA) is an unsupervised multivariate technique, such as principal component analysis (PCA), which aims to separate the data into principal components (PCx). Each PC[1] contributes to explaining the variability of the data[2] with several variables correlated in each one. Therefore, the more information that is described with the fewest number of PCs, the better the variables describe the model. In the same way, MFA and PCA can also be graphically displayed, plotting the projections of the observations and the loadings of the variables onto the components (the so-called 'circle of correlations').

However, when the observations are described by several groups with a different number of variables in each one – subsets of data – e.g. on longitudinal studies, to perform a PCA could be erroneous, giving weight to the set of variables with the largest number and not to the one that better explains the model. To solve this problem, MFA is able to combine multiple data sets regardless of the quantity and nature – quantitative/qualitative – of the variables [1]. In fact, MFA could be employed in several disciplines [2] and contexts [3].

Two steps are necessary to perform an MFA: First, for each subset of variables a PCA is computed if the variables are quantitative; or a multiple correspondence analysis (MCA) if the variables are qualitative. This will be followed by the normalization of each data table, dividing all its elements by the first singular value obtained from its PCA (the square root of an eigenvalue can be considered a variance) to make variables comparable.

1 These variables are called, depending upon the context, principal components (PCs), factors, eigenvectors, singular vectors or loadings.

2 The importance of each component is expressed by the variance (i.e. eigenvalue) of its projections or by the proportion of the variance explained. In this context, PCA is interpreted as an orthogonal decomposition of the variance (also called inertia) of a data table.

The second step is to build up a grand matrix with all normalized data tables, analysed by a (non-normalized) PCA, which gives a set of factor scores for the observations and loadings of each variable. In addition, MFA provides a set of partial factor scores for the observations of each data table that reflects the specific 'viewpoint' of this data table [4]. New variables are created as linear combinations of the original variables and can be interpreted using variables' correlations or contributions together with a set of graphical outputs.

Overall, different entities are represented sharing dimensions:

A. Plot for groups of variables versus the two first principal components.
B. Individual factor map represents the observations with the partial values of each group.
C. Variables correlation circle, in which the circle's radius represents how the variable (radius) is correlated in each PC (axes). The more a vector has a magnitude close to 1, the better the projection is.
D. Partial axes of each group in the first two PCs.

Clustering analysis

One common statistical step in biomedicine studies is to group objects by similarity – clustering analysis. Several methods are available to fit different inputs. However, there is not a general best-performing approach, it depends on the data set [5].

Most of the algorithms require the user to identify the number of clusters before the clustering analysis. Foreseeably, this step is not straightforward and different procedures could be used to address it. In 1985, Milligand and Cooper evaluated different cluster solutions, suggesting the Calinski-Harabasz index (1974) as one of the most effective [6].

The Calinski-Harabasz index ensures the compactness and isolation of the cluster through the minimum sum of distances between the objects of the cluster and their

fictitious cluster centre – the centroid – and the maximum distance between the clusters, based on the average between- and within- cluster sum of squares.

Moreover, as an unsupervised learning task, one of the most important issues is to evaluate the goodness of the data partition. This is possible through internal and external clustering validation indexes. Internal validation would judge the cluster quality on the basis of certain intrinsic statistical properties of the clustering itself [7], without any external information not present in the data that external validation relies upon [8]. In the same way, different measures are equivalent to deal with the validation step. In this study, the Calinski-Harabasz index would solve internal validation.

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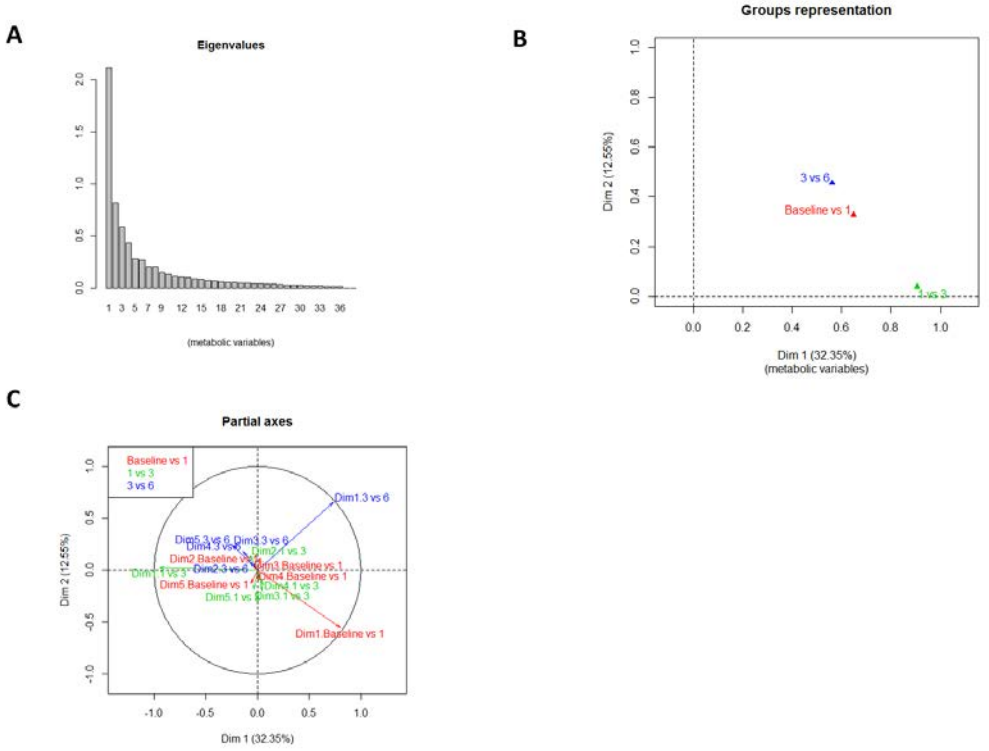
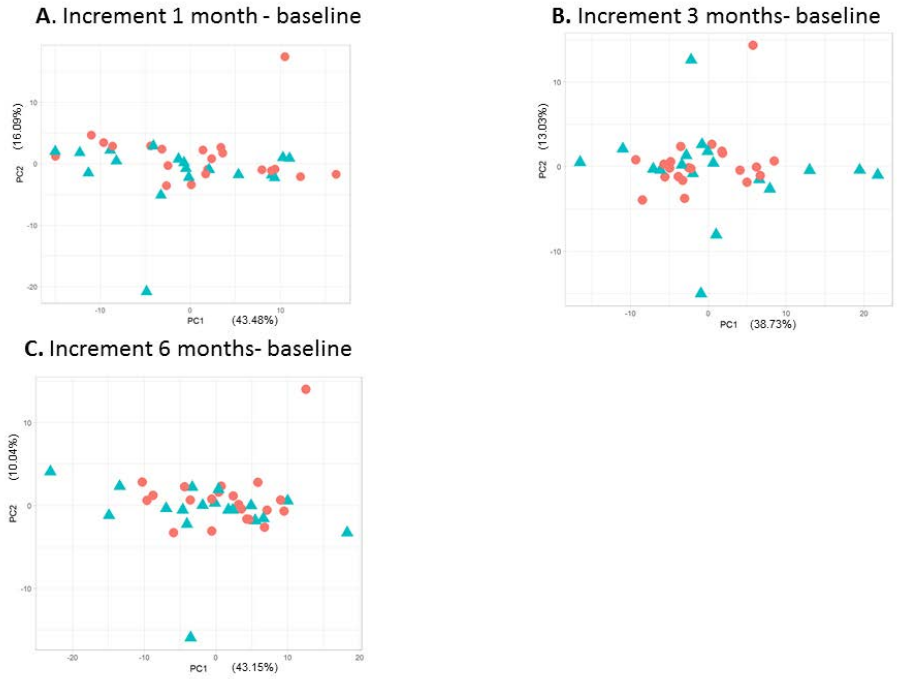
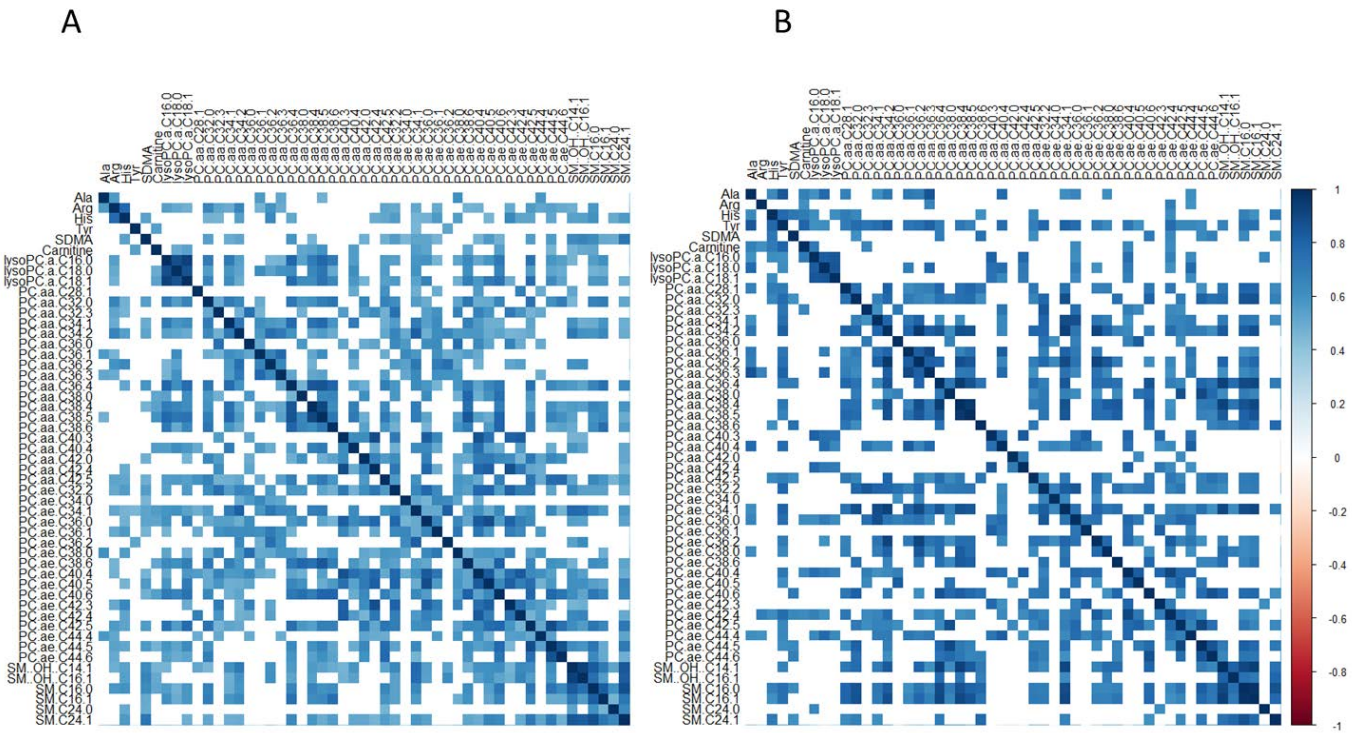
S1 Fig. Multifactor analysis graphical outputs. A. Eigenvalues of the different principal components (PCx) of the analysis. B. Plot for groups of variables versus the two first principal components. C. Partial axes of each group in the first two PCx. The increment 1 month – baseline is highly correlated with the first dimension and negatively correlated with the second dimension. The increment 3 months – 1 month is negatively correlated with the first dimension. The increment 6 months – 3 months is positively explained by the first component and second component.



S2 Fig. Scatter plot of the metabolically healthy obese (red dots) and metabolically unhealthy (blue triangles) individuals in the following increments: A: T1–T0, B: T3–T0 and C: T6–T0.



S3 Fig. Metabolic correlation matrix of the discriminant metabolites of the K-means cluster analysis at the increment T6-T0 A) correlations between metabolites in phenotype 1.B) correlations between metabolites in phenotype 2. Only those correlations with $p<0.05$ are shown.



S4 Fig. Bubble plot of the most impacted metabolite clusters in metabotype 1 (A) and 2 (B) respectivetly. Chemical enrichment statistics was calculated by applying the Kolmogorov-Smirnov test on the metabolites at the increment of time T6-T0. Clusters are generated by chemical similarity and ontology mapping. Cluster colors give the proportion of increased or decreased compounds (red=increased, blue=decreased). *P*-values were corrected for multiple testing by false discovery rate and only those clusters with $p<0.05$ are shown.

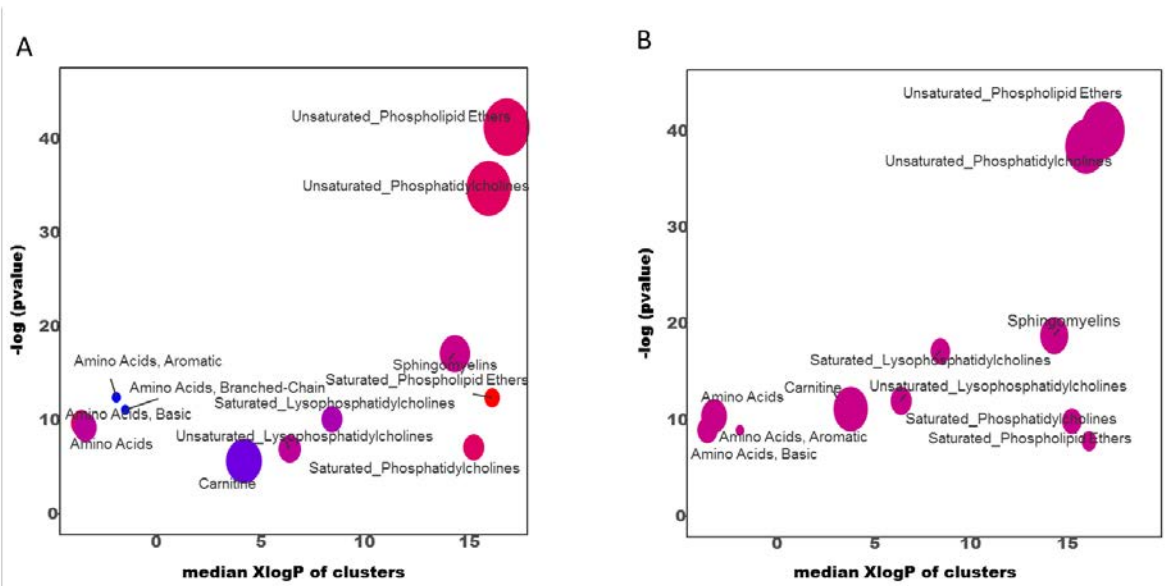


Table S1. Summary of the metabolites (semi-)quantified in blood serum by MS/MS and those excluded. Metabolites are grouped into classes based on their metabolic function or structural similarities

Class	Metabolite abbreviation	Metabolite	Platform	Data type
aminoacids	Ala	Alanine	LC-MS/MS	Quantified
aminoacids	Arg	Arginine	LC-MS/MS	Quantified
aminoacids	Asn	Asparagine	LC-MS/MS	Quantified
aminoacids	Asp	Aspartate	LC-MS/MS	Quantified
aminoacids	Cit	Citrulline	LC-MS/MS	Quantified
aminoacids	Gln	Glutamine	LC-MS/MS	Quantified
aminoacids	Glu	Glutamate	LC-MS/MS	Quantified
aminoacids	Gly	Glycine	LC-MS/MS	Quantified
aminoacids	His	Histidine	LC-MS/MS	Quantified
aminoacids	Ile	Isoleucine	LC-MS/MS	Quantified
aminoacids	Leu	Leucine	LC-MS/MS	Quantified
aminoacids	Lys	Lysine	LC-MS/MS	Quantified
aminoacids	Met	Methionine	LC-MS/MS	Quantified
aminoacids	Orn	Ornitine	LC-MS/MS	Quantified
aminoacids	Phe	Phenylalanine	LC-MS/MS	Quantified
aminoacids	Pro	Proline	LC-MS/MS	Quantified
aminoacids	Ser	Serine	LC-MS/MS	Quantified
aminoacids	Thr	Threonine	LC-MS/MS	Quantified
aminoacids	Trp	Tryptophan	LC-MS/MS	Quantified
aminoacids	Tyr	Tyrosine	LC-MS/MS	Quantified
aminoacids	Val	Valine	LC-MS/MS	Quantified
biogenic amines	Ac-Orn	Acetylornithine	LC-MS/MS	< LOD
biogenic amines	ADMA	Asymmetric dimethylarginine	LC-MS/MS	< LOD
biogenic amines	alpha-AAA	alpha-Aminoadipic acid	LC-MS/MS	< LOD
biogenic amines	c4-OH-Pro	cis-4-Hydroxyproline	LC-MS/MS	< LOD
biogenic amines	Carnosine	Carnosine	LC-MS/MS	< LOD
biogenic amines	Creatinine	Creatinine	LC-MS/MS	Quantified
biogenic amines	DOPA	DOPA	LC-MS/MS	< LLOQ
biogenic amines	Dopamine	Dopamine	LC-MS/MS	< LLOQ
biogenic amines	Histamine	Histamine	LC-MS/MS	< LLOQ
biogenic amines	Kynurenine	Kynurenine	LC-MS/MS	Quantified
biogenic amines	Met-SO	Methioninesulfoxide	LC-MS/MS	< LOD
biogenic amines	Nitro-Tyr	Nitrotyrosine	LC-MS/MS	< LOD
biogenic amines	PEA	Phenylethylamine	LC-MS/MS	< LOD
biogenic amines	Putrescine	Putrescine	LC-MS/MS	Quantified
biogenic amines	Sarcosine	Sarcosine	LC-MS/MS	Quantified
biogenic amines	Serotonin	Serotonin	LC-MS/MS	Quantified
biogenic amines	Spermidine	Spermidine	LC-MS/MS	< LLOQ
biogenic amines	Spermine	Spermine	LC-MS/MS	QC-CV>25%
biogenic amines	t4-OH-Pro	trans-4-Hydroxyproline	LC-MS/MS	< LLOQ
biogenic amines	Taurine	Taurine	LC-MS/MS	Quantified
biogenic amines	SDMA	Symmetric dimethylarginine	LC-MS/MS	Quantified
acylcarnitines	C0	Carnitine	FIA-MS/MS	Quantified
acylcarnitines	C2	Acetylcarnitine	FIA-MS/MS	Quantified
acylcarnitines	C3	Propionylcarnitine	FIA-MS/MS	< LLOQ
acylcarnitines	C3:1	Propenoylcarnitine	FIA-MS/MS	QC-CV>25%
acylcarnitines	C3-OH	Hydroxypropionylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C4	Butyrylcarnitine	FIA-MS/MS	< LLOQ
acylcarnitines	C4:1	Butenylcarnitine	FIA-MS/MS	< LOD
acylcarnitines	C4-OH (C3-DC)	Hydroxybutyrylcarnitine	FIA-MS/MS	< LOD
acylcarnitines	C5	Valerylcarnitine	FIA-MS/MS	< LLOQ

acylcarnitines	C5:1	Tiglylcarnitine	FIA-MS/MS	QC-CV>25%
acylcarnitines	C5:1-DC	Glutaconylcarnitine	FIA-MS/MS	QC-CV>25%
acylcarnitines	C5-DC (C6-OH)	Glutarylcarnitine* (Hydroxyhexanoylcarnitine)	FIA-MS/MS	Semi-quantified
acylcarnitines	C5-M-DC	Methylglutarylcarnitine	FIA-MS/MS	QC-CV>25%
acylcarnitines	C5-OH (C3-DC-M)	Hydroxyvalerylcarnitine (Methylmalonylcarnitine)	FIA-MS/MS	QC-CV>25%
acylcarnitines	C6 (C4:1-DC)	Hexanoylcarnitine (Fumarylcarnitine)	FIA-MS/MS	Semi-quantified
acylcarnitines	C6:1	Hexenoylcarnitine	FIA-MS/MS	< LOD
acylcarnitines	C7-DC	Pimelylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C8	Octanoylcarnitine	FIA-MS/MS	Quantified
acylcarnitines	C9	Nonaylcarnitine	FIA-MS/MS	QC-CV>25%
acylcarnitines	C10	Decanoylcarnitine	FIA-MS/MS	Quantified
acylcarnitines	C10:1	Decenoylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C10:2	Decadienylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C12	Dodecanoylcarnitine	FIA-MS/MS	< LLOQ
acylcarnitines	C12:1	Dodecenoylcarnitine	FIA-MS/MS	< LOD
acylcarnitines	C12-DC	Dodecanedioylcarnitine	FIA-MS/MS	< LOD
acylcarnitines	C14	Tetradecanoylcarnitine	FIA-MS/MS	< LLOQ
acylcarnitines	C14:1	Tetradecenoylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C14:1-OH	Hydroxytetradecenoylcarnitine	FIA-MS/MS	QC-CV>25%
acylcarnitines	C14:2	Tetradecadienylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C14:2-OH	Hydroxytetradecadienylcarnitine	FIA-MS/MS	QC-CV>25%
acylcarnitines	C16	Hexadecanoylcarnitine	FIA-MS/MS	< LLOQ
acylcarnitines	C16:1	Hexadecenoylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C16:1-OH	Hydroxyhexadecenoylcarnitine	FIA-MS/MS	QC-CV>25%
acylcarnitines	C16:2	Hexadecadienylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C16:2-OH	Hydroxyhexadecadienylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C16-OH	Hydroxyhexadecanoylcarnitine	FIA-MS/MS	QC-CV>25%
acylcarnitines	C18	Octadecanoylcarnitine	FIA-MS/MS	< LLOQ
acylcarnitines	C18:1	Octadecenoylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C18:1-OH	Hydroxyoctadecenoylcarnitine	FIA-MS/MS	Semi-quantified
acylcarnitines	C18:2	Octadecadienylcarnitine	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C14:0	lysoPC a C14:0	FIA-MS/MS	< LOD
glycerophospholipids	lysoPC a C16:0	lysoPC a C16:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C16:1	lysoPC a C16:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C17:0	lysoPC a C17:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C18:0	lysoPC a C18:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C18:1	lysoPC a C18:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C18:2	lysoPC a C18:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C20:3	lysoPC a C20:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C20:4	lysoPC a C20:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C24:0	lysoPC a C24:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C26:0	lysoPC a C26:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C26:1	lysoPC a C26:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C28:0	lysoPC a C28:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	lysoPC a C28:1	lysoPC a C28:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C24:0	PC aa C24:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C26:0	PC aa C26:0	FIA-MS/MS	< LOD
glycerophospholipids	PC aa C28:1	PC aa C28:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C30:0	PC aa C30:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C30:2	PC aa C30:2	FIA-MS/MS	< LOD
glycerophospholipids	PC aa C32:0	PC aa C32:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C32:1	PC aa C32:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C32:2	PC aa C32:2	FIA-MS/MS	< LOD

glycerophospholipids	PC aa C32:3	PC aa C32:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C34:1	PC aa C34:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C34:2	PC aa C34:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C34:3	PC aa C34:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C34:4	PC aa C34:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C36:0	PC aa C36:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C36:1	PC aa C36:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C36:2	PC aa C36:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C36:3	PC aa C36:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C36:4	PC aa C36:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C36:5	PC aa C36:5	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C36:6	PC aa C36:6	FIA-MS/MS	QC-CV>25%
glycerophospholipids	PC aa C38:0	PC aa C38:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C38:1	PC aa C38:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C38:3	PC aa C38:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C38:4	PC aa C38:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C38:5	PC aa C38:5	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C38:6	PC aa C38:6	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C40:1	PC aa C40:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C40:2	PC aa C40:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C40:3	PC aa C40:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C40:4	PC aa C40:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C40:5	PC aa C40:5	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C40:6	PC aa C40:6	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C42:0	PC aa C42:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C42:1	PC aa C42:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C42:2	PC aa C42:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C42:4	PC aa C42:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C42:5	PC aa C42:5	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC aa C42:6	PC aa C42:6	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C30:0	PC ae C30:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C30:1	PC ae C30:1	FIA-MS/MS	< LOD
glycerophospholipids	PC ae C30:2	PC ae C30:2	FIA-MS/MS	< LOD
glycerophospholipids	PC ae C32:1	PC ae C32:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C32:2	PC ae C32:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C34:0	PC ae C34:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C34:1	PC ae C34:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C34:2	PC ae C34:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C34:3	PC ae C34:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C36:0	PC ae C36:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C36:1	PC ae C36:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C36:2	PC ae C36:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C36:3	PC ae C36:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C36:4	PC ae C36:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C36:5	PC ae C36:5	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C38:0	PC ae C38:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C38:1	PC ae C38:1	FIA-MS/MS	QC-CV>25%
glycerophospholipids	PC ae C38:2	PC ae C38:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C38:3	PC ae C38:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C38:4	PC ae C38:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C38:5	PC ae C38:5	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C38:6	PC ae C38:6	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C40:1	PC ae C40:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C40:2	PC ae C40:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C40:3	PC ae C40:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C40:4	PC ae C40:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C40:5	PC ae C40:5	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C40:6	PC ae C40:6	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C42:0	PC ae C42:0	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C42:1	PC ae C42:1	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C42:2	PC ae C42:2	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C42:3	PC ae C42:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C42:4	PC ae C42:4	FIA-MS/MS	Semi-quantified

glycerophospholipids	PC ae C42:5	PC ae C42:5	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C44:3	PC ae C44:3	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C44:4	PC ae C44:4	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C44:5	PC ae C44:5	FIA-MS/MS	Semi-quantified
glycerophospholipids	PC ae C44:6	PC ae C44:6	FIA-MS/MS	Semi-quantified
sphingolipids	SM (OH) C14:1	SM (OH) C14:1	FIA-MS/MS	Semi-quantified
sphingolipids	SM (OH) C16:1	SM (OH) C16:1	FIA-MS/MS	Semi-quantified
sphingolipids	SM (OH) C22:1	SM (OH) C22:1	FIA-MS/MS	Semi-quantified
sphingolipids	SM (OH) C22:2	SM (OH) C22:2	FIA-MS/MS	Semi-quantified
sphingolipids	SM (OH) C24:1	SM (OH) C24:1	FIA-MS/MS	Semi-quantified
sphingolipids	SM C16:0	SM C16:0	FIA-MS/MS	Semi-quantified
sphingolipids	SM C16:1	SM C16:1	FIA-MS/MS	Semi-quantified
sphingolipids	SM C18:0	SM C18:0	FIA-MS/MS	Semi-quantified
sphingolipids	SM C18:1	SM C18:1	FIA-MS/MS	Semi-quantified
sphingolipids	SM C20:2	SM C20:2	FIA-MS/MS	Semi-quantified
sphingolipids	SM C22:3	SM C22:3	FIA-MS/MS	< LOD
sphingolipids	SM C24:0	SM C24:0	FIA-MS/MS	Semi-quantified
sphingolipids	SM C24:1	SM C24:1	FIA-MS/MS	Semi-quantified
sphingolipids	SM C26:0	SM C26:0	FIA-MS/MS	QC-CV>25%
sphingolipids	SM C26:1	SM C26:1	FIA-MS/MS	QC-CV>25%
sugars	H1	hexose	FIA-MS/MS	< LOD

According to manufacturer guidelines the detected MRM signal for lipid measurements is a sum of several isobaric/isomeric lipids. "For example: the signal of PC aa C36:6 can arise from at least 15 different lipid species that have different fatty acid composition (e.g. PC 16:1/20:5 versus PC 18:4/18:2), various positioning of fatty acids sn-1/sn-2 (e.g. PC 18:4/18:2 versus PC 18:2/18:4) and different double bond positions and stereochemistry in those fatty acid chains (e.g. PC(18:4(6Z,9Z,12Z,15Z)/18:2(9Z,12Z)) versus PC (18:4(9E,11E,13E,15E)/18:2(9Z,12Z)))".

QC-CV>25%: high analytical variances in the quality control replicates (coeficient of variation >25%); LOD: limit of detection; LLOQ: limit of quantification

Table S2. Anthropometric, biochemical and clinical characteristics of metabolically healthy (MH) and unhealthy (MU) individuals before the intervention[1].

	MH (n=21)	**MU (n=18)**	***P***
Gender, nF(nM)	13 (8)	14 (4)	0,32
Type of surgery, RYGB (nSG)	11 (10)	15 (3)	0.50
Age, y	39.29 ± 8.87	42.56 ± 10.94	0.37
Weight, kg	135.90 ± 28.45	144.83 ± 25.88	0.27
BMI, kg/m^2	48.81 ± 9.12	52.51 ± 7.14	0.14
Waist-hip ratio	0.96 ± 0.22	0.91 ± 0.13	0.46
Waist circumference, cm	133.47 ± 21.32	137.83 ± 21.18	0.53
Hip circumference, cm	142.53 ± 21.74	151.39 ± 16.66	0.18
Fasting glucose, mmol/ml	94.29 ± 13.65	120.72 ± 40.68	<0.01
Fasting insulin, µU/ml	15.51 ± 8.49	20.04 ± 9.96	0.11
HOMA-IR, AU	3.77 ± 2.43	6.19 ± 3.84	<0.05
HBA1c, %	5.51 ± 0.23	5.73 ± 0.34	0.06
SBP, mmHg	130 ± 17	137 ± 17	0.22
DBP mmHg	79 ± 15	88 ± 12	0.06
C-LDL, mg/dl	126.55 ± 38.44	130.31 ± 34.57	<0.01
C-HDL, mg/dl	52.48 ± 17.48	46.88 ± 19.48	0.43
VLDL, mg/dl	20.33 ± 8.98	32.20 ± 11.51	0.66
TG, mg/dl	101.67 ± 44.88	161.00 ± 57.55	0.29
CHOL, mg/dl	201.14 ± 43.38	213.06 ± 44.34	<0.01
CRP, mg/l	11.75 ± 9.05	8.64 ± 7.84	0.52
GOT, U/l	21.10 ± 9.52	27.17 ± 19.09	0.27
GPT, U/l	38.10 ± 11.48	52.06 ± 31.21	0.14
GGT, U/l	28.58 ± 15.65	34.39 ± 19.56	0.30
Cardiometabolic risk factors, n (%)			
Waist circumference	16 (76.2%)	18 (100%)	
TG	2 (9.5%)	10 (55.6%)	
HDL	8 (38.1%)	11 (61.1%)	
BP	5 (23.8%)	13 (72.2%)	
Fasting glucose	1 (4.8%)	9 (50.0%)	

[1] Values are shown as Mean ± SD. *P* values were determined by independent t-test after log-transformated the variables * *P* values were determined by fisher's exact test

AU, arbitrary units; BMI, body mass index; BP, blood pressure; CHOL, total cholesterol; C- LDL, low-density lipoproteins cholesterol; C-HDL, high-density lipoproteins cholesterol; CRP, C-reactive protein; DBP, diastolic blood pressure; GOT, aspartate transaminase; GPT, alanine transaminase; GGT, gamma glutamyl transferase; HbA1c, glycated haemoglobin A1c; HOMA-IR, insulin resitance calculated by homeostatic model assessment; ; RYGP, Roux-en-Y gastric bypass SBP, systolic blood pressure; SG, sleeve gastrectomyTG, triglycerides. Cardiometabolic risk factors Adult Treatment Panel III criteria): Waist circumference >102 cm for male and >88 for female; TG over 150 mg/dl; HDL ≤40 for male and ≤50 for female; BP, SBP>130mmHg or DBP>85mmHg; fasting glucose over 110 mmol/ml.

Table S3. Concentrations of metabolites in metabolically healthy (MH) and unhealthy (MU) individuals before the intervention [1].

	MH (n=21)	MU (n=18)	p-value	p-adjusted[2]
Isoleucine, µM	94.30 ± 27.20	104.44 ± 23.94	0.19	0.55
Leucine, µM	180.19 ± 47.98	191.06 ± 44.76	0.47	0.68
Valine, µM	306.19 ± 82.38	332.61 ± 77.87	0.29	0.57
Alanine, µM	436.48 ± 144.21	513.17 ± 94.33	<0.05	0.55
Proline, µM	207.48 ± 60.41	266.28 ± 118.42	0.05	0.55
Glycine, µM	282.62 ± 61.34	300.94 ± 115.59	0.74	0.88
Serine, µM	153.79 ± 29.23	170.33 ± 51.25	0.30	0.57
Tryptophan, µM	64.19 ± 16.72	70.76 ± 21.37	0.34	0.58
Phenylalanine, µM	84.27 ± 22.91	91.22 ± 20.02	0.29	0.57
Methionine, µM	29.65 ± 7.01	29.71 ± 8.83	0.90	0.95
Ornitine, µM	99.45 ± 34.93	105.86 ± 39.18	0.60	0.78
Arginine, µM	139.71 ± 28.79	158.65 ± 42.61	0.14	0.55
Histidine, µM	93.23 ± 17.49	102.87 ± 27.09	0.22	0.55
Asparagine, µM	50.24 ± 10.04	47.13 ± 14.17	0.29	0.57
Aspartate, µM	18.68 ± 7.03	28.31 ± 16.70	0.06	0.55
Glutamate, µM	59.90 ± 24.69	113.59 ± 92.91	<0.01	0.37
Glutamine, µM	755.38 ± 162.36	743.61 ± 301.04	0.48	0.70
Citrulline, µM	34.36 ± 9.78	30.90 ± 11.10	0.21	0.55
Tyrosine, µM	88.76 ± 26.34	96.77 ± 27.03	0.33	0.58
Threonine, µM	148.28 ± 40.81	155.29 ± 61.04	0.90	0.95
Lysine, µM	367.81 ± 72.91	398.06 ± 89.99	0.28	0.57
Creatinine, µM	78.28 ± 19.27	82.94 ± 21.98	0.55	0.73
Kynurenine, µM	3.09 ± 1.12	3.55 ± 1.27	0.21	0.55
Putrescine, µM	0.20 ± 0.09	0.24 ± 0.06	0.13	0.55
Sarcosine, µM	8.59 ± 3.55	8.71 ± 4.14	1.00	1.00
Serotonin, µM	0.69 ± 0.46	0.77 ± 0.28	0.36	0.60
Taurine, µM	107.41 ± 36.90	110.36 ± 47.12	0.90	0.95
Symmetric dimethylarginine, µM	1.30 ± 0.29	1.33 ± 0.31	0.77	0.88
Carnitine, µM	43.95 ± 11.84	47.52 ± 15.81	0.54	0.73
Acetylcarnitine, µM	7.97 ± 3.73	7.51 ± 4.58	0.40	0.64
Hydroxypropionylcarnitine, µM	0.24 ± 0.05	0.26 ± 0.05	0.13	0.55
Hexanoylcarnitine, µM	0.11 ± 0.06	0.11 ± 0.02	0.97	0.98
Glutarylcarnitine, µM	0.05 ± 0.02	0.05 ± 0.01	0.65	0.81
Pimelylcarnitine, µM	0.05 ± 0.01	0.05 ± 0.01	0.87	0.95
Octanoylcarnitine, µM	0.52 ± 0.14	0.56 ± 0.12	0.30	0.57
Decanoylcarnitine, µM	0.46 ± 0.13	0.60 ± 0.15	<0.01	0.37
Decenoylcarnitine, µM	0.22 ± 0.07	0.28 ± 0.08	<0.05	0.55
Decadienylcarnitine, µM	0.15 ± 0.03	0.17 ± 0.05	0.16	0.55
Tetradecenoylcarnitine, µM	0.18 ± 0.06	0.21 ± 0.06	0.07	0.55
Tetradecadienylcarnitine, µM	0.12 ± 0.04	0.13 ± 0.03	0.12	0.55
Hexadecenoylcarnitine, µM	0.11 ± 0.04	0.12 ± 0.03	0.34	0.58
Hexadecadienylcarnitine, µM	0.04 ± 0.01	0.05 ± 0.02	0.23	0.56
Hydroxyhexadecenoylcarnitine, µM	0.04 ± 0.02	0.05 ± 0.02	0.12	0.55
Octadecenoylcarnitine, µM	0.16 ± 0.06	0.18 ± 0.06	0.43	0.67
Hydroxyoctadecenoylcarnitine, µM	0.04 ± 0.01	0.05 ± 0.01	0.45	0.68
Octadecadienylcarnitine, µM	0.10 ± 0.03	0.11 ± 0.03	0.45	0.68
LysoPC a C16:0, µM	133.85 ± 37.43	172.62 ± 62.86	<0.05	0.55
LysoPC a C16:1, µM	3.69 ± 1.36	4.97 ± 2.19	<0.05	0.55
LysoPC a C17:0, µM	1.95 ± 0.64	2.28 ± 1.01	0.29	0.57
LysoPC a C18:0, µM	45.80 ± 16.60	56.01 ± 27.49	0.22	0.55
LysoPC a C18:1, µM	30.46 ± 11.45	34.49 ± 12.98	0.30	0.57
LysoPC a C18:2, µM	42.56 ± 11.84	45.28 ± 22.29	0.92	0.95

LysoPC a C20:3, µM	4.04 ± 2.15	4.76 ± 1.90	0.19	0.55
LysoPC a C20:4, µM	10.71 ± 4.23	10.85 ± 4.16	0.87	0.95
LysoPC a C24:0, µM	0.44 ± 0.12	0.56 ± 0.21	<0.05	0.55
LysoPC a C26:0, µM	0.41 ± 0.23	0.66 ± 0.53	0.06	0.55
LysoPC a C26:1, µM	0.32 ± 0.12	0.36 ± 0.15	0.34	0.58
LysoPC a C28:0, µM	0.36 ± 0.13	0.57 ± 0.46	0.08	0.55
LysoPC a C28:1, µM	0.47 ± 0.15	0.59 ± 0.31	0.15	0.55
PC aa C24:0, µM	0.21 ± 0.06	0.28 ± 0.18	0.14	0.55
PC aa C28:1, µM	2.94 ± 0.72	3.10 ± 0.79	0.53	0.72
PC aa C30:0, µM	1.77 ± 0.70	2.04 ± 0.77	0.25	0.57
PC aa C32:0, µM	10.20 ± 3.03	11.27 ± 3.46	0.33	0.58
PC aa C32:1, µM	10.25 ± 6.21	13.55 ± 6.79	0.06	0.55
PC aa C32:3, µM	0.22 ± 0.08	0.25 ± 0.07	0.21	0.55
PC aa C34:1, µM	132.83 ± 61.11	146.53 ± 43.02	0.19	0.55
PC aa C34:2, µM	332.90 ± 160.62	345.39 ± 123.31	0.67	0.84
PC aa C34:3, µM	9.64 ± 5.37	11.05 ± 4.71	0.21	0.55
PC aa C34:4, µM	0.69 ± 0.43	0.72 ± 0.40	0.76	0.88
PC aa C36:0, µM	1.43 ± 0.66	1.73 ± 1.03	0.30	0.57
PC aa C36:1, µM	18.46 ± 8.82	18.16 ± 5.40	0.76	0.88
PC aa C36:2, µM	129.15 ± 54.69	122.13 ± 46.44	0.72	0.88
PC aa C36:3, µM	75.56 ± 38.85	81.83 ± 28.23	0.33	0.58
PC aa C36:4, µM	120.18 ± 42.12	120.35 ± 44.19	0.89	0.95
PC aa C36:5, µM	9.03 ± 5.27	10.24 ± 4.54	0.29	0.57
PC aa C38:0, µM	3.15 ± 1.42	3.38 ± 1.15	0.46	0.68
PC aa C38:1, µM	1.23 ± 0.55	1.80 ± 1.52	0.22	0.55
PC aa C38:3, µM	40.04 ± 22.18	43.29 ± 16.58	0.35	0.59
PC aa C38:4, µM	93.81 ± 33.35	90.67 ± 37.13	0.68	0.84
PC aa C38:5, µM	28.26 ± 9.57	29.31 ± 10.44	0.75	0.88
PC aa C38:6, µM	60.23 ± 27.85	66.13 ± 28.17	0.43	0.67
PC aa C40:1, µM	0.50 ± 0.09	0.87 ± 0.88	0.13	0.55
PC aa C40:2, µM	0.34 ± 0.10	0.99 ± 1.65	0.13	0.55
PC aa C40:3, µM	0.64 ± 0.24	1.14 ± 1.30	0.13	0.55
PC aa C40:4, µM	2.44 ± 0.85	3.17 ± 1.53	0.11	0.55
PC aa C40:5, µM	5.06 ± 1.87	5.32 ± 2.18	0.68	0.84
PC aa C40:6, µM	21.55 ± 9.74	22.97 ± 9.11	0.49	0.71
PC aa C42:0, µM	0.70 ± 0.20	0.82 ± 0.38	0.31	0.58
PC aa C42:1, µM	0.35 ± 0.11	0.48 ± 0.33	0.14	0.55
PC aa C42:2, µM	0.28 ± 0.10	0.55 ± 0.67	0.10	0.55
PC aa C42:4, µM	0.21 ± 0.06	0.49 ± 0.66	0.08	0.55
PC aa C42:5, µM	0.32 ± 0.11	0.46 ± 0.31	0.08	0.55
PC aa C42:6, µM	0.36 ± 0.11	0.45 ± 0.25	0.21	0.55
PC ae C30:0, µM	0.31 ± 0.07	0.34 ± 0.08	0.24	0.57
PC ae C30:2, µM	0.10 ± 0.02	0.15 ± 0.11	0.11	0.55
PC ae C32:1, µM	2.23 ± 0.60	2.20 ± 0.84	0.75	0.88
PC ae C32:2, µM	0.68 ± 0.21	0.72 ± 0.21	0.59	0.77
PC ae C34:0, µM	0.51 ± 0.17	0.52 ± 0.15	0.78	0.89
PC ae C34:1, µM	5.26 ± 1.66	5.02 ± 1.20	0.76	0.88
PC ae C34:2, µM	7.78 ± 3.51	6.91 ± 3.04	0.38	0.63
PC ae C34:3, µM	4.83 ± 1.98	4.29 ± 2.05	0.31	0.58
PC ae C36:0, µM	0.95 ± 0.32	1.07 ± 0.38	0.27	0.57
PC ae C36:1, µM	3.03 ± 1.04	4.97 ± 5.05	0.14	0.55
PC ae C36:2, µM	5.93 ± 2.13	5.76 ± 1.65	0.91	0.95
PC ae C36:3, µM	4.05 ± 2.12	3.54 ± 1.58	0.41	0.65
PC ae C36:4, µM	9.55 ± 3.86	7.98 ± 3.77	0.13	0.55
PC ae C36:5, µM	6.95 ± 2.45	6.28 ± 2.89	0.29	0.57
PC ae C38:0, µM	1.57 ± 0.69	1.69 ± 0.48	0.39	0.63
PC ae C38:2, µM	1.66 ± 0.66	4.02 ± 6.55	0.20	0.55
PC ae C38:3, µM	1.62 ± 0.65	2.95 ± 3.30	0.13	0.55
PC ae C38:4, µM	5.89 ± 1.86	5.54 ± 1.90	0.53	0.72
PC ae C38:5, µM	10.16 ± 3.70	8.68 ± 3.76	0.14	0.55
PC ae C38:6, µM	4.17 ± 1.59	3.79 ± 1.50	0.42	0.67
PC ae C40:1, µM	1.15 ± 0.42	1.64 ± 1.13	0.10	0.55
PC ae C40:2, µM	1.17 ± 0.31	2.01 ± 2.02	0.09	0.55

PC ae C40:3, µM	0.92 ± 0.23	2.34 ± 3.42	0.12	0.55
PC ae C40:4, µM	2.04 ± 0.59	2.78 ± 1.94	0.19	0.55
PC ae C40:5, µM	1.92 ± 0.57	2.78 ± 2.14	0.16	0.55
PC ae C40:6, µM	2.33 ± 0.68	2.29 ± 0.71	0.84	0.95
PC ae C42:1, µM	0.43 ± 0.15	0.84 ± 0.95	0.08	0.55
PC ae C42:2, µM	0.50 ± 0.16	0.77 ± 0.77	0.17	0.55
PC ae C42:3, µM	0.79 ± 0.22	1.15 ± 1.05	0.21	0.55
PC ae C42:4, µM	1.03 ± 0.27	1.17 ± 0.59	0.53	0.72
PC ae C42:5, µM	2.28 ± 0.58	2.51 ± 0.99	0.50	0.71
PC ae C44:3, µM	0.16 ± 0.05	0.33 ± 0.43	0.10	0.55
PC ae C44:4, µM	0.49 ± 0.15	0.53 ± 0.22	0.62	0.79
PC ae C44:5, µM	2.35 ± 0.67	1.96 ± 0.70	0.07	0.55
PC ae C44:6, µM	1.72 ± 0.44	1.52 ± 0.59	0.16	0.55
SM (OH) C14:1, µM	3.83 ± 1.08	3.61 ± 1.06	0.51	0.72
SM (OH) C16:1, µM	1.56 ± 0.44	1.45 ± 0.47	0.45	0.68
SM (OH) C22:1, µM	6.21 ± 2.38	5.26 ± 1.94	0.21	0.55
SM (OH) C22:2, µM	5.48 ± 1.83	5.32 ± 1.12	0.98	0.99
SM (OH) C24:1, µM	0.60 ± 0.20	0.52 ± 0.21	0.19	0.55
SM C16:0, µM	77.67 ± 21.62	71.48 ± 20.64	0.33	0.58
SM C16:1, µM	12.63 ± 3.71	12.51 ± 3.82	0.85	0.95
SM C18:0, µM	11.55 ± 4.69	11.33 ± 3.68	0.93	0.95
SM C18:1, µM	6.61 ± 2.46	6.67 ± 2.27	0.88	0.95
SM C20:2, µM	0.28 ± 0.10	0.29 ± 0.08	0.91	0.95
SM C24:0, µM	9.41 ± 3.83	8.46 ± 2.49	0.53	0.72
SM C24:1, µM	31.49 ± 11.35	28.98 ± 7.47	0.60	0.78

[1] Values are shown as Mean ± SD (µM)

[2] *P* values derived from *t* test after log-transformed the variables, p-adjusted are *P* values corrected for multiple testing by the false discovery rate

aa, acyl-acyl; ae, acyl-alkyl; LPC a, lysophosphatidylcholines; Cx:y, where x is the number of carbons in the fatty acid side chain; y is the number of double bonds in the fatty acid side chain; DC, decarboxyl; M, methyl; OH, hydroxyl; PC, phophatidylcholine; SM, sphingomyelin.

Table S4. Anthropometric and clinical characteristics of metabolically healthy (MH) and unhealthy (MU) individuals over time[1].

		After surgery, mo				
	Before surgery	**1**	**3**	**6**	**p-time[2]**	**p-time x cluster[2]**
Fasting glucose, µU/mL	106.49 ± 31.89	89.17 ± 11.25	83.76 ± 12.09*	84.31 ± 11.06	<0.001	0.20
MH	94.29 ± 13.65	86.45 ± 9.69	82.62 ± 13.40	86.53 ± 10.87		
MU	120.72 ± 40.68	92.56 ± 12.41	85.18 ± 10.48	81.80 ± 11.09		
Fasting insulin, µU/mL	17.60 ± 9.36	13.62 ± 8.25*	9.94 ± 3.82*	8.95 ± 3.78***	<0.001	0.60
MH	15.51 ± 8.49	11.70 ± 6.77	9.67 ± 3.73	7.80 ± 3.51		
MU	20.04 ± 9.96	16.02 ± 9.46	10.25 ± 4.01	10.18 ± 3.77		
HOMA-IR, AU	4.89 ± 3.35	3.06 ± 1.99*	2.05 ± 0.86**	1.89 ± 0.86***	<0.001	0.50
MH	3.77 ± 2.43	2.57 ± 1.58	1.95 ± 0.87	1.72 ± 0.87		
MU	6.19 ± 3.84	3.68 ± 2.31	2.15 ± 0.87	2.07 ± 0.85		
HBA1c, %	5.59 ± 0.29	5.15 ± 0.26***	5.21 ± 0.29**	5.20 ± 0.23	<0.001	0.57
MH	5.51 ± 0.23	5.14 ± 0.28	5.24 ± 0.21	5.19 ± 0.23		
MU	5.73 ± 0.34	5.15 ± 0.24	5.17 ± 0.39	5.23 ± 0.26		
Weight, kg	140.03 ± 27.31	124.50 ± 23.49***	113.99 ± 22.31***	100.41 ± 19.53***	<0.001	0.61
MH	135.90 ± 28.45	122.35 ± 25.32	112.82 ± 24.49	98.64 ± 21.77		
MU	144.83 ± 25.88	127.31 ± 21.32	115.44 ± 19.92	102.18 ± 17.54		
BMI, kg/m2	50.52 ± 8.37	45.09 ± 7.19***	41.28 ± 6.80***	36.42 ± 6.14***	<0.001	0.64
MH	48.81 ± 9.12	43.93 ± 7.78	40.44 ± 7.30	35.54 ± 6.93		
MU	52.51 ± 7.14	46.61 ± 6.25	42.32 ± 6.20	37.31 ± 5.31		
Waist-hip ratio	0.93 ± 0.18	0.87 ± 0.11	0.90 ± 0.21	0.85 ± 0.07	0.31	0.57
MH	0.96 ± 0.22	0.89 ± 0.10	0.87 ± 0.09	0.85 ± 0.07		
MU	0.91 ± 0.13	0.85 ± 0.11	0.93 ± 0.30	0.85 ± 0.08		
Waist circumference, cm	135.71 ± 21.05	123.92 ± 16.06***	116.54 ± 13.94***	108.71 ± 13.87***	<0.001	0.85
MH	133.47 ± 21.32	123.57 ± 16.74	115.86 ± 15.79	107.00 ± 14.15		
MU	137.83 ± 21.18	124.38 ± 15.66	117.44 ± 11.50	110.53 ± 13.79		
Hip circumference, cm	147.09 ± 19.53	141.65 ± 14.82	131.84 ± 18.98	125.58 ± 13.60*	<0.001	0.59
MH	142.53 ± 21.74	139.10 ± 16.49	132.62 ± 15.33	123.29 ± 15.59		
MU	151.39 ± 16.66	145.00 ± 11.97	130.81 ± 23.44	128.00 ± 11.10		
Diastolic pressure, mmHg	84 ± 14	76 ± 13	78 ± 12	77 ± 10	<0.01	0.49
MH	79 ± 15	73 ± 12	78 ± 11	76 ± 9		
MU	88 ± 12	80 ± 14	79 ± 14	78 ± 11		
Systolic pressure, mmHg	134 ± 17	124 ± 19	127 ± 17	126 ± 17	0.12	0.85
MH	130 ± 17	122 ± 17	123 ± 12	125 ± 13		
MU	137 ± 17	128 ± 21	132 ± 20	128 ± 20		
C-LDL, mg/dL	128.23 ± 36.32	101.50 ± 29.79**	106.35 ± 29.90*	106.07 ± 36.70	<0.001	0.70

MH	126.55 ± 38.44	100.49 ± 31.47	105.51 ± 28.13	106.78 ± 34.55		
MU	130.31 ± 34.57	102.86 ± 28.47	107.44 ± 32.98	105.14 ± 40.75		
C-HDL, mg/dL	49.97 ± 18.36	38.34 ± 10.16***	44.11 ± 10.89	46.44 ± 13.26	<0.001	0.69
MH	52.48 ± 17.48	40.37 ± 11.29	48.24 ± 12.00	50.24 ± 15.49		
MU	46.88 ± 19.48	35.94 ± 8.35	39.00 ± 6.65	42.13 ± 8.81		
VLDL, mg/dL	25.81 ± 11.73	23.90 ± 7.77	20.50 ± 8.05	18.88 ± 8.30	<0.01	0.49
MH	20.33 ± 8.98	20.52 ± 6.09	19.25 ± 8.92	18.62 ± 9.73		
MU	32.20 ± 11.51	27.96 ± 7.86	22.09 ± 6.87	19.16 ± 6.90		
TG, mg/dL	129.05 ± 58.65	121.89 ± 38.22	109.82 ± 38.63	95.56 ± 38.43	<0.001	0.23
MH	101.67 ± 44.88	105.95 ± 31.28	102.57 ± 43.00	90.94 ± 41.26		
MU	161.00 ± 57.55	141.81 ± 37.50	118.76 ± 31.40	100.80 ± 35.63		
CHOL, mg/dL	206.64 ± 43.66	163.75 ± 36.92***	170.08 ± 37.09**	169.72 ± 45.16	<0.001	0.56
MH	201.14 ± 43.38	161.85 ± 39.19	173.52 ± 34.92	175.94 ± 43.50		
MU	213.06 ± 44.34	166.13 ± 34.99	165.82 ± 40.27	162.67 ± 47.47		
CRP, mg/l	10.13 ± 8.41	6.69 ± 5.05*	5.55 ± 5.46	2.55 ± 2.79**	<0.001	0.53
MH	11.75 ± 9.05	7.46 ± 5.12	8.59 ± 6.66	4.14 ± 3.80		
MU	8.64 ± 7.84	6.06 ± 5.12	3.26 ± 2.96	1.48 ± 1.08		
Leptin, mg/ml	78.33 ± 35.38	41.15 ± 26.40***	32.84 ± 16.28***	23.83 ± 10.28***	<0.001	0.60
MH	77.61 ± 35.50	39.86 ± 34.79	32.74 ± 15.48	23.89 ± 9.18		
MU	78.85 ± 36.53	42.22 ± 18.38	32.90 ± 17.48	23.79 ± 11.35		
Adiponectin, mcg/ml	7.70 ± 4.11	9.66 ± 4.16*	10.98 ± 7.16*	12.76 ± 7.71**	<0.001	0.79
MH	7.82 ± 4.44	9.56 ± 4.03	11.40 ± 4.85	12.46 ± 6.19		
MU	7.61 ± 3.99	9.75 ± 4.47	10.68 ± 8.69	13.01 ± 9.07		
GOT, IU/L	23.90 ± 14.84	34.23 ± 17.18**	27.61 ± 18.79	21.47 ± 9.32	<0.001	0.60
MH	21.10 ± 9.52	31.21 ± 15.02	28.43 ± 22.56	22.12 ± 11.14		
MU	27.17 ± 19.09	37.81 ± 19.32	26.59 ± 13.35	20.73 ± 7.03		
GPT, U/L	44.54 ± 23.56	61.60 ± 28.91**	45.95 ± 25.98	36.22 ± 13.44	<0.001	0.96
MH	38.10 ± 11.48	57.05 ± 28.69	43.38 ± 29.54	34.47 ± 15.82		
MU	52.06 ± 31.21	67.00 ± 29.15	49.12 ± 21.23	38.20 ± 10.30		
GGT, U/L	31.41 ± 17.66	32.31 ± 15.44	30.89 ± 55.01	19.03 ± 9.89*	<0.001	0.91
MH	28.58 ± 15.65	30.58 ± 15.51	35.05 ± 72.10	17.53 ± 6.58		
MU	34.39 ± 19.56	34.38 ± 15.59	25.76 ± 21.23	20.86 ± 12.88		
Uric, mmol/L	5.50 ± 1.25	6.53 ± 2.52	5.02 ± 1.23	4.46 ± 1.16	<0.001	0.44
MH	5.44 ± 1.46	6.14 ± 2.93	5.05 ± 1.34	4.48 ± 1.17		
MU	5.57 ± 0.97	7.01 ± 1.86	4.98 ± 1.11	4.44 ± 1.18		
Creatinine, mmol/L	0.79 ± 0.15	0.77 ± 0.20	0.67 ± 0.17**	0.72 ± 0.15	<0.001	0.71
MH	0.78 ± 0.12	0.77 ± 0.17	0.66 ± 0.17	0.74 ± 0.16		
MU	0.81 ± 0.18	0.77 ± 0.24	0.68 ± 0.17	0.69 ± 0.13		
Urea, mg/dL	31.38 ± 9.79	21.97 ± 8.09**	23.61 ± 8.28*	25.17 ± 7.96	<0.001	0.69
MH	32.05 ± 8.58	23.95 ± 8.43	25.13 ± 8.62	26.73 ± 9.48		

MU	30.61 ± 11.25	19.50 ± 7.14	21.82 ± 7.72	23.50 ± 5.83

[1] Values are shown as Mean ± SD. Total n 39 patients, separate in metabollicaly health (MH, n=21) and metabollicaly abnormal (MU, n=18).

[2] *P* values represent changes over time (p-time) and time x group interaction (p-time x group) derived from linear mixed model after log-transformate the variables and corrected for multiple testing by false discovery rate. Taking into acount the co-founders: age, gender and type of surgery

*, ** ,*** represents $p<0.05$, $p<0.01$ and $p<0.0001$ respectivetly at 1 month, 3 months or 6 months after surgery vs baseline estimated in linear mixed-effects models, corrected for multiple testing by the false discovery rate

#, $p<0.05$; ##, $p<0.01$ and ###, $p<0.0001$ represents change over time differently between MH and MU

AU, arbitrary units; BMI, body mass index; CHOL, total cholesterol; C- LDL, low-density lipoproteins cholesterol; C-HDL, high-density lipoproteins cholesterol; CRP, c-reactive protein; GOT, Aspartate transaminase; GPT, Alanine transaminase; GGT, Gamma glutamyl transferase; HbA1c, glycated haemoglobin A1c ; HOMA-IR, insulin resitance calculated by homeostatic model assessment; TG, trilglicerides

Table S5. Concentration of metabolites of metabolically healthy (MH) and unhealthy (MU) individuals after surgery[1].

		After surgery, mo				
	Before surgery	**1**	**3**	**6**	**p-time[2]**	**p-time x cluster[2]**
Isoleucine, µM	98.98 ± 25.92	91.04 ± 27.60	86.17 ± 37.44*	74.60 ± 22.03*	<0.001	0.78
MH	94.30 ± 27.20	85.04 ± 23.28	75.37 ± 23.47	67.80 ± 17.37		
MU	104.44 ± 23.94	98.46 ± 31.27	98.33 ± 46.50	81.92 ± 24.76		
Leucine, µM	185.21 ± 46.24	143.54 ± 44.53***	148.73 ± 61.90***	132.32 ± 34.72**	<0.001	0.57
MH	180.19 ± 47.98	133.75 ± 38.46	128.27 ± 36.15	124.99 ± 34.49		
MU	191.06 ± 44.76	155.62 ± 49.56	171.76 ± 76.69	140.22 ± 34.53		
Valine, µM	318.38 ± 80.39	225.95 ± 67.91***	253.06 ± 110.01***	214.55 ± 54.29**	<0.001	0.60
MH	306.19 ± 82.38	211.81 ± 52.71	216.89 ± 51.67	204.00 ± 53.97		
MU	332.61 ± 77.87	243.41 ± 81.27	293.75 ± 142.35	225.92 ± 54.41		
Alanine, µM	471.87 ± 128.17	374.82 ± 110.87	415.12 ± 157.67	405.59 ± 105.49	<0.01	0.57
MH	436.48 ± 144.21	372.38 ± 94.69	372.83 ± 114.01	383.14 ± 68.44		
MU	513.17 ± 94.33	377.82 ± 131.16	462.69 ± 188.15	429.77 ± 133.47		
Proline, µM	234.62 ± 95.27	207.54 ± 73.35	241.71 ± 116.27	207.00 ± 51.87	<0.05	0.53
MH	207.48 ± 60.41	203.16 ± 62.54	206.17 ± 49.79	197.36 ± 44.77		
MU	266.28 ± 118.42	212.94 ± 86.59	281.69 ± 153.99	217.38 ± 58.59		
Glycine, µM	291.08 ± 89.69	365.18 ± 136.59**	378.97 ± 180.20	366.74 ± 109.32	<0.05	0,57
MH	282.62 ± 61.34	395.76 ± 153.18	350.33 ± 94.03	369.36 ± 101.25		
MU	300.94 ± 115.59	327.41 ± 105.23	411.19 ± 243.57	363.92 ± 121.53		
Serine, µM	161.42 ± 41.16	180.66 ± 54.05	193.22 ± 75.32	189.81 ± 45.84	0.65	0.74
MH	153.79 ± 29.23	190.82 ± 59.72	174.54 ± 38.31	176.50 ± 24.87		
MU	170.33 ± 51.25	168.11 ± 44.66	215.63 ± 101.00	204.15 ± 58.77		
Tryptophan, µM	67.22 ± 19.04	44.04 ± 15.65**	55.54 ± 24.50	49.70 ± 18.26	<0.001	0.39
MH	64.19 ± 16.72	45.98 ± 15.93	52.19 ± 15.60	54.76 ± 16.47		
MU	70.76 ± 21.37	41.64 ± 15.44	59.31 ± 31.88	44.26 ± 19.14		
Phenylalanine, µM	87.48 ± 21.63	64.25 ± 21.72***	70.40 ± 28.08***	65.37 ± 17.77*	<0.001	0.56
MH	84.27 ± 22.91	63.73 ± 22.19	60.91 ± 16.48	63.79 ± 16.08		
MU	91.22 ± 20.02	64.90 ± 21.80	81.08 ± 34.59	67.07 ± 19.94		
Methionine, µM	29.68 ± 7.73	24.39 ± 7.91	27.70 ± 11.93	25.02 ± 7.18	0.60	0.81
MH	29.65 ± 7.01	24.72 ± 6.09	25.48 ± 7.82	25.27 ± 5.01		
MU	29.71 ± 8.83	24.00 ± 9.82	30.37 ± 15.39	24.76 ± 9.18		
Ornitine, µM	102.41 ± 36.60	78.10 ± 31.99*	94.57 ± 52.94	88.49 ± 24.37	<0.001	0.60
MH	99.45 ± 34.93	85.75 ± 35.42	86.66 ± 30.13	86.40 ± 17.10		
MU	105.86 ± 39.18	68.65 ± 25.03	103.48 ± 70.55	90.75 ± 30.97		
Arginine, µM	148.45 ± 36.61	118.73 ± 29.13*	140.55 ± 49.74	137.67 ± 42.34	<0.01	0.61
MH	139.71 ± 28.79	118.95 ± 27.67	125.11 ± 27.80	130.17 ± 30.43		

MU	158.65 ± 42.61	118.46 ± 31.70	157.91 ± 62.93	145.74 ± 52.39		
Histidine, µM	97.68 ± 22.65	76.90 ± 18.28**	98.00 ± 39.38	94.81 ± 24.85	<0.001	0.57
MH	93.23 ± 17.49	75.44 ± 17.56	84.51 ± 20.36	87.16 ± 18.05		
MU	102.87 ± 27.09	78.70 ± 19.53	113.18 ± 49.78	103.04 ± 29.05		
Asparagine, µM	48.81 ± 12.05	41.26 ± 10.39*	46.34 ± 18.58*	46.79 ± 11.79	<0.01	0.60
MH	50.24 ± 10.04	42.60 ± 8.87	43.78 ± 9.74	43.96 ± 6.31		
MU	47.13 ± 14.17	39.59 ± 12.07	49.23 ± 25.20	49.85 ± 15.45		
Aspartate, µM	23.12 ± 13.21	18.55 ± 11.20	20.98 ± 11.21	20.02 ± 11.41	<0.05	0.57
MH	18.68 ± 7.03	18.35 ± 12.60	16.14 ± 6.38	17.63 ± 8.81		
MU	28.31 ± 16.70	18.82 ± 9.46	26.78 ± 13.11	22.81 ± 13.73		
Glutamate, µM	84.68 ± 70.13	70.10 ± 54.31	64.69 ± 48.23	59.26 ± 67.14	<0.001	0.50
MH	59.90 ± 24.69	63.73 ± 50.13	49.44 ± 21.98	44.60 ± 16.51		
MU	113.59 ± 92.91	77.97 ± 59.67	81.85 ± 63.06	75.05 ± 94.61		
Glutamine, µM	749.95 ± 233.35	796.32 ± 248.09	792.84 ± 204.31	855.67 ± 235.37	0.05	0.20
MH	755.38 ± 162.36	828.71 ± 211.14	827.89 ± 171.26	829.43 ± 169.87		
MU	743.61 ± 301.04	756.29 ± 289.01	744.31 ± 241.69	883.92 ± 295.14		
Citrulline, µM	32.76 ± 10.42	25.11 ± 9.22*	34.21 ± 14.04	34.87 ± 9.35	<0.001	0.53
MH	34.36 ± 9.78	27.27 ± 8.25	32.86 ± 7.65	33.86 ± 7.20		
MU	30.90 ± 11.10	22.44 ± 9.90	35.74 ± 19.04	35.96 ± 11.44		
Tyrosine, µM	92.46 ± 26.62	62.16 ± 19.16***	72.85 ± 37.90**	65.83 ± 17.42*	<0.001	0.76
MH	88.76 ± 26.34	60.90 ± 15.98	65.30 ± 21.15	63.74 ± 13.67		
MU	96.77 ± 27.03	63.72 ± 22.92	81.34 ± 50.08	68.08 ± 21.09		
Threonine, µM	151.52 ± 50.55	128.79 ± 42.46	129.37 ± 60.03	121.20 ± 33.88	<0.01	0.72
MH	148.28 ± 40.81	134.17 ± 46.53	124.11 ± 27.53	125.80 ± 28.09		
MU	155.29 ± 61.04	122.14 ± 37.13	135.29 ± 83.66	116.25 ± 39.77		
Lysine, µM	381.77 ± 81.57	314.79 ± 83.31	328.21 ± 111.39	312.30 ± 82.29	0.13	0.73
MH	367.81 ± 72.91	314.19 ± 82.87	304.06 ± 69.78	297.14 ± 74.17		
MU	398.06 ± 89.99	315.53 ± 86.41	357.20 ± 144.21	328.62 ± 90.32		
Creatinine, µM	80.43 ± 20.42	76.94 ± 24.42	76.23 ± 30.25	73.75 ± 18.00	0.52	0.69
MH	78.28 ± 19.27	78.27 ± 27.21	69.82 ± 15.13	75.62 ± 19.91		
MU	82.94 ± 21.98	75.30 ± 21.19	83.44 ± 40.61	71.73 ± 16.25		
Kynurenine, µM	3.30 ± 1.20	2.16 ± 0.85**	2.60 ± 1.41**	2.39 ± 0.89	<0.001	0.20
MH	3.09 ± 1.12	2.23 ± 0.74	2.31 ± 0.61	2.42 ± 0.94		
MU	3.55 ± 1.27	2.07 ± 0.97	2.91 ± 1.91	2.34 ± 0.87		
Putrescine, µM	0.22 ± 0.08	0.24 ± 0.07	0.23 ± 0.10	0.26 ± 0.09	0.29	0.49
MH	0.20 ± 0.09	0.22 ± 0.06	0.23 ± 0.09	0.25 ± 0.08		
MU	0.24 ± 0.06	0.25 ± 0.08	0.23 ± 0.12	0.26 ± 0.10		
Sarcosine, µM	8.65 ± 3.78	7.78 ± 4.50	7.49 ± 3.75	7.53 ± 2.46	0.69	0.91
MH	8.59 ± 3.55	8.39 ± 5.22	7.40 ± 2.71	7.98 ± 2.74		
MU	8.71 ± 4.14	7.02 ± 3.43	7.59 ± 4.76	7.05 ± 2.10		

Serotonin, µM	0.73 ± 0.39	0.57 ± 0.28	0.78 ± 0.57	0.87 ± 0.58	0.11	0.82
MH	0.69 ± 0.46	0.62 ± 0.27	0.82 ± 0.52	0.89 ± 0.46		
MU	0.77 ± 0.28	0.51 ± 0.29	0.74 ± 0.63	0.85 ± 0.69		
Taurine, µM	108.77 ± 41.38	106.12 ± 36.43	97.20 ± 44.76	100.63 ± 33.64	<0.05	0.39
MH	107.41 ± 36.90	108.48 ± 37.60	102.46 ± 46.36	101.42 ± 37.00		
MU	110.36 ± 47.12	103.35 ± 35.95	89.75 ± 43.25	99.84 ± 31.43		
Symmetric dimethylarginine, µM	1.32 ± 0.30	1.45 ± 0.50	1.49 ± 0.64	1.38 ± 0.31	0.50	0.60
MH	1.30 ± 0.29	1.38 ± 0.36	1.34 ± 0.28	1.41 ± 0.30		
MU	1.33 ± 0.31	1.52 ± 0.63	1.66 ± 0.87	1.35 ± 0.33		
Carnitine, µM	45.60 ± 13.74	35.27 ± 12.16	39.69 ± 17.42*	39.67 ± 11.49	<0.001	0.60
MH	43.95 ± 11.84	37.95 ± 12.84	37.22 ± 9.95	38.17 ± 8.21		
MU	47.52 ± 15.81	31.96 ± 10.72	42.46 ± 23.23	41.29 ± 14.40		
Acetylcarnitine, µM	7.76 ± 4.10	14.26 ± 8.03**	10.98 ± 6.94	8.79 ± 3.69	<0.001	0.82
MH	7.97 ± 3.73	13.80 ± 8.61	9.68 ± 3.47	8.83 ± 3.57		
MU	7.51 ± 4.58	14.83 ± 7.47	12.45 ± 9.39	8.74 ± 3.97		
Hydroxypropionylcarnitine, µM	0.25 ± 0.05	0.24 ± 0.07	0.25 ± 0.07	0.25 ± 0.06	0.98	0.20
MH	0.24 ± 0.05	0.27 ± 0.07	0.22 ± 0.05	0.24 ± 0.07		
MU	0.26 ± 0.05	0.22 ± 0.06	0.28 ± 0.08	0.25 ± 0.06		
Hexanoylcarnitine, µM	0.11 ± 0.04	0.12 ± 0.05	0.11 ± 0.03	0.12 ± 0.04	0.29	0.60
MH	0.11 ± 0.06	0.12 ± 0.05	0.10 ± 0.02	0.12 ± 0.06		
MU	0.11 ± 0.02	0.13 ± 0.05	0.12 ± 0.03	0.11 ± 0.03		
Glutarylcarnitine, µM	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.98	0.32
MH	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.01		
MU	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.02		
Pimelylcarnitine, µM	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.02	0.71	0.60
MH	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.02		
MU	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.02		
Octanoylcarnitine, µM	0.54 ± 0.13	0.56 ± 0.18	0.55 ± 0.15	0.54 ± 0.16	0.98	0.20
MH	0.52 ± 0.14	0.59 ± 0.21	0.50 ± 0.10	0.55 ± 0.21		
MU	0.56 ± 0.12	0.52 ± 0.12	0.61 ± 0.18	0.53 ± 0.07		
Decanoylcarnitine, µM	0.53 ± 0.16	0.59 ± 0.23*	0.60 ± 0.24	0.57 ± 0.24	0.37	0.20
MH	0.46 ± 0.13	0.61 ± 0.24	0.54 ± 0.16	0.59 ± 0.32		
MU	0.60 ± 0.15	0.58 ± 0.23	0.68 ± 0.30	0.53 ± 0.10		
Decenoylcarnitine, µM	0.25 ± 0.08	0.27 ± 0.10	0.26 ± 0.09	0.25 ± 0.10	0.38	0.28
MH	0.22 ± 0.07	0.27 ± 0.10	0.24 ± 0.07	0.27 ± 0.14		
MU	0.28 ± 0.08	0.26 ± 0.10	0.29 ± 0.10	0.24 ± 0.04		
Decadienylcarnitine, µM	0.16 ± 0.04	0.16 ± 0.06	0.16 ± 0.04	0.16 ± 0.04	0.90	0.20
MH	0.15 ± 0.03	0.17 ± 0.07	0.15 ± 0.04	0.16 ± 0.05		
MU	0.17 ± 0.05	0.13 ± 0.03	0.17 ± 0.05	0.15 ± 0.03		
Tetradecenoylcarnitine, µM	0.19 ± 0.06	0.23 ± 0.08*	0.21 ± 0.09	0.19 ± 0.06	<0.05	0.23

MH	0.18 ± 0.06	0.23 ± 0.09	0.19 ± 0.04	0.20 ± 0.07		
MU	0.21 ± 0.06	0.22 ± 0.07	0.24 ± 0.11	0.18 ± 0.04		
Tetradecadienylcarnitine, µM	0.12 ± 0.04	0.13 ± 0.05	0.12 ± 0.04	0.12 ± 0.03	0.78	0.30
MH	0.12 ± 0.04	0.14 ± 0.06	0.11 ± 0.03	0.12 ± 0.04		
MU	0.13 ± 0.03	0.12 ± 0.03	0.14 ± 0.05	0.12 ± 0.03		
Hexadecenoylcarnitine, µM	0.11 ± 0.03	0.12 ± 0.04	0.11 ± 0.03	0.11 ± 0.03	0.59	0.32
MH	0.11 ± 0.04	0.13 ± 0.04	0.11 ± 0.02	0.11 ± 0.03		
MU	0.12 ± 0.03	0.11 ± 0.03	0.12 ± 0.04	0.10 ± 0.02		
Hexadecadienylcarnitine, µM	0.04 ± 0.01	0.05 ± 0.02	0.05 ± 0.03	0.04 ± 0.01	0.80	0.39
MH	0.04 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.01		
MU	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.04	0.04 ± 0.01		
Hydroxyhexadecenoylcarnitine, µM	0.04 ± 0.02	0.05 ± 0.01*	0.05 ± 0.01	0.05 ± 0.01	0.72	0.31
MH	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.01		
MU	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.02	0.05 ± 0.01		
Octadecenoylcarnitine, µM	0.17 ± 0.06	0.25 ± 0.09***	0.23 ± 0.10	0.19 ± 0.05	<0.001	0.57
MH	0.16 ± 0.06	0.25 ± 0.09	0.20 ± 0.05	0.19 ± 0.05		
MU	0.18 ± 0.06	0.24 ± 0.09	0.25 ± 0.13	0.20 ± 0.06		
Hydroxyoctadecenoylcarnitine, µM	0.05 ± 0.01	0.05 ± 0.02	0.05 ± 0.02	0.04 ± 0.01	0.98	0.49
MH	0.04 ± 0.01	0.05 ± 0.03	0.04 ± 0.01	0.05 ± 0.01		
MU	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	0.04 ± 0.01		
Octadecadienylcarnitine, µM	0.11 ± 0.03	0.12 ± 0.07	0.11 ± 0.03	0.10 ± 0.03	0.14	0.60
MH	0.10 ± 0.03	0.13 ± 0.09	0.10 ± 0.02	0.10 ± 0.03		
MU	0.11 ± 0.03	0.11 ± 0.03	0.12 ± 0.04	0.10 ± 0.03		
lysoPhosphatidylcholine acyl C16:0, µM	151.74 ± 53.75	146.86 ± 51.52	172.71 ± 74.45	159.56 ± 63.64	0.31	0.56
MH	133.85 ± 37.43	146.91 ± 48.16	141.17 ± 26.80	142.62 ± 37.33		
MU	172.62 ± 62.86	146.79 ± 56.91	208.20 ± 94.04	177.79 ± 81.06		
lysoPhosphatidylcholine acyl C16:1, µM	4.28 ± 1.88	3.19 ± 1.44	4.27 ± 2.22	4.16 ± 1.93	<0.01	0.53
MH	3.69 ± 1.36	3.28 ± 1.41	3.57 ± 1.08	3.47 ± 0.90		
MU	4.97 ± 2.19	3.08 ± 1.51	5.06 ± 2.87	4.91 ± 2.45		
lysoPhosphatidylcholine acyl C17:0, µM	2.10 ± 0.84	1.76 ± 0.76	2.29 ± 1.08	2.25 ± 0.79	<0.05	0.50
MH	1.95 ± 0.64	1.81 ± 0.72	1.90 ± 0.41	2.14 ± 0.63		
MU	2.28 ± 1.01	1.70 ± 0.83	2.73 ± 1.41	2.36 ± 0.94		
lysoPhosphatidylcholine acyl C18:0, µM	50.51 ± 22.58	35.96 ± 16.81	45.86 ± 17.99	46.25 ± 19.06	<0.001	0.61
MH	45.80 ± 16.60	37.15 ± 15.36	39.93 ± 7.55	42.44 ± 12.36		
MU	56.01 ± 27.49	34.49 ± 18.83	52.53 ± 23.61	50.36 ± 24.21		
lysoPhosphatidylcholine acyl C18:1, µM	32.32 ± 12.19	31.99 ± 14.11	41.08 ± 19.27	42.94 ± 19.35	<0.05	0.61
MH	30.46 ± 11.45	33.11 ± 13.79	35.63 ± 6.96	37.51 ± 13.28		
MU	34.49 ± 12.98	30.59 ± 14.79	47.22 ± 26.20	48.79 ± 23.43		
lysoPhosphatidylcholine acyl C18:2, µM	43.82 ± 17.26	34.47 ± 15.48	45.21 ± 19.61	47.53 ± 23.02	<0.01	0.72
MH	42.56 ± 11.84	35.86 ± 12.36	42.44 ± 10.14	42.24 ± 14.95		

MU	45.28 ± 22.29	32.75 ± 18.91	48.31 ± 26.65	53.22 ± 28.96		
lysoPhosphatidylcholine acyl C20:3, µM	4.37 ± 2.04	2.19 ± 1.08**	2.87 ± 1.06*	3.08 ± 1.69*	<0.001	0.60
MH	4.04 ± 2.15	2.30 ± 1.24	2.65 ± 0.91	2.47 ± 0.56		
MU	4.76 ± 1.90	2.06 ± 0.87	3.13 ± 1.19	3.74 ± 2.23		
lysoPhosphatidylcholine acyl C20:4, µM	10.77 ± 4.15	11.81 ± 4.34	12.75 ± 6.56	12.41 ± 6.72	0.50	0.60
MH	10.71 ± 4.23	11.78 ± 4.20	10.58 ± 2.81	10.29 ± 2.47		
MU	10.85 ± 4.16	11.86 ± 4.63	15.19 ± 8.59	14.68 ± 8.97		
lysoPhosphatidylcholine acyl C24:0, µM	0.50 ± 0.18	0.41 ± 0.15	0.44 ± 0.13	0.44 ± 0.14	<0.01	0.20
MH	0.44 ± 0.12	0.45 ± 0.19	0.39 ± 0.09	0.42 ± 0.09		
MU	0.56 ± 0.21	0.36 ± 0.08	0.49 ± 0.15	0.47 ± 0.19		
lysoPhosphatidylcholine acyl C26:0, µM	0.53 ± 0.41	0.38 ± 0.36*	0.41 ± 0.30*	0.38 ± 0.32	<0.001	0.32
MH	0.41 ± 0.23	0.42 ± 0.46	0.30 ± 0.10	0.29 ± 0.15		
MU	0.66 ± 0.53	0.33 ± 0.18	0.51 ± 0.39	0.47 ± 0.42		
lysoPhosphatidylcholine acyl C26:1, µM	0.34 ± 0.14	0.33 ± 0.17	0.33 ± 0.18	0.32 ± 0.16	0.80	0.39
MH	0.32 ± 0.12	0.37 ± 0.20	0.26 ± 0.12	0.28 ± 0.15		
MU	0.36 ± 0.15	0.30 ± 0.13	0.41 ± 0.20	0.37 ± 0.15		
lysoPhosphatidylcholine acyl C28:0, µM	0.46 ± 0.34	0.34 ± 0.29*	0.36 ± 0.26	0.38 ± 0.26	<0.001	0.62
MH	0.36 ± 0.13	0.37 ± 0.37	0.28 ± 0.04	0.29 ± 0.11		
MU	0.57 ± 0.46	0.30 ± 0.12	0.44 ± 0.36	0.49 ± 0.33		
lysoPhosphatidylcholine acyl C28:1, µM	0.52 ± 0.24	0.38 ± 0.26*	0.43 ± 0.23**	0.42 ± 0.17	<0.001	0.28
MH	0.47 ± 0.15	0.43 ± 0.32	0.35 ± 0.09	0.38 ± 0.12		
MU	0.59 ± 0.31	0.32 ± 0.14	0.52 ± 0.29	0.47 ± 0.21		
PC aa C24.0, µM	0.25 ± 0.14	0.23 ± 0.20	0.23 ± 0.16	0.21 ± 0.11	0.09	0.39
MH	0.21 ± 0.06	0.25 ± 0.25	0.17 ± 0.03	0.18 ± 0.05		
MU	0.28 ± 0.18	0.20 ± 0.08	0.28 ± 0.21	0.24 ± 0.15		
PC aa C28.1, µM	3.01 ± 0.75	2.11 ± 0.58**	2.41 ± 0.82**	2.30 ± 0.74	<0.001	0.56
MH	2.94 ± 0.72	2.16 ± 0.55	2.29 ± 0.61	2.45 ± 0.72		
MU	3.10 ± 0.79	2.05 ± 0.62	2.55 ± 1.00	2.13 ± 0.75		
PC aa C30.0, µM	1.90 ± 0.74	1.45 ± 0.50	1.62 ± 0.69	1.52 ± 0.89	<0.01	0.72
MH	1.77 ± 0.70	1.40 ± 0.46	1.50 ± 0.64	1.56 ± 1.08		
MU	2.04 ± 0.77	1.51 ± 0.54	1.75 ± 0.74	1.47 ± 0.68		
PC aa C32.0, µM	10.69 ± 3.23	11.82 ± 3.48	13.13 ± 6.13	12.21 ± 4.88	0.30	0.56
MH	10.20 ± 3.03	11.68 ± 2.79	11.60 ± 3.04	12.22 ± 3.15		
MU	11.27 ± 3.46	12.00 ± 4.27	14.85 ± 8.14	12.20 ± 6.38		
PC aa C32.1, µM	11.77 ± 6.61	7.11 ± 3.19	9.33 ± 5.12	8.16 ± 4.23	<0.001	0.50
MH	10.25 ± 6.21	7.14 ± 3.09	8.94 ± 5.15	8.19 ± 4.26		
MU	13.55 ± 6.79	7.07 ± 3.39	9.78 ± 5.23	8.14 ± 4.37		
PC aa C32.3, µM	0.24 ± 0.07	0.22 ± 0.07	0.23 ± 0.09	0.20 ± 0.06	0.09	0.60
MH	0.22 ± 0.08	0.21 ± 0.06	0.21 ± 0.06	0.21 ± 0.06		
MU	0.25 ± 0.07	0.23 ± 0.07	0.26 ± 0.12	0.20 ± 0.07		

PC aa C34.1, µM	139.15 ± 53.30	136.42 ± 42.27	154.44 ± 70.31	144.93 ± 54.67	0.89	0.57
MH	132.83 ± 61.11	137.92 ± 39.62	142.43 ± 52.60	146.85 ± 41.79		
MU	146.53 ± 43.02	134.57 ± 46.50	167.96 ± 85.87	142.85 ± 67.64		
PC aa C34.2, µM	338.67 ± 142.90	277.00 ± 79.15	330.50 ± 145.75	307.78 ± 140.63	0.06	0.60
MH	332.90 ± 160.62	276.67 ± 69.14	318.17 ± 123.21	319.21 ± 125.39		
MU	345.39 ± 123.31	277.41 ± 92.26	344.38 ± 170.71	295.46 ± 159.68		
PC aa C34.3, µM	10.29 ± 5.06	5.46 ± 2.61**	7.52 ± 3.95	7.24 ± 3.68	<0.001	0.61
MH	9.64 ± 5.37	5.70 ± 2.66	7.44 ± 3.72	7.43 ± 4.18		
MU	11.05 ± 4.71	5.15 ± 2.59	7.61 ± 4.31	7.02 ± 3.20		
PC aa C34.4, µM	0.70 ± 0.41	0.27 ± 0.13***	0.38 ± 0.22***	0.39 ± 0.26*	<0.001	0.93
MH	0.69 ± 0.43	0.28 ± 0.13	0.36 ± 0.22	0.40 ± 0.31		
MU	0.72 ± 0.40	0.26 ± 0.14	0.40 ± 0.22	0.37 ± 0.21		
PC aa C36.0, µM	1.57 ± 0.85	1.06 ± 0.59**	1.37 ± 0.65	1.37 ± 0.67	<0.001	0.61
MH	1.43 ± 0.66	1.08 ± 0.67	1.22 ± 0.37	1.35 ± 0.60		
MU	1.73 ± 1.03	1.04 ± 0.49	1.53 ± 0.85	1.40 ± 0.77		
PC aa C36.1, µM	18.32 ± 7.35	11.50 ± 5.56**	14.06 ± 5.38*	14.54 ± 5.35	<0.001	0.76
MH	18.46 ± 8.82	12.27 ± 5.88	14.20 ± 5.20	14.99 ± 3.96		
MU	18.16 ± 5.40	10.55 ± 5.16	13.91 ± 5.75	14.07 ± 6.68		
PC aa C36.2, µM	125.91 ± 50.51	77.67 ± 35.78***	99.07 ± 38.03*	104.34 ± 44.03	<0.001	0.78
MH	129.15 ± 54.69	81.07 ± 32.29	100.81 ± 31.69	111.41 ± 35.45		
MU	122.13 ± 46.44	73.46 ± 40.28	97.13 ± 45.11	96.73 ± 52.13		
PC aa C36.3, µM	78.45 ± 34.07	44.88 ± 18.58**	58.84 ± 24.77*	59.76 ± 27.49	<0.001	0.61
MH	75.56 ± 38.85	46.97 ± 19.76	57.26 ± 25.89	61.20 ± 25.68		
MU	81.83 ± 28.23	42.29 ± 17.24	60.63 ± 24.15	58.22 ± 30.30		
PC aa C36.4, µM	120.26 ± 42.52	127.91 ± 42.11	127.46 ± 66.07	111.14 ± 52.68	0.21	0.57
MH	120.18 ± 42.12	119.30 ± 38.76	109.92 ± 38.76	108.41 ± 30.59		
MU	120.35 ± 44.19	138.55 ± 44.78	147.20 ± 84.35	114.09 ± 70.58		
PC aa C36.5, µM	9.59 ± 4.92	6.53 ± 4.18*	7.41 ± 5.02*	5.77 ± 2.80*	<0.001	0.85
MH	9.03 ± 5.27	5.64 ± 2.04	6.45 ± 3.19	5.73 ± 3.06		
MU	10.24 ± 4.54	7.63 ± 5.74	8.50 ± 6.45	5.82 ± 2.63		
PC aa C38.0, µM	3.26 ± 1.29	2.62 ± 0.99	3.08 ± 1.56	3.07 ± 1.29	<0.05	0.60
MH	3.15 ± 1.42	2.65 ± 0.75	2.80 ± 0.72	3.12 ± 1.22		
MU	3.38 ± 1.15	2.58 ± 1.24	3.41 ± 2.14	3.02 ± 1.41		
PC aa C38.1, µM	1.49 ± 1.12	0.98 ± 0.72**	1.13 ± 0.78	1.30 ± 0.66	<0.01	0.82
MH	1.23 ± 0.55	0.96 ± 0.82	0.99 ± 0.49	1.26 ± 0.37		
MU	1.80 ± 1.52	1.00 ± 0.61	1.28 ± 1.01	1.35 ± 0.90		
PC aa C38.3, µM	41.54 ± 19.61	21.09 ± 8.54***	25.80 ± 9.83***	25.56 ± 11.95	<0.001	0.60
MH	40.04 ± 22.18	21.79 ± 8.91	24.96 ± 9.96	26.03 ± 10.99		
MU	43.29 ± 16.58	20.24 ± 8.25	26.74 ± 9.93	25.06 ± 13.34		
PC aa C38.4, µM	92.36 ± 34.71	77.19 ± 28.58	82.38 ± 37.56*	77.39 ± 32.93	0.08	0.60

MH	93.81 ± 33.35	76.28 ± 27.79	74.74 ± 19.37	77.43 ± 18.23		
MU	90.67 ± 37.13	78.32 ± 30.35	90.97 ± 50.31	77.34 ± 44.60		
PC aa C38.5, µM	28.74 ± 9.86	24.68 ± 8.74	28.88 ± 13.73	27.16 ± 11.08	0.29	0.67
MH	28.26 ± 9.57	24.68 ± 8.74	25.94 ± 7.21	26.69 ± 6.80		
MU	29.31 ± 10.44	24.68 ± 9.01	32.19 ± 18.26	27.67 ± 14.68		
PC aa C38.6, µM	62.95 ± 27.79	72.99 ± 28.59	74.38 ± 45.68	61.91 ± 30.63	<0.05	0.70
MH	60.23 ± 27.85	69.79 ± 27.57	68.95 ± 37.95	62.11 ± 32.03		
MU	66.13 ± 28.17	76.96 ± 30.17	80.48 ± 53.70	61.68 ± 30.34		
PC aa C40.1, µM	0.69 ± 0.64	0.55 ± 0.35	0.58 ± 0.33	0.58 ± 0.28	<0.05	0.49
MH	0.50 ± 0.09	0.56 ± 0.43	0.43 ± 0.05	0.49 ± 0.11		
MU	0.87 ± 0.88	0.55 ± 0.20	0.76 ± 0.43	0.71 ± 0.40		
PC aa C40.2, µM	0.64 ± 1.15	0.47 ± 0.82*	0.46 ± 0.75*	0.48 ± 0.68	<0.01	0.56
MH	0.34 ± 0.10	0.45 ± 0.92	0.25 ± 0.06	0.32 ± 0.10		
MU	0.99 ± 1.65	0.49 ± 0.69	0.70 ± 1.06	0.66 ± 0.96		
PC aa C40.3, µM	0.87 ± 0.92	0.69 ± 0.67	0.72 ± 0.60*	0.73 ± 0.65	<0.05	0.50
MH	0.64 ± 0.24	0.70 ± 0.82	0.52 ± 0.13	0.60 ± 0.14		
MU	1.14 ± 1.30	0.67 ± 0.45	0.96 ± 0.82	0.87 ± 0.92		
PC aa C40.4, µM	2.78 ± 1.25	1.90 ± 0.84*	2.25 ± 1.05*	2.22 ± 0.94	<0.001	0.56
MH	2.44 ± 0.85	1.97 ± 0.85	1.90 ± 0.55	2.06 ± 0.53		
MU	3.17 ± 1.53	1.82 ± 0.85	2.64 ± 1.33	2.39 ± 1.24		
PC aa C40.5, µM	5.18 ± 1.99	3.88 ± 1.73*	4.72 ± 2.02	4.50 ± 1.85	<0.01	0.70
MH	5.06 ± 1.87	3.97 ± 1.87	4.29 ± 1.26	4.44 ± 1.25		
MU	5.32 ± 2.18	3.76 ± 1.60	5.20 ± 2.59	4.56 ± 2.40		
PC aa C40.6, µM	22.21 ± 9.35	20.34 ± 9.48	21.26 ± 10.61	19.00 ± 9.41	0.12	0.75
MH	21.55 ± 9.74	19.91 ± 9.09	20.46 ± 8.51	19.38 ± 9.73		
MU	22.97 ± 9.11	20.87 ± 10.21	22.16 ± 12.81	18.59 ± 9.43		
PC aa C42.0, µM	0.76 ± 0.30	0.71 ± 0.25	0.76 ± 0.34	0.74 ± 0.25	0.50	0.39
MH	0.70 ± 0.20	0.73 ± 0.25	0.66 ± 0.14	0.75 ± 0.20		
MU	0.82 ± 0.38	0.68 ± 0.25	0.88 ± 0.46	0.74 ± 0.30		
PC aa C42.1, µM	0.41 ± 0.24	0.37 ± 0.20	0.40 ± 0.21	0.40 ± 0.16	0.25	0.20
MH	0.35 ± 0.11	0.39 ± 0.23	0.32 ± 0.06	0.39 ± 0.10		
MU	0.48 ± 0.33	0.35 ± 0.14	0.49 ± 0.28	0.40 ± 0.21		
PC aa C42.2, µM	0.41 ± 0.47	0.31 ± 0.36*	0.32 ± 0.33*	0.33 ± 0.33	<0.01	0.53
MH	0.28 ± 0.10	0.29 ± 0.38	0.22 ± 0.04	0.26 ± 0.08		
MU	0.55 ± 0.67	0.32 ± 0.35	0.43 ± 0.46	0.41 ± 0.47		
PC aa C42.4, µM	0.34 ± 0.47	0.25 ± 0.31	0.29 ± 0.38	0.30 ± 0.39	<0.01	0.56
MH	0.21 ± 0.06	0.23 ± 0.27	0.19 ± 0.04	0.20 ± 0.05		
MU	0.49 ± 0.66	0.28 ± 0.35	0.41 ± 0.53	0.41 ± 0.55		
PC aa C42.5, µM	0.38 ± 0.23	0.35 ± 0.16	0.41 ± 0.23	0.42 ± 0.25	0.10	0.49
MH	0.32 ± 0.11	0.34 ± 0.16	0.33 ± 0.13	0.38 ± 0.13		

MU	0.46 ± 0.31	0.36 ± 0.17	0.50 ± 0.28	0.47 ± 0.34		
PC aa C42.6, µM	0.40 ± 0.19	0.31 ± 0.14	0.35 ± 0.16*	0.36 ± 0.16	<0.05	0.59
MH	0.36 ± 0.11	0.31 ± 0.17	0.30 ± 0.08	0.32 ± 0.09		
MU	0.45 ± 0.25	0.31 ± 0.09	0.40 ± 0.20	0.40 ± 0.21		
PC ae C30.0, µM	0.33 ± 0.07	0.24 ± 0.07**	0.29 ± 0.08	0.28 ± 0.08	<0.001	0.44
MH	0.31 ± 0.07	0.26 ± 0.08	0.28 ± 0.07	0.28 ± 0.08		
MU	0.34 ± 0.08	0.22 ± 0.04	0.30 ± 0.09	0.27 ± 0.07		
PC ae C30.2, µM	0.12 ± 0.08	0.10 ± 0.08	0.11 ± 0.07	0.10 ± 0.06	0.21	0.57
MH	0.10 ± 0.02	0.11 ± 0.10	0.08 ± 0.02	0.09 ± 0.03		
MU	0.15 ± 0.11	0.09 ± 0.04	0.14 ± 0.10	0.11 ± 0.08		
PC ae C32.1, µM	2.22 ± 0.71	2.06 ± 0.59	2.49 ± 1.11	2.44 ± 0.74	0.17	0.56
MH	2.23 ± 0.60	2.08 ± 0.43	2.27 ± 0.49	2.56 ± 0.54		
MU	2.20 ± 0.84	2.02 ± 0.75	2.73 ± 1.53	2.30 ± 0.91		
PC ae C32.2, µM	0.70 ± 0.21	0.63 ± 0.17	0.80 ± 0.39	0.74 ± 0.22	<0.05	0.32
MH	0.68 ± 0.21	0.65 ± 0.14	0.70 ± 0.11	0.77 ± 0.17		
MU	0.72 ± 0.21	0.62 ± 0.20	0.91 ± 0.54	0.71 ± 0.26		
PC ae C34.0, µM	0.51 ± 0.16	0.39 ± 0.14*	0.48 ± 0.20	0.49 ± 0.19	<0.01	0.57
MH	0.51 ± 0.17	0.39 ± 0.11	0.44 ± 0.13	0.51 ± 0.14		
MU	0.52 ± 0.15	0.40 ± 0.17	0.52 ± 0.26	0.47 ± 0.24		
PC ae C34.1, µM	5.15 ± 1.45	4.72 ± 1.55	5.59 ± 2.33	5.56 ± 1.65	0.08	0.60
MH	5.26 ± 1.66	4.98 ± 1.38	5.31 ± 1.30	5.86 ± 1.29		
MU	5.02 ± 1.20	4.41 ± 1.74	5.91 ± 3.12	5.24 ± 1.97		
PC ae C34.2, µM	7.38 ± 3.29	4.71 ± 1.57**	6.55 ± 2.94	6.62 ± 2.13	<0.001	0.78
MH	7.78 ± 3.51	4.98 ± 1.47	6.33 ± 1.53	7.01 ± 1.83		
MU	6.91 ± 3.04	4.38 ± 1.67	6.80 ± 4.03	6.21 ± 2.42		
PC ae C34.3, µM	4.58 ± 2.00	3.57 ± 1.27*	5.20 ± 2.50	5.25 ± 2.01	<0.001	0.69
MH	4.83 ± 1.98	3.75 ± 1.17	4.96 ± 1.44	5.37 ± 1.66		
MU	4.29 ± 2.05	3.36 ± 1.38	5.47 ± 3.35	5.13 ± 2.39		
PC ae C36.0, µM	1.01 ± 0.35	0.91 ± 0.28	0.99 ± 0.39	0.99 ± 0.34	0.30	0.53
MH	0.95 ± 0.32	0.89 ± 0.26	0.87 ± 0.15	0.98 ± 0.26		
MU	1.07 ± 0.38	0.94 ± 0.31	1.12 ± 0.53	0.99 ± 0.42		
PC ae C36.1, µM	3.92 ± 3.60	2.92 ± 2.63*	3.32 ± 2.78	3.21 ± 2.32	<0.001	0.57
MH	3.03 ± 1.04	2.98 ± 3.08	2.56 ± 0.69	2.80 ± 0.72		
MU	4.97 ± 5.05	2.84 ± 2.02	4.18 ± 3.87	3.65 ± 3.28		
PC ae C36.2, µM	5.85 ± 1.90	4.21 ± 1.55**	5.44 ± 2.29	5.43 ± 2.04	<0.001	0.72
MH	5.93 ± 2.13	4.47 ± 1.54	5.24 ± 1.69	5.68 ± 1.70		
MU	5.76 ± 1.65	3.89 ± 1.54	5.66 ± 2.87	5.16 ± 2.39		
PC ae C36.3, µM	3.81 ± 1.88	2.27 ± 0.88***	3.11 ± 1.30*	3.19 ± 1.06	<0.001	0.72
MH	4.05 ± 2.12	2.45 ± 0.82	3.06 ± 0.83	3.40 ± 0.92		
MU	3.54 ± 1.58	2.04 ± 0.91	3.18 ± 1.72	2.95 ± 1.18		

PC ae C36.4, µM	8.83 ± 3.85	5.80 ± 1.95**	7.44 ± 3.43*	7.74 ± 2.64	<0.001	0.60
MH	9.55 ± 3.86	6.00 ± 1.68	6.84 ± 1.65	7.93 ± 1.58		
MU	7.98 ± 3.77	5.55 ± 2.26	8.13 ± 4.68	7.53 ± 3.52		
PC ae C36.5, µM	6.64 ± 2.65	5.35 ± 1.93	7.21 ± 3.56	7.31 ± 3.07	<0.01	0.64
MH	6.95 ± 2.45	5.38 ± 1.45	6.57 ± 1.56	7.39 ± 1.43		
MU	6.28 ± 2.89	5.31 ± 2.45	7.93 ± 4.92	7.24 ± 4.27		
PC ae C38.0, µM	1.62 ± 0.60	1.20 ± 0.52*	1.46 ± 0.82	1.32 ± 0.56	<0.01	0.85
MH	1.57 ± 0.69	1.17 ± 0.48	1.37 ± 0.65	1.32 ± 0.63		
MU	1.69 ± 0.48	1.22 ± 0.58	1.57 ± 0.98	1.31 ± 0.50		
PC ae C38.2, µM	2.75 ± 4.57	1.45 ± 1.76**	1.71 ± 2.37**	1.82 ± 2.29	<0.001	0.64
MH	1.66 ± 0.66	1.47 ± 1.98	1.10 ± 0.36	1.35 ± 0.49		
MU	4.02 ± 6.55	1.43 ± 1.51	2.38 ± 3.36	2.31 ± 3.25		
PC ae C38.3, µM	2.24 ± 2.35	1.54 ± 1.97**	1.72 ± 2.22**	1.67 ± 1.82	<0.001	0.53
MH	1.62 ± 0.65	1.47 ± 2.05	1.11 ± 0.41	1.26 ± 0.32		
MU	2.95 ± 3.30	1.63 ± 1.93	2.40 ± 3.12	2.10 ± 2.58		
PC ae C38.4, µM	5.73 ± 1.86	4.47 ± 1.31*	5.04 ± 1.92*	5.02 ± 1.56	<0.01	0.57
MH	5.89 ± 1.86	4.61 ± 1.24	4.66 ± 1.19	5.26 ± 0.79		
MU	5.54 ± 1.90	4.31 ± 1.42	5.46 ± 2.47	4.76 ± 2.12		
PC ae C38.5, µM	9.48 ± 3.75	8.31 ± 3.06	9.77 ± 4.58	10.09 ± 3.85	0.13	0.60
MH	10.16 ± 3.70	8.53 ± 2.64	9.06 ± 2.39	10.47 ± 2.61		
MU	8.68 ± 3.76	8.02 ± 3.58	10.56 ± 6.19	9.68 ± 4.94		
PC ae C38.6, µM	4.00 ± 1.54	2.95 ± 1.13*	3.85 ± 1.75	3.78 ± 1.49	<0.01	0.70
MH	4.17 ± 1.59	2.96 ± 0.97	3.60 ± 1.00	3.90 ± 1.26		
MU	3.79 ± 1.50	2.93 ± 1.35	4.14 ± 2.33	3.66 ± 1.74		
PC ae C40.1, µM	1.37 ± 0.85	1.06 ± 0.78	1.18 ± 0.66	1.18 ± 0.58	<0.01	0.61
MH	1.15 ± 0.42	1.09 ± 0.91	0.97 ± 0.23	1.05 ± 0.28		
MU	1.64 ± 1.13	1.03 ± 0.59	1.42 ± 0.88	1.33 ± 0.77		
PC ae C40.2, µM	1.56 ± 1.43	1.25 ± 0.65	1.40 ± 0.89	1.39 ± 0.85	0.17	0.53
MH	1.17 ± 0.31	1.24 ± 0.71	1.08 ± 0.31	1.22 ± 0.40		
MU	2.01 ± 2.02	1.27 ± 0.58	1.75 ± 1.17	1.58 ± 1.15		
PC ae C40.3, µM	1.57 ± 2.41	1.14 ± 1.61*	1.25 ± 1.93*	1.27 ± 1.84	<0.001	0.49
MH	0.92 ± 0.23	1.06 ± 1.60	0.72 ± 0.15	0.79 ± 0.19		
MU	2.34 ± 3.42	1.22 ± 1.65	1.85 ± 2.73	1.79 ± 2.60		
PC ae C40.4, µM	2.38 ± 1.42	1.78 ± 0.96*	2.07 ± 1.33*	2.13 ± 1.13	<0.001	0.53
MH	2.04 ± 0.59	1.78 ± 1.01	1.64 ± 0.43	1.96 ± 0.35		
MU	2.78 ± 1.94	1.78 ± 0.93	2.54 ± 1.80	2.32 ± 1.60		
PC ae C40.5, µM	2.32 ± 1.55	2.24 ± 1.26	2.45 ± 1.81	2.46 ± 1.46	0.83	0.56
MH	1.92 ± 0.57	2.09 ± 0.85	1.83 ± 0.45	2.12 ± 0.39		
MU	2.78 ± 2.14	2.43 ± 1.64	3.14 ± 2.46	2.83 ± 2.04		
PC ae C40.6, µM	2.31 ± 0.68	2.11 ± 0.69	2.35 ± 1.03	2.34 ± 0.80	0.44	0.60

MH	2.33 ± 0.68	2.12 ± 0.60	2.15 ± 0.55	2.41 ± 0.68		
MU	2.29 ± 0.71	2.10 ± 0.82	2.57 ± 1.38	2.26 ± 0.92		
PC ae C42.1, µM	0.62 ± 0.67	0.47 ± 0.51	0.51 ± 0.45	0.54 ± 0.42	<0.01	0.56
MH	0.43 ± 0.15	0.48 ± 0.61	0.37 ± 0.11	0.42 ± 0.11		
MU	0.84 ± 0.95	0.46 ± 0.37	0.67 ± 0.62	0.67 ± 0.58		
PC ae C42.2, µM	0.62 ± 0.54	0.47 ± 0.45	0.56 ± 0.37	0.55 ± 0.35	<0.01	0.63
MH	0.50 ± 0.16	0.51 ± 0.56	0.47 ± 0.15	0.49 ± 0.13		
MU	0.77 ± 0.77	0.43 ± 0.28	0.66 ± 0.51	0.63 ± 0.49		
PC ae C42.3, µM	0.96 ± 0.74	0.73 ± 0.58**	0.84 ± 0.60*	0.85 ± 0.55	<0.001	0.56
MH	0.79 ± 0.22	0.74 ± 0.61	0.67 ± 0.17	0.76 ± 0.22		
MU	1.15 ± 1.05	0.72 ± 0.56	1.03 ± 0.83	0.96 ± 0.76		
PC ae C42.4, µM	1.09 ± 0.45	0.76 ± 0.32**	0.93 ± 0.40*	0.98 ± 0.39	<0.001	0.60
MH	1.03 ± 0.27	0.82 ± 0.35	0.85 ± 0.23	0.98 ± 0.21		
MU	1.17 ± 0.59	0.69 ± 0.28	1.03 ± 0.53	0.98 ± 0.53		
PC ae C42.5, µM	2.39 ± 0.79	2.47 ± 0.74	2.70 ± 1.26	2.79 ± 0.98	0.45	0.39
MH	2.28 ± 0.58	2.49 ± 0.63	2.26 ± 0.54	2.70 ± 0.49		
MU	2.51 ± 0.99	2.44 ± 0.86	3.20 ± 1.63	2.89 ± 1.34		
PC ae C44.3, µM	0.23 ± 0.30	0.18 ± 0.20	0.19 ± 0.23	0.22 ± 0.26	<0.01	0.60
MH	0.16 ± 0.05	0.17 ± 0.19	0.13 ± 0.04	0.15 ± 0.04		
MU	0.33 ± 0.43	0.19 ± 0.22	0.25 ± 0.32	0.28 ± 0.37		
PC ae C44.4, µM	0.51 ± 0.19	0.38 ± 0.14**	0.41 ± 0.14**	0.45 ± 0.16	<0.001	0.32
MH	0.49 ± 0.15	0.42 ± 0.16	0.38 ± 0.08	0.47 ± 0.14		
MU	0.53 ± 0.22	0.32 ± 0.10	0.45 ± 0.19	0.43 ± 0.19		
PC ae C44.5, µM	2.17 ± 0.70	2.22 ± 0.79	2.50 ± 1.23	2.48 ± 0.77	0.24	0.39
MH	2.35 ± 0.67	2.39 ± 0.65	2.29 ± 0.62	2.68 ± 0.59		
MU	1.96 ± 0.70	2.02 ± 0.92	2.73 ± 1.67	2.27 ± 0.91		
PC ae C44.6, µM	1.63 ± 0.52	1.44 ± 0.48	1.65 ± 0.72	1.70 ± 0.57	<0.05	0.50
MH	1.72 ± 0.44	1.53 ± 0.39	1.54 ± 0.38	1.84 ± 0.46		
MU	1.52 ± 0.59	1.32 ± 0.55	1.78 ± 0.97	1.56 ± 0.65		
SM (OH) C14:1, µM	3.73 ± 1.06	3.45 ± 1.08	3.94 ± 1.65	3.81 ± 1.22	0.30	0.53
MH	3.83 ± 1.08	3.48 ± 1.05	3.64 ± 1.04	4.05 ± 1.09		
MU	3.61 ± 1.06	3.40 ± 1.15	4.27 ± 2.12	3.56 ± 1.33		
SM (OH) C16:1, µM	1.51 ± 0.45	1.69 ± 0.53	1.77 ± 0.77	1.63 ± 0.52	0.30	0.56
MH	1.56 ± 0.44	1.70 ± 0.46	1.63 ± 0.50	1.67 ± 0.41		
MU	1.45 ± 0.47	1.67 ± 0.62	1.92 ± 0.98	1.58 ± 0.64		
SM (OH) C22:1, µM	5.77 ± 2.22	2.83 ± 1.13***	3.31 ± 1.27***	3.61 ± 2.05*	<0.001	0.76
MH	6.21 ± 2.38	2.97 ± 1.07	3.43 ± 1.30	4.09 ± 2.33		
MU	5.26 ± 1.94	2.66 ± 1.20	3.16 ± 1.28	3.09 ± 1.64		
SM (OH) C22:2, µM	5.41 ± 1.52	3.98 ± 1.15**	4.63 ± 1.51**	4.58 ± 1.55	<0.001	0.60
MH	5.48 ± 1.83	4.12 ± 1.11	4.38 ± 1.21	4.80 ± 1.71		

MU	5.32 ± 1.12	3.79 ± 1.21	4.91 ± 1.79	4.35 ± 1.38		
SM (OH) C24:1, µM	0.57 ± 0.20	0.38 ± 0.14***	0.44 ± 0.16**	0.44 ± 0.18	<0.001	0.57
MH	0.60 ± 0.20	0.38 ± 0.13	0.42 ± 0.14	0.47 ± 0.19		
MU	0.52 ± 0.21	0.38 ± 0.15	0.46 ± 0.18	0.40 ± 0.17		
SM C16:0, µM	74.81 ± 21.13	76.98 ± 21.22	85.12 ± 31.63	83.88 ± 31.41	0.62	0.57
MH	77.67 ± 21.62	78.39 ± 20.55	79.64 ± 17.22	86.01 ± 21.78		
MU	71.48 ± 20.64	75.25 ± 22.53	91.28 ± 42.29	81.58 ± 40.16		
SM C16:1, µM	12.58 ± 3.71	12.72 ± 3.74	14.06 ± 5.74	12.08 ± 3.65	0.34	0.59
MH	12.63 ± 3.71	12.42 ± 3.68	12.98 ± 3.27	12.52 ± 3.12		
MU	12.51 ± 3.82	13.09 ± 3.90	15.27 ± 7.58	11.60 ± 4.23		
SM C18:0, µM	11.45 ± 4.20	16.06 ± 5.57**	14.89 ± 6.96	12.91 ± 5.76	<0.001	0.69
MH	11.55 ± 4.69	15.56 ± 5.06	13.48 ± 5.30	12.46 ± 3.99		
MU	11.33 ± 3.68	16.67 ± 6.24	16.48 ± 8.34	13.39 ± 7.35		
SM C18:1, µM	6.64 ± 2.34	8.98 ± 2.96**	9.02 ± 4.14	7.36 ± 2.46	<0.001	0.73
MH	6.61 ± 2.46	8.79 ± 2.97	8.14 ± 2.87	7.30 ± 1.97		
MU	6.67 ± 2.27	9.22 ± 3.02	10.01 ± 5.13	7.42 ± 2.99		
SM C20:2, µM	0.28 ± 0.09	0.28 ± 0.09	0.30 ± 0.13	0.26 ± 0.12	0.48	0.60
MH	0.28 ± 0.10	0.27 ± 0.09	0.27 ± 0.11	0.26 ± 0.13		
MU	0.29 ± 0.08	0.29 ± 0.10	0.33 ± 0.14	0.27 ± 0.12		
SM C24:0, µM	8.97 ± 3.27	5.25 ± 1.85***	6.02 ± 1.84***	6.45 ± 3.68	<0.001	0.72
MH	9.41 ± 3.83	5.33 ± 1.85	6.01 ± 1.94	6.92 ± 4.37		
MU	8.46 ± 2.49	5.15 ± 1.91	6.04 ± 1.79	5.95 ± 2.85		
SM C24:1, µM	30.33 ± 9.71	31.81 ± 10.19	34.66 ± 12.98	33.99 ± 14.22	0.51	0.57
MH	31.49 ± 11.35	32.16 ± 9.97	32.10 ± 10.37	33.71 ± 11.47		
MU	28.98 ± 7.47	31.36 ± 10.74	37.53 ± 15.24	34.29 ± 17.18		

[1] Values are shown as Mean ± SD. Total n 39 patients, separate in metabollicaly health (MH, n=21) and metabollicaly abnormal (MU, n=18).

[2] *P* values represent changes over time (p-time) and time x group interaction (p-time x group) derived from linear mixed model after log-transformed variables and corrected for multiple testing by false discovery rate. Taking into account the co-founders: age, gender and type of surgery.

#, p<0.05; ##, p<0.01 and ###, p<0.0001 represents change over time differently between MH and MU

aa, acyl-acyl; ae, acyl-alkyl; LPC a, lysophosphatidylcholines; Cx:y, where x is the number of carbons in the fatty acid side chain; y is the number of double bonds in the fatty acid side chain; DC, decarboxyl; M, methyl; OH, hydroxyl; PC, phophatidylcholine; SM, sphingomyelin.

Table S6. Changes in metabolite concentrations of both clusters after surgery[1].

		After surgery, mo				
	Before surgery	1	3	6	p-time[2]	p-time x cluster[2]
Isoleucine, µM	98,98 ± 25,92	91,04 ± 27,60	86,17 ± 37,44	74,60 ± 22,03	<0,001	0,11
Cl-1	91,34 ± 25,15	89,08 ± 31,70	86,70 ± 41,88	79,43 ± 24,33		
Cl-2	111,19 ± 22,92	94,05 ± 20,45	85,32 ± 30,52	67,57 ± 16,81#		
Leucine, µM	185,21 ± 46,24	143,54 ± 44,53**	148,73 ± 61,90*	132,32 ± 34,72*	<0,001	0,07
Cl-1	171,30 ± 42,45	143,67 ± 52,57	145,73 ± 66,29	140,54 ± 35,99		
Cl-2	207,47 ± 44,50	143,33 ± 29,97	153,58 ± 56,33	120,37 ± 30,42##		
Valine, µM	318,38 ± 80,39	225,95 ± 67,91**	253,06 ± 110,01*	214,55 ± 54,29**	<0,001	0,06
Cl-1	290,13 ± 62,18	228,35 ± 81,51	243,29 ± 100,48	226,63 ± 57,12		
Cl-2	363,60 ± 87,34	222,27 ± 41,50	268,85 ± 126,56	196,99 ± 46,85##		
Alanine, µM	471,87 ± 128,17	374,82 ± 110,87	415,12 ± 157,67	405,59 ± 105,49	<0,01	<0,01
Cl-1	419,42 ± 104,03	387,09 ± 122,01	416,24 ± 162,05	428,06 ± 106,98		
Cl-2	555,80 ± 120,59	356,00 ± 92,03#	413,31 ± 156,81	372,91 ± 98,90##		
Proline, µM	234,62 ± 95,27	207,54 ± 73,35	241,71 ± 116,27	207,00 ± 51,87	<0,05	<0,05
Cl-1	199,75 ± 48,13	205,28 ± 79,05	226,00 ± 74,71	217,19 ± 54,99		
Cl-2	290,40 ± 124,15	211,00 ± 66,17	267,08 ± 163,55	192,18 ± 45,29##		
Glycine, µM	291,08 ± 89,69	365,18 ± 136,59*	378,97 ± 180,20*	366,74 ± 109,32**	<0,01	<0,05
Cl-1	275,25 ± 74,88	374,91 ± 161,03	397,86 ± 209,00	412,81 ± 114,08		
Cl-2	316,40 ± 107,26	350,27 ± 90,37	348,46 ± 121,91	299,73 ± 56,69##		
Serine, µM	161,42 ± 41,16	180,66 ± 54,05	193,22 ± 75,32	189,81 ± 45,84	0,62	0,35
Cl-1	151,10 ± 29,94	179,92 ± 61,75	189,57 ± 78,99	203,31 ± 50,21		
Cl-2	177,93 ± 51,49	181,80 ± 41,54	198,83 ± 72,07	170,18 ± 31,11		
Tryptophan, µM	67,22 ± 19,04	44,04 ± 15,65*	55,54 ± 24,50	49,70 ± 18,26	<0,001	<0,01
Cl-1	60,27 ± 13,65	48,15 ± 16,62	54,37 ± 24,06	55,68 ± 18,31		
Cl-2	78,35 ± 21,49	37,73 ± 11,95#	57,43 ± 26,07	41,01 ± 14,94###		
Phenylalanine, µM	87,48 ± 21,63	64,25 ± 21,72**	70,40 ± 28,08**	65,37 ± 17,77	<0,001	<0,05
Cl-1	80,91 ± 21,95	66,67 ± 23,93	67,24 ± 28,87	69,97 ± 17,50		
Cl-2	97,99 ± 16,91	60,55 ± 17,97	75,51 ± 27,06	58,68 ± 16,68##		
Methionine, µM	29,68 ± 7,73	24,39 ± 7,91	27,70 ± 11,93	25,02 ± 7,18	0,57	0,33
Cl-1	26,67 ± 5,40	24,72 ± 8,77	27,99 ± 12,46	27,47 ± 6,59		
Cl-2	35,23 ± 8,48	23,91 ± 6,73	27,26 ± 11,54	21,47 ± 6,72		
Ornitine, µM	102,41 ± 36,60	78,10 ± 31,99	94,57 ± 52,94	88,49 ± 24,37	<0,001	<0,05
Cl-1	91,93 ± 30,26	83,54 ± 36,26	87,81 ± 40,45	96,29 ± 22,33		
Cl-2	119,18 ± 40,52	69,75 ± 22,71#	105,50 ± 69,07	77,15 ± 23,61##		
Arginine, µM	148,45 ± 36,61	118,73 ± 29,13	140,55 ± 49,74	137,67 ± 42,34	<0,001	<0,05
Cl-1	139,95 ± 37,84	124,29 ± 31,13	137,74 ± 53,93	152,95 ± 41,76		

Cl-2	162,07 ± 31,02	110,21 ± 24,31	145,08 ± 43,84	115,44 ± 33,59##		
Histidine, µM	97,68 ± 22,65	76,90 ± 18,28*	98,00 ± 39,38	94,81 ± 24,85	<0,001	<0,05
Cl-1	86,53 ± 12,95	77,15 ± 21,92	93,60 ± 41,94	98,05 ± 20,92		
Cl-2	115,52 ± 23,72	76,51 ± 11,31	105,10 ± 35,28	90,09 ± 30,13##		
Asparagine, µM	48,81 ± 12,05	41,26 ± 10,39	46,34 ± 18,58	46,79 ± 11,79	<0,01	0,11
Cl-1	45,84 ± 9,65	41,78 ± 11,17	46,45 ± 21,10	50,49 ± 12,47		
Cl-2	53,55 ± 14,22	40,45 ± 9,38	46,16 ± 14,39	41,42 ± 8,65#		
Aspartate, µM	23,12 ± 13,21	18,55 ± 11,20	20,98 ± 11,21	20,02 ± 11,41	<0,05	0,23
Cl-1	22,10 ± 12,38	19,47 ± 12,57	20,79 ± 10,93	20,43 ± 9,73		
Cl-2	24,75 ± 14,73	17,05 ± 8,73	21,26 ± 12,08	19,36 ± 14,25		
Glutamate, µM	84,68 ± 70,13	70,10 ± 54,31	64,69 ± 48,23	59,26 ± 67,14*	<0,001	0,19
Cl-1	75,57 ± 63,92	76,23 ± 61,11	65,34 ± 51,45	63,41 ± 79,15		
Cl-2	99,25 ± 79,17	60,69 ± 42,06	63,65 ± 44,53	53,23 ± 47,50		
Glutamine, µM	749,95 ± 233,35	796,32 ± 248,09	792,84 ± 204,31	855,67 ± 235,37	0,09	0,87
Cl-1	699,63 ± 177,96	809,61 ± 281,67	784,89 ± 232,67	939,50 ± 258,82		
Cl-2	830,47 ± 290,65	775,93 ± 193,02	805,42 ± 158,04	733,73 ± 126,33		
Citrulline, µM	32,76 ± 10,42	25,11 ± 9,22	34,21 ± 14,04	34,87 ± 9,35	<0,001	<0,05
Cl-1	29,01 ± 7,81	26,43 ± 9,32	33,03 ± 13,85	37,59 ± 8,07		
Cl-2	38,77 ± 11,48	23,07 ± 9,00#	36,12 ± 14,69	30,92 ± 10,04##		
Tyrosine, µM	92,46 ± 26,62	62,16 ± 19,16**	72,85 ± 37,90**	65,83 ± 17,42	<0,001	<0,01
Cl-1	82,47 ± 21,05	63,76 ± 21,07	68,00 ± 30,67	72,01 ± 17,58		
Cl-2	108,45 ± 27,38	59,71 ± 16,17#	80,68 ± 47,70	56,85 ± 13,25###		
Threonine, µM	151,52 ± 50,55	128,79 ± 42,46*	129,37 ± 60,03	121,20 ± 33,88	<0,01	0,13
Cl-1	141,53 ± 41,30	122,42 ± 45,10	132,97 ± 62,11	129,26 ± 30,00		
Cl-2	167,50 ± 60,74	138,55 ± 37,41	123,55 ± 58,50	109,47 ± 37,15		
Lysine, µM	381,77 ± 81,57	314,79 ± 83,31	328,21 ± 111,39	312,30 ± 82,29	0,1	0,17
Cl-1	355,83 ± 63,70	321,22 ± 91,94	335,05 ± 135,39	337,00 ± 86,21		
Cl-2	423,27 ± 91,63	304,93 ± 69,93	316,25 ± 50,22	276,36 ± 63,74		
Creatinine, µM	80,43 ± 20,42	76,94 ± 24,42	76,23 ± 30,25	73,75 ± 18,00	0,52	<0,05
Cl-1	76,91 ± 22,42	79,27 ± 27,81	77,93 ± 34,81	80,48 ± 17,53		
Cl-2	86,07 ± 15,84	73,38 ± 18,41	73,47 ± 21,99	63,95 ± 14,27##		
Kynurenine, µM	3,30 ± 1,20	2,16 ± 0,85*	2,60 ± 1,41	2,39 ± 0,89	<0,001	<0,001
Cl-1	2,85 ± 0,93	2,25 ± 0,92	2,56 ± 1,44	2,55 ± 1,05		
Cl-2	4,02 ± 1,25	2,02 ± 0,72#	2,65 ± 1,42	2,09 ± 0,38###		
Putrescine, µM	0,22 ± 0,08	0,24 ± 0,07	0,23 ± 0,10	0,26 ± 0,09*	0,22	0,06
Cl-1	0,21 ± 0,09	0,24 ± 0,06	0,25 ± 0,11	0,29 ± 0,09		
Cl-2	0,24 ± 0,04	0,24 ± 0,07	0,21 ± 0,08	0,21 ± 0,06#		
Sarcosine, µM	8,65 ± 3,78	7,78 ± 4,50	7,49 ± 3,75	7,53 ± 2,46	0,67	0,07
Cl-1	7,89 ± 3,83	8,10 ± 4,61	7,36 ± 2,78	7,80 ± 2,73		
Cl-2	9,86 ± 3,49	7,28 ± 4,45#	7,70 ± 5,07	7,14 ± 2,06#		

Serotonin, µM	0,73 ± 0,39	0,57 ± 0,28	0,78 ± 0,57	0,87 ± 0,58	0,09	0,26
Cl-1	0,60 ± 0,31	0,60 ± 0,29	0,78 ± 0,53	0,87 ± 0,44		
Cl-2	0,96 ± 0,44	0,52 ± 0,27	0,79 ± 0,64	0,87 ± 0,75		
Taurine, µM	108,77 ± 41,38	106,12 ± 36,43	97,20 ± 44,76	100,63 ± 33,64	<0,05	0,97
Cl-1	103,31 ± 38,47	104,37 ± 35,94	98,85 ± 48,52	106,63 ± 32,66		
Cl-2	117,51 ± 45,65	108,70 ± 38,25	94,51 ± 39,92	92,45 ± 34,76		
Symmetric dimethylarginine, µM	1,32 ± 0,30	1,45 ± 0,50	1,49 ± 0,64	1,38 ± 0,31	0,47	<0,05
Cl-1	1,23 ± 0,24	1,43 ± 0,40	1,51 ± 0,69	1,54 ± 0,27		
Cl-2	1,45 ± 0,34	1,47 ± 0,63	1,46 ± 0,57	1,15 ± 0,20##		
Carnitine, µM	45,60 ± 13,74	35,27 ± 12,16	39,69 ± 17,42	39,67 ± 11,49	<0,001	<0,01
Cl-1	40,84 ± 11,49	38,64 ± 12,80	36,78 ± 11,21	43,76 ± 12,54		
Cl-2	53,21 ± 13,96	30,10 ± 9,29##	44,38 ± 24,22	33,73 ± 6,45###		
Acetylcarnitine, µM	7,76 ± 4,10	14,26 ± 8,03**	10,98 ± 6,94	8,79 ± 3,69	<0,001	<0,01
Cl-1	7,71 ± 3,77	12,59 ± 7,96	11,70 ± 7,62	10,30 ± 2,94		
Cl-2	7,85 ± 4,71	16,82 ± 7,69	9,83 ± 5,78	6,59 ± 3,69		
Hydroxypropionylcarnitine, µM	0,25 ± 0,05	0,24 ± 0,07	0,25 ± 0,07	0,25 ± 0,06	0,96	0,1
Cl-1	0,23 ± 0,05	0,26 ± 0,06	0,23 ± 0,05	0,25 ± 0,08		
Cl-2	0,27 ± 0,06	0,23 ± 0,08	0,28 ± 0,09	0,24 ± 0,04		
Hexanoylcarnitine, µM	0,11 ± 0,04	0,12 ± 0,05*	0,11 ± 0,03	0,12 ± 0,04	0,28	0,07
Cl-1	0,10 ± 0,05	0,13 ± 0,05	0,12 ± 0,03	0,12 ± 0,05		
Cl-2	0,12 ± 0,02	0,12 ± 0,05	0,11 ± 0,03	0,10 ± 0,03#		
Glutarylcarnitine, µM	0,05 ± 0,01	0,05 ± 0,01	0,05 ± 0,01	0,05 ± 0,02	0,98	0,06
Cl-1	0,05 ± 0,02	0,05 ± 0,02	0,05 ± 0,01	0,05 ± 0,02		
Cl-2	0,06 ± 0,01	0,04 ± 0,01#	0,05 ± 0,01	0,05 ± 0,01		
Pimelylcarnitine, µM	0,05 ± 0,01	0,05 ± 0,01	0,06 ± 0,02	0,05 ± 0,02	0,68	0,33
Cl-1	0,05 ± 0,01	0,05 ± 0,01	0,06 ± 0,02	0,06 ± 0,01		
Cl-2	0,05 ± 0,01	0,05 ± 0,01	0,06 ± 0,02	0,04 ± 0,02		
Octanoylcarnitine, µM	0,54 ± 0,13	0,56 ± 0,18	0,55 ± 0,15	0,54 ± 0,16	0,98	0,25
Cl-1	0,52 ± 0,13	0,59 ± 0,20	0,54 ± 0,15	0,58 ± 0,19		
Cl-2	0,57 ± 0,13	0,52 ± 0,14	0,57 ± 0,17	0,49 ± 0,08		
Decanoylcarnitine, µM	0,53 ± 0,16	0,59 ± 0,23	0,60 ± 0,24	0,57 ± 0,24	0,4	0,13
Cl-1	0,49 ± 0,13	0,60 ± 0,25	0,60 ± 0,25	0,63 ± 0,28		
Cl-2	0,59 ± 0,18	0,58 ± 0,22	0,60 ± 0,24	0,47 ± 0,12#		
Decenoylcarnitine, µM	0,25 ± 0,08	0,27 ± 0,10	0,26 ± 0,09	0,25 ± 0,10	0,35	<0,01
Cl-1	0,22 ± 0,07	0,26 ± 0,11	0,26 ± 0,09	0,27 ± 0,12		
Cl-2	0,29 ± 0,08	0,28 ± 0,09	0,26 ± 0,09	0,21 ± 0,04###		
Decadienylcarnitine, µM	0,16 ± 0,04	0,16 ± 0,06	0,16 ± 0,04	0,16 ± 0,04	0,91	<0,05
Cl-1	0,15 ± 0,04	0,16 ± 0,05	0,16 ± 0,04	0,16 ± 0,05		
Cl-2	0,18 ± 0,04	0,15 ± 0,07#	0,16 ± 0,05	0,14 ± 0,02#		
Tetradecenoylcarnitine, µM	0,19 ± 0,06	0,23 ± 0,08	0,21 ± 0,09	0,19 ± 0,06	<0,05	0,09

Cl-1	0,19 ± 0,06	0,23 ± 0,09	0,22 ± 0,09	0,22 ± 0,05		
Cl-2	0,20 ± 0,06	0,22 ± 0,06	0,20 ± 0,08	0,15 ± 0,04#		
Tetradecadienylcarnitine, µM	0,12 ± 0,04	0,13 ± 0,05	0,12 ± 0,04	0,12 ± 0,03	0,82	0,32
Cl-1	0,12 ± 0,04	0,13 ± 0,05	0,13 ± 0,04	0,12 ± 0,03		
Cl-2	0,13 ± 0,04	0,12 ± 0,04	0,12 ± 0,05	0,11 ± 0,03		
Hexadecenoylcarnitine, µM	0,11 ± 0,03	0,12 ± 0,04	0,11 ± 0,03	0,11 ± 0,03	0,67	0,13
Cl-1	0,11 ± 0,03	0,12 ± 0,04	0,12 ± 0,03	0,11 ± 0,03		
Cl-2	0,12 ± 0,03	0,10 ± 0,03#	0,10 ± 0,03	0,10 ± 0,02		
Hexadecadienylcarnitine, µM	0,04 ± 0,01	0,05 ± 0,02	0,05 ± 0,03	0,04 ± 0,01	0,82	0,7
Cl-1	0,04 ± 0,01	0,05 ± 0,02	0,05 ± 0,03	0,05 ± 0,01		
Cl-2	0,05 ± 0,01	0,04 ± 0,01	0,04 ± 0,01	0,04 ± 0,01		
Hydroxyhexadecenoylcarnitine, µM	0,04 ± 0,02	0,05 ± 0,01	0,05 ± 0,01	0,05 ± 0,01	0,67	0,33
Cl-1	0,04 ± 0,02	0,05 ± 0,01	0,05 ± 0,01	0,05 ± 0,01		
Cl-2	0,05 ± 0,02	0,04 ± 0,01	0,05 ± 0,01	0,04 ± 0,01		
Octadecenoylcarnitine, µM	0,17 ± 0,06	0,25 ± 0,09***	0,23 ± 0,10**	0,19 ± 0,05	<0,001	<0,01
Cl-1	0,16 ± 0,06	0,24 ± 0,10	0,23 ± 0,12	0,22 ± 0,05		
Cl-2	0,19 ± 0,06	0,25 ± 0,07	0,21 ± 0,05	0,15 ± 0,03##		
Hydroxyoctadecenoylcarnitine, µM	0,05 ± 0,01	0,05 ± 0,02	0,05 ± 0,02	0,04 ± 0,01	0,98	0,15
Cl-1	0,04 ± 0,01	0,05 ± 0,02	0,05 ± 0,02	0,05 ± 0,01		
Cl-2	0,05 ± 0,01	0,04 ± 0,02#	0,04 ± 0,01	0,04 ± 0,01		
Octadecadienylcarnitine, µM	0,11 ± 0,03	0,12 ± 0,07	0,11 ± 0,03	0,10 ± 0,03	0,12	0,14
Cl-1	0,10 ± 0,03	0,13 ± 0,09	0,11 ± 0,04	0,11 ± 0,03		
Cl-2	0,11 ± 0,03	0,11 ± 0,03	0,10 ± 0,03	0,09 ± 0,02		
lysoPhosphatidylcholine acyl C16:0, µM	151,74 ± 53,75	146,86 ± 51,52	172,71 ± 74,45	159,56 ± 63,64*	0,26	<0,001
Cl-1	128,78 ± 34,78	152,62 ± 56,05	170,95 ± 73,33	178,54 ± 66,61		
Cl-2	188,48 ± 59,20	138,03 ± 44,04#	175,55 ± 79,17	131,94 ± 49,58###		
lysoPhosphatidylcholine acyl C16:1, µM	4,28 ± 1,88	3,19 ± 1,44	4,27 ± 2,22	4,16 ± 1,93	<0,01	<0,01
Cl-1	3,53 ± 1,23	3,50 ± 1,41	4,23 ± 2,18	4,42 ± 1,29		
Cl-2	5,49 ± 2,15	2,72 ± 1,38##	4,34 ± 2,37	3,78 ± 2,63###		
lysoPhosphatidylcholine acyl C17:0, µM	2,10 ± 0,84	1,76 ± 0,76	2,29 ± 1,08	2,25 ± 0,79*	<0,01	<0,01
Cl-1	1,81 ± 0,56	1,94 ± 0,85	2,24 ± 0,97	2,48 ± 0,74		
Cl-2	2,56 ± 1,01	1,49 ± 0,51##	2,37 ± 1,28	1,91 ± 0,76###		
lysoPhosphatidylcholine acyl C18:0, µM	50,51 ± 22,58	35,96 ± 16,81	45,86 ± 17,99	46,25 ± 19,06	<0,001	<0,001
Cl-1	41,17 ± 14,12	38,98 ± 18,14	45,09 ± 16,52	51,70 ± 19,30		
Cl-2	65,45 ± 25,83	31,33 ± 13,86##	47,10 ± 20,79#	38,33 ± 16,41###		
lysoPhosphatidylcholine acyl C18:1, µM	32,32 ± 12,19	31,99 ± 14,11	41,08 ± 19,27**	42,94 ± 19,35***	<0,01	<0,01
Cl-1	26,88 ± 8,22	34,88 ± 15,81	41,90 ± 20,93	48,78 ± 21,14		
Cl-2	41,04 ± 12,64	27,55 ± 9,93##	39,77 ± 16,98	34,45 ± 13,04###		
lysoPhosphatidylcholine acyl C18:2, µM	43,82 ± 17,26	34,47 ± 15,48	45,21 ± 19,61	47,53 ± 23,02	<0,01	<0,01
Cl-1	36,69 ± 9,85	38,20 ± 17,97	44,88 ± 18,91	54,44 ± 25,41		

Cl-2	55,22 ± 20,57	28,75 ± 8,24##	45,73 ± 21,47	37,47 ± 14,90###		
lysoPhosphatidylcholine acyl C20:3, µM	4,37 ± 2,04	2,19 ± 1,08*	2,87 ± 1,06	3,08 ± 1,69	<0,001	<0,001
Cl-1	3,46 ± 1,36	2,48 ± 1,20	2,91 ± 1,03	3,55 ± 1,94		
Cl-2	5,83 ± 2,14	1,75 ± 0,69###	2,82 ± 1,16#	2,40 ± 0,98###		
lysoPhosphatidylcholine acyl C20:4, µM	10,77 ± 4,15	11,81 ± 4,34	12,75 ± 6,56	12,41 ± 6,72	0,47	<0,05
Cl-1	9,08 ± 3,09	11,81 ± 5,09	12,99 ± 7,80	13,92 ± 7,98		
Cl-2	13,48 ± 4,27	11,82 ± 3,00	12,36 ± 4,11	10,21 ± 3,61###		
lysoPhosphatidylcholine acyl C24:0, µM	0,50 ± 0,18	0,41 ± 0,15	0,44 ± 0,13	0,44 ± 0,14	<0,01	<0,01
Cl-1	0,42 ± 0,13	0,44 ± 0,18	0,43 ± 0,14	0,44 ± 0,08		
Cl-2	0,62 ± 0,18	0,37 ± 0,10##	0,44 ± 0,11#	0,45 ± 0,21##		
lysoPhosphatidylcholine acyl C26:0, µM	0,53 ± 0,41	0,38 ± 0,36	0,41 ± 0,30	0,38 ± 0,32	<0,001	0,13
Cl-1	0,40 ± 0,17	0,41 ± 0,45	0,40 ± 0,36	0,38 ± 0,19		
Cl-2	0,72 ± 0,58	0,33 ± 0,17	0,43 ± 0,21	0,38 ± 0,45#		
lysoPhosphatidylcholine acyl C26:1, µM	0,34 ± 0,14	0,33 ± 0,17	0,33 ± 0,18	0,32 ± 0,16	0,85	0,24
Cl-1	0,30 ± 0,12	0,35 ± 0,20	0,29 ± 0,17	0,33 ± 0,17		
Cl-2	0,41 ± 0,14	0,32 ± 0,12	0,40 ± 0,17	0,31 ± 0,14		
lysoPhosphatidylcholine acyl C28:0, µM	0,46 ± 0,34	0,34 ± 0,29	0,36 ± 0,26	0,38 ± 0,26	<0,001	<0,05
Cl-1	0,36 ± 0,14	0,37 ± 0,34	0,39 ± 0,33	0,37 ± 0,16		
Cl-2	0,58 ± 0,47	0,29 ± 0,12#	0,32 ± 0,14	0,42 ± 0,38#		
lysoPhosphatidylcholine acyl C28:1, µM	0,52 ± 0,24	0,38 ± 0,26	0,43 ± 0,23	0,42 ± 0,17	<0,001	<0,05
Cl-1	0,43 ± 0,14	0,41 ± 0,32	0,41 ± 0,25	0,44 ± 0,15		
Cl-2	0,68 ± 0,29	0,34 ± 0,12##	0,45 ± 0,19	0,40 ± 0,20##		
PC aa C24.0, µM	0,25 ± 0,14	0,23 ± 0,20	0,23 ± 0,16	0,21 ± 0,11	0,1	0,2
Cl-1	0,21 ± 0,08	0,25 ± 0,24	0,23 ± 0,20	0,19 ± 0,06		
Cl-2	0,29 ± 0,18	0,20 ± 0,07	0,22 ± 0,08	0,23 ± 0,17		
PC aa C28.1, µM	3,01 ± 0,75	2,11 ± 0,58*	2,41 ± 0,82	2,30 ± 0,74	<0,001	<0,001
Cl-1	2,71 ± 0,55	2,28 ± 0,58	2,46 ± 0,80	2,65 ± 0,70		
Cl-2	3,50 ± 0,78	1,85 ± 0,48##	2,33 ± 0,86#	1,79 ± 0,45###		
PC aa C30.0, µM	1,90 ± 0,74	1,45 ± 0,50	1,62 ± 0,69	1,52 ± 0,89	<0,01	<0,05
Cl-1	1,73 ± 0,64	1,52 ± 0,56	1,70 ± 0,80	1,83 ± 1,04		
Cl-2	2,16 ± 0,83	1,33 ± 0,37	1,49 ± 0,45	1,06 ± 0,24##		
PC aa C32.0, µM	10,69 ± 3,23	11,82 ± 3,48	13,13 ± 6,13*	12,21 ± 4,88**	0,24	<0,001
Cl-1	10,00 ± 2,62	12,18 ± 3,92	13,76 ± 6,81	14,92 ± 4,53		
Cl-2	11,80 ± 3,88	11,27 ± 2,73	12,10 ± 4,92	8,27 ± 1,46###		
PC aa C32.1, µM	11,77 ± 6,61	7,11 ± 3,19	9,33 ± 5,12	8,16 ± 4,23	<0,001	<0,01
Cl-1	10,26 ± 5,73	7,65 ± 2,82	9,62 ± 5,17	9,65 ± 4,35		
Cl-2	14,19 ± 7,39	6,28 ± 3,61##	8,87 ± 5,22	6,01 ± 3,09###		
PC aa C32.3, µM	0,24 ± 0,07	0,22 ± 0,07	0,23 ± 0,09	0,20 ± 0,06	0,09	<0,01
Cl-1	0,21 ± 0,06	0,22 ± 0,07	0,23 ± 0,08	0,23 ± 0,06		
Cl-2	0,27 ± 0,07	0,23 ± 0,06	0,24 ± 0,11	0,16 ± 0,05###		

PC aa C34.1, µM	139,15 ± 53,30	136,42 ± 42,27	154,44 ± 70,31	144,93 ± 54,67**	0,82	<0,001
Cl-1	127,55 ± 47,58	143,43 ± 44,42	159,60 ± 76,30	174,06 ± 50,87		
Cl-2	157,72 ± 58,24	125,68 ± 37,64#	146,11 ± 61,41	102,55 ± 23,58###		
PC aa C34.2, µM	338,67 ± 142,90	277,00 ± 79,15	330,50 ± 145,75	307,78 ± 140,63	<0,05	<0,001
Cl-1	316,79 ± 138,46	288,52 ± 84,83	334,76 ± 140,05	373,81 ± 143,16		
Cl-2	373,67 ± 147,63	259,33 ± 68,54	323,62 ± 160,15	211,73 ± 59,58###		
PC aa C34.3, µM	10,29 ± 5,06	5,46 ± 2,61*	7,52 ± 3,95	7,24 ± 3,68	<0,001	<0,001
Cl-1	9,03 ± 4,05	6,18 ± 2,50	7,28 ± 3,21	8,68 ± 4,07		
Cl-2	12,30 ± 5,96	4,34 ± 2,45##	7,92 ± 5,05	5,13 ± 1,44###		
PC aa C34.4, µM	0,70 ± 0,41	0,27 ± 0,13**	0,38 ± 0,22**	0,39 ± 0,26	<0,001	<0,01
Cl-1	0,59 ± 0,31	0,30 ± 0,13	0,36 ± 0,21	0,46 ± 0,32		
Cl-2	0,89 ± 0,49	0,23 ± 0,12##	0,41 ± 0,25	0,28 ± 0,07###		
PC aa C36.0, µM	1,57 ± 0,85	1,06 ± 0,59	1,37 ± 0,65	1,37 ± 0,67	<0,001	<0,001
Cl-1	1,30 ± 0,57	1,15 ± 0,70	1,37 ± 0,57	1,53 ± 0,60		
Cl-2	2,01 ± 1,05	0,93 ± 0,33##	1,37 ± 0,80#	1,14 ± 0,73###		
PC aa C36.1, µM	18,32 ± 7,35	11,50 ± 5,56*	14,06 ± 5,38	14,54 ± 5,35	<0,001	<0,001
Cl-1	15,91 ± 5,91	12,70 ± 5,53	14,19 ± 5,43	17,04 ± 4,87		
Cl-2	22,17 ± 7,97	9,66 ± 5,26##	13,85 ± 5,53	10,91 ± 3,78###		
PC aa C36.2, µM	125,91 ± 50,51	77,67 ± 35,78*	99,07 ± 38,03	104,34 ± 44,03	<0,001	<0,001
Cl-1	111,68 ± 39,57	86,66 ± 36,07	98,60 ± 34,07	126,25 ± 41,36		
Cl-2	148,67 ± 58,74	63,88 ± 31,65##	99,84 ± 45,17	72,47 ± 24,28###		
PC aa C36.3, µM	78,45 ± 34,07	44,88 ± 18,58*	58,84 ± 24,77	59,76 ± 27,49	<0,001	<0,001
Cl-1	70,38 ± 30,83	49,54 ± 18,28	59,39 ± 24,63	72,83 ± 28,16		
Cl-2	91,37 ± 36,04	37,73 ± 17,21##	57,96 ± 25,97	40,75 ± 10,20###		
PC aa C36.4, µM	120,26 ± 42,52	127,91 ± 42,11	127,46 ± 66,07	111,14 ± 52,68	0,19	<0,01
Cl-1	111,80 ± 36,63	120,34 ± 45,16	131,74 ± 73,71	130,10 ± 57,12		
Cl-2	133,79 ± 48,81	139,53 ± 35,25	120,55 ± 53,52	83,57 ± 30,19###		
PC aa C36.5, µM	9,59 ± 4,92	6,53 ± 4,18	7,41 ± 5,02	5,77 ± 2,80	<0,001	<0,01
Cl-1	8,80 ± 5,50	6,90 ± 4,96	7,12 ± 4,44	6,64 ± 3,23		
Cl-2	10,86 ± 3,64	5,95 ± 2,65#	7,89 ± 6,02	4,51 ± 1,36###		
PC aa C38.0, µM	3,26 ± 1,29	2,62 ± 0,99	3,08 ± 1,56	3,07 ± 1,29	<0,01	<0,001
Cl-1	2,84 ± 1,19	2,82 ± 1,06	3,07 ± 1,58	3,52 ± 1,42		
Cl-2	3,93 ± 1,18	2,32 ± 0,81##	3,11 ± 1,59	2,42 ± 0,71###		
PC aa C38.1, µM	1,49 ± 1,12	0,98 ± 0,72	1,13 ± 0,78	1,30 ± 0,66	<0,01	0,45
Cl-1	1,27 ± 0,65	1,10 ± 0,85	1,14 ± 0,79	1,39 ± 0,47		
Cl-2	1,87 ± 1,60	0,80 ± 0,42	1,09 ± 0,79	1,18 ± 0,88		
PC aa C38.3, µM	41,54 ± 19,61	21,09 ± 8,54**	25,80 ± 9,83**	25,56 ± 11,95	<0,001	<0,001
Cl-1	35,27 ± 14,13	23,25 ± 8,23	26,30 ± 10,02	30,47 ± 13,04		
Cl-2	51,57 ± 23,25	17,79 ± 8,20##	24,99 ± 9,88#	18,43 ± 4,68###		
PC aa C38.4, µM	92,36 ± 34,71	77,19 ± 28,58	82,38 ± 37,56	77,39 ± 32,93	<0,05	<0,001

Cl-1	81,85 ± 24,68	76,36 ± 30,11	82,54 ± 39,12	90,95 ± 33,48		
Cl-2	109,19 ± 42,15	78,47 ± 27,04	82,12 ± 36,46	57,65 ± 20,39###		
PC aa C38.5, µM	28,74 ± 9,86	24,68 ± 8,74	28,88 ± 13,73	27,16 ± 11,08	0,2	<0,01
Cl-1	26,58 ± 9,01	25,76 ± 9,76	29,33 ± 14,04	32,17 ± 11,19		
Cl-2	32,20 ± 10,47	23,03 ± 6,88	28,16 ± 13,73	19,87 ± 5,73###		
PC aa C38.6, µM	62,95 ± 27,79	72,99 ± 28,59*	74,38 ± 45,68	61,91 ± 30,63	<0,05	<0,001
Cl-1	58,39 ± 27,80	72,42 ± 33,98	75,82 ± 49,63	73,98 ± 33,53		
Cl-2	70,25 ± 27,07	73,88 ± 18,56	72,05 ± 40,30	44,35 ± 13,40###		
PC aa C40.1, µM	0,69 ± 0,64	0,55 ± 0,35	0,58 ± 0,33	0,58 ± 0,28	<0,01	<0,001
Cl-1	0,52 ± 0,23	0,60 ± 0,43	0,56 ± 0,35	0,57 ± 0,15		
Cl-2	0,85 ± 0,86	0,48 ± 0,19#	0,64 ± 0,31#	0,62 ± 0,48###		
PC aa C40.2, µM	0,64 ± 1,15	0,47 ± 0,82	0,46 ± 0,75	0,48 ± 0,68	<0,001	<0,01
Cl-1	0,39 ± 0,49	0,53 ± 0,97	0,45 ± 0,75	0,45 ± 0,40		
Cl-2	1,03 ± 1,71	0,37 ± 0,53##	0,49 ± 0,78#	0,54 ± 0,97###		
PC aa C40.3, µM	0,87 ± 0,92	0,69 ± 0,67	0,72 ± 0,60	0,73 ± 0,65	<0,01	<0,001
Cl-1	0,62 ± 0,33	0,76 ± 0,79	0,72 ± 0,59	0,74 ± 0,27		
Cl-2	1,27 ± 1,36	0,58 ± 0,43##	0,73 ± 0,65#	0,72 ± 1,00###		
PC aa C40.4, µM	2,78 ± 1,25	1,90 ± 0,84	2,25 ± 1,05	2,22 ± 0,94	<0,001	<0,001
Cl-1	2,29 ± 0,82	2,04 ± 0,80	2,20 ± 0,94	2,51 ± 0,83		
Cl-2	3,56 ± 1,44	1,69 ± 0,88##	2,33 ± 1,23#	1,79 ± 0,95###		
PC aa C40.5, µM	5,18 ± 1,99	3,88 ± 1,73	4,72 ± 2,02	4,50 ± 1,85	<0,001	<0,001
Cl-1	4,51 ± 1,40	4,20 ± 1,84	4,81 ± 2,05	5,36 ± 1,88		
Cl-2	6,25 ± 2,36	3,38 ± 1,49##	4,57 ± 2,04#	3,24 ± 0,84###		
PC aa C40.6, µM	22,21 ± 9,35	20,34 ± 9,48	21,26 ± 10,61	19,00 ± 9,41	0,07	<0,001
Cl-1	20,31 ± 8,90	21,05 ± 11,04	21,51 ± 11,49	22,85 ± 10,28		
Cl-2	25,25 ± 9,56	19,25 ± 6,64	20,85 ± 9,45	13,41 ± 3,69###		
PC aa C42.0, µM	0,76 ± 0,30	0,71 ± 0,25	0,76 ± 0,34	0,74 ± 0,25	0,48	<0,01
Cl-1	0,66 ± 0,19	0,75 ± 0,28	0,77 ± 0,33	0,83 ± 0,23		
Cl-2	0,91 ± 0,37	0,65 ± 0,18#	0,76 ± 0,38	0,62 ± 0,22###		
PC aa C42.1, µM	0,41 ± 0,24	0,37 ± 0,20	0,40 ± 0,21	0,40 ± 0,16	0,21	<0,01
Cl-1	0,33 ± 0,11	0,39 ± 0,23	0,40 ± 0,22	0,43 ± 0,13		
Cl-2	0,53 ± 0,34	0,33 ± 0,12#	0,40 ± 0,21	0,35 ± 0,19###		
PC aa C42.2, µM	0,41 ± 0,47	0,31 ± 0,36	0,32 ± 0,33	0,33 ± 0,33	<0,001	<0,001
Cl-1	0,28 ± 0,16	0,32 ± 0,38	0,31 ± 0,26	0,32 ± 0,16		
Cl-2	0,61 ± 0,70	0,28 ± 0,35##	0,35 ± 0,42#	0,35 ± 0,49###		
PC aa C42.4, µM	0,34 ± 0,47	0,25 ± 0,31	0,29 ± 0,38	0,30 ± 0,39	<0,01	<0,001
Cl-1	0,22 ± 0,18	0,27 ± 0,31	0,26 ± 0,31	0,29 ± 0,23		
Cl-2	0,52 ± 0,69	0,23 ± 0,30##	0,33 ± 0,47#	0,33 ± 0,57###		
PC aa C42.5, µM	0,38 ± 0,23	0,35 ± 0,16	0,41 ± 0,23	0,42 ± 0,25**	<0,05	<0,001
Cl-1	0,31 ± 0,11	0,37 ± 0,17	0,42 ± 0,22	0,48 ± 0,20		

Cl-2	0,50 ± 0,32	0,31 ± 0,15##	0,40 ± 0,25#	0,34 ± 0,31###		
PC aa C42.6, µM	0,40 ± 0,19	0,31 ± 0,14	0,35 ± 0,16	0,36 ± 0,16	<0,01	<0,01
Cl-1	0,34 ± 0,10	0,33 ± 0,17	0,34 ± 0,16	0,39 ± 0,14		
Cl-2	0,49 ± 0,25	0,28 ± 0,08##	0,36 ± 0,15	0,32 ± 0,18###		
PC ae C30.0, µM	0,33 ± 0,07	0,24 ± 0,07*	0,29 ± 0,08	0,28 ± 0,08	<0,001	<0,001
Cl-1	0,30 ± 0,06	0,25 ± 0,08	0,29 ± 0,09	0,30 ± 0,08		
Cl-2	0,38 ± 0,07	0,22 ± 0,05##	0,29 ± 0,07	0,24 ± 0,05###		
PC ae C30.2, µM	0,12 ± 0,08	0,10 ± 0,08	0,11 ± 0,07	0,10 ± 0,06	0,23	0,3
Cl-1	0,10 ± 0,03	0,11 ± 0,10	0,11 ± 0,08	0,10 ± 0,03		
Cl-2	0,16 ± 0,11	0,09 ± 0,04	0,11 ± 0,07	0,10 ± 0,08		
PC ae C32.1, µM	2,22 ± 0,71	2,06 ± 0,59	2,49 ± 1,11	2,44 ± 0,74*	0,12	<0,01
Cl-1	2,00 ± 0,48	2,15 ± 0,55	2,53 ± 1,19	2,86 ± 0,62		
Cl-2	2,56 ± 0,90	1,92 ± 0,63#	2,42 ± 1,03	1,82 ± 0,38###		
PC ae C32.2, µM	0,70 ± 0,21	0,63 ± 0,17	0,80 ± 0,39	0,74 ± 0,22*	<0,05	<0,01
Cl-1	0,62 ± 0,17	0,66 ± 0,18	0,80 ± 0,38	0,85 ± 0,16		
Cl-2	0,82 ± 0,20	0,60 ± 0,15#	0,79 ± 0,42	0,57 ± 0,18###		
PC ae C34.0, µM	0,51 ± 0,16	0,39 ± 0,14	0,48 ± 0,20	0,49 ± 0,19	<0,01	<0,001
Cl-1	0,46 ± 0,14	0,42 ± 0,12	0,49 ± 0,22	0,60 ± 0,16		
Cl-2	0,59 ± 0,16	0,36 ± 0,16##	0,46 ± 0,17	0,33 ± 0,10###		
PC ae C34.1, µM	5,15 ± 1,45	4,72 ± 1,55	5,59 ± 2,33	5,56 ± 1,65**	<0,05	<0,001
Cl-1	4,80 ± 1,29	5,07 ± 1,55	5,67 ± 2,41	6,55 ± 1,24		
Cl-2	5,71 ± 1,56	4,20 ± 1,46#	5,46 ± 2,27	4,12 ± 0,97###		
PC ae C34.2, µM	7,38 ± 3,29	4,71 ± 1,57*	6,55 ± 2,94	6,62 ± 2,13	<0,001	<0,001
Cl-1	6,61 ± 2,39	5,07 ± 1,61	6,48 ± 2,58	7,83 ± 1,63		
Cl-2	8,61 ± 4,16	4,16 ± 1,39#	6,66 ± 3,57	4,87 ± 1,44###		
PC ae C34.3, µM	4,58 ± 2,00	3,57 ± 1,27	5,20 ± 2,50	5,25 ± 2,01**	<0,001	<0,01
Cl-1	4,25 ± 1,61	3,79 ± 1,39	5,26 ± 2,35	6,22 ± 1,76		
Cl-2	5,11 ± 2,48	3,24 ± 1,01	5,11 ± 2,82	3,85 ± 1,47###		
PC ae C36.0, µM	1,01 ± 0,35	0,91 ± 0,28	0,99 ± 0,39	0,99 ± 0,34*	0,2	<0,001
Cl-1	0,88 ± 0,25	0,93 ± 0,28	0,99 ± 0,41	1,15 ± 0,27		
Cl-2	1,21 ± 0,41	0,88 ± 0,29#	0,98 ± 0,37	0,75 ± 0,29###		
PC ae C36.1, µM	3,92 ± 3,60	2,92 ± 2,63	3,32 ± 2,78	3,21 ± 2,32	<0,001	<0,001
Cl-1	2,99 ± 1,60	3,24 ± 3,10	3,06 ± 1,90	3,38 ± 1,41		
Cl-2	5,41 ± 5,21	2,43 ± 1,65##	3,74 ± 3,87	2,97 ± 3,31###		
PC ae C36.2, µM	5,85 ± 1,90	4,21 ± 1,55	5,44 ± 2,29	5,43 ± 2,04	<0,001	<0,001
Cl-1	5,30 ± 1,78	4,63 ± 1,60	5,33 ± 1,87	6,34 ± 1,88		
Cl-2	6,74 ± 1,81	3,57 ± 1,26##	5,61 ± 2,93	4,10 ± 1,49###		
PC ae C36.3, µM	3,81 ± 1,88	2,27 ± 0,88*	3,11 ± 1,30	3,19 ± 1,06	<0,001	<0,01
Cl-1	3,40 ± 1,39	2,46 ± 0,92	3,02 ± 1,06	3,71 ± 0,92		
Cl-2	4,48 ± 2,38	1,98 ± 0,74#	3,26 ± 1,66	2,42 ± 0,73###		

PC ae C36.4, µM	8,83 ± 3,85	5,80 ± 1,95*	7,44 ± 3,43	7,74 ± 2,64	<0,001	<0,01
Cl-1	7,86 ± 2,59	6,06 ± 1,87	7,21 ± 3,09	8,94 ± 2,22		
Cl-2	10,37 ± 5,00	5,39 ± 2,06#	7,82 ± 4,03	5,99 ± 2,27###		
PC ae C36.5, µM	6,64 ± 2,65	5,35 ± 1,93	7,21 ± 3,56	7,31 ± 3,07*	<0,01	<0,01
Cl-1	6,00 ± 1,76	5,43 ± 1,99	7,13 ± 3,41	8,51 ± 3,06		
Cl-2	7,67 ± 3,48	5,22 ± 1,90	7,35 ± 3,94	5,57 ± 2,19###		
PC ae C38.0, µM	1,62 ± 0,60	1,20 ± 0,52	1,46 ± 0,82	1,32 ± 0,56	<0,001	<0,01
Cl-1	1,46 ± 0,61	1,30 ± 0,58	1,42 ± 0,71	1,52 ± 0,61		
Cl-2	1,89 ± 0,49	1,04 ± 0,40##	1,53 ± 0,99	1,02 ± 0,29###		
PC ae C38.2, µM	2,75 ± 4,57	1,45 ± 1,76	1,71 ± 2,37	1,82 ± 2,29	<0,001	<0,001
Cl-1	1,76 ± 1,58	1,67 ± 2,04	1,57 ± 2,02	1,76 ± 1,17		
Cl-2	4,34 ± 6,94	1,12 ± 1,22##	1,92 ± 2,93#	1,90 ± 3,40###		
PC ae C38.3, µM	2,24 ± 2,35	1,54 ± 1,97*	1,72 ± 2,22	1,67 ± 1,82	<0,001	<0,001
Cl-1	1,84 ± 2,05	1,70 ± 2,19	1,69 ± 2,27	1,84 ± 1,93		
Cl-2	2,86 ± 2,72	1,30 ± 1,62##	1,76 ± 2,24#	1,41 ± 1,70###		
PC ae C38.4, µM	5,73 ± 1,86	4,47 ± 1,31	5,04 ± 1,92	5,02 ± 1,56	<0,01	<0,001
Cl-1	5,04 ± 1,17	4,55 ± 1,21	4,96 ± 1,84	5,70 ± 1,38		
Cl-2	6,83 ± 2,24	4,34 ± 1,49#	5,16 ± 2,10	4,02 ± 1,29###		
PC ae C38.5, µM	9,48 ± 3,75	8,31 ± 3,06	9,77 ± 4,58	10,09 ± 3,85*	0,09	<0,01
Cl-1	8,52 ± 2,47	8,56 ± 3,08	9,74 ± 4,66	11,76 ± 3,60		
Cl-2	11,01 ± 4,91	7,92 ± 3,10	9,81 ± 4,62	7,67 ± 2,87###		
PC ae C38.6, µM	4,00 ± 1,54	2,95 ± 1,13	3,85 ± 1,75	3,78 ± 1,49	<0,001	<0,01
Cl-1	3,60 ± 1,27	3,06 ± 1,20	3,76 ± 1,67	4,43 ± 1,46		
Cl-2	4,63 ± 1,76	2,78 ± 1,05#	4,00 ± 1,92	2,84 ± 0,96###		
PC ae C40.1, µM	1,37 ± 0,85	1,06 ± 0,78	1,18 ± 0,66	1,18 ± 0,58	<0,001	<0,01
Cl-1	1,11 ± 0,46	1,16 ± 0,90	1,13 ± 0,60	1,27 ± 0,50		
Cl-2	1,79 ± 1,15	0,92 ± 0,55#	1,28 ± 0,76	1,06 ± 0,69###		
PC ae C40.2, µM	1,56 ± 1,43	1,25 ± 0,65	1,40 ± 0,89	1,39 ± 0,85	0,13	<0,001
Cl-1	1,27 ± 0,71	1,36 ± 0,73	1,42 ± 0,95	1,56 ± 0,71		
Cl-2	2,02 ± 2,09	1,08 ± 0,46#	1,36 ± 0,80	1,16 ± 1,01###		
PC ae C40.3, µM	1,57 ± 2,41	1,14 ± 1,61	1,25 ± 1,93	1,27 ± 1,84	<0,001	<0,001
Cl-1	1,20 ± 1,82	1,24 ± 1,78	1,21 ± 1,96	1,35 ± 1,88		
Cl-2	2,17 ± 3,10	0,97 ± 1,34#	1,32 ± 1,96	1,15 ± 1,87###		
PC ae C40.4, µM	2,38 ± 1,42	1,78 ± 0,96	2,07 ± 1,33	2,13 ± 1,13	<0,001	<0,001
Cl-1	1,95 ± 1,02	1,85 ± 1,02	2,02 ± 1,31	2,29 ± 0,98		
Cl-2	3,06 ± 1,71	1,67 ± 0,89##	2,14 ± 1,42#	1,90 ± 1,33###		
PC ae C40.5, µM	2,32 ± 1,55	2,24 ± 1,26	2,45 ± 1,81	2,46 ± 1,46*	0,74	<0,01
Cl-1	1,89 ± 1,05	2,25 ± 1,09	2,30 ± 1,48	2,68 ± 1,42		
Cl-2	2,99 ± 1,97	2,23 ± 1,53#	2,68 ± 2,31	2,15 ± 1,53###		
PC ae C40.6, µM	2,31 ± 0,68	2,11 ± 0,69	2,35 ± 1,03	2,34 ± 0,80	0,34	<0,01

Cl-1	2,11 ± 0,62	2,16 ± 0,75	2,30 ± 1,03	2,69 ± 0,80		
Cl-2	2,62 ± 0,69	2,03 ± 0,62	2,43 ± 1,08	1,83 ± 0,45###		
PC ae C42.1, µM	0,62 ± 0,67	0,47 ± 0,51	0,51 ± 0,45	0,54 ± 0,42	<0,001	<0,01
Cl-1	0,45 ± 0,27	0,52 ± 0,62	0,50 ± 0,46	0,56 ± 0,32		
Cl-2	0,89 ± 0,99	0,41 ± 0,30#	0,53 ± 0,45#	0,52 ± 0,55###		
PC ae C42.2, µM	0,62 ± 0,54	0,47 ± 0,45	0,56 ± 0,37	0,55 ± 0,35	<0,001	<0,001
Cl-1	0,46 ± 0,20	0,53 ± 0,55	0,55 ± 0,38	0,59 ± 0,22		
Cl-2	0,88 ± 0,79	0,38 ± 0,25##	0,58 ± 0,37#	0,51 ± 0,49###		
PC ae C42.3, µM	0,96 ± 0,74	0,73 ± 0,58	0,84 ± 0,60	0,85 ± 0,55	<0,001	<0,001
Cl-1	0,74 ± 0,27	0,79 ± 0,62	0,77 ± 0,45	0,89 ± 0,34		
Cl-2	1,30 ± 1,08	0,65 ± 0,53##	0,94 ± 0,80	0,80 ± 0,77###		
PC ae C42.4, µM	1,09 ± 0,45	0,76 ± 0,32	0,93 ± 0,40	0,98 ± 0,39	<0,001	<0,001
Cl-1	0,93 ± 0,29	0,82 ± 0,32	0,91 ± 0,38	1,07 ± 0,30		
Cl-2	1,35 ± 0,54	0,67 ± 0,31##	0,97 ± 0,45	0,86 ± 0,48###		
PC ae C42.5, µM	2,39 ± 0,79	2,47 ± 0,74	2,70 ± 1,26	2,79 ± 0,98**	0,31	<0,01
Cl-1	2,13 ± 0,59	2,55 ± 0,71	2,66 ± 1,27	3,10 ± 0,96		
Cl-2	2,80 ± 0,92	2,35 ± 0,77#	2,77 ± 1,29	2,33 ± 0,85###		
PC ae C44.3, µM	0,23 ± 0,30	0,18 ± 0,20	0,19 ± 0,23	0,22 ± 0,26	<0,01	<0,05
Cl-1	0,17 ± 0,10	0,19 ± 0,21	0,18 ± 0,17	0,20 ± 0,13		
Cl-2	0,34 ± 0,46	0,17 ± 0,19#	0,21 ± 0,30	0,24 ± 0,39#		
PC ae C44.4, µM	0,51 ± 0,19	0,38 ± 0,14*	0,41 ± 0,14	0,45 ± 0,16	<0,001	<0,01
Cl-1	0,46 ± 0,13	0,41 ± 0,16	0,42 ± 0,14	0,51 ± 0,13		
Cl-2	0,59 ± 0,23	0,33 ± 0,09#	0,41 ± 0,15	0,38 ± 0,18###		
PC ae C44.5, µM	2,17 ± 0,70	2,22 ± 0,79	2,50 ± 1,23	2,48 ± 0,77**	0,19	<0,01
Cl-1	2,04 ± 0,54	2,36 ± 0,84	2,52 ± 1,34	2,85 ± 0,60		
Cl-2	2,37 ± 0,88	2,02 ± 0,69	2,47 ± 1,09	1,94 ± 0,68###		
PC ae C44.6, µM	1,63 ± 0,52	1,44 ± 0,48	1,65 ± 0,72	1,70 ± 0,57*	<0,05	<0,01
Cl-1	1,49 ± 0,39	1,50 ± 0,45	1,67 ± 0,74	1,95 ± 0,47		
Cl-2	1,86 ± 0,62	1,33 ± 0,51#	1,63 ± 0,71	1,35 ± 0,52###		
SM (OH) C14:1, µM	3,73 ± 1,06	3,45 ± 1,08	3,94 ± 1,65	3,81 ± 1,22	0,23	<0,001
Cl-1	3,55 ± 1,02	3,58 ± 1,19	4,01 ± 1,73	4,48 ± 0,96		
Cl-2	4,01 ± 1,10	3,25 ± 0,90	3,81 ± 1,55	2,85 ± 0,84###		
SM (OH) C16:1, µM	1,51 ± 0,45	1,69 ± 0,53	1,77 ± 0,77	1,63 ± 0,52	0,28	<0,01
Cl-1	1,45 ± 0,45	1,76 ± 0,59	1,84 ± 0,88	1,90 ± 0,45		
Cl-2	1,61 ± 0,44	1,57 ± 0,42	1,64 ± 0,54	1,23 ± 0,34###		
SM (OH) C22:1, µM	5,77 ± 2,22	2,83 ± 1,13***	3,31 ± 1,27***	3,61 ± 2,05	<0,001	<0,01
Cl-1	5,32 ± 1,76	3,01 ± 1,18	3,29 ± 1,31	4,18 ± 2,27		
Cl-2	6,50 ± 2,71	2,55 ± 1,02#	3,33 ± 1,27	2,78 ± 1,40###		
SM (OH) C22:2, µM	5,41 ± 1,52	3,98 ± 1,15	4,63 ± 1,51	4,58 ± 1,55	<0,001	<0,001
Cl-1	4,91 ± 1,43	4,24 ± 1,17	4,72 ± 1,54	5,16 ± 1,59		

Cl-2	6,21 ± 1,36	3,58 ± 1,04##	4,47 ± 1,52#	3,74 ± 1,05###		
SM (OH) C24:1, µM	0,57 ± 0,20	0,38 ± 0,14*	0,44 ± 0,16	0,44 ± 0,18	<0,001	<0,05
Cl-1	0,54 ± 0,18	0,41 ± 0,14	0,45 ± 0,17	0,49 ± 0,18		
Cl-2	0,61 ± 0,23	0,34 ± 0,12#	0,42 ± 0,14	0,36 ± 0,15##		
SM C16:0, µM	74,81 ± 21,13	76,98 ± 21,22	85,12 ± 31,63	83,88 ± 31,41**	0,52	<0,001
Cl-1	69,65 ± 18,15	80,70 ± 22,97	87,47 ± 35,44	99,69 ± 29,88		
Cl-2	83,08 ± 23,49	71,28 ± 17,41#	81,33 ± 25,15	60,88 ± 15,63###		
SM C16:1, µM	12,58 ± 3,71	12,72 ± 3,74	14,06 ± 5,74	12,08 ± 3,65	0,3	<0,01
Cl-1	11,58 ± 3,05	12,53 ± 4,13	14,27 ± 6,24	13,81 ± 3,16		
Cl-2	14,17 ± 4,21	13,02 ± 3,17	13,72 ± 5,06	9,55 ± 2,81###		
SM C18:0, µM	11,45 ± 4,20	16,06 ± 5,57***	14,89 ± 6,96*	12,91 ± 5,76	<0,001	<0,05
Cl-1	11,29 ± 4,63	16,55 ± 6,31	15,83 ± 8,12	15,50 ± 6,18		
Cl-2	11,70 ± 3,54	15,31 ± 4,28	13,38 ± 4,37	9,15 ± 1,61##		
SM C18:1, µM	6,64 ± 2,34	8,98 ± 2,96**	9,02 ± 4,14**	7,36 ± 2,46	<0,001	<0,05
Cl-1	6,37 ± 2,22	8,95 ± 3,37	9,41 ± 4,61	8,51 ± 2,43		
Cl-2	7,07 ± 2,54	9,04 ± 2,32	8,40 ± 3,31	5,68 ± 1,30##		
SM C20:2, µM	0,28 ± 0,09	0,28 ± 0,09	0,30 ± 0,13	0,26 ± 0,12	0,48	0,11
Cl-1	0,27 ± 0,09	0,29 ± 0,11	0,31 ± 0,14	0,30 ± 0,14		
Cl-2	0,31 ± 0,09	0,26 ± 0,07	0,29 ± 0,11	0,21 ± 0,06#		
SM C24:0, µM	8,97 ± 3,27	5,25 ± 1,85**	6,02 ± 1,84**	6,45 ± 3,68	<0,001	<0,001
Cl-1	7,76 ± 2,50	5,50 ± 2,05	5,98 ± 1,89	7,46 ± 4,34		
Cl-2	10,91 ± 3,51	4,87 ± 1,48##	6,09 ± 1,84#	4,99 ± 1,73###		
SM C24:1, µM	30,33 ± 9,71	31,81 ± 10,19	34,66 ± 12,98	33,99 ± 14,22*	0,45	<0,01
Cl-1	28,53 ± 9,16	33,82 ± 11,59	35,89 ± 14,54	40,13 ± 15,22		
Cl-2	33,20 ± 10,20	28,71 ± 6,80#	32,67 ± 10,21	25,07 ± 5,49###		

[1] Values are shown as Mean ± SD. Total n 39 patients, separate in Cluster 1 (Cl-1, n=24) and Cluster 2 (Cl-2, n=15) according K-means clustering at increment 6 month vs baseline.

[2] *P* values represent changes over time (p-time) and time x group interaction (p-time x group) derived from linear mixed model after log-transformed variables and corrected for multiple testing by the false discovery rate. Taking into account the co-founders: age, sex and cluster.

#, p<0.05; ##, p<0.01 and ###, p<0.0001 represents change over time differently between Cl-1 and Cl-2

Table S7. Concentrations of metabolites in both clusters before the intervention[1]

	Cluster 1 (n=24)	Cluster 2 (n=15)	p-adjusted[2]
Isoleucine, µM	91.34 ± 25.15	111.19 ± 22.92	0,08
Leucine, µM	171.30 ± 42.45	207.47 ± 44.50	0,08
Valine, µM	290.13 ± 62.18	363.60 ± 87.34	0,06
Alanine, µM	419.42 ± 104.03	555.80 ± 120.59	<0.05
Proline, µM	199.75 ± 48.13	290.40 ± 124.15	0,07
Glycine, µM	275.25 ± 74.88	316.40 ± 107.26	0,28
Serine, µM	151.10 ± 29.94	177.93 ± 51.49	0,17
Tryptophan, µM	60.27 ± 13.65	78.35 ± 21.49	0,06
Phenylalanine, µM	80.91 ± 21.95	97.99 ± 16.91	0,06
Methionine, µM	26.67 ± 5.40	35.23 ± 8.48	0,05
Ornitine, µM	91.93 ± 30.26	119.18 ± 40.52	0,11
Arginine, µM	139.95 ± 37.84	162.07 ± 31.02	0,14
Histidine, µM	86.53 ± 12.95	115.52 ± 23.72	<0.05
Asparagine, µM	45.84 ± 9.65	53.55 ± 14.22	0,17
Aspartate, µM	22.10 ± 12.38	24.75 ± 14.73	0,66
Glutamate, µM	75.57 ± 63.92	99.25 ± 79.17	0,42
Glutamine, µM	699.63 ± 177.96	830.47 ± 290.65	0,22
Citrulline, µM	29.01 ± 7.81	38.77 ± 11.48	0,06
Tyrosine, µM	82.47 ± 21.05	108.45 ± 27.38	0,05
Threonine, µM	141.53 ± 41.30	167.50 ± 60.74	0,25
Lysine, µM	355.83 ± 63.70	423.27 ± 91.63	0,08
Creatinine, µM	76.91 ± 22.42	86.07 ± 15.84	0,23
Kynurenine, µM	2.85 ± 0.93	4.02 ± 1.25	0,05
Putrescine, µM	0.21 ± 0.09	0.24 ± 0.04	0,19
Sarcosine, µM	7.89 ± 3.83	9.86 ± 3.49	0,19
Serotonin, µM	0.60 ± 0.31	0.96 ± 0.44	0,08
Taurine, µM	103.31 ± 38.47	117.51 ± 45.65	0.40
Symmetric dimethylarginine, µM	1.23 ± 0.24	1.45 ± 0.34	0,11
Carnitine, µM	40.84 ± 11.49	53.21 ± 13.96	0,06
Acetylcarnitine, µM	7.71 ± 3.77	7.85 ± 4.71	0,95
Hydroxypropionylcarnitine, µM	0.23 ± 0.05	0.27 ± 0.06	0,13
Hexanoylcarnitine, µM	0.10 ± 0.05	0.12 ± 0.02	0,28
Glutarylcarnitine, µM	0.05 ± 0.02	0.06 ± 0.01	0,21
Pimelylcarnitine, µM	0.05 ± 0.01	0.05 ± 0.01	0,19
Octanoylcarnitine, µM	0.52 ± 0.13	0.57 ± 0.13	0,31
Decanoylcarnitine, µM	0.49 ± 0.13	0.59 ± 0.18	0,14
Decenoylcarnitine, µM	0.22 ± 0.07	0.29 ± 0.08	0,08
Decadienylcarnitine, µM	0.15 ± 0.04	0.18 ± 0.04	0,06
Tetradecenoylcarnitine, µM	0.19 ± 0.06	0.20 ± 0.06	0,56
Tetradecadienylcarnitine, µM	0.12 ± 0.04	0.13 ± 0.04	0,28
Hexadecenoylcarnitine, µM	0.11 ± 0.03	0.12 ± 0.03	0,25
Hexadecadienylcarnitine, µM	0.04 ± 0.01	0.05 ± 0.01	0,92
Hydroxyhexadecenoylcarnitine, µM	0.04 ± 0.02	0.05 ± 0.02	0,49
Octadecenoylcarnitine, µM	0.16 ± 0.06	0.19 ± 0.06	0,18
Hydroxyoctadecenoylcarnitine, µM	0.04 ± 0.01	0.05 ± 0.01	0,23
Octadecadienylcarnitine, µM	0.10 ± 0.03	0.11 ± 0.03	0,51
LysoPC a C16:0, µM	128.78 ± 34.78	188.48 ± 59.20	<0.05
LysoPC a C16:1, µM	3.53 ± 1.23	5.49 ± 2.15	0,05
LysoPC a C17:0, µM	1.81 ± 0.56	2.56 ± 1.01	0,07
LysoPC a C18:0, µM	41.17 ± 14.12	65.45 ± 25.83	0,05
LysoPC a C18:1, µM	26.88 ± 8.22	41.04 ± 12.64	<0.05
LysoPC a C18:2, µM	36.69 ± 9.85	55.22 ± 20.57	0,05
LysoPC a C20:3, µM	3.46 ± 1.36	5.83 ± 2.14	<0.05
LysoPC a C20:4, µM	9.08 ± 3.09	13.48 ± 4.27	<0.05
LysoPC a C24:0, µM	0.42 ± 0.13	0.62 ± 0.18	<0.05
LysoPC a C26:0, µM	0.40 ± 0.17	0.72 ± 0.58	0,15

LysoPC a C26:1, µM	0.30 ± 0.12	0.41 ± 0.14	0,07
LysoPC a C28:0, µM	0.36 ± 0.14	0.58 ± 0.47	0,19
LysoPC a C28:1, µM	0.43 ± 0.14	0.68 ± 0.29	0,05
PC aa C24:0, µM	0.21 ± 0.08	0.29 ± 0.18	0,27
PC aa C28:1, µM	2.71 ± 0.55	3.50 ± 0.78	<0.05
PC aa C30:0, µM	1.73 ± 0.64	2.16 ± 0.83	0,19
PC aa C32:0, µM	10.00 ± 2.62	11.80 ± 3.88	0,21
PC aa C32:1, µM	10.26 ± 5.73	14.19 ± 7.39	0,18
PC aa C32:3, µM	0.21 ± 0.06	0.27 ± 0.07	0,07
PC aa C34:1, µM	127.55 ± 47.58	157.72 ± 58.24	0,19
PC aa C34:2, µM	316.79 ± 138.46	373.67 ± 147.63	0,31
PC aa C34:3, µM	9.03 ± 4.05	12.30 ± 5.96	0,16
PC aa C34:4, µM	0.59 ± 0.31	0.89 ± 0.49	0,13
PC aa C36:0, µM	1.30 ± 0.57	2.01 ± 1.05	0,09
PC aa C36:1, µM	15.91 ± 5.91	22.17 ± 7.97	0,07
PC aa C36:2, µM	111.68 ± 39.57	148.67 ± 58.74	0,13
PC aa C36:3, µM	70.38 ± 30.83	91.37 ± 36.04	0,16
PC aa C36:4, µM	111.80 ± 36.63	133.79 ± 48.81	0,23
PC aa C36:5, µM	8.80 ± 5.50	10.86 ± 3.64	0,25
PC aa C38:0, µM	2.84 ± 1.19	3.93 ± 1.18	0,06
PC aa C38:1, µM	1.27 ± 0.65	1.87 ± 1.60	0,28
PC aa C38:3, µM	35.27 ± 14.13	51.57 ± 23.25	0,09
PC aa C38:4, µM	81.85 ± 24.68	109.19 ± 42.15	0,11
PC aa C38:5, µM	26.58 ± 9.01	32.20 ± 10.47	0,19
PC aa C38:6, µM	58.39 ± 27.80	70.25 ± 27.07	0,28
PC aa C40:1, µM	0.52 ± 0.23	0.85 ± 0.86	0,25
PC aa C40:2, µM	0.39 ± 0.49	1.03 ± 1.71	0,26
PC aa C40:3, µM	0.62 ± 0.33	1.27 ± 1.36	0,18
PC aa C40:4, µM	2.29 ± 0.82	3.56 ± 1.44	0,05
PC aa C40:5, µM	4.51 ± 1.40	6.25 ± 2.36	0,08
PC aa C40:6, µM	20.31 ± 8.90	25.25 ± 9.56	0,2
PC aa C42:0, µM	0.66 ± 0.19	0.91 ± 0.37	0,09
PC aa C42:1, µM	0.33 ± 0.11	0.53 ± 0.34	0,12
PC aa C42:2, µM	0.28 ± 0.16	0.61 ± 0.70	0,18
PC aa C42:4, µM	0.22 ± 0.18	0.52 ± 0.69	0,21
PC aa C42:5, µM	0.31 ± 0.11	0.50 ± 0.32	0,13
PC aa C42:6, µM	0.34 ± 0.10	0.49 ± 0.25	0,11
PC ae C30:0, µM	0.30 ± 0.06	0.38 ± 0.07	<0.05
PC ae C30:2, µM	0.10 ± 0.03	0.16 ± 0.11	0,15
PC ae C32:1, µM	2.00 ± 0.48	2.56 ± 0.90	0,12
PC ae C32:2, µM	0.62 ± 0.17	0.82 ± 0.20	0,05
PC ae C34:0, µM	0.46 ± 0.14	0.59 ± 0.16	0,07
PC ae C34:1, µM	4.80 ± 1.29	5.71 ± 1.56	0,16
PC ae C34:2, µM	6.61 ± 2.39	8.61 ± 4.16	0,19
PC ae C34:3, µM	4.25 ± 1.61	5.11 ± 2.48	0,31
PC ae C36:0, µM	0.88 ± 0.25	1.21 ± 0.41	0,07
PC ae C36:1, µM	2.99 ± 1.60	5.41 ± 5.21	0,19
PC ae C36:2, µM	5.30 ± 1.78	6.74 ± 1.81	0,08
PC ae C36:3, µM	3.40 ± 1.39	4.48 ± 2.38	0,21
PC ae C36:4, µM	7.86 ± 2.59	10.37 ± 5.00	0,18
PC ae C36:5, µM	6.00 ± 1.76	7.67 ± 3.48	0,19
PC ae C38:0, µM	1.46 ± 0.61	1.89 ± 0.49	0,08
PC ae C38:2, µM	1.76 ± 1.58	4.34 ± 6.94	0,26
PC ae C38:3, µM	1.84 ± 2.05	2.86 ± 2.72	0,3
PC ae C38:4, µM	5.04 ± 1.17	6.83 ± 2.24	0,06
PC ae C38:5, µM	8.52 ± 2.47	11.01 ± 4.91	0,18
PC ae C38:6, µM	3.60 ± 1.27	4.63 ± 1.76	0,15
PC ae C40:1, µM	1.11 ± 0.46	1.79 ± 1.15	0,13
PC ae C40:2, µM	1.27 ± 0.71	2.02 ± 2.09	0,28
PC ae C40:3, µM	1.20 ± 1.82	2.17 ± 3.10	0,36
PC ae C40:4, µM	1.95 ± 1.02	3.06 ± 1.71	0,11
PC ae C40:5, µM	1.89 ± 1.05	2.99 ± 1.97	0,15
PC ae C40:6, µM	2.11 ± 0.62	2.62 ± 0.69	0,09

PC ae C42:1, µM	0.45 ± 0.27	0.89 ± 0.99	0,19
PC ae C42:2, µM	0.46 ± 0.20	0.88 ± 0.79	0,15
PC ae C42:3, µM	0.74 ± 0.27	1.30 ± 1.08	0,15
PC ae C42:4, µM	0.93 ± 0.29	1.35 ± 0.54	0,06
PC ae C42:5, µM	2.13 ± 0.59	2.80 ± 0.92	0,08
PC ae C44:3, µM	0.17 ± 0.10	0.34 ± 0.46	0,25
PC ae C44:4, µM	0.46 ± 0.13	0.59 ± 0.23	0,15
PC ae C44:5, µM	2.04 ± 0.54	2.37 ± 0.88	0,28
PC ae C44:6, µM	1.49 ± 0.39	1.86 ± 0.62	0,14
SM (OH) C14:1, µM	3.55 ± 1.02	4.01 ± 1.10	0,28
SM (OH) C16:1, µM	1.45 ± 0.45	1.61 ± 0.44	0,36
SM (OH) C22:1, µM	5.32 ± 1.76	6.50 ± 2.71	0,23
SM (OH) C22:2, µM	4.91 ± 1.43	6.21 ± 1.36	0,06
SM (OH) C24:1, µM	0.54 ± 0.18	0.61 ± 0.23	0,36
SM C16:0, µM	69.65 ± 18.15	83.08 ± 23.49	0,16
SM C16:1, µM	11.58 ± 3.05	14.17 ± 4.21	0,13
SM C18:0, µM	11.29 ± 4.63	11.70 ± 3.54	0,84
SM C18:1, µM	6.37 ± 2.22	7.07 ± 2.54	0,47
SM C20:2, µM	0.27 ± 0.09	0.31 ± 0.09	0,28
SM C24:0, µM	7.76 ± 2.50	10.91 ± 3.51	0,05
SM C24:1, µM	28.53 ± 9.16	33.20 ± 10.20	0,25

[1]**Values are shown as Mean ± SD**

[2] p derived from *t* test after log-transformed variables and corrected for multiple testing by false discovery rate

PUBLICACION 6

Characterization of Metabolomic Profile Associated with Metabolic Improvement after Bariatric Surgery in Subjects with Morbid Obesity

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RESUMEN PUBLICACION 6

Objetivos: El objetivo de esta publicación fue identificar metabolitos séricos asociados a la mejora metabólica de la cirugía bariátrica en sujetos con obesidad mórbida.

Metodología: Se aplicó un flujo metabolómico no-dirigido mediante LC-ESI-TripleTOF-MS en 18 muestras de suero de sujetos con obesidad mórbida metabólicamente *enfermos* y 21 muestras de suero de sujetos con obesidad mórbida metabólicamente *sanos*, antes y uno, tres y seis meses después de una intervención de cirugía bariátrica. La evolución temporal de los pacientes se estudio mediante el análisis de factores múltiples. El *t* test pareado y el análisis lineal mixto se utilizó para identificar los metabolitos discriminantes en cada grupo de estudio.

Resultados: Después de la cirugía, el grupo de pacientes *enfermos* y *sanos* evolucionan hacia un punto metabólico común, identificado a los tres meses. Los cambios metabólicos más importantes que se producen después de la cirugía discriminativos entre los pacientes *sanos* y *enfermos* son cambios en los niveles del ácido hidroxipropiónico, ácidos grasos hidroxilados de cadena media y larga y ácidos biliares glucuronizados. Los metabolitos del grupo de los indoles y derivados y de los amino ácidos son los grupos más alterados en los pacientes *enfermos*. Se observó una correlación negativa entre los cambios de la circunferencia de la cintura y los niveles de colesterol y el metabolismo de los amino ácidos. Los cambios en el perfil de amino ácidos y los cambios en el metabolismo de los lípidos (diacilglicéridos, ácidos grasos hidroxilados y ácidos grasos de cadena media) estaban asociados con el ácido hidroxifeniláctico.

Conclusiones: Este estudio abre nuevas ideas en los cambios fisiológicos de la cirugía bariática que podrían ayudar a entender las mejoras metabólicas que se producen. La metabolómica no-dirigida ha demostrado su potencial en el estudio de la obesidad y sus perfiles metabólicos asociados.

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Article

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Characterization of Metabolomic Profile Associated with Metabolic Improvement after Bariatric Surgery in Subjects with Morbid Obesity

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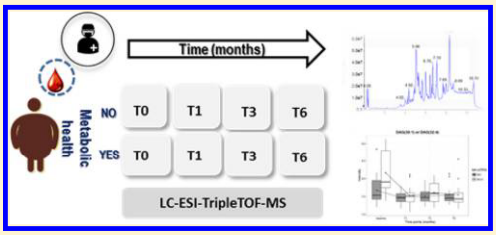
Supporting Information

ABSTRACT: The exact impact of bariatric surgery in metabolically "healthy" (MH) or "unhealthy" (MU) phenotypes for the study of the metabolic improvement is still unknown. We applied an untargeted LC-ESI-TripleTOF-MS-driven metabolomics approach in serum samples from 39 patients with morbid obesity (MH and MU) 1, 3, and 6 months after bariatric surgery. Multiple factor analysis, along with correlation and enrichment analyses, was carried out to distinguish those metabolites associated with metabolic improvement. Hydroxypropionic acids, medium-/long-chain hydroxy fatty acids, and bile acid glucuronides were the most discriminative biomarkers of response between MH and MU phenotypes. Hydroxypropionic (hydroxyphenyllactic-related) acids, amino acids, and glycerolipids were the most significant clusters of metabolites altered after bariatric surgery in MU ($p < 0.001$). After surgery, MU and MH changed toward a common metabolic state 3 months after surgery. We observed a negative correlation with changes in waist circumference and cholesterol levels with metabolites of lipid metabolism. Glycemic variables were correlated with hexoses, which, in turn, correlated with gluconic acid and amino acid metabolism. Finally, we noted that hydroxyphenyllactic acid was associated with amino acid and lipid metabolism. Microbial metabolism of amino acid and BA glucuronidation pathways may be the key points of metabolic rearrangement after surgery.



KEYWORDS: *untargeted metabolomics, microbiota metabotype, bariatric surgery, obesity, metabolically healthy, indole metabolites, mass spectrometry, hydroxyphenyllactic*

INTRODUCTION

Obesity is predictive of all-cause mortality and increases the risk of developing metabolic-related diseases such as type 2 diabetes. Obesity is associated with insulin resistance, dyslipidaemia, hypertension, and high cardiometabolic risk.[1]

Paradoxically, 10−30% of people with obesity are characterized by low cardiometabolic risk (higher insulin sensitivity, normal blood pressure, lower lipid levels of triglycerides (TGs) and low-density lipoproteins, and higher levels of high-density lipoproteins), and thus they are defined as metabolically healthy (MH).[2] Hence, apart from confounders such as age, gender, ethnicity, and lifestyle, being or not being MH depends on insulin resistance or the number of metabolic abnormalities present in the individual.[3] These subjects present a lower risk of mortality and of developing metabolic complications.[4]

Despite several attempts to describe the mechanisms of metabolic balance in MH obesity, such as those based on inflammatory response,[5] visceral adipose tissue regulation, or diet/lifestyle,[6] the door is still open to achieve the complete

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understanding of the metabolic health in obesity. Therefore, a change in the study of this paradigm is required to work out the causes of the existence of diverse metabolic profiles in obesity but especially which specific pathways are involved.

Studying the metabolic changes after the employment of a weight loss strategy such as bariatric surgery may be a tool to unveil insights into metabolic deregulation. Several studies have reported that bariatric surgery is a metabolic modifier, from the dissipation of metabolic syndrome status a few days after the intervention to the remission of type 2 diabetes.[7]

The metabolome, the set of metabolites in a biological sample, mirrors the products of the genome, transcriptome, and proteome and reflects any environmental or endogenous manifestations.[8] A comprehensive metabolic profiling of a human serum could provide an exhaustive view of the evolution of the subjects after surgery[9] and unveil the connection between obesity and its related complications.[10]

To date, few metabolomics approaches have been used to study the metabolic consequences of the current strategies for weight loss. Among these, a targeted metabolomics approach is dominant, focused on the quantification of specific groups of metabolites, for instance, amino acids,[11,12] acylcarnitines,[12] and phospholipids.[13] Thus the use of untargeted metabolomics for a non-hypothesis-driven approach may expand the assessment of the onset of metabolic improvements after a weight loss intervention underlying unknown mechanisms involved. The potential use of metabolomics in the biomedical field has increased the discovery of new biomarkers of prognosis or diagnosis of a disease.[14]

The aim of this work is to identify serum metabolites associated with metabolic improvement in subjects with morbid obesity after undergoing bariatric surgery. To this end, we adopted an untargeted metabolomics approach to analyze serum samples of subjects with MH and metabolically unhealthy (MU) obesity both before bariatric surgery intervention and 1, 3, and 6 months later through liquid chromatography coupled to triple quadrupole time-of-flight mass spectrometry (HPLC-qTOF-MS).

■ MATERIAL AND METHODS

The protocol was approved by the local Ethics and Research Committee (Hospital Universitario Virgen de la Victoria, Malaga), and all participants provided written informed consent.

Subjects and Study Design

Serum samples from 39 patients with morbid obesity (body mass index (BMI) > 40 kg/m^2) (>10 year history of obesity) who underwent bariatric surgery (n = 26 roux-en-Y gastric bypass; n = 13 sleeve gastrectomy) were collected before and 1, 3, and 6 months after surgery at the Virgen de la Victoria University Hospital and Carlos Haya Hospital (Malaga, Spain).

Subjects were stratified according to their degree of metabolic syndrome, as defined by the Adult Treatment Panel III criteria:[15] metabolically healthy (MH) subjects with ≤2 criteria (n = 21) and metabolically unhealthy (MU) subjects with ≥3 criteria (n = 18).

All of the patients were adults (between 19 and 59 years old), and they comprised 27 females and 12 males. The exclusion criteria were the intake of antidiabetic, corticosteroid, or antibiotic drugs, the presence of acute or chronic infection, a history of alcohol abuse or drug dependence, and a history of cancer. Other treatments, including anti-inflammatory, anti-hypertensive, and anticholesterolemic agents, were recorded.

Anthropometric and Biochemical Parameters

Anthropometric and biochemical parameters were measured in each period of time using standardized techniques, as previously described.[16−18] In this work, we report: (a) anthropometric markers: body weight, BMI, waist circumference, hip circumference, and waist-hip index; (b) markers of glucose regulation: glycated hemoglobin A1c (HbA1c), plasma concentrations of fasting glucose, fasting insulin, and calculated Homeostatic Model Assessment (HOMA-IR index = fasting insulin × fasting glucose/22.5 arbitrary units); (c) blood pressure markers (BP): diastolic and systolic blood pressure; (d) blood lipid markers: total cholesterol (CHOL), very low-density lipoprotein (VLDL), low-density lipoprotein (c-LDL), high-density lipoprotein cholesterol (c-HDL), and TGs; and (e) liver enzyme activities: gamma glutamyl transferase (GGT), aspartate transaminase (GOT), and alanine transaminase (GPT); and (f) C-reactive protein (CRP).

Serum Metabolomic Analysis

Sample Treatment and Data Acquisition. Fasting morning serum was stored at −80 °C until analysis. Serum samples (50 μL) were subjected to an in-plate hybrid extraction for deproteinization and phospholipid removal with solid-phase extraction, as previously described.[19] Liquid chromatography (LC) was performed on a Shimadzu Nexera X2 series HPLC system (Kyoto, Japan) using a 50 × 2.1 mm, 5 μm (Waters) Atlantis T3 reverse-phase column, with a sample injection volume of 5 μL. A linear gradient elution was performed with a binary system consisting of [A] Milli-Q water 0.1% HCOOH (v/v) and [B] methanol (v/v) at a constant flow rate of 600 μL min^{-1}. The gradient elution (v/v) of [B] used was as follows (time, min; B, %): (0, 0), (2, 0), (4.5, 85), (7, 100), (9.9, 100), (10, 0), (13, 0). The HPLC system was online coupled to a hybrid quadrupole-TOF mass spectrometer TripleTOF 6600 System (AB Sciex, Toronto, Ontario, Canada) equipped with a Turbo Spray IonDrive source operating in positive (ESI+) or negative (ESI−) ion electrospray modes. Full data acquisition was performed by scanning from 70 to 850 m/z in both ionization modes. LC−MS data were acquired in two successive batches of analysis, and the sequences of injections were randomized to avoid possible bias. Three types of quality control (QC) were included in the injection plate design to check for the analytical quality grade: QC1, Milli-Q water samples; QC2, aqueous solution of a standard metabolite mix (5 ppm final standard concentration, described in ref. 20); and QC3, reinjected selected biological samples within the same batch. The QC samples were analyzed throughout the run, every 20 injections, to provide a measurement of the stability and performance of the system and evaluate the quality of the acquired data. Information-dependent acquisition was performed in high-sensitivity and low-cycle time mode, recording masses with an ion count greater than 500 cps, not excluding former target ions, and excluding isotopes within 4 Da, with an ion tolerance of 50 ppm, a maximum of seven spectra of candidate ions per cycle, and with a dynamic background subtraction.

Standards and Reagents. Internal standard mix of glycocholic acid-(glycine 1-^{13}C) and 1-O-stearoyl-sn-glycero-3-phosphocholine (negative control), external standard mix of acetyl-d_3-L-carnitine and indole-3-acetic-2,2-d_2 acid (Sigma-Aldrich, St. Louis, MO), and QC metabolite standard mix were

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prepared as previously described.[20] 2-Hydroxy-hexanoic acid, 3-(4-hydroxyphenyl)-2-hydroxypropanoic acid, 3-(4-hydroxyphenyl)-propanoic acid, 3-hydroxy-dodecanoic acid, 3-hydroxy-octanoic acid, 3-hydroxy-tetradecanoic acid, 4-hydroxybutyric acid, acetylcarnitine, caffeine, choline, citric acid, creatine, docosapentaenoic acid (osbond acid), docosatetraenoic acid (adrenic acid), dodecanoic acid (lauric acid), gluconic acid, hexoses, indole-3-carboxaldehyde, indolelactic acid, isocitric acid, L-arginine, L-citrulline, L-glutamate, L-histidine, L-leucine, L-ornithine, L-tryptophan, L-tyrosine, octadecatrienoic acid (linolenic acid), palmitoylmonoglyceride, phe−phe, retinol, tetradecanoic acid (miristic acid), theobromine, and uric acid (Sigma) and octadecenoylcarnitine and octadecadienylcarnitine (Larodan, Solna, Sweden) were used to confirm the identity of metabolites.

Data Analysis and Metabolite Identification. Mass extraction and alignment of the peaks were performed using MarkerView 1.3.0.1 software (AB Sciex); parameters were optimized separately for positive and negative ionization modes (Table S-1). The subsequent analyses were executed in R version 3.3.2, unless otherwise noted.

Data were filtered out to remove any ion that did not appear in >20% of the samples of each group separately.[21] Data were log-transformed and Pareto-scaled for multivariate analysis.[22]

Because of the potential effect of age,[23] gender,[24] and type of surgery[25] on the metabolome, data were adjusted by these variables in all statistical analyses.

Paired *t* tests were used to identify discriminant features at each time point (6 months (T6), 3 months (T3), and 1 month (T1)) with respect to the baseline (T6−T0, T3−T0, and T1−T0) for the MH and MU groups separately. To control the false discovery rate (FDR) associated with multiple hypothesis testing, *p* values were adjusted to allow a maximum 5% probability of false-positive detection based on the Benjamini-Hochberg procedure.[26] A two-way hierarchical cluster analysis based on Pearson correlation and Ward's distance method was performed to determine clusters of mass features originating from the same metabolite and thereby reducing the queried masses (PermutMatrix 1.9.3 software). Levels of evidence in the annotation of discriminant metabolites were assigned in accordance with Metabolomics Standards Initiative criteria.[27] Putatively annotated compounds were carried out by matching mass features with mass spectral databases (Human Metabolome Database,[28] Metlin,[29] MetFrag[30]) with a mass error tolerance of ±10 mDa (assigning a level 2 of the evidence in the identification). When peak chromatographic and MS responses of the identified metabolites matched with peak chrompatographic and MS responses of commercial reference standards, level 1 of evidence in the identification was assigned.

Multivariate analyses were applied to the identified metabolites to recognize those most responsible for the changes at each time point. Initially, an exploratory analysis was performed through multiple factor analysis (MFA), taking into account each single time point (*FactoMineR* R package).[31] Afterward, a sparse partial least-squares discriminant analysis (sPLS-DA) was used to distinguish the most discriminative metabolites of MH and MU in T6−T0, T3−T0, and T1−T0 (*spls* R package).[32] The sparsity (*eta*) and number of hiding components (*K*) were chosen using a 10-fold cross-validation procedure, and the predictability of the models was calculated on a leave-one-out basis. Principal components analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) were used to visualize the separation of the groups. Finally, linear mixed models were employed to obtain the effect of the interaction time × metabolic state.

Pearson correlation analysis was carried out between log-normalized metabolite levels and anthropometric/clinical variables to evaluate the relationship between the metabolite change and the metabolic improvement for each increment of time. Only those correlations of discriminant metabolites at T3−T0, with *p* values adjusted by multiple testing by the FDR < 0.1 and correlation coefficients > |0.5|, were visualized (Cytoscape 3.5.1). MFA was also performed with clinical variables and the discriminative metabolites of MH/MU in T3−T0 to show the relation between these variables.

Enrichment analysis was performed using the bioinformatics tool ChemRich (Chemical Similarity Enrichment Analysis for Metabolomics). The ChemRich statistical approach compares chemical similarities using the Medial Subject Headings database and Tanimoto chemical similarity coefficients to cluster metabolites into nonoverlapping chemical groups. *p* values are obtained by employing the Kolmogorov−Smirnov test using the created clusters and adjusted by the FDR.[33]

■ RESULTS

Anthropometric and Clinical Parameters

The characteristics of the subjects are presented in Table S-2. MH and MU were balanced in terms of gender, age, and anthropometric parameters. At baseline, MU presented higher levels of fasting glucose, HOMA-IR, c-LDL, and CHOL (*p* value <0.05). In general, after the surgery the aforementioned variables normalize, already observed 1 month after the intervention. Table S-3 shows the evolution of anthropometric and clinical parameters in both groups after surgery. At 3 months after surgery, seven of the MU groups were still "unhealthy" and three of the MH groups had crossed the line of "unhealthy" obesity. Six months after the initial point, five patients from the MU group were still "unhealthy" and four of the MH groups had changed to being "unhealthy".

The availability of the serum samples before surgery and 1, 3, and 6 months after surgery was as follows: MH: 21, 20, 20, and 17, respectively; MU: 18, 16, 17, and 15, respectively.

Data Acquisition Quality

The analytical quality was first examined through a PCA displaying the classification of serum samples, confirming that no batches were overlapping, along with the absence of carryover in QC and the detection of no outliers in positive and negative ionization modes (Figure S-1). The quality of the hybrid extraction was checked by monitoring the internal standards in the chromatogram. LC−MS analytical stability was evaluated by controlling external standards over time. As a result, one sample in the positive ionization mode and other in negative ionization mode did not pass the analytical quality; therefore, they were removed. As shown in Table S-4, metabolite components of QC met the quality criteria proposed for the metabolomics analysis protocol: retention time shifts ≤0.05 min, mass accuracy deviations <10 mDa, and peak areas with CV < 25%.

Selection and Identification of Discriminatory Metabolites Related to Metabolic Improvement

A first selection of the most discriminative features between groups and time points was performed using univariate analyses. From the initial 3000 features obtained in each ionization mode, the only features that met the criteria (*p* <

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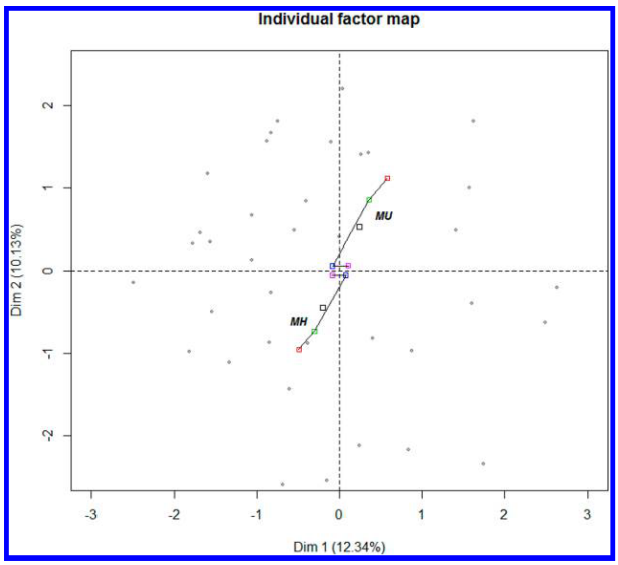

Figure 1. Projection of the individual mean (metabolically healthy (MH) and metabolically unhealthy (MU)) for each period of time onto the global analysis based on the identified metabolites in positive and negative operating modes. The scatter plot was created with the first two dimensions of the multiple factor analysis (MFA). Periods of time are represented by red, green, blue, and pink dots corresponding to the baseline (T0), 1 month, 3 months, and 6 months after the surgery, respectively, and linked by a black line with the partial positions of each point. Gray dots indicate the position of each subject in the scatter plot.

0.05) were considered for identifying metabolites. The complete list of identified metabolites and their fold changes is available in Table S-5. Metabolites lacking chemical annotation were not considered for subsequent analyses.

MFA was applied to all identified metabolites irrespective of which time period was statistically significant. The first two dimensions explained 22.47% of the total variance. Figure 1 shows the mean evolution of both groups converging to a common point over time, observed 3 months after surgery, corresponding to the origin of the MFA score plot. Global differences after the surgery are shown in Figure S-2, and the separation of MH and MU in each time point is represented in Figure S-3.

Discriminant metabolites in each increment of time, T6−T0, T3−T0, and T1−T0, were explored through sPLS-DA using the optimal parameters to obtain a minimum classification error (Table S-6). The changes between the groups of patients were mainly acute (T1) and derived from middle-term (T3) adaptations. At 6 months, no additional significant metabolites between MH and MU were found (Figure S-4). Discriminant changes in metabolite levels between MH and MU in the convergent point (T3−T0) are shown in Table 1. In brief, diglycerides (DGs) and hexoses enabled discrimination between MU and MH in each increment of time, displaying major fold changes in MU. Acute changes were characterized by modifications in fatty acids, hydroxy fatty acids, amino acids, and their microbiota-related compounds (hydroxyphenyl-propionic derivatives). Nevertheless, steroid conjugates and acylcarnitines were mainly discriminative of both groups in the time increment T3−T0. Confirmation of this common steady metabolic state postsurgery in both groups was obtained by comparing whether the levels of the discriminant metabolites in MH were statistically equal to those from MU at 3 months ($p > 0.05$). At 3 months MU group was different from MH group at baseline in terms of tyrosine, leucine, glycochenodeoxycholic acid-3 glucuronide, hydroxy-indolepropanoic acid, indole-3-carboxaldehyde, and lysophosphoethanolamines (LPE) metabolites (Table 1).

Enrichment Analysis and Correlation Analysis for Metabolic Improvement

Correlation between metabolite and clinical variables in T3−T0 showed that changes in waist circumference and cholesterol levels were negatively correlated with LPE and fatty acid metabolism. 3-(4-Hydroxyphenyl)-2-hydroxypropanoic or hydroxyphenyllactic acid was the link between amino acids and lipid metabolism. Changes in 3-(4-hydroxyphenyl)-2-hydroxypropanoic levels also positively correlated with hexoses. In addition, hexoses associated with glycaemic variables (Figure 2).

We mapped the 46 discriminative metabolites between groups in the increment T3−T0 in ChemRich to detect those chemical families that were most responsible for the metabolic changes in each group (Table 2). Indoles and derivatives, amino acids, glycerolipids, glycerophospholipids, and fatty acids were altered most in MU subjects. On the contrary, not a single chemical family reached statistical significance in the MH group.

MFA of the identified metabolites and clinical variables confirmed the separation of MH and MU in T3−T0 by the first dimension of the scatterplot (Figure 3A,C). The third dimension explained the separation between clinical and metabolic variables (Figure 3B). Correlations of the variables with the main dimensions are shown in Figure 3D,E. The first

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Table 1. Discriminant Serum Metabolites between Metabolically Healthy and Metabolically Unhealthy Subjects in the Time Increment T3−T0 (in accordance with the results of sPLS-DA[a,b])

potential marker	m/z[c]	error (mDa)	RT (min)	assignation	level[d]	discriminant[e] T1	T3	T6	MU (n = 18)[f]	MH (n = 21)[f]	MU T3 vs MH T0[g]
CHO Metabolism											
hexoses	179.0649	−8.8	0.32	$[M-H]^-$	1	√	√	√	−0.19 ± 0.15[i]	0.00 ± 0.19	
hexoses	203.0524	0.2	0.31	$[M+Na]^+$					−0.18 ± 0.19[h]	0.01 ± 0.13	
gluconic acid	195.0620	−11	0.33	$[M-H]^-$	1	√	√		−0.23 ± 0.23[i]	−0.07 ± 0.38	
citric acid	191.0189	0.8	0.79	$[M-H]^-$	1		√		0.20 ± 0.44[h]	0.39 ± 0.51[j]	
Amino Acids											
L-tryptophan	203.0819	0.7	3.58	$[M-H]^-$	1		√		−0.53 ± 0.57[i]	−0.27 ± 0.36	
L-tryptophan	205.0966	0.6	3.54	$[M+H]^+$					−0.41 ± 0.46[i]	−0.17 ± 0.36	
L-tyrosine	180.0662	0.4	0.95	$[M-H]^-$	1		√		−0.50 ± 0.46[i]	−0.27 ± 0.41[i]	i
L-arginine	173.1144	−10	0.30	$[M-H]^-$	1	√	√		−0.12 ± 0.20[i]	−0.07 ± 0.15	
L-arginine	175.1183	0.7	0.30	$[M+H]^+$					−0.10 ± 0.24	−0.02 ± 0.15	
L-citrulline	198.084	0.9	0.31	$[M+Na]^+$	1	√	√		−0.09 ± 0.24	0.06 ± 0.26	
L-glutamate	146.0581	−12.2	0.32	$[M-H]^-$	1	√	√		−0.12 ± 0.14[i]	−0.02 ± 0.12	
L-histidine	154.0707	−8.5	0.31	$[M-H]^-$	1		√		−0.10 ± 0.21	−0.09 ± 0.18	
L-leucine	130.0869	0.5	0.90	$[M-H]^-$	1		√		−0.41 ± 0.38[i]	−0.30 ± 0.36[h]	h
L-ornithine	131.0878	−5.2	0.33	$[M-H]^-$	1		√		−0.05 ± 0.23	−0.02 ± 0.22	
Dipeptides											
phenylalanylphenylalanine	313.1553	−0.6	4.06	$[M+H]^+$	1	√	√		−0.39 ± 0.88	−0.25 ± 0.78	
gamma-glutamyl-L-isoleucine	259.1120	17.9	0.31	$[M-H]^-$	2		√	√	−0.22 ± 0.54	−0.01 ± 0.25	
Acylcarnitines											
hexadecenoylcarnitine	398.3254	1.1	5.16	$[M+H]^+$	2		√		−0.08 ± 0.66	0.21 ± 0.54	
octadecadienylcarnitine (linoleyl carnitine)	424.3412	0.9	5.21	$[M+H]^+$	1		√		0.01 ± 0.67	0.15 ± 0.40	
octadecenoylcarnitine (elaidic carnitine)	426.3565	1.3	5.28	$[M+H]^+$	1		√		0.21 ± 0.59	0.35 ± 0.36[j]	
Fatty Acids											
3-hydroxy-octanoic acid	159.1017	1	4.88	$[M-H]^-$	1	√	√		−0.09 ± 0.51	0.11 ± 0.46	
3-hydroxy-dodecanoic acid	215.1647	0.6	5.39	$[M-H]^-$	1	√	√		0.15 ± 0.47	0.22 ± 0.50[i]	
hydroxy-octadecatrienoic acid (HOTE)	295.2243	2.5	5.61	$[M+H]^+$	2		√		−0.06 ± 0.30	0.00 ± 0.21	
hydroxy-octadecadienoic acid (HODE)	301.2119	2.5	6.22	$[M+Na]^+$	2		√		−0.41 ± 0.63[h]	−0.03 ± 0.48	
dodecanoic acid (lauric acid)	199.1698	0.6	5.80	$[M-H]^-$	1	√	√		−0.27 ± 0.49	−0.07 ± 0.44	
tetradecanoic acid (myristic acid)	227.2009	0.8	6.22	$[M-H]^-$	1		√		−0.25 ± 0.45	−0.05 ± 0.44	
octadecatrienoic acid (linolenic acid)	277.2157	1.6	6.26	$[M-H]^-$	1		√		−0.43 ± 0.59	−0.12 ± 0.54	
docosapentaenoic acid (osbond acid)	331.2625	0.6	6.54	$[M+H]^+$	1	√	√		0.02 ± 0.61	0.21 ± 0.48[i]	
Diglycerides											
DG(30:1) or DG(32:4)	561.4482	0.7 or 3.2	8.10	$[M+Na/H]^+$	2	√	√	√	−0.57 ± 0.67[h]	−0.15 ± 0.46	
DG(32:3) or DG(34:6)	585.4494	−0.5	8.19	$[M+Na/H]^+$	2		√		4.49 ± 2.86[i]	1.59 ± 3.12	
DG(32:2) or DG(34:5)	587.4647	−0.1	8.18	$[M+Na/H]^+$	2	√	√	√	−0.69 ± 0.65[i]	−0.27 ± 0.50	
DG(32:1) or DG(34:4)	589.4804	−0.2	8.44	$[M+Na/H]^+$	2	√	√	√	−0.45 ± 0.47[i]	−0.12 ± 0.31	
DG(34:4) or DG(36:7)	611.4647	−0.1	8.10	$[M+Na/H]^+$	2		√		−0.94 ± 0.76[i]	−0.46 ± 0.81	
DG(40:7) or DG(42:10)	689.512	−0.5	8.50	$[M+Na/H]^+$	2	√	√		−0.11 ± 0.60	0.03 ± 0.49	
Steroids/Bile Acid Derivatives											
glycochenodeoxycholic acid 3-glucuronide	606.3233	4.5	5.25	$[M-H_2O-H]^-$	2		√		0.09 ± 0.54	−0.20 ± 1.16	h
cholic acid glucuronide	583.3073	5.1	5.35	$[M-H]^-$	2		√		0.34 ± 1.73	1.14 ± 1.92[i]	
dihydroxyandrostenone sulfate	383.1528	0.6	6.01	$[M-H]^-$	2		√		0.35 ± 0.77	0.47 ± 0.63[i]	
Microbiota Derivatives											
3-(4-hydroxyphenyl)-propionic acid	165.055	0.7	4.88	$[M-H]^-$	1	√	√		0.56 ± 1.91	1.66 ± 2.49[i]	
3-(4-hydroxyphenyl)-2-hydroxypropanoic acid	181.0494	1.2	3.76	$[M-H]^-$	1	√	√		−0.49 ± 0.38[i]	−0.25 ± 0.38	

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Table 1. continued

potential marker	m/z[c]	error (mDa)	RT (min)	assignation	level[d]	discriminant[e] T1	T3	T6	MU (n = 18)[f]	MH (n = 21)[f]	MU T3 vs MH T0[g]
Microbiota Derivatives											
2-hydroxy-3-(3-indolyl) propionic acid	204.0659	0.7	4.31	$[M-H]^-$	1	√	√		−0.53 ± 0.48[i]	−0.46 ± 0.32[i]	[h]
Purine Derivatives											
uric acid	167.0299	−8.8	0.41	$[M-H]^-$	1	√	√		−0.19 ± 0.17[i]	−0.13 ± 0.13[i]	
Cofactors and Vitamins											
retinol	269.2261	0.8	6.29	$[M-H_2O+H]$	1	√	√		−0.48 ± 0.50[h]	−0.10 ± 0.23	
choline	104.1066	0.4	0.31	$[M+H]^+$	1		√		−0.25 ± 0.24[i]	−0.11 ± 0.24	
Other Metabolites											
LPE (20:3)	502.2932	0.7	6.11	$[M-H]^-$	2	√	√		−0.98 ± 0.83[i]	−0.66 ± 0.87[i]	[i]
LPE (18:3) or LPE (16:0)	476.2739	3.3	6.05	$[M+H]^+$/ $[M+Na]^+$	2	√	√		0.24 ± 0.42	0.36 ± 0.34[i]	[i]
LPE (16:1)	450.2596	3	5.82	$[M-H]^-$	2	√	√		−0.55 ± 0.78[i]	−0.17 ± 0.59	
palmitoylmonoglyceride	353.2650	1.2	6.32	$[M+Na]^+$	1	√	√	√	−0.19 ± 0.33[h]	−0.03 ± 0.36	
theobromine	181.0724	−0.4	3.57	$[M+H]^+$	1		√	√	−0.86 ± 0.72[i]	−0.51 ± 0.91	
indole-3-carboxaldehyde	144.0446	0.9	4.34	$[M-H]^-$	1		√		−0.37 ± 0.48[i]	−0.59 ± 0.98[i]	[i]

[a]Abbreviations: MU, metabolically unhealthy; MH, metabolically healthy; RT, retention time; sPLS-DA, sparse partial least-squares discriminant analysis. [b]Identified metabolites listed according to metabolic classes and with increasing m/z of the compound. [c]Data obtained by LC-ESI-qTOF-MS. [d]Level of metabolite identification according to metabolomics Standard Initiative, Sumner et al.[27] [e]√ if the compound is discriminative between MH and MU in sPLS-DA analysis in the respective increments of time: T1−T0, T3−T0, and T6−T0. [f]Values are shown as mean ± sd. Mean(log(T3) − log(T0)). p values were determined by paired t test comparing T3 and T0 of each group after adjusted by gender, age, and type of surgery and false discovery rate by Benjamini-Hochberg procedure. Total n = 39 patients, separated in metabolically healthy (MH, n = 21) and metabolically abnormal (MU, n = 18) at baseline. At the different points of time, the availability of the samples was: for MH 20, 20, and 17 and for MU 16, 17, and 15 at 1, 3, and 6 months, respectively. [g]p values were determined by independent t test comparing MH at baseline versus MU 3 months of surgery after adjusted by gender, age, and type of surgery and false discovery rate by Benjamini-Hochberg procedure. [h]$p < 0.1$. [i]$p < 0.05$. [j]$p < 0.001$.

dimension was highly correlated with glycaemia and weight parameters. These variables hardly overlapped with the acylcarnitines, and propionic acids were near CHOL and c-LDL with the DGs (Figure 3E). 3-(4-Hydroxyphenyl)-2-hydroxypropanoic acid presented high correlation with the first dimension and proximity with amino acids and DGs.

■ DISCUSSION

Through an untargeted metabolomics approach, we explored an integrative global metabolic evolution of patients with morbid obesity after a weight-loss intervention. We also identified metabolic fingerprints of changes in MH and MU after surgery. We observed that microbial-related metabolites such as hydroxy fatty acids, BAs, and hydroxypropionic acid (indole derivatives) are the most important biomarkers to reach a stable metabolic state after bariatric surgery regardless of the health status of the patient at baseline.

MFA revealed that MU and MH change after surgery toward a common metabolic state 6 months after surgery. In fact, a steady metabolic state was observed in the groups as early as 3 months after the intervention. For this reason, the postidentification analyses have been focused on this time point.

Acute Changes in Microbial and Amino-Acid-Related Metabolites

A depletion in amino acid levels was observed as early as 1 month after the intervention. This phenomenon has also been noticed with the use of different strategies for weight loss and is generally greater after a surgical intervention than after diet/lifestyle changes.[12] High levels of amino acids are a biosignature of subjects with obesity. Out of all of the amino acids, branched-chain amino acids (BCAAs) such as leucine, isoleucine, and valine and aromatic amino acids such as tryptophan, tyrosine, and histidine have been described as being correlated with glicemia disruption.[34] Indeed, BCAAs have been associated with different cardiometabolic complications, from insulin resistance to coronary artery disease.[35]

We also identified a drop in the levels of dipeptides after the intervention. Although LC−MS did not allow them to be fully characterized and a chemical standard was not available, thanks to the MS/MS spectra and databases we have proposed phenylalanine and BCAA derivative dipeptides. However, little is known about their role in obesity and cardiometabolic diseases.

The decrease in the levels of amino acids was also mirrored by changes in gut microbiota metabolism products. We found that indole metabolites were the most important chemical family of metabolites that changes in MU after bariatric surgery. (Hydroxyphenyl-)hydroxypropionic acid and 2-hydroxy-3-(3-indolyl)propanoic acid are gut microbiota-related compounds of the amino acid metabolism. In 2017, the reductive pathway of *Clostridium sporongenes*, a gut bacterium from the phylum Firmicutes, was demonstrated to produce phenylpropionic acid, hydroxyphenylpropionic acid, and indolepropionic acid through the metabolization of phenylalanine, tyrosine, and tryptophan, respectively.[36] Moreover, the degradation of phenylalanine and tyrosine by lactic acid bacteria increases the levels of hydroxyl-propionic acid metabolites.[37] The catabolism of tryptophan produces hydroxyl-indolepropionic acid. Wlodarska et al. suggested that the stimulation of indole derivative production could promote an anti-inflammatory response, improving the mucus layer.[38]

Correlation analysis suggested that these microbiota metabolites could like the metabolism of amino acids, glucose,

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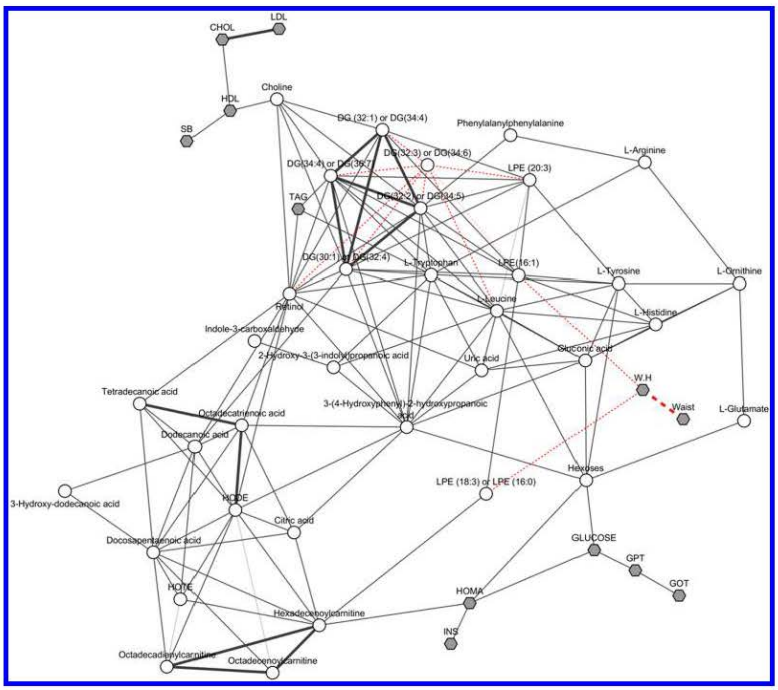

Figure 2. Visualization of Pearson correlations between metabolite−metabolite and metabolite−clinical variables (in gray) of the discriminative metabolites between MH and MU in the time increment T3−T0 (Cytoscape). Only statistical correlations after adjustment of the false discovery rate are shown ($p < 0.1$). Correlations with $r > 0.8$ are represented by thicker lines. Negative correlations are shown in red.

Table 2. Enrichment Analysis Based on Chemical Similarity Enrichment Clustering Performed by ChemRICH[a]

		MU			MH		
cluster name	cluster size	hits	altered ratio	p	hits	altered ratio	p
indole and derivatives	3	3	1	<0.001	2	0.7	0.068
amino acids, peptides, and analogues	7	4	0.6	<0.001	1	0.1	1
glycerolipids	5	4	0.8	0.001	0	0	1
glycerophospholipids	3	2	0.7	0.002	2	0.7	0.076
fatty acyls	9	3	0.3	0.051	2	0.2	0.410

[a]Significant clusters of metabolites generated by chemical similarity and ontology mapping by ChemRICH are shown. Hits mean the altered metabolites in metabolically unhealthy (MU) and metabolically healthy (MH) subjects, respectively. Chemical enrichment statistics were calculated by applying the Kolmogorov−Smirnov test on the 48 discriminant metabolites at the increment of time T3−T0. p values were adjusted using the Benjamini-Hochberg method to control the false discovery rate.

fatty acid, and DGs. Thus (hydroxyphenyl-)hydroxypropionic acid and 2-hydroxy-3-(3-indolyl)propanoic acid could be key in metabolic regulation after surgery.

On the other side, the increase in the number of BA glucuronides and steroid sulfates suggests the involvement of phase II (detoxifying) glucuronyl- and sulfate-transferase enzymes. Glucuronide conjugates represent up to 10% of the circulating pool of BAs in healthy individuals. However, conjugated BAs with amino acids are such relatively strong acids that even glucuronidation, which is the major phase II biotransformation pathway for exogenous components such as drugs, remains a minor pathway for endogenous BAs.[39]

In the same way, the adaptive changes postsurgery could be responsible for the decrease in bacteria with glucuronidase activity. In addition, the alteration of dietary patterns, intestinal motility, and mucosal hyperplasia postsurgery may contribute to modifying the composition of BAs.[40]

How BAs affect the metabolism of an individual has already been described. BAs are known to regulate the metabolism of glucose and energy through the farnesoid-X receptor (FXR) and G-protein-coupled receptor TGR5, respectively.[41] Nevertheless, the biological activity of the different forms of BAs is still unknown. Whereas glycine-conjugated BAs are as efficient as the unconjugated form in activating FXR, Trottier et al. demonstrated that glucuronide conjugates suppress the biological activity of BAs.[42]

Certain studies have attempted to link BAs with metabolic improvement. These studies were mainly targeted approaches

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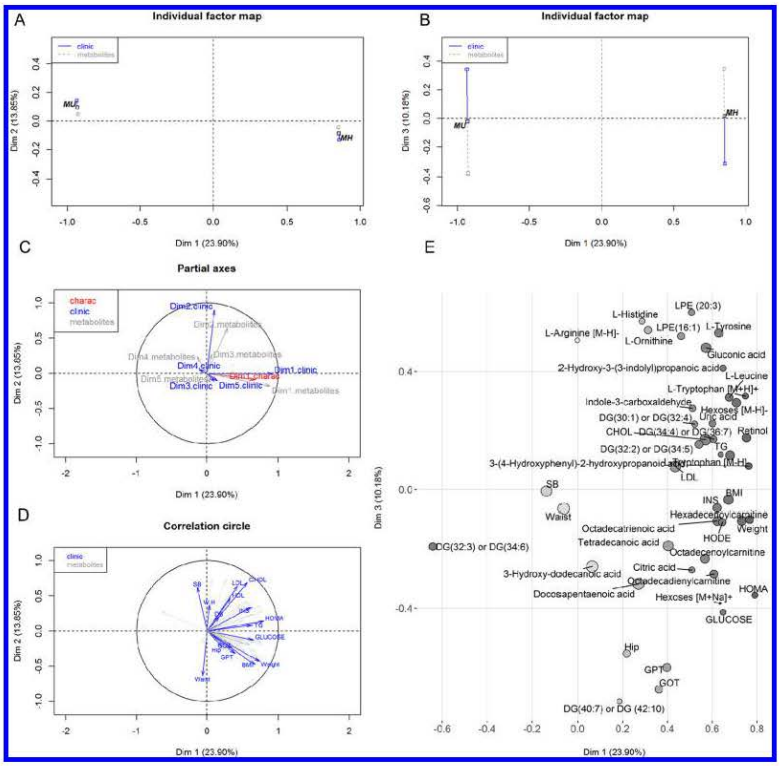

Figure 3. Multiple factor analysis (MFA) of clinical variables and identified metabolites in T3−T0. (A) Projection of the mean individual (metabolically healthy (MH) and metabolically unhealthy (MU)) onto the global analysis based on the clinical variables and identified metabolites in T3−T0 in positive and negative operating modes. The scatter plot was created with the first two dimensions of the MFA. (B) The scatter plot was created with the first and the third dimensions of the MFA. (C) Partial axes of each group of variables (clinical, metabolic) in the first two dimensions and the position of the health status in the plot. (D) Correlation circle of variables into the first and second dimensions. Only clinical variables are shown. (E) Correlation of the variables into the first (*x* axis and darkness of the bubbles), second (size of the bubbles), and third (*y* axis) dimensions of the scatter plot. Clinical, metabolic, and health status variables are represented by blue, gray, and red, respectively, except in panel E, where all of the variables are represented by gray.

based on specific BA structures[43] or the global profile of primary/secondary or conjugated/unconjugated BAs,[44] but they excluded those derived from alternative metabolisms such as glucuronidation or sulphatation. Although no significant correlations were observed in T3−T0, we suggest that BAs may be potential biomarkers in regulating the metabolic state of an individual.

Steady Changes in Lipid and Gluconeogenic Metabolism

MU subjects present greater changes in levels of lipid metabolism than subjects classified as healthy. In fact, lipid species and derivatives interfere with different metabolic pathways to increase insulin resistance. Our results indicate that there are three interconnected major groups of metabolites that reflect mechanism-induced insulin resistance: fatty acids, DGs, and acylcarnitines. It is worth noting that different studies have reported inconsistent trends in specific lipid metabolites and cardiovascular diseases. For instance, diacyl-phosphatidilcholine (PC) C32:1 has been independently associated with increased risk of T2D, and acyl-alkyl-PC C34:3 has been associated with a decreased risk.[45] Therefore, each PC, fatty acid, or derivate should be considered an entity in itself. Generalizing about the role of a pathway in the development of an unhealthy phenotype could be inaccurate, so a specific emphasis on each lipid structure is required.

A higher delivery of fatty acids to the muscle and liver is associated with lower rates of intracellular fat oxidation or the conversion of fatty acids to neutral lipids. The main causes that may contribute to this decontrol are an excess of caloric intake, defects in adipocyte metabolism, alterations in mitochondrial fatty acid oxidation, and inhibition of lipoprotein lipase activity.[46] We noted that from the first month after bariatric surgery, both DGs and fatty acids, especially long-chain fatty acids and those that are hydroxylated, decreased in MU. 3-Hydroxy-octanoic acid and 3-hydroxy-dodecanoic acid increased in MH.

In MH, an increase in the number of long-chain acylcarnitines was observed after surgery. Increases in acylcarnitines after the first month may be caused by incomplete long-chain fatty acid beta-oxidation and an altered

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tricarboxylic cycle, as already characterized in diabetes. Acetylcarnitine is a product of fatty acid beta-oxidation and glucose oxidation and can be used by the citric acid cycle for energy generation. In the same vein, we also observed the effect of the gluconeogenic metabolism through changes in citric acid, gluconic acid, and total hexose metabolites. In MU, levels of citric acid increase, presumably to compensate for the decrease in hexose levels and gluconic acid.

Translating our results into the physiopathology of obesity, these findings may indicate that different metabolic mechanisms may lead to the development of related diseases, from impaired mitochondrial oxidation, reflected by an increase in levels of fatty acids, DGs, and acylcarnitines, to acute changes in amino-acid-related microbiota metabolites and conjugated/unconjugated BA.

Strengths and Limitations

The removal of proteins and glycerophospholipids by this validated method has improved the quality of the MS signals for the identification of specific molecules. We were aware that this could provoke the lost of specific groups of metabolites. The interpretation and validation of our results is still limited to the completeness of chemical annotation in databases, the characteristics of LC−MS data, and the availability of standards in the market.

To reduce the chance of false-positive findings from the low number of patients enrolled in the study, rigorous statistical strategies were applied. For instance, a strict data mining approach was applied to reduce the dimensionality of the data, and multivariate discriminate analyses were used to select the most important metabolites to classify the groups. It is worth noting that stronger results would be obtained by using an independent validation cohort.

■ CONCLUSIONS

This study opens new insights into the physiological changes after bariatric surgery toward a distinct metabolic status in humans. Microbial indole-related products, as well as the BA glucuronidation pathway, may be the key in the regulation of metabolic health in subjects with obesity. The great potential of an untargeted metabolomics approach has to be exploited to understand the obesity and metabolic profiles.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00144.

Figure S-1. PCA score plot of samples (dots): QC1 (Milli-Q water samples), QC2 (aqueous solution of a standard), and QC3 (replicates). Figure S-2. Group score plot representation of each period of time. Figure S-3. PCA and OPLS-DA score plot representation. Figure S-4. Venn diagram of the discriminative metabolites in each increment of time (T1−T0, T3−T0, T6−T0) according to the results of the sPLS-DA. Table S-1. Data preprocessing parameters. Table S-2. Anthropometric, biochemical, and clinical characteristics of metabolically healthy (MH) and unhealthy (MU) individuals before the intervention. Table S-3. Anthropometric, biochemical, and clinical characteristics of metabolically healthy (MH) and unhealthy (MU) individuals over time. Table S-4. Variation in retention time, peak area, peak height, and detection mass in aqueous QC2 samples injected in randomized order throughout the analysis. Table S-5. Identified metabolites of changes after surgery in metabolically healthy (MH) and metabolically unhealthy (MU) subjects with obesity at each period of time. Table S-6. Sparse partial least-squares discriminant analysis parameters chosen for each increment of time and % of correct classification. (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AU, arbitrary unit; BA, bile acid; BCAA, branched-chain amino acid; BMI, body mass index; BP, blood pressure; c-HDL, high-density lipoprotein cholesterol; c-LDL, low-density lipoprotein cholesterol; CRP, C-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; DG, diglyceride; ESI−, electrospray operating in negative ionization mode; ESI+, electrospray operating in positive ionization mode; FDR, false discovery rate; FXR, farnesoid-X receptor; GGT, gamma glutamyl transferase; GOT, aspartate transaminase; GPT, alanine transaminase; HbA1c, glycated hemoglobin A1; HOMA-IR index, insulin resistance calculated by homeostatic model assessment index; LC, liquid chromatography; LPE, lysophosphoethanolamine; MFA, multiple factor analysis; MH, metabolically healthy; MS, mass spectrometry; MU, metabolically unhealthy; PC, phosphatidylcholine; PCA, principal component analysis; Phe−Phe, phenylalanine−phenylalanine; QC, quality control; qTOF, quadrupole time-of-flight; SBP, systolic blood pressure; sPLS-DA, sparse partial least-squares discriminant analysis; TG, triglyceride; VLDL, very-low-density lipoprotein.

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Material Suplementario

PUBLICACION 6

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Figure S-1. Principal component analysis (PCA) score plot of samples (dots): QC1 (Milli-Q water samples), QC2 (aqueous solution of a standard), QC3 (replicates). Panel A represents Turbo Spay IonDrive source operating in positive mode (ESI+) and panel B in negative mode (ESI-). Data were normalized and Pareto-scaled before analysis. The scatter plot was created with the first two dimensions of the PCA.

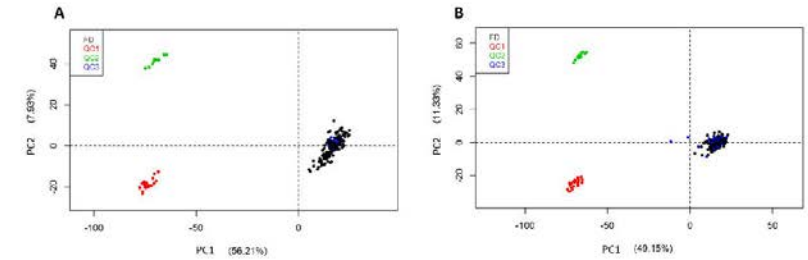

Figure S-2. Group score plot representation of each period of time: baseline (T0, red) and 1 month (green), 3 months (blue) and 6 months (pink) after the surgery. The scatter plot was created with the first two main dimensions of the PCA.

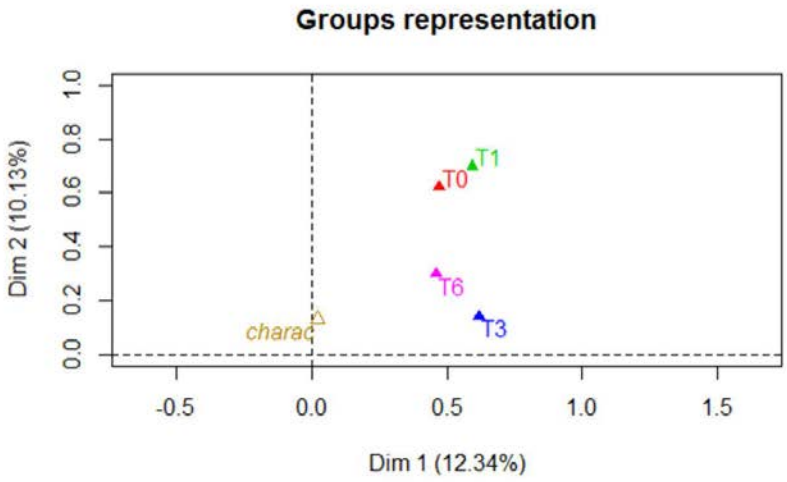

Figure S-3. PCA score plot representation of each period of time: baseline (T0, red) and 1 month (green), 3 months (blue) and 6 months (pink) after the surgery (**A**) and OPLS-DA score plot representation of metabolically healthy (MH) subjects with obesity (black points) between those subjects with metabolically unhealthy (MU) obesity before surgery (**B**) (red points), 1 month after surgery (**C**), 3 months after surgery (**D**) and 6 months after surgery (**E**).

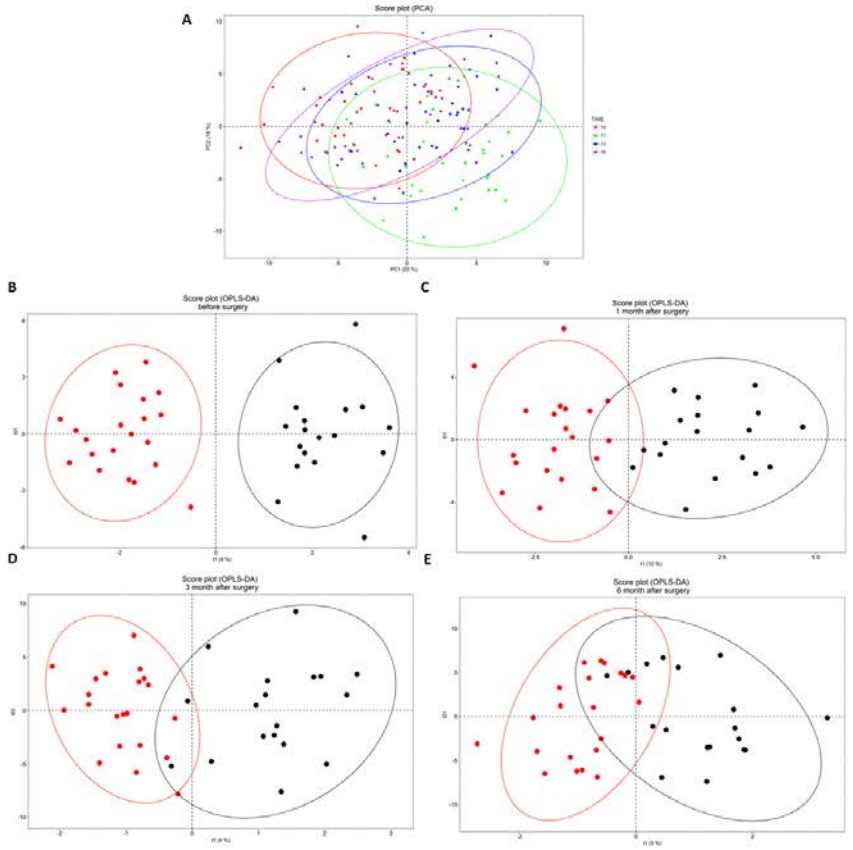

Figure S-4. Venn diagram of the discriminative metabolites in each increment of time (T1–T0, T3–T0, T6–T0) according to the results of the sPLS-DA

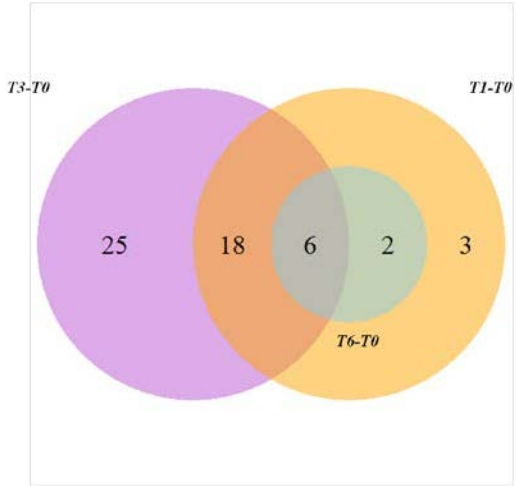

Table S-1. Data pre-processing parameters

Parameter	Description	Value ESI+	Value ESI-
Peak finding[a]	Subtraction offset (scans)	15	15
	Subtraction multiplication factor	1,5	1,5
	Minimum spectral peak width (ppm)	1	1
	Minimum retention time peak width (scans)	3	3
	Noise threshold	5	5
Peak alignment[a]	Retention time tolerance (min)	0,18	0,08
	Mass tolerance (Da)	0,02	0,02
	Intensity threshold	5	5
	Maximum number of peaks	3000	3000

[a]MarkerViewTM 1.3.0.1 software (AB Sciex, Toronto, Ontario, Canada).

Table S-2. Anthropometric, biochemical and clinical characteristics of metabolically healthy (MH) and unhealthy (MU) individuals before the intervention[1]

	MH (n=21)	**MU (n=18)**	***P***
Gender, nF(nM)	13 (8)	14 (4)	0,32
Type of surgery, RYGB (nSG)	11 (10)	15 (3)	0.50
Age, y	39.29 ± 8.87	42.56 ± 10.94	0.37
Weight, kg	135.90 ± 28.45	144.83 ± 25.88	0.27
BMI, kg/m^2	48.81 ± 9.12	52.51 ± 7.14	0.14
Waist-hip ratio	0.96 ± 0.22	0.91 ± 0.13	0.46
Waist circumference, cm	133.47 ± 21.32	137.83 ± 21.18	0.53
Hip circumference, cm	142.53 ± 21.74	151.39 ± 16.66	0.18
Fasting glucose, mmol/ml	94.29 ± 13.65	120.72 ± 40.68	<0.01
Fasting insulin, µU/ml	15.51 ± 8.49	20.04 ± 9.96	0.11
HOMA-IR, AU	3.77 ± 2.43	6.19 ± 3.84	<0.05
HBA1c, %	5.51 ± 0.23	5.73 ± 0.34	0.06
SBP, mmHg	130 ± 17	137 ± 17	0.22
DBP mmHg	79 ± 15	88 ± 12	0.06
C-LDL, mg/dl	126.55 ± 38.44	130.31 ± 34.57	<0.01
C-HDL, mg/dl	52.48 ± 17.48	46.88 ± 19.48	0.43
VLDL, mg/dl	20.33 ± 8.98	32.20 ± 11.51	0.66
TG, mg/dl	101.67 ± 44.88	161.00 ± 57.55	0.29
CHOL, mg/dl	201.14 ± 43.38	213.06 ± 44.34	<0.01
CRP, mg/l	11.75 ± 9.05	8.64 ± 7.84	0.52
GOT, U/l	21.10 ± 9.52	27.17 ± 19.09	0.27
GPT, U/l	38.10 ± 11.48	52.06 ± 31.21	0.14
GGT, U/l	28.58 ± 15.65	34.39 ± 19.56	0.30
Cardiometabolic risk factors, n (%)			
Waist circumference	16 (76.2%)	18 (100%)	
TG	2 (9.5%)	10 (55.6%)	
HDL	8 (38.1%)	11 (61.1%)	
BP	5 (23.8%)	13 (72.2%)	
Fasting glucose	1 (4.8%)	9 (50.0%)	

[1] Values are shown as Mean ± SD. *P* values were determined by independent t-test after log-transformated the variables * *P* values were determined by fisher's exact test

AU, arbitrary units; BMI, body mass index; BP, blood pressure; CHOL, total cholesterol; C- LDL, low-density lipoproteins cholesterol; C-HDL, high-density lipoproteins cholesterol; CRP, C-reactive protein; DBP, diastolic blood pressure; GOT, aspartate transaminase; GPT, alanine transaminase; GGT, gamma glutamyl transferase; HbA1c, glycated haemoglobin A1c; HOMA-IR, insulin resitance calculated by homeostatic model assessment; ; RYGP, Roux-en-Y gastric bypass SBP, systolic blood pressure; SG, sleeve gastrectomyTG, triglycerides. Cardiometabolic risk factors Adult Treatment Panel III criteria): Waist circumference >102 cm for male and >88 for female; TG over 150 mg/dl; HDL ≤40 for male and ≤50 for female; BP, SBP>130mmHg or DBP>85mmHg; fasting glucose over 110 mmol/ml.

Table S-3. Anthropometric, biochemical and clinical characteristics of metabolically healthy (MH) and unhealthy (MU) individuals over time[1]

		After surgery, mo				
	Before surgery	**1**	**3**	**6**	**p-time[2]**	**p-time x cluster[2]**
Fasting glucose, µU/mL	106.49 ± 31.89	89.17 ± 11.25	83.76 ± 12.09*	84.31 ± 11.06	<0.001	0.20
MH	94.29 ± 13.65	86.45 ± 9.69	82.62 ± 13.40	86.53 ± 10.87		
MU	120.72 ± 40.68	92.56 ± 12.41	85.18 ± 10.48	81.80 ± 11.09		
Fasting insulin, µU/mL	17.60 ± 9.36	13.62 ± 8.25*	9.94 ± 3.82*	8.95 ± 3.78***	<0.001	0.60
MH	15.51 ± 8.49	11.70 ± 6.77	9.67 ± 3.73	7.80 ± 3.51		
MU	20.04 ± 9.96	16.02 ± 9.46	10.25 ± 4.01	10.18 ± 3.77		
HOMA-IR, AU	4.89 ± 3.35	3.06 ± 1.99*	2.05 ± 0.86**	1.89 ± 0.86***	<0.001	0.50
MH	3.77 ± 2.43	2.57 ± 1.58	1.95 ± 0.87	1.72 ± 0.87		
MU	6.19 ± 3.84	3.68 ± 2.31	2.15 ± 0.87	2.07 ± 0.85		
HBA1c, %	5.59 ± 0.29	5.15 ± 0.26***	5.21 ± 0.29**	5.20 ± 0.23	<0.001	0.57
MH	5.51 ± 0.23	5.14 ± 0.28	5.24 ± 0.21	5.19 ± 0.23		
MU	5.73 ± 0.34	5.15 ± 0.24	5.17 ± 0.39	5.23 ± 0.26		
Weight, kg	140.03 ± 27.31	124.50 ± 23.49***	113.99 ± 22.31***	100.41 ± 19.53***	<0.001	0.61
MH	135.90 ± 28.45	122.35 ± 25.32	112.82 ± 24.49	98.64 ± 21.77		
MU	144.83 ± 25.88	127.31 ± 21.32	115.44 ± 19.92	102.18 ± 17.54		
BMI, kg/m2	50.52 ± 8.37	45.09 ± 7.19***	41.28 ± 6.80***	36.42 ± 6.14***	<0.001	0.64
MH	48.81 ± 9.12	43.93 ± 7.78	40.44 ± 7.30	35.54 ± 6.93		
MU	52.51 ± 7.14	46.61 ± 6.25	42.32 ± 6.20	37.31 ± 5.31		
Waist-hip ratio	0.93 ± 0.18	0.87 ± 0.11	0.90 ± 0.21	0.85 ± 0.07	0.31	0.57
MH	0.96 ± 0.22	0.89 ± 0.10	0.87 ± 0.09	0.85 ± 0.07		
MU	0.91 ± 0.13	0.85 ± 0.11	0.93 ± 0.30	0.85 ± 0.08		
Waist circumference, cm	135.71 ± 21.05	123.92 ± 16.06***	116.54 ± 13.94***	108.71 ± 13.87***	<0.001	0.85
MH	133.47 ± 21.32	123.57 ± 16.74	115.86 ± 15.79	107.00 ± 14.15		
MU	137.83 ± 21.18	124.38 ± 15.66	117.44 ± 11.50	110.53 ± 13.79		
Hip circumference, cm	147.09 ± 19.53	141.65 ± 14.82	131.84 ± 18.98	125.58 ± 13.60*	<0.001	0.59
MH	142.53 ± 21.74	139.10 ± 16.49	132.62 ± 15.33	123.29 ± 15.59		
MU	151.39 ± 16.66	145.00 ± 11.97	130.81 ± 23.44	128.00 ± 11.10		
Diastolic pressure, mmHg	84 ± 14	76 ± 13	78 ± 12	77 ± 10	<0.01	0.49
MH	79 ± 15	73 ± 12	78 ± 11	76 ± 9		
MU	88 ± 12	80 ± 14	79 ± 14	78 ± 11		
Systolic pressure, mmHg	134 ± 17	124 ± 19	127 ± 17	126 ± 17	0.12	0.85
MH	130 ± 17	122 ± 17	123 ± 12	125 ± 13		
MU	137 ± 17	128 ± 21	132 ± 20	128 ± 20		

C-LDL, mg/dL	128.23 ± 36.32	101.50 ± 29.79**	106.35 ± 29.90*	106.07 ± 36.70	<0.001	0.70
MH	126.55 ± 38.44	100.49 ± 31.47	105.51 ± 28.13	106.78 ± 34.55		
MU	130.31 ± 34.57	102.86 ± 28.47	107.44 ± 32.98	105.14 ± 40.75		
C-HDL, mg/dL	49.97 ± 18.36	38.34 ± 10.16***	44.11 ± 10.89	46.44 ± 13.26	<0.001	0.69
MH	52.48 ± 17.48	40.37 ± 11.29	48.24 ± 12.00	50.24 ± 15.49		
MU	46.88 ± 19.48	35.94 ± 8.35	39.00 ± 6.65	42.13 ± 8.81		
VLDL, mg/dL	25.81 ± 11.73	23.90 ± 7.77	20.50 ± 8.05	18.88 ± 8.30	<0.01	0.49
MH	20.33 ± 8.98	20.52 ± 6.09	19.25 ± 8.92	18.62 ± 9.73		
MU	32.20 ± 11.51	27.96 ± 7.86	22.09 ± 6.87	19.16 ± 6.90		
TG, mg/dL	129.05 ± 58.65	121.89 ± 38.22	109.82 ± 38.63	95.56 ± 38.43	<0.001	0.23
MH	101.67 ± 44.88	105.95 ± 31.28	102.57 ± 43.00	90.94 ± 41.26		
MU	161.00 ± 57.55	141.81 ± 37.50	118.76 ± 31.40	100.80 ± 35.63		
CHOL, mg/dL	206.64 ± 43.66	163.75 ± 36.92***	170.08 ± 37.09**	169.72 ± 45.16	<0.001	0.56
MH	201.14 ± 43.38	161.85 ± 39.19	173.52 ± 34.92	175.94 ± 43.50		
MU	213.06 ± 44.34	166.13 ± 34.99	165.82 ± 40.27	162.67 ± 47.47		
CRP, mg/l	10.13 ± 8.41	6.69 ± 5.05*	5.55 ± 5.46	2.55 ± 2.79**	<0.001	0.53
MH	11.75 ± 9.05	7.46 ± 5.12	8.59 ± 6.66	4.14 ± 3.80		
MU	8.64 ± 7.84	6.06 ± 5.12	3.26 ± 2.96	1.48 ± 1.08		
Leptin, mg/ml	78.33 ± 35.38	41.15 ± 26.40***	32.84 ± 16.28***	23.83 ± 10.28***	<0.001	0.60
MH	77.61 ± 35.50	39.86 ± 34.79	32.74 ± 15.48	23.89 ± 9.18		
MU	78.85 ± 36.53	42.22 ± 18.38	32.90 ± 17.48	23.79 ± 11.35		
Adiponectin, mcg/ml	7.70 ± 4.11	9.66 ± 4.16*	10.98 ± 7.16*	12.76 ± 7.71**	<0.001	0.79
MH	7.82 ± 4.44	9.56 ± 4.03	11.40 ± 4.85	12.46 ± 6.19		
MU	7.61 ± 3.99	9.75 ± 4.47	10.68 ± 8.69	13.01 ± 9.07		
GOT, IU/L	23.90 ± 14.84	34.23 ± 17.18**	27.61 ± 18.79	21.47 ± 9.32	<0.001	0.60
MH	21.10 ± 9.52	31.21 ± 15.02	28.43 ± 22.56	22.12 ± 11.14		
MU	27.17 ± 19.09	37.81 ± 19.32	26.59 ± 13.35	20.73 ± 7.03		
GPT, U/L	44.54 ± 23.56	61.60 ± 28.91**	45.95 ± 25.98	36.22 ± 13.44	<0.001	0.96
MH	38.10 ± 11.48	57.05 ± 28.69	43.38 ± 29.54	34.47 ± 15.82		
MU	52.06 ± 31.21	67.00 ± 29.15	49.12 ± 21.23	38.20 ± 10.30		
GGT, U/L	31.41 ± 17.66	32.31 ± 15.44	30.89 ± 55.01	19.03 ± 9.89*	<0.001	0.91
MH	28.58 ± 15.65	30.58 ± 15.51	35.05 ± 72.10	17.53 ± 6.58		
MU	34.39 ± 19.56	34.38 ± 15.59	25.76 ± 21.23	20.86 ± 12.88		
Uric, mmol/L	5.50 ± 1.25	6.53 ± 2.52	5.02 ± 1.23	4.46 ± 1.16	<0.001	0.44
MH	5.44 ± 1.46	6.14 ± 2.93	5.05 ± 1.34	4.48 ± 1.17		
MU	5.57 ± 0.97	7.01 ± 1.86	4.98 ± 1.11	4.44 ± 1.18		
Creatinine, mmol/L	0.79 ± 0.15	0.77 ± 0.20	0.67 ± 0.17**	0.72 ± 0.15	<0.001	0.71
MH	0.78 ± 0.12	0.77 ± 0.17	0.66 ± 0.17	0.74 ± 0.16		
MU	0.81 ± 0.18	0.77 ± 0.24	0.68 ± 0.17	0.69 ± 0.13		
Urea, mg/dL	31.38 ± 9.79	21.97 ± 8.09**	23.61 ± 8.28*	25.17 ± 7.96	<0.001	0.69

MH	32.05 ± 8.58	23.95 ± 8.43	25.13 ± 8.62	26.73 ± 9.48
MU	30.61 ± 11.25	19.50 ± 7.14	21.82 ± 7.72	23.50 ± 5.83

[1] Values are shown as Mean ± SD. Total n 39 patients, separate in metabollicaly health (MH, n=21) and metabollicaly abnormal (MU, n=18).

[2] *P* values represent changes over time (p-time) and time x group interaction (p-time x group) derived from linear mixed model after log-transformate the variables and corrected for multiple testing by false discovery rate. Taking into acount the co-founders: age, gender and type of surgery

*, ** ,*** represents $p<0.05$, $p<0.01$ and $p<0.0001$ respectivetly at 1 month, 3 months or 6 months after surgery vs baseline estimated in linear mixed-effects models, corrected for multiple testing by the false discovery rate

#, $p<0.05$; ##, $p<0.01$ and ###, $p<0.0001$ represents change over time differently between MH and MU

AU, arbitrary units; BMI, body mass index; CHOL, total cholesterol; C- LDL, low-density lipoproteins cholesterol; C-HDL, high-density lipoproteins cholesterol; CRP, c-reactive protein; GOT, Aspartate transaminase; GPT, Alanine transaminase; GGT, Gamma glutamyl transferase; HbA1c, glycated haemoglobin A1c ; HOMA-IR, insulin resitance calculated by homeostatic model assessment; TG, trilglicerides

Table S4. Variation in retention time, peak area, peak height and detection mass in aqueous QC2 samples injected in randomized order throughout the analysis

Analyte Name	Detection mass (m/z)			Retention Time (min)		Intensity (peak area)		
	Exact Mass (theoretical)	Mean (Markerview)	Error (mDa)	Mean	Shift (max - min)	Mean	SD	CV (%)
Quality controls								
Negative mode								
Gallic acid	169,0142	169,0132	1,04	1,50	(1.52 - 1.50)	39882,66667	2847,02	7,14
α-hydroxyisobutyric acid (2-hydroxybutyric acid)	103,0401	103,0397	0,36	1,15	(1.18 - 1.16)	5514,59	253,23	4,59
L-Phenylalanine	164,0717	164,0713	0,39	2,26	(2.28 - 2.26)	13780,25	692,57	5,03
L-Tryptophan	203,0826	203,0823	0,29	3,59	(3.64 - 3.59)	33103,92	2666,16	8,05
4-Hydroxyhippuric acid	194,0459	194,0451	0,77	3,54	(3.60 - 3.54)	29072,42	2232,44	7,68
Syringic acid	197,0455	197,0450	0,53	4,08	(4.12 - 4.07)	27863,42	2126,17	7,63
Epicatechin	289,0718	289,0708	0,95	3,98	(4.03 - 3.99)	153559,17	12582,69	8,19
Naringenin	271,0612	271,0597	1,48	4,74	(4.76 - 4.73)	293984,17	30295,45	10,31
Glycochenodeoxycholic acid	448,3068	448,3037	3,12	5,50	(5.53 - 5.50)	178916,67	15212,55	8,50
Palmitic acid	255,2329	255,2322	0,73	6,65	(6.71 - 6.64)	3702,97	495,82	13,39
Dodecanedioic acid	229,1445	229,1437	0,82	5,10	(5.12 - 5.09)	159406,67	17509,57	10,98
Stearic acid	283,2642	283,2635	0,73	7,09	(7.15 - 7.07)	2889,46	356,72	12,35
Positive mode								
L-Carnitine hydrochloride	162,1125	162,1128	-0,34	0,29	(0.30 - 0.29)	77338,58	12374,62	16,00
L-Citrulline	176,1030	176,1029	0,06	0,32	(0.32 - 0.31)	7122,73	943,87	13,25
Acetylcholine (chloride)	146,1175	146,1174	0,14	0,37	(0.38 - 0.36)	35469,17	6580,05	18,55
Acetyl-L-carnitine	204,1230	204,1231	-0,08	0,51	(0.51 - 0.40)	91533,00	12080,36	13,20
L-Valine	118,0862	118,0852	1,04	0,32	(0.45 - 0.42)	1959,13	263,14	13,43
L-Leucine	132,1019	132,1016	0,29	0,92	(1.00 - 0.91)	12674,17	1616,86	12,76
L-Isoleucine	132,1019	132,1016	0,29	0,92	(1.00 - 0.91)	12674,17	1616,86	12,76
L-Phenylalanine	166,0862	166,0861	0,14	2,23	(2.41 - 2.24)	29629,67	3888,47	13,12
L-Tryptophan	205,0971	205,0969	0,24	3,54	(3.56 - 3.54)	30551,92	3731,70	12,21
4-Hydroxyhippuric acid	196,0604	196,0600	0,42	3,48	(3.50 - 3.47)	3733,21	526,08	14,09
Epicatechin	291,0863	291,0863	0,00	3,94	(3.97 - 3.94)	39298,92	6040,95	15,37
Naringenin	273,0761	273,0761	0,00	4,70	(4.72 - 4.70)	83740,25	10098,48	12,06
Glycochenodeoxycholic acid (-H2O)	432,3108	432,3094	1,41	5,46	(5.49 - 5.45)	10071,03	2353,38	23,37
Internal and external standards								
Negative mode								
Glycocholic acid 13C	465,31039	465,30290	7,49	5,33	(5.35-5.30)	161703980,77	549527055,52	29,51

Indole-3-acetic-2,2-d_2 acid	176,07337	176,068	5,37	4.40	(4.70-4.0)	7180,59	647,01	9,01
Positive mode								
Glycocholic acid 13C	467,32495	467,31900	5,95	5,28	(5.31-5.28)	2290,59	638,6	27,88
Acetyl-d3-L-carnitine hydrochloride	207,14901	207,1429	6,11	0,39	(0.41-0.39)	56374,25	14062,74	24,95

Table S-5. Identified metabolites of changes after surgery in metabolically healthy (MH) and metabolically unhealthy (MU) subjects with obesity at each period of time[a)]

Potential marker	m/z [b)]	Δm/z [mDa]	rt [min]	Assignation	[e)]	1 month vs baseline [d)]		3 month vs baseline [d)]		6 month vs baseline [d)]		time [e)]	time x status [e)]	Adducts
						MH	MU	MH	MU	MH	MU			
CHO metabolism														
Hexoses	203,0524	0,2	0,31	[M+Na]+	1	-0.04 ± 0.16	**-0.12 ± 0.14**	0.01 ± 0.13	**-0.18 ± 0.19***	0.01 ± 0.13	**-0.26 ± 0.17****	<0.001	<0.05	204.06 13C [M+Na]+, 383.12 2 [M+Na]+
	179,0649	-8,8	0,32	[M-H]-		-0.07 ± 0.16	**-0.14 ± 0.12**	0 ± 0.19	**-0.19 ± 0.15**	0 ± 0.23	**-0.17 ± 0.17**	<0.001	0.46	215.05 [M+Cl]-, 216.05 13C[M+Cl]-
Gluconic acid	195,062	-11	0,33	[M-H]-	1	-0.12 ± 0.27	**-0.27 ± 0.25**	-0.07 ± 0.38	**-0.23 ± 0.23**	-0.09 ± 0.32	-0.18 ± 0.23	<0.001	0.73	
Citric acid	191,0189	0,8	0,79	[M-H]-	1	**0.57 ± 0.54**	**0.49 ± 0.39**	0.39 ± 0.51	0.2 ± 0.44	0.09 ± 0.42	0.21 ± 0.46	<0.001	0.72	
Isocitric acid	191,034	-14,3	0,41	[M-H]-	1	**0.42 ± 0.49**	**0.42 ± 0.44**	0.26 ± 0.45	0.26 ± 0.37	0.04 ± 0.37	0.17 ± 0.48	<0.001	0.98	192.03 13C[M-H]-, 173.02[M-H2O-H]-
Amino acids														
L-Tryptophan	205,0966	0,6	3,54	[M+H]+	1	**-0.36 ± 0.37**	**-0.43 ± 0.44**	-0.17 ± 0.36	-0.41 ± 0.46	-0.13 ± 0.25	-0.52 ± 0.6	<0.001	0.54	
	203,0819	0,7	3,58	[M-H]-		**-0.36 ± 0.45**	**-0.5 ± 0.4**	-0.27 ± 0.36	-0.53 ± 0.57	-0.32 ± 0.48	-0.57 ± 0.7	<0.001	0.73	429.15 [2M+Na-2H]-, 272.07 13C[M-H+CHOONa]-, 271.07 [M-H+CHOONa]-, 225.06 [M+Na-2H]-, 339.05 [M-H+2CHOONa]-, 204.08 13C[M-H]-
L-Tyrosine	180,0662	0,4	0,95	[M-H]-	1	**-0.46 ±0.39**	**-0.52 ± 0.40**	**-0.27 ± 0.41**	-0.50 ± 0.46	-0.32 ± 0.39	**-0.36 ± 0.44**	<0.001	0,71	
L-Arginine	175,1183	0,7	0,3	[M+H]+	1	**-0.17 ± 0.19**	**-0.26 ± 0.19**	-0.02 ± 0.15	-0.1 ± 0.24	0.03 ± 0.22	-0.12 ± 0.25	<0.001	0.62	
	173,1144	-10	0,3	[M-H]-	1	**-0.09 ± 0.13**	**-0.22 ± 0.18**	-0.07 ± 0.15	-0.12 ± 0.2	0.01 ± 0.19	-0.07 ± 0.19	<0.001	1	
L-Citrulline	198,084	0,9	0,31	[M+Na]+	1	-0.23 ± 0.28	**-0.35 ± 0.29**	0.06 ± 0.26	-0.09 ± 0.24	0.06 ± 0.31	0.06 ± 0.31	<0.001	0.49	
L-Glutamate	146,0581	-12,2	0,32	[M-H]-	1	0.02 ± 0.19	-0.15 ± 0.22	-0.02 ± 0.12	-0.12 ± 0.14	-0.14 ± 0.42	**-0.25 ± 0.2**	<0.01	0.7	

L-Histidine	154,0707	-8,5	0,31	[M-H]-	1	**-0.19 ± 0.19**	**-0.2 ± 0.21**	-0.09 ± 0.18	-0.1 ± 0.21	-0.03 ± 0.18	-0.04 ± 0.19	<0.001	0.98	
L-Leucine	130,0869	0,5	0,9	[M-H]-	1	**-0.38 ± 0.32**	**-0.35 ± 0.34**	-0.3 ± 0.36	**-0.41 ± 0.38**	-0.37 ± 0.29	-0.33 ± 0.48	<0.001	0.96	198.07 [M+CHOONa-H]-
L-Ornithine	131,0878	-5,2	0,33	[M-H]-	1	-0.18 ± 0.25	**-0.26 ± 0.24**	-0.02 ± 0.22	-0.05 ± 0.23	0.01 ± 0.21	0.09 ± 0.23	<0.001	0,7	
Dipeptides														
Phenylalanylphenylalanine	313,1553	-0,6	4,06	[M+H]+	1	-0.21 ± 0.73	**-0.66 ± 0.66**	-0.25 ± 0.78	-0.39 ± 0.88	-0.28 ± 0.8	-0.54 ± 0.81	<0.05	0.61	314.16 13C[M+H]+
	311,1399	0,2	4,11	[M-H]-		-0.2 ± 0.62	**-0.63 ± 0.67**	-0.25 ± 0.76	-0.39 ± 0.91	-0.32 ± 0.71	-0.56 ± 0.78	<0.05	0.77	312.14 13C[M-H]-, 645.27 [2M+Na-2H]-, 380.13 13C[M-H+CHOONa]-, 379.13 [M-H+CHOONa]-
Gamma-glutamyl-L-isoleucine	259,112	17,9	0,31	[M-H]-	2	-0.18 ± 0.38	-0.22 ± 0.46	-0.01 ± 0.25	-0.22 ± 0.54	-0.09 ± 0.41	**-0.36 ± 0.34**	<0.01	0.7	
Acylcarnitines														
Acetylcarnitine	204,1226	0,4	0,39	[M+H]+	1	0.44 ± 0.85	**0.95 ± 0.84**	0.24 ± 0.53	0.17 ± 0.76	-0.06 ± 0.54	0.07 ± 0.54	<0.001	0.35	205.13 13C[M+H]+
Hydroxy-tetradecenoylcarnitine	386,2896	0,48	4,89	[M+H]+	2	**0.58 ± 0.63**	0.31 ± 1.13	0.56 ± 0.72	0.37 ± 1.22	0.16 ± 0.48	0.35 ± 0.59	<0.01	0.54	
Hexadecenoylcarnitine	398,3254	1,1	5,16	[M+H]+	2	0.46 ± 0.62	**0.48 ± 0.49**	0.21 ± 0.54	-0.08 ± 0.66	-0.08 ± 0.56	-0.19 ± 0.47	<0.001	0.48	
Octadecadienylcarnitine (linoleyl carnitine)	424,3412	0,9	5,21	[M+H]+	1	0.43 ± 0.54	**0.42 ± 0.44**	0.15 ± 0.4	0.01 ± 0.67	-0.04 ± 0.44	-0.04 ± 0.4	<0.001	0.71	
Octadecenoylcarnitine (elaidic carnitine)	426,3565	1,3	5,28	[M+H]+	1	**0.56 ± 0.49**	**0.54 ± 0.39**	0.35 ± 0.36	0.21 ± 0.59	0.14 ± 0.46	0.02 ± 0.3	<0.001	0.61	427.36 13C[M+H]+, 448.34 [M+Na]+
Fatty acids														
Hydroxy-butyric acid	103,0408	-0,7	1,08	[M-H]-	1	**2.82 ± 3.26**	**3.13 ± 2.89**	1.67 ± 2.81	**1.98 ± 2.02**	0.53 ± 3.2	1.17 ± 2.46	<0.001	0.98	
Hydroxy-hexanoic acid	131,0704	1	4,16	[M-H]-	1	-0.22 ± 0.36	-0.07 ± 0.61	-0.25 ± 0.46	-0.27 ± 0.88	-0.34 ± 0.5	**-0.64 ± 0.59**	<0.01	0.77	
3-Hydroxy-octanoic acid	159,1017	1	4,88	[M-H]-	1	-0.09 ± 0.47	**-0.33 ± 0.31**	0.11 ± 0.46	-0.09 ± 0.51	-0.11 ± 0.36	-0.16 ± 0.36	0.13	0.77	
3-Hydroxy-dodecanoic acid	215,1647	0,6	5,39	[M-H]-	1	**0.33 ± 0.42**	**0.45 ± 0.4**	0.22 ± 0.5	0.15 ± 0.47	-0.11 ± 0.4	-0.05 ± 0.55	<0.001	0.82	283.15 [M-H+CHOONa]-
Hydroxy-tetradecanoic acid	243,1969	-0,3	5,69	[M-H]-	1	0.2 ± 0.33	**0.44 ± 0.44**	0.13 ± 0.41	0.07 ± 0.49	-0.22 ± 0.36	-0.07 ± 0.45	<0.001	0.7	
Hydroxy-octadecatrienoic acid (HOTE)	295,2243	2,5	5,61	[M+H]+	2	0.07 ± 0.24	0.02 ± 0.25	0 ± 0.21	-0.06 ± 0.3	-0.14 ± 0.18	**-0.24 ± 0.17**	<0.001	0.64	
Hydroxy-octadecadienoic acid (HODE)	301,2119	2,5	6,22	[M+Na]+	2	0.21 ± 0.58	0.28 ± 0.56	-0.03 ± 0.48	-0.41 ± 0.63	-0.66 ± 0.6	**-0.66 ± 0.49**	<0.001	0.29	302.22 13C [M+Na]+, 323.19 [M+2Na-H]+, 279.23 [M+H-H2O]+
Dihydroxy-octadecenoic acid (DiHOME)	313,2368	1,6	5,43	[M-H]-	2	**-0.37 ± 0.31**	-0.21 ± 0.41	-0.26 ± 0.33	-0.27 ± 0.46	-0.23 ± 0.28	-0.32 ± 0.31	<0.001	0.77	
Dodecanoic acid (lauric acid)	199,1698	0,6	5,8	[M-H]-	1	-0.04 ± 0.34	0.06 ± 0.43	-0.07 ± 0.44	-0.27 ± 0.49	-0.3 ± 0.28	**-0.38 ± 0.35**	<0.001	0.7	267.16 [M-H+CHOONa]-
Tetradecanoic acid (myristic	227,2009	0,8	6,22	[M-H]-	1	0.13 ± 0.53	0.13 ± 0.52	-0.05 ± 0.44	-0.25 ± 0.45	-0.33 ± 0.45	**-0.38 ± 0.32**	<0.001	0.82	228.2 13C[M+Na]+

acid)													
Octadecatrienoic acid (linolenic acid)	277,2157	1,6	6,26	[M-H]-	1	0.14 ± 0.63	0.22 ± 0.61	-0.12 ± 0.54	-0.43 ± 0.59	-0.68 ± 0.58	**-0.67 ± 0.49**	<0.001	0.71	278.22 13C [M-H]-, 346.21 13C [M-H+CHOONa]-, 345.2 [M-H+CHOONa]-
Docosatetraenoic acid (adrenic acid)	355,2611	-0,4	6,71	[M+Na]+	1	**0.52 ± 0.6**	**0.66 ± 0.53**	0.31 ± 0.54	0.25 ± 0.64	-0.01 ± 0.69	0.13 ± 0.53	<0.001	0.61	377.24 [M+2Na-H]+
	331,2621	2,2	6,8	[M-H]-		0.53 ± 0.87	**0.73 ± 0.78**	0.27 ± 0.82	0.24 ± 0.85	-0.06 ± 0.81	0.15 ± 0.71	<0.001	0.96	332.26 13C [M-H]-, 400.25 13C[M-H+CHOONa]-, 399.25 [M-H+CHOONa]-
Docosapentaenoic acid (osbond acid)	331,2625	0,6	6,54	[M+H]+	1	0.39 ± 0.62	**0.58 ± 0.57**	0.21 ± 0.48	0.02 ± 0.61	-0.26 ± 0.57	-0.1 ± 0.63	<0.001	0.45	354.25 13C[M+Na/H]+, 353.25 [M+Na]+
	329,247	1,6	6,58	[M-H]-		0.41 ± 0.78	**0.57 ± 0.65**	0.18 ± 0.67	0.08 ± 0.72	-0.68 ± 1.94	-0.62 ± 1.76	<0.001	0.98	397.23 [M-H+CHOONa]-
Diglycerids														
DG(40:7) or DG (42:10)	689,512	-0,5	8,5	[M+Na/H]+	2	0.22 ± 0.73	**0.67 ± 0.6**	0.03 ± 0.49	-0.11 ± 0.6	-0.25 ± 0.57	-0.44 ± 0.92	<0.001	0.2	690.52 13C[M+Na/H]+
DG(34:4) or DG(36:7)	611,4647	-0,1	8,1	[M+Na/H]+	2	-0.55 ± 0.89	-0.54 ± 0.74	-0.46 ± 0.81	**-0.94 ± 0.76**	-0.52 ± 0.82	**-1.12 ± 0.81**	<0.001	0.54	612.47 13C[M+Na/H]+
DG(32:3) or DG(34:6)	585,4494	-0,5	8,19	[M+Na/H]+	2	2.61 ± 3.22	2.49 ± 3.19	1.59 ± 3.12	**4.49 ± 2.86**	2.21 ± 3.31	**4.11 ± 2.43**	<0.001	0.2	
DG(32:2) or DG(34:5)	587,4647	-0,1	8,18	[M+Na/H]+	2	-0.36 ± 0.5	**-0.67 ± 0.58**	-0.27 ± 0.5	**-0.69 ± 0.65**	-0.25 ± 0.44	**-0.75 ± 0.64**	<0.001	0.31	588.47 13C[M+Na/H]+
DG(30:1) or DG(32:4)	561,4482	0.7 or 3.2	8,1	[M+Na/H]+	2	-0.21 ± 0.44	**-0.67 ± 0.56**	-0.15 ± 0.46	-0.57 ± 0.67	-0.07 ± 0.33	**-0.65 ± 0.6**	<0.001	0.05	562.45 13C[M+Na/H]+
DG (38:4) or DG(40:7)	667,5277	0,61	8,79	[M+Na/H]+	2	-0.68 ± 0.88	-0.58 ± 0.8	-0.76 ± 1.55	**-0.58 ± 0.55**	-0.39 ± 0.89	-0.88 ± 0.92	<0.001	0.54	
DG (32:1) or DG(34:4)	589,4804	-0,2	8,44	[M+Na/H]+	2	-0.17 ± 0.28	**-0.52 ± 0.39**	-0.12 ± 0.31	-0.45 ± 0.47	-0.13 ± 0.24	**-0.53 ± 0.42**	<0.001	<0.05	590.48 13C[M+Na/H]+
Steroids/Bile acids derivates														
Glycochenodeoxycholic acid 3-glucuronide	606,3233	4,5	5,25	[M-H_2O-H]-	2	**0.33 ± 1.2**	**0.69 ± 0.67**	-0.2 ± 1.16	0.09 ± 0.54	-0.16 ± 0.98	0.11 ± 0.63	<0.05	0.96	628.31 [M+Na-H2O-2H]-
Cholic acid glucuronide	583,3073	5,1	5,35	[M-H]-	2	1.28 ± 1.77	**1.48 ± 1.45**	1.14 ± 1.92	0.34 ± 1.73	1.34 ± 1.45	0.85 ± 1.22	<0.001	0.77	566.3 13C [M-H2O-H]-, 565.3 [M-H2O-H]-
Deoxycholic acid	415,2814	0,5	6,23	[M+Na]+	2	-0.11 ± 0.41	-0.03 ± 0.36	-0.07 ± 0.19	-0.29 ± 0.33	-0.19 ± 0.27	**-0.31 ± 0.24**	<0.001	0.31	
Dihydroxyandrostenone sulfate	383,1528	0,6	6,01	[M-H]-	2	0.56 ± 0.77	0.69 ± 0.87	0.47 ± 0.63	0.35 ± 0.77	0.26 ± 0.78	**0.55 ± 0.5**	<0.001	0.87	
Microbiota derivatates														
2-Hydroxy-3-(3-indolyl)propanoic acid	204,0659	0,7	4,31	[M-H]-	1	**-0.39 ± 0.35**	-0.29 ± 0.48	**-0.46 ± 0.32**	**-0.53 ± 0.48**	-0.44 ± 0.29	**-0.78 ± 0.59**	<0.001	0.46	272.05 [M-H+CHOONa]-
3-(4-Hydroxyphenyl)-2-hydroxypropanoic acid	181,0494	1,2	3,76	[M-H]-	1	**-0.22 ± 0.25**	-0.14 ± 0.32	-0.25 ± 0.38	**-0.49 ± 0.38**	-0.34 ± 0.3	**-0.59 ± 0.43**	<0.001	0.41	
3-(4-Hydroxyphenyl)-	165,055	0,7	4,88	[M-H]-	1	**1.78 ± 2.09**	0.99 ± 1.86	1.66 ± 2.49	0.56 ± 1.91	0.81 ± 2.2	0.54 ± 1.04	<0.001	0.71	

propionic acid													
Purine/pirimidine derivates													
Uric acid	167,0299	-8,8	0,41	[M-H]-	1	**-0.15 ± 0.18**	-0.03 ± 0.18	**-0.13 ± 0.13**	**-0.19 ± 0.17**	-0.16 ± 0.14	**-0.26 ± 0.18**	<0.001	0.41	168.03 13C [M-H]-, 335.07 2[M-H]-357.06 2M-2H+ Na
Creatine	132,0762	0,6	0,37	[M+H]+	1	**-0.72 ± 0.56**	**-0.84 ± 0.58**	-0.42 ± 0.63	-0.48 ± 0.59	-0.38 ± 0.6	-0.45 ± 0.67	<0.001	0.88	
Cofactors and vitamins													
Retinol	269,2261	0,8	6,29	[M-H20+H]	1	-0.18 ± 0.42	0.02 ± 0.49	-0.1 ± 0.23	**-0.48 ± 0.5**	-0.27 ± 0.21	**-0.55 ± 0.41**	<0.001	<0.05	
Choline	104,1066	0,4	0,31	[M+H]+	1	-0.23 ± 0.28	**-0.34 ± 0.25**	-0.11 ± 0.24	**-0.25 ± 0.24**	-0.14 ± 0.26	**-0.28 ± 0.24**	<0.001	0.55	
Other metabolites													
LPE (20:3)	502,2932	0,7	6,11	[M-H]-	2	**-1.09 ± 1.24**	**-1.8 ± 1.55**	-0.66 ± 0.87	**-0.98 ± 0.83**	-0.45 ± 0.71	-0.7 ± 0.79	<0.001	0.7	
LPE (18:3) or LPE (16:0)	476,2739	3,3	6,05	[M+H]+ / [M+Na]+	2	**0.34 ± 0.39**	0.02 ± 0.51	**0.36 ± 0.34**	0.24 ± 0.42	0.33 ± 0.35	0.33 ± 0.39	<0.001	0.29	
LPE(16:1)	450,2596	3	5,82	[M-H]-	2	-0.24 ± 0.67	**-0.7 ± 0.7**	-0.17 ± 0.59	-0.55 ± 0.78	-0.11 ± 0.42	-0.28 ± 0.8	<0.001	0.7	
Palmitoylmonoglyceride	353,265	1,2	6,32	[M+Na]+	1	0.02 ± 0.31	-1.2 ± 2.67	-0.03 ± 0.36	-0.19 ± 0.33	0.02 ± 0.34	**-0.39 ± 0.29**	0.13	0.21	354.27 13C[M+Na]+
Caffeine	195,0877	0	3,93	[M+H]+	1	**-2.32 ± 2.22**	**-2.64 ± 2.16**	-1.06 ± 2.51	-1.27 ± 1.4	-0.7 ± 2.44	-2.19 ± 2.33	<0.001	0.54	196.09 13C [M+H]+
Theobromine	181,0724	-0,4	3,57	[M+H]+	1	**-0.79 ± 0.54**	**-0.97 ± 0.77**	-0.51 ± 0.91	**-0.86 ± 0.72**	-0.26 ± 0.71	**-0.95 ± 0.68**	<0.001	0.34	
	179,0567	0,7	3,83	[M-H]-		**-1.39 ± 1.22**	**-1.6 ± 1.29**	-0.64 ± 1.41	-0.43 ± 1.25	0 ± 1.21	0.16 ± 1.46	<0.001	0.96	
Indole-3-carboxaldehyde	144,0446	0,9	4,34	[M-H]-	1	**-0.42 ± 0.53**	**-0.33 ± 0.36**	-0.59 ± 0.98	-0.37 ± 0.48	-0.32 ± 0.32	-0.46 ± 0.58	<0.001	0.86	

Abbreviations: MU, Metabolically Unhealthy, MH, Metabolically Healthy; RT, Retention Time; sPLS-DA, Sparse Partial Least Squares Discriminant Analysis

a. Identified metabolites listed in accordance with metabolic classes.

b. Data obtained by LC-ESI-qTOF-MS.

c. Level of identification evidence according to metabolomics Standard Initiative, Sumner et al.(1)

d. Values are shown as mean ± sd. Mean (log(T3)-log(T0)). *P* values were determined by paired *t*-test comparing T3 and T0 of each group after adjusted by gender, age and type of surgery and corrected by multiple comparisons with false discovery rate method.

e. *P* values represent changes over time (p-time) and time x group interaction (p-time x group) derived from linear mixed model and corrected by multiple comparation with false discovery rate method taking into accound all the 3000 features. The model considers the co-founders: age, gender and type of surgery.

* p<0.1, **p<0.05, ***p<0.001

Table S-6. Sparse Partial Least Squares discriminant analysis paramaters chosen for each increment of time and % of correct classification

	T1-T0	**T3-T0**	**T6-T0**
eta	0,4	0,2	0,7
K	1	1	1
% of correct classification	82,05	76,92	74,36

T1-T0, T3-T0 and T6-T0 are the increments of time 1 month, 3 months and 6 months respect baseline respectivetly
Optimal parameters to obtain a minimum clasification error chosen by (v-fold) cross-validation. *K*, Number of hidden components; *eta*, sparsity tning parameter.

5. DISCUSIONES

La relación epidemiológica entre obesidad y diabetes es una realidad mundialmente conocida. Sin embargo, los mecanismos implicados en la fisiopatología del órgano adiposo, su funcionalidad y su influencia sobre el estado metabólico general de un individuo no están completamente identificados. Por otro lado, la aparición de un fenotipo atípico, de sujetos con obesidad metabólicamente *sana*, definidos por tener un bajo número de factores de riesgo de síndrome metabólica, y un subgrupo de sujetos con normopeso pero con un número elevado de factores de riesgo, indicaría que la asociación obesidad y resistencia a la insulina no es estrictamente predictiva.

Ante esta situación, el objetivo principal de esta tesis doctoral ha consistido en la identificación de marcadores metabolómicos de resistencia a la insulina, dependientes e independientes de la presencia de obesidad. Para abordar este desafío se han utilizado dos perspectivas complementarias que configuran las dos hipótesis del trabajo: la primera, que el estudio de fenotipos discordantes obesidad/resistencia a la insulina, nos permitirá identificar biomarcadores dependientes e independientes de obesidad y diabetes y la segunda, que la cirugía bariátrica y los cambios en el estilo de vida mediante dieta y ejercicio comparten perfiles metabólicos que pueden aportar información sobre los mecanismos implicados en el desarrollo de la diabetes y enfermedades asociadas.

La cirugía bariátrica es conocida como la intervención más eficaz para alcanzar un descenso de peso considerable, duradero y efectivo. En los últimos años, se ha utilizado el término *cirugía metabólica*, para dar nombre a esta cirugía cuando su propósito va más allá de la pérdida de peso y se utiliza para tratar sus comorbilidades, en especial la DT2. Paradójicamente, la remisión de la DT2 o la mejora de factores de síndrome metabólico, se observan pocos días después de la intervención, hecho que reflejaría la implicación de un factor independiente al peso en el desarrollo de sus comorbilidades. También se han observado beneficios sobre la salud metabólica con un cambio en el estilo de vida, combinando una dieta hipocalórica y ejercicio físico, aunque los resultados son de menor proporción y menos duraderos. Por esto, conocer las bases moleculares de las dos estrategias de pérdida de peso permitiría identificar nuevas dianas de estudio.

Al iniciar esta investigación, en enero de 2013, habían publicados 18 estudios, que identificaban metabolitos asociados a la respuesta a la cirugía bariátrica, 7 artículos que identificaban metabolitos asociados a la pérdida de peso con cambios en el estilo de vida y 5 trabajos que comparaban los dos tratamientos (**Revisión 2**). A lo largo del desarrollo de esta tesis, se han publicado alrededor de 20 trabajos más que han abordado esta problemática [142–156]. No obstante, la mayoría de los estudios publicados emplean una aproximación metabolómica dirigida por LC-MS o una aproximación no-dirigida por GC-MS y resonancia magnética nuclear, limitando los resultados al estudio de unas rutas biológicas concretas sin beneficiarse del potencial de una estrategia LC-MS para obtener una visión global de los mecanismos moleculares asociados a las perturbaciones del metaboloma endógeno.

En la **Publicación 5** el objetivo principal fue identificar diferencias en la respuesta global post-cirugía según el estado metabolómico basal de los sujetos con obesidad, metabólicamente *sanos* o *enfermos* utilizando una aproximación metabolómica dirigida. Este enfoqué incluyó la cuantificación de 188 metabolitos de las rutas biológicas de los amino ácidos, aminas biógenas, acilcarnitinas, hexosas, glicerofosfolípidos y esfingolípidos.

Debido a la falta de consenso entre las diferentes guías clínicas en la definición de salud metabólica, se optó por la utilización de una estratégica estadística no-supervisada, para identificar subgrupos de pacientes según su respuesta post-cirugía. En esta primera aproximación no se identificaron patrones de respuesta definidos por los diferentes criterios de salud metabólica, género, edad o tipo de cirugía bariátrica. Se identificaron dos fenotipos (metabotipos), definidos por la respuesta a los 6 meses (T6-T0). Clínicamente estos metabotipos cambian en la resistencia a la insulina, los niveles de ácido úrico, colesterol y LDL independientemente del peso perdido, el género y el tipo de cirugía. Este estudio evidenció diferentes respuestas a una misma intervención quirúrgica y la necesidad de una monitorización continuada de los pacientes.

También observado en la **Publicación 6**, todos los individuos presentaban un patrón común de cambio post-cirugía, caracterizado especialmente por metabolitos de los

amino ácidos. En concreto, se observaron amino ácidos de cadena ramificada (leucina, isoleucina y valina), aminoácidos aromáticos, ornitina, arginina y sus derivados. De hecho, diferentes estudios han apuntado altos niveles de amino ácidos en sujetos con obesidad[157]. Además, los amino ácidos de cadena ramificada y los aromáticos han sido considerados como potenciales biomarcadores de pronóstico de DT2[158,159]. Cambios en los niveles de estos amino ácidos reflejan que la cirugía bariátrica comporta perturbaciones en el metaboloma, descritos en menor magnitud, después de una intervención dietética[75].

Después de la cirugía bariátrica también se identificaron compuestos de degradación de los amino ácidos: tirosina, fenilalanina y triptófano, por la microbiota intestinal (**Publicación 4**). En concreto, se observó una disminución del ácido hidroxifenil-láctico (hidroxi-(hidroxifenil)propanóico), un aumento del ácido fenil-láctico (hidroxifenilpropanóico) y una disminución del ácido índole-láctico respectivamente. Aunque en la **Publicación 4,** no se observaron cambios en los niveles de los amino ácidos, hecho que podría estar vinculado en que las mujeres presentaban obesidad clasificada como metabólicamente *sana*, también se han identificado, una disminución en metabolitos producidos por las bacterias intestinales, como el ácido fenil-láctico y el ácido hidroxifenil-láctico, especialmente en aquellos sujetos con pérdidas de peso superiores al 10%. Los niveles de estos metabolitos han correlacionado positivamente con cambios en el colesterol total y el nHDL. Las bacterias del género Clostridium[160] y Peptostreptococcus [161] entre otras, ambas del Filo de las Firmicutes, son las principales productoras de estos co-metabolitos. Abundantes concentraciones de las bacterias del Filo de las Firmicutes se han observado en pacientes con obesidad y DT2 (**Revisión 1**), y disminuyen después de la cirugía[162] o una dieta hipocalórica y ejercicio físico[163]. Por otra parte, cabe destacar que ambas estrategias de pérdida de peso comportan un cambio en la composición de la dieta, sea por el seguimiento de una dieta post-cirugía en los primeros meses después de la intervención, como el cambio implícito a una intervención por cambios en el estilo de vida, respectivamente. Estos cambios podrían estar relacionados con una disminución del aporte proteico y consecuentemente una disminución de su correspondiente perfil metabólico.

Las **Publicaciones 1-6** desvelan alteraciones en el perfil lipídico en la obesidad y la DT2, y en ambas estrategias de pérdida de peso. Particularmente, se observó cambios en el metabolismo de los esteroides, fosfolípidos, esfingomielinas y en el caso de la cirugía bariátrica también en los ácidos grasos y diacilglicéridos.

Los niveles de esteroles (ácidos biliares y hormonas estrogénicas) han sido modulados de manera diferente según la estrategia de pérdida de peso, especialmente sus formas glucuronizadas o sulfatadas (**Publicación 4**). Se reconoce un papel de las hormonas estrogéncias en el balance energético y en la saciedad del individuo[164,165], aunque su implicación en la patología de la obesidad es contradictoria entre los estudios[166,167].

Los niveles de ácidos biliares primarios y secundarios se han propuesto como moléculas claves en la interconexión de la microbiota intestinal con la obesidad-diabetes (**Revisión 2).** Un cambio en la microbiota intestinal en estos sujetos podría explicar cambios en la conversión de los ácidos biliares primarios a los secundarios. Los ácidos biliares modulan el metabolismo de la glucosa y los lípidos, hecho que la variación de sus niveles después de la intervención tendría un impacto global en el organismo.

El metabolismo de los fosfolípidos es modulado de manera interconecta con el metabolismo de los esfingolípidos y de los ácidos grasos. Los fosfolípidos, o también llamados glicerofosfolípidos, son componentes estructurales y funcionales de las membranas celulares y precursores de diferentes mediadores lipídicos[168]. Estas moléculas están formadas por la unión de ácidos grasos unidos a la posición sn1 y sn2 del glicerol. El proceso de síntesis lo constituye dos etapas: la primera, llamada ruta de *novo* (Kennedy pathway), dónde el fosfolípido se forma a partir de la esterificación del glicerol-3-fosfato y metabolización a DG y la segunda etapa de remodelación (Lands' cycle). En esta etapa actúa la fosfalipasa y la lisofosfolípido aciltransferasa (LPLATs) formando los diferentes tipos de fosfolípidos (fosfatidilcolina (PC), fosfatidiletanolamina (PE) y fosfatidilserina (PS)). Los fosfolípidos PC y PE son los mayores constituyentes de la membrana plasmática mientras que los PI y PS son menos abundantes. Las encimas del ciclo de Lands son

selectivas de ácidos grasos. LPLATs es conocido por estar sobrerregulado durante la abiogénesis y se asocia con la diferenciación de los adipocitos[169]. La fosfolipasa rompe el enlace de un ácido graso de la cadena de glicerol, generando los lisolípidos a partir de los fosfolípidos. El cambio en la composición de la membrana lipídica en sujetos con obesidad podría asociarse con una inflamación del tejido adiposo y al desarrollo de las enfermedades relacionadas a la obesidad, aunque el efecto de la remodelación lipídica de la membrana plasmática aún es controvertido. De hecho, después de la cirugía los sujetos metabólicamente *enfermos* presentaban mayores cambios en el perfil de fosfolípidos que los *sanos* **(Publicación 5)**.

La conexión entre los ácidos grasos, los DGs y las acilcarnitinas se ha evidenciado en la **Publicación 6**. Esta conexión podría ser responsable de la mejora del estado de elevada resistencia a la insulina en estos sujetos. Una mayor entrega de ácidos grasos al músculo e hígado se encuentra relacionada con menores tasas de oxidación de grasa intracelular o de conversión de ácidos grasos a lípidos neutros. Las principales causas que contribuyen a este estado pueden deberse a un exceso de la ingesta calórica, defectos en el metabolismo de los adipocitos, alteraciones en la oxidación mitocondrial de ácidos grasos e inhibición de las lipasas. En cambio los sujetos *sanos* aumentan los niveles del ácido hidroxioctanoico y el ácido hidroxidodecanoico después de la intervención.

Por otra parte los DG son esenciales en la síntesis de SM a partir de las ceramidas. Las esfingomielinas SM (d18:0/22:0) y SM (d18:0/20:0, d16:0/22:0) fueron moléculas discriminantes entre los sujetos que perdieron más del 10% de peso y los que perdieron menos en la intervención con la Dieta Mediterránea y ejercicio físico. En la aproximación metabolómica dirigida no se incluían la identificación de estas esfingomielinas, no obstante después de la intervención se observaron cambios en los niveles de esta molécula.

Los DG también son el substrato para la síntesis del TG y almacenados en el hígado. La incorporación de determinados ácidos grasos podría afectar a la composición de los ácidos grasos disponibles para la síntesis de fosfolípidos, durante la movilización

del tejido adiposo. Estudiar todos estos procesos podría desvelar la maquinaria que hay detrás en el desarrollo de la obesidad.

En el tejido adiposo visceral, en sujetos con obesidad y elevada resistencia a la insulina, se ha identificado un perfil específico de fosfolípidos (**Publicación 3)**. Estos sujetos presentaban niveles más bajos de fosfolípidos de cadena de ácido grasos de 18 átomos de carbono respecto a los sujetos con obesidad y sensibilidad a la insulina. Esta característica fue distintiva de este fenotipo y no se caracterizó cuando la resistencia a la insulina era diagnosticada en sujetos delgados.

El perfil metabólico asociado a la obesidad, independiente de la resistencia a la insulina, presentó una relación negativa con los fosfolípidos PC 36:3, PE P-36:1 y PE P-36:2. Estos resultados reflejan los cambios observados por Pietiläinen *et al*. en la remodelación de los adipocitos en gemelos monozigotos con y sin obesidad. El gemelo con obesidad presentaba mayores proporciones de ácido palmitoleico (C16:1) y araquidónico (C20:4) en su tejido adiposo y bajos niveles de ácidos grasos saturados, ácido linoleico (C18:2) y linolénico (C 18:3). Otros estudios han mostrado que especies araquidonil- incrementan durante la diferenciación de los adiposos mientras que las especias con linolénico disminuyen debido a la actividad de la aciltransferasa. Cadenas de ácido araquidónico son convertidas en metabolitos pro-inflamatorios como prostaglandinas y eicosanoides.

Este hecho crea un ambiente pro-inflamatorio vulnerable a los adipocitos y favorece la respuesta inflamatoria y la oxidación, factor clave en el desarrollo de resistencia a la insulina, por el contrario mientras las especies lipídicas derivadas del n-6 PUFA confieren una función inflamatoria, los n-3 PUFA poseen una acción anti-inflamatoria[170]. En la **Publicación 2** se han identificado mayores niveles en suero de n6 PUFAs en los sujetos que presentaban obesidad y resistencia a la insulina comparado con el grupo control (normo peso y sensibilidad a la insulina). Reforzando los resultados observados en VAT.

La creación de un modelo multimetabolito a partir de los metabolitos seleccionados con la regresión LASSO ha mostrado una elevada especificidad y sensibilidad para clasificar sujetos con obesidad y sensibilidad a la insulina entre sujetos con obesidad

[AUC 96.7% (91.9-1009] y la población en general [AUC 87.6% (79.0-96.2%)]. Este resultado nos ha permitido aproximar estos resultados a la práctica clínica.

Los cambios en la lipidómica del VAT se reflejaron en suero mediante el análisis por enfoque dirigido. Especies de esfingomielinas, con cadenas saturadas, se asociaron con el patrón característico de cadenas de ácidos grasos de 18 átomos de carbonos (SM C18:0. Cer d18:1/C18:0, dihidroCer d18:0/C18:0 y d18:0/C22:0). Estos resultados van más allá de la literatura actual [171–173] e indicarían un rasgo común de los sujetos con resistencia a la insulina, reforzando los resultados observados en el tejido adiposo. En estudios de metabolómicos de gran escala se han identificado (liso)lípidos, como el lisoPC C18:2, como potencial biomarcadores de diabetes[174,175]. En la **Publicación 1** se identificó una disminución del perfil de (liso)glicerofosfolípidos caracterizado por el fenotipo de obesidad severa independiente del grado de glicemia. En línea con los resultados de la P**ublicación 6**, alteraciones en el metabolismo de los lípidos podría estar asociado con la expansión del tejido adiposo. Estos resultados también correlacionarían con los identificados en la **Publicación 2** que se observaron alteraciones en los niveles de los DG y los ácidos grasos en presencia de elevada resistencia a la insulina y mayores niveles de ácido araquidónico, palmitoleico en obesidad. El análisis de enriquecimiento nos ha mostrado que los metabolitos procedentes de la clase de w-6 PUFAs serían los predominantes en la obesidad, estos lípidos actuarían de mediadores perpetuando la inflamación de bajo grado.

En este proyecto se identificó un modelo multimetabolito con elevada sensibilidad y especificidad formado por los niveles de 15 DGs, del ácido adrénico y del ácido úrico. Este marcador permitió discriminar aquellos sujetos con elevada resistencia a la insulina entre toda la población [AUC 80.1% (68.9-91.4)].

Trasladando nuestros resultados en la fisiopatología de la obesidad, en la presente tesis doctoral se han identificado perfiles metabólicos asociados e independientes de la funcionalidad y composición del tejido adiposo visceral. Las mejoras metabólicas después de una intervención de peso reflejan una modulación del perfil de los amino ácidos y de los lípidos (DG, acilcarnitinas, ácidos grasos y fosfolípidos) que podrían

estar vinculado con los mecanismos implicados a la resistencia a la insulina, productos de la microbiota intestinal derivados de los amino ácidos y ácidos biliares conjugados podrían tener un papel relevante. La confirmación de estos resultados en cohortes más grandes y en estudios prospectivos es necesaria para confirmar nuestros resultados.

Empresas como Metabolon (www.metabolon.com) han utilizado el potencial de la metabolómica para la identificación y posteriormente, de la comercialización de un panel de marcadores para el reconocimiento de un estado de resistencia a la insulina en un estado temprano. Este panel de marcadores se compone del hidroxibutirato, un ácido orgánico que actúa como marcador de la oxidación de los ácidos grasos; del linoleoil-glicerofosfocolina, un lípido que actúa como marcador de la síntesis de ácidos grasos en el hígado y la inflamación y del ácido oleico, un ácido grasos libre que actúa como marcador de la lipólisis y de los ácidos grasos plasmáticos totales. Este panel de 'diagnóstico' ha estado validado en 13 países europeos y probado en más de 1200 sujetos *sanos* (no-diabéticos).

6. CONCLUSIONES

Teniendo en cuenta los objetivos de la presente tesis doctoral abordados a lo largo de los diferentes trabajos presentados, se extraen las siguientes conclusiones generales:

1. Se han caracterizado los perfiles metabólicos séricos y de tejido adiposo visceral de los fenotipos concordantes y discordantes de obesidad y resistencia a la insulina por metabolómica dirigida, no-dirigida y semidirigida. El metaboloma sérico de los sujetos con obesidad se ha distinguido por alteraciones en el metabolismo de los (liso-)fosfolípidos. Un **estado de elevada resistencia a la insulina** independiente de la obesidad se ha determinado por cambios en el perfil de los diacilglicéridos y de los niveles del amino ácido valina y dependiendo de la obesidad por cambios en los niveles de la glicina, del glutamato y de metabolitos relacionados con el metabolismo del estrés oxidativo y de la inflamación. Se ha identificado cambios en la composición del tejido adiposo visceral en la obesidad, por metabolitos de las rutas de los amino ácidos de cadena ramificada, del ciclo de Krebs, del metabolismo del glutatión, de la glicólisis, de la gluconeogénesis y del metabolismo de los fosfolípidos. Los fosfolípidos con cadenas de ácidos grasos de 18 átomos de carbono, se han identificado como rasgo característico de los sujetos con obesidad y sensibilidad a la insulina.

2. Se ha modelado un biomarcador sensible y específico de la obesidad con sensibilidad a la insulina en tejido adiposo visceral, combinando la fosfatidiletanolamina (18:2), el fosfatidilinositol (18:0), la fosfatidilserina (18:0), la fosfatidilcolina (18:0/18:1), la fosfatidilcolina (18:2/18:2) y la fosfatidilcolina (18:2/18:3). Este biomarcador ha discriminado sujetos con obesidad y sensibilidad a la insulina, de la población total y con mayor especificidad y sensibilidad de los sujetos con obesidad. En las muestras de suero se ha desarrollado un biomarcador, formado por diacilglicéridos, ácido úrico y ácido adrénico con una elevada especificidad y sensibilidad para clasificar sujetos con obesidad y elevada resistencia a la insulina de la población con resistencia a la insulina, de la población con obesidad y de la población total.

3. Una pérdida de peso superior al 10%, con una elevada adherencia a la dieta Mediterránea hipocalórica y un incremento de la actividad física durante 12 meses en mujeres con obesidad metabólicamente *sana* se ha asociado con cambios en el metaboloma plasmático de productos de la microbiota intestinal del metabolismo de los amino ácidos y cambios en los niveles de especies lipídicas.

4. Se han caracterizado los perfiles metabólicos séricos que subyacen a las adaptaciones metabólicas pos-cirugía bariátrica mediante una aproximación metabolómica dirigida y no dirigida. Se ha diferenciado dos metabotipos o fenotipos metabólicos, definidos por la respuesta metabólica a los 6 meses de la cirugía bariátrica, e independientes del estado basal del individuo, edad, género y tipo de cirugía utilizada. El metabotipo con mayores mejoras en los niveles de sensibilidad a la insulina y de los factores cardiovasculares, son aquellos con menores niveles de precursores de la gluconeogénesis, de metabolitos del metabolismo de la urea y menores cambios de composición de los microcomponentes de las lipoproteínas. La cirugía bariátrica se ha asociado con cambios en el metabolismo de fosfolípidos, de amino ácidos aromáticos y de cadena ramificada, de carbohidratos, de diacilglicéridos y ácidos biliares. Estas alteraciones se han relacionado con la mejora metabólica de los pacientes.

5. Se han definido perfiles metabólicos séricos de las respuestas a la cirugía bariátrica dependiendo del estado metabólico basal del paciente mediante técnicas metabolómicas dirigida y no-dirigida. El análisis de factores múltiples ha revelado que los sujetos con obesidad metabólicamente *sana* o *enferma* cambian después de la cirugía hacia un estado metabólico común, ya observado a los 3 meses post-cirugía y se mantiene hasta los 6 meses. El metabolismo de los productos del metabolismo microbiano así como de la ruta de los ácidos biliares glucuronizados puede ser la clave en la regulación de la salud metabólica en sujetos con obesidad después de la cirugía bariátrica.

El estudio metabolómico en un modelo de fenotipos concordantes y discordantes ha demostrado aportar nuevas piezas en el estudio de la obesidad y sus enfermedades metabólicas asociadas. El metabolismo de los lípidos y de los amino ácidos podría

estar alterado en pacientes con obesidad y elevada resistencia a la insulina. Estos cambios podrían reflejar parte de los cambios en la composición y la funcionalidad del tejido adiposo visceral en el desarrollo de la obesidad. Después de una intervención de pérdida de peso se ha evidenciado una afectación metabólica global. Las estrategias de pérdida de peso como modelo de estudio de la obesidad han ofrecido un enfoque complementario sobre las rutas biológicas subyacentes a la enfermedad. Se han observado beneficios metabólicos en ambas estrategias de pérdida de peso estudiadas, la cirugía bariátrica o el cambio en el estilo de vida. Aunque la magnitud de los cambios después de la cirugía bariátrica han sido superiores al de una intervención en el estilo de vida a largo plazo, la cirugía bariátrica y los cambios en el estilo de vida para la pérdida de peso en sujetos con obesidad comparten perfiles metabólicos relacionados al metabolismo de los lípidos y a la degradación de los amino ácidos por la microbiota intestinal. Se ha observado que modificaciones en el estilo de vida con cambios en la composición nutricional de la dieta podrían originar cambios adicionales sobre la salud independientemente del estado metabólico basal del paciente.

Los efectos de la cirugía bariátrica y los cambios en el estilo de vida, combinando dieta y actividad física, sobre la salud metabólica abren nuevas perspectivas e hipótesis en el entendimiento de la obesidad y sus enfermedades asociadas. Las especies lipídicas, especialmente el estudio del grado de redundancia en la composición de las cadenas de ácidos grasos de los diacilglicéridos, fosfolípidos y esfingolípidos, así como, el estudio de la degradación de los amino ácidos por la microbiota intestinal podrían abrir nuevas vías de investigación en el campo de la obesidad.

CONCLUSIONS

Taking into account the objectives of this doctoral thesis addressed throughout the different articles presented, the following conclusions can be drawn:

1. The serum and visceral adipose tissue metabolic profiles of the concordant and discordant phenotypes of obesity and insulin resistance have been characterized by directed, non-directed and semi-directed metabolomics. The serum metabolome of subjects with obesity has been distinguished by alterations in the metabolism of (lyso-)phospholipids. A state of high insulin resistance independent of obesity has been determined by changes in the profile of diglycerides and the valine amino acid levels and associated with obesity due to changes in the levels of glycine, glutamate and metabolites related to the metabolism of oxidative stress and inflammation. Changes in the composition of visceral adipose tissue in obesity have been identified by metabolites of the branched-chain amino acid pathway, the Krebs cycle, the glutathione metabolism, glycolysis, gluconeogenesis and the metabolism of phospholipids. Phospholipids with fatty acid chains of 18 carbon atoms have been identified as a feature of subjects with obesity and insulin sensitivity.

2. A sensitive and specific biomarker of obesity with insulin sensitivity in visceral adipose tissue has been modelled, combining phosphatidylethanolamine (18:2), phosphatidylinositol (18:0), phosphatidylserine (18:0), phosphatidylcholine (18:0/18:1), phosphatidylcholine (18:2/18:2) and phosphatidylcholine (18:2/18:3). This biomarker has identified subjects with obesity and insulin sensitivity from the total population and with greater specificity and sensitivity among subjects with obesity. In serum, a biomarker formed by diglycerides, uric acid and adrenal acid with a high specificity and sensitivity able to classify subjects with obesity and high insulin resistance among the population with insulin resistance, the population with obesity and the total population.

3. A weight loss greater than 10%, with a high adherence to a hypocaloric Mediterranean diet and increased physical activity after 12 months in women with metabolically healthy obesity has been associated with changes in the plasma metabolome of products of degradation of amino acids by the intestinal microbiota and changes in the levels of lipid species.

4. The metabolic serum profiles underlying the metabolic adaptations after bariatric surgery have been characterized by a targeted and non-targeted metabolomic approach. Two metabotypes or metabolic phenotypes have been differentiated by the metabolic response six months after bariatric surgery, independently of the baseline status of the subjects, age, gender and type of surgery carried out. The metabotypes with the greatest improvements in levels of insulin sensitivity and cardiovascular factors are those with lower levels of precursors of gluconeogenesis and metabolites of urea metabolism, and minor composition changes of lipoprotein microcomponents. Bariatric surgery has been associated with changes in the metabolism of phospholipids, aromatic and branched-chain amino acids, carbohydrates, diglycerides and bile acids. These alterations have been related to metabolic improvement among patients.

5. Serum metabolic profiles of responses to bariatric surgery have been defined depending on the basal metabolic state of the patient through targeted and non-targeted metabolomic techniques. Multiple factor analysis has revealed that subjects with metabolically healthy or diseased obesity change after surgery to a common metabolic state, observed as early as three months post-surgery and maintained until six months. The metabolism of microbial metabolism products, as well as the pathway of glucuronized bile acids, may be the key in the regulation of metabolic health in subjects with obesity after bariatric surgery.

The metabolomic study in a model of concordant and discordant phenotypes has been shown to contribute in unveail new pieces in the study of obesity and its associated metabolic diseases. The metabolism of lipids and amino acids can be altered in patients with obesity and high insulin resistance. These changes may reflect some of the changes in the composition and functionality of visceral adipose tissue in the development of obesity. After a weight loss intervention, a global metabolic affectation was evidenced. The study of the effects after a weight loss strategy as a model for the study of obesity has offered us a complementary approach to the biological pathways underlying the disease. Metabolic benefits have been observed in terms of weight loss strategies studied, bariatric surgery and changes in lifestyle. Although the magnitude of the changes after bariatric surgery

was superior to those changes observed in the long-term lifestyle intervention, bariatric surgery and lifestyle changes in regard to weight loss in obese subjects share metabolic profiles related to the metabolism of lipids and the degradation of amino acids by the gut microbiota. It has been observed that changes in lifestyle along with changes in the nutritional composition of the diet could lead to additional changes in health, regardless of the basal metabolic state of the patient.

The effects of bariatric surgery and changes in lifestyle, combined with diet and physical activity, on metabolic health open new perspectives and hypotheses in the understanding of obesity and its related diseases. Lipid species, especially the study of the degree of redundancy in the composition of the fatty acid chains of diglycerides, phospholipids and sphingolipids, as well as the study of the degradation of amino acids by the gut microbiota, could open new research channels in the field of obesity.

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ANEXOS

Concordant vs Discordant Phenotypes for Obesity and Diabetes. Shared and Exclusive Metabolic Alterations and Phenotypic Predictors

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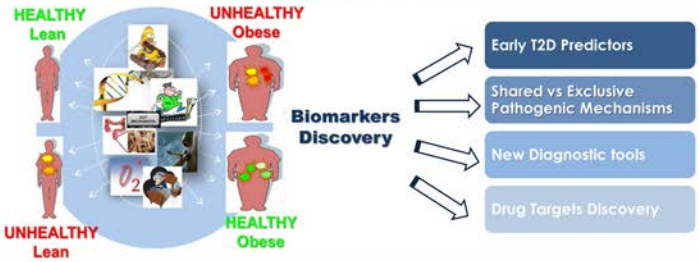
BACKGROUND

Discordant metabolic phenotypes for obesity and insulin resistance (IR) or type 2 diabetes (T2D) provide a unique and still unexploited opportunity to examine the interrelations between these associated metabolic disorders.

GOAL

To identify the metabolic traits of the obese or the prediabetic phenotype separately, so to have insights on the pathogenic mechanisms underlying both diseases, and check for their predictive power



Material and Methods

Subjects and STUDY DESIGN

Cross-sectional observational study

N=64 Caucasic adults (19M / 45F, 22-71 y)
Assignment to 4 sex- and age-matched phenotypic groups

^Subjects at risk of T2D were defined based on impaired fasting glucose (≥100 mg/dL and ≤126 mg/dL) and insulin resistance (HOMA-IR > 3.4)
* At least a 10-year history of obesity at the time of recruitment

Protocol approved by the local Ethics and Research Committees, Virgen de la Victoria and Carlos Haya Hospitals, Malaga, Spain

Metabolic profiling & Data Analysis

WHAT? ✓ 81 POLAR metabolites (AAs, BAs, ACs, Total Hexoses)
✓ 399 NON-POLAR metabolites (Phospholipids, Sphingolipids)

HOW? ✓ ESI-QqQ-MS TSQ Vantage™ (Thermo Scientific)
✓ AbsoluteIDQ p180 Kit (Biocrates Life Sciences AG)

- LC-MS/MS: • Hypersil GOLD 3.0 µm 2.1 x 100 mm HPLC column (Thermo Scientific), 5 µl Inj. vol., ESI+
- FIA-MS/MS: • 10 µl inj. vol., ESI+ and ESI-

Quality Checks ✓ Randomized run sequence
✓ QC1, QC2, QC3, Zero and Blank samples every 20 samples

Quantification ✓ MRM, 7-point calibration curves, ISTD correction

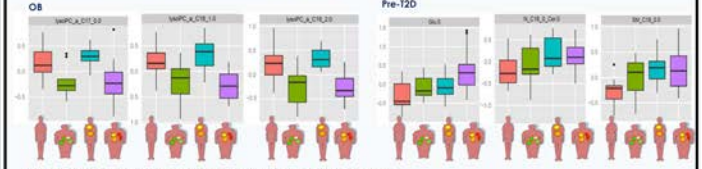
Pre-processing ✓ Noisy variables & age and drug-related confounders removal
✓ Missing data imputation (k-NN averaging)
✓ Data normalization

Inter-group comparison (UVA) ✓ ANOVA, HSD Tukey contrast, p=.05, q=.05

Phenotipic prediction (MVA): ✓ Feature selection (F-test, Kruskal test, Limma test, Random Forest)
✓ Group classification (DLDA, LDA, QDA, PLSDA, SCDA)
✓ Spearman's rank-order correlation coefficients
✓ PCA

RESULTS

Plot 1. Box-plots of significantly discriminating markers



Plot 2. Correlation plots among metabolic and clinical markers

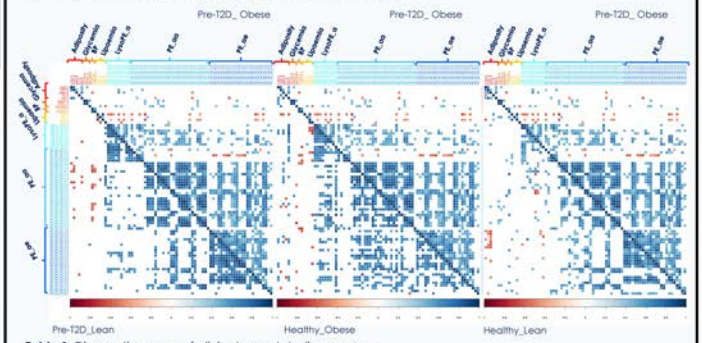


Table 1. Diagnostic power of clinical vs metabolic measures

		Clinical		Metabolic		Clinical		Metabolic		Clinical		Metabolic	
		[all subjects]				[healthy only, obese (n=12) versus lean (n =19)]				[pre-T2D only, obese (n=21) versus lean (n =12)]			
		misclass.	probab.*	misclass.	probab.*	misclass.	probab.*	misclass.	probab.*	misclass.	probab.*	misclass.	probab.*
Obesity	DLDA	0.02	0.98	**0.22**	0.78	0.00	1.00	**0.21**	0.79	0.03	0.98	**0.22**	0.78
	LDA	0.01	0.98	**0.20**	0.76	0.01	0.99	**0.37**	0.62	0.05	0.94	**0.19**	0.79
	QDA	0.03	0.97	**0.26**	0.72	0.03	0.97	**0.37**	0.63	0.09	0.91	**0.20**	0.79
	PLSDA	0.02	0.96	**0.17**	0.82	0.04	0.96	**0.19**	0.77	0.06	0.94	**0.23**	0.73
	SCDA	0.02	0.94	**0.20**	0.79	0.00	0.98	**0.23**	0.76	0.03	0.95	**0.22**	0.77
		[all subjects]				[lean only, pre-T2D (n=12) versus non pre-T2D (n =19)]				[obese only, pre-T2D (n=21) versus non pre-T2D (n =12)]			
		misclass.	probab.*	misclass.	probab.*	misclass.	probab.*	misclass.	probab.*	misclass.	probab.*	misclass.	probab.*
Pre-T2D	DLDA	0.08	0.91	**0.39**	0.61	0.06	0.95	**0.23**	0.77	0.06	0.94	**0.50**	0.50
	LDA	0.04	0.96	**0.40**	0.58	0.08	0.92	**0.35**	0.64	0.06	0.94	**0.52**	0.48
	QDA	0.04	0.96	**0.40**	0.56	0.09	0.90	**0.42**	0.58	0.06	0.94	**0.48**	0.51
	PLSDA	0.07	0.92	**0.42**	0.57	0.08	0.92	**0.30**	0.66	0.10	0.87	**0.50**	0.49
	SCDA	0.09	0.89	**0.39**	0.59	0.05	0.94	**0.26**	0.73	0.06	0.91	**0.41**	0.52

* average probability

CONCLUSIONS

The quantification of individual targeted metabolites gave a more granular measure of the individuals' metabolic phenotype beyond standard clinical parameters alone, enabling to depict shared and exclusive metabolic alterations associated to obesity and pre-T2D and hence identify early markers of T2D risk, but was not predictive by itself. Since cause and effect relationships between altered metabolites and metabolic impairment were not elucidated, future research on potential causal pathways are strongly aimed.

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MAKING SENSE OF METABOLOMIC DATA: COMPREHENSIVE ANALYSIS OF ALTERED METABOLIC PATHWAYS IN DIABETES AND OBESITY

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INTRODUCTION

Bioinfomatic tools for enrichment and visualization of 'omics'data

- Hypothesis about biological systems
- Disease biomarkers and therapeutic targets
- Improve biological interpretation of data

Are they adequate for metabolomic datasets?

OBJECTIVES

Describe the suitability of bioinformatic tools to interpret metabolomic datasets: type 1 and 2 diabetes mellitus and obesity as case study

MATERIAL AND METHODS

Datasets

Datasets of metabolic diseases generated in metabolomics studies (LC-MS, NMR and GC-MS) were obtained from MetabolomeXchange, a repository of metabolomics datasets.

MetabolomeXchange

Datasets contained potential biomarkers of type 1 and type 2 diabetes mellitus and obesity [1,2], among others.

Metabolites

Datasets included a wide range of chemical classes: amino acids, carbohydrates, organic acids, lipids, etc.

Identifiers

Codes (ID) of each compound were searched in several metabolite databases (KEGG, PubChem, HMDB, etc.) so they could be analysed and visualized.

Bioinformatic tools used

Libraries of metabolic pathways

Online portals and software programs

» Different enrichment analysis and visualization outputs were obtained.
» Only the most representative outputs are shown.

RESULTS AND DISCUSSION

Which are the most affected pathways in my condition of study?

OVER-REPRESENTATION ANALYSIS

Over-representation (ORA) analysis is always the first enrichment analysis carried out.

Table 1: ORA performed with MBRole:

Metabolic pathway	Hits/total metabolites	P-value
Type 1 diabetes		
Aminoacyl-tRNA biosynthesis	5/75	1.34E-06
Valine, leucine and isoleucine degradation	4/40	4.37E-06
Valine, leucine and isoleucine biosynthesis	3/27	6.80E-05
Glutathione metabolism	2/38	6.40E-03
Nitrogen metabolism	2/39	6.74E-03
Type 2 diabetes and obesity		
Fatty acid biosynthesis	4/49	1.19E-03
Starch and sucrose metabolism	4/50	1.29E-03
Aminoacyl-tRNA biosynthesis	4/75	5.76E-03
Amino sugar and nucleotide sugar metabolism	4/88	1.01E-02
Valine, leucine and isoleucine biosynthesis	2/27	2.87E-02

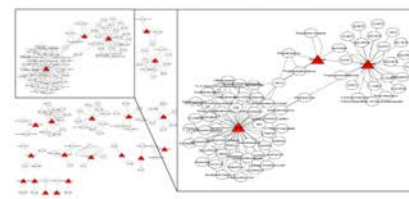
» ORA provides a list of the most altered (over/underexpressed) metabolic pathways in our study.

Is my target compound closely related to other interesting metabolites?

NETWORK ANALYSIS

Metabolite networks are simplified representations of relationships between molecules. They represent complex cellular processes and comprise metabolite interactions. They contain information not covered in metabolic pathway databases and can help in identifying altered neighbourhoods.

Figure 1: Network of the metabolites of the dataset (red) and related metabolites (white) in type 2 diabetes and obesity. The network was built with MetScape, a plug-in for Cytoscape.



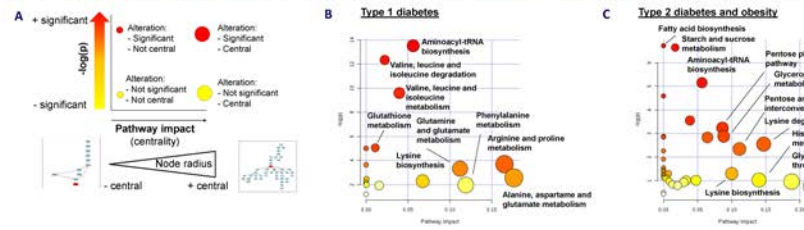
» Metabolite networks provide valuable information how metabolites correlate and show these metabolites that could be also altered in the condition of study, e.g. diabetes, and may be potential disease biomarkers or therapeutical targets.

How important is the alteration of my pathway?

TOPOLOGY PATHWAY ANALYSIS

Topology pathway analysis correlates the degree of the metabolic alteration (p-value) and how central the metabolite in the pathway is. This information is shown as 'metabolomics views'.

Figure 2: Panel A shows how to interpret the 'metabolomics views', and panels B and C the 'metabolomics views' of type 1 diabetes and type2 diabetes + obesity. They were created with MetaboAnalyst.



» The integration of ORA and pathway impact results provides information about how important a metabolite alteration is, and how could it affect to our metabolism.

REFERENCES

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[2] PLoS ONE 5 (2010) 5(12):e15234..

CONCLUSIONS

The study of altered metabolic pathways allowed us interpreting data from metabolomics studies and extracting very valuable information from them that might help identifying disease biomarkers and possible metabolic alterations related to diseases. This information could be translated to the clinical practice to predict metabolic alterations before the onset of diseases.

ACKNOWLEDGEMENTS

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Untargeted profiling of concordant/discordant phenotype sof high insulin resistance and obesity to predict a biomarker model for the risk of developing diabetes

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BACKGROUND

Discordant metabolic phenotypes for obesity and insulin resistance (IR) or type 2 diabetes (T2D) provide a unique and still unexploited opportunity to examine the interrelations between these associated metabolic disorders.

GOALS

1) **To explore the metabolic profiles of high IR and obesity**
2) **To identify differences between concordant/discordant phenotypes of high IR and obesity**
3) **To define a predictive model for the risk of developing of diabetes**

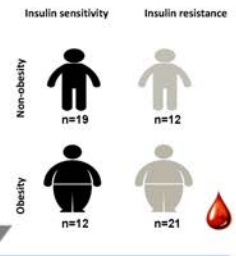
METHODOLOGY

Study design and sample collection

N=64 Caucasic adults (19M / 45F, 22-71 y)
Assignment to 4 sex- and age-matched phenotypic groups

Insulin resistance: ≥100 mg/dL and ≤126 mg/dL and insulin resistance HOMA-IR > 3.4
* At least a 10-year history of obesity at the time of recruitment

Protocol approved by the local Ethics and Research Committees, Virgen de la Victoria and Carlos Haya Hospitals, Malaga, Spain



UNTARGETED METABOLOMICS LC-ESI-qTOF-MS

TripleTOF® 5600 Hybrid quadrupole-TOF mass spectrometer (AB Sciex)

Data acquisition
- ✓ Randomized run sequence
- ✓ QC1, QC2, QC3, Zero and Blank samples every 20 samples

Data analysis
- ✓ Noisy variables & age and drug-related confounders removal
- ✓ Missing data imputation (k-NN averaging)
- ✓ Data normalization
- ✓ Multivariate:
  - OSC-PLS-DA, R^2X (cum), R^2Y (cum) and Q^2 (cum) parameters,
  - CV and permutation tests (n=200)
  - VIP≥2

Biomarker identification

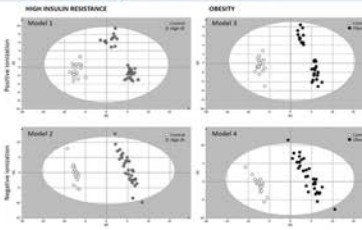
Biological interpretation
- ✓MetaboAnalyst
- ✓ChemRich (http://chemrich.fiehnlab.ucdavis.edu/)

Predictive models
- ✓model = λ_1 * metabolite$_1$ + λ_2 * metabolite$_2$ + ... + λ_n * metabolite$_n$
- ✓The LASSO regression method
- ✓**ROC curves**: AUC(CIs 95%)

RESULTS AND DISCUSSIONS

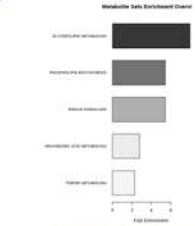
Separation of the control group from subjects with high IR (models 1 and 2) and subjects with obesity (models 3 and 4) in both ionization modes

OSC-PLS-DA score plots. White circles refer to the control group (subjects with IS and non-obesity), grey circles to high IR and black circles to obesity.

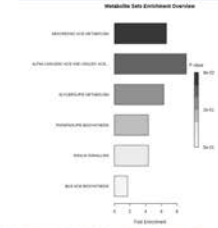


Pathways involved in High IR and Obesity

HIGH INSULIN RESISTANCE

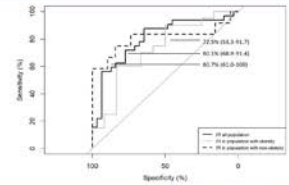


OBESITY



P-values were calculated by applying hipergeometric test. Only the metabolites that presented VIP≥2 and adjusted p-value≤0.05 were used

Multimetabolite biomarker



The biomarker model was formed by the arithmetic mean of the Eiheon DAGs identified (IDG), adrenic acid and uric acid.

CONCLUSIONS

Through an untargeted metabolomic-driven approach we have explored the metabolic profiles of concordant and discordant phenotypes of subjects high IR and/or obesity. Large alterations in lipid metabolism, oxidative stress and inflammation were unveiled. In addition, these results allowed to build a multimetabolite biomarker model to predict high IR regardless of obesity that includes the measurement of DGs, uric acid and adrenic acid. It might be also employed to predict the risk of developing diabetes. These findings provide new insights in the research of metabolic diseases and unveil new potential targets in therapeutic treatments of diabetes and obesity

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