Estudio metabolómico de biomarcadores nutricionales en estudios de intervención mediante resonancia magnética nuclear. Intervención con alimentos, complementos y patrones alimentarios

Rosa Vázquez Fresno

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UNIVERSIDAD DE BARCELONA

FACULTAD DE FARMACIA

DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA

ESTUDIO METABOLÓMICO DE BIOMARCADORES NUTRICIONALES EN ESTUDIOS DE INTERVENCIÓN MEDIANTE RESONANCIA MAGNÉTICA NUCLEAR. INTERVENCIÓN CON ALIMENTOS, COMPLEMENTOS Y PATRONES ALIMENTARIOS

ROSA VÁZQUEZ FRESNO

2015

UNIVERSIDAD DE BARCELONA

FACULTAD DE FARMACIA

PROGRAMA DE DOCTORADO
ALIMENTACIÓN Y NUTRICIÓN

2010-2014

ESTUDIO METABOLÓMICO DE BIOMARCADORES NUTRICIONALES EN ESTUDIOS DE INTERVENCIÓN MEDIANTE RESONANCIA MAGNÉTICA NUCLEAR. INTERVENCIÓN CON ALIMENTOS, COMPLEMENTOS Y PATRONES ALIMENTARIOS

Memoria presentada por ROSA VÁZQUEZ FRESNO para optar al título de doctora por la Universidad de Barcelona

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(Director)

ROSA VÁZQUEZ FRESNO

2015

"El mejor científico está abierto a la experiencia, y esta empieza con un romance, es decir, la idea de que todo es posible".

Ray Bradbury (1920-2012), Escritor estadounidense.

Este trabajo ha sido financiado por:

Ministerio de Economía y Competitividad y Fondo Europeo de Desarrollo Regional (FEDER)

Proyecto AGL2006-14228-C03-02

Proyecto AGL 2009-13906-C02-01

Beca de Formación Personal Investigador (FPI) 2010-2014 (BES-2010-031313)

- Beca de Estancia FPI 2013 (EEBB-I-14-07889)

Programa Ingenio-Consolider FUN-C-FOOD (CSD2007-063)

Acción Complementaria AGL-2010-10084-E

Agencia de Gestión de Ayudas Universitarias y de Investigación (AGAUR) – Generalitat de Catalunya

Ayuda de apoyo a las actividades de Grupos de Investigación 2009-SGR-724 y 2014-SGR-1566

AGRADECIMIENTOS

Primero de todo me gustaría agradecer a mis dos directores de Tesis, en los que he tenido un apoyo continuo desde que empecé este camino. Cristina, gracias por la confianza que depositaste en mí hace ya 5 años, echando la vista atrás veo la cantidad de cosas que he podido aprender de ti. Rafa, gracias por todo lo que me has enseñado durante este camino, que ha sido muchísimo, conmigo te estrenaste como director de tesis y ahora ya llevas tres!! Muchas gracias por todo lo que he aprendido de vosotros, tanto en el ámbito profesional como de crecimiento personal.

A mis compañeros y amigos en el laboratorio. A las Marías: Maria Rotchés de tu he après tota la practicitat que et caracteritza, has sigut un suport enorme els anys que hem compartit al laboratori. Maria Boto, esa chica leonesa risueña con la que hemos pasado grandes tardes y noches de cañas, y que se ha convertido en una gran amiga. Montse, gràcies per les "hores de café" i les xerradetes cafeteres, ets una persona excepcional! Dra Tulipani, esa alegría y buen humor que inunda el laboratorio y esas carcajadas que contagian cuando las oyes. Mar, la meva companya de metabolómica, quina energia!, quina vitalitat i perseverancia! Ets incansable!. Mireia y Olha, siempre estáis ahí para cualquier duda o pregunta que me surja, siempre con un buen consejo! Francesca, ciao bellísima! contigo nos adentramos en el maravilloso mundo de la resonancia magnética nuclear. También tengo grandes recuerdos en el laboratorio con el resto de mis compañeros Ximena, Enrique, Ascen y ahora más recientes Marisa y Fran. Sin olvidarme de compañeros tanto en Farmacia como en Torribera, Anna Tresserra, Paola, Miriam, Mariángeles, Natalia. También recuerdo aquellos que cuando yo estaba empezando, ellos acabando..Alex, Gallardo y Mariluz.

A mis amigos que han estado siempre apoyándome en los buenos y malos momentos: Ángeles, que siempre has estado, estas y estarás ahí, Bea "la gallega", Bertix, Ana, a mis amigos del cole, a los "biofriends" con los que llevamos 10 años juntos, y suma y sigue!, Atilla, Marcel y Gregg mis supermegaguays compañeros de piso, y a esa gran familia de "*tusamigossonmisamigos*".

Thanks to all the people that I met in Canada. David thank you soooo much for welcome me in your lab, also thanks Rupa and Trent. Lu, Hong, Edison, thank you for your friendship, Karolina and Helena was a pleasure meeting you in Canada I hope to see you again in Europe or Australia. Philip, I'm very grateful to you!! I was feeling in Edmonton like being at home thanks to you. I hope to see you again!

Arnau, gràcies a aquest doctorat ens vam poder conèixer i després de moltes tardes cerveseres, viatges i tirades de "tetines" hem compartit molt bons moments. Gràcies per tot el recolzament que he tingut amb tu des de l'inici fins a ara, i en els pròxims viatges que emprenguem!

A mi familia, Mamá, tú eres un ejemplo de superación, de ti he aprendido a luchar en la vida por lo que quiero. Papá, de ti puedo decir muchas cosas, pero la principal es que tienes toda mi admiración. No sería lo que soy sin vosotros, siempre me habéis apoyado y ayudado en todo y habéis estado ahí, de forma incondicional. Al resto de mi familia, gracias por todo el apoyo y respaldo.

A todos vosotros, MUCHAS GRACIAS!

LISTA DE ABREVIATURAS

2 D	2 dimensiones
4-HPA	4-hidroxifenilacetato
ANOVA	Análisis de la Variancia
AUC	Área bajo la curva (del inglés, *Area under the curve*)
COSY	Espectroscopia de correlación (del inglés, CO*rrelation SpectroscopY*)
CVD	Enfermedad cardiovascular (del inglés, *Cardiovascular Disease*)
d	doblete
EtG	Etilglucurónido
FDR	Tasa de falsos positivos (del inglés, *False Discovery Rate)*
FFQ	Cuestionario de frecuencia de consumo (del inglés, *Food Frequency Questionnaire*)
FID	Decaimiento libre de inducción (del inglés, *Free Induction Decay)*
FM	Metaboloma proveniente de los alimentos (del inglés, *Food Metabolome*)
HCA	Análisis de clúster jerarquizado (del inglés, *Hierarchical Clustering Analysis*)
HMDB	Base de datos del metaboloma humano (del inglés, *Human Metabolome DataBase*)
CI	Intervalo de confianza (del inglés, *confidence interval*)
IMC	Índice de masa corporal
IQR	Rango intercuartil (del inglés, *interquartile range***)**

m	multiplete
MT	Metabotipo
MD	Dieta mediterránea (del inglés, *mediaterranean diet*)
NOESY	Espectroscopia de efecto nuclear *Overhauser* (del inglés, *Nuclear Overhauser Effect SpectroscopY*).
OSC	Corrección ortogonal de las señales (del inglés "*Ortogonal Signal Correction"*)
PCA	Análisis de componentes principales (del inglés "*Principal Component Analysis*")
PLS-DA	Análisis discriminante por mínimos cuadrados parciales (del inglés, *Partial least squares Discriminant Analysis*).
PREDIMED	Prevención con dieta Mediterránea
RMN	Resonancia magnética nuclear (en inglés, *NMR*)
ROC	Curva de rendimiento diagnóstico (del inglés R*eceiver Operating Characteristic)*
s	singlete
t	triplete
T2D	*Diabetes Mellitus* tipo 2
U	No identificado/desconocido (del inglés U*nidentified/Unknown*)
UFC	Unidades formadoras de colonias
VIP	Importancia de las variables en la proyección (del inglés, *Variable Importance in the Projection)*
WIP	Ingesta de polifenoles del vino (del inglés, *Wine Polyphenols Intake*)
WIS	Estudio de intervención con vino (del inglés, *Wine Interventional Study)*

ÍNDICE

1. INTERÉS…… 1

HIGHLIGHT OVERVIEW AND IMPACT OF THE THESIS...…….. 7

2. ANTECEDENTES BIBLIOGRÁFICOS…… 11

2.1 LA METABOLÓMICA…… 14

2.1.1 Introducción……..... 14

2.1.2 Diagrama de flujo en metabolómica……..... 17

- Recolección y preparación de la muestra biológica…... 18
- Adquisición de los datos……. 18
- Análisis de datos…….. 20
- Identificación metabólica……..... 20
- Interpretación biológica……..... 20

2.1.3 Resonancia Magnética Nuclear.
Antecedentes de la técnica analítica……..... 22
2.1.3.1 Principios físicos de la técnica analítica..... 24
2.1.3.2 Componentes de un espectrómetro de RMN 31

2.2 NUTRIMETABOLÓMICA: ESTUDIO DE BIOMARCADORES Y PERFILES NUTRICIONALES… 33

2.2.1 Nutrimetabolómica……... 33

2.2.1.1 Estudios nutricionales……..... 33

- Estudios de intervención agudos, de media/larga duración……..... 33
- Estudios cohorte observacionales…… 34

2.2.1.2 Tipologia de componentes nutricionales para el estudio de biomarcadores..... 36

I. Compuestos bioactivos……..... 36
II. Alimentos individuales……..... 37
III. Complementos alimenticios……..... 37
IV. Patrones alimentarios……..... 39

2.2.2 Biomarcadores nutricionales…….. 42

2.2.2.1 Biomarcadores para la nutrición:
efecto de la digestión, metabolismo y microbiota intestinal……..... 42

I. Tipo de biomarcadores nutricionales

- Biomarcadores de ingesta o exógenos……. 43
- Biomarcadores de efecto o endógenos…… 43

II. Criterios de validación biológica y analítica par biomarcadores nutricionales…….. 45

2.2.2.2 Biomarcadores para la salud:
Concepto de fenotipo metabólico (metabotipo)……. 46

2.3 APLICACIÓN DE LA NUTRIMETABOLÓMICA EN EL ESTUDIO DE BIOMARCADORES NUTRICIONALES E IMPACTO EN LA SALUD

2.3.1 Biomarcadores nutricionales de exposición a un alimento.. 48

Capítulo de libro: Olha Khymenets, Rosa Vázquez-Fresno, Rafael Llorach, Mireia Urpi- Sarda, Mar Garcia-Aloy, Sara Tulipani, Ascensión Lupianez-Barbero, Cristina Andres-Lacueva. "Metabolomic approach to finding targets of wine benefits in human health", capítol 16 del llibre "Wine: Safety, Consumer Preferences and impact on Human Health". Springer, 2014

2.3.2 Biomarcadores nutricionales de exposición a componentes bioactivos e impacto en la salud …… 95

Capítulo de libro: Rosa Vázquez-Fresno, Sara Tulipani, Olha Khymenets, Mireia Urpi-Sarda, Mar Garcia-Aloy, Montse Rabassa, Maria Boto-Ordoñez, Maria Rotches-Ribalta, Rafael Llorach and Cristina Andres-Lacueva. Emerging Applications of Metabolomics to Polyphenols and CVD Biomarker Discovery, capítol 79 del llibre "*Polyphenols in Human Health and Disease*" (Volumen 2), (Academic Press, Editorial Elsevier, 2014).

3. HIPÓTESIS Y OBJETIVOS

HYPOTHESES AND AIMS

3.1 HIPÓTESIS……. 119

HYPOTHESES…….. *120*

3.2 OBJETIVOS…….. 121

AIMS…… *122*

4. METODOLOGÍA

4.1. ANÁLISIS METABOLÒMICO NO DIRIGIDO POR RESONANCIA MAGNÉTICA NUCLEAR

4.1.1 METODOLOGÍA ANALÍTICA:

4.1.1.1. Preparación de la muestra..... 125

4.1.1.2 Adquisición de los datos y procesado de los espectres..... 127

4.1.1.3. Integración de picos..... 129

4.1.2 METODOLOGÍA BIOESTADÍSTICA

4.1.2.1 Métodos de pre-tratamiento……... 131

4.1.2.2 Métodos quimiométricos y análisis estadístico

I. Análisis univariante..... 134

II Análisis multivariante..... 136

- Análisis componentes principales (PCA)
- *Heatmap y Hierarchical clustering analysis* (HCA)
- Agrupación *K-means*
- Análisis discriminante por mínimos cuadrados parciales (PLS-DA)

III. Regresión logística y curvas ROC……..... 145

IV. Análisis de correlaciones……..... 148

4.1.2.3 Identificación y cuantificación de metabolitos…. 150

I. Identificación de metabolitos……..... 150

II. Cuantificación metabólica……..... 150

4.1.2.4 Interpretación biológica……..... 151

4.2 METODOLOGÍA CLÍNICA:

Estudios clínicos para el desarrollo de biomarcadores nutricionales

4.2.1 Estudio del efecto de la intervención con un alimento..... 152

4.2.2 Estudio del efecto de la intervención con un complemento alimenticio..... 159

4.2.3 Estudio de patrones alimentarios..... 161

5. RESULTADOS

5.1 BIOMARCADORES NUTRICIONALES TRAS EL CONSUMO DE UN ALIMENTO

I. Estudio de un alimento y del efecto de la matriz alimentaria..... 167

Publicación 1: Vázquez-Fresno R, Llorach R, Alcaro F, Rodríguez MA, Vinaixa M, Chiva-Blanch G, Estruch R, Correig X, Andrés-Lacueva C. 1H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors. *Electrophoresis.* 2012, 33, 2345–2354. doi: 10.1002/elps.201100646.

II. Estudio de la sensibilidad y especificidad de los biomarcadores nutricionales 179

Publicación 2: Vázquez-Fresno R, Llorach R, Urpi-Sarda M, Khymenets O, Bulló M, Corella D, Fitó M, Martínez-González MA, Estruch R, Andres-Lacueva C. An NMR metabolomics approach reveals a combined-biomarkers model in a wine interventional trial with validation in free-living individuals of the PREDIMED study. *Metabolomics [In press]* doi:10.1007/s11306-014-0735-x.

III. Estudio del fenotipo metabólico después del consumo de un alimento..... 195

Publicación 3: Vázquez-Fresno R, Llorach R, Perera A, Rupasri Mandal, Tinahones FJ, Wishart DS, Andrés-Lacueva C. Clinical phenotype clustering in cardiovascular risk patients for the identification of responsive metabotypes after red wine polyphenol intake. [*En proceso de publicación*].

5.2 BIOMARCADORES NUTRICIONALES DE SALUD TRAS LA INGESTA DE COMPLEMENTOS ALIMENTICIOS

Evaluación de un probiótico sobre la huella metabólica en una población de mujeres con mastitis..... 237

Publicación 4: Vázquez-Fresno R, Llorach R, Marinic J, Tulipani S, Garcia-Aloy M, Espinosa-Martos I, Jiménez E, Rodríguez JM, Andres-Lacueva C. Urinary metabolomic fingerprinting after consumption of a probiotic strain in women with mastitis. *Pharmacological Research* 2014 Sep;87:160-5. doi: 10.1016/j.phrs.2014.05.010.

5.3 BIOMARCADORES NUTRICIONALES Y DE SALUD TRAS EL SEGUIMIENTO DE PATRONES ALIMENTARIOS

Determinación de patrones dietéticos en el perfil urinario de una población después de 3 años de seguimiento de intervención..... 249

Publicación 5: Vázquez-Fresno R, Llorach, R, Urpi-Sarda M, Lupianez-Barbero A, Estruch R, Corella D, Fitó M, Arós F, Ruiz-Canela M, Salas-Salvadó J, Andres-Lacueva C. Metabolomic pattern analysis after Mediterranean diet intervention in a non-diabetic population: a 1- and 3-year follow-up in the PREDIMED study. *Journal of Proteome Research* 2015, 14 (1), pp 531–540. DOI: 10.1021/pr5007894

6. DISCUSIÓN GENERAL..... 269

7. CONCLUSIONES..... 285

CONCLUSIONS..... 287

8. REFERENCIAS BIBLIOGRÁFICAS…… 289

9. ANEXOS

9.1 OTRAS PUBLICACIONES EN REVISTAS…… 311

Revisión bibliográfica: Llorach R, Garcia-Aloy M, Tulipani S, Vázquez-Fresno R, Andres-Lacueva C. Nutrimetabolomic strategies to develop new biomarkers of intake and health effects. *Journal of Agricultural and Food Chemistry*. 2012; 60(36):8797-8808. doi: 10.1021/jf301142b.

Publicación 1: Khymenets O, Andres-Lacueva C, Urpi-Sarda M, Vazquez-Fresno R, Mercader Mart M, Reglero G, Torres M, Llorach R. Metabolic fingerprint after acute and under sustained consumption of a functional beverage based on grape skin extract in healthy human subjects. *Food & Function [Accepted]. doi: 10.1039/C4FO00684D*

9.2 OTROS CAPÍTULOS DE LIBROS…….. 348

Capítulo I: Trespalacios MP, Tulipani S, Garcia-Aloy M; Zamora-Ros R, Rotchés-Ribalta M, Rabassa M, Urpi-Sarda M, Boto M, Vázquez R, Llorach R, Andrés-Lacueva C. Nutritional Biomarkers: Applications and Challenges. En: Morrisson JL (editor). *Food Intake: Regulation, Assessing and Controlling.*Nova Science Publishers, Inc.; 2013. ISBN: 978-1-61324-183-7

Capítulo II: Rabassa M, Zamora-Ros R, Tulipani S, Khymenets O, Urpi-Sarda M, Garcia-Aloy M, Boto M, Vázquez R, Rotches-Ribalta M; Chiva G, Trespalacios MP, Llorach R, Andres-Lacueva C. Flavonoids from food and its implication in human health. A: Yamane K, Kato Y (editors): *Handbook of flavonoids: dietary sources, properties and health benefits*. Nova Science Publishers; 2012. ISBN: 978-1-61942-052-6

Capítulo III: Rotches-Ribalta M, Urpi-Sarda M, Tulipani S, Trespalacios P, Rabassa-Bonet M, Boto-Ordóñez M, Vázquez-Fresno R, Garcia-Aloy M, Llorach R, Andres-Lacueva C. Metabolism and pharmacokinetics of resveratrol: sources and food matrix on the bioavailability of resveratrol. A: Delmas D (editor): *Resveratrol: sources, production, and health benefits.* Nova Science Publishers; 2012. ISBN: 978-1-62081-997-5

9.3 COMUNICACIONES EN CONGRESOS……. 353

Resumen

Entender la influencia de la alimentación en la salud y enfermedades humanas es uno de los mayores objetivos de la nutrición moderna. La nutrimetabolómica se define como la disciplina "ómica" que estudia cómo afecta la dieta al conjunto del metaboloma; siendo propuesta como poderosa herramienta para explorar la relación entre nutrición y salud.

Esta Tesis Doctoral tiene como objetivo principal contribuir en la identificación de biomarcadores nutricionales de consumo (biomarcadores de ingesta) así como su impacto en la salud (biomarcadores de efecto), y la huella metabólica que producen sobre el perfil urinario desde distintas aproximaciones metabolómicas mediante Resonancia Magnética Nuclear. Para la consecución de este objetivo se estudió el metaboloma desde la ingesta de diferentes componentes alimentarios (alimento, complemento alimenticio y patrones alimentarios).

En el caso del estudio en un alimento se utilizó vino, vino desalcoholizado y ginebra para evaluar el efecto de la matriz alimentaria de las diferentes fracciones del alimento. El análisis del metaboloma mostró tanto metabolitos provenientes del *food metabolome* (tartrato, etanol, manitol), endógenos (3-metil-2-oxovalerato), y derivados de la acción de la microbiota intestinal (hipurato y 4-hidroxifenilacetato). En cuanto a la matriz alimentaria, se observó una posible interacción entre el alcohol y los biomarcadores relacionados con los metabolitos de la microbiota intestinal. Seguidamente, se propuso estudiar los biomarcadores de ingesta de un alimento, y para ello se detectaron en un estudio de intervención (vino) y se determinó el poder predictivo mediante la evaluación de su sensibilidad y especificidad. Se evaluaron de forma individual, observando finalmente que la combinación de dos de ellos (tartrato+etilglucurónido)

ofrecía un mayor poder predictivo (área bajo la curva [AUC]:90.7%) que individualmente. Se realizó una validación en otra población (estudio observacional), viéndose una reproducibilidad de los resultados (AUC: 92.4%) con una alta sensibilidad (≥84%) y especificidad (>90%) en ambas poblaciones.

Para evaluar sobre el fenotipo metabólico el efecto de una intervención con un alimento, se estratificó una población con alto riesgo cardiovascular atendiendo a sus parámetros clínicos. Se evaluó el efecto de la ingesta de polifenoles del vino en los clúster más discriminantes ("obesos y diabéticos" *vs.* "sanos con riesgo"). Se observó en el metaboloma un comportamiento diferencial para la excreción del 4-hidroxifenilacetato (microbiota intestinal) en el clúster que se clasificó como "obeso y diabético" frente al clúster "sano con riesgo", exhibiéndose en los primeros una posible alteración de la microbiota intestinal después del consumo de vino.

En la evaluación de los patrones alimentarios, se analizó el perfil metabólico de una población al inicio, 1 año y 3 años de seguimiento de un patrón de Dieta Mediterránea (MD) suplementada con aceite de oliva/nueces frente a una dieta baja en grasa. Se detectaron varios metabolitos endógenos relacionados con el metabolismo energético (ciclo de los ácidos tricarboxílicos, β-oxidación), derivados de la microbiota, así como también del *food metabolome*. Asimismo, se pudo asociar ciertos metabolitos con patrones de dieta, viéndose asociadas las dos MD a un mayor consumo de vegetales, mientras que la dieta baja en grasa presentaba una mayor asociación al consumo de carne roja.

En la evaluación del impacto en la salud, se administró un complemento alimenticio (probiótico) a mujeres lactantes con mastitis y se observó una

mejoría y una reducción del estado inflamatorio (seguimiento médico), lo que provocó una deserción voluntaria de fármacos (ibuprofeno y paracetamol), hecho que se reflejó en el metaboloma urinario. También se vio un aumento en la excreción de creatina y de metabolitos derivados de la microbiota intestinal (hipurato y trimetilamina-N-óxido). Esta Tesis Doctoral presenta diferentes estrategias nutrimetabolómicas para el desarrollo y estudio de biomarcadores nutricionales y su impacto en la salud.

Abstract

Understanding the link between nutrition and health is one of the major goals of modern nutrition. Nutrimetabolomics is an "omics" science that studies the effect of diet through the metabolome. It is a powerful tool for exploring the relationship between nutrition and health. The aim of this PhD thesis is to contribute to the identification of biomarkers related to food consumption (biomarkers of intake) and their impact on health (biomarkers of effect).

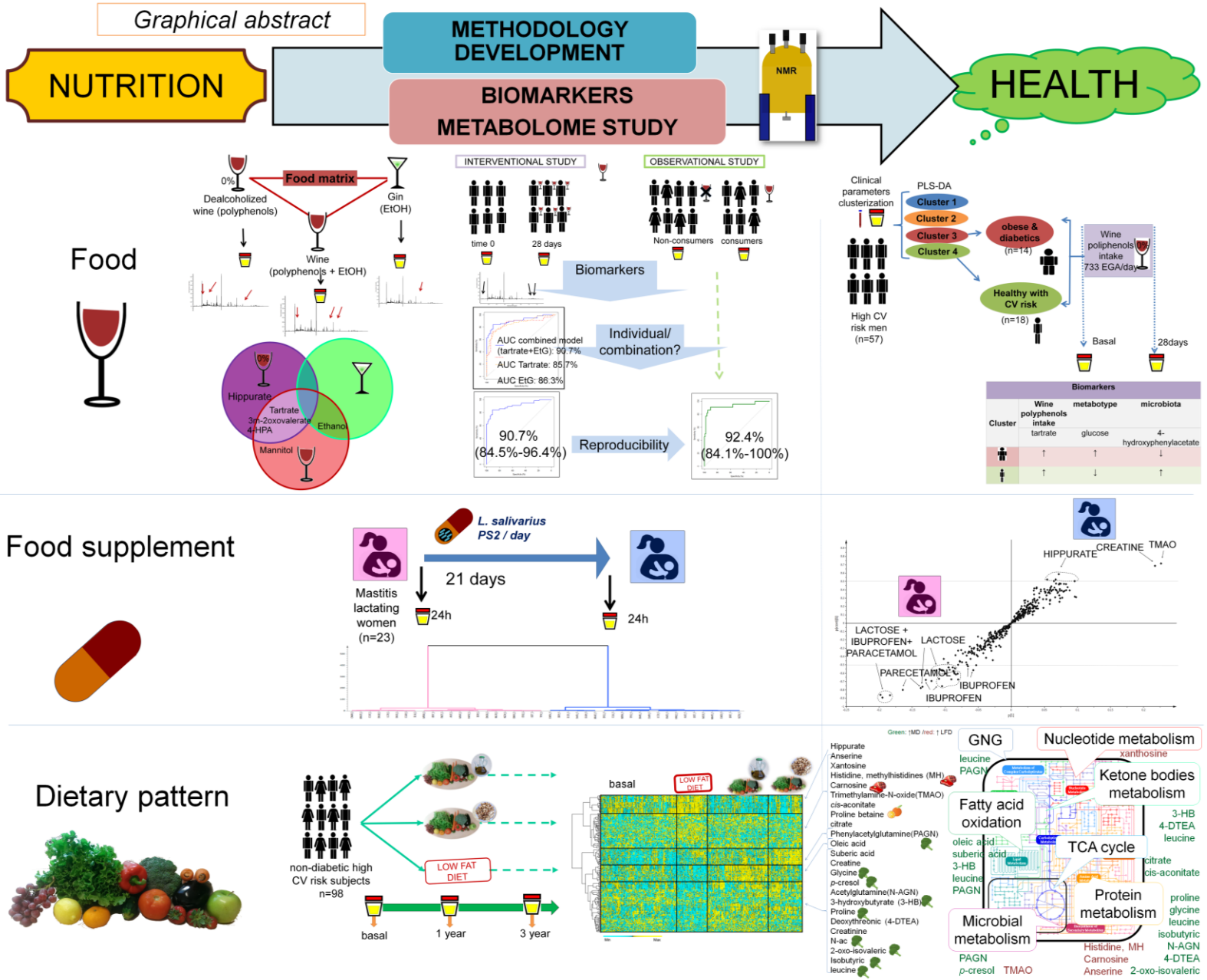
To achieve this goal, the human metabolome was studied by nuclear magnetic resonance (NMR) after the intake of different food components (food, supplements and dietary patterns). Wine, dealcoholized wine and gin were used to evaluate the food matrix effect. Results showed metabolites from the *food metabolome*, endogenous and gut microbiota, pointing to a potential interaction between alcohol and gut microbiota.

To study food exposure biomarkers, the predictive power in the wine interventional study concluded that a model combining two biomarkers (tartrate+ethyl glucuronide) has greater predictive power than the individual markers. Reproducible results also were shown in epidemiological data (AUC>90%). Furthermore, high cardiovascular risk individuals were stratified based on their clinical parameters.

The effect of wine polyphenols intake in the metabolic phenotype for the two most discriminant clusters ("obese-diabetics" vs. "healthier") was evaluated, and different excretion of gut microbiota metabolite 4-hydroxyphenylacetate was observed. Concerning the dietary patterns, the metabolic footprint after 3-years follow-up with Mediterranean diet vs. low-fat pattern was analyzed. Several metabolites (i.e. energy pathway-

metabolites), and dietary patterns association with certain metabolites were also observed.

To assess the impact on health, a probiotic supplement was administered to breastfeeding women with mastitis. The reduction of inflammation (medical test) and voluntary desertion of pharmacological drugs was revealed in the urinary metabolome. This Doctoral Thesis presents different nutrimetabolomics strategies to study nutritional biomarkers and improve the understanding of their impact on human health.


Graphical abstract
NUTRITION
METHODOLOGY DEVELOPMENT
BIOMARKERS METABOLOME STUDY
NMR
HEALTH
Food
0%
Dealcoholized wine (polyphenols)
Food matrix
Gin (EtOH)
Wine (polyphenols + EtOH)
Hippurate
Tartrate
3m-2oxovalerate
4-HPA
Ethanol
Mannitol
INTERVENTIONAL STUDY
OBSERVATIONAL STUDY
time 0
28 days
Non-consumers
consumers
Biomarkers
AUC combined model (tartrate+EtG): 90.7%
AUC Tartrate: 85.7%
AUC EtG: 86.3%
Individual/ combination?
90.7% (84.5%-96.4%)
Reproducibility
92.4% (84.1%-100%)
Clinical parameters clusterization
PLS-DA
Cluster 1
Cluster 2
Cluster 3
Cluster 4
High CV risk men (n=57)
obese & diabetics (n=14)
Healthy with CV risk (n=18)
Wine poliphenols intake 733 EGA/day
Basal
28days
Biomarkers
Cluster
Wine polyphenols intake
metabotype
microbiota
tartrate
glucose
4-hydroxyphenylacetate
Food supplement
L. salivarius PS2 / day
21 days
Mastitis lactating women (n=23)
24h
24h
HIPPURATE
CREATINE
TMAO
LACTOSE + IBUPROFEN+ PARACETAMOL
LACTOSE
PARECETAMOL
IBUPROFEN
IBUPROFEN
Dietary pattern
non-diabetic high CV risk subjects n=98
LOW FAT DIET
basal
1 year
3 year
basal
LOW FAT DIET
Hippurate
Anserine
Xantosine
Histidine, methylhistidines (MH)
Carnosine
Trimethylamine-N-oxide(TMAO)
cis-aconitate
Proline betaine
citrate
Phenylacetylglutamine(PAGN)
Oleic acid
Suberic acid
Creatine
Glycine
p-cresol
Acetylglutamine(N-AGN)
3-hydroxybutyrate (3-HB)
Proline
Deoxythreonic (4-DTEA)
Creatinine
N-ac
2-oxo-isovaleric
Isobutyric
leucine
GNG
leucine
PAGN
Nucleotide metabolism
xanthosine
Ketone bodies metabolism
3-HB
4-DTEA
leucine
Fatty acid oxidation
oleic acid
suberic acid
3-HB
leucine
PAGN
TCA cycle
citrate
cis-aconitate
Protein metabolism
proline
glycine
leucine
isobutyric
N-AGN
4-DTEA
Microbial metabolism
PAGN
p-cresol
TMAO
Histidine, MH
Carnosine
Anserine
2-oxo-isovaleric

INTERÉS

HIGHLIGHT OVERVIEW AND IMPACT OF THE THESIS

Entender el rol de la alimentación en la determinación de la salud y enfermedades humanas es uno de los mayores objetivos de la nutrición moderna. La **metabolómica** en el ámbito de la nutrición, se ha definido como una herramienta útil para la evaluación del estado nutricional de un individuo, el consumo de un alimento, o las consecuencias biológicas derivadas del seguimiento de una intervención nutricional, así como también la posible detección de perturbaciones moleculares inducidas por la dieta y capaces de predecir potenciales implicaciones en la salud (Claus *et al.* 2013).

A diferencias de otras ciencias "-ómicas" como la genómica, la transcriptómica, o la proteómica, la metabolómica es la -ómica que estudia el conjunto de metabolitos; los metabolitos son el resultado final de los genes, que reflejan más directamente el comportamiento de la célula u organismos a nivel del fenotipo. Las variaciones en el metabolismo primario suceden en término de segundos, permitiendo que el metaboloma sea una medida sensible e inmediata del fenotipo de un individuo. Se podría decir que los metabolitos serían como firmas directas de la actividad bioquímica de nuestro organismo (Patti *et al.* 2012).

El conjunto de metabolitos o metaboloma de un individuo está compuesto por diferentes partes o fracciones: (1) el metaboloma endógeno, provenientes del metabolismo de las células y tejidos del individuo; (2) el "*food metabolome*" procedente de los metabolitos derivados de la digestión de los compuestos nutricionales esenciales y no esenciales del metabolismo derivado del individuo (o huésped) y de su microbiota intestinal; (3) el "*drug metabolome*" procedente de compuestos xenobióticos derivados de fármacos, etc. y (4) el metabolismo derivado del ambiente, polución, etc. "*pollutant metabolome*" (Scalbert *et al.* 2014). Solo los dos

primeros: el metaboloma endógeno y el *food metabolome* se ven influenciados por la dieta.

La metabolómica nutricional o **nutrimetabolómica** se define como la disciplina ómica que estudia cómo afecta la dieta al conjunto del metaboloma (Zhang *et al.* 2008), y ha sido propuesta como una poderosa herramienta para explorar la relación entre la nutrición y la salud (McNiven *et al.* 2011).

Como cualquier componente exógeno, el *food metabolome*, puede influir o modular el metaboloma endógeno; y, a su vez, el conjunto del metaboloma puede evaluarse por la detección o cambio en las concentraciones de metabolitos en los biofluídos. La Resonancia Magnética Nuclear (RMN) es una técnica analítica considerablemente utilizada en metabolómica en estudios de evaluación nutricional (Heinzmann *et al.* 2011; O'Sullivan *et al.* 2011; Rasmussen *et al.* 2012). Se caracteriza por ser una técnica robusta, no destructiva y altamente reproducible (Beckonert *et al.* 2007; Bothwell *et al.* 2011). Por tanto, mediante RMN podemos encontrar cambios producidos en el metaboloma debidos a una intervención nutricional, así como el estudio y evaluación de biomarcadores nutricionales fruto de dicha intervención. Un biomarcador nutricional es cualquier indicador bioquímico, funcional o clínico medido en una muestra biológica que refleja el estado nutricional respecto a la ingesta o al metabolismo de los componentes de la dita, así como las consecuencias biológicas de la ingesta alimentaria (Potischman *et al.* 2003)

Los biomarcadores en nutrición y salud se pueden clasificar en: **Biomarcadores de ingesta/exposición o exógenos** propios de un alimento, compuesto bioactivo, componente alimenticio o conjunto de

alimentos que provienen directamente de la digestión, absorción intestinal y biotransformación de éstos; y **biomarcador de efecto o endógenos**; son aquellos que se ven modulados por la dieta y muestran cambios de la respuesta biológica e impacto en la salud.

Los biomarcadores nutricionales de exposición, ante la necesidad de obtener una valoración precisa de la ingesta pueden proporcionar una medida objetiva de la ingesta y estado nutricional (Llorach *et al.* 2012), y ser un importante complemento a los datos dietéticos clásicos a menudo con limitaciones y errores metodológicos.

En el estudio de los biomarcadores nutricionales, es muy importante además, una cuidadosa validación (Andersen 2013; Jenab *et al.* 2009). Sin embargo, hay relativamente pocos alimentos con biomarcadores de ingesta o exposición cuidadosamente validados (Hedrick *et al.* 2012; Scalbert *et al.* 2014). Por ello, es necesaria una adecuada evaluación para considerarlos unos biomarcadores sensibles, específicos y reproducibles en varios tipos de poblaciones (Jenab *et al.* 2009). En este caso, estudiar el poder predictivo y precisión de éstos mediante estrategias como el uso de modelos predictivos mediante curvas ROC es una forma de evaluar la sensibilidad y especificidad de dichos biomarcadores, siendo una estrategia emergente en estudios de evaluación de biomarcadores nutricionales (Garcia-Aloy *et al.* 2014; Rothwell *et al.* 2014).

En cuanto a los biomarcadores de efecto y salud, mediante la nutrimetabolómica, podremos medir aquellos metabolitos que se hayan visto modulados por la dieta, que hayan producido un cambio bioquímico y fisiológico en el organismo, con un posible impacto en la salud (McNiven *et al.* 2011). Además, y siguiendo en este ámbito, la nutrimetabolómica puede ser utilizada para caracterizar el fenotipo metabólico de los individuos ya sea debidos interacciones metabólicas con

bacterias comensales y simbióticas de la microbiota intestinal, o por factores ambientales y de comportamiento, incluyendo así también las preferencias nutricionales. Por ello, permite evaluar la variabilidad intra- e inter-individual de una misma población y el resultado producido tras una intervención nutricional.

En esta Tesis Doctoral se ha realizado el estudio de biomarcadores nutricionales de ingesta y efecto en estudios de intervención con un alimento, un complemento alimenticio o unos patrones dietéticos concretos y, a su vez, se ha estudiado la huella metabólica que producen en el perfil metabólico urinario mediante resonancia magnética nuclear. Se ha evaluado el efecto de las diferentes fracciones de una bebida como en este caso es el vino (contenido polifenólico y alcohol); se ha determinado el poder predictivo de biomarcadores nutricionales mediante la evaluación de la sensibilidad y especificidad en estudios intervencionales y observacionales. Igualmente, no solo se han evaluado los biomarcadores de ingesta sinó también los biomarcadores de efecto en un alimento, en un complemento alimenticio (en este caso un probiótico) y el perfil metabólico después de una intervención de larga duración con tres patrones dietéticos concretos. Así como también se ha diferenciado el fenotipo metabólico dentro de una población y explorado el efecto de la intervención nutrición sobre este fenotipo.

Se presenta una Tesis Doctoral, por tanto, dónde se consigue englobar las diferentes estrategias establecidas en nutrimetabolómica (Llorach *et al.* 2012): (a) la evaluación de una intervención nutricional, (b) la exposición dietética y la monitorización del consumo de un alimento/complemento alimenticio o conjunto de alimentos (patrón nutricional) (c) el fenotipado e impacto metabólico de una dieta sobre la salud.

HIGHLIGHT OVERVIEW AND IMPACT OF THE THESIS

Understanding the role of nutrition in human health and disease is one of the major goals of modern nutrition. Metabolomics in the field of nutrition "Nutrimetabolomics" is a useful tool for the evaluation of the nutritional status of individuals, food consumption, or the biological consequences of following a nutritional intervention, as well as the possible detection of molecular perturbations induced by diet, and is able to predict potential health implications (Claus *et al.* 2013).

Metabolomics differentiates itself from the other "*-omics*" families of sciences (such as genomics, transcriptomics and proteomics) by studying metabolites (small molecules) or sets of metabolites. Metabolites are the end result of genes, which directly reflect the behavior of cells or organisms at a phenotype level. Since variations occur in the primary metabolism within seconds, the metabolome is an incredibly sensitive measure of the immediate phenotype of an individual. It could be said that metabolites are the direct signatures of the biochemical activity of our organism (Patti *et al.* 2012).

The metabolome, or the set of metabolites of an individual, is composed of different parts: (1) the endogenous metabolome, derived from the metabolism of cells and tissues that take part of an individual; (2) the "food metabolome", metabolites derived from digestion of essential and non-essential nutritional compounds from the individual (host) and gut microflora metabolism; (3) the "drug metabolome", xenobiotic compounds from derivatives of drugs, nutraceuticals, etc. and (4) the metabolic derivatives of environmental pollution the "pollutant metabolome" (Scalbert *et al.* 2014). Only the endogenous metabolome and food metabolome are influenced by diet.

Nutrimetabolomics is the discipline that studies how diet affects the whole metabolome (Zhang *et al.* 2008) and has been proposed as a powerful tool to explore the relationship between nutrition and health (McNiven *et al.* 2011).

Like any exogenous component, the *food metabolome*, can influence and modulate the endogenous metabolome. The entire metabolome can be assessed by detecting changes in the concentrations of metabolites in biofluids (blood, urine, saliva, etc.). The Nuclear Magnetic Resonance (NMR) is an analytical technique widely used in metabolomics studies of nutritional assessment (Heinzmann *et al.* 2011; O'Sullivan *et al.* 2011; Rasmussen *et al.* 2012) and is a robust, non-destructive and highly reproducible technique (Beckonert *et al.* 2007; Bothwell *et al.* 2011). Using NMR we can search for changes in the metabolome due to nutritional intervention studies and evaluate the resulting nutritional biomarkers. Nutritional biomarkers are any biochemical, functional or clinical indicators measured in biological samples. They reflect the nutritional status regarding intake or metabolism of dietary compounds and the biological consequences of food intake (Potischman *et al.* 2003).

Biomarkers of nutrition and health can be classified into two groups. (1) Biomarkers of intake/exposure (exogenous metabolites): bioactive compounds, food components, or group of foods that come directly from digestion, intestinal absorption and biotransformation. (2) Biomarkers of effect (endogenous): intrinsic biomarkers modulated by diet that show changes in biological response may cause or reflect physiological changes.

Given the need for an accurate assessment of intake, nutritional biomarkers of exposure can provide an objective measure of intake and nutritional status (Llorach *et al.* 2012). Nutrimetabolomics can be an important complement to conventional dietary data, which has its limitations and methodological errors.

When studying nutritional biomarkers it is important to carefully validate each biomarker (Andersen 2013; Jenab *et al.* 2009). However, there are relatively few carefully validated food intake/exposure biomarkers (Hedrick *et al.* 2012; Scalbert *et al.* 2014). A proper biomarker assessment will consider specific, sensitive and reproducible biomarkers in several populations(Jenab *et al.* 2009). To evaluate the predictive power and accuracy of biomarkers as model predictors, receiver operator curves (ROC curves) are commonly used. They are an emerging strategy in evaluating the sensitivity and specificity in nutritional biomarker studies (Garcia-Aloy *et al.* 2014; Rothwell *et al.* 2014).

Biomarkers of effect and health can be discovered using nutrimetabolomics by measuring metabolites that produce a biochemical and physiological change in the body. Nutrimetabolomics can further characterize the metabolic phenotype of individuals through detecting metabolic interactions with commensal bacteria and symbiotic intestinal microbiota, environmental and behavioral factors, including nutritional preferences. The comprehensiveness of nutrimetabolomics allows for both *intra-* and *inter-*individual variability studies before, during, and after nutritional interventions.

This Thesis is focused on the study of nutritional biomarkers of intake and effect in intervention studies with a food, a food supplement or specific dietary patterns through the urinary metabolic fingerprint by

nuclear magnetic resonance. To measure the effect of biomarkers of intake, different fractions of wine were evaluated by measuring the alcohol and polyphenolic content. The predictive power of nutritional biomarkers has been determined by evaluating the sensitivity and specificity in interventional and observational studies. Similarly, biomarkers of effect were evaluated by studying the metabolites of a food, a probiotic food supplement and a long term intervention with three specific dietary patterns. Differential metabolic phenotypes were discovered in a population and the effect of nutritional intervention on these phenotypes was explored.

The following Doctoral Thesis encompasses different strategies established in nutrimetabolomics (Llorach *et al.* 2012): (a) the assessment of nutritional intervention, (b) the dietary exposure and consumption monitoring of a food/food supplement or food group (nutritional pattern) (c) the phenotype and metabolic impact of diet on health.

ANTECEDENTES BIBLIOGRÁFICOS

Los antecedentes bibliográficos que se presentan a continuación como parte introductoria de la Tesis Doctoral permiten situar la presente tesis en el contexto científico en el que se desarrolla. En primer lugar se sitúa la metabolómica en todo su conjunto, para después centrarse en los dos puntos importantes que rodean esta tesis: nutrición y metabolómica. Ambos conceptos desembocan en el término nutrimetabolómica. Dentro de la nutrimetabolómica se debe hablar del concepto de biomarcador, su clasificación, y de los biomarcadores utilizados en nutrimetabolómica. Seguidamente, creo imprescindible hacer una pincelada sobre los fundamentos en los que se basa la técnica para entender mejor el funcionamiento y análisis con los que se han obtenido los resultados de la presente Tesis. Finalmente, y para acabar este apartado introductorio, se presenta la aplicación de la nutrimetabolómica en la búsqueda de biomarcadores. Está aplicación se expone mediante dos ejemplos consistentes en dos capítulos de libro. En ellos se presenta una recopilación de estudios dónde se exponen una serie de biomarcadores mediante la aproximación nutrimetabolómica.

2. 1. LA METABOLÓMICA

2.1.1 INTRODUCCIÓN

En esta era post-genómica, dónde se ha generado una especial necesidad de asignar una función a cada gen con el objetivo de encontrar potenciales dianas terapéuticas y descubrir nuevos biomarcadores, han ido creciendo otras ciencias "ómicas" también, como es el caso de la Metabolómica.
"*Omics*" proviene de la palabra "ome" que en latín significa "masivo". En este momento concurren más de 200 disciplinas que contienen este sufijo, algunos ejemplos serían: la genómica (referente a genes), transcriptómica (ARN transcrito), proteómica (proteínas), metabolómica (metabolitos), lipidómica (lípidos), glicómica (oligosacáridos), fluxómica (mapas de flujo), fenómica (comportamiento celular o fenotipo) (Lindon 2007).

Existe una conexión directa entre la metabolómica y el fenotipo. Los metabolitos son el resultado final de los genes y reflejan más directamente el comportamiento de la célula a nivel del fenotípico (**Figura 2.1**). Algunas de las razones (Kell 2006) por las que la metabolómica se diferenciaría de otras "ómicas" son:

- El número de genes en el genoma humano es de 31.896 genes (hhtp://eugenes.org/), con los consiguientes procesos de transcripción y "*splicing*" se pueden llegar a producir alrededor de 10^6 proteínas diferentes (Oh *et al.* 2004); por otra parte, actualmente se han detectados aproximadamente unos 7.000 metabolitos en biofluídos en humanos (Wishart *et al.* 2013), aunque compuestos esperables o susceptibles de ser encontrados en esos biofluídos, pueden ser unos 50.000 (Wishart *et al.* 2013).

- Uno de los problemas en transcriptómica en muchos casos es la dificultad de relacionar los cambios en la expresión génica con los puntos finales del proceso, como aquellos utilizados en el diagnóstico de enfermedades o de evaluación farmacológica. En cambio, el perfil metabólico observa los efectos bioquímicos en un organismo, y esto representa una aproximación más cercana a los procesos finales reales en la cascada biológica (Lindon 2007).
- A diferencia de una proteína o de un transcrito, un metabolito es igual en todos los organismos (Kell *et al.* 2005).
- El perfil metabólico es un análisis más rápido que los análisis proteómicos y transcriptómicos. Además, se ha estimado que los análisis metabolómicos son de 2 a 3 veces más económicos que los experimentos proteómicos y transcriptómicos (Dunn *et al.* 2005).
- Por último, la tecnología utilizada en este campo es muy versátil y genérica.

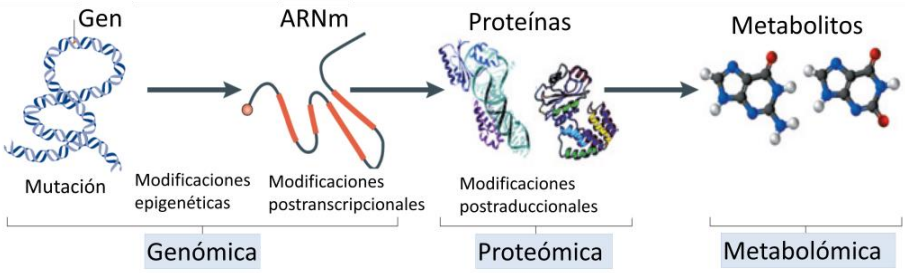


Figura 2.1. **Cascada Ómica**. Genes y proteínas están sujetos a mutación, procesos de regulación epigenética y modificaciones post-traduccionales, respectivamente. Los metabolitos representan los productos finales de la cascada, los cuales están más cercanos al fenotipo. Adaptación de Patti *et al.* 2012.

A menudo, existe una controversia en el uso de los términos **metabonómica** y **metabolómica**. El término ***metabonómica*** fue definido formalmente por primera vez en 1999 por J. Nicholson *et al.*

(Nicholson *et al.* 1999) como "la medida cuantitativa de las respuestas dinámicas metabólicas multi-paramétricas de los sistemas vivos a los estímulos fisiopatológicos o modificaciones genéticas". Por otro lado, el término ***metabolómica*** fue introducido por Oliver Fiehn (Fiehn 2002) con la siguiente definición: "el análisis amplio y cuantitativo de los metabolitos en un sistema". Esto indica que mientras la metabonómica se centra en los cambios de los niveles de ciertos metabolitos con la influencia de los estímulos externos, la metabolómica trata la identificación no sesgada de los metabolitos de un sistema biológico. Posteriormente, en 2009 David Wishart complementó la definición de metabolómica como "el estudio sistemático de un gran número de metabolitos de bajo peso molecular generados por procesos celulares bajo condiciones específicas" (Wishart 2009) considerando los conceptos de ambas definiciones anteriormente mencionadas. Actualmente, las metodologías se superponen y ambos términos se utilizan indistintamente entre los miembros de la comunidad científica.

La información proporcionada por ambos conceptos nos da el término "**metaboloma**", que se define como "el conjunto de metabolitos de bajo peso molecular (<1.500 daltons) presente en un fluido biológico, en una célula u organismo, bajo unas condiciones fisiológicas y/o perturbaciones determinadas (variaciones genéticas, estados patológicos o respuestas a estímulos externos)" (Kell *et al.* 2005). El metaboloma está formado por diferentes partes o fracciones: (1) el metaboloma endógeno, provenientes del metabolismo de las células y tejidos del individuo; (2) el "*food metabolome*" procedente de los metabolitos derivados de la digestión de los compuestos nutricionales esenciales y no esenciales, del metabolismo derivado del individuo (o huésped) y de su microbiota intestinal; (3) el "*drug metabolome*" procedente de compuestos xenobióticos derivados de

fármacos, etc. y (4) el metabolismo derivado del ambiente, polución, etc. "*pollutant metabolome*" (Scalbert *et al.* 2014). Dónde solo los dos primeros: el **metaboloma endógeno** y el ***food metabolome*** se ven influenciados por la dieta, y en los cuales nos centraremos con especial atención a lo largo de esta Tesis Doctoral.

2.1.2 DIAGRAMA DE FLUJO EN METABOLÓMICA

El diagrama de flujo de trabajo que se aplica comúnmente en metabolómica, una vez realizada la hipótesis de partida y establecido el diseño del estudio, se categoriza por 5 pasos principales (**Figura 2.2**):

- Recolección y preparación de las muestras biológicas
- Adquisición de los datos
- Análisis de los datos
- Identificación metabólica
- Interpretación biológica

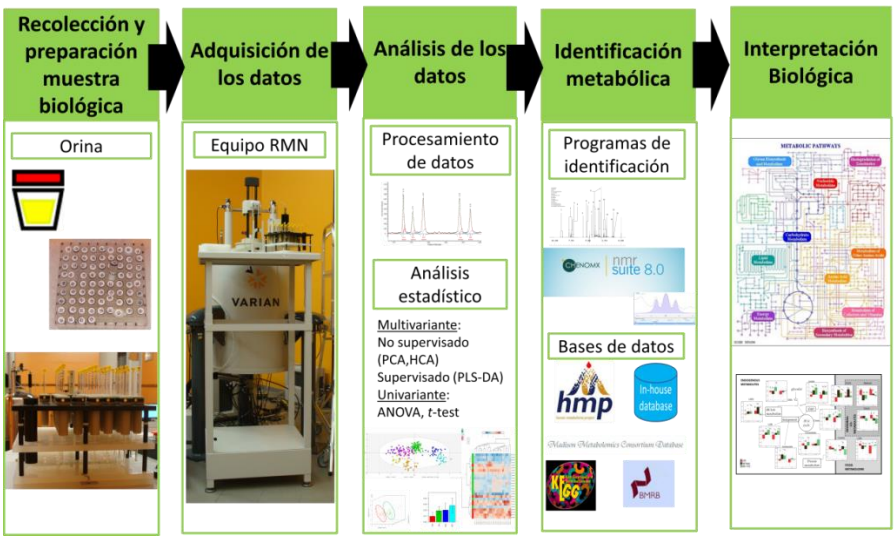


Figura 2.2. Diagrama de flujo del proceso de trabajo en metabolómica

A continuación, se describen en detalle:

- Recolección de los biofluídos y preparación de las muestras

Se utilizan gran variedad de biofluídos para estudios metabolómicos desde la orina, el plasma, la saliva, fluidos seminales, líquido amniótico, fluido cerebroespinal, líquido sinovial, fluidos digestivos, aspirados pulmonares (Claus *et al.* 2013), siendo la orina y la sangre los menos invasivos (Gibney *et al.* 2005). Además, las aguas fecales ofrecen la posibilidad de estudiar los metabolitos de la microbiota intestinal.

La preparación de las muestras consiste en la extracción de los analitos provenientes de las matrices biológicas, con la consiguiente adecuación de la muestra a la técnica analítica correspondiente, la eliminación de los componentes que puedan interferir en el análisis, etc. (Beckonert *et al.* 2007; Dettmer *et al.* 2007). Los metabolitos que se encuentran en bajas concentraciones requieren una concentración previa de la muestra en el procedimiento de extracción para conseguir llegar a los límites de detección de la técnica empleada en cada caso.

- Adquisición de los datos: análisis dirigido y análisis no dirigido

Para la adquisición de los datos se utilizan habitualmente dos aproximaciones metabolómicas: La **aproximación dirigida** o **perfil metabólico ("*metabolic profiling*")** permite el análisis cuantitativo de un grupo de metabolitos predefinido pertenecientes a una determinada clase de compuestos o a una ruta metabólica concreta. Cada vez más estas aproximaciones dirigidas son complementadas con aproximaciones no dirigidas. Además, las aproximaciones no dirigidas, mediante la aportación de información cualitativa o semi-cuantitativa, tienen la potencialidad de generar un marcado aumento en la identificación de potenciales biomarcadores.

Las **aproximaciones no-dirigidas o huella metabólica ("*metabolic fingerprinting*")** pretenden cubrir el metaboloma tan ampliamente como

sea posible, con la detección del máximo número de metabolitos incluyendo aquellos metabolitos poco caracterizados o desconocidos (en inglés denominados "*unknowns*"). Por tanto, el enfoque no dirigido nos da una visión más amplia de forma cualitativa o semi-cuantitativa del metaboloma, con el objetivo de detectar el máximo número de metabolitos en una muestra, incluyendo aquellos poco caracterizados o totalmente desconocidos. Esta técnica es una herramienta necesaria en la búsqueda de biomarcadores y es entorno a la que gira la presente Tesis Doctoral.

El metaboloma incluye una gran variedad de clases de compuestos tanto endógenos como exógenos, con diferencia de tamaño, polaridad y concentración. Por ello, una sola técnica analítica no abarca o no es representativa de la diversidad que alberga el metaboloma. Las técnicas más comúnmente utilizadas para la adquisición de datos en metabolómica son la espectrometría de masas (en inglés *Mass Spectrometry*, MS) y la Resonancia Magnética Nuclear (RMN), muchas veces utilizadas de forma combinada (Zhang *et al.* 2012). La primera de ellas, normalmente va combinada con otras técnicas de separación analítica como la cromatografía de gases (de sus siglas en inglés GC-MS) o la cromatografía líquida (de sus siglas en inglés LC-MS). Por otro lado, la RMN es una técnica con una sensibilidad más limitada pero con una alta reproducibilidad de los resultados. La combinación de ambas técnicas analítica proporciona una mayor recopilación de información y sensibilidad en el análisis (Lanza *et al.* 2010).

- Análisis de datos, identificación e interpretación biológica

Los análisis metabolómicos generan una gran cantidad de datos. Estos datos pueden ser tratados por análisis estadísticos univariantes (*t*-student, ANOVA [test paramétricos], Mann Whitney, Wilcoxon [test no paramétricos], etc.) o, por análisis estadísticos multivariantes, en estos últimos, el objetivo es de reducir la dimensionalidad de los datos. Dentro de los análisis multivariantes podemos encontrar análisis mediante métodos supervisados y análisis mediante métodos no supervisados (Goodacre *et al.* 2004). Los métodos no supervisados como el análisis de componentes principales (en inglés *Principal Component Analysis*, PCA) o el análisis de agrupación jerárquica (en inglés, *Hierarchical Clustering Analysis*, HCA), determinan patrones de datos de acuerdo con sus propiedades y sin conocimiento previo de la clase a la que pertenecen. Son también utilizados para la identificación de agrupamientos o "*clusters*" dentro de una muestra poblacional (Madsen *et al.* 2010). Dentro de los métodos supervisados, entre otros, se incluyen: el análisis discriminante por mínimos cuadrados parciales (en inglés, *Partial Least Square Discriminant Analysis*, PLS-DA), el análisis discriminante por mínimos cuadrados parciales ortogonal (del inglés, *Orthogonal Partial Least Square Discriminant Analysis*, OPLS-DA), o el análisis discriminante por mínimos cuadrados parciales con el filtro de corrección de señales ortogonales (en inglés *Orthogonal Signal Correction –Partial Least Square Discriminant Analysis*, OSC-PLS-DA).

Una vez realizado el análisis de los datos, el siguiente paso es la identificación de los metabolitos discriminantes o significativos. La identificación metabólica se realiza mediante una serie de aproximaciones analíticas y computacionales. Para la MS, se pueden realizar experimentos adicionales como experimentos de fragmentación en tándem (MS/MS) o fragmentación multiple (MS^n); para RMN, experimentos de dos

dimensiones 2D, como *COSY, TOCSY, HSQC,* etc.; y en ambas técnicas analíticas siendo comparados estructuralmente con estándares si están disponibles, o con información disponible que proporcionan bases de datos metabolómicas (Cui *et al.* 2008; Degtyarenko *et al.* 2008; Fahy *et al.* 2007; Smith *et al.* 2005; Taguchi *et al.* 2007; Ulrich *et al.* 2008; Wang *et al.* 2009; Williams 2008; Wishart *et al.* 2013). A parte de estas bases de datos que corresponden mayoritariamente a compuestos químicos, también hay otras bases de datos correspondientes a rutas metabólicas dónde se pueden situar estos metabolitos en las rutas, y ayudar a la interpretación biológica de los resultados obtenidos (Frolkis *et al.* 2010; Kanehisa *et al.* 2006; Krummenacker *et al.* 2005; Pico *et al.* 2008).

2.1.3 RESONANCIA MAGNÉTICA NUCLEAR.

Antecedentes de la técnica analítica

La RMN es una técnica utilizada en metabolómica principalmente por ser una técnica cualitativa y cuantitativa, no destructiva y altamente reproducible que permite la detección de metabolitos presentes en concentraciones relativamente altas (μM-mM) en varios biofluídos (p.ej. orina, plasma, suero y saliva) y tejidos (Beckonert *et al.* 2007). Las principales limitaciones de esta técnica son la menor sensibilidad comparado con la MS y un mayor volumen de muestra requerido para el análisis. Una particularidad de esta técnica está en el proceso de identificación y es que tiene la ventaja de que la mayoría de metabolitos tienen una huella de desplazamiento químico característica o única, es decir, mediante RMN es muy difícil encontrar dos compuestos con idénticas características (idéntico número de picos, idéntico desplazamiento químico, intensidades de los picos, constante de acoplamiento) (Wishart 2009).

Brevemente, la espectrometría de masas consiste en la separación de los iones (cargados en modo positivo o negativo) de acuerdo con su relación masa/carga (m/z). Al igual que para RMN, la MS también tiene una serie de desventajas, principalmente: requiere una extensa preparación para la extracción de los metabolitos dependiendo de la matriz biológica con la consiguiente posible pérdida de ciertos compuestos en el proceso. Estas series de ventajas e inconvenientes para cada técnica se presentan a continuación (**Tabla 2.1**).

Tabla 2.1. Comparativa de las dos técnicas analíticas utilizadas en metabolómica

RMN		**MS**	
VENTAJAS	**DESVENTAJAS**	**VENTAJAS**	**DESVENTAJAS**
✓Preparación de la muestra rápida y sencilla	×Baja sensibilidad (bajo límite de detección: ~1-5μM) -y selectividad	✓ Mayor sensibilidad (mayor número de metabolitos 500-1000)	×Necesita separación cromatográfica previa (GC-, LC-)
✓No requiere separación cromatográfica previa	×Volumen de muestra relativamente grande (~500μl)	✓ Pequeños volúmenes de muestra	×Puede requerir larga preparación muestra para extracción de metabolitos, depende matriz biológica, posible pérdida de compuestos
✓Mayor robustez, no sesgada	× No puede detectar sales ni iones inorgánicos	✓ Muy flexible	× Destructiva
✓Alta reproducibilidad	×No puede detectar compuestos no protonados	✓Capacidad detectar gran parte del metaboloma	
✓Versátil. Es posible el análisis directo de sólidos y estudios *in-vivo*	× control del pH		
✓ No requiere derivatización			
✓No destructiva			

Extraído de Álvarez-Sánchez 2012; Wishart 2008; Wishart 2009. GC: cromatografía en gas; LC: cromatografía líquida.

2.1.3.1 Principios físicos de la técnica analítica

La RMN permite la detección en una muestra, de todos los compuestos que contienen protones (^{1}H). El protón es el núcleo más utilizado en RMN debido a su gran abundancia natural (~99.985%), (Moco *et al.* 2007). Generalmente los estudios realizados en metabolómica mediante RMN son altamente reproducibles y en muchos casos solo se necesita una única réplica analítica por cada tiempo (Beckonert *et al.* 2007). La RMN ha jugado un papel central en la comprensión del metabolismo y los procesos metabólicos durante más de 30 años (Wilson *et al.* 1974). De hecho, tanto *in vivo* como *in vitro* han sido utilizados experimentos de RMN de ^{1}H, ^{13}C, ^{31}P en miles de estudios de metabolismo microbiano, animal, o vegetal (Shulman *et al.* 2001). El uso inicial de la RMN en estos estudios metabólicos ha permitido a la RMN ser una de las primeras tecnologías adoptadas en los primeros días de la metabolómica y la metabonómica (Nicholson *et al.* 1999; Reo 2002).

La espectroscopia de RMN se basa en medir la absorción de la radiación electromagnética en la región de radiofrecuencias, de alrededor de 4 a 900 MHz. En contraste con la absorción ultravioleta, visible e infrarroja, los núcleos de los átomos son los que participan en el proceso de absorción en vez de los electrones exteriores. Para que en los núcleos se formen los estados de energía que hagan posible la absorción, es necesario colocar el analito en un campo magnético intenso (Skoog 2007). La técnica de RMN utiliza ondas de radiofrecuencia para proporcionar información sobre los núcleos magnéticos. El término espectroscopia se utiliza para describir cualquier técnica en la que se usa la radiación electromagnética, y la espectroscopia por RMN es solo una entre un rango de métodos espectroscópicos (**Figura 2.3**).

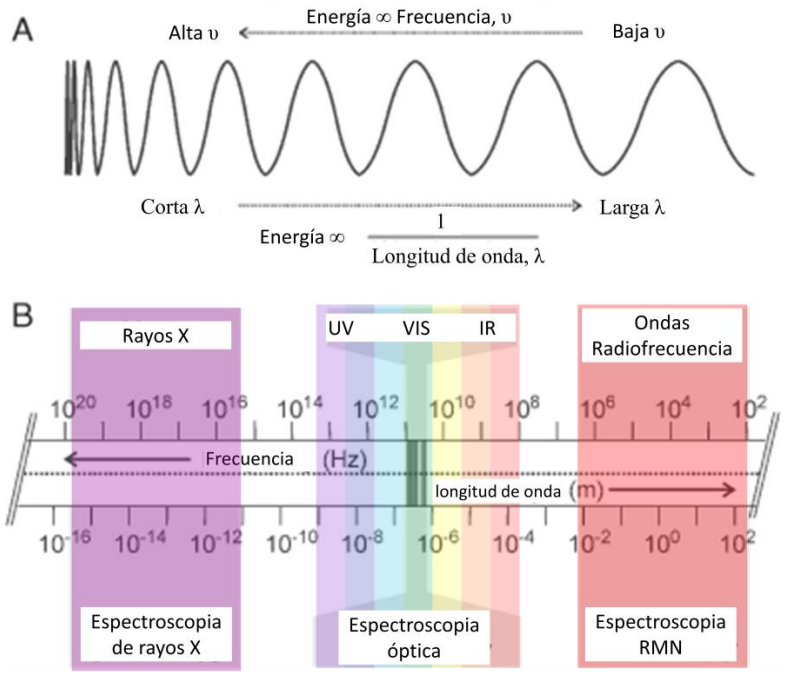


Figura 2.3. Propiedades físicas de los espectros electromagnéticos. (**A**) La radiación electromagnética se encuentra empaquetada en fotones. Cada fotón tiene una frecuencia asociada *ν,* y longitud de onda, λ. La materia solo puede absorber radiación electromagnética a longitudes de onda discretas, debido a que la energía, E, de cualquier haz de radiación está relacionado con la longitud de onda, λ, por una constante, h, en la fórmula E= h/λ. (**B**) Diferentes regiones del espectro de radiación electromagnética se usan para diferentes tipos de espectroscopia. UV: ultravioleta; Vis: visible; IR: infrarroja; RMN, resonancia magnética nuclear (Adaptación de Bothwell *et al.* 2011).

La técnica se basa en que los ^{1}H en diferentes moléculas exhiben diferentes frecuencias de absorción. Por tanto, mide el magnetismo nuclear o el cambio en el magnetismo nuclear de una molécula. El funcionamiento es mediante la medición de la absorción de luz (ondas de radiofrecuencia, RF) debido a los cambios en la orientación de los espines nucleares, y para ello se requieren grandes campos magnéticos (300MHz, 400MHz, 500MHz, 600MHz, 800MHz). Se caracteriza porqué cada

compuesto puede definirse por un único patrón característico en el espectro de RMN.

La finalidad de esta técnica es determinar las estructuras de los compuestos orgánicos. Esta técnica puede usarse sólo para estudiar núcleos atómicos con un número impar de protones o neutrones (o ambos), esto se da en los átomos de ^{1}H, ^{13}C, ^{19}F y ^{31}P. Este tipo de núcleos son magnéticamente activos, es decir, poseen espín, igual que los electrones, ya que los núcleos poseen carga positiva y poseen un movimiento de rotación sobre un eje que hace que se comporten como pequeños imanes. Por ello, estos átomos mencionados anteriormente son los más utilizados en experimentos de RMN, especialmente el ^{1}H, por su gran abundancia en la naturaleza.

En ausencia de un campo magnético, los espines nucleares se orientan al azar (**Figura 2.4a**). En cambio, cuando una muestra se coloca en un campo magnético, los núcleos con espín positivo se orientan en la misma dirección del pulso de energía, en un estado de mínima energía denominado estado de espín α, mientras que los núcleos con espín negativo se orientan en dirección opuesta a la del pulso de energía, en un estado de mayor energía denominado estado de espín β (**Figura 2.4b**). Existen más núcleos en el estado de espín α que en el β pero aunque la diferencia de población no es enorme sí que es suficiente para establecer las bases de la espectroscopia de RMN, que son, la diferencia de energía entre los dos estados de espín α y β, dependiendo de la fuerza del campo magnético aplicado (B_0). Cuanto mayor sea el campo magnético, mayor diferencia energética habrá entre los dos estados de espín, por ello serán mejores grandes campos magnéticos (**Figura 2.4c**).

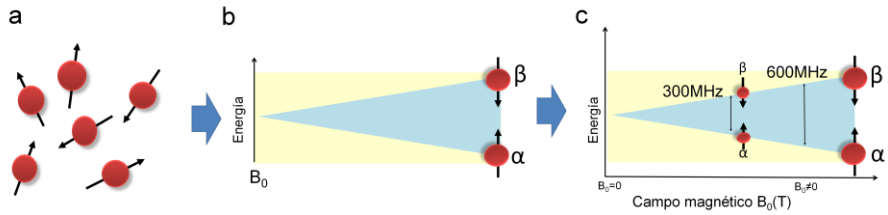


Figura 2.4. (a) Espines orientados al azar; (b) muestras bajo un campo magnético (B_0), núcleos con espín positivo se orientan en la misma dirección que el campo (espín α), núcleos con espín negativo se orientan en dirección opuesta a la del campo magnético, en un estado de mayor energía (espín β). (c) dos estados de espín tienen la misma energía cuando el campo externo es cero, pero divergen a medida que el campo aumenta; a mayor intensidad de campo, mayor separación energética entre los dos estados.

Un tubo que contiene un compuesto orgánico o mezcla de compuestos se hace girar alrededor de su eje vertical, y el campo magnético se mantiene constante mientras es irradiada brevemente por un pulso intenso de radiación. En aquel momento, los núcleos en el estado de espín α tienden a ir al estado β. Cuando cesa la onda irradiante, los núcleos vuelven a su estado inicial emitiendo ondas de RF, cuya frecuencia depende de las diferencias de energía (ΔE) entre los estados de espín α y β. Esa señal de RF es en el dominio del tiempo, es decir, la intensidad de esta frecuencia disminuye con el tiempo a medida que todos los núcleos vuelven a su estado inicial. Ésta se denomina señal de decaimiento libre de inducción (FID, por sus siglas en inglés *Free Induction Decay*), la cual proporciona información sobre la muestra irradiada. La FID puede detectarse con una bobina radiorreceptora. La señal de decaimiento libre de inducción (intensidad respecto a tiempo) es recogida en un ordenador. Una vez recolectados las FID, mediante la Transformada de Fourier la intensidad respecto a tiempo se transforma matemáticamente a intensidad respecto a frecuencia, obteniendo así el espectro común de RMN (**Figura 2.5**)(Bothwell *et al.* 2011).

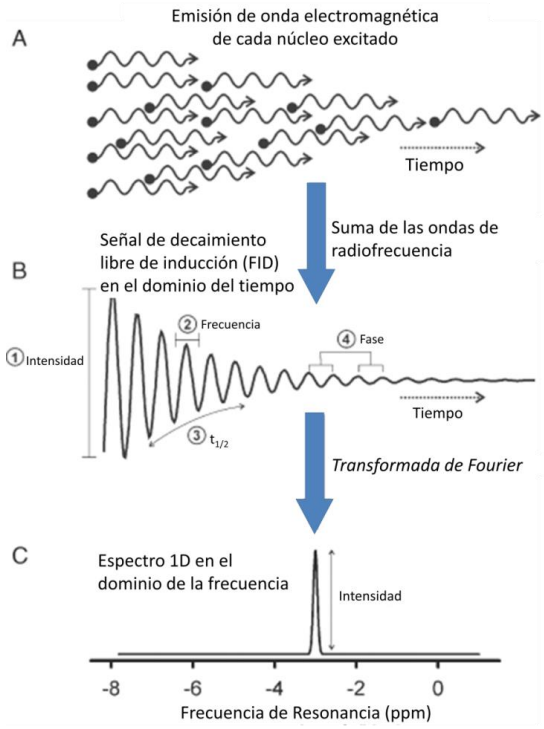


Figura 2.5. Construcción de las señales de decaimiento (del inglés, *Free induction decay, FID*) en un espectro unidimensional (1D) sobre los cuatro parámetros comunes de la RMN. (**A**) Núcleos (círculos negros) que han sido excitados a un estado de espín superior, los cuales se relajaran de nuevo con el tiempo (eje x) a una menor estado de energía a través de la emisión de fotones de radiación electromagnética (líneas onduladas). (**B**) Cuando estos fotones de radiación electromagnética son agregados a un conjunto de átomos de relajación, el decaimiento en la intensidad de señal emitida de la radiación electromagnética en el tiempo producirá la FID. Las FIDs se pueden describir utilizando estos cuatro parámetros: (1) intensidad, (2) frecuencia, (3) vida media o $t_{1/2}$ y (4) fase. La RMN es una de las pocas técnicas espectroscópicas que permite que se extraiga la información de todos estos cuatro parámetros. (**C**) Utilizando la técnica denominada transformación de Fourier, las FIDs, o los gráficos de intensidad *versus* tiempo en el dominio del tiempo, se convierten en gráficos de intensidad *versus* frecuencia en el dominio de frecuencia. Adaptación de Bothwell *et al.* 2011.

Los núcleos, en realidad, como pueden ser los protones o los carbonos que forman las moléculas orgánicas, no se encuentran aislados sino que están rodeados de electrones que los protegen parcialmente del campo magnético externo al que se ven sometidos. Los electrones se mueven

generando un pequeño campo magnético inducido que se opone al campo magnético externo. El resultado de este hecho es que el campo magnético que realmente llega al núcleo es más débil que el campo externo, por tanto, se dice que el núcleo está protegido o apantallado. Este apantallamiento es muy importante desde el punto de vista experimental ya que el campo magnético efectivo que siente un protón dentro de una molécula es siempre menor que el campo externo, y por lo tanto, para que el núcleo entre en resonancia dicho campo externo debe ser mayor. Si todos los protones (^{1}H) de una molécula orgánica estuvieran apantallados de igual forma, todos entrarían en resonancia con la misma combinación de frecuencia y campo magnético. Sin embargo, los protones se hallan dentro de entornos electrónicos diferentes y, por tanto, se encuentran diferentemente protegidos o apantallados. Por lo general, los efectos de protección, o apantallamiento, de las nubes electrónicas que rodean a cada protón son diferentes, lo que provoca diferentes frecuencias de emisión. El resultado es un espectro de diversas frecuencias donde cada conjunto de núcleos específicos da origen a una señal única de RMN. Así pues, un espectro de RMN es una gráfica de la intensidad de señal en función de la frecuencia de la energía electromagnética que liberan los diversos núcleos de una muestra. Las variaciones en las frecuencias de absorción de resonancia magnética nuclear, que tienen lugar debido al distinto apantallamiento de los núcleos, reciben el nombre de **desplazamientos químicos** (y se representan mediante el símbolo δ y su unidad es el ppm). El compuesto de referencia más común en resonancia magnética nuclear para soluciones acuosas es el ácido 3-(trimetilsilil)propanoico ($C_6H_{14}O_2Si$), TSP. Otro compuesto de referencia frecuentemente utilizado en experimentos de RMN es el ácido 4,4-dimetil-4-silapentano-1-sulfonico (DSS). Como el silicio es menos electronegativo que el carbono, los

grupos metilo del TSP son relativamente ricos en electrones, es decir, sus protones están fuertemente apantallados. Como consecuencia de este apantallamiento, estos protones absorben a una intensidad de campo mayor que el resto de protones enlazados al carbono o a otros elementos, de manera que casi todas las señales de resonancia magnética nuclear aparecen a campos más bajos (hacia la izquierda de la señal de TSP). Además debido a la simetría de la molécula, todos los protones del TSP absorben con el mismo desplazamiento químico dando una intensa y única absorción.

Las escala más común de desplazamiento químico es la escala δ (delta) en la que la absorción del tetrametilsilano (TSP) se define como δ 0.00. La mayor parte de los protones absorben a campos menores que el TSP, de modo que la escala δ aumenta hacia los campos menores. En el caso de los protones (^{1}H), la mayoría de las señales varían entre 0 y 12 δ, mientras que las señales del ^{13}C varían del 0 a 250 δ.

Otro término importante a la hora de la identificación de compuestos en un espectro de RMN a parte del desplazamiento químico, es la **multiplicidad** o número de picos de una señal. La multiplicidad viene dada por la regla n+1, dónde n es el número de protones equivalentes que desdoblan una señal (Skoog 2007). La multiplicidad en cada desplazamiento químico podrá denominarse: singlete (si se trata de un pico, *s*), doblete (si son dos picos, *d*), triplete (3, *t*), cuadruplete (4, *q*), […], multiplete (varios, *m*). Tanto el desplazamiento como la multiplicidad son dos parámetros característicos de cada compuesto, es decir cada compuesto tendrá unos desplazamientos químicos con una determinada multiplicidad de cada desplazamiento, esto nos dará información sobre qué molécula se trata. Las distancias entre los picos de los multipletes dan información estructural. A la distancia entre los picos de un multiplete (medida en Herzios) se le llama **constante de acoplamiento** entre los

protones magnéticamente acoplados. Se simboliza como J_{ab} donde H_a y H_b son los protones que acoplan entre sí. Las constantes de acoplamiento pueden ayudar a distinguir entre los posibles isómeros de un compuesto.

2.1.3.2 Componentes de un espectrómetro de RMN

El equipo consta de las siguientes partes (**Figura 2.6**):

1. Un imán estable que produce un campo magnético. Se introduce la muestra para analizar en la sonda que se encuentra dentro del imán.
2. Un transmisor de radiofrecuencias. Una consola controlada por un ordenador genera los pulsos de radiofrecuencia que son aplicados a la muestra. También contiene un detector para medir la absorción de energía de radiofrecuencia de la muestra.
3. Un ordenador que controla la consola y registra todos los parámetros.

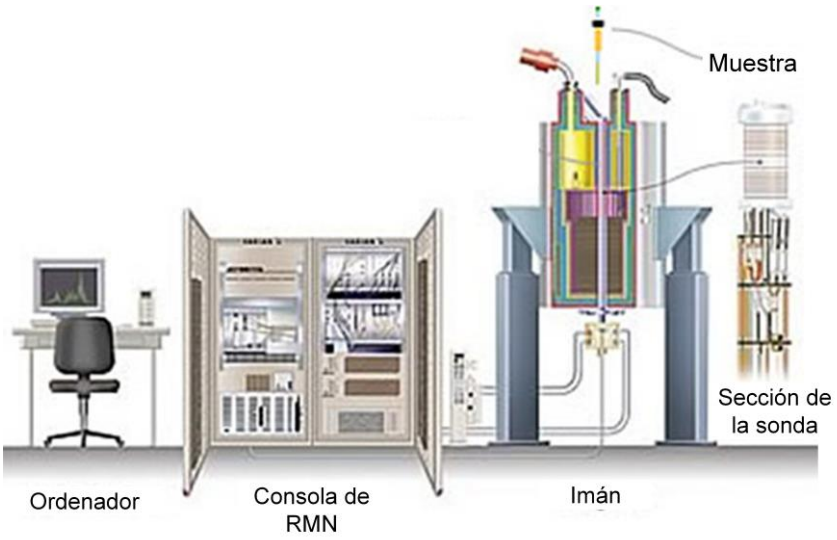


Figura 2.6. Componentes de un espectrómetro de Resonancia Magnética Nuclear. Adaptación de Agilent (www.agilent.com/labs/features/2011_101_nmr.html)

Para finalizar este apartado, se muestra la sección longitudinal (**Figura 2.7**) de un espectrómetro de Resonancia Magnética Nuclear con las diferentes partes.

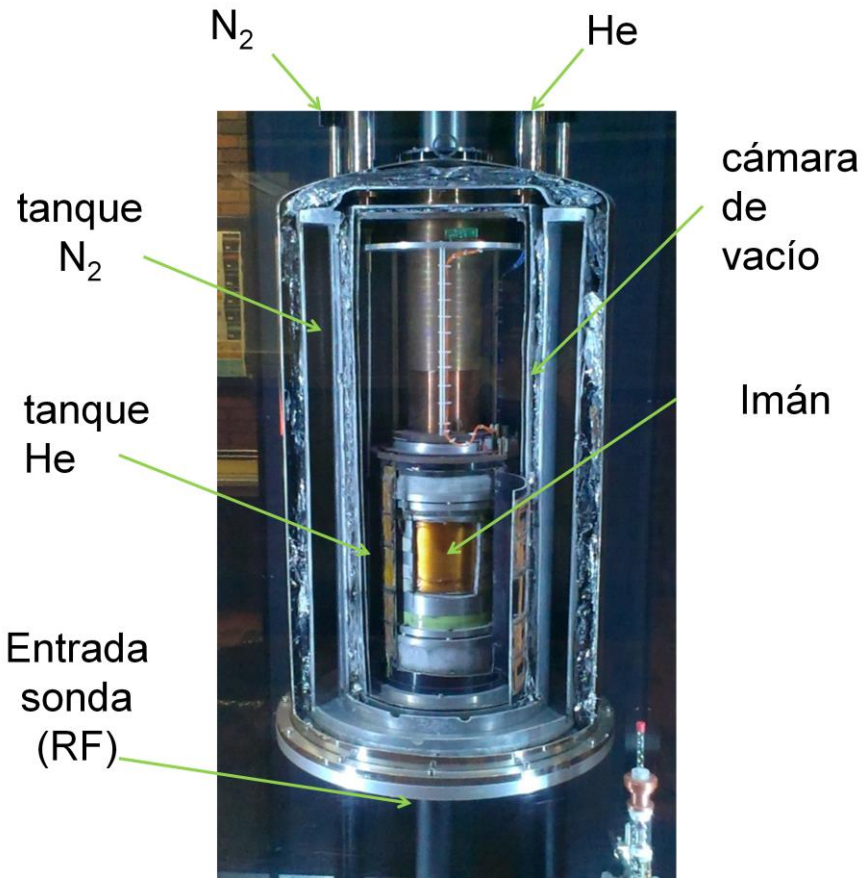


Figura 2.7. Sección longitudinal de un equipo de Resonancia Magnética Nuclear. (Equipo 400MHz, Facultad de Química, Universidad de Alberta, Canadá). Para generar estos elevados campos magnéticos, es necesario utilizar materiales superconductores, y estos deben estar enfriados mediante helio (He) a temperaturas alrededor de 4K (-269ºC). Por tanto, el interior del equipo contendrá dos camisas de refrigeración: una con helio líquido para enfriar el electroimán y la otra de nitrógeno líquido (N_2) para enfriar el helio. RF: radiofrecuencia.

2.2 NUTRIMETABOLÓMICA: ESTUDIO DE BIOMARCADORES Y PERFILES NUTRICIONALES

2.2.1 NUTRIMETABÓLOMICA

La aplicación de la metabolómica a la investigación nutricional, **nutrimetabolómica**, es más reciente pero con ello, no menos prometedora comparado con otros campos ya más explorados como el de la farmacología y toxicología. La nutrimetabolómica ha sido propuesta como una poderosa herramienta para explorar la relación entre la nutrición y la salud (Llorach *et al.* 2012). Se centra en la evaluación del metaboloma relacionado con la dieta, tanto exógeno (también llamado en inglés "*food metabolome*") como endógeno, con el objetivo de identificar los biomarcadores potenciales del consumo de alimentos tanto de forma aguda como crónica, y del efecto bajo una intervención dietética (Gibney *et al.* 2005; McNiven *et al.* 2011; Oresic 2009).

2.2.1.1 Estudios nutricionales

I. Estudios de intervención (agudos/media-larga duración)

En los **estudios de intervención** los individuos consumen el alimento en una única ingesta (estudios **agudos**) o en repetidas ingestas durante un periodo de tiempo (estudios **de media- o larga duración**) (Scalbert *et al.* 2014); de pocos días a meses o años. El biofluído a analizar se recoge después de la ingesta hasta un período de 24h post-ingesta. La limitación que pueden tener intervenciones de corta duración puede ser que no sea suficientemente específico el alimento analizado en la población estudiada,

ya que las dietas ordinarias pueden contener otros alimentos que contengan precursores de este biomarcador. Se ha demostrado en un estudio transversal a partir de un estudio de intervención de dieta global, que solo se pudo verificar el 23% de los biomarcadores potenciales observados en estudios previos (Andersen *et al.* 2014a).

II. Estudios de cohorte u observacionales

Por otra parte, los **estudios transversales de cohorte u observacionales** pueden jugar un papel importante en la búsqueda de biomarcadores. En estos estudios, se seleccionan los consumidores bajos (o no consumidores) y altos (o consumidores habituales) de los datos proporcionados de ingesta de alimentos, u otras herramientas dietéticas, que previamente debemos obtener. El comparar estos grupos permite la identificación de biomarcadores que reflejen el consumo habitual del alimento que se desea estudiar. A pesar de la potencialidad de los estudios transversales para la búsqueda de biomarcadores, se ha de tener en cuenta el hecho de que muchos alimentos están o pueden estar altamente correlacionados entre sí, por tanto puede haber un riesgo al identificar un biomarcador hasta que no quede establecida su identidad y especificidad en ese alimento (Scalbert *et al.* 2014).

Tabla 2.2 Comparación de los diferentes tipos de estudios clínicos nutricionales Adaptación de Jenab *et al.* 2009; Pujos-Guillot *et al.* 2013; Scalbert *et al.* 2014.

	Estudio intervención agudo	**Estudio intervención media / larga- duración**	**Estudio cohorte**
Nivel de control dieta	+++	++	+
Número metabolitos discriminantes	+++	++	+
Heterogeneidad de la población	+	++	+++
Riesgo falsos positivos (correlaciones entre alimentos)	+	++	+++
	farmacocinética	farmacocinética estabilidad del metabolito	insuficiente especificidad
			validación en estudios de intervención

+++: Máximo; +: Mínimo.

2.2.1.2 Tipología de componentes nutricionales para el estudio de biomarcadores

Los perfiles metabólicos nutricionales dependerán del componente nutricional evaluado, por tanto como componentes nutricionales a evaluar tendremos:

I. Compuestos bioactivos

Los alimentos vegetales contienen una colección de compuestos bioactivos que han sido ampliamente estudiados por su potencial saludables (Hooper *et al.* 2006), incluyendo compuestos fenólicos (polifenoles los que se dividen en diferentes clases como las flavonas, flavonoles, flavan-3-oles, isoflavonas, flavanonas, antocianidinas y lignanos), esteroles y estanoles vegetales, salicilatos y glucosinolatos (Hooper *et al.* 2006; Manach *et al.* 2009). Varios ejemplos de estudios realizados sobre este tipo de compuestos desde una aproximación metabolómica son por ejemplo, polifenoles del té (Daykin *et al.* 2005), del vino y de la uva (Jacobs *et al.* 2012; van Dorsten *et al.* 2010; van Velzen *et al.* 2009; van Velzen *et al.* 2008) las isoflavonas de la soja (Solanky *et al.* 2005; Solanky *et al.* 2003), en cuanto a polifenoles y compuestos fenólicos. Por otra parte, otro estudio metabolómico realizó una intervención dietética con una bebida enriquecida en esteroles vegetales (Szymańska *et al.* 2012a). En otro ejemplo, un estudio agudo reveló cambios en el perfil metabolómico en humanos después del consumo de una bebida compuesta por manzana, zanahoria y fresas (fitoquímicos dietéticos) (Walsh *et al.* 2007),

Es importante estudiar estos compuestos bioactivos aislados para poder estudiar el efecto que ejercen en la salud, pero además, como estos

compuestos no se encuentran aislados en nuestra alimentación sino que forman parte de los alimentos, y que por ello, pueden interaccionar con otros compuestos provenientes del alimento dónde se encuentran de forma favorable (sinérgica) o desfavorable (antagónica), además de poder existir un efecto sobre la matriz alimentaria dónde se encuentran, es necesario estudiarlos también en la forma en la que consumimos, en el alimento completo.

II. Alimentos individuales

Se han realizado tanto estudios metabolómicos de intervención agudos, de media/larga duración así como estudios observacionales, para identificar la presencia de biomarcadores específicos de alimentos o la monitorización de cambios en concentraciones de metabolitos relacionados con la dieta. Hasta la fecha, la aplicación de la metabolómica ha identificado un número de biomarcadores de ingesta de ciertos alimentos incluyendo el salmón y la carne roja, cereales integrales de trigo, fresas y frambuesas, crucíferas, brócoli, cebolla, cítricos, bebidas como el café, té, manzanilla, vino, cacao, frutos secos (nueces, almendras, avellanas), y fibra dietética (Scalbert *et al.* 2014).

III. Complementos alimenticios

Los complementos alimenticios son productos alimenticios con la finalidad de complementar la ingesta de alimentos de la dieta normal, ya que aunque en circunstancias normales, una dieta adecuada y equilibrada proporciona todos los nutrientes necesarios para el normal desarrollo y mantenimiento de un organismo sano, las investigaciones realizadas demuestran que esta situación ideal no se da en la práctica para todos los nutrientes, ni para todos los grupos de población. Los complementos alimenticios pueden componerse de vitaminas, minerales (Real Decreto

1487/2009), ácidos grasos, aminoácidos y otros compuestos nitrogenados, péptidos y enzimas, flavonoides y carotenoides, nucleótidos, polisacáridos y oligosacáridos, y otras sustancia como germen de trigo, polen, jalea real, levadura de cerveza, lecitina de soja, propóleos, (ampliación anexo Real Decreto 1487/2009) o compuestos encapsulados pre- y probióticos.

Hay varios estudios metabolómicos referentes a complementos alimenticios con vitaminas. En un estudio de intervención se utilizó harina de trigo fortificada con ácido fólico+ vitamina B1, B2, edetato de sodio férrico y óxido de zinc (Jiang *et al.* 2011) durante 8 meses en mujeres de edad reproductiva para reducir el riesgo en defectos en el tubo neural. Otros estudios consistieron en la suplementación con: vitamina D (O'Sullivan *et al.* 2011a); vitamina E (Johnson *et al.* 2012), betaína (Kim, Jung et al. 2008), suplementación de ácidos grasos poliinsaturados en formato de cápsulas de aceite de pescado (Rudkowska, Paradis et al. 2013), o suplementación de aminoácidos (en cerdos) (He, Kong et al. 2009).

Los probióticos son "microorganismos que administrados en cantidades adecuadas, confieren un beneficio saludable en el huésped" según la FAO/WHO (WHO/FAO 2002). Generalmente se establece como un producto probiótico con $<10^6$-10^8 UFC/g o $>10^6$-10^{10} UFC/día de células viables para ser clínicamente eficaces. Pero no hay unos niveles de recuento celular establecidos para garantizar un efecto saludable (Champagne *et al.* 2011). Un microorganismo para poder ser llamado probiótico debe cumplir los siguientes criterios: 1) Su cultivo puede producirse a escala industrial; 2) Puede sobrevivir durante el proceso de producción y almacenamiento; 3) Puede tolerar el ambiente en el estómago del huésped; 4) Ejerce un efecto saludable para el consumidor (WHO/FAO 2002). Algunos de los géneros utilizados en productos

probióticos son: 1) bacterias productoras de ácido láctico (LAB): *Lactobacillus, Bifidobacterium, Streptococcus;* 2) Bacterias no productoras de ácido láctico: *Bacillus, Propionibacterium*; 3) levaduras no patógenas: *Saccharomyces*; 4) bacilos no formadores de esporas y no flagelados o *Coccobacilli*.

Los probióticos se pueden suministrar en forma de alimento o en forma de complemento alimenticio (cápsula, polvo, etc.). Existen varios estudios sobre el efecto de los probióticos en estudios clínicos (Homayoni Rad *et al.* 2012). Un ejemplo metabolómico de este caso es un estudio en heces de 16 sujetos mediante RMN con la administración durante un mes de un complejo probiótico simbiótico basado en *Lactobacillus acidophilus*, *Bifidobacterium longum* y fructooligosacáridos (Ndagijimana *et al.* 2009). Otro estudio con suplementación probiótica de *Lactobacillus rhamnosus* (NCC4007) se realizó en diferentes órganos o compartimentos en ratones para ver el efecto producido sobre la microbiota intestinal (Martin *et al.* 2009).

IV. Patrones alimentarios

Cada día comemos una gran variedad de alimentos de forma conjunta, no solo un alimento o nutriente. Hasta la fecha, la mayoría de estudios metabolómicos se focalizan en analizar un solo alimento o complemento alimentario, pero hay un creciente interés entre los investigadores en estudiar la dieta completa de los individuos, caracterizando la ingesta dietética como patrones alimentarios, con el objetivo de determinar los biomarcadores característicos o discriminantes del tipo de dieta y ver su efecto en la salud. En este campo, los biomarcadores metabolómicos pueden ser útiles para reflejar dichos patrones alimentarios.

De los primeros estudios en metabolómica que trataron la modulación dietética y diferencias metabolómicas entre varios tipos de dieta está el trabajo de Stella y colaboradores (Stella *et al.* 2006), dónde evaluó en un estudio de intervención en un período de 15 días los cambios en el metaboloma urinario de tres tipos de dietas: dieta vegetariana, dieta de bajo contenido cárnico y dieta de alto contenido cárnico. Ejemplos de otros análisis metabolómicos en el estudio de patrones alimentarios son por ejemplo el realizado por Andersen et al.(Andersen *et al.* 2014b) en el que utiliza la aproximación metabolómica no dirigida como herramienta de detección de patrones alimentarios entre una dieta danesa media y una dieta nórdica. Xu *et al.* (Xu *et al.* 2010) realizó un análisis comparando población que consumía una dieta omnívora frente a una intervención dietética de patrón lactovegetariano consistente en 480 g/día de vegetales, 350 g/día de fruta (incluido zumos), 360 g/día de tofu o alimentos de soja, 220 g/día de leche, 240 g/día de setas, y 350 g/día (mujeres) o 600 g/día (hombres) de arroz, asimismo comparando también hombres y mujeres. May *et al.* estudió el perfil metabólico en individuos después de una intervención nutricional rica en fitoquímicos (cítricos, crucíferas, soja) frente a una dieta sin frutas ni verduras (basal) (May *et al.* 2013). Un estudio de intervención centrado en dieta rica en crucíferas fue conducido por Andersen et al. (Andersen *et al.* 2013), dónde demostró que se pueden encontrar marcadores específicos de exposición a determinados alimentos aun formando parte de dietas complejas.

Estudios observacionales han identificado múltiples metabolitos que discriminan tipos de patrones alimentarios, sobretodo en patrones de dieta occidental (caracterizada por una alta ingesta de carne). Un trabajo realizado por O'Sullivan *et al.* (O'Sullivan *et al.* 2011b) analizó el patrón alimentario de 135 individuos clasificando la población en grupos de

individuos, dando como resultado 3 grupos diferenciados por su perfil de ingesta nutricional y reflejándose en el metaboloma urinario. Peré-Trepat *et al.* también desarrollaron una estrategia para la evaluación entre datos dietéticos provenientes de cuestionarios de frecuencia de alimentos y perfiles metabolómicos (Peré-Trepat *et al.* 2010). En este caso, los patrones alimentarios se definieron por análisis multivariante y se identificaron metabolitos asociados a patrones alimentarios en individuos que consumían una dieta basada en alimentos de origen animal vs. dieta basada en alimentos de origen vegetal. Bouchar-Mereier observó una correlación positiva entre patrones de dieta occidental y un conjunto de aminoácidos y acilcarnitinas de cadena corta (Bouchard-Mercier *et al.* 2013). En un estudio en gemelas adultas de Reino Unido el patrón de fruta y verdura fue correlacionado con varios metabolitos en las rutas de los gicerofosfo- y esfingolípidos; por otro lado, también se identificó otro patrón de dieta de baja energía "o de régimen" (Menni *et al.* 2013). En uno de los estudios más exhaustivos hasta la fecha, Floegel et al. usó el modelo multivariante de regresión de rangos reducidos "reduced rank regression" (RRR) para crear patrones alimentarios los cuales explicaban la variación máxima de los diferentes metabolitos (Floegel *et al.* 2013). Otro ejemplo es el proporcionado por Altmaier *et al.* que identificó siete patrones alimentarios (Altmaier *et al.* 2011); y los resultados mostraron que ciertos patrones alimentarios estaban altamente asociados con concentraciones de metabolitos en sangre; también definió el concepto de dieta "saludable" rica en pescado, pollo, arroz, vegetales cocinados y fruta fresca, yogurt, queso y leche, y bajos niveles de carne y "snacks", asociado a bajos niveles de saturación de ácidos grasos provenientes de los glicerofosfolípidos. Estos estudios dan una buena evidencia del potencial de la aplicación metabolómica para definir un perfil de biomarcadores que reflejan un patrón alimentario habitual.

2.2.2 BIOMARCADORES NUTRICIONALES

Los biomarcadores han sido definidos por el grupo de trabajo del *National Institute of Health (NIH)* de Estados Unidos como las características medidas de forma objetiva y evaluadas como indicadores de procesos biológicos normales, procesos patológicos y respuestas biológicas a intervenciones terapéuticas (Group. 2001). Los metabolitos juegan un papel importante en sistemas biológicos y comparado con las aproximaciones mediante el diagnóstico clásico y los biomarcadores clínicos clásicos, ofrece potenciales ventajas en sensibilidad y especificidad (Monteiro *et al.* 2013) la cual apoya la viabilidad de las aproximaciones metabolómicas en la búsqueda y descubrimiento de nuevos biomarcadores.

2.2.2.1 Biomarcadores para la nutrición: Efecto de la digestión, metabolismo y microbiota intestinal

El uso de biomarcadores nutricionales puede ser utilizado como medida del estado nutricional y de la exposición a compuestos bioactivos en los alimentos; como indicador de la ingesta de alimentos; y validación de las medidas de ingesta nutricional. Los biomarcadores también son útiles cuando hay datos escasos de la composición de los alimentos.

I. Tipos de Biomarcadores Nutricionales

- ***Biomarcadores de ingesta o exógenos***

El *food metabolome* se define como aquella parte del metaboloma humano que proviene directamente de la digestión, absorción intestinal y biotransformación de los alimentos y sus constituyentes ya sea por acción de los tejidos humanos (metabolismo del huésped) y/o por la microbiota intestinal (metabolismo microbiano) (Scalbert *et al.* 2014). Más de 25.000 compuestos conocidos son pertenecientes a alimentos, siendo la mayoría metabolizados en el organismo después de su ingestión. Los biomarcadores detectados en el metaboloma derivados del consumo o ingesta de un determinado alimento o patrón alimentario, son los **biomarcadores de ingesta** o **exógeno**s.

- ***Biomarcadores de efecto o endógenos***

Además, pueden también detectarse compuestos endógenos que se han visto modulados por la ingesta del alimento, patrón alimentario, etc. y que nos proporcionan información del estado fisiológico del organismo (Rezzi *et al.* 2007). Por tanto esos metabolitos que se encuentran en un biofluído de forma habitual pero se ha visto modificada su excreción por la intervención dietética, se denomina **biomarcadores de efecto** o endógenos. Por ejemplo, el consumo de té verde se ha visto que causa un cambio inmediato en la concentración de metabolitos relacionados con el metabolismo de la glucosa, el ciclo de los ácidos tricarboxílicos (TCA) o ciclo de *Krebs* y aminoácidos (Law *et al.* 2008). La RMN en este sentido, es una técnica muy adecuada, ya que se pueden detectar de forma habitual gran cantidad de compuestos endógenos presentes de forma habitual en orina (Bouatra *et al.* 2013). La presencia de varios componentes endógenos de una misma ruta metabólica puede sernos de gran ayuda

para la interpretación biológica cuando se quiere evaluar el efecto que ha tenido la intervención dietética en el organismo, el alimento subministrado o el patrón dietético. A continuación se muestra un mapa con los metabolitos asignados a rutas metabólicas (endógenos), o bien provenientes de algún alimento (exógenos), que se han visto influidos después de tres intervenciones dietéticas diferentes, y las posibles conexiones que puede haber entre ellos (**Figura 2.8**).

Este apartado se desarrolla con más profundidad en el punto *2.3* dónde se presenta un capítulo de libro de biomarcadores nutricionales de ingesta y efecto frente al riesgo de padecer una determinada enfermedad o patología (p. ej. en este caso enfermedad cardiovascular).

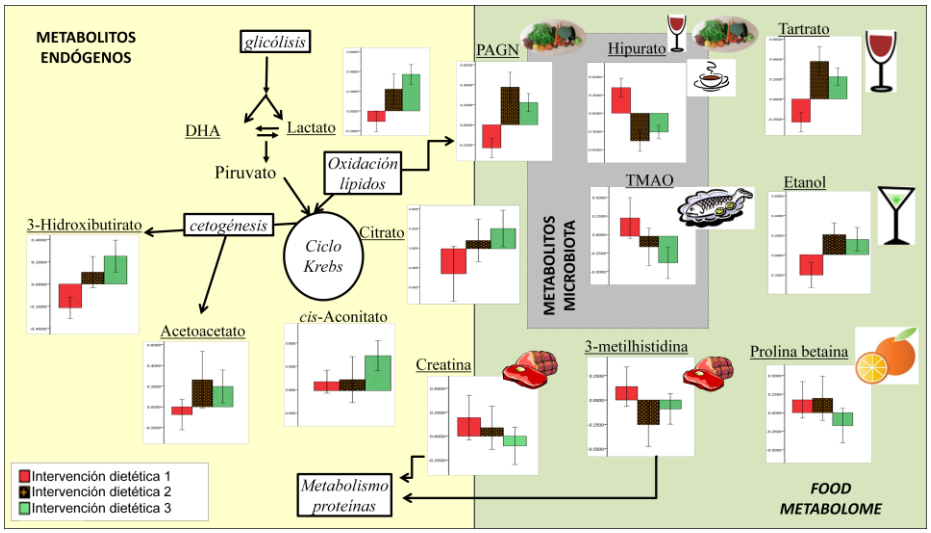


Figura 2.8. Metaboloma urinario: Metabolitos endógenos situados en diferentes rutas metabólicas (biomarcadores de efecto), metabolitos que provienen de diferentes alimentos (biomarcadores de ingesta), y metabolitos provenientes de la dieta y metabolizados por la microbiota intestinal. TMAO: trimetilamina-N-óxido. PAGN: fenilacetilglutamina, DHA: dihidroxiacetona.

II. Criterios de validación biológica y analítica para biomarcadores nutricionales. Adaptación de Andersen 2013 [Tesis Doctoral].

A) Validación biológica:

- Relación conocida con la exposición: Se ha de comprobar que la asociación con la exposición al alimento y el marcador de exposición es causal.
- Sensibilidad y especificidad: El marcador analizado debe ser exclusivo (o tanto como sea posible) a la exposición estudiada. Esto implica que el porcentaje de verdaderos positivos (sensibilidad) a la exposición, como los verdaderos negativos (especificidad) a la exposición, debe ser tan alta como sea posible.
- Dosis-respuesta: Tiene que haber una correlación positiva entre el nivel de exposición y el nivel del marcador que se ha medido.
- Variación Inter-individual: Debe ser investigada la principal fuente de variabilidad inter-individual, como el genotipo, edad, sexo, tabaquismo, microbiota intestinal, etc.
- Tiempo de exposición: Un marcador de exposición debe ser preciso, que refleje únicamente la ingesta reciente o de larga duración de un alimento, el número días, meses, años de ingesta. El tipo de exposición de un marcador debe ser determinada.

B) Validación analítica:

- Muestras: Se debe definir el tiempo y tipo de muestra, como también las condiciones de almacenamiento y la preparación de las muestras.
- Medidas definidas: El marcador debe ser cuantificable por un método definido y el error analítico debe ser conocido.

2.2.2.2. Biomarcadores para la salud: Concepto de fenotipo metabólico (metabotipo)

El fenotipado metabólico (en inglés, "*metabolic phenotyping*") o metabotipo es el resultado de la interacción entre diferentes factores (dieta, estilo de vida, microbiota intestinal, genética, etc.) (Calvani *et al.* 2010; Illig *et al.* 2010) y describe un perfil metabólico característico el cual refleja la bioquímica, el estado fisiológico y la exposición ambiental de una población (Holmes *et al.* 2008; Rezzi *et al.* 2007).

Los metabotipos humanos están determinados por la combinación de factores genéticos y ambientales, como pueden ser la dieta y el estilo de vida, representando una gran fuente de biodiversidad. La determinación del metabotipo en poblaciones humanas puede ser de gran interés en relacionar datos fisiológicos cuantitativos y bioquímicos con fenotípicos, genéticos y variaciones ambientales. La medición de la influencia dietética en el metabotipo puede proporcionar información muy valiosa y un grado de respuesta a la modulación dietética mostrada por los individuos. Esto puede, dar idea del papel que juega la dieta y la nutrición en el desarrollo de enfermedades y proporcionar información incluso para una posible nutrición personalizada (Rezzi *et al.* 2007).

En nutrimetabolómica, los diferentes metabotipos individuales o nutritipos, pueden ayudar a entender mejor el vínculo que pueda existir entre la nutrición y la salud/enfermedad.

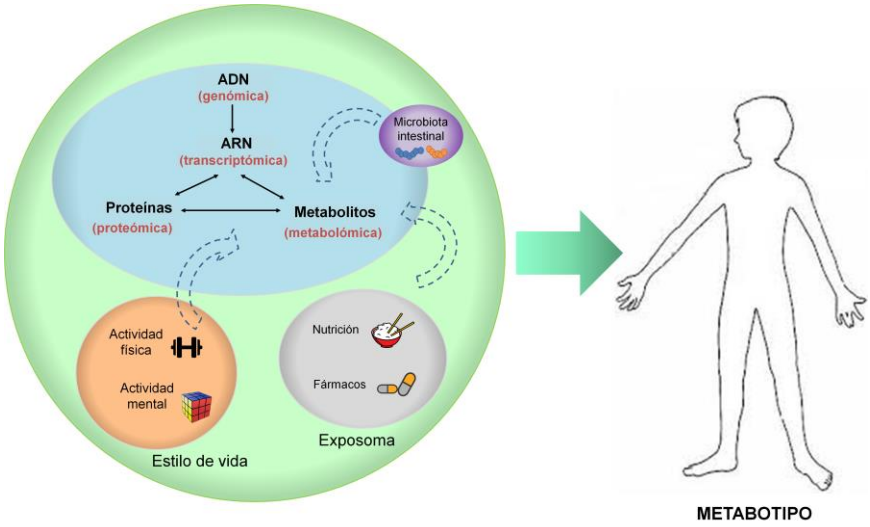


Figura 1.9. El metabotipo está influido por factores genéticos y de regulación a nivel de genes, ARN y proteínas; por la microbiota intestinal de cada individuo; por factores de exposición (exposoma) como los alimentos y los fármacos; y por factores ambientales y de estilo de vida como el ejercicio físico o mental. Adaptación Claus *et al.* 2013.

2.3 APLICACIÓN DE LA NUTRIMETABOLÓMICA EN EL ESTUDIO DE BIOMARCADORES NUTRICIONALES E IMPACTO EN LA SALUD

Los antecedentes bibliográficos que se presentan a continuación permiten ver la utilización y aplicación de la nutrimetabolómica y la búsqueda de biomarcadores de consumo y efecto mediante dos capítulos de libro dentro del contexto de los polifenoles, como biomarcadores nutricionales, y su efecto beneficioso en la salud.

A continuación se presentan dos capítulos de libro: el primero se centra en el estudio de un alimento, el vino y los polifenoles del vino en estudios dirigidos y no dirigidos mediante diferentes enfoques metabolómicos. El segundo, engloba la aplicación de la metabolómica para la investigación de biomarcadores de ingesta y efecto sobre el consumo de polifenoles y la enfermedad cardiovascular.

2.3.1 BIOMARCADORES NUTRICIONALES DE EXPOSICIÓN A UN ALIMENTO

En este parte de la introducción presentada en forma de capítulo, se pretende introducir la aproximación nutrimetabolómica en el ámbito de la investigación de un alimento concreto, en este caso el vino, ya que está presente en gran parte del contenido de resultados de la presente Tesis Doctoral.

En estos últimos años, las aproximaciones metabolómicas han iniciado sus investigaciones para analizar el impacto metabólico del vino y sus componentes (principalmente polifenoles del vino) en el organismo. Este

capítulo de libro pretende ilustrar mediante un enfoque metabolómico la interacción entre el vino y la salud. Para tratar este tema, se ha querido clasificar este análisis desde tres puntos de vista o enfoques: (1) Investigación metabolómica con vino y polifenoles derivados del vino; (2) Descubrimiento de biomarcadores metabolómicos de ingesta y efecto tras el consumo de vino; (3) Investigación dirigida y perfil metabólico del resveratrol sobre la interacción metabólica entre el vino y la microbiota intestinal. De este modo, se pretenden abarcar e ilustrar todas las aproximaciones realizadas en este tema.

Así, este capítulo de libro se titula "Metabolomic approach to finding targets of wine benefits in human health", capítulo 16 del libro "Wine: Safety, Consumer Preferences and impact on Human Health". Springer, 2014.

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Chapter 16: Metabolomic approaches in the study of wine benefits in human health.

Book: "Wine: Safety, Consumer Preferences and Impact on Human Health"

Editors: M.V. Moreno-Arribas and B. Bartolomé

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Abstract:

Metabolomics is one of the emerging "-omics" technologies that has great advantages for studying metabolic interaction between diet and human health. Over the last decade it has been successfully used in various wine nutritional studies, applying both hypothesis-driven and exploratory strategies; as a result, various aspects of the wine-human health interaction have been addressed. The exploration-driven findings provided original information on the complex metabolic relationship between wine and the human organism, reflected in changes in homeostatic metabolism and/or in immediate metabolic events, as well as in metabolic changes related to the efficiency and activity of gut microbiota. Targeted along with untargeted profiling studies provided new aspects on the bioavailability of wine-derived compounds and suggested the application of certain metabolic patterns as markers of wine dietary exposure. Finally, the outcomes of these studies showed the advantage and great potential of the application of a metabolomic approach in wine nutrition research with regard to understanding and revealing novel mechanisms underlying the health benefits of wine consumption and in searching for targets of wine benefits in human health.

Keywords: Wine biomarkers, Combined biomarkers, Biomarker of consumption, Biomarker of effect

1. Introduction

The past few decades were characterized by revolutionary developments in life science technologies. High throughput and efficient, robust and rapid "-omics" technologies brought many advantages to research, allowing global analysis of samples on different system levels (Kussmann et al. 2008). They technically enabled the profiles of specific types of molecules (DNA, RNA, proteins, lipids, small compounds, etc.) to be studied and came to be used as holistic research approaches, such as transcriptomics, proteomics, lipidomics, glycomics, metabolomics, etc., providing overall information about the status of the organism. Driven by constantly accumulating technical and methodological advances, the growth in "-omics" research has promoted its practical implementation in all the existing fields of life science, and nutrition is no exception (Kussmann et al. 2008; Kussmann et al. 2006). In fact, nutrition is probably one of the research fields where "-omics" approaches can bring many advantages, promote progression and open up new perspectives (Ordovas Munoz 2013). On the whole, the application of "-omics" technologies in nutrition is expected to stimulate exploration of the physiological processes involved in the interaction between nutrition and health and help to identify potential markers of this relationship. Basically, the principal

goal of their application is to support the accomplishment of the main objective of nutrition research – to comprehensively understand the role of diet in human health. In this context, metabolomics is seen as one of the most powerful of the "-omics" approaches, and can be widely applied in nutrition research (Kussmann et al. 2008; Kussmann et al. 2006; Ordovas Munoz 2013; Llorach et al. 2012; Scalbert et al. 2014; Primrose et al. 2011; Gibney et al. 2005; Brennan 2013).

Compared to other "-omics" sciences (i.e., genomics, transcriptomics, proteomics), a metabolomic approach has several potential advantages. Metabolomics involves the study of small molecules or metabolites present in biological samples (Brennan 2013). The metabolome, a whole set of metabolites, can be characterized by two components: (i) endogenous – metabolites defined by genetic background, age, sex, etc., and (ii) exogenous – metabolites appearing in our organism from the environment, where the most important external factor is diet (Scalbert et al. 2014). On the whole, the metabolome is very complex and is constantly varying, as it is under continuous influence from numerous extrinsic and intrinsic factors (Gibney et al. 2005). In this sense, the study of metabolites reveals useful biological information since metabolites represent biological intermediates and/or end points of biological pathways and, therefore, are implicated in the health

status of the organism (Brennan 2013; Llorach et al. 2012; Ordovas Munoz 2013). Thus, the characterization of metabolites in response to any kind of stimulus provides us with a snapshot of the metabolism or a molecular fingerprint of it, and this can serve as an index of the biological state of the organism (Astarita and Langridge 2013).

Currently nutritional research is experiencing a remarkable transformation driven by this new technological tool (Brennan 2013; Astarita and Langridge 2013; Gibney et al. 2005; Ismail et al. 2013; Jones et al. 2012; Llorach et al. 2012). Thus, a metabolomic approach has been increasingly applied in the analysis of food components, the identification of diet-derived metabolites, the evaluation of their bioavailability and metabolism, the analysis of gut microbiota metabolic activities, and the determination of physiological response to a particular diet regimen, food or nutraceuticals (Astarita and Langridge 2013; Scalbert et al. 2014). Finally, the application of metabolomics to nutrition research promotes the emergence and expansion of a novel "-omics" discipline, "nutrimetabolomics", devoted to the exploration of the complex metabolic relationship between nutrition and health at metabolome level. As a result, nutrimetabolomics forms an essential part of nutrition system biology (Panagiotou and Nielsen 2009; Jones et al. 2012), which is a new

integrating approach to studying the impact of nutrition on biological systems by measuring and integrating genomic, proteomic and metabolic data.

In this chapter, we would like to focus on the achievements and perspectives to studying the impact of wine consumption on human well-being and health using this new methodological approach, metabolomics. Therefore, in the following sections we will consider the specificities of metabolomic analysis and we will offer an overview of the findings provided by applying a nutrimetabolomic approach in current wine research, emphasizing its relevance to understanding the complexity of the interaction between wine and health.

2 Specificity of metabolomic studies

Metabolomics in nutrition attempts to decipher metabolic alterations in response to a specific food ingredient, food product or diet (Kussmann et al. 2008; Scalbert et al. 2014). Depending on the research question, two analytical strategies are used in metabolomic studies: targeted and non-targeted (Patti et al. 2012; Zulyniak and Mutch 2011). Targeted analysis is a hypothesis-driven approach and is applied when a study is focused on the analysis of already known metabolites. It provides high-quality (quantitative) data on compounds using dedicated and

optimized analytical methods. Historically targeted analysis was focused on studying a single or several resemble metabolites. Current technical progress has significantly improved the accuracy, sensitivity and capacity of analytical platforms, which has permitted the analysis of complex metabolite systems, such as pathways and classes, driven by both specific (targeted profiling) and exploratory (non-targeted profiling and fingerprinting) interests (Fernie et al. 2004). In contrast to targeted analysis, non-targeted analysis is a data-driven approach, aimed at analysing the metabolome as broadly as possible, providing a holistic overview of the metabolic changes occurring under different conditions. Such wide-ranging metabolome screening provides only qualitative data, which is used for the generation of new hypotheses. Both approaches can complement each other in respect of providing question data that is relevant to research; thus, certain information about metabolites and related pathways that could not be gained through a targeted approach due to its methodological restrictions could be explored by untargeted analysis; and vice versa, qualitative information obtained from a data-driven approach could be validated and confirmed by targeted analysis.

Metabolome responsiveness to external and internal stimuli is the fundamental asset of nutrimetabolomic studies; however, on the other

hand, it is a major challenge from a methodological standpoint. Multiple sources of metabolic variability, such as genetic polymorphisms, environmental conditions and feeding patterns (Kim et al. 2014; Nicholson et al. 2011; Floegel et al. 2013) determined by a variety of biological, cultural, socio-economic, psychological and behavioural factors (Kussmann et al. 2008), need to be carefully considered in the experimental design of any nutrimetabolomic study in order to reduce biological components in metabolic variations that are not linked to the dietary intervention itself (Zulyniak and Mutch 2011). On the other hand, the workflow procedure should be standardized as well to minimize the technical variability component in the global system of metabolic variation (Kim et al. 2014; Burton et al. 2008; Zulyniak and Mutch 2011; Issaq et al. 2009).

A five-step workflow is typically applied in metabolomic studies, including nutrimetabolomic ones (Llorach et al. 2012; Brennan 2013): (i) sample collection; (ii) sample preparation; (iii) data acquisition; (iv) data analysis; and (v) biological interpretation of the obtained results.

The choice of sample type, preparation, separation platform and analytical instrumentation should be considered in respect of obtaining high-quality data used for analysis, especially in untargeted

metabolomic analysis, and will be defined by the objectives of the study (Patti et al. 2012; Zulyniak and Mutch 2011; Tulipani et al. 2013; Issaq et al. 2009). Blood (serum and plasma), reflecting mainly instantaneous metabolic events and dynamics of nutrient and metabolite flow in the inter-organ metabolism (Kussmann et al. 2008) or information stemming from prolonged nutrient exposure (Zulyniak and Mutch 2011), and urine, providing a time-averaged representation of recent homeostatic metabolic changes and of gut microbial metabolic activities (Kussmann et al. 2008; Bouatra et al. 2013), are among the first-choice samples used in nutrition research. In addition to their relevant physiological meaning for nutrition, these biofluids are obtained in a non-invasive way using well-standardized sampling protocols, which make them widely used in human intervention and epidemiological studies and, consequently, in nutrimetabolomic studies. However, depending on the initial biological question, other types of samples could be used in nutrition research (Gibney et al. 2005; Jenab et al. 2009), including faecal samples, increasingly applied to study information on metabolic gut health, digestive efficiency and the activity of gut microbiota (Kussmann et al. 2008).

Any type of biosample is characterized by its inherent metabolome complexity. Sample preparation methods depend on the sensitivity and

specificity of the selected analytical techniques and consist of extracting analytes from the biological matrix and bringing them into a format compatible with the analytical technique used (Fernández-Peralbo and Luque de Castro 2012; Tulipani et al. 2013; Issaq et al. 2009). Various technical platforms are applied in metabolome analysis, among which mass spectrometry (MS), coupled to separation techniques such as gas (GC) or liquid (LC) chromatography, and nuclear magnetic resonance spectrometry (NMR) are the most commonly used (Ismail et al. 2013; Kussmann et al. 2008; Zulyniak and Mutch 2011; Issaq et al. 2009). However, none of the currently existing analytical platforms are able to measure the whole metabolome (Brennan 2013; Issaq et al. 2009). Each technology has its own advantages and disadvantages, reflected in its capacity to detect specific types of compounds (Issaq et al. 2009), and therefore they provide different sets of information, some parts of which could be overlapped. To obtain optimal coverage of the metabolome, especially in non-targeted analysis, a combination of several platforms could be used in metabolomic studies (van Dorsten et al. 2010; Jacobs et al. 2012a).

Metabolomic studies, especially non-targeted ones, generate huge amounts of data, which should be carefully preprocessed prior to being analysed in order to transform the data in the data set into more

comparable formats in order to ease and improve its analysis (Smolinska et al. 2012; Issaq et al. 2009). The quality of generated data is monitored by applying specific controls (Gika et al. 2008). While data obtained from targeted profiling normally could be treated by applying univariate statistical analysis, the complexity of metabolic profiling very often requires data mining techniques based on multivariate statistical methods (Issaq et al. 2009; Trygg et al. 2007). A set of unsupervised methods (pattern recognition and clustering analysis, without prior knowledge of sample class) and supervised methods (several discriminant analyses based on previously predefined classes of samples) could be applied to data in order to evaluate alteration in metabolic signatures or assess possible metabolic markers, respectively. Any supervised modelling used for identifying metabolites that differ between studied groups should be validated, in order to minimize false positive discoveries. The differential signals recognized by multivariate analysis should be further identified, by applying a series of analytical and computational approaches, combined in a multistep identification process (Llorach-Asuncion et al. 2010; Smolinska et al. 2012). In many cases, metabolites can be positively identified if their orthogonal measures (retention time, mass spectrum, accurate mass, MS/MS fragmentation, isotope abundance pattern or

chemical shifts, peak intensities and spin coupling patterns) match a known standard or literature values, otherwise they are marked as unknowns, which could be previously reported but not identified or *de novo* described (Wishart 2011). Identified altered metabolites should be investigated in terms of their correspondence to specific metabolic pathways in order to make a biological interpretation of the metabolic alteration and to draw conclusions about its relevance to human health.

3. Metabolomic approach in studying the interaction between wine and health

Over the last decade, the metabolomic approach also started to be applied in the investigation of the metabolic impact of wine and its components (principally wine polyphenols) on the human organism. Several human metabolomic studies, as shown in **Table 16.1,** have so far been conducted in wine nutrition research targeting diverse subjects. For reviewing purposes we have combined them according to the methodological approaches applied, and research focus addressed, into: wine- and grape-derived polyphenols (PPh) metabolomic research (Section 3.1), discovery of wine consumption and effect biomarkers (Section 3.2), resveratrol metabolism profiling (Section 3.3) and targeted research on the metabolic interaction between microbiota and wine (Section 3.4).

Suggested place for Table 16.1

3.1. Metabolic imprints of wine polyphenol consumption

A number of metabolomics studies were conducted by a group from the Netherlands in which the effect of red wine- and grape-derived PPh consumption on human host and gut bacterial metabolism was studied using ^{1}H-NMR spectroscopy on its own or in combination with GC-MS, LC-MS untargeted and targeted approaches in order to increase the coverage of the metabolome and to expand the interpretation of nutritional intervention (van Dorsten et al. 2010; Jacobs et al. 2012b; Jacobs et al. 2012a; Jacobs et al. 2008). In their first work (Jacobs et al. 2008) (**Table 16.1, N1**), designed as a methodological study aimed at developing and validating the NMR-based untargeted metabolome profiling of faeces, the impact of wine and grape juice extracts on human faecal metabolome was evaluated in samples collected in a midterm randomized crossover intervention study. Methodologically, the study was performed by using differential polarity extraction procedures, which enabled the detection and analysis of a relatively wide range of metabolites with different physicochemical properties, including polar compounds such as short-chain fatty acids (SCFA), organic acids, amino acids, trimethylamine, ethanol, glycerol and

glucose, and less polar compounds such as cholate, lipids, bile acids and phenolic acids. As a result, more elaborated NMR-based profiling of faecal samples provided a holistic view on the fermentation products of dietary components and the activity of microbiota. The application of an advanced approach revealed that, in contrast to GJX (grape juice extract), a 4 wk consumption of MIX (mix of grape juice and wine extracts) induced significant changes in NMR metabolome profiles of faeces. Some interesting results on the interaction between wine polyphenols and microbiota were reported. Thus, the reduction of isobutyrate was monitored after MIX consumption, which was related by authors to the inhibition of protein fermentation by wine extract PPh. The wine part of the MIX extract contained roughly 100 times more catechin than the grape juice part. Since no such changes were observed after GJX consumption, the catechin content of the wine extract was linked to the isobutyrate effect, and thus it was suggested that it was able to modulate the microbial ecology of the gut. Other interesting observations were related to the variability of metabolomic profiles. Thus, interindividual variability was related to metabolite concentration rather than to metabolite composition, suggesting that different colonic microbiota share general biochemical characteristics, whereas high intra-individual variability in faeces metabolome

indicated that diet and lifestyle greatly affect the metabolome, suggesting that they should be considered carefully in the design of nutrimetabolomic studies.

A global metabolite profiling of 24 h urine was studied by the group in a subsequent study (van Dorsten et al. 2010) (**Table 16.1, N2**) in order to investigate the urinary excretion of phenolic acids of microbiota origin and to study changes in other urinary metabolites that could signal an impact of grape PPh intake on (metabolic) health status. As in the previous study, contrasting MIX with GJX intervention enabled researchers to set aside those effects provoked by the consumption of wine-derived PPh. The multivariate analysis of global ^{1}H-NMR profiling revealed that the consumption of wine PPh within complex extract resulted in significant increase of hippuric acid (HipAc) and 4-hydroxyhippuric acid (4-OHHipAc) in 24 h urine. Due to the limited sensitivity of the NMR technique in the detection of phenolic acids, a more sensitive GC-MS targeted approach (Grun et al. 2008) was used to complement the analysis. As a result, about 18 different phenolic acids were found to be significantly elevated due to both interventions. However, HipAc (already in agreement with the NMR findings), 3-hydroxyphenylpropionic acid (3-OHPhPrAc), 3,4-dihydroxyphenylpropionic acid (3,4-diOHPhPrAc) and 1,2,3-

trihydroxybenzen (1,2,3-triOHBenzen) were associated only with the consumption of MIX (Table 16.1, N2), which was related to its higher wine-derived procyanidin and catechin content. In addition to giving insights into the metabolic degradation of grape and wine PPh in humans, this metabolomic study has also demonstrated changes in endogenous urinary metabolites. A slightly different effect between consumption of the two extracts on endogenous metabolite excretion was also related to differences in the PPh content of these interventions. Thus, NMR detected an increase in urinary excretion of citrate after MIX intake, suggesting that wine PPh could have an impact on mitochondrian tricarboxylic acid (TCA) cycle turnover. The GC-MS targeted profiling further advocated changes in the TCA cycle by monitoring the elevation of other urinary TCA intermediates, such as isocitrate. In addition, NMR metabolome profiling showed that MIX supplementation was associated with raised excretion of betaine, an important methyl donor and a regulator of homocysteine homeostasis and to a lesser extent trigolline, a metabolite of niacin (vit B3). 4-hydroxymandelic (4-OH-mandelic) and 3-methoxy-4-hydroxymandelic (3-MO-4-OH-mandelic, vanillylmandelic) acids, metabolites of catecholamines, detected by GC-MS, were also elevated due to the MIX consumption. Thus, authors suggested that endogenous biological

pathways, such as phenylalanine metabolism, biogenic amines and catecholamine metabolisms, could be modulated by the consumption of wine PPh.

In a subsequent study (Jacobs et al. 2012a) (**Table 16.1, N3**), the group tried to identify phenolic metabolites, known to originate from gut microbial fermentation, in both urine and plasma after 4-day consumption of a mix of wine and grape juice extracts (WGM) similar to the previous study . A combination of specific sample preparation with both targeted and untargeted mass-spectrometric approaches was used to achieve optimum profiling of interesting metabolites. Urine profiling by targeted GC-MS detected several phenolic acids as the most relevant for gut microbiota fermentation of the consumed PPh; however, the origin (wine or grape juice extract) of the detected phenolic acids remained unclear. A wide range of metabolites involved in many different metabolic pathways was analysed in this study with the aim of generating new hypotheses on the mechanism of action of ingested polyphenols. The most noticeable were changes in microbial fermentation products of aromatic amino acids (Table 16.1, N3), which were in line with changes observed in faeces (Jacobs et al. 2008). In addition, the reduction, detected in this study, in urinary 1-methylhistidine, a metabolite of dietary protein, and in tyrosine,

threonine and lysine plasmatic levels, also supported the idea of the ability of PPh to influence protein digestibility. These data led the authors to the hypothesis that wine and grape PPh or their microbial metabolites may benefit human health by reducing colonic protein fermentation and/or changing microbial amino acid metabolism. These results encourage the follow-up and study of the cross-talk between the gut microbial metabolism and host metabolic response following PPh and protein consumption. A subtle alteration in the urinary levels of 3,4-dihydroxyphenylglycol (3,4-diOHPhglycol) was observed (Table 16.1, N3) that suggested that wine PPh, most probably due to the presence of tyramine, might influence, through norepinephrine, turnover of catecholamine metabolism. The reduction of plasmatic sphingomyelin, two lysophosphatidylcholines and two phosphatidylcholines was detected and this, according to the authors, might indicate that wine and grape PPh affect the inflammatory signalling cascade, possibly at the mucosal gastrointestinal barrier.

The main pitfalls of conventional NMR metabolome profiling, i.e. limited sensitivity and signal overlapping, could be partially overcome by the application of SPE sub-profiling of urine, which was demonstrated in the latest study of the group (Jacobs et al. 2012b) (**Table 16.1, N4**). The methodological upgrade consisted in the

application of different polarity fractions obtained by automated SPE to NMR analysis in order to enable more accurate quantification and less ambiguous identification of metabolites. This methodological improvement was tested on 24-hour urine samples collected in a short-term (4 days) crossover placebo-controlled intervention trial in which volunteers consumed a PPh-rich mixture of red wine and grape juice extracts (WGM) in two different food matrixes: water and soy drink. As a result, the increase in urinary excretion of several microbiota-derived phenolic acids (Table 16.1, N4), normally undetectable by conventional NMR, was monitored. These changes were related to WGM consumption, independently of the food matrix applied. However, on the other hand, it was observed that soy drink induced an excretion of aconitate, citrate and trigonelline, suggesting that endogenous metabolism was affected possibly via the tricarboxylic cycle (TCA). This could be very important, since earlier studies (van Dorsten et al. 2010) suggested that the TCA cycle was a putative target for wine PPh consumption. Consequently, the issue of the suitability of soy drink as a potential carrier for wine and grape PPh should be studied further. In this respect, some metabolic issues should be additionally clarified, since even under recent upgrades, the methods had restricted sensitivity to some lower abundant metabolites of WGM

origin, endogenous compounds and some soy-derived PPh metabolites. On the whole, the study opened encouraging prospects for the technical improvement of NMR-based compound detection and analysis, aimed at providing better access for the analysis of metabolites involved in the interaction between diet and health.

3.2 Impact of wine consumption on human metabolome

In parallel to nutrimetabolomic studies focused principally on the PPh component of wine and grape juice extracts, a couple of untargeted studies on complex wine were performed by our group (Vázquez-Fresno et al. 2014; Vazquez-Fresno et al. 2012) using a conventional ^{1}H-NMR-based untargeted approach (**Table 16.1, N5** and **N6**). The individual and combined impacts of the two principal bioactive parts of wine, alcohol and PPh, on human urinary metabolome were evaluated in the first study (Vazquez-Fresno et al. 2012) (**Table 16.1, N5**) by analysing 24-hour urine samples after midterm (4 wk) intervention with red wine (RWA) at real-life doses, which was performed in parallel with dealcoholized red wine (RWD) (the same PPh content) and gin (ethanol control) treatments in a randomized crossover study. The detection of tartaric acid, a major acid in grapes and also a chief component of wine, after RWA and RWD intake was related to wine

PPh consumption, whereas ethanol was related to alcohol component of wine, and was obviously also detected after gin consumption. Two recognized colonic microbiota PPh metabolites, hippuric acid (HipAc) and 4-hydroxyphenylacetic acid (4-OHPhAtAc), were related to the consumption of the PPh content of wine (Table 13.1, N5). However, in contrast to 4-OHPhAtAc, which also increased after RWA consumption, HipAc did not change as a result of this treatment, suggesting that the alcohol content of wine might have some impact on the metabolic pathway related to the production of this compound. As a result, the study reported on the following markers of red wine consumption: tartaric acid, as a wine-derived compound, 4-OHPhAtAc related to the gut metabolism of wine PPh, and ethanol as the principal metabolite of wine alcohol. Shortly after this study, urinary tartaric acid was confirmed as a sensitive and specific dietary biomarker of wine consumption in a randomized crossover feeding trial (Regueiro et al. 2014). Finally, based on the obtained results, it was also projected that a combination of tartrate and ethanol markers could also be used in the monitoring of global compliance to wine dietary intervention. In fact, in our second study (Vázquez-Fresno et al. 2014), the combined biomarker model was successfully developed based on the previous intervention study. Ethanol glucuronide, a principal metabolite of

ethanol, was identified and in combination with tartaric acid was shown to be a strong NMR-detected biomarker for wine consumption not only in a controlled intervention study with wine but also in a cohort study involving free-living subjects (Table 16.1, N6). Moreover, this "tartrate-ethyl glucuronide" model, being more sensitive than individual markers themselves, showed a promising performance since its sensitivity accounted for discrimination of up to 3 days post-consumption of one glass of wine in a free-living population. So far, only resveratrol and resveratrol metabolites fulfil the criteria to be considered as nutritional biomarkers of wine consumption for application in epidemiological studies (Zamora-Ros et al. 2009). These findings provided wine nutritional research with promising biomarkers, which could be used alone or in combination with classical dietary assessment methods in clinical and epidemiological studies in order to be able to estimate more precisely the relationship between wine and health.

An endogenous product of first-step catabolism of branch-chain amino acids (BCAA), 3-methyl-2-oxovalerate, was identified in urine after consumption of wine in an intervention study (Vazquez-Fresno et al. 2012; Vázquez-Fresno et al. 2014) (Table 16.1, N5 & N6). Interestingly, this metabolite was also elevated in the group of wine

consumers from the epidemiological study (Vázquez-Fresno et al. 2014) (Table 16.1, N6), thus behaving really as a robust marker of the effects of wine consumption. In general, elevated levels of BCAA could be related to a decrease in their metabolic rate, which is associated with health-compromising statuses such as insulin resistance, diabetes, cardiovascular disease, etc. (Batch et al. 2014). The increase of urinary 3-methyl-2-oxovalerate levels detected in both studies could suggest that the induction of BCAA catabolism by wine PPh components has benefits for health, which should be studied in further investigations. On the whole, the results of these two studies showed the capacity of a conventional NMR-based untargeted approach to obtain a comprehensive metabolome picture including food metabolome and endogenous biomarkers of moderate wine intake, thereby fully supporting the application of this approach in complex clinical or epidemiological studies.

3.3 Metabolomics in Resveratrol Research

Resveratrol is a natural stilbene with specific physicochemical properties responsible for its biological activity (Szekeres et al. 2010). Interest in resveratrol as a principal bioactive compound of wine lies in a reductionist approach, which investigates the beneficial health

impacts of individual wine PPh as the main factors responsible for the "French paradox" effect (Renaud et al. 1998; Chiva-Blanch et al. 2013). In this respect, resveratrol has been and is extensively studied for its biological activity in relation to the observed benefits of wine consumption for health (Delmas et al. 2011). Although this PPh is also present in some other foods, wines are the most important source of resveratrol in people´s diet (Zamora-Ros et al. 2008). Thus, resveratrol was shown to be a good marker of dietary intake of wine in both interventional (Zamora-Ros et al. 2006) and epidemiological studies (Zamora-Ros et al. 2009). In fact, the latter study (Zamora-Ros et al. 2009) showed that using resveratrol metabolites, instead of single resveratrol, could increase the ability to discriminate between wine consumers and non-wine consumers even in a free-living population.

Previous studies showed an extensive metabolic conversion of dietary resveratrol consumption (Delmas et al. 2011; Andres-Lacueva et al. 2009). Consequently, the role of resveratrol metabolism became recognized both in relation to its biological activity and human health interactions (Delmas et al. 2011) and as a good marker of wine consumption (Zamora-Ros et al. 2009). In contrast to other wine PPh, resveratrol metabolites could not be monitored by conventional non-targeted metabolomic approaches (NMR- and MS-based), principally

due to the relatively low concentrations of bioavailable resveratrol metabolites, in addition to specific technical limitations of these approaches. In this respect, more sensitive targeted profiling of metabolites is a method that can provide a comprehensive overview of the complexity of resveratrol metabolism. Recently two LC-MS-based targeted studies evaluating complex profiles of metabolites provided updated information on resveratrol metabolism in humans (Rotches-Ribalta et al. 2012b; Rotches-Ribalta et al. 2012a). In the first study (**Table 16.1, N7**), the pharmacokinetics of plasmatic and urinary resveratrol metabolic profiles was studied after moderate consumption of red wine. Seventeen metabolites of resveratrol (12 metabolites) and piceid (5 metabolites) were identified (Table 16.1, N7). This was the first pharmacokinetics study to consider such a large metabolic profile of resveratrol and piceid in humans after an acute intake of RWA in moderate doses. Later, in a second study (Rotches-Ribalta et al. 2012b) (**Table 13.1, N8**), resveratrol metabolite profiling was extended by refining the sample preparation procedure. Thanks to the availability of some standards, an optimized methodology permitted more accurate identification and quantification of sulphoconjugated metabolites (e.g. disulphated and sulphoglucuronidated), which were previously underestimated, but whose impact could be significantly relevant to

human health through the modification of resveratrol stability, chemical structure, transport and metabolism at the cellular level of the organism (Delmas et al. 2011; Patel et al. 2013). In total, 21 different resveratrol metabolites, including those formed by gut and microbiota metabolism, were included in the targeted resveratrol profiling of 24 h urine samples from the crossover intervention study with a 4 wk intake of red wine (RWA) and dealcoholized red wine (RWD) with the same PPh content. Targeted profiling of urinary resveratrol metabolites showed that the alcohol component of wine did not affect the resveratrol bioavailability, either in total or as a composition of its various metabolites. On the whole, these studies presented a focused resveratrol metabolism profiling approach, which is not only able to provide a global sum of the total metabolites, which is of important value for resveratrol bioavailability estimation, but also show the importance of each individual resveratrol metabolite in metabolism and the health effect of wine-derived resveratrol.

3.4 Application of targeted metabolomics in exploring wine-microbiota interaction

Recent interest in the role of colonic microbiota in the bioavailability and bioactivity of dietary PPh (Cardona et al. 2013; Etxeberria et al.

2013) has encouraged further investigation into the metabolic fate of grape- and wine-derived PPh in the human organism. Previously, studies tended to evaluate the microbiota-mediated interaction between wine and health mainly through a reductionist approach, focusing on single components of wine as its primary PPh, such as resveratrol (Bode et al. 2013), catechin and procyanidins (Gonthier et al. 2003), anthocyanin (Forester and Waterhouse 2008), etc., or vice versa on activities of specific strains of colonic microbes (Barroso et al. 2013). However, the high complexity of gut microbiome and its high interindividual variability challenge such evaluations; in addition, the impact of wine as a complex food could not be explained. The application of metabolomic approaches opens up the opportunity to have a more holistic look at the issue (Moco et al. 2012).

The composition of microbial phenolic metabolites in faecal samples collected after regular consumption of either red wine (RWA), dealcoholized red wine (RWD) or gin (GIN) was analysed using targeted LC-MS (Jimenez-Giron et al. 2013) in a pilot crossover study (**Table 16.1, N9**). For this purpose, the method for detecting and quantifying 60 different phenolic metabolites in faeces was earlier developed and validated (Sanchez-Patan et al. 2011). The analysis showed that the microbial metabolic profile of faeces was substantially

modified after moderate intake of red wine, either dealcoholized or not, and 8 phenolic acids were significantly increased. Metabolites derived from the catabolism of both flavan-3-ols and anthocyanins seemed to contribute to these changes. According to the results of the study, the bioavailability and biotransformation of red wine PPh by gut microbiota, and likewise resveratrol (Rotches-Ribalta et al. 2012b), were not affected by the alcoholic matrix of the wine. Large inter-individual differences in the formation of microbial metabolites after each red wine intervention, but not after gin intervention, were related to variations in the microbiota composition among subjects.

The developed targeted analysis was applied by the same group in a larger study with 41 volunteers (Munoz-Gonzalez et al. 2013) (**Table 16.1, N10**). This time, changes in the microbial-derived phenolic metabolite profile of faeces due to moderate 4-week red wine consumption were monitored in a parallel-armed study. Of 60 targeted compounds, 35 were identified in all faeces samples. No detectable amounts of the phenolic compounds present in wine, such as anthocyanins, flavan-3-ols and stilbenes, were found in the faecal samples, suggesting their extensive catabolism by colonic microbiota. Ten compounds, mainly benzoic and 4-hydroxyvaleric (4-OH-valeric) acids (Table 16.1, N10), showed a statistically significant increase after

the wine intake. Authors reported that red wine consumption increased the total phenolic metabolite content of faeces to varying degrees. According to their gut microbial capacity to metabolize wine PPh, individuals were tentatively classified into three "metabotypes" (Bolca et al. 2013) by μg of wine-derived total PPh detected per g of faeces: 1) low (<500 μg /g), 2) moderate (500–1000 μg /g), and 3) high (>1000 μg /g) metabolizers. Thus, these results supported a hypothesis on the existence in the human population of different PPh-metabolizing phenotypes (van Velzen et al. 2009). Although these data should be confirmed and better explored in a larger intervention study, they already carry great significance when considering individual PPh-metabolizing phenotypes in wine-health interaction studies. In general, these two targeted metabolomic studies on faeces confirmed that gut microbiota is among the principal objects of wine PPh action.

The scientific community has become aware that the microbe-derived metabolites of PPh represent a large proportion of wine PPh intake, impacting on their bioavailability and potentially exhibiting some bioactive effects (Forester and Waterhouse 2009; Monagas et al. 2010). Analysing bioavailability through monitoring host and microbiota-derived metabolites together, we can reveal the main protagonists of corporative interaction among wine PPh and microbiota and human

health, in addition to obtaining valuable information on the microbiota catabolic activity on wine PPh. Led by this idea, the host and microbiota metabolites of consumed red wine PPh formed within and excreted in urine from our organism were studied using a metabolomic targeted approach in our group (Boto-Ordonez et al. 2013) **(Table 16.1, N11).** The wide-ranging urinary metabolomic fingerprint of phenolics (n=61) was monitored by a specially developed LC-MS technique in enzymatically hydrolyzed and non-hydrolyzed 24 h urine samples collected before and after a regular 4 wk intake of dealcoholized red wine (RWD). As a result, 37 enzymatically deconjugated and 24 conjugated metabolites were detected, of which 49 were increased after RWD consumption, pointing to a considerable complexity of wine PPh microbial catabolism (Table 16.1, N11). So far, this targeted profiling has provided the most complete urinary phenolic metabolic fingerprint after wine consumption. The highest percentage of increase corresponds to microbial metabolites derived from flavan-3-ol and anthocyanin degradation. Some of the detected wine PPh microbial catabolites were recently shown to exert specific microbiota-directed bioactivities in *in vitro* (Sanchez-Patan et al. 2012) and *in vivo* (Boto-Ordonez et al. 2014) studies. In this way, wine PPh can beneficially interplay with microbiota activity with respect to human health. Some

such interplays were recently reported when substantial changes in the gut miocrobiota provoked by wine consumption were associated with certain health-relevant changes in the human organism (Queipo-Ortuno et al. 2012; Clemente-Postigo et al. 2013). Therefore, microbiota could be seen not only as a new metabolic organ of wine PPh but also as a target of their action with respect to health benefits for humans.

As a final point, these studies showed the potential of a targeted metabolomic approach in wine-microbiota research focused primarily on evaluating the complexity of wine PPh metabolism and on detecting wine colonic metabolites as potentially bioactive molecules within the human organism both systemically and at the level of colon.

Suggested place for Figure 16.1

4 Conclusions

The nutrimetabolomics studies, so far performed in wine nutrition research, showed that the consumption of wine and wine-derived PPh impact strongly on our metabolism, provoking global changes in both exogenous and endogenous metabolites. Thus, alterations in homeostatic metabolism and in immediate metabolic events were observed. Moreover, the gut microbiota metabolic efficiency and

activity were also affected. These findings provided information on the complex metabolic relationship between wine and the human organism. In addition, targeted metabolomic studies on wine PPh and their metabolites suggested the application of metabolic patterns of resveratrol and other wine-derived PPh as markers of wine dietary exposure, which is an important step forward toward more accurate assessment of wine effects in future clinical and epidemiological studies. The main findings thus far of wine metabolomic research are briefly summarized in **Figure 16.1**. Finally, the outcomes of these studies showed the advantage and great potential of the application of a metabolomic approach in wine nutrition research, especially in understanding and revealing novel mechanisms underlying the health benefits of wine consumption and in searching for targets of wine benefits to human health.

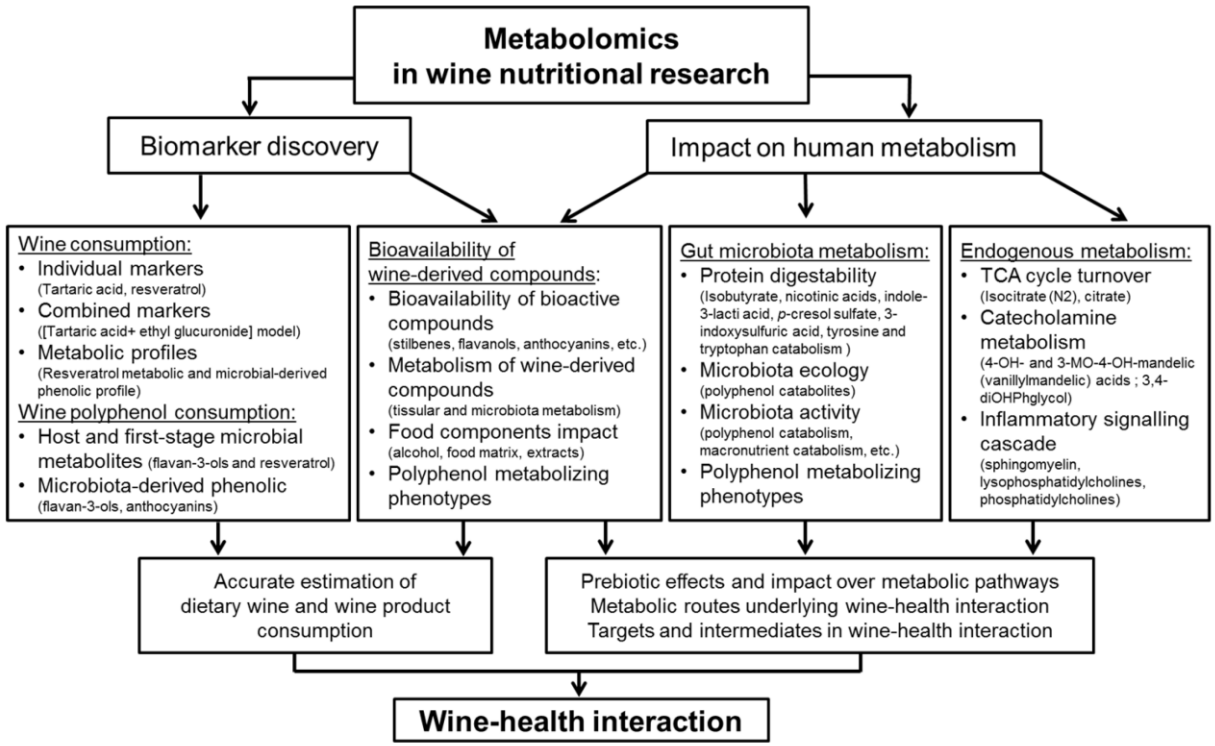

Figure 16.1: Applications of a metabolomic approach to wine nutrition research and the relevance of its finding to understanding wine-health interaction.

Table 16.1. Application of metabolomics in studies with wine and wine-derived products in humans

N	Intervention and dosage	Study design & duration	Subjects & health status	Samples analysed	Metabolomic approach	Main findings	Ref
1	MIX (GJX+WX) vs. GJX (800 mg GAE PPh/d)	PC, RM, CO (e.g. B/A) MTI (4 wk)	31 ♂ 22 ♀ Mildly hypertensive non-smokers	Faeces	Untargeted ^{1}H-NMR	- detection of wide range of compounds: SCFA, organic and amino Acs, PhAcs, lipid components, etc.; - ↓isobutyrate (GJX+WX) - interindividual variability in faeces related to concentration rather than to composition of detected metabolites	(Jacobs et al. 2008)
2	MIX (GJX+WX) vs. GJX (800 mg GAE PPh/d)	PC, RM, CO (e.g. B/A) MTI (4 wk)	33 ♂ 25 ♀ Mildly hypertensive non-smokers	24 h urine	Untargeted ^{1}H-NMR & NMR (HipAc)	-↑HipAc (35%), ↑4-OHHipAc, betaine, ↑trigonelline, and unidentified aromatic compounds (GJX+WX) - ↑citrate (GJX+WX)	(van Dorsten et al. 2010)
					Targeted GC-MS-based PhAcs profiling	- ↑18 PhAcs (GJX+WX and GJX): SyrAc, 3- and 4-OHHipAc; 3-OHPhAtAc, 4-OHmandelic and vanillmandelic Acs; - ↑ HipAc, 3-OHPhPrAc, 3,4-diOHPhPrAc and 1,2,3-triOHBenzen (GJX+WX); - ↑ 4-OHPhAtAc, homovanillic and diOHFer Acs and phenylglutamine (GJX) - ↑ TCA: isocitrate (GJX+WX), *cis*-aconitate and oxaloacetate (GJX)	
3	WGM (870 mg red wine + 540 mg grape juice extracts)	PC, RM, CO STI (4 d)	35 ♂ Healthy	24 h urine	Targeted GC-MS-based PhAcs profiling & NMR (HipAc)	- ↑ SyrAc, 3-OHHipAc; pyrogallol; 3-OHPhAtAc; 3-(3-OHPh)PrAc and FerAc; trans-CinAc; VanAc	(Jacobs et al. 2012a)
			Subpopulation 21 ♂ Healthy	24 h urine & plasma	Untargeted GC-MS- & LC-MS/MS-based polar and non-polar fractions profiling	Urine metabolites (n = 17): - Phenolic metabolites: ↑3-OHHipAc; pyrogallol; 3-OHPhAtAc; HipAc; catechol, 4-OHHipAc; 3,4-diOHHipAc; VanAc; trans-FerAc; - Endogenous metabolites: ↑ indole-3-lactic acid; glucose-1-phosphate; sucrose; nicotinic acid; 1-methylhistidine; ↓3-indoxylsulphuric Ac; p-cresol Sulf; 3,4-diOHPhglycol; Plasma metabolites (n = 12): ↓tyrosine; sphingomyelin; dehydroepiandrosterone Sulf; two lysophophatidylcholines; two phosphaticylcholines; threonine; ↑ oleic acid; phosphaticylcholine, phosphate (lipid fraction).	
					Targeted SPE-LC-MS/MS-based catecholamines and steroids profiling		
4	WGM (870 mg red wine	PC, RM, CO (e.g. B/A)	35 ♂ Healthy	24 h urine	Untargeted ^{1}H-NMR	Sub-profiles of SPE fractioned urine: - Fraction of aromatic compounds, e.g. microbiota PPh	(Jacobs et al. 2012b)

	+ 540 mg grape juice extracts) in 200 mL water vs. WGM_Soy (870 mg red wine + 540 mg grape juice extracts) in 200 mL soy drink	STI (4 d)				metabolites: ↑ 3-OHPhAtAc; homovanillate; 3-(3-OHPh)-3-OHPrAc; 4-OHHipAc, HipAc, 3-OHHipAc, caprate, 2-PhPrAc, tryptophan, several unknowns (WGM vs PC independently of food matrix) – no impact of tested food matrix on WGX polyphenol metabolism. - Fraction of semi-polar compounds: ↑*cis*- and *trans*-aconitates (WGM_Soy vs PC); ↑*cis*-aconitate ↓-tryptophan (water vs soy matrix in WGM treated) - Fraction of polar metabolites, e.g. endogenous origin: ↑ - citrate, cis-aconitate, trigolline (WGM_Soy vs PC) Non-fractioned urine profiles: ↑2-OHHipAc,HipAc and unknown (WGM vs PC); ↑2-OHHipAc, HipAc, *cis*-aconitate; citrate (WGM_Soy vs PC); ↑ *cis*-aconitate (water vs soy matric in WGM treatments)	
5	RWA (272 mL/d, 30 g EtOH/d, 2933 PPh in mEqGAE/L) vs. RWD (272 mL/d, 0 g EtOH/d, 2694 PPh in mEqGAE/L) vs. GIN (100 mL/d, 38 g EtOH/d, 0 PPh in mEqGAE/L)	RM, CO (e.g. B/A) MTI (4 wk)	61♂ High risk coronary heart disease	24 h urine	Untargeted ^{1}H-NMR	<u>Wine markers:</u> - food metabolome markers: tartrate (RWA & RWD), ethanol and mannitol (RWA); - endogenous markers: 3-methyl-oxovalerate (first-stage catabolite of branched-chain amino acid (BCAA)); - gut microbiota markers: HipAc, 4-OHPhAtAc;	(Vazquez-Fresno et al. 2012)
6	RWA (272 mL/d, 30 g EtOH/d, 2933 PPh in mEqGAE/L) & PREDIMED (epidemiological study)	RM, CO MTI (4 wk) CS (free-living subjects): Non-consumers vs. Consumers	56 ♂ High risk coronary heart disease 91 (♂+♀) High risk coronary heart disease	24 h urine	Untargeted ^{1}H-NMR	<u>Wine intake individual markers (by AUC%):</u> Ethyl glucuronide (86.3%); Tartrate (85.7%); Ethanol (75.6%); 2,3-butanediol (75.6%); 3-methyl-2-oxovalerate (70.8%); Mannitol (67.4%); Three unknown compounds (range 73.8–76.5 %). <u>Wine intake combined markers (by AUC%):</u> [Tartrate + Ethyl glucuronide] marker (90.7%) in AI study [Tartrate + Ethyl glucuronide] marker (92.4%) in cohort study. <u>Markers of wine effect:</u> 3-methyl-2-oxovalerate (both AI and cohort study)	(Vázquez-Fresno et al. 2014)

7	RWA (375 mL) vs. GE (15 tablets/400 mL water)	PA (e.g. B/A) AI	10♂ (7♂ RW and 3♂ GE) Healthy non-smokers	Plasma kinetics Urine kinetics	Targeted LC-ESI-MS/MS resveratrol metabolism profiling	- 17 identified Resv and piceid metabolites: Resv- glucoside, phase II metabolites of *cis*- and *trans*-Resv, phase II metabolites of piceid and phase II metabolites of diH-Resv; - Plasma and urinary pharmacokinetics of RW-derived piceid and Resv host and microbial metabolites;	(Rotches-Ribalta et al. 2012a)
8	RWA (272 mL, 30 g EtOH/d) vs. RWD (272 mL, 0 g EtOH/d)	RM, CO MTI (4 wk)	59 (♂+♀) High cardiovascular risk	24 h urine	Targeted LC-ESI-MS/MS resveratrol metabolism profiling	- improve detection of sulfated Resv metabolites; - 21 identified host and microbiota-derived Resv metabolites; - bioavailability of Resv is not affected by alcohol	(Rotches-Ribalta et al. 2012b)
9	RWA (272 mL/d, 30 g EtOH/d, 2933 PPh in mEqGAE/L) & RWD (272 mL/d, 0 g EtOH/d, 2694 PPh in mEqGAE/L) & GIN (100 mL/d, 38 g EtOH/d, 0 PPh in mEqGAE/L)	RM, CO MTI (4 wk)	8 Healthy	Faeces	Targeted UPLC-ESI-MS/MS microbial PhAcs profiling	- ↑8 phenolic acids: 3,5-diOHBzAc, protocatechuic Ac, p-coumaric Ac; 3-O-MetGalAc, VanAc, SyrAc; 4-OH-5-PhValAc, PhPrAc; - alcohol does not influence the formation of RW phenolic metabolites by the gut microbiota. - evaluated "basal" profiles of faeces phenolic compounds - interindividual variability in faeces RW-derived phenolic content reflects variation in colonic microbiota	(Jimenez-Giron et al. 2013)
10	RWA (250 mL/d, ≈450 mg total PPh/d, where ~270 mg/L monomeric & oligomeric flavan-3-ols)	PA, RM MTI (4 wk)	41 (19♂+22♀) -33 intervention - 8 control	Faeces	Targeted UPLC-ESI-MS/MS microbial PhAcs profiling	- 35 quantified metabolites - no wine original phenolics detected in faeces - high "basal" interindividual variability in faeces phenolic content - ↑ total phenolic metabolite content of faeces after RW - Subject grouping by total RW-derived PPh detected in faeces: 1) <500, 2) 500–1000, and 3) >1000 μg/g. - ↑10 compounds: 3,5-diOHBzAc, protocatechuic Ac, 3-O-MetGalAc, VanAc; 3-OHPhAtAc; SyrAc; 4-OH-5-(3´,4´-diOHPh)ValAc; 4-OH-5-PhValAc, 5-(3´-OHPh)-γ-valerolactone, 3-PhPrAc.	(Munoz-Gonzalez et al. 2013)
11	RWD (272 mL, 0 g EtOH 2694 mEqGAE/L)	B/A MTI (4 wk)	36 ♂ High cardiovascular risk	24 h urine	Targeted UPLC-ESI-MS/MS microbial-derived phenolic profiling	- identified 37 deconjugated and 24 conjugated metabolites; - ↑21 phase II metabolites: 11 flavan-3-ols (Sulfs, Glucs of epicatechin and Met-epicatechins); 4 OHBzAcs (Met-GalAc-Sulf and ethylgallate-Sulf; 2 OHBzAc-Glucs); 7 phase II OHPh-valerolactone metabolites (DHPV- and MHPV-Sulfs and -Glucs); - ↑28 microbial metabolites: 13 OHBzAc derivatives (2,4-, 2,6-, 2,5- 3,5-diOHBzAcs; protocatechuic Ac; SyrAc; 4- and 3-OHBzAcs; 4- and 3-OHHipAcs; GalAc and Met-GalAc; ethylgallate); 5 OHPhAtAc derivatives (PhAtAc; 3- and 2-	(Boto-Ordonez et al. 2013)

						OHPhAtAcs: 3,4-diOHPhAtAc; homovanillic acid); 6 OH-cinnamic Acs (*m*-, *o*- and *p*-coumaric Acs; CafAc; FerAc; SinAc); 3 OHPhPrAc derivatives (3-(4-OHPh)- and 3-(3-OHPh)- PrAc; dihydro-CafAc); 2 glycines (vanilloyl- and feruloyl-glycines); 2 DHPVs; enterolacone and pyrogallol.	

AI – acute intervention (single dose); AtAc – acetic acid; AUC – area under curve of receiver operating characteristics (ROC); B/A – before/after design of the study; BzAc – benzoic acid; CafAc – caffeic acid; CinAc – cinnamic acid; CO – crossover design; CS – cohort study; DHPV – dihydroxyphenylvalerolactone; EtOH – ethanol; FerAc – ferulic acid; GAE – gallic acid equivalents; GalAc – gallic acid; GE – grape extract; GIN – gin; GJX – grape juice extract; Gluc – glucuronide; H – hydro; HipAc – hippuric acid; OH – hydroxy; LLE – liquid-liquid extraction; Met – methyl; MHPV – methylhydroxyphenylvalerolactone; MIX – the wine (WX) and grape juice (GJX) extracts mix; MTI – midterm intervention; PA – parallel arm design; PC – placebo-controlled study; Ph – phenyl; PhAc(s) – phenolic acid(s); PPh – polyphenols; PrAc – propionic acid; Resv – resveratrol; RM – randomized intervention; RWA – red wine (containing alcohol); RWD – dealcoholized red wine; SCFA – short-chain fatty acids; SinAc – sinapic acid; SPE – solid-phase extraction; STI – short-term intervention; Sulf – sulfate; SyrAc – syringic acid; ValAc – valeric acid; VanAc – vanillic acid; WGM – mix of wine- and grape juice-derived extracts; WX – wine extract

Bibliography

Andres-Lacueva C, Urpi-Sarda M, Zamora-Ros R, Lamuela-Raventos RM (2009) Bioavailability and Metabolism of Resveratrol. In: Plant Phenolics and Human Health. John Wiley & Sons, Inc., pp 265-297. doi:10.1002/9780470531792.ch13

Astarita G, Langridge J (2013) An emerging role for metabolomics in nutrition science. J Nutrigenet Nutrigenomics 6 (4-5):181-200. doi:10.1159/000354403

Barroso E, Sanchez-Patan F, Martin-Alvarez PJ, Bartolome B, Moreno-Arribas MV, Pelaez C, Requena T, van de Wiele T, Martinez-Cuesta MC (2013) Lactobacillus plantarum IFPL935 favors the initial metabolism of red wine polyphenols when added to a colonic microbiota. J Agric Food Chem 61 (42):10163-10172. doi:10.1021/jf402816r

Batch BC, Hyland K, Svetkey LP (2014) Branch chain amino acids: biomarkers of health and disease. Curr Opin Clin Nutr Metab Care 17 (1):86-89. doi:10.1097/MCO.0000000000000010

Bode LM, Bunzel D, Huch M, Cho GS, Ruhland D, Bunzel M, Bub A, Franz CM, Kulling SE (2013) In vivo and in vitro metabolism of trans-resveratrol by human gut microbiota. Am J Clin Nutr 97 (2):295-309. doi:10.3945/ajcn.112.049379

Bolca S, Van de Wiele T, Possemiers S (2013) Gut metabotypes govern health effects of dietary polyphenols. Curr Opin Biotechnol 24 (2):220-225. doi:10.1016/j.copbio.2012.09.009

Boto-Ordonez M, Urpi-Sarda M, Queipo-Ortuno MI, Corella D, Tinahones FJ, Estruch R, Andres-Lacueva C (2013) Microbial metabolomic fingerprinting in urine after regular dealcoholized red wine consumption in humans. J Agric Food Chem 61 (38):9166-9175. doi:10.1021/jf402394c

Boto-Ordonez M, Urpi-Sarda M, Queipo-Ortuno MI, Tulipani S, Tinahones FJ, Andres-Lacueva C (2014) High levels of Bifidobacteria are associated with increased levels of anthocyanin microbial metabolites: a randomized clinical trial. Food Funct. doi:10.1039/c4fo00029c

Bouatra S, Aziat F, Mandal R, Guo AC, Wilson MR, Knox C, Bjorndahl TC, Krishnamurthy R, Saleem F, Liu P, Dame ZT, Poelzer J, Huynh J, Yallou FS, Psychogios N, Dong E, Bogumil R, Roehring C, Wishart DS (2013) The human urine metabolome. PLoS One 8 (9):e73076. doi:10.1371/journal.pone.0073076

Brennan L (2013) Metabolomics in nutrition research: current status and perspectives. Biochem Soc Trans 41 (2):670-673. doi:10.1042/BST20120350

Burton L, Ivosev G, Tate S, Impey G, Wingate J, Bonner R (2008) Instrumental and experimental effects in LC-MS-based metabolomics. J Chromatogr B Analyt Technol Biomed Life Sci 871 (2):227-235. doi:10.1016/j.jchromb.2008.04.044

Cardona F, Andres-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuno MI (2013) Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem 24 (8):1415-1422. doi:10.1016/j.jnutbio.2013.05.001

Chiva-Blanch G, Arranz S, Lamuela-Raventos RM, Estruch R (2013) Effects of wine, alcohol and polyphenols on cardiovascular disease risk factors: evidences from human studies. Alcohol Alcohol 48 (3):270-277. doi:10.1093/alcalc/agt007

Clemente-Postigo M, Queipo-Ortuno MI, Boto-Ordonez M, Coin-Araguez L, Roca-Rodriguez MM, Delgado-Lista J, Cardona F, Andres-Lacueva C, Tinahones FJ (2013) Effect of acute and chronic red wine consumption on lipopolysaccharide concentrations. Am J Clin Nutr 97 (5):1053-1061. doi:10.3945/ajcn.112.051128

Delmas D, Aires V, Limagne E, Dutartre P, Mazue F, Ghiringhelli F, Latruffe N (2011) Transport, stability, and biological activity of resveratrol. Ann N Y Acad Sci 1215:48-59. doi:10.1111/j.1749-6632.2010.05871.x

Etxeberria U, Fernandez-Quintela A, Milagro FI, Aguirre L, Martinez JA, Portillo MP (2013) Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. J Agric Food Chem 61 (40):9517-9533. doi:10.1021/jf402506c

Fernández-Peralbo MA, Luque de Castro MD (2012) Preparation of urine samples prior to targeted or untargeted metabolomics mass-spectrometry analysis. TrAC Trends in Analytical Chemistry 41 (0):75-85. doi:http://dx.doi.org/10.1016/j.trac.2012.08.011

Fernie AR, Trethewey RN, Krotzky AJ, Willmitzer L (2004) Metabolite profiling: from diagnostics to systems biology. Nat Rev Mol Cell Biol 5 (9):763-769. doi:10.1038/nrm1451

Floegel A, von Ruesten A, Drogan D, Schulze MB, Prehn C, Adamski J, Pischon T, Boeing H (2013) Variation of serum metabolites related to habitual diet: a targeted metabolomic approach in EPIC-Potsdam. Eur J Clin Nutr 67 (10):1100-1108. doi:10.1038/ejcn.2013.147

Forester SC, Waterhouse AL (2008) Identification of Cabernet Sauvignon anthocyanin gut microflora metabolites. J Agric Food Chem 56 (19):9299-9304. doi:10.1021/jf801309n

Forester SC, Waterhouse AL (2009) Metabolites are key to understanding health effects of wine polyphenolics. J Nutr 139 (9):1824S-1831S. doi:10.3945/jn.109.107664

Gibney MJ, Walsh M, Brennan L, Roche HM, German B, van Ommen B (2005) Metabolomics in human nutrition: opportunities and challenges. Am J Clin Nutr 82 (3):497-503

Gika HG, Theodoridis GA, Wilson ID (2008) Liquid chromatography and ultra-performance liquid chromatography-mass spectrometry fingerprinting of human urine: sample stability under different handling and storage conditions for metabonomics studies. J Chromatogr A 1189 (1-2):314-322. doi:10.1016/j.chroma.2007.10.066

Gonthier MP, Cheynier V, Donovan JL, Manach C, Morand C, Mila I, Lapierre C, Remesy C, Scalbert A (2003) Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. J Nutr 133 (2):461-467

Grun CH, van Dorsten FA, Jacobs DM, Le Belleguic M, van Velzen EJ, Bingham MO, Janssen HG, van Duynhoven JP (2008) GC-MS methods for metabolic profiling of microbial fermentation products of dietary polyphenols in human and in vitro intervention studies. J Chromatogr B Analyt Technol Biomed Life Sci 871 (2):212-219. doi:10.1016/j.jchromb.2008.04.039

Ismail NA, Posma JM, Frost G, Holmes E, Garcia-Perez I (2013) The role of metabonomics as a tool for augmenting nutritional information in epidemiological studies. Electrophoresis 34 (19):2776-2786. doi:10.1002/elps.201300066

Issaq HJ, Van QN, Waybright TJ, Muschik GM, Veenstra TD (2009) Analytical and statistical approaches to metabolomics research. J Sep Sci 32 (13):2183-2199. doi:10.1002/jssc.200900152

Jacobs DM, Deltimple N, van Velzen E, van Dorsten FA, Bingham M, Vaughan EE, van Duynhoven J (2008) (1)H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. NMR Biomed 21 (6):615-626. doi:10.1002/nbm.1233

Jacobs DM, Fuhrmann JC, van Dorsten FA, Rein D, Peters S, van Velzen EJ, Hollebrands B, Draijer R, van Duynhoven J, Garczarek U (2012a)

Impact of short-term intake of red wine and grape polyphenol extract on the human metabolome. J Agric Food Chem 60 (12):3078-3085. doi:10.1021/jf2044247

Jacobs DM, Spiesser L, Garnier M, de Roo N, van Dorsten F, Hollebrands B, van Velzen E, Draijer R, van Duynhoven J (2012b) SPE-NMR metabolite sub-profiling of urine. Anal Bioanal Chem 404 (8):2349-2361. doi:10.1007/s00216-012-6339-2

Jenab M, Slimani N, Bictash M, Ferrari P, Bingham SA (2009) Biomarkers in nutritional epidemiology: applications, needs and new horizons. Hum Genet 125 (5-6):507-525. doi:10.1007/s00439-009-0662-5

Jimenez-Giron A, Queipo-Ortuno MI, Boto-Ordonez M, Munoz-Gonzalez I, Sanchez-Patan F, Monagas M, Martin-Alvarez PJ, Murri M, Tinahones FJ, Andres-Lacueva C, Bartolome B, Moreno-Arribas MV (2013) Comparative study of microbial-derived phenolic metabolites in human feces after intake of gin, red wine, and dealcoholized red wine. J Agric Food Chem 61 (16):3909-3915. doi:10.1021/jf400678d

Jones DP, Park Y, Ziegler TR (2012) Nutritional metabolomics: progress in addressing complexity in diet and health. Annu Rev Nutr 32:183-202. doi:10.1146/annurev-nutr-072610-145159

Kim K, Mall C, Taylor SL, Hitchcock S, Zhang C, Wettersten HI, Jones AD, Chapman A, Weiss RH (2014) Mealtime, temporal, and daily variability of the human urinary and plasma metabolomes in a tightly controlled environment. PLoS One 9 (1):e86223. doi:10.1371/journal.pone.0086223

Kussmann M, Raymond F, Affolter M (2006) OMICS-driven biomarker discovery in nutrition and health. J Biotechnol 124 (4):758-787. doi:10.1016/j.jbiotec.2006.02.014

Kussmann M, Rezzi S, Daniel H (2008) Profiling techniques in nutrition and health research. Curr Opin Biotechnol 19 (2):83-99. doi:10.1016/j.copbio.2008.02.003

Llorach-Asuncion R, Jauregui O, Urpi-Sarda M, Andres-Lacueva C (2010) Methodological aspects for metabolome visualization and characterization: a metabolomic evaluation of the 24 h evolution of human urine after cocoa powder consumption. J Pharm Biomed Anal 51 (2):373-381. doi:10.1016/j.jpba.2009.06.033

Llorach R, Garcia-Aloy M, Tulipani S, Vazquez-Fresno R, Andres-Lacueva C (2012) Nutrimetabolomic strategies to develop new biomarkers of intake and health effects. J Agric Food Chem 60 (36):8797-8808. doi:10.1021/jf301142b

Moco S, Martin FP, Rezzi S (2012) Metabolomics view on gut microbiome modulation by polyphenol-rich foods. J Proteome Res 11 (10):4781-4790. doi:10.1021/pr300581s

Monagas M, Urpi-Sarda M, Sanchez-Patan F, Llorach R, Garrido I, Gomez-Cordoves C, Andres-Lacueva C, Bartolome B (2010) Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. Food Funct 1 (3):233-253. doi:10.1039/c0fo00132e

Munoz-Gonzalez I, Jimenez-Giron A, Martin-Alvarez PJ, Bartolome B, Moreno-Arribas MV (2013) Profiling of microbial-derived phenolic metabolites in human feces after moderate red wine intake. J Agric Food Chem 61 (39):9470-9479. doi:10.1021/jf4025135

Nicholson G, Rantalainen M, Maher AD, Li JV, Malmodin D, Ahmadi KR, Faber JH, Hallgrimsdottir IB, Barrett A, Toft H, Krestyaninova M, Viksna J, Neogi SG, Dumas ME, Sarkans U, The Molpage C, Silverman BW, Donnelly P, Nicholson JK, Allen M, Zondervan KT, Lindon JC, Spector TD, McCarthy MI, Holmes E, Baunsgaard D, Holmes CC (2011) Human metabolic profiles are stably controlled by genetic and environmental variation. Mol Syst Biol 7:525. doi:10.1038/msb.2011.57

Ordovas Munoz JM (2013) Predictors of obesity: the "power" of the omics. Nutr Hosp 28 Suppl 5:63-71. doi:10.3305/nh.2013.28.sup5.6919

Panagiotou G, Nielsen J (2009) Nutritional systems biology: definitions and approaches. Annu Rev Nutr 29:329-339. doi:10.1146/annurev-nutr-080508-141138

Patel KR, Andreadi C, Britton RG, Horner-Glister E, Karmokar A, Sale S, Brown VA, Brenner DE, Singh R, Steward WP, Gescher AJ, Brown K (2013) Sulfate metabolites provide an intracellular pool for resveratrol generation and induce autophagy with senescence. Sci Transl Med 5 (205):205ra133. doi:10.1126/scitranslmed.3005870

Patti GJ, Yanes O, Siuzdak G (2012) Innovation: Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol 13 (4):263-269. doi:10.1038/nrm3314

Primrose S, Draper J, Elsom R, Kirkpatrick V, Mathers JC, Seal C, Beckmann M, Haldar S, Beattie JH, Lodge JK, Jenab M, Keun H, Scalbert A (2011) Metabolomics and human nutrition. Br J Nutr 105 (8):1277-1283. doi:10.1017/S0007114510004812

Queipo-Ortuno MI, Boto-Ordonez M, Murri M, Gomez-Zumaquero JM, Clemente-Postigo M, Estruch R, Cardona Diaz F, Andres-Lacueva C, Tinahones FJ (2012) Influence of red wine polyphenols and ethanol

on the gut microbiota ecology and biochemical biomarkers. Am J Clin Nutr 95 (6):1323-1334. doi:10.3945/ajcn.111.027847

Regueiro J, Vallverdu-Queralt A, Simal-Gandara J, Estruch R, Lamuela-Raventos RM (2014) Urinary tartaric acid as a potential biomarker for the dietary assessment of moderate wine consumption: a randomised controlled trial. Br J Nutr 111 (9):1680-1685. doi:10.1017/S0007114513004108

Renaud SC, Gueguen R, Schenker J, d'Houtaud A (1998) Alcohol and mortality in middle-aged men from eastern France. Epidemiology 9 (2):184-188

Rotches-Ribalta M, Andres-Lacueva C, Estruch R, Escribano E, Urpi-Sarda M (2012a) Pharmacokinetics of resveratrol metabolic profile in healthy humans after moderate consumption of red wine and grape extract tablets. Pharmacol Res 66 (5):375-382. doi:10.1016/j.phrs.2012.08.001

Rotches-Ribalta M, Urpi-Sarda M, Llorach R, Boto-Ordonez M, Jauregui O, Chiva-Blanch G, Perez-Garcia L, Jaeger W, Guillen M, Corella D, Tinahones FJ, Estruch R, Andres-Lacueva C (2012b) Gut and microbial resveratrol metabolite profiling after moderate long-term consumption of red wine versus dealcoholized red wine in humans by an optimized ultra-high-pressure liquid chromatography tandem mass spectrometry method. J Chromatogr A 1265:105-113. doi:10.1016/j.chroma.2012.09.093

Sanchez-Patan F, Cueva C, Monagas M, Walton GE, Gibson GR, Quintanilla-Lopez JE, Lebron-Aguilar R, Martin-Alvarez PJ, Moreno-Arribas MV, Bartolome B (2012) In vitro fermentation of a red wine extract by human gut microbiota: changes in microbial groups and formation of phenolic metabolites. J Agric Food Chem 60 (9):2136-2147. doi:10.1021/jf2040115

Sanchez-Patan F, Monagas M, Moreno-Arribas MV, Bartolome B (2011) Determination of microbial phenolic acids in human faeces by UPLC-ESI-TQ MS. J Agric Food Chem 59 (6):2241-2247. doi:10.1021/jf104574z

Scalbert A, Brennan L, Manach C, Andres-Lacueva C, Dragsted LO, Draper J, Rappaport SM, van der Hooft JJ, Wishart DS (2014) The food metabolome: a window over dietary exposure. Am J Clin Nutr 99 (6):1286-1308. doi:10.3945/ajcn.113.076133

Smolinska A, Blanchet L, Buydens LM, Wijmenga SS (2012) NMR and pattern recognition methods in metabolomics: from data acquisition to

biomarker discovery: a review. Anal Chim Acta 750:82-97. doi:10.1016/j.aca.2012.05.049

Szekeres T, Fritzer-Szekeres M, Saiko P, Jager W (2010) Resveratrol and resveratrol analogues--structure-activity relationship. Pharm Res 27 (6):1042-1048. doi:10.1007/s11095-010-0090-1

Trygg J, Holmes E, Lundstedt T (2007) Chemometrics in metabonomics. J Proteome Res 6 (2):469-479. doi:10.1021/pr060594q

Tulipani S, Llorach R, Urpi-Sarda M, Andres-Lacueva C (2013) Comparative analysis of sample preparation methods to handle the complexity of the blood fluid metabolome: when less is more. Anal Chem 85 (1):341-348. doi:10.1021/ac302919t

van Dorsten FA, Grun CH, van Velzen EJ, Jacobs DM, Draijer R, van Duynhoven JP (2010) The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics. Mol Nutr Food Res 54 (7):897-908. doi:10.1002/mnfr.200900212

van Velzen EJ, Westerhuis JA, van Duynhoven JP, van Dorsten FA, Grun CH, Jacobs DM, Duchateau GS, Vis DJ, Smilde AK (2009) Phenotyping tea consumers by nutrikinetic analysis of polyphenolic end-metabolites. J Proteome Res 8 (7):3317-3330. doi:10.1021/pr801071p

Vazquez-Fresno R, Llorach R, Alcaro F, Rodriguez MA, Vinaixa M, Chiva-Blanch G, Estruch R, Correig X, Andres-Lacueva C (2012) (1)H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors. Electrophoresis 33 (15):2345-2354. doi:10.1002/elps.201100646

Vázquez-Fresno R, Llorach R, Urpi-Sarda M, Khymenets O, Bulló M, Corella D, Fitó M, Martínez-González M, Estruch R, Andres-Lacueva C (2014) An NMR metabolomics approach reveals a combined-biomarkers model in a wine interventional trial with validation in free-living individuals of the PREDIMED study. Metabolomics:1-10. doi:10.1007/s11306-014-0735-x

Wishart DS (2011) Advances in metabolite identification. Bioanalysis 3 (15):1769-1782. doi:10.4155/bio.11.155

Zamora-Ros R, Andres-Lacueva C, Lamuela-Raventos RM, Berenguer T, Jakszyn P, Martinez C, Sanchez MJ, Navarro C, Chirlaque MD, Tormo MJ, Quiros JR, Amiano P, Dorronsoro M, Larranaga N, Barricarte A, Ardanaz E, Gonzalez CA (2008) Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer

and Nutrition (EPIC)-Spain cohort. Br J Nutr 100 (1):188-196. doi:10.1017/S0007114507882997

Zamora-Ros R, Urpi-Sarda M, Lamuela-Raventos RM, Estruch R, Martinez-Gonzalez MA, Bullo M, Aros F, Cherubini A, Andres-Lacueva C (2009) Resveratrol metabolites in urine as a biomarker of wine intake in free-living subjects: The PREDIMED Study. Free Radic Biol Med 46 (12):1562-1566. doi:10.1016/j.freeradbiomed.2008.12.023

Zamora-Ros R, Urpi-Sarda M, Lamuela-Raventos RM, Estruch R, Vazquez-Agell M, Serrano-Martinez M, Jaeger W, Andres-Lacueva C (2006) Diagnostic performance of urinary resveratrol metabolites as a biomarker of moderate wine consumption. Clin Chem 52 (7):1373-1380. doi:10.1373/clinchem.2005.065870

Zulyniak MA, Mutch DM (2011) Harnessing metabolomics for nutrition research. Curr Pharm Biotechnol 12 (7):1005-1015

2.3.2 BIOMARCADORES NUTRICIONALES DE EXPOSICIÓN A COMPUESTOS BIOACTIVOS E IMPACTO EN LA SALUD

En los últimos años, un gran número de estudios de intervención y estudios epidemiológicos ha proporcionado nuevas evidencias sobre las propiedades beneficiosas de los polifenoles de la dieta en la reducción del riesgo de enfermedad cardiovascular. Aun así, es necesario que se invierta en la investigación para descubrir y validar nuevos biomarcadores de la ingesta de polifenoles y su efecto en fomentar la salud cardiovascular. Para afrontar el reto con éxito, el uso de las tecnologías –ómicas emergentes y los enfoques generadores de hipótesis son muy esperanzadores. Particularmente, la metabolómica, a pesar de que es la más joven de las ómicas, ha tenido grandes éxitos en varias aplicaciones para la identificación de potenciales biomarcadores relacionados con la predicción, el diagnóstico temprano y la prognosis de la enfermedad cardiovascular (ECV). Este capítulo pretende hacer una visión global de la aplicación de las aproximaciones en metabolómica para el descubrimiento de biomarcadores de polifenoles y ECV, además de sus implicaciones en la evaluación de riesgo cardiovascular.

Así, este capítulo de libro se titula "Emerging Applications of Metabolomics to Polyphenols and CVD Biomarker Discovery" y ha sido publicado en el libro que lleva como título "Polyphenol in Human Health and Disease" (Volumen 2), editado por Ronald Ross Watson, Victor R. Preedy y Sherma Zibaldi, en Academic Press, de la editorial Elsevier. (2014). ISBN: 978-0-12-398472-2.

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CHAPTER

79

Emerging Applications of Metabolomics to Polyphenols and CVD Biomarker Discovery

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1. INTRODUCTION

The World Health Organization reported that 17.3 million deaths in 2008 were caused by cardiovascular events, where over 80% took place in low- and middle-income countries (http://www.who.int/cardiovascular_diseases/en/). Thus, the early identification of individuals who will develop cardiovascular diseases (CVD) is paramount when prioritizing preventive resources; thereby; the identification of novel early biomarkers of CVD risk is a widespread focus for research.

The impact of nutrition on CVD development and prevention is commonly accepted.[1,2] Twenty-five to forty percent of all vascular disease and cancer deaths worldwide are caused by unhealthy diets and obesity.[3] For instance, the INTERHEART study[4] demonstrated that low consumption of fruits and vegetables was among the nine factors accounting for ≥90% of the myocardial infarction risk in populations from 52 countries representing all regions of the world.[4–6] Plant origin dietary food such fruits, vegetables, cereals, tea, coffee and wine are common sources of a wide variety of natural compounds (e.g., polyphenols, which are present in high amounts in the diets of European and US populations).[7]

Polyphenols are divided into two main groups: so-called non-flavonoids and flavonoids. The basic structure of flavonoids is the 2-fenilbenzopirona (C6–C3–C6), and they are classified into eight subclasses: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols, anthocyanidins, isoflavones, and proanthocyanidins. The non-flavonoids group is classified according to the number of carbons it contains and comprises the following subclasses: simple phenols, benzoic acids, hydrolyzable tannins, acetophenones and phenylacetic acids, cinnamic acids, coumarins, benzophenones, xanthones, stilbenes, chalcones, lignans, and secoiridoids.[8]

A growing catalogue of evidence from epidemiological studies indicates a positive association between the consumption of polyphenol-rich foods and the reduction in the incidences of CVD.[9–14] A 7-year follow-up epidemiological study[15] has revealed that a high flavonoid intake was related to a lower risk of fatal CVD in the US adult population; and the anthocyanidins, flavan-3-ols, flavones, flavonols, and procyanidins were identified as the principal classes of polyphenols responsible for lowering the risk of CVD mortality. The association between dietary intakes of flavonones, anthocyanidins, and specific flavonoid-rich foods and a decreased risk of death caused by CVD was also observed in a prospective study with more than 30,000 post-menopausal women.[16] A recent meta-analysis on six prospective cohort studies reported that a flavonol intake was inversely associated with stroke incidence, whereas even small amounts of flavonoid-rich foods were seen to have beneficial effects on cardiovascular health.[17]

Polyphenols in Human Health and Disease.
DOI: http://dx.doi.org/10.1016/B978-0-12-398456-2.00079-7

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Plenty of *in vitro* and human interventional studies have also been carried out to understand the mechanisms of action underlying the health-promoting properties of polyphenols on the vascular system. They consider possible impacts of polyphenols at different levels: the induction of antioxidant defenses,[18,19] blood-pressure-lowering effects,[20–26] the improvement of endothelial function,[23,27–36] the inhibition of platelet aggregation[22,37–39] and low-density lipoprotein (LDL) oxidation,[19,40] the modulation of inflammatory response,[41–43] and many others. Altogether, the findings of these studies point to a complexity of interactions between dietary polyphenols and the human organism.

Over the latest decades of research into polyphenols and CVD, based on numerous animal and human studies, several classes of health-promoting polyphenols were identified. Anthocyanins from red wine, purple grapes, pomegranate, certain berries and other fruits and vegetables exhibit anti-inflammatory[44,45] and antihypertensive[46] effects, insulin sensitivity,[47] antiatherosclerotic effects;[48] inhibit platelet aggregation;[22,49] and increase vascular function.[50,51] Flavanones, mainly found in oranges and grapes, are recognized to have the ability to improve blood lipid profiles in subjects suffering from dyslipemia and coronary atherosclerosis.[52,53] Aside from their anti-inflammatory, anti-proliferative and antioxidative effects,[54,55] flavonols of wine, tomatoes, onions, apples, tea, berries, broccoli, lettuce and other vegetables, and beverage origins are implicated in the direct inhibition of platelet cell signaling, platelet aggregation, and thrombus formation.[56] Flavan-3-ols monomers and polymers (proanthocyanidins) from green tea and cocoa products are reported to have beneficial effects which range from reduction of blood pressure and insulin resistance,[23,57] to the improvement of endothelial[32] and inmuno-modulatory function.[41] Stilbenes, particularly resveratrol present in grapes, red wine, and peanuts improves cholesterol metabolism (modulating lipids and lipoprotein metabolism) and, therefore, protects against atherosclerosis.[12] Isoflavones such as genistein and daidzein found in soybeans and legumes are associated with beneficial actions on the vasculature, the myocardium, and the circulating lipid profile.[58,59] Additionally, hydrolyzable tannins such as ellagitannins, especially those found in nuts, tea, grapes, berries, and some fruits such as pomegranate, peach and apricot,[60] are recognized due to their antiatherogenic effects.[61] Finally, tyrosol and hydroxytyrosol, mainly of olive oil origin,[62] exhibit antioxidant, antihypertensive, blood lipid and endothelial function improving effects.[63,64]

Despite growing scientific evidence, it is still very difficult to make nutritional recommendations for dietary polyphenols. In the last decade, it has become increasingly apparent that the limited number of traditional biomarkers of polyphenol effects may fail to produce accurate exploration, explanation or prediction of polyphenol effect on health.[65,66] In order to unambiguously identify the actual bioavailability and potential bioactivity in health promotion and disease prevention phenolic components of diet, and to possess the tools necessary to monitor dietary intakes, the identification and validation of new biomarkers of consumption and effect has become of central importance.

To estimate dietary intake of polyphenols, specific databases have been generated and are constantly updated, collecting all reported information on the polyphenols present in food and beverages as well as on their metabolites after intake.[67–69] For instance, Phenol Explorer emerged as the first comprehensive web-based database on polyphenol content in food, and currently it contains information on more than 500 polyphenols in more than 450 types of food and beverages (http://www.phenol-explorer.eu).[7] Furthermore, the Phenol Explorer database 2.0[70] was recently updated to include the concentrations of polyphenols and polyphenol metabolites of phenol-rich sources and around 375 polyphenol metabolites identified in urine and plasma on polyphenol metabolism in humans and experimental animals.

The discovery and assessment of nutritional biomarkers (biomarkers of food intake and/or effect) is greatly dependent on the use of appropriate analytical tools (able to detect metabolites present in different biological matrices) and can be subjected to slight changes. If until now targeted approaches represented the "classical" way for biomarker discovery, the fast development of the high-throughput technologies has recently made the application of non-targeted "-omic" approaches a complementary tool which promises important advances in this area.[65,71]

2. METABOLOMICS AND BIOMARKERS DISCOVERY

Biomarkers have been defined by the National Institutes of Health (NIH) working group as a "characteristic that is measured in an objective way and is evaluated as an indicator of normal biological processes, pathogen processes and biological answers to a therapeutic intervention."[72] Metabolomics has emerged as an important tool for the discovery of new biomarkers. Metabolites are important players in biological systems and compared to classical diagnostic approaches and conventional clinical biomarkers, offer potential advantages in sensitivity and specificity[73]

that support the feasibility of metabolomic approaches in biomarkers discovery.

The Metabolomics Society defines metabolomics as the "newly emerging field of -omics research concerned with the comprehensive characterization of the small molecule metabolites in biological systems which can provide an overview of the metabolic status and global biochemical events associated with a cellular or biological system" (http://www.metabolomicssociety.org).

In respect to other "omics" science (i.e., genomics, transcriptomics, proteomics), several have the potential advantages of the metabolomic approach. Metabolomics tries to define the changes in the metabolite concentration, related to genetic, biologic or environmental alterations. Therefore, the metabolome is closer to the phenotype of the organism and gives us a better profile of its biological state.[74–76] The variations in the primary metabolism occur in terms of seconds, allowing the metabolome to be a sensible and immediate measure of the phenotype of a system. Hundreds of samples could be analyzed in a matter of days, depending on the analytical tool used. Therefore experiments on a large scale are possible. The metabolome of a system (cell, biofluid, tissue,) can provide information on the state of a clinical risk, by the appearance, progression and/or regression of the disease; therefore it can serve us as a diagnostic tool. Thus the metabolomics approach may be applied on the first stage of the biomarkers discovery, which later should be validated using other approaches.

2.1 The Metabolomic Workflow

The workflow typically applied in metabolomics has been broadly categorized into five main steps which start after the biological question has been posed and the study design established. It includes: sample collection and preparation, data acquisition, and data analysis followed by the biological interpretation of the obtained results (Figure 79.1).[77]

2.1.1 Sample Collection and Preparation

According to the initial biological question, the scientific interest could be directed towards the analysis of the metabolome of different biological matrices, such as plasma, serum, urine, fecal water, cerebrospinal fluid, breath, saliva, and tissue.[78] However, blood and urine are the most common biosamples analyzed in human metabolomic studies, due to the less invasive method of obtaining them.[78] Fecal water offers the possibility to study gut microbiota metabolites but it cannot indicate those metabolites that are actually absorbed by the host. Other biofluids such as cerebrospinal fluid or tissue are collected in a more invasive way. Saliva is not widely used in metabolomics, but its potential to discriminate metabolic profiles induced by diet is attractive for further explorations.[78]

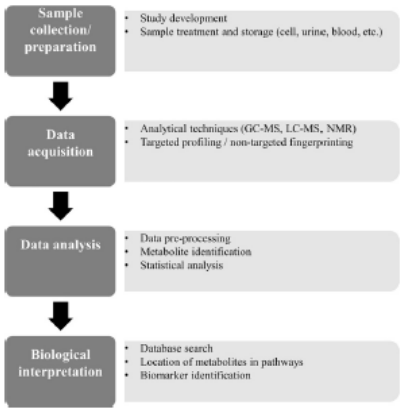

FIGURE 79.1 **Schematic representation of a nutrimetabolomic experimental workflow.** Biological samples are collected and subsequently analyzed using several analytical platforms. The different analytical platforms are used for data acquisition. The data are preprocessed and statistically analyzed. Finally, the results are biologically interpreted. *Reprinted with permission from Llorach R, Garcia-Aloy M, Tulipani S, Vazquez-Fresno R, Andres-Lacueva C. Nutrimetabolomic strategies to develop new biomarkers of intake and health effects. J Agric Food Chem 2012;60(36):8797–8808. Copyright 2012, American Chemical Society.*

Sample preparation consists in extracting the analytes from the biological matrix and brings them into a format compatible with the analytical technique used while removing components that will interfere with the analysis.[79,80] Low-abundant metabolites require including a pre-concentration step in the extraction procedure to achieve the detection limits of the technique applied. The quality of sample preparation is an important factor for the achievement of each analytical procedure; however, it should be noted that any kind of sample preparation will cause analyte losses.[80]

2.1.2 Data Acquisition: Targeted and Non-targeted Approaches

Two metabolomic approaches have been described for the acquisition of metabolomic data. The targeted approach enables us to quantitatively analyze a predefined metabolite group which belongs to a particular class of compounds or a metabolic pathway, and can also be used to validate a biomarker after it has been discovered.[81] Being a hypothesis-driven approach, certain information about the metabolic pathway of an organism and its relations with certain physiologic and

pathologic states could not be achieved. Consequently, targeted approaches are increasingly complemented with non-targeted approaches, which, although providing qualitative or semi-quantitative information, have the potentialities to drive a marked increase in the identification of potential biomarkers.

The non-targeted approach aims to cover the metabolome as broadly as possible, detecting the maximum number of metabolites, including the detection of previously unknown or poorly characterized metabolites. This provides us with an unbiased "pattern" of a disease, lifestyle or environmental conditions without knowing the represented metabolites. Thus, the non-targeted approach gives a wider but qualitative or semi-quantitative vision of the metabolome with the objective to detect the maximum number of metabolites in a sample, including the ones that are unknown or very little known.

2.1.2.1 ANALYTICAL TECHNIQUES

Metabolome includes a huge variety of endogenous and exogenous classes of compounds, with differences in size, polarity and compound concentration. Consequently, no single analytical method is able to represent in a unique snapshot such diversity.

The most common techniques used in metabolomics for data acquisition are nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), often applied in combination to extract from samples as much information as possible.[82] The first one has a high reproducibility but limited sensitivity; the second one is normally combined with other techniques such as gas chromatography (GC-MS) or liquid chromatography (LC-MS), and this achieves high sensitivity in the analysis.[76] NMR is used in metabolomics predominantly because it is a quantitative, highly reproducible non-destructive technique[83] and it allows the detection of metabolites present in relatively high concentrations in several biofluids (i.e., urine, plasma, serum) and tissues.[80] The limitation of this analytical tool lies in its inherent insensitivity compared to MS and requires relatively large sample volumes.

MS consists in separating ions (charged in positive or negative mode) according to their mass-to-charge ratio (m/z). MS requires separation techniques depending on the class of substance. GC-MS is the oldest tool for qualitative metabolic profiling, providing high chromatographic resolution. This technique consists in an electron impact ionization wherein the GC column eluants are introduced, the source ionized and fragmented to generate a characteristic fragmentation pattern and mass spectrum that is typically used for chemical identification. LC-MS provides metabolite separation by equilibration between a mobile liquid phase and a stationary solid (or liquid) phase. This technique differs from GC-MS in distinct ways (lower analysis temperatures, and sample volatility not required) and this simplifies sample preparation. Finally, capillary electrophoresis-mass spectrometry (CE-MS) is another analytical tool consisting in the separation of metabolites firstly by CE, based on charge and size, and then selectively detected using MS by monitoring a large range of m/z values. CE is appropriate for the separation of polar and charged compounds and can provide complementary information to LC-MS on the biological composition of samples, detecting and quantifying cationic and anionic metabolites.

However, as in the case of NMR, MS has a number of weaknesses: MS analysis may require extensive sample preparation for metabolite extraction, depending on the biological matrix studied, which can result in the loss of certain compounds. In summary, for metabolomic studies both analytical techniques can provide useful approaches and highly complementary information. The use of both techniques is often necessary for full metabolome characterization and, therefore, is increasingly applied in metabolomic trials.[76,84,85]

2.1.3 *Data Analysis and Biological Interpretation*

As recently reviewed, a growing number of bioinformatics tools have been proposed for data analysis and biological interpretation.[86–95]

Usually metabolomic studies generate huge amounts of data, which are subsequently treated by univariate statistical analysis (i.e., Student's t-test or one-way ANOVA) or more commonly by multivariate methods, in order to improve the statistical power of the multidimensional datasets gathered. Several methods of data analysis are generally employed, including unsupervised and supervised strategies.[96] Unsupervised methods such as principal component analysis, and hierarchical clustering analysis determine pattern sets according to their properties and without prior knowledge of the sample class, and are typically employed to identify clustering within a sample population.[88] Supervised methods such as principal component analysis and discriminant analysis, partial least square discriminant analysis, or orthogonal least square discriminant analysis are the most commonly used, being powerful statistical tools that use additional information on the datasets, such as clinical data, to determine similarities and differences between predefined classes of samples.

Once a metabolite results in significantly differentiating classes of samples, the next step lies in its identification. Currently, the efforts in metabolite identification are supported by a series of analytical and computational approaches. The most used approaches include the obtention of further structural information by additional experiments such as tandem

(MS/MS) or multiple (MS^n) fragmentation experiments, to enable structural comparisons with authentic standards, when commercially available,[97,98] or with information contained in open metabolite databases.[99]

There are several metabolomics databases with suitable information for identification and characterization of metabolites, such as ChEBI[100] (which contains biological interesting compounds), PubChem[101] (which contains a collection of known organic compounds which link to PubMed articles), ChemSpider[102] (which contains chemical compounds), Lipid Maps[103] (which contains biological lipids), METLIN database[104] (which contains known human metabolites), the BioMagResBank or BMRB[105] (which contains experimental NMR spectra of mammalian metabolites), MassBank[106] (which contains MS spectra of a variety of metabolites, drugs, and toxic compounds), the Madison Metabolomics Consortium Database (MMCD)[107] (which contains experimental and predicted NMR spectra of *Arabidopsis* metabolites), and HMDB 3.0[108] (which contains most of the known or measurable endogenous metabolites in humans). These databases tend to have a major focus on chemicals with a lesser focus on biological data. In nutrimetabolomic human studies metabolic pathway databases are very useful tools to locate metabolites identified in a specific human pathway. These databases include Kyoto Encyclopedia of Genes and Genomes (KEGG),[109] the "Cyc" databases,[110] Reactome database,[111] WikiPathways,[112] the Small Molecule Pathway Databases or SMPDB,[91] and the Medical Biochemistry Page (themedicalbiochemistrypage.org/). Clearly, metabolite identification and interpretation of the metabolomic data with these bioinformatics tools will have a potential application in clinical research. In addition, newly emerging software tools exploiting these databases are useful in disease diagnosis and disease treatment.

3. NUTRIMETABOLOMICS AND BIOMARKERS OF POLYPHENOL EXPOSURE

The application of metabolomics to nutrition research (nutrimetabolomics) is more recent but no less promising compared to already-explored fields such as pharmacology and toxicology. Nutrimetabolomics has been proposed as a powerful tool for exploring the complex relation between nutrition and health.[77] It is focused on the evaluation of the endogenous and exogenous metabolome related to diet (food metabolome), with the goal of identifying potential biomarkers of food consumption (acute or chronic) and/or the effect of a dietetic intervention.[78,113–117]

As recently reviewed by our group,[77] most of the recent nutrimetabolomic studies aimed to evaluate the impact of phytochemical-rich food, particularly polyphenols, on the human metabolome in the framework of human interventional trials. Table 79.1 summarizes non-targeted studies conducted so far on the consumption of polyphenol-containing food.

The first nutrimetabolomic study was designed to evaluate the effects of soy isoflavones on plasma metabolome.[118] Two years later, the same authors conducted a similar study in which they assessed the effect of daily intake of soy isoflavones in conjugated form due to phase II metabolism or unconjugated form in the urinary metabolome.[119] Both studies suggested that soy isoflavones had an inhibitory effect on glycolysis associated with a general alteration in energy metabolism. Most food products analyzed from a metabolomic approach are infusions. For instance, Wang *et al.*[120] initially studied the biological response to the ingestion of chamomile in 14 healthy volunteers, proposing that the lower levels of creatinine after chamomile consumption resulted from the antioxidative activity of chamomile. In the same year, Daykin *et al.*[121] evaluated the effects of acute consumption of 3 g of decaffeinated black tea extract, corresponding approximately to six cups of tea, in four healthy volunteers. A year later, the different metabolic effects of caffeine, green tea and black tea were evaluated in a randomized crossover intervention trial.[122] Recently, van Velzen *et al.*[123] proposed an integrated approach using a kinetic metabolomics and nutritional intake assessment of/on sharp black tea through an intervening cross randomized and double blind trial. Results showed that hippuric acid was predominantly excreted after black and green tea ingestion,[121,122,123] after a mixture of wine and grape juice extract,[128,129] and after chamomile ingestion.[120] Furthermore, our research group has studied the urinary metabolome modifications after acute intake of cocoa[124] and almond skin extract[125] in two randomized placebo trials where phenylvaleric acids and phenylvalerolactone have been identified after consumption of these food sources of flavan-3-ols. Hydroxyhippuric, hydroxyphenylacetic, and hydroxyphenylpropionic acids are other metabolites produced by gut microbiota after consumption of almond skin extract,[125] black tea,[122,123] and wine and grape juice extract.[128,129] Subsequently, we evaluated urinary changes after eating nuts for 12 weeks in patients with metabolic syndrome,[97,131] identifying phase II metabolites of medium-chain polysaturated fatty acids (C10–C13). In addition, alterations in amino acid metabolism and other microbial-derived metabolites were determined following the consumption of nuts, so conjugates of urolithins were detected. Moreover, urinary markers of serotonin metabolism

TABLE 79.1 Nutrimetabolomic Non-Targeted Studies with Polyphenol-containing Foods in Human Dietary Interventions

Subjects (*n*)	Study Design	Intervention (Dose Per Day)	Duration	Samples	Analytical Technique	Ref.
5 healthy	Single group	60 g soy protein (45 mg isoflavones)	1 complete menstrual cycle	plasma	^{1}H-NMR	118
9 healthy	Parallel	**a.** 60 g textured vegetable protein (45 mg conjugated isoflavone glucoside)	1 complete menstrual cycle	24 hr urine	^{1}H-NMR	119
		b. 50 g miso (25 mg unconjugated isoflavones)				
14 healthy	Single group	200 mL chamomile tea (5 g powder in 200 mL water)	2 weeks	urine	^{1}H-NMR	120
3 healthy	Single group	3 g decaffeinated black-tea extract	acute	urine	^{1}H-NMR[a]	121
17 healthy	Crossover randomized	**a.** 6 g black tea in water	2 days each period	24 hr urine plasma	^{1}H-NMR	122
		b. 6 g green tea in water				
		c. 360 mg caffeine in gelatin capsules (control)				
20 healthy	Crossover randomized double-blind	>200 mL water with a capsule containing 2500 mg of:	acute	48 hr urine	^{1}H-NMR	123
		a. dried black-tea extract powder (800 mg polyphenols)				
		b. sucrose (placebo)				
10 healthy	Crossover randomized	**a.** 40 g cocoa powder + 250 mL water	acute	24 hr urine	HPLC-q-ToF-MS	124
		b. 40 g cocoa powder + 250 mL milk				
		c. 250 mL (control)				
24 healthy	Parallel randomized blind	10 capsules containing:	acute	24 hr urine	LC-q-ToF-MS	125
		a. almond skin extract (3.5 g almond extract + 0.5 g microcrystalline cellulose)				
		b. 4 g microcrystalline cellulose (placebo)				
42 metabolic syndrome	Parallel randomized	**a.** Low-fat diet with mixed nuts (30 g:15 g walnuts, 7.5 g almonds + 7.5 g hazelnuts)	3 months	24 hr urine	LC-ESI-q-ToF-MS	97
		b. Low-fat diet (control)				
21 healthy	Single group	4 × 100 mL apple, carrot and strawberry drinks	2 days	urine	^{1}H-NMR HPLC-q-ToF-MS	126
20 healthy	Single group	500 g broccoli and Brussels sprouts	2 weeks	48 hr urine	^{1}H-NMR	127
29 mildly HTA	Crossover doubled-blind	**a.** Mix of wine and grape juice extract (800 mg polyphenols)	1 month each period	24 hr urine	^{1}H-NMR	128
		b. microcrystalline cellulose (placebo)				
58 mildly HTA	Crossover randomized double-blind	**a.** Mix of wine and grape juice extract, or red grape juice extract (800 mg polyphenols)	1 month each period	24 hr urine	^{1}H-NMR[b]	129
		b. Microcrystalline cellulose (placebo)				
39[c] mildly HTA	Crossover randomized double-blind	**a.** Mix of wine and grape juice extract, or grape juice extract (800 mg polyphenols)	1 month each period	feces	^{1}H-NMR	130
		b. Microcrystalline cellulose (placebo)				

[a]*HPLC NMR-MS was used to identify an unknown metabolite.*
[b]*GC-MS was used for targeted profiling of urinary phenolic acids.*
[c]*Although there were 53 volunteers who participated in the study, researchers analyzed fecal samples from 39 subjects.*
Adapted with permission from Llorach R, Garcia-Aloy M, Tulipani S, Vazquez-Fresno R, Andres-Lacueva C. Nutrimetabolomic strategies to develop new biomarkers of intake and health effects. J Agric Food Chem 2012;60(36):8797–8808. Copyright 2012, American Chemical Society.

were also related with the nut intake. Other nutrimetabolomic studies with foods rich in polyphenols tried to evaluate the effect of fruit and vegetable beverages,[126] cruciferous,[127] or an extract of wine and grape juice in urine[128,129] and feces samples.[130] As alteration in amino acids' metabolism was observed after an intake of vegetable and fruit beverages, and changes in carbohydrate and lipid metabolism have been detected after the intake of green and black tea.[129] Furthermore, higher levels of S-methyl-L-cysteine sulfoxide (SMCSO) were found after consumption of broccoli and Brussels sprouts, proposing SMCSO as a biomarker of cruciferous consumption.[127]

4. RECENT APPLICATIONS OF METABOLOMICS IN CVD RISK ASSESSMENT

As the result of a multifactorial etiology and a complex set of interactions between genetic and environmental factors, the development of CVD determines important changes in the biochemical and metabolic profile in the body,[132] thus providing potential markers for early diagnosis. Clinical markers of CVD risk assessment are widely described in the bibliography.[133–135] Although the major clinical risk factors explain a large proportion of the risk of developing CVD, a significant proportion of subjects had few or no traditional risk factors and a low rate (score) in current prediction models. In addition, the current biochemical-clinical predictors are useful in the measurement of CVD risk in proximity to overt disease, but provide very little information on the pathophysiological mechanisms involved in the development of the disorder. Consequently, there is a clear need for new strategies to assist the diagnosis, to reduce cardiovascular complications and improve the quality of life. A recent review showed an overview of studies assessing the clinical biomarkers of CVD risk and type-2 diabetes mellitus, on the basis of hypothesis-driven selection as well as hypothesis-free approaches from novel "*-omics*" technologies. As a result, integrated approaches, combining biomarkers from genomics, transcriptomics, proteomics, and metabolomics, as well as serial measurements of biochemical biomarkers, are required to make a complete assessment of the potential clinical usefulness of biomarkers for risk prediction of CVD.[136]

In 2008, the *Journal of the American College of Cardiology* published a review about the state of the art of the application of metabolomics as a powerful strategy to obtain new cardiovascular biomarkers.[81] As a final conclusion, the authors proposed that even though this approximation is still in development, the use of the metabolomics, together with other "omics," will transform the capacity to elucidate and discover valuable clinical biomarkers and obtain a deeper understanding of the biological mechanisms underlying the association of solid risk parameters and the development of CVD. In recent years, a growing number of publications have demonstrated that both the targeted and non-targeted metabolomic approximations may allow the discovery of new potential biomarkers of CVD outcomes, giving a quick and non-invasive early diagnosis and prognosis of the severity of cardiovascular events.[135,137–142] Following are some examples of the most recent metabolomic studies focused on both chronic and acute cardiovascular diseases. Details of each study and the main metabolites found are presented in Table 79.2.

4.1 Coronary Artery Disease

The main plasma metabolic changes associated with coronary artery disease (CAD) and hypertension involve the lipids and lipoproteins family, particularly LDL, and very-low-density lipoprotein (VLDL) cholesterol, as well as choline-containing compounds.[138] The first cardiovascular metabolomic studies carried out by the NMR approach were published by Brindle *et al.*[143] Several serum spectral regions distinguished the metabolome of healthy and disease groups, reflecting differences in lipoproteins (a decrease of high-density lipoprotein (HDL) cholesterol and an increase in LDL and VLDL cholesterol in subjects of the severe atherosclerotic group). Other studies described some sugars and fatty acids,[144] glucose, pyruvate, ketonic acids, branched-chain amino acids (BCAAs), long-chain fatty acids, and tricarboxylic acids cycle (TCA) metabolites,[85] which are mostly related to insulin resistance. Two studies from this pathological stage observed that the metabolism of BCAAs and the TCA were the two principal metabolic pathways associated with the presence of coronary artery disease.[85,145]

4.2 Myocardial Ischemia

An increase in lactate, BCAAs, and glucose levels and a decrease in lipid levels were found in subjects with myocardial ischemia by an NMR metabolomic study.[146] A non-targeted analysis using LC-MS technique identified some intermediates of TCA, nucleotides, and amino acids with inducible myocardial ischemia during exercise stress.[147] Similarly, Lewis and co-workers found changes in the plasma of the purine degradation products (adenosine diphosphate (ADP), adenosine triphosphate (AMP), inosine, hypoxanthine, and xanthine), intermediates of anaerobic glycolysis

TABLE 79.2 Human Cardiovascular Biomarkers by Metabolomic Methodologies

Pathological Stage	Study Design	Technique	Approach	Biofluid	Metabolite and Excretion with the Illness*	Ref.
Coronary artery disease	Severe CAD ($n = 36$), control ($n = 30$)	^{1}H-NMR	Non-targeted	Serum	HDL(↓), LDL(↑), VLDL-cholesterol(↑), choline (↓)	143
	Atherosclerosis ($n = 16$), healthy ($n = 28$)	GC-MS	Targeted	Plasma	Palmitate (↑), stearate(↑), 1-monolinoleoylglycerol (↑)	144
	Atherosclerosis ($n = 9$); healthy subjects ($n = 10$).	GC-MS, ^{1}H-NMR	Non-targeted	Plasma	Glucose (↑), pyruvate (↓), fructose, myo-inositol (↓), 2,3, 4-trihydroxybutyrate (↑), lactate (↓), 3-hydroxybutyrate (↑), acetoacetate (↑), (valine, isoleucine and leucine (↑)), glycerol (↑), long-chain fatty acids ↑), TCA metabolites (↓: citrate, isocitrate, succinate and malate), other amino acids (↓), 2-hydroxybutyrate (↑), 4-hydroxyproline (↓).	85
	Initial: CAD ($n = 174$), control ($n = 174$);	GC-MS	Targeted	Plasma	Leucine/isoleucine (↑); glutamate/glutamine (↑), long-chain acylcarnitines (↓)	145
	Replication: CAD ($n = 140$); control ($n = 140$)					
Myocardial ischemia	CAD ($n = 22$)	^{1}H-NMR	Non-targeted	Serum	Lactate (↑), lipids (↓), glucose (↑), valine (↑), leucine (↑), isoleucine (↑)	146
	Acute myocardial ischemia ($n = 18$), controls ($n = 18$)	HPLC-MS	Targeted?	Plasma	Citrate (↓), γ-aminobutyrate (↓), oxaloacetate (↓), citrulline (↓), argininosuccinate (↓), uric acid (↓),	147
	PMI ($n = 20$), control ($n = 16$);	HPLC-MS, LC-MS	Targeted	Plasma	Aconitate (↓), hypoxanthine (↑), TMAO (↓), threonine (↓), aminoisobutyric (↑), inosine(↑), isoleucine/leucine (↑), malonic acid (↑), 1-methylhistamine (↑), xanthine (↑), alanine (↓), choline (↓), proline (↓), serine (↓)	81
	SM I ($n = 12$), control ($n = 9$)					
	Swine ($n = 9$), MIS patients (acute myocardial ischemia) ($n = 20$), and control patients ($n = 10$)	^{1}H-NMR	Targeted	Serum	Glucose (↑), lactate (↑), glutamine (↑), glycine (↑), glycerol (↑), phenylalanine (↑), tyrosine (↑), phosphoethanolamine (↑), choline-containing compounds (↓), triacylglycerols (↓)	142
	CAD ($n = 12$), LVD ($n = 10$), control ($n = 17$)	FI-MS	Targeted	Plasma	Acetylcarnitine (↓), 3-hydroxybytyrylcarnitine (↓)	148
Heart failure (HF)	HF patients ($n = 15$) and healthy controls ($n = 20$)	^{1}H-NMR	Targeted	Urine	(↑): Acetone, acetoacetate, acetate. methylmalonate, PAG, hippurate, betaine, TMAO, DMA, cytosine, glutarate; (↓): citrate, succinate, cis-aconitate. 1-methylnicotinamide, 2-hydroxyisobutyrate, creatinine, valine, lactate, 1-methylnicotinamide, 2-oxoglutarate, adenine, glutamine, histidine, phenylalanine, pyruvate	149
	LVD or HF ($n = 52$), controls ($n = 57$)	GC-ToF-MS	Non-targeted	Serum	(↑): Pseudouridine, 2-oxoglutarate, 2-hydroxy, 2-methylpropanoic acid, erythritol, 2,4,6-trihydroxypyrimidine	150
Atrial fibrillation	Persistent AF: AF ($n = 8$), control ($n = 8$); Post-operative: AF: -AF ($n = 18$), control ($n = 19$)	^{1}H-NMR	Targeted	Atrial (cardiac) tissue	(↑): 3-hydroxybutyrate , tyrosine, leucine, glycine	151

AAA	Small aneurysm ($n = 15$); large aneurysm ($n = 15$); healthy ($n = 11$)	LC-QTOF-MS, LC-MS/MS	Non-targeted	Plasma	(↓): Sphingolipids, lysophospholipids, cholesterol metabolites, and AcylCN	[141]
ACS	NSTEACS ($n = 9$), atherosclerosis ($n = 10$) vs. healthy ($n = 10$)	GC-MS	Non-targeted	Plasma	4-hydroxyproline (↓), citrate (↓), aspartate (↓), fructose (↓), lactate (↑), urea(↑), glucose (↑), valine (↑)	[152]
	ACS (acute coronary syndrome) ($n = 19$ NSTEACS), healthy subjects ($n = 6$)	GC-MS	Target and non-targeted	Plasma	(↑): Glucose, fructose, myo-inositol, pyruvate, lactate, citrate, isocitrate, succinate, malate; oxalacetate; cysteine, 4-hydroxyproline, 3-hydroxybutyrate and 2,3,4-hydroxybutyrate; (↓): alanine, serine, glycine, threonine, aspartate, tryptophan, tyrosine, urea, 2-aminobutyrate creatinine, aminomalonate	[153]
Obesity and insulin resistance	Obese ($n = 74$), lean ($n = 67$)	MS/MS	Targeted	Serum	(↑): Alanine, valine, leucine/isoleucine, phenylalanine, tyrosine, glutamate/glutamine, aspartate/asparagine, arginine, AcylCN; (↓): glycine.	[154]
		GC-MS		Urine	(↑) Ethylmalonate, isobutyryl; (↓): glycine, isovalerylglycine, 2-oxoglutarate	
Obesity	Healthy ($n = 15$), healthy obese patients ($n = 15$)	MS/MS	Targeted	Serum	(↑): Glycine, glutamine , and glycero-phosphatidylcholine (PC) 42:0; (↓):PCaa 32:0, PCaa 32:1, PCaa 40:5	[155]
Cerebral infarction	Healthy ($n = 47$); stroke ($n = 54$)	^{1}H-NMR	Targeted	Plasma	(↑): Lactate, pyruvate, glycolate, formate. (↓): glutamine and methanol	[156]
	Healthy ($n = 30$) stroke ($n = 28$)			Urine	(↓) Citrate, hippurate, and glycine	
Diabetes	Type 1 diabetic patients ($n = 9$), non-diabetic controls ($n = 9$)	LC-MS/MS	Targeted	Plasma	20 Amino acids (also BCAAs) (↑) (↑): glucose, 3-hydroxybutyrate, lactate, acetate, acetone, acetoacetate, citrate, allantoin	[76]
	Diabetic individuals ($n = 40$), controls ($n = 60$)	UHPLC/MS/MS2; GC-MS, ^{1}H-NMR	Non-targeted	Plasma	1,5-anhydroglucitol (↓), 3-hydroxybutyrate (↑), medium-chain-length fatty acids (↑/↓), BCAAs (↑); PC (↓); PE (↑)	[84]
Insuline resistance	Cohort non-diabetic subjects, $n = 399$	UHPLC-MS/MS (+/− ESI) and GC-MS (+ EI)	Non-targeted	Plasma	α-hydroxybutyrate (marker of insulin resistance); amino acids, lysophospholipid, acylCN, fatty acids (associated to insulin sensivity)	[157]
Obesity and diabetes	Normal weight (NW, $n = 39$), obese (OB, $n = 64$), and T2D ($n = 17$) adolescents	MS/MS	Targeted	Plasma	(↓) T2D: Medium-to- short-chain AcylCN species, amino acids	[158]
CVD risk in healthy individuals	Healthy with CVD risk ($n = 864$)	^{1}H-NMR	Non-targeted	Plasma	High risk:2-oxoglutarate(↑), dimethylglycine(↑), serine(↑); Low risk: (↑)3-hydroxybutyrate, threonine(↓), creatinine(↓)	[159]
Diabetes and CVD risk	Cohort study participants ($n = 1015$)	LC-MS	Targeted	Plasma	Glutamine(↓), glutamate(↑), BCAAs (↑), tryptophan break down products (kynurenic acid, anthranilic acid) (↑), nucleotide metabolites (xanthosine, *n*-carbamoyl-β-alanine) (↑)	[160]

**Arrows indicate the increase or decrease of this specific metabolite with the illness.*

NSTEACS, non-ST segment elevation acute coronary syndrome patients; ACS, acute coronary syndrome; MIS, CAD, coronary artery disease; PMI, planned myocardial infarction; SMI, spontaneous myocardial infarction; FI, flow injection; AF, atrial fibrillation; AAA, abdominal aortic aneurysm; CVD, cardiovascular disease; GC-ToF-MS, gas chromatography-time of flight-mass spectrometry; LVD, left ventricular dysfunction; UHPLC-MS/MS (± ESI), ultra-high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry; LC-QToF-MS, liquid chromatography-quadrupole-time of flight-mass spectrometry; TMAO, trimethylamin-N-oxide; PAG, phenylacetylglycine, DMA, dimethylamine; BCAAs, branched-chain amino acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; AcylCN, acylcarnitine; T2D, type 2 diabetes; ↑/↓, depends on the compound.

(lactate) and the TCA cycle metabolites (succinic acid, malic and aconitic acid), and also amino acids known to be concentrated within cardiomyocytes after planned myocardial infarction.[81] Also, Bodi *et al.*[142] found an increase in circulating levels of glucose and lactate as well as in glutamine, glycine, glycerol, phenylalanine, tyrosine, and phosphoethanolamine; the decrease in choline-containing compounds was also present in this trial. After an ischemia-reperfusion injury, the study findings[148] were consistent with a general decrease in oxidative fuel metabolism and a greater reliance on anaerobic metabolism of glucose for energy.

4.3 Heart Failure

A recent study conducted by Kang and colleagues showed that it was possible to detect changes between heart failure (HF) patients and controls by comparing both groups using NMR spectroscopy. The metabolite profiles of the HF patients exhibited increased urinary levels of ketone bodies accompanied by reduced levels of TCA intermediates, suggesting altered energy metabolism. Furthermore, they observed perturbations in metabolic pathways involved in methylmalonate and nucleotide metabolism.[149] Similarly, Dunn and co-workers[150] measured serum metabolites of 52 individuals with heart failure and 57 controls through a GC-MS-based platform, identifying pseudouridine as the most discriminatory metabolite of the disease stage. Also, markers of a stroke were analyzed by Jung and coworkers in a recent work.[156]

4.4 Atrial Fibrillation

Recent data from Mayr *et al.*[151] evidenced elevated levels of 3-hydroxybutyrate, ketogenic amino acids (tyrosine and leucine) and glycine, in persistent atrial fibrillation in cardiac tissue. It has highlighted an association between greater atrial tissue concentrations of ketogenic amino acids and 3-hydroxybutyrate and persistant atrial fibrillation, denoting a significant low ratio of glucose to acetate and suggesting that ketone bodies are important as an energy source in arrhythmia states. Another example of chronic state is the abdominal aortic aneurysm also recently submitted to study in a metabolomic interventional trial.[141]

4.5 Acute Coronary Syndromes

A recent study by Vallejo *et al.*[152] was performed comparing patients with acute coronary syndromes and healthy volunteers, finding, mainly, that 4-hydroxyproline is an interesting marker, presenting inferior levels in patients with acute coronary syndrome. Two years later, Teul and co-workers presented similar results.[153]

In last few years, the targeted and non-targeted metabolomic approaches have also begun to be applied to clinical research on other CVD risk factors besides the above-mentioned, including diabetes mellitus type 2 and obesity.[154,155] Both interventional[76] and epidemiological studies[84] have been carried out for the identification of a series of known and also novel, deregulated metabolites that associate with diabetes and the early appearance of insulin resistance and glucose intolerance in non-diabetic subjects.[157,158] Finally, Bernini and colleagues studied in a recent metabolomic trial early biomarkers of CVD in a healthy CVD risk population.[159] Furthermore, another recent study investigated in the Framingham Heart Study participant's pathways associated with cardiometabolic risk.[160]

Summarizing, with regard to biofluid samples blood was the most widely used biological sample, using plasma in 17 articles,[76,84,85,141,144,147,148,153,154–156,157,158] and serum in four studies,[142,143,146,155] whereas one was performed on urine,[149] and two used both urine and blood samples.[156,159] Furthermore, one study analyzed cardiac tissue samples.[151] With regard to the analytical platform, MS was extensively applied,[141,144,145,147,148,153,155,158,160,156–158,160] but NMR has also been employed in seven studies,[142,143,146,149,151,156,159] and in three articles both platforms were applied.[76,84,85] Referent to metabolites, there are some metabolites in common in the pathological stages of the cardiovascular disease: amino acids. Concretely branched-chain amino acids are commonly presented in several studies,[76,84,85,146,149,151,154,157,158] also choline and choline-containing metabolites,[142,143,149,155] lactate,[142,146,153,156,158] and some other tricarboxylic cycle intermediates,[85,147,149,153,156,159,157,158] ketone bodies such as 3-hydroxybutyrate[84,151,153,159] and 4-hydroxyproline.[85,153,158] Also, compounds in the pathway of lipid metabolism as carnitine derivatives.[147,148,154,157,158] The characteristics of these studies and the list of metabolites found are presented in Table 79.2.

No single biomarker will be specific enough to be used on its own as the only marker of cardiovascular disease. The development of clinically useful biomarkers will require a more comprehensive understanding of how exogenous factors can impact on the metabolome (e.g., diet, medication) and what intra- and inter-individual variability has normal values in these metabolites. A recent study published in *Nature*[161] describes the current potential of metabolomics in unambiguously identifying metabolic profiles of CVD risk prediction. Particularly, the metabolomics

approach used for the first time led to the elucidation of the biochemical mechanisms probably underlying the link between specific dietary components (namely phosphatidylcholines) and the risk of atherosclerosis, through the generation of pro-atherosclerotic metabolites (choline, trimethylamine-N-oxide and betaine) by the intestinal microbiota. Besides supporting the interrelation between diet, gut microbiota, and the human metabolome, the study demonstrated the potentialities of metabolomic biomarker discovery to elucidate novel pathways of the disease. In fact, unlike the well-established relationship between levels of plasma triglycerides and cholesterol with cardiovascular risk, little was known, until now, of the role of the third major category of lipids (phospholipids) in the pathogenesis of atherosclerotic disease.

5. INTERMEDIATE BIOMARKERS OF CVD RISK RELATED TO DIET EXPOSURE

In the past few years a considerable number of interventional studies have been published, most of them evaluating the effect of an acute or chronic intake of a particular food in the CVD risk population. Below, there are some examples of polyphenol intake interventional or epidemiological studies in high-cardiovascular-risk subjects. A summary of selected studies is presented in Table 79.3.

In a long-term interventional cocoa feeding trial with subjects at high risk of cardiovascular disease[162] there were several phenolic compounds in urine, including phase II conjugated metabolites, glucuronides and sulfate conjugates of epicatechin and epicatechin derivates identified, as well as valerolactones, considered as metabolome biomarkers of cocoa intake. Plasma was also evaluated, showing an increase in glucuronide conjugates of dihydroxyphenylvalerolactones after the same intake. These findings exhibit biomarkers of cocoa intake and could be of interest with regards to CVD risk, because some of these phenolic acids have been recently proved to inhibit the secretion of pro-inflammatory cytokines involved in the early stages of the atherosclerosis process.

A recent trial has also studied the urinary metabolomic changes observed in male subjects with cardiovascular risk factors following the moderate consumption of red wine, dealcoholized red wine, and gin.[163] Samples were analyzed by an ^{1}H-NMR-driven non-targeted approach and both potential biomarkers of wine consumption (as tartrate) and wine effect on subjects were reported (an increase of 3-methyl-2-oxovalerate). Increased plasma levels of BCAAs (valine, leucine, and isoleucine) have been linked to cardiovascular and diabetic disorders.[154] Therefore, the observed increase in urinary 3-methyl-2-oxovalerate (a BCAA metabolite) suggested a possible up-regulation of the BCAA catabolic pathway after both red wine and dealcoholized red wine intake. Also hippurate and 4-hydroxyphenylacetate were identified as gut miocrobiota biomarkers after red wine consumption.[128,166,167]

Metabolic syndrome (MetS) is a constellation of metabolic disturbances which represent known risk factors for the development of atherosclerotic cardiovascular disease.[168] Interestingly, nuts have been recently proposed as promising functional foods for managing both long- and short-term cardiometabolic risk factors.[169–171] In this context, Tulipani *et al.*,[97] applying an HPLC-Q-ToF-MS-driven non-targeted approach, studied the urinary changes in volunteers with metabolic syndrome following 12 weeks of nut consumption (walnuts, hazelnuts, and almonds: 30 g/day) in comparison to individuals given a control diet. The multivariate analysis discriminated relevant mass features from the urinary metabolome corresponding to the nut-enriched diet. This study revealed 20 nut intake biomarkers, including fatty acid conjugated metabolites, phase II and microbial-derived phenolic metabolites, and serotonin metabolites. Using a targeted approach, the same authors further quantified the increased excretion of urolithins, namely represented by metabolites derived from the microbial metabolism of the ellagitannins,[131] and came to the conclusion that the role of nut polyphenols on clinical parameters of metabolic syndrome cannot be discarded.

Recently, large-scale metabolomic studies have been carried out to correlate specific dietary patterns (from a particular geographical community) with CVD and risk-related factors. Holmes and co-workers[164] evaluated with an NMR technique the metabolic phenotype diversity and the association with diet and blood pressure in a study with a huge number of individuals from China, Japan, the UK, and USA. The study found differences between the patterns of the East Asian and Western populations. Diets, diet-related major risk factors and coronary heart disease were significantly different in the East Asian diet (Chinese and Japanese phenotypes), where the main differences were noticed in the vegetables and animal proteins consumed, and in blood pressure markers. Curiously, they found that Japanese living in Japan and Japanese Americans are also differentiated in a multivariate analysis (orthogonal least square discriminant analysis) by discriminatory metabolites. Referring to metabolic biomarkers they found an association between urinary excretion of several metabolites such as alanine, formate, hippurate and N-methylnicotinate and differences in systolic and

TABLE 79.3 Biomarkers of Polyphenol Intake in CVD Human Studies by Metabolomic Methodologies

Subjects of the Study	Polyphenol Intake	Metabolomic Technique	Biofluid	Metabolite	Excretion in Biofluid	Biomarkers Information	Reference
High-risk cardiovascular subjects ($n = 42$)	a. 20 g of cocoa powder/day with 250 mL of skimmed milk	LC-MS/MS	Urine	Epicatechin-*O*-glucuronide, epicatechin-*O*-sulfate, *O*-methyl-epicatechin-*O*-glucuronide, *O*-methyl-epicatechin-*O*-sulfate, vanillic, 3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic acids, DHPV *O*-glucuronide, PHPV-sulfate, MHPV *O*-glucuronide, MHPV *O*-sulfate	↑	Biomarkers of regular cocoa intake (flavanols, and colonic microbiota-derived metabolites from epicatechin/procyanidins)	162
	b. 500 mL/day skimmed milk alone		Plasma	DHPV *O*-glucuronide, MHPV *O*-glucuronide	↑		
Metabolic syndrome: nuts group ($n = 22$); control group ($n = 20$)	a. 30 g/day of unpeeled mixed nuts (15 g of walnuts, 7.5 g of almonds and 7.5 g of hazelnuts)	HPLC-Q-ToF-MS	Urine	10-hydroxy-decene-4,6-diynoic acid sulfate, tridecadienoic/tridecynoic acid glucuronide, 4-coumaryl alcohol glucuronide, urolithin A sulfoglucuronide, 4-coumaryl alcohol sulfate, N-acetylserotonin sulfate, hydroxyindoleacetic acid, dodecanedioic acid, pyrogallol sulfate, urolithin A glucuronide, urolithin A sulfate	↑	Biomarkers of nut consumption	97
	b. Control diet						
Metabolic syndrome: nuts group ($n = 22$); control group ($n = 20$)	a. 30 g/day of unpeeled mixed nuts (15 g of walnuts, 7.5 g of almonds and 7.5 g of hazelnuts)	HPLC-Q-ToF-MS	Urine	Ellagitannins and procyanidins	↑	Biomarkers of nut consumption	131
				Urolithins A and B derived from ellagitannins	↑	Microbial-derived metabolites of ellagitannins (nut consumption)	
	b. Control diet						
Cardiovascular risk subjects ($n = 61$)	a. 272 mL/day red wine (R)	1 H-NMR	Urine	Tartrate (W, D)	↑	Wine intake biomarker	163
				Ethanol (W, G)	↑	Alcohol from beverage	

				Hippurate (D) 4-hydroxyphenylacetate (D)	↑	Gut microbiota biomarkers and/or polyphenol metabolites	
	b. 272 mL/day dealcoholized red wine (D)			3-methyl-2-oxovalerate (W, D)	↑	Endogenous metabolites from BCAAs	
	c. 100 mL/day gin (G)						
China ($n = 832$), Japan ($n = 1138$), UK ($n = 496$) and USA ($n = 2164$) population	– (Geographical diet-associated changes)	1 H-NMR	Urine	Formate , alanine , hippurate	Formate ↓, alanine ↑, hippurate ↓	Metabolites related with systolic and diastolic blood pressure	[164]
North China ($n = 523$);	–	1 H NMR	Urine	Dimethylglycine, alanine, lactate, isoleucine, leucine, valine, *N*-acetyls of glycoprotein fragments (including uromodulin), *N*-acetyl neuraminic acid, pentanoic/heptanoic acid, methylguanidine	↑ North China	TCA cycle, fatty-acid metabolism, methylmalonate and nucleotide metabolism	[165]
South China ($n = 244$)	(Geographical diet-associated changes)			Hippurate, 4-cresyl sulfate, phenylacetylglutamine; 2-hydroxyisobutyrate, succinate, creatine, *scyllo*-inositol, prolinebetaine, *trans*-aconitate	↑ South China		

DHPV, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; MHPV, 5-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone; HPLC-Q-TOF-MS, high performance liquid chromatography-quadrupole-time of flight-mass spectrometry; TCA, tricarboxylic acids cycle; BCAAs, branched-chain amino acids; W, red wine; G, gin; D, dealcoholized red wine.

diastolic blood pressure. Alanine was higher in people consuming a predominantly animal diet compared with a predominantly vegetable diet and it was directly associated positively with both systolic and diastolic blood pressure. As reported in experimental animal models, alanine modulates cardiovascular responses to circulating catecholamines and increases blood pressure.[172] Formate and sodium urinary excretion positively correlated, suggesting formate plays a role in blood pressure regulation. Furthermore, the inverse association of hippurate with blood pressure reflected a definite association between diet and dietary polyphenol sources.[173] These epidemiological results demonstrate that the metabolic phenotype of the population could discriminate different subgroups within that population, and could identify putative biomarkers of diet linked to hypertension.

The second study investigated the association between dietary habits and blood pressure, identifying discriminant biomarkers between north and south Chinese subjects at different risks of CVD.[165] Urinary discriminating metabolites included dimethylglycine, alanine, lactate, BCAAs N-acetyls of glycoprotein fragments, N-acetyl neuraminic acid, petranoic/heptanoic acid and methylguanidine (higher in the north than in the south). The metabolites that were higher in the south than in the north were gut microbial co-metabolites such as hippurate, 4-cresyl sulfate, phenylacetylglutamine, 2-hydroxyisobutyrate, succinate, creatine, scyllo-inositol, prolinebetaine, and transaconitate. In accordance with cardiovascular risk factors, systolic and diastolic blood pressure were higher in the northern sample population; as were body mass index, smoking rates and lower physical activity. These findings indicate the importance of environmental influences (e.g., diet), endogenous metabolism, and mammalian-gut microbial cometabolism, which may help explain north–south China differences in CVD risk.

Currently, the metabolomic approach is also applied to identify groups of individuals (metabolic phenotypes) which have a differential response to dietary interventions, which is a promising tool for better understanding the mechanisms of actions underlying the inter-individual difference in the response to dietary challenges and CVD risk.[174] As an example, Martin *et al.*[175] evaluated the metabolic responses of 30 subjects in free-living conditions, ranked by an anxiety phenotype after a daily intake of 40 g of black chocolate for 14 days. At the beginning, during and at the end of the study plasma and urine samples were collected and these were analyzed by NMR and MS. The results showed a metabolic effect after a daily consumption of chocolate, which can modify the metabolism of subjects with different levels of anxiety. As a result, it indicated that the metabolic effects were statistically significant only in subjects with a "high anxiety phenotype." In fact, black chocolate intake resulted in a decrease in urinary levels of catecholamines and cortisol in subjects with high stress. In another study, O'Sullivan *et al.*[176] applied this strategy in a double-blind, randomized, placebo-controlled dietary intervention study. The authors wondered whether metabolic phenotype could identify responders to a supplementation of vitamin D in relation to metabolic syndrome markers. After 4 weeks of intervention with 15 g of vitamin D or a placebo, urine and plasma samples were analyzed by 1H-NMR, and k-means clustering of biochemical parameters was performed. The primary analysis did not show any effect of supplementation on markers of the metabolic syndrome, but k-means clustering was able to group together responders to vitamin D supplementation.

However, to the best of our knowledge large-scale nutrimetabolomic studies specifically focused on assessing the effects of polyphenol-rich food consumption in the prevention and/or reversion of CVD by non-targeted approach are still missing. "Intermediate biomarkers," namely biomarkers of early metabolic diagnosis of disease onset, which in addition are strictly related to food exposure, should be further explored.

The "*meet-in-the middle*" approach was first mentioned by Vineis and Perera in 2007.[177] It was applied in order to fortify a new research line on the role of dietary factors in prevention or development of a pathologic state. As an example of its application, Chadeau-Hyam *et al.*[178] aimed to identify potential intermediate plasma metabolomic biomarkers related to both the occurrence of colon and breast cancer and the exposure to specific dietary components in a subcohort study. The study identified significant inverse correlations between dietary fiber intake and potential biomarkers of colon cancer development, strengthening the evidence on fiber intake as the most established protective factor against colon cancer. The findings of this study prove the potentiality of the research focused on identification of novel relevant intermediate biomarkers linking dietary exposure to disease development, and on understanding the metabolic pathways explaining the observed relations. The application of this approach could make a key contribution in deciphering the dietary polyphenols effects on CVD prevention.

6. CONCLUSIONS AND FUTURE CHALLENGES

Nutrimetabolomics is an emerging approach in nutritional sciences, holding the potential to increase

the discovery and identification rate of novel biomarkers of food intake (food metabolome) and food effects on CVD prevention. The application of nutrimetabolomic studies to large-scale cohort studies is highly hoped for in order to: (1) clarify the effect of dietary polyphenols as an instrument in CVD prevention, and (2) fill the gap between clinical and nutritional research and establish new correlations and a deeper understanding of the impact of polyphenol intake on health preservation. The integration of metabolomic technologies and the clinical disease risk assessment will allow us a better approach to the biomedical problem, and it will have a positive impact on public health.

Acknowledgments

The authors are grateful for the support granted by the Spanish government: CICYT AGL2009–13906-C02–01 and FUN-C Food CSD2007–063 from the Ingenio-CONSOLIDER program. R.V.-F; M.R; M.B-O; S. T.; O.K.; R. L. and M. U.-S would like to thank the FPI and FPU fellowships, the "Juan de la Cierva" and the "Ramon y Cajal" programs of the Spanish Government and the Fondo Social Europeo, respectively. M. G.-A and M.R-R thanks the Generalitat de Catalunya's Agency for Management of University and Research Grants (AGAUR) for the FI-DGR fellowship.

References

1. De Caterina R, Zampolli A, Del Turco S, Madonna R, Massaro M. Nutritional mechanisms that influence cardiovascular disease. *Am J Clin Nutr* 2006;**83**(2):421S–6S.
2. Ignarro LJ, Balestrieri ML, Napoli C. Nutrition, physical activity, and cardiovascular disease: an update. *Cardiovasc Res* 2007;**73**(2):326–40.
3. de Roos B. Nutrition proteomics and biomarker discovery. *Expert Rev Proteomics* 2009;**6**(4):349–51.
4. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* 2004;**364**(9438):937–52.
5. Levenson JW, Skerrett PJ, Gaziano JM. Reducing the global burden of cardiovascular disease: the role of risk factors. *Prev Cardiol* 2002;**5**(4):188–99.
6. Yusuf S, Reddy S, Ôunpuu S, Anand S. Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. *Circulation* 2001;**104**(22):2746–53.
7. Neveu V, Perez-Jiménez J, Vos F, Crespy V, du Chaffaut L, Mennen L, et al. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. *Database (Oxford)* 2010;**2010**:bap024.
8. Andrés-Lacueva C, Medina-Remon A, Llorach R, Urpi-Sarda M, Khan N, Chiva-Blanch G, et al. Phenolic compounds: chemistry and occurrence in fruits and vegetables. In: de la Rosa EA-P LA, González-Aguilar GA, editors. *Fruit and vegetable phytochemicals: chemistry, nutritional value and stability*. Ames, IA: Blackwell Publishing; 2009. p. 53–88.
9. Scalbert A, Manach C, Morand C, Remesy C, Jimenez L. Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr* 2005;**45**(4):287–306.
10. Cassidy A, Mukamal KJ, Liu L, Franz M, Eliassen AH, Rimm EB. High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. *Circulation* 2013;**127**(2):188–96.
11. Jennings A, Welch AA, Fairweather-Tait SJ, Kay C, Minihane AM, Chowienczyk P, et al. Higher anthocyanin intake is associated with lower arterial stiffness and central blood pressure in women. *Am J Clin Nutr* 2012;**96**(4):781–8.
12. Zamora-Ros R, Urpi-Sarda M, Lamuela-Raventós RM, Martínez-González MÁ, Salas-Salvadó J, Arós F, et al. High urinary levels of resveratrol metabolites are associated with a reduction in the prevalence of cardiovascular risk factors in high-risk patients. *Pharmacol Res* 2012;**65**(6):615–20.
13. Cassidy A, Rimm EB, O'Reilly ÉJ, et al. Dietary flavonoids and risk of stroke in women. *Stroke* 2012;**43**(4):946–51.
14. Cassidy A, O'Reilly ÉJ, Kay C, Chiuve SE, Resrode KM. Habitual intake of flavonoid subclasses and incident hypertension in adults. *Am J Clin Nutr* 2011;**93**(2):338–47.
15. McCullough ML, Peterson JJ, Patel R, Jacques PF, Shah R, Dwyer JT. Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults. *Am J Clin Nutr* 2012;**95**(2):454–64.
16. Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, et al. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *Am J Clin Nutr* 2007;**85**(3):895–909.
17. Hollman PC, Geelen A, Kromhout D. Dietary flavonol intake may lower stroke risk in men and women. *J Nutr* 2010;**140**(3):600–4.
18. Rein D, Lotito S, Holt RR, Keen CL, Schmitz HH, Fraga CG. Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status. *J Nutr* 2000;**130**(8S Suppl):2109S–14S.
19. Wan Y, Vinson JA, Etherton TD, Proch J, Lazarus SA, Kris-Etherton PM. Effects of cocoa powder and dark chocolate on LDL oxidative susceptibility and prostaglandin concentrations in humans. *Am J Clin Nutr* 2001;**74**(5):596–602.
20. Hooper L, Kroon PA, Rimm EB, Cohn JS, Harvey I, Le Cornu KA, et al. Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. *Am J Clin Nutr* 2008;**88**(1):38–50.
21. Desch S, Schmidt J, Kobler D, Sonnabend M, Eitel I, Sareban M, et al. Effect of cocoa products on blood pressure: systematic review and meta-analysis. *Am J Hypertens* 2010;**23**(1):97–103.
22. Erlund I, Koli R, Alfthan G, Marniemi J, Puukka P, Mustonen P, et al. Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. *Am J Clin Nutr* 2008;**87**(2):323–31.
23. Grassi D, Necozione S, Lippi C, Croce G, Valeri L, Pasqualetti P, et al. Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. *Hypertension* 2005;**46**(2):398–405.
24. Taubert D, Roesen R, Lehmann C, Jung N, Schömig E. Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide: a randomized controlled trial. *JAMA* 2007;**298**(1):49–60.
25. Taubert D, Roesen R, Schömig E. Effect of cocoa and tea intake on blood pressure: a meta-analysis. *Arch Intern Med* 2007;**167**(7):626–34.
26. Park YK, Kim JS, Kang MH. Concord grape juice supplementation reduces blood pressure in Korean hypertensive men: double-blind, placebo controlled intervention trial. *Biofactors* 2004;**22**(1–4):145–7.
27. Heiss C, Dejam A, Kleinbongard P, Schewe T, Sies H, Kelm M. Vascular effects of cocoa rich in flavan-3-ols. *JAMA* 2003;**290**(8):1030–1.
28. Heiss C, Finis D, Kleinbongard P, Hoffmann A, Rassaf T, Kelm M, et al. Sustained increase in flow-mediated dilation after daily intake of high-flavanol cocoa drink over 1 week. *J Cardiovasc Pharmacol* 2007;**49**(2):74–80.

29. Heiss C, Kleinbongard P, Dejam A, Perré S, Schroeter H, Sies H, et al. Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. *J Am Coll Cardiol* 2005;**46**(7):1276–83.
30. Engler MB, Engler MM, Chen CY, Malloy MJ, Browne A, Chiu EY, et al. Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults. *J Am Coll Nutr* 2004;**23**(3):197–204.
31. Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, et al. (−)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci USA* 2006;**103**(4):1024–9.
32. Wang-Polagruto JF, Villablanca AC, Polagruto JA, Lee L, Holt RR, Schrader HR, et al. Chronic consumption of flavanol-rich cocoa improves endothelial function and decreases vascular cell adhesion molecule in hypercholesterolemic postmenopausal women. *J Cardiovasc Pharmacol* 2006;**47**(Suppl 2):S177–86 [discussion S206–9].
33. Grassi D, Mulder TP, Draijer R, Desideri G, Molhuizen HO, Ferri C. Black tea consumption dose-dependently improves flow-mediated dilation in healthy males. *J Hypertens* 2009;**27**(4):774–81.
34. Widlansky ME, Hamburg NM, Anter E, Holbrook M, Kahn DF, Elliott JG, et al. Acute EGCG supplementation reverses endothelial dysfunction in patients with coronary artery disease. *J Am Coll Nutr* 2007;**26**(2):95–102.
35. Cuevas AM, Guasch V, Castillo O, Irribarra V, Mizon C, San Martin A, et al. A high-fat diet induces and red wine counteracts endothelial dysfunction in human volunteers. *Lipids* 2000;**35**(2):143–8.
36. Papamichael C, Karatzis E, Karatzi K, Aznaouridis K, Papaioannou T, Protogerou A, et al. Red wine's antioxidants counteract acute endothelial dysfunction caused by cigarette smoking in healthy nonsmokers. *Am Heart J* 2004;**147**(2):E5.
37. Pearson DA, Paglieroni TG, Rein D, Wun T, Schramm DD, Wang JF, et al. The effects of flavanol-rich cocoa and aspirin on ex vivo platelet function. *Thromb Res* 2002;**106**(4–5):191–7.
38. Rein D, Paglieroni TG, Wun T, Pearson DA, Schmitz HH, Gosselin R, et al. Cocoa inhibits platelet activation and function. *Am J Clin Nutr* 2000;**72**(1):30–5.
39. Keevil JG, Osman HE, Reed JD, Folts JD. Grape juice, but not orange juice or grapefruit juice, inhibits human platelet aggregation. *J Nutr* 2000;**130**(1):53–6.
40. Mathur S, Devaraj S, Grundy SM, Jialal I. Cocoa products decrease low density lipoprotein oxidative susceptibility but do not affect biomarkers of inflammation in humans. *J Nutr* 2002;**132**(12):3663–7.
41. Mao TK, van de Water J, Keen CL, Schmitz HH, Gershwin ME. Modulation of TNF-alpha secretion in peripheral blood mononuclear cells by cocoa flavanols and procyanidins. *Dev Immunol* 2002;**9**(3):135–41.
42. Schramm DD, Karim M, Schrader HR, Holt RR, Kirkpatrick NJ, Polagruto JA, et al. Food effects on the absorption and pharmacokinetics of cocoa flavanols. *Life Sci* 2003;**73**(7):857–69.
43. Schramm DD, Wang JF, Holt RR, Ensunsa JL, Gonsalves JL, Lazarus SA, et al. Chocolate procyanidins decrease the leukotriene-prostacyclin ratio in humans and human aortic endothelial cells. *Am J Clin Nutr* 2001;**73**(1):36–40.
44. Zhu Y, Ling W, Guo H, Song F, Ye Q, Zou T, et al. Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: a randomized controlled trial. *Nutr Metab Cardiovasc Dis* 2012. Epub ahead of print.
45. Hassellund SS, Flaa A, Kjeldsen SE, Seljeflot I, Karlsen A, Erlund I, et al. Effects of anthocyanins on cardiovascular risk factors and inflammation in pre-hypertensive men: a double-blind randomized placebo-controlled crossover study. *J Hum Hypertens* 2013;**27**(2):100–6.
46. Shaughnessy KS, Boswall IA, Scanlan AP, Gottschall-Pass KT, Sweeney MI. Diets containing blueberry extract lower blood pressure in spontaneously hypertensive stroke-prone rats. *Nutr Res* 2009;**29**(2):130–8.
47. Takikawa M, Inoue S, Horio F, Tsuda T. Dietary Anthocyanin-Rich bilberry extract Ameliorates Hyperglycemia and insulin sensitivity via activation of AMP-activated protein kinase in diabetic mice. *J Nutr* 2010;**140**(3):527–33.
48. Wu X, Kang J, Xie C, Burris R, Ferguson ME, Badger TM, et al. Dietary blueberries attenuate atherosclerosis in apolipoprotein e-deficient mice by upregulating antioxidant enzyme expression. *J Nutr* 2010;**140**(9):1628–32.
49. Naruszewicz M, Laniewska I, Millo B, Dłuzniewski M. Combination therapy of statin with flavonoids rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients after myocardial infraction (MI). *Atherosclerosis* 2007;**194**(2):e179–184.
50. Chou EJ, Keevil JG, Aeschlimann S, Wiebe DA, Folts JD, Stein JH. Effect of ingestion of purple grape juice on endothelial function in patients with coronary heart disease. *Am J Cardiol* 2001;**88**(5):553–5.
51. Vita JA. Polyphenols and cardiovascular disease: effects on endothelial and platelet function. *Am J Clin Nutr* 2005;**81**(1):292S–7S.
52. Kurowska EM, Spence JD, Jordan J, Wetmore S, Freeman DJ, Piché LA, et al. HDL-cholesterol-raising effect of orange juice in subjects with hypercholesterolemia. *Am J Clin Nutr* 2000;**72**(5):1095–100.
53. Gorinstein S, Caspi A, Libman I, Lerner HT, Huang D, Leontowicz H, et al. Red grapefruit positively influences serum triglyceride level in patients suffering from coronary atherosclerosis: studies in vitro and in humans. *J Agric Food Chem* 2006;**54**(5):1887–92.
54. Boots AW, Haenen GRMM, Bast A. Health effects of quercetin: From antioxidant to nutraceutical. *Eur J Pharmacol* 2008;**585**(2–3):325–37.
55. Bischoff SC. Quercetin: potentials in the prevention and therapy of disease. *Curr Opin Clin Nutr Metab Care* 2008;**11**(6):733–40.
56. Hubbard GP, Wolffram S, Lovegrove JA, Gibbins JM. Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. *J Thromb Haemost* 2004;**2**(12):2138–45.
57. Hooper L, Kay C, Abdelhamid A, Kroon PA, Cohn JS, Rimm EB, et al. Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. *Am J Clin Nutr* 2012;**95**(3):740–51.
58. de Kleijn MJJ, van der Schouw YT, Wilson PWF, Grobbee DE, Jacques PF. Dietary intake of phytoestrogens is associated with a favorable metabolic cardiovascular risk profile in postmenopausal US women: the Framingham study. *J Nutr* 2002;**132**(2):276–82.
59. Squadrito F, Altavilla D, Crisafulli A, Saitta A, Cucinotta D, Morabito N, et al. Effect of genistein on endothelial function in postmenopausal women: a randomized, double-blind, controlled study. *Am J Med* 2003;**114**(6):470–6.
60. Clifford MN, Scalbert A. Ellagitannins – nature, occurrence and dietary burden. *J Sci Food Agric* 2000;**80**(7):1118–25.
61. Larrosa M, García-Conesa MT, Espín JC, Tomás-Barberán FA. Ellagitannins, ellagic acid and vascular health. *Mol Aspects Med* 2010;**31**(6):513–39.
62. Miró-Casas E, Covas MI, Fitó M, Farré-Albadalejo M, Marrugat J, de la Torre R. Tyrosol and hydroxytyrosol are absorbed from moderate and sustained doses of virgin olive oil in humans. *Eur J Clin Nutr* 2003;**57**(1):186–90.

63. Covas MI, Nyyssönen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, et al. The effect of polyphenols in olive oil on heart disease risk factors, a randomized trial. *Ann Intern Med* 2006;**145**(5):333–41.
64. Moreno-Luna R, Muñoz-Hernandez R, Miranda ML, Costa AF, Jimenez-Jimenez L, Vallejo-Vaz AJ, et al. Olive oil polyphenols decrease blood pressure and improve endothelial function in young women with mild hypertension. *Am J Hypertens* 2012;**25**(12):1299–304.
65. Zamora-Ros R, Rabassa M, Llorach R, González CA, Andres-Lacueva C. Application of dietary phenolic biomarkers in epidemiology: past present and future. *J Agric Food Chem* 2012;**60**(27):6648–57.
66. Andres-Lacueva C, Zamora-Ros R. Wanted: specific nutritional biomarkers for food consumption for the study of its protective role in health. *Br J Nutr* 2010;**103**(3):307–8.
67. US Department of Agriculture. *USDA database for the proanthocyanidin content of selected foods*. Beltsville, MD; 2004
68. US Departament of Agriculture. *USDA database for the isoflavone content of selected foods*, release 2.0 ed.; Beltsville, MD; 2008.
69. US Departament of Agriculture. *USDA database for the flavonoid content of selected foods*, release 3.0 ed.; Beltsville, MD; 2011.
70. Rothwell JA, Urpi-Sarda M, Boto-Ordoñez M, Knox C, Llorach R, Eisner R, et al. Phenol-Explorer 2.0: a major update of the Phenol-Explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals. *Database (Oxford)* 2012;**2012**:bas031.
71. Pérez-Jiménez J, Hubert J, Hooper L, Cassidy A, Manach C, Williamson G, et al. Urinary metabolites as biomarkers of polyphenol intake in humans: a systematic review. *Am J Clin Nutr* 2010;**92**(4):801–9.
72. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001;**69**(3):89–95.
73. Monteiro MS, Carvalho M, Bastos ML, Guedes de Pinho P. Metabolomics analysis for biomarker discovery: advances and challenges. *Curr Med Chem* 2013;**20**(2):257–71.
74. McKillop AM, Flatt PR. Emerging applications of metabolomic and genomic profiling in diabetic clinical medicine. *Diabetes Care* 2011;**34**(12):2624–30.
75. Bain JR, Stevens RD, Wenner BR, Ilkayeva O, Muoio DM, Newgard CB. Metabolomics applied to diabetes research: moving from information to knowledge. *Diabetes* 2009;**58**(11):2429–43.
76. Lanza IR, Zhang S, Ward LE, Karakelides H, Raftery D, Nair KS. Quantitative metabolomics by H-NMR and LC-MS/MS confirms altered metabolic pathways in diabetes. *PLoS One* 2010;**5**(5):e10538.
77. Llorach R, Garcia-Aloy M, Tulipani S, Vazquez-Fresno R, Andres-Lacueva C. Nutrimetabolomic strategies to develop new biomarkers of intake and health effects. *J Agric Food Chem* 2012;**60**(36):8797–808.
78. Gibney MJ, Walsh M, Brennan L, Roche HM, German B, van Ommen B. Metabolomics in human nutrition: opportunities and challenges. *Am J Clin Nutr* 2005;**82**(3):497–503.
79. Dettmer K, Aronov PA, Hammock BD. Mass spectrometry-based metabolomics. *Mass Spectrom Rev* 2007;**26**(1):51–78.
80. Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc* 2007;**2**(11):2692–703.
81. Lewis GD, Wei R, Liu E, Yang E, Shi X, Martinovic M, et al. Metabolite profiling of blood from individuals undergoing planned myocardial infarction reveals early markers of myocardial injury. *J Clin Invest* 2008;**118**(10):3503–12.
82. Zhang A, Sun H, Wang P, Han Y, Wang X. Modern analytical techniques in metabolomics analysis. *Analyst* 2012;**137**(2):293–300.
83. Bothwell JH, Griffin JL. An introduction to biological nuclear magnetic resonance spectroscopy. *Biol Rev Camb Philos Soc* 2011;**86**(2):493–510.
84. Suhre K, Meisinger C, Döring A, Altmaier E, Belcredi P, Gieger C, et al. Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. *PLoS One* 2010;**5**(11):e13953.
85. Teul J, Rupérez FJ, Garcia A, Vaysse J, Balayssac S, Gilard V, et al. Improving metabolite knowledge in stable atherosclerosis patients by association and correlation of GC-MS and 1H NMR fingerprints. *J Proteome Res* 2009;**8**(12):5580–9.
86. Chagoyen M, Pazos F. MBRole: enrichment analysis of metabolomic data. *Bioinformatics* 2011;**27**(5):730–1.
87. Xia J, Wishart DS. Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. *Nat Protoc* 2011;**6**(6):743–60.
88. Madsen R, Lundstedt T, Trygg J. Chemometrics in metabolomics – A review in human disease diagnosis. *Analytica Chimica Acta* 2010;**659**(1–2):23–33.
89. Cottret L, Wildridge D, Vinson F, Barrett MP, Charles H, Sagot MF, et al. MetExplore: a web server to link metabolomic experiments and genome-scale metabolic networks. *Nucleic Acids Res* 2010;**38**(Web Server issue):W132–7.
90. Eliasson M, Rannar S, Trygg J. From data processing to multivariate validation – essential steps in extracting interpretable information from metabolomics data. *Curr Pharm Biotechnol* 2011;**12**(7):996–1004.
91. Frolkis A, Knox C, Lim E, et al. SMPDB: The small molecule pathway database. *Nucleic Acids Res* 2010;**38**(Suppl. 1):D480–7.
92. Karnovsky A, Weymouth T, Hull T, Tarcea VG, Scardoni G, Laudanna C, et al. Metscape 2 bioinformatics tool for the analysis and visualization of metabolomics and gene expression data. *Bioinformatics* 2012;**28**(3):373–80.
93. Suhre K, Schmitt-Kopplin P. MassTRIX: mass translator into pathways. *Nucleic Acids Res* 2008;**36**(Suppl. 2):W481–4.
94. Tohge T, Fernie AR. Web-based resources for mass-spectrometry-based metabolomics: A user's guide. *Phytochemistry* 2009;**70**(4):450–6.
95. van Iersel MP, Kelder T, Pico AR, Hanspers K, Coort S, Conklin BR, et al. Presenting and exploring biological pathways with PathVisio. *BMC Bioinformatics* 2008;**9**:399.
96. Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB. Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends Biotechnol* 2004;**22**(5):245–52.
97. Tulipani S, Llorach R, Jáuregui O, López-Uriarte P, Garcia-Aloy M, Bullo M, et al. Metabolomics unveils urinary changes in subjects with metabolic syndrome following 12-week nut consumption. *J Proteome Res* 2011;**10**(11):5047–58.
98. Ceglarek U, Leichtle A, Brügel M, Kortz L, Brauer R, Bresler K, et al. Challenges and developments in tandem mass spectrometry based clinical metabolomics. *Mol Cell Endocrinol* 2009;**301**(1–2):266–71.
99. Draper J, Enot DP, Parker D, Beckmann M, Snowdon S, Lin W, et al. Metabolite signal identification in accurate mass metabolomics data with MZedDB, an interactive m/z annotation tool utilising predicted ionisation behaviour "rules". *BMC Bioinformatics* 2009;**10**(1):227.
100. Degtyarenko K, de Matos P, Ennis M, Hastings J, Zbinden M, McNaught A, et al. ChEBI: a database and ontology for chemical entities of biological interest. *Nucleic Acids Res* 2008;**36**(Database issue):D344–50.

101. Wang Y, Xiao J, Suzek TO, Zhang J, Wang J, Bryant SH. PubChem:a public information system for analyzing bioactivities of small molecules. *Nucleic Acids Res* 2009;**37**(Web Server issue):W623–33.
102. Williams AJ. Public chemical compound databases. *Curr Opin Drug Discov Devel* 2008;**11**(3):393–404.
103. Fahy E, Sud M, Cotter D, Subramaniam S. LIPID MAPS online tools for lipid research. *Nucleic Acids Res* 2007;**35**(Web Server issue):W606–12.
104. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, et al. METLIN: a metabolite mass spectral database. *Ther Drug Monit* 2005;**27**(6):747–51.
105. Ulrich EL, Akutsu H, Doreleijers JF, Harano Y, Ioannidis YE, Lin J, et al. BioMagResBank. *Nucleic Acids Res* 2008;**36**(Database issue):D402–408.
106. Taguchi R, Nishijima M, Shimizu T. Basic analytical systems for lipidomics by mass spectrometry in Japan. *Methods Enzymol* 2007;**432**:185–211.
107. Cui Q, Lewis IA, Hegeman AD, Anderson ME, Li J, Schulte CF, et al. Metabolite identification via the Madison metabolomics consortium database. *Nat Biotechnol* 2008;**26**(2):162–4.
108. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, et al. HMDB 3.0—The human metabolome database in 2013. *Nucleic Acids Res* 2013;**41**(D1):D801–7.
109. Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, et al. From genomics to chemical genomics: new developments in KEGG. *Nucleic Acids Res* 2006;**34**(Database issue):D354–7.
110. Krummenacker M, Paley S, Mueller L, Yan T, Karp PD. Querying and computing with BioCyc databases. *Bioinformatics* 2005;**21**(16):3454–5.
111. Joshi-Tope G, Gillespie M, Vastrik I, D'Eustachio P, Schmidt E, de Bono B, et al. Reactome: a knowledgebase of biological pathways. *Nucleic Acids Res* 2005;**33**(Database issue): D428–32.
112. Pico AR, Kelder T, van Iersel MP, Hanspers K, Conklin BR, Evelo C. WikiPathways: pathway editing for the people. *PLoS Biol* 2008;**6**(7):e184.
113. McNiven EM, German JB, Slupsky CM. Analytical metabolomics: nutritional opportunities for personalized health. *J Nutr Biochem* 2011;**22**(11):995–1002.
114. Primrose S, Draper J, Elsom R, Kirkpatrick V, Mathers JC, Seal C, et al. Metabolomics and human nutrition. *Br J Nutr* 2011;**105**(08):1277–83.
115. Wishart DS. Metabolomics: applications to food science and nutrition research. *Trends Food Sci Technol* 2008;**19**(9):482–93.
116. Oresic M. Metabolomics, a novel tool for studies of nutrition, metabolism and lipid dysfunction. *Nutr Metab Cardiovasc Dis* 2009;**19**(11):816–24.
117. Rezzi S, Ramadan Z, Fay LB, Kochhar S. Nutritional metabonomics: applications and perspectives. *J Proteome Res* 2007;**6**(2):513–25.
118. Solanky KS, Bailey NJ, Beckwith-Hall BM, Davis A, Bingham S, Holmes E, et al. Application of biofluid 1H nuclear magnetic resonance-based metabonomic techniques for the analysis of the biochemical effects of dietary isoflavones on human plasma profile. *Anal Biochem* 2003;**323**(2):197–204.
119. Solanky KS, Bailey NJ, Beckwith-Hall BM, Bingham S, Davis A, Holmes E, et al. Biofluid 1H NMR-based metabonomic techniques in nutrition research – metabolic effects of dietary isoflavones in humans. *J Nutr Biochem* 2005;**16**(4):236–44.
120. Wang Y, Tang H, Nicholson JK, Hylands PJ, Sampson J, Holmes E. A metabonomic strategy for the detection of the metabolic effects of chamomile (*Matricaria recutita* L.) ingestion. *J Agric Food Chem* 2005;**53**(2):191–6.
121. Daykin CA, Van Duynhoven JP, Groenewegen A, Dachtler M, Van Amelsvoort JM, Mulder TP. Nuclear magnetic resonance spectroscopic based studies of the metabolism of black tea polyphenols in humans. *J Agric Food Chem* 2005;**53**(5):1428–34.
122. Van Dorsten FA, Daykin CA, Mulder TP, Van Duynhoven JP. Metabonomics approach to determine metabolic differences between green tea and black tea consumption. *J Agric Food Chem* 2006;**54**(18):6929–38.
123. van Velzen EJ, Westerhuis JA, van Duynhoven JP, van Dorsten FA, Grün CH, Jacobs DM, et al. Phenotyping tea consumers by nutrikinetic analysis of polyphenolic end-metabolites. *J Proteome Res* 2009;**8**(7):3317–30.
124. Llorach R, Urpi-Sarda M, Jauregui O, Monagas M, Andres-Lacueva C. An LC-MS-based metabolomics approach for exploring urinary metabolome modifications after cocoa consumption. *J Proteome Res* 2009;**8**(11):5060–8.
125. Llorach R, Garrido I, Monagas M, Urpi-Sarda M, Tulipani S, Bartolome B, et al. Metabolomics study of human urinary metabolome modifications after intake of almond (*Prunus dulcis* (Mill.) D.A. Webb) skin polyphenols. *J Proteome Res* 2010;**9**(11):5859–67.
126. Walsh MC, Brennan L, Pujos-Guillot E, Sébédio JL, Scalbert A, Fagan A, et al. Influence of acute phytochemical intake on human urinary metabolomic profiles. *Am J Clin Nutr* 2007;**86**(6):1687–93.
127. Edmands WM, Beckonert OP, Stella C, Campbell A, Lake BG, Lindon JC, et al. Identification of human urinary biomarkers of cruciferous vegetable consumption by metabonomic profiling. *J Proteome Res* 2011;**10**(10):4513–21.
128. van Velzen EJ, Westerhuis JA, van Duynhoven JP, van Dorsten FA, Hoefsloot HC, Jacobs DM, et al. Multilevel data analysis of a crossover designed human nutritional intervention study. *J Proteome Res* 2008;**7**(10):4483–91.
129. van Dorsten FA, Grün CH, van Velzen EJ, Jacobs DM, Draijer R, van Duynhoven JP. The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics. *Mol Nutr Food Res* 2010;**54**(7):897–908.
130. Jacobs DM, Deltimple N, van Velzen E, van Dorsten FA, Bingham M, Vaughan EE, et al. (1)H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. *NMR Biomed* 2008;**21**(6):615–26.
131. Tulipani S, Urpi-Sarda M, García-Villalba R, Rabassa M, López-Uriarte P, Bulló M, et al. Urolithins are the main urinary microbial-derived phenolic metabolites discriminating a moderate consumption of nuts in free-living subjects with diagnosed metabolic syndrome. *J Agric Food Chem* 2012;**60**(36):8930–40.
132. Schnackenberg LK, Beger RD. Metabolomic biomarkers: their role in the critical path. *Drug Discov Today: Technol* 2007;**4**(1):13–6.
133. Koenig W. Cardiovascular biomarkers: Added value with an integrated approach? *Circulation* 2007;**116**(1):3–5.
134. Halim SA, Newby LK, Ohman EM. Biomarkers in cardiovascular clinical trials past, present, future. *Clin Chem* 2012;**58**(1):45–53.
135. Gilstrap LG, Wang TJ. Biomarkers and cardiovascular risk assessment for primary prevention: an update. *Clin Chem* 2012;**58**(1):72–82.
136. Herder C, Karakas M, Koenig W. Biomarkers for the prediction of type 2 diabetes and cardiovascular disease. *Clin Pharmacol Ther* 2011;**90**(1):52–66.
137. Griffin JL, Atherton H, Shockcor J, Atzori L. Metabolomics as a tool for cardiac research. *Nat Rev Cardiol* 2011;**8**(11):630–43.
138. Senn T, Hazen SL, Tang WH. Translating metabolomics to cardiovascular biomarkers. *Prog Cardiovasc Dis* 2012;**55**(1):70–6.
139. Rhee EP, Gerszten RE. Metabolomics and cardiovascular biomarker discovery. *Clin Chem* 2012;**58**(1):139–47.

140. Friedrich N. Metabolomics in diabetes research. *J Endocrinol* 2012;**215**(1):29–42.
141. Ciborowski M, Teul J, Martin-Ventura JL, Egido J, Barbas C. Metabolomics with LC-QTOF-MS permits the prediction of disease stage in aortic abdominal aneurysm based on plasma metabolic fingerprint. *PLoS One* 2012;**7**(2):e31982.
142. Bodi V, Sanchis J, Morales JM, et al. Metabolomic profile of human myocardial ischemia by nuclear magnetic resonance spectroscopy of peripheral blood serum: a translational study based on transient coronary occlusion models. *J Am Coll Cardiol* 2012;**59**(18):1629–41.
143. Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW, et al. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1H-NMR-based metabonomics. *Nat Med* 2002;**8**(12):1439–44.
144. Chen X, Liu L, Palacios G, Gao J, Zhang N, Li G, et al. Plasma metabolomics reveals biomarkers of the atherosclerosis. *J Sep Sci* 2010;**33**(17–18):2776–83.
145. Shah SH, Bain JR, Muehlbauer MJ, Stevens RD, Crosslin DR, Haynes C, et al. Association of a peripheral blood metabolic profile with coronary artery disease and risk of subsequent cardiovascular events. *Circ Cardiovasc Genet* 2010;**3**(2):207–14.
146. Barba I, de León G, Martín E, Cuevas A, Aguade S, Candell-Riera J, et al. Nuclear magnetic resonance-based metabolomics predicts exercise-induced ischemia in patients with suspected coronary artery disease. *Magn Reson Med* 2008;**60**(1):27–32.
147. Sabatine MS, Liu E, Morrow DA, Heller E, McCarroll R, Wiegand R, et al. Metabolomic identification of novel biomarkers of myocardial ischemia. *Circulation* 2005;**112**(25):3868–75.
148. Turer AT, Stevens RD, Bain JR, Muehlbauer MJ, van der Westhuizen J, Mathew JP, et al. Metabolomic profiling reveals distinct patterns of myocardial substrate use in humans with coronary artery disease or left ventricular dysfunction during surgical ischemia/reperfusion. *Circulation* 2009;**119**(13):1736–46.
149. Kang SM, Park JC, Shin MJ, Lee H, Oh J, Ryu do H, et al. (1)H nuclear magnetic resonance based metabolic urinary profiling of patients with ischemic heart failure. *Clin Biochem* 2011;**44**(4):293–9.
150. Dunn W, Deepak S, Buch M, McDowell G, Spasic I, Ellis D, et al. Serum metabolomics reveals many novel metabolic markers of heart failure, including pseudouridine and 2-oxoglutarate. *Metabolomics* 2007;**3**(4):413–26.
151. Mayr M, Yusuf S, Weir G, Chung YL, Mayr U, Yin X, et al. Combined metabolomic and proteomic analysis of human atrial fibrillation. *J Am Coll Cardiol* 2008;**51**(5):585–94.
152. Vallejo M, García A, Tuñón J, García-Martínez D, Angulo S, Martin-Ventura JL, et al. Plasma fingerprinting with GC-MS in acute coronary syndrome. *Anal Bioanal Chem* 2009;**394**(6):1517–24.
153. Teul J, Garcia A, Tuñón J, Martin-Ventura JL, Tarín N, Bescós LL, et al. Targeted and non-targeted metabolic time trajectory in plasma of patients after acute coronary syndrome. *J Pharm Biomed Anal* 2011;**56**(2):343–51.
154. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* 2009;**9**(4):311–26.
155. Oberbach A, Blüher M, Wirth H, Till H, Kovacs P, Kullnick Y, et al. Combined proteomic and metabolomic profiling of serum reveals association of the complement system with obesity and identifies novel markers of body fat mass changes. *J Proteome Res* 2011;**10**(10):4769–88.
156. Jung JY, Lee HS, Kang DG, Kim NS, Cha MH, Bang OS, et al. 1H-NMR-based metabolomics study of cerebral infarction. *Stroke* 2011;**42**(5):1282–8.
157. Gall WE, Beebe K, Lawton KA, et al. alpha-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. *PLoS ONE* 2010;**5**(5):e10883.
158. Mihalik SJ, Michaliszyn SF, de las Heras J, Bacha F, Lee S, Chace DH, et al. Metabolomic profiling of fatty acid and amino acid metabolism in youth with obesity and type 2 diabetes: evidence for enhanced mitochondrial oxidation. *Diabetes Care* 2012;**35**(3):605–11.
159. Bernini P, Bertini I, Luchinat C, Tenori L, Tognaccini A. The cardiovascular risk of healthy individuals studied by NMR metabonomics of plasma samples. *J Proteome Res* 2011;**10**(11):4983–92.
160. Cheng S, Rhee EP, Larson MG, Lewis GD, McCabe EL, Shen D, et al. Metabolite profiling identifies pathways associated with metabolic risk in humans. *Circulation* 2012;**125**(18):2222–31.
161. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* 2011;**472**(7341):57–63.
162. Urpi-Sarda M, Monagas M, Khan N, Llorach R, Lamuela-Raventós RM, Jáuregui O, et al. Targeted metabolic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 2009;**1216**(43):7258–67.
163. Vázquez-Fresno R, Llorach R, Alcaro F, Rodríguez MÁ, Vinaixa M, Chiva-Blanch G, et al. (1)H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors. *Electrophoresis* 2012;**33**(15):2345–54.
164. Holmes E, Loo RL, Stamler J, Bictash M, Yap IK, Chan Q, et al. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature* 2008;**453**(7193):396–400.
165. Yap IK, Brown IJ, Chan Q, Wijeyesekera A, Garcia-Perez I, Bictash M, et al. Metabolome-wide association study identifies multiple biomarkers that discriminate north and south Chinese populations at differing risks of cardiovascular disease: INTERMAP study. *J Proteome Res* 2010;**9**(12):6647–54.
166. Grün CH, van Dorsten FA, Jacobs DM, Le Belleguic M, van Velzen EJ, Bingham MO, et al. GC-MS methods for metabolic profiling of microbial fermentation products of dietary polyphenols in human and in vitro intervention studies. *J Chromatogr B Analyt Technol Biomed Life Sci* 2008;**871**(2):212–9.
167. Saura-Calixto F, Pérez-Jiménez J, Touriño S, Serrano J, Fuguet E, Torres JL, et al. Proanthocyanidin metabolites associated with dietary fibre from *in vitro* colonic fermentation and proanthocyanidin metabolites in human plasma. *Mol Nutr Food Res* 2010;**54**(7):939–46.
168. 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) Final Report. *Circulation* 2002;**106**(25):3143–421.
169. Ros E. Nuts and novel biomarkers of cardiovascular disease. *Am J Clin Nutr* 2009;**89**(5):1649S–56S.
170. Sabaté J, Ang Y. Nuts and health outcomes: new epidemiologic evidence. *Am J Clin Nutr* 2009;**89**(5):1643S–8S.
171. Li TY, Brennan AM, Wedick NM, Mantzoros C, Rifai N, Hu FB. Regular consumption of nuts is associated with a lower risk of cardiovascular disease in women with type 2 diabetes. *J Nutr* 2009;**139**(7):1333–8.
172. Conlay LA, Maher TJ, Wurtman RJ. Alanine increases blood pressure during hypotension. *Pharmacol Toxicol* 1990;**66**(5):415–6.
173. Mulder TP, Rietveld AG, van Amelsvoort JM. Consumption of both black tea and green tea results in an increase in the excretion of hippuric acid into urine. *Am J Clin Nutr* 2005;**81**(1):256S–60S.

174. Rezzi S, Ramadan Z, Martin FP, Fay LB, van Bladeren P, Lindon JC, et al. Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals. *J Proteome Res* 2007;**6**(11):4469–77.
175. Martin FP, Rezzi S, Peré-Trepat E, Kamlage B, Collino S, Leibold E, et al. Metabolic effects of dark chocolate consumption on energy, gut microbiota, and stress-related metabolism in free-living subjects. *J Proteome Res* 2009;**8**(12):5568–79.
176. O'Sullivan A, Gibney MJ, Connor AO, Mion B, Kaluskar S, Cashman KD, et al. Biochemical and metabolomic phenotyping in the identification of a vitamin D responsive metabotype for markers of the metabolic syndrome. *Mol Nutr Food Res* 2011;**55**(5):679–90.
177. Vineis P, Perera F. Molecular epidemiology and biomarkers in etiologic cancer research: the new in light of the old. *Cancer Epidemiol Biomarkers Prev* 2007;**16**(10):1954–65.
178. Chadeau-Hyam M, Athersuch TJ, Keun HC, De Iorio M, Ebbels TM, Jenab M, et al. Meeting-in-the-middle using metabolic profiling – a strategy for the identification of intermediate biomarkers in cohort studies. *Biomarkers* 2011;**16**(1):83–8.

HIPÓTESIS Y OBJECTIVOS

HYPOTHESES AND AIMS

HIPÓTESIS

Esta Tesis Doctoral se desarrolla a partir de las siguientes hipótesis de partida:

- Los biomarcadores nutricionales evaluados en estudios clínicos de intervención nos proporcionarán medidas del estado nutricional y de salud de los individuos. El estudio de biomarcadores nutricionales mediante una aproximación metabolómica con resonancia magnética muclear nos permitirá observar cambios en el perfil metabólico de los individuos.

- El poder predictivo de los biomarcadores nutricionales evaluados en una población se reproducirá en otras poblaciones. La identificación y validación de biomarcadores nutricionales en diferentes poblaciones será una herramienta robusta de medida de ingesta alimentaria y de hábitos dietéticos.

- En una misma población existirán diferentes respuestas de modulación de la dieta debido a las diferencias interindividuales. Estas diferencias interindividuales se caracterizaran por diferentes fenotipos metabólicos que permitirán evaluar el efecto de una intervención nutricional de manera personalizada. Este hecho ayudará a una mejor comprensión entre la relación nutrición y salud.

HYPOTHESES

This doctoral thesis presents the following hypotheses:

- Nutritional biomarkers evaluated in clinical intervention studies will provide us with a measure of nutritional and health status. The study of nutritional biomarkers using a metabolomics approach based on nuclear magnetic resonance will reveal metabolic profile changes in the individuals.

- The predictive power of nutritional biomarkers evaluated in a population will be replicated in other populations. The identification and validation of nutritional biomarkers in different populations will be a robust tool for measuring food intake and dietary habits.

- The same population will have different responses to modulations in diet due to interindividual differences. Their interindividualities will be characterized by different metabolic phenotypes that reflect the effect of personalized nutritional intervention. These studies will help to better understand the link between nutrition and health.

-

OBJETIVOS

El **objetivo principal** de esta Tesis Doctoral es identificar biomarcadores de ingesta y de efecto en perfiles metabólicos urinarios desde un enfoque metabolómico por Resonancia Magnética Nuclear (RMN).

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

- Desarrollar e implementar una metodología óptima mediante RMN para la preparación, procesado y análisis de muestras de orina en estudios nutricionales.
- Analizar el metaboloma urinario después de una intervención nutricional y evaluar el efecto que tiene la matriz alimentaria sobre él (publicación 1).
- Determinar el poder predictivo de biomarcadores nutricionales en estudios de intervención y en estudios observacionales (publicación 2).
- Diferenciar fenotipos metabólicos dentro una misma población a partir de marcadores bioquímicos clásicos y explorar el efecto de una intervención dietética sobre dichos fenotipos (publicación 3).
- Estudiar el metaboloma urinario tras un período de intervención con un complemento alimenticio (probiótico) y su impacto en la salud (publicación 4).
- Establecer marcadores de patrones nutricionales concretos, observados en el perfil urinario de una población con una intervención dietética de larga duración (3 años de seguimiento) (publicación 5).

AIMS

The main objective of this thesis is to identify biomarkers of intake and their effect on urinary metabolomic profiles through a metabolomics approach using nuclear magnetic resonance (NMR).

To address this main objective, the following specific objectives have been raised:

- Develop and implement an optimal NMR methodology for the preparation, preprocessing and analysis of urine samples in nutritional studies.
- Analyze the urinary metabolome after nutritional intervention and assess matrix effects (Paper 1).
- Determine the predictive power of nutritional biomarkers in intervention and observational studies (Paper 2).
- Differentiate metabolic phenotypes within the same population from classical biochemical markers and explore the effect of dietary intervention on these phenotypes (Paper 3).
- Study the urinary metabolome and its impact on health after a period of intervention with a probiotic food supplement (Paper 4).
- Establish specific markers of nutritional patterns observed in the urinary profile of a population with a long-term dietary intervention (3-year follow-up) (Paper 5).

METODOLOGÍA

4.1 ANÁLISIS METABOLÓMICO NO DIRIGIDO POR RESONANCIA MAGNÉTICA NUCLEAR

4.1.1 METODOLOGÍA ANALÍTICA

4.1.1.1 Preparación de las muestras

El análisis de las muestras biológicas provenientes de los estudios presentados se realizó siguiendo el protocolo metodológico de Beckonert *et al.* (Beckonert *et al.* 2007), se desarrolló y optimizó para los objetivos de la presente Tesis Doctoral. El método consiste en la preparación de la muestra para su posterior análisis mediante el espectrómetro de RMN (500MHz) instalado en los servicios científico-técnicos del *Parc Científic* de Barcelona.

Se descongelaron las muestras de orina de los voluntarios gradualmente a 4º C dentro de frigorífico, se agitaron mediante "vortex" y se centrifugaron a 13.200 revoluciones por minuto (rpm) durante 5 min y una temperatura de 4º C. La centrifugación se utiliza para evitar la presencia de cualquier compuesto particulado que pueda afectar a la calidad del espectro (Beckonert *et al.* 2007). Se cogió el sobrenadante de la centrifugación, a la que se le adicionó 120 μL de solución tampón compuesta por: 1,5 M de fosfato potásico monobásico (KH_2PO_4), 2 mM de azida sódica (NaN_3) y 0.1% de *3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt* (TSP) (0.97 mM), 98 atom % D, ajustada dicha solución tampón previamente a pH 7.0. El primer elemento nombrado de la solución tampón se utiliza para amortiguar el pH de la solución, el

segundo, es un inhibidor del crecimiento bacteriano y finalmente el TSP se añade como compuesto de referencia para el procesado de los espectros.

Para los análisis metabolómicos mediante RMN se necesitan volúmenes relativamente grandes en comparación con otras técnicas analíticas como la espectrometría de masas. En este caso se partió de un volumen de 600 μL de orina para los estudios de un alimento y de un complemento alimenticio y de 300 μL de orina para las muestras pertenecientes al estudio PREDIMED (por no disponer de suficiente volumen de muestra). Para este último estudio se mezcló dicho volumen (300 μL de orina) con 120μL de solución tampón, y se adicionaron 200 μL de agua/agua deuterada (8:2) para conseguir un volumen total de 620 μL (300 μl + 120 μl + 200 μl) suficiente para tener la altura necesaria para poder ser analizado por el espectrómetro de RMN. Debido a que se analizan y comparan muestras similares para ver diferencias significativas, es necesario mantener todas las muestras al mismo pH, ya que el desplazamiento químico de algunos metabolitos es muy sensible a cambios de pH. Además, el análisis de las muestras mediante RMN requiere que el biofluído a analizar esté en un tubo transparente dónde puedan incidir las radiaciones electromagnéticas, por tanto se utilizaron tubos de cristal de 5mm de diámetro especiales para RMN.

4.1.1.2 Adquisición de datos y procesado de los espectros

El equipo de RMN utilizado fue un *Varian Inova-500MHz* con intercambiador de muestras automático.

i) Adquisición de datos

- ***Experimentos 1D. Uso del programa NOESY con presaturación para la supresión del agua***

Experimentos de 1D-NOESY (*Nuclear Overhauser Enhancement SpectroscopY*) con la adquisición de la señal de presaturación del agua. Este programa consiste en el primer incremento de la secuencia de pulso NOESY con la presaturación de la señal del agua, durante el tiempo de relajación entre barridos/tiempo de espera del barrido (o tiempo de relajación) y durante el tiempo de mezcla. Para cada muestra, los espectros se obtuvieron con 128 barridos (en inglés, "*scans*") y 32 K puntos de datos sobre una anchura espectral de 14 ppm, resultando en un tiempo de adquisición de 2 s, un tiempo de relajación de 5 s y un tiempo de mezcla de 100 ms. A continuación se muestran los parámetros de adquisición de las muestras.

Tabla 4.1. Parámetros de procesado de los espectros 1D para todos los estudios de la presente Tesis Doctoral

Experimento NOESY con presaturación	**Abreviación**	**Valor**
Puntos de datos	np	32052
Número de barridos	nt	128
Anchura espectral	sw[ppm]	14
Tiempo de adquisición	at [s]	2
Tiempo de relajación	d1[s]	5
Tiempo de mezcla t_m	mixN [ms]	100
Poder de presaturación	satpwr	2

Durante la adquisición la temperatura se mantuvo constante a 298 K con un factor de ampliación de la línea de 0.3 Hz, previo a la transformada de Fourier.

- ***Experimentos 2D***

Para aportar mayor información estructural durante el proceso de identificación de las muestras, se utilizaron experimentos COSY (en inglés "*COrrelation SpectroscopY*"). El fundamento de los experimentos 2D se basa en la inclusión de un tiempo variable en la secuencia de pulsos. Los experimentos 2D se realizaron en un equipo de RMN *Bruker Avance III de 600MHz*.

El experimento COSY, significa espectroscopia de correlación homonuclear protón-protón (^{1}H-^{1}H), determina qué hidrógenos se acoplan entre si y permite calcular el valor de la constante de acoplamiento *J*. La diagonal es el espectro 1D y las señales que están fuera de la diagonal reflejan los hidrógenos que están acoplados, es decir, aquéllos con los que correlaciona (**Figura 4.1**).

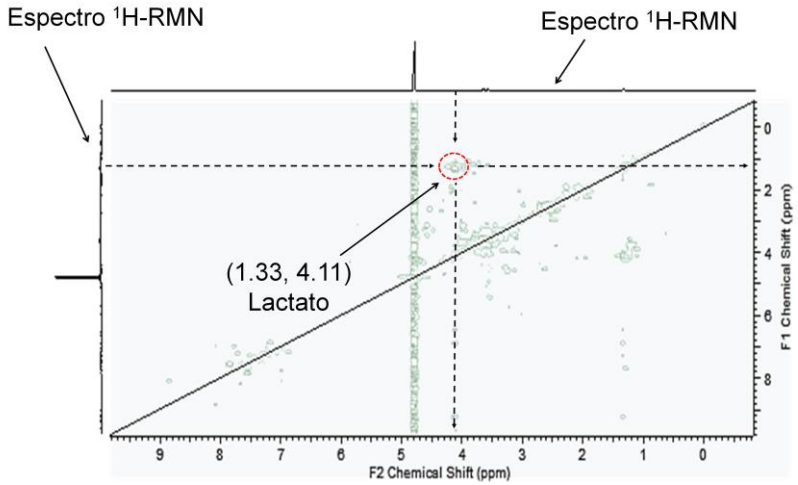

Figura 4.1. Espectro 2-D COSY

Tabla 4.2. Parámetros de procesado de los espectros 2D COSY

Parámetros COSY	Abreviación	Valor
Secuencia de pulso	*cosygp*	-
Número de barridos	NS	48
Anchura espectral	SWH(Hz)	6410.26
Tiempo de relajación	D1[s]	1.1
Tamaño de adquisición	TD/1TD	(2048, 512)
Tamaño espectral	SI/1SI	(1024, 1024)

ii) Procesado de espectros

Posterior a la adquisición de los espectros, se requiere un pre-procesado de los espectros que consiste en el faseado (corrección de la fase), el referenciado (en este caso con la señal de TSP a desplazamiento químico [δ] 0.0) y la corrección de la línea base. El procesado de los espectros se realizó con el programa TopSpin 3.0 (Bruker Biospin Ltd.).

4.1.1.3 Integración de picos

Inicialmente se probaron dos tipos de integraciones (Publicación 1). La primera fue mediante segmentación del espectro en porciones, en inglés *buckets* o *bins*. El proceso de partición de cada espectro se llama *binning* (**Figura 4.2**). En este caso, cada espectro de cada voluntario fue segmentado en porciones de 0.005 ppm de anchura entre el rango espectral 0-10 ppm y las áreas correspondientes fueron integradas usando el programa ACD/NMR Processor 12.0 (Advanced Chemistry Development, Toronto, Canada). Este método de reducción de datos

también tiene la propiedad de ocultar cualquier variación de posición proporcionando una modelización más robusta (Cloarec *et al.* 2004). El inconveniente de ello es que la interpretación de los metabolitos identificados no es fácil ya que es necesario volver a examinar varias veces el espectro de RMN para identificar los metabolitos.

Otro método utilizado, que fue también testado en publicación 1 es el de la integración del perfil metabólico de los individuos. Es decir, se selecciona metabolito a metabolito, y se integran los correspondientes picos. Así, se obtiene una integración completa del pico, el inconveniente que posee es que se puede no considerar metabolitos en el proceso de integración que puedan ser importantes en el estudio. Este método de integración se realizó mediante el software AMIX 3.8 (Bruker Biospin Ltd).

En los sucesivos objetivos se optó por el primer método debido al mayor aporte de información y facilidad del proceso, ya que después de comparar ambos métodos (publicación 1), se vieron parecidos resultados en cuanto a los metabolitos identificados y analizados.

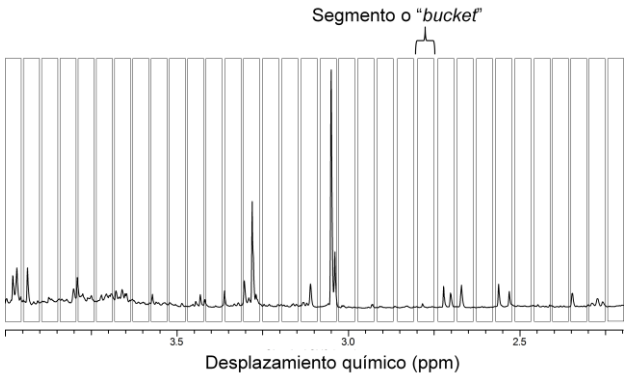

Figura 4.2. Ilustración de la segmentación espectral o *binning* aplicada a un espectro de RMN.

4.1.2 METODOLOGÍA BIOESTADÍSTICA:

Integración, métodos de pre-tratamiento de la matriz de datos, métodos quimiométricos y análisis estadístico

4.1.2.1 Métodos de pre-tratamiento de la matriz de datos

Para el análisis de todos los trabajos de esta Tesis Doctoral se ha utilizado para el procesado de los datos y la normalización, la plataforma web de análisis metabolómico *Metaboanalyst* (www.metaboanalyst.ca). Esta plataforma incluye métodos de procesado de datos, normalización, análisis estadístico y herramientas para la interpretación de resultados. En el caso de los trabajos realizados en la publicación 1 y 3 también se ha utilizado para el análisis estadístico mediante ANOVA y correlaciones entre las señales mediante correlaciones de *Pearson*.

Los datos obtenidos a partir de una aproximación metabolómica no dirigida pueden presentar diferentes fuentes de variación aparte de la variación biológica que se quiere estudiar. Estas variaciones pueden deberse a: 1) diferencias en el orden de magnitud entre las intensidades de los picos (los metabolitos que se encuentran a concentraciones más elevadas no necesariamente son más importantes que los que se encuentran a concentraciones más bajas); 2) diferencias en las magnitudes de los cambios en las concentraciones de los metabolitos, 3) variación biológica no inducida (bajo condiciones experimentales idénticas algunos metabolitos muestran grandes fluctuaciones en sus concentraciones); 4)

variaciones técnicas debidas a la preparación de las muestras o errores analíticos, 5) la heterocedasticidad, es decir, cuando la variación de las señales no es constante en todas las muestras (van den Berg *et al.* 2006). Por tanto, el objetivo del pre-tratamiento de los datos es minimizar al máximo las variaciones debidas a otras causas que no sea propiamente la biológica.

i) Filtrado de datos

El filtrado de datos en metabolómica se utiliza para prescindir de aquellas señales que no aportan información (sino ruido). Dichas señales se pueden clasificar en tres grupos (Xia *et al.* 2011): (1) señales muy bajas que se acercan al límite de detección; (2) señales que solo se detectan en muy pocas muestras; y (3) aquellas señales que son prácticamente constantes. El proceso de filtrado mejora el análisis y reduce el ratio de posibles falsos positivos en el análisis estadístico. Hay varias formas de detectarlas, en la presente Tesis si utilizó el método de filtrado **IQR** (en inglés "*InterQuartil Range*") un filtro que excluye datos que muestran poca variación entre las condiciones experimentales en función de su rango inter-cuantil. Esta función se implementó en *Metaboanalyst* con posterioridad a la publicación del primer artículo (publicación 1), y por ello no se utilizó en él, pero sí para los siguientes artículos.

ii) Normalización

El término normalización aquí se refiere a la normalización muestra a muestra. A menudo en metabolómica hay una variación no deseada entre muestras. Por ejemplo, en estudios metabolómicos de orina puede haber un efecto de dilución. Hay diferentes técnicas para normalizar los datos, también llamada *row-wise normalization*. La técnica *row-wise normalization* utilizada en los estudios de la presente Tesis fue la normalización por

suma, dónde se divide cada intensidad por la suma de las intensidades totales del espectro (Craig *et al.* 2006). La normalización por suma se suele utilizar en datos quimiométricos no cuantitativos (Xia *et al.* 2011).

iii) Transformación de los datos

Son conversiones no lineales de los datos para corregir la heterocedasticidad, con el objetivo de convertir relaciones multiplicativas en relaciones aditivas, y para hacer distribuciones sesgadas más simétricas (van den Berg *et al.* 2006). La transformación de los datos se utiliza para normalizar las variables y que éstas sigan una distribución normal. En la presente Tesis, cuando los datos no seguían una distribución normal, para utilizar un test estadístico paramétrico, se transformaron los datos mediante la raíz cúbica (Xia *et al.* 2011) ya que generó una mejor transformación de los datos que una transformación logarítmica. Esta opción también la proporciona el programa *Metaboanalyst* en su interfaz. Cuando se realizaron test no paramétricos no se realizó este procedimiento, ya que no es necesario que los datos sigan una distribución normal.

iv) Escalado

El escalado de los datos consiste en dividir cada variable de cada metabolito de la matriz de datos por un factor escalar, el cual es diferente para cada variable y normalmente es una medida de la dispersión para esa variable. Los datos presentados en esta Tesis Doctoral se han escalado mediante un método llamado *Pareto*, que consiste en dividir cada variable por la raíz cuadrada de su desviación estándar, de este modo los grandes cambios son menos predominantes (van den Berg *et al.* 2006).

4.1.2.2 Métodos quimiométricos y análisis estadístico

Los métodos quimiométricos se pueden definir como la aplicación de métodos matemáticos, estadísticos, gráficos o simbólicos para maximizar la información la cual puede ser extraída de datos químicos o espectrales (Wishart 2007). En las aproximaciones quimiométricas no es necesario que se hayan identificado previamente los compuestos. Una propiedad esencial de la mayoría de estudios metabolómicos es la multidimensionalidad de los datos, es decir, el número de muestras es bastante menor que el número de variables (o metabolitos), que asegura que el conjunto de datos es sustancialmente complejo. Por ello hay varios métodos, mayoritariamente de análisis multivariante, para reducir esa dimensionalidad. A continuación se presentan los tipos de análisis estadísticos realizados bien de forma conjunta o de manera individual, en los trabajos que conforman esta Tesis Doctoral.

I) Análisis univariante

Para comparar las medias entre dos grupos se empleó el test *t* de Student para datos que seguían una distribución normal, y el test de Mann-Whitney para datos que no seguían una distribución normal (pruebas no paramétricas). Cuando existían más de dos grupos de comparación se utilizó el análisis de la varianza de un factor (ANOVA, del inglés *ANalysis Of VAriance)*.

El ANCOVA es un análisis ANOVA al que a la variable dependiente se le ha eliminado el efecto predicho por una o más covariables mediante regresión lineal múltiple. La inclusión de covariables puede aumentar la potencia estadística porque a menudo reduce la variabilidad. Este análisis se aplicó al estudio del que deriva la publicación 5, dónde se utilizó la

ANCOVA para ajustar cada variable por su variable a tiempo inicial (estado basal).

Estos análisis inicialmente se realizaron con *Metaboanalyst* (para ANOVA en la publicación 1 y 3). En el resto de estudios, Mann-Whitney, *t*-Student y ANCOVA se calculó mediante SPSS 18 (Chicago, IL, USA).

Correcciones para comparaciones múltiples

Para análisis univariante se ha de llevar especial cuidado cuando se realizan comparaciones en metabolómica ya que se al tener tal volumen de datos de análisis, por ejemplo comparar dos grupos (casos *vs.* controles) y tener 100 metabolitos a comparar, este hecho puede dar falsos positivos en el análisis; es decir, puede no haber un efecto biológico pero únicamente por azar dar datos significativos. Por ello, se realizan métodos de corrección como Bonferroni o *False Discovery Rate* (FDR) en el caso de análisis univariante. En el caso del análisis multivariante este problema está solventado ya que todas las variables se consideran simultáneamente, pero aparecen otros problemas como es la dimensionalidad (este concepto se comenta más adelante en el apartado de análisis multivariante). La tasa de falsos positivos, o FDR, y la corrección por Bonferroni son dos métodos de ajuste del nivel de significación. Bonferroni es un método muy conservador y bastante restrictivo, mientras que FDR es un método menos conservador (Benjamini *et al.* 1995).

Visualización de los análisis univariante: Diagramas de cajas o "Boxplots"

Hay varias formas para visualizar los datos en los análisis univariante, para los resultados de esta Tesis se han utilizado los diagramas de cajas o "*boxplots*" (**Figura 4.3**). Es un gráfico que suministra información sobre los valores mínimo y máximo, los cuartiles Q1 (25% de los datos), Q2 o

mediana (50% de los datos) y Q3 (75% de los datos), y sobre la existencia de valores atípicos y la simetría de la distribución. Está compuesto por un rectángulo, la "caja", y dos brazos, los "bigotes".

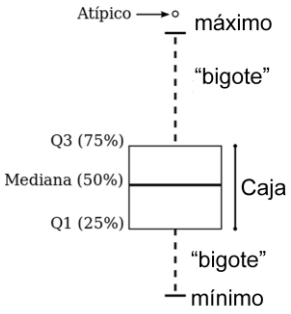

Figura 4.3 Diagrama de cajas o "*boxplot*"

II) Análisis multivariante

El análisis multivariante supone la observación y análisis estadístico simultáneo de más de dos variables. Los análisis multivariantes utilizados en esta Tesis Doctoral fueron tanto análisis que incluyen métodos no supervisados como el análisis de componentes principales (PCA), el análisis de agrupación jerarquizado (del inglés, *hierarchical clustering analysis*, HCA), *k-means*, y métodos supervisados como el PLS-DA (en su variante OSC-PLS-DA). Todos estos análisis mencionados con anterioridad se han utilizado en un momento u otro del proceso estadístico para los resultados de la presente Tesis.

- ***Análisis de componentes principales (PCA)***

El análisis más comúnmente usado de reducción de la dimensionalidad es conocido como análisis de componentes principales (del inglés "*Principal Component Analysis*", PCA). El PCA no es una técnica de clasificación sino de agrupación no supervisado. El PCA es particularmente útil para

identificar cómo difiere una muestra de otra, qué variables contribuyen más a las diferencias y qué variables contribuyen de la misma manera (es decir, están correlacionadas) (Trygg *et al.* 2007; Wishart 2007).

Este análisis tiene como objetivo proyectar complejos datos de altas dimensiones a nuevos sistemas de coordenadas con menos dimensiones. La dirección de la proyección se calcula para maximizar la varianza de los datos en solo las primeras dimensiones (llamadas componentes principales). El primer componente principal describe la máxima varianza del conjunto de datos con la consiguiente componente principal que contiene la correspondiente cantidad de varianza no contemplada por la componente principal anterior (Trygg *et al.* 2007). El PCA es un buen método para revelar la estructura interna de los datos respecto a la varianza. También es una buena prueba de análisis exploratorio de los datos. Los resultados que se pueden obtener de un PCA pueden ser unos gráficos de dispersión llamados "*score-plot*" y "*loading-plot*". Los "*scores*" representan los datos originales en el nuevo espacio de coordenadas, es decir las muestras analizadas, y pueden así ser interpretadas en términos de similitudes o diferencias entre estas muestras; y los "*loadings*" es el peso aplicado a los datos originales durante el proceso de proyección, es decir, los metabolitos analizados. El PCA es muy sensible a los valores extremos o "*outliers"*, por ello muchas veces se utiliza como proceso exploratorio para detectarlos (**Figura 4.4**). Los "*outliers*" pueden ser debidos a problemas técnicos en el proceso analítico, valores incorrectos, o datos mal etiquetados o valores atípicos. Con este objetivo se aplicó para el estudio de patrones dietéticos (publicación 5), ya que pueden ayudar a visualizar los datos preliminarmente y detectar posibles *outliers,* como fueron las señales de acetaminofén (comúnmente conocido como paracetamol), las cuales no eran objeto de nuestro estudio.

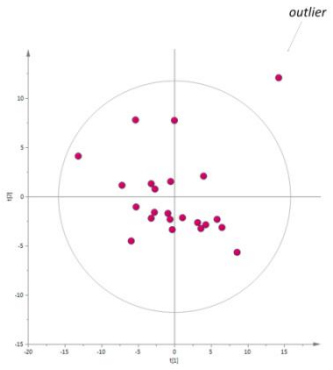

Figura 4.4 Ejemplo de PCA para la evaluación de valores *outliers* dentro de un mismo grupo

- ***Heatmap* y *Hierarchical clustering Analysis* (HCA)**

El *hierarchical clustering analysis* es otro método multivariante no supervisado que también permite una visión exploratoria global de los datos. El h*eatmap* es un método utilizado en las ciencias ómicas para visualizar grandes cantidades de datos. Para calcular un clúster jerárquico es necesario definir de qué forma se forman los clúster, cuántos clústers se forman y cómo se define la distancia entre los grupos. Hay diferentes medidas de distancias en el HCA, como también diferentes algoritmos de agrupación. En la presente tesis, en el estudio de un complemento alimenticio (publicación 4) se realizó un HCA representado con un dendrograma con las voluntarias para ver su separación al inicio y final de la intervención. En cambio para el estudio de patrones dietéticos (publicación 5), se utilizó un HCA dónde se visualizaron los individuos y metabolitos mediante un *heatmap*, utilizando como método de medida de distancias, la distancia Euclídea y como algoritmo, el algoritmo de Ward. El dendrograma se representó con el programa SIMCA-P+ y el *heatmap* mediante el programa PermutMatrix v1.9.3

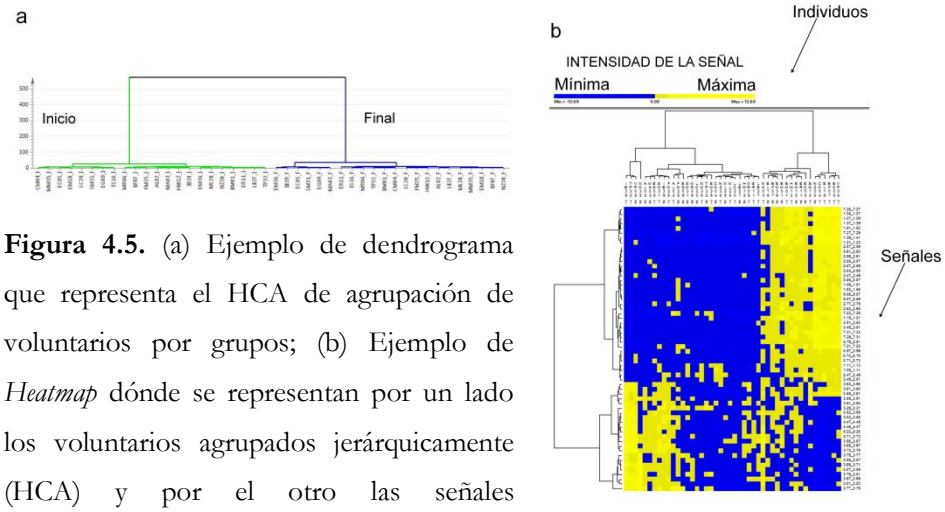

Figura 4.5. (a) Ejemplo de dendrograma que representa el HCA de agrupación de voluntarios por grupos; (b) Ejemplo de *Heatmap* dónde se representan por un lado los voluntarios agrupados jerárquicamente (HCA) y por el otro las señales discriminantes también agrupadas.

- ***Agrupación k-means***

En este caso, se preestablecen unos grupos determinados, a continuación se asigna cada individuo a uno de estos grupos (o clusters) de forma aleatoria. Seguidamente se calculan los llamados centroides, y se asigna cada individuo al centroide más cercano. Esto puede hacer que algunos individuos cambien de grupo. Esta operación se lleva a cabo de forma iterativa hasta que los individuo dejan de cambiar de grupos (Martínez-González 2006). Este análisis se calculó usando la librería *k-means algorithm* del paquete R v.2.14.1 y la librería *clValid* para el análisis de validación de clúster. También se realizaron análisis para detectar la agrupación más óptima: el análisis de *Dunn* para la coherencia interna, y el análisis de *FOM* para la homogeneidad externa.

- ***Análisis discriminante por mínimos cuadrados parciales (PLS-DA)***

A diferencia del PCA o el HCA, ésta es una técnica de análisis supervisado, esto significa que la información correspondiente a las diferentes clases que divide la población es conocida previamente y facilitada antes de realizar el análisis. El análisis de PLS-DA es utilizado para la separación entre grupos de observaciones, básicamente mediante la rotación de los componentes del PCA para obtener la máxima separación de clases.

El PLS-DA proyecta los datos (X) en espacio de pocas dimensiones que maximiza la separación entre grupos en las primeras dimensiones o "*variables latentes*". Estas variables latentes se ordenan según la cantidad de varianza explicada en las clases o variables Y. La R^2 estima la bondad de ajuste o porcentaje de variación explicada. R^2 tiene valores dentro del rango de 0 a 1. Valores cercanos a 1 indican que el modelo es capaz de clasificar correctamente las muestras en las clases que se han definido. En el análisis PLS-DA los resultados se visualizan de igual forma que en el análisis PCA, por gráficos de dispersión "scores plot" y gráficos "loading plot" y tienen la misma funcionalidad anteriormente especificada (**Figura 4.6**).

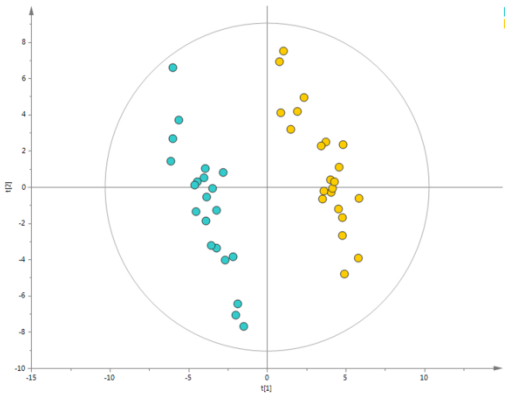

Figura 4.6. Ejemplo de PLS-DA *Score plot* que muestra la separación entre dos grupos de sujetos.

Filtro de corrección ortogonal de las señales (OSC)

En estudios clínicos con número de muestras relativamente elevado dónde existen grandes variaciones inter-individuales, el efecto que estamos buscando se ve enmascarado por otra variación no deseada. En este caso, suele ser necesario aplicar filtros multivariantes de pre-procesado de los datos antes de realizar el PLS-DA. El filtro utilizado en los PLS-DA realizados en este trabajo corresponde al filtro OSC (del inglés, *Orthogonal Signal Correction*) o filtro de corrección ortogonal de las señales, el cual consiste en eliminar la información de las señales (X) que no está relacionada (o correlacionada) con la clase (Y) (Llorach *et al.* 2009; Pujos-Guillot *et al.* 2013).

Estrategias de validación de modelos

La aplicación del PLS-DA conlleva tener en cuenta que puede llevar a un sobreajuste o "*overfitting*" del modelo, que significa que aunque el modelo PLS-DA pueda clasificar correctamente las muestras, para nuevas muestras que se quiera clasificar, éstas pueden no ser asignadas a la clase correcta. Para evitar esto es importante realizar pruebas de validación cruzada y pruebas de permutación (Westerhuis *et al.* 2008).

La **validación cruzada**, o en inglés *cross-validation*, consiste en dividir aleatoriamente el conjunto de muestras en grupo de entrenamiento y grupo de validación. Mediante el grupo de entrenamiento o "*training set*" se crea el modelo, mientras que el grupo de validación o "*validation set*" sirve para probar el modelo. Esto se repite varias veces (iteraciones). Este procedimiento nos proporciona la Q^2.

La Q^2 es la habilidad de predicción que tiene el modelo. Así pues, mientras que la R^2 nos dará la variación explicada, el valor Q^2 o error de predicción nos indicará lo bien que se puede predecir una clase si se introducen nuevos datos en el modelo. El valor de Q^2 va de 0 a 1, siendo 0 indicador de que el modelo no tiene capacidad predictiva, y 1 el valor con máxima capacidad predictiva (Westerhuis *et al.* 2008). Una $Q^2 \geq 0.5$ indica un buen modelo (muy utilizado en datos metabolómicos), mientras que $Q^2=0.9$, corresponde a un modelo excelente (más utilizado en calibraciones)(Eriksson 2006).

La **prueba de permutación** evalúa si la clasificación de las muestras que se ha realizado para el modelo es mejor que cualquier otra clasificación escogida al azar (Westerhuis *et al.* 2008). De este modo, durante la prueba de permutación (que se realiza de forma iterativa un número *n* de veces predeterminado) se puede calcular un nuevo modelo mediante una clasificación aleatoria, la cual nos proporcionará también un nuevo valor R^2 y Q^2, los cuales podrán ser comparados con los del modelo real PLS-DA con los que se está evaluando. El grafico resultante de la prueba de permutación, nos da en el extremo superior derecho los valores R^2 y Q^2 originales, y a la izquierda y cuanto más inferior mejor, los valores R^2 y Q^2 finales obtenidos en el test de permutación (valores de límite de intercepción) (**Figura 4.7**). Se pueden considerar para un modelo válido unos valores de límite de intercepción de R^2 y Q^2 menores de 0.4 y 0.05, respectivamente (van der Voet 1994).

Para los resultados que se presentan en esta Tesis Doctoral se realizaron pruebas de permutación de n=200 y de validación cruzada de 7 iteraciones, es decir, el conjunto de los datos se dividió en 7 grupos, uno de los cuales se consideró como grupo de validación y el resto como

grupo de entrenamiento, repitiendo este proceso 7 veces, siendo cada vez uno de los grupos el grupo de validación.

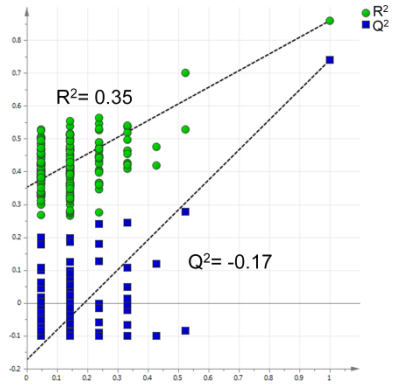

Figura 4.7. Ejemplo de prueba de permutación.

Para una **doble validación cruzada**, se puede utilizar un grupo de entrenamiento (cogiendo por ejemplo el 75%) y utilizar como grupo de validación, la población restante (el 25%). Por lo tanto, con el grupo de entrenamiento se crea el modelo, mientras que con el grupo restante se utiliza para validar el modelo creado previamente. Este procedimiento se repite varias veces hasta que todas las muestras hayan entrado una vez en el grupo de validación (del mismo modo que en la validación cruzada simple, aquí la única diferencia es el tamaño de los grupos de entrenamiento y validación). Si el modelo encaja razonablemente en el grupo de validación, entonces, se puede considerar un modelo válido (Szymańska *et al.* 2012a). Este procedimiento se utilizó para el OSC-PLS-DA del estudio de patrones alimentarios (publicación 5).

Selección de las variables discriminantes

La selección de variables es una importante estrategia en metabolómica. Puede utilizarse para construir un modelo (con el objetivo de que sea mejor y más parsimonioso), o bien para la selección de biomarcadores. Hay varias estrategias para la selección de variables, entre ellas el valor VIP, o el *p(corr)* (o la correlación entre la variable X y el valor *p* o peso de

cada metabolito), y una forma de visualizarlos es por ejemplo mediante los gráficos S-plot.

Los valores **VIP** (en inglés, *Variable Importance in the Projection*) es una medida de la importancia de una variable, concretamente es una suma ponderada de los cuadrados del "*loading*" PLS que tiene en cuenta la cantidad de varianza explicada - Y de cada componente (Xia *et al.* 2011). Expresado de otra manera, es la contribución que tiene cada variable en el modelo PLS-DA. Por lo tanto, valores VIP más altos, indican una mayor contribución de esa variable, es decir será más importante en el modelo. En la mayoría de estudios metabolómicos se utiliza normalmente un valor VIP>1, considerándose este valor apropiado para la selección de las variables como discriminantes (Chong *et al.* 2005; Eriksson 2006; Gosselin *et al.* 2010). Para las muestras utilizadas en esta Tesis, se han seleccionado valores más elevados, VIP >1.5, siendo de este modo, más restrictiva la selección de dichas variables, reduciendo la posibilidad de obtener falsos positivos.

El gráfico **S-plot** es un tipo de gráfico dónde muestran el peso *(p)* que cada señal presenta dentro del modelo y la correlación entre la variable X y el valor p [*p(corr)*]. Se denominan S-plot debido a que habitualmente estos gráficos tienen una forma que tiende a recordar a una S. El *p(corr)* toma valores entre -1 y +1. Para nuestro estudio hemos considerado valores $|p(corr)| \geq 0.5$ como punto de corte para la selección de variables (Llorach *et al.* 2009) (**Figura 4.8**).

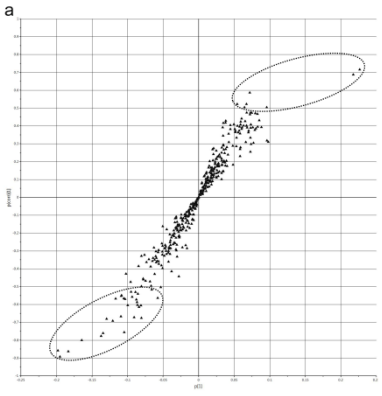

Figura 4.8. Ejemplo de *S-plot* para la selección de las variables. Los círculos indican las señales discriminantes.

III) Regresión logística y curvas ROC

Para el estudio de la sensibilidad y especificidad de biomarcadores nutricionales (publicación 2), el análisis se dividió en 3 pasos: (1) la selección de biomarcadores, (2) la evaluación de dichos biomarcadores, (3) la creación de un modelo.

Para la selección de los biomarcadores se utilizó el test no paramétrico de Mann-Whitney. De él, los biomarcadores con un *p*-valor <0.05 fueron seleccionados para ser evaluados. El siguiente paso fue la evaluación de dichos biomarcadores. Para ello se testaron uno a uno mediante curvas ROC y se evaluaron mediante el área bajo la curva (sus siglas en inglés corresponden a *AUC*) de dichas curvas.

A continuación se procedió a la creación de un modelo que incluyera varios marcadores, pero que fueran los mínimos necesarios y que dieran la mayor información posible como biomarcadores con el objetivo de discriminar la población entre consumidores/no consumidores. Para la creación de dicho modelo se utilizó la regresión logística por pasos "*stepwise*".

- ***Regresión logística por pasos (stepwise)***

La regresión logística es un tipo de análisis de regresión utilizado para predecir el resultado de una variable categórica (una variable que puede adoptar un número limitado de categorías) en función de las variables independientes o predictoras. Se basa en la siguiente ecuación:

$$y = \alpha + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_{k,}$$

dónde y es la variable dependiente, y x_k son las variables independientes, a es una constante, mientras que β es el coeficiente de regresión o pendiente de la variable x_k. Para realizar una regresión logística es necesario que la variable dependiente sea dicotómica; siendo 0 los controles y 1 los casos (Martínez-González 2006).

El método por pasos (o *stepwise*) es una estrategia de selección de variables. Consiste en seleccionar, en cada paso, aquella variable que mejor cumple un criterio prefijado basado en la significación estadística de inclusión (para añadirla al modelo) o el criterio de exclusión (para eliminarla).

- ***Curvas ROC***

Los gráficos de curvas ROC (del inglés, "*receiving operating characteristics*") muestran el poder predictivo y de discriminación del modelo, y por tanto, son útiles para visualizar, organizar y seleccionar los clasificadores. La forma de cuantificar el poder discriminatorio del modelo es a través de la determinación del área bajo la curva ROC (AUC, del inglés *Area Under the Curve*).

Para una mayor aproximación en la evaluación de biomarcadores es importante considerar la frecuencia en que el test produce verdaderos positivos (en inglés t*rue positives* [*TP*]), verdaderos negativos (en inglés *true negatives [TN*]), falsos positivos (*false positives*, *FP*) y falsos negativos (*false negatives, FN*). La **sensibilidad** es la capacidad que posee el modelo logístico de predicción para identificar correctamente a los positivos (casos). Es decir, a la probabilidad de que un sujeto en este caso consumidor sea clasificado correctamente como tal. Por otro lado, la **especificidad** será la capacidad que dispone el modelo para clasificar correctamente a los sujetos negativos (controles, o no-consumidores en este caso).

$$\text{Sensibilidad} = TP/(TP+FN)$$

$$\text{Especificidad} = TN/(TN+FP)$$

Si todas las muestras positivas se clasifican correctamente, e igual sucede con las negativas (es decir, un perfecto clasificador), la AUC tiene como valor 1. Una AUC de 0.5 es equivalente a una clasificación aleatoria de los sujetos.

Por tanto, la curva ROC es una curva representada en un diagrama de 2 ejes, dónde en este caso, el eje Y es la sensibilidad, y el eje X es la especificidad (**Figura 4.9**). La línea diagonal del gráfico de curva ROC representa la clasificación aleatoria de una variable. Por consiguiente, se puede clasificar la utilidad de los biomarcadores basándose en la curva AUC en función del rango en el que se encuentre (Xia *et al.* 2013). Si la curva AUC se encuentra entre el rango:

0.9-1.0 =excelente
0.8-0.9 = buena
0.7-0.8= regular
0.6-0.7= pobre
0.5-0.6= mala

A partir de la fórmula:

$$d = \text{raíz cuadrada } [(1-\text{Sensibilidad})^2 + (1-\text{Especificidad})^2].$$

Donde *d* es la distancia desde la esquina superior izquierda a cualquier punto de la curva ROC. El mínimo valor de *d* corresponderá al óptimo punto de corte el cual determina la sensibilidad y especificidad para ese punto.

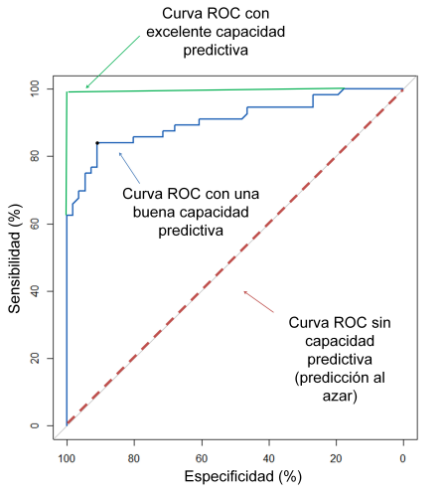

Figura 4.9. Ejemplo de curva ROC con excelente (línea verde), buena (azul), y sin capacidad predictiva (roja discontinua).

La regresión logística por pasos se calculó mediante el programa *SPSS*. Para calcular la curva ROC y la comparación entre las dos curvas ROC del modelo en el estudio de intervención y en la población de vida libre se utilizó la librería *p*ROC (Robin *et al.* 2011) del paquete estadístico R, que además del análisis y la visualización de las curvas, también permite la comparación entre dos curvas ROC, devolviéndote una *p* de significancia. Este paquete fue utilizado para analizar, visualizar y comparar las curvas generadas entre ambas poblaciones.

IV) Análisis de correlaciones

La finalidad de la correlación es examinar la dirección y la magnitud de la asociación entre dos variables, es decir, cómo varían conjuntamente. Por tanto, podremos tener una correlación positiva (ambas variables aumentan de la misma manera) o bien negativamente (una variable aumenta mientras que la otra disminuye). Hay dos coeficientes de correlación que se usan frecuentemente: el de *Pearson* (paramétrico) y el de *Spearman* (no paramétrico).

El coeficiente de correlación *r* puede ir de -1 a 1, por tanto:

r<0 correlación negativa;

r=0 no existe correlación;

r>0 correlación positiva.

Y la correlación puede clasificarse en:

$|r| < 0.3$ asociación débil

$0.3 \leq |r| \leq 0.7$ asociación moderada

$|r| > 0.7$ asociación fuerte

En los trabajos aquí expuestos se han utilizado los análisis de correlaciones de la siguiente manera: (a) para la identificación: correlaciones entre picos correspondientes a una misma señal; (b) correlaciones entre metabolitos y alimentos mediante datos provenientes del cuestionario de frecuencia de consumo de alimentos (del inglés *food frequency questionnaire, FFQ*).

Para datos que seguían una distribución normal se utilizaron las correlaciones de *Pearson*, en cambio, para datos no normales, se utilizó la correlación de *Spearman*, dependiendo con que programa se realizaron el resto de análisis se utilizó *Metaboanalyst* o *SPSS*. Para las correlaciones con los datos provenientes del cuestionario FFQ, solo se aplicó *SPSS*, ya que los datos descriptivos de ingesta no se podían introducir en *Metaboanalyst*.

Para correlaciones entre picos dentro de un mismo metabolito se utilizó como valor de corte aceptable que las señales tuvieran una correlación $r \geq 0.7$. Para correlaciones con datos provenientes de cuestionarios de ingesta de alimentos, se consideró un valor a partir de $r \geq 0.3$ para considerar una correlación moderada entre un alimento y un metabolito correlacionado con éste (Scalbert *et al.* 2014).

4.1.2.3 Identificación y cuantificación de metabolitos

I) Identificación de metabolitos

La identificación de los metabolitos de los resultados que se exponen en la presente Tesis se realizó mediante el uso o combinación de 3 métodos diferentes:

1) Utilizando el programa de identificación *Chenomx NMR Suite 7.0 profiler* (Chenomx Edmonton, Canadá). Chenomx es un programa de "*spiking*" o fortificación virtual que permite la identificación de compuestos en espectros de 1 dimensión (1D). Contiene una biblioteca de referencia espectral interna con más de 260 compuestos teniendo en cuenta el pH y la frecuencia de trabajo del equipo utilizado.

2) Mediante la búsqueda del desplazamiento químico y la asignación de picos para cada metabolito, en bibliografía existente de datos espectrales de RMN de libre uso. Algunos ejemplos son "*Human Metabolome Database*" (HMDB, www.hmdb.ca), "*Biological Magnetic Resonance Data Bank*" (BMRB, http://www.bmrb.wisc.edu) y la "*Madison Metabolomics Consortium Database*" (MMCD, mmcd.nmrfam.wisc.edu)

3) Finalmente en algunos de los últimos análisis se realizaron pruebas adicionales con técnicas 2D de espectroscopia ya comentadas anteriormente (apartado 4.1.1.2).

II) Cuantificación metabólica

Los compuestos son identificados y cuantificados antes del análisis estadístico. Este método ha sido utilizado en el estudio presentado en la publicación 3.

En este caso, los compuestos son cuantificados mediante el programa de identificación y cuantificación *Chenomx NMR Profiler 7.0* (Edmonton, Canadá). Es un programa de *"spiking*" o fortificación virtual con una base de datos espectrales de RMN de metabolitos puros recolectado a diferentes pH y frecuencias espectrométricas (500, 600, 800MHz).

4.1.2.4 Interpretación biológica

Una vez se ha procedido a identificar los metabolitos relevantes es necesario una interpretación biológica para entender y pretender responder la hipótesis generada. Hay varias herramientas que permiten llevar a cabo este objetivo. La HMDB a parte de proporcionarte información química del compuesto, te da una breve descripción, así como información de otros estudios científicos dónde se ha descrito, además te proporciona páginas de enlace a otros programas de identificación e interpretación. También ha sido de gran utilidad el uso de bases de datos que permiten situar los metabolitos en las correspondientes rutas metabólicas como la *KEGG (Kyoto Encyclopedia of Genes and Genomes*) (Kanehisa *et al.* 2006), y así ver una visión global de la ruta/s metabólica/s que pertenece el metabolito y las posibles interacciones en esa misma ruta con otros metabolitos. Paralelamente, se ha buscado en la bibliografía científica información perteneciente a los metabolitos de interés y las rutas relacionadas, así como estudios similares y/o relacionados.

4.2 MEDOLOGÍA CLÍNICA:

Estudios clínicos para el desarrollo de biomarcadores nutricionales

4.2.1 ESTUDIO DEL EFECTO DE LA INTERVENCIÓN CON UN ALIMENTO:

ESTUDIO EN VINO

Este estudio se realizó en el marco del proyecto AGL2006-14228-C03-02, coordinado entre la Universidad de Barcelona y el Hospital Clínico de Barcelona. Este estudio se ha utilizado para diferentes objetivos: (1) Aplicación de la Resonancia Magnética Nuclear (RMN) a un estudio de intervención y búsqueda de marcadores de consumo y efecto mediante y el efecto de la matriz alimentaria (publicación 1). (2) Análisis de paneles de biomarcadores de exposición mediante la determinación de la especificidad y sensibilidad de los biomarcadores hallados (publicación 2). (3) Evaluar el efecto del vino desalcoholizado sobre distintos fenotipos metabólicos dentro de una misma población a partir de marcadores bioquímicos y antropomórficos (publicación 3).

I. Estudio de un alimento y el efecto sobre la matriz alimentaria

Se trata de un estudio de intervención aleatorizado, cruzado y controlado, se llevó a cabo en períodos de 4 semanas. Se analizaron un total de 61 individuos mayores de 55 años con elevado riesgo cardiovascular. Los criterios de inclusión fueron: voluntarios consumidores moderados de alcohol (1-3 copas al día) y tener *Diabetes Mellitus* y/o 3 o más de los siguientes factores de riesgo de enfermedad cardiovascular: consumo habitual de tabaco, hipertensión, hipercolesterolemia ([LDL] plasmático ≥160 mg/dL, [HDL] plasmático ≤40 mg/dL, obesidad (IMC≥30 kg/m^2) y/o antecedentes familiares de enfermedad cardiovascular. Los criterios de exclusión fueron haber padecido anteriores episodios de enfermedad cardiovascular y/o alcoholismo. Los voluntarios siguieron un período de lavado durante 15 días, en el cual se pidió que siguieran una dieta sin bebidas alcohólicas ni productos derivados de la uva. Se recogió la orina de 24h de los voluntarios al inicio y después de 28 días de cada tratamiento: 30 g de alcohol/día (272 mL) de vino tinto, 272 mL al día de vino tinto desalcoholizado (igual contenido de polifenoles en ambos vinos), o 100 mL al día de ginebra (30 g de alcohol/día), durante 4 semanas (28 días) en cada intervención. En todos los períodos se siguieron las mismas restricciones dietéticas que en el período de lavado. Una vez transcurridas estas cuatro semanas, la intervención se repitió en el orden inverso, y así hasta completarlo con las tres bebidas del estudio (**ver Figura 4.10**).

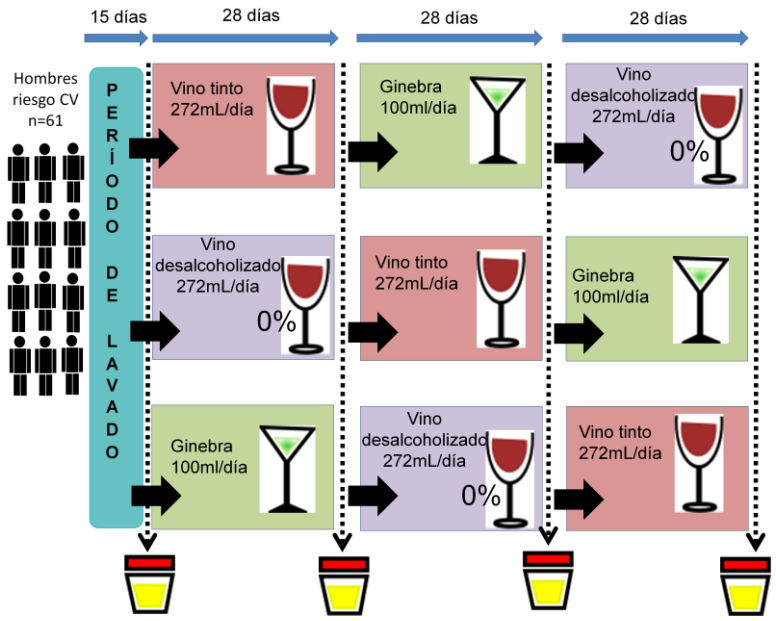

Figura 4.10. **Estudio en un alimento y el efecto sobre la matriz alimentaria.** Diseño del estudio controlado, aleatorizado y cruzado de intervención en humanos con consumo de vino tinto, vino tinto desalcoholizado y ginebra. CV: cardiovascular.

II. Estudio de la sensibilidad y especificidad de los biomarcadores nutricionales

Para este objetivo se ha utilizado (1) un estudio de intervención y (2) un estudio cohorte sin intervención (individuos en estado basal del estudio PREDIMED).

Se incluyeron 56 participantes del estudio de intervención con vino (descrito en el apartado anterior) en estado basal (después del período de lavado)- orina basal, y después de la intervención de 28 días con vino tinto. Además, se consideró una sub-muestra aleatoria de los participantes del estudio PREDIMED (http://www.predimed.es). Esta sub-muestra se obtuvo gracias a la cesión de las muestras de los nodos de Barcelona y Valencia correspondientes al estudio PREDIMED en el marco del proyecto AGL2009-13906-C02-01/02. Para la población cohorte PREDIMED (población 2), se analizó la orina de primera hora de la mañana en condiciones de ayuno de los individuos en estado basal, en el momento de la inclusión al estudio (antes de iniciar la intervención dietética), para evitar cualquier efecto de la intervención nutricional durante el transcurso del estudio. Para la división de individuos en consumidores *vs* no consumidores de vino se utilizó un cuestionario de frecuencia de consumo de alimentos (del inglés *food frequency questionnaire, FFQ*) dónde se recogían los hábitos alimentarios de los participantes. Además se utilizó un recordatorio de 24 horas, dónde se recolectaron los datos de ingesta dónde aparecía la frecuencia de consumo de vino de los participantes. Para la evaluación de la detección de biomarcadores en tiempo transcurrido después de la última copa, se consideraron voluntarios con ingesta de vino el día anterior (n=53), entre 2-3 días antes (n=14) y más de 3 días (n=15) frente a los no consumidores (n=69).

La estratificación del estudio cohorte (n=205) fue del siguiente modo: no consumidores de vino (0 ml/día, n=69), consumidores intermitentes (<180 ml/día, n=87), y consumidores habituales (≥180 ml/día, n=22). Los participantes no consumidores de vino pero sí consumidores de otras bebidas alcohólicas no fueron evaluados (n=27). Para este estudio se analizaron los consumidores diarios de vino y los no consumidores, por tanto, los consumidores no habituales (intermitentes) tampoco fueron considerados (**Figura 4.11**).

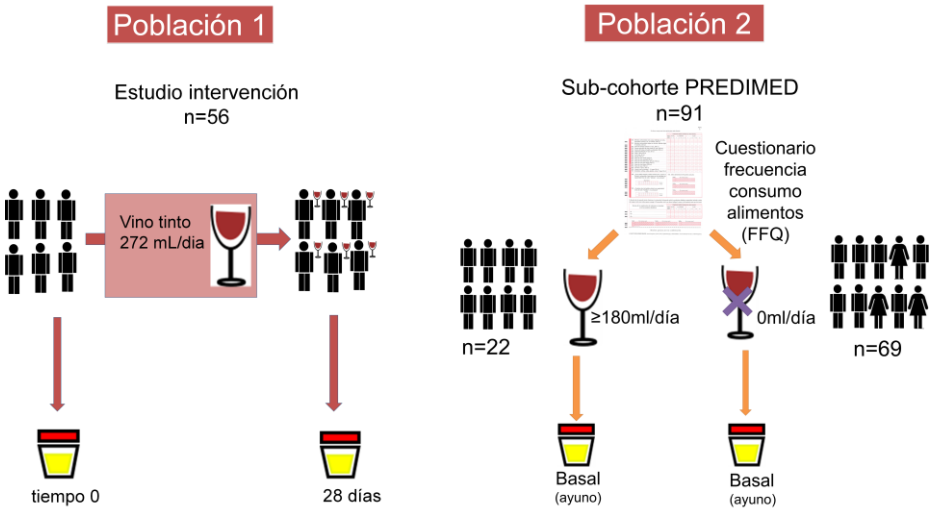

Figura 4.11. Estudio de la sensibilidad y especificidad de biomarcadores nutricionales. Diseño del estudio con las dos poblaciones: estudio de intervención de vino (población 1) y población cohorte consumidores/no consumidores de vino del estudio PREDIMED (población 2).

III. Estudio del fenotipo metabólico tras el consumo de un alimento

En este caso los individuos son divididos en 4 grupos según su perfil bioquímico a partir de los datos plasmáticos y urinarios mediante el algoritmo de *k-means,* con el objetivo de observar diferencias metabólicas tras el consumo de polifenoles provenientes de la ingesta de vino, 733 Equivalentes de Ácido Gálico [GAE/día], en forma de vino tinto desalcoholizado (**Figura 4.12**).

Los parámetros antropométricos y bioquímicos que se utilizaron fueron la medida del Índice de Masa Corporal (IMC), el ratio cintura-cadera (estos dos parámetros para evaluar la obesidad): niveles de glucosa, colesterol total, HDL, LDL, ratio HDL/LDL, triacilglicéridos, diuresis 24h, creatina plasmática, ácido úrico, aminotransferasas, bilirrubina, ferritina, y otras proteínas como albúmina o proteína C reactiva, enzimas como la fosfatasa alcalina, la lactato deshidrogenasa, e iones (sodio, potasio, etc.), así como también globulinas, niveles de apolipoproteínas, hemoglobina y recuento de glóbulos rojos, a la vez que algunos parámetros de coagulación como la protrombina, trombina y fibrinógeno.

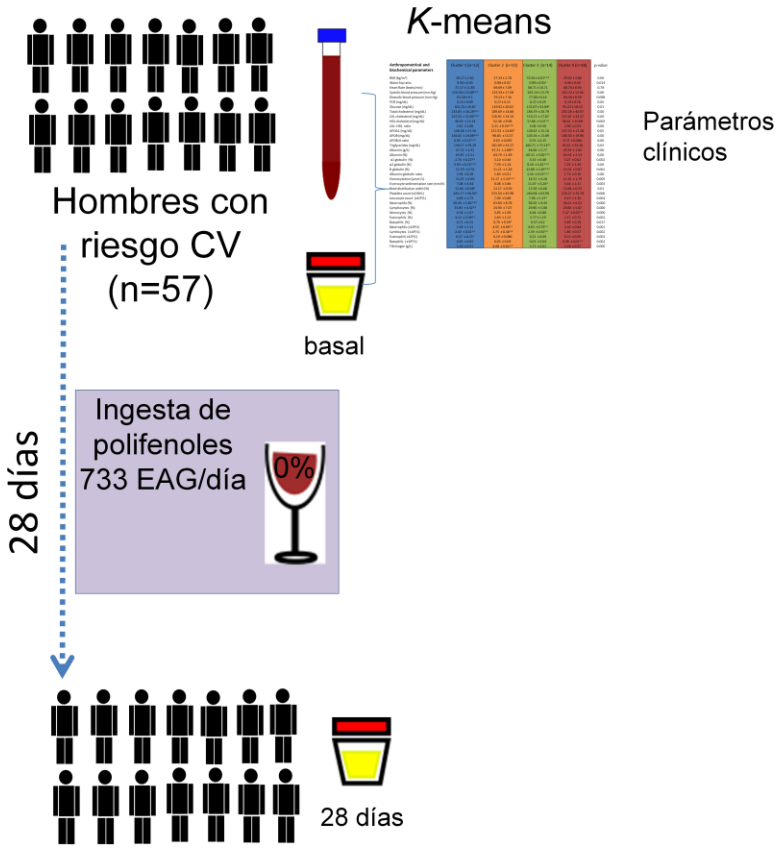

Figura 4.12. Estudio del fenotipo metabólico tras el consumo de un alimento. Formación de grupos de similitud mediante *k-means* a partir de los datos clínicos de los individuos. CV: cardiovascular; EAG: equivalentes de ácido gálico

4.3.2 ESTUDIO DEL EFECTO DE LA INTERVENCIÓN CON UN COMPLEMENTO ALIMENTICIO:

Estudio de un complemento alimenticio probiótico

Este estudio se realizó en el marco del programa Ingenio-CONSOLIDER FUN-C-FOOD (CSD2007-063) y con una colaboración de la Universidad de Barcelona y la Universidad Complutense de Madrid (Dr. Juan Miguel Rodríguez).

En este estudio de intervención se reclutaron 30 mujeres con síntomas de mastitis. De las 30, 7 de ellas abandonaron la intervención durante el estudio debido a varios motivos: 2 mujeres sufrieron anemia severa, 4 de ellas estuvieron tomando antibióticos, y por último, una se mudó a otro país por lo que no pudo finalizar el estudio. Las 23 mujeres que completaron el estudio debían cumplir las siguientes características: sufrir inflamación mamaria, lactancia dolorosa, un recuento de bacterias de la leche $>3\log_{10}$UFC/mL, y un recuento de leucocitos de la leche $> 6\ \log_{10}$ células/mL. Ninguna de ellas consumió probióticos comerciales ni suplementos durante la intervención. Los criterios de exclusión fueron aquellas mujeres con abscesos mamarios (colección de pus en la mama sin apertura para drenar), síndrome de Raynaud's (falta de riego sanguíneo en la mama) o cualquier otra patología mamaria.

El diseño del estudio consistió en la ingestión diaria de una cápsula con 200 mg de probiótico liofilizado el cual contenía $\sim 9\ \log_{10}$ UFC de la especie *L. Salivarius PS2* durante 21 días. Se recolectó la orina durante 24h al inicio del estudio (día cero, basal) y al final del estudio (día 21) (**Figura 4.13**). Además, para ver la evolución de las participantes, se les pidió que

mientras durara el estudio rellenaran un cuestionario relacionado con la evolución de la mastitis y la presencia de cualquier potencial efecto secundario efecto del tratamiento con el probiótico.

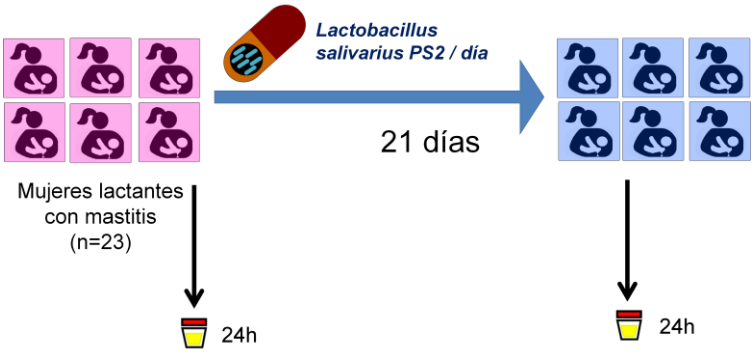

Figura 4.13. Estudio de un complemento alimenticio: Estudio de un probiótico. Diseño del estudio de intervención de consumo de probiótico en mujeres con mastitis. ufc: unidades formadoras de colonias.

4.3.3 ESTUDIO DE PATRONES ALIMENTARIOS:

Estudio PREDIMED

El estudio PREDIMED es un ensayo clínico aleatorizado y multicéntrico de intervención, de una media de 5 años de duración que tiene como principal objetivo la evaluación de los efectos de la Dieta Mediterránea en la prevención primaria de la enfermedad cardiovascular en individuos con alto riesgo (Estruch *et al.* 2013). Este estudio consiste en la evaluación del efecto de la dieta Mediterránea suplementada bien con aceite de oliva virgen extra o con una mezcla de frutos secos comparadas ambas con una dieta control baja en grasa (**Figura 4.14**). El diseño de este estudio metabolómico se realizó en el marco del proyecto nacional AGL2009-13906-C02-01/02 "Desarrollo de biomarcadores de consumo y de efecto de un patrón de alimentación Mediterránea en la prevención de la enfermedad cardiovascular: una aproximación metabolómica" coordinado en colaboración entre la Universidad de Barcelona y la Fundació Clínic.

Los participantes del estudio PREDIMED eran hombres de edades comprendidas entre los 55 y 80 años, y mujeres desde los 60 hasta los 80 años de edad. Ninguno de los participantes presentaba enfermedad cardiovascular diagnosticada y debían presentar al menos alguno de los siguientes criterios: Diabetes Mellitus tipo II, y/o presentar al menos tres factores de riesgo cardiovascular: fumadores de tabaco habituales, hipertensión, hipercolesterolemia, IMC ≥25 kg/m2, o antecedentes familiares de enfermedad cardiovascular. Como criterios de exclusión no debían presentar enfermedad cardiovascular, o cualquier enfermedad crónica de carácter grave, drogadicción o alcoholismo, antecedentes de alergia o intolerancia al aceite de oliva o frutos secos.

En el presente estudio se seleccionaron 98 participantes evaluados clínicamente como no diabéticos con alto riesgo cardiovascular (**Figura 4.15**). Como parámetros de inclusión debían tener al menos los tres factores de riesgo cardiovascular (comentados anteriormente). Como criterios de exclusión fue ser diagnosticado con Diabetes Mellitus tipo II (para este estudio concreto), además de los criterios de exclusión del estudio clínico anteriormente comentados. Los participantes asignados a la dieta baja en grasa se les aconsejó reducir todos los tipos de grasa y se les dio las recomendaciones por escrito de acuerdo con las directrices de la *American Heart Association.* Por otra parte, los participantes asignados a los grupos de dieta mediterránea (tanto el grupo de aceite de oliva como el de frutos secos) recibieron instrucciones según el cuestionario de 14 puntos de medida de adherencia a la dieta mediterránea, que incluía el aceite de oliva extra virgen para cocinar y aliñar los platos, el aumento de consumo de vegetales, frutos secos y pescado; el consumo de carne blanca en lugar de carne roja o procesada, y con un moderado consumo de vino tinto. No se sugirió ni la restricción energética ni la promoción de actividad física para ningún grupo participante en la intervención. Se proporcionó a los participantes para que pudieran seguir el estudio 1 L/semana de aceite de oliva extra-virgen/una mezcla de frutos secos (30 g/día) siendo 15 g nueces, 7.5 g de avellanas y 7.5 g de almendras. Además, todos los participantes tenían libre acceso a los nutricionistas durante el transcurso del estudio.

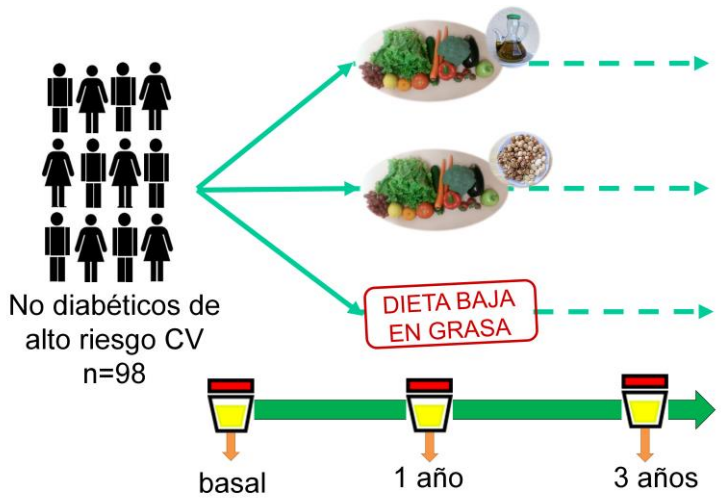

FIGURA 4.14. Estudio de patrones alimentarios. Estudio PREDIMED. Estudio de intervención con dos intervenciones dietéticas con dieta Mediterránea frente a una dieta control baja en grasa. CV: cardiovascular

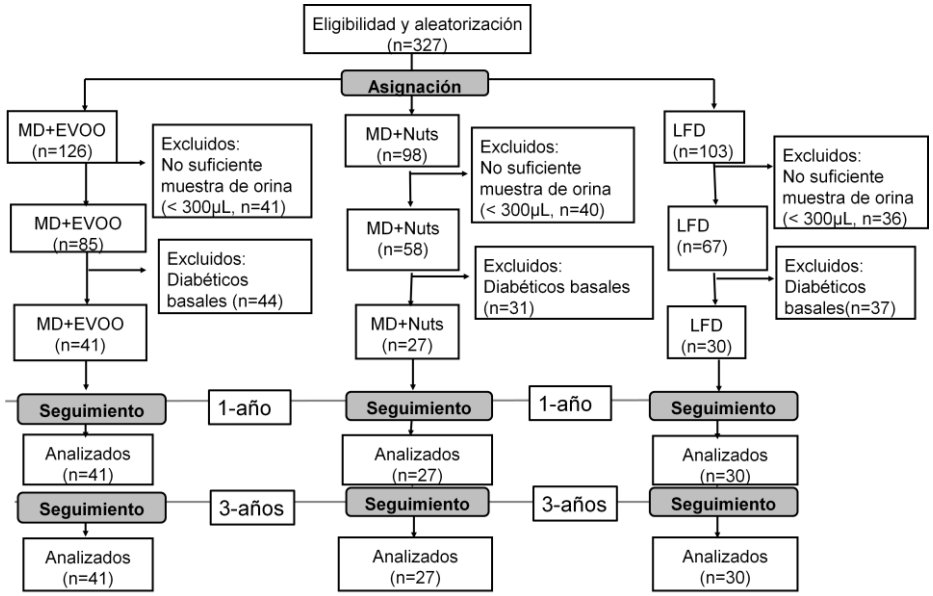

Figura 4.15. Diagrama de flujo de los participantes

RESULTADOS

5.1. BIOMARCADORES NUTRICIONALES TRAS EL CONSUMO DE UN ALIMENTO

I. Estudio de un alimento y el efecto de la matriz alimentaria

Los resultados de este estudio se publicaron en la revista *Electrophoresis* indexada en el *Journal Citation Reports* con un factor de impacto de 3.161 (2013) y situada en el primer cuartil de la categoría CHEMISTRY, ANALYTICAL (16/76) (2013).

> Rosa Vázquez-Fresno, Rafael Llorach, Francesca Alcaro, Miguel Ángel Rodríguez, Maria Vinaixa, Gemma Chiva-Blanch, Ramon Estruch, Xavier Correig, Cristina Andrés-Lacueva C. ^{1}H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors. *Electrophoresis*. 2012, 33, 2345–2354. doi: 10.1002/elps.201100646.

Resumen:

El consumo moderado de vino se ha visto asociado a una actividad promotora de la salud. Se utilizó un enfoque metabolómico basado en la ^{1}H-RMN para identificar diferencias metabólicas urinarias tras el consumo moderado de vino en el marco de un estudio prospectivo aleatorizado, cruzado y controlado. Sesenta y un voluntarios varones con factores de alto riesgo cardiovascular siguieron tres intervenciones dietéticas (28 días): de vino tinto (RWA) (30 g alcohol, 272 mL/día), vino tinto desalcoholizado (RWD) (272 mL/día, control de polifenoles), y ginebra (GIN) (100 ml/día, control de alcohol). Después de cada período, se recogieron y se analizaron mediante ^{1}H-RMN las muestras de orina de 24h. De acuerdo con los resultados del análisis ANOVA, los biomarcadores significativos fueron agrupados en cuatro categorías: marcadores relacionados con el alcohol (etanol); marcadores relacionados con ginebra; marcadores relacionados con vino; y

marcadores de la microbiota intestinal. Los metabolitos del vino se clasificaron en dos grupos; en primer lugar, los metabolitos del *food metabolome*: tartrato (RWA y RWD), etanol y manitol (RWA); y en segundo lugar, biomarcadores que se relaciona con modificaciones endógenas después del consumo de vino, que comprende un metabolito derivado de aminoácidos de cadena ramificada (3-metil-oxovalerato). Además, se ha identificado una posible interacción entre el alcohol y los biomarcadores relacionados con los metabolitos derivados de la microbiota intestinal (hipurato y ácido 4-hidroxifenilacetato). Desde nuestro conocimiento, esta es la primera vez que este enfoque ha sido aplicado en una intervención nutricional con vino tinto. Los resultados muestran la capacidad de este método para obtener una imagen completa del metaboloma incluyendo tanto el *food metabolome* como biomarcadores endógenos del consumo moderado de vino.

Electrophoresis 2012, *33*, 2345–2354

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Received November 24, 2011
Revised March 16, 2012
Accepted April 2, 2012

Research Article

[1]H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors

Moderate wine consumption is associated with health-promoting activities. An H-NMR-based metabolomic approach was used to identify urinary metabolomic differences of moderate wine intake in the setting of a prospective, randomized, crossover, and controlled trial. Sixty-one male volunteers with high cardiovascular risk factors followed three dietary interventions (28 days): dealcoholized red wine (RWD) (272mL/day, polyphenol control), alcoholized red wine (RWA) (272mL/day) and gin (GIN) (100mL/day, alcohol control). After each period, 24-h urine samples were collected and analyzed by ^{1}H-NMR. According to the results of a one-way ANOVA, significant markers were grouped in four categories: alcohol-related markers (ethanol); gin-related markers; wine-related markers; and gut microbiota markers (hippurate and 4-hydroxphenylacetic acid). Wine metabolites were classified into two groups; first, metabolites of food metabolome: tartrate (RWA and RWD), ethanol, and mannitol (RWA); and second, biomarkers that relates to endogenous modifications after wine consumption, comprising branched-chain amino acid (BCAA) metabolite (3-methyl-oxovalerate). Additionally, a possible interaction between alcohol and gut-related biomarkers has been identified. To our knowledge, this is the first time that this approach has been applied in a nutritional intervention with red wine. The results show the capacity of this approach to obtain a comprehensive metabolome picture including food metabolome and endogenous biomarkers of moderate wine intake.

Keywords:
Biomarkers / Metabolomics / Nuclear Magnetic Resonance / Urinary profile / Wine intake
DOI 10.1002/elps.201100646

1 Introduction

Cardiovascular disease (CVD) is one of the main causes of mortality in western countries. Diet and lifestyle are recognized as the major modifiable risk factors in CVD [1]. The low incidence of coronary heart disease (CHD) in Mediterranean countries has been partly ascribed to the dietary habits of their inhabitants [2, 3]. As one of the main components of Mediterranean diet, wine and its constituents, especially polyphenols, may provide additional health-promoting benefits [4–6]. Results of several clinical and epidemiological studies have showed the protective effect of a moderate wine consumption against CVD [7], particularly with regards to oxidative stress [8, 9], inflammation, and vascular function [10–12], the main causes behind the development of CVD [13]. However, there are discrepancies on the effects of the different types of alcoholic drinks (wine, beer, and liquors) on the cardiovascular system and whether the possible protective mechanisms of alcoholic beverages are due to their alcoholic component (ethanol), their nonalcoholic constituents, mainly polyphenols, or both [14, 15]. Therefore, new biomarkers of wine and alcohol consumption are needed in order to increase the knowledge on the effects of these beverages on the cardiovascular system and to clarify their protective mechanisms of CVD.

Foodomics is considered a new discipline that studies food and nutrition fields through advanced omics technology application: genomic, transcriptomic, proteomic, and /or metabolomic [16] using sensitive methods (NMR,

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Abbreviations: δ, chemical shift; **BCAA**, brached-chain amino acids; **CHD**, coronary heart disease; **CVD**, cardiovascular disease; **d**, doublet; **m**, multiplet; **q**, quadruplet; **RWA**, red wine alcoholized; **RWD**, dealcoholized red wine; **s**, singlet; **t**, triplet

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GC-MS, LC-MS, CE) to detect and quantify changes in dietary patterns [17]. ^{1}H-NMR-based metabolomics is a very robust technique for metabolomic applications enabling the simultaneous detection of a wide range of structurally different metabolites [18], and it can facilitate the discovery of new candidates for biomarkers of disease risk [19–21]. Similarly, NMR-based metabolomics can provide information, in combination with dietary intake, about the development of different diseases, as published by Vinaixa et al., who assessed the effect of dietary cholesterol on the progressive development of fatty liver disease [22]. As far as red wine polyphenol intake is concerned, van Dorsten et al. used a dry mix of red wine and red grape juice extracts to assess the metabolic fate of red wine polyphenols in humans by GC-MS and NMR metabolomics [23]. Despite this use of extracts from red wine, as far we know, no reports on metabolomic studies using wine (as a beverage) in a nutritional intervention with either animals or humans are available.

As a result, the aim of the present study is to apply, for the first time to our knowledge, an ^{1}H-NMR-based metabolomic strategy in order to investigate the effect of wine intake on the human metabolome in a urinary profile, identifying the most relevant markers of consumption and the endogenous effect of this beverage on human volunteers.

2 Material and methods

2.1 Subjects and study design

A total of 61 high-risk subjects aged ≥55 years without documented CHD (CHD: ischemic heart disease—angina/recent or past myocardial infarction/previous or cerebral vascular accident, peripheral vascular disease) were recruited for the study. The subjects included had diabetes mellitus or more than three of the following CHD risk factors: tobacco smoking, hypertension, hypercholesterolemia, plasma LDL cholesterol ≥160 mg/dL, plasma HDL cholesterol <40 mg/dL, obesity (BMI (in kg/m^2) ≥30), and/or a family history of premature CHD (first-line male relatives <55 years or females <65 years). Participants had to voluntarily give signed informed consent. Subjects with a previous history of CVD, any severe chronic disease, alcoholism, or other toxic abuse were excluded. The study was a prospective, randomized, crossover, and controlled trial. As a result of random computer-generated selection, participants were asked to take either, 272mL/day of red wine (hereafter, RWA) (about 30g ethanol/day), 272mL/day of dealcoholized red wine (hereafter, RWD, polyphenols control), or 100mL/day of gin (hereafter, GIN, alcohol control), every day for 4 weeks (28 days). Twenty-four-hour urine samples were collected on the last day of each period as well as the basal time. In order to evaluate the metabolic profiles, urine samples were analyzed by ^{1}H-NMR after each intake. All participants received all three interventions. Subjects had forbidden other alcoholic beverages 15 days before the first intervention and during the study, also asked not to change their dietary pattern during the study. A 7-day food record questionnaire was validated in our population [24] monitoring nutrient intake at the beginning of the study and after each intervention, also a medical record and Minnesota Leisure Time Physical Activity Questionnaire validated in Spain [25] were performed. The wine used for the interventions (RWA and RWD) was elaborated with Merlot grape variety. The daily dose of alcohol was the same in both the RWA and GIN periods and the polyphenol composition of the wine is detailed in Table 1 [26]. The total phenolic content of the three beverages was determined by the Folin–Ciocalteu method [27], the phenolic profile of RWA and RWD and resveratrol and piceid contents were measured by using HPLC-diode-array detection [28], [29].

2.2 Sample preparation

The urine samples were thawed, vortexed, and centrifuged at 13 200 rpm for 5 min. The supernatant (600 μL) from each urine sample was mixed with an internal standard solution (120 μL, consisting of 0.1% TSP (3-(trimethylsilyl)-proprionate-2,2,3,3-d4, chemical shift reference), 2 mM of sodium azide (NaN_3, bacteriostatic agent), and 1.5 M KH_2PO_4 in 99% deuterium water (D_2O)). The optimized pH of the buffer was set at 7.0, with a potassium deuteroxide (KOD) solution, to minimize variations in the chemical shifts of the NMR resonances. This mixture was transferred to a 5-mm NMR tube.

2.3 ^{1}H-NMR data acquisition and processing

The ^{1}H-NMR spectra were acquired on a Varian-Inova-500 MHz NMR Spectrometer with presaturation of the water resonance using a NOESYPRESAT pulse sequence. During the acquisition, the internal temperature was kept constant at 298 K. An exponential window function was applied to the free induction decay (FID) with a line-broadening factor of 0.3 Hz prior to the Fourier transformation. For each sample, FIDs were collected into 32 K data points (128 scans) with a spectral width of 14 ppm, an acquisition time of 2 s, relaxation delay of 5 s, and a mixing time of 100 ms. NMR spectra were phased, baseline corrected, and calibrated (TSP, 0.0 ppm) using TopSpin software (version 3.0, Bruker, BioSpin, Germany). After baseline correction, two different approaches were subsequently used to summarize spectral data. On the one hand, original spectral data were bucketed in equal-size domains of 0.005 ppm [30–32] using ACD/NMR Processor 12.0 software (Advanced Chemistry Development, Toronto, Canada). On the other hand, intensities of each 1H-NMR region conveniently identified in the urine 1D-NMR spectra were integrated for each sample entering the study (profiling integration) using the AMIX 3.8 software package (Bruker). In both cases, the spectral region between 4.68 and 5.08 ppm was excluded from the dataset to avoid spectral interference from residual water.

Table 1. Phenolic composition of beverages used in the study

	RWA	RWD	GIN
Alcohol (%)	14.2	0.42	38
Total phenols (mEqGA/L)	2933.35 ± 377.31	2694.92 ± 86.79	ND
Gallic acid (mg/L)	68.48 ± 6.40	73.17 ± 7.01	ND
Protocatechuic acid (mg/L)	5.22 ± 0.62	5.85 ± 0.51	ND
Tyrosol (mg/L)	43.59 ± 4.73	47.81 ± 3.90	ND
Catechin (mg/L)	123.51 ± 11.30	126.45 ± 13.35	ND
Epicatechin (mg/L)	67.86 ± 7.74	70.57 ± 8.22	ND
trans-Caftaric (mg/L)	18.62 ± 1.45	19.21 ± 1.62	ND
trans-Caffeic (mg/L)	11.50 ± 0.79	12.18 ± 0.92	ND
trans-Coutaric (mg/L)	5.21 ± 0.45	5.62 ± 0.52	ND
2-S-glutationylcaftaric (mg/L)	10.30 ± 1.00	10.76 ± 1.26	ND
Quercetin-3-glucuronide (mg/L)	11.88 ± 1.38	11.25 ± 1.42	ND
Quercetin (mg/L)	26.66 ± 0.78	23.82 ± 2.37	ND
Isorhamnetin (mg/L)	3.34 ± 0.27	2.96 ± 0.14	ND
Delphinidin-3-glucoside (mg/L)	15.25 ± 0.89	14.71 ± 1.62	ND
Petunidin-3-glucoside (mg/L)	12.29 ± 1.06	12.04 ± 1.15	ND
Peonidin-3-glucoside (mg/L)	6.78 ± 0.62	6.68 ± 0.57	ND
Malvidin-3-glucoside (mg/L)	48.83 ± 4.45	49.86 ± 4.27	ND
Malvidin-(6-acetyl)-3-glucoside (mg/L)	10.97 ± 0.96	10.41 ± 1.20	ND
Malvidin-(6-coumaroyl)-3-glucoside (mg/L)	4.15 ± 0.27	3.54 ± 0.33	ND
trans-Resveratrol (mg/L)	2.92 ± 0.36	2.73 ± 0.23	ND
cis-Resveratrol (mg/L)	2.79 ± 0.15	2.75 ± 0.15	ND
trans-Piceid (mg/L)	9.41 ± 1.12	10.53 ± 0.96	ND
cis-Piceid (mg/L)	7.71 ± 0.34	7.08 ± 0.87	ND

RWA, RWD, and GIN (nonsignificant differences were found for any of the phenolic compounds between RWA and RWD (student's *t*-test for independent samples). Mean ± SD.

2.4 Statistical analysis

Datasets derived from the two above-mentioned integrations were submitted to MetaboAnalyst, a web-based platform for comprehensive analysis of metabolomic data [33]. The two different matrices were row-wise normalized (rows were samples) by the sum of the intensities of the spectra [34] and column-wise normalized (columns were metabolites) using Pareto scaling [21] prior to being analyzed by ANOVA test. Fisher's LSD test for multiple comparisons was applied as a post hoc test; a $p < 0.05$ was considered to indicate statistical significance. In addition, we performed a correlation test (Pearson's correlation) [33] for verification when two or more signals came from the same metabolite. Correlation values close to 1 indicate a strong correlation, while values lower than 0.5 indicate a weak correlation, and values very close to 0 indicate no correlation.

2.5 Metabolite identification

Metabolite identification was performed according to Chenomx NMR Suite 7.0 profiler (Chenomx Edmonton, Canada) by comparing NMR spectral data to those available in databases such as the Human Metabolome Database (HMDB; http://www.hmdb.ca), the Biological Magnetic Resonance Data Bank (BMRB, http://www.bmrb.wisc.edu), and the Madison Metabolomics Consortium Database (MMCD, www.mmcd.nmrfam.wisc.edu), along with the existing NMR-based metabolomics literature [35–37].

3 Results and discussions

The total phenolic content of the three beverages used in this study, the phenolic profile of RWA and RWD, and resveratrol and piceid content are presented in Table 1. ^{1}H- NMR resonance assignments with chemical shifts and multiplicity of the signals elucidated in ^{1}H- NMR spectra of urine of the participants are shown in Table 2. Results performed by multivariate techniques were not consistent and difficult to interpret so we choose ANOVA test to analyze selected NMR signals to determine statistical differences between interventions of the individuals [38, 39]. ANOVA test is effective determining whether a given set of NMR signals contains interesting information. The significant bins returned by the ANOVA test ($p < 0.05$) for both techniques are shown in Table 3. Results showed that significant bins from the two above-mentioned integrations were in consonance. The box plots with the Fisher's LSD test for multiple comparisons are shown in Fig. 1. Additionally, in order to present an overview of the whole representative spectrum, the significant metabolites of each spectrum in basal and the three dietary interventions are shown (Fig. 2). Identified markers were grouped in

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Table 2. ^{1}H-NMR resonance assignments with chemical shifts for signals identified in samples of human urine

Code	Metabolite	δ (H^1 shift) ppm	Multiplicity
1	Acetate	1.93	s
2	Acetoacetate	2.27	s
3	Acetone	2.24	s
4	cis-Aconitate	3.11; 5.75	s; s
5	Acetylcarnitine	2.15	s
6	Alanine	1.49	d
7	Betaine	3.27	s
8	Carnitine	3.23	s
9	Citrate	2.68 + 2.55	d + d
10	Creatine	3.94, 3.04	s; s
11	Creatinine	3.06; 4.06	s; s
12	Dimethylamine (DMA)	2.72	s
13	Ethanol	1.19; 3.69	t; q
14	Formate	8.46	s
15	Fucose	5.20	d
16	Glycine	3.57	s
17	Glycylproline	3.94	s
18	Glucose	3.50; 4.66; 5.25	m; d; d
19	Hippurate	3.98; 7.57; 7.64; 7.84	d; tt; t; dd
20	Histidine	7.08	s
21	2-Hydroxyisobutyrate	1.36	s
22	3-Hydroxyisovalerate	1.26	s
23	3-Hydroxymandelate	6.82	s
24	4-Hydroxyphenylacetate	6.87	d
25	Indole-3-acetate	7.51	d
26	Lactate	1.33	d
27	Leucine	0.96	t
28	Lysine	1.73	m
29	Malonate	3.12	s
30	Mannitol	3.69; 3.77; 3.80; 3.87	dd; m; d; dd
31	n-Methylhistidine	7.09	s
32	1-Methylnicotinamide	4.47	s
33	Methylsuccinate	1.07	d
34	3-Methyl-2-oxovalerate	1.10	d
35	N-N Dimethylglycine (DMG)	2.93	s
36	Tartrate	4.35	s
37	N-Phenylacetylglycine (PAG)	7.35; 7.43	m; m
38	Succinate	2.41	s
39	Taurine	3.43; 3.26	t; t
40	Trigonelline	4.43; 8.08; 8.85; 9.13	s; m; m; s
41	Trimethylamine (TMA)	2.89	s
42	Trimethylamine-N-oxide (TMAO)	3.27	s
43	Threonine	1.33	d
44	Tyrosine	6.90	d
45	Urea	5.75–5.90	m
46	Valine	0.98; 1.05	d; d

s: singlet; d: doublet; dd: double doublet; t: triplet; tt: double triplet; q: quadruplet; m: multiplet.

four categories, namely: alcohol-related markers; gin-related markers; wine-related markers; gut microbiota markers.

3.1 Alcohol-related markers

A significant presence of ethanol (δ1.19 (t), and δ3.69 (q)) was detected in the urine of participants after RWA and GIN consumption, which suggests that this presence may correspond to the ethanol from the beverage (both gin and red wine). In fact, ethanol has been found in wine by NMR [40]. With regard to human samples, ethanol has been identified in the urine of healthy Caucasian volunteers who, after 2 days of abstinence from alcoholic beverages, consumed sake and rice wine [41]. The presence of ethanol in urine was recently used as an indicator of noncompliance of dietary protocol

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Table 3. Metabolites detected after the three treatments

Compound	^{1}H-NMR Chemical shift (ppm)		After RWA intake	After RWD intake	After GIN intake	*p*-value		Metabolites information
	0.005 ppm integration	Profiling integration				0.005 ppm integration	Profiling integration	
Tartrate	4.35	4.35	↑	↑	–	4.81×10^{-9}	1.0147×10^{-7}	Wine compound
Mannitol	3.77	3.69–3.87	↑	–	–	3.68×10^{-2}	3.43×10^{-2}	Wine alcoholized compound
	3.87					4.69×10^{-2}		
Ethanol	1.19	1.19	↑	–	↑	1.43×10^{-4}	3.0324×10^{-4}	Alcohol from beverage
	3.69					8.60×10^{-4}		
3-Methyl-2-oxovalerate	1.10	1.10	↑	↑	–	1.63×10^{-5}	3.48×10^{-2}	Valine, leucine, and isoleucine degradation
4-Hydroxyphenylacetate	6.87	6.87	↑	↑	–	1.89×10^{-2}	4.31×10^{-2}	Phenylalanine and tyrosine metabolism Gut microbiota
Hippurate	7.84	7.84	–	↑	–	6.56×10^{-4}	1.26×10^{-2}	Phenylalanine metabolism
	3.98	3.98				2.59×10^{-2}	1.49×10^{-2}	Gut microbiota
	7.64	7.64				2.58×10^{-3}	3.05×10^{-2}	
	7.57	7.57				1.04×10^{-2}	3.29×10^{-2}	

-: no changes.
p-values of each metabolite ($p < 0.05$; ANOVA).

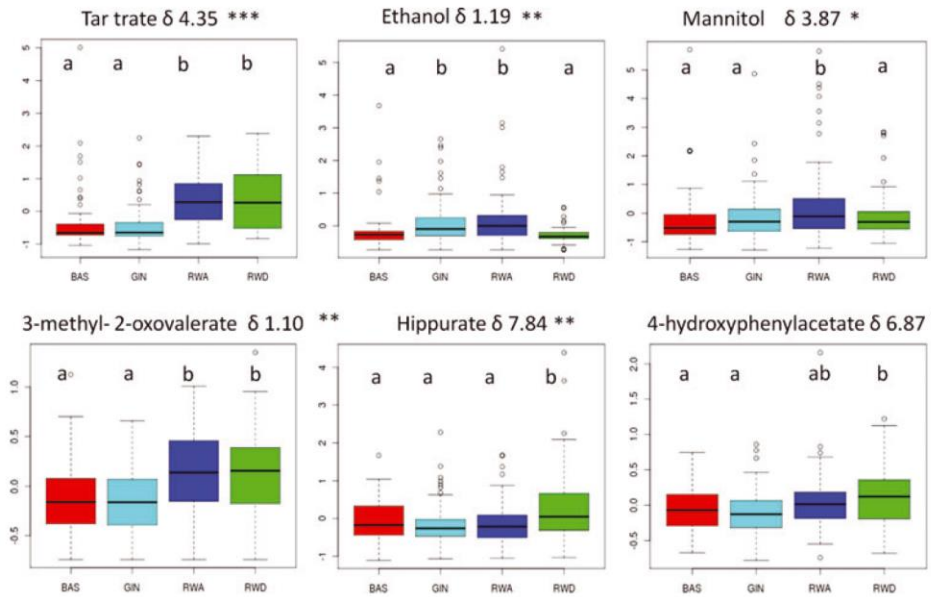

Figure 1. Box plots of the intensities of significant metabolites. ***$p < 0.00001$; **$p < 0.005$; *$p < 0.05$. Different letters indicate significant differences between interventions. BAS (basal time); RWA (red wine), RWD (dealcoholized red wine), and GIN (Gin).

in a metabolomic experiment in which alcohol intake was forbidden [42]. In our case, we view the presence of ethanol in the interventions that has the alcoholic component in the dietary protocol (RWA and GIN), concluding that the identified ethanol could be considered as a biomarker of the overall compliance of dietary interventions.

3.2 Gin-related markers

Two correlating signals (δ2.13 (s) and 5.69 (s), Fig. 3A) were excreted in statistically higher concentrations after GIN intake. These two signals are also present in some volunteers in basal time and after a RWA or RWD period, without a

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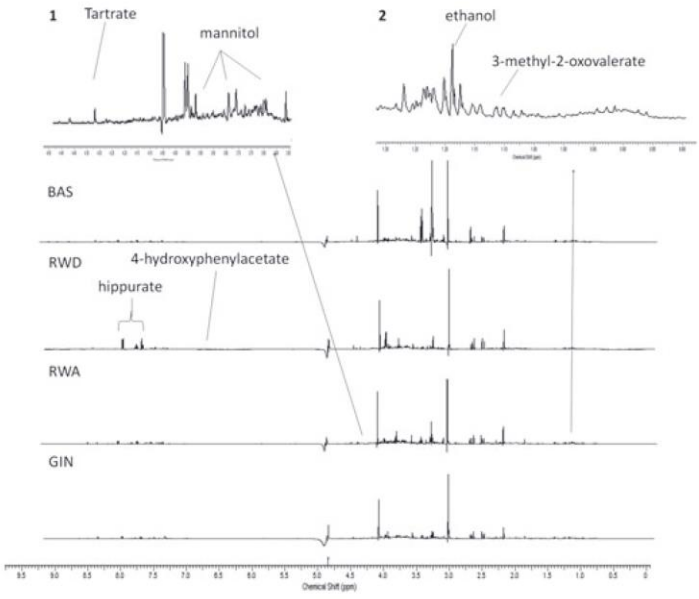

Figure 2. Representative 500-MHz ^{1}H-NMR region of spectra in basal time (BAS), RWD (dealcoholized red wine); RWA (red wine), GIN (gin). The figure exhibits the significant metabolites in ANOVA test. Zoomed regions are performed. Spectral area zoomed between 3.55 and 4.50 ppm (1); 0.80–1.30 ppm interval area (2).

clear pattern in terms of volunteers and interventions followed. This behavior indicates either possible gin compound or an endogenous compound because not all gin volunteers presented these peaks in their urinary metabolome. Querying to the NMR database failed to confirm the assignation of these signals. However, the δ2.13 peak could suggest the presence of acetylcholine or acetylcarnitine, but this hypothesis could not be confirmed by comparing the sample with a urine sample spiked with commercial standards, so it has been rejected. Therefore, we have not been able to identify these two correlated signals and they have been termed as "unknown 1."

3.3 Wine-related markers

This group comprises those markers related to the intake of red wine (RWA and/or RWD). With regards to these two classes of wine (RWA and RWD) intake, a chemical shift at 4.35 ppm (s), corresponding to tartrate, was detected. The box plot in Fig. 1 shows the significant differences between the wine, basal, and gin periods, with no differences between wines. Tartaric acid is the major acid in grapes and so it is also present in wine [40, 43]. Lord et al. tested the frequent ingestion of grape juice (28 mL) in a human population producing urinary tartrate concentrations > 300 g/mg creatinine [44]. Dietary sources strongly influence the concentration of urinary tartrate, and its production by intestinal yeast or bacteria is insignificant because the majority of tartaric acid is destroyed by microbial action [45, 46], indicating that the urinary tartrate in our samples was provided by the wine composition. In addition, Yamashita et al. demonstrated that tartaric acid has the beneficial effect of enhancing the bioavailability of wine polyphenols [47].

In this context, an endogenous product of the degradation of branched-chain amino acids (BCAA) termed

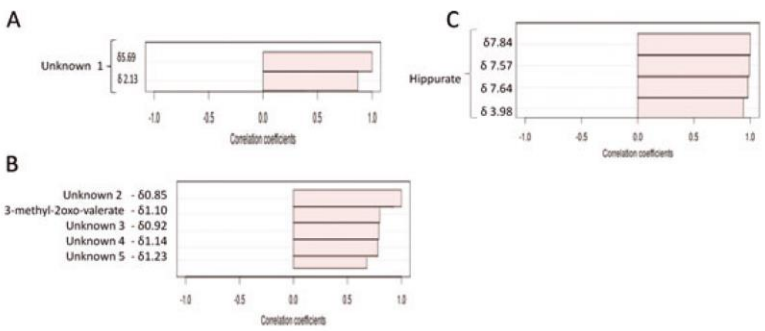

Figure 3. Correlation plot from integration bucketing corresponding to signals of unknown 1 (A); Aliphatic region metabolites, 0.84–0.86: unknown 2; 1.09–1.11: methyl-2-oxovalerate; 0.90–0.92: unknown 3; 1.13–1.15: unknown 4; 1.23–1.24: unknown 5 (B); and Hippurate (C). Correlation coefficient >0.5, for all the figures. The compounds are represented as horizontal bars. Light pink color indicates positive correlations.

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3-methyl-2-oxovalerate (δ1.10, d) was identified in urines after the intake of RWA and RWD (Fig. 1, Table 2). This identification was confirmed by spiking urine samples with the commercial standard. According to the results obtained, Connor et al. found an increase in urinary levels of the BCAA valine and its metabolite 3-methyl-2-oxovalerate in diabetic mice [48]. Moreover, it was discovered that cardiovascular subjects such as diabetic [48, 49] and obese subjects [50, 51] have elevated plasma levels of BCAAs [52], because of the reduction in BCAA catabolism. The first step in the catabolism of BCAAs is a reversible step and is carried out in the brain and several nonhepatic tissues by branched-chain aminotransferase (BCAT) to convert BCAAs into branched-chain alpha ketoacids (BCKAs) such as 3-methyl-2-oxovalerate. Our results show an increase in 3-methyl-2-oxovalerate in urine as a result of the first step of BCAA catabolism, suggesting a possible upregulation of this pathway carried out by BCAT enzyme after RWA and RWD intake.

In the aliphatic region comprising 0.85–1.24 ppm, where 3-methyl-2-oxovalerate is also present, there were statistical differences between wine intake (RWA and RWD) and basal or gin treatment. An increase in several signals in this region (see nomenclature in Fig. 3B: unknown 2: δ0.85 (s), unknown 3: δ0.92 (s), unknown 4: δ1.15 (d); unknown 5: δ1.24 (d+d)) was detected after wine consumption, with a good correlation between them and with 3-methyl-2-oxovalerate (Fig. 3B). The identification of these unassigned signals (overall the 0.80–1.00 ppm region) was hampered by their low signal intensities, a broadband signal area, and the absence of other resonance patterns.

With regard to RWA intake, mannitol (δ3.78, dd) showed a significant increase in the sugar region. Mannitol is a polyol produced by heterofermentative lactic acid bacteria from the reduction of fructose [53] and is present in wine. Liu et al. tested mannitol concentration in different wines and fruit juices, finding higher values in Cabernet Sauvignon, a red wine from the same family as Merlot (the wine used in this study) [54], than in sweet white, medium-dry white, or Pinot noir wine. The presence of this polyol in urine samples after RWA intake could be explained by the fact that it is largely eliminated from the body before any metabolism can take place [55], and the absence after RWD intake suggests a possible elimination of this compound during the process of dealcoholization.

3.4 Gut microbiota markers

In samples from the RWD intake period, the intensity signals of hippurate (δ3.98 (d), δ7.57 (tt), δ 7.64 (t), δ7.84 (dd), Fig. 3C) increased in comparison with GIN and basal time. An interesting significant difference was observed between RWA and RWD (Fig. 1 and Table 3). Hippurate is a metabolite belonging to the phenylalanine and tyrosine degradation pathways and it has also been related to polyphenol microbiota catabolism. There have been several studies in rats and humans showing an increase in hippurate excretion after wine polyphenol consumption [56, 57]. Another gut microbiota metabolite, 4-hydroxyphenylacetate (δ6.87), showed an effect of wine intake, with no significant differences between RWD and RWA; however, it did show significantly higher levels after RWD intake compared to the GIN and basal period. This metabolite has been identified in urine and faeces [32, 56] after consumption of polyphenolic compounds in wine [57, 58], it could explain no differences in these results between wines (RWD and RWA) in the current results. In addition, this metabolite is also related to tyrosine metabolism. An interesting result was that no significant differences between the alcohol-containing diets (RWA and GIN) were detected (Fig. 1, Table 3).

The two markers related to gut microbiota showed a similar behavior (Fig. 1); however, slight differences were observed. The similarities were found in relation to the influence of alcohol intake where for both metabolites, RWA and GIN showed no significant difference. This fact suggests a possible impact of ethanol on the metabolic pathways related to the production of both compounds, probably modifying the relationship between microbiota and human metabolism. A recent study conducted by Gao et al. [59] compared urine from Wistar and Spraguel-Dawley rats after ethanol administration. The authors demonstrated a different excretion pattern in some metabolites affected by alcohol intake. Concretely, hippurate showed less excretion after ethanol administration, and in another hand, excretion of 4-hydroxyphenylacetate increased with alcohol administration. With regard to differences in the present results, while hippurate content was significantly different when comparing RWA and RWD, the amount of 4-hydroxyphenylacetic acid did not differ significantly between wine diets (RWA and RWD). This behavior could be explained because 4-hydroxyphenylacetate is a compound increased with wine consumption, counteracting with the effect of alcohol administration. As a result, no differences between wines (RWD and RWA) (Fig. 1, Table 2) and between alcohol-containing beverages (RWA and GIN) were observed. Hippurate could arise from different sources, such as diet (mainly polyphenols), oxidative stress, and intestinal microbiota [20]. In contrast, in the case of 4-hydroxyphenylacetic acid, this behavior (no differences between wine diets) suggests that an important part of this compound should be associated with wine intake from the intervention.

4 Concluding remarks

The results of the current work show the capability of an NMR-based metabolomic approach to detect significant changes in metabolites after moderate wine consumption. Wine-related biomarkers may be classified into two groups. The first group comprises those metabolites coming from the metabolism of food components (food metabolome), where mannitol is related to an RWA diet, and tartrate is a biomarker of wine intake (RWA and RWD). These results support the notion that this compound (tartrate) could be considered to be a possible marker of wine intake. The second group

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comprises those markers related to endogenous modifications after wine consumption: BCAA metabolites and other signals in the same spectral area. In spite of this, more about BCAAs and wine intake needs to be known in order to understand the connection between the catabolic pathway of BCAA and moderate wine intake.

Despite these biomarkers, ethanol was a robust biomarker of alcohol consumption as it was related to GIN and RWA diets. In fact, combining tartrate and ethanol, we observed a global compliance of dietary intervention, important factor in metabolomics studies that other authors also has been investigated [60]. Finally, the gut microbiota metabolites, 4-hydroxyphenylacetate, and hippurate, showed a particular effect in combination with alcohol, providing new insights into the assimilation of polyphenol metabolites.

One of the main limitations of our study is that there we have no washout periods between interventions. Washout periods between interventions would have extended the study 6 weeks more, which would have made it difficult to ensure compliance and any increase in study length may increase participant dropout rate [61]. Previous studies carried out at the Hospital Clinic of Barcelona confirmed that changes in cellular and endothelial adhesion molecules due to the intervention were already observed after 15 days of treatment [62,63], and no carryover effect was observed, the absence of a washout period would probably not have changed the results. Therefore, the results observed at the end of the 4-week period could be attributed to the intervention and should only be compared with those observed at the end of the other interventions.

In conclusion, to our knowledge, this is the first time that this approach has been applied in a nutritional intervention with red wine. The results showed that H-NMR-based metabolomics is a powerful strategy for obtaining biomarkers in nutritional intervention studies. In fact, the results obtained generate new perspectives on understanding the relationship between moderate wine intake and human health.

This research was supported by Spanish National Grants: CICYT-AGL 2006-14228-C03-02 (Spanish Ministry of Education and Science), as well as AGL2009-13906-C02-01, AGL 2010-10084-E, CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD (CSD2007-063) from the MICINN, Spain and the three grants ICTS-2009-43 from the MICINN, Spain; for the utilization of the NMR equipment in Parc Científic of Barcelona. RVF would like to thank the FPI fellowship from the Spanish Ministry of Science and Innovation (MICINN). RLL would like to thank MICINN and the European Social Funds for their financial contribution to the R. Llorach Ramón y Cajal contract (Ramon y Cajal Programme, MICINN-RYC). FA was supported by the Leonardo da Vinci Programme 2010, the European Commission's Lifelong Learning Programme. We thank the participants for their collaboration in the Study. Torres SA provided the red wine and dealcoholized red wine used in the study, and Gin Xoriguer provided the gin used in the study.

The authors have declared no conflict of interest.

5 References

[1] Leifert, W. R., Abeywardena, M. Y., *Nutr. Res.* 2008, *28*, 729–737.

[2] Joshipura, K. J., Hu, F. B., Manson, J. E., Stampfer, M. J., Rimm, E. B., Speizer, F. E., Colditz, G., Ascherio, A., Rosner, B., Spiegelman, D., Willett, W. C., *Ann. Intern. Med.* 2001, *134*, 1106–1114.

[3] Dauchet, L., Amouyel, P., Dallongeville, J., *Nat. Rev. Cardiol.* 2009, *6*, 599–608.

[4] Estruch, R., *Food Res. Int.* 2000, *33*, 219–226.

[5] Zamora-Ros, R., Urpí-Sardà, M., Lamuela-Raventós, R. M., Estruch, R., Martínez-González, M. A., Bulló, M., Arós, F., Cherubini, A., Andres-Lacueva, C., *Free Radic. Biol. Med.* 2009, *46*, 1562–1566.

[6] Lindberg, M. L., Amsterdam, E. A., *Clin. Cardiol.* 2008, *31*, 347–351.

[7] Renaud, S., de Lorgeril, M., *Lancet* 1992, *339*, 1523–1526.

[8] Estruch, R., Sacanella, E., Mota, F., Chiva-Blanch, G., Antúnez, E., Casals, E., Deulofeu, R., Rotilio, D., Andres-Lacueva, C., Lamuela-Raventos, R. M., de Gaetano, G., Urbano-Marquez, A., *Nutr. Metab. Cardiovasc. Dis.* 2011, *21*, 46–53.

[9] Tian, L., Wang, H., Abdallah, A. M., Prinyawiwatkul, W., Xu, Z., *J. Agric. Food Chem.* 2011, *59*, 6453–6458.

[10] Botden, I. P., Langendonk, J. G., Meima, M. E., Boomsma, F., Seynhaeve, A. L., ten Hagen, T. L., Jan Danser, A. H., Sijbrands, E. J., *Am. J. Hypertens.* 2011, *24*, 162–168.

[11] Vazquez-Prieto, M. A., Renna, N. F., Diez, E. R., Cacciamani, V., Lembo, C., Miatello, R. M., *Am. J. Hypertens.* 2011, *24*, 234–240.

[12] Huang, P.-H., Chen, Y.-H., Tsai, H.-Y., Chen, J.-S., Wu, T.-C., Lin, F.-Y., Sata, M., CHen, J.-W., Lin, S.-J., *Arterioscler. Thromb. Vasc. Biol.* 2010, *30*, 869–877.

[13] Mukamal, K., Conigrave, K. M., Mittleman, M.A., Camargo, C.A., Jr., Stampfer, M.J., Willett, W.C., Rimm, E.B., *New Engl. J. Med.* 2003, *348*, 109–118.

[14] Beulens, J. W., Algra, A., Soedamah-Muthu, S. S., Visseren, F. L., Grobbee, D. E., van der Graaf, Y., Group, S. S., *Atherosclerosis* 2010, *212*, 281–286.

[15] Spaak, J., Tomlinson, G., McGowan, C. L., Soleas, G. J., Morris, B. L., Picton, P., Notarius, C. F., Floras, J. S., *Am. J. Physiol. Heart Circ. Physiol.* 2010, *298*, H2226–H2231.

[16] Herrero, M., Simó, C., García-Cañas, V., Ibáñez, E., Cifuentes, A., *Mass Spectrom Rev.* 2011, *31*, 49–69.

[17] Puiggròs, F., Solà, R., Bladé, C., Salvadó, M. J., Arola, L., *J. Chromatogr. A* 2011, *1218*, 7399–7414.

[18] Beckonert, O., Keun, H. C., Ebbels, T. M., Bundy, J., Holmes, E., Lindon, J. C., Nicholson, J. K., *Nat. Protoc.* 2007, *2*, 2692–2703.

[19] Holmes, E., Loo, R. L., Stamler, J., Bictash, M., Yap, I. K., Chan, Q., Ebbels, T., De Iorio, M., Brown, I. J., Veselkov, K. A., Daviglus, M. L., Kesteloot, H., Ueshima, H., Zhao, L., Nicholson, J. K., Elliott, P., *Nature* 2008, *453*, 396–400.

[20] Carrola, J., Rocha, C. M., Barros, A. S., Gil, A. M., Goodfellow, B. J., Carreira, I. M., Bernardo, J., Gomes, A., Sousa, V., Carvalho, L., Duarte, I. F., *J. Proteome Res.* 2011, *10*, 221–230.

[21] Kang, S. M., Park, J. C., Shin, M. J., Lee, H., Oh, J., Ryu, d. H., Hwang, G. S., Chung, J. H., *Clin. Biochem.* 2011, *44*, 293–299.

[22] Vinaixa, M., Rodríguez, M. A., Rull, A., Beltrán, R., Bladé, C., Brezmes, J., Cañellas, N., Joven, J., Correig, X., *J. Proteome Res.* 2010, *9*, 2527–2538.

[23] van Dorsten, F. A., Grün, C. H., van Velzen, E. J., Jacobs, D. M., Draijer, R., van Duynhoven, J. P., *Mol. Nutr. Food Res.* 2010, *54*, 897–908.

[24] Schröder, H., Covas, M. I., Marrugat, J., Vila, J., Pena, A., Alcántara, M., Masiá, R., *Clin Nutr.* 2001, *20*, 429–437.

[25] Elosua, R., Marrugat, J., Molina, L., Pons, S., Pujol, E., *Am. J. Epidemiol.* 1994, *139*, 1197–1209.

[26] Chiva-Blanch, G., Urpi-Sarda, M., Llorach, R., Rotches-Ribalta, M., Guillén, M., Casas, R., Arranz, S., Valderas-Martinez, P., Portoles, O., Corella, D., Tinahones, F., Lamuela-Raventos, R. M., Andres-Lacueva, C., Estruch, R., *Am. J. Clin. Nutr.* 2012, *95*, 326–334.

[27] Singleton, V., Rossi, J., *Am. J. Enol. Vitic.* 1965, *16*, 144–158.

[28] Ibern-Gomez, M., Andres-Lacueva, C., Lamuela-Raventos, R., Waterhouse, A., *Am. J. Enol. Vitic.* 2002, *53*, 218–227.

[29] Romero-Pérez, A. I., Ibern-Gómez, M., Lamuela-Raventós, R. M., de La Torre-Boronat, M. C., *J. Agric. Food Chem.* 1999, *47*, 1533–1536.

[30] Yap, I. K., Brown, I. J., Chan, Q., Wijeyesekera, A., Garcia-Perez, I., Bictash, M., Loo, R. L., Chadeau-Hyam, M., Ebbels, T., De Iorio, M., Maibaum, E., Zhao, L., Kesteloot, H., Daviglus, M. L., Stamler, J., Nicholson, J. K., Elliott, P., Holmes, E., *J. Proteome Res.* 2010, *9*, 6647–6654.

[31] Zhao, L., Liu, X., Xie, L., Gao, H., Lin, D., *Anal. Sci.* 2010, *26*, 1277–1282.

[32] Jacobs, D. M., Deltimple, N., van Velzen, E., van Dorsten, F. A., Bingham, M., Vaughan, E. E., van Duynhoven, J., *NMR Biomed.* 2008, *21*, 615–626.

[33] Xia, J., Wishart, D. S., *Nat. Protoc.* 2011, *6*, 743–760.

[34] Rezzi, S., Martin, F. P., Alonso, C., Guilarte, M., Vicario, M., Ramos, L., Martínez, C., Lobo, B., Saperas, E., Malagelada, J. R., Santos, J., Kochhar, S., *J. Proteome Res.* 2009, *8*, 4799–4809.

[35] Saude, E., Sykes, B. D., *Metabolomics* 2007, *3*, 19–27.

[36] Mi Park, E., Lee, E., Jin Joo, H., Oh, E., Lee, J., Lee, J. S., *Clin. Chem. Lab. Med.* 2009, *47*, 188–194.

[37] Zuppi, C., Messana, I., Forni, F., Rossi, C., Pennacchietti, L., Ferrari, F., Giardina, B., *Clin. Chim. Acta* 1997, *265*, 85–97.

[38] Le Gall, G., Colquhoun, I. J., Davis, A. L., Collins, G. J., Verhoeyen, M. E., *J. Agric. Food Chem.* 2003, *51*, 2447–2456.

[39] Le Gall, G., Colquhoun, I. J., Defernez, M., *J. Agric. Food Chem.* 2004, *52*, 692–700.

[40] Son, H. S., Hwang, G. S., Kim, K. M., Ahn, H. J., Park, W. M., Van Den Berg, F., Hong, Y. S., Lee, C. H., *J. Agric. Food Chem.* 2009, *57*, 1481–1490.

[41] Teague, C., Holmes, E., Maibaum, E., Nicholson, J., Tang, H., Chan, Q., Elliott, P., Stamler, J., Ueshima, H., Zhou, B., Wilson, I., *Analyst* 2004, *129*, 259–264.

[42] Edmands, W. M., Beckonert, O. P., Stella, C., Campbell, A., Lake, B. G., Lindon, J. C., Holmes, E., Gooderham, N. J., *J. Proteome Res.* 2011, *10*, 4513–4521.

[43] Son, H. S., Kim, K. M., van den Berg, F., Hwang, G. S., Park, W. M., Lee, C. H., Hong, Y. S., *J. Agric. Food Chem.* 2008, *56*, 8007–8016.

[44] Lord, R. S., Burdette, C. K., Bralley, J. A., *Clin. Chem.* 2005, *51*, 672–673.

[45] Fonseca, A., *Can. J. Microbiol.* 1992, *38*, 1242–1251.

[46] Fonseca, A., Fell, J. W., Kurtzman, C. P., Spencer-Martins, I., *Int. J. Syst. Evol. Microbiol.* 2000, *50*(Pt 1), 389–394.

[47] Yamashita, S., Sakane, T., Harada, M., Sugiura, N., Koda, H., Kiso, Y., Sezaki, H., *Ann. NY Acad. Sci.* 2002, *957*, 325–328.

[48] Connor, S. C., Hansen, M. K., Corner, A., Smith, R. F., Ryan, T. E., *Mol. Biosyst.* 2010, *6*, 909–921.

[49] Wijekoon, E. P., Skinner, C., Brosnan, M. E., Brosnan, J. T., *Can. J. Physiol. Pharmacol.* 2004, *82*, 506–514.

[50] Newgard, C. B., An, J., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Lien, L. F., Haqq, A. M., Shah, S. H., Arlotto, M., Slentz, C. A., Rochon, J., Gallup, D., Ilkayeva, O., Wenner, B. R., Yancy, W. S., Eisenson, H., Musante, G., Surwit, R. S., Millington, D. S., Butler, M. D., Svetkey, L. P., *Cell Metab.* 2009, *9*, 311–326.

[51] She, P., Van Horn, C., Reid, T., Hutson, S. M., Cooney, R. N., Lynch, C. J., *Am. J. Physiol. Endocrinol. Metab.* 2007, *293*, E1552–E1563.

[52] Shah, S. H., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Crosslin, D. R., Haynes, C., Dungan, J., Newby, L. K., Hauser, E. R., Ginsburg, G. S., Newgard, C. B., Kraus, W. E., *Circ. Cardiovasc. Genet.* 2010, *3*, 207–214.

[53] Pilone, G. J., Clayton, M. G., Van Duivendoden, R. J., *Am. J. Enol. Vitic.* 1991, *42*, 153.

[54] Liu, S. Q., David, C. R., *Am. J. Enol. Vitic.* 1994, *45*, 229–233.

[55] Lee, J., Chung, B. C., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2006, *831*, 126–131.

[56] Grün, C. H., van Dorsten, F. A., Jacobs, D. M., Le Belleguic, M., van Velzen, E. J., Bingham, M. O., Janssen, H. G., van Duynhoven, J. P., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2008, *871*, 212–219.

[57] van Velzen, E. J., Westerhuis, J. A., van Duynhoven, J. P., van Dorsten, F. A., Hoefsloot, H. C., Jacobs, D. M., Smit, S., Draijer, R., Kroner, C. I., Smilde, A. K., *J. Proteome Res.* 2008, *7*, 4483–4491.

[58] Saura-Calixto, F., Pérez-Jiménez, J., Touriño, S., Serrano, J., Fuguet, E., Torres, J. L., Goñi, I., *Mol. Nutr. Food Res.* 2010, *54*, 939–946.

[59] Gao, X., Zhao, A., Mingmei, Z., Lin, J., Qiu, Y., Su, M., Jia, W., *Metabolomics* 2011, *7*, 363–374.

[60] Loo, R. L., Chan, Q., Brown, I. J., Robertson, C. E., Stamler, J., Nicholson, J. K., Holmes, E., Elliott, P., INTERMAP Research Group, *Am. J. Epidemiol.* 2012, *175*, 348–358.

[61] Welch, R. W., Antoine, J. M., Berta, J. L., Bub, A., de Vries, J., Guarner, F., Hasselwander, O., Hendriks, H., Jäkel, M.,

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Koletzko, B. V., Patterson, C. C., Richelle, M., Skarp, M., Theis, S., Vidry, S., Woodside, J. V., International Life Sciences Institute Europe Functional Foods Task Force, *Br. J. Nutr.* 2011, *106*(Suppl 2), S3–S15.

[62] Estruch, R., Sacanella, E., Badia, E., Antúnez, E., Nicolás, J. M., Fernández-Solá, J., Rotilio, D., de Gaetano, G., Rubin, E., Urbano-Márquez, A., *Atherosclerosis* 2004, *175*, 117–123.

[63] Vázquez-Agell, M., Sacanella, E., Tobias, E., Monagas, M., Antúnez, E., Zamora-Ros, R., Andrés-Lacueva, C., Lamuela-Raventós, R. M., Fernández-Solá, J., Nicolás, J. M., Estruch, R., *J. Nutr.* 2007, *137*, 2279–2284.

II. Estudio de la sensibilidad y especificidad de los biomarcadores nutricionales

Este estudio se ha publicado en la revista *Metabolomics* con un factor de impacto de 3.965 (2013) perteneciente al segundo cuartil ENDOCRINOLOGY & METABOLISM (35/125)

Rosa Vázquez-Fresno, Rafael Llorach, Mireia Urpi-Sarda, Olha Khymenets, Mònica Bulló, Dolores Corella, Montserrat Fitó, Miguel Angel Martínez-González, Ramon Estruch, Cristina Andres-Lacueva. An NMR metabolomics approach reveals a combined-biomarkers model in a wine interventional trial with validation in free-living individuals of the PREDIMED study. *Metabolomics* [*In press*] doi:10.1007/s11306-014-0735-x.

RESUMEN:

El desarrollo de biomarcadores robustos de consumo puede mejorar la clasificación de los individuos con respecto a su exposición dietética. Además, la validación de ellos en una población de vida libre sigue siendo un importante reto. El objetivo de este estudio es evaluar los biomarcadores de ingesta de vino utilizando un enfoque metabolómico por RMN para medir la utilidad de estos biomarcadores en un estudio de intervención con vino (WIS, n = 56), además de evaluar en una población de individuos de vida libre (estudio PREDIMED, n = 91). Se encontraron nueve metabolitos con una mayor excreción urinaria después de la ingesta de vino en el estudio WIS: cinco metabolitos pertenecientes al *food metabolome* (tartrato, etil glucurónido [EtG], 2,3-butanediol, manitol y etanol); uno relacionado con la respuesta endógena a la exposición de vino (3-metil-2-oxovalerato) y tres compuestos no identificados. Se evaluó cada metabolito mediante curvas ROC y el área bajo la curva (AUC) exhibió entre el 67,4% y el 86,3% cuando se evaluaron de forma individual. A continuación, con estos metabolitos, se generó un modelo de combinación de biomarcadores mediante una regresión logística. El modelo generado incluyó la combinación tartrato-EtG, mostrando una AUC de 90,7% en el estudio WIS. Del mismo modo el AUC en el estudio

PREDIMED fue de 92,4%. Así, los resultados mostraron que un modelo con la combinación de biomarcadores tartrato-EtG es más útil para evaluar la exposición a vino que biomarcadores individuales, tanto en el estudio de intervención como en los datos epidemiológicos. A nuestro entender, esta es la primera vez que un modelo de biomarcador combinado usando una plataforma basada en RMN para la investigación de biomarcadores de vino así como su reproducción en una población de vida libre.

Metabolomics
DOI 10.1007/s11306-014-0735-x

ORIGINAL ARTICLE

An NMR metabolomics approach reveals a combined-biomarkers model in a wine interventional trial with validation in free-living individuals of the PREDIMED study

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Received: 2 July 2014 / Accepted: 23 September 2014
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Abstract The development of robust biomarkers of consumption would improve the classification of participants with regard to their dietary exposure. In addition, validation of them in free-living individuals remains an important challenge. The aim of this study is to assess wine intake biomarkers using an NMR metabolomic approach to measure the utility of these biomarkers in a wine interventional study (WIS, $n = 56$) and also to evaluate them in a free-living individuals (PREDIMED study, $n = 91$). Nine metabolites showed a significantly higher presence in urinary excretion in WIS after wine intake: five food metabolome metabolites (tartrate, ethyl glucuronide [EtG], 2,3-butanediol, mannitol, and ethanol); one related to the endogenous response to wine exposure (3-methyl-2-oxovalerate) and three unidentified compounds. Receiver operating characteristic (ROC) curve for each single metabolite were evaluated and exhibited areas under the curves (AUC) between 67.4 and 86.3 % when they were evaluated individually. Then, a logistic regression model was fitted to generate a combined-biomarkers model using these metabolites. The model generated which included tartrate-EtG, showed an AUC of 90.7 % in WIS. Similarly, the AUC in the PREDIMED study was 92.4 %. Results showed that a model combining tartrate-EtG is more useful for evaluating exposure to wine than single biomarkers, both in interventional studies and epidemiological data. To our knowledge, this is the first time that a combined-biomarker model using an NMR platform in wine biomarkers'

Electronic supplementary material The online version of this article (doi:10.1007/s11306-014-0735-x) contains supplementary material, which is available to authorized users.

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Published online: 11 October 2014

research has been generated and reproduced in a free-living population.

Keywords Biomarkers · Nutrimetabolomics · NMR · Wine · Interventional study · Cohort study

Abbreviations

AUC	Area under the curve
CI	Confidence interval
COSY	Correlation spectroscopy
d	Doublet
EtG	Ethyl glucuronide
FFQ	Food frequency questionnaires
FM	Food metabolome
IQR	Interquartile range
J	J-coupling
m	Multiplet
ROC	Receiver operating characteristic
s	Singlet
t	Triplet
U	Unassigned compound
WIS	Wine interventional study

1 Introduction

The food metabolome is very complex and variable and could constitute an extremely useful source of information on the human diet. Accurate characterization of the food metabolome should allow precise monitoring of dietary exposure and identification of foods that influence disease risk (Scalbert et al. 2014). Nowadays, evaluation of food intake is a big challenge, particularly if the relation between diet and health is taken into consideration. In this context, the moderate wine intake included in the Mediterranean food pattern has been related to some health benefits (Estruch 2000). In addition, wine is of special interest in Mediterranean countries because it is a common constituent of the daily pattern of Mediterranean diets. Therefore, in the biomarker research field, studies testing biomarker exposure conducted in countries with a regular wine intake are an interesting focus for research.

Metabolomics offers a conceptual breakthrough for biomarker discovery (Pujos-Guillot et al. 2013). NMR is a robust technique for metabolomic applications enabling the simultaneous detection of a wide range of structurally different metabolites. As it is a non-hypothesis-driven approach, this enables new biomarkers to be discovered in the field of food research (O'Sullivan et al. 2011). Several studies apply metabolomic approaches to identify novel dietary biomarkers (Llorach et al. 2012), while the number of metabolomics studies that use an ROC curve procedure is still very limited (<2 % in the last 10 years) (Xia et al. 2013), and only a few are applied in food biomarker research (Lloyd et al. 2013). To date, robust NMR-based metabolomics has rarely been applied to identify new biomarkers of wine intake. However, two metabolomics studies have evaluated a mix of red wine and red grape juice extracts to assess the wine polyphenols in humans subjected to microbial metabolism by NMR analysis (Jacobs et al. 2008; van Dorsten et al. 2010).

Traditionally, in nutrition a single biomarker has been used to define exposure to complex foods (e.g., red wine) or complex dietary patterns in interventional trials and/or a free-living population. However, in clinical research, the evaluation of a combination of several biomarkers by a logistic regression model is frequently applied. For instance, Hwa and co-workers demonstrated that a combination of more than one biomarker with a logistic regression model can improve the predictive sensitivity of breast cancer and lymph node metastatic status (Hwa et al. 2008). The goal of biomarker development in metabolomics is to create a predictive model from a collection of multiple compounds, which can be used to classify individuals into specific groups with optimal sensitivity and specificity (Xia et al. 2013). All this suggests a change in biomarker discovery research conventionally focused on a single biomarker, shifting towards a combination of biomarkers to assess dietary exposure.

A few limited studies have been carried out to determine biomarkers of wine consumption in human individuals, reporting gallic acid, 4-O-methylgallic acids (Mennen et al. 2006), caffeic acid (Simonetti et al. 2001) and catechins (Donovan et al. 2002) as biomarkers of wine intake in interventional studies, with only resveratrol and resveratrol metabolites assessed in epidemiological data (Zamora-Ros et al. 2009). Moreover, procyanidin metabolites derived from the microbiota are well described after wine polyphenol intake (Gonthier et al. 2003). In this context, human gut microbiota variability and the presence in other procyanidin dietary sources such as green tea (Roowi et al. 2009), cocoa products (Urpi-Sarda et al. 2009b) or almond skin (Bartolomé et al. 2010; Urpi-Sarda et al. 2009a) increase the variability between subjects and decrease the specificity of these metabolites as candidate biomarker of wine intake.

The purpose of the present study is to identify wine intake biomarkers in the context of a Mediterranean wine interventional study using the robust and reproducible ^{1}H-nuclear magnetic resonance spectroscopy analysis, and subsequently measure the utility of these biomarkers by evaluating them in a free-living independent Mediterranean sub-cohort.

2 Materials and methods

2.1 Subjects and study design

2.1.1 Wine interventional study (WIS)

Wine intervention study was a prospective, randomized, crossover and controlled trial (Chiva-Blanch et al. 2012). High-risk subjects aged ≥55 years without documented CHD (CHD: ischemic heart disease—angina/recent or past myocardial infarction/previous or cerebral vascular accident, peripheral vascular disease) were recruited for the study. The subjects included had diabetes mellitus or more than three of the following CHD risk factors: tobacco smoking, hypertension, hypercholesterolemia, plasma LDL cholesterol ≥160 mg/dL, plasma HDL cholesterol <40 mg/dL, obesity (BMI (in kg/m2) ≥30), and/or a family history of premature CHD (first-line male relatives <55 years or females <65 years). Participants had to voluntarily give signed informed consent. Subjects with a previous history of CVD, any severe chronic disease, alcoholism, or other toxic abuse were excluded. The study was a prospective, randomized, crossover, and controlled trial. As a result of random computer-generated selection, participants were asked to take either, 272 mL/day of red wine (about 30 g ethanol/day), 272 mL/day of dealcoholized red wine (polyphenols control), or 100 mL/day of gin (alcohol control), every day for 4 weeks (28 days). Twenty-four-hour urine samples were collected on the last day of each period as well as the basal time. All participants received all three interventions. To fulfil the objectives of the present study, we evaluated the urinary metabolome modifications from 56 participants between baseline and after 28 days of red wine intake, analysed by ^{1}H-NMR spectroscopy.

2.1.2 PREDIMED sub-cohort study

A random subsample of participants in the PREDIMED study was used to test the WIS results. The PREDIMED study is a parallel-group, single-blind, multicentre, randomized, controlled, 5-year feeding trial assessing the effects of the Mediterranean diet (MD) supplemented either with extra virgin olive oil (MD + EVOO) or mixed nuts (MD + Nuts) on the primary prevention of CHD compared to advice on a low-fat diet (LFD). The participants from the present study had between 53 and 79 years of age and fulfilled more than three cardiovascular risk factors (current smoking, hypertension, hypercholesterolemia, body mass index (BMI) ≥25 kg/m^2, or a family history of premature cardiovascular disease). Exclusion criteria were type 2 diabetes mellitus, cardiovascular disease, any severe chronic illness, drug or alcohol addiction, history of allergy, or intolerance to olive oil or nuts. The sub-cohort consisted of a random sample of 205 participants at high cardiovascular risk, recruited from the Barcelona and Valencia PREDIMED centres. Urinary metabolome was analysed at baseline (before to start the dietary intervention). The PREDIMED study design and 137-item validated food frequency questionnaire used have been reported elsewhere (Estruch et al. 2006, 2013; Fernández-Ballart et al. 2010). Data reported included information on drinking habits, such as amount, frequency and type of alcohol intake. The question "how long ago did you consume your last glass of wine?" was also asked.

2.2 Untargeted ^{1}H-NMR metabolomics analysis

2.2.1 Wine interventional study (WIS)

A procedure based on previous published methodology (Vázquez-Fresno et al. 2012) was applied for the metabolomics analysis. The urine samples were thawed, vortexed, and centrifuged at 13,200 rpm for 5 min. The supernatant (600 µL) from each urine sample was mixed with an internal standard solution (120 µL, consisting of 0.1 % TSP (3-(trimethylsilyl)-proprionate-2,2,3,3-d4, chemical shift reference), 2 mM of sodium azide (NaN3, bacteriostatic agent), and 1.5 M KH_2PO_4 in 99 % deuterium water (D_2O)).

2.2.2 PREDIMED sub-cohort study

Briefly, the initial volume of urine from the sub-cohort study participants was 300 µL of urine sample diluted with 200 µL of H_2O/D_2O (8:2) and mixed with the internal standard solution. (120 µL, consisting of 0.1 % TSP (3-(trimethylsilyl)-proprionate-2,2,3,3-d4, chemical shift reference), 2 mM of sodium azide (NaN3, bacteriostatic agent), and 1.5 M KH_2PO_4 in 99 % deuterium water (D_2O)).

For both studies, the optimized pH of the buffer was set at 7.0, with a potassium deuteroxide (KOD) solution, to minimize variations in the chemical shifts of the NMR resonances. Mixture was transferred to a 5-mm NMR tube. The spectral data processed were intelligent bucketed in domains of 0.005 ppm (Sousa et al. 2013) and integrated using ACD/NMR Processor 12.0 software (Advanced Chemistry Development, Inc.). The spectral region between 4.75 and 5.00 ppm was excluded from the data set to avoid spectral interference from residual water. Significant unassigned compounds were further characterized using 2-D NMR experiments in 600 MHz Bruker Avance III: a previous ^{1}H-NMR experiment, followed by a ^{1}H-^{1}H homonuclear $'$H/$'$H correlation spectroscopy (COSY) experiment. The ^{1}H-NMR experiment was processed with 128 scans with a spectral

width of 14 ppm, an acquisition time of 3.2 s and a relaxation delay of 3 s. In the COSY analysis, 512 × 1,024 data points were collected and processed in a 1,024 × 1,024 matrix using 48 scans per increment, a relaxation delay of 1.1 s, an acquisition time of 0.16 s and 16 dummy scans. To exclude data points showing little variance across experimental conditions, data were interquartile range (IQR) filtered, and row-wise normalized by sum to reduce systematic bias during sample collection (Xia and Wishart 2011).

2.3 Metabolite identification

Discriminant metabolites were identified using the Chenomx NMR Suite 7.5 (Chenomx Inc., Edmonton, Canada) library, by comparing NMR spectral data to those available in databases such as the Human Metabolome Database (HMDB; www.hmdb.ca), the Biological Magnetic Resonance Data Bank (BMRB, www.bmrb.wisc.edu) and the Madison Metabolomics Consortium Database (MMCD, mmcd.nmrfam.wisc.edu).

2.4 Classification of the free-living population in the cohort study in terms of wine intake

The population from the cohort study ($n = 205$) was stratified using food frequency questionnaires (FFQs) for non-wine consumers (0 mL/d, $n = 69$), intermittent wine consumers (<180 mL/d, $n = 87$) and wine consumers (≥180 mL/d, $n = 22$), based on a daily wine intake (Teissedre and Landrault 2000). Wine consumers concern individuals that were drinking red, white, rosé or Muscat wine. No sparkling wines were considered. Non-wine consumers that drink other kinds of alcohol-containing beverages were excluded from the study ($n = 27$). Besides, the intermittent wine consumers were not considered for the assessment of biomarkers of wine intake. Alcohol intake was assessed at baseline with a validated 137-item semi-quantitative FFQ. In the validation study for the Spanish version of this questionnaire, the intra-class correlation coefficient between alcohol intake from the FFQ and repeated food records was 0.82 (Fernández-Ballart et al. 2010). The use of FFQ in metabolomics (Menni et al. 2013) and the stratification of the cohort individuals by consumption is reported as being an effective strategy for discovering sensitive biomarkers of consumption (Pujos-Guillot et al. 2013).

Because very few participants consumed wine on a daily basis, a skewed consumption frequency pattern emerged (Lloyd et al. 2013), resulting in a different number of samples in the sub-cohort study tested for each group. In this context, the receiver operating characteristic (ROC) curve is a non-parametric measure of biomarker utility and there is no need for the two distributions to have an equal number of individuals and equal variance (Xia et al. 2013). In the analysis of the last time of wine consumption, the population analysed were participants who consumed their last glass of wine the day before ($n = 53$), between 2 and 3 days before ($n = 14$), and up to 3 days ($n = 15$) among the non-wine consumers group ($n = 69$). Each group was compared individually with the non-wine consumers group.

2.5 Statistical analysis of potential biomarkers

The Mann–Whitney U test was used to explore differences in the NMR data to compare baseline and wine intake periods in the WIS, and in PREDIMED sub-cohort consumers and non-consumers. Significant metabolites from WIS study which were tested in ROC curve analysis were those previously found (Vázquez-Fresno et al. 2012) and those significant after Bonferroni correction. ROC curves for each metabolite that was statistically significant were calculated (each ROC curve was constructed using a dichotomous variable of wine consumption).Then, a logistic regression model was generated by forward stepwise selection procedures as previously described (Bahado-Singh et al. 2012; Murabito et al. 2009). The area under the curve (AUC) was calculated and used to assess the adequacy of the prediction model in the WIS. Then, this model was evaluated in the sub-cohort PREDIMED study with the subsequent ROC analysis to test the accuracy of the model in the free-living population. Spearman's rank correlation coefficient was calculated to estimate correlations between wine intake from FFQ and the combined model. The Mann–Whitney test was also applied in the analysis of "last time of drinking wine" among the groups of consumers and the non-consumers. Mann–Whitney U test analysis and a logistic regression model were conducted using SPSS 20 and statistical significance was set at $p < 0.05$. AUC in ROC curve and p value resulting from comparing AUCs were calculated with the pROC package from www.r-project.org (Robin et al. 2011).

3 Results and discussion

Descriptive baseline characteristics of the participants from both studies are summarized in Online Resource 1. Composition of wine provided for WIS study is detailed in Online Resource 2.

3.1 Detection of potential wine intake biomarkers

Statistically significant metabolites evaluated at baseline and after consumption of wine in the WIS study were food metabolome biomarkers related to wine (tartrate, ethyl glucuronide, 2,3 butanediol, ethanol and mannitol), the

Table 1 Significant metabolites resulting from the analysis comparing the baseline values and the values after red wine intake (WIS study; $n = 56$) and PREDIMED sub-cohort (epidemiological data, $n = 91$) with the Mann–Whitney U statistical test

Biomarker	NMR assignment		WIS	PREDIMED sub-cohort	Metabolite information
	δ	^{1}H-^{1}H COSY[a]	p	p	
EtG	1.24 (t, $J = 7.10$ Hz) 4.48 (d, $J = 7.97$ Hz)	–	3.40×10^{-11}	5.84×10^{-9}	Ethanol metabolite (FM)
Tartrate	4.35 (s)	–	7.43×10^{-11}	6.86×10^{-8}	Wine compound (FM)
Ethanol	1.19 (t, $J = 7.05$ Hz)	–	2.97×10^{-6}	0.008	Compound of alcohol-containing beverages (FM)
2,3-butanediol	1.15 (d, $J = 5.90$ Hz)	–	1.15×10^{-6}	0.004	Wine compound (FM)
3-methyl-2-oxovalerate	1.10 (d, $J = 6.36$ Hz)	–	1.45×10^{-4}	0.002	Branched-chain amino acid metabolites
Mannitol	3.69 (dd, $J = 6.27$ Hz, 11.93 Hz)	–	0.001	0.006	Wine compound (FM)
U1	1.26 (t, $J = 7.21$ Hz)	4.171	1.37×10^{-6}	7.24×10^{-6}	–
U2	0.85 (d, $J = 6.80$ Hz)	1.863	3.42×10^{-6}	0.009	–
U3	0.92 (d, $J = 6.58$ Hz)	1.867	1.42×10^{-5}	0.01	–

$p < 0.05$ in Mann–Whitney U test

EtG ethyl glucuronide, *FM* food metabolome, δ chemical shift, in parenthesis multiplicity and *J*-coupling, *U* unassigned compound, *WIS* wine interventional study, *d* doublet, *t* triplet, *s* singlet, *J* *J*-coupling

[a] 2D analysis from unidentified compounds

endogenous metabolite 3-methyl-2-oxovalerate, and three unidentified compounds (U1, U2, U3). Two metabolites previously described associated with dealcoholized wine (Vázquez-Fresno et al. 2012) and derived from gut microbiota were hippurate and 4-hydroxyphenylacetate. These two metabolites were also tested with wine versus baseline participants showing no significant association after logistic regression analysis ($p > 0.05$). Significant metabolites comparing the baseline values and the values after the intake of red wine in WIS (crossover intervention, $n = 56$), and in the PREDIMED sub-cohort (epidemiological data, $n = 91$), are presented in Table 1.

The subsequent ROC curve analysis for each single metabolite tested in WIS study, revealed the best AUC for the ethyl glucuronide (EtG) (AUC = 86.3 %) and tartrate (AUC = 85.7 %) metabolites, followed by 2,3-butanediol (AUC = 76.7 %), ethanol signal (AUC = 75.6 %) and the unassigned metabolites U1 (AUC = 76.5 %), U2 (AUC = 75.4 %) and U3 (AUC = 73.8 %), 3-methyl-2-oxovalerate (AUC = 70.8 %) and mannitol (AUC = 69.9 %). Sensitivity and specificity in each case were also determined (Online Resource 3).

3.2 Combined biomarkers model in the wine intake study

Subsequently, to achieve greater accuracy with the potential wine biomarkers, the significant metabolites in the WIS study were subjected to a forward logistic regression analysis to create a model in order to optimally allocate each individual as a wine/non-wine intake subject. The resulting model included tartrate and EtG biomarkers of wine intake. The ROC curves for tartrate and EtG within the model and separately are presented in Fig. 1.

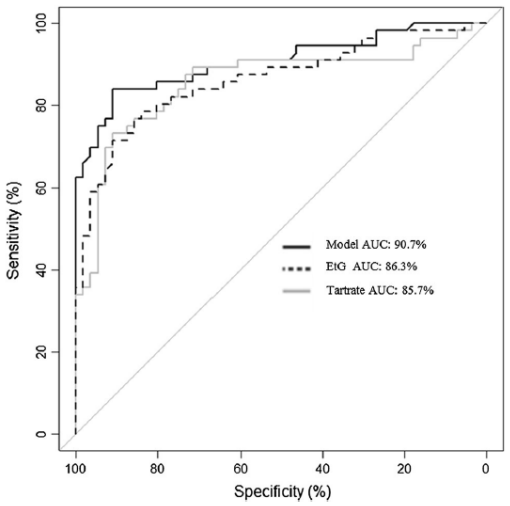

Fig. 1 Comparison of ROC curves for combined-biomarkers model and for individual metabolites (WIS study). Area under the curve (AUC) is calculated for each case

3.3 Combined biomarkers model in the PREDIMED sub-cohort study

To assess the reproducibility our experimental findings, the resulting model for potential biomarkers of wine intake was tested in the sub-cohort of the PREDIMED study. A significant correlation of daily wine intake reported in PREDIMED study and the combined model ($r = 0.6$, $p < 0.001$) was observed. The result of the ROC curve analysis in this case, was a comparable and slightly greater AUC with very similar sensitivity and specificity to those obtained in the WIS. The ROC curves for the logistic regression in the WIS and the prediction of consumers and non-consumers from the free-living population are presented in Fig. 2. The equation generated from the logistic regression and the AUCs from both the model in the WIS and the PREDIMED study with their sensitivity and specificity are shown in Table 2. A comparison between the curves was also made, and the results exhibited no statistical differences ($p = 0.7$) between the both ROC curves. The similar AUC curve for both studies could be explained by the similar consumption of wine among the participants for both studies. The mean ± SD reported consumption of daily wine consumers ($n = 22$) in the PREDIMED study was 343.07 ± 139.41 ml, while the WIS study participants' consumption was fixed at 272 mL/day. Figure 3 shows the distribution by boxplot analysis and the statistical significance of the metabolites that generated the model comparing both studies.

The additional information concerning to the last time that the participants consumed wine before urine collection (1 day, between 2 and 3 days, and >3 days), and the results of combined wine biomarkers (tartrate-EtG) comparing wine consumers and non-wine consumers, are presented in Fig. 4. Statistically significant were the participants who had their last drink of wine one day before (1 day) the urine collection ($p = 4 \times 10^{-11}$) and between the second and third day (2–3 days) ($p = 0.01$), both compared with the non-wine consumers group. No differences were shown between non-wine consumers and consumers whose last glass of wine was reported more than 3 days before. Therefore, using these data the applied methodology could allow the detection of wine consumers up to 3 days after the last glass of wine consumed.

3.4 Biomarkers associated with wine exposure and classification of wine consumers in both WIS and PREDIMED studies

The obtained results showed that several metabolites, both exogenous (food metabolome) and endogenous, were associated with wine consumers. Tartrate, 2,3-butanediol, ethanol, ethyl glucuronide and mannitol are described compounds present in wine (Liu 2002; Son et al. 2008, 2009), while 3-methyl-2-oxovalerateis a product of degradation of branched-chain amino acids. Elsewhere, in a previous work conducted by our group, hippurate and 4-hydroxyphenylacetate were significantly associated with

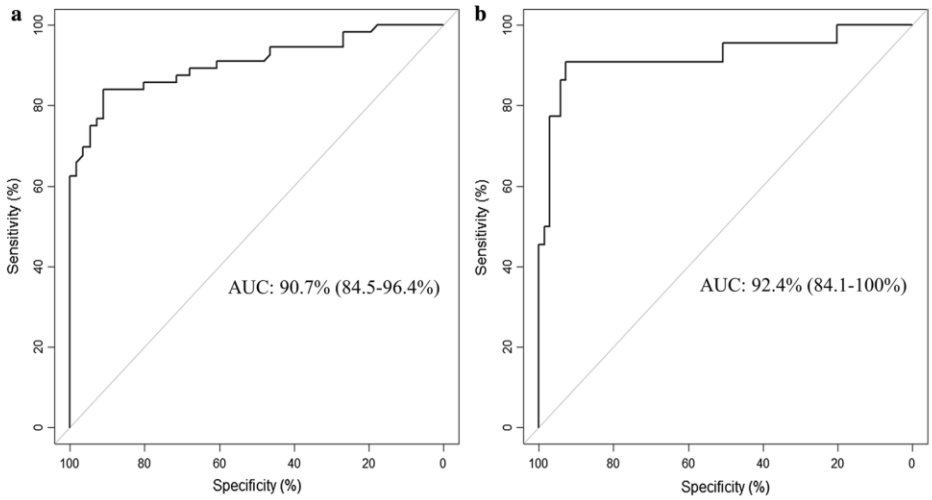

Fig. 2 Receiver operating characteristic (ROC) curve analysis using model (**a**) in the intervention WIS study, and applying the same model to the PREDIMED sub-cohort study (**b**), with the AUC (CI) discriminating between wine consumers and non-wine consumers. Comparison between both curves, $p = 0.7$

Table 2 Metabolites included in the logistic classification model from the Mann–Whitney U test

Regression model	AUC (95 % CI)	Sensitivity (%)	Specificity (%)
Intervention study			
Metabolites (tartrate-EtG)[a]	90.7 (84.5–96.4)	83.9	91.1
Sub-cohort study			
Metabolites (tartrate-EtG)[a]	92.4 (84.1–100)	90.9	92.8

Equation of the model = −5.400 + (4.65 × (δ EtG)) + (2.315 × (δ tartrate))

AUC area under the ROC curve, *CI* confidence interval

[a] Metabolites included in the model

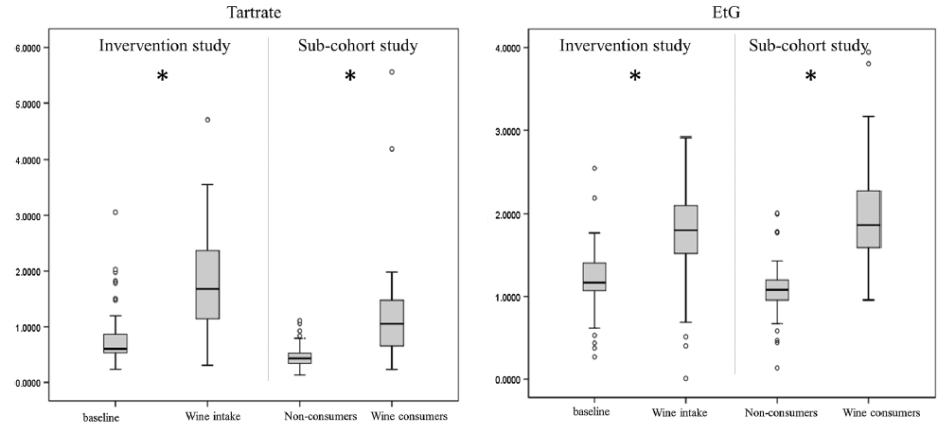

Fig. 3 Boxplot of urinary tartrate and ethyl glucuronide (EtG) at baseline and after wine intake in the WIS intervention study; and in wine and non-wine consumers from the sub-cohort PREDIMED study. *$p < 0.001$

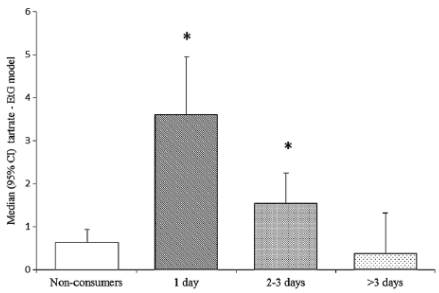

Fig. 4 Combined wine biomarkers model (tartrate-EtG) comparing consumers that consume their last glass of wine 1 day, between 2 and 3 days, and more than 3 days before the urine collection, between the non-wine consumers in the PREDIMED sub-cohort. **Mann–Whitney U test ($p < 0.01$)*

consumers of dealcoholized wine (Vázquez-Fresno et al. 2012). This fact suggests a possible impact of ethanol on the metabolic pathways related to the excretion of both compounds.

With regard to the AUC curves, the results show that wine and non-wine consumers can be classified with good accuracy using a combination of two biomarkers. One of them, tartrate, is the major acid in grapes and is also present in wine (Son et al. 2008). Even though tartrate is also used in the food industry as an additive (Bemrah et al. 2012), the amount present in wine is considerably higher (2-8 g/L) in red wine (Pérez-Magariño and González-San José 2004), white wine (Darias-Martín et al. 2000; López-Tamames et al. 1996; Recamales et al. 2006), rosé (Salinas et al. 2005) and Muscatel wines (Roussis et al. 2005). In addition, some human studies have detected tartrate in urine after wine consumption (Heinzmann et al. 2011; Vázquez-Fresno et al. 2012). The other metabolite present in the model, ethyl glucuronide, is a metabolite of ethanol and has received much recent attention as a sensitive and specific biological marker of acute ethanol consumption after intake of alcoholic beverages (Lande and Marin 2013). In addition, the results demonstrated the good accuracy of the classification of wine consumers, in spite of including non-wine consumers who drink other kinds of alcohol-containing beverages in the non-consumer group.

The combination of tartrate and ethyl glucuronide exhibited good accuracy in interventional data. Furthermore, our analysis of the epidemiological data showed good classification of wine consumers reported from the FFQs and confirmed by NMR urinary biomarker excretion. These results are in line with those published by Zamora-Ros et al.(Zamora-Ros et al. 2009) using an LC–MS/MS targeted approach. The authors showed that using resveratrol metabolites, instead of single resveratrol, could increase the ability to discriminate between wine consumers and non-wine consumers (Zamora-Ros et al. 2009). Compared with NMR-based metabolomics studies, potential biomarkers of citrus consumption were evaluated in an interventional as well as in an epidemiological assay (Heinzmann et al. 2010), showing similar results in terms of accuracy to the model currently being used for wine intake.

3.5 The use of the modelling process

With regard to the modelling process, several studies have used this approach in metabolomics-based clinical biomarker investigation, obtaining a variety in terms of the number of biomarkers included. An NMR-based study on epithelial cancer detection (Odunsi et al. 2005) had a perfect-fitting model with an AUC = 1.0 combining two signals. In this context, Bahado-Singh et al. (2012) reported that with three metabolites cases can be distinguished from controls. Altogether, these findings demonstrate that there are several results on combining biomarkers in a model, although it is important to find the simplest combination of metabolites that can produce a proper effective outcome (Xia et al. 2013). The development of robust food biomarkers has important goals aimed at providing better classification of individuals in terms of dietary exposure (Garcia-Aloy et al. 2014), and in turn this would improve assessment of the relationship between diet and chronic disease (O'Gorman et al. 2013).

3.6 Detection times of biomarkers from the model

The concentration of EtG in urine after a given dose of ethanol may vary considerably between, but also within, individuals as it is influenced by several factors besides the amount of alcohol consumed, such as urine dilution and time of voiding (Høiseth et al. 2007). Detection times for EtG ranging from <24 h to >90 h were demonstrated after alcohol ingestion depending on the ethanol ingested (Beck et al. 2007). Our data reported a mean ± SD alcohol intake in the total wine consumers of 16.92 ± 19.85 g/day with a mean ± SD 78.25 ± 13.32 g/kg of body weight, therefore, a mean alcohol intake of 0.22 g/kg of body weight. Weinmann et al. confirmed by mass spectrometry that after 9 g of ethanol intake (0.16 g/kg), EtG reached a maximum concentration at 8–10 h and is detectable over 30 h (Weinmann et al. 2004), this fact is in line with our results. Similarly other studies reported detection up to 48 h (~0.5 g/kg) (Wojcik and Hawthorne 2007)and up to 130 h (~5 days) in extreme cases of alcohol intoxication (Helander et al. 2009).

Tartrate is reported excreted over 24 h after acute intake of grape juice (Stalmach et al. 2013). Further, Lord and co-workers reported urinary tartrate excretion after restricted tartrate intake and after ingestion of 280 mL of grape juice the day after (Lord et al. 2005). As far as we know, there are no further studies testing tartrate excretion beyond the day after its consumption. In any case, the values of the combined model of tartrate-EtG of consumers ≥24 h and <72 h are in keeping with the kinetics results of studies presented above (Helander et al. 2009; Stalmach et al. 2013; Weinmann et al. 2004; Wurst et al. 2006).

4 Concluding remarks

This study shows the potential for identifying wine intake biomarkers in interventional studies using an NMR-based metabolomics approach with a good accuracy and reproducibility in free-living individuals. Also, these results confirm that a model combining tartrate and EtG is a better biomarker of exposure to wine than a single biomarker. Additionally, these combined wine biomarkers could be still descriminative up to 3 days after the last consumption of wine. To our knowledge, this is the first time that the strategy of combined biomarkers defined on the base of NMR-based untargeted approximation was successfully applied in wine consumption research. Such robust metabolomic approach should be of great interest to future studies planning to use NMR technique to discriminate between individuals in terms of their dietary patterns in the search for new biomarkers and their association with health.

Acknowledgments Supported by the Spanish National Grants from Ministry of Economy and Competitiveness (MINECO) and co-founded by FEDER (Fondo Europeo de Desarrollo Regional): AGL2006-14228-C03-02/ALI, AGL2009-13906-C02-01, AGL2010-10084-E, the CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD (CSD2007-063), CIberOBN, as well as PI13/01172 Project, (Plan N de I+D+i 2013-2016) by ISCII-Subdirección General de Evaluación y Fomento de la Investigación. We also thank the award of 2014SGR1566 from the Generalitat de Catalunya's Agency AGAUR. R.V.-F, O.K, M.U.-S and R. Ll. would like to thank the FPI fellowship, the "Juan de la Cierva" and the "Ramon y Cajal" programmes of the Spanish Government and the Fondo Social Europeo. We thank the participants for their collaboration in the study.

Conflict of interest All the authors declare no competing financial interest.

Compliance with ethical requirements *WIS study*. The study received the ethical approval Institutional Review Board of the Hospital Clinic of Barcelona. All participants had signed an informed

consent. This trial has been registered in the Current Controlled Trials in London, International Standard Randomized Controlled Trial Number (ISRCTN88720134).

PREDIMED study. The trial protocol was conducted according to the Declaration of Helsinki and was approved by the institutional review boards of all the centres involved. All participants had signed an informed consent. This trial has been registered in the Current Controlled Trials in London, International Standard Randomized Controlled Trial Number (ISRCTN35739639).

References

Bahado-Singh, R. O., Akolekar, R., Mandal, R., et al. (2012). Metabolomics and first-trimester prediction of early-onset pre-eclampsia. *The journal of maternal-fetal & neonatal medicine, 25*, 1840–1847.

Bartolomé, B., Monagas, M., Garrido, I., et al. (2010). Almond (Prunus dulcis (Mill.) D.A. Webb) polyphenols: From chemical characterization to targeted analysis of phenolic metabolites in humans. *Archives of Biochemistry and Biophysics, 501*, 124–133.

Beck, O., Stephanson, N., Böttcher, M., et al. (2007). Biomarkers to disclose recent intake of alcohol: potential of 5-hydroxytryptophol glucuronide testing using new direct UPLC-tandem MS and ELISA methods. *Alcohol and Alcoholism, 42*, 321–325.

Bemrah, N., Vin, K., Sirot, V., et al. (2012). Assessment of dietary exposure to annatto (E160b), nitrites (E249-250), sulphites (E220-228) and tartaric acid (E334) in the French population: the second French total diet study. *Food additives and contaminants Part A, 29*, 875–885.

Chiva-Blanch, G., Urpi-Sarda, M., Llorach, R., et al. (2012). Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial. *American Journal of Clinical Nutrition, 95*, 326–334.

Darias-Martín, J. J., Rodríguez, O., Díaz, E., & Lamuela-Raventós, R. M. (2000). Effect of skin contact on the antioxidant phenolics in white wine. *Food Chemistry, 71*, 483–487.

Donovan, J. L., Kasim-Karakas, S., German, J. B., & Waterhouse, A. L. (2002). Urinary excretion of catechin metabolites by human subjects after red wine consumption. *British Journal of Nutrition, 87*, 31–37.

Estruch, R. (2000). Wine and cardiovascular disease. *Food Research International, 33*, 219–226.

Estruch, R., Martínez-González, M. A., Corella, D., et al. (2006). Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Annals of Internal Medicine, 145*, 1–11.

Estruch, R., Ros, E., Salas-Salvadó, J., et al. (2013). Primary prevention of cardiovascular disease with a Mediterranean diet. *New England Journal of Medicine, 368*, 1279–1290.

Fernández-Ballart, J. D., Piñol, J. L., Zazpe, I., et al. (2010). Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *British Journal of Nutrition, 103*, 1808–1816.

Garcia-Aloy, M., Llorach, R., Urpi-Sarda, M., et al. (2014). Nutrimetabolomics fingerprinting to identify biomarkers of bread exposure in a free-living population from the PREDIMED study cohort. *Metabolomics,* 1–11. doi:10.1007/s11306-014-0682-6.

Gonthier, M.-P., Cheynier, V., Donovan, J. L., et al. (2003). Microbial Aromatic Acid Metabolites Formed in the Gut Account for a Major Fraction of the Polyphenols Excreted in Urine of Rats Fed Red Wine Polyphenols. *Journal of Nutrition, 133*, 461–467.

Heinzmann, S. S., Brown, I. J., Chan, Q., et al. (2010). Metabolic profiling strategy for discovery of nutritional biomarkers: proline betaine as a marker of citrus consumption. *American Journal of Clinical Nutrition, 92*, 436–443.

Heinzmann, S. S., Merrifield, C. A., Rezzi, S., et al. (2011). Stability and Robustness of Human Metabolic Phenotypes in Response to Sequential Food Challenges. *Journal of Proteome Research, 11*, 643–655.

Helander, A., Böttcher, M., Fehr, C., Dahmen, N., & Beck, O. (2009). Detection Times for Urinary Ethyl Glucuronide and Ethyl Sulfate in Heavy Drinkers during Alcohol Detoxification. *Alcohol and Alcoholism, 44*, 55–61.

Høiseth, G., Bernard, J. P., Karinen, R., et al. (2007). A pharmacokinetic study of ethyl glucuronide in blood and urine: Applications to forensic toxicology. *Forensic Science International, 172*, 119–124.

Hwa, H.-L., Kuo, W.-H., Chang, L.-Y., et al. (2008). Prediction of breast cancer and lymph node metastatic status with tumour markers using logistic regression models. *J Eval Clin Pract, 14*, 275–280.

Jacobs, D. M., Deltimple, N., van Velzen, E., et al. (2008). (1)H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. *NMR in Biomedicine, 21*, 615–626.

Lande, R. G., & Marin, B. (2013). A Comparison of Two Alcohol Biomarkers in Clinical Practice: Ethyl Glucuronide Versus Ethyl Sulfate. *Journal of Addictive Diseases, 32*, 288–292.

Liu, S. Q. (2002). Malolactic fermentation in wine – beyond deacidification. *Journal of Applied Microbiology, 92*, 589–601.

Llorach, R., Garcia-Aloy, M., Tulipani, S., Vazquez-Fresno, R., & Andres-Lacueva, C. (2012). Nutrimetabolomic Strategies To Develop New Biomarkers of Intake and Health Effects. *Journal of Agriculture and Food Chemistry, 60*, 8797–8808.

Lloyd, A. J., Beckmann, M., Haldar, S., et al. (2013). Data-driven strategy for the discovery of potential urinary biomarkers of habitual dietary exposure. *American Journal of Clinical Nutrition, 97*, 377–389.

López-Tamames, E., Puig-Deu, M. A., Teixeira, E., & Buxaderas, S. (1996). Organic acids, sugars, and glycerol content in white winemaking products determined by HPLC: Relationship to climate and varietal factors. *American Journal of Enology and Viticulture, 47*, 193–198.

Lord, R. S., Burdette, C. K., & Bralley, J. A. (2005). Significance of Urinary Tartaric Acid. *Clinical Chemistry, 51*, 672–673.

Mennen, L. I., Sapinho, D., Ito, H., et al. (2006). Urinary flavonoids and phenolic acids as biomarkers of intake for polyphenol-rich foods. *British Journal of Nutrition, 96*, 191–198.

Menni, C., Zhai, G., Macgregor, A., et al. (2013). Targeted metabolomics profiles are strongly correlated with nutritional patterns in women. *Metabolomics, 9*, 506–514.

Murabito, J. M., Keyes, M. J., Guo, C.-Y., et al. (2009). Cross-sectional relations of multiple inflammatory biomarkers to peripheral arterial disease: The Framingham Offspring Study. *Atherosclerosis, 203*, 509–514.

O'Gorman, A., Gibbons, H., & Brennan, L. (2013). Metabolomics in the identification of biomarkers of dietary intake. *Computational and Structural Biotechnology Journal*, *4*, e201301004. doi:10.5936/csbj.201301004.

Odunsi, K., Wollman, R. M., Ambrosone, C. B., et al. (2005). Detection of epithelial ovarian cancer using 1H-NMR-based metabonomics. *International Journal of Cancer, 113*, 782–788.

O'Sullivan, A., Gibney, M. J., & Brennan, L. (2011). Dietary intake patterns are reflected in metabolomic profiles: potential role in

dietary assessment studies. *American Journal of Clinical Nutrition, 93*, 314–321.

Pérez-Magariño, S., & González-San José, M. L. (2004). Evolution of Flavanols, Anthocyanins, and Their Derivatives during the Aging of Red Wines Elaborated from Grapes Harvested at Different Stages of Ripening. *Journal of Agriculture and Food Chemistry, 52*, 1181–1189.

Pujos-Guillot, E., Hubert, J., Martin, J.-F., et al. (2013). Mass Spectrometry-based Metabolomics for the Discovery of Biomarkers of Fruit and Vegetable Intake: Citrus Fruit as a Case Study. *Journal of Proteome Research, 12*, 1645–1659.

Recamales, Á. F., Sayago, A., González-Miret, M. L., & Hernanz, D. (2006). The effect of time and storage conditions on the phenolic composition and colour of white wine. *Food Research International, 39*, 220–229.

Robin, X., Turck, N., Hainard, A., et al. (2011). pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics, 12*, 77.

Roowi, S., Stalmach, A., Mullen, W., et al. (2009). Green Tea Flavan-3-ols: Colonic Degradation and Urinary Excretion of Catabolites by Humans. *Journal of Agriculture and Food Chemistry, 58*, 1296–1304.

Roussis, I. G., Lambropoulos, I., & Papadopoulou, D. (2005). Inhibition of the decline of volatile esters and terpenols during oxidative storage of Muscat-white and Xinomavro-red wine by caffeic acid and N-acetyl-cysteine. *Food Chemistry, 93*, 485–492.

Salinas, M. R., Garijo, J., Pardo, F., Zalacain, A., & Alonso, G. L. (2005). Influence of prefermentative maceration temperature on the colour and the phenolic and volatile composition of rosé wines. *Journal of the Science of Food and Agriculture, 85*, 1527–1536.

Scalbert, A., Brennan, L., Manach, C., et al. (2014). The food metabolome: a window over dietary exposure. *American Journal of Clinical Nutrition, 99*, 1286–1308.

Simonetti, P., Gardana, C., & Pietta, P. (2001). Caffeic acid as biomarker of red wine intake. *Methods in Enzymology, 335*, 122–130.

Son, H.-S., Hwang, G.-S., Kim, K. M., et al. (2009). Metabolomic Studies on Geographical Grapes and Their Wines Using 1H NMR Analysis Coupled with Multivariate Statistics. *Journal of Agriculture and Food Chemistry, 57*, 1481–1490.

Son, H. S., Kim, K. M., van den Berg, F., et al. (2008). 1H nuclear magnetic resonance-based metabolomic characterization of wines by grape varieties and production areas. *Journal of Agriculture and Food Chemistry, 56*, 8007–8016.

Sousa, S. A. A., Magalhães, A., & Ferreira, M. M. C. (2013). Optimized bucketing for NMR spectra: Three case studies. *Chemometrics and Intelligent Laborary Systems, 122*, 93–102.

Stalmach, A., Edwards, C. A., Wightman, J. D., & Crozier, A. (2013). Colonic catabolism of dietary phenolic and polyphenolic compounds from Concord grape juice. *Food & Function, 4*, 52–62.

Teissedre, P.-L., & Landrault, N. (2000). Wine phenolics: contribution to dietary intake and bioavailability. *Food Research International, 33*, 461–467.

Urpi-Sarda, M., Garrido, I., Monagas, M., et al. (2009a). Profile of Plasma and Urine Metabolites after the Intake of Almond [Prunus dulcis (Mill.) D.A. Webb] Polyphenols in Humans. *Journal of Agriculture and Food Chemistry, 57*, 10134–10142.

Urpi-Sarda, M., Monagas, M., Khan, N., et al. (2009b). Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats. *Analytical and Bioanalytical Chemistry, 394*, 1545–1556.

van Dorsten, F. A., Grün, C. H., van Velzen, E. J., et al. (2010). The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics. *Molecular Nutrition & Food Research, 54*, 897–908.

Vázquez-Fresno, R., Llorach, R., Alcaro, F., et al. (2012). (1) H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors. *Electrophoresis, 33*, 2345–2354.

Weinmann, W., Schaefer, P., Thierauf, A., Schreiber, A., & Wurst, F. M. (2004). Confirmatory analysis of ethylglucuronide in urine by liquid-chromatography/electrospray ionization/tandem mass spectrometry according to forensic guidelines. *Journal of the American Society for Mass Spectrometry, 15*, 188–193.

Wojcik, M. H., & Hawthorne, J. S. (2007). Sensitivity of commercial ethyl glucuronide (ETG) testing in screening for alcohol abstinence. *Alcohol and Alcoholism, 42*, 317–320.

Wurst, F. M., Dresen, S., Allen, J. P., et al. (2006). Ethyl sulphate: a direct ethanol metabolite reflecting recent alcohol consumption. *Addiction, 101*, 204–211.

Xia, J., Broadhurst, D., Wilson, M., & Wishart, D. (2013). Translational biomarker discovery in clinical metabolomics: an introductory tutorial. *Metabolomics, 9*, 280–299.

Xia, J., & Wishart, D. S. (2011). Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. *Nature Protocols, 6*, 743–760.

Zamora-Ros, R., Urpí-Sardà, M., Lamuela-Raventós, R. M., et al. (2009). Resveratrol metabolites in urine as a biomarker of wine intake in free-living subjects: The PREDIMED Study. *Free Radical Biology and Medicine, 46*, 1562–1566.

SUPPLEMENTAL MATERIAL

Online Resource 1. Baseline characteristics of the participants in both studies examined.

	WIS	PREDIMED sub-cohort			
	Acute wine intake (n=56)	Non-wine consumers (n=69)	Intermittent wine consumers (n=87)	Daily wine consumers (n=22)	*P*
Mean ± SD age, years	60.5 ± 8.4	67.8 ± 5.4	67.1 ± 6.1	64.2 ± 4.4	0.03
Gender (%)	-[a]	79.7 (f); 20.3 (m)	66 (f); 35 (m)	22.7(f);77.3(m)	<0.001
Mean ± SD BMI, kg/m^2	29.6 ± 4.0	30.5 ± 3.9	30.2 ± 4.3	29.8 ± 3.4	0.54
% Current smokers	25.0	8.7	8.0	27.3	0.02
% Type 2 diabetes mellitus	23.2	56.5	54.0	54.5	0.95
% Hypertension	48.2	87.0	81.6	86.4	0.63
% Dyslipidemia	30.4	78.3	74.7	63.6	0.39

WIS: wine interventional study; f: female, m: male. P<0.05, comparing the three groups of consumers in the cohort study in a Chi-square test to compare qualitative traits and analysis of variance test to compare means of quantitative variables. [a]*only men were recruited in the study.*

Online Resource 2. Composition of wine used in WIS study. Mean ± SD.

Alcohol (%)	14.2
Tartaric acid (g/L)	5.20
Total phenols (mEqGA/L)	2933.35 ± 377
Gallic acid (mg/L)	68.48 ± 6.40
Protocatechuic acid (mg/L)	5.22 ± 0.62
Tyrosol (mg/L)	43.59 ± 4.73
Catechin (mg/L)	123.51 ± 11.30
Epicatechin (mg/L)	67.86 ± 7.74
trans-Caftaric (mg/L)	18.62 ± 1.45
trans-Caffeic (mg/L)	11.50 ± 0.79
trans-Coutaric (mg/L)	5.21 ± 0.45
2-S-glutationylcaftaric (mg/L)	10.30 ± 1.00
Quercetin-3-glucuronide (mg/L)	11.88 ± 1.38
Quercetin (mg/L)	26.66 ± 0.78
Isorhamnetin (mg/L)	3.34 ± 0.27
Delphinidin-3-glucoside (mg/L)	15.25 ± 0.89
Petunidin-3-glucoside (mg/L)	12.29 ± 1.06
Peonidin-3-glucoside (mg/L)	6.78 ± 0.62
Malvidin-3-glucoside (mg/L)	48.83 ± 4.45
Malvidin-(6-acetyl)-3-glucoside (mg/L)	10.97 ± 0.96
Malvidin-(6-coumaroyl)-3-glucoside (mg/L)	4.15 ± 0.27
trans-Resveratrol (mg/L)	2.92 ± 0.36
cis-Resveratrol (mg/L)	2.79 ± 0.15
trans-Piceid (mg/L)	9.41 ± 1.12
cis-Piceid (mg/L)	7.71 ± 0.34

Online Resource 3. ROC curve analysis for each significant metabolite.

Biomarker	**AUC (95% CI)**	**Sensitivity (%)**	**Specificity (%)**
EtG	86.3 (79.4-93.2)	78.57	83.93
Tartrate	85.7 (78.2-93.1)	76.79	85.71
Ethanol	75.6 (66.4-84.8)	75.0	76.79
2,3 butanediol	76.7 (67.6-85.7)	71.42	73.21
U1	76.5 (67.6-80.5)	69.64	73.21
U2	75.4 (66.3-84.6)	73.21	71.43
U3	73.8 (64.4-83.2)	69.64	73.21
3-methyl-2-oxovalerate	70.8 (61.1-80.6)	69.64	64.29
mannitol	67.4 (57.3-77.5)	78.57	60.71

EtG: ethyl glucuronide. AUC: area under the ROC curve. CI: confidence interval. U: unassigned compound

III. Estudio del fenotipo metabólico en una población tras el consumo de un alimento

Los resultados de este estudio se han enviado a una revista indexada en el *Journal Citation Reports* para su publicación y actualmente se encuentra en proceso de revisión.

> Rosa Vázquez-Fresno, Rafael Llorach, Alexandre Perera, Rupasri Mandal, Miguel Feliz, Francisco J Tinahones, David S Wishart, Cristina Andrés-Lacueva. Clinical phenotype clustering in cardiovascular risk patients for the identification of responsive metabotypes after red wine polyphenol intake. [*En proceso de revisión*].

RESUMEN

Los fenotipos metabólicos (o metabotipos) de los individuos son el resultado de las interacciones entre los genes, el medio ambiente, el estilo de vida, la dieta y la microbiota intestinal. El objetivo de este estudio es evaluar el fenotipo clínico y metabólico en la identificación de la respuesta diferencial a una intervención dietética para la mejora de la condición cardiometabólica. Se realizó el fenotipado clínico de 57 voluntarios varones con altos factores de riesgo cardiovascular mediante análisis de *k-means* en función de 69 parámetros bioquímicos y antropométricos en plasma y orina. Se realizó un análisis de validación de clúster basado en el análisis de coherencia interna de *Dunn,* y el análisis de *FOM* para la homogeneidad externa. El análisis de *k*-means produjo cuatro grupos de individuos con perfiles clínicos diferenciados. Se exploraron y validaron las diferencias basales en la orina entre los perfiles metabolómicos entre fenotipos clínicos mediante modelos OSC-PLS-DA. El análisis multivariante (OSC-PLS-DA) de datos espectrales de ^{1}H-NMR reveló que el modelo comparando el "clúster de obesos y diabéticos" (OD-c) frente al "clúster más saludable" (H-c) mostró la mejor previsibilidad y robustez en términos de explicar las diferencias por pares entre los grupos en los perfiles metabólicos basales. Al considerar estos dos clústers, después de seguir una intervención con la ingesta de polifenoles del vino (WPI, 733 equivalentes de ácido gálico [GAE/día] durante 28 días), se observaron dos grupos diferentes de metabolitos. Las diferencias entre los valores

basales y después de la intervención de los datos metabolómicos se analizaron mediante ANOVA. Aquel grupo de metabolitos *asociados a un metabotipo específico* (OD-c), como la glucosa como significativamente característica del grupo (corrección por FDR, p <0,01), y siguiendo una tendencia, el lactato, la betaína y la dimetilamina; así como el grupo de metabolitos *asociados a la intervención* con polifenoles del vino (OD-c_WPI y H-c_WPI), tartrato como característico del grupo (corrección por FDR, p <0,001), y con una tendencia similar en su perfil, manitol, metanol treonina, fucosa y 3-hidroxifenilacetato. Por otro lado, el 4-hidroxifenilacetato (metabolito derivado del metabolismo microbiano del intestino después de la ingesta de polifenoles de vino) aumentó significativamente (corrección por FDR, p <0,05) para H-c_WPI en comparación con OD-c_WPI y los puntos basales (H-c_BAS y OD-c_BAS), el cual muestra un *efecto de la intervención sobre el metabotipo*. Con ello, este estudio proporciona estrategias eficientes para caracterizar la heterogeneidad en la respuesta del individuo a la intervención dietética y la identificación de los beneficios para la salud en grupos específicos de la población.

Clinical phenotype clustering in cardiovascular risk patients for the identification of responsive metabotypes after red wine polyphenol

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KEYWORD: metabolic phenotype, metabolomics, metabotype, cardiovascular disease, wine polyphenols, NMR, 4-hydroxyphenylacetate, gut microbiota

ABSTRACT

Metabolic phenotypes of individuals are the result of genes, environment, lifestyle, diet and gut microbiota interactions. The aim of this study is to evaluate the robustness of clinical and metabolic phenotyping in identifying differential responsiveness to dietary strategies in the improvement of cardiometabolic status. Clinical phenotyping of 57 male volunteers with high cardiovascular risk factors was performed using *K*-means cluster analysis based on 69 anthropometric and plasma biochemical parameters. Cluster validation analysis based on Dunn analysis for internal coherence and FOM analysis for external homogeneity was applied. The *K*-means analysis produced four clusters with particularly significant clinical profiles. Basal differences on the urine metabolomic profiles among clinical phenotypes were explored and validated by OSC-PLS-DA models. Multivariate analysis (OSC-PLS-DA) of ^{1}H-NMR spectra revealed that the model comparing the "obese and diabetic cluster" (OD-c) against the "healthier cluster" (H-c) showed the best predictability and robustness in terms of explaining the pairwise differences between clusters. When considering just these two clusters, two different groups of differentiating metabolites were observed following an intervention with wine polyphenol intake (WPI, 733 Equivalents of Gallic Acid

[GAE/day] per 28 days). Glucose was significantly associated with the OD-c metabotype (FDR correction, $p<0.01$,) while lactate, betaine and dimethylamine showed a less significant trend. Tartarate was significantly associated with wine polyphenol intervention (OD-c_WPI and H-c_WPI) (FDR correction $p<0.001$), while mannitol, threonine methanol, fucose and 3-hydroxyphenylacetate exhibited a less significant trend. 4-HPA (a metabolite derived from gut microbial metabolism after wine polyphenol intake) significantly increased (FDR correction, $p<0.05$) for H-c_WPI compared to OD-c_WPI and compared to both basal states (H-c_BAS and OD-c_BAS), thereby exhibiting a clear metabotypic intervention effect. This study illustrates how metabolomics can be used to characterize the heterogeneity in individual responsiveness to dietary intervention and the identification of health benefits in specific population groups.

1 INTRODUCTION

Metabolic phenotypes (metabotypes) are the result of interactions among several different factors (diet, lifestyle, gut microbiota, genetics, etc.), and describe characteristic metabolic profiles reflecting the biochemistry, the physiological status, and the environmental exposure in a population (Rezzi, Ramadan et al. 2007; Holmes, Wilson et al. 2008). Applications of metabolic phenotyping in nutrition research could be very useful in terms of assigning individuals to a particular metabolic phenotype. This could help improve our understanding of the linkage between both diet and disease with the different individual metabotypes (McNiven, German et al. 2011; Kinross, Li et al. 2014).

Metabolomic technologies permit the characterization of large numbers of small molecules in human biofluids. ^{1}H-NMR-based metabolomics is a very robust technique for performing metabolomic studies, enabling the simultaneous detection and quantification of a wide range of different metabolites. Because of this, NMR-based metabolomics has been applied in a variety of disciplines. In the field of nutrition, NMR-based metabolomics has been used to identify the most significant changes in a metabolic profile arising from dietary intervention studies, dietary biomarker studies and diet related disease studies (Brennan 2014). It can also be used to identify new small

molecule candidates for disease biomarkers, including conditions such as cardiovascular disease (Rupérez, Ramos-Mozo et al. 2012; Yang, Wang et al. 2013)

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, with CVD-associated deaths rising very quickly in low-to-middle income countries. Modifiable risk factors for CVD—which include hypertension, smoking, abdominal obesity, abnormal lipids, diabetes mellitus, stress, low consumption of fruits and vegetables, and lack of regular physical activity—are the major contributors to CVD morbidity and mortality (Dahlöf 2010). Additionally, reduced plasma HDL levels and elevated plasma TAG concentrations are known to be significant risk factors for ischemic heart disease (IHD) (Lewington, Whitlock et al. 2007; Frikke-Schmidt, Nordestgaard et al. 2008). C-reactive protein (CRP) is another CVD risk marker (Ridker, Danielson et al. 2009). In addition, high plasma levels of homocysteine are considered to be a risk factor for vascular disease, heart failure and strokes (Kaptoge, Di Angelantonio et al. 2010). Another important risk factor is type 2 diabetes mellitus (T2D). The prevalence of T2D is increasing rapidly around the world. Clinical predictors such as body mass index (BMI), fat distribution measured by Waist-hip ratio (WHR), CRP and fasting blood glucose levels can be helpful in measuring

diabetes risk (Pradhan, Manson et al. 2001; Wilson, Meigs et al. 2007; Wannamethee, Papacosta et al. 2010; Wang, Larson et al. 2011).

The low incidence of cardiovascular and coronary heart disease (CHD) in Mediterranean countries has been partly assigned to their distinct dietary habits (Dauchet, Amouyel et al. 2009). Several studies have shown an inverse association between the Mediterranean diet and the incidence of CVD (Estruch, Ros et al. 2013). As one of the main constituents of Mediterranean diet, wine and its components, especially polyphenols, may provide additional health benefits (Chiva-Blanch, Urpi-Sarda et al. 2013). In particular, the regular consumption of wine polyphenols used in this study appears to mitigate CVD risk factors, leading to reduced blood pressure (Chiva-Blanch, Urpi-Sarda et al. 2012) and inflammatory parameters (Chiva-Blanch, Urpi-Sarda et al. 2012). The health benefits of polyphenols provided by wine intake are of particular interest. In the present study, a long-term feeding trial was performed to determine changes in urinary metabolites between different metabotypes. Therefore, the aim of the present work was to classify a specific population into phenotypic groups according to their biochemical characteristics, and then to use ^{1}H-NMR-based urinary metabolomics to observe the different metabolic responses after red wine polyphenols intake.

2 MATERIAL AND METHODS

2. 1 Subjects and Study Design

The study was a prospective, randomized, crossover, and controlled trial (Chiva-Blanch, Urpi-Sarda et al. 2012). High-risk subjects aged ≥55 years without documented CHD (CHD: ischemic heart disease—angina/recent or past myocardial infarction/previous or cerebral vascular accident, peripheral vascular disease) were recruited for the study. The subjects included had diabetes mellitus or more than three of the following CHD risk factors: tobacco smoking, hypertension, hypercholesterolemia, plasma LDL cholesterol ≥160 mg/dL, plasma HDL cholesterol <40 mg/dL, obesity (BMI (in kg/m2) ≥30), and/or a family history of premature CHD (first-line male relatives <55 years or females <65 years). Participants had to voluntarily give signed informed consent. Subjects with a previous history of CVD, any severe chronic disease, alcoholism, or other toxic substance abuse were excluded.

To fulfil the objectives of the present study, we used ^{1}H-NMR spectroscopy to evaluate the urinary metabolomes from 57 participants between baseline and after 28 days of red wine polyphenols intake (WPI, polyphenol content:733 Equivalents of Gallic Acid [EGA/day])

in form of dealcoholized wine from a Merlot grape variety. Results of polyphenol composition analysis of the beverages are shown in Supplemental material, Table S1. The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent before participation in the study. The trial has been registered in the Current Controlled Trials in London, International Standard Randomized Controlled Trial Number (ISRCTN88720134).

2.2 Anthropometric Measurements and Biochemical Analyses

Anthropometric measurements and biochemical analyses were performed using standardized methods (Estruch, Martínez-González et al. 2006). BMI and WHR were measured in all the participants to evaluate their obesity status. Systolic and diastolic blood pressures as well as heart rate were also measured. Clinical parameters were tested in the blood and urine of participants at the beginning of the study (baseline) in order to characterize the biochemical status of each participant. Blood glucose levels, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), LDL/HDL ratio, triacylglycerides (TAG), 24h-diuresis, plasmatic creatine, uric acid, aminotransferases, bilirubin, ferritin, C-reactive protein, albumin, enzymes (alkaline phosphatase, lactate

dehydrogenase), ions (Na^+, K^+), as well as globulins, apolipoprotein levels, hemoglobin and red blood cell count; with several coagulation parameters (prothrombin, thrombin, fibrinogen) were measured. In total, 69 anthropometric and biochemical baseline parameters were evaluated. These are shown in Table 1.

2.3 Biochemical Biomarkers and Clinical Phenotype by a *k*-means Algorithm

The final data set contained 69 variables from 57 samples (of the initial set of 61 individuals, 4 were excluded because of incomplete data regarding clinical and anthropometric parameters). Prior to *k*-means analysis all variables were typified. All cluster metrics were computed with 1000 different random initializations of the *k*-means algorithm in order to avoid local minima. A maximum number of 100 iterations were allowed in the *k*-means calculations. All computations were carried out using the R package for Statistical Computing v. 2.14.1. This included the statistics package for the *k*-means algorithm and the clValid package for the cluster validation analysis. Dunn analysis for internal coherence and FOM analysis for external homogeneity were applied to the dataset employing Euclidean distances and a *k*-means clustering algorithm. Our results suggest that a cluster solution

consisting of 4 centers or groups (4 clusters) showed the optimal properties of internal coherence and grouping stability (the detailed methodology and the validation procedure are in the supplemental material).

2. 4 Metabolomic NMR Spectroscopy

2.4.1 ^{1}H-NMR sample preparation, data acquisition and processing

The protocols used for this work were based on previously published methodology (Vázquez-Fresno, Llorach et al. 2012). The urine samples were thawed, vortexed, and centrifuged at 13,200 rpm for 5 min. The supernatant (600 μL) from each urine sample was mixed with an internal standard solution (120 μL, consisting of 0.1 % TSP (3-(trimethylsilyl)-proprionate-2,2,3,3-d4, chemical shift reference), 2 mM of sodium azide (NaN_3, bacteriostatic agent), and 1.5 M KH_2PO_4 in 99 % deuterium water (D_2O)). The optimized pH of the buffer was set at 7.0, with a potassium deuteroxide (KOD) solution, to minimize variations in the chemical shifts of the NMR resonances. The mixture was transferred to a 5-mm NMR tube. The processed spectral data were bucketed in domains of 0.005 ppm and integrated using ACD/NMR Processor 12.0 software (Advanced Chemistry Development, Inc.). The

spectral region between 4.75 and 5.00 ppm was excluded from the data set to avoid spectral interference from residual water.

2.5 Statistical Analysis

2.5.1 Biochemical biomarkers and phenotyping cluster differences

Clusters were performed using *k*-means cluster analysis as described previously. A Kolmogorov-Smirnov test ($p<0.05$) was used to test the normality of the all variables using SPSS, version 18.0 for Windows (SPSS®, Chicago, IL, USA). ANOVA analysis was performed to evaluate differences in the mean biochemical measurements across clusters where statistical differences were analyzed ($p<0.05$). Comparisons between clusters were assessed using a Tukey post-hoc multiple comparison test. In the case of non-parametric variables, a Kruskall Wallis test was used to test significant differences. Additionally, a Mann-Whitney test was used to detect significances between clusters. All these tests were performed by SPSS, version 18.0 for Windows (SPSS®, Chicago, IL, USA).

2.5.2 Metabolomic cluster analysis- OSC-PLS-DA multivariate analysis

Data generated from the NMR spectral integration were submitted to MetaboAnalyst (Xia, Mandal et al. 2012). Data were normalized using the sum of the spectral intensities, then log transformed and Pareto scaled. Data were then analyzed using the SIMCA-P+ 13 software (Umetrics, Umea, Sweden) by multivariate discriminant analysis OSC-PLS-DA. A pairwise comparison analysis between the four clusters was carried out. The quality of the models was evaluated by the goodness-of-fit parameter (R^2X), the proportion of the variance of the response variable that is explained by the model (R^2Y) and the predictive ability parameter (Q), which was calculated using seven-fold internal cross-validation (Vázquez-Fresno, Llorach et al. 2014). Validation of the OSC-PLS-DA models was carried out by a permutation test (n=200). Additional information about the methodology is provided in the supplementary data. After untargeted analysis with baseline samples which characterized two most discriminant clusters, then, the quantification of the samples was performed for these two clusters.

2.5. 3 Metabolomic phenotype analysis by ANOVA

Quantified data were submitted to MetaboAnalyst in order to find possible differences between clusters after WPI and normalized (24-h urine volume normalization, cube root transformed and Pareto scaled) (Xia, Mandal et al. 2012) before further analysis. Metabolites were analyzed by a one-way ANOVA test followed by Fisher's LSD test for multiple comparisons. The false discovery rate (FDR) test, a statistical approach to the problem of multiple comparisons, was used in this study to counter the effect of multiple testing and verify the most discriminating metabolites (Benjamini and Hochberg 1995). Box-plots were used to show the statistical differences between treatments with *P* values <0.05 being considered significant. **Figure 1** displays a summary of the steps followed in this study.

2.6 Metabolite Identification and Quantification

The methyl singlet produced by a known quantity of TSP (0.97mM) was used as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. The ^{1}H-NMR spectra were analyzed using the Chenomx NMR Suite Professional Software package (version 7.8; Chenomx Inc, Edmonton, ALB, Canada), which permitted both

identification and quantification by manually fitting the NMR spectra to an internal metabolite database.

3 RESULTS AND DISCUSSION

3. 1 Characterization of Clinical Phenotypes.

61 participants were initially recruited into this study; of these, 57 participants were included in the final cluster analysis (4 were removed from the study because an incomplete biochemical profile). Of the 69 baseline biochemical parameters, *k*-means cluster analysis classified 4 distinct phenotypic groups: cluster 1 (n= 12), cluster 2 (n= 13), cluster 3 (n= 14) and cluster 4 (n= 18). Age, smoking habits, mean dietary intake, mean concentrations of biochemical parameters and statistical tests for each cluster are presented in **Table 1**. Cluster 1 was defined by a significantly lower systolic blood pressure (mmHg), α2-globulin (%) and neutrophil levels (%); higher total cholesterol (mg/dL), LDL cholesterol (mg/dL), apolipoprotein B (mg/dL), and apolipoprotein B/apolipoprotein A ratio (APOB/APOA), compared with all other clusters. Cluster 2 showed lower LDL/HDL ratio compared with all other clusters and significantly higher blood glucose levels compared to cluster 4. Cluster 3 was characterized by significantly higher BMI values, α2-globulin (%), β-globulin (%),albumin/globulin ratio, and

homocysteine (µmol/L) levels and a lower albumin percentage (%) compared with the other clusters. In addition, CRP values were the highest in cluster 3 and statistically significant compared with clusters 1 and 4. Furthermore, glucose levels were significantly higher in cluster 3 (>126mg/dL) compared with cluster 4 (<110mg/dL). More than >126mg/dL is diagnostic of T2D following the American Diabetes Association (ADA) criteria. Cluster 3 had the highest but not statistically significant values of the WHR index, a measure of fat distribution and also a BMI>30 indicating an obese participants cluster (Apovian and Gokce 2012). There is a strong positive association between obesity (measured by BMI) and risk of T2D in men (Wannamethee, Papacosta et al. 2010). In epidemiological studies, high plasma levels of homocysteine (hyperhomocysteinemia) are considered to be a risk factor for vascular disease (Welch and Loscalzo 1998), heart failure and strokes (Collaboration 2002). Among persons with T2D, the association between homocysteine levels and cardiovascular disease may be stronger than that in non-diabetic individuals (Ndrepepa, Kastrati et al. 2008). Moreover, cluster 3 showed the lowest HDL-cholesterol levels and the highest TAG levels compared to the other clusters. These features are considered risk factors for IHD (Lewington, Whitlock et al. 2007; Frikke-Schmidt, Nordestgaard et al.

2008). Finally, cluster 4 showed significantly lower concentrations of TAG (mg/dL), leucocyte count ($x10^9$/L), neutrophils ($x10^9$/L), lymphocytes ($x10^9$/L) and erythrocyte sedimentation rate (mm/h) than cluster 3; also K (mEq/L) presented lower levels compared to other clusters. The elevated circulating white blood cell count (neutrophils, lymphocytes and monocytes) has been proposed as one of a few biomarkers of potential utility for cardiovascular disease risk prediction (Horne, Anderson et al. 2005). Moreover, the erythrocyte sedimentation rate (<10 mm/h) may be indicative of inflammation and a useful additional diagnostic criterion for coronary heart disease (Yayan 2012). On the other hand, low serum potassium levels (<4 mEq/L) in a propensity-matched study was associated with higher mortality and chronic heart failure (Ahmed, Zannad et al. 2007). Overall, cluster 4 had lower levels of cardiovascular disease biomarkers than all other clusters (Table 1).

3.2 Clinical Phenotypes and NMR-based Metabolomic Profiles

After separation of the participants into 4 biochemically distinct clusters, an OSC-PLS-DA analysis was performed to discriminate the clusters by their NMR-derived urinary profiles. The results obtained by OSC-PLS-DA showed that the most strongly discriminated clusters

were cluster 3 *versus* cluster 4 (see Supplemental Material); for this reason, all subsequent analyses were focused on further characterizing cluster 3 which was named the "obese and diabetic cluster" (OD-c), versus cluster 4 or named the "healthier cluster" (H-c).

3. 3 Metabolomic Phenotype Analysis to Responses to Wine Polyphenol Intake

Table 2 presents the results from the multiple comparison ANOVA analysis comparing cluster OD-c and H-c before and after wine polyphenols intake (WPI). Several metabolites exhibited to be associated to wine polyphenols intervention including tartrate, 4-hydroxyphenylacetate (4-HPA), 3-hydroxyphenylacetate (3-HPA), mannitol, methanol threonine and fucose. Further, 4 metabolites presented an association to metabolic phenotype (OD-c) which includes glucose, lactate betaine and dimethylamine, associated to obesity and TD2 (Xie, Waters et al. 2012; Menni, Fauman et al. 2013).

After false discovery rate (FDR) correction, tartrate, glucose and 4-hydroxyphenylacetate (4-HPA) exhibited significant results. Different patterns of response were observed for these 3 metabolites: Tartrate was higher for both clusters after WPI (OD-c_WPI and H-c_WPI) (*wine polyphenols intervention metabolite*). Glucose was higher in the

baseline group and after intervention in cluster OD-c (OD-c_BAS, OD-c_WPI) compared with cluster H-c (H-c_BAS and H-c_WPI) (*metabolic phenotype related metabolite*) characteristic for cluster OD-c. Finally, 4-HPA showed higher urinary excretion after WPI among subjects H-c_WPI than those in OD-c_WPI and at baseline (OD-c_BAS, H-c_BAS), exhibiting a distinct post-intervention metabolic response in individuals for different clusters (*metabotypic intervention effect)*. Box-plots show the statistical differences observed for these metabolites by multiple comparison ANOVA analysis (**Figure 2**).

Tartrate is the major organic acid in grapes and so it is also present in wine (Son, Kim et al. 2008; Son, Hwang et al. 2009). Recently, it has been proposed to be a biomarker of wine consumption for both interventional and epidemiological studies (Vázquez-Fresno, Llorach et al. 2014). The quantity of excreted urinary tartarate for OD-c_WPI was 1.06±0.19 mmols (84.57±14.58 µM/mM creatinine), as well as H-c_WPI 1.29±0.29 µmols (107.89±16.69 µM/mM creatinine) in 24h-urine samples (**Table 2**). Similar to our results, a recent study reported a tartrate concentration of 91.8µg/mg creatinine (73.69 µM/mM creatinine) measured after 10h of acute wine intake (200 ml) (Regueiro, Vallverdú-Queralt et al. 2013). Additionally, the presence of this metabolite at statistically significant levels in both groups after WPI

demonstrated a global compliance by all individuals in this intervention study.

The presence of glucose in urine has long been used as an indicator of diabetes mellitus (Urakami, Kubota et al. 2005). T2D is characterized by the presence of glucose in urine, obesity, as well as high serum levels of homocysteine and CRP, which are all characteristics of the OD-c subjects (Table 1). Moreover, there is a strong positive association between obesity (measured by BMI) and T2D risk (Wannamethee, Papacosta et al. 2010). The amount of glucose excreted for volunteers corresponding to the OD-c were 14.04±7.56 mmols (2157.79±1108.56 µM/mM creatinine) and 13.78±7.85 mmols (1613.70±1042.12 µM/mM creatinine) in 24h-urine samples for the OD-c_BAS and OD-c_WPI groups, respectively. These values were significantly higher than concentrations found in normal urine (12.5 - 58.4 µM/mM creatinine) (Bouatra, Aziat et al. 2013). When reported values of glucose in urine are ≥100 mg/dl (5.5 mM) it is considered to be a positive test for diabetes (Urakami, Kubota et al. 2005).

Lastly, 4-hydroxyphenylacetate (4-HPA) is a metabolite involved in tyrosine and phenylalanine metabolism. 4-HPA is also a compound that is known to be increased in urine after consumption of wine (Vázquez-

Fresno, Llorach et al. 2012), chocolate (Martin, Rezzi et al. 2009), or cranberries (Prior, Rogers et al. 2010). This is because it is also a metabolic byproduct of polyphenol degradation by gut microbiota (Moco, Martin et al. 2012), particularly *F. prausnitzii, Bifidobacterium, Clostridium difficile, Subdoligranulum, Lactobacillus sp.* are thought to be responsible for the metabolism of 4-HPA (Nicholson, Holmes et al. 2012). The amount of 4-HPA excreted for the differential response cluster (H-c_WPI) was 0.28±0.03 mmols (25.35±1.48 µM/mM creatinine), significantly higher than excretion values described in the literature under normal conditions (1.4-14.6 µM/mM creatinine) (Bouatra, Aziat et al. 2013). It has been suggested that obese and diabetic people experience changes in gut microbial metabolites as a result of cardiovascular related pathologies (Shen, Obin et al. 2013). Several studies have found a decrease in *Bifidobacterium, F. prausnitzii,* and certain species of *Clostridium and Lactobacillus* in obese (Tagliabue and Elli 2013) and diabetic subjects (Everard and Cani 2013). These findings are in agreement with our results, as lower levels of 4-HPA were found in cluster OD-c than in cluster H-c after wine polyphehols intake.

4 CONCLUSIONS

The present study has shown that phenotypic analysis using an unsupervised clustering technique (*k-means* analysis) can identify clusters according with their biochemical profiles. The two most discriminating clusters were named according to their clinical parameters and identified as the "obese and diabetic cluster" (OD-c) and the "healthier cluster" (H-c). Moreover, metabolomic phenotyping using NMR detected a distinct metabolic response between individuals grouped in these phenotypic clusters. In particular, comparisons between OD-c and H-c exhibited different levels of excretion of 4-HPA after wine polyphenols intake. Likewise, a metabolite linked with a specific metabotype (glucose) and another metabolite linked with dietary intervention (tartrate) were also observed. According to our results, the subjects in OD-c appear to have an altered gut metabolism compared to individuals in H-c. Lastly, this approach showed efficient strategies for characterizing the heterogeneity of inter-individual responses to a dietary intervention and for identifying associations with health, and health benefits in specific population groups.

ACKNOWLEDGEMENTS

Supported by the Spanish National Grants from Ministry of Economy and Competitiveness (MINECO) and cofounded by FEDER (Fondo Europeo de Desarrollo Regional): AGL2009-13906-C02-01 as well as PI13/01172 Project, (Plan N de I+D+i 2013-2016) by ISCII-Subdirección General de Evaluación y Fomento de la Investigación and the JPI HDHL FOODBALL (PCIN-2014-133) and TEC2013-44666-R. We also thank the award of 2014SGR1566 from the Generalitat de Catalunya's Agency AGAUR. R.V.-F, and R. Ll. would like to thank the FPI fellowship and the "Ramon y Cajal" programmes of the Spanish Government and the Fondo Social Europeo. CIBER-OBN and -BBN are initiative of the Spanish ISCIII. We thank the participants for their collaboration in the study. The authors have declared no conflict of interest

Abbreviations:

WPI: wine polyphenols intake; BAS: baseline period; H-c: Healthier cluster; OD-c: obese and diabetic cluster; CVD: cardiovascular disease; CHD: coronary heart disease; T2D: type 2 Diabetes Mellitus; LDL: low density lipoprotein; HDL: high density lipoprotein; BMI: body mass index; TAG: triacylglycerides; WHR: waist-to-hip index; OSC-PLS-DA: Orthogonal signal correction partial least-squares discriminant analysis; 4-HPA: 4-hydroxyphenylacetate; FDR: false discovery rate

5 REFERENCES

Ahmed, A., F. Zannad, et al. (2007). "A propensity-matched study of the association of low serum potassium levels and mortality in chronic heart failure." Eur Heart J **28**(11): 1334-1343.

Apovian, C. M. and N. Gokce (2012). "Obesity and cardiovascular disease." Circulation **125**(9): 1178-1182.

Benjamini, Y. and Y. Hochberg (1995). "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing." Journal of the Royal Statistical Society. Series B (Methodological) **57**(1): 289-300.

Bouatra, S., F. Aziat, et al. (2013). "The Human Urine Metabolome." PLoS ONE **8**(9): e73076.

Brennan, L. (2014). "NMR-based metabolomics: From sample preparation to applications in nutrition research." Progress in Nuclear Magnetic Resonance Spectroscopy **83**(0): 42-49.

Chiva-Blanch, G., M. Urpi-Sarda, et al. (2012). "Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial." The American Journal of Clinical Nutrition **95**(2): 326-334.

Chiva-Blanch, G., M. Urpi-Sarda, et al. (2012). "Dealcoholized Red Wine Decreases Systolic and Diastolic Blood Pressure and Increases Plasma Nitric Oxide: Short Communication." Circulation Research **111**(8): 1065-1068.

Chiva-Blanch, G., M. Urpi-Sarda, et al. (2013). "Effects of red wine polyphenols and alcohol on glucose metabolism and the lipid profile: A randomized clinical trial." Clinical Nutrition **32**(2): 200-206.

Collaboration, H. S. (2002). "Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis." JAMA **288**(16): 2015-2022.

Dahlöf, B. (2010). "Cardiovascular Disease Risk Factors: Epidemiology and Risk Assessment." The American Journal of Cardiology **105**(1, Supplement): 3A-9A.

Dauchet, L., P. Amouyel, et al. (2009). "Fruits, vegetables and coronary heart disease." Nat Rev Cardiol **6**(9): 599-608.

Estruch, R., M. A. Martínez-González, et al. (2006). "Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial." Ann Intern Med **145**(1): 1-11.

Estruch, R., E. Ros, et al. (2013). "Primary Prevention of Cardiovascular Disease with a Mediterranean Diet." New England Journal of Medicine **368**(14): 1279-1290.

Everard, A. and P. D. Cani (2013). "Diabetes, obesity and gut microbiota." Best practice & research. Clinical gastroenterology **27**(1): 73-83.

Frikke-Schmidt, R., B. G. Nordestgaard, et al. (2008). "Association of loss-of-function mutations in the ABCA1 gene with high-density lipoprotein

cholesterol levels and risk of ischemic heart disease." JAMA **299**(21): 2524-2532.

Frikke-Schmidt, R., B. G. Nordestgaard, et al. (2008). "Association of loss-of-function mutations in the ABCA1 gene with high-density lipoprotein cholesterol levels and risk of ischemic heart disease." JAMA **299**(21): 2524-2532.

Holmes, E., I. D. Wilson, et al. (2008). "Metabolic phenotyping in health and disease." Cell **134**(5): 714-717.

Horne, B. D., J. L. Anderson, et al. (2005). "Which white blood cell subtypes predict increased cardiovascular risk?" J Am Coll Cardiol **45**(10): 1638-1643.

Kaptoge, S., E. Di Angelantonio, et al. (2010). "C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis." Lancet **375**(9709): 132-140.

Kinross, J., J. V. Li, et al. (2014). "Nutritional modulation of the metabonome: applications of metabolic phenotyping in translational nutritional research." Current opinion in gastroenterology **30**(2): 196-207.

Lewington, S., G. Whitlock, et al. (2007). "Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths." Lancet **370**(9602): 1829-1839.

Lewington, S., G. Whitlock, et al. (2007). "Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths." Lancet **370**(9602): 1829-1839.

Martin, F.-P. J., S. Rezzi, et al. (2009). "Metabolic Effects of Dark Chocolate Consumption on Energy, Gut Microbiota, and Stress-Related Metabolism in Free-Living Subjects." Journal of Proteome Research **8**(12): 5568-5579.

McNiven, E. M. S., J. B. German, et al. (2011). "Analytical metabolomics: nutritional opportunities for personalized health." The Journal of Nutritional Biochemistry **22**(11): 995-1002.

Menni, C., E. Fauman, et al. (2013). "Biomarkers for Type 2 Diabetes and Impaired Fasting Glucose Using a Nontargeted Metabolomics Approach." Diabetes **62**(12): 4270-4276.

Moco, S., F.-P. J. Martin, et al. (2012). "Metabolomics View on Gut Microbiome Modulation by Polyphenol-rich Foods." Journal of Proteome Research **11**(10): 4781-4790.

Ndrepepa, G., A. Kastrati, et al. (2008). "Circulating homocysteine levels in patients with type 2 diabetes mellitus." Nutr Metab Cardiovasc Dis **18**(1): 66-73.

Nicholson, J. K., E. Holmes, et al. (2012). "Host-Gut Microbiota Metabolic Interactions." Science **336**(6086): 1262-1267.

Pradhan, A. D., J. E. Manson, et al. (2001). "C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus." JAMA **286**(3): 327-334.

Prior, R. L., T. R. Rogers, et al. (2010). "Urinary Excretion of Phenolic Acids in Rats Fed Cranberry†." Journal of Agricultural and Food Chemistry **58**(7): 3940-3949.

Regueiro, J., A. Vallverdú-Queralt, et al. (2013). "Development of a LC–ESI-MS/MS Approach for the Rapid Quantification of Main Wine Organic Acids in Human Urine." Journal of Agricultural and Food Chemistry **61**(27): 6763-6768.

Rezzi, S., Z. Ramadan, et al. (2007). "Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals." J Proteome Res **6**(11): 4469-4477.

Ridker, P. M., E. Danielson, et al. (2009). "Reduction in C-reactive protein and LDL cholesterol and cardiovascular event rates after initiation of rosuvastatin: a prospective study of the JUPITER trial." Lancet **373**(9670): 1175-1182.

Rupérez, F., P. Ramos-Mozo, et al. (2012). "Metabolomic study of plasma of patients with abdominal aortic aneurysm." Analytical and Bioanalytical Chemistry **403**(6): 1651-1660.

Shen, J., M. S. Obin, et al. (2013). "The gut microbiota, obesity and insulin resistance." Molecular Aspects of Medicine **34**(1): 39-58.

Son, H. S., G. S. Hwang, et al. (2009). "Metabolomic studies on geographical grapes and their wines using 1H NMR analysis coupled with multivariate statistics." J Agric Food Chem **57**(4): 1481-1490.

Son, H. S., K. M. Kim, et al. (2008). "1H nuclear magnetic resonance-based metabolomic characterization of wines by grape varieties and production areas." J Agric Food Chem **56**(17): 8007-8016.

Tagliabue, A. and M. Elli (2013). "The role of gut microbiota in human obesity: Recent findings and future perspectives." Nutrition, Metabolism and Cardiovascular Diseases **23**(3): 160-168.

Urakami, T., S. Kubota, et al. (2005). "Annual Incidence and Clinical Characteristics of Type 2 Diabetes in Children as Detected by Urine Glucose Screening in the Tokyo Metropolitan Area." Diabetes Care **28**(8): 1876-1881.

Vázquez-Fresno, R., R. Llorach, et al. (2012). "1H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors." ELECTROPHORESIS **33**(15): 2345-2354.

Vázquez-Fresno, R., R. Llorach, et al. (2014). "An NMR metabolomics approach reveals a combined-biomarkers model in a wine interventional trial with validation in free-living individuals of the PREDIMED study." Metabolomics: 1-10.

Vázquez-Fresno, R., R. Llorach, et al. (2014). "Metabolomic Pattern Analysis after Mediterranean Diet Intervention in a Nondiabetic Population: A

1- and 3-Year Follow-up in the PREDIMED Study." Journal of Proteome Research.

Wang, T. J., M. G. Larson, et al. (2011). "Metabolite profiles and the risk of developing diabetes." Nat Med **17**(4): 448-453.

Wannamethee, S. G., O. Papacosta, et al. (2010). "Assessing prediction of diabetes in older adults using different adiposity measures: a 7 year prospective study in 6,923 older men and women." Diabetologia **53**(5): 890-898.

Welch, G. N. and J. Loscalzo (1998). "Homocysteine and atherothrombosis." N Engl J Med **338**(15): 1042-1050.

Wilson, P. W., J. B. Meigs, et al. (2007). "Prediction of incident diabetes mellitus in middle-aged adults: the Framingham Offspring Study." Arch Intern Med **167**(10): 1068-1074.

Xia, J., R. Mandal, et al. (2012). "MetaboAnalyst 2.0—a comprehensive server for metabolomic data analysis." Nucleic Acids Research **40**(W1): W127-W133.

Xie, B., M. J. Waters, et al. (2012). "Investigating potential mechanisms of obesity by metabolomics." Journal of biomedicine & biotechnology **2012**: 805683.

Yang, Y., L. Wang, et al. (2013). "Study of metabonomic profiles of human esophageal carcinoma by use of high-resolution magic-angle spinning 1H NMR spectroscopy and multivariate data analysis." Analytical and Bioanalytical Chemistry **405**(10): 3381-3389.

Yayan, J. (2012). "Erythrocyte sedimentation rate as a marker for coronary heart disease." Vasc Health Risk Manag **8**: 219-223.

TABLES

Table 1. Biochemical and anthropometrical parameters of subjects and mean baseline concentration of individuals clusters. *P*-values of ANOVA test (*) for parametric variables and Kruskall Wallis test (♦) for non-parametric variables, (p<0.05) for both tests. Superscript numbers (^{1,2,3,4}) indicate differences between number cluster shown, in Tukey post-hoc test (for parametrical variables) and Mann-Whitney test.(for non-parametrical variables).

	Cluster 1 (n=12)	**Cluster 2 (n=13)**	**Cluster 3 (n=14)**	**Cluster 4 (n=18)**	***p*-value**
Characteristics					
Age (y)	59.83 ±8.62	62.08 ± 10.84	61.21 ± 5.98	59.78 ± 8.13	0.87^{+}
Current smokers (%)	1.75 ± 0.45	1.85 ± 0.38	1.86 ± 0.36	1.72 ± 0.46	0.14$^{♦}$
Dietary Data					
Energy (MJ)	8074.70 ± 211.65	8046.42± 1738.63	8347.50± 1909.31	7387.47 ± 1838.73	0.54^{+}
TE protein (%)	21.37 ± 1.97	20.55 ± 3.17	19.86 ± 4.04	21.12 ± 3.94	0.70^{+}
TE carbohydrates (%)	43.19 ± 7.02	40.57 ± 8.58	43.05 ± 8.27	41.90 ±7.03	0.83^{+}
TE fat (%)	33.68 ± 6.60	37.31 ± 7.36	36.44 ± 6.26	36.22 ± 4.54	0.56^{+}

Anthropometrical and biochemical parameters					
BMI (kg/m^2)	28.17 ± 2.62	27.33 ± 2.78	33.56 ± 4.01^{1,2,4}	29.02 ± 3.86	<0.001^{+}
Waist-hip ratio	0.94 ± 0.05	0.98 ± 0.03	0.99 ± 0.05^{1}	0.96 ± 0.04	0.01^{+}
Heart Rate (beats/min)	72.17 ± 11.85	69.69 ± 7.09	68.71 ± 10.71	68.78 ± 8.94	0.78^{+}
Systolic blood pressure (mm Hg)	124.58 ± 15.08^{2,3,4}	153.54 ± 17.58	143.14 ± 13.79	141.22 ± 12.06	<0.001^{+}
Diastolic blood pressure (mm Hg)	81.58 ± 9.1	79.23 ± 7.36	77.00 ± 9.14	84.50 ± 8.59	0.098^{+}
CRP (mg/dL)	0.15 ± 0.09	0.27 ± 0.31	0.37 ± 0.29^{1,4}	0.14 ± 0.18^{2,3}	0.028$^{\blacklozenge}$
Glucose (mg/dL)	101.25 ± 20.87	119.92 ± 28.62	132.07 ± 53.84^{1,4}	95.22 ± 18.10^{2,3}	0.01$^{\blacklozenge}$
Diuresi 24h (mL)	1587.50 ± 621.35	1946.15 ±753.45^{4}	1470.00 ± 441.46	1339.56± 425.43	0.03^{+}
Plasmatic creatinine (mg/dL)	1.01 ± 0.17	0.96 ± 0.19	1.02 ± 0.11	0.96 ± 0.11	0.52^{+}
Uric acid (mg/dL)	6.58 ± 1.47	6.10 ± 0.85	6.89 ± 1.38	6.11 ± 0.78	0.19^{+}
Na (mEq/L)	141.75 ± 2.14	141.15 ± 1.77	141.36 ± 2.06	140.72 ± 1.49	0.50^{+}
K (mEq/L)	4.28 ± 0.23	4.19 ± 0.36	4.24 ± 0.33	3.90 ± 0.24^{1,2,3}	0.002^{+}

P (mEq/L)	3.66 ± 0.61[2]	3.02 ± 0.49	3.40 ± 0.58	3.09 ± 0.67	0.03[+]
Mg (mEq/L)	2.07 ± 0.19	2.21 ± 0.31	2.02 ± 0.15	2.12 ± 0.16	0.13[+]
Fe (mEq/L)	85.00 ± 23.65	99.38 ± 26.96	75.43 ± 21.18	94.89 ± 30.19	0.084[+]
Total cholesterol (mg/dL)	243.83±36.28[2,3,4]	189.69 ± 14.68	184.79 ± 20.79	203.28 ± 40.97	<0.001[+]
LDL cholesterol (mg/dL)	167.33 ±32.82[2,3,4]	116.92 ± 14.14	114.71 ± 17.87	137.67 ± 33.57	<0.001[+]
HDL cholesterol (mg/dL)	48.92 ± 13.14	52.46 ± 9.98	37.86 ± 5.07[1,2]	46.61 ± 10.09	0.003[+]
LDL cholesterol:HDL cholesterol ratio	3.61 ± 1.06	2.31 ± 0.54[1,3,4]	3.06 ± 0.50	2.98 ± 0.53	<0.001[+]
Triglycerides (mg/dL)	138.17 ± 59.29	101.69 ± 33.27	160.71 ± 73.14[2,4]	95.61 ± 35.28	0.03[+]
Apolipoprotein APOA1 (mg/dL)	146.58 ± 23.36	157.92 ± 14.83[3]	138.43 ± 15.16	147.33 ± 17.28	0.05[+]
Apolipoprotein APOB (mg/dL)	136.42 ± 24.98[2,3,4]	98.85 ± 13.37	103.36 ± 13.89	106.50 ± 19.96	<0.001[+]
APOB/A ratio	0.95 ± 0.23[2,3,4]	0.63 ± 0.095	0.76 ± 0.15	0.72 ± 0.086	<0.001[+]
ASAT (UI/L)	30.08 ± 13.56	23.23 ± 4.92	28.14 ± 10.6	24.39 ± 6.05	0.203[♦]
ALAT (UI/L)	44.42 ± 41.39	26.15 ± 8.97	31.21 ± 16.33	28.83 ± 15.03	0.230[♦]

GGT (UI/L)	42.00 ± 33.68	26.08 ± 7.95	36.71 ± 25.78	27.22 ± 17.36	0.450♦
Bilirubin (mg/dL)	0.77 ± 0.31	0.85 ± 0.26[3]	0.56 ± 0.12[2]	0.73 ± 0.25	0.024♦
Alkaline phosphatase (UI/L)	135.00 ± 20.69	162.54 ± 53.67	127.29 ± 31.30	135.28±34.03	0.082+
Lactate DH (UI/L)	327.83 ± 64.77	305.31 ± 16.25	340.50 ± 63.66	357.39±46.26	0.104+
Total proteins (g/L)	74.08 ± 2.64	73.46 ± 3.69	72.14 ± 2.69	69.94±2.75[1,2]	0.001+
Albumin (g/L)	47.33 ± 1.92	47.31 ± 1.88[3,4]	44.00 ± 2.77	43.39 ± 1.82	<0.001+
Albumin (%)	65.87 ± 2.11	64.79 ± 2.49	60.32 ± 3.82[1,2,4]	63.36 ± 2.53	<0.001+
α1 globulin (%)	2.76 ± 0.22[3,4]	3.10 ± 0.46	3.53 ± 0.48	3.27 ± 0.62	0.002+
α2 globulin (%)	5.93 ± 0.74[2,3,4]	7.39 ± 1.14	9.10 ± 1.62[1,2,4]	7.25 ± 1.49	<0.001+
B globulin (%)	11.19 ± 0.76	11.21 ± 1.28	12.80 ± 1.83[1,2,4]	11.02 ± 0.87	0.001+
Γ globulin (%)	14.25 ± 1.99	13.52 ± 2.04	14.25 ± 2.68	15.10 ± 1.77	0.25+
Albumin:globulin ratio	1.94 ± 0.18	1.85 ± 0.21	1.54 ± 0.24[1,2,4]	1.74 ± 0.19	<0.001+
Ferritine(ng/mL)	167.92 ± 102.02	187.85 ± 166.93	179.07 ± 144.77	208.56±152.61	0.89+

Folic acid (serum) (ng/mL)	9.50 ± 3.43	10.98 ± 2.79	8.51 ± 3.52	10.27 ± 4.65	0.36[+]
Intra-erythrocytary folic acid (ng/mL)	333.27 ± 79.13	401.54 ± 69.95	417.57 ± 107.39	374.38 ± 80.27	0.08[+]
Vitamin B-12 (pg/mL)	525.58 ± 368.70	428.85 ± 145.05	377.07 ± 139.37	380.44 ±110.63	0.29[♦]
Homocysteine (µmol /L)	11.07 ± 2.60	11.17 ± 1.24[1,2,4]	14.31 ± 4.28	11.26 ± 1.79	0.005[+]
Hemoglobin concentration (g/L)	152.75 ± 9.92	151.00 ± 11.77	143.57 ± 9.15	147.33 ± 8.09	0.08[+]
Hematocrit (L/L)	0.45 ± 0.03	0.45 ± 0.03	0.43 ± 0.03	0.44 ± 0.025	0.16[+]
Erythrocyte mean corpuscular volume (fL)	89.80 ± 2.59	91.05 ± 3.73	90.35 ± 5.30	88.78 ± 3.35	0.42[+]
Mean corpuscular hemoglobin (pg)	30.20 ± 0.93	30.82 ± 0.98	30.31 ± 1.89	29.88 ± 1.16	0.28[+]
Mean corpuscular hemoglobin concentration (g/L)	336.25 ± 5.15	338.45 ± 4.74	335.57 ± 7.44	336.56 ± 9.99	0.78[+]
Erytrocyte sedimentation rate (mm/h)	7.08 ± 4.44	8.08 ± 3.86	11.07 ± 5.26[1,4]	5.44 ± 2.31[2,3]	0.003[♦]

Reed distribution width (%)	13.06 ± 0.58[4]	13.17 ± 0.59	13.59 ± 0.66	13.68 ± 0.53	0.01[+]
Hemoglobin distribution width (g/L)	25.74 ± 1.46	24.49 ± 1.96	25.51 ± 2.65	26.03 ± 2.18	0.24[+]
Platelet count (x109/L)	282.17 ± 56.36[4]	233.54 ± 45.98	264.64 ± 63.99	220.17 ± 33.78	0.006[+]
Mean platelet volume (fL)	8.27 ± 0.47	9.11 ± 0.74	8.85 ± 0.72	8.89 ± 0.99	0.05[+]
Leucocyte count (x10^9/L)	6.84 ± 1.73	7.09 ± 0.88	7.96 ± 1.23[4]	6.15 ± 1.30	0.004[+]
Neutrophils (%)	50.45 ± 5.82[2,3,4]	63.84 ± 8.78	58.26 ± 4.43	58.21 ± 6.32	<0.001[+]
Lymphocytes (%)	35.83 ± 4.42[2,4]	24.90 ± 7.07	29.90 ± 5.06	29.83 ± 5.87	<0.001[+]
Monocytes (%)	6.58 ± 1.07	5.85 ± 1.09	6.44 ± 0.88	7.47 ± 0.63[2,3]	<0.001[+]
Eosinophils (%)	4.12 ± 2.04[2,4]	2.65 ± 1.23	2.77 ± 1.19	2.15 ± 0.72	0.002[+]
Basophils (%)	0.71 ± 0.25	0.79 ± 0.39[4]	0.57 ± 0.2	0.49 ± 0.19	0.01[+]
Unclassified cells (%)	2.16 ± 0.95	1.99 ± 0.93	2.05 ± 0.50	1.86 ± 0.56	0.726[+]
Neutrophils (x10^9/L)	3.49 ± 1.11	4.55 ± 0.89[1,4]	4.63 ± 0.79[1,4]	3.56 ± 0.84	0.001[+]
Lymphocytes (x10^9/L)	2.43 ± 0.61[2,4]	1.75 ± 0.46[1,3]	2.39 ± 0.56[2,4]	1.85 ± 0.57	0.002[+]

Monocytes (x10^9/L)	0.45 ± 0.12	0.42 ± 0.10	0.52 ± 0.13	0.45 ± 0.10	0.24$^{♦}$
Eosinophil (x10^9/L)	0.27 ± 0.15	0.19 ± 0.086	0.21 ± 0.09	0.13 ± 0.05^{1,2,3}	0.001$^{♦}$
Basophils (x10^9/L)	0.05 ± 0.03	0.05 ± 0.04	0.03 ± 0.04	0.08 ± 0.01^{1,2}	0.002$^{♦}$
Unclassified cells (x10^9/L)	0.16 ± 0.06	0.15 ± 0.06	0.17 ± 0.05^{4}	0.11 ± 0.05^{1,3}	0.029$^{♦}$
Prothrombin time (%)	97.58 ± 3.68^{4}	95.54 ± 5.72	96.00 ± 4.93	92.50 ± 5.83	0.126$^{♦}$
Prothrombin time (seg)	13.07 ± 0.45	13.23 ± 0.78	13.29 ± 0.47	13.56 ± 0.67	0.186^{+}
Thromboplastin partial time (seg)	28.48 ± 0.89	26.65 ± 7.97	26.38 ± 7.55	29.35 ± 2.11	0.386^{+}
Fibrinogen (g/L)	3.18 ± 0.51	4.08 ± 0.82^{1,4}	3.73 ± 0.62	3.18 ± 0.37	<0.001^{+}

All values are mean ± SD, *p*-values are based on simple ANOVA test ($^{+}$) for parametric variables and Kruskall Wallis test ($^{♦}$) for non-parametric variables, (p<0.05 for both tests); Superscript numbers adjacent to values from each cluster denote a significant difference between indicated cluster number based on Tukey post-hoc test (for parametrical variables) and Mann-Whitney test.(for non-parametrical variables). TE protein %: protein as percentage of total energy intake; TE carbohydrate%: carbohydrate as percentage of total energy intake; TE fat %: fat as percentage of total energy intake. PCR: c-reactive protein; BMI: body mass index; ASAT: aspartate aminotranspherase; ALAT: alanine aminotransferase; GGT:gamma-glutamyl transpeptidase; Na: sodium; K: potassium; P: phosphorous; Mg: Magnessium; Fe: iron. DH: dehydrogenase.

Table 2. Urinary excretion amounts [mmols, 24h-urine] of metabolites after ANOVA analysis, in parenthesis µM/mM creatinine. OD-c_BAS: obese and diabetic cluster in basal period; H-c_BAS: healthier cluster in basal period; OD-c_WPI: obese and diabetic cluster after wine polyphenols intake; H-c_WPI: healthier cluster after wine polyphenol intake. WPI: wine polyphenols intake; MTP: metabotype.4-HPA: 4-hydroxyphenylacetate; 3-HPA: 3-hydroxyphenylacetate. DMA: dimethylamine.

	MEAN ± SEM; mmols, 24h (µM/mM creatinine)						
Metabolite	**OD-c_BAS**	**H-c_BAS**	**OD-c_WPI**	**H-c_WPI**	***p*-value**	***FDR p-value***	**Metabolite information**
Tartrate	0.140 ± 0.052 (14.16 ± 5.78)	0.451 ± 0.019 (39.83 ± 14.74)	1.065 ± 0.19 (84.57 ± 14.58)	1.292 ± 0.285 (107.89 ± 16.69)	7.09E-07	6.02E-05	WPI
Glucose	14.044 ± 7.562 (2157.79 ± 1108.56)	0.210 ± 0.019 (19.95 ± 1.61)	13.786± 7.850 (1613.70 ± 1042.12)	0.188± 0.038 (16.53 ± 2.08)	0.0001	0.005	MTP
4-HPA	0.170 ± 0.025 (17.23 ± 2.04)	0.163 ± 0.013 (15.52 ± 1.13)	0.184 ± 0.022 (16.23 ± 1.95)	0.280 ± 0.029 (25.35 ± 1.48)	0.0008	0.02	WPI-MTP
3-HPA	0.056 ± 0.01 (40.71± 8.34)	0.062 ± 0.005 (48.39± 3.99)	0.0856 ± 0.016 (56.04± 10.97)	0.101 ± 0.012 (75.7± 10.43)	0.01	0.18	WPI
Mannitol	0.556 ± 0.112 (462.62+142.13)	0.782 ± 0.144 (646.87± 132.08)	1.223 ± 0.157 (804.66± 117.30)	1.312 ± 0.255 (957.04± 210.15)	0.005	0.99	WPI
Threonine	0.094 ± 0.014 (9.07 ± 1.17)	0.107 ± 0.013 (10.33 ± 1.51)	0.141 ± 0.013 (12.64 ± 2.19)	0.173 ± 0.032 (15.00 ± 1.99)	0.02	0.18	WPI
Methanol	0.473 ± 0.077 (46.83 ± 6.80)	0.436 ± 0.050 (39.99 ± 3.72)	0.682 ± 0.133 (51.02 ± 5.07)	0.676 ± 0.076 (59.91 ± 3.82)	0.03	0.30	WPI
Fucose	0.32 ± 0.04 (30.58 ± 2.36)	0.327 ± 0.023 (30.55 ± 1.81)	0.503 ± 0.069 (39.01 ± 4.56)	0.495 ± 0.081 (42.84 ± 3.87)	0.04	0.31	WPI
Lactate	0.516 ± 0.19 (65.56 ± 34.17)	0.212 ± 0.075 (19.77 ± 1.37)	0.432 ± 0.087 (37.83 ± 11.02)	0.294 ± 0.030 (26.09 ± 1.80)	0.04	0.33	MTP
Betaine	0.549 ± 0.217 (56.01 ± 21.61)	0.207 ± 0.030 (19.36 ± 2.64)	0.676 ± 0.245 (53.77 ± 18.74)	0.214 ± 0.037 (18.83 ± 2.28)	0.02	0.18	MTP
DMA	0.469 ± 0.021 (51.68 ± 7.82)	0.503 ± 0.048 (45.89 ± 3.20)	0.905 ± 0.230 (65.36 ± 8.11)	0.568 ± 0.078 (51.21 ± 4.62)	0.02	0.18	MTP

FIGURE LEGEND

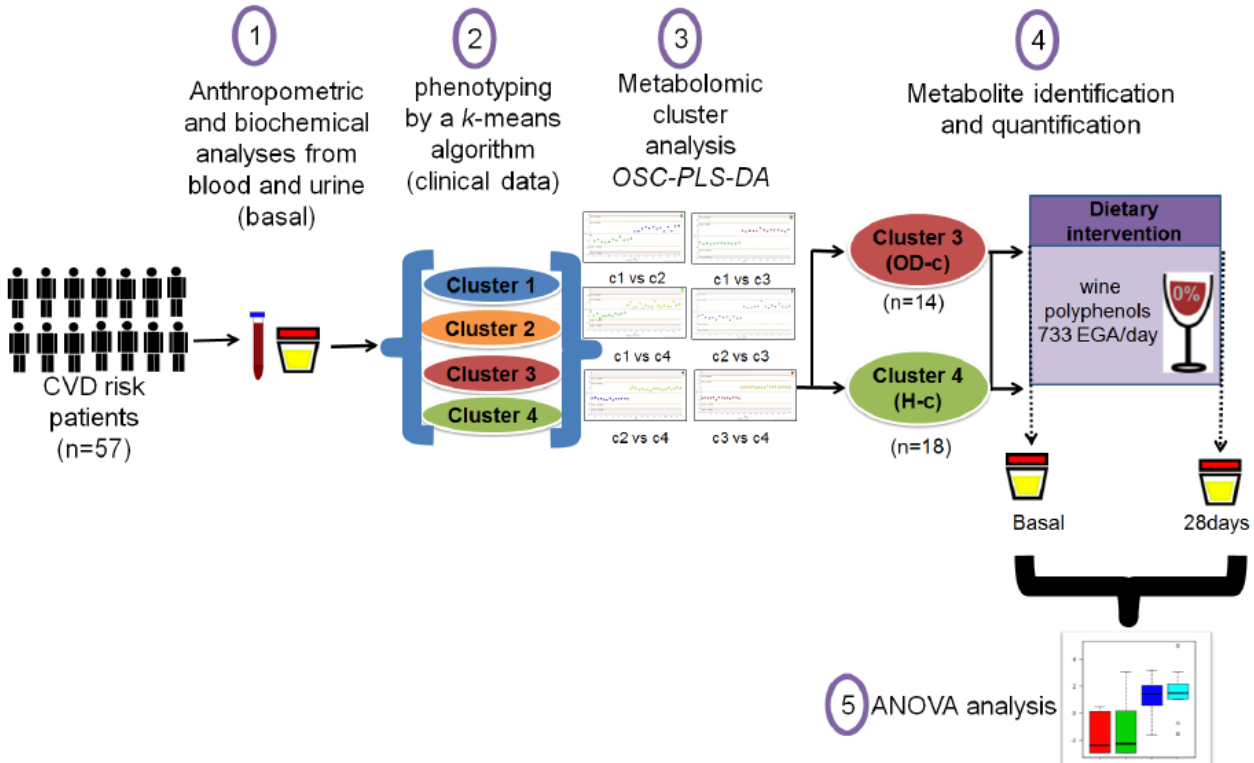


Figure 1. Methodological strategy steps followed in the present study. CVD: cardiovascular disease; c: cluster; OD-c: obese and diabetic cluster; H-c: healthier cluster. OSC-PLS-DA: partial least-squares discriminant analysis with orthogonal signal correction. EGA: equivalents of gallic acid

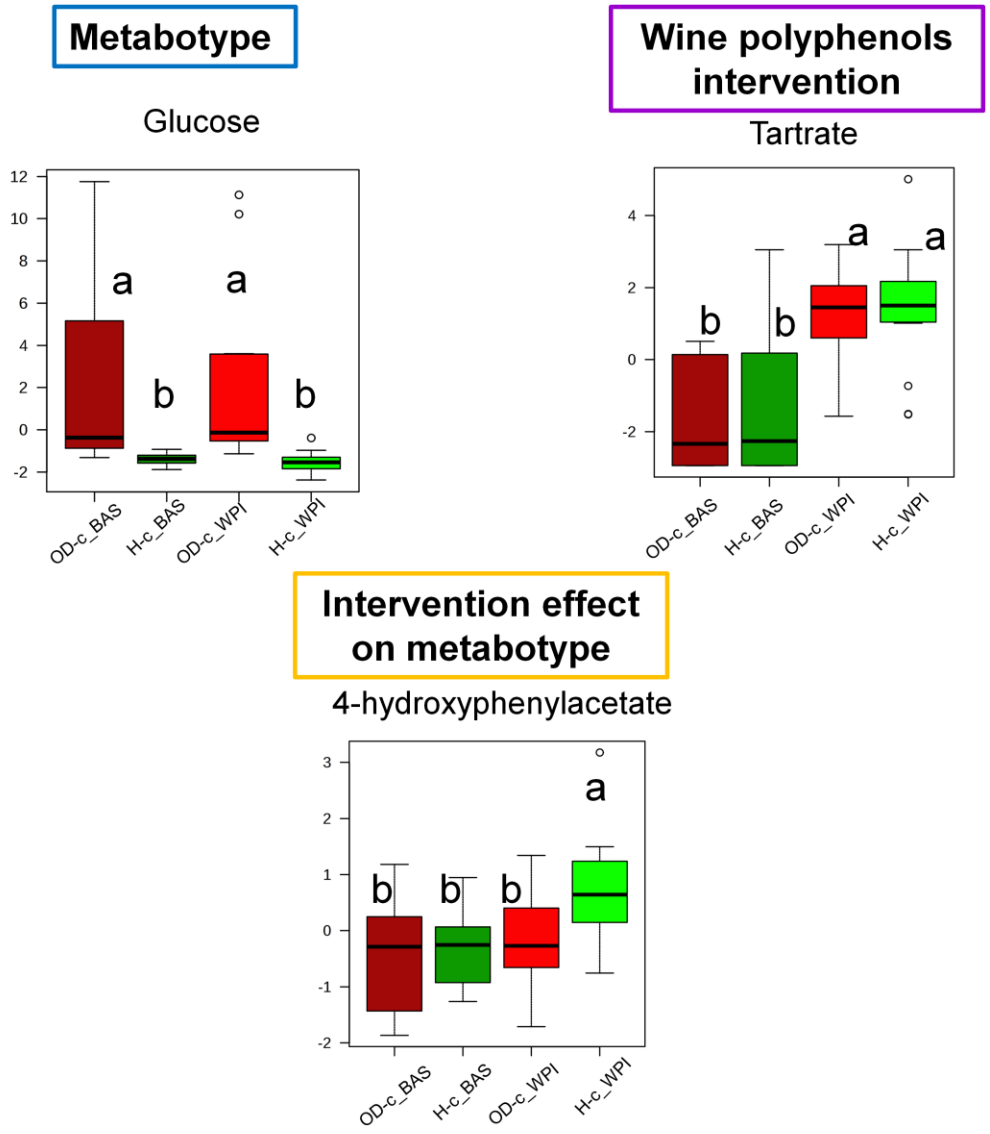


Figure 2. Box-plots of the metabolites derived from ANOVA test ($p<0.05$ after FDR correction). Different letters indicate significant differences between interventions. OD-c_BAS: obese and diabetic cluster in basal period; H-c_BAS: healthier cluster in basal period; OD-c_WPI: obese and diabetic cluster after wine polyphenols intake; H-c_WPI: healthier cluster after wine polyphenols intake.

Supplemental Material

Table S1. Phenolic composition of wine polyphenols from the study. Mean ± SD.

Alcohol (%)	0.42
Total phenols (mEqGA/L)	2694.92 ± 86.79
Gallic acid (mg/L)	73.17 ± 7.01
Protocatechuic acid (mg/L)	5.85 ± 0.51
Tyrosol (mg/L)	47.81 ± 3.90
Catechin (mg/L)	126.45 ± 13.35
Epicatechin (mg/L)	70.57 ± 8.22
trans-Caftaric	19.21 ± 1.62
trans-Caffeic (mg/L)	12.18 ± 0.92
trans- Coutaric (mg/L)	5.62 ± 0.52
2-S-glutationylcaftaric (mg/L)	10.76 ± 1.26
Quercetin-3-glucuronide (mg/L)	11.25 ± 1.42
Quercetin (mg/L)	23.82 ± 2.37
Isorhamnetin (mg/L)	2.96 ± 0.14
Delphinidin-3-glucoside (mg/L)	14.71 ± 1.62
Petunidin-3-glucoside (mg/L)	12.04 ± 1.15
Peonidin-3-glucoside (mg/L)	6.68 ± 0.57
Malvidin-3-glucoside (mg/L)	49.86 ±4.27
Malvidin-(6-acetyl)-3-glucoside (mg/L)	10.41 ± 1.20
Malvidin-(6-coumaroyl)-3-glucoside (mg/L)	3.54 ± 0.33
trans-Resveratrol (mg/L)	2.73 ± 0.23
cis-Resveratrol	2.75 ± 0.15
trans-Piceid (mg/L)	10.53 ± 0.96
cis-Piceid (mg/L)	7.08 ± 0.87

Supplemental Methodology

1.- K-means clustering

Given the experimental structure, there is no prior knowledge on the number of possible clusters in the dataset. Thus, a cluster-complexity or number-of-clusters analysis has been performed by taking into consideration the number of clusters ranging from 2 to 6, limiting the analysis to 6 clusters in order to obtain at least 10 samples/cluster.

The cluster complexity analysis consisted of two principal measures. The first index is an internal clustering measurement proposed by J.C. Dunn (Dunn 1974). This index is based on the principle that clusters should be compact and separated, as measured in (1),

$$D = \min_{1 \le i \le n} \left(\min_{1 \le j \le n, i \ne j} \left(\frac{d(c_i, c_j)}{\max_{1 \le k \le (d'(c_k))}} \right) \right) \qquad (1)$$

where n is the number of clusters, $d(c_i,c_j)$ is the distance function between cluster i and j (distance between centroids), and $d'(c_k)$ is the intracluster distance for cluster k (mean sum of distances between the objects in the same cluster, sometimes approximated as its maximum). For a given clustering solution, a higher Dunn index indicates a better cluster structure.

The second index is the Figure of Merit (FOM), which is an estimate of the predictive power of a clustering algorithm. FOM was proposed by Yeung et al. to compare the overall homogeneity of clusters formed by different clustering methods on the same data (Yeung, Haynor et al. 2001). Thus, it deems the cluster solution that generates the most homogenous clusters as optimal. FOM is based on the assumption that validity of a cluster is increasing in value if in a second experiment the same samples would group together and reveal similar values. (2),

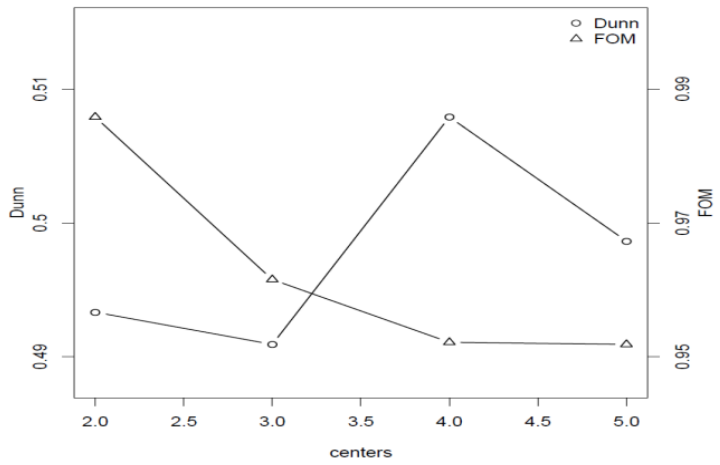
$$FOM(l, K) = \sqrt{\frac{1}{N} \sum_{k=1}^{K} \sum_{i \in C_k(l)} d(x_{i,l}, \mu_{C_k}(l))} \qquad (2)$$

where $\mu_{C_k}(l)$ is the mean peak value for cluster k, $x_{i,l}$ is the measurement of the variable l for sample i. The expression in (2) is

adjusted with a factor ($\sqrt{\frac{N}{N-K}}$) before it is aggregated over all variables. In contrast to the Dunn index, smaller values indicate better homogeneities.

Both analyses (the Dunn analysis for internal coherence and FOM analysis for external homogeneity are shown in Figure 1) were applied to the dataset employing Euclidean distances and a k-means clustering algorithm (MacQueen 1967). Results suggest that a cluster solution of 4 centers is revealing some group structure from the dataset showing optimal properties of internal coherence and grouping stability.

Figure 1. Results of Dunn analysis for internal coherence and FOM analysis for external homogeneity



2.- OSC-PLS-DA.

The dataset derived from the peak integration was submitted to MetaboAnalyst. The dataset was normalized by the sum of the intensities of the spectra. A normalized dataset was then submitted to SIMCA-P+ 11.5 (Umetrics,Umea, Sweden). To improve the data analysis, a preprocessing filter, termed an orthogonal signal correction (OSC) was selected. The OSC filter can selectively remove the variation of data X (NMR data set) having no correlation with Y (cluster assignation). This filter was carried out by the SIMCA-P software.

The data set was log transformed and Pareto-scaled before partial least squares discriminant analysis with orthogonal signal correction (OSC-PLS-DA). Pairwise comparison between the four clusters was carried out. The quality of the models was evaluated by parameters largely used in most metabolomics studies, namely the goodness-of-fit parameter (R2X), the proportion of the variance of the response variable that is explained by the model (R2Y) and the predictive ability parameter (Q2), which was calculated by a seven-round internal cross-validation of the data using a default option of the SIMCA-P+ 11.5 software. Values of Q2 < 0 suggests a model with no predictive ability, with the reliability increasing as Q2 approaches 1. The results obtained by OSC-PLS-DA showed that the most discriminated clusters were 3 versus 4 (Supplemental Table 1).

Supplemental Table 1. OSC-PLS-DA scores based on ^{1}H-NMR signals of urine samples comparing the four clusters (c1, c2, c3 and c4).

R^2X	R^2Y	Q^2(cum)	Comparison	Permutation test, n=200 (intercept R^2, Q^2)
0.103	0.881	0.581	c1 vs c2	0.67, -0.13
0.165	0.985	0.914	c1 vs c3	0.53, -0.20
0.077	0.858	0.458	c1 vs c4	0.64, -0.08
0.138	0.942	0.834	c2 vs c3	0.57, -0.19
0.117	0.976	0.880	c2 vs c4	0.58, -0.20
0.207	0.991	0.962	c3 vs c4	0.40, -0.23*

***** The y-axis intercept (R^2 and Q^2 when the correlation coefficient is zero) is an indication of over fit; lowest R^2- intercept limit and a negative Q^2-intercept limit for comparison of c3 vs c4, indicating a valid model (Llorach-Asunción, Jauregui et al. 2010; Pujos-Guillot, Hubert et al. 2013).

REFERENCES

Dunn, J.C. *J Cybernetics*. 1974; 4(1): 95-104

Llorach-Asunción, et al. J *Pharmaceut Biomed.* 2010**;** 51(2): 373-381

MacQueen, J. *Some methods for classification and analysis of multivariate observations*. Proc. Fifth Berkeley Symp. on Math. Statist. and Prob., Univ. of Calif. Press.1967

Pujos-Guillot, et al. 2013. *J Proteome Res.* 12(4): 1645-1659.

Yeung, et al. 2001. *Bioinformatics.* 17(4): 309-318

5.2. BIOMARCADORES NUTRICIONALES DE SALUD TRAS LA INGESTA DE COMPLEMENTOS ALIMENTICIOS

Evaluación de un probiótico sobre la huella metabólica en una población de mujeres con mastitis

La determinación de la huella metabólica en la orina de mujeres con mastitis después de la ingesta de un probiótico después de 21 días se ha publicado en la revista *Pharmacological Research* indexada en el Journal Citation Reports con un factor de impacto de 3.976 (2013) y situada en el primer cuartil de la categoría PHARMACOLOGY & PHARMACY (38/256).

Rosa Vázquez-Fresno, Rafael Llorach, Jelena Marinic, Sara Tulipani, Mar Garcia-Aloy, Irene Espinosa-Martos, Esther Jiménez, Juan Miguel Rodríguez, Cristina Andres-Lacueva. Urinary metabolomic fingerprinting after consumption of a probiotic strain in women with mastitis. *Pharmacological Research* 2014 Sep;87:160-5. doi: 10.1016/j.phrs.2014.05.010.

RESUMEN:

La mastitis infecciosa es una condición común entre las mujeres lactantes, siendo los estafilococos y estreptococos los principales agentes etiológicos. En este contexto, algunas cepas de lactobacilos aislados a partir de la leche materna parecen ser particularmente eficaces para el tratamiento de mastitis y, por lo tanto, constituyen una alternativa atractiva a la medicación con antibióticos. Se aplicó un enfoque metabolómico basado en ^{1}H-RMN para detectar diferencias metabolómicas después del consumo de una cepa probiótica (*Lactobacillus salivarius* PS2) en mujeres con mastitis. Se recogió la orina de 24 h de las mujeres lactantes con mastitis al inicio del estudio (estado basal) y después

de 21 días de la administración del probiótico (PB). Los análisis multivariantes (OSC-PLS-DA y *hierarchical clustering analysis*) mostraron diferencias en el metaboloma después del tratamiento con PB. Los metabolitos discriminantes detectados al inicio del estudio fueron la lactosa, el ibuprofeno y el acetaminofén (paracetamol), dos fármacos (anti-inflamatorio y analgésico) utilizados comúnmente para el dolor producido por la mastitis. Mientras que, después de la ingesta de PB, se detectó la creatina y los co-metabolitos derivados de la microbiota intestinal hipurato y TMAO. Además, se observó una deserción voluntaria de los fármacos después de la administración del probiótico. La aplicación de la metabolómica basada en la RMN permitió una caracterización de los efectos globales de consumo de probióticos en las mujeres que sufrían mastitis y destacó el potencial de este enfoque en la evaluación de los resultados del consumo de probióticos. Hasta la fecha, esta es la primera vez que este enfoque ha sido aplicado en mujeres con mastitis durante la lactancia.

Pharmacological Research 87 (2014) 160–165

Contents lists available at ScienceDirect

Pharmacological Research

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Urinary metabolomic fingerprinting after consumption of a probiotic strain in women with mastitis

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ARTICLE INFO

Article history:
Received 25 April 2014
Received in revised form 18 May 2014
Accepted 19 May 2014
Available online 29 May 2014

Keywords:
Nutrimetabolomics
Metabolic biomarker
Mastitis
NMR
Probiotics
Lactobacillus salivarius

ABSTRACT

Infectious mastitis is a common condition among lactating women, with staphylococci and streptococci being the main aetiological agents. In this context, some lactobacilli strains isolated from breast milk appear to be particularly effective for treating mastitis and, therefore, constitute an attractive alternative to antibiotherapy. A ^{1}H NMR-based metabolomic approach was applied to detect metabolomic differences after consuming a probiotic strain (Lactobacillus salivarius PS2) in women with mastitis. 24 h urine of women with lactational mastitis was collected at baseline and after 21 days of probiotic (PB) administration. Multivariate analysis (OSC-PLS-DA and hierarchical clustering) showed metabolome differences after PB treatment. The discriminant metabolites detected at baseline were lactose, and ibuprofen and acetaminophen (two pharmacological drugs commonly used for mastitis pain), while, after PB intake, creatine and the gut microbial co-metabolites hippurate and TMAO were detected. In addition, a voluntary desertion of the pharmacological drugs ibuprofen and acetaminophen was observed after probiotic administration. The application of NMR-based metabolomics enabled the identification of the overall effects of probiotic consumption among women suffering from mastitis and highlighted the potential of this approach in evaluating the outcomes of probiotics consumption. To our knowledge, this is the first time that this approach has been applied in women with mastitis during lactation.

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Introduction

Mastitis is a common disease during lactation with an incidence of up to 33% of lactating mothers [1], and 74–95% of cases observed in the first 3 months post-partum [2]. This condition is usually defined as an inflammation of the mammary gland, characterized by a variety of local and, sometimes, systemic symptoms [3]. These symptoms of mastitis are accompanied by changes in the biochemical and immunological composition of milk, and in its sensorial properties.

The infectious aetiology of lactational mastitis is usually so high that some authors define the term "mastitis" as an infectious process of the mammary gland, involving staphylococci, streptococci and/or corynebacteria [2,4]. Traditionally, *Staphylococcus aureus* has been considered as the main aetiological agent of acute mastitis, although *Staphylococcus epidermidis* is emerging as the leading cause of both subacute and chronic mastitis in human medicine [5–7]. Multiresistance to antibiotics and/or formation of biofilms is very common among clinical isolates of these two staphylococcal species. This explains why this condition used to be elusive to

Abbreviations: A, acetaminophen; CFU, colony-forming unit; FID, free induction decay; HCA, hierarchical cluster analysis; IB, ibuprofen; NMR, nuclear magnetic resonance; OSC-PLS-DA, orthogonal signal correction partial least squares-discriminant analysis; PB, probiotic; TMA, trimethylamine; TMAO, trimethylamine-N-oxide; TSP, 3-(trimethylsilyl)-proprionate-2,2,3,3-d4.

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http://dx.doi.org/10.1016/j.phrs.2014.05.010
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antibiotic therapy. In addition, maternal antibiotic therapy may disturb the normal microbiota of the respiratory and digestive tracts of the mother and breastfed infant pair, a fact that may itself further inhibit defence against infection [8]. Therefore, there is a need to develop strategies that represent an alternative to the use of antibiotics and, in this context; probiotics seem to be an appealing approach.

Dysbiosis is the change in the balance of microbiota composition such that it may become harmful to host health [9]. Mammary dysbiosis may lead to mastitis, a condition that represents the first medical cause for undesired weaning [3,10]. Probiotics which have been defined by the WHO/FAO as "live microorganisms that when administered in adequate amounts confer a health benefit on the consumer, can potentially influence systemic health with several mechanisms of action" [11]. In this regards, probiotics in several studies are focused of research [9].

Human milk is a source of bacteria to the infant gut. In addition, modulation of maternal gut microbiota during pregnancy and lactation could have a direct effect on infant health [10]. Recent studies indicate that the mammary gland contains its own microbiota during late pregnancy and lactation. Particularly, selected strains isolated from breast milk can be good candidates for use as probiotics [12,13]. Noticeably, probiotic bacteria that are originally isolated from human milk are specifically attractive organisms since they would fulfil some of the main criteria generally recommended for human probiotics, such as human origin, a history of safe prolonged intake by a particularly sensitive population (neonates, infants), and adaptation to mucosal and dairy substrates [14].

The advent of "-omics" approaches, particularly, metabolomics analysis promises to accelerate progress in our understanding to discern the molecular pathways and biochemical mechanisms under the influence of the microbiota [15]. ^{1}H nuclear magnetic resonance (^{1}H NMR) based on metabolomics, combined with multivariate statistical analysis, has proven to be a powerful technology for providing profiles of numerous components present in biological fluids, enabling non-discriminant, non-destructive, highly reproducible, high-throughput analysis [16]. In this regard, metabolite profiling strategies have shown a remarkable potential to differentiate and characterize infectious diseases caused by microorganisms of different strains, species and genera [17], to obtain a metabolic footprint of microorganisms [18], and to assess the effects of probiotics on the mammalian metabolism [19].

This potential prompted us to examine the applicability of NMR-based metabolomics for identifying the metabolomics changes occurring in biofluids (i.e. urine) of these women as a reflect of overall biochemical effects that could occur among women suffering from lactational mastitis after probiotic consumption, an approach that has not been documented so far. In this context, a metabolomic approach based on ^{1}H NMR spectroscopy coupled with multivariate statistical analysis was used in order to investigate the metabolite composition of urine samples collected from nursing women with mastitis, before and after the oral administration of the probiotic strain *Lactobacillus salivarius* PS2.

Materials and methods

Subjects and study design

Initially, a total of 30 women with mastitis symptoms were screened and, of these, seven dropped out during the study. The reasons for dropping out, after the first analysis, were as follows: two women were suffering from severe anaemia (as assessed by blood analysis), four were taking antibiotics, and the last one moved to another country (Denmark) and could not deliver the final sample. Finally, 23 women finished the study (characteristics of the participants are reported in Supplementary Material, Table S1). All met the following criteria: breast inflammation, painful breastfeeding, milk bacterial count >3 $\log_{10}$ colony-forming unit (CFU)/mL, and milk leucocyte count >6 $\log_{10}$ cells/mL. None of them ingested commercial probiotic foods or supplements during the study. Women with mammary abscesses, Raynaud's syndrome or any other mammary pathology were excluded. All volunteers gave written informed consent to the protocol, which was approved by the Ethical Committee of the Hospital Clínico of Madrid (Spain). The study was registered in the ClinicalTrials.gov database (NCT00716183). All volunteers were asked to follow a controlled diet for 48 h (see Supplementary Material, Table S2) before the collection of the samples in order to facilitate metabolite detection in the urine samples. Women were asked to fill in a record questionnaire related to the evolution of mastitis and the presence of any potential secondary effect of probiotic treatment. The records were used to monitor adherence to the study protocol. All the 23 women reported daily ingestion of a capsule over the 21 days with the exception of two women who reported ingestion over 20 days.

The study lasted 21 days and, during this period, women consumed daily a capsule with 200 mg of a freeze-dried probiotic (PB) containing ~9 $\log_{10}$ CFU of *L. Salivarius*PS2. The capsules were kept at 4 °C throughout the study. Urine (24 h) samples from all the women were collected at the beginning (day 0, baseline) and at the end of the study (day 21, after PB) (see in Supplementary Material: *Collection Procedures*).

Sample preparation

A total of 46 urine samples (from the beginning and end of the intervention) from the 23 women were analyzed. The urine samples were thawed, vortexed and centrifuged at 13,200 rpm for 5 min. The supernatant (600 μl) from each urine sample was mixed with an internal standard solution [120 μL, consisting of 0.1% TSP (3-(trimethylsilyl)-proprionate-2,2,3,3-d4, chemical shift reference), 2 mM of sodium azide (NaN3, bacteriostatic agent) and 1.5 M KH_2PO_4, in 99% deuterium water (D2O)]. The optimized pH of the buffer was set at 7.0, with a potassium deuteroxide solution, to minimize variations in the chemical shifts of the NMR resonances [20]. This mixture was transferred to a 5 mm NMR tube.

Urine ^{1}H NMR spectra acquisition and processing

^{1}H NMR spectra were acquired on a Varian Inova-500 MHz spectrometer (Varian Inc.) operating at a frequency of 500 MHz and a temperature of 298 K. A NOESY-presat pulse sequence was applied to suppress the residual water signal. Free induction decay (FID) were collected into 32 K data points (128 scans) with a spectral width of 14 ppm, an acquisition time of 2 s, relaxation delay of 5 s, and a mixing time of 100 ms [20]. A 0.3 Hz line broadening function was applied to raw FIDs prior to Fourier transformation. The resulting NMR spectra were manually phased, baseline corrected and calibrated (TSP, 0.0 ppm) using ACD Labs 1D NMR Processor 12.0 software (Advanced Chemistry Development Inc., Toronto, Canada). Prior to integration, each spectrum was segmented into 0.025 ppm chemical shift bins (buckets). The residual water resonance signal (δ 4.73–5.00) was excluded, and the resulting data set in the range from 0 to 10 ppm was then submitted to MetaboAnalyst 2.0, a web-based platform for comprehensive analysis of metabolomic data (www.metaboanalyst.ca). The matrix was row-wise normalized (rows were samples) by the sum of the intensities to reduce systematic bias during sample collection.

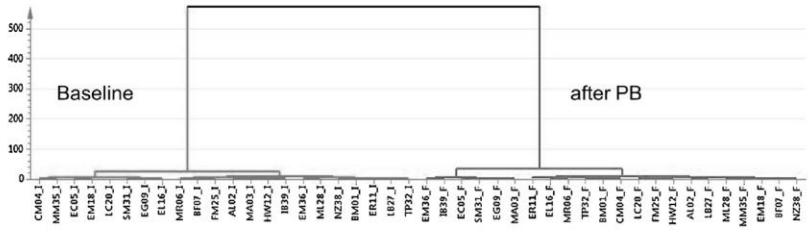


Fig. 1. Tree plot of sample of hierarchical clustering analysis (HCA) with Ward's aggregation method and sorted by size. Samples of women with mastitis, at the beginning of the intervention (baseline), and after probiotic intake (after PB).

Multivariate statistical analysis

The multivariate analysis was performed using the SIMCA-P+13.0 software (Umetrics, Umea, Sweden). The data set was Pareto-scaled (each variable weighted according to $1/\sqrt{SD}$), and then pre-processed using Orthogonal Signal Correction (OSC) filtration before PLS-DA analysis to reduce the variability not associated with the intervention effect [21,22]. The application of this filter was by a corresponding tool provided by SIMCA-P+13.0 software. The ability to classify each individual in the correct group was assessed by R^2Y and the prediction power of the model was assessed by the Q^2 parameter. The OSC-PLS-DA model was calculated by a seven-round internal cross-validation of the data. Validation of the models and the evaluation of the degree of overfitting were then crucial to ensure that models were robust and not overfitted. For this purpose, a response permutation test ($n = 200$) was performed, and the correlation coefficient between the original Y and the permuted Y plotted against the cumulative R^2 and Q^2 was calculated. Generally the R^2- and Q^2-intercept limits for a valid model should be less than 0.4 and 0.05, respectively [23]. Hierarchical clustering analysis (HCA), where distance between clusters in the multivariate space is measured according to Ward's aggregation method and sorted by size, was also applied and results were plotted as a tree plot.

The contribution of the signals to the separation of the classes was visualized by using S-plot associated with the OSC-PLS-DA, which graphically combine the weight (p) of each metabolite within the model and its correlation (p(corr)) with the modelled class designation, thus helping in the selection of statistically significant and potentially relevant metabolites. Chemical shifts showing high correlation coefficients in the S-plots (p(corr) ≥ 0.5 and (p(corr) ≤ -0.5) arbitrary cut-off, previously adopted by our research group [21,24], were included in the list of candidate markers explaining the separation between baseline and after probiotic intake, which was then submitted to the metabolite identification procedure.

Metabolite identification

Discriminant metabolites were identified using the Chenomx NMR Suite 7.5 (Chenomx Inc., Edmonton, Canada) library, by comparing NMR spectral data to those available in databases such as the Human Metabolome Database (HMDB; www.hmdb.ca), the Biological Magnetic Resonance Data Bank (BMRB, www.bmrb.wisc.edu) and the Madison Metabolomics Consortium Database (MMCD, mmcd.nmrfam.wisc.edu). Metabolites from acetaminophen and ibuprofen not available in such libraries and databases are identified by bibliographic support [25,26]. Further, Pearson's correlation test was performed to assess correlations between signals corresponding to the same metabolite. Additionally, the Mann–Whitney test was used to test the significance for identified metabolites ($p < 0.05$).

Results

Microbiological and clinical outcomes

The staphylococcal/streptococcal mean count at day 0 was 3.60 log CFU/mL (3.34–3.85; 95% CI), while that after the probiotic treatment was 2.83 CFU/mL (2.59–3.07). This difference was statistically significant (p-value <0.001; Paired t *student* test, $p < 0.05$). Clinically, most of the women (>85%) that participated in the assay had improved completely or notably after the administration of the probiotic strain. The evolution of the symptoms was evaluated at day 0 (baseline) and at the end of the study by midwives from women's health centres. At both times, the volunteers were asked to score their breast pain from 0 (extremely painful) to 10 (no pain). At the end of the study, midwives reported that 18 women had no clinical symptoms of mastitis while the remaining 5 women had improved but still displayed mild symptoms. The mean score of breast pain reported by the women at day 0 was 2.35 ± 1.29 (SD), while it improved in all the participants at the end of the study (8.56 ± 2.07).

Multivariate analysis of urinary profile and metabolite identification

A total number of 22 volunteers, after detecting and excluding one outlier spectral of a volunteer, were considered for analysis (Supplementary Material Fig. S1). The OSC-PLS-DA analysis resulted in a model characterized by good robustness and predictability to explain the differences between the two sample periods, $R^2Y = 0.9$ and $Q^2 = 0.7$, respectively. In addition, a permutation test ($n = 200$) was carried out to validate the model. The y-axis intercept (R^2 and Q^2 when the correlation coefficient is zero) is an indication of overfit. This test showed an R^2 intercept of 0.34 and a Q^2 intercept of −0.16, validating the model. The samples were subsequently analyzed using a hierarchical cluster analysis (HCA), and closer inspection revealed that the clustering of groups corresponded to before (baseline cluster) and after probiotic intake periods (Fig. 1).

To better visualize the contribution of signals in the separation of the classes, an S-plot was used. The markers with higher p and p(corr) values were the most relevant signals. The lower-left quadrant of the S-plot in Fig. 2 displays the most important signals in the urine samples at baseline (p(corr) ≤ -0.5) and the upper-right quadrant of the S-plot displays the most important signals after PB intake p(corr) ≥ 0.5). The signals in the middle of the figure did not show any relevance in the model. The tentative metabolites identified between both time periods with p(corr) derived from the S-plot and the p-value from the Mann–Whitney test are shown in Table 1.

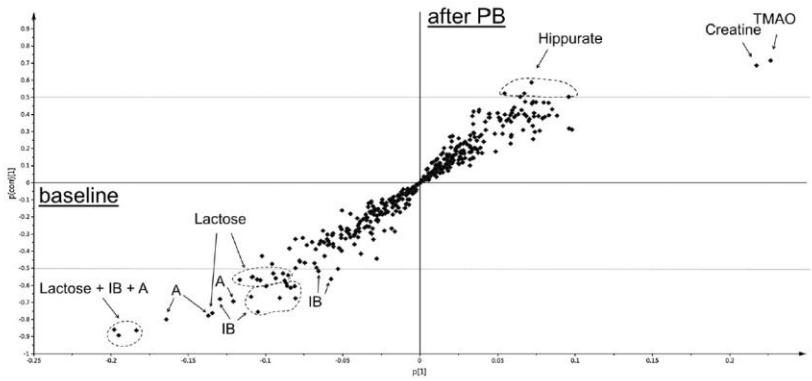


Fig. 2. *S*-plot associated with the OSC-PLS-DA score plot obtained in women with mastitis at the beginning of the intervention and after 21 days of probiotic intake intervention. The lower-left quadrant of the *S*-plot displays the discriminative chemical shifts before the 21-day probiotic intervention (baseline) and the upper-right quadrant of the *S*-plot displays the discriminative chemical shifts after 21 days of intervention (after PB). Dashed lines mark the threshold for each period: $p(\text{corr}) = +0.5$ and $p(\text{corr}) = -0.5$. IB: ibuprofen + ibuprofen metabolites. A: acetaminophen + acetaminophen metabolites; TMAO: Trimethylamine-N-oxide. Lactose + IB + A indicates chemical shifts overlapped for lactose, ibuprofen and acetaminophen metabolites, achieving the greater values of the *S*-plot at baseline.

Discussion

The baseline discriminant metabolites found in the urine of women with mastitis were lactose, ibuprofen and acetaminophen. Lactose is the major carbohydrate present in breast milk, providing a primary source of energy for infant growth. Moreover, its excretion in urine in pregnancy and lactation periods is well documented [27]. Excretion of lactose in urine is a measure of increased permeability of the lactating breast during inflammation [28]. Likewise, Fetherston et al. [28], in an attempt to identify possible predictors of mastitis in lactating mothers in the first 3 months post-partum, observed a significant elevation in the concentration of lactose in urine over a 24-h period, with the highest levels observed when breast inflammation symptoms were more severe. These

Table 1
The significant metabolites derived from the *S*-plot in OSC-PLS-DA comparing women with mastitis at the beginning of the study (baseline) and after probiotic administration (after PB). ↑, indicates higher levels after probiotic intake ($p(\text{corr}) \geq 0.5$); ↓, indicates lower levels after probiotic intake ($p(\text{corr}) \leq -0.5$). *p-Value from *Mann–Whitney test*.

Metabolites	Chemical shift (multiplicity)	After PB	Metabolic information	$p(\text{corr})$	p^*
Lactose	3.55 (m)[a]	↓	Major carbohydrate present in breast milk	−0.76	5.74×10^{-7}
	3.66 (m)[a]			−0.57	6.59×10^{-5}
	3.73 (t)[a]			−0.55	8.86×10^{-5}
	3.85 (m)[a]			−0.57	0.001
	4.45(d, J = 7.83 Hz)[a]			−0.57	3.26×10^{-5}
	4.67 (d, J = 7.83 Hz)[a]			−0.55	0.001
	5.23 (d, J = 3.91 Hz)[a]			−0.53	6.60×10^{-5}
IB/IB metabolite	0.73 (d, J = 6.60 Hz)[b]	↓	Anti-inflammatory drug	−0.56	0.002
	1.07 (d, J = 6.60 Hz)[b]			−0.68	1.68×10^{-6}
	1.38 (d, J = 7.12 Hz)[b]			−0.67	5.16×10^{-8}
	1.85 (m)[b]			−0.57	3.00×10^{-3}
	2.58 (d, J = 7.34 Hz)[b]			−0.68	3.26×10^{-5}
	2.84 (d, J = 7.34 Hz)[b]			−0.67	1.73×10^{-4}
	3.50(s)[b]			−0.60	1.90×10^{-4}
A/A metabolites	2.16 (s)[c]	↓	Analgesic drug	−0.80	1.33×10^{-6}
	7.13 (d, J = 8.80 Hz)[c]			−0.78	3.36×10^{-6}
Lactose + IB + A	3.62 (m)[‡]	↓	–	−0.89	1.77×10^{-8}
TMAO	3.27 (s)[d]	↑	Product of choline metabolism by gut microbiota	0.72	2.65×10^{-8}
Hippurate	7.56 (t, J = 7.41 Hz)[e]	↑	Glycine conjugate of benzoic acid associated with the microbial degradation of certain dietary components (gut microbiota co-metabolite)	0.50	0.001
	7.66 (t, J = 7.41 Hz)[e]			0.59	2.51×10^{-4}
	8.54 (br)[e]			0.52	0.002
Creatine	3.03 (s)[f]	↑	Energy maintenance	0.69	1.74×10^{-5}

PB: probiotic; $^*p < 0.05$, Mann–Whitney test; IB: ibuprofen; A: Acetaminophen; superscript numbers indicate correlation among the signals–same number indicates same metabolite with a correlation $r \geq 0.90$. [b]These signals also correlate ($r \geq 0.90$) with other signals of IBU/IBU metabolites (δ 0.85, δ 1.21, δ 5.56, δ 7.26, δ 7.31). [c]These signals also correlate ($r \geq 0.90$) with other signals of A (δ 5.10), TMAO: Trimethylamine-N-oxide; d: doublet, s: singlet, t: triplet, br: broad band, J: J-coupling value. [‡]Overlapping signal: identifications match with Chenomx library and [25]. [a,d,e,f]Identification match with Chenomx library and with standard compound from BMRB database. [b,c]Identification agrees with [25] and [26] and HMDB spectra. The chemical shift corresponding to creatine at δ 3.93, as well as hippurate at δ 3.96, not shown in this table, might be affected by interference of the ibuprofen and lactose resonances. Lactose + IB + A: chemical shifts overlapped containing lactose, ibuprofen and acetaminophen metabolites

observations led to the conclusion that urinary excretion of lactose could be used as a reliable indicator of mastitis and a predictor of the degree and types of symptom experienced by mothers during this condition. Decreased concentration of lactose in urine following probiotic intake might, therefore, favourably affect the milk volume and the milk composition but may also represent an indicator of changes in breast physiology that allow the recovery of a mastitis mammary gland to its normal lactating state.

Higher urinary excretion of ibuprofen (IB) and acetaminophen (A) and their metabolites at baseline, and lower urinary levels after 21 days, exhibited a free desertion (without a medical prescription) of the anti-inflammatory and analgesic drugs intake in women after probiotic administration. These pharmacological drugs are widely used in the amelioration of mastitis pain [2,29]. Additionally, acetaminophen, ibuprofen and their related metabolites are readily identifiable in ^{1}H NMR urine spectra, and their spectral signatures are distinctive in human urinary metabolome after their ingestion [25,30].

After PB administration, the discriminant metabolites found were trimethylamine-N-oxide (TMAO), hippurate and creatine. Trimethylamine-N-oxide is an oxidation product of trimethylamine, and both are products of choline metabolism. Choline from the diet is converted by gut microbiota to form trimethylamine (TMA), which is mainly oxidized to TMAO [31]. Choline requirements are very important during pregnancy and lactation. The requirements of choline are also likely to increase during pregnancy and lactation because large amounts of choline must be delivered to the foetus across the placenta [32] and secreted into breast milk [33] from maternal circulation. The increased levels of TMAO in urine could suggest a beneficial probiotic effect in the choline metabolism of the lactating women from the present study. Additionally, the presence of TMAO in human urine may also be related with high meat [34] and fish [35] intake.

Many factors have been found to be associated with changes in the urinary excretion of hippurate (a metabolite strongly associated with diet and intestinal microbiota) [36]. To our knowledge, though, no studies evaluating women with mastitis have found a link with hippurate; however, a study of cows' milk metabolites found lower levels of this metabolite in mastitis cows [37]. Thus, an increase in the levels of this metabolite after probiotic intake in urine could be reflected by increased levels of hippurate in milk, in consonance with an improvement in the health of women from the present study.

Several studies have demonstrated the importance of gut microbiota in contributing to the excretion of metabolites such as TMAO and hippurate. For instance, their excretion was not observed in germ-free animals; however, after the exposure to intestinal microbiota, hippurate and TMAO excretion was detected [38]. Moreover, some studies reported a reduction in the urinary excretion of both hippurate and TMAO in rats treated with antibiotics [39,40]. In addition, this fact supports the non-antibiotic intake from the participants during the study. In this regard, research is ongoing concerning the important effects of gut microbiota on human health [41,42].

Finally, the creatine pool is important for maintaining ATP levels when energy demand is transiently greater than the rate of ATP synthesis [43]. Under physiological conditions, creatine is spontaneously converted to creatinine, which is subsequently excreted in urine [44]. However, elevated urinary levels of creatine and creatinine are also used as biomarkers of protein intake, associated with meat consumption [45].

A recent study reported a higher excretion of creatine in the breastfeeding stage, which indicates the ability of breastfeeding women to adjust to these metabolic demands imposed by breastfeeding [46]. The increased urinary level of creatine found after probiotic intervention could exhibit an increased endogenous synthesis of creatine and may reflect a re-establishment of the normal lactation stage in these women after the intervention.

A possible lack of the present study is a proper control group of mastitis women without the administration of the probiotic. However, the objective of this study was to assess the urinary metabolomics changes in mastitis women before and after the probiotic administration. The amelioration of mastitis symptoms was previously tested in two randomly selected groups of women with mastitis: one group consuming probiotic and the other without probiotic administration (control group) [13], concluding that the probiotic administration appear to be an efficient alternative for the treatment of lactational infectious mastitis during lactation.

Conclusions

Characterization of the urine metabolic profile of the nursing women with mastitis at the end of the intervention by an NMR-based metabolomic approach showed that probiotic supplementation decreased lactose excretion which could suggest a normalization of breast permeability. Additional studies, examining the correlation of lactose with inflammatory and immunological mediators, along with the mother's health status, are needed to confirm this possibility.

The reduction in the urine excretion of ibuprofen and acetaminophen demonstrates the voluntary desertion of anti-inflammatory and analgesic drugs intake in these women at the end of the study. Moreover, an increase of TMAO, creatine and hippurate might reflect changes in choline metabolism, in energetic pathways and in gut microbiota metabolism, in line with a reported improvement in the health status of the women at the end of the intervention.

By profiling urinary metabolites we have demonstrated the applicability of NMR-based metabolomics for identifying the overall biochemical effects of oral probiotic administration to women suffering from lactational mastitis, and highlighted the potential of this approach in evaluating the outcomes of probiotics consumption.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgements

We thank the participants for their collaboration in the study. This research was supported by Spanish National Grants from the Spanish Ministry of Science and Innovation (MICINN): CICYT-AGL 2006-14228-C03-02, AGL2009-13906-C02-01, AGL2010-10084-E, AGL2010-15420 and CONSOLIDER INGENIO 2010 Programme FUN-C-FOOD (CSD2007-063); and a research contract from the Nutricia/Danone Research Centre for Specialized Nutrition (Utrecht, The Netherlands). RVF, ST and RLl would like to thank the, FPI 2010 fellowship, "Juan de la Cierva", and "Ramon y Cajal" programmes respectively from MICINN and the European Social Funds. MGA and MJ would like to thank the predoctoral fellowships awarded by the Generalitat de Catalunya (FI-DGR 2011), and the Faculty of Medicine, University of Rijeka TransMedRi-256686, and EUFP7-REGPOT-2010-5 grants, respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2014.05.010.

References

[1] Foxman B, D'Arcy H, Gillespie B, Bobo JK, Schwartz K. Lactation mastitis: occurrence and medical management among 946 breastfeeding women in the United States. Am J Epidemiol 2002;155:103–14.
[2] WHO. Mastitis: causes and management; 2000.
[3] Lawrence RLRM. Breastfeeding. A guide for the medical profession. 6th ed. St. Luis: Elsevier Mosby; 2005.
[4] Barbosa-Cesnik C, Schwartz K, Foxman B. Lactation mastitis. JAMA 2003;289:1609–12.
[5] Delgado S, Arroyo R, Martín R, Rodríguez JM. PCR-DGGE assessment of the bacterial diversity of breast milk in women with lactational infectious mastitis. BMC Infect Dis 2008;8:51.
[6] Delgado S, Arroyo R, Jiménez E, Marín ML, del Campo R, Fernández L, et al. Staphylococcus epidermidis strains isolated from breast milk of women suffering infectious mastitis: potential virulence traits and resistance to antibiotics. BMC Microbiol 2009;9:82.
[7] Cusack L, Brennan M. Lactational mastitis and breast abscess – diagnosis and management in general practice. Aust Fam Physician 2011;40:976–9.
[8] Kummeling I, Stelma FF, Dagnelie PC, Snijders BE, Penders J, Huber M, et al. Early life exposure to antibiotics and the subsequent development of eczema, wheeze, and allergic sensitization in the first 2 years of life: the koala birth cohort study. Pediatrics 2007;119:e225–31.
[9] Walker AW, Lawley TD. Therapeutic modulation of intestinal dysbiosis. Pharmacol Res 2013;69:75–86.
[10] Fernández L, Langa S, Martín V, Maldonado A, Jiménez E, Martín R, et al. The human milk microbiota: origin and potential roles in health and disease. Pharmacol Res 2013;69:1–10.
[11] Lee IC, Tomita S, Kleerebezem M, Bron PA. The quest for probiotic effector molecules – unraveling strain specificity at the molecular level. Pharmacol Res 2013;69:61–74.
[12] Arroyo R, Martin V, Maldonado A, Jimenez E, Fernandez L, Rodriguez JM. Treatment of infectious mastitis during lactation: antibiotics versus oral administration of lactobacilli isolated from breast milk. Clin Infect Dis 2010;50:1551–8.
[13] Jiménez E, Fernández L, Maldonado A, Martín R, Olivares M, Xaus J, et al. Oral administration of lactobacillus strains isolated from breast milk as an alternative for the treatment of infectious mastitis during lactation. Appl Environ Microbiol 2008;74:4650–5.
[14] Collins JK, Thornton G, Sullivan GO. Selection of probiotic strains for human applications. Int Dairy J 1998;8:487–90.
[15] Martin F-PJ, Collino S, Rezzi S. 1H NMR-based metabonomic applications to decipher gut microbial metabolic influence on mammalian health. Magn Reson Chem 2011;49:S47–54.
[16] Lenz EM, Wilson ID. Analytical strategies in metabonomics. J Proteome Res 2007;6:443–58.
[17] Slupsky CM, Cheypesh A, Chao DV, Fu H, Rankin KN, Thomas JM, et al. Streptococcus pneumoniae and staphylococcus aureus pneumonia induce distinct metabolic responses. J Proteome Res 2009;8:3029–36.
[18] Sulek K, Frandsen H, Smedsgaard J, Skov T, Wilcks A, Licht T. Metabolic footprint of lactobacillus acidophilus NCFM at different pH. Metabolomics 2012;8:244–52.
[19] Martin FP, Wang Y, Sprenger N, Yap IK, Lundstedt T, Lek P, et al. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. Mol Syst Biol 2008;4:157.
[20] Vazquez-Fresno R, Llorach R, Alcaro F, Rodriguez MA, Vinaixa M, Chiva-Blanch G, et al. (1)H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors. Electrophoresis 2012;33:2345–54.
[21] Tulipani S, Llorach R, Jáuregui O, López-Uriarte P, Garcia-Aloy M, Bullo M, et al. Metabolomics unveils urinary changes in subjects with metabolic syndrome following 12-week nut consumption. J Proteome Res 2011;10:5047–58.
[22] Pujos-Guillot E, Hubert J, Martin J-F, Lyan B, Quintana M, Claude S, et al. Mass spectrometry-based metabolomics for the discovery of biomarkers of fruit and vegetable intake: citrus fruit as a case study. J Proteome Res 2013;12:1645–59.
[23] Van Der Voet H. Comparing the predictive accuracy of models using a simple randomization test. Chemometr Intell Lab Syst 1994;25:313–23.
[24] Llorach R, Urpi-Sarda M, Jauregui O, Monagas M, Andres-Lacueva C. An LC–MS-based metabolomics approach for exploring urinary metabolome modifications after cocoa consumption. J Proteome Res 2009;8:5060–8.
[25] Holmes E, Loo RL, Cloarec O, Coen M, Tang H, Maibaum E, et al. Detection of urinary drug metabolite (xenometabolome) signatures in molecular epidemiology studies via statistical total correlation (NMR) spectroscopy. Anal Chem 2007;79:2629–40.
[26] Neunzig I, Göhring A, Drăgan C-A, Zapp J, Peters FT, Maurer HH, et al. Production and NMR analysis of the human ibuprofen metabolite 3-hydroxyibuprofen. J Biotechnol 2012;157:417–20.
[27] Date JW. The excretion of lactose and some monosaccharides during pregnancy and lactation. Scand J Clin Lab Invest 1964;16:589–96.
[28] Fetherston CM, Lai CT, Hartmann PE. Relationships between symptoms and changes in breast physiology during lactation mastitis. Breastfeed Med 2006;1:136–45.
[29] Strong GD. Provider management and support for breastfeeding pain. J Obstet Gynecol Neonatal Nurs 2011;40:753–64.
[30] Loo RL, Coen M, Ebbels T, Cloarec O, Maibaum E, Bictash M, et al. Metabolic profiling and population screening of analgesic usage in nuclear magnetic resonance spectroscopy-based large-scale epidemiologic studies. Anal Chem 2009;81:5119–29.
[31] Russell WR, Hoyles L, Flint HJ, Dumas M-E. Colonic bacterial metabolites and human health. Curr Opin Microbiol 2013;16:246–54.
[32] Welsch F. Studies on accumulation and metabolic fate of (N-Me3h)choline in human term placenta fragments. Biochem Pharmacol 1976;25:1021–30.
[33] Holmes-McNary MQ, Cheng WL, Mar MH, Fussell S, Zeisel SH. Choline and choline esters in human and rat milk and in infant formulas. Am J Clin Nutr 1996;64:572–6.
[34] Stella C, Beckwith-Hall B, Cloarec O, Holmes E, Lindon JC, Powell J, et al. Susceptibility of human metabolic phenotypes to dietary modulation. J Proteome Res 2006;5:2780–8.
[35] Svensson BG, Åkesson B, Nilsson A, Paulsson K. Urinary excretion of methylamines in men with varying intake of fish from the baltic sea. J Toxicol Environ Health 1994;41:411–20.
[36] Lees HJ, Swann JR, Wilson ID, Nicholson JK, Holmes E. Hippurate The natural history of a mammalian–microbial cometabolite. J Proteome Res 2013;12:1527–46.
[37] Sundekilde UK, Poulsen NA, Larsen LB, Bertram HC. Nuclear magnetic resonance metabonomics reveals strong association between milk metabolites and somatic cell count in bovine milk. J Dairy Sci 2013;96:290–9.
[38] Nicholls AW, Mortishire-Smith RJ, Nicholson JK. NMR spectroscopic-based metabonomic studies of urinary metabolite variation in acclimatizing germ-free rats. Chem Res Toxicol 2003;16:1395–404.
[39] Swann JR, Tuohy KM, Lindfors P, Brown DT, Gibson GR, Wilson ID, et al. Variation in antibiotic-induced microbial recolonization impacts on the host metabolic phenotypes of rats. J Proteome Res 2011;10:3590–603.
[40] Yap IKS, Li JV, Saric J, Martin F-P, Davies H, Wang Y, et al. Metabonomic and microbiological analysis of the dynamic effect of vancomycin-induced gut microbiota modification in the mouse. J Proteome Res 2008;7:3718–28.
[41] Nicholson JK, Holmes E, Wilson ID. Gut microorganisms, mammalian metabolism and personalized health care. Nat Rev Microbiol 2005;3:431–8.
[42] Leser TD, Mølbak L. Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. Environ Microbiol 2009;11:2194–206.
[43] da Silva RP, Nissim I, Brosnan ME, Brosnan JT. Creatine synthesis: hepatic metabolism of guanidinoacetate and creatine in the rat in vitro and in vivo. Am J Physiol Endocrinol Metab 2009;296(2):E256–61.
[44] Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev 2000;80:1107–213.
[45] Rasmussen LG, Winning H, Savorani F, Toft H, Larsen TM, Dragsted LO, et al. Assessment of the effect of high or low protein diet on the human urine metabolome as measured by NMR. Nutrients 2012;4:112–31.
[46] Sachse D, Bærug A, Sletner L, Birkeland IK, Nakstad B, Jenum AK, et al. Urine NMR metabolomics analysis of breastfeeding biomarkers during and after pregnancy in a large prospective cohort study. Scand J Clin Lab Invest 2014;74(3):264–72.

Supplementary Material

Collection Procedures for urine samples

Women were asked to collect 24 h urine samples as follows:

- On day 1, urinate into the toilet when you get up in the morning. Note the exact time (e.g., 6:15 AM).
- Afterwards, collect all urine in the provided container for the next 24 h.
- On day 2, urinate into the container when you get up in the morning. This should be within ten minutes before or after the time of the first morning void on the first day (which was flushed). In this example, you would try to void between 6:05 and 6:25 on the second day.
- Cap the container. Keep it in the refrigerator (4–8 ºC) during the whole collection period.
- Label the container with your name, the date and the time of completion, and return it as instructed.

No preservative was used for urine collection. However, urine bacterial concentration (CFU/ml) did not significantly change between 0 and 24 h samples (100 µl) as assessed by BHI (total count), MCK (enterobacteriaceae) and CNA (bacteria with fastidious growth) agar plate cultures.

Table S1. Characteristics of the study women (n = 23).

Characteristic	n
1. Age	
<32	**7**
32–37	**10**
>37 years	**6**
2. Number of children	
1	**16**
2	**5**
3	**2**
3. Type of delivery	
Vaginal	**21**
C-section	**2**
4. Lactation (days)	
≤ 30 days	**7**
30<days<72	**9**
≥72 days	**7**

Table S2. Controlled diet for 48 h prior to the volunteers' urine collection.

Food Restricted (from day 3 before to the day of the study, both included)	**Drinks Prohibited** (from day 3 to the day of the study, both included)	**Foods allowed**
Fruits, vegetables, jams, garlic, parsley, olives, virgin / extra virgin olive oil, potatoes, nuts (walnuts, almonds, peanuts etc.), honey, legumes, soy products, mushrooms, chocolate and confectionery containing it.	Tea, coffee, beer, wine, champagne, smoothies, cocoa, cider, fruit juices, colas, orangeade, and other fruit soft drinks.	Dairy products, meat and meat products, poultry, eggs, rice, pasta, white bread and pastries without chocolate, fish, seafood.

Proposed menu for the day before the study day

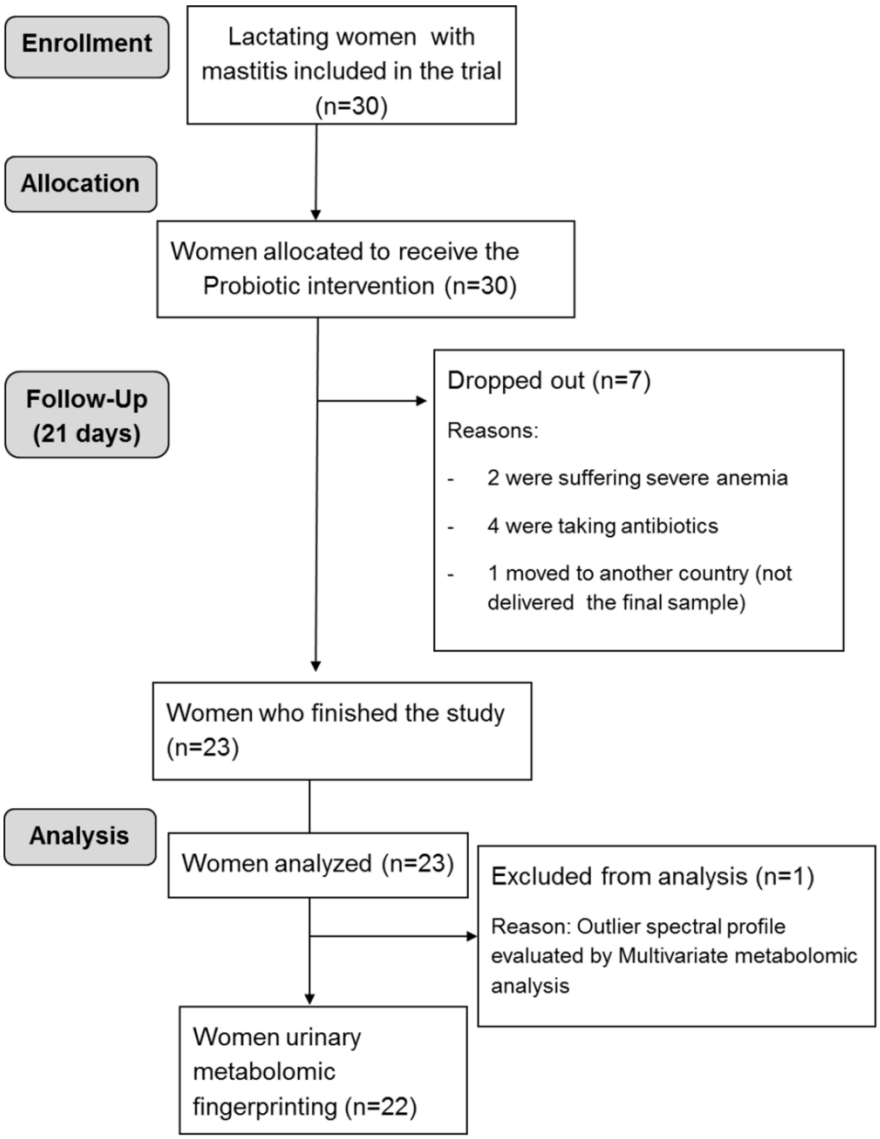
Breakfast:
A choice of:
-cookies or pastries (croissant, brioche buns ...) Not whole bread, **without** chocolate or fruit, jam or preserves.
-sandwich or snack (sliced bread or white bread (not whole or cereal bread.)) with ham and / or cheese and / or ham (no tomato).
- Glass of white milk or yogurt or any other dairy product (no chocolate, coffee or fruit).

Lunch (choice of)
Pasta carbonara (cream, bacon, cheese)
- Meat or fish, baked, boiled to taste, but remember not to use extra virgin / virgin olive oil. White rice can be used as garnish.
- Omelette (or shrimps, cheese, tuna ...) or fried eggs, or boiled.
- White bread or sliced bread (not whole or cereal bread.)
- Egg custard or rice pudding or plain yogurt.

Dinner:
A choice of:
- Rice with ham, prawns, crab sticks and omelettes (not olive oil).
- Omelette with ham and / or cheese
- Chicken or escalope and / or tuna
- White bread or sliced bread (not whole or cereal bread)
- Egg custard or rice pudding or plain yogurt

Mid-morning and afternoon snack (in the case of people who are used to doing this): no chocolate, coffee or tea.
- Dairy products
- White bread or toast with butter and / or ham or any other sausage (no mortadella with olives)
- - Pastries /cakes (no chocolate, sweets or fruit).

Figure S1. Flow-chart of women participants through the present study involving to the probiotic administration trial.



5.3. BIOMARCADORES NUTRICIONALES Y DE SALUD TRAS EL SEGUIMIENTO DE PATRONES ALIMENTARIOS

Determinación de patrones dietéticos en el perfil urinario de una población tras 3 años de seguimiento de intervención

Este artículo ha sido publicado en la revista *Journal of Proteome Research* indexa con un factor de impacto de 5.001 (2013) perteneciente al primer cuartil de BIOCHEMICAL RESEARCH METHODS (9/78)

Rosa Vázquez-Fresno, Rafael Llorach, Mireia Urpi-Sarda, Ascension Lupianez-Barbero, Ramon Estruch, Dolores Corella, Montserrat Fitó, Fernando Arós, Miguel Ruiz-Canela, Jordi Salas-Salvadó, Cristina Andres-Lacueva. Metabolomic pattern analysis after Mediterranean diet intervention in a non-diabetic population: a 1- and 3-year follow-up in the PREDIMED study. *Journal of Proteome Research* 2015, 14 (1), pp 531–540. doi: 10.1021/pr5007894

RESUMEN:

La dieta mediterránea (MD) se considera un patrón de dieta con efectos beneficiosos sobre la salud humana. El objetivo de este estudio fue evaluar el efecto de la MD en el metaboloma urinario mediante la comparación de los sujetos en 1 y 3 años de seguimiento, después de un MD suplementada con aceite de oliva virgen extra (MD + EVOO) o frutos secos (MD + Nuts), frente a un grupo que siguió una dieta control baja en grasa (LFD). Se evaluaron desde un enfoque metabolómico a noventa y ocho voluntarios no diabéticos, correspondiente a los grupos MD+EVOO (n = 41), MD+Nuts (n = 27) o LFD (n = 30). Se examinaron los perfiles urinarios de ^{1}H RMN al inicio del estudio, después de 1 y 3 años de seguimiento. Se utilizaron análisis de datos multivariante (OSC-PLS-DA y HCA) cómo métodos para la identificación de los potenciales grupos de biomarcadores discriminantes, exhibiendo una separación visible en el metaboloma urinario entre los grupos de MD

frente al estado inicial y LFD, y por tanto, un efecto de la intervención nutricional sobre el metaboloma urinario. Los resultados revelaron que los grupos de intervención con MD estaban relacionados con el metabolismo de hidratos de carbono (3-hidroxibutirato, citrato, *cis*-aconitato), creatina, creatinina, aminoácidos (prolina, N-acetilglutamina, glicina, aminoácidos de cadena ramificada y metabolitos derivados), lípidos (ácidos oleico y subérico) y co-metabolitos microbianos (fenilacetilglutamina, *p*-cresol). De lo contrario, hipurato, trimetilamina-N-óxido, histidina y derivados (metilhistidinas, carnosina, anserina) y xantosina fueron predominantes después de la intervención con dieta baja en grasa. Estos resultados muestran que la aplicación de la metabolómica basada en RMN ha permitido la clasificación de los individuos atendiendo a su patrón de dieta y esto pone de relieve el potencial de este enfoque para la evaluación de los cambios en el metaboloma urinaria en diferentes puntos de seguimiento en respuesta a las intervenciones dietéticas específicas.

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Metabolomic Pattern Analysis after Mediterranean Diet Intervention in a Nondiabetic Population: A 1- and 3-Year Follow-up in the PREDIMED Study

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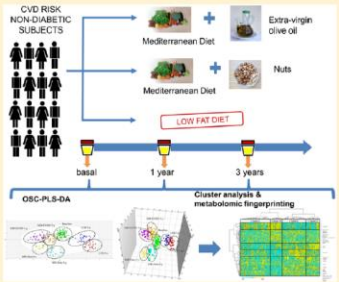
S *Supporting Information*

ABSTRACT: The Mediterranean diet (MD) is considered a dietary pattern with beneficial effects on human health. The aim of this study was to assess the effect of an MD on urinary metabolome by comparing subjects at 1 and 3 years of follow-up, after an MD supplemented with either extra-virgin olive oil (MD + EVOO) or nuts (MD + Nuts), to those on advice to follow a control low-fat diet (LFD). Ninety-eight nondiabetic volunteers were evaluated, using metabolomic approaches, corresponding to MD + EVOO (n = 41), MD + Nuts (n = 27), or LFD (n = 30) groups. The ^{1}H NMR urinary profiles were examined at baseline and after 1 and 3 years of follow-up. Multivariate data analysis (OSC-PLS-DA and HCA) methods were used to identify the potential biomarker discriminating groups, exhibiting a urinary metabolome separation between MD groups against baseline and LFD. Results revealed that the most prominent hallmarks concerning MD groups were related to the metabolism of carbohydrates (3-hydroxybutyrate, citrate, and *cis*-aconitate), creatine, creatinine, amino acids (proline, *N*-acetylglutamine, glycine, branched-chain amino acids, and derived metabolites), lipids (oleic and suberic acids), and microbial cometabolites (phenylacetylglutamine and *p*-cresol). Otherwise, hippurate, trimethylamine-*N*-oxide, histidine and derivates (methylhistidines, carnosine, and anserine), and xanthosine were predominant after LFD. The application of NMR-based metabolomics enabled the classification of individuals regarding their dietary pattern and highlights the potential of this approach for evaluating changes in the urinary metabolome at different time points of follow-up in response to specific dietary interventions.



KEYWORDS: *Nutrimetabolomics, Mediterranean diet, NMR, low-fat diet, olive oil, nuts*

INTRODUCTION

Nowadays, the importance of a healthy diet is widely highlighted, along with the lifestyle needed to prevent several metabolic disorders and nutrition-linked illnesses such as obesity, diabetes, and cardiovascular disease. Traditionally, studies centered on diet have focused on a single nutrient or dietary component in the evaluation of dietary intake.[1,2] However, the assessment of dietary patterns to elucidate the beneficial effects in complex disease processes is increasing. For instance, the Mediterranean diet (MD) is a dietary pattern that has recently shown beneficial effects on human health.[3] This

Special Issue: Environmental Impact on Health

Received: July 31, 2014
Published: October 29, 2014

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particular diet is based on the consumption of vegetables, fish, legumes, nuts, and cereals. Other representative features are the consumption of olive oil as the main source of fat, moderate wine intake, relatively low amounts of meat (mainly poultry, instead of beef and pork), and low to moderate consumption of dairy products.[4] Likewise, recently studies reported that MD supplemented with extra-virgin olive oil (EVOO) or nuts can reduce the incidence and prevalence of major cardiovascular events.[3,5−8] Moreover, frequency of nut consumption was recently shown to be inversely associated with the prevalence of obesity, metabolic syndrome, and diabetes[9] and lower incidence of mortality[10] in subjects at high cardiovascular risk from the PREDIMED study. Similarly, EVOO consumption was recently reported to be inversely related to cardiovascular disease mortality[11] and reduced the risk of atrial fibrillation.[12]

Metabolomics is an interesting tool for assessing the nutritional status of an individual, as well as for studying the biological consequences or metabolic mechanisms following a nutritional intervention.[13−15] For instance, population-based studies have shown marked differences in metabolic profiles within and between populations that reflect dietary differences.[16,17] A number of metabolic profiling studies have been undertaken to assess the metabolic phenotype of humans due to their dietary pattern.[18,19] Moreover, a metabolomic approach has been proposed to evaluate the intricate relationship between nutrition and health.[20]

In the present study, we aimed to assess the impact on urine metabolites of a long-term intervention with a complex diet in a cohort study population. Specifically, we evaluated the effect on the urine metabolome of a 1- and 3-year dietary intervention with an MD plus either EVOO or nuts as supplements in a subcohort from PREDIMED nondiabetic subjects using high-throughput screening ^{1}H NMR spectroscopy. To our knowledge, this is the first time that a long-term MD interventional study has been evaluated on the metabolic profile of individuals using a metabolomic approach.

■ MATERIAL AND METHODS

Study Design and Participants

The PREDIMED study is a parallel-group, single-blind, multicenter, randomized, controlled, 5-year feeding trial assessing the effects of a Mediterranean diet (MD) supplemented either with EVOO (MD + EVOO) or mixed nuts (MD + Nuts) on the primary prevention of cardiovascular disease (CVD) compared to a low-fat diet (LFD). Details of the study protocol were published previously.[21−23] The present substudy is an analysis using 98 clinically evaluated nondiabetic subjects at high CVD risk, recruited from the Barcelona (IDIBAPS) and Valencia centers of the PREDIMED study[3] (ISRCTN 35739639). These participants had at least three cardiovascular risk factors (current smoking, hypertension, hypercholesterolemia, body mass index (BMI) $\geq$ 25 kg/m^2, or a family history of premature cardiovascular disease). Exclusion criteria were type 2 diabetes mellitus, cardiovascular disease, any severe chronic illness, drug or alcohol addiction, history of allergy, or intolerance to olive oil or nuts. Urine samples were obtained at baseline and after 1 and 3 years of the intervention period, along with other clinical and dietary data, collected according to the PREDIMED study protocols.

Dietary Intervention

Participants allocated to the low-fat diet were advised to reduce all types of fat and were given written recommendations according to the American Heart Association guidelines. MD group participants received instructions directed at upscaling the 14-item score measuring adherence to MD, including the use of EVOO for cooking and dressing; increased consumption of vegetables, nuts and fish products; consumption of white meat instead of red or processed meat; and following a moderate pattern of red wine consumption (Supporting Information, Table S1). Neither energy restriction nor physical activity promotion were suggested for any intervention group. MD group subjects were given free allotments of EVOO (1 L/week) or mixed nuts (30 g/day, as 15 g of walnuts, 7.5 g of hazelnuts, and 7.5 g of almonds). All participants had free access to their dietitian throughout the study. The fatty acid and minor component composition of the EVOO and nuts employed in the study was published elsewhere.[21] The local research ethics committee approved the study protocol, and all participants provided written informed consent.

Metabolomic Analysis of Urine Samples

^{1}H-NMR Sample Preparation Data Acquisition and Processing. A procedure based on previously published methodology was applied for the metabolomic analysis. Briefly, the initial volume of urine samples from the study participants was 300 μL diluted with 200 μL of H_2O/D_2O (8:2) and mixed with a buffer solution.[24] The spectral data processed were intelligent, bucketed in domains of 0.005 ppm, and integrated using ACD/NMR Processor 12.0 software (Advanced Chemistry Development, Inc.). The spectral region between 4.75 and 5.00 ppm was excluded from the data set to avoid spectral interference from residual water. The ^{1}H NMR experiment was processed with 128 scans with a spectral width of 14 ppm, an acquisition time of 3.2 s, and a relaxation delay of 3 s. To exclude data points showing little variance across experimental conditions, data were interquartile range (IQR) filtered, row-wise normalized by sum to reduce systematic bias during sample collection, and cube root transformed by a corresponding tool provided by MetaboAnalyst.[25]

Exploratory Data Analysis and Orthogonal Signal Correction Partial Least-Squares Discriminant Analysis (OSC-PLS-DA). Data were imported to SIMCA-P+13.0 software (Umetrics, Umeå, Sweden) and Pareto-scaled prior to being analyzed. An exploratory data analysis, principal component analysis (PCA), which provides a summary overview of all observations in the data, was performed. After the initial overview of the data, the aim was to build a model that could be useful for classifying new samples and at the same time could allow identification of biomarkers from the distinct groups.[26] Then, the data set was preprocessed using orthogonal signal correction (OSC) filtration before PLS-DA analysis to reduce the variability not associated with the diet effect.[27] The ability to classify each individual in the correct group was assessed by the proportion of the variance of the response variable that is explained by the model (R^2Y) and the predictive ability parameter (Q^2), which was calculated by a seven-round internal cross-validation of the data using a default option of the SIMCA-P+13.0 software. The values of $Q^2 < 0$ suggest a model with no predictive ability, and $0 < Q^2 < 1$ suggests some predictive character, with the reliability increasing as Q^2 approaches 1.[28] Validation of the models and the evaluation of the degree of overfitting were then crucial to ensure that models were robust and not overfitted, as well as to exclude models that are just due to chance. For this purpose, a response permutation test (n = 200) was performed, and the correlation

dx.doi.org/10.1021/pr5007894 | J. Proteome Res. 2015, 14, 531−540

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Table 1. Tentative Discriminant Metabolites Derived from the Multivariate Analysis (VIP > 1.5) of NMR Signal Intensities in Urine[a]

MD associated metabolites		1-year ANCOVA (p)			3-year ANCOVA (p)		
metabolite ID	δ (multiplicity)	E vs L	N vs L	E vs N	E vs L	N vs L	E vs N
3-HB	1.20 (d)	↑*	↑*	-	↑*	↑*	-
leucine	0.93 (d)	↑*	↑*	-	↑*	↑*	-
isobutyric acid[§,#]	1.06 (d)	↑*	↑*	-	↑*	↑*	-
2-oxoisovaleric[⊥,†]	1.14 (d)	-	-	-	↑*	-	-
4-DTEA[◊]	1.22 (d)	↑*	↑*	-	↑*	↑*	-
N-Ac[‡]	2.06 (s)	↑*	-	-	↑*	-	-
glycine	3.57 (s)	-	-	-	↑	↑	-
p-cresol[#]	2.34 (s)	↑*	-	-	-	↑*	↓*
	7.22 (d)						
	7.27 (d)						
suberic acid	1.29 (m)	↑*	↑*	-	↑*	↑*	↓*
	1.60 (t)						
oleic acid	1.33 (m)	↑*	↑*	-	↑*	↑*	-
	1.63 (m)						
	1.80 (m)						
	2.00 (m)						
proline	1.95 (m)	↑*	↑*	-	↑*	↑*	-
	2.06 (m)						
	3.38dt						
	4.12dd						
U3.46	3.46 (s)	↑*	↑	-	↑*	↑*	-
U3.33	3.33 (m)	-	-	-	-	-	-
U3.50	3.50 (s)	-	-	-	↑*	↑*	-
U3.81	3.81(s)	-	↑	-	↑*	↑*	-
MD + Nuts associated metabolites		1-year ANCOVA (p)			3-year ANCOVA (p)		
metabolite ID	δ (multiplicity)	N vs E	N vs L	E vs L	N vs E	N vs L	E vs L
PAGN[○]	1.93 (m)	↑*	↑*	↑*	-	↑*	↑*
	2.25 (t)						
	3.67 (m)						
	4.18 (m)						
	7.36**						
	7.42 (t)						
N-AGN	2.08 (m)	↑*	↑*	↑*	↑	↑*	↑*
	2.26 (m)						
	4.18 (m)						
creatine	3.04 (s)	-	-	-	↑*	↑*	-
	3.93 (s)						
U5.02	5.02 (t)	↓*	↓*	-	-	-	-
U5.15	5.15 (t)	↓*	↓*	-	-	↑*	↑*
U6.76	6.76 (m)	-	↑*	-	↑*	↑	-
MD + EVOO associated metabolites		1-year ANCOVA (p)			3-year ANCOVA (p)		
metabolite ID	δ (multiplicity)	E vs N	E vs L	N vs L	E vs N	E vs L	N vs L
creatinine	3.05 (s)	↑	↑	-	↑*	↑*	-
	4.06 (s)						
citrate	2.55 (dd)	↑*	↑*	-	-	↑*	-
	2.69 (dd)						
cis-aconitate	5.74 (s)	↑*	-	↑*	-	-	-
	3.12 (s)						
U7.50	7.50 (d)	↓*	↓*	-	-	-	-
U8.49	8.49 (s)	-	↓	-	-	↓*	-
LFD associated metabolites		1-year ANCOVA (p)			3-year ANCOVA (p)		
metabolite ID	δ (multiplicity)	L vs E	L vs N	E vs N	L vs E	L vs N	E vs N
hippurate	3.97 (d)	↑*	↑*	-	↑*	↑*	↓*
	7.54 (t)						
	7.64 (t)						
	7.83 (d)						
	8.54 (bb)						

dx.doi.org/10.1021/pr5007894 | J. Proteome Res. 2015, 14, 531−540

Table 1. continued

	LFD associated metabolites	1-year ANCOVA (p)			3-year ANCOVA (p)		
metabolite ID	δ (multiplicity)	L vs E	L vs N	E vs N	L vs E	L vs N	E vs N
TMAO	3.28 (s)	-	-	↓	↑*	-	↓*
anserine	3.78 (s)	↑*	↑	-	↓*	-	-
	8.28 (s)						
histidine†	7.08 (s)	↑*	↑*	-	-	-	-
	7.93 (s)						
3-MH†	7.05 (s)	↑*	-	↓*	-	-	-
	7.95(s)						
1-MH†	7.73 (s)	-	↑	↓	-	-	-
	7.01 (s)						
carnosine	8.11 (s)	-	↑	-	↑*	-	-
	7.18 (s)						
proline betaine¥	3.11 (s)	-	-	-	↑*	↑*	↑*
	3.30 (s)						
xanthosine	5.87 (d)	-	↑*	↑*	↑*	-	-

[a]For the direction of change in ANCOVA analysis, (↑) indicates a relatively higher metabolite urinary excretion; (↓) indicates a relatively lower excretion. A dash indicates no statistical significance in ANCOVA analysis ($p < 0.05$). *Statistical significance from the baseline (*t-test*, $p < 0.05$). 3-HB, 3-hydroxybutyrate; 4-DTEA, 4-deoxythreonic acid; E, MD + EVOO; L, LFD; N, MD + Nuts; N-Ac, *N*-acetylglycoproteins; N-AGN, *N*-acetylglutamine; bb, broad band; d, doublet; m, multiplet; MH, methylhistidine; PAGN: phenylacetylglutamine; s, singlet; t, triplet; TMAO, trimethylamine-N-oxide; U, unassigned signals. **Signal excluded from the analysis because it overlapped with a signal of acetaminophen. Identifications agree with §,[76] #,[71] ⊥,[77] ◊,[78] ÷,[79,80,82] ¥,[63] †,[81] ○,[16] HMDB, and Chenomx NMR Suite software.

coefficient between the original Y and the permuted Y plotted against the cumulative R^2 and Q^2 was calculated. Generally, the R^2 and Q^2 intercept limits for a valid model should be less than 0.4 and 0.05, respectively.[29] Despite this validation, the predictive ability of the OSC-PLS-DA models was also evaluated. In this context, the analysis with a training set (75% of the samples), removing 25% of the population (as validation set), was repeated four times, and the ability to classify each individual in the correct group was also assessed each time.[30] The percentage of correctly classified individuals for the validation set was further evaluated. Potential markers of interest were extracted from variable importance in projection (VIP) scores that were constructed from the OSC-PLS-DA analysis, and markers were chosen based on their contribution to the variation and correlation within the data set. VIP value is defined as the influence that each variable has in the PLS-DA model. Thus, the higher the VIP value, the more relevant is the variable in the model. Variables with a VIP value >1.5 were considered important in discriminating between groups[31] and were selected as the most relevant to explain the differences in metabolic profile. While a VIP > 1 threshold is generally accepted,[32−34] the cutoff applied in this study is more restrictive, reducing the possibility of obtaining false positive results.

Two-Way Hierarchical Clustering Analysis (Two-Way HCA). The data set composed of the important signals (VIP > 1.5) obtained from the OSC-PLS-DA model was submitted to two-way HCA using PermutMatrix version 1.9.3.[35] PermutMatrix is a freely available program (http://www.lirmm.fr/~caraux/PermutMatrix/EN/index.html). Two-way clustering means that the NMR signals (rows) and urine samples/individuals (columns) are clustered simultaneously to obtain groups of samples (individuals) and NMR signals that behave as similarly as possible.[36] Groups of individuals with similar patterns are adjacent in this tree.[37] Two-way HCA was carried out using Euclidean distance, and aggregation of the observations was performed using Ward's method. A heat map of the peak intensities of the metabolites and samples of the individuals was obtained to visualize and characterize urinary metabolomes constructed based on the potential candidates of importance.

Statistical Analysis of Covariance (ANCOVA). Before submitting the data to ANCOVA analysis, the normality of continuous variables derived from the VIP scores was assessed by the Kolmogorov−Smirnov test. Then, statistical analysis was used to evaluate differences among the three intervention groups and the time periods during the study. Changes among interventions were analyzed by ANCOVA (covariance test) with a subsequent Bonferroni post hoc test. Potential confounding was controlled by baseline values. After 1 and 3 years, changes for each treatment were assessed using a *t*-test. Pearson correlation was also applied for significant metabolites and FFQ data in order to find significant correlations between metabolites and food records ($r > 0.3$, $p < 0.05$). The SPSS 20 statistical package (SPSS, Chicago, IL, USA) was used for the analysis. Differences were considered significant at $p < 0.05$.

Metabolite Identification and Interpretation. Significant metabolites were tentatively identified using the Chenomx NMR Suite 7.6 profiler software (Chenomx, Inc., Edmonton, Canada), by comparing NMR spectral data to those available in databases such as the Human Metabolome Database (HMDB), the Biological Magnetic Resonance Data Bank (BMRB), and the Madison Metabolomics Consortium Database (MMCD), along with the existing NMR-based metabolomics literature. Second, features were correlated using Pearson's correlation coefficient ($r \geq 0.7$, $p < 0.001$) to identify clusters of features that were likely related. Finally, the biological interpretation was carried out using information from the HMDB and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases and the published research reports.

■ RESULTS

The current study was assessed with 98 nondiabetic men and women, aged 53 to 79 years. A flowchart of the participants allocated in the present study (Figure S1) and descriptive data

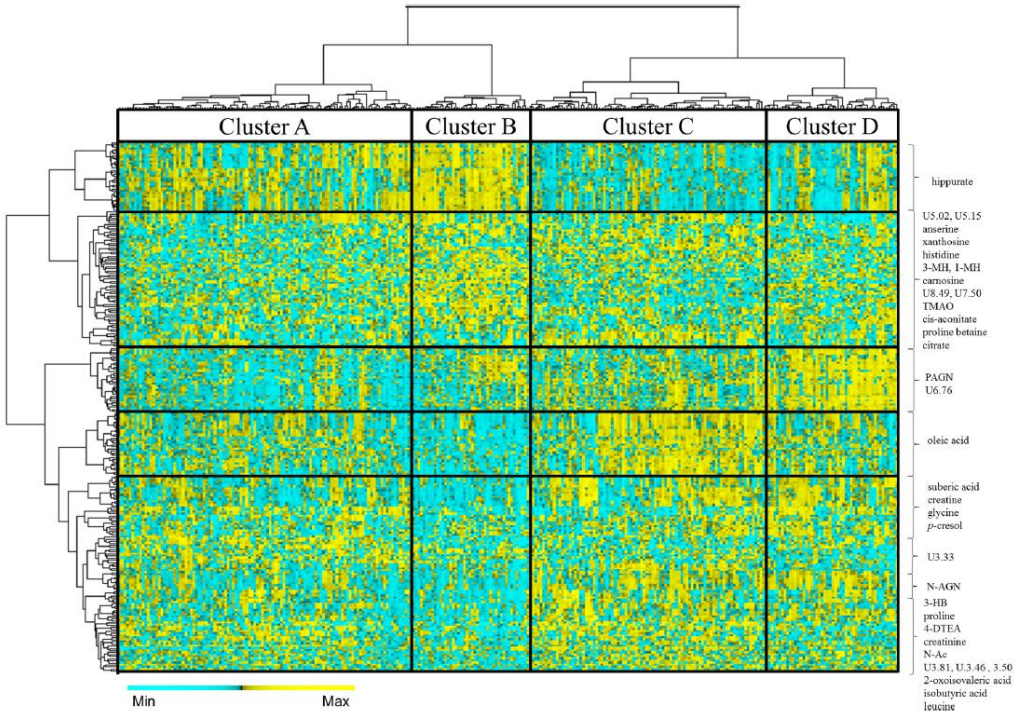

Figure 1. Two-way hierarchical clustering analysis (processed with PermutMatrix according to the Euclidean distance and Ward's aggregation method). Heat map representation of the clustered data matrix in which each colored cell represents the intensity of appropriate NMR signals, according to the color scale at the bottom of the figure. Rows: NMR signals (253, VIP > 1.5). Columns: urine samples baseline and LFD (1y + 3y); MD + EVOO (1y + 3y), and MD + Nuts (1y + 3y).

of the initially eligible individuals (Table S2) are presented in the Supporting Information.

Metabolomic Differences between Baseline and 1 and 3 Years of Follow-Up

Orthogonal Signal Correction-PLS-DA (OSC-PLS-DA) Analysis. The initial overview of the ^{1}H NMR urine data by PCA analysis showed substantial separation of signals from the data corresponding to acetaminophen and derived metabolites (Supporting Information Figure S2). To allow differentiation of spectra without the overriding influence of the strong acetaminophen peaks, these signals from the NMR data were removed before performing further metabolomic analysis.[38] Then, the OSC-PLS-DA analysis resulted in a model with R^2Y = 0.75 and Q^2 = 0.58. The permutation test displayed an R^2 intercept of 0.28 and a Q^2 intercept of −0.28. To further assess the modeling quality process, a training subset of the individuals was taken (75% of participants) and a validation subset (25%) was used to test the model. This analysis was repeated four times, with each sample appearing once in the validation set. The mean value of R^2Y from this analysis was 0.75, and the mean Q^2(cum) was 0.54. The mean percentage of correctly classified individuals performed in the validation set was 96.94%. The validation set fitted reasonably, confirming the validation of the model (Supporting Information, Table S3). VIP values, which indicate the contribution of metabolites to the classification of samples, were calculated for each variable in the OSC-PLS-DA model. Discriminant individuals of MD vs LFD and baseline groups were observed on the score plot graphic (Supporting Information, Figure S3).

Identification of Features from the Multivariate Analysis. The selected features (VIP > 1.5) for the partial least-squares discriminant analysis were tentatively identified using the procedure explained above. The information concerning the tentative identification, chemical shifts, and multiplicity of each metabolite is provided in Table 1.

Two-Way Hierarchical Cluster Analysis (HCA) and Heat Map Visualization. For HCA, the samples were classified into two main groups corresponding with MD diets against baseline and LFD samples. Subsequently, these clusters were divided into two cluster levels: on the one hand, clusters A and B, and on the other hand, clusters C and D (Figure 1). Cluster A was predominantly formed by baseline individuals. Cluster B exhibited LFD individuals (1- and 3-year follow-up). The other two clusters corresponded to MD groups: cluster C was the MD + EVOO (1- and -3 year follow-up) and cluster D was the MD + Nuts (1- and 3-year follow-up) group. The classification table of the individuals in the corresponding cluster for the HCA is shown in Supporting Information (Table S4).

ANCOVA and *t* Test Analysis to Assess Urinary Metabolomic Differences. After the identification procedure, metabolites were submitted to statistical analysis comparing their urinary excretion between interventions (using ANCOVA analysis) and between time periods (using *t*-test analysis). The

dx.doi.org/10.1021/pr5007894 | J. Proteome Res. 2015, 14, 531−540

significant metabolites were divided according to their excretion into MD-associated metabolites (observed in both MD groups), MD + Nuts-associated metabolites, MD + EVOO-associated metabolites, and LFD-associated metabolites (Table 1). The MD-associated metabolites group concerned ketone bodies such as 3-hydroxybutyrate (3-HB), amino acids such as proline, glycine, the branched-chain amino acid (BCAA) leucine, and its derived metabolites (isobutyric acid and 2-oxoisovaleric acid), the threonine metabolite 4-deoxythreonic acid (4-DTEA), the *N*-acetyl groups of glycoproteins (N-Ac), gut microbiota cometabolite *p*-cresol, and also the fatty acid (oleic acid), and its breakdown product (suberic acid), along with the unidentified metabolites U3.46, U3.50, and U3.81. The MD + Nuts-associated metabolites compress metabolites of the amino acid glutamine (phenylacetylglutamine [PAGN] and *N*-acetylglutamine [N-AGN]), creatine, and four unknown metabolites. PAGN and N-AGN were statistically significantly more highly excreted in both MD groups than LFD; however, they were also higher in MD + Nuts versus MD + EVOO. The MD + EVOO-related metabolites included creatinine and two intermediates of the tricarboxylic acid cycle (TCA), citrate, and *cis*-aconitate, with higher excretion in MD + EVOO than in the other groups. Finally, LFD-associated metabolites involved a higher excretion in particular of hippurate (in both time periods), but also of trimethyl-*N*-oxide (TMAO) and histidine and its derived metabolites (3-methylhistidine [3-MH], 1-methylhistidine [1-MH], carnosine, and anserine), as well as proline-betaine and xanthosine.

In general, results showed more differences between the MD groups than the LFD group, particularly comparing the MD + EVOO and LFD groups, which had the most predominant changes in statistical analysis (lower *p*-values; see Supporting Information, Table S5). Moreover, most MD-associated metabolites were excreted in 1 and 3 years with the same trend. However, some metabolites, particularly from LFD, are characteristic of one of the two time points evaluated.

■ DISCUSSION

The present results showed that the 1- and 3-year intervention follow-ups presented a marked effect on urinary metabolomic phenotype in the volunteers.

Metabolites from Protein, Lipid, and Carbohydrate Metabolic Pathways in MD Groups

BCAA leucine and the products of BCAA reflect differences in gluconeogenesis (GNG)[16] in MD compared to the control diet (LFD). Moreover, these results could suggest a particular importance of BCAAs in lipid oxidation and ketone body synthesis. 4-DTEA is a diastereoisomer of 2,3-dihydroxybutanoic acid. 4-DTEA is a breakdown product of threonine also involved in amino acid catabolism. Threonine deaminase converts threonine to α-ketobutyrate, a precursor of isoleucine (a BCAA), and this is further modified by reductases to 4-deoxyerythronic acid and its diastereoisomer 4-DTEA.[39] The similar results with 3-HB excretion could suggest a possible relationship between these threonine catabolic products and ketone body production, consistent with the ketogenic nature of the amino acid threonine.[40]

The increase in urinary excretion of the ketone body 3-HB in MD could suggest an increase in fatty acid oxidation (FAO).[41] FAO is indispensable for the conversion of lipids (dietary or from lipid storage) to ketone bodies.[42] Moreover, PAGN is endogenously formed in humans by phenylacetate, which could be related to oxidation of phenyl containing fatty acids[43] or through the exogenous intake contained in plant food sources.[44] Differences in excretion of PAGN are described that reflect changes in GNG and the tricarboxylic acid (TCA) cycle.[45]

Higher levels of creatine and creatinine present in MD groups are observed. The high excretion of creatine reflects increased turnover in the creatine/creatinine pathway.[46] Furthermore, changes in citrate and *cis*-aconitate suggest a different modulation of the TCA cycle.[47] Also, proline is the catabolite of peptide degradation and is a precursor of pyruvate. Pyruvate can be converted into acetyl-CoA, which is the main input for a series of reactions such as the TCA cycle. Additionally, decreases in levels of proline and citrate were observed in diet-induced hyperlipidemia in rats.[48]

Food Intake Metabolites (Food Metabolome) Particular for Each Intervention Group

PAGN and NAG, both derived from the amino acid glutamine, were found associated within the MD groups, particularly in the MD-Nuts group, suggesting an important implication of glutamine metabolism in this group of individuals. Additionally, glutamic acid, whose conversion into glutamine takes place in tissues such as the liver, skeletal muscle, and brain,[49] is the major amino acid from walnuts,[50] hazelnuts,[51] and almonds,[52] in consonance with the higher excretion in the MD + Nuts group from our results.

Oleic acid (highly excreted in the MD groups, particularly in MD-EVOO) is the major fatty acid in olive oil (75.0% $\pm$ 0.8) and is also highly present in nuts (hazelnuts 72.1% $\pm$ 0.2, almonds 61.2% $\pm$ 0.4, and walnuts 14.0% $\pm$ 0.3; data extracted from the composition of virgin olive oil and nuts used in the present trial[21]). Oleic acid is mainly metabolized in the body by FAO; however, amounts of it could be excreted[53] and detected[54] in urine. It is reported that the upregulated short-chain dicarboxylic acids and long-chain fatty acids (such as oleic acid) in the urine of rats showed an increasing pressure on energy utilization from the catalysis of the fatty acid pathway in fasting.[55] Further, suberic acid is a dicarboxylic acid, which is a metabolic breakdown product derived from oleic acid.[56] The increased intake of PUFAs (polyunsaturated fatty acids, contained within nuts) and MUFAs (monounsaturated fatty acids, contained in EVOO and nuts) resulted in greater excretion of FAO products in the urine. Thus, it is suggested that a supplementation with EVOO and nuts in the MD groups manifested an increase in their FAO metabolism and turnover. These results are in line with other results published after 12 weeks of nut consumption in an interventional study.[57] Similarly, it is described that MUFA and PUFA upregulate FAO[4] by activating ligands for peroxisome proliferator-activated receptors (PPARs), as fatty acids are natural PPAR ligands.[58] Activation of PPARs by dietary fatty acids such as oleic acid makes tissues more dependent on FAO by stimulating fatty acid utilization pathways including transport, esterification, and oxidation,[59] thereby improving fatty acid utilization.

3-MH, 1-M, carnosine, and anserine are compounds derived from the histidine metabolic pathway related to muscle protein breakdown[60] and also biomarkers of meat consumption.[8] Likewise, in the LFD group, a moderate association ($r > 0.4$, $p < 0.05$) was found with reported red meat intake from the FFQ for histidine, 3-MH, and carnosine. Some studies exhibited metabolites being discriminant among lactovegetarian and

dx.doi.org/10.1021/pr5007894 | J. Proteome Res. 2015, 14, 531−540

omnivorous people, showing a decreased excretion of them in people following vegetarian diets, while an increase of N-Ac, citrate, glycine, and PAGN is evidenced[18,19] in vegetarian diets. Similarly, a moderate association ($r > 0.3$, $p < 0.05$) was found with some MD-associated metabolites (3-HB, leucine, isobutyric, 2-oxoisovaleric acid, N-Ac, proline, glycine *p*-cresol, and PAGN) with vegetable, legume, and fruit intake in MD groups from the FFQ.

Finally, xanthosine is a metabolite involved in purine metabolism. Decreased levels of urinary xanthosine are found in rats with myocardial infarction compared with controls.[61] Also, xanthosine is a discriminant compound indicating different diets found in the urine of rats fed by a normal diet and a turkey-based diet.[62] However, proline betaine is a marker of citrus consumption based on orange juice intake.[63] Moreover, the proline betaine signals of the present results correlate with the orange consumption ($r > 0.5$, $p < 0.0001$) reported in the FFQ in the LFD group but also in the MD groups, exhibiting a moderate association of citrus fruit consumption by all the population.

Metabolites Derived from Microbial Metabolism

A number of studies have demonstrated the importance of the gut microbiota in contributing to the excretion of metabolites such as PAGN and TMAO, hippurate, and *p*-cresol, which are often referred to as urinary microbial cometabolites.[64−66] Overall, our results suggest an increase in the activity of microbiota in an MD associated with PAGN and *p*-cresol, while, however, in the LFD group an increase in the gut microbiota-related pathway associated with hippurate and TMAO is shown. Gut microbiota also extensively catabolize protein and aromatic amino acids including phenylalanine and tyrosine, to form PAGN and *p*-cresol.[67,68] Gut microbiota facilitate host energy recovery from dietary sources (which suggests that gut microbiota are an important environmental factor that affects energy harvest from the diet and energy storage in the host).[69] *p*-Cresol is a microbial metabolite associated with both dietary polyphenols and aromatic amino acids (from dietary proteins).[70] The presence of PAGN and *p*-cresol in MD groups may highlight a relationship between microbiota activity and the host metabolism of aromatic compounds.[71]

TMAO was also associated with fish intake; however, no correlation in our samples has been shown with any kind of fish or seafood intake in the LFD group. In addition, TMAO has recently been proposed as a pro-atherogenic species;[72] in this regard, low levels of this metabolite in MD + EVOO present after 3 years of intervention are an interesting focus to explore. Hippurate is a gut microbial mammalian cometabolite of benzoic acid that can be generated by a range of gut microbes from low-molecular-weight aromatic compounds and polyphenols in the gut.[73] Urinary levels of hippurate have been shown to correlate with the obese phenotype in different animal models.[74] Hippurate has also been inversely linked to blood pressure, suggesting a further connection with diet and obesity.[17] However, no correlation with body mass index, hypercholesterolemia, or hypertension has been associated with these signals in the present results. We conclude that differences in hippurate in the LFD group suggest a disturbance of the intestinal microbiota associated with hippurate metabolism. Likewise, higher urinary excretion of metabolites such as hippurate and TMAO was observed in heart failure patients, whereas metabolites such as creatinine, citrate, and *cis*-aconitate (higher in MD groups) were relatively lower.[75]

The present results show important changes in urinary metabolome predominantly at 1-year follow-up for all three groups. Some urinary excretion changes are maintained at the 3-year follow-up. The marked changes are a predominant enhancement in an MD of catabolic-associated pathways. Results suggest a persistent effect of an MD, particularly MD + EVOO, on urinary metabolome for the 3-year follow-up (see significance in Supporting Information Table S1). Further, MD + Nuts also had the same excretion pattern tendency as the MD + EVOO group. Likewise, compliance with both Mediterranean diets was assessed on the whole population of the PREDIMED study with compliance biomarkers for both MD groups,[3] exhibiting an overall good compliance in the interventions. In addition, future steps along similar lines will be taken to evaluate changes in metabolome in a diabetic population linked to Mediterranean diets.

■ CONCLUSIONS

These results have shown that the interventions monitored after 3 years of follow-up had an observed effect on human urinary metabolome. MD groups had a different metabolic fingerprinting compared to baseline and the control (LFD) groups. The most prominent hallmarks of these changes concerning MD groups were related to carbohydrate and lipid metabolism, amino acids (and derived metabolites), and also microbial cometabolites (PAGN and *p*-cresol). Finally, LFD-associated metabolites were hippurate and metabolites related to histidine metabolism, as well as xanthosine. Furthermore, food metabolome metabolites have enabled to associate intervention groups with particular food intakes. MD groups exhibited a moderate association with vegetable and fruit intake. Additionally, a moderate association with meat intake biomarkers was observed in the LFD group. The application of NMR-based metabolomics enabled the classification of individuals regarding their dietary pattern and highlighted the potential of this approach in evaluating changes in the urinary metabolomic fingerprinting of these individuals at different time points of follow-up in response to a particular intervention.

■ ASSOCIATED CONTENT

Supporting Information

Quantitative score of adherence to the Mediterranean diet (Table S1). Baseline characteristics of the participants (Table S2). Summary of parameters for assessing OSC-PLS-DA modeling quality in the training and validation set (Table S3). *P*-values in the ANCOVA test of metabolites derived from multivariate analysis (Table S4). Flowchart of participants evaluated in the subsample of the PREDIMED trial (Figure S1). PCA exploratory data analysis: (A) 3D score plot from individuals and (B) 3D loading plot from the NMR signals before removing acetaminophen signals; (C) 3D score plot from the individuals and (D) 3D loading plot from the NMR signals after removing the acetaminophen signals (Figure S2). Two-dimensional and 3D OSC-PLS-DA score plots of NMR signals deriving from the urine samples collected at 0 days (baseline) MD + EVOO (1-year and 3-year), MD + Nuts (1-year and 3-year), and LFD (1-year and 3-year) (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

dx.doi.org/10.1021/pr5007894 | *J. Proteome Res.* 2015, 14, 531−540

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Supported by the Spanish National Grants from Ministry of Economy and Competitiveness (MINECO) and cofounded by FEDER (Fondo Europeo de Desarrollo Regional): AGL2006-14228-C03-02/ALI, AGL2009-13906-C02-01, AGL2010-10084-E, and AGL2010-22319-C03-03, the CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD (CSD2007-063), CIberOBN an initiative of SCIII, and as well as CNIC-06, PI11/02505, PI13/01172 Project (Plan N de I+D+i 2013-2016) by ISCII-Subdirección General de Evaluación y Fomento de la Investigación. We also thank the award of 2014SGR1566 from the Generalitat de Catalunya's Agency AGAUR. R.V.-F., M.U.-S. and R. Ll. would like to thank the FPI fellowship, the "Ramon y Cajal" programmes of the Spanish Government and the Fondo Social Europeo. We thank the participants for their collaboration in the study.

ABBREVIATIONS

1-MH, 1-methylhistidine; 3-HB, 3-hydroxybutyrate; 3-MH, 3-methylhistidine; 4-DTEA, 4-deoxythreonic acid; ANCOVA, analysis of covariance; BCAA, branched-chain amino acids; CVD, cardiovascular disease; FAO, fatty acid oxidation; EVOO, extra-virgin olive oil; FFQ, food frequency questionnaire; GNG, gluconeogenesis; HCA, hierarchical clustering analysis; IQR, interquartile range; LFD, low-fat diet; MD, Mediterranean diet; MUFA, monounsaturated fatty acids; N-Ac, *N*-acetylglycoproteins; OSC-PLS-DA, partial least-squares discriminant analysis with orthogonal signal correction; PAGN, phenylacetylglutamine; PCA, principal component analysis; PPPAR, proliferator-activated receptor; PUFA, polyunsaturated fatty acids; TCA, tricarboxylic acids; TMAO, trimethylamine-*N*-oxide; U, unidentified; VIP, variable importance in projection

REFERENCES

(1) Solanky, K. S.; Bailey, N. J.; Beckwith-Hall, B. M.; Bingham, S.; Davis, A.; Holmes, E.; Nicholson, J. K.; Cassidy, A. Biofluid 1H NMR-based metabonomic techniques in nutrition research: metabolic effects of dietary isoflavones in humans. *J. Nutr. Biochem.* **2005**, *16* (4), 236–244.

(2) Van Dorsten, F. A.; Daykin, C. A.; Mulder, T. P. J.; Van Duynhoven, J. P. M. Metabonomics approach to determine metabolic differences between green tea and black tea consumption. *J. Agric. Food Chem.* **2006**, *54* (18), 6929–6938.

(3) Estruch, R.; Ros, E.; Salas-Salvadó, J.; Covas, M. I.; Corella, D.; Arós, F.; Gómez-Gracia, E.; Ruiz-Gutiérrez, V.; Fiol, M.; Lapetra, J.; Lamuela-Raventos, R. M.; Serra-Majem, L.; Pintó, X.; Basora, J.; Muñoz, M. A.; Sorlí, J. V.; Martínez, J. A.; Martínez-González, M. A.; Investigators, P. S. Primary prevention of cardiovascular disease with a Mediterranean diet. *N. Engl. J. Med.* **2013**, *368* (14), 1279–90.

(4) Bergouignan, A.; Momken, I.; Schoeller, D. A.; Simon, C.; Blanc, S. Metabolic fate of saturated and monounsaturated dietary fats: the Mediterranean diet revisited from epidemiological evidence to cellular mechanisms. *Prog. Lipid Res.* **2009**, *48* (3–4), 128–47.

(5) Salas-Salvadó, J.; Fernández-Ballart, J.; Ros, E.; et al. Effect of a mediterranean diet supplemented with nuts on metabolic syndrome status: One-year results of the predimed randomized trial. *Arch. Intern. Med.* **2008**, *168* (22), 2449–2458.

(6) Ruiz-Canela, M.; Estruch, R.; Corella, D.; Salas-Salvadó, J.; Martínez-González, M. A. Association of mediterranean diet with peripheral artery disease: The predimed randomized trial. *J. Am. Med. Assoc.* **2014**, *311* (4), 415–417.

(7) Ortega-Azorín, C.; Sorlí, J. V.; Estruch, R.; Asensio, E. M.; Coltell, O.; González, J. I.; Martínez-González, M. Á.; Ros, E.; Salas-Salvadó, J.; Fitó, M.; Arós, F.; Lapetra, J.; Serra-Majem, L.; Ruiz-Gutiérrez, V.; Gómez-Gracia, E.; Fiol, M.; Flores, G.; Pintó, X.; Saiz, C.; Ordovás, J. M.; Corella, D. Amino acid change in the carbohydrate response element binding protein is associated with lower triglycerides and myocardial infarction incidence depending on level of adherence to the Mediterranean diet in the PREDIMED trial. *Circ.: Cardiovasc. Genet.* **2014**, *7* (1), 49–58.

(8) Dragsted, L. O. Biomarkers of meat intake and the application of nutrigenomics. *Meat Sci.* **2010**, *84* (2), 301–307.

(9) Ibarrola-Jurado, N.; Bulló, M.; Guasch-Ferré, M.; Ros, E.; Martínez-González, M. A.; Corella, D.; Fiol, M.; Wärnberg, J.; Estruch, R.; Román, P.; Arós, F.; Vinyoles, E.; Serra-Majem, L.; Pintó, X.; Covas, M.-I.; Basora, J.; Salas-Salvadó, J.; the, P. S. I. Cross-sectional assessment of nut consumption and obesity, metabolic syndrome and other cardiometabolic risk factors: the PREDIMED study. *PLoS One* **2013**, *8* (2), e57367.

(10) Guasch-Ferre, M.; Bullo, M.; Martinez-Gonzalez, M.; Ros, E.; Corella, D.; Estruch, R.; Fito, M.; Aros, F.; Warnberg, J.; Fiol, M.; Lapetra, J.; Vinyoles, E.; Lamuela-Raventos, R.; Serra-Majem, L.; Pinto, X.; Ruiz-Gutierrez, V.; Basora, J.; Salas-Salvado, J. Frequency of nut consumption and mortality risk in the PREDIMED nutrition intervention trial. *BMC Med.* **2013**, *11* (1), 164.

(11) Guasch-Ferre, M.; Hu, F.; Martinez-Gonzalez, M.; Fito, M.; Bullo, M.; Estruch, R.; Ros, E.; Corella, D.; Recondo, J.; Gomez-Gracia, E.; Fiol, M.; Lapetra, J.; Serra-Majem, L.; Munoz, M.; Pinto, X.; Lamuela-Raventos, R.; Basora, J.; Buil-Cosiales, P.; Sorli, J.; Ruiz-Gutierrez, V.; Martinez, J.; Salas-Salvado, J. Olive oil intake and risk of cardiovascular disease and mortality in the PREDIMED Study. *BMC Med.* **2014**, *12* (1), 78.

(12) Martínez-González, M. Á.; Toledo, E.; Arós, F.; Fiol, M.; Corella, D.; Salas-Salvadó, J.; Ros, E.; Covas, M. I.; Fernández-Crehuet, J.; Lapetra, J.; Muñoz, M. A.; Fitó, M.; Serra-Majem, L.; Pintó, X.; Lamuela-Raventós, R. M.; Sorlí, J. V.; Babio, N.; Buil-Cosiales, P.; Ruiz-Gutierrez, V.; Estruch, R.; Alonso, A. Extra-virgin olive oil consumption reduces risk of atrial fibrillation: the PREDIMED trial. *Circulation* **2014**, *130*, 18–26.

(13) Wishart, D. S. Metabolomics: applications to food science and nutrition research. *Trends Food Sci. Technol.* **2008**, *19* (9), 482–493.

(14) Manach, C.; Hubert, J.; Llorach, R.; Scalbert, A. The complex links between dietary phytochemicals and human health deciphered by metabolomics. *Mol. Nutr. Food Res.* **2009**, *53* (10), 1303–15.

(15) Oresic, M. Metabolomics, a novel tool for studies of nutrition, metabolism and lipid dysfunction. *Nutr., Metab. Cardiovasc. Dis.* **2009**, *19* (11), 816–24.

(16) Yap, I. K.; Brown, I. J.; Chan, Q.; Wijeyesekera, A.; Garcia-Perez, I.; Bictash, M.; Loo, R. L.; Chadeau-Hyam, M.; Ebbels, T.; De Iorio, M.; Maibaum, E.; Zhao, L.; Kesteloot, H.; Daviglus, M. L.; Stamler, J.; Nicholson, J. K.; Elliott, P.; Holmes, E. Metabolome-wide association study identifies multiple biomarkers that discriminate north and south Chinese populations at differing risks of cardiovascular disease: INTERMAP study. *J. Proteome Res.* **2010**, *9* (12), 6647–54.

(17) Holmes, E.; Loo, R. L.; Stamler, J.; Bictash, M.; Yap, I. K.; Chan, Q.; Ebbels, T.; De Iorio, M.; Brown, I. J.; Veselkov, K. A.; Daviglus, M. L.; Kesteloot, H.; Ueshima, H.; Zhao, L.; Nicholson, J. K.; Elliott, P. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature* **2008**, *453* (7193), 396–400.

(18) O'Sullivan, A.; Gibney, M. J.; Brennan, L. Dietary intake patterns are reflected in metabolomic profiles: potential role in dietary assessment studies. *Am. J. Clin. Nutr.* **2011**, *93* (2), 314–21.

(19) Xu, J.; Yang, S.; Cai, S.; Dong, J.; Li, X.; Chen, Z. Identification of biochemical changes in lactovegetarian urine using 1H NMR

spectroscopy and pattern recognition. *Anal. Bioanal. Chem.* **2010**, *396* (4), 1451−63.
(20) McNiven, E. M. S.; German, J. B.; Slupsky, C. M. Analytical metabolomics: nutritional opportunities for personalized health. *J. Nutr. Biochem.* **2011**, *22* (11), 995−1002.
(21) Estruch, R.; Martínez-González, M. A.; Corella, D.; Salas-Salvadó, J.; Ruiz-Gutiérrez, V.; Covas, M. I.; Fiol, M.; Gómez-Gracia, E.; López-Sabater, M. C.; Vinyoles, E.; Arós, F.; Conde, M.; Lahoz, C.; Lapetra, J.; Sáez, G.; Ros, E.; PREDIMED Study Investigators. Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Ann. Int. Med.* **2006**, *145* (1), 1−11.
(22) Fernández-Ballart, J. D.; Piñol, J. L.; Zazpe, I.; Corella, D.; Carrasco, P.; Toledo, E.; Perez-Bauer, M.; Martínez-González, M. Á.; Salas-Salvadó, J.; Martín-Moreno, J. M. Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *Br. J. Nutr.* **2010**, *103* (12), 1808−1816.
(23) Martínez-González, M. Á.; Corella, D.; Salas-Salvadó, J.; Ros, E.; Covas, M. I.; Fiol, M.; Wärnberg, J.; Arós, F.; Ruíz-Gutiérrez, V.; Lamuela-Raventós, R. M.; Lapetra, J.; Muñoz, M. Á.; Martínez, J. A.; Sáez, G.; Serra-Majem, L.; Pintó, X.; Mitjavila, M. T.; Tur, J. A.; Portillo, M. d. P.; Estruch, R. Cohort profile: design and methods of the PREDIMED study. *Int. J.Epidemiol.* **2012**, *41* (2), 377−385.
(24) Vázquez-Fresno, R.; Llorach, R.; Alcaro, F.; Rodríguez, M.; Vinaixa, M.; Chiva-Blanch, G.; Estruch, R.; Correig, X.; Andrés-Lacueva, C. (1) H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors. *Electrophoresis* **2012**, *33* (15), 2345−54.
(25) Xia, J.; Mandal, R.; Sinelnikov, I. V.; Broadhurst, D.; Wishart, D. S. MetaboAnalyst 2.0: a comprehensive server for metabolomic data analysis. *Nucleic Acids Res.* **2012**, *40*, W127−33.
(26) Madsen, R.; Lundstedt, T.; Trygg, J. Chemometrics in metabolomic: a review in human disease diagnosis. *Anal. Chim. Acta* **2010**, *659* (1−2), 23−33.
(27) Llorach-Asunción, R.; Jauregui, O.; Urpi-Sarda, M.; Andres-Lacueva, C. Methodological aspects for metabolome visualization and characterization: A metabolomic evaluation of the 24 h evolution of human urine after cocoa powder consumption. *J. Pharm. Biomed. Anal.* **2010**, *51* (2), 373−381.
(28) Weljie, A. M.; Dowlatabadi, R.; Miller, B. J.; Vogel, H. J.; Jirik, F. R. An inflammatory arthritis-associated metabolite biomarker pattern revealed by 1H NMR spectroscopy. *J. Proteome Res.* **2007**, *6* (9), 3456−3464.
(29) Van Der Voet, H. Comparing the predictive accuracy of models using a simple randomization test. *Chemom. Intell. Lab. Syst.* **1994**, *25* (2), 313−323.
(30) Szymańska, E.; Saccenti, E.; Smilde, A.; Westerhuis, J. Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics* **2012**, *8* (1), 3−16.
(31) O'Sullivan, A.; Gibney, M. J.; Brennan, L. Dietary intake patterns are reflected in metabolomic profiles: potential role in dietary assessment studies. *Am. J. Clin. Nutr.* **2011**, *93* (2), 314−321.
(32) Eriksson, L.; Johansson, E.; Kettaneh-Wold, N.; Trygg, J.; Wikström, C.; Wold, S. *Multi- and Megavariate Data Analysis*; Umetrics: Umeå, Sweden, 2001.
(33) Chong, I.-G.; Jun, C.-H. Performance of some variable selection methods when multicollinearity is present. *Chemom. Intell. Lab. Syst.* **2005**, *78* (1−2), 103−112.
(34) Gosselin, R.; Rodrigue, D.; Duchesne, C. A Bootstrap-VIP approach for selecting wavelength intervals in spectral imaging applications. *Chemom. Intell. Lab. Syst.* **2010**, *100* (1), 12−21.
(35) Caraux, G.; Pinloche, S. PermutMatrix: a graphical environment to arrange gene expression profiles in optimal linear order. *Bioinformatics* **2005**, *21* (7), 1280−1281.
(36) Hageman, J. A.; Berg, R. A.; Westerhuis, J. A.; Werf, M. J.; Smilde, A. K. Genetic algorithm based two-mode clustering of metabolomics data. *Metabolomics* **2008**, *4* (2), 141−149.
(37) Li, T.; Dai, L.; Li, L.; Hu, X.; Dong, L.; Li, J.; Salim, S. K.; Fu, J.; Zhong, H. Typing of unknown microorganisms based on quantitative analysis of fatty acids by mass spectrometry and hierarchical clustering. *Anal. Chim. Acta* **2011**, *684* (1−2), 112−20.
(38) Holmes, E.; Loo, R. L.; Cloarec, O.; Coen, M.; Tang, H.; Maibaum, E.; Bruce, S.; Chan, Q.; Elliott, P.; Stamler, J.; Wilson, I. D.; Lindon, J. C.; Nicholson, J. K. Detection of urinary drug metabolite (xenometabolome) signatures in molecular epidemiology studies via statistical total correlation (NMR) spectroscopy. *Anal. Chem.* **2007**, *79* (7), 2629−2640.
(39) Ballevre, O.; Prugnaud, J.; Houlier, M. L.; Arnal, M. Assessment of threonine metabolism in vivo by gas chromatography/mass spectrometry and stable isotope infusion. *Anal. Biochem.* **1991**, *193* (2), 212−9.
(40) Diaz, S. O.; Barros, A. S.; Goodfellow, B. J.; Duarte, I. F.; Galhano, E.; Pita, C.; Almeida, M. d. C.; Carreira, I. M.; Gil, A. M. Second trimester maternal urine for the diagnosis of trisomy 21 and prediction of poor pregnancy outcomes. *J. Proteome Res.* **2013**, *12* (6), 2946−2957.
(41) Diaz, S. l. O.; Pinto, J.; Graça, G. a.; Duarte, I. F.; Barros, A. n. S.; Galhano, E. l.; Pita, C.; Almeida, M. d. C. u.; Goodfellow, B. J.; Carreira, I. M.; Gil, A. M. Metabolic biomarkers of prenatal disorders: an exploratory NMR metabonomics study of second trimester maternal urine and blood plasma. *J. Proteome Res.* **2011**, *10* (8), 3732−3742.
(42) Houten, S. M.; Wanders, R. J. A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation. *J. Inherited Metab. Dis.* **2010**, *33* (5), 469−77.
(43) Nicholls, A. W.; Nicholson, J. K.; Haselden, J. N.; Waterfield, C. J. A metabonomic approach to the investigation of drug-induced phospholipidosis: an NMR spectroscopy and pattern recognition study. *Biomarkers* **2000**, *5* (6), 410−423.
(44) Wightman, F.; Lighty, D. L. Identification of phenylacetic acid as a natural auxin in the shoots of higher plants. *Phys. Plant.* **1982**, *55* (1), 17−24.
(45) Zhao, X.; Fritsche, J.; Wang, J.; Chen, J.; Rittig, K.; Schmitt-Kopplin, P.; Fritsche, A.; Häring, H.-U.; Schleicher, E.; Xu, G.; Lehmann, R. Metabonomic fingerprints of fasting plasma and spot urine reveal human pre-diabetic metabolic traits. *Metabolomics* **2010**, *6* (3), 362−374.
(46) Wyss, M.; Kaddurah-Daouk, R. Creatine and creatinine metabolism. *Physiol. Rev.* **2000**, *80* (3), 1107−1213.
(47) Yang, D.; Brunengraber, H. Glutamate, a window on liver intermediary metabolism. *J. Nutr.* **2000**, *130* (4), 991−991.
(48) Miao, H.; Chen, H.; Zhang, X.; Yin, L.; Chen, D.-Q.; Cheng, X.-L.; Bai, X.; Wei, F. Urinary metabolomics on the biochemical profiles in diet-induced hyperlipidemia rat using ultraperformance liquid chromatography coupled with quadrupole time-of-flight SYNAPT high-definition mass spectrometry. *J. Anal. Methods Chem.* **2014**, *2014*, 9.
(49) Newsholme, P.; Procopio, J.; Lima, M. M. R.; Pithon-Curi, T. C.; Curi, R. Glutamine and glutamate: their central role in cell metabolism and function. *Cell Biochem. Funct.* **2003**, *21* (1), 1−9.
(50) Savage, G. P. Chemical composition of walnuts (*Juglans regia* L.) grown in New Zealand. *Plant Foods Hum. Nutr.* **2001**, *56* (1), 75−82.
(51) Köksal, A. İ.; Artik, N.; Şimşek, A.; Güneş, N. Nutrient composition of hazelnut (*Corylus avellana* L.) varieties cultivated in Turkey. *Food Chem.* **2006**, *99* (3), 509−515.
(52) Seron, L. H.; Poveda, E. G.; Prats Moya, M. S.; Martín Carratalá, M. L.; Berenguer-Navarro, V.; Grané-Teruel, N. Characterisation of 19 almond cultivars on the basis of their free amino acids composition. *Food Chem.* **1998**, *61* (4), 455−459.
(53) Zhang, Q.; Wang, G.; Du, Y.; Zhu, L.; A, J. GC/MS analysis of the rat urine for metabonomic research. *J. Chromatogr. B* **2007**, *854* (1−2), 20−25.
(54) Bouatra, S.; Aziat, F.; Mandal, R.; Guo, A. C.; Wilson, M. R.; Knox, C.; Bjorndahl, T. C.; Krishnamurthy, R.; Saleem, F.; Liu, P.; Dame, Z. T.; Poelzer, J.; Huynh, J.; Yallou, F. S.; Psychogios, N.; Dong, E.; Bogumil, R.; Roehring, C.; Wishart, D. S. The human urine metabolome. *PLoS One* **2013**, *8* (9), e73076.

(55) Gao, X.; Zhao, A.; Zhou, M.; Lin, J.; Qiu, Y.; Su, M.; Jia, W. GC/MS-based urinary metabolomics reveals systematic differences in metabolism and ethanol response between Sprague−Dawley and Wistar rats. *Metabolomics* **2011**, *7* (3), 363−374.
(56) Jin, S. J.; Tserng, K. Y. Metabolic origins of urinary unsaturated dicarboxylic acids. *Biochemistry* **1990**, *29* (37), 8540−8547.
(57) Tulipani, S.; Llorach, R.; Jáuregui, O.; López-Uriarte, P.; Garcia-Aloy, M.; Bullo, M.; Salas-Salvadó, J.; Andrés-Lacueva, C. Metabolomics unveils urinary changes in subjects with metabolic syndrome following 12-week nut consumption. *J. Proteome Res.* **2011**, *10* (11), 5047−5058.
(58) Luo, D. X.; Cao, D. L.; Xiong, Y.; Peng, X. H.; Liao, D. F. A novel model of cholesterol efflux from lipid-loaded cells. *Acta Pharmacol. Sin.* **2010**, *31* (10), 1243−57.
(59) Mandard, S.; Müller, M.; Kersten, S. Peroxisome proliferator-activated receptor alpha target genes. *Cell. Mol. Life Sci.* **2004**, *61* (4), 393−416.
(60) Aranibar, N.; Vassallo, J. D.; Rathmacher, J.; Stryker, S.; Zhang, Y.; Dai, J.; Janovitz, E. B.; Robertson, D.; Reily, M.; Lowe-Krentz, L.; Lehman-McKeeman, L. Identification of 1- and 3-methylhistidine as biomarkers of skeletal muscle toxicity by nuclear magnetic resonance-based metabolic profiling. *Anal. Biochem.* **2011**, *410* (1), 84−91.
(61) Jiang, P.; Dai, W.; Yan, S.; Chen, Z.; Xu, R.; Ding, J.; Xiang, L.; Wang, S.; Liu, R.; Zhang, W. Potential biomarkers in the urine of myocardial infarction rats: a metabolomic method and its application. *Mol. BioSyst.* **2011**, *7* (3), 824−831.
(62) Gu, H.; Chen, H.; Pan, Z.; Jackson, A. U.; Talaty, N.; Xi, B.; Kissinger, C.; Duda, C.; Mann, D.; Raftery, D.; Cooks, R. G. Monitoring diet effects via biofluids and their implications for metabolomics studies. *Anal. Chem.* **2006**, *79* (1), 89−97.
(63) Heinzmann, S. S.; Brown, I. J.; Chan, Q.; Bictash, M.; Dumas, M.-E.; Kochhar, S.; Stamler, J.; Holmes, E.; Elliott, P.; Nicholson, J. K. Metabolic profiling strategy for discovery of nutritional biomarkers: proline betaine as a marker of citrus consumption. *Am. J. Clin. Nutr.* **2010**, *92* (2), 436−443.
(64) Holmes, E.; Li, J. V.; Marchesi, J. R.; Nicholson, J. K. Gut microbiota composition and activity in relation to host metabolic phenotype and disease risk. *Cell Metabolism* **2012**, *16* (5), 559−564.
(65) Lees, H. J.; Swann, J. R.; Wilson, I. D.; Nicholson, J. K.; Holmes, E. Hippurate: the natural history of a mammalian-microbial cometabolite. *J. Proteome Res.* **2013**, *12* (4), 1527−1546.
(66) Martin, F. P.; Wang, Y.; Sprenger, N.; Yap, I. K.; Lundstedt, T.; Lek, P.; Rezzi, S.; Ramadan, Z.; van Bladeren, P.; Fay, L. B.; Kochhar, S.; Lindon, J. C.; Holmes, E.; Nicholson, J. K. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. *Mol. Syst. Biol.* **2008**, *4*, 157.
(67) Díaz, E.; Ferrández, A.; Prieto, M. a. A.; García, J. L. Biodegradation of aromatic compounds by *Escherichia coli*. *Microbiol. Mol. Biol. Rev.* **2001**, *65* (4), 523−569.
(68) Selmer, T.; Andrei, P. I. *p*-Hydroxyphenylacetate decarboxylase from *Clostridium difficile*. *Eur. J. Biochem.* **2001**, *268* (5), 1363−1372.
(69) Bäckhed, F.; Ding, H.; Wang, T.; Hooper, L. V.; Koh, G. Y.; Nagy, A.; Semenkovich, C. F.; Gordon, J. I. The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (44), 15718−15723.
(70) Lord, R. S.; Bralley, J. A. Clinical applications of urinary organic acids. Part 2. Dysbiosis markers. *Altern. Med. Rev.* **2008**, *13* (4), 292−306.
(71) Rezzi, S.; Ramadan, Z.; Martin, F.-P. J.; Fay, L. B.; van Bladeren, P.; Lindon, J. C.; Nicholson, J. K.; Kochhar, S. Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals. *J. Proteome Res.* **2007**, *6* (11), 4469−4477.
(72) Koeth, R. A.; Wang, Z.; Levison, B. S.; Buffa, J. A.; Org, E.; Sheehy, B. T.; Britt, E. B.; Fu, X.; Wu, Y.; Li, L.; Smith, J. D.; DiDonato, J. A.; Chen, J.; Li, H.; Wu, G. D.; Lewis, J. D.; Warrier, M.; Brown, J. M.; Krauss, R. M.; Tang, W. H.; Bushman, F. D.; Lusis, A. J.; Hazen, S. L. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat. Med.* **2013**, *19* (5), 576−85.
(73) Nicholson, J. K.; Holmes, E.; Wilson, I. D. Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.* **2005**, *3* (5), 431−8.
(74) Calvani, R.; Miccheli, A.; Capuani, G.; Tomassini Miccheli, A.; Puccetti, C.; Delfini, M.; Iaconelli, A.; Nanni, G.; Mingrone, G. Gut microbiome-derived metabolites characterize a peculiar obese urinary metabotype. *Int. J. Obes.* **2010**, *34* (6), 1095−8.
(75) Kang, S. M.; Park, J. C.; Shin, M. J.; Lee, H.; Oh, J.; Ryu do, H.; Hwang, G. S.; Chung, J. H. (1)H nuclear magnetic resonance based metabolic urinary profiling of patients with ischemic heart failure. *Clin. Biochem.* **2011**, *44* (4), 293−9.
(76) Jiang, C. Y.; Yang, K. M.; Yang, L.; Miao, Z. X.; Wang, Y. H.; Zhu, H. B. A H NMR-based metabonomic investigation of time-related metabolic trajectories of the plasma, urine and liver extracts of hyperlipidemic hamsters. *PLoS One* **2013**, *8* (6), e66786.
(77) Holmes, E.; Foxall, P. J.; Spraul, M.; Farrant, R. D.; Nicholson, J. K.; Lindon, J. C. 750 MHz 1H NMR spectroscopy characterisation of the complex metabolic pattern of urine from patients with inborn errors of metabolism: 2-hydroxyglutaric aciduria and maple syrup urine disease. *J. Pharm. Biomed. Anal.* **1997**, *15* (11), 1647−59.
(78) Appiah-Amponsah, E.; Shanaiah, N.; Nagana Gowda, G. A.; Owusu-Sarfo, K.; Ye, T.; Raftery, D. Identification of 4-deoxythreonic acid present in human urine using HPLC and NMR techniques. *J. Pharm. Biomed. Anal.* **2009**, *50* (5), 878−885.
(79) Martin, F.-P. J.; Dumas, M.-E.; Wang, Y.; Legido-Quigley, C.; Yap, I. K. S.; Tang, H.; Zirah, S.; Murphy, G. M.; Cloarec, O.; Lindon, J. C.; Sprenger, N.; Fay, L. B.; Kochhar, S.; van Bladeren, P.; Holmes, E.; Nicholson, J. K. A top-down systems biology view of microbiome-mammalian metabolic interactions in a mouse model. *Mol. Syst. Biol.* **2007**, *3* (1), n/a−n/a.
(80) Wang, Y.; Lawler, D.; Larson, B.; Ramadan, Z.; Kochhar, S.; Holmes, E.; Nicholson, J. K. Metabonomic investigations of aging and caloric restriction in a life-long dog study. *J. Proteome Res.* **2007**, *6* (5), 1846−1854.
(81) Pechlivanis, A.; Kostidis, S.; Saraslanidis, P.; Petridou, A.; Tsalis, G.; Mougios, V.; Gika, H. G.; Mikros, E.; Theodoridis, G. A. 1H NMR-based metabonomic investigation of the effect of two different exercise sessions on the metabolic fingerprint of human urine. *J. Proteome Res.* **2010**, *9* (12), 6405−6416.
(82) Xu, J.; Liu, C.; Cai, S.; Dong, J.; Li, X.; Feng, J.; Chen, Z. Metabolomic profilings of urine and serum from high fat-fed rats via 1H NMR spectroscopy and pattern recognition. *Appl. Biochem. Biotechnol.* **2013**, *169* (4), 1250−1261.

Supporting Information

Table S1. Quantitative Score of Adherence to the Mediterranean Diet

Food and Frequency of Consumption	Criteria for 1 Point*
1. Do you use olive oil as main culinary fat?	Yes
2. How much olive oil do you consume in a given day (including oil used for frying, salads, out-of-house meals, etc.)?	4 or more tablespoons
3. How many vegetable servings do you consume per day? (1 serving = 200g [consider side dishes as 1/2 serving])	2 or more (at least 1 portion raw or as salad)
4. How many fruit units (including natural fruit juices) do you consume per day?	3 or more
5. How many servings of red meat, hamburger or meat products (ham, sausage, etc.) do you consume per day? (1 serving = 100-150 g)	Less than 1
6. How many servings of butter, margarine or cream do you consume per day? (1 serving = 12g)	Less than 1
7. How many sweet or carbonated beverages do you drink per day?	Less than 1
8. How much wine do you drink per week?	7 or more glasses
9. How many servings of legumes do you consume per week? (1 serving = 150 g)	3 or more
10. How many servings of fish or shellfish do you consume per week? (1 serving = 100-150 g of fish or 4-5 units or 200 g of shellfish)	3 or more
11. How many times per week do you consume commercial sweets or pastries (not home-made), such as cakes, cookies, biscuits or custard?	Less than 2
12. How many servings of nuts (including peanuts) do you consume per week? (1 serving = 30 g)	3 or more
13. Do you preferentially consume chicken, turkey or rabbit meat instead of veal, pork, hamburger,or sausage?	Yes
14. How many times per week do you consume vegetables, pasta, rice or other dishes seasoned with *sofrito* (sauce made with tomato and onion, leek or garlic and simmered with olive oil)?	2 or more

* 0 points if these criteria are not met.

Table S2. Baseline characteristic of the non-diabetic sub-cohort (n = 98) evaluated in the present study

	Baseline Characteristics
Mean age, years (SD)	66.6 (5.7)
Men, n (%)	28 (28.7)
Energy intake, kcal/d (SD)	2411.9 (615.5)
BMI, Mean (SD) Kg/m^2	30.2 (3.8)
Current smokers, n (%)	10 (10.2)
Hypertension, n (%)	89 (90.8)
Hypercholesterolemia, n (%)	84 (85.7)

SD: standard deviation.

Supporting Information

Table S3. Summary of parameters for assessing OSC-PLS-DA modelling quality after removing samples (25%). This procedure has been repeated four times using different samples each time as validation set

Training set (75%)	N[a]	R^2Y_{cum}[b]	Q^2_{cum}[b]	Correctly classified individuals in validation set (%)
Training set 1	6	0.752	0.541	91.9%
Training set 2	6	0.744	0.527	97.3%
Training set 3	6	0.758	0.557	100%
Training set 4	6	0.751	0.531	98.63%
Mean ± SD	6	0.751±0.005	0.539±0.013	96.94%

[a]N, number of components.

[b]R^2Y_{cum} is the cumulative modeled variations in the Y matrix, and Q^2Y_{cum} is the cumulative predicted variation in the Y matrix.

SD: standard deviation

Supporting Information

Table S4. Misclassification table of subjects resulting from hierarchical cluster analysis (HCA) (selected features performed with VIP>1.5 from the OSC-PLS-DA model)

Individuals from each group		HCA clusters	Members in each HCA cluster				Correct	Misclassified
			B	LFD (1+3y)	MD+EVOO (1+3y)	MD+Nuts (1+3y)		
B	98	Cluster A	**84**	19	3	5	86%	14%
LFD	60[1]	Cluster B	0	**41**	2	0	68%	32%
MD+EVOO	82[1]	Cluster C	10	0	**76**	3	93%	7%
MD+Nuts	54[1]	Cluster D	4	0	1	**46**	85%	15%
Total	294	Total	98	60	82	54	84%	16%

B, baseline; [1]sum of the individuals with the same treatment in 1 and 3 years of follow-up. 1+3y, sum of 1- and 3-year periods.

Supporting Information

Table S5. *P*-values in ANCOVA test of metabolites derived from multivariate analysis. For direction of change in ANCOVA analysis, (↑) indicates a relatively higher metabolite urinary excretion, (↓) indicates a relatively lower excretion. Dashed line indicates no statistical significance in ANCOVA analysis ($p<0.05$). * Statistical significance from the baseline (t-test, $p<0.05$).

Metabolite ID	**δ (multiplicity)**	**1-year ANCOVA (*p*)**			**3-year ANCOVA (*p*)**		
MD associated metabolites		**E vs. L**	**N vs. L**	**E vs. N**	**E vs. L**	**N vs. L**	**E vs. N**
3-HB	1.20 (d)	(↑)$4.47x10^{-7*}$	(↑)0.003^{*}	-	(↑)$1.75x10^{-5*}$	(↑)0.006^{*}	-
Leucine	0.93 (d)	(↑)0.002^{*}	(↑)0.007^{*}	-	(↑)0.03^{*}	(↑)0.01^{*}	-
Isobutyric acid	1.06 (d)	(↑)0.02^{*}	(↑)0.009^{*}	-	(↑)$3.79x10^{-7*}$	(↑)$3.52x10^{-5*}$	-
2-oxoisovaleric	1.14 (d)	-	-	-	(↑)0.004^{*}	-	-
4-DTEA	1.22 (d)	(↑)$1.93x10^{-5*}$	(↑)$6.71x10^{-4*}$	-	(↑)$3.84x10^{-5*}$	(↑)$3.05x10^{-7*}$	-
N-Ac	2.06 (m)	(↑)0.001^{*}	-	-	(↑)$7.26\ x10^{-5*}$	-	-
Glycine	3.57 (s)	-	-	-	(↑)0.03	(↑)0.03	-
***p*-cresol**	2.34 (s) 7.22 (d) 7.27 (d)	(↑)0.001^{*}	-	-	-	(↑)0.02^{*}	(↓)0.001^{*}
Suberic acid	1.29 (m) 1.60 (t)	(↑)$9.33x10^{-6*}$	(↑)0.02^{*}	-	(↑)0.001^{*}	(↑)$2.06x10^{-6*}$	(↓)0.02^{*}
Oleic acid	1.33 (m) 1.63 (m) 1.80 (m) 2.00 (m) 0.89 (t) 2.37 (t)	(↑)$6.68x10^{-5*}$	(↑)0.01^{*}	-	(↑)0.001^{*}	(↑)0.01^{*}	-
Proline	1.95 (m) 2.06 (m) 3.38dt 4.12dd	(↑)$1.84x10^{-5*}$	(↑)$1.19x10^{-4*}$	-	(↑)0.002^{*}	(↑)$6.14x10^{-5*}$	-
U3.46	3.46 (s)	(↑)0.001^{*}	(↑)0.009	-	(↑)0.04^{*}	(↑)0.02^{*}	-
U3.33	3.33 (m)	-	-	-	-	-	-
U3.50	3.50(s)	-	-	-	(↑)$3x10^{-6*}$	(↑)$1.8\ x10^{-4*}$	-
U3.81	3.81(s)	-	(↑)0.001	-	(↑)0.002^{*}	(↑)$3\ x10^{-5*}$	-
Metabolite ID	**δ** (multiplicity)	**1-year ANCOVA (*p*)**			**3-year ANCOVA (*p*)**		
MD+Nuts associated		**N vs. E**	**N vs. L**	**E vs. L**	**N vs. E**	**N vs. L**	**E vs. L**

metabolites							
PAGN	1.93 (m) 2.25 (t) 3.67 (m) 4.18 (m) 7.36[##] 7.42 (t)	(↑)0.01[*]	(↑)6.90x10^{-7*}	(↑)0.004[*]	-	(↑)7.56x10^{-6*}	(↑)6.90x10^{-7*}
N-AGN	2.08 (m) 2.26 (m) 4.18 (m)	(↑)0.001[*]	(↑)3.27x10^{-9*}	(↑)0.001[*]	(↑)0.06[*]	(↑)4.04x10^{-11*}	(↑)3.56x10^{-8*}
Creatine	3.04 (s) 3.93 (s)	-	-	-	(↑)9.07x10^{-7*}	(↑)1.47x10^{-6*}	-
U5.02	5.02 (t)	(↓)1.05x10^{-5*}	(↓)2.44x10^{-8*}	-	-	-	-
U5.15	5.15 (t)	(↓)0.00001[*]	(↓)2.18x10^{-4*}	-	-	(↑)0.001[*]	(↑)0.004[*]
U6.76	6.76 (m)	-	(↑)0.003[*]	-	(↑)7.66x10^{-7*}	(↑)6.83x10^{-5}	-
Metabolite ID	**δ** (multiplicity)	**1-year ANCOVA (*p*)**			**3-year ANCOVA (*p*)**		
MD+EVOO associated metabolites		**E vs. N**	**E vs. L**	**N vs. L**	**E vs. N**	**E vs. L**	**N vs. L**
Creatinine	3.05 (s) 4.06 (s)	(↑)0.001	(↑)0.008	-	(↑)0.002[*]	(↑)4.49x10^{-4*}	-
Citrate	2.55 (dd) 2.69 (dd)	(↑)3.41x10^{-6*}	(↑)0.005[*]	-	-	(↑)2.3x10^{-4*}	-
cis-aconitate	5.74 (s) 3.12 (s)	(↑)0.002[*]	-	(↑)0.001[*]	-	-	-
U7.50	7.50 (d)	(↓)1.0x10^{-5*}	(↓)3.9x10^{-4*}	-	-	-	-
U8.49	8.49(s)	-	(↓)0.03	-	-	(↓)0.02[*]	-
Metabolite ID	**δ** (multiplicity)	**1-year ANCOVA (*p*)**			**3-year ANCOVA (*p*)**		
LFD associated metabolites		**L vs. E**	**L vs. N**	**E vs. N**	**L vs. E**	**L vs. N**	**E vs. N**
Hippurate	3.97 (d) 7.54 (t) 7.64 (t) 7.83 (d) 8.54 (bb)	(↑)5.23x10^{-15*}	(↑)1.7x10^{-16*}	-	(↑)2.13x10^{-11*}	(↑)0.001[*]	(↓)5.27x10^{-4*}
TMAO	3.28 (s)	-	-	(↓)0.003	(↑)9.56x10^{-5*}	-	(↓)0.03[*]
Anserine	3. 78 (s) 8.28 (s)	(↑)1.67x10^{-5*}	(↑)0.01	-	(↓)0.006[*]	-	-
Histidine	7.08 (s) 7.93 (s)	(↑)0.002[*]	(↑)0.007[*]	-	-	-	-
3-MH	7.05 (s) 7.95(s)	(↑)3.59x10^{-6*}	-	(↓)0.02[*]	-	-	-
1-MH	7.73 (s) 7.01 (s)	-	0.01(↑)	0.03(↓)	-	-	-
Carnosine	8.11 (s) 7.18(s)	-	(↑)0.04	-	(↑)0.03[*]	-	-
Proline betaine	3.11 (s) 3.30 (s)	-	-	-	(↑)5.87x10^{-7*}	(↑)6.53x10^{-13*}	(↑)0.001[*]
Xanthosine	5.87 (d)	-	(↑)0.005[*]	(↑)0.02[*]	(↑)0.01[*]	-	-

Footnotes: 3-HB: 3-hydroxybutyrate; 4-DTEA: 4 deoxythreonic acid; bb: broad band; d: doublet; E: MD+EVOO; L: LFD; N: MD+Nuts; N-Ac: N-acetylglycoproteins; N-AGN: N-acetylglutamine; m: multiplet; MH: methylhistidine; PAGN: phenylacetylglutamine; s: singlet; t: triplet; TMAO: trimethylamine-N-oxide; U: unassigned signals. [**]signal excluded from the analysis because it overlapped with a signal of acetaminophen.

Supporting Information

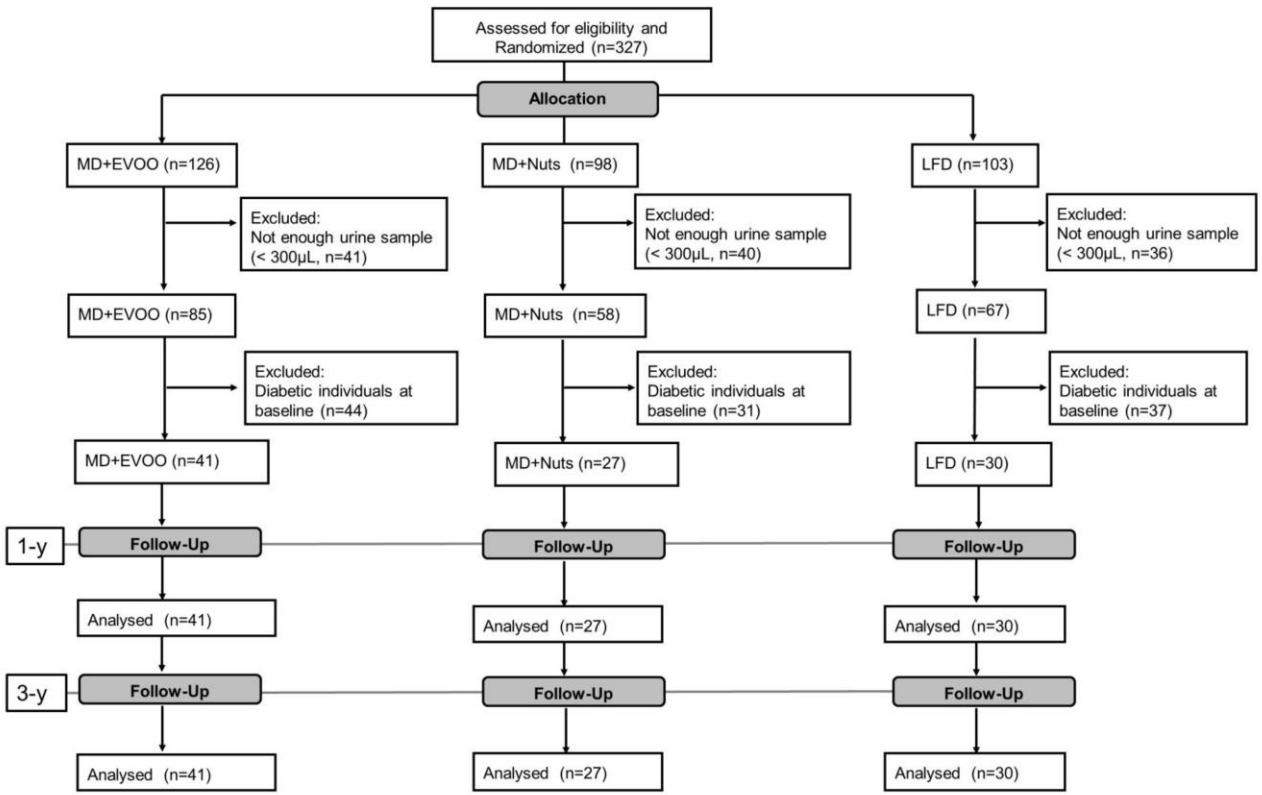

Figure S1. Flow chart of participants evaluated in the subsample of the PREDIMED trial.

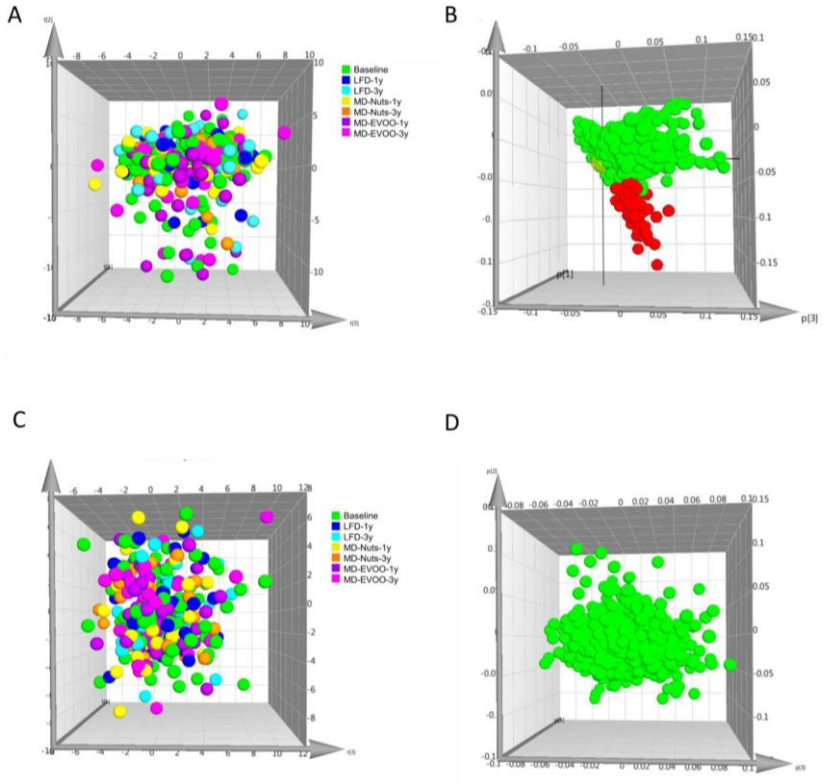

Figure S2. PCA exploratory data analysis. (A) 3D score plot from individuals before removing acetaminophen signals, and (B) 3D loading plot from the NMR signals before removing acetaminophen signals. (C) 3D Score plot from the individuals after removing the acetaminophen signals, and (D) 3D loading plot from the NMR signals after removing the acetaminophen signals. Red spheres from (B) indicate the acetaminophen signals.

Supporting Information

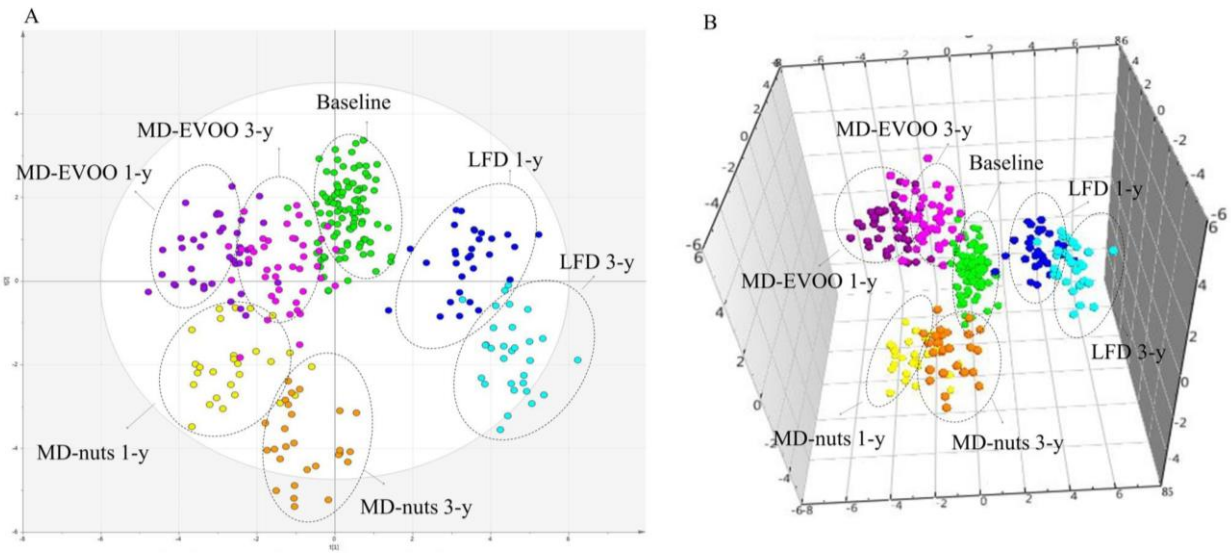

Figure S3. 2D OSC-PLS-DA score plot (A) and 3D OSC-PLSDA score plot (B) of NMR signals derived from the urine samples collected at 0 days (baseline, green sphere), MD+EVOO (1-year: purple spheres; 3-year: orange spheres), MD+Nuts (1-year: yellow spheres; 3-year: light blue) and LFD (1-year: dark blue spheres; 3-year: red spheres).

DISCUSIÓN GENERAL

Si bien se presenta una discusión individual para los diferentes estudios que componen los resultados de esta Tesis Doctoral (ver compendio de publicaciones en el apartado Resultados), este capítulo pretende ofrecer una visión global a modo de discusión general de todos los resultados obtenidos.

Desarrollo e implementación de la metodología mediante RMN para la preparación, procesado y análisis de muestras de orina en estudios nutricionales.

La primera parte de la presente Tesis Doctoral comprende el desarrollo e implementación del método de preparación de muestra, los parámetros del equipo para su procesado mediante RMN y el análisis de los datos. Para tal efecto, la metodología de preparación de las muestras, y los parámetros del equipo, fueron implementados a partir de muestras de un estudio cruzado aleatorizado y controlado tras la intervención con vino tinto, vino tinto desalcoholizado y ginebra en individuos varones de un rango de edad concreto (>55 años) (publicación 1). El hecho de ser un estudio cruzado aleatorizado y controlado posee una serie de ventajas, como ser un estudio estadísticamente eficaz, además de reducirse la posible influencia por las covariables confusoras ya que cada paciente sirve propiamente como control. Este tipo de estudios dan resultados más precisos (Elbourne *et al.* 2002) por lo que son recomendables en estudios nutricionales (Scalbert *et al.* 2009). Además, al tener solo individuos varones de un determinado rango de edad hay un mayor control sobre la variabilidad interindividual ya que no se ve influida aquélla asociada al género y edad (Kochhar *et al.* 2006), y por ello obtener unos resultados metodológicos más robustos y reproducibles. Un análisis univariante es un análisis supervisado, así, cualquier marcador identificado por esta aproximación ha de validarse en otro estudio independientemente

(Scalbert *et al.* 2014). Inicialmente se realizó un análisis univariante en el estudio con vino (publicación 1)(Vázquez-Fresno *et al.* 2012) para tener una aproximación global del perfil metabolómico, y después se realizó una aproximación mediante la predicción con regresión logística y curvas ROC en ese mismo estudio, con la consiguiente reproducción en un estudio epidemiológico con otra población (publicación 2) (Vázquez-Fresno *et al.* 2014b).

Seguidamente se pretendió aplicar las aproximaciones metabolómicas multivariantes para reducir la multidimensionalidad de los datos que nos proporciona los estudios metabolómicos con RMN en intervenciones nutricionales. Para la aplicación de técnicas multivariantes que discriminan clases o grupos, es preferible iniciar la aplicación en un estudio sencillo, un modelo sencillo con únicamente dos clases (Westerhuis *et al.* 2008). Por ello, se aplicó en el estudio de intervención con el complemento alimenticio el cual constaba de dos puntos de muestreo (es decir, dos clases): antes y después de la intervención.

Un aspecto a destacar fue el siguiente paso metodológico que consistió en adaptar la metodología analítica a menores volúmenes y ser aplicada a estudios clínicos y epidemiológicos con un gran número de muestras (>250 muestras) como fue el caso del estudio PREDIMED. Además, en estudios nutricionales complejos, puede ser útil el uso combinado de análisis multivariante y univariante (PLS-DA y ANOVA) (Fardet *et al.* 2008; Ross *et al.* 2013) como se ha aplicado en el estudio de intervención de patrones de alimentos (publicación 5).

El uso de técnicas no supervisadas como el *k-means,* para clasificar individuos en grupos, cuando *a priori* pertenecen a un mismo grupo, es una técnica de clasificación aplicada en ciertos estudios metabolómicos y

de nutrición (Kong *et al.* 2014; O'Sullivan *et al.* 2011a). Este método fue utilizado para el estudio de los fenotipos metabólicos de los individuos de una misma población (de alto riesgo cardiovascular) e intervención con ingesta de polifenoles del vino (publicación 3).

Por último, en cuanto a la metodología seguida durante el transcurso de esta Tesis Doctoral, después de la identificación de los biomarcadores discriminantes, el paso final es dar un valor cuantitativo al efecto o cambio que se ha producido (Wishart 2008). Por ello, gracias a una estancia predoctoral en el *Metabolomics Inovation Center* liderado por el Dr. David Wishart se realizó la cuantificación de las muestras para el estudio de los fenotipos metabólicos, el cual queda reflejado en la publicación 3.

Biomarcadores en estudios nutricionales: Análisis del metaboloma urinario y evaluación del efecto matriz

En el primer estudio realizado, el análisis del metaboloma urinario mostró tanto metabolitos que correspondían al *food metabolome* (tartrato, etanol, manitol), metabolitos endógenos (3-metil-2-oxovalerato), así como derivados de la acción de la microbiota intestinal (hipurato y 4-hidroxifenilacetato). En cuanto a la matriz alimentaria, no se vieron diferencias en el marcador de consumo de vino (tartrato) para ninguno de los dos vinos, e igual ocurrió con el etanol en el caso del vino con alcohol y la ginebra. En cambio, se observó una posible interacción entre el alcohol y los biomarcadores relacionados con los metabolitos derivados de la microbiota intestinal, viéndose así un posible efecto por parte de la matriz alcohólica en los dos tipos de vinos con igual contenido de polifenoles.

El efecto de la matriz alimentaria se ha venido analizando en varios estudios de intervención nutricional como por ejemplo el efecto de la

matriz en cacao (Urpi-Sarda *et al.* 2009), en tomate y salsas de tomate (Tulipani *et al.* 2012) o el efecto matriz en vino sobre parámetros clínicos (Chiva-Blanch *et al.* 2012). En otro estudio realizado en nuestro grupo con el mismo diseño clínico que se muestra en la publicación 1, no se observaron diferencias en el contenido de resveratrol y metabolitos del resveratrol para los dos tipos de vino (Rotches-Ribalta *et al.* 2012). Del mismo modo, en el estudio del que deriva la publicación 1 tampoco se observaron diferencias en el contenido de tartrato entre el consumo de vino con alcohol y desalcoholizado; en cambio, en otro estudio focalizado en la microbiota intestinal con el mismo diseño de estudio, sí que se vieron diferencias significativas referente a la composición de la población bacteriana de los individuos después de cada intervención, este hecho puede por lo tanto indicar que también se pueda ver modificado el perfil metabólico derivado de la acción de la microbiota intestinal (Queipo-Ortuño *et al.* 2012) de metabolitos que se producen por medio de estas bacterias.

El efecto del alcohol sobre la excreción de metabolitos de la microbiota intestinal se ha examinado en otros estudios (Gika *et al.* 2013), dónde la administración de alcohol provocaba cambios en la excreción de metabolitos como el 4-hidroxifenilacetato, sugiriendo un efecto especifico según la población bacteriana. Un estudio en ratas comparó los efectos después de la administración de alcohol, observando varios metabolitos que presentaron un patrón de excreción diferencial tras la ingesta de alcohol, entre ellos algunos derivados de la microbiota intestinal (Gao *et al.* 2011). En este aspecto, en cuanto al posible efecto que podría tener la fracción alcohólica en el metabolismo microbiano serían necesarios más estudios que confirmaran esta hipótesis.

Determinación del poder predictivo de biomarcadores nutricionales en estudios de intervención y en estudios observacionales.

Tradicionalmente, se han utilizado biomarcadores individuales para caracterizar la exposición dietética, como por ejemplo la vitamina C para el consumo de frutas y verduras, aunque su contenido varía en función de la especie, variedad, etc. La combinación de diferentes biomarcadores puede proporcionar medidas más precisas de la exposición dietética (Scalbert *et al.* 2014).

En esta Tesis Doctoral, en el trabajo realizado sobre el estudio de biomarcadores de consumo de vino (publicación 2) (Vázquez-Fresno *et al.* 2014b) se evalúan biomarcadores de exposición a la ingesta de vino de forma individual, llegando finalmente a la conclusión que la combinación de dos de ellos ofrece más poder predictivo que cada uno de ellos por separado. En este trabajo se propone el uso de un modelo con la combinación de biomarcadores para una mayor predicción de ingesta de un alimento en individuos que han consumido dicho alimento frente al estado inicial (sin ingesta del alimento). En el mismo estudio también se realizó una validación en otra población con datos epidemiológicos, observándose una reproducibilidad de los resultados del estudio de intervención, obteniendo en ambas poblaciones una muy alta sensibilidad y especificidad de los biomarcadores estudiados.

Estudio del fenotipo metabólico de una misma población tras el consumo de un alimento

Una misma población puede tener diferentes agrupaciones según su fenotipo metabólico (o metabotipo). La estrategia seguida en este estudio usa diferentes herramientas de clasificación y discriminación también utilizadas en otros estudios nutricionales (O'Sullivan *et al.* 2011b).

Después de la segregación en diferentes grupos o "clúster" de los individuos, se observó un comportamiento diferencial en los grupos comparados después de la intervención nutricional, para la excreción de un metabolito previamente observado después del consumo de vino, el 4-hidroxifenilacetato. En el clúster que se clasificó como diabético y obeso según los datos bioquímicos y antropométricos, no se vio aumentada la excreción de este metabolito respecto a los niveles basales. En cambio en el clúster aparentemente más sano en cuanto a sus datos clínicos, se observó una mayor excreción frente al estado basal y frente al otro grupo (diabético y obeso) comparado, tal y como aparece en los resultados sobre la población general del estudio (Vázquez-Fresno *et al.* 2012). Este hecho sugiere, que el grupo de individuos diabéticos y obesos parece mostrar una posible alteración en el metabolismo proveniente de la microbiota intestinal en la ruta metabólica del 4-hidroxifenilacetato, un derivado de la degradación de polifenoles (Moco *et al.* 2012), compuesto excretado después del consumo de vino y polifenoles del vino y de la uva, y detectado mediante RMN (van Dorsten *et al.* 2010; Vázquez-Fresno *et al.* 2012). Se ha descrito que en individuos con obesidad y diabetes, existe un cambio en las poblaciones microbianas que alberga el intestino en géneros como *Bifidobacterium, F. prausnitzii,* y algunas especies de *Clostridium* y *Lactobacillus* en individuos obesos (Tagliabue *et al.* 2013) y diabéticos (Everard *et al.* 2013) (poblaciones bacterianas que interviene en el metabolismo del 4-hidroxifenilacetato)(Nicholson *et al.* 2012) este hecho está en línea y sustenta los resultados obtenidos.

Patrones alimentarios en una población con una intervención dietética de larga duración

Los biomarcadores metabolómicos reflejan patrones alimentarios habituales (Steffen *et al.* 2014). Así se ha manifestado en el estudio sobre biomarcadores metabolómicos en patrones alimentarios después de una intervención dietética con dos variantes de dieta mediterránea y una dieta baja en grasa durante un seguimiento de 3 años de duración (Vázquez-Fresno *et al.* 2014c). Además de marcadores que puedan reflejar el consumo de ciertos alimentos, como por ejemplo glutamina y sus derivados en el consumo de frutos secos, ácidos oleico y subérico en el caso de aceite de oliva y frutos secos, propios de la intervención nutricional ya que las recomendaciones dietéticas eran el seguimiento de una dieta mediterránea suplementada con estos dos tipos de alimentos.

También, se han podido asociar ciertos metabolitos con patrones de dieta, viéndose la dieta mediterránea asociada a un mayor consumo de vegetales; y por otro lado, la dieta baja en grasa asociada a un mayor consumo de carne roja. Estas asociaciones se han visto mediante el cuestionario de frecuencia de consumo de alimentos (FFQ) realizado a la población durante este período y contrastados con otros estudios de patrones alimentarios en poblaciones con patrones de dieta vegetariana (O'Sullivan *et al.* 2011a; Stella *et al.* 2006; Xu *et al.* 2010) y estudios de consumo de carne de intervención y observaciones (Bertram *et al.* 2007; Dragsted 2010; Stella *et al.* 2006).

Asimismo, después de las intervenciones con dieta mediterránea, se han descrito un gran número de metabolitos endógenos, predominantemente aquellos relacionados con el metabolismo energético (glucólisis, ciclo de los ácidos tricarboxílicos, *β*-oxidación), siendo coherente ya que tenían un

mayor contenido en lípidos que la dieta baja en grasa. Además, los ácidos grasos como el ácido linoleico, α-linolénico (mayoritario en nueces), araquidónico, docosahexanóico, eicosapentaenóico, oleico, y eláidico se ha descrito que puedan favorecer el proceso de oxidación de ácidos grasos (*β*-oxidación) mediante la estimulación de las rutas de utilización, transporte, esterificación y oxidación de los ácidos grasos, por ser ligandos del receptor nuclear *peroxisome proliferator-activated receptor* (PPAR) (Luo *et al.* 2010) y de este modo mejorar su utilización. Estos resultados están en consonancia con otros realizados en un estudio de intervención con consumo de nueces, dónde también se determinaron metabolitos derivados de la oxidación de ácidos grasos como el ácido dodecanedióico (Tulipani *et al.* 2011).

Complementos alimenticios: Ingesta de un probiótico y su impacto en la salud

Mediante la administración de un probiótico como complemento alimenticio en mujeres lactantes con mastitis se observó una mejora en el estado de las mujeres (observación mediante seguimiento médico) con lo que provocó una deserción voluntaria de fármacos antiinflamatorios y analgésicos, hecho que se reflejó en el metaboloma urinario después del período de intervención. Además, también se vio un aumento en la excreción de creatina y metabolitos derivados de la acción de la microbiota intestinal (hipurato y trimetilamina-N-óxido) después del período de intervención. Este hecho puede explicarse por una acción microbiana producida por el probiótico sobre la microbiota intestinal, es decir el hecho de la ingestión de cepas probióticas puede modificar la acción de la propia microbiota intestinal (Delzenne *et al.* 2011). Por otro lado al inicio se detectó lactosa en orina, una característica propia de las mujeres lactantes pero también descrita como incrementada en períodos

de mastitis, con mayor excreción en período de mayor inflamación (Fetherston *et al.* 2006). La utilización de probióticos como complementos alimenticios se ha administrado para varias dolencias como por ejemplo en desórdenes inflamatorios, infecciosos, alérgicos, y digestivos (diarreas, síndrome del colon irritable) (Quigley 2010).

Biomarcadores de efecto en estudios nutricionales

Referente a los biomarcadores de ingesta cabe destacar el tartrato y etil glucurónido como biomarcadores del consumo de vino con una buena reproducibilidad en diferentes poblaciones; además de otros posibles marcadores como los derivados de la glutamina (N-acetilglutamina y fenilacetilglutamina) con el consumo de frutos secos; y el ácido oleico y derivados con el consumo de aceite de oliva. En estos dos últimos casos, será necesario corroborarlos en estudios futuros. En cuanto a los biomarcadores endógenos modulados por el efecto de la intervención dietética; en el caso de los estudios en vino (Vázquez-Fresno *et al.* 2012; Vázquez-Fresno *et al.* 2014b), el 3-metil-2-oxovalerato fue descrito como marcador endógeno con mayor excreción después del consumo de vino. Igualmente, en el estudio con probióticos (Vázquez-Fresno *et al.* 2014a) se han encontrado marcadores endógenos (creatina) y derivados de la acción de la microbiota intestinal (trimetilamina-N-óxido, hipurato). Referente al estudio de intervención con dieta mediterránea (Vázquez-Fresno *et al.* 2014c), es dónde han aparecido más metabolitos endógenos, mayoritariamente relacionados con el metabolismo energético (carbohidratos, lípidos y proteínas), junto también con algunos metabolitos de ingesta y derivados de la microbiota. El hecho de encontrar un mayor número de marcadores endógenos en este estudio, puede ser debido a la duración de la intervención dietética. Al ser una intervención de larga duración (evaluada durante el primer y tercer año)

podría ser un período de tiempo suficientemente largo para poder observar cambios generales de larga duración en rutas metabólicas.

También cabe destacar el efecto que se ha evidenciado en varios trabajos aquí presentados sobre la microbiota intestinal, una fracción del metaboloma humano aún poco explorado. Prácticamente en todos los trabajos de esta tesis doctoral aparecen metabolitos derivados de la acción de la microbiota intestinal (publicación 1, publicación 3, publicación 4 y publicación 5) exhibiendo un importante papel de esta fracción del metaboloma en el metaboloma humano. Se conocen cerca de 100 compuestos que son metabolitos específicos microbianos y otros debidos a la biotransformación de la microbiota tanto de compuestos endógenos como de exógenos. Por ello, la importancia de la microbiota intestinal en la contribución del metaboloma humano es un emergente foco de estudio (Nicholson *et al.* 2012).

Asimismo, referente al xenometaboloma, fracción del metaboloma humano proveniente de fármacos y otros compuestos xenobióticos, en el caso del estudio de la ingesta de un complemento alimenticio probiótico (Vázquez-Fresno *et al.* 2014a), se analizaron estas señales y, de hecho, aparecieron como discriminantes en el estado inicial de la intervención (antes del consumo del probiótico), ya que eran mujeres que partían de una enfermedad o patología (mastitis), por lo que era interesante monitorizar todas las variables relacionadas con el estado de salud. En cambio, en el estudio con dieta mediterránea, no se consideraron relevantes para el análisis las señales de acetaminofén (paracetamol) y derivados, debido a que podían interferir en el análisis metabolómico para la observación del efecto de la dieta, por tanto, una vez observadas en la orina de algunos voluntarios, se decidió excluirlas del análisis.

Perspectivas de futuro

Los estudios metabolómicos no dirigidos son esenciales para la búsqueda de biomarcadores de exposición y de efecto de una intervención nutricional (Scalbert *et al.* 2014), en este sentido, debido a que aún hay muy pocos estudios dónde poder comparar los resultados, es necesario disponer de un mayor número de estudios que abarquen diferentes diseños, poblaciones y estrategias de análisis para ampliar el panel de biomarcadores así como también poder comparar y confirmar los biomarcadores ya encontrados. Es necesario que los biomarcadores proporcionen medidas objetivas del estado nutricional; por ello, las investigaciones futuras también deben ir direccionadas a refinar los biomarcadores existentes, el establecimiento de nuevos indicadores de ingesta de alimentos específicos y el desarrollo de técnicas que puedan ser medidas más precisas de la situación nutricional (Hedrick *et al.* 2012).

A pesar de que sigue siendo un reto obtener datos absolutos de cuantificación mediante análisis no dirigidos en lugar de datos relativos, se debe seguir trabajando en este aspecto. Las medidas cuantitativas pueden mejorar la aplicabilidad así como la interpretación de los resultados.

Asimismo, es importante para obtener una comprensión más completa del impacto que tiene nuestra alimentación en la salud, el hecho de explorar los fenotipos metabólicos de una misma población y las diferentes respuestas que se puedan obtener después de una intervención nutricional; siendo, de este modo una potencial herramienta en el camino de la nutrición personalizada.

CONCLUSIONES

CONCLUSIONS

De los diferentes trabajos que constituyen la Tesis Doctoral derivan las siguientes conclusiones:

1. Se ha implementado un método rápido, altamente reproducible y aplicable a gran número de muestras para determinar el perfil metabólico urinario e identificar biomarcadores de ingesta, así como metabolitos endógenos derivados de una intervención dietética ya sea de un alimento, complemento alimenticio o patrón alimentario.
2. La aplicación de la metodología ha permitido la clasificación de los individuos atendiendo a sus patrones metabolómicos mediante diferentes estrategias de análisis (análisis discriminante multivariante, univariantes, y modelos de predicción mediante curvas ROC). En este aspecto cabe destacar la robustez de esta aproximación en la caracterización de los cambios en el metaboloma urinario asociados a una intervención dietética de corta y larga duración.
3. La combinación de biomarcadores de exposición nutricional observada en estudios de intervención mejora la capacidad predictiva respecto a la consideración de éstos mismos de forma individual. Ofrecen así, un alto poder predictivo (con alta sensibilidad y especificidad), siendo posible su validación en datos epidemiológicos. Se confirma, por tanto, como herramienta eficaz para la búsqueda de biomarcadores. Concretamente, la combinación de dos marcadores de consumo de vino (tartrato-etil glucurónido) confieren un mejor poder predictivo como marcadores de consumo de este alimento.
4. Se han observado marcadores que reflejan el *exposome/food metabolome* tales como metabolitos derivados de la microbiota intestinal (TMAO, PAGN *p*-cresol, hipurato, 4-hidroxifenilacetato) y del xenometaboloma procedente de la ingesta de fármacos (paracetamol

e ibuprofeno), así como una metabolitos asociados al consumo de frutas y verduras (3-HB, leucina, isobutírico, 2-oxoisovalérico, glicina, N-Ac, prolina, *p*-cresol, PAGN), de naranja (prolina betaina) y de carne roja (3-MH, histidina, carnosina).

5. Las diferencias en el fenotipo metabólico o metabotipo mediante la estratificación por parámetros clínicos y bioquímicos de los individuos de una misma población antes de una intervención, ha revelado un efecto diferencial dieta-fenotipo en el metaboloma urinario después de una intervención dietética.

6. Después de una intervención de larga duración (≥1 año) se han podido separar los individuos según su huella metabólica atendiéndose a los patrones alimentarios seguidos durante ese período. Además ha permitido determinar conjuntamente metabolitos provenientes de diversas rutas metabólicas, tanto del metabolismo energético y rutas metabólicas afines (ciclo de los ácidos tricarboxílicos, oxidación de ácidos grasos, gluconeogénesis, metabolismo de cuerpos cetónicos), metabolismo de nucleótidos, así como aminoácidos y derivados, o metabolitos provenientes de la microbiota intestinal. Estos resultados podrán permitir, en futuros estudios, el análisis dirigido de rutas metabólicas interconectadas y la consiguiente integración en redes metabólicas, para una mejor comprensión e interpretación del estado global de los individuos.

CONCLUSIONS

From the work presented in this thesis, the conclusions are as follows:

1. A fast, high throughput, highly reproducible, widely applicable methodology to determine the urinary metabolic profile and identify biomarkers of intake and endogenous metabolites derived from dietary interventions (including a food, a food supplement or dietary pattern) has been applied.

2. The application of this methodology has allowed the classification of individuals according to their metabolic patterns using different strategies (discriminant multivariate and univariate analysis, and predictive models by ROC curves analysis). The reproducibility and robustness of this approach in characterizing changes in the urinary metabolome associated with a dietary intervention of short- and long-term interventions has been highlighted.

3. The combination of biomarkers of nutritional exposure offers a high sensitivity and specificity, leading to greater predictive power in the study of individual biomarkers. Epidemiological data was used to validate the method and confirmed the initial results, establishing this method as an effective tool for biomarker discovery. Precisely, the combination of two markers of wine consumption (tartrate-ethyl glucuronide) as the best predictive power markers of consumption.

4. Markers reflecting the exposome/food metabolome have been observed, such as metabolites derived from the gut microbiota (TMAO, PAGN *p*-cresol, hippurate, 4-hydroxyphenylacetate), and from the xenometabolome coming from drug intake (paracetamol and ibuprofen), as well as metabolites associated with consumption of fruits and vegetables (3-HB, leucine, isobutyric, 2- oxoisovaleric, glycine, N-Ac, proline, *p*-cresol, PAGN), orange (proline betaine) and red meat intake (3-MH, histidine, carnosine).

5. Differences in metabolic phenotype/metabotypes within a population (considering clinical/biochemical parameters at baseline of individuals before an intervention) reveal a differential effect in the diet-phenotype urinary metabolome after dietary intervention.

6. After a long-term intervention (≥1 year), the separation of individuals according to their metabolomic fingerprint by following particular dietary patterns was shown. This has allowed us to identify metabolites from various metabolic pathways, including energy metabolism and related pathways (tricarboxylic acids cycle, fatty acid oxidation, gluconeogenesis, ketone bodies metabolism), amino acid metabolism (and their derivatives), nucleotide metabolism, and metabolites from gut microbiota. These results may allow targeted analysis of interconnected metabolic pathways, which can be subsequently integrated into metabolic networks. In the future, this work will allow a better understanding and interpretation of the overall health status of individuals.

REFERENCIAS BIBLIOGRÁFICAS

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Altmaier, E., G. Kastenmüller, W. Römisch-Margl, B. Thorand, K. Weinberger, T. Illig, J. Adamski, A. Döring and K. Suhre (2011). "Questionnaire-based self-reported nutrition habits associate with serum metabolism as revealed by quantitative targeted metabolomics." *European Journal of Epidemiology* 26(2): 145-156.

Álvarez-Sánchez, B. (2012). "Nuevas plataformas analíticas en metabolómica" [Tesis doctoral], Universidad de Córdoba (España).

Andersen, M.-B., M. Kristensen, C. Manach, E. Pujos-Guillot, S. Poulsen, T. Larsen, A. Astrup and L. Dragsted (2014a). "Discovery and validation of urinary exposure markers for different plant foods by untargeted metabolomics." *Analytical and Bioanalytical Chemistry* 406(7): 1829-1844.

Andersen, M.-B., H. Reinbach, Å. Rinnan, T. Barri, C. Mithril and L. Dragsted (2013). "Discovery of exposure markers in urine for Brassica-containing meals served with different protein sources by UPLC-qTOF-MS untargeted metabolomics." *Metabolomics* 9(5): 984-997.

Andersen, M.-B. S. (2013). "Discovery of food exposure markers in urine and evaluation of dietary compliance by untargeted LC-MS metabolomics" [Tesis doctoral], Universidad de Copenhagen (Dinamarca).

Andersen, M.-B. S., Å. Rinnan, C. Manach, S. K. Poulsen, E. Pujos-Guillot, T. M. Larsen, A. Astrup and L. O. Dragsted (2014b). "Untargeted Metabolomics as a Screening Tool for Estimating Compliance to a Dietary Pattern." *Journal of Proteome Research* 13(3): 1405-1418.

Beckonert, O., H. C. Keun, T. M. D. Ebbels, J. Bundy, E. Holmes, J. C. Lindon and J. K. Nicholson (2007). "Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts." *Nature Protocols* 2(11): 2692-2703.

Benjamini, Y. and Y. Hochberg (1995). "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing." *Journal of the Royal Statistical Society. Series B (Methodological)* 57(1): 289-300.

Bertram, H. C., C. Hoppe, B. O. Petersen, J. O. Duus, C. Molgaard and K. F. Michaelsen (2007). "An NMR-based metabonomic investigation on effects of milk and meat protein diets given to 8-year-old boys." *The British Journal of Nutrition* 97(4): 758-763.

Bothwell, J. H. F. and J. L. Griffin (2011). "An introduction to biological nuclear magnetic resonance spectroscopy." *Biological Reviews* 86(2): 493-510.

Bouatra, S., F. Aziat, R. Mandal, A. C. Guo, M. R. Wilson, C. Knox, T. C. Bjorndahl, R. Krishnamurthy, F. Saleem, P. Liu, Z. T. Dame, J. Poelzer, J. Huynh, F. S. Yallou, N. Psychogios, E. Dong, R. Bogumil, C. Roehring and D. S. Wishart (2013). "The Human Urine Metabolome." *PLoS One* 8(9): e73076.

Bouchard-Mercier, A., I. Rudkowska, S. Lemieux, P. Couture and M. C. Vohl (2013). "The metabolic signature associated with the Western dietary pattern: a cross-sectional study." *Nutrition Journal* 12: 158.

Calvani, R., A. Miccheli, G. Capuani, A. Tomassini Miccheli, C. Puccetti, M. Delfini, A. Iaconelli, G. Nanni and G. Mingrone (2010). "Gut microbiome-derived metabolites characterize a peculiar obese urinary metabotype." *International Journal of Obesity* 34(6): 1095-1098.

Champagne, C. P., R. P. Ross, M. Saarela, K. F. Hansen and D. Charalampopoulos (2011). "Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices." *International Journal of Food Microbiology* 149(3): 185-193.

Chiva-Blanch, G., M. Urpi-Sarda, R. Llorach, M. Rotches-Ribalta, M. Guillén, R. Casas, S. Arranz, P. Valderas-Martinez, O. Portoles, D. Corella, F. Tinahones, R. M. Lamuela-Raventos, C. Andres-Lacueva and R. Estruch (2012). "Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial." *The American Journal of Clinical Nutrition* 95(2): 326-334.

Chong, I.-G. and C.-H. Jun (2005). "Performance of some variable selection methods when multicollinearity is present." *Chemometrics and Intelligent Laboratory Systems* 78(1–2): 103-112.

Claus, S. P. and J. R. Swann (2013). "Nutrimetabonomics:Applications for Nutritional Sciences, with Specific Reference to Gut Microbial Interactions." *Annual Review of Food Science and Technology* 4(1): 381-399.

Cloarec, O., M. E. Dumas, J. Trygg, A. Craig, R. H. Barton, J. C. Lindon, J. K. Nicholson and E. Holmes (2004). "Evaluation of the Orthogonal Projection on Latent Structure Model Limitations Caused by Chemical Shift Variability and Improved Visualization of Biomarker Changes in 1H NMR Spectroscopic Metabonomic Studies." *Analytical Chemistry* 77(2): 517-526.

Craig, A., O. Cloarec, E. Holmes, J. K. Nicholson and J. C. Lindon (2006). "Scaling and Normalization Effects in NMR Spectroscopic Metabonomic Data Sets." *Analytical Chemistry* 78(7): 2262-2267.

Cui, Q., I. A. Lewis, A. D. Hegeman, M. E. Anderson, J. Li, C. F. Schulte, W. M. Westler, H. R. Eghbalnia, M. R. Sussman and J. L. Markley (2008). "Metabolite identification via the Madison Metabolomics Consortium Database." *Nature Biotechnology* 26(2): 162-164.

Daykin, C. A., J. P. Van Duynhoven, A. Groenewegen, M. Dachtler, J. M. Van Amelsvoort and T. P. Mulder (2005). "Nuclear magnetic resonance spectroscopic based studies of the metabolism of black tea polyphenols in humans." *Journal of Agricultural and Food Chemistry* 53(5): 1428-1434.

Degtyarenko, K., P. de Matos, M. Ennis, J. Hastings, M. Zbinden, A. McNaught, R. Alcántara, M. Darsow, M. Guedj and M. Ashburner (2008). "ChEBI: a database and ontology for chemical entities of biological interest." *Nucleic Acids Research* 36(Database issue): D344-350.

Delzenne, N. M., A. M. Neyrinck, F. Backhed and P. D. Cani (2011). "Targeting gut microbiota in obesity: effects of prebiotics and probiotics." *Nature Reviews Endocrinology* 7(11): 639-646.

Dettmer, K., P. A. Aronov and B. D. Hammock (2007). "Mass spectrometry-based metabolomics." *Mass Spectrometry Reviews* 26(1): 51-78.

Dragsted, L. O. (2010). "Biomarkers of meat intake and the application of nutrigenomics." *Meat Science* 84(2): 301-307.

Dunn, W. B. and D. I. Ellis (2005). "Metabolomics: Current analytical platforms and methodologies." *TrAC Trends in Analytical Chemistry* 24(4): 285-294.

Elbourne, D. R., D. G. Altman, J. P. Higgins, F. Curtin, H. V. Worthington and A. Vail (2002). "Meta-analyses involving cross-over trials: methodological issues." *International Journal of Epidemiology* 31(1): 140-149.

Eriksson, L; Johansson, E; Kettaneh-Wold, J; Trygg, J; Wikström, C; Wold, S. Ed. (2006). *Multi- and Megavariate Data Analysis: Principles and Applications*. Umeå. ISBN: 978-91-973730-2-9.

Estruch, R., E. Ros, J. Salas-Salvadó, M.-I. Covas, D. Corella, F. Arós, E. Gómez-Gracia, V. Ruiz-Gutiérrez, M. Fiol, J. Lapetra, R. M. Lamuela-Raventos, L. Serra-Majem, X. Pintó, J. Basora, M. A. Muñoz, J. V. Sorlí, J. A. Martínez and M. A. Martínez-González (2013). "Primary Prevention of Cardiovascular Disease with a Mediterranean Diet." *New England Journal of Medicine* 368(14): 1279-1290.

Everard, A. and P. D. Cani (2013). "Diabetes, obesity and gut microbiota." *Best practice & Research. Clinical gastroenterology* 27(1): 73-83.

Fahy, E., M. Sud, D. Cotter and S. Subramaniam (2007). "LIPID MAPS online tools for lipid research." *Nucleic Acids Research* 35(Web Server issue): W606-612.

Fardet, A., R. Llorach, A. Orsoni, J.-F. Martin, E. Pujos-Guillot, C. Lapierre and A. Scalbert (2008). "Metabolomics Provide New Insight on the Metabolism of Dietary Phytochemicals in Rats." *The Journal of Nutrition* 138(7): 1282-1287.

Fetherston, C. M., C. T. Lai and P. E. Hartmann (2006). "Relationships between symptoms and changes in breast physiology during lactation mastitis." *Breastfeed Medicine* 1(3): 136-145.

Fiehn, O. (2002). "Metabolomics – the link between genotypes and phenotypes." *Plant Molecular Biology* 48(1-2): 155-171.

Floegel, A., A. von Ruesten, D. Drogan, M. B. Schulze, C. Prehn, J. Adamski, T. Pischon and H. Boeing (2013). "Variation of serum metabolites related to habitual diet: a targeted metabolomic approach in EPIC-Potsdam." *European Journal of Clinical Nutrition* 67(10): 1100-1108.

Frolkis, A., C. Knox, E. Lim, T. Jewison, V. Law, D. D. Hau, P. Liu, B. Gautam, S. Ly, A. C. Guo, J. Xia, Y. Liang, S. Shrivastava and D. S. Wishart (2010). "SMPDB: The Small Molecule Pathway Database." *Nucleic Acids Research* 38(suppl 1): D480-D487.

Gao, X., A. Zhao, M. Zhou, J. Lin, Y. Qiu, M. Su and W. Jia (2011). "GC/MS-based urinary metabolomics reveals systematic differences in metabolism and ethanol response between Sprague–Dawley and Wistar rats." *Metabolomics* 7(3): 363-374.

Garcia-Aloy, M., R. Llorach, M. Urpi-Sarda, S. Tulipani, R. Estruch, M. A. Martínez-González, D. Corella, M. Fitó, E. Ros, J. Salas-Salvadó and C. Andres-Lacueva (2014). "Novel Multimetabolite Prediction of Walnut Consumption by a Urinary Biomarker Model in a Free-Living Population: the PREDIMED Study." *Journal of Proteome Research* 13(7): 3476-3483.

Gibney, M. J., M. Walsh, L. Brennan, H. M. Roche, B. German and B. van Ommen (2005). "Metabolomics in human nutrition: opportunities and challenges." *American Journal of Clinical Nutrition* 82(3): 497-503.

Gika, H. G. and I. D. Wilson (2013). "Global metabolic profiling for the study of alcohol-related disorders." *Bioanalysis* 6(1): 59-77.

Goodacre, R., S. Vaidyanathan, W. B. Dunn, G. G. Harrigan and D. B. Kell (2004). "Metabolomics by numbers: acquiring and understanding global metabolite data." *Trends in Biotechnology* 22(5): 245-252.

Gosselin, R., D. Rodrigue and C. Duchesne (2010). "A Bootstrap-VIP approach for selecting wavelength intervals in spectral imaging applications." *Chemometrics and Intelligent Laboratory Systems* 100(1): 12-21.

Group., B. D. W. (2001). "Biomarkers and surrogate endpoints: preferred definitions and conceptual framework." *Clinical Pharmacology & Therapeutics* 69(3): 89-95.

Hedrick, V. E., A. M. Dietrich, P. A. Estabrooks, J. Savla, E. Serrano and B. M. Davy (2012). "Dietary biomarkers: advances, limitations and future directions." *Nutrition Journal* 11: 109.

Heinzmann, S. S., C. A. Merrifield, S. Rezzi, S. Kochhar, J. C. Lindon, E. Holmes and J. K. Nicholson (2011). "Stability and Robustness of Human Metabolic Phenotypes in Response to Sequential Food Challenges." *Journal of Proteome Research* 11(2): 643-655.

Holmes, E., I. D. Wilson and J. K. Nicholson (2008). "Metabolic Phenotyping in Health and Disease." *Cell* 134(5): 714-717.

Homayoni Rad, A., F. Akbarzadeh and E. V. Mehrabany (2012). "Which are more important: prebiotics or probiotics?" *Nutrition* 28(11-12): 1196-1197.

Hooper, L. and A. Cassidy (2006). "A review of the health care potential of bioactive compounds." *Journal of the Science of Food and Agriculture* 86(12): 1805-1813.

Illig, T., C. Gieger, G. Zhai, W. Romisch-Margl, R. Wang-Sattler, C. Prehn, E. Altmaier, G. Kastenmuller, B. S. Kato, H.-W. Mewes, T. Meitinger, M. H. de Angelis, F. Kronenberg, N. Soranzo, H. E. Wichmann, T. D. Spector, J. Adamski and K. Suhre (2010). "A genome-wide perspective of genetic variation in human metabolism." *Nature Genetics* 42(2): 137-141.

Jacobs, D. M., J. C. Fuhrmann, F. A. van Dorsten, D. Rein, S. Peters, E. J. J. van Velzen, B. Hollebrands, R. Draijer, J. van Duynhoven and U. Garczarek (2012). "Impact of Short-Term Intake of Red Wine and Grape Polyphenol Extract on the Human Metabolome." *Journal of Agricultural and Food Chemistry* 60(12): 3078-3085.

Jenab, M., N. Slimani, M. Bictash, P. Ferrari and S. Bingham (2009). "Biomarkers in nutritional epidemiology: applications, needs and new horizons." *Human Genetics* 125(5-6): 507-525.

Kanehisa, M., S. Goto, M. Hattori, K. F. Aoki-Kinoshita, M. Itoh, S. Kawashima, T. Katayama, M. Araki and M. Hirakawa (2006). "From genomics to chemical genomics: new developments in KEGG." *Nucleic Acids Research* 34(Database issue): D354-357.

Kell, D. B. (2006). "Systems biology, metabolic modelling and metabolomics in drug discovery and development." *Drug Discovery Today* 11(23–24): 1085-1092.

Kell, D. B., M. Brown, H. M. Davey, W. B. Dunn, I. Spasic and S. G. Oliver (2005). "Metabolic footprinting and systems biology: the medium is the message." *Nature Reviews Microbiology* 3(7): 557-565.

Kochhar, S., D. M. Jacobs, Z. Ramadan, F. Berruex, A. Fuerholz and L. B. Fay (2006). "Probing gender-specific metabolism differences in humans by nuclear magnetic resonance-based metabonomics." *Analytical Biochemistry* 352(2): 274-281.

Kong, L. C., B. A. Holmes, A. Cotillard, F. Habi-Rachedi, R. Brazeilles, S. Gougis, N. Gausserès, P. D. Cani, S. Fellahi, J.-P. Bastard, S. P. Kennedy, J. Doré, S. D. Ehrlich, J.-D. Zucker, S. W. Rizkalla and K. Clément (2014). "Dietary Patterns Differently Associate with Inflammation and Gut Microbiota in Overweight and Obese Subjects." *PLoS One* 9(10): e109434.

Krummenacker, M., S. Paley, L. Mueller, T. Yan and P. D. Karp (2005). "Querying and computing with BioCyc databases." *Bioinformatics* 21(16): 3454-3455.

Lanza, I. R., S. Zhang, L. E. Ward, H. Karakelides, D. Raftery and K. S. Nair (2010). "Quantitative metabolomics by H-NMR and LC-MS/MS confirms altered metabolic pathways in diabetes." *PLoS One* 5(5): e10538.

Law, W. S., P. Y. Huang, E. S. Ong, C. N. Ong, S. F. Y. Li, K. K. Pasikanti and E. C. Y. Chan (2008). "Metabonomics investigation of human urine after ingestion of green tea with gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry and 1H NMR spectroscopy." *Rapid Communications in Mass Spectrometry* 22(16): 2436-2446.

Lindon J.C., Nicholson. J. K., Holmes E. , Ed. (2007). *The Handbook of Metabonomics and Metabolomics*, Elsevier. ISBN: 9780444528414.

Llorach, R., M. Garcia-Aloy, S. Tulipani, R. Vazquez-Fresno and C. Andres-Lacueva (2012). "Nutrimetabolomic Strategies To Develop New Biomarkers of Intake and Health Effects." *Journal of Agricultural & Food Chemistry*.

Llorach, R., M. Urpi-Sarda, O. Jauregui, M. Monagas and C. Andres-Lacueva (2009). "An LC-MS-based metabolomics approach for exploring urinary metabolome modifications after cocoa consumption." *Journal of Proteome Research* 8(11): 5060-5068.

Luo, D. X., D. L. Cao, Y. Xiong, X. H. Peng and D. F. Liao (2010). "A novel model of cholesterol efflux from lipid-loaded cells." *Acta Pharmacologica Sinica* 31(10): 1243-1257.

Madsen, R., T. Lundstedt and J. Trygg (2010). "Chemometrics in metabolomics--a review in human disease diagnosis." *Analytica Chimica Acta* 659(1-2): 23-33.

Manach, C., J. Hubert, R. Llorach and A. Scalbert (2009). "The complex links between dietary phytochemicals and human health deciphered by metabolomics." *Molecular Nutrition & Food Research* 53(10): 1303-1315.

Martin, F.-P. J., N. Sprenger, I. K. S. Yap, Y. Wang, R. Bibiloni, F. Rochat, S. Rezzi, C. Cherbut, S. Kochhar, J. C. Lindon, E. Holmes and J. K. Nicholson (2009). "Panorganismal Gut Microbiome−Host Metabolic Crosstalk." *Journal of Proteome Research* 8(4): 2090-2105.

Martínez-González, M. A., Ed. (2006). *Bioestadística Amigable*, Pamplona: Díaz de Santos. ISBN: 8479787910.

May, D. H., S. L. Navarro, I. Ruczinski, J. Hogan, Y. Ogata, Y. Schwarz, L. Levy, T. Holzman, M. W. McIntosh and J. W. Lampe (2013). "Metabolomic profiling of urine: response to a randomised, controlled feeding study of select fruits and vegetables, and application to an observational study." *British Journal of Nutrition* 110(10): 1760-1770.

McNiven, E. M., J. B. German and C. M. Slupsky (2011). "Analytical metabolomics: nutritional opportunities for personalized health." *Journal of Nutritional Biochemistry* 22(11): 995-1002.

Menni, C., G. Zhai, A. Macgregor, C. Prehn, W. Romisch-Margl, K. Suhre, J. Adamski, A. Cassidy, T. Illig, T. D. Spector and A. M. Valdes (2013). "Targeted metabolomics profiles are strongly correlated with nutritional patterns in women." *Metabolomics* :9(2): 506-514.

Moco, S., F. P. Martin and S. Rezzi (2012). "Metabolomics view on gut microbiome modulation by polyphenol-rich foods." *Journal of Proteome Research* 11(10): 4781-4790.

Moco, S., J. Vervoort, R. J. Bino, R. C. H. De Vos and R. Bino (2007). "Metabolomics technologies and metabolite identification." *TrAC Trends in Analytical Chemistry* 26(9): 855-866.

Monteiro, M. S., M. Carvalho, M. L. Bastos and P. Guedes de Pinho (2013). "Metabolomics Analysis for Biomarker Discovery: Advances and Challenges." *Current Medicinal Chemistry* 20(2): 257-271.

Ndagijimana, M., L. Laghi, B. Vitali, G. Placucci, P. Brigidi and M. E. Guerzoni (2009). "Effect of a synbiotic food consumption on human gut metabolic profiles evaluated by 1H Nuclear Magnetic Resonance spectroscopy." *International Journal of Food Microbiology* 134(1–2): 147-153.

Nicholson, J. K., E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia and S. Pettersson (2012). "Host-Gut Microbiota Metabolic Interactions." *Science* 336(6086): 1262-1267.

Nicholson, J. K., J. C. Lindon and E. Holmes (1999). "'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data." *Xenobiotica* 29(11): 1181-1189.

O'Sullivan, A., M. J. Gibney, A. O. Connor, B. Mion, S. Kaluskar, K. D. Cashman, A. Flynn, F. Shanahan and L. Brennan (2011a). "Biochemical and metabolomic phenotyping in the identification of a vitamin D responsive metabotype for markers of the metabolic syndrome." *Molecular Nutrition & Food Research* 55(5): 679-690.

O'Sullivan, A., M. J. Gibney and L. Brennan (2011b). "Dietary intake patterns are reflected in metabolomic profiles: potential role in dietary assessment studies." *The American Journal of Clinical Nutrition* 93(2): 314-321.

Oh, J. E., K. Krapfenbauer, M. Fountoulakis, T. Frischer and G. Lubec (2004). "Evidence for the existence of hypothetical proteins in human bronchial epithelial, fibroblast, amnion, lymphocyte, mesothelial and kidney cell lines." *Amino Acids* 26(1): 9-18.

Oresic, M. (2009). "Metabolomics, a novel tool for studies of nutrition, metabolism and lipid dysfunction." *Nutrition Metabolism & Cardiovascular Disease* 19(11): 816-824.

Patti, G. J., O. Yanes and G. Siuzdak (2012). "Innovation: Metabolomics: the apogee of the omics trilogy." *Nature Reviews. Molecular Cell Biology* 13(4): 263-269.

Peré-Trepat, E., A. B. Ross, F.-P. Martin, S. Rezzi, S. Kochhar, A. L. Hasselbalch, K. O. Kyvik and T. I. A. Sørensen (2010). "Chemometric strategies to assess metabonomic imprinting of food habits in epidemiological studies." *Chemometrics and Intelligent Laboratory Systems* 104(1): 95-100.

Pico, A. R., T. Kelder, M. P. van Iersel, K. Hanspers, B. R. Conklin and C. Evelo (2008). "WikiPathways: pathway editing for the people." *PLoS Biology* 6(7): e184.

Potischman, N. and J. L. Freudenheim (2003). "Biomarkers of Nutritional Exposure and Nutritional Status: An Overview." *The Journal of Nutrition* 133(3): 873S-874S.

Pujos-Guillot, E., J. Hubert, J.-F. Martin, B. Lyan, M. Quintana, S. Claude, B. Chabanas, J. A. Rothwell, C. Bennetau-Pelissero, A. Scalbert, B. Comte, S. Hercberg, C. Morand, P. Galan and C. Manach (2013). "Mass Spectrometry-based Metabolomics for the Discovery of Biomarkers of Fruit and Vegetable Intake: Citrus Fruit as a Case Study." *Journal of Proteome Research* 12(4): 1645-1659.

Queipo-Ortuño, M. I., M. Boto-Ordóñez, M. Murri, J. M. Gomez-Zumaquero, M. Clemente-Postigo, R. Estruch, F. Cardona Diaz, C. Andrés-Lacueva and F. J. Tinahones (2012). "Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers." *The American Journal of Clinical Nutrition* 95(6): 1323-1334.

Quigley, E. M. (2010). "Prebiotics and probiotics; modifying and mining the microbiota." *Pharmacological Research* 61(3): 213-218.

Rasmussen, L. G., H. Winning, F. Savorani, C. Ritz, S. B. Engelsen, A. Astrup, T. M. Larsen and L. O. Dragsted (2012). "Assessment of dietary exposure related to dietary GI and fibre intake in a nutritional metabolomic study of human urine." *Genes & Nutrition* 7(2): 281-293.

Reo, N. V. (2002). "NMR-based metabolomics." *Drug and Chemical Toxicology* 25(4): 375-382.

Rezzi, S., Z. Ramadan, F. P. Martin, L. B. Fay, P. van Bladeren, J. C. Lindon, J. K. Nicholson and S. Kochhar (2007). "Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals." *Journal of Proteome Research* 6(11): 4469-4477.

Robin, X., N. Turck, A. Hainard, N. Tiberti, F. Lisacek, J. C. Sanchez and M. Muller (2011). "pROC: an open-source package for R and S+ to analyze and compare ROC curves." *BMC Bioinformatics* 12: 77.

Ross, A. B., E. Pere-Trépat, I. Montoliu, F.-P. J. Martin, S. Collino, S. Moco, J.-P. Godin, M. Cléroux, P. A. Guy, I. Breton, R. Bibiloni, A. Thorimbert, I. Tavazzi, L. Tornier, A. Bebuis, S. J. Bruce, M. Beaumont, L.-B. Fay and S. Kochhar (2013). "A Whole-Grain–Rich Diet Reduces Urinary Excretion of Markers of Protein Catabolism and Gut Microbiota Metabolism in Healthy Men after One Week." *The Journal of Nutrition* 143(6): 766-773.

Rotches-Ribalta, M., M. Urpi-Sarda, R. Llorach, M. Boto-Ordoñez, O. Jauregui, G. Chiva-Blanch, L. Perez-Garcia, W. Jaeger, M. Guillen, D. Corella, F. J. Tinahones, R. Estruch and C. Andres-Lacueva (2012). "Gut and microbial resveratrol metabolite profiling after moderate long-term consumption of red wine versus dealcoholized red wine in humans by an optimized ultra-high-pressure liquid chromatography tandem mass spectrometry method." *Journal of Chromatography A* 1265(0): 105-113.

Rothwell, J. A., Y. Fillâtre, J.-F. Martin, B. Lyan, E. Pujos-Guillot, L. Fezeu, S. Hercberg, B. Comte, P. Galan, M. Touvier and C. Manach (2014). "New Biomarkers of Coffee Consumption Identified by the Non-Targeted Metabolomic Profiling of Cohort Study Subjects." *PLoS One* 9(4): e93474.

Scalbert, A., L. Brennan, O. Fiehn, T. Hankemeier, B. Kristal, B. van Ommen, E. Pujos-Guillot, E. Verheij, D. Wishart and S. Wopereis (2009). "Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research." *Metabolomics* 5(4): 435-458.

Scalbert, A., L. Brennan, C. Manach, C. Andres-Lacueva, L. O. Dragsted, J. Draper, S. M. Rappaport, J. J. van der Hooft and D. S. Wishart (2014). "The food metabolome: a window over dietary exposure." *The American Journal of Clinical Nutrition* 99(6): 1286-1308.

Shulman, R. G. and D. L. Rothman (2001). "13C NMR OF INTERMEDIARY METABOLISM: Implications for Systemic Physiology." *Annual Review of Physiology* 63(1): 15-48.

Skoog, Douglas A., F. James Holler, and Stanley R. Crouch. 2007. *Principles of instrumental analysis*. Belmont, CA: Thomson Brooks/Cole. ISBN: 978-607-481-390-6.

Smith, C. A., G. O'Maille, E. J. Want, C. Qin, S. A. Trauger, T. R. Brandon, D. E. Custodio, R. Abagyan and G. Siuzdak (2005). "METLIN: a metabolite mass spectral database." *Therapeutic Drug Monitoring* 27(6): 747-751.

Solanky, K. S., N. J. Bailey, B. M. Beckwith-Hall, S. Bingham, A. Davis, E. Holmes, J. K. Nicholson and A. Cassidy (2005). "Biofluid 1H NMR-based metabonomic techniques in nutrition research - metabolic effects of dietary isoflavones in humans." *Journal of Nutritional Biochemistry* 16(4): 236-244.

Solanky, K. S., N. J. Bailey, B. M. Beckwith-Hall, A. Davis, S. Bingham, E. Holmes, J. K. Nicholson and A. Cassidy (2003). "Application of biofluid 1H nuclear magnetic resonance-based metabonomic techniques for the analysis of the biochemical effects of dietary isoflavones on human plasma profile." *Analytical Biochemistry* 323(2): 197-204.

Steffen, L., Y. Zheng and B. Steffen (2014). "Metabolomic Biomarkers Reflect Usual Dietary Pattern: A Review." *Current Nutrition Reports* 3(1): 62-68.

Stella, C., B. Beckwith-Hall, O. Cloarec, E. Holmes, J. C. Lindon, J. Powell, F. van der Ouderaa, S. Bingham, A. J. Cross and J. K. Nicholson (2006). "Susceptibility of Human Metabolic Phenotypes to Dietary Modulation." *Journal of Proteome Research* 5(10): 2780-2788.

Szymańska, E., F. van Dorsten, J. Troost, I. Paliukhovich, E. J. van Velzen, M. W. B. Hendriks, E. Trautwein, J. M. van Duynhoven, R. Vreeken and A. Smilde (2012a). "A lipidomic analysis approach to evaluate the response to cholesterol-lowering food intake." *Metabolomics* 8(5): 894-906.

Szymańska, E., E. Saccenti, A. Smilde and J. Westerhuis (2012b). "Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies." *Metabolomics* 8(1): 3-16.

Tagliabue, A. and M. Elli (2013). "The role of gut microbiota in human obesity: Recent findings and future perspectives." *Nutrition, Metabolism and Cardiovascular Diseases* 23(3): 160-168.

Taguchi, R., M. Nishijima and T. Shimizu (2007). "Basic analytical systems for lipidomics by mass spectrometry in Japan." *Methods in Enzymology* 432: 185-211.

Trygg, J., E. Holmes and T. Lundstedt (2007). "Chemometrics in metabonomics." *Journal of Proteome Research* 6(2): 469-479.

Tulipani, S., R. Llorach, O. Jáuregui, P. López-Uriarte, M. Garcia-Aloy, M. Bullo, J. Salas-Salvadó and C. Andrés-Lacueva (2011). "Metabolomics unveils urinary changes in subjects with metabolic syndrome following 12-week nut consumption." *Journal of Proteome Research* 10(11): 5047-5058.

Tulipani, S., M. Martinez Huelamo, M. Rotches Ribalta, R. Estruch, E. E. Ferrer, C. Andres-Lacueva, M. Illan and R. M. Lamuela-Raventós (2012). "Oil matrix effects on plasma exposure and urinary excretion of phenolic compounds from tomato sauces: Evidence from a human pilot study." *Food Chemistry* 130(3): 581-590.

Ulrich, E. L., H. Akutsu, J. F. Doreleijers, Y. Harano, Y. E. Ioannidis, J. Lin, M. Livny, S. Mading, D. Maziuk, Z. Miller, E. Nakatani, C. F. Schulte, D. E. Tolmie, R. Kent Wenger, H. Yao and J. L. Markley (2008). "BioMagResBank." *Nucleic Acids Research* 36(Database issue): D402-408.

Urpi-Sarda, M., M. Monagas, N. Khan, R. Llorach, R. M. Lamuela-Raventos, O. Jauregui, R. Estruch, M. Izquierdo-Pulido and C. Andres-Lacueva (2009). "Targeted metabolic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography-tandem mass spectrometry." *Journal of Chromatography A* 1216(43): 7258-7267.

van den Berg, R. A., H. C. Hoefsloot, J. A. Westerhuis, A. K. Smilde and M. J. van der Werf (2006). "Centering, scaling, and transformations: improving the biological information content of metabolomics data." *BMC Genomics* 7: 142.

van der Voet, H. (1994). "Comparing the predictive accuracy of models using a simple randomization test." *Chemometrics and Intelligent Laboratory Systems* 25(2): 313-323.

van Dorsten, F. A., C. H. Grün, E. J. van Velzen, D. M. Jacobs, R. Draijer and J. P. van Duynhoven (2010). "The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics."*Molecular Nutrition & Food Research* 54(7):897-908.

van Velzen, E. J., J. A. Westerhuis, J. P. van Duynhoven, F. A. van Dorsten, C. H. Grun, D. M. Jacobs, G. S. Duchateau, D. J. Vis and A. K. Smilde (2009). "Phenotyping tea consumers by nutrikinetic analysis of polyphenolic end-metabolites." *Journal of Proteome Research* 8(7): 3317-3330.

van Velzen, E. J., J. A. Westerhuis, J. P. van Duynhoven, F. A. van Dorsten, H. C. Hoefsloot, D. M. Jacobs, S. Smit, R. Draijer, C. I. Kroner and A. K. Smilde (2008). "Multilevel data analysis of a crossover designed human nutritional intervention study." *Journal of Proteome Research* 7(10): 4483-4491.

Vázquez-Fresno, R., R. Llorach, F. Alcaro, M. Rodríguez, M. Vinaixa, G. Chiva-Blanch, R. Estruch, X. Correig and C. Andrés-Lacueva (2012). "(1) H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors." *Electrophoresis* 33(15): 2345-2354.

Vázquez-Fresno, R., R. Llorach, J. Marinic, S. Tulipani, M. Garcia-Aloy, I. Espinosa-Martos, E. Jiménez, J. M. Rodríguez and C. Andres-Lacueva (2014a). "Urinary metabolomic fingerprinting after consumption of a probiotic strain in women with mastitis." *Pharmacological Research* 87(0): 160-165.

Vázquez-Fresno, R., R. Llorach, M. Urpi-Sarda, O. Khymenets, M. Bulló, D. Corella, M. Fitó, M. Martínez-González, R. Estruch and C. Andres-Lacueva (2014b). "An NMR metabolomics approach reveals a combined-biomarkers model in a wine interventional trial with validation in free-living individuals of the PREDIMED study." *Metabolomics*: 1-10. doi:10.1007/s11306-014-0735-x.

Vázquez-Fresno, R., R. Llorach, M. Urpi-Sarda, A. Lupianez-Barbero, R. Estruch, D. Corella, M. Fitó, F. Arós, M. Ruiz-Canela, J. Salas-Salvadó and C. Andres-Lacueva (2014c). "Metabolomic pattern analysis after Mediterranean diet intervention in a non-diabetic population: a 1- and 3-year follow-up in the PREDIMED study." *Journal of Proteome Research*. 14 (1), pp 531–540.

Walsh, M. C., L. Brennan, E. Pujos-Guillot, J. L. Sebedio, A. Scalbert, A. Fagan, D. G. Higgins and M. J. Gibney (2007). "Influence of acute phytochemical intake on human urinary metabolomic profiles." *American Journal of Clinical Nutrition* 86(6): 1687-1693.

Wang, Y., J. Xiao, T. O. Suzek, J. Zhang, J. Wang and S. H. Bryant (2009). "PubChem: a public information system for analyzing bioactivities of small molecules." *Nucleic Acids Research* 37(Web Server issue): W623-633.

Westerhuis, J., H. J. Hoefsloot, S. Smit, D. Vis, A. Smilde, E. J. van Velzen, J. M. van Duijnhoven and F. van Dorsten (2008). "Assessment of PLSDA cross validation." *Metabolomics* 4(1): 81-89.

WHO/FAO (2002). "Guidelines for the evaluation of probiotics in food." *Food and Agricuoture Organization of the Unitaed Nations and World Health Organization Expert Consultation Report*.

Williams, A. J. (2008). "Public chemical compound databases." *Current Opinion in Drug Discovery & Development* 11(3): 393-404.

Wilson, D. M., A. L. Burlingame, T. Cronholm and J. Sjövall (1974). "Deuterium and carbon-13 tracer studies of ethanol metabolism in the rat by 2H, 1H-decoupled 13C nuclear magnetic resonance." *Biochemical and Biophysical Research Communications* 56(3): 828-835.

Wishart, D. S. (2007). "Current Progress in computational metabolomics." *Briefings in Bioinformatics* 8(5): 279-293.

Wishart, D. S. (2008). "Metabolomics: applications to food science and nutrition research." *Trends in Food Science & Technology* 19(9): 482-493.

Wishart, D. S. (2009). "Computational strategies for metabolite identification in metabolomics." *Bioanalysis* 1(9): 1579-1596.

Wishart, D. S., T. Jewison, A. C. Guo, M. Wilson, C. Knox, Y. Liu, Y. Djoumbou, R. Mandal, F. Aziat, E. Dong, S. Bouatra, I. Sinelnikov, D. Arndt, J. Xia, P. Liu, F. Yallou, T. Bjorndahl, R. Perez-Pineiro, R. Eisner, F. Allen, V. Neveu, R. Greiner and A. Scalbert (2013). "HMDB 3.0—The Human Metabolome Database in 2013." *Nucleic Acids Research* 41(D1): D801-D807.

Xia, J., D. Broadhurst, M. Wilson and D. Wishart (2013). "Translational biomarker discovery in clinical metabolomics: an introductory tutorial." *Metabolomics* 9(2): 280-299.

Xia, J. and D. S. Wishart (2011). "Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst." *Nature Protocols* 6(6): 743-760.

Xu, J., S. Yang, S. Cai, J. Dong, X. Li and Z. Chen (2010). "Identification of biochemical changes in lactovegetarian urine using 1H NMR spectroscopy and pattern recognition." *Analytical and Bioanalytical Chemistry* 396(4): 1451-1463.

Zhang, A., H. Sun, P. Wang, Y. Han and X. Wang (2012). "Modern analytical techniques in metabolomics analysis." *Analyst* 137(2): 293-300.

Zhang, X., Y. Yap, D. Wei, G. Chen and F. Chen (2008). "Novel omics technologies in nutrition research." *Biotechnology Advances* 26(2): 169-176.

ANEXOS

9.1 OTRAS PUBLICACIONES EN REVISTAS

Revisión bibliográfica:

Llorach R, Garcia-Aloy M, Tulipani S, Vázquez-Fresno R, Andres-Lacueva C. Nutrimetabolomic strategies to develop new biomarkers of intake and health effects. *Journal of Agricultural and Food Chemistry*. 2012; 60(36):8797-8808. doi: 10.1021/jf301142b.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Review

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Nutrimetabolomic Strategies To Develop New Biomarkers of Intake and Health Effects

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ABSTRACT: Correctly assessing the metabolic status of subjects after consumption of specific diets is an important challenge for modern nutrition. Recently, metabolomics has been proposed as a powerful tool for exploring the complex relationship between nutrition and health. Nutritional metabolomics, through investigating the role that dietary components play in the maintenance of health and development of risk disease, aims to identify new biomarkers that allow the intake of these compounds to be monitored and related to their expected biological effects. This review offers an overview of the application of nutrimetabolomic strategies in the discovery of new biomarkers in human nutritional research, suggesting three main categories: (1) assessment of nutritional and dietary interventions; (2) diet exposure and food consumption monitoring; and (3) health phenotype and metabolic impact of diet. For this purpose, several examples of these applications will be used to provide evidence and to discuss the advantages and drawbacks of these nutrimetabolomic strategies.

KEYWORDS: *metabolomics, nutrimetabolomics, diet, biomarkers, nutrition, health phenotype*

INTRODUCTION

In the postgenomic era the integrated study of human physiology and pathology has emerged as a fundamental approach. In this regard, the Institute for Systems Biology has defined the term "systems biology" as "the study of an organism, viewed as an *integrated* and *interacting network* of genes, proteins and biochemical reactions which give rise to life", focusing the study on one organism and on all its components and the interactions among them, rather than studying them separately.[1] In the study of systems biology the "omics cascade" is a central concept.[2] It comprises the data obtained by genomics, transcriptomics, proteomics, and metabolomics, with the aim of integrating them to study the biochemical and biological mechanisms and elucidating the impact of diseases or environmental factors on the organism. Among ~omics levels, metabolomics is the one allocated at the end point of the omics cascade, because it is the nearest to the phenotype. Thus, it is an interesting tool in the study of phenotypic changes caused by the impact of environmental agents on several diseases.[2] The Metabolomics Society defines metabolomics as the "newly emerging field of omics research concerned with the comprehensive characterization of the small molecule metabolites in biological systems which can provide an overview of the metabolic status and global biochemical events associated with a cellular or biological system".[3] Two terms, close in meaning, have been used: metabonomics and metabolomics. Although both focus their research on the analysis of metabolites in the organism, metabonomics[4] focuses its efforts on studying the metabolic changes in an integrated biological system, whereas metabolomics[5] also considers the study of metabolites in cells, tissues, organs, and organisms. This discrepancy is also due to the origin of the word: the "metabolomics" concept is based on the metabolome, whereas the term "metabonomics" comes from the Greek roots "meta" and "nomos", which mean "change" and "rules" or "laws", respectively, in reference to the analysis of changes in metabolism.[6] In this regard, "metabolome" has been defined as the complete set of metabolites or chemicals that can be found in a cell, organ, or organism.[2,7] The term "metabolomics" is the one employed in this review. Two factors that influence the metabolome have been described: the endogenous metabolome, which involves all intrinsic metabolites related to the primary and intermediary metabolism, and the exogenous metabolome, which refers to all metabolites arising from extrinsic factors such as diet (i.e., food metabolome), microbiota, physical activity, stress, or drugs.[8,9] Specifically, "food metabolome" has been defined as all metabolites deriving from the ingestion of food.[10]

Metabolomic studies have shown a typical pipeline that has been broadly categorized into five main steps: sample collection, sample preparation, data acquisition, data analysis, and biological interpretation (Figure 1).[2,11] In addition, it is also necessary to highlight the importance of applying a quality control pipeline in metabolomic studies. A metabolomic approach has been applied in different kinds of samples, including single cells,[12] tissues, and biofluids. In this context, blood and urine are the most likely sources of biofluids for human metabolomics.[8] Other biofluids, such as saliva[13] and cerebrospinal fluid,[14] have also been used but to a lesser extent. A picture of the whole metabolome cannot be measured using a single analytical technology; however, bibliographic references about the use of multianalytical platforms are scarce. This means that various analytical techniques have emerged for

Special Issue: 5th International Conference on Polyphenols and Health

Received: March 15, 2012
Revised: May 17, 2012
Accepted: May 18, 2012
Published: May 18, 2012

ACS Publications © 2012 American Chemical Society

dx.doi.org/10.1021/jf301142b | *J. Agric. Food Chem.* 2012, 60, 8797−8808

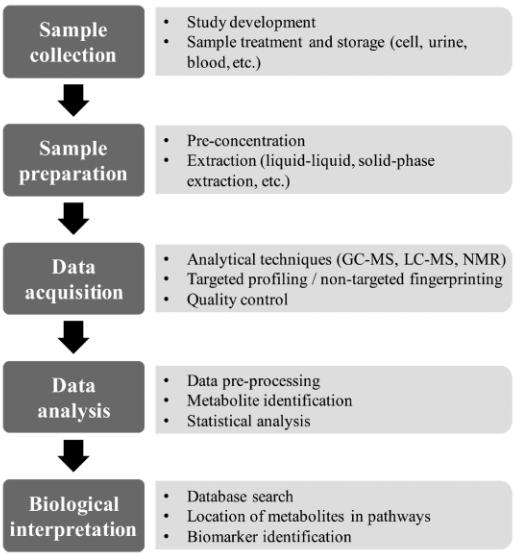

Figure 1. Metabolomics workflow scheme.

studying the metabolome. Among them, mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the most commonly applied in metabolomic analysis. An explanation and evaluation of their advantages and disadvantages have been reviewed previously.[2,7,9,15,16] With regard to data analysis and biological interpretation, comprehensive reviews and descriptions of bioinformatic tools have been published.[17−26]

Two approaches have been described for the study of metabolomic data.[2,16] One of them is known as "targeted profiling", which is based on the analysis of a predefined metabolite group associated with a particular class of compounds or metabolic pathway. It provides quantitative results. Nevertheless, with this approach some information related to the whole metabolic network and its relationship with pathophysiological status might be lost. Instead, "non-targeted fingerprinting" attempts to obtain a broad picture of a metabolome with the aim of detecting the maximum number of metabolites in a sample, including those that are unknown or poorly characterized, giving a global approach of the whole metabolome of an organism. Its aim is to study metabolite patterns in response to a disease or an environmental or lifestyle factor, elucidating the new roles of some metabolites. This second metabolomic approach provides qualitative data and is considered to be a hypothesis-generating approach, whereas the first one is regarded as a hypothesis-driven method.[2]

Metabolomics, Health, and Nutrition. Metabolomics offers a wide range of applications in biomedical research. One of these is the capacity to predict or detect the stages of diseases and their progression or to monitor the compliance and response to therapy through the use of biomarkers.[2,3] Recent reviews have highlighted the relevance of metabolomics for obtaining biomarkers when this approach was applied in clinical conditions such as cardiovascular disease, cancer, or inborn errors of metabolism.[27] In this context, two phases in the discovery of biomarkers through metabolomics have been described.[27] The first one is the discovery phase, which provides potential biomarkers using nontargeted metabolomic approaches. Between 10 and 100 samples controlled by confounding factors are required in this stage. Fewer samples could be sufficient if samples were available before and after the perturbation of the same individual that was studied, allowing each subject to act as his own control, thereby reducing interindividual variability. Once the putative biomarker is identified, the process of validation in a general population is necessary. The aim of the second phase is to evaluate the specificity and selectivity of the biomarker using an absolute quantification by a targeted metabolic approach of a range of 100−1000 samples.

Currently, the main causes of morbidity and mortality worldwide are those pathologies known as noncommunicable diseases.[28] They include cardiovascular diseases, diabetes, cancers, and chronic respiratory diseases. All of them are often related to aging and are more influenced by lifestyle factors, such as tobacco use, insufficient physical activity, or unhealthy diet, than by genetic determinants. Due to the multifactorial etiology of these diseases, and because it has been established that a disease is characterized by exhibiting a particular pattern of metabolites, a new concept for biomarkers has emerged.[29,30] This focuses on the development of biomarker patterns, or metabolite signatures, instead of isolated biomarkers, as has been used traditionally in clinical practice.[30] This change means that more knowledge about perturbed pathways in pathological states is required. Furthermore, more complex technologies are needed to adequately characterize the novel biomarkers. Consequently, the combined use of both metabolomic approaches described previously offers an interesting scenario for the research of new biomarkers related to these pathologies.[2,27,29,31,32]

Focusing on the nutrition sciences, metabolomics is an interesting tool for assessing the nutritional status of an individual, the food consumption, the biological consequences of following a nutritional intervention, or the study of metabolic mechanisms in response to a diet depending on a particular metabolic phenotype.[7,10,16] With regard to the metabolome, which has been defined above, it should be noted that feeding induces alterations in the endogenous metabolome, and they can be evaluated by the detection of exogenous metabolites in biofluids.[9] They can be used as potential biomarkers of food consumption (i.e., food metabolome) and/or the effects of a dietetic intervention. The use of biomarkers in this field is interesting because they are objective and accurate tools. Recently, metabolomics has been proposed as a powerful tool for exploring the complex relationship between nutrition and health.[33] Several studies and reviews have pointed out the interest in applying metabolomics to the nutritional field of work.[7−9,16,31,33,34]

The aim of this review is to offer an overview of the state of the art of the application of nutrimetabolomic strategies in the discovery of new biomarkers in human health nutritional research.

■ REVIEW STRATEGY

To assess the current status of the application of nutrimetabolomic strategies in biomarker discovery, we have conducted an exhaustive search of the scientific bibliography. Our search was applied to the PubMed and Web of Science electronic databases. The search strategy used for the PubMed database was ("Metabolome"[Mesh] OR "Metabolomics"[-

Mesh]) AND ("Diet"[Mesh] OR "Food"[Mesh] OR "Nutritional Sciences"[Mesh]) AND ("Biological Markers"[Mesh]) AND ("Humans"[Mesh]). The search strategy used for the Web of Science database was (TS=(metabolom* OR metabonom*) AND TS=(diet* OR food* OR nutrition*) AND TS=(biomarker* OR biological marker*)) AND Document Types=(Article). The last search was run on January 25, 2012. Furthermore, to identify additional studies we also analyzed the reference lists of the publications selected. We included papers that used the metabolomics technology applied in nutritional studies. These had to be original investigations carried out in humans. No language or publication date restrictions were imposed. The search of the PubMed and Web of Science databases provided 24 and 146 publications, respectively. After adjusting for duplicates, 159 remained. Of these, we excluded 34 reviews and 7 other document types (congresses, comments, letters, etc.). The titles and abstracts of the remaining 118 articles were screened. Of these, 57 were excluded for the following reasons: 49 used animal models, 5 evaluated food matrices, 2 were conducted on bacteria, and 1 was an in vitro study. The 61 remaining studies were conducted in humans, but 25 were excluded because they were not related to the topic. Finally, we did not include 1 study because it used lipidomics technology, 7 studies because they used a targeted analysis, and 2 because they did not apply chemometric methods. Additionally, 14 publications in the field were detected among the references of the papers selected. Therefore, 40 studies were included in this review.

After reviewing the selected articles, we propose a classification with three principal categories (Figure 2), namely,

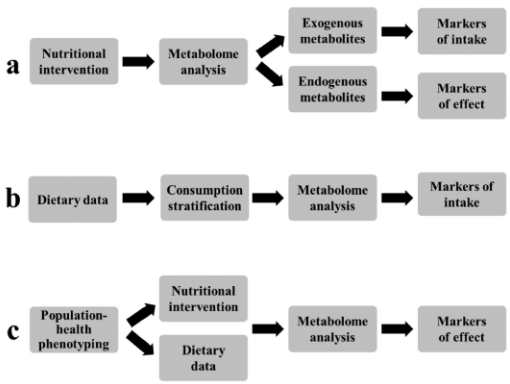

Figure 2. Main nutrimetabolomic approaches implemented: (a) assessment of nutritional and dietary interventions; (b) diet exposure and food consumption monitoring; (c) health phenotype and metabolic impact of diet.

"assessment of nutritional and dietary interventions", "diet exposure and food consumption monitoring", and "health phenotype and metabolic impact of diet". As well as these categories we have also considered a heterogeneous category termed "other studies".

■ NUTRIMETABOLOMIC STRATEGIES TO DEVELOP NEW BIOMARKER ASSESSMENT

An important challenge for modern nutrition is to correctly assess the metabolic status of subjects after consumption of specific nutrients, foods, or diets, with the aim of promoting health. Dietary components have been associated with an important diversity of mechanisms of action and biological effects in consumers. Furthermore, it is assumed that food consumption has an effect on the human metabolome. Consequently, the concept of nutritional metabolomics, or nutrimetabolomics, has emerged with the introduction of metabolomic technology into nutritional research.[35]

Assessment of Nutritional and Dietary Interventions. In the past few years a considerable number of studies using this approach have been published, most of them focused on evaluating the effect of an acute or chronic intake of a particular food (Figure 2a). Nevertheless, to our knowledge, there is only one human intervention study designed to evaluate the influence of dietary pattern interventions on the metabotype.[36]

A summary of selected studies is presented in Table 1. Almost all of the studies were performed on healthy adults,[36−46] whereas others were performed on healthy children,[47] pregnant women,[48] or mildly hypertensive,[49−51] metabolic syndrome,[52] or cancer[53] patients. The publications selected involved different types of design. Most of them were prospective studies,[36−39,44−54] but in five of them the volunteers consumed an acute dose of the food item evaluated.[40−43,55] The length of each intervention period in the prospective studies varied from 2 days[44,54] to 8 months.[48] Urine was the most widely used biological sample,[36,38−45,49,50,52,55] whereas three studies were performed on blood samples,[37,48,53] and two used both urine and blood samples.[47,54] Furthermore, two studies analyzed feces.[46,51] With regard to the analytical platform, ^{1}H NMR was the most common analytical technique used in these publications,[36−41,45−47,49−51,53,54] but MS has also been employed in five studies,[42,43,48,52,55] and in one article both platforms were applied.[44]

Effects on Metabolome and Biological Interpretation. The metabolic changes observed in these studies, together with their biological interpretation, are summarized in Table 2. This table shows several modifications either in food or in the endogenous metabolome. Broadly speaking, the effects on the food metabolome were more widely detected in urine samples, whereas blood samples showed the most changes in the endogenous metabolome. With regard to the modifications on the food metabolome, these can be classified into two classes: those directly derived from the digestion of food components by the human organism and those derived from the metabolism of human gut microbiota.

With regard to metabolites directly derived from the digestion of food components by the human organism, urinary levels of creatine, creatinine, and carnitine increased after the intake of meat.[36,47] High concentrations of creatine and carnitine are found in meats, and creatinine is formed from biodegradation of creatine and is carried to the kidneys by blood plasma to be eliminated by urine.[56] These metabolites have been proposed as potential biomarkers of dietary meat intake, although they have not yet been validated.[56] In support of this, carnitine was almost totally absent after 15 days of following a vegetarian diet,[36] and reduced levels of urinary creatinine were observed following the intake of chamomile tea[39] and fruit and vegetable juice.[44] However, Wang et al.[39] proposed that the lower levels of creatinine after chamomile consumption resulted from the antioxidative activity of chamomile. Among the reviewed studies, methylhistidine and anserine were associated with salmon consumption,[55] although

Table 1. Characteristics of Nutrimetabolomic Studies Assessing the Effect of Dietary Interventions in Humans

subjects: *n*	study design	intervention (dose per day)	duration	sample	analytical technique	ref
12 healthy	crossover randomized	(a) low-meat diet (60 g of red meat; 65 g of protein) (b) high-red-meat diet (420 g of red meat; 143–150 g of protein) (c) vegetarian diet (143–150 g of protein from nonmeat sources)	15 days each period	24 h urine	^{1}H NMR	36
5 healthy	single group	60 g of soy protein (45 mg of isoflavones)	1 complete menstrual cycle	plasma	^{1}H NMR	37
9 healthy	parallel	(a) 60 g tof extured vegetable protein (45 mg of conjugated isoflavone glucosides) (b) 50 g of miso (25 mg of unconjugated isoflavones)	1 complete menstrual cycle	24 h urine	^{1}H NMR	38
14 healthy	single group	200 mL of chamomile tea (5 g of powder in 200 mL of water)	2 weeks	urine	^{1}H NMR	39
3 healthy	single group	3 g of decaffeinated black tea extract	acute	urine	^{1}H NMR[a]	40
17	crossover randomized	(a) 6 g of black tea in water (b) 6 g of green tea in water (c) 360 mg of caffeine in gelatin capsules (control)	2 days each period	24 h urine plasma	^{1}H NMR	54
20 healthy	crossover randomized double-blind	>200 mL of water with a capsule containing 2500 mg of (a) dried black tea extract powder (800 mg of polyphenols) (b) sucrose (placebo)	acute	48 h urine	^{1}H NMR	41
10 healthy	crossover randomized	(a) 40 g of cocoa powder + 250 mL of water (b) 40 g of cocoa powder + 250 mL of milk (c) 250 mL of milk (control)	acute	24 h urine	LC-q-ToF-MS	42
24 healthy	parallel randomized blind	10 capsules containing (a) almond skin extract (3.5 g of almond extract + 0.5 g of microcrystalline cellulose) (b) 4 g of microcrystalline cellulose (placebo)	acute	24 h urine	LC-q-ToF-MS	43
42 metabolic syndrome	parallel randomized	(a) low-fat diet with mixed nuts (30 g: 15 g of walnuts + 7.5 g of almonds + 7.5 g of hazelnuts) (b) low-fat diet (control)	3 months	24 h urine	LC-q-ToF-MS	52
21 healthy	single group	4 × 100 mL of apple, carrot, and strawberry drinks	2 days	urine	^{1}H NMR LC-q-ToF-MS	44
20 healthy	single group	500 g of broccoli and Brussels sprouts	2 weeks	48 h urine	^{1}H NMR	45
29 mildly HTA	crossover double-blind	(a) mix of wine and grape juice extract (800 mg of polyphenols) (b) microcrystalline cellulose (placebo)	1 month each period	24 h urine	^{1}H NMR	49

dx.doi.org/10.1021/jf301142b | *J. Agric. Food Chem.* 2012, 60, 8797−8808

Table 1. continued

subjects: *n*	study design	intervention (dose per day)	duration	sample	analytical technique	ref
58 mildly HTA	crossover randomized double-blind	(a) mix of wine and grape juice extract or red grape juice extract (800 mg of polyphenols) (b) microcrystalline cellulose (placebo)	1 month each period	24 h urine	^{1}H NMR[b]	50
39[c] mildly HTA	crossover randomized double-blind	(a) mix of wine and grape juice extract or grape juice extract (800 mg of polyphenols) (b) microcrystalline cellulose (placebo)	1 month each period	feces	^{1}H NMR	51
96 healthy	parallel	(a) fortified wheat flour (300 g: 20 mg of folic acid + 3.5 mg of vitamin B_1 + 3.5 mg of vitamin B_2 + 30 mg of ferric sodium edetate +25 mg of zinc oxide/kg) (b) placebo	8 months	serum	UPLC-ToF-MS	48
17 prostate cancer	crossover randomized	(a) diet rich in whole-grain rye and rye bran products (485 g) (b) diet of refined whole-grain products with added cellulose (485 g) (control)	1.5 months each period	plasma	^{1}H NMR	53
16 healthy	single group	synbiotic (0.5 g of fructooligosaccharides + 10^9 CFU *B. longum* + 10^9 CFU *L. acidophilus* per serving, twice a day)	1 month	feces	^{1}H NMR	46
24 healthy	parallel	(a) 1.5 L of skimmed milk (53 g of protein) (b) 250 g of low-fat meat (53 g of protein)	1 week	24 h urine serum	^{1}H NMR	47
36	crossover randomized	orange juice, tea with skimmed milk and sugar, butter croissant, and (a) cornflakes with milk (control) (b) smoked salmon trimmings (60 g) (c) steamed broccoli florets (200 g) (d) raspberries (200 g) (e) 2 biscuits (37.5 g) with 125 mL of semiskimmed milk	acute	4.5 h urine	FIE-MS[d]	55

[a]HPLC NMR-MS was used to identify an unknown metabolite. [b]GC-MS was used for targeted profiling of urinary phenolic acids. [c]Although there were 53 volunteers who participated in the study, researchers analyzed fecal samples from 39 subjects. [d]GC-ToF-MS was used for targeted analysis.

dx.doi.org/10.1021/jf301142b | J. Agric. Food Chem. 2012, 60, 8797−8808

Journal of Agricultural and Food Chemistry Review

Table 2. Effects Observed on Metabolome following a Nutritional Intervention and Their Biological Interpretation

effects on metabolome[a]	biological interpretation[b]	ref
↑ creatine (high-meat), ↑ creatinine (high-meat)	markers of meat intake	36
↑ carnitine (high-meat), ↓ carnitine (vegetarian)	markers of red-meat intake/↑ β-oxidation	
↑ trimethylamine-*N*-oxide (high-meat)	marker of meat intake/metabolite from microbiota	
↑ taurine (high-meat), ↑ *N*-acetyl-5-hydroxytryptamine (high-meat)	markers of protein intake	
↑ *p*-hydroxyphenylacetic acid (vegetarian)	metabolite from microbiota	
↓ N^6,N^6,3N^6-trimethyllysine (vegetarian)	↑ synthesis of carnitine from N^6,N^6,N^6-trimethyllysine	
↑ acetylcarnitine (high-meat), ↑ glutamine (high-meat)		
↑ lactate	↑ anaerobic metabolism/↓ gluconeogenesis	37
↓ carbohydrate concentrations	↑ anaerobic metabolism	
subject-specific changes in isoleucine, valine, triglycerols, choline, acetate, acetoacetate, and alanine	changes in carbohydrate and energy metabolism	
↑ *N*-acetyl glycoproteins	changes in protein turnover	
↑ 3-hydroxybutyrate, changes in lipoproteins		
↑ trimethylamine-*N*-oxide	improved glomerular function or general kidney function/ metabolite from microbiota	38
changes in choline and betaine	improved glomerular function or general kidney function/ metabolite from microbiota/changes in lipid and cholesterol metabolism and transport	
changes in creatinine and creatine	improved glomerular function or general kidney function	
↑ glutamate, ↑ glutamine	changes in tricarboxylic acid cycle/↑ protein breakdown	
↓ citrate, changes in sugar region	↓ glycolysis	
changes in methylamines, glycine, and acetate	changes in lipid and cholesterol metabolism and transport	
↑ hippuric acid	metabolite from microbiota	39
↑ unknown metabolite	metabolite from chamomile	
↓ creatinine	↑ antioxidative activity	
↑ glycine		
↑ hippuric acid, ↑ 1,3-dihydroxyphenyl-2-*O*-sulfate	metabolites from microbiota	40
↑ gallic acid (tentative identification), ↑ para-substituted aromatic compound		
urine: ↓ tyrosine (black tea), ↓ alanine (black tea), ↓ glutamine/glutamate, ↑ taurine (black tea), ↑ glycine, ↑ valine, ↑ methionine (black tea), ↑ pyruvate, ↑ α-ketoglutarate, ↑ glucose (black tea), ↑ betaine (green tea), ↑ dimethylamine, ↑ *N*-acetyl glycoproteins; changes in unknown metabolites		54
↑ hippuric acid, ↑ 1,3-dihydroxyphenyl-2-*O*-sulfate, ↑ unidentified aromatic compounds (green tea)	metabolites from microbiota	
↑ citrate (green tea), ↑ succinate, ↑ oxaloacetate (green tea), ↑ 2-oxoglutarate	↑ oxidative energy metabolism/changes in fatty acid biosynthesis, gluconeogenesis, and glyceroneogenesis/↑ insulin secretion	
↑ β-hydroxybutyrate (black tea)	changes in ketogenesis and fatty acid oxidation	
plasma: ↓ acetate (black tea), ↑ acetate (green tea), ↑ β-hydroxybutyrate (green tea); changes in lipoprotein distribution		
↓ glucose	↑ insulin activity	
↓ lactate (green tea), ↓ alanine (green tea)	↓ anaerobic glycolysis	
↑ hippuric acid, ↑ 4-hydroxyhippuric acid, ↑ 1,3-dihydroxyphenyl-2-*O*-sulfate	metabolites from microbiota	41
↑ 6-amino-5-[*N*-methylformylamino]-1-methyluracil, ↑ methyluric acids, ↑ 3,7-dimethyluric acid, ↑ methylxanthines, ↑ theobromine, ↑ caffeine	purine alkaloid derivatives	42
↑ vanillic acid, ↑ vanilloylglicine, ↑ epicatechin-*O*-sulfate, ↑ *O*-methylepicatechin	markers of cocoa or chocolate intake	
↑ 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid, ↑ phenylvalerolactone derivatives	metabolites from microbiota	
↑ 3,5-diethyl-2-methylpyrazine, ↑ hydroxyacetophenone, ↑ cocoa diketopiperazines	cocoa flavor and taste compounds	
↑ hydroxynicotinic acid, ↑ trigonelline	nicotinic acid derivatives	
↑ tyrosine	amino acid from cocoa	
↑ flavonoid conjugates, ↑ phenylvalerolactones, ↑ 4-hydroxy-5-(phenyl)valeric acid conjugates, ↑ hydroxyphenylacetic acid conjugates, ↑ hydroxyphenylpropionic acid conjugates, ↑ vallinic acid glucuronide, ↑ hydroxyhippuric acid, ↑ ferulic acid glucuronide	metabolites from microbiota and almond skin extract	43
↑ 10-hydroxydecene-4,6-diynoic acid sulfate, ↑ tridecadienoic/tridecynoic acid glucuronide, ↑ dodecanedioic acid	↑ β-oxidative metabolism and turnover	52
↑ pyrogallol sulfate, ↑ *p*-coumaryl alcohol glucuronide, ↑ *p*-coumaryl alcohol sulfate, ↑ conjugates of urolithin A	metabolites from microbiota	
↑ *N*-acetylserotonin sulfate, ↑ hydroxyindolacetic acid	tryptophan/serotonin metabolites	
↑ hippuric acid	marker of benzoic acid intake/metabolite from microbiota	44
↓ methylhistidine	marker of myofibrillar protein turnover	
↓ creatinine	changes in muscle metabolism	
↑ unknown metabolite		
↑ SMCSO, ↑ 3 metabolites structurally related to SMCSO	markers of cruciferous vegetable intake	45
↑ hippuric acid, ↑ 4-hydroxyhippuric acid, ↑ 4-hydroxyphenylacetic acid, ↑ unknown aromatic metabolite	metabolites from microbiota	49
↑ formic acid, ↓ alanine, ↓ unknown metabolite		
↑ 3- and 4-hydroxyhippuric acid, ↑ 3-hydroxyphenylpropionic acid, ↑ 3- and 4-hydroxyphenylacetic acid, ↑ hippuric acid (mix)	metabolites from microbiota	50
↑ citrate (mix)	changes in mitochondrial tricarboxylic acid cycle turnover	
↑ betaine (mix)	changes in homocysteine homeostasis	

dx.doi.org/10.1021/jf301142b | J. Agric. Food Chem. 2012, 60, 8797−8808

Table 2. continued

effects on metabolome[a]	biological interpretation[b]	ref
↓ isobutyrate; changes in unknown metabolites	↓ protein fermentation/modulation of the gut microbial ecology	51
↑ fructose 6-phosphate, ↑ lactic acid	↑ glycolysis/↑ antioxidative capacity	48
↑ sphingosine 1-phosphate	↑ pentose phosphate pathway → ↑ normal development of the nervous system	
↑ docosahexaenoic acid	↑ neuroprotection	
↓ hexadecanoic acid	↑ biosynthesis pathway of unsaturated fatty acids	
↑ sebacic acid, ↓ indoleacrylic acid, ↑ L-aspartyl-L-phenylalanine, ↓ harderoporphyrin, ↑ glycolithocholate, ↑ tryptophan, ↓ C18:2, ↑ C18:3, ↑ C20:1, ↓ C20:3, ↓ C20:4, ↑ C20:5, ↓ C22:4, ↓ C22:5, ↑ C24:1		
↑ 3-hydroxybutyric acid, ↑ acetone	↑ energy metabolism	53
↑ betaine	marker whole grain and bran intake/changes in homocysteine metabolism	
↑ *N*,*N*-dimethylglycine	changes in homocysteine metabolism	
↑ dimethyl sulfone	metabolite from microbiota	
↓ tyrosine, ↓ phenylalanine, ↓ alanine, ↓ glutamate, ↓ lysine, ↓ glycine, ↓ valine, ↓ isoleucine	↑ amino acid assimilation	46
↑ propionate, ↑ butyrate	metabolite from microbiota	
↓ succinate, ↑ acetate, ↑ lactate, changes in unknown metabolites		
urine: ↑ urea (meat), ↑ unknown metabolite (meat)		47
↓ hippuric acid (milk)	metabolites from microbiota	
↑ creatine (meat), ↑ histidine (meat)	markers of meat intake	
serum: minor changes in lipid profile (milk)		
↑ 1-methylhistidine (salmon), ↑ anserine (salmon)	metabolites of histidine and histidine-derived peptides	55
↑ trimethylamine-*N*-oxide (salmon)	degradation product of carnitine/marker of seafood consumption	
↑ ascorbate (broccoli and raspberry)	marker of fruit and vegetable intake	
↑ tetronic acid and derivatives (broccoli)	putative ascorbate catabolism products	
caffeoyl sulfate (raspberry), methylepicatechin sulfate (raspberry)	phase II metabolism products	

[a]Biofluids not specified in studies of this table are in Table 1. [b]Biological interpretation corresponds to author's discussions about the effects observed on metabolome including endogenous and exogenous metabolites.

both metabolites could be markers of meat, chicken, or fish intake.[56] Several markers related to cocoa flavor and taste have also been identified.[42] Higher levels of *S*-methyl-L-cysteine sulfoxide (SMCSO) and three other metabolites structurally related to SMCSO were observed after 14 days of a daily consumption of broccoli and Brussels sprouts, and the authors proposed the use of SMCSO as a biomarker of cruciferous vegetable consumption.[45] In another study, consumption of broccoli and raspberries was associated with higher urinary excretion of ascorbate and derivatives of this metabolite, which could be used as markers of fruit and vegetable intake.[55] However, tetronic acids were specific to broccoli intake, whereas caffeoyl sulfate and methylepicatechin sulfate were characteristics of raspberry consumption. In a recent publication, Tulipani et al.[52] identified phase II metabolites of medium-chain polyunsaturated fatty acids (C10−C13), which were the most significant urinary markers following a 12 week nut consumption. Moreover, urinary markers of serotonin metabolism were also related, for the first time, to nut intake, although their origin (dietary or endogenous) remains to be elucidated.[52]

Changes in microbiota-derived metabolites were observed in urine samples after the consumption of vegetables or plant-derived products. In most NMR analyses of urinary metabolome, modifications in hippuric acid excretion have been detected. The excretion pattern of this metabolite showed an increase after the consumption of chamomile,[39] black and green tea,[40,41,54] fruit and vegetable juice,[44] and a mix of wine and grape juice extract.[49,50] Hydroxyhippuric, hydroxyphenylacetic, and hydroxyphenylpropionic acids are other metabolites from dietary polyphenols produced by gut microbiota[57] the urinary concentrations of which have also been raised following the consumption of a vegetarian diet,[36] black tea,[41] almond skin extract,[43] and wine and grape juice extract or grape juice extract.[49,50] Additionally, phenylvaleric acids and phenylvalerolactone have been identified after consumption of food sources of flavan-3-ols.[42,43] Some of these compounds were still being excreted in urine 24 h after the intake of a single dose.[42,43] Other microbial-derived metabolites, such as conjugates of urolithin A, were detected in the urinary metabolome after the intake of nuts.[52] In other studies, which are not included in this review, these metabolites have also been detected after the intake of cocoa, almonds, or nuts.[58,59]

With regard to endogenous metabolite alterations, Solanky et al.[37,38] suggested that soy isoflavones had an inhibitory effect on glycolysis associated with a general alteration in energy metabolism. More changes in carbohydrate and lipid metabolism have been detected with the intake of green and black tea[54] and fortified wheat flour.[48] Alterations in amino acid metabolism were determined following the consumption of nuts,[52] a fruit and vegetable drink,[44] and a synbiotic product.[46] It is also important to bear in mind that similar food items with different chemical compositions could have different effects on the modifications of metabolome. In this field, Solanky et al.[38] observed a difference in the magnitude of the biological effects of soy isoflavones depending on their conjugation. Furthermore, in the study of van Dorsten et al.,[50] the effects observed on the urinary metabolome following consumption of grape juice extract or a mix of wine and grape juice extract differed slightly, suggesting that a small change in the phytochemical composition of a food item could have a different impact on the metabolome.

Diet Exposure and Food Consumption Monitoring. As depicted in Figure 2b, this strategy focuses on applying the

metabolomic approach to explore the relationship between dietary information and metabolic profiles in producing new insights (biomarkers) that allow the intake of a particular dietary pattern or component to be assessed. It is important to note that this approach could be applied in interventional studies and in observational studies in which participants are in free-living conditions with ad libitum diets.

In large epidemiological studies in which it is possible to obtain a large amount of information about dietary habits (e.g., either food frequency questionnaires (FFQs) or 24 h recall), metabolomics may be used to evaluate this information.[33] Pere-Trepat et al.[60] described a strategy to discriminate between different dietary patterns and ^{1}H NMR plasma metabolic profiles in a cohort of Danish twins. After a principal component analysis (PCA) of FFQs, five dietary patterns emerged: energy intake, plant versus animal-based diet, "traditional diet" versus sugar-rich diet, "traditional" versus "modern" diets, and consumption of skimmed versus whole dairy products. Moreover, metabolic phenotypes related to the dietary patterns obtained by partial least squares discriminant analysis (PLS-DA) were essentially based on differences in lipids and amino acid profiles in plasma.[60] Another recent study also focused on the relationship between dietary patterns and metabolomic profiles.[61] Recorded 3 day food diaries and biofluids (plasma and urine) from 160 subjects were collected in a double-blind, randomized, placebo-controlled dietary intervention study. The authors analyzed the data by *K*-means clustering for dietary information and ^{1}H NMR for biofluids. Among the results, ^{1}H NMR spectra of urine allowed the identification of metabolites associated with different dietary patterns. In addition, the authors identified several biomarkers of particular food groups, for instance, phenylacetylglutamine for vegetable intake.[61]

With regard to those studies related to the application of metabolomics to identify a particular biomarker or biomarker pattern that could help us to check the adherence to the intervention in large epidemiological studies or the free-living population intake of a particular dietary component, Heinzmann et al.[62] have developed a strategy for food biomarker discovery that combines nutritional intervention with metabolic phenotyping and biomarker validation in a large-scale epidemiological study. The authors identified the urinary excretion of proline betaine (a metabolite from citrus) as a specific and sensitive biomarker of citrus fruit intake. First, they identified proline betaine as a urinary biomarker of citrus consumption in a study with eight participants. Then the authors quantified the relative concentrations of proline betaine in citrus products and evaluated the urinary excretion profile after orange juice consumption. Finally, the validation was carried out, generating a receiver operating characteristic (ROC) curve in U.K. participants in the INTERMAP study in which citrus intake was established from four 24 h dietary recalls per person. The area under the curve (AUC) was 92.3% with a specificity and sensitivity of 90.6% and 86.3%, respectively. The platform selected to produce the untargeted urinary metabolic profiles was ^{1}H NMR.[62] A strong relationship between citrus intake and excretion of proline betaine has also been observed by Lloyd et al.[63] In this work, the authors used nontargeted metabolite urinary fingerprinting (FIE-MS and FT-ICR-MS) to study the relationships between citrus exposure in free-living human subjects, estimated by a FFQ, and the chemical content of urine. The results showed that an acute intake of orange juice resulted in the appearance of proline betaine and several biotransformed metabolites in postprandial urine. In addition, a process of biomarker validation showed sensitivities of 80.8−92.2% and specificities of 74.2−94.1% (false discovery rate-adjusted P values < 0.05) for elevated proline betaine in those volunteers who reported a high consumption of citrus.[63]

Health Phenotype and Metabolic Impact of Diet. An overall representation of this strategy is illustrated in Figure 2c. Usually, nutritional studies collect anthropometrical, biochemical, and medical data (i.e., health data) that could be used to split the population into phenotypes, allowing a deeper study of either the dietary intervention or dietary pattern effect. Selection of phenotypes could be carried out intuitively, for instance, by selecting only those who were diabetic and obese from the obese population and then studying the effect of a particular food consumption, or it could be achieved by using unsupervised multiparametric statistical algorithms such as PCA or *K*-means clustering.[64]

In this context, Martin et al.[65] studied the metabolic responses of free-living subjects (n = 30), classified into low- and high-anxiety trait phenotypes, to a daily consumption of 40 g of dark chocolate for up to 14 days. Plasma and urine samples were collected at the beginning, in the middle, and at the end of a 2 week study and were analyzed by NMR and MS. Results indicated that a daily intake of chocolate was strongly dependent on the dispositional stress state of the individuals, as noted with statistically significant metabolic effects only in subjects with a high-anxiety trait. In fact, intake of dark chocolate resulted in the diminution of the levels of catecholamines and cortisol in the urine from subjects with high dispositional stress.[65] In another study, O'Sullivan et al.[64] applied this kind of strategy in a double-blind, randomized, placebo-controlled dietary intervention to study whether metabolic phenotyping could identify responders to vitamin D supplementation in terms of the metabolic syndrome. After 4 weeks of intervention with 15 g of vitamin D or placebo, a *K*-means clustering of basal biochemical parameters of metabolic syndrome was carried out. With this analysis, authors defined five metabolic phenotypes with different biochemical profiles. In addition, a ^{1}H NMR analysis of urine and plasma was performed. First results did not show any significant effects of vitamin D supplementation on biomarkers of metabolic syndrome. However, fasting insulin concentrations, homeostatic model assessment (HOMA) score, and C-reactive protein (CRP) concentrations were significantly reduced following supplementation with vitamin D in the metabotype characterized by low concentrations of vitamin D and altered adipokine profile.[64]

Other Studies. As described above, this category groups several studies showing particular approaches to the application of metabolomics in nutritional studies. Population-based studies in the field of nutrimetabolomics showed differences in the urinary metabolome among subjects from different geographic zones,[66−69] leading to the hypothesis that diet greatly influences the endogenous urinary metabolome.[66] After analysis of the urinary metabolome of four populations (Japan, China, United States, and United Kingdom) from the INTERMAP study, two metabolic phenotypes (i.e., metabotypes) were differentiated.[67] Each corresponded to East Asian and Western populations, showing contrasting dietary patterns between them. Furthermore, two differentiated metabotypes among Chinese populations were observed.[67,68] In line with these results, previous analyses of these populations showed

differences in metabolite profiles among them.[69] Thus, consumption information should be available to allow successful interpretation of metabolomic data.[66]

The major subgroup of this heterogeneous category involves a wide range of study designs. They include novel approaches ranging from the integration of high-dimensional biological data[70] to the exploration of a new biomarker concept around the connection between exposition and disease,[71] through the evaluation of associations between metabotypes and dietary preferences,[72] the metabolic responses induced by a nutritional intervention following physical exercise,[73,74] or the comparison of the extent of dietary and physiological effects on the urinary metabolome.[75] Bakker et al.[70] proposed the simultaneous study of biochemical parameters, gene expression, proteins, and metabolites in a nutritional intervention to study the effects of specific dietary components in overweight men. This global approach allows an integrated biological interpretation of the data obtained to be carried out, with the aim of studying the molecular processes involved in a particular health or disease status. Chadeau-Hyam et al.[71] applied a "meet-in-the-middle" approach in a subcohort of the EPIC study. This approach was focused on the identification of putative intermediate metabolic biomarkers related to both exposure and disease outcomes, with the aim of reinforcing the knowledge of dietary factors that influence disease development. In the analyzed EPIC cohort, authors identified plasma metabolomic signatures linking colon cancer with dietary fiber. The strategy of Rezzi et al.[72] showed different metabotypes according to chocolate preferences independent of the ingested food. With these observations, the authors suggested that specific dietary preferences may influence metabolic status and gut microbiota, which may have long-term consequences on health status. Finally, Xu et al.[75] explored the differences in the magnitude of the effect of diet and gender on the metabolic urinary profiles of men and women who followed a lactovegetarian or omnivorous diet. Researchers concluded that their results indicated that food patterns played a larger role in the metabolome than gender.

As well as these examples of nutrimetabolomic studies, we have included in this category a subgroup of recent works focused on the development and validation of methods and protocols in nutrimetabolomic studies. In this context, a three-step validation approach was suggested for identifying dietary biomarkers in complex metabolomic data sets influenced by inter- and intraindividual variation.[76] Pereira et al.[77] developed and validated a simple and robust UPLC-MS method for the nutrimetabolomic analysis of human plasma samples. They tested and compared five methods including three protein precipitation procedures and two anticoagulants. Their results showed that the method utilizing heparin as an anticoagulant and protein precipitation with methanol provided the most reproducible results. Fave et al.[78] proposed a standardized protocol designed to unify experimental strategies in the study of intake biomarkers with the aim of minimizing the potential confounding effects of pathophysiological and environmental factors. They described a robust and reproducible protocol for analyzing urinary metabolite profiles from individuals in fasting and different feeding statuses (during the period between the evening meal and breakfast and after food ingestion), suggesting that this could be a tool for obtaining data from habitual diet or specific food consumption. The novelty of this study is that this protocol would help to obtain homogeneous data in nutrimetabolomic studies and would facilitate the comparison of results obtained in different studies.[78]

Summary of Nutrimetabolomics Studies Findings. Modifications on food metabolome included variations in the concentrations of metabolites directly derived from the ingestion of food components and from human gut microbiota. These metabolites could be used as isolated biomarkers, or biomarker patterns, of the exposure to specific dietary components, foods, or dietary patterns to discover the actual ingestion of a subject or population. Most published studies refer to the analysis of changes or differences in metabolome according to nutritional data. To obtain more specific details of potential biomarkers of intake, a second approach for nutrimetabolomic studies has been described. Along with this metabolomic approach, some intake biomarkers of specific foods have been proposed, for instance, proline betaine (marker of citrus consumption). The strong relationship between citrus intake and this metabolite has been confirmed in different population samples by different research groups, validating its biological robustness. However, in most cases the corresponding validation required to evaluate its specificity and sensitivity is missing. Therefore, it might be concluded that the research of food metabolome biomarkers in the nutrimetabolomic field is currently in the previously described first stage, that is, the discovery phase. To improve both phases, the extensive bibliography derived from published studies about metabolism and bioavailability should be taken into account. Additionally, because the nontargeted fingerprinting approach is first used to discover novel biomarker candidates of intake and health effects, it is also necessary to implement a targeted approach postdiscovery phase with the aim of obtaining more detailed data of these potential biomarkers. Furthermore, in the scientific bibliography we can also find studies demonstrating that the metabotype could have an impact on nutritional responses.

Alterations in carbohydrate, lipid, amino acid, and/or energy metabolism have been detected through the increase or reduction of endogenous metabolites. These metabolites could be potential biomarkers of the effects on human health that exert a nutritional intervention, showing more specific data about the relationship between diet and pathophysiological states. The generated biomarkers could be used globally in all subjects or specifically for a target population group according to phenotyping criteria.

■ CURRENT LIMITATIONS

The application of nutrimetabolomics in large epidemiological studies opens new perspectives in the field of food metabolome biomarker discovery and validation. An important point in these studies is that very often it is necessary to realize that it is not possible to obtain a homogeneous metabolome sample collection, mainly because the metabolome will be different as a result of different factors, for example, the elapsed time since the last meal. This point is especially significant in observational studies in which there are no restrictions or parameters such as diet or food intake.

Most of the analytical methods used in nutrimetabolomic studies have been NMR spectroscopy, which is characterized by a high reproducibility but with a limited sensitivity, followed by MS techniques, which are more sensitive. Therefore, there is no analytical platform that is capable of identifying all of the metabolites present in a metabolome. Furthermore, there are still unknown signals, and currently it is still difficult to unify data from different nutrimetabolomic studies.

PERSPECTIVES

Differences in metabolome have been observed among different populations, leading to the hypothesis that food patterns could have a key role. Although the application of metabolomics in the nutritional field is quite recent, numerous studies suggesting different approaches have been published. The most used approach until now is that which assesses the changes observed in the endogenous and food metabolome after a nutritional intervention.

The bibliographic data about the excretion behavior of microbiota metabolites (present in urine even after 24 h of a single-dose intake) could be a new source of food intake biomarkers. Therefore, increasing the ability to identify these metabolites, for example, by using highly specialized bioinformatic tools, could open new windows in the field of monitoring the consumption of certain foods as a prerequisite for its association with health. An important advantage of this hypothesis is that some gut microbial metabolites are quite specific for particular compounds (i.e., urolithin for ellagitannins or hydroxyphenylvalerolactones for flavan-3-ols). Even if we consider that several dietary sources provide flavan-3-ols (i.e., tea or cocoa), the phenylvalerolactone and phenylvaleric acid profiles could discriminate between tea and cocoa consumers.

It is also important to extend the understanding of the role and weight exerted by endogenous factors such as gender, age, circadian rhythm, lifestyle, and microbiota. This knowledge will enable researchers to take these factors into account and thus reduce the intra- and interindividual variations of metabolic profiles. In this way, with the characterization of the physiological endogenous metabolome (i.e., metabotype), researchers will be able to classify individuals in accordance with their metabolite concentrations. More specifically in nutritional sciences, it is interesting to have a complete description of the metabolites present in humans depending on their nutritional status or food pattern. Therefore, future nutrimetabolomic studies should use standardized diets and well-defined populations in randomized, placebo-controlled, and double-blind crossover trials. This fact will also help in the evaluation of the prognostic and development of new therapeutic nutritional strategies. At the same time, it is also necessary to improve the technology around metabolomics.

This evolution will allow metabolomics to be introduced in the personalized nutritional field, with the aim of either recommending the best dietary choices for maintaining health or implementing a particular nutritional risk-lowering or therapeutic treatment, as well as to measure the compliance and response of dietary treatments. Furthermore, with technology advances their efficiency and precision will be enhanced, and therefore they will become more economical. These facts will facilitate the use of metabolomics in personalized nutrition. Personalized dietary recommendations are usually associated with the employment of functional foods, and the application of metabolomics in the functional food sector is also interesting. It can be used in the improvement of traditional products or in the development of new ones, with the aim of adapting their nutritional value to the requirements of specific consumer groups. The nutritional properties and health benefits of these products could be investigated, and the derived information would enable the reporting of nutritional and health claims.

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Funding

R.L. thanks the "Ramón y Cajal" program of the Spanish Ministry of Science and Innovation (MICINN) and Fondo Social Europeo (FSE). M.G.-A. thanks the Generalitat de Catalunya's Agency for Management of University and Research Grants (AGAUR) for the predoctoral FI-DGR 2011 fellowship. S.T. thanks the MICINN for a postdoctoral fellowship for the transfer of foreign researchers to Spain, and R.V.-F. thanks the MICINN for a predoctoral FPI fellowship. This work has been supported by grants from MICINN (Ingenio-CONSOLIDER program FUN-C-Food CSD2007-063, AGL2009-13906-C02-01, AGL2010-10084-E, and RYC-2010-07334) and from Merck Serono Research Grants 2010 (*Fundación Salud 2000*).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CFU, colony-forming unit; ESI, electrospray ionization; FFQ, food frequency questionnaire; FIE, flow infusion electrospray ionization; FT-ICR, Fourier-transform ion cyclotron resonance; GC, gas chromatography; ^{1}H NMR, hydrogen-1 nuclear magnetic resonance; HPLC, high-performance liquid chromatography; HTA, hypertensive; LC, liquid chromatography; MS, mass spectrometry; PCA, principal component analysis; q, quadrupole; SMCSO, *S*-methyl-L-cysteine sulfoxide; ToF, time-of-flight; UPLC, ultraperformance liquid chromatography.

REFERENCES

(1) Institute for Systems Biology. Systems Biology – the 21st Century Science; http://www.systemsbiology.org/Intro_to_Systems_Biology/Systems_Biology_--_the_21st_Century_Science (accessed Dec 1, 2011).

(2) Dettmer, K.; Aronov, P. A.; Hammock, B. D. Mass spectrometry-based metabolomics. *Mass Spectrom. Rev.* **2007**, *26*, 51–78.

(3) Metabolomics Society. Metabolomics; http://www.metabolomicssociety.org/metabolomics.html (accessed Dec 1, 2011).

(4) Nicholson, J. K.; Lindon, J. C.; Holmes, E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **1999**, *29*, 1181–1189.

(5) Oliver, S. G. Functional genomics: lessons from yeast. *Philos. Trans. R. Soc. London B: Biol. Sci.* **2002**, *357*, 17–23.

(6) Lindon, J. C.; Holmes, E.; Bollard, M. E.; Stanley, E. G.; Nicholson, J. K. Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. *Biomarkers* **2004**, *9*, 1–31.

(7) Wishart, D. S. Metabolomics: applications to food science and nutrition research. *Trends Food Sci Technol.* **2008**, *19*, 482–493.

(8) Gibney, M. J.; Walsh, M.; Brennan, L.; Roche, H. M.; German, B.; van Ommen, B. Metabolomics in human nutrition: opportunities and challenges. *Am. J. Clin. Nutr.* **2005**, *82*, 497–503.

(9) Scalbert, A.; Brennan, L.; Fiehn, O.; Hankemeier, T.; Kristal, B. S.; van Ommen, B.; Pujos-Guillot, E.; Verheij, E.; Wishart, D.; Wopereis, S. Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. *Metabolomics* **2009**, *5*, 435–458.

(10) Manach, C.; Hubert, J.; Llorach, R.; Scalbert, A. The complex links between dietary phytochemicals and human health deciphered by metabolomics. *Mol. Nutr. Food Res.* **2009**, *53*, 1303−1315.
(11) Visioli, F.; De La Lastra, C. A.; Andres-Lacueva, C.; Aviram, M.; Calhau, C.; Cassano, A.; D'Archivio, M.; Faria, A.; Fave, G.; Fogliano, V.; Llorach, R.; Vitaglione, P.; Zoratti, M.; Edeas, M. Polyphenols and human health: a prospectus. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 524−546, DOI: 10.1080/10408391003698677.
(12) Amantonico, A.; Urban, P. L.; Zenobi, R. Analytical techniques for single-cell metabolomics: state of the art and trends. *Anal. Bioanal. Chem.* **2010**, *398*, 2493−2504.
(13) Bertram, H. C.; Eggers, N.; Eller, N. Potential of human saliva for nuclear magnetic resonance-based metabolomics and for health-related biomarker identification. *Anal. Chem.* **2009**, *81*, 9188−9193.
(14) Wuolikainen, A.; Moritz, T.; Marklund, S. L.; Antti, H.; Andersen, P. M. Disease-related changes in the cerebrospinal fluid metabolome in amyotrophic lateral sclerosis detected by GC/TOFMS. *PLoS One* **2011**, *6*, e17947.
(15) Mishur, R. J.; Rea, S. L. Applications of mass spectrometry to metabolomics and metabonomics: detection of biomarkers of aging and of age-related diseases. *Mass Spectrom. Rev.* **2012**, *31*, 70−95.
(16) Oresic, M. Metabolomics, a novel tool for studies of nutrition, metabolism and lipid dysfunction. *Nutr. Metab. Cardiovasc. Dis.* **2009**, *19*, 816−824.
(17) Chagoyen, M.; Pazos, F. MBRole: enrichment analysis of metabolomic data. *Bioinformatics* **2011**, *27*, 730−731.
(18) Cottret, L.; Wildridge, D.; Vinson, F.; Barrett, M. P.; Charles, H.; Sagot, M. F.; Jourdan, F. MetExplore: a web server to link metabolomic experiments and genome-scale metabolic networks. *Nucleic Acids Res.* **2010**, *38*, W132−137.
(19) Eliasson, M.; Rannar, S.; Trygg, J. From data processing to multivariate validation − essential steps in extracting interpretable information from metabolomics data. *Curr. Pharm. Biotechnol.* **2011**, *12*, 996−1004.
(20) Frolkis, A.; Knox, C.; Lim, E.; Jewison, T.; Law, V.; Hau, D. D.; Liu, P.; Gautam, B.; Ly, S.; Guo, A. C.; Xia, J.; Liang, Y.; Shrivastava, S.; Wishart, D. S. SMPDB: The Small Molecule Pathway Database. *Nucleic Acids Res.* **2010**, *38*, D480−D487.
(21) Karnovsky, A.; Weymouth, T.; Hull, T.; Tarcea, V. G.; Scardoni, G.; Laudanna, C.; Sartor, M. A.; Stringer, K. A.; Jagadish, H. V.; Burant, C.; Athey, B.; Omenn, G. S. Metscape 2 bioinformatics tool for the analysis and visualization of metabolomics and gene expression data. *Bioinformatics* **2012**, *28*, 373−380.
(22) Madsen, R.; Lundstedt, T.; Trygg, J. Chemometrics in metabolomics − a review in human disease diagnosis. *Anal. Chim. Acta* **2010**, *659*, 23−33.
(23) Suhre, K.; Schmitt-Kopplin, P. MassTRIX: mass translator into pathways. *Nucleic Acids Res.* **2008**, *36*, W481−484.
(24) Tohge, T.; Fernie, A. R. Web-based resources for mass-spectrometry-based metabolomics: a user's guide. *Phytochemistry* **2009**, *70*, 450−456.
(25) van Iersel, M. P.; Kelder, T.; Pico, A. R.; Hanspers, K.; Coort, S.; Conklin, B. R.; Evelo, C. Presenting and exploring biological pathways with PathVisio. *BMC Bioinformatics* **2008**, *9*, 399.
(26) Xia, J.; Wishart, D. S. Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. *Nat. Protoc.* **2011**, *6*, 743−760.
(27) Mamas, M.; Dunn, W. B.; Neyses, L.; Goodacre, R. The role of metabolites and metabolomics in clinically applicable biomarkers of disease. *Arch. Toxicol.* **2011**, *85*, 5−17.
(28) World Health Organization. Global status report on non-communicable diseases 2010 [online]; http://whqlibdoc.who.int/publications/2011/9789240686458_eng.pdf (accessed Feb 7 2012).
(29) Schnackenerg, L.; Beger, R. Metabolomic biomarkers: their role in the critical path. *Drug Discovery Today: Technol.* **2007**, *4*, 13−16.
(30) van der Greef, J.; Stroobant, P.; van der Heijden, R. The role of analytical sciences in medical systems biology. *Curr. Opin. Chem. Biol.* **2004**, *8*, 559−565.
(31) Rezzi, S.; Ramadan, Z.; Fay, L. B.; Kochhar, S. Nutritional metabonomics: applications and perspectives. *J. Proteome Res.* **2007**, *6*, 513−525.
(32) Griffin, J. L.; Atherton, H.; Shockcor, J.; Atzori, L. Metabolomics as a tool for cardiac research. *Nat. Rev. Cardiol.* **2011**, *8*, 630−643.
(33) McNiven, E. M.; German, J. B.; Slupsky, C. M. Analytical metabolomics: nutritional opportunities for personalized health. *J. Nutr. Biochem.* **2011**, *22*, 995−1002.
(34) Zivkovic, A. M.; German, J. B. Metabolomics for assessment of nutritional status. *Curr. Opin. Clin. Nutr. Metab. Care* **2009**, *12*, 501−507.
(35) Zhang, X.; Yap, Y.; Wei, D.; Chen, G.; Chen, F. Novel omics technologies in nutrition research. *Biotechnol. Adv.* **2008**, *26*, 169−176.
(36) Stella, C.; Beckwith-Hall, B.; Cloarec, O.; Holmes, E.; Lindon, J. C.; Powell, J.; van der Ouderaa, F.; Bingham, S.; Cross, A. J.; Nicholson, J. K. Susceptibility of human metabolic phenotypes to dietary modulation. *J. Proteome Res.* **2006**, *5*, 2780−2788.
(37) Solanky, K. S.; Bailey, N. J.; Beckwith-Hall, B. M.; Davis, A.; Bingham, S.; Holmes, E.; Nicholson, J. K.; Cassidy, A. Application of biofluid ^{1}H nuclear magnetic resonance-based metabonomic techniques for the analysis of the biochemical effects of dietary isoflavones on human plasma profile. *Anal. Biochem.* **2003**, *323*, 197−204.
(38) Solanky, K. S.; Bailey, N. J.; Beckwith-Hall, B. M.; Bingham, S.; Davis, A.; Holmes, E.; Nicholson, J. K.; Cassidy, A. Biofluid ^{1}H NMR-based metabonomic techniques in nutrition research − metabolic effects of dietary isoflavones in humans. *J. Nutr. Biochem.* **2005**, *16*, 236−244.
(39) Wang, Y.; Tang, H.; Nicholson, J. K.; Hylands, P. J.; Sampson, J.; Holmes, E. A metabonomic strategy for the detection of the metabolic effects of chamomile (*Matricaria recutita* L.) ingestion. *J. Agric. Food Chem.* **2005**, *53*, 191−196.
(40) Daykin, C. A.; Van Duynhoven, J. P.; Groenewegen, A.; Dachtler, M.; Van Amelsvoort, J. M.; Mulder, T. P. Nuclear magnetic resonance spectroscopic based studies of the metabolism of black tea polyphenols in humans. *J. Agric. Food Chem.* **2005**, *53*, 1428−1434.
(41) van Velzen, E. J.; Westerhuis, J. A.; van Duynhoven, J. P.; van Dorsten, F. A.; Grun, C. H.; Jacobs, D. M.; Duchateau, G. S.; Vis, D. J.; Smilde, A. K. Phenotyping tea consumers by nutrikinetic analysis of polyphenolic end-metabolites. *J. Proteome Res.* **2009**, *8*, 3317−3330.
(42) Llorach, R.; Urpi-Sarda, M.; Jauregui, O.; Monagas, M.; Andres-Lacueva, C. An LC-MS-based metabolomics approach for exploring urinary metabolome modifications after cocoa consumption. *J. Proteome Res.* **2009**, *8*, 5060−5068.
(43) Llorach, R.; Garrido, I.; Monagas, M.; Urpi-Sarda, M.; Tulipani, S.; Bartolome, B.; Andres-Lacueva, C. Metabolomics study of human urinary metabolome modifications after intake of almond (*Prunus dulcis* (Mill.) D.A. Webb) skin polyphenols. *J. Proteome Res.* **2010**, *9*, 5859−5867.
(44) Walsh, M. C.; Brennan, L.; Pujos-Guillot, E.; Sebedio, J. L.; Scalbert, A.; Fagan, A.; Higgins, D. G.; Gibney, M. J. Influence of acute phytochemical intake on human urinary metabolomic profiles. *Am. J. Clin. Nutr.* **2007**, *86*, 1687−1693.
(45) Edmands, W. M.; Beckonert, O. P.; Stella, C.; Campbell, A.; Lake, B. G.; Lindon, J. C.; Holmes, E.; Gooderham, N. J. Identification of human urinary biomarkers of cruciferous vegetable consumption by metabonomic profiling. *J. Proteome Res.* **2011**, *10*, 4513−4521.
(46) Ndagijimana, M.; Laghi, L.; Vitali, B.; Placucci, G.; Brigidi, P.; Guerzoni, M. E. Effect of a synbiotic food consumption on human gut metabolic profiles evaluated by (1)H nuclear magnetic resonance spectroscopy. *Int. J. Food Microbiol.* **2009**, *134*, 147−153.
(47) Bertram, H. C.; Hoppe, C.; Petersen, B. O.; Duus, J. O.; Molgaard, C.; Michaelsen, K. F. An NMR-based metabonomic investigation on effects of milk and meat protein diets given to 8-year-old boys. *Br. J. Nutr.* **2007**, *97*, 758−763.
(48) Jiang, Z.; Liang, Q.; Wang, Y.; Zheng, X.; Pei, L.; Zhang, T.; Luo, G. Metabonomic study on women of reproductive age treated with nutritional intervention: screening potential biomarkers related to neural tube defects occurrence. *Biomed. Chromatogr.* **2011**, *25*, 767−774.

(49) van Velzen, E. J.; Westerhuis, J. A.; van Duynhoven, J. P.; van Dorsten, F. A.; Hoefsloot, H. C.; Jacobs, D. M.; Smit, S.; Draijer, R.; Kroner, C. I.; Smilde, A. K. Multilevel data analysis of a crossover designed human nutritional intervention study. *J. Proteome Res.* **2008**, *7*, 4483−4491.

(50) van Dorsten, F. A.; Grun, C. H.; van Velzen, E. J.; Jacobs, D. M.; Draijer, R.; van Duynhoven, J. P. The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics. *Mol. Nutr. Food Res.* **2010**, *54*, 897−908.

(51) Jacobs, D. M.; Deltimple, N.; van Velzen, E.; van Dorsten, F. A.; Bingham, M.; Vaughan, E. E.; van Duynhoven, J. (1)H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. *NMR Biomed.* **2008**, *21*, 615−626.

(52) Tulipani, S.; Llorach, R.; Jauregui, O.; Lopez-Uriarte, P.; Garcia-Aloy, M.; Bullo, M.; Salas-Salvado, J.; Andres-Lacueva, C. Metabolomics unveils urinary changes in subjects with metabolic syndrome following 12-week nut consumption. *J. Proteome Res.* **2011**, *10*, 5047−5058.

(53) Moazzami, A. A.; Zhang, J. X.; Kamal-Eldin, A.; Aman, P.; Hallmans, G.; Johansson, J. E.; Andersson, S. O. Nuclear magnetic resonance-based metabolomics enable detection of the effects of a whole grain rye and rye bran diet on the metabolic profile of plasma in prostate cancer patients. *J. Nutr.* **2011**, *141*, 2126−2132.

(54) Van Dorsten, F. A.; Daykin, C. A.; Mulder, T. P. J.; Van Duynhoven, J. P. M. Metabonomics approach to determine metabolic differences between green tea and black tea consumption. *J. Agric. Food Chem.* **2006**, *54*, 6929−6938.

(55) Lloyd, A. J.; Fave, G.; Beckmann, M.; Lin, W.; Tailliart, K.; Xie, L.; Mathers, J. C.; Draper, J. Use of mass spectrometry fingerprinting to identify urinary metabolites after consumption of specific foods. *Am. J. Clin. Nutr.* **2011**, *94*, 981−991.

(56) Dragsted, L. O. Biomarkers of meat intake and the application of nutrigenomics. *Meat Sci.* **2010**, *84*, 301−307.

(57) Rechner, A. R.; Kuhnle, G.; Bremner, P.; Hubbard, G. P.; Moore, K. P.; Rice-Evans, C. A. The metabolic fate of dietary polyphenols in humans. *Free Radical Biol. Med.* **2002**, *33*, 220−235.

(58) Monagas, M.; Urpi-Sarda, M.; Sanchez-Patan, F.; Llorach, R.; Garrido, I.; Gomez-Cordoves, C.; Andres-Lacueva, C.; Bartolome, B. Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food Funct.* **2010**, *1*, 233−253.

(59) Cerda, B.; Periago, P.; Espin, J. C.; Tomas-Barberan, F. A. Identification of urolithin a as a metabolite produced by human colon microflora from ellagic acid and related compounds. *J. Agric. Food Chem.* **2005**, *53*, 5571−5576.

(60) Pere-Trepat, E.; Ross, A. B.; Martin, F. P.; Rezzi, S.; Kochhar, S.; Hasselbalch, A. L.; Kyvik, K. O.; Sorensen, T. I. A. Chemometric strategies to assess metabonomic imprinting of food habits in epidemiological studies. *Chemom. Intell. Lab. Syst.* **2010**, *104*, 95−100.

(61) O'Sullivan, A.; Gibney, M. J.; Brennan, L. Dietary intake patterns are reflected in metabolomic profiles: potential role in dietary assessment studies. *Am. J. Clin. Nutr.* **2011**, *93*, 314−321.

(62) Heinzmann, S. S.; Brown, I. J.; Chan, Q.; Bictash, M.; Dumas, M. E.; Kochhar, S.; Stamler, J.; Holmes, E.; Elliott, P.; Nicholson, J. K. Metabolic profiling strategy for discovery of nutritional biomarkers: proline betaine as a marker of citrus consumption. *Am. J. Clin. Nutr.* **2010**, *92*, 436−443.

(63) Lloyd, A. J.; Beckmann, M.; Fave, G.; Mathers, J. C.; Draper, J. Proline betaine and its biotransformation products in fasting urine samples are potential biomarkers of habitual citrus fruit consumption. *Br. J. Nutr.* **2011**, *106*, 812−824.

(64) O'Sullivan, A.; Gibney, M. J.; Connor, A. O.; Mion, B.; Kaluskar, S.; Cashman, K. D.; Flynn, A.; Shanahan, F.; Brennan, L. Biochemical and metabolomic phenotyping in the identification of a vitamin D responsive metabotype for markers of the metabolic syndrome. *Mol. Nutr. Food Res.* **2011**, *55*, 679−690.

(65) Martin, F. P.; Rezzi, S.; Pere-Trepat, E.; Kamlage, B.; Collino, S.; Leibold, E.; Kastler, J.; Rein, D.; Fay, L. B.; Kochhar, S. Metabolic effects of dark chocolate consumption on energy, gut microbiota, and stress-related metabolism in free-living subjects. *J. Proteome Res.* **2009**, *8*, 5568−5579.

(66) Lenz, E. M.; Bright, J.; Wilson, I. D.; Hughes, A.; Morrisson, J.; Lindberg, H.; Lockton, A. Metabonomics, dietary influences and cultural differences: a ^{1}H NMR-based study of urine samples obtained from healthy British and Swedish subjects. *J. Pharm. Biomed. Anal.* **2004**, *36*, 841−849.

(67) Holmes, E.; Loo, R. L.; Stamler, J.; Bictash, M.; Yap, I. K.; Chan, Q.; Ebbels, T.; De Iorio, M.; Brown, I. J.; Veselkov, K. A.; Daviglus, M. L.; Kesteloot, H.; Ueshima, H.; Zhao, L.; Nicholson, J. K.; Elliott, P. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature* **2008**, *453*, 396−400.

(68) Yap, I. K.; Brown, I. J.; Chan, Q.; Wijeyesekera, A.; Garcia-Perez, I.; Bictash, M.; Loo, R. L.; Chadeau-Hyam, M.; Ebbels, T.; De Iorio, M.; Maibaum, E.; Zhao, L.; Kesteloot, H.; Daviglus, M. L.; Stamler, J.; Nicholson, J. K.; Elliott, P.; Holmes, E. Metabolome-wide association study identifies multiple biomarkers that discriminate north and south Chinese populations at differing risks of cardiovascular disease: INTERMAP study. *J. Proteome Res.* **2010**, *9*, 6647−6654.

(69) Dumas, M. E.; Maibaum, E. C.; Teague, C.; Ueshima, H.; Zhou, B.; Lindon, J. C.; Nicholson, J. K.; Stamler, J.; Elliott, P.; Chan, Q.; Holmes, E. Assessment of analytical reproducibility of ^{1}H NMR spectroscopy based metabonomics for large-scale epidemiological research: the INTERMAP Study. *Anal. Chem.* **2006**, *78*, 2199−2208.

(70) Bakker, G. C.; van Erk, M. J.; Pellis, L.; Wopereis, S.; Rubingh, C. M.; Cnubben, N. H.; Kooistra, T.; van Ommen, B.; Hendriks, H. F. An antiinflammatory dietary mix modulates inflammation and oxidative and metabolic stress in overweight men: a nutrigenomics approach. *Am. J. Clin. Nutr.* **2010**, *91*, 1044−1059.

(71) Chadeau-Hyam, M.; Athersuch, T. J.; Keun, H. C.; De Iorio, M.; Ebbels, T. M.; Jenab, M.; Sacerdote, C.; Bruce, S. J.; Holmes, E.; Vineis, P. Meeting-in-the-middle using metabolic profiling − a strategy for the identification of intermediate biomarkers in cohort studies. *Biomarkers* **2011**, *16*, 83−88.

(72) Rezzi, S.; Ramadan, Z.; Martin, F. P.; Fay, L. B.; van Bladeren, P.; Lindon, J. C.; Nicholson, J. K.; Kochhar, S. Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals. *J. Proteome Res.* **2007**, *6*, 4469−4477.

(73) Chorell, E.; Moritz, T.; Branth, S.; Antti, H.; Svensson, M. B. Predictive metabolomics evaluation of nutrition-modulated metabolic stress responses in human blood serum during the early recovery phase of strenuous physical exercise. *J. Proteome Res.* **2009**, *8*, 2966−2977.

(74) Lee, R.; West, D.; Phillips, S. M.; Britz-McKibbin, P. Differential metabolomics for quantitative assessment of oxidative stress with strenuous exercise and nutritional intervention: thiol-specific regulation of cellular metabolism with *N*-acetyl-L-cysteine pretreatment. *Anal. Chem.* **2010**, *82*, 2959−2968.

(75) Xu, J.; Yang, S.; Cai, S.; Dong, J.; Li, X.; Chen, Z. Identification of biochemical changes in lactovegetarian urine using ^{1}H NMR spectroscopy and pattern recognition. *Anal. Bioanal. Chem.* **2010**, *396*, 1451−1463.

(76) Montoliu, I.; Martin, F. P.; Guy, P. A.; Tavazzi, I.; Bruce, S.; Collino, S.; Galindo-Cuspinera, V.; Genick, U.; Martin, N.; Le Coutre, J.; Kochhar, S.; Rezzi, S. Validation on high variance metabolic profiles: taste stratification in a free living population. *Chemom. Intell. Lab. Syst.* **2010**, *104*, 8−19.

(77) Pereira, H.; Martin, J. F.; Joly, C.; Sebedio, J. L.; Pujos-Guillot, E. Development and validation of a UPLC/MS method for a nutritional metabolomic study of human plasma. *Metabolomics* **2010**, *6*, 207−218.

(78) Fave, G.; Beckmann, M.; Lloyd, A. J.; Zhou, S.; Harold, G.; Lin, W.; Tailliart, K.; Xie, L.; Draper, J.; Mathers, J. C. Development and validation of a standardized protocol to monitor human dietary exposure by metabolite fingerprinting of urine samples. *Metabolomics* **2011**, *7*, 469−484.

Publicación 1:

Khymenets O, Andres-Lacueva C, Urpi-Sarda M, Vazquez-Fresno R, Mercader Mart M, Reglero G, Torres M, Llorach R. Metabolic fingerprint after acute and under sustained consumption of a functional beverage based on grape skin extract in healthy human subjects. *Food & Function [Accepted]*. doi: 10.1039/C4FO00684D

Metabolic fingerprint after acute and under sustained consumption of a functional beverage based on grape skin extract in healthy human subjects

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Abstract:

Grape-derived polyphenols are considered to be one of the most promising ingredients for functional food due to their health-promoting activities. We applied an HPLC-MS-based untargeted metabolomics approach in order to evaluate the impact of a functional food based on grape skin polyphenols on the urinary metabolome of healthy subjects. Thirty-one volunteers participated in two dietary crossover randomized intervention studies: with a single-dose intake (187 mL) and with a 15-day sustained consumption (twice per day, 187 mL/day in total) of a functional beverage (FB). Postprandial (4-hour) and 24-hour urine samples collected after acute consumption and on the last day of sustained FB consumption, respectively, were explored using an untargeted HPLC-qTOF-MS approach. Multivariate modelling with subsequent application of an S-plot revealed differential mass features related to acute and prolonged consumption of FB. More than half of the mass features were shared between the two types of samples, among which several phase II metabolites of grape-derived polyphenols were identified at confidence level II. Prolonged consumption of FB was specifically reflected in urine metabolome by the presence of first-stage microbial metabolites of flavanols: hydroxyvaleric acid and hydroxyvalerolactone derivatives. Overall, several epicatechin and phenolic acid metabolites both of tissular and microbiota origin were the most representative markers of FB consumption. To our knowledge, this is one of the first studies where an untargeted LC-MS metabolomics approach has been applied in nutrition research on a grape-derived FB.

Introduction

Plant phytochemicals are considered to be one of the most important health-promoting compounds present in our diet.[1] Therefore, due to their strong bioactive potential, it is proposed that phytochemical extracts from fruits and vegetables serve as a principal component in a variety of nutraceuticals, e.g. in functional foods.[2] Over the recent decades, the concept of functional food, a food designed to allow consumers to eat enriched foods close to their natural state,[2] has attracted much interest and popularity, since it was proposed as a novel therapeutic

approach to prevent or attenuate diet-related diseases such as obesity, diabetes, atherosclerosis, and neurodegeneration.[3]

Grape and grape-derived food products are well-known sources of bioactive compounds, which are mainly represented by a wide range of polyphenols, and, therefore, they are among the most commonly used in the development of functional food.[4, 5] In general, grape and grape-derived products are some of the most important natural sources of polyphenols in our diet, and their dietary consumption is directly related to various beneficial health effects.[6] Grape skin, being an edible part of the berry, has an important antioxidant capacity due to its high polyphenol content, among which the most abundant are flavonoids, anthocyanins, proanthocyanidins, procyanidins, phenolic acids and stilbenes.[6, 7] Consequently, extracts from grape skin are first-choice candidates for the development of functional foods and nutraceuticals to be used as alternative treatment in the management of several diet-related diseases.[4, 5, 8]

Current progress in the development and implementation of omics' technologies has opened new perspectives in nutrition research, [9] e.g. food metabolome analysis,[10] and, consequently, they have become widely applied in nutraceutical investigation.[11] Today, metabolomics is being applied successfully in quality control,[12] in metabolism evaluation[13, 14] and also in the assessment of the beneficial effects of functional food.[11, 15] In the latest perspective, untargeted metabolomics is foreseen as one of the most promising approaches for understanding the complex relationship between diet and human health due to its potential for data-driven exploration.[16] Recently, in a couple of studies, an untargeted metabolomics approach was applied in the evaluation of the impact of grape-derived food and extract consumption on human urinary metabolome using NMR technology alone[17] or in combination with GC-MS and LC-MS/MS approaches.[14, 18] The results from these studies suggested that the metabolic fate of grape-derived polyphenols and other bioactive compounds could be clearly perceived by examining overall urinary metabolome profiles.[14, 18, 19] However, due to the limitations of an NMR technical approach, mainly related to its relatively low sensitivity (µmole – mmole range),[20] just a few compounds could be identified and related to the dietary consumption of grape-derived products.[14, 17] Another methodological approach extensively used in untargeted

metabolomics, mass spectrometry coupled with liquid chromatography (LC-MS), offers highly sensitive, reproducible and quasi-quantitative metabolite analyses.[20, 21] Therefore, this technique has become widely applied in nutritional research.[22] However, so far, no studies using an LC-MS untargeted metabolomics approach have been performed in human intervention studies with grape-derived products or functional foods.

The objective of the present study was to elucidate the impact of acute and sustained consumption of a functional beverage based on grape skin extracts on the urinary metabolome (i.e. food metabolome) of healthy subjects by applying an untargeted HPLC-MS metabolomics approach.

Experimental

Chemicals and reagents

The following chemicals were obtained commercially: L-phenylalanine, 1-methyl-L-histidine, L-tryptophan, 20-deoxyguanosine, glycochenodeoxycholic acid, (-)-epicatechin and procyanidin B2 (Sigma-Aldrich, St Louis, MO), 4-hydroxyhippuric acid (PhytoLab GmbH & Co., KG) and naringenin (Extrasynthèse, Genay, France). HPLC-grade acetonitrile and formic acid 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).

Human intervention study

Thirty-one healthy adult volunteers (15 men and 16 women, aged 25–55 years) participated in a double-blind, placebo-controlled, randomized crossover intervention study with a drink containing grape skin extract. The study protocol was approved by the Ethics Committee of the Hospital Universitario La Paz, Madrid. The detailed study design was reported earlier.[23] Briefly, one week prior to and during the 46 days of the study the participants maintained a low-polyphenol diet. For the acute study, after a seven-day washout period, the subjects consumed 187 mL of the FB (functional beverage), containing a grape skin extract, or the CB (control beverage), as a placebo, in randomized order together with a standard breakfast consisting of white bread, cheese and yoghurt. After that, they consumed the corresponding beverage twice a

day (at lunch and dinner), 187 mL per day in total, for the next 15 days. The same procedure was repeated after a second seven-day washout period according to the crossover design.[23] On the last day of each washout and intervention period, 24-hour urine was collected from volunteers. For the acute study, the urine collected during the first four postprandial hours was used. All urine samples were stored in aliquots at -80 ºC prior to analysis.

Both the placebo (CB) and the intervention (FB) beverages had the same macronutrient composition of carbohydrates (sugars), 10 g/L, and energy, 40 kcal/L. The FB contained a grape extract preparation obtained from red grape skins using alcohol extraction in different temperature conditions. Therefore, due to the grape skin extract component, the FB contained hydroxycinnamic (4280 µg/L) and hydroxybenzoic (83 mg/L) acids, anthocyanins (16 mg/L), flavanols (96 mg/L) and stilbenes (5.7 mg/L), whereas the CB contained no polyphenols.[23] The FB had a total polyphenol index (I280) of 116 (arbitrary units), which is much higher than an average wine index.[24]

LC-MS metabolomics analysis

The sample preparation was conducted according to a previously published methodology.[25-27] HPLC-MS analysis was performed using an Agilent 1200 Series Rapid Resolution HPLC system coupled to a hybrid quadrupole TOF QSTAR Elite (Applied Biosystems/MDS Sciex) according to the protocol for untargeted metabolomics analysis established in our group.[25-27] Thus, chromatographic separation was performed using RP 18 Luna 5 µm, 50 × 230 mm column (Phenomenex, Torrance, CA) in mobile phases consisted of (A) 0.1% HCOOH and (B) CAN 0.1% HCOOH, at a flow rate of 600 µL/min with the following linear gradient elution (t, %B): (0, 1), (4, 20), (6, 95), (7.5, 95), (8, 1), (12, 1). q-TOF spray parameters were set as previously described[27] and full data acquisition was performed in the scan range 70–700 m/z in positive ionization mode. LC-MS data were acquired in three successive equilibrated batches. In addition, samples and sequences of injections were randomized in order to avoid possible bias. Four types of quality control (QC) were analysed along with samples in each batch in order to monitor the stability and functionality of the system: QC1 – Milli-Q water; QC2 – 5 µg/mL of exogenous standard mixture consisting of 4-hydroxyhippuric acid, epicatechin, procyanidin B

and naringenin; QC3 – 5 µg/mL of endogenous standard mixture consisting of methyl-L-histidine, deoxyguanosine, glycochenodeoxycholic acid, phenylalanine and tryptophan; and QC4 – about 10% of all samples were reinjected within the same batch. The QC data were used in order to monitor the system's stability and functionality, guaranteeing the reliability of the acquired data.[25, 27] Raw LC-MS data are deposited at the laboratory of Biomarkers & Nutrimetabolomic Research Group. Nutrition & Food Science Department., Pharmacy School-University of Barcelona, and can be available upon request.

Data preprocessing

LC-MS raw data were processed using MarkerView TM 1.2.1. software (Applied Biosystems, MDS Sciex, Toronto, Ontario, Canada). Peak detection was performed using a minimum spectral peak width of 1 ppm, a minimum RT peak width of 3 scans, a noise threshold of 5 and a subtraction multiple factor of 1.5. The retention time tolerance was 0.1 min and the mass tolerance was 0.05 Da.

Multivariate analysis (MVA) of data

The MVA was performed using SIMCA-P+ 13.0 software (Umetrics, Umea, Sweden). The data set was log-transformed and Pareto-scaled before unsupervised principal component analysis (PCA), and supervised statistical modelling by simple and orthogonal partial least squares discriminant analysis (PLS-DA and OPLS-DA, respectively). The PCA was used to gain an overview of the quality of the data acquisition step, while the PLS-DAs were applied for the exploration of the differences in metabolomes among the sample groups. OPLS separates the system variation in X (LC-MS data) into two parts, one that is linearly correlated (predictive) and one that is uncorrelated (orthogonal) to Y. This separation facilitates the model interpretation as the variation between classes can be interpreted from the predictive component, and the variation within classes can be interpreted from the orthogonal component.[28] The type of beverage (CB vs FB) and time of urine collection (washout versus intervention) were used as categorical Y variables in both the PLS-DA and OPLS-DA. The quality of the supervised models was evaluated by the goodness-of-fit parameter (R^2X), the proportion of the variance of the response variable that is explained by the model (R^2Y_{cum}) and the predictive ability parameter

($Q2_{cum}$), which was calculated by a seven-round internal cross-validation of the data using a default option of the SIMCA-P+ 13.0 software. The OPLS-DA models were tested for overfitting with an ANOVA based on the cross-validated predictive residuals (CV-ANOVA)[29] using SIMCA software, while the PLS-DA model was tested by the permutation test (n=200).[30] Potential markers of interest were extracted from the S-plot, a scatter plot that graphically combined the covariance and correlation loading profiles resulting from the constructed OPLS models.[31] Pairwise discrimination between the FB and CB classes for acute and sustained chronic consumption of beverages was carried out by two distinct S-plots and differential features were chosen based on their correlations in each data set. Only mass features showing correlation coefficients (|p(corr)|) in both S-plots of ≥ 0.5 [27] were included in the list of candidate markers explaining the separation between FB and CB interventions.

Metabolite identification

Markers contributing to the discrimination between diets were identified through a multiple-step procedure.[26, 32] First, a two-way hierarchical clustering analysis using the Pearson distance and Ward's method (PermutMatrix 1.9.3.0 software)[33] was applied to both sets of differential signals obtained from the S-plots. Mass features and urine samples were clustered simultaneously in order to group the observations by their similarities. The mass features corresponded to the same metabolite - protonated molecules,[13] C isotopes, adducts and in-source generated fragments,[34] - were identified as clusters based on a heat map of intensities and of a correlation matrix.[34] Second, identification of compounds was carried out on the basis of their exact mass (±5 mDa of accepted mass difference), which was compared to those registered in an in-house database[35] that mainly focused on the food metabolome, and in freely available external databases: the Human Metabolome Database (HMDB; www.hmdb.ca) and the MetLin database.[36] In addition, the in-source fragmentation behaviour of compounds, represented as ions clusters aggregated by two-way HCA, was used as complementary information to identify the metabolites.[37] Finally, the metabolites with the two highest levels of confidence identity, levels I and II (when at least two orthogonal physicochemical properties, such as mass, fragmentation or retention time, corresponded with authentic standards or with public/in-house

spectral libraries, respectively),[38] were taken into further consideration. The biological relevance of the identified metabolites was evaluated based on information extracted from earlier published reports accessed through free search engines.

Results

Data acquisition quality

PCA analysis of the three QC classes revealed a clear separation between distinct control samples belonging to QC1, QC2 and QC3 (**Figure 1S (A)**, Supplementary Information). Neither carry-over nor apparent clustering due to the sequence of injections was detected. No artificial trends took place in the study samples during data acquisition, since QC4 replicates were tightly clustered in the PCA scores plot (**Figure 1S (B)** Supplementary Information). The quality control results were in agreement with criteria recently proposed for metabolomics studies, [25, 27] suggesting that the overall quality of the acquired data was good.

Analysis of urinary profiles in acute and midterm sustained consumption studies

To assess the intervention-dependent changes in the urinary metabolome in both consumption studies, an OPLS-DA using two classes, FB and CB, was applied separately to the acute and sustained studies' data sets. The robust, high-quality 1+2 OPLS-DA models were obtained with regard to the separation of samples by type of beverage consumption for both studies (**Figure 1 A** and **B**). Both OPLS-DA models were further applied for metabolite discriminant analysis.

The metabolic profiles of 4-hour postprandial urines and 24-hour urines collected on the last day of the one-week washout and of the 15-day sustained intervention periods were assessed using PLS-DA modelling. In contrast to both acute and sustained FB consumption, where changes in urinary metabolic profiles were observed, no important alterations in urinary metabolic profiles caused by any intervention with the CB were noticed (**Figure 2S (A),** Supplementary Information). The differences in 24-hour urinary metabolome between washout and under sustained FB intervention were similar to those observed in comparative analysis between the FB and CB beverages (**Figure 2S (B),** Supplementary Information).

The S-plots[31] associated with the OPLS-DA were generated to reveal the statistically significant metabolites responsible for FB consumption. The differential mass feature selection using a reliability cut-off of $p(corr) \geq |0.5|$, indicating a significant relation to the consumption of the FB (upper-right quadrants) or CB (lower-left quadrants) beverages, is presented in **Figure 2 A** and **B**, for acute and prolonged intervention studies, respectively**.**

Comparative analysis of metabolome alteration due to acute and sustained interventions

The comparison of metabolome modifications induced by both acute and sustained FB intake was performed and aggrupation of m/z features are represented on a Venn diagram (**Figure 3**). The mass features positively affected by FB intake ($p(corr) \geq 0.5$) were classified into three groups: specifically related to acute consumption, specifically related to sustained consumption, and a common group. In general, about 70% of acute consumption markers were also detected in samples from the sustained study, whereas 44% of markers were exclusively related to the prolonged FB consumption in contrast to about 30% for the acute.

Characterization and identification of FB consumption biomarkers

A number of differential compounds unique and common to acute and sustained consumption studies detected as clusters composed of different kinds of ions were identified. Eighteen metabolites, identified at least with confidence level II, are summarized in **Table 1** according to their main characteristics: retention time, detected mass and postulated identification. The identity of 4-hydroxyhippuric acid was confirmed comparing its mass-chromatographic behaviour with the authentic standard present in QC 2 (**Figure 3S,** Supplementary Information). The rest of the metabolites were annotated at confidence level II. All eighteen metabolites from both studies had a higher relative abundance in the samples related to the consumption of the FB. These metabolites were considered in relation to their metabolic origin: as host (represented principally by phase II metabolites of polyphenols originally present in grape skin) and microbial (compounds derived from grape polyphenols due to the catabolic activities of intestinal microbiota). With regard to the differences in metabolic profiles observed between acute and prolonged consumption of the FB, the sustained consumption was clearly associated with the presence of metabolites of an exclusively microbial origin.[27] On the other hand, only

one postprandial-specific metabolite was identified, while other metabolites were common for both sample types (**Table 1**).

Discussion

The current study shows that acute and 15-day sustained consumption of an FB based on grape skin extracts produces significant changes in urinary metabolome. Using an LC-MS-based untargeted approach with subsequent multivariate analysis, we identified a range of metabolites that are chiefly related to the metabolism of grape skin polyphenols, a principal active component of the tested FB.

Metabolite 1 (**Table 1**) was annotated as ***tyrosine***, based on its LC-MS behaviour. It was the only discriminative metabolite related specifically to acute intervention with an FB. This non-essential amino acid was earlier also described as an important contributor to the 6-hour postprandial urinary metabolome changes provoked by acute consumption of cocoa powder.[27] According to the phytochemical database of Dr Duke (http://www.ars-grin.gov/duke/plants.html), grape berries can contain about 120–617 ppm of tyrosine. Therefore, it could have been extracted along with other polyphenols and consumed within the FB at much more elevated concentrations, thus appearing excreted in 4-hour postprandial urine. Nevertheless, the endogenous origin of the detected tyrosine must also be considered.

Elevated excretion of ***4-hydroxyhippuric acid*** (**Table 1**, metabolite 2) after consumption of the functional beverage was noticed in both acute and under prolonged consumption of FB. Recently, elevated 4-hydroxyhippurate was detected by untargeted NMR, along with its 3-hydroxyl isoform, in 24-hour urine after grape-derived product consumption.[18] Consumption of other polyphenol-rich foods, such as tea[39] and cocoa, [40] was also related to its elevated excretion, which was associated mainly with the microbial metabolism of consumed proanthocyanidins and flavanols. On the other hand, whether directly from grape skin[41] or released from pelargonidin,[42] 4-hydroxybenzoic acid may directly undergo conjugation with glycine in the liver under its absorption in the small intestine,[43] forming 4-hydroxyhippuric acid. Thus, this may explain its postprandial appearance after FB consumption. Therefore, we relate

24-hour excretion of 4-hydroxyhippuric acid under chronic consumption to both *p*-hydroxybenzoic glycination and microbial catabolic activities.

Common to acute and sustained consumption of FB, metabolites 3 and 6 (**Table 1**) were annotated as ***glucuronides of hydroxy-dimethoxybenzoic*** **acid** based on their LC-MS behaviour. Their mass fragmentation patterns suggest to us that they are conjugates of syringic and dimethylgallic acids, however no specific assignment could be made due to the lack of standards. Syringic acids could directly derive from grape extract due to their relative abundance in the berry skin,[44] or could originate in the body as microbiota catabolic products[45] derived from corresponding *O*-methylated flavonoid syringetin and anthocyanidin malvidin, normally abundant in red grapes.[46] Therefore, syringic acid may be glucuroconjugated directly upon absorption or later after its release by microbiota, and thus may be available in the body during the first postprandial hours and over a prolonged time after ingestion. Earlier, syringic acid was reported to be elevated after sustained consumption of grape-derived food.[14, 18, 47] With regard to dimethylgallic acid, very little information is available on this metabolite in relation to polyphenol metabolism. Thus, the 3,4-dimethyl derivative of gallic acid was proposed as a putative marker of black tea consumption,[48] in contrast to its 3,5-dimethyl isoform (syringic acid), suggesting that the two isomers detected in our samples could arrive by different ways. However, both of them might also have a common origin related to 3-*O*-methylated gallic acid,[48, 49] originating from the corresponding anthocyanin, petunidin, abundant in grape skin [46] or as a minor *O*-methylated metabolite[49] of grape-derived gallic acid. However, due to the limited information available, it is difficult to be conclusive with certainty about the origins of dimethylgallic acid with respect to our intervention.

Metabolites 4 and 5 (**Table 1**), positively changed in both postprandial and after prolonged FB consumption urines, were annotated as ***vanillic acid glucuronide*** and ***vanilloylglycine***, respectively, based on their LC-MS characteristics. Recently, vanilloylglycine was detected in 24-hour urines after regular dealcoholized red wine consumption.[47] Both compounds were related to the colonic microbiota metabolism of dietary polyphenols, principally of hydroxycinnamates[50, 51] and flavan-3-ols,[27, 52] also quite abundant in grape berry skins.[44]

However, in our study we have already detected these metabolites in 4-hour postprandial urine. This could be explained by the fact that vanillic acid was probably already present in the FB polyphenol extract, as was shown for red grape skin.[44] Thus, we hypothesize that on the one hand, both metabolites could be directly related to FB polyphenol extract, and on the other, they could be related to the microbial metabolism of more complex grape skin polyphenols, therefore being excreted as glucuronidated and glycinated derivatives of vanillic acid in urines obtained both after acute and sustained consumption.

Metabolite 18 (**Table 1**), common to both acute and sustained FB consumption, was annotated as ***dihydrosinapic acid glucuronide*** according to its LC-MS behaviour. It is a metabolite of sinapic acid via reduction of the double bond in the cinnamic acid side chain.[50, 51] Sinapic acid is among the most abundant hydroxycinnamates found in red grapes and derived products,[53] and recently its increase in 24-hour urine was related to consumption of dealcoholized red wine.[47] However, no data on the detection of its hydrated metabolite, dihydrosinapic acid, in relation to a dietary consumption of polyphenols, have been reported yet. Recently, it was proposed that reduction activity on cinnamates is not restricted only to intestinal microflora activity, as tissular metabolism[54] might also be involved. Therefore, we postulated that dihydrosinapic acid detected in acute samples might also be generated as a phase I metabolite of sinapic acid, and later, upon prolonged ingestion of an FB, appear in urine as a microbial metabolite as well.

Metabolites 11, 16 and 17 (**Table 1**,) were annotated as a ***glucuronide*** and ***two sulphates of epicatechin***, respectively, based on their LC-MS behaviour. The increase of epicatechin in urinary host metabolites during both acute and sustained consumption of FB, was obviously related to the intake of flavan-3-ols, which are the most abundant class of flavonoids present in grape and related products.[46, 53] In general, epicatechin phase II metabolites are recognized markers of consumption of flavanol-rich food,[26, 27, 55, 56] especially for postprandial stages. Their relation to the consumption of grape and derived food products was recently reported by our group in a targeted metabolomics study on the consumption of dealcoholized wine.[47] Relatively short excretion times of epicatechin phase II metabolites enable their identification in both of our sample types: 4-hour postprandial and 24-hour urines. In contrast, the metabolites 7, 8, 14

and metabolites 9, 10, 12, 15 and 13 (**Table 1**) were identified as principal discriminant metabolites of prolonged FB consumption. Based on their exact masses, retention times and in-source fragmentation patterns, these compounds were annotated as belonging to first-stage flavan-3-ol microbial metabolism[57] derivatives of ***hydroxyl-(dihydroxy)-valeric acid*** (two glucuronides and sulphate), and derivatives of ***dihydroxyphenyl-*** (two glucuronides), ***hydroxyl-methoxyphenyl-*** (two glucuronides) and ***hydroxyphenyl-valerolactones*** (one glucuronide), respectively. Earlier, various phase II hydroxyvaleric acid and hydroxyvalerolactone derivatives, including those identified in the present study, were also reported to be markers of consumption of flavanol-rich food such as almond skin,[55] cocoa[26, 27] and tea.[52, 58] Although grapes, especially red ones, are known to be very rich in flavanols, so far no studies have reported on their microbial metabolites in relation to grape polyphenol consumption. Only recently, our group suggested them as markers of chronic grape-derived product consumption (dealcoholized red wine) using targeted LC-MS analysis,[47] and the present study, applying an untargeted approach, has ratified that the phase II metabolites of microbiota-derived hydroxyvaleric acid and hydroxyvalerolactones could serve as principal markers of prolonged grape skin flavanol consumption.

Similarities between urinary metabolome under acute and prolonged intakes of grape skin polyphenols, both as differential mass features (**Figure 2**) and identified metabolites (**Table 1**), were observed. This is in line with the notion that the urine under sustained consumption should also contain compounds related to postprandial metabolism, since the 24-hour urine samples were collected on the last intervention day when the last two doses of the FB had been consumed at lunch and dinner. Differences in the metabolic profiles of the continuous consumption were clearly associated with the presence of metabolites of an exclusively microbial origin (**Table 1**). According to the study design and analysis, all unique mass features detected under sustained consumption, including unidentified ones, should be related either directly to microbial metabolism or to effects provoked by prolonged grape skin polyphenol consumption.

In contrast to prolonged consumption, we identified only one postprandial-specific metabolite. As we explained earlier, we expected mass features unique to the 4-hour postprandial sample to also be present in 24-hour urinary samples, however they could not be detected due to the metabolome-diluting effect. Thus, in this study the acute samples were used for two purposes: (i) to evaluate 4-hour postprandial urinary metabolome after FB intake; and (ii) to contrast those metabolites related specifically to 24-hour urine under prolonged grape skin consumption.

The potential health effects of grape polyphenols are of particular interest from the nutraceutical and functional food viewpoints.[4, 5, 7, 8] The mechanisms by which grape polyphenols exert their protective effects are not entirely clear, but they are conditioned by polyphenol bioavailability within the human organism.[53] The detection of polyphenol metabolites in urine following their dietary ingestion approximates their availability and recapitulates their fate in an organism, linking it to their biological effects. Previously, such evaluations were usually studied applying (targeted) analysis that was specifically focused on the main or on grape-specific polyphenols.[23, 47,59, 60] Thus, our previous study characterized in detail the resveratrol metabolism following ingestion of the functional beverage tested in the present study by applying a targeted LC-MS approach.[23] Since, the resveratrol metabolites could not be detected in the present study, probably due to the technical limitation of the applied untargeted approach, the results of the targeted study could be considered as complementary data to our results, confirming the bioavailability of ingested polyphenols. Our study, applying an untargeted LC-MS approach and data-driven analysis, was able to show that after dietary ingestion of grape skin polyphenols in the form of a functional beverage, the main alterations in urinary metabolites were related to polyphenol metabolism. As a result, we were able to assess the specificity of these alterations under sustained consumption in respect of the postprandial period.

Conclusion

In conclusion, our study showed that single and prolonged consumption of a grape skin polyphenol-based FB promoted significant changes in the profiles of urinary low-weight

metabolites. To our knowledge, this is the first study to assess the urinary metabolic fingerprint of dietary ingested grape polyphenols applying an untargeted LC-MS approach. Although our study was performed with a limited (for untargeted metabolomics) number of subjects living within close geographical area, the application of the crossover study design and highly sensitive HPLC-qTOF-MS technology enabled us to detect that the principal changes in urinary metabolome were related to the grape skin polyphenol metabolism. Thus, on the one hand, various metabolites of grape skin-derived phenolic acids and epicatechins were mutually identified in 4-hour postprandial and 24-hour urine after acute and under prolonged sustained consumption of FB, respectively. These metabolites could be recognized as generic markers of grape skin polyphenol consumption. On the other hand, several first-stage microbial metabolites represented by the phase II metabolites of hydroxylvaleric acid and hydroxyvalerolactone derivatives were also associated with high grape skin flavanol content. They were exclusively present in 24-hour urine during sustained consumption of the FB, therefore we would suggest them as principal microbiota-derived markers of prolonged consumption of grape skin polyphenols. However, it should be mentioned that despite of the controlled dietary conditions, which were applied in the present study, these metabolites cannot be considered solely as biomarkers of grape skin polyphenol intake since most of them have also been identified after consumption of other sources of flavan-3-ols, such as cocoa and almond,[26, 27, 55] and tea.[61] Furthermore, we should admit that the use of only positive ionization conditioned our research to discover principally positively ionized markers. This should be reconsidered in future studies, since negative ionization, although being less significant in the gathering of metabolic information,[62] could provide complementary metabolic data. In addition, a multimetabolite marker approach recently introduced in nutrimetabolomic [32] could provide more accurate evaluation of the exposure to grape-derived polyphenols, but this should be specifically tested. Therefore, we would suggest that the health impact of the tested functional beverage consumption, as well as other foods with grape skin-derived components, should be studied considering the complexity of polyphenol metabolism.

Acknowledgements

This work has been supported by Spanish public grants AGL2006-14228-C03-02 and AGL2009-13906-C02-01 and the CONSOLIDER-INGENIO 2010 Programme, FUN-C-FOOD (CSD2007-063) from the Spanish Ministry of Economy and Competitiveness (MINECO), the PI13/01172 Project, (Plan N de I+D+i 2013-2016) by ISCII-Subdirección General de Evaluación y Fomento de la Investigación as co-funded by FEDER (Fondo Europeo de Desarrollo Regional); the National Strategic Consortia for Technical Research Programme, in collaboration with the Bosch i Gimpera Foundation, FBG305132 and FBG305273; and Miguel Torres S.A. (Proyecto CENIT HIGEA CEN-20072003). We also thank the award of 2014SGR1566 from the Generalitat de Catalunya's Agency AGAUR. The Juan de la Cierva postdoctoral fellowship contract from the Spanish government awarded by the Spanish Ministry to Olha Khymenets is acknowledged. Rosa Vazquez-Fresno, Rafael Llorach and Mireia Urpi-Sarda would like to thank, respectively, the FPI 2010 fellowship and the Ramon y Cajal programmes from MICINN and the European Social Funds.

Table 1: Metabolites which positively correlated with acute and sustained consumption of a functional beverage (FB)

Metabolite cluster	RT (min)	Detected mass (m/z^+)	Assignation	Identification	Elemental formula	Level[a] of identification	Biological relevance to intervention	Acute	Sustained
1	0.47	182.0833	$[M+H]^+$	tyrosine	$C_9H_{11}NO_3$	II	FB or endogenously derived tyrosine	↑	-
2	2.87	196.0598 121.0274 122.0297	$[M+H]^+$ $[M+H-Glyc]^+$ $^{13}C[M+H-Glyc]^+$	4-hydroxyhippuric acid	$C_9H_9NO_4$	I	Hydroxybenzoic acid and/or microbial polyphenol metabolism	↑	↑
3	3.15	392.1206 199.0618 155.0687	$[M+NH4]^+$ $[M+H-Gluc]^+$ $[M+H-Gluc-CO_2]^+$	hydroxy-dimethoxybenzoic acid glucuronide[b]	$C_{15}H_{18}O_{11}$	II	Hydroxybenzoic acid and/or microbial polyphenol metabolism	↑	↑
4	3.28	345.0833 362.1113 169.0499 125.0585	$[M+H]^+$ $[M+NH_4]^+$ $[M+H-Gluc]^+$ $[M+H-Gluc-CO_2]^+$	vanillic acid glucuronide	$C_{14}H_{16}O_{10}$	II	Phenolic acid and polyphenol microbial metabolism	↑	↑
5	3.52	226.0727 227.0753 151.042 123.0439	$[M+H]^+$ $^{13}C[M+H]^+$ $[M+H-Glyc]^+$ $[M+H-Glyc-CO]^+$	vanilloylglycine	$C_{10}H_{11}NO_5$	II	Phenolic acid and polyphenol microbial metabolism	↑	↑
6	3.62	375.0973 392.1247 199.0624 155.0691 140.0481	$[M+H]^+$ $[M+NH_4]^+$ $[M+H-Gluc]^+$ $[M+H-Gluc-CO_2]^+$ $[M+H-Gluc-CO_2-CH_3]^+$	hydroxy-dimethoxybenzoic acid glucuronide[b]	$C_{15}H_{18}O_{11}$	II	Hydroxybenzoic acid and/or microbial polyphenol metabolism	↑	↑
7	3.72	420.1501 209.081	$[M+NH_4]^+$ $[M+H-Gluc-H_2O]^+$	4-hydroxy-5-(dihydroxyphenyl)-valeric acid glucuronide	$C_{17}H_{22}O_{11}$	II	Microbial metabolism of flavan-3-ols	-	↑
8	3.88	403.125 420.1523 209.0812 191.0706 149.0561	$[M+H]^+$ $[M+NH_4]^+$ $[M+H-Gluc-H_2O]^+$ $[M+H-Gluc-H_2O-H_2O]^+$ $[M+H-Gluc-H_2O-H_2O-C_2H_2O]^+$	4-hydroxy-5-(dihydroxyphenyl)-valeric acid glucuronide	$C_{17}H_{22}O_{11}$	II	Microbial metabolism of flavan-3-ols	-	↑

Table 1: Continuation

Metabolite cluster	RT (min)	Detected Mass (m/z+)	Assignation	Identification	Elemental formula	Level[a] of identification	Biological relevance to intervention	Acute	Sustained
9	4.37	385.1151	$[M+H]^+$	5-(dihydroxyphenyl)-γ-valerolactone glucuronide	$C_{17}H_{20}O_{10}$	II	Microbial metabolism of flavan-3-ols	-	↑
		407.0959	$[M+Na]^+$						
		402.1401	$[M+NH_4]^+$						
		403.1444	$^{13}C[M+NH_4]^+$						
		209.0819	$[M+H\text{-}Gluc]^+$						
		210.0839	$^{13}C[M+H\text{-}Gluc]^+$						
		191.0707	$[M+H\text{-}Gluc\text{-}H_2O]^+$						
10	4.57	385.113	$[M+H]^+$	5-(dihydroxyphenyl)-γ -valerolactone glucuronide	$C_{17}H_{20}O_{10}$	II	Microbial metabolism of flavan-3-ols	-	↑
		386.1153	$^{13}C[M+H]^+$						
		407.0927	$[M+Na]^+$						
		402.1382	$[M+NH_4]^+$						
		403.1403	$^{13}C[M+NH_4]^+$						
		367.1009	$[M+H\text{-}H_2O]^+$						
		209.0813	$[M+H\text{-}Gluc]^+$						
		210.083	$^{13}C[M+H\text{-}Gluc]^+$						
		191.0698	$[M+H\text{-}Gluc\text{-}H_2O]^+$						
		192.0723	$^{13}C[M+H\text{-}Gluc\text{-}H_2O]^+$						
		149.0581	$[M+H\text{-}Gluc\text{-}C_2H_3O_2]^+$						
		131.0468	$[M+H\text{-}Gluc\text{-}C_2H_3O_2\text{-}H_2O]^+$						
		123.0416	$[M+H\text{-}Gluc\text{-}C_2H_3O_2\text{-}C_2H_2]^+$						
11	4.67	467.1165	$[M+H]^+$	(epi)catechin glucuronide[c]	$C_{21}H_{22}O_{12}$	II	Epicatechin metabolism	↑	↑
		291.0894	$[M+H\text{-}Gluc]^+$						
		139.0393	$[M+H\text{-}Gluc\text{-}C_8H_8O_3$[c]$]^+$						
12	4.73	399.1325	$[M+H]^+$	5-(hydroxyl-methoxy-phenyl)-γ-valerolactone glucuronide	$C_{18}H_{22}O_{10}$	II	Microbial metabolism of flavan-3-ols	-	↑
		416.159	$[M+NH_4]^+$						
		417.1613	$^{13}C[M+NH_4]^+$						
		223.0982	$[M+H\text{-}Gluc]^+$						
		224.1004	$^{13}C[M+H\text{-}Gluc]^+$						
		205.087	$[M+H\text{-}Gluc\text{-}H_2O]^+$						
		163.0756	$[M+H\text{-}Gluc\text{-}H_2O\text{-}C_2H_2O]^+$						

Table 1: Continuation

Metabolite cluster	RT (min)	Detected Mass (m/z+)	Assignation	Identification	Elemental formula	Level[a] of identification	Biological relevance to intervention	Acute	Sustained
13	4.85	369.1195 370.1229 351.1092 193.0867 194.0895 133.0641	$[M+H]^+$ $^{13}C[M+H]^+$ $[M+H-H_2O]^+$ $[M+H-Gluc]^+$ $^{13}C[M+H-Gluc]^+$ $[M+H-Gluc-C_2H_3O_2]^+$	5-(hydroxyphenyl)-γ-valerolactone glucuronide	$C_{17}H_{20}O_9$	II	Microbial metabolism of flavan-3-ols	-	↑
14	5	307.0513 289.0379	$[M+H]^+$ $[M+H-H_2O]^+$	4-hydroxy-5-(dihydroxyphenyl)-valeric acid sulphate	$C_{11}H_{14}O_8S$	II	Microbial metabolism of flavan-3-ols	-	↑
15	5.17	223.095	$[M+H-Gluc]^+$	5'-(hydroxy-methoxy-phenyl)-γ-valerolactone glucuronide	$C_{12}H_{14}O_4$	II	Microbial metabolism of flavan-3-ols	-	↑
16	5.45	371.043	$[M+H]^+$	(epi)catechin sulphate-2	$C_{15}H_{14}O_9S$	II	Epicatechin metabolism	↑	↑
17	5.9	371.0445	$[M+H]^+$	(epi)catechin sulphate-3	$C_{15}H_{14}O_9S$	II	Epicatechin metabolism	↑	↑
18	6.03	420.1493 227.0904 228.0937 155.0709 140.0471	$[M+NH_4]^+$ $[M+H-Gluc]^+$ $^{13}C[M+H-Gluc]^+$ $[M+H-Gluc-C_2H_4COO]^+$ $[M+H-Gluc-C_2H_4COO-CH_3]^+$	dihydrosinapic acid glucuronide	$C_{17}H_{22}O_{11}$	II	Phenolic acid and polyphenol microbial metabolism	↑	↑

Gluc – glucuronide residue; Glyc – glycine residue; [a] – levels of identification are reported according to Sumner et al. 2007[38]; [b] – either syringic or dimethylgallic acid glucuronide, no specific assignment could be made due to the lack of standards; [c] – fragment of (epi)catechin, according to Flamini R. [63]; ↑ - indicates higher levels after FB consumption in comparison to CB consumption ($p[corr] \leq 0.05$); - - no changes were observed.

Figure legends:

Figure 1: Score plots of orthogonal partial least squares discriminant (OPLS-DA) modelling for acute (**A**) and 15-day sustained (**B**) intervention studies with control (samples marked as blue and green cycles, respectively) and functional (samples marked as red and yellow cycles, respectively) beverages. N – number of components in OPLS-DA modelling; O – orthogonal component; P – predictive component; R^2X_{cum} and R^2Y_{cum} are the cumulative modelled variation in the X and Y matrix, respectively; Q^2_{cum} is the cumulative predicted variation in the Y matrix; the *P*-value from CV-ANOVA, based on the cross-validated predictive residuals, is listed for both models (significant result indicates a valid model).

Figure 2: S-plots associated with OPLS-DA score plots for data obtained from urine LC-MS analysis for acute (**A**) and sustained (**B**) intervention studies on the consumption of both (FB and CB) beverages. Dashed lines represent p(corr) $\geq|0.5|$ cut-offs used for the selection of putative markers coloured in red and yellow for acute and sustained consumptions, respectively.

Figure 3: Diagram showing the overlap among the m/z features and those metabolites which were identified and annotated according to the levels I and II of the confidence of identity[38] in both acute and sustained intervention studies with functional (FB) beverage. The corresponding listed metabolites´ mass features are presented in Table 1.

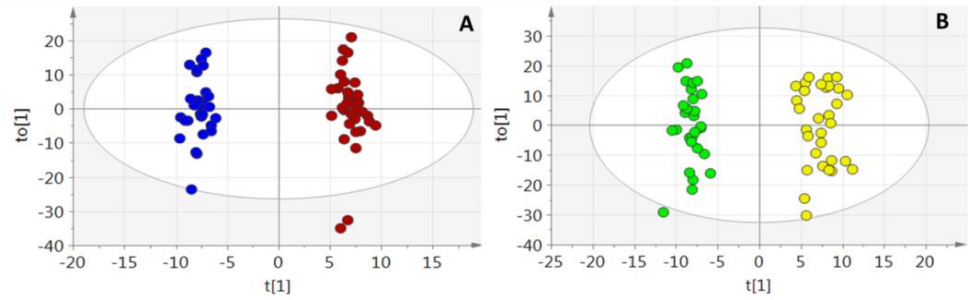
References

1. R. H. Liu, *Am J Clin Nutr*, 2003, **78**, 517S-520S.
2. N. Jain and K. Ramawat, in *Natural Products*, ed. K. G. Ramawat and J.-M. Mérillon, Springer Berlin Heidelberg, 2013, ch. 70, pp. 2559-2580.
3. T. Magrone, F. Perez de Heredia, E. Jirillo, G. Morabito, A. Marcos and M. Serafini, *Can J Physiol Pharmacol*, 2013, **91**, 387-396.
4. A. P. Gollucke, *Recent Pat Food Nutr Agric*, 2010, **2**, 105-109.
5. V. Georgiev, A. Ananga and V. Tsolova, *Nutrients*, 2014, **6**, 391-415.
6. J. Yang and Y.-Y. Xiao, *Critical Reviews in Food Science and Nutrition*, 2013, **53**, 1202-1225.
7. M. Nassiri-Asl and H. Hosseinzadeh, *Phytother Res*, 2009, **23**, 1197-1204.
8. M. De Nisco, M. Manfra, A. Bolognese, A. Sofo, A. Scopa, G. C. Tenore, F. Pagano, C. Milite and M. T. Russo, *Food Chem*, 2013, **140**, 623-629.
9. E. Trujillo, C. Davis and J. Milner, *J Am Diet Assoc*, 2006, **106**, 403-413.
10. A. Scalbert, L. Brennan, C. Manach, C. Andres-Lacueva, L. O. Dragsted, J. Draper, S. M. Rappaport, J. J. van der Hooft and D. S. Wishart, *Am J Clin Nutr*, 2014, **99**, 1286-1308.
11. X. Zhang, W. Wang and K. Xiao, in *Genomics, Proteomics, and Metabolomics in Nutraceuticals and Functional Foods*, Wiley-Blackwell, 2010, pp. 11-22.
12. M. Farag, *Recent Pat Biotechnol*, 2013.
13. K. Lan, G. Xie and W. Jia, *Evid Based Complement Alternat Med*, 2013, **2013**, 819147.
14. F. A. van Dorsten, C. H. Grun, E. J. van Velzen, D. M. Jacobs, R. Draijer and J. P. van Duynhoven, *Mol Nutr Food Res*, 2010, **54**, 897-908.
15. A. M. Knab, D. C. Nieman, N. D. Gillitt, R. A. Shanely, L. Cialdella-Kam, D. A. Henson and W. Sha, *Int J Sport Nutr Exerc Metab*, 2013, **23**, 150-160.
16. L. Brennan, *Biochem Soc Trans*, 2013, **41**, 670-673.
17. R. Vazquez-Fresno, R. Llorach, F. Alcaro, M. A. Rodriguez, M. Vinaixa, G. Chiva-Blanch, R. Estruch, X. Correig and C. Andres-Lacueva, *Electrophoresis*, 2012, **33**, 2345-2354.
18. D. M. Jacobs, J. C. Fuhrmann, F. A. van Dorsten, D. Rein, S. Peters, E. J. van Velzen, B. Hollebrands, R. Draijer, J. van Duynhoven and U. Garczarek, *J Agric Food Chem*, 2012, **60**, 3078-3085.
19. D. M. Jacobs, L. Spiesser, M. Garnier, N. de Roo, F. van Dorsten, B. Hollebrands, E. van Velzen, R. Draijer and J. van Duynhoven, *Anal Bioanal Chem*, 2012, **404**, 2349-2361.
20. N. W. Lutz, J. V. Sweedler and R. A. Wevers, *Methodologies for Metabolomics: Experimental Strategies and Techniques*, Cambridge University Press, 2013.
21. B. Zhou, J. F. Xiao, L. Tuli and H. W. Ressom, *Mol Biosyst*, 2012, **8**, 470-481.
22. R. Llorach, M. Garcia-Aloy, S. Tulipani, R. Vazquez-Fresno and C. Andres-Lacueva, *J Agric Food Chem*, 2012, **60**, 8797-8808.
23. M. Rotches-Ribalta, M. Urpi-Sarda, M. M. Marti, G. Reglero and C. Andres-Lacueva, *Electrophoresis*, 2013.
24. X. Ceto, J. M. Gutierrez, M. Gutierrez, F. Cespedes, J. Capdevila, S. Minguez, C. Jimenez-Jorquera and M. del Valle, *Anal Chim Acta*, 2012, **732**, 172-179.
25. S. Tulipani, R. Llorach, O. Jauregui, P. Lopez-Uriarte, M. Garcia-Aloy, M. Bullo, J. Salas-Salvado and C. Andres-Lacueva, *J Proteome Res*, 2011, **10**, 5047-5058.
26. R. Llorach, M. Urpi-Sarda, S. Tulipani, M. Garcia-Aloy, M. Monagas and C. Andres-Lacueva, *Mol Nutr Food Res*, 2013, **57**, 962-973.
27. R. Llorach, M. Urpi-Sarda, O. Jauregui, M. Monagas and C. Andres-Lacueva, *J Proteome Res*, 2009, **8**, 5060-5068.
28. S. Wiklund, E. Johansson, L. Sjostrom, E. J. Mellerowicz, U. Edlund, J. P. Shockcor, J. Gottfries, T. Moritz and J. Trygg, *Anal Chem*, 2008, **80**, 115-122.
29. L. Eriksson, J. Trygg and S. Wold, *Journal of Chemometrics*, 2008, **22**, 594-600.

30. J. Westerhuis, H. J. Hoefsloot, S. Smit, D. Vis, A. Smilde, E. J. van Velzen, J. M. van Duijnhoven and F. van Dorsten, *Metabolomics*, 2008, **4**, 81-89.
31. Y. Wang, J. Wang, M. Yao, X. Zhao, J. Fritsche, P. Schmitt-Kopplin, Z. Cai, D. Wan, X. Lu, S. Yang, J. Gu, H. U. Haring, E. D. Schleicher, R. Lehmann and G. Xu, *Anal Chem*, 2008, **80**, 4680-4688.
32. M. Garcia-Aloy, R. Llorach, M. Urpi-Sarda, S. Tulipani, R. Estruch, M. A. Martinez-Gonzalez, D. Corella, M. Fito, E. Ros, J. Salas-Salvado and C. Andres-Lacueva, *J Proteome Res*, 2014, **13**, 3476-3483.
33. G. Caraux and S. Pinloche, *Bioinformatics*, 2005, **21**, 1280-1281.
34. R. Llorach-Asuncion, O. Jauregui, M. Urpi-Sarda and C. Andres-Lacueva, *J Pharm Biomed Anal*, 2010, **51**, 373-381.
35. F. Fernandez-Albert, R. Llorach, C. Andres-Lacueva and A. Perera, *Bioinformatics*, 2014.
36. C. A. Smith, G. O'Maille, E. J. Want, C. Qin, S. A. Trauger, T. R. Brandon, D. E. Custodio, R. Abagyan and G. Siuzdak, *Ther Drug Monit*, 2005, **27**, 747-751.
37. R. S. Plumb, K. A. Johnson, P. Rainville, B. W. Smith, I. D. Wilson, J. M. Castro-Perez and J. K. Nicholson, *Rapid Communications in Mass Spectrometry*, 2006, **20**, 1989-1994.
38. L. W. Sumner, A. Amberg, D. Barrett, M. H. Beale, R. Beger, C. A. Daykin, T. W. Fan, O. Fiehn, R. Goodacre, J. L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A. N. Lane, J. C. Lindon, P. Marriott, A. W. Nicholls, M. D. Reily, J. J. Thaden and M. R. Viant, *Metabolomics : Official journal of the Metabolomic Society*, 2007, **3**, 211-221.
39. E. J. van Velzen, J. A. Westerhuis, J. P. van Duynhoven, F. A. van Dorsten, C. H. Grun, D. M. Jacobs, G. S. Duchateau, D. J. Vis and A. K. Smilde, *J Proteome Res*, 2009, **8**, 3317-3330.
40. M. Urpi-Sarda, R. Llorach, N. Khan, M. Monagas, M. Rotches-Ribalta, R. Lamuela-Raventos, R. Estruch, F. J. Tinahones and C. Andres-Lacueva, *J Agric Food Chem*, 2010, **58**, 4706-4711.
41. S. Khadem and R. J. Marles, *Molecules*, 2010, **15**, 7985-8005.
42. M. A. El Mohsen, J. Marks, G. Kuhnle, K. Moore, E. Debnam, S. Kaila Srai, C. Rice-Evans and J. P. Spencer, *Br J Nutr*, 2006, **95**, 51-58.
43. C. P. Badenhorst, E. Erasmus, R. van der Sluis, C. Nortje and A. A. van Dijk, *Drug Metab Rev*, 2014.
44. P. Mattila, J. Hellstrom and R. Torronen, *J Agric Food Chem*, 2006, **54**, 7193-7199.
45. G. Williamson and M. N. Clifford, *Br J Nutr*, 2010, **104 Suppl 3**, S48-66.
46. R. Flamini, F. Mattivi, M. De Rosso, P. Arapitsas and L. Bavaresco, *Int J Mol Sci*, 2013, **14**, 19651-19669.
47. M. Boto-Ordonez, M. Urpi-Sarda, M. I. Queipo-Ortuno, D. Corella, F. J. Tinahones, R. Estruch and C. Andres-Lacueva, *J Agric Food Chem*, 2013, **61**, 9166-9175.
48. J. M. Hodgson, L. W. Morton, I. B. Puddey, L. J. Beilin and K. D. Croft, *J Agric Food Chem*, 2000, **48**, 2276-2280.
49. A. N. Booth, M. S. Masri, D. J. Robbins, O. H. Emerson, F. T. Jones and F. De Eds, *J Biol Chem*, 1959, **234**, 3014-3016.
50. L. A. Griffiths, *Biochem J*, 1969, **113**, 603-609.
51. A. Fardet, R. Llorach, A. Orsoni, J. F. Martin, E. Pujos-Guillot, C. Lapierre and A. Scalbert, *J Nutr*, 2008, **138**, 1282-1287.
52. J. J. van der Hooft, R. C. de Vos, V. Mihaleva, R. J. Bino, L. Ridder, N. de Roo, D. M. Jacobs, J. P. van Duynhoven and J. Vervoort, *Anal Chem*, 2012, **84**, 7263-7271.
53. J. Yang and Y. Y. Xiao, *Critical Reviews in Food Science and Nutrition*, 2013, **53**, 1202-1225.
54. M. Y. Moridani, H. Scobie and P. J. O'Brien, *Toxicol Lett*, 2002, **133**, 141-151.
55. R. Llorach, I. Garrido, M. Monagas, M. Urpi-Sarda, S. Tulipani, B. Bartolome and C. Andres-Lacueva, *J Proteome Res*, 2010, **9**, 5859-5867.

56. H. Ito, M. P. Gonthier, C. Manach, C. Morand, L. Mennen, C. Remesy and A. Scalbert, *Br J Nutr*, 2005, **94**, 500-509.
57. M. Monagas, M. Urpi-Sarda, F. Sanchez-Patan, R. Llorach, I. Garrido, C. Gomez-Cordoves, C. Andres-Lacueva and B. Bartolome, *Food Funct*, 2010, **1**, 233-253.
58. J. van Duynhoven, J. J. van der Hooft, F. A. van Dorsten, S. Peters, M. Foltz, V. Gomez-Roldan, J. Vervoort, R. C. de Vos and D. M. Jacobs, *J Proteome Res*, 2014.
59. M. Rotches-Ribalta, M. Urpi-Sarda, R. Llorach, M. Boto-Ordonez, O. Jauregui, G. Chiva-Blanch, L. Perez-Garcia, W. Jaeger, M. Guillen, D. Corella, F. J. Tinahones, R. Estruch and C. Andres-Lacueva, *J Chromatogr A*, 2012, **1265**, 105-113.
60. A. Jimenez-Giron, M. I. Queipo-Ortuno, M. Boto-Ordonez, I. Munoz-Gonzalez, F. Sanchez-Patan, M. Monagas, P. J. Martin-Alvarez, M. Murri, F. J. Tinahones, C. Andres-Lacueva, B. Bartolome and M. V. Moreno-Arribas, *J Agric Food Chem*, 2013, **61**, 3909-3915.
61. J. van Duynhoven, J. J. van der Hooft, F. A. van Dorsten, S. Peters, M. Foltz, V. Gomez-Roldan, J. Vervoort, R. C. de Vos and D. M. Jacobs, *J Proteome Res*, 2014, **13**, 2668-2678.
62. G. Xie, X. Zheng, X. Qi, Y. Cao, Y. Chi, M. Su, Y. Ni, Y. Qiu, Y. Liu, H. Li, A. Zhao and W. Jia, *J Proteome Res*, 2010, **9**, 125-133.
63. R. Flamini, *ISRN Spectroscopy*, 2013, **2013**, 45.

Figure 1



N	R^2X_{cum}	R^2Y_{cum}	Q^2_{cum}	*P-value**
1 P +2 O	0.171	0.985	0.77	1.41e-13

N	R^2X_{cum}	R^2Y_{cum}	Q^2_{cum}	*P-value**
1 P +2 O	0.211	0.962	0.74	1.28e-13

Figure 2

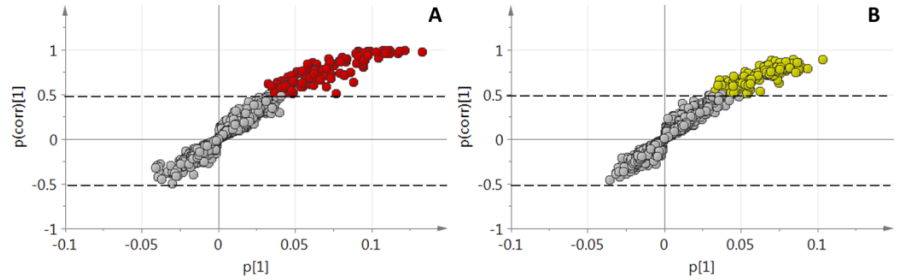
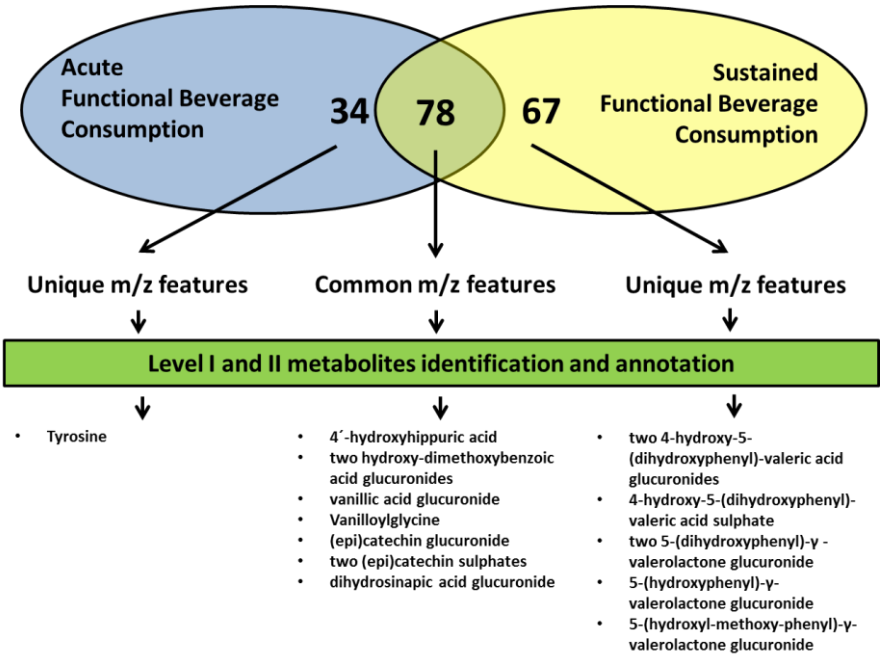


Figure 3



9.2 OTROS CAPÍTULOS DE LIBROS

Capítulo I: Trespalacios MP, Tulipani S, Garcia-Aloy M; Zamora-Ros R, Rotchés-Ribalta M, Rabassa M, Urpi-Sarda M, Boto M, Vázquez R, Llorach R, Andrés-Lacueva C. Nutritional Biomarkers: Applications and Challenges. En: Morrisson JL (editor). *Food Intake: Regulation, Assessing and Controlling.* Nova Science Publishers, Inc.; 2013. ISBN: 978-1-61324-183-7

Chapter 6

NUTRITIONAL BIOMARKERS: APPLICATIONS AND CHALLENGES

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ABSTRACT

Diet is implicated in a myriad of diseases, ranging from events in the first weeks of life to chronic and age-related illnesses, for example, cancer and cardiovascular disease. A primary goal of nutrition research is to optimize health via dietary interventions, by prevention, delay, or reduction in the severity of disease.

To date, several methods for dietary assessment have been used, such as food frequency questionnaires, food diaries, food records, or 24-h dietary recalls. However, they are associated with different measurement errors between diet observed and true intake, which may obscure some disease risk associations. Thus, nutrition epidemiologists started using different biomarkers in biological samples that are more objective measures and are independent of errors related to dietary assessment methods. A nutritional biomarker can be any biological compound that is an indicator of nutritional status with respect to the intake or metabolism of dietary constituents. An ideal nutritional biomarker would accurately reflect its dietary intake level and it would be specific, sensitive and applicable to many populations. It should also be reproducible, validated and reliable.

The discovery and assessment of nutritional biomarkers highly depend on appropriate analytical tools. An ideal technique for biomarkers discovery and monitoring, unfortunately, is not currently available, the critical limiting factor being the lack of

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Capítulo II:

Rabassa M, Zamora-Ros R, Tulipani S, Khymenets O, Urpi-Sarda M, Garcia-Aloy M, Boto M, Vázquez R, Rotches-Ribalta M; Chiva G, Trespalacios MP, Llorach R, Andres-Lacueva C. Flavonoids from food and its implication in human health. En: Yamane K, Kato Y (editores): *Handbook of flavonoids: dietary sources, properties and health benefits*. Nova Science Publishers; 2012. ISBN: 978-1-61942-052-6

FLAVONOIDS FROM FOOD AND ITS IMPLICATION IN HUMAN HEALTH

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ABSTRACT

Flavonoids comprise a large group of phenolic compounds synthesized by plants as secondary metabolites, with a common diphenylpropane skeleton (C6-C3-C6). Over 9,000 structurally distinct flavonoids have been identified in nature, although less than 200 were found in a sample French population. Flavonoids can be classified according to their chemical structure into eight subclasses: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols or flavanols, anthocyanidins, isoflavones and proanthocyanidins.

Flavonoids occur ubiquitously in plants and plant products. They are generally found in glycosylated forms, mainly with glucose or rhamnose moieties, but other sugars may also be involved. Flavonoids in foods contribute to their flavor, providing color, astringency and bitterness. Flavonols are the most frequent flavonoids in foods, with

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onions, apples and tea being the main food sources. Flavones are widely distributed in plants, but in lower amounts than flavonols. The richest sources are herbs and spices. Dihydroflavonols have been identified in a few foods, such as wines and oregano. Flavanones are characteristic compounds of citrus fruits and their derived juices. Anthocyanidins are responsible for the colours of fruits and vegetables, like pink, red, blue or purple. They are abundant in berries and their derived products, such as juices and wines. Flavan-3-ols are found as aglycones in foods. Catechin and epicatechin are the main flavan-3-ol contributors, with tea, apples, red wine and chocolate being their most common food sources. Proanthocyanidins are oligomeric and polymeric flavan-3-ols, also known as condensed tannins, and are responsible for the astringency of various fruits or beverages and for the bitterness of chocolate. Theaflavins and thearubigins are derived flavanol compounds that are only found in tea. Isoflavones are found almost exclusively in leguminous plants, particularly in soybeans and their processed products.

The bioavailability of flavonoids varies greatly among and within their subclasses, in terms of their chemical structure and sugar moiety type. Flavonoids are usually absorbed by both the small intestine and colon as aglycones after being hydrolyzed by either enzymes or the microbiota with a high intra- and inter-individuality. After absorption, they are quickly metabolized in the intestinal cells or in the liver. Therefore, the biological activity of flavonoids may be due to their conjugated forms or the aglycones that may be set free in target tissues. Finally, they are eliminated by renal and/or liver excretion. Most flavonoids are known to exert similar biological effects – such as antioxidant, anticarcinogenic, anti-inflammatory, antiobesity, antidiabetic, antiallergic, hepatoprotective, and gastroprotective activities – in vitro. But each individual compound has different effective concentrations, may use different mechanism of actions and even exert different effects.

Epidemiological studies have suggested that flavonoids may provide protection against several chronic diseases, such as cardiovascular and neurodegenerative diseases, diabetes, obesity and some cancers. However, scientific evidence on the role of flavonoid intake in disease prevention is promising but not conclusive, and further clinical and epidemiological studies are needed.

Capítulo III: Rotches-Ribalta M, Urpi-Sarda M, Tulipani S, Trespalacios P, Rabassa-Bonet M, Boto-Ordóñez M, Vázquez-Fresno R, Garcia-Aloy M, Llorach R, Andres-Lacueva C. Metabolism and pharmacokinetics of resveratrol: sources and food matrix on the bioavailability of resveratrol. En: Delmas D (editor): *Resveratrol: sources, production, and health benefits.* Nova Science Publishers; 2012. ISBN: 978-1-62081-997-5

SOURCES AND FOOD MATRIX ON THE BIOAVAILABILITY OF RESVERATROL

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ABSTRACT

Resveratrol consumption has been positively associated with the prevention of a number of diseases, mainly due to its reported antioxidant activity and anti-inflammatory and antitumor effects, and has, therefore, the potential to be evaluated for therapeutic use. However, scarce studies have been carried out in humans and the results yielded are still controversial. Before making nutritional recommendations regarding this promising molecule, other aspects such as dietary intake and bioavailability should be considered.

Resveratrol and other stilbenes, such as piceid, have been identified in a large number of plants, although they have been found in a few dietary

components, with red wine and grape products as the major sources. Therefore, urinary resveratrol metabolites have been described as nutritional biomarkers of wine consumption, providing an additional tool for clinical and epidemiological studies, since they reflect a more objective assessment of nutrient intake than self-reported dietary data. Its estimated intake or exposure is essential in order to relate resveratrol consumption with its possible health effects *in vivo*. However, resveratrol is rapidly metabolized, and therefore the bioactivity of this compound will be related to its metabolites. Identification and measurement of metabolic forms are key prerequisites for understanding the role of these compounds, since they will be the ones that reach the peripheral circulation and tissues where they could exert their biological effect. Phase II metabolism of resveratrol has been widely studied, with the glucuronide and sulfate forms being the main resveratrol conjugates. However, few studies have focused on the microbial metabolism of resveratrol, which leads to dihydroresveratrol formation, although this could give a better idea about effects of long-term resveratrol consumption. Furthermore, the matrix in which resveratrol is delivered could play an important role in its bioavailability.

This chapter, therefore, provides an overview of the metabolism of resveratrol after short- and long-term consumption and the microbiota and food matrix effects on its bioavailability. This needs to be considered in order to gain a better understanding of the links between resveratrol intake and health.

9.3 COMUNICACIONES EN CONGRESOS

Durante el período predoctoral se han presentado 5 conferencias invitada en congresos y una comunicación oral de las cuales presentan algunos de los resultados recogidos en la presente Tesis Doctoral. También se han presentado 3 pósters en 3 congresos internacionales: el "*V International Conference on Polyphenols and Health (ICPH)"*, el *International Workshop on Metabolomics 'Technology and Applications' CIC bioGUNE*, el "*X Congreso Internacional de Barcelona sobre la Dieta Mediterránea".*

CONFERENCIA INVITADA:

Andrés-Lacueva C, Urpi-Sarda M, Llorach R, Rotchés-Ribalta M, Chiva-Blanch G, Zamora-Ros R, Boto-Ordóñez M, Vázquez R, Rabassa-Bonet M, Tulipani S, Trespalacios P, García-Aloy M, Lamuela-Raventos RM, Estruch R. Metabolomic and targeted study of biomarkers in an interventional clinical study on wine consumption. *Wine active compounds: international conference*. Beaune (Francia). Marzo 2011

Llorach R, Urpi-Sarda M, Tulipani S, Jauregui O, Garcia-Aloy M, Vazquez R, Rabassa M, Fernandez F, Perera A, Andres-Lacueva C. Polyphenols-rich diets consumption and cardiovascular-health promoting. Application of diferent nutrimetabolomics strategies to develop new biomarkers of intake and health-effects. *The 5th International Conference on Polyphenols and Health (ICPH)*. Sitges, Barcelona (España). Octubre 2011

Andres-Lacueva C, Urpi-Sarda M, Vazquez-Fresno R, Tulipani S, Rotches-Ribalta M, Boto-Ordonez M, Queipo-Ortuno M, Chiva G, Estruch R, Tinahones FJ, Llorach R. Red wine polyphenols and human

health. *20th International Congress of Nutrition*. Granada (España). Septiembre 2013

Andres-Lacueva C.; Mireia Urpi-Sarda,; García-Aloy, M,; Vazquez-Fresno, R,; Estruch, R,;Corella, D,;Martínez-González,MA,; Salas-Salvadó, J.;Covas, MI,; Serra-Majem, L.; Drapers, J,;Llorach, R. New metabolomics strategies in clinical nutrition research: from diet to revealing disease risk biomarkers. *20th International Congress of Nutrition*. Granada (España). Septiembre 2013

Andres-Lacueva C, Urpi-Sarda M, García-Aloy, M, Vazquez-Fresno R, Estruch R, Corella, D, Martínez-González MA, Salas-Salvadó J, Covas MI, Serra-Majem L, Drapers J, Llorach R. Metabolomic strategies in clinical nutrition research: from polyphenol rich diet to revealing disease risk biomarkers. *VI International Conference on Polyphenols and Health (ICPH-6)*. Buenos Aires (Argentina). Octubre 2013

COMUNICACIÓN ORAL:

Llorach, R; Vázquez-Fresno, R; Perera, A; Chiva, G; Tinahones, F; Estruch, R; Andres-Lacueva, C Metabotyping in cardiovascular risk subjects reveals differences in their urinary profiles after wine intake. *9th Annual International Conference of the Metabolomics Society*. Glasgow (Reino Unido). Julio 2013

^{1}H-NMR-based Metabolomic Approach to Evaluate the Effect of Moderate Wine Intake in a Human Population

Rosa Vázquez-Fresno[1], Rafael Llorach[1] Francesca Alcaro[1], Miguel Ángel Rodríguez[2], Maria Vinaixa[2], Gemma Chiva[3], Ramon Estruch[3], Xavier Correig[2] Cristina Andrés-Lacueva[1]*

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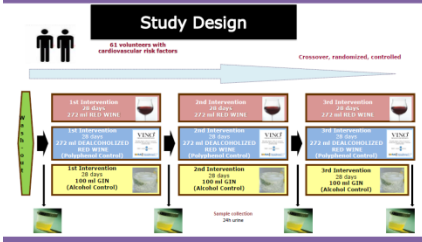
INTRODUCTION

Moderate wine intake is associated with health promoting activities. NMR allows the detection of a wide range of metabolites for metabolomic applications . The aim of the present study is to apply a NMR-metabolomic strategy in order to investigate the human metabolic effect of wine intake in urinary profile identifying the most relevant metabolic changes after moderate intake of red wine (RW).

MATERIAL AND METHODS

Study Design



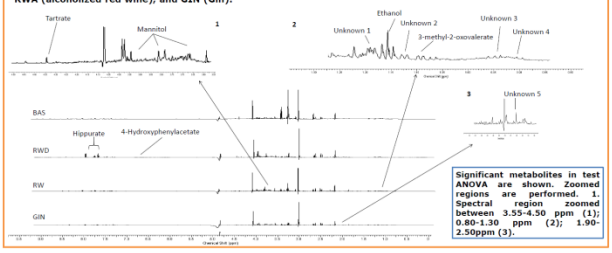
Metabolomics workflow

RESULTS

Table 1: Metabolites significant in ANOVA test after treatments. Chemical shifts and p-values of each metabolite ($p<0.05$) [a]wine with alcohol, [b]wine without alcohol, [c]not modified.

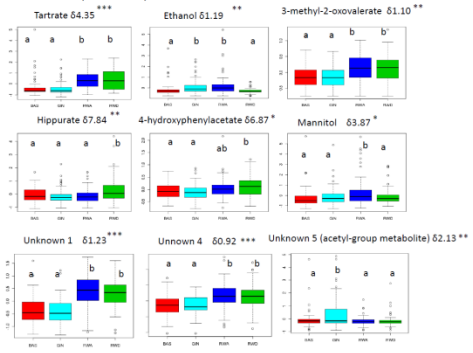
Compound	^{1}H NMR Chemical shift (ppm)		After consumption of	P-value		Metabolites information
	Variable size bucketing	0.005ppm bucketing	wine	Variable size bucketing	0.005ppm bucketing	
Tartrate	4.35	4.35	$\uparrow^{ab}$	1.0147×10^{-7}	4.81×10^{-9}	Wine compound
Unknown 1	1.24	1.24	$\uparrow^{ab}$	3.0143×10^{-8}	2.54×10^{-17}	Unknown
Ethanol	1.19	1.19 3.67	$\uparrow^{a}$	3.0324×10^{-4}	1.43×10^{-4} 8.60×10^{-4}	Alcohol from bevarage
Unknown 2	1.15	1.15	$\uparrow^{ab}$	4.38×10^{-3}	2.97×10^{-3}	Unknown
3-methyl-2-oxovalerate	-	1.10	$\uparrow^{ab}$	3.48×10^{-2}	1.63×10^{-3}	Valine, leucine and isoleucine degradation
4-hydroxyphenylacetate	6.87	6.87	$\uparrow^{b}$	4.31×10^{-2}	1.89×10^{-2}	Phenylalanine and Tyrosine metabolism. Gut microbiota
Hippurate	7.84 3.98 7.64 7.57	7.84 3.98 7.64 7.57	$\uparrow^{b}$	1.26×10^{-2} 1.49×10^{-2} 3.05×10^{-2} 3.29×10^{-2}	6.56×10^{-4} 2.59×10^{-2} 2.58×10^{-3} 1.04×10^{-2}	Phenylalanine metabolism. Gut microbiota
Mannitol	[3.69-3.87]	3.77 3.87	$\uparrow^{a}$	3.43×10^{-2}	3.68×10^{-2} 4.69×10^{-2}	Wine alcoholized compound
Unknown 3	-	0.85	$\uparrow^{ab}$	-	7.01×10^{-8}	Unknown
Unknown 4	-	0.92	$\uparrow^{ab}$	-	4.31×10^{-7}	Unknown
Unknown 5	2.13	2.13 5.69	$\downarrow^{c}$	2.85×10^{-2}	8.78×10^{-4} 2.94×10^{-3}	Endogenous acetylated metabolite

Figure 2. Representative 500MHz ^{1}H-NMR. region of spectra in BAS (basal period);RWD (dealcoholized red wine) RWA (alcoholized red wine), and GIN (Gin).



Tartrate was identified associated to wine intake (DRW and RW). Several peaks in aliphatic region of spectrum (unknown 1, 2, 3 and 4) showed a significant increase after wine intake. In this region, the methyl-2-oxovalerate, a branched-chain aminoacids (BCAA) metabolite, was identified. DRW intake increased the excretion of hippurate and hydroxyphenylacetate, two metabolites from gut microbiota, compared with GIN. Moreover, ethanol was associated to GIN and RW consumption.

Figure 1. Box plots of the intensities of significant metabolites in ANOVA test. Different letters indicate significant differences.
*****P<0.00001; **P<0.005; *P<0.05 .**



CONCLUSIONS

Presence of tartrate after wine-related diets and ethanol after alcohol-related diets (GIN and RW) indicates a global compliance. Furthermore, it is proposed tartrate as a biomarker of wine intake. More about BCAAs and wine intake it will be known to understand the relation between catabolic pathway of BCAA and wine intake. It is suspected an alcohol interaction with the excretion of gut microbiota metabolites but more studies are needed to clarify the behaviour of these two gut microbiota metabolites with alcohol intake.

REFERENCES

•Estruch, R., E. Sacanella, et al. (2011). Nutr Metab Cardiovasc Dis **21**(1): 46-53.
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•Connor, S. C., M. K. Hansen, et al. (2010). Mol Biosyst **6**(5): 909-921.
•Kang, S. M., J. C. Park, et al. (2011). Clin Biochem **44**(4): 293-299.

ACKNOWLEDGMENTS

This research was supported by Spanish national grants: CICYT-AGL 2006-14228-C03-02 (Spanish Ministry of Education and Science), as well as AGL2009-13906-C02-01 and CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD (CSD2007-063) from the MICINN, Spain. R.Vazquez-Fresno would like to thank to the FPI fellowship from the Spanish Ministry of Science and Innovation (MICINN). R L.l thanks MICINN and The European Social Funds for its financial contribution to the R. Ll Ramón y Cajal contract (Ramon y Cajal Programme, MICINN-RYC). F.Alcaro was supported by a *Leonardo da Vinci Programme 2010* , European Commission's Lifelong Learning Programme.

1H-NMR BASED METABOLOMIC ANALYSIS OF THE EFFECT OF MODERATE WINE CONSUMPTION IN SUBJECTS WITH CARDIOVASCULAR RISK FACTORS

Rosa Vázquez-Fresno[1], Rafael Llorach[1] Francesca Alcaro[1], Miguel Ángel Rodríguez[2], Maria Vinaixa[2], Gemma Chiva[3], Ramon Estruch[3], Xavier Correig[2] Cristina Andrés-Lacueva[1]*

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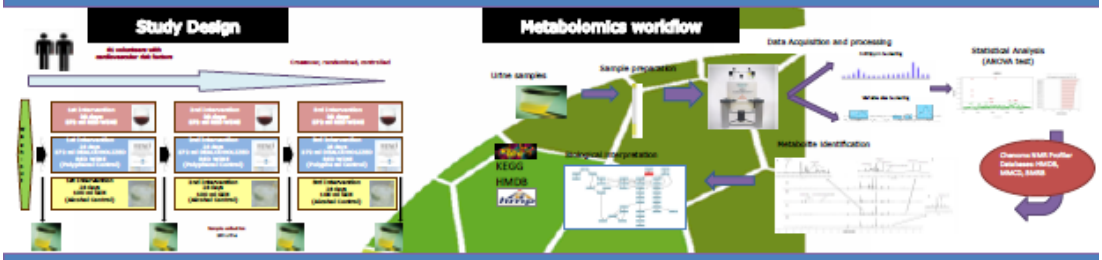
INTRODUCTION

Moderate wine intake is associated with health promoting activities. NMR allows the detection of a wide range of metabolites for metabolomic applications . The aim of the present study is to apply a NMR-metabolomic strategy in order to investigate the human metabolic effect of wine intake in urinary profile identifying the most relevant metabolic changes after moderate intake of red wine (RWA).

MATERIAL AND METHODS



RESULTS

Table 1: Metabolites significant in ANOVA test after treatments. Chemical shifts and p-values of each metabolite (p<0.05) *wine with alcohol, *wine without alcohol, *not modified.

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4-hydroxyphenylacetate	6.87	6.87	↑*	$4.31x10^{-5}$	$1.89 x10^{-5}$	Phenylalanine and Tyrosine metabolism. Gut microbiota
Hippurate	7.84	7.84	↑*	$1.26 x10^{-5}$	$6.56 x10^{-6}$	Phenylalanine metabolism. Gut microbiota
	3.98	3.98		$1.49 x10^{-5}$	$2.59 x10^{-7}$	
	7.64	7.64		$3.05 x10^{-5}$	$2.58 x10^{-6}$	
	7.57	7.57		$8.28 x10^{-5}$	$1.04 x10^{-5}$	
Mannitol	[3.69-3.87]	3.77	↑*	$3.43 x10^{-5}$	$3.68 x10^{-6}$	Wine alcoholized compound
		3.87			$4.69 x10^{-6}$	
Unknown 3	-	0.85	↑**	-	$7.01 x10^{-8}$	Unknown
Unknown 4	-	0.92	↑**	-	$4.31 x10^{-7}$	Unknown
Unknown 5	2.13	2.13	↓	$2.85 x10^{-4}$	$8.78 x 10^{-5}$	Endogenous acetylated metabolite
		5.69			$2.94 x10^{-5}$	

Tartrate was identified associated to wine intake (DRW and RWA). Several peaks in alliphatic region of spectrum (unknown 1, 2, 3 and 4) showed a significant increase after wine intake. In this region, the methyl-2-oxovalerate, a branched-chain aminoacids (BCAA) metabolite, was identified. DRW intake increased the excretion of hippurate and hydroxyphenylacetate, two metabolites from gut microbiota, compared with GIN. Moreover, ethanol was associated to GIN and RW consumption.

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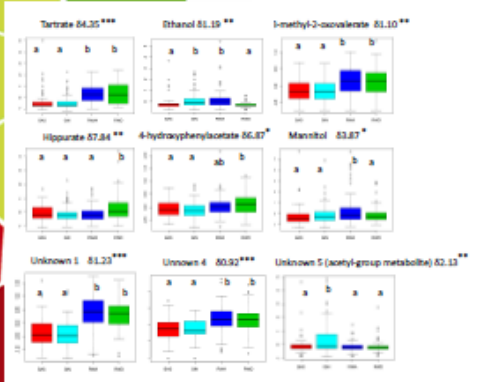

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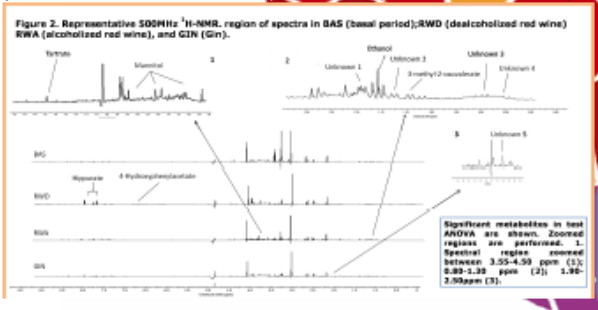

Significant metabolites in test ANOVA are shown. Zoomed regions are performed. 1. Spectral region zoomed between 3.55-4.50 ppm (1); 0.80-1.30 ppm (2); 1.90-2.50ppm (3).

CONCLUSIONS

Presence of tartrate after wine-related diets and ethanol after alcohol-related diets (GIN and RWA) indicates a global compliance. Furthermore, it is proposed tartrate as a biomarker of wine intake. More about BCAAs and wine intake it will be known to understand the relation between catabolic pathway of BCAA and wine intake. It is suspected an alcohol interaction with the excretion of gut microbiota metabolites but more studies are needed to clarify the behaviour of these two gut microbiota metabolites with alcohol intake.

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Nutrimetabolomic strategy using an untargeted NMR approach to find wine exposure biomarkers: From interventional randomized clinical trials to an observational study on free-living subjects

Vázquez-Fresno R[1,2], Llorach R[1,2*], Urpi-Sarda M[1,2], Khymenets O[1,2], Bulló M[3,4], Corella D[4,5], Fitó M[4,6], Martínez-González MA[4,7], Estruch R[4,8], Andres-Lacueva C[1,2*].

[1]Biomarkers and Nutrimetabolomic Lab, Nutrition and Food Science Department, XaRTA, INSA, Torribera Campus, Pharmacy Faculty, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. [2]INGENIO-CONSOLIDER Program, Fun-C-Food CSD2007-063, Ministry of Science and Innovation, Barcelona, Spain. [3]Human Nutrition Unit, Biochemistry and Biotechnology Department and Hospital Universitari de Sant Joan de Reus, Institut d'Investigació Sanitaria Pere Virgili, Universitat Rovira I Virgili, Reus, Spain. [4]CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III, Madrid, Spain. [5]Department of Preventive Medicine and Public Health, University of Valencia, Valencia, Spain. [6]Cardiovascular Epidemiology Unit, Municipal Institute for Medical Research (IMIM), Barcelona, Spain. [7]Department of Preventive Medicine and Public Health, University of Navarra, Pamplona, Spain. [8]Department of Internal Medicine, Hospital Clinic, Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Barcelona, Spain.. *Corresponding author: rafallorach@ub.edu – rafallorach@ub.edu- http:www.nutrimetabolomics.com

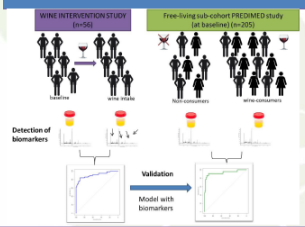
INTRODUCTION

A comprehensive characterization of the food metabolome should enable accurate monitoring of dietary exposure and identification of foods influencing disease risks. In this field, few studies have been conducted to identify biomarkers of wine exposure, particularly using metabolomics platforms. Moreover, the development of robust biomarkers of consumption would improve the classification of subjects with regard to their dietary exposure. In addition, validation of potential biomarkers in free-living subjects remains an important challenge.

OBJECTIVE

To assess wine intake biomarkers using nuclear magnetic resonance (NMR) in the context of an interventional study (WIS), to measure the utility of these biomarkers (single or combined) and validate them in a free-living population (PREDIMED)[1].

MATERIAL AND METHODS

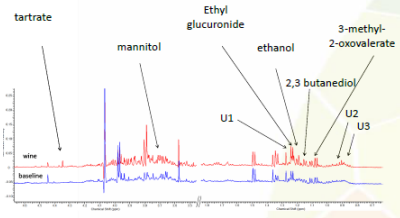


1. Detection of significant metabolites in wine interventional study (WIS study) after wine exposure using ^{1}H-NMR.
2. ROC curve analysis[2] using the significant metabolites: single/combined biomarkers.
3. Validation in a sub-sample randomly selected from the baseline cohort of the PREDIMED study*.
4. Detection of wine consumer last intake, using the Model (tartrate-EtG).

* Population from PREDIMED study stratified using FFQs for non-wine consumers (0ml/day, n=96), intermittent wine consumers (<180 ml/day, n=87) and daily wine consumers (≥180ml/day, n=22). ROC curves were performed with non-wine consumers vs. daily wine consumers.

RESULTS

1. Detection of potential wine exposure biomarkers in interventional study



2. ROC curve analysis: Single/combined biomarkers of WIS study

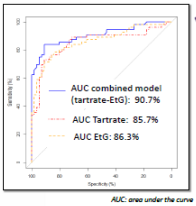
ROC curves for each significant metabolite

Biomarker	AUC (95% CI)	WIS *P-value*	PREDIMED *P-value*
Ethyl glucuronide (EtG)	86.3 (79.4-93.2)	3.40×10^{-11}	6.52×10^{-10}
Tartrate	85.7 (78.2-93.1)	7.43×10^{-11}	1.79×10^{-8}
Ethanol	75.6 (66.4-84.8)	2.97×10^{-6}	0.003
2,3 butanediol	76.7 (67.6-85.7)	1.15×10^{-6}	0.001
3-methyl-2-oxovalerate	70.8 (61.1-80.6)	1.45×10^{-4}	0.001
mannitol	67.4 (57.3-77.5)	0.001	0.006
U1	76.5 (67.6-80.5)	1.37×10^{-6}	1.68×10^{-4}
U2	75.4 (66.3-84.6)	3.42×10^{-6}	0.01
U3	73.8 (64.4- 83.2)	1.42×10^{-5}	0.014

AUC: area under the curve; CI: confidence interval; U: unidentified compound

Step-wise logistic regression to create a combined biomarkers model [3]

Candidate biomarkers after logistic regression to perform the model: **tartrate – Ethylglucuronide (EtG)**

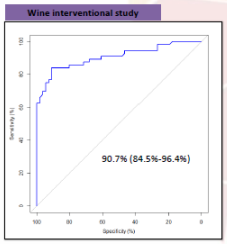


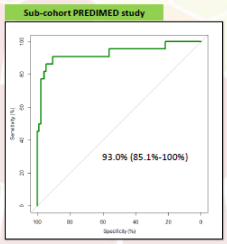
AUC: area under the curve

3. Validation of combined wine exposure biomarkers in the sub-cohort PREDIMED study

The results show that wine and non-wine consumers can be classified with **excellent** [4] accuracy using a combination of 2 biomarkers.
The validation exhibited similar AUC in both studies, with no statistical significance in AUC between the ROC curves from interventional and epidemiological study (p=0.6).

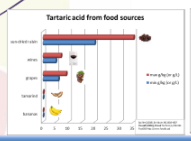
Utility of biomarker based on AUC [4]:
0.9–1.0 = excellent
0.8–0.9 = good
0.7–0.8 = fair
0.6–0.7 = poor
0.5–0.6 = fail

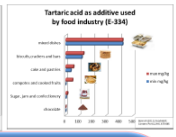




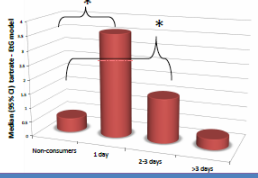
AUC: area under the curve (95% CI)

Validation in free-living population





4. "Last time of drinking wine" question before urine collection



Combined wine biomarkers model (tartrate-EtG) comparing consumers that consume their last glass of wine 1 day, between 2 and 3 days, and more than 3 days before the urine collection, between the non-wine consumers in the PREDIMED sub-cohort. *p<0.01

The applied methodology could allow the detection of wine consumers up to 2-3 days after the last glass of wine consumed

CONCLUSIONS

- **Tartrate** and EtG have been detected and validated as powerful biomarkers of **wine intake**
- This study confirms that a model combining tartrate and EtG is a better biomarker of exposure to wine than a single biomarker.
- NMR-based metabolomics approach has the potential for identifying wine intake biomarkers in interventional studies with good accuracy and reproducibility in free-living subjects.
- Additionally, these combined wine biomarkers could be detected in urine up 2-3 days after the last consumption of wine.
- Interesting for future studies to discriminate between individuals in terms of their dietary patterns in the search for new biomarkers and their association with health

ACKNOWLEDGMENTS

This research was supported by Spanish National Grants: CICYT-AGL2006-14228-C03-02/ALI, AGL2008-13906-C02-01, AGL2010-10084-E, the CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD (CSD2007-063), and CiberOBN from the MICINN, Spain. R.V.-F. O.K. M.U.-S and R. LI. would like to thank the FPI fellowship, the "Juan de la Cierva" and the "Ramon y Cajal" programmes of the Spanish Government and the Fondo Social Europeo. We thank the participants for their collaboration in the study.

REFERENCES:
(1). Estruch, R. et. (2006). Ann Intern Med 145(1): 1-11.
(2). Heinzmann, et al. (2010). Am J Clin Nutr. 92(2): 436-443.
(3). Hwa, H.-L., W.-H. Kuo, et al. (2008). J Eval Clin Pract. 14(2): 275-280.
(4). Xia, J., D. Broadhurst, et al. (2013). Metabolomics 9(2): 280-299