Dietary exposure biomarkers in nutritional intervention and observational studies to discover biomarkers of intake and disease-risk through a HPLC-QToF-MS metabolomics approach

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Seminari de Recerca de la Facultat de Farmàcia
Barcelona, 19 d'abril de 2016

Department of Nutrition & Food Science
Pharmacy School
University of Barcelona

"BIOMARKERS AND NUTRITIONAL & FOOD METABOLOMICS" RESEARCH GROUP

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Location

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FUNDING | **COLLABORATIONS**

Unión Europea
Fondo Europeo de Desarrollo Regional
Una manera de hacer Europa

UNIVERSITAT DE BARCELONA

BIOMARKERS & NUTRIMETABOLOMICS

Alimentos Funcionales
Consolider Fun-C-Food
Ingenio 2010

Ministry of Economy and Competitiveness

European Regional Development Fund (ERDF)

Project AGL 2009-13906-C02-01

Program Ingenio-Consolider FUN-C-FOOD (CDS 2007-063)

Complementary Action AGL2010-10084-E

Agència de Gestió d'Ajuts Universitaris i de Recerca
AGAUR

Agency for Management of University and Research Grants (AGAUR) - Generalitat de Catalunya

Grants for universities and research centres for the recruitment of new research personnel (FI-DGR 2011)

CCiT UB
Centres Científics i Tecnològics
UNIVERSITAT DE BARCELONA
O Jáuregui

UNIVERSITAT ROVIRA I VIRGILI
J Salas-Salvadó
M Bulló

FUNDACIÓ CLÍNIC BARCELONA
R Estruch

Predimed
Prevención con Dieta Mediterránea
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D Corella
E Ros
MA Martínez-González

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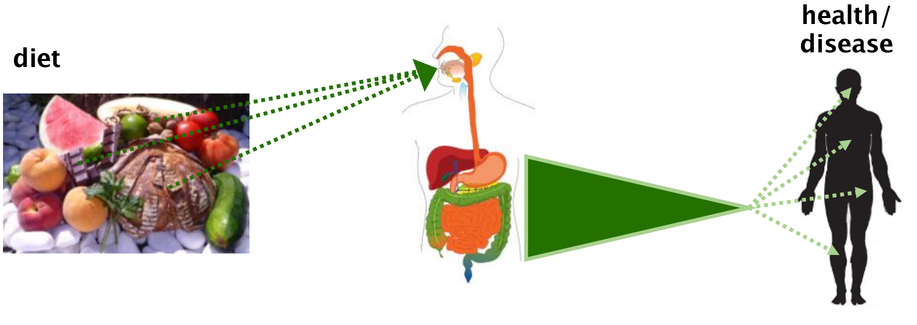
ACCURATE MEASUREMENT OF FOOD INTAKE



(Bingham *Public Health Nutr*, 2002; Livingstone & Black *J Nutr*, 2003; Tucker *Nutr Metab Cardiovasc Dis*, 2007)

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ACCURATE MEASUREMENT OF FOOD INTAKE

↓

FOOD SURVEYS

FFQ
R24h
DIETARY RECORDS

LIMITATIONS ↓

- Misreport of intake
- Limitation of the food list
- Errors derived from the conversion food → compounds by composition tables
- Underestimation of daily and seasonal variability

(Bingham *Public Health Nutr*, 2002; Livingstone & Black *J Nutr*, 2003; Tucker *Nutr Metab Cardiovasc Dis*, 2007)

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ACCURATE MEASUREMENT OF FOOD INTAKE

↓

FOOD SURVEYS

↓

NUTRITIONAL BIOMARKERS

Biochemical, functional or clinical index measured in a biological specimen which reveals the nutritional status of intake or metabolism of a dietary constituent, and the biologic consequences of dietary intake.

Joint Programming Initiative
A Healthy Diet
for a Healthy Life
(JPI HDHL), 2014

(Potischman & Freudenheim *J Nutr*, 2003; van Ommen *et al. Mol Nutr Food Res*, 2009; Raiten *et al. Am J Clin Nutr*, 2011)

NUTRITIONAL BIOMARKERS

Biological validation

- Known relation to the exposure
- Sensitivity and specificity
- Dose-response
- Inter-individual variation
- Time of exposure
- Type of population

Analytical validation

- Type/conditions biological specimen
- Precision and accuracy from defined measure

(Andersen 2013, from Spencer *et al. Br J Nutr* 2008, Jenab *et al. Hum Genet* 2009, Manach *et al. Mol Nutr Food Res* 2009)

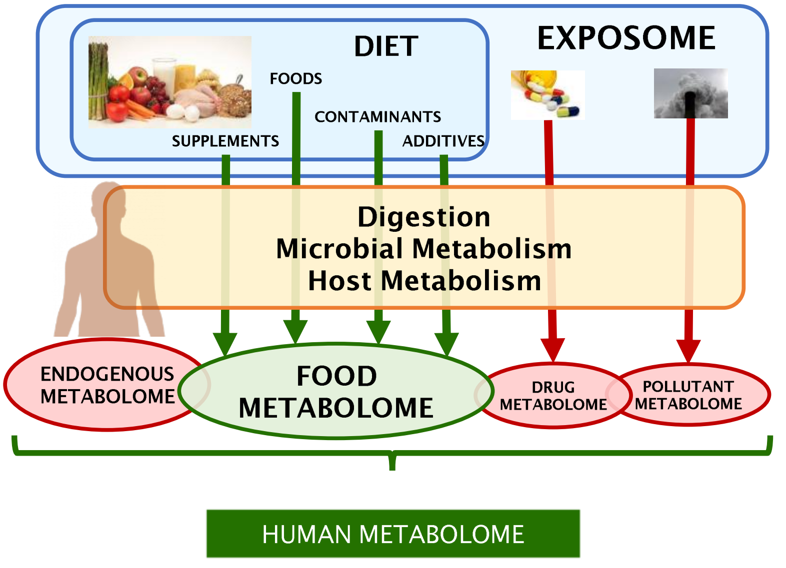
NUTRITIONAL BIOMARKERS

↑

METABOLOMICS

Studies the set of low molecular weight compounds (metabolites) present in a biological system (cell, tissue or fluid) in order to evaluate the biochemical processes that are associated with specific phenotypes.

(Nicholson *et al. Nature*, 2010; Patti *et al. Nat Rev Mol Cell Biol*, 2012)

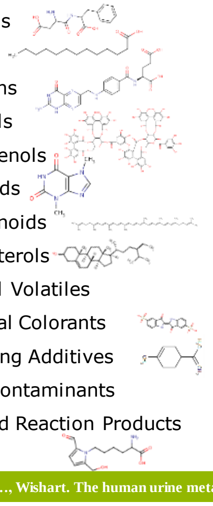


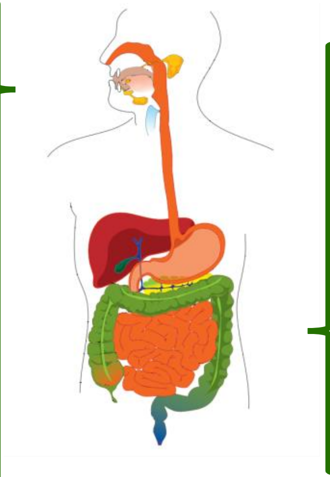
(Bouatra, …, Wishart. The human urine metabolome. *Plos One*, 2013; Wishart *et al. Nucleic Acids Res*, 2013; Scalbert *et al. Am J Clin Nutr*, 2014)

FOOD METABOLOME: **COMPLEXITY & VARIABILITY**

(>25.000 compounds in foods)

Carbohydrates
Proteins
Lipids
Vitamins
Minerals
Polyphenols
Alkaloids
Carotenoids
Phytosterols
Natural Volatiles
Artificial Colorants
Flavoring Additives
Food Contaminants
Maillard Reaction Products
…





Tissue & Microbial Biotransformations

Oxidation,
Reduction,
Hydrolysis,
Dehydrogenation,
Methylation,
Sulfation,
Acetylation,
Glucuronidation,
Amino acid conjugation,
Glutathione conjugation,
…

(Bouatra, …, Wishart. The human urine metabolome. *Plos One*, 2013; Wishart *et al. Nucleic Acids Res*, 2013; Scalbert *et al. Am J Clin Nutr*, 2014)

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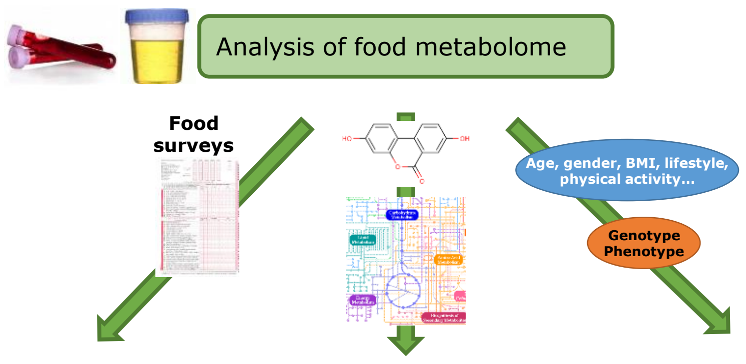
FOOD METABOLOME: APLICATIONS



Dietary assessment
- **Food intake**
- **Nutritional exposure**

- **New metabolites**
- **New potential food bioactives**

- **Segmentation of poor or high absorbers & metabolizers**

(Manach, Glasgow 2013; Scalbert *et al. Am J Clin Nutr*, 2014)

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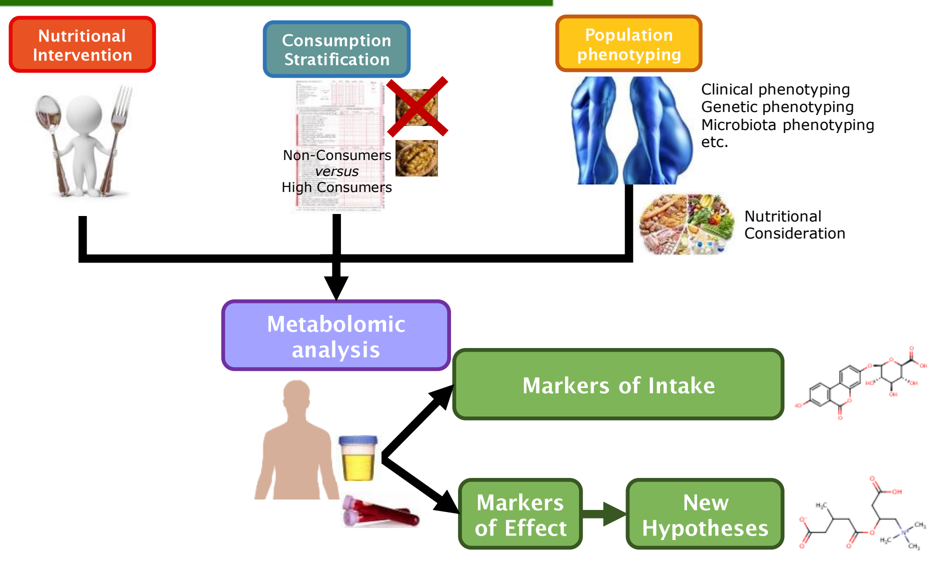
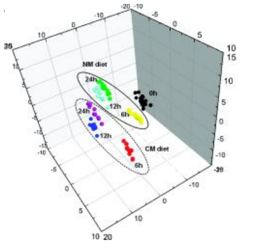
NUTRIMETABOLOMICS: APPROACHES

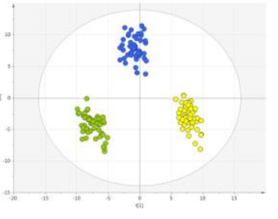


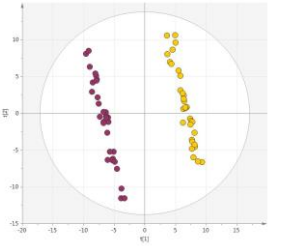
(Llorach R *et al. J Agric Food Chem*, 2012)

NUTRITIONAL STUDY DESIGNS

ACUTE INTERVENTION STUDY	CHRONIC INTERVENTION STUDY	OBSERVATIONAL STUDY







Level of control of the diet

Heterogeneity of the population

Number of discriminant metabolites

(Llorach R *et al. J Agric Food Chem*, 2012; Pujos-Guillot *et al. J Proteome Res*, 2013)

Narrative Review

The food metabolome: a window over dietary exposure[1–3]

Augustin Scalbert, Lorraine Brennan, Claudine Manach, Cristina Andres-Lacueva, Lars O Dragsted, John Draper, Stephen M Rappaport, Justin JJ van der Hooft, and David S Wishart

The American Journal of Clinical Nutrition

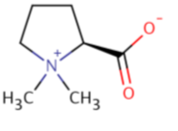
1 FOOD ←→
(>) 1 BIOMARKER
INDIVIDUALLY ASSESSED

TABLE 3
Tentative dietary biomarkers identified through untargeted metabolomic approaches in human dietary intervention studies and cross-sectional studies[1]

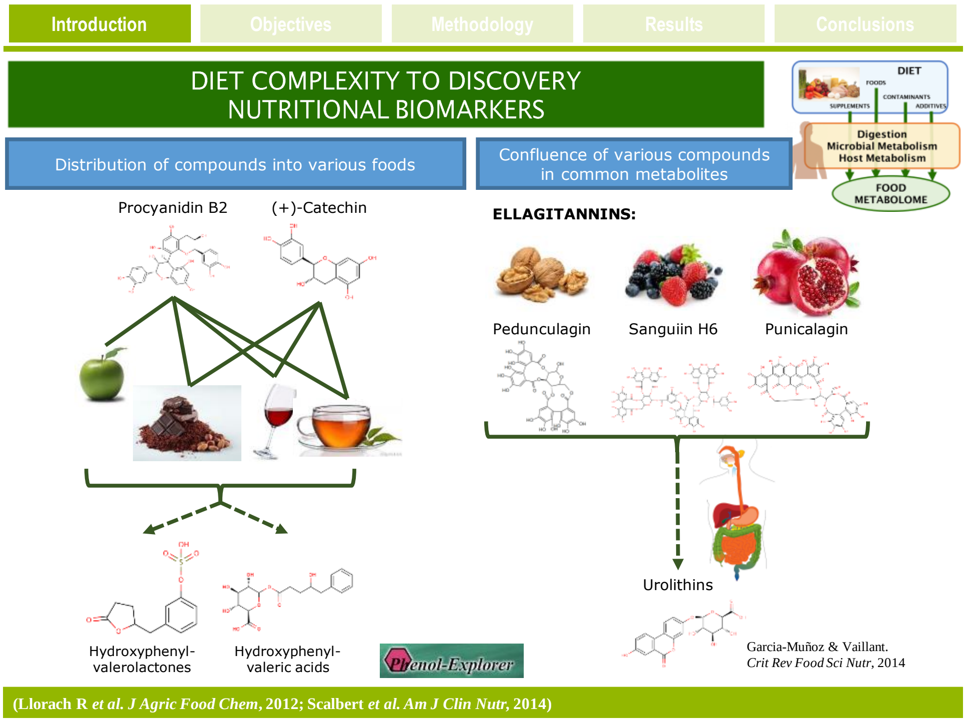
Dietary factor and study type	No. of subjects	Comparison	Dietary assessment tool	Biospecimen	Analytic technique	Biomarker	Reference
Citrus fruit							
CS	499	Consumers/ nonconsumers	24-h dietary record	U (24-h)	NMR	Proline betaine	(80)
CS	12	H/M/L	FFQ	U (fasting)	FIE-FTICR-MS	Proline betaine, 4-hydroxyproline betaine	(44)
Orange juice							
AI	4	Consumers/ control	NA	U (kinetics)	LC-Q-Tof, LTQ-Orbitrap	Proline betaine, limonene-8,9-diol-glucuronide,* nootkatone-13, 14-diol-glucuronide,* hesperetin-3′- glucuronide, hydroxyproline betaine, *N*-methyltyramine-sulfate,* naringenin-7-*O*-glucuronide	(49)
SMTI	12	Consumers/ control	NA	U (24-h)	LC-Q-Tof, LTQ-Orbitrap		
Citrus fruit							
CS	80	H/L	FFQ and 24-h dietary record	U (spot)	LC-Q-Tof, LTQ-Orbitrap		
CS	107	Consumers/ nonconsumers	24-h dietary record	U (24-h)	LC-Q-Tof	Proline betaine, hesperetin-3-glucuronide*	(81)

Orange

Proline betaine



(Scalbert *et al. Am J Clin Nutr*, 2014;99(6):1286-1308)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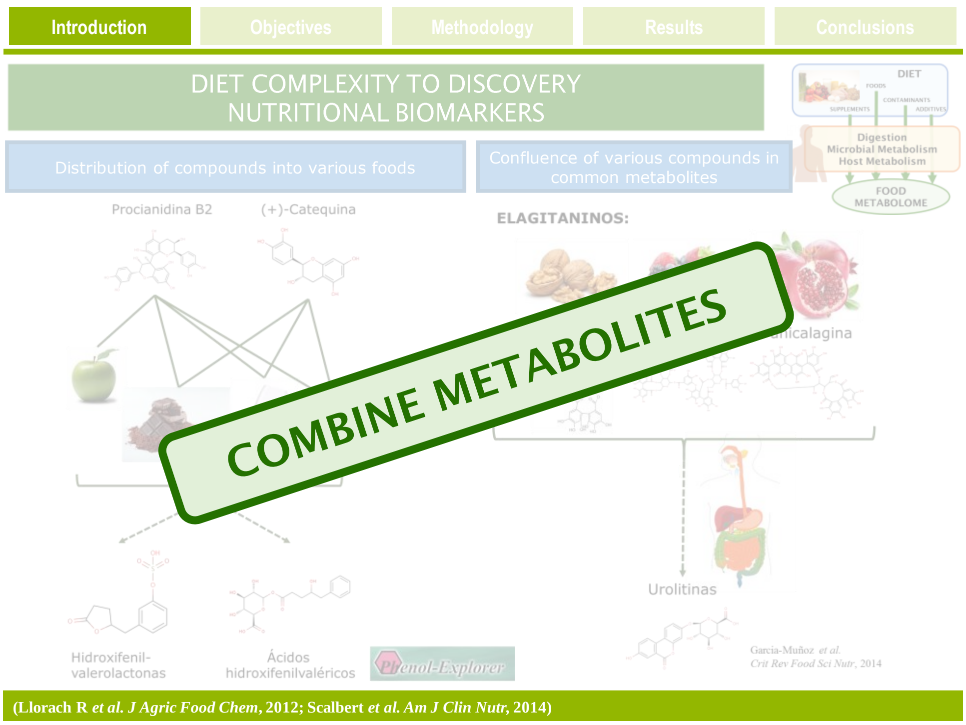
Garcia-Muñoz & Vaillant. Crit Rev Food Sci Nutr, 2014
(Llorach R et al. J Agric Food Chem, 2012; Scalbert et al. Am J Clin Nutr, 2014)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Distribution of compounds into various foods
Confluence of various compounds in common metabolites
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(+)-Catequina
ELAGITANINOS:
COMBINE METABOLITES
Urolitinas
Hidroxifenil-valerolactonas
Ácidos hidroxifenilvaléricos
Phenol-Explorer
Garcia-Muñoz et al. Crit Rev Food Sci Nutr, 2014
(Llorach R et al. J Agric Food Chem, 2012; Scalbert et al. Am J Clin Nutr, 2014)

HYPOTHESIS

Since metabolomics offers a new approach for the determination of biomarkers of dietary exposure, we will observe differences in metabolic fingerprints associated with the consumption of food, which will allow us to predict its intake.

MAIN OBJECTIVE

Identify biomarkers related to the intake of certain foods (**markers of consumption**) and its possible association with health (**markers of effect**) by the application of an HPLC-QToF-MS nontargeted metabolomic strategy in nutritional studies with different designs .

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SPECIFIC OBJECTIVES

Characterize urinary metabolic fingerprint associated with the intake of widely consumed foods: bread, nuts, cocoa.

Replicate characterized biomarkers of exposure in controlled clinical trials in a free-living population.

Develop predictive models for determining usual intake and compare its predictive ability with respect to the ability of the metabolites evaluated individually.

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HPLC-QToF-MS UNTARGETED METABOLOMIC ANALYSIS

Biological Samples ▶ **Data Acquisition** ▶ **Data Analysis** ▶ **Biomarker Identification** ▶ **Biological Interpretation**

Urine

HPLC-QToF-MS

ESI+
ESI–

Data processing

- Marker detection
- Alignment

Multivariate analysis

- Unsupervised techniques (PCA)
- Supervised techniques (PLS-DA)

Ion Characterization

Metabolite Identification

hmp

Metabolome.jp

MassBank

BIOMARKERS & NUTRIMETABOLOMICS

Universitat de Barcelona

Quality Control Analysis of Critical Points:

! Robustness analysis

! Validation of multivariate models

! Multistep procedure

(Llorach *et al. J Pharm Biomed Anal*, 2010; Want *et al.*, *Nature Protocols*, 2010; Dunn *et al. Nature Protocols*, 2011; Llorach R *et al. J Agric Food Chem*, 2012)

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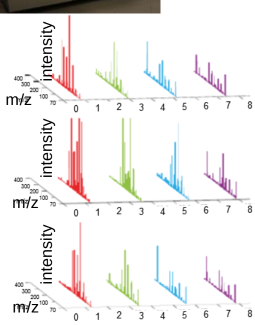
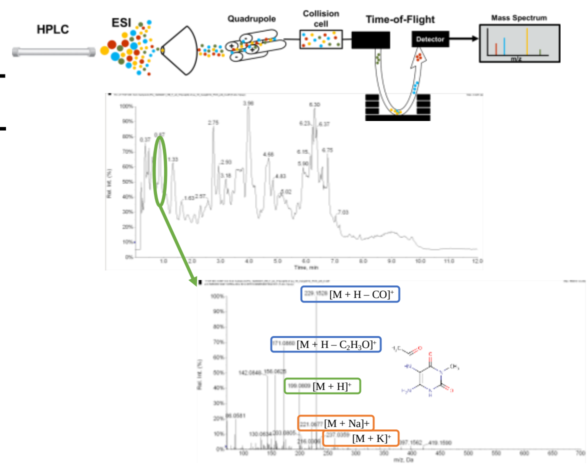
HPLC-QToF-MS

ESI+
ESI–





(Llorach-Asuncion *et al. J Pharm Biomed Anal*, 2010)

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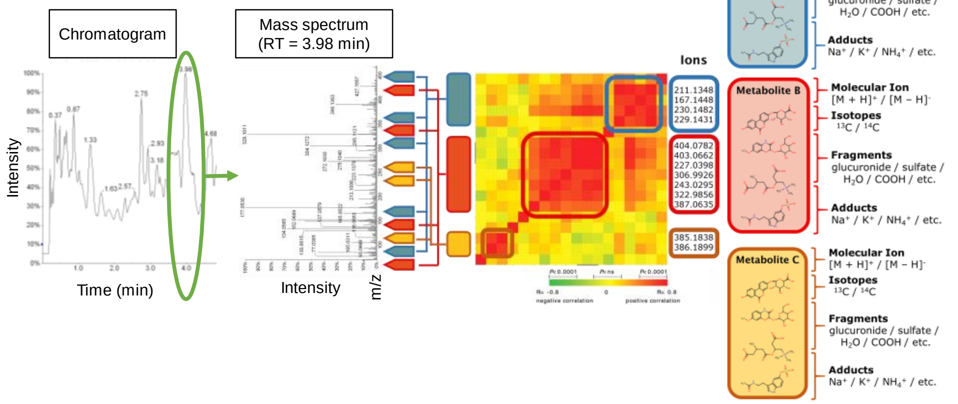
HPLC-QToF-MS UNTARGETED METABOLOMIC ANALYSIS

Biological Samples ▶ **Data Acquisition** ▶ Data Analysis ▶ Biomarker Identification ▶ Biological Interpretation

COELUTION & CORRELATION



(Llorach-Asuncion *et al. J Pharm Biomed Anal*, 2010; Fernández-Albert *et al. Anal Chem*, 2014)

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HPLC-QToF-MS UNTARGETED METABOLOMIC ANALYSIS

Biological Samples ▶ **Data Acquisition** ▶ Data Analysis ▶ Biomarker Identification ▶ Biological Interpretation

Quality control

- QC1: Milli-Q water
- QC2: pool of phenolic compounds
- QC3: pool of endogenous compounds
- QC4: reinjection of opposite samples

Equilibration between plates

Randomization in plates

Distribution of samples & QCs in plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10-QC4	S11	S12
B	S13	S14	S15	S16	S17	S18	S19	S20	QC1	QC2	QC3	S21
C	S22	S23	S24	S25	S26	S27	S28	S29	S30-QC4	S31	S32	S33
D	S34	S35	S36	S37	S38	S39	S40	QC1	QC2	QC3	S41	S42
E	S43	S44	S45	S46	S47	S48	S49	S50-QC4	S51	S52	S53	S54
F	S55	S56	S57	S58	S59	S60	QC1	QC2	QC3	S61	S62	S63
G	S64	S65	S66	S67	S68	S69	S70-QC4	S71	S72	S73	S74	S75
H	S76	S77	S78	S79	S80	QC1	QC2	QC3				

(Llorach *et al. J Proteome Res*, 2009; Llorach *et al. J Proteome Res*, 2010; Llorach-Asuncion *et al. J Pharm Biomed Anal*, 2010)

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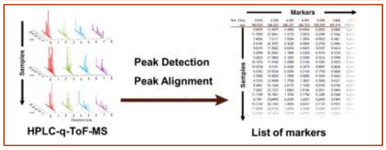
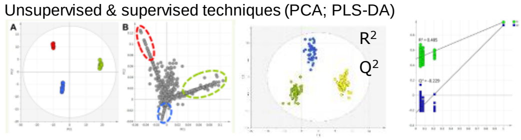
Data processing

Marker detection & Alignment



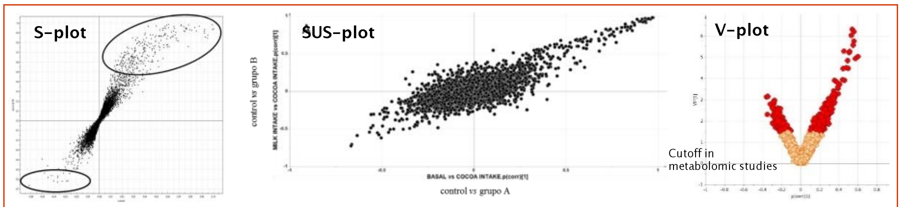
Multivariate analysis

Unsupervised & supervised techniques (PCA; PLS-DA)



(cross-validation / permutation test)

Visualization of results from metabolome



(Llorach-Asuncion *et al. J Pharm Biomed Anal*, 2010)

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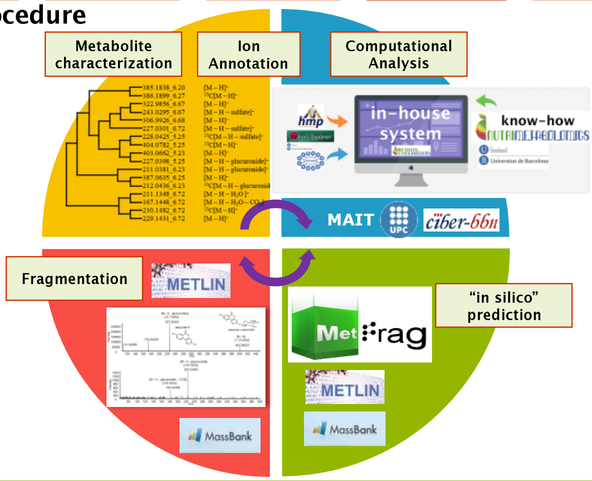
HPLC-QTOF-MS UNTARGETED METABOLOMIC ANALYSIS

Biological Samples ▶ Data Acquisition ▶ Data Analysis ▶ **Biomarker Identification** ▶ Biological Interpretation

Multi-step procedure



(Garcia-Aloy M *et al. J Proteome Res*, 2014; Garcia-Aloy M *et al. Metabolomics*, 2015; Semba *et al.* Andres-Lacueva *JAMA Intern Med*, 2014; Urpi-Sarda *et al. Mol Nutr Food Res*, 2014; Llorach *et al. Mol Nutr Food Res*, 2013; Tulipani *et al. Anal Chem*, 2013; Tulipani *et al. J Proteome Res*, 2011; Llorach *et al. J Pharm Biomed Anal*, 2010; Fernandez-Albert *et al. Anal Chem*, 2014; Fernandez-Albert *et al. Bioinformatics*, 2014; Llorach *et al. J Proteome Res*, 2010; Llorach *et al. J Proteome Res*, 2009)

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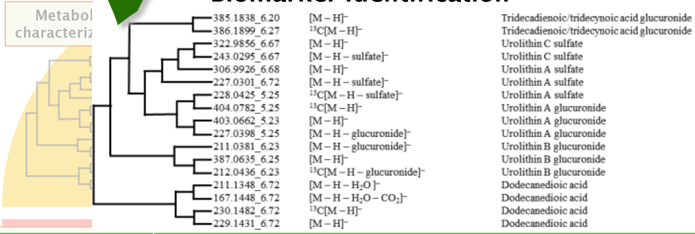
Multi-step procedure

Biomarker Identification



Level	Confidence of identity	Level of evidence
1	Identified compounds	Comparison of ≥2 orthogonal properties with an authentic compound analyzed under identical experimental conditions.
2	Putatively annotated compounds	Based upon property similarity with public/commercial databases, without chemical reference standard.
3	Putatively characterized compound classes	Based upon properties of known compounds of a chemical class.
4	Unknown compounds	Unidentified compounds.

(Sumner *et al*. Proposed minimum reporting standards for chemical analysis (CAWG-MSI). *Metabolomics*, 2007)

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HPLC-QToF-MS UNTARGETED METABOLOMIC ANALYSISQ

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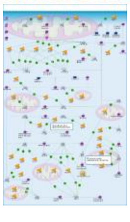
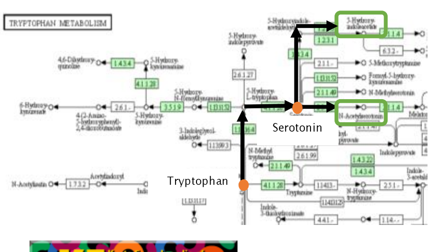
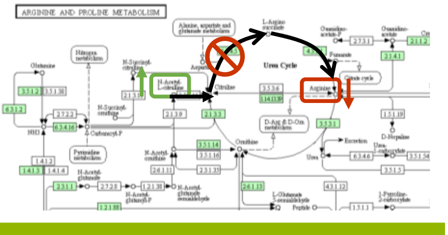
MetaCore

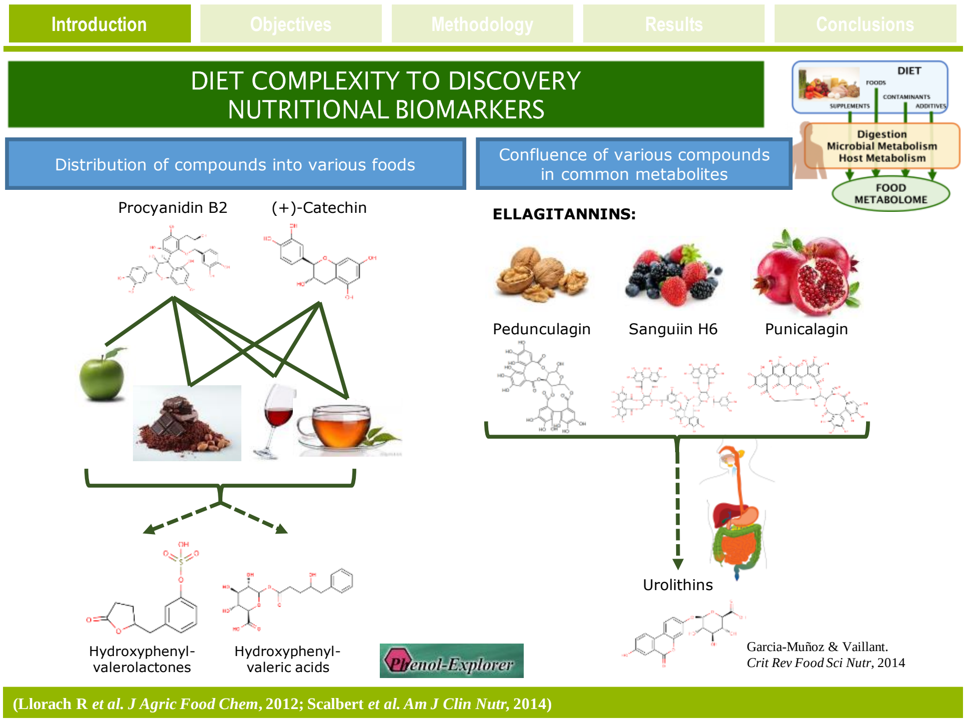






PubMed

(Llorach-Asuncion *et al*. *J Pharm Biomed Anal*, 2010)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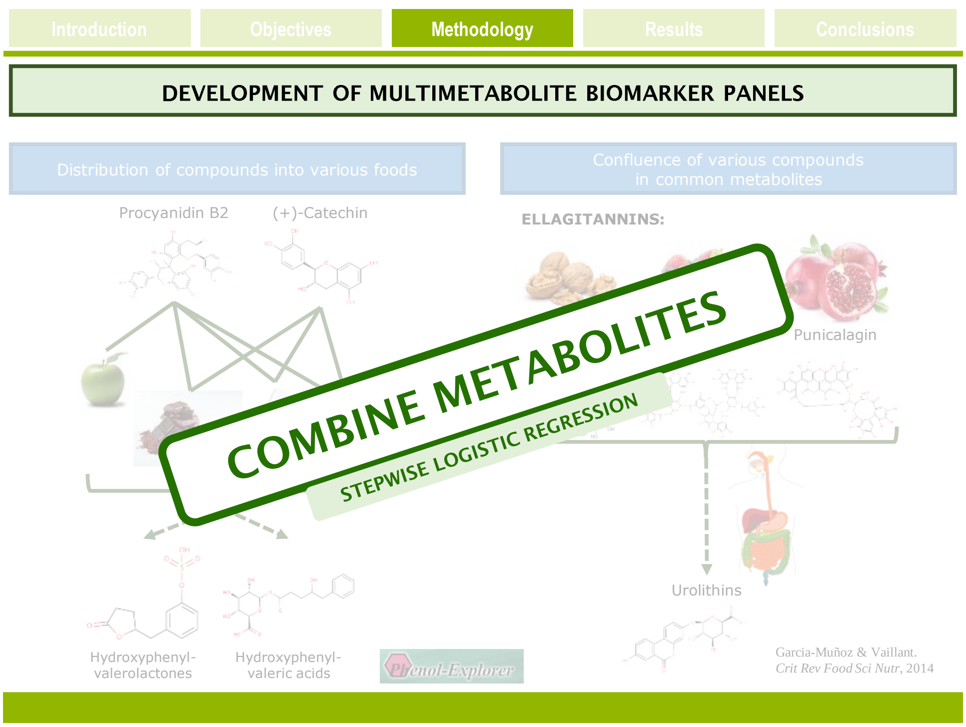
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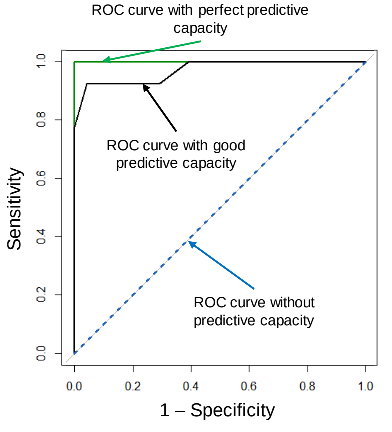
Garcia-Muñoz & Vaillant. Crit Rev Food Sci Nutr, 2014

DEVELOPMENT OF MULTIMETABOLITE BIOMARKER PANELS

PREDICTIVE CAPACITY OF BIOMARKERS: ROC CURVES

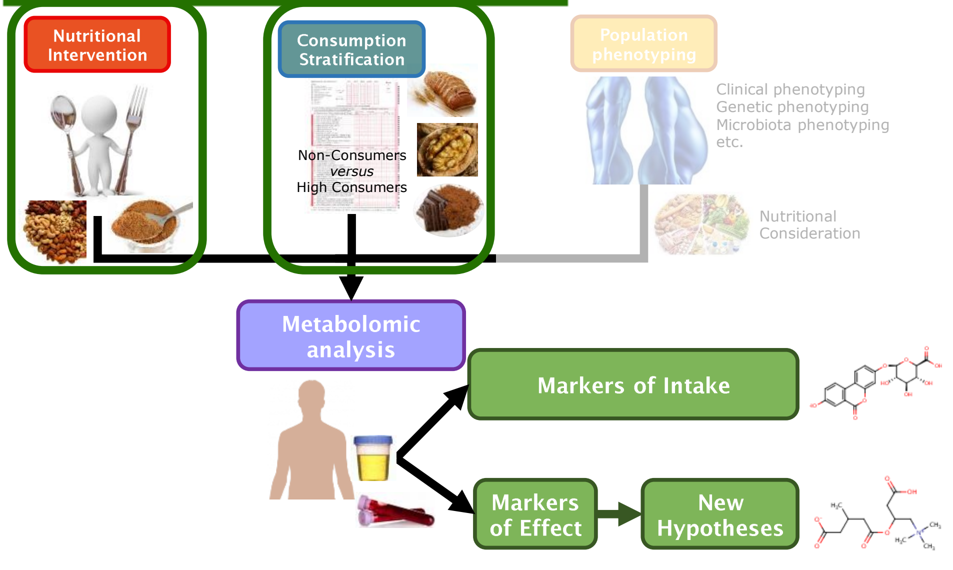


AUCs

90%–100% = EXCELLENT
80%–90% = GOOD
70%–80% = FAIR
60%–70% = POOR
50%–60% = FAIL

(Xia *et al*. *Metabolomics*, 2013)

NUTRIMETABOLOMICS: APPROACHES



(Llorach R *et al*. *J Agric Food Chem*, 2012)

BREAD

STRATIFICATION OF POPULATION:
CROSS-SECTIONAL ANALYSIS

PREDIMED COHORT (N=7447)

Subsample (n=275)

BREAD consumption stratification

Excluded (n=120):
- not meet the stratification criteria

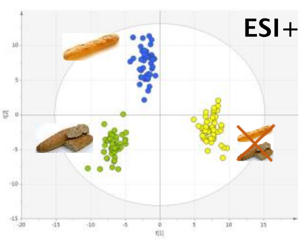
Non-consumers (n=56)	White-bread consumers (n=48)	Whole-grain bread consumers (n=51)

(Garcia-Aloy M *et al. Metabolomics*, 2015)

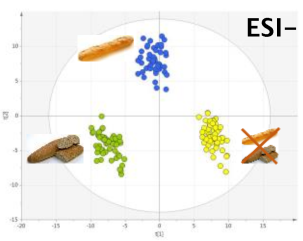
Introduction | Objectives | Methodology | **Results** | Conclusions

BREAD

Multivariate analysis: OSC-PLS-DA

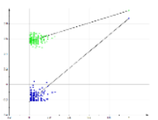


$R^2Y(cum)=0.934$; $Q^2(cum)=0.733$



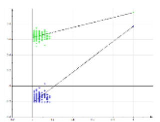
$R^2Y(cum)=0.939$; $Q^2(cum)=0.700$

Permutation test



$R^2=0.566$; $Q^2=-0.226$

Permutation test



$R^2=0.627$; $Q^2=-0.219$

(Garcia-Aloy M *et al. Metabolomics*, 2015)

BREAD

RT (min)	DETECTED MASS (m/z)	ASSIGNATION	IDENTIFICATION	vs	vs	vs
0.88	188.0049	$[M-H]^-$	2-Aminophenol sulphate	↑	↑	–
1.48	328.1036	$[M+H]^+$	HPAA glucuronide	↑	↑	–
	326.0851	$[M-H]^-$		–	↑	–
2.07	168.0609	$[M+H]^+$	HHPAA	–	↑	↑
3.40	372.0925	$[M+H]^+$	HMBOA glucuronide	↑	–	–
	370.0772	$[M-H]^-$		↑	↑	–
3.68	326.0922	$[M-H]^-$	HBOA glycoside	–	↑	↑
3.72	152.0671	$[M+H]^+$	HPPA	–	↑	–
4.78	196.0596	$[M+H]^+$	HMBOA	↑	↑	–
	194.0410	$[M-H]^-$		↑	↑	–
2.85	357.0791	$[M-H]^-$	DHPPA glucuronide	↑	↑	↑
3.12	233.0118	$[M-H]^-$	3,5-Dihydroxyphenylethanol sulphate	–	↑	–
5.75	289.0412	$[M-H]^-$	DHPPTA sulphate	–	↑	↑
3.67	313.0558	$[M-H]^-$	Hydroxybenzoic acid glucuronide	↑	↑	–
4.72	275.0219	$[M-H]^-$	Dihydroferulic acid sulphate	–	↑	↑
6.32	299.1278	$[M+H-GlcA]^+$	Enterolactone glucuronide	–	↑	↑
	473.1447	$[M-H]^-$		–	↑	↑
2.73	255.1345	$[M+H]^+$	Pyrraline	–	↑	–
	253.1172	$[M-H]^-$		–	↑	↑
3.25	338.0871	$[M+H]^+$	3-Indolecarboxylic acid glucuronide	–	↑	↑
	336.0697	$[M-H]^-$		–	↑	↑
4.65	377.1475	$[M+H]^+$	Riboflavine	↑	↑	↑
0.63	218.1140	$[M+H]^+$	N-α-Acetylcitrulline	–	↓	–
4.20	338.0882	$[M+H]^+$	2,8-Dihydroxyquinoline glucuronide	–	↑	↑
	160.0382	$[M-H-GlcA]^-$		–	↑	↑

(Garcia-Aloy M *et al. Metabolomics*, 2015)

BREAD

AUCs	vs	vs	vs
HPAA glucuronide	73.5% (63.8%-83.2%)	64.0% (53.3%-74.6%)	
HHPAA		67.8% (57.7%-77.9%)	69.7% (59.3%-80.1%)
HMBOA glucuronide	68.2% (57.8%-78.7%)		
HPPA		69.9% (59.8%-79.9%)	
HMBOA	68.4% (57.8%-79.0%)	66.3% (55.6%-77.0%)	
Enterolactone glucuronide		69.6% (59.7%-79.5%)	73.0% (63.0%-83.1%)
Pyrraline		65.8% (55.6%-76.0%)	
3-Indolecarboxylic acid glucuronide		67.2% (57.0%-77.4%)	65.5% (54.6%-76.5%)
Riboflavin	64.2% (53.4%-75.0%)	73.2% (63.7%-82.8%)	62.9% (51.5%-74.4%)
2-Aminophenol sulphate	66.4% (56.0%-76.7%)	68.9% (59.0%-78.9%)	
HPAA glucuronide		62.0% (51.7%-72.4%)	
HMBOA glucuronide	66.1% (55.9%-76.3%)	61.0% (50.5%-71.5%)	
HBOA glycoside		73.0% (63.6%-82.4%)	63.4% (52.6%-74.2%)
HMBOA	69.2% (59.2%-79.3%)	66.8% (56.8%-76.7%)	
DHPPA glucuronide	64.9% (54.4%-75.4%)	78.4% (69.8%-87.1%)	65.1% (54.5%-75.8%)
3,5-Dihidroxifeniletanol sulphate		67.0% (56.8%-77.2%)	
DHPPTA sulphate		76.7% (67.6%-85.7%)	76.1% (67.1%-85.1%)
Hydroxybenzoic acid glucuronide	67.4% (57.2%-77.6%)	61.3% (50.8%-71.7%)	
Dihydroferulic acid sulphate		74.3% (65.0%-83.6%)	74.6% (65.0%-84.2%)
Enterolactone glucuronide		65.6% (55.4%-75.7%)	62.8% (52.2%-73.4%)
Pyrraline		64.8% (54.7%-75.0%)	62.5% (51.6%-73.3%)
3-Indolecarboxylic acid glucuronide		66.8% (56.9%-76.7%)	63.0% (52.3%-73.7%)

90%–100% = excellent; 80%–90% = good; 70%–80% = fair; 60%–70% = poor; y 50%–60% = fail

(Garcia-Aloy M *et al. Metabolomics*, 2015)

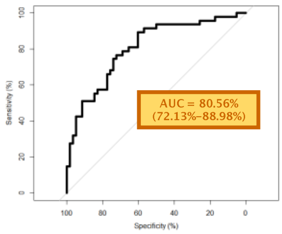
Introduction | Objectives | Methodology | **Results** | Conclusions

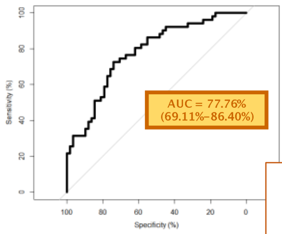
BREAD

Predimed

MULTIMETABOLITE COMBINED MODELS

	Coef.	Err. Est.	*p*
HPAA glucuronide	1,565	0,542	0,004
HHPAA			
HMBOA glucuronide			
HPPA			
HMBOA	1,639	0,556	0,003
Enterolactone glucuronide			
Pyrraline			
3-Indolecarboxylic acid glucuronide			
Riboflavin	0,842	0,340	0,013
2-Aminophenol sulphate	1,359	0,401	0,001
HPAA glucuronide			
HMBOA glucuronide			
HBOA glycoside			
HMBOA	1,816	0,445	<0,001
DHPPA glucuronide			
3,5-Dihidroxifeniletanol sulphate			
DHPPTA sulphate			
Hydroxybenzoic acid glucuronide			
Dihydroferulic acid sulphate			
Enterolactone glucuronide			
Pyrraline			
3-Indolecarboxylic acid glucuronide			





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(Garcia-Aloy M *et al. Metabolomics*, 2015)

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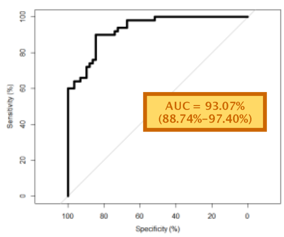
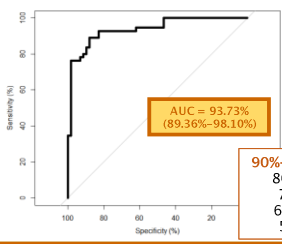
Predimed

MULTIMETABOLITE COMBINED MODELS

	Coef.	Err. Est.	*p*
HPAA glucuronide			
HHPAA	1,750	0,787	0,026
HMBOA glucuronide			
HPPA	1,361	0,579	0,019
HMBOA	1,362	0,674	0,043
Enterolactone glucuronide	1,642	0,559	0,003
Pyrraline	1,436	0,636	0,024
3-Indolecarboxylic acid glucuronide	1,617	0,556	0,004
Riboflavin	1,921	0,524	<0,001
2-Aminophenol sulphate			
HPAA glucuronide			
HMBOA glucuronide			
HBOA glycoside			
HMBOA	1,856	0,659	0,005
DHPPA glucuronide	1,289	0,439	0,003
3,5-Dihidroxifeniletanol sulphate			
DHPPTA sulphate	1,685	0,481	<0,001
Hydroxybenzoic acid glucuronide			
Dihydroferulic acid sulphate	0,911	0,438	0,037
Enterolactone glucuronide	1,157	0,581	0,047
Pyrraline	1,397	0,502	0,005
3-Indolecarboxylic acid glucuronide	0,980	0,449	0,029





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(Garcia-Aloy M *et al. Metabolomics*, 2015)

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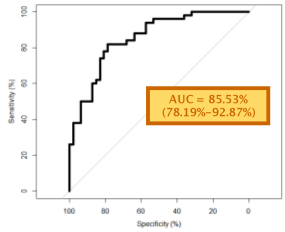
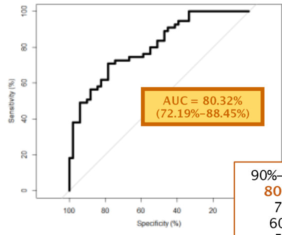
MULTIMETABOLITE COMBINED MODELS

vs

	Coef.	Err. Est.	*p*
HPAA glucuronide			
HHPAA	2,923	0,924	0,002
HMBOA glucuronide			
HPPA			
HMBOA			
Enterolactone glucuronide	2,009	0,500	<0,001
Pyrraline	1,248	0,536	0,020
3-Indolecarboxylic acid glucuronide			
Riboflavin			



2-Aminophenol sulphate			
HPAA glucuronide			
HMBOA glucuronide			
HBOA glycoside			
HMBOA			
DHPPA glucuronide			
3,5-Dihidroxifeniletanol sulphate			
DHPPTA sulphate	1,159	0,327	<0,001
Hydroxybenzoic acid glucuronide			
Dihydroferulic acid sulphate	1,077	0,355	0,002
Enterolactone glucuronide			
Pyrraline			
3-Indolecarboxylic acid glucuronide			



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(Garcia-Aloy M *et al. Metabolomics*, 2015)

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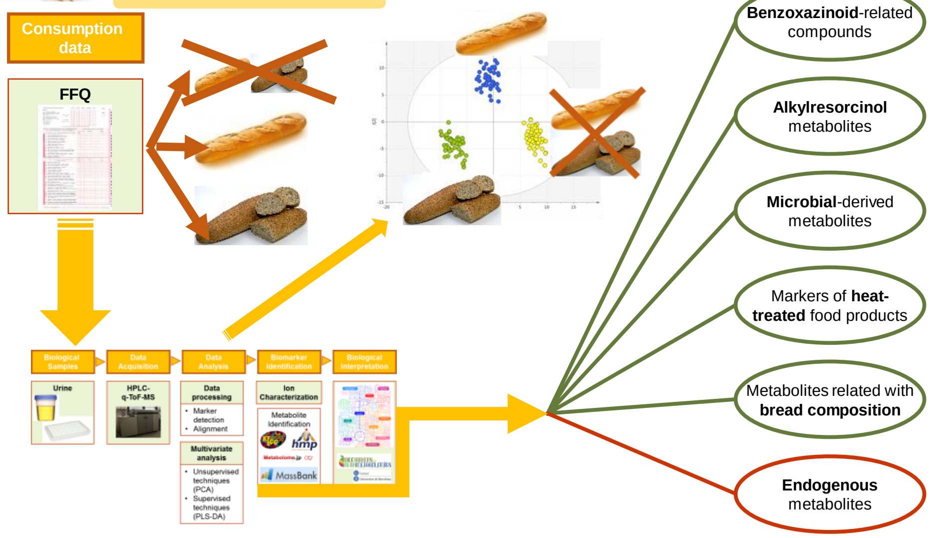
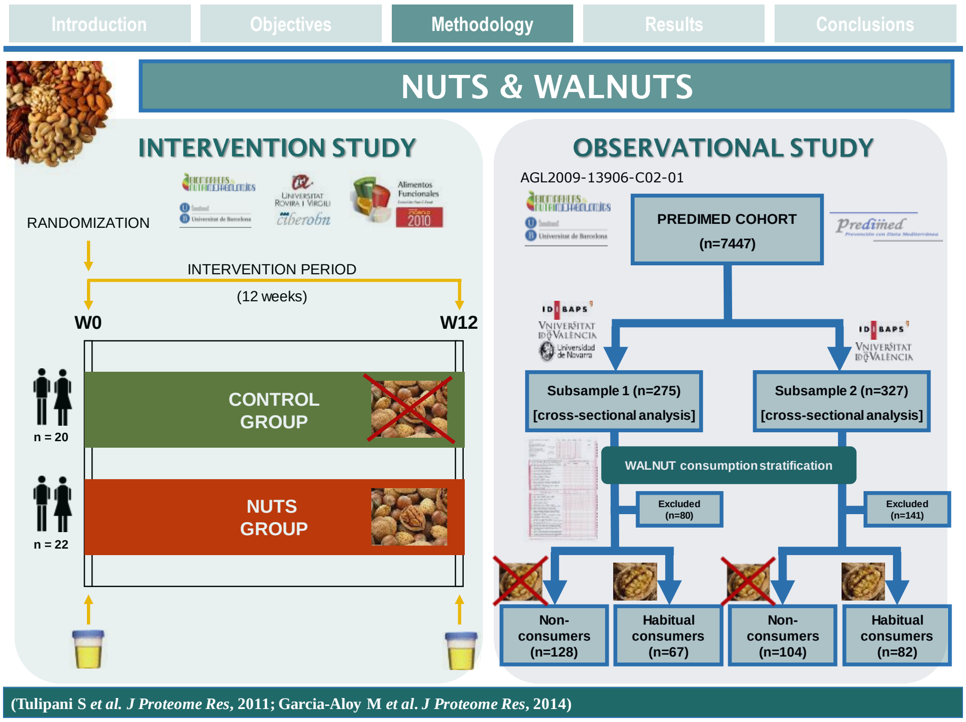
ABSTRACT



(Garcia-Aloy M *et al. Metabolomics*, 2015)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W12
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NUTS GROUP
n = 22
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[cross-sectional analysis]
Subsample 2 (n=327)
[cross-sectional analysis]
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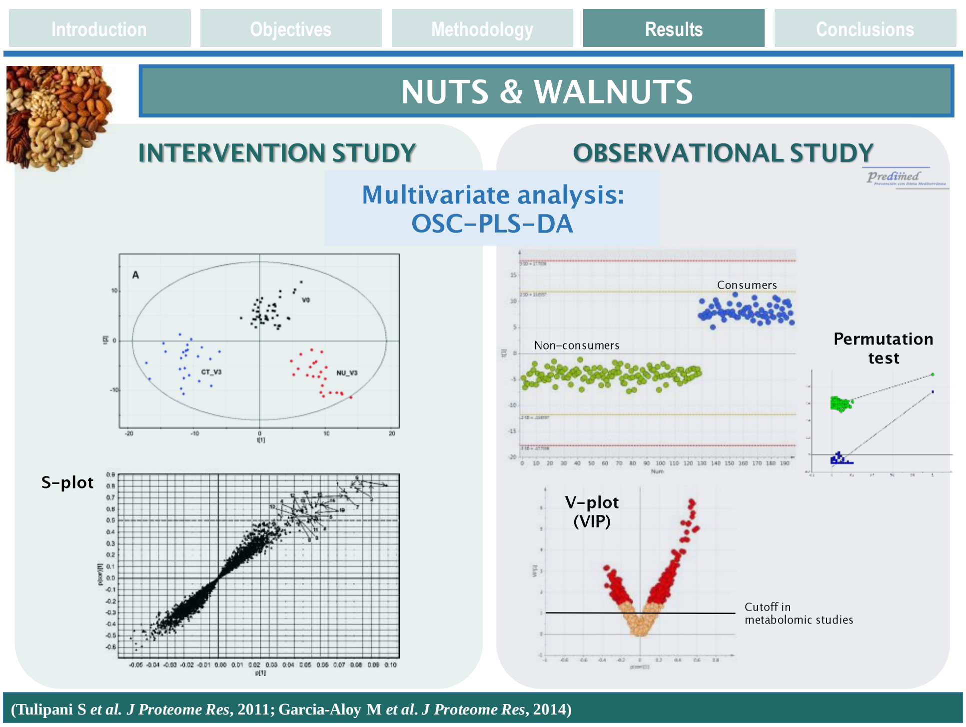
Excluded
(n=80)
Excluded
(n=141)
Non-consumers
(n=128)
Habitual consumers
(n=67)
Non-consumers
(n=104)
Habitual consumers
(n=82)
(Tulipani S et al. J Proteome Res, 2011; Garcia-Aloy M et al. J Proteome Res, 2014)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Cutoff in
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(Tulipani S et al. J Proteome Res, 2011; Garcia-Aloy M et al. J Proteome Res, 2014)

NUTS & WALNUTS

INTERVENTION STUDY

RT (min)	DETECTED MASS (m/z)	ASSIGNATION	IDENTIFICATION
4.80	257.0085	[M – H]$^-$	10-Hydroxy-decene-4,6-diynoic acid sulfate
	177.0545	[M – H – sulfate]$^-$	
6.25	385.1844	[M – H]$^-$	Tridecadienoic/tridecynoic acid glucuronide
	386.1880	^{13}C[M – H]$^-$	
	387.2011	[M + H]$^+$	
	211.1688	[M + H – GlcA]$^+$	
	193.1576	[M + H – GlcA – H_2O]$^+$	
6.72	229.1403	[M – H]$^-$	Dodecanedioic acid
	230.1441	^{13}C[M – H]$^-$	
	211.1314	[M – H – H_2O]$^-$	
	167.1433	[M – H – H_2O – CO_2]$^-$	
2.55	204.9827	[M – H]$^-$	Pyrogallol sulfate
	233.0118	[HSO_3 – H]$^-$	
5.10	325.0890	[M – H]$^-$	*p*-Coumaryl alcohol glucuronide
	326.0987	^{13}C[M – H]$^-$	
5.28	403.0627	[M – H]$^-$	Urolithin A glucuronide
	404.0654	^{13}C[M – H]$^-$	
	227.0357	[M – H – GlcA]$^-$	
	405.0817	[M + H]$^+$	
	229.0495	[M + H – GlcA]$^+$	
5.30	483.0195	[M – H]$^-$	Urolithin A sulfoglucuronide
6.55	229.0197	[M – H]$^-$	*p*-Coumaryl alcohol sulfate
	230.0221	^{13}C[M – H]$^-$	
	149.0615	[M – H – sulfate]$^-$	
	150.0646	^{13}C[M – H – sulfate]$^-$	
6.75	306.9885	[M – H]$^-$	Urolithin A sulfate
4.30	297.0560	[M – H]$^-$	*N*-Acetylserotonin sulfate
4.62	190.0505	[M – H]$^-$	Hydroxyindoleacetic acid
	146.0614	[M – H – CO_2]$^-$	
	192.0648	[M + H]$^+$	
	174.0539	[M + H – H_2O]$^+$	
	146.0592	[M + H – CH_2O_2]$^+$	

OBSERVATIONAL STUDY

Predimed

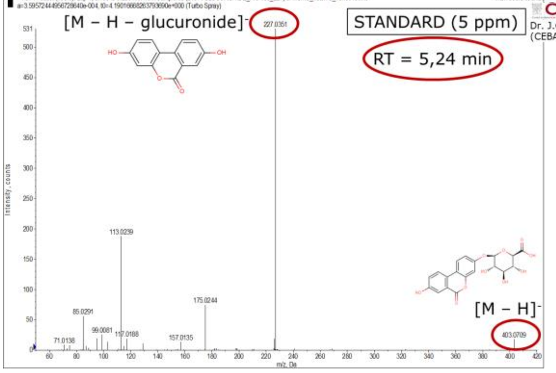
RT (min)	MASA DETECTADA (m/z)	ASIGNACIÓN	IDENTIFICACIÓN
4.62	257.0149	[M – H]$^-$	10-Hydroxy-decene-4,6-diynoic acid sulfate
6.20	385.1838	[M – H]$^-$	Tridecadienoic/tridecynoic acid glucuronide
	386.1899	^{13}C[M – H]$^-$	
	387.1995	[M + H]$^+$	
	388.2035	^{13}C[M + H]$^+$	
	211.1668	[M + H – GlcA]$^+$	
5.22	419.0618	[M – H]$^-$	Urolithin C glucuronide
5.25	403.0662	[M – H]$^-$	Urolithin A glucuronide
	404.0677	^{13}C[M – H]$^-$	
	227.0398	[M – H – GlcA]$^-$	
	228.0425	^{13}C[M – H – GlcA]$^-$	
	405.0830	[M + H]$^+$	
	422.1100	[M + NH_4]$^+$	
	229.0490	[M + H – GlcA]$^+$	
5.35	483.0227	[M – H]$^-$	Urolithin A sulfoglucuronide
6.25	387.0770	[M – H]$^-$	Urolithin B glucuronide
	211.0381	[M – H – GlcA]$^-$	
	212.0436	^{13}C[M – H – GlcA]$^-$	
	389.0864	[M + H]$^+$	
	213.0534	[M + H – GlcA]$^+$	
6.34	473.1491	[M – H]$^-$	Enterolactone glucuronide
	474.1525	^{13}C[M – H]$^-$	
	297.1127	[M – H – GlcA]$^-$	
	492.1842	[M + NH_4]$^+$	
6.67	243.0295	[M – H – sulfate]$^-$	Urolithin C sulfate
6.72	306.9915	[M – H]$^-$	Urolithin A sulfate
	227.0348	[M – H – sulfate]$^-$	
3.23	336.0751	[M – H]$^-$	3-Indolecarboxylic acid glucuronide
	338.0854	[M + H]$^+$	
3.83	270.0081	[M – H]$^-$	Hydroxyindoleacetic acid sulfate
4.20	297.0561	[M – H]$^-$	*N*-Acetylserotonin sulfate

(Tulipani S *et al. J Proteome Res*, 2011; Garcia-Aloy M *et al. J Proteome Res*, 2014)

NUTS & WALNUTS

INTERVENTION STUDY

RT	m/z	ASSIGNATION	IDENTIFICATION
5.28	403.0627	[M – H]$^-$	Urolithin A glucuronide
	404.0654	^{13}C[M – H]$^-$	
	227.0357	[M – H – GlcA]$^-$	
	405.0817	[M + H]$^+$	
	229.0495	[M + H – GlcA]$^+$	



OBSERVATIONAL STUDY

Predimed

RT	m/z	ASSIGNATION	IDENTIFICATION
5.25	403.0662	[M – H]$^-$	Urolithin A glucuronide
	404.0677	^{13}C[M – H]$^-$	
	227.0398	[M – H – GlcA]$^-$	
	228.0425	^{13}C[M – H – GlcA]$^-$	
	405.0830	[M + H]$^+$	
	[illegible]00	[M + NH_4]$^+$	
	[illegible]90	[M + H – GlcA]$^+$	

CSIC
Dr. J.C. Espín (CEBAS-CSIC)

(Tulipani S *et al. J Proteome Res*, 2011; Garcia-Aloy M *et al. J Proteome Res*, 2014)

Introduction | Objectives | Methodology | **Results** | Conclusions

NUTS & WALNUTS

OBSERVATIONAL STUDY

Predimed Prevención con Dieta Mediterránea

AUCs	Subsample 1	Subsample 2
10-Hydroxy-decene-4,6-diynoic acid sulfate	74.4% (66.4%-82.5%)	72.6% (65.0%-80.3%)
Tridecadienoic/tridecynoic acid glucuronide	**85.1% (79.8%-90.4%)**	77.2% (70.4%-84.0%)
Urolithin C glucuronide	75.4% (67.7%-83.0%)	71.4% (63.8%-79.0%)
Urolithin A glucuronide	**82.0% (75.7%-88.4%)**	**83.2% (77.3%-89.1%)**
Urolithin A sulfoglucuronide	70.4% (62.0%-78.7%)	79.0% (72.1%-85.9%)
Urolithin B glucuronide	59.1% (50.6%-67.7%)	67.7% (59.6%-75.8%)
Enterolactone glucuronide	62.3% (54.1%-70.5%)	66.3% (58.4%-74.2%)
Urolithin C sulfate	69.7% (61.5%-78.0%)	73.3% (65.7%-80.9%)
Urolithin A sulfate	78.7% (71.3%-86.1%)	79.2% (72.5%-85.9%)
3-Indolecarboxylic acid glucuronide	73.7% (66.2%-81.3%)	60.2% (52.0%-68.4%)
Hydroxyindoleacetic acid sulfate	61.0% (52.5%-69.6%)	68.8% (61.2%-76.3%)
N-Acetylserotonin sulfate	64.5% (56.2%-72.8%)	64.5% (56.5%-72.5%)

90%–100% = excellent; **80%–90% = good**; 70%–80% = fair; 60%–70% = poor; y 50%–60% = fail

(Garcia-Aloy M *et al. J Proteome Res*, 2014)

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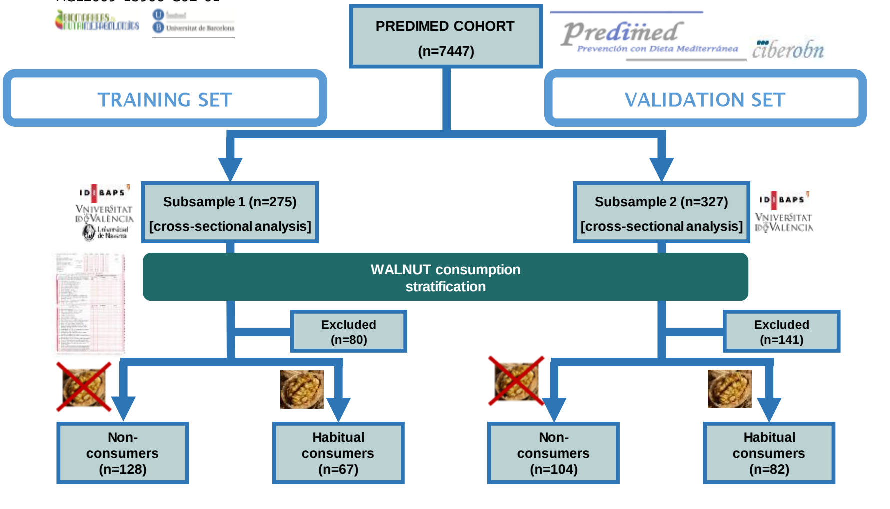
NUTS & WALNUTS

OBSERVATIONAL STUDY



(Garcia-Aloy M *et al. J Proteome Res*, 2014)

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NUTS & WALNUTS

OBSERVATIONAL STUDY

Predimed

STEPWISE LOGISTIC REGRESSION

TRAINING SET

	Coefficient	Standard Error	*p*
10-Hydroxy-decene-4,6-diynoic acid sulfate	1,041	0,431	0,016
Tridecadienoic/tridecynoic acid glucuronide	2,212	0,491	<0,001
Urolithin C glucuronide			
Urolithin A glucuronide	0,778	0,305	0,011
Urolithin A sulfoglucuronide			
Urolithin B glucuronide			
Enterolactone glucuronide			
Urolithin C sulfate			
Urolithin A sulfate	0,812	0,395	0,040
3-Indolecarboxylic acid glucuronide	0,945	0,306	0,002
Hydroxyindoleacetic acid sulfate			
N-Acetylserotonin sulfate			

(Garcia-Aloy M *et al. J Proteome Res*, 2014)

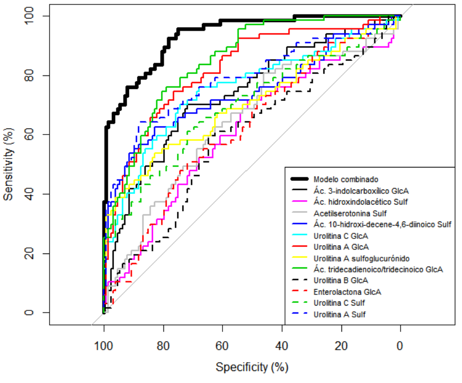
Introduction | Objectives | Methodology | Results | Conclusions

NUTS & WALNUTS

MULTIMETABOLITE COMBINED MODELS

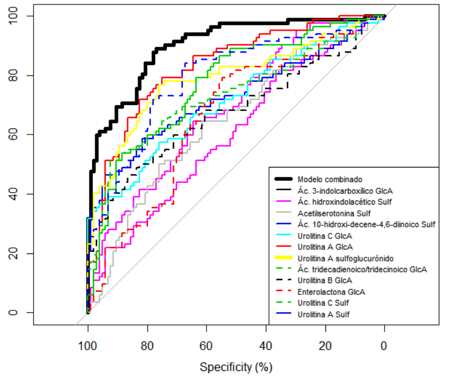
Predimed

Training set



AUC = 93.45% (90.08%–96.81%)

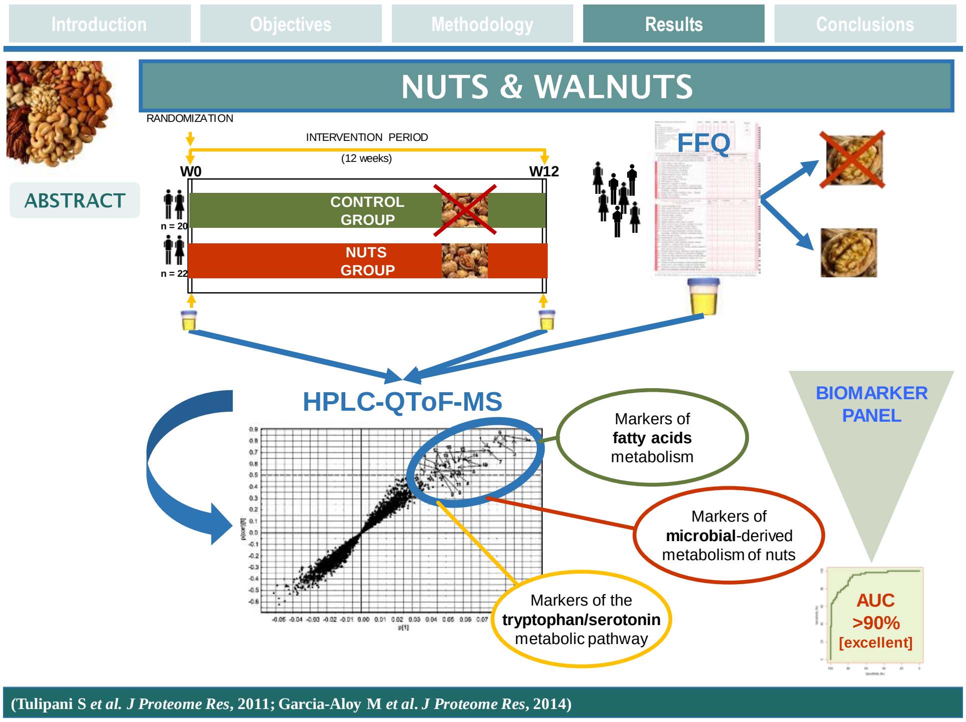
Validation set



AUC = 90.22% (85.87%–94.57%)

90%–100% = excellent; 80%–90% = good; 70%–80% = fair; 60%–70% = poor; y 50%–60% = fail

(Garcia-Aloy M *et al. J Proteome Res*, 2014)



Introduction
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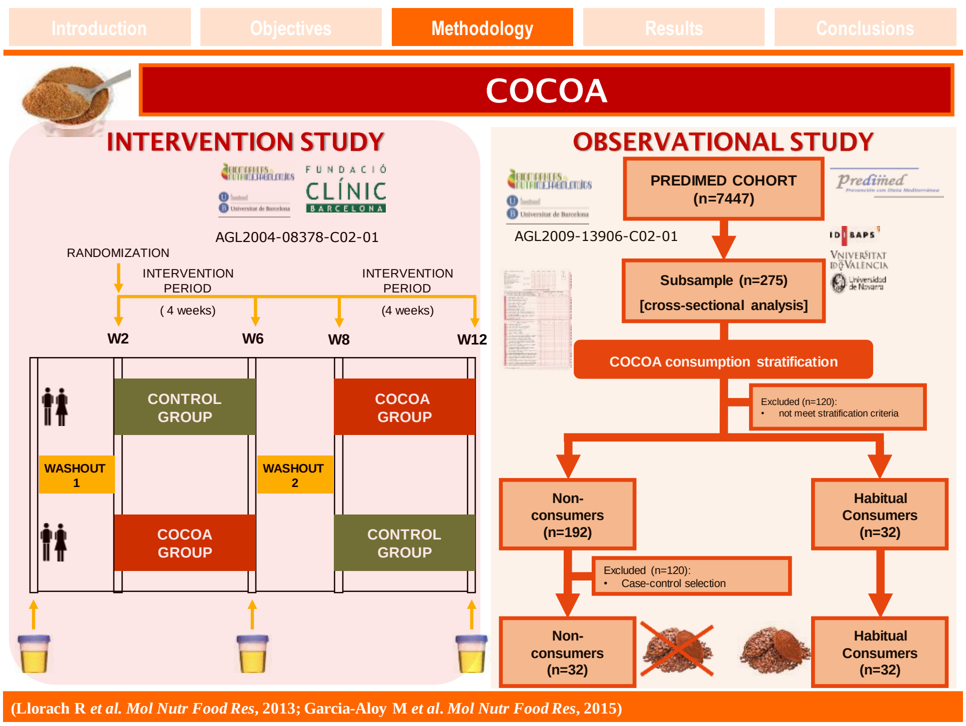
NUTS & WALNUTS
ABSTRACT
RANDOMIZATION
INTERVENTION PERIOD
(12 weeks)
W0
W12
CONTROL GROUP
n = 20
NUTS GROUP
n = 22
FFQ
HPLC-QToF-MS
Markers of fatty acids metabolism
Markers of microbial-derived metabolism of nuts
Markers of the tryptophan/serotonin metabolic pathway
BIOMARKER PANEL
AUC >90% [excellent]
(Tulipani S et al. J Proteome Res, 2011; Garcia-Aloy M et al. J Proteome Res, 2014)



Introduction
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COCOA
INTERVENTION STUDY
FUNDACIÓ CLÍNIC BARCELONA
AGL2004-08378-C02-01
RANDOMIZATION
INTERVENTION PERIOD
(4 weeks)
INTERVENTION PERIOD
(4 weeks)
W2
W6
W8
W12
CONTROL GROUP
COCOA GROUP
WASHOUT 1
WASHOUT 2
COCOA GROUP
CONTROL GROUP
OBSERVATIONAL STUDY
PREDIMED COHORT (n=7447)
AGL2009-13906-C02-01
Subsample (n=275) [cross-sectional analysis]
COCOA consumption stratification
Excluded (n=120):
• not meet stratification criteria
Non-consumers (n=192)
Habitual Consumers (n=32)
Excluded (n=120):
• Case-control selection
Non-consumers (n=32)
Habitual Consumers (n=32)
(Llorach R et al. Mol Nutr Food Res, 2013; Garcia-Aloy M et al. Mol Nutr Food Res, 2015)

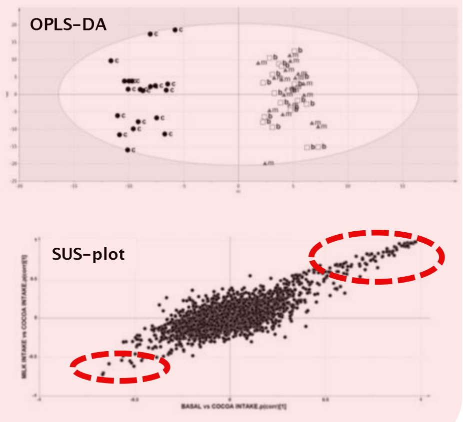
COCOA

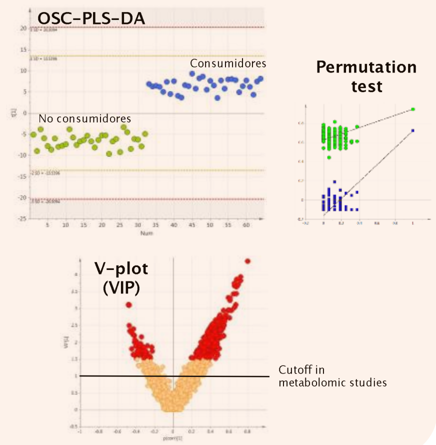
INTERVENTION STUDY

OBSERVATIONAL STUDY

Predimed

Multivariate analysis





(Llorach R *et al. Mol Nutr Food Res*, 2013; Garcia-Aloy M *et al. Mol Nutr Food Res*, 2015)

COCOA

INTERVENTION STUDY

RT (min)	DETECTED MASS (m/z)	ASSIGNATION	IDENTIFICATION
0.87	199.0832/197.0691	$[M + H]^+/[M - H]^-$	AMMU
1.08	183.0493	$[M + H]^+$	7-Methyluric acid
1.22	199.0844/197.0701	$[M + H]^+/[M - H]^-$	AMMU *isomer*
1.68	183.0516	$[M + H]^+$	3-Methyluric acid
2.05	167.0575	$[M + H]^+$	7-Metilxanthine
2.47	167.0570/165.0429	$[M + H]^+/[M - H]^-$	3-Metilxanthine
2.80	197.0688/195.0526	$[M + H]^+/[M - H]^-$	3,7-Dimethyluric acid
3.37	181.0719	$[M + H]^+$	Theobromine
3.67	343.0684	$[M - H]^-$	Vanillic acid glucuronide
3.85	226.0711/224.0592	$[M + H]^+/[M - H]^-$	Vanilloglycine
4.95	465.1071	$[M - H]^-$	(Epi)catechin glucuronide
5.15	423.0280	$[M - H]^-$	Vanillic acid sulfoglucuronide
5.58	545.0614	$[M - H]^-$	(Epi)catechin sulfoglucurónido
6.02	383.0486	$[M - H]^-$	Methyl(epi)catechin sulfate
6.32	383.0459	$[M - H]^-$	Methyl(epi)catechin sulfate
4.05	401.1112	$[M - H]^-$	HDHPVA glucuronide
4.23	401.1090	$[M - H]^-$	HDHPVA glucuronide
4.35	223.0972	$[M + H]^+$	MHPV
4.38	415.1270	$[M - H]^-$	HHMPVA sulfate
4.87	385.1143/383.0995	$[M + H]^+/[M - H]^-$	DHPV glucuronide
5.03	397.1165	$[M - H]^-$	MHPV glucuronide
5.10	463.0584	$[M - H]^-$	DHPV sulfoglucuronide
5.12	289.0365	$[M + H]^+$	DHPV sulfate
5.13	367.1025	$[M - H]^-$	HPV glucuronide
5.17	305.0335	$[M - H]^-$	HDHPVA sulfate
5.45	397.1127	$[M - H]^-$	MHPV glucuronide
5.53	289.0379	$[M - H]^-$	DHPV sulfate
5.72	287.0221	$[M - H]^-$	DHPV sulfate
6.12	289.0374/287.0188	$[M + H]^+/[M - H]^-$	DHPV sulfate
6.45	271.0309	$[M - H]^-$	HPVA sulfate
6.50	301.0416	$[M - H]^-$	MHPV sulfate
7.12	289.0406	$[M - H]^-$	DHPVA sulfate
7.17	273.0453	$[M - H]^-$	HPVA sulfate
0.62	140.0328	$[M + H]^+$	Hydroxynicotinic acid
2.83	169.0941	$[M + H]^+$	Cyclo(propylalanyl)
3.08	151.1227	$[M + H]^+$	3,5-Diethyl-2-methylpyrazine
4.67	278.0698	$[M - H]^-$	*N*-[4′-Hydroxycinnamoyl]-L-aspartic acid
5.07	308.0782	$[M - H]^-$	*N*-[4′-Hydroxy-3′-methoxy-cinnamoyl]-aspartic
1.22	262.0359	$[M + H]^+$	Tyrosine sulfate
1.97	232.1547	$[M + H]^+$	Butyrylcarnitine
2.28	290.1600	$[M + H]^+$	Methylglutarylcarnitine

OBSERVATIONAL STUDY

Predimed

RT (min)	DETECTED MASS (m/z)	ASSIGNATION	IDENTIFICATION
0.63	151.0259	$[M - H]^-$	Xanthine
0.67	199.0816	$[M + H]^+$	AMMU
0.87	199.0785	$[M + H]^+$	AMMU *isomer*
1.13	183.0509/181.0325	$[M + H]^+/[M - H]^-$	3-Methyluric acid
1.37	167.0568	$[M + H]^+$	7-Metilxanthine
1.62	167.0597/165.0416	$[M + H]^+/[M - H]^-$	3-Metilxanthine
1.85	197.0678/195.0500	$[M + H]^+/[M - H]^-$	3,7-Dimethyluric acid
2.75	181.0707	$[M + H]^+$	Theobromine
4.38	230.9982	$[M - H]^-$	Vanillin sulfate
4.48	465.1013	$[M + H]^+$	(Epi)catechin glucuronide
4.85	167.0365	$[M - H]^-$	Vanillic acid
5.37	369.0252	$[M - H]^-$	(Epi)catechin sulfate
3.73	401.1072	$[M - H]^-$	HDHPVA glucuronide
3.90	415.1237	$[M - H]^-$	HHMPVA glucuronide
3.90	223.0925	$[M + H]^+$	MHPV
4.15	287.0229	$[M - H - GlcA]^-$	DHPV sulfoglucuronide
4.20	383.1005	$[M - H]^-$	DHPV glucuronide
4.30	225.0736	$[M - H]^-$	HDHPVA
4.37	305.0291	$[M - H]^-$	HDHPVA sulfate
4.42	385.1105/383.0972	$[M + H]^+/[M - H]^-$	DHPV glucuronide
4.60	319.0495	$[M - H]^-$	HHMPVA sulfate
4.60	397.1101	$[M - H]^-$	MHPV glucuronide
4.70	367.0990	$[M - H]^-$	HPV glucuronide
5.22	289.0343	$[M + H]^+$	DHPV sulfate
5.62	191.0678	$[M - H - sulfato]^-$	HPV sulfate
6.54	289.0391	$[M - H]^-$	DHPVA sulfate
6.64	273.0454	$[M - H]^-$	HPVA sulfate
1.88	170.0449	$[M + H]^+$	Furoylglycine
4.72	261.0872	$[M - H]^-$	Cyclo(aspartyl-phenylalanyl)
4.73	281.1135/279.0943	$[M + H]^+/[M - H]^-$	Aspartyl-Phenylalanine
1.87	290.1590	$[M + H]^+$	Methylglutarylcarnitine

(Llorach R *et al. Mol Nutr Food Res*, 2013; Garcia-Aloy M *et al. Mol Nutr Food Res*, 2015)

COCOA

ACUTE INTERVENTION	LONG-TERM INTERVENTION	FREE-LIVING POPULATION

research articles Journal of proteome research

An LC-MS-Based Metabolomics Approach for Exploring Urinary Metabolome Modifications after Cocoa Consumption

Rafael Llorach, Mireia Urpi-Sarda, Olga Jauregui, Maria Monagas, and Cristina Andres-Lacueva

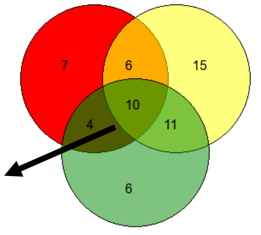
Research Article

Metabolomic fingerprint in patients at high risk of cardiovascular disease by cocoa intervention

Rafael Llorach, Mireia Urpi-Sarda, Sara Tulipani, Mar Garcia-Aloy, Maria Monagas and Cristina Andres-Lacueva

Untargeted metabolomics approach to obtain a metabolic footprint of regular dietary consumption by designing models of combined urinary biomarkers: Cocoa product intake in free-living subjects from the PREDIMED study

Mar Garcia-Aloy, Rafael Llorach, Mireia Urpi-Sarda, Olga Jáuregui, Dolores Corella, Miguel A. Martínez-González, Jordi Salas-Salvadó, Montserrat Fitó, Emilio Ros, Ramon Estruch, Cristina Andres-Lacueva. *[submitted]*



Theobromine Metabolism

- AMMU
- AMMU *isomer*
- 3-Methyluric acid
- 7-Metilxanthine
- 3-Metilxanthine
- 3,7-Dimethyluric acid
- Theobromine

Polyphenol metabolites produced by microbiota

- Methoxyhydroxyphenylvalerolactone
- 5-(3',4'-Dihydroxiphenyl)-valerolactone glucuronide
- 5-(3',4'- Dihydroxiphenyl)-valerolactone sulfate

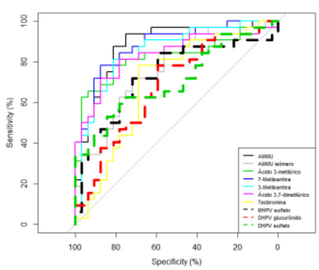
(Garcia-Aloy M *et al. Mol Nutr Food Res*, 2015)

COCOA

OBSERVATIONAL STUDY

	AUCs
AMMU	88.18% (79.47%-96.90%)
AMMU isomer	76.66% (65.05%-88.27%)
3-Methyluric acid	82.23% (71.23%-93.22%)
7-Metilxanthine	88.28% (80.09%-96.48%)
3-Metilxanthine	85.16% (75.59%-94.72%)
3,7-Dimethyluric acid	83.59% (73.28%-93.91%)
Theobromine	69.82% (56.45%-83.20%)
Methoxyhydroxyphenylvalerolactone	73.44% (60.63%-86.24%)
5-(3',4'-Dihydroxiphenyl)-valerolactone GlcA	68.26% (55.02%-81.51%)
5-(3',4'- Dihydroxiphenyl)-valerolactone sulfate	71.09% (58.27%-83.92%)

ROC



90%-100% = excellent; **80%-90% = good;** 70%-80% = fair; 60%-70% = poor; y 50%-60% = fail

(Garcia-Aloy M *et al. Mol Nutr Food Res*, 2015)

Introduction | Objectives | Methodology | **Results** | Conclusions

COCOA

MULTIMETABOLITE COMBINED MODELS

Predimed

STEPWISE LOGISTIC REGRESSION

TRAINING SET

	Coefficient	Standard Error	*p*
AMMU			
AMMU isomer			
3-Methyluric acid			
7-Metilxanthine	5,563	1,899	0,003
3-Metilxanthine			
3,7-Dimethyluric acid			
Theobromine			
Methoxyhydroxyphenylvalerolactone			
5-(3',4'-Dihydroxiphenyl)-valerolactone GlcA	4,081	1,559	0,009
5-(3',4'- Dihydroxiphenyl)-valerolactone sulfate			

(Garcia-Aloy M *et al. Mol Nutr Food Res*, 2015)

Introduction | Objectives | Methodology | **Results** | Conclusions

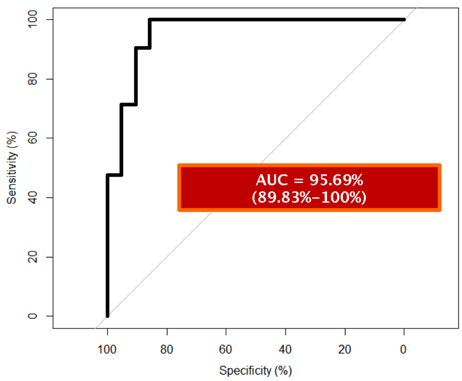
COCOA

MULTIMETABOLITE COMBINED MODELS

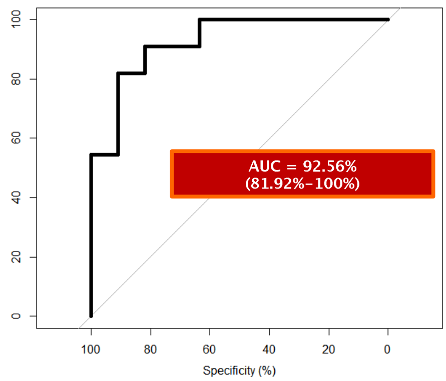
Predimed

AUCs

TRAINING SET



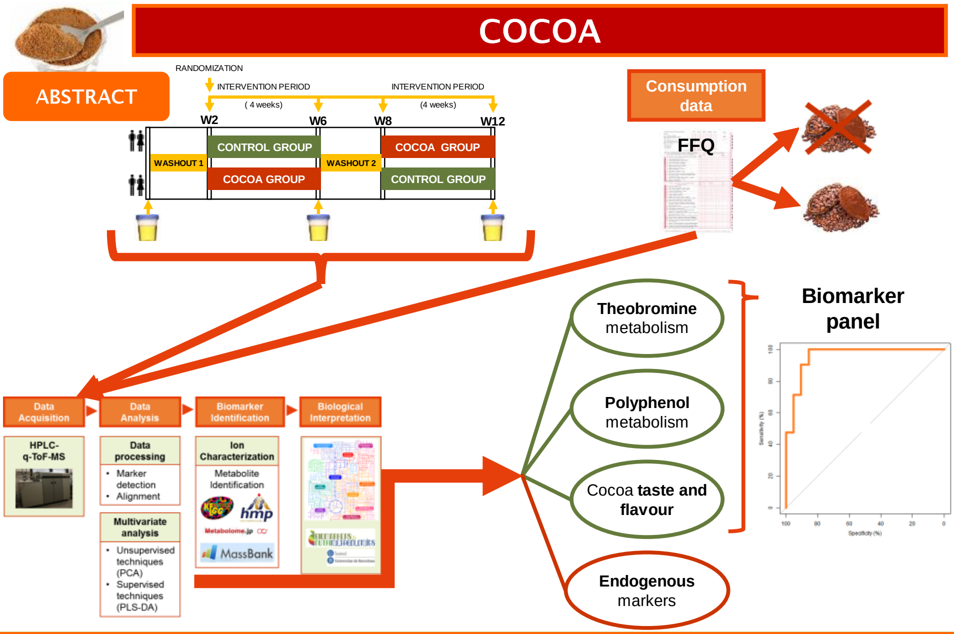
VALIDATION SET



90%-100% = excellent; 80%-90% = good; 70%-80% = fair; 60%-70% = poor; y 50%-60% = fail

(Garcia-Aloy M *et al. Mol Nutr Food Res*, 2015)

COCOA



(Llorach R *et al. Mol Nutr Food Res*, 2013; Garcia-Aloy M *et al. Mol Nutr Food Res*, 2015)

1. Metabolic footprint of daily consumption of BREAD is characterized by compounds from own cereal phytochemicals, such as benzoxazinoids and alkylresorcinols metabolites; by compounds produced by the microbiota, as the metabolites of the enterolactones, hydroxybenzoic acid and dihydroferulic acid; as well as other compounds such as pyrraline and 3-indolecarboxylic acid glucuronide. Furthermore, among consumers of whole-grain bread showed increased and decreased excretion of 2,8-dihydroxyquinoline glucuronide and acetylcitrulline, respectively, which might be involved in the beneficial effects associated with the intake of bread previously observed in epidemiological studies.

2. Metabolic footprint of regular consumption of NUTS, particularly WALNUTS, is characterized by markers of fatty acid metabolism, compounds derived from the metabolism of ellagitannins by the microbiota, as well as compounds of tryptophan and serotonin metabolic pathway. The importance of the identification of the latter class of compounds is in the role of serotonin in the regulation of energy balance.

3. Metabolic footprint of habitual consumption of COCOA is characterized by compounds of theobromine and polyphenol metabolism, as well as metabolites related to the processing of cocoa. Cocoa consumption has also been associated with reduced urinary excretion of metabolites related to the metabolism of acylcarnitines and tyrosine sulfation, which may be related to cardiovascular disease.

4. Many of the characterized biomarkers in clinical trials of nutritional intervention have been replicated in free-living subjects evaluated in observational conditions.

5. Analysis of stepwise logistic regression allows the combination of different metabolites with discriminatory capacity for consumption of certain foods that are characterized by being formed by compounds of different nature that might provide additional information.

6. The predictive ability of dietary exposure through the combined multi-metabolite models is improved compared to the ability of these compounds evaluated individually. The combined models could be useful in improving the accuracy in the assessment of dietary intake.

7. The nutrimetabolomics allows us to reveal possible mechanisms of action to explain the effect of diet observed in epidemiological studies and, thus, contribute to the generation of new hypotheses in the field of food and health.

THANK YOU VERY MUCH!!!