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
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“BIOMARKERS AND NUTRITIONAL & FOOD METABOLOMICS” RESEARCH GROUP

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Location
Dept. of Nutrition & Food Science
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University of Barcelona (Spain)
Diagonal Campus, Av. Diagonal, 643 Av. Joan XXIII s/n (Barcelona)
Introduction

Ministry of Economy and Competitiveness
European Regional Development Fund (ERDF)
Project AGL 2009-13906-C02-01
Program Ingenio-ConsoliderFUN-C-FOOD (CDS 2007-063)
Complementary Action AGL2010-10084-E

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)

Objectives

Methodology

Results

Conclusions

ACCURATE MEASUREMENT OF FOOD INTAKE

diet

health/disease

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

Introduction
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ACCURATE MEASUREMENT OF FOOD INTAKE

FOOD SURVEYS

R24h

Dietary Records

LIMITATIONS

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


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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.

NUTRITIONAL BIOMARKERS

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

Type/conditions biological specimen
Precision and accuracy from defined measure

BIOLOGICAL VALIDATION

Analytical validation


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.


**Introduction**

**Methodology**

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**Conclusions**
**FOOD METABOLOME: APPLICATIONS**

Analysis of food metabolome

Dietary assessment
- Food intake
- Nutritional exposure

- New metabolites
- New potential food bioactives

- Segmentation of poor or high absorbers & metabolizers


**NUTRIMENTABOLOMICS: APPROACHES**

Clinical phenotyping
- Genetic phenotyping
- Microbiota phenotyping etc.

Nutritional Consideration

Metabolomic analysis

Markers of Intake

Markers of Effect

New Hypotheses

NUTRITIONAL STUDY DESIGNS

INTRODUCTION

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ACUTE INTERVENTION STUDY

CHRONIC INTERVENTION STUDY

OBSERVATIONAL STUDY

Level of control of the diet

Heterogeneity of the population

Number of discriminant metabolites


Table 3

The American Journal of Clinical Nutrition

Orange

Proline betaine

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**Conclusions**

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**Diet Complexity to Discover Nutritional Biomarkers**

**Distribution of compounds into various foods**

**Confluence of various compounds in common metabolites**

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**ELLAGITANNINS:**

- Pedunculagin
- Sanguin H6
- Punicalagin

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**ELAGITANNINOS:**

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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.

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.
HPLC-QToF-MS UNTARGETED METABOLOMOMIC ANALYSIS

**Quality Control Analysis of Critical Points:**
- Robustness analysis
- Validation of multivariate models
- Multistep procedure


HPLC-QToF-MS UNTARGETED METABOLOMOMIC ANALYSIS

(Barter-Asuncion et al., J Pharm Biomed Anal, 2010)
**HPLC-QToF-MS UNTARGETED METABOLOMIC ANALYSIS**

**COELUTION & CORRELATION**

- Chromatogram
- Mass spectrum (RT = 3.98 min)

**Quality control**

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

**Distribution of samples & QCs in plates**


HPLC-QToF-MS UNTARGETED METABOLOMOMIC ANALYSIS

**Methodology**

- **Data processing**
  - Marker detection & Alignment
  
  ![HPLC-qToF-MS](image)
  
  List of markers

- **Multivariate analysis**
  - Unsupervised & supervised techniques (PCA; PLS-DA)
  
  ![Visualization of results from metabolome](image)

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

**Introduction**

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**Data Acquisition**

**Biomarker Identification**

**Biological Interpretation**

**Results**

**Conclusions**

---

**HPLC-QToF-MS UNTARGETED METABOLOMOMIC ANALYSIS**

**Methodology**

- **Data processing**
  - Metabolite characterization
  - Ion Annotation
  - Computational Analysis

- **Multi-step procedure**
  - Fragmentation
  - "in silico" prediction

**Introduction**

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**References**

- Llorach et al. *J Proteome Res*, 2010
**HPLC-QToF-MS UNTARGETED METABOLOMICS ANALYSIS**

Multi-step procedure

### Level of confidence of identity

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<th>Level of evidence</th>
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<td>1</td>
<td>Identified compounds</td>
<td>Comparison of ≥2 orthogonal properties with an authentic compound analyzed under identical experimental conditions.</td>
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<td>2</td>
<td>Putatively annotated compounds</td>
<td>Based upon property similarity with public/commercial databases, without chemical reference standard.</td>
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<td>3</td>
<td>Putatively characterized compound classes</td>
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<td>Unknown compounds</td>
<td>Unidentified compounds.</td>
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</table>

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

**Objectives**

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**Results**

**Conclusions**

### Distribution of compounds into various foods

<table>
<thead>
<tr>
<th>Proanthocyanidin B2</th>
<th>(+)-Catechin</th>
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<tr>
<td>Hydroxyphenyl-valerolactones</td>
<td>Hydroxyphenyl-valeric acids</td>
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### Confluence of various compounds in common metabolites

**ELLagitannins:**

- Pedunculagin
- Sanguiin H6
- Punicalagin


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### Development of multimetabolite biomarker panels

**Introduction**

**Objectives**

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### Combine metabolites

**Stepwise Logistic Regression**


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**DEVELOPMENT OF MULTIMETABOLITE BIOMARKER PANELS**

**PREDICTIVE CAPACITY OF BIOMARKERS: ROC CURVES**

- **ROC curve with perfect predictive capacity**
- **ROC curve with good predictive capacity**
- **ROC curve without predictive capacity**

**AUCs**

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

(Xia et al. Metabolomics, 2013)

**NUTRIMETABOLOMICS: APPROACHES**

- **Nutritional Intervention**
- **Consumption Stratification**
- **Population phenotyping**
  - Clinical phenotyping
  - Genetic phenotyping
  - Microbiota phenotyping etc.
- **Metabolic analysis**
- **Markers of Intake**
- **Markers of Effect**
- **New Hypotheses**

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)

Introduction

Methodology

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

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>DETECTED MASS (m/z)</th>
<th>ASSIGNATION</th>
<th>IDENTIFICATION</th>
<th>AUCs</th>
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<td>0.88</td>
<td>188.0049 M-1</td>
<td>2-Aminophenol sulphate</td>
<td>↑</td>
<td>76.1% (67.1%)</td>
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<tr>
<td>1.48</td>
<td>328.1036 M+H+</td>
<td>HPPA glucuronide</td>
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<td>68.9% (59.0%)</td>
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<td>2.07</td>
<td>168.0609 M+H+</td>
<td>HHPAA</td>
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<tr>
<td>3.40</td>
<td>370.0772 M-H-</td>
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<td>6.32</td>
<td>299.1278 M+H+ - GlcA</td>
<td>Enterolactone glucuronide</td>
<td>↑</td>
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<td>473.1447</td>
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<td>2.73</td>
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<td>66.8% (56.9%)</td>
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(Garcia-Aloy M et al., Metabolomics, 2015)

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

**MULTIMETABOLITE COMBINED MODELS**

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(Garcia-Aloy M et al., Metabolomics, 2015)
### MULTIMETABOLITE COMBINED MODELS

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</table>

### Introduction

**Objectives**

- Consumption data
  - FFQ

**Methodology**

- Benzoazinoid-related compounds
- Alkylresorcinol metabolites
- Microbial-derived metabolites
- Markers of heat-treated food products
- Metabolites related with bread composition
- Endogenous metabolites

**Results**

- AUC = 0.83 (72.1%-84.6%
- 90%-100% = excellent
- 80%-90% = good
- 70%-80% = fair
- 60%-70% = poor
- 50%-60% = fail

**Conclusions**

(Garcia-Alloy M et al. Metabolomics, 2015)
**NUTS & WALNUTS**

**INTERVENTION STUDY**

- RANDOMIZATION
- INTERVENTION PERIOD
  - W0 (baseline)
  - W12 (12 weeks)

- CONTROL GROUP: n = 20
- NUTS GROUP: n = 22

**OBSERVATIONAL STUDY**

- PREDIMED COHORT (n=7447)

- Subsample 1 (n=279) (cross-sectional analysis)
- Subsample 2 (n=327) (cross-sectional analysis)

- WALNUT consumption stratification
  - Excluded (n=80)
  - Excluded (n=141)
  - Habitual consumers
    - Non-consumers (n=128)
    - Habitual consumers (n=67)
  - Habitual consumers (n=82)

**Objectives**

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

---

**NUTS & WALNUTS**

**INTERVENTION STUDY**

- Multivariate analysis: OSC-PLS-DA

- S-plot

**OBSERVATIONAL STUDY**

- Permutation test
- V-plot (VIP)

- Cutoff in metabolomic studies

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

**INTERVENTION STUDY**

<table>
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<td>[M - H - sulfate]</td>
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<td>[M + H - GlcA]</td>
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**OBSERVATIONAL STUDY**

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<td>Urolithin A glucuronide</td>
</tr>
</tbody>
</table>

**RESULTS**

**CONCLUSIONS**

Objectives

- Evaluate the effects of nut consumption on metabolic markers.
- Identify and quantify specific metabolites.

Methodology

- Utilized liquid chromatography-mass spectrometry (LC-MS) for metabolite detection.
- Analyzed samples from intervention and observational studies.

**RESULTS**

- Significant increases in metabolites such as Urolithin A glucuronide.
- Reduction in markers associated with inflammation and oxidation.

**CONCLUSIONS**

- Nut consumption positively impacts metabolic health.
- Nut consumption may offer therapeutic benefits in metabolic disorders.

(Tulipani S et al. J Proteome Res, 2011; García-Aloy M et al., J Proteome Res, 2014)
**AGL2009-13906-C02-01**

**PREDIMED COHORT**
(n=7447)

**TRAINING SET**

**VALIDATION SET**

**WALNUT consumption stratification**

- **Subsample 1 (n=275)**
  - Cross-sectional analysis
- **Subsample 2 (n=327)**
  - Cross-sectional analysis

- **Non-consumers**
  - (n=128)
- **Habitual consumers**
  - (n=104)

- **Excluded**
  - (n=141)

**Introduction**

**Methodology**

**Results**

**Conclusions**

---

**NUTS & WALNUTS**

**OBSERVATIONAL STUDY**

**AUCs**

<table>
<thead>
<tr>
<th><strong>Subsample 1</strong></th>
<th><strong>Subsample 2</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>10-Hydroxy-decane-4,6-dienoic acid sulfate</td>
<td>74.4% (66.4%-82.5%)</td>
</tr>
<tr>
<td>Tridecadienoic/tridecynoic acid glucuronide</td>
<td>85.1% (79.8%-90.4%)</td>
</tr>
<tr>
<td>Urolithin C glucuronide</td>
<td>75.4% (67.7%-83.0%)</td>
</tr>
<tr>
<td><strong>Urolithin A glucuronide</strong></td>
<td><strong>82.0% (75.7%-88.4%)</strong></td>
</tr>
<tr>
<td>Urolithin A sulfoglucuronide</td>
<td>70.4% (62.0%-78.7%)</td>
</tr>
<tr>
<td>Urolithin B glucuronide</td>
<td>59.1% (50.6%-67.7%)</td>
</tr>
<tr>
<td>Enterodiol glucononide</td>
<td>62.3% (54.1%-70.5%)</td>
</tr>
<tr>
<td>Urolithin C sulfamate</td>
<td>69.7% (61.5%-78.0%)</td>
</tr>
<tr>
<td>Urolithin A sulfamate</td>
<td>78.7% (71.3%-86.1%)</td>
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<tr>
<td>3-Indolecarboxylic acid glucuronide</td>
<td>73.7% (66.2%-81.3%)</td>
</tr>
<tr>
<td>Hydroxyindoleacetic acid sulfate</td>
<td>61.0% (52.5%-69.6%)</td>
</tr>
<tr>
<td>N'-Acetylserotonin sulfate</td>
<td>64.5% (56.2%-72.8%)</td>
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</table>

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

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

**Methodology**

**Results**

**Conclusions**

---

**NUTS & WALNUTS**

**OBSERVATIONAL STUDY**

**STEPWISE LOGISTIC REGRESSION**

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<td>2,212</td>
<td>0,491</td>
<td>&lt;0,001</td>
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---

Urolithin C glucuronide

Urolithin A glucuronide

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-Acetylserytonin sulfate

---

**NUTS & WALNUTS**

**MULTIMETABOLITE COMBINED MODELS**

**Training set**

**Validation set**

AUC = 93.45% (90.08%–96.81%)

AUC = 90.22% (85.87%–94.57%)

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

---

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

**Methodology**

**Results**

**Conclusions**

---

**NUTS & WALNUTS**

**Abstract**

**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)

---

**Cocoa**

**Intervention Study**

**Observational Study**

**Predimed Cohort**

(n=7447)

Subsample (n=275)

[Cross-sectional analysis]

**Cocoa consumption stratification**

Non-consumers (n=192)

Habitual Consumers (n=32)

Non-consumers (n=32)

Habitual Consumers (n=32)

**Introduction**

**Objectives**

**Methodology**

**Results**

**Conclusions**

---

**COCOA**

**INTERVENTION STUDY**

OBSERVATIONAL STUDY

**Multivariate analysis**

---

**DETECTED MASS (m/z)**

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(Loorach R et al. Mol Nutr Food Res. 2013; Garcia-Alloy M et al. Mol Nutr Food Res. 2015)
Introduction

Methodology

Results

Conclusions

**COCOA**

**ACUTE INTERVENTION**

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

**LONG-TERM INTERVENTION**

**FREE-LIVING POPULATION**

Objectives

AMMU

AMMU isomer

3-Methyluric acid

7-Metilxantheme

3-Metilxantheme

3,7-Dimethyluric acid

Theobromine

Methoxyhydroxyphenylvalerolactone

5-[(3',4')-Dihydroxypheyl]-valerolactone glucuronide

5-[(3',4')-Dihydroxypheyl]-valerolactone sulfate

Polyphenol metabolites produced by microbiota

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

**OBSERVATIONAL STUDY**

AUCs

Theobromine

88.18% (79.47%-96.90%)

AMMU isomer

76.66% (65.05%-88.27%)

3-Methyluric acid

82.23% (71.23%-93.22%)

7-Metilxantheme

88.28% (80.09%-96.48%)

3-Metilxantheme

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')-Dihydroxypheyl]-valerolactone GlcA

68.20% (55.02%-81.51%)

5-[(3',4')-Dihydroxypheyl]-valerolactone sulfate

51.09% (58.27%-83.92%)

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
MULTI-METABOLITE COMBINED MODELS

**STEPWISE LOGISTIC REGRESSION**

<table>
<thead>
<tr>
<th>AMMU</th>
<th>AMMU isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methyluric acid</td>
<td></td>
</tr>
<tr>
<td>7-Methylxanthine</td>
<td>5.563</td>
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</tr>
<tr>
<td>3,7-Dimethyluric acid</td>
<td></td>
</tr>
<tr>
<td>Theobromine</td>
<td></td>
</tr>
</tbody>
</table>

Methoxyhydroxyphenylvalerolactone

| 5-(3',4')-Dihydroxyphenyl-valerolactone ClcA | 4.081 | 1.559 | 0.009 |
| 5-(3',4')-Dihydroxyphenyl-valerolactone sulfate |

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

COCOA
MULTI-METABOLITE COMBINED MODELS

**AUCs**

<table>
<thead>
<tr>
<th>TRAINING SET</th>
<th>VALIDATION SET</th>
</tr>
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<tbody>
<tr>
<td><img src="image" alt="AUC Graph" /></td>
<td><img src="image" alt="AUC Graph" /></td>
</tr>
</tbody>
</table>

| AUC | 95.69% (89.8%-100%) | AUC | 92.56% (81.92%-100%) |

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

(Garcia-Aloy M et al., Mol Nutr Food Res, 2015)
Theobromine metabolism
Polyphenol metabolism
Cocoa taste and flavour
Endogenous markers

Biomarker panel

Introduction
Methodology
Results
Conclusions

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 pyrroline and 3-indolecarboxylic acid glucuronide. Furthermore, among consumers of whole-grain bread showed increased and decreased excretion of 2,3-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!!!