“Impact of smoking cessation on the metabolic profile of former smokers”

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ST05
Overview

- Introduction to metabolomics
- Smoking cessation study
- Untargeted metabolic profiling of biofluids
- Targeted analysis of plasma fatty acids
- Summary and outlook
The **metabolome** represents the entity of all ‘small’ molecules (< 1500 Dalton) and is most predictive of the phenotype of an organism.

**Metabolomics** is the study of the entire set of small molecules in a biological sample.

- Identification
- Quantification
- Validation
Why metabolomics?

Differences in metabolic pathways

At risk

Healthy

BIOMARKERS

At risk

Healthy

Differences in metabolic pathways
Metabolomic strategies

Metabolomic fingerprinting:
- Untargeted screening and identification of as many metabolites in a sample as possible
- Ideally identification of the related metabolic pathways.

Metabolomic profiling:
- Identification and quantification of a selective number of predefined metabolites, which are normally related to a specific metabolic pathway.
GC-TOF-MS: Analytical workflow for the untargeted biomarker identification

**Biological Sample**
- Urine, Plasma, Saliva

**Sample clean-up**
- IS-spiking (d₄-ttMA)
- Urea digestion (only Urine)
- Protein precipitation
- Methoximation, Silylation

**MS Analysis**
- GC-TOF-MS (Almsco)
- GC: Dimethyl-polysiloxane (30m x 250µm i.d.)

**Data processing**
- Baseline correction ProtoTOF (Almsco)
- Mass detection, peak alignment (Mzmine)
- Normalization to IS

**Statistical analysis & target hit identification**

**Statistics:**
- PLS-DA
- Mann-Whitney-U test, fold change

**Identification:**
- Deconvolution,
- Databases (NIST, Golm)
- Reference compounds

_Mueller et al. JPR, Dec 2013._
_Müller et al. JCB, Mar 2014._
Objectives of the smoking cessation study

- Clinical study: smoking cessation
- Untargeted metabolomic fingerprinting by GC-TOF-MS
  - In plasma, saliva and urine
- Identify altered biochemical pathways and biomarkers for smoking cessation
- Establish and apply targeted methods (e.g.: fatty acids, amino acids, eicosanoids)
Study design of smoking cessation study

- 60 healthy volunteers
  - Male: age 20-50
  - BMI 18-29
  - Smoker: >15 cigarettes / day during the last year
  - Strong intention to stop smoking

- 3 months:
  - Study start (first group in): January 14th, 2015
  - Study end (last group out): June 23rd, 2015

- After first day subjects have to quit smoking
- 4 x 24 hour stationary visits
  - Controlled diet
- Several ambulant visits to assess compliance of the subjects
Study timelines

1\textsuperscript{st} stationary visit
TP0

2\textsuperscript{nd} stationary visit
TP1

3\textsuperscript{rd} stationary visit
TP2

4\textsuperscript{th} stationary visit
TP3

5 ambulant visits

3 ambulant visits

6 ambulant visits

1 \textsuperscript{st} day

1 week

1 month

3 months
Study design: stationary visits (N=4)

- 24 h-urine sample from each subject generated from the 3 fractions
- EDTA-plasma: cooled vacutainer, immediately centrifuged 4°C, frozen with dry ice
- Saliva: modified unstimulated spitting method by Navazesh, ANN NY ACAD SCI, 1993.
Study completion

Included subjects: 60
Drop outs: 21
Compliant subjects 39

Dropout reasons:

- Protocol violation (e.g. too high COex) 4
- Started smoking (self reported and/or detected) 12
- Withdrawn study agreement 3
- Missed visit 2
Summary: saliva fingerprinting

**TP0 vs TP1 (Smoker vs after 1 week of cessation)**
- Arachidonic acid
- (Amino acid metabolism)
- (Energy metabolism)

**TP0 vs TP2 (Smoker vs after 1 month of cessation)**
- Arachidonic acid
- (Amino acid metabolism)
- (Energy metabolism)

**TP0 vs TP3 (Smoker vs after 3 months of cessation)**
- Arachidonic acid
- (Amino acid metabolism)
- (Energy metabolism)

**General findings**
- Decreasing number of metabolites over time of cessation
- 24 different metabolites over all points in time
- Overall highest number of metabolites
Summary: urine fingerprinting

**TP0 vs TP1 (Smoker vs after 1 week of cessation)**
- Tryptophan metabolism
  - Kynurenic acid

**TP0 vs TP2 (Smoker vs after 1 month of cessation)**
- Tryptophan metabolism
  - Kynurenic acid

**TP0 vs TP3 (Smoker vs after 3 months of cessation)**
- Tryptophan metabolism
  - Kynurenic acid

**General findings**
- Increasing number of metabolites over time of cessation
- 16 different metabolites over all points in time
- Overall lowest number of metabolites
Plasma Group separation PLS-DA

Study 1 Plasma S/NS

Study 2 Plasma TP0/TP3
Summary: plasma fingerprinting

**TP0 vs TP1 (Smoker vs after 1 week of cessation)**
- Amino acid metabolism
  - Tryptophan metabolism
- Glycolysis

**TP0 vs TP2 (Smoker vs after 1 month of cessation)**
- Amino acid metabolism
  - Tryptophan metabolism
- Fatty acid metabolism
  - Arachidonic acid

**TP0 vs TP3 (Smoker vs after 3 months of cessation)**
- Amino acid metabolism
  - Tryptophan metabolism
- Fatty acid metabolism

**General findings**
- Increasing number of metabolites over time of cessation
- 33 different metabolites over all points in time
Conclusion fingerprinting

Several metabolites could be identified by the fingerprinting approach:

- Overall 102 statistically significant alterations were found thereof 64 different
  - 33 different metabolites in plasma
  - 24 different metabolites in saliva
  - 16 different metabolites in urine

Most interesting finding were alterations in:

- Fatty acid metabolism (plasma)
  - Arachidonic acid metabolism (plasma + saliva) → precursor to eicosanoids
- Amino acid metabolism (plasma)
- Tryptophan metabolism (plasma + urine)
Targeted analysis of fatty acids by GC-TOF-MS

- 44 FAMEs
- Chainlength C 4-32
- Butyric acid to dotriacontanoic acid
- Saturated and unsaturated FA
- Cis and trans separation (FA 18:1 C9 (17) und 18:1 t9 (18))
Relative fatty acid profile

FA C22:6...
FA C22:5 c7,c10,c13,c16,c19
FA C24:1 c15
FA C22:4 c7,c10,c13,c16
FA C24:0
FA C20:5 c5,c8,c11,c14,c17
FA C23:0
FA C20:4 c8,c11,c14,c17
FA C22:1 c13
FA C20:4 c5,c8,c11,c14
FA C22:0
FA C20:3 c8,c11,c17
FA C20:2 c11,c14
FA C20:1 c11
FA C18:3 c9,c12c,c15
FA C20:0
FA C18:3 c6,c9,c12
FA C18:2 c9,c12
FA C18:1 c11
FA C18:1 c9
FA C18:0
FA C17:0
FA C16:1 c9
FA C16:0
FA C15:0
FA C14:1 c9
FA C14:0
FA C12:0
FA C10:0

Relative fatty acid concentration [%]
Fatty acid method: statistical evaluation

**Pre Analysis**
- **Gaussian distribution**
  - QQ plot
  - No gaussian distribution

**Statistical tests**
- Quades Test
  - Test for up to five points in time
  - Group analysis of:
    - Paired data
    - No gaussian distribution
    - $\alpha = 5\%$
- Wilcoxon signed rank test
  - Test for two Groups
    - Timeseries $\rightarrow$ correction for significance
    - Bonferroni correction $\alpha = 5\% \rightarrow 1.25\%$
    - Paired data
    - No gaussian distribution
# Fatty acid method: statistical evaluation

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Trend</th>
<th>Quade test p-value</th>
<th>Wilcoxon signed rank test p-value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TP0-TP1-TP2-TP3</td>
<td>TP0/1</td>
</tr>
<tr>
<td>FA C12:0</td>
<td>▲</td>
<td>0.005214</td>
<td>0.009348</td>
</tr>
<tr>
<td>FA C14:0</td>
<td>▲</td>
<td>0.000001</td>
<td>0.000002</td>
</tr>
<tr>
<td>FA C16:0</td>
<td>(▼)</td>
<td>0.022240</td>
<td>0.027830</td>
</tr>
<tr>
<td>FA C17:0</td>
<td>▼</td>
<td>0.001951</td>
<td>0.001468</td>
</tr>
<tr>
<td>FA C18:1 c9</td>
<td>▼</td>
<td>0.000059</td>
<td>0.714500</td>
</tr>
<tr>
<td>FA C18:3 c6,c9,c12</td>
<td>▲</td>
<td>0.000531</td>
<td>0.000068</td>
</tr>
<tr>
<td>FA C18:3 c9,c12c,c15</td>
<td>▼</td>
<td>0.049430</td>
<td>0.018950</td>
</tr>
<tr>
<td>FA C20:3 c8,c11,c17</td>
<td>▲</td>
<td>0.003908</td>
<td>0.038580</td>
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<tr>
<td>FA C20:4 c5,c8,c11,c14</td>
<td>▲</td>
<td>0.000175</td>
<td>0.511600</td>
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<tr>
<td>FA C22:1 c13</td>
<td>▼</td>
<td>0.001822</td>
<td>0.006844</td>
</tr>
<tr>
<td>FA C20:5 c5,c8,c11,c14,c17</td>
<td>▲</td>
<td>0.010380</td>
<td>0.008191</td>
</tr>
<tr>
<td>FA C24:0</td>
<td>▼</td>
<td>0.010190</td>
<td>0.418500</td>
</tr>
<tr>
<td>FA C24:1 c15</td>
<td>▼</td>
<td>0.003966</td>
<td>0.009348</td>
</tr>
<tr>
<td>FA C22:6 c4,c7,c10,c13,c16,c19</td>
<td>▲</td>
<td>0.004862</td>
<td>0.120600</td>
</tr>
</tbody>
</table>

**Findings:**
- Increased levels in saturated and polyunsaturated fatty acids
- Decreased levels monounsaturated
- Recovery of certain FA after increase or decrease (unspecific)
Fatty acid method: statistical evaluation
FA C18:1 c9

<table>
<thead>
<tr>
<th></th>
<th>TP0 [%]</th>
<th>TP1 [%]</th>
<th>TP2 [%]</th>
<th>TP3 [%]</th>
</tr>
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<tbody>
<tr>
<td>Mean</td>
<td>24.27</td>
<td>24.19</td>
<td>23.19</td>
<td>22.71</td>
</tr>
<tr>
<td>Median</td>
<td>23.75</td>
<td>24.29</td>
<td>23.84</td>
<td>22.99</td>
</tr>
<tr>
<td>SD</td>
<td>3.05</td>
<td>2.61</td>
<td>2.87</td>
<td>2.39</td>
</tr>
</tbody>
</table>
Fatty acids: Discussion

Mueller et al. JPR, Dec 2013; Müller et al. JCB, Mar 2014; Göttel et al. 2016 (in preparation)

- Partial recovery of MUFA and SFA levels upon smoking cessation.
  *Altered endogenous desaturation in smokers; possibly caused by an upregulation SCD1.*

- Increased levels of PUFAs upon smoking cessation.
  *Free radicals and other tobacco smoke components have been shown to ineract with various cell components such as lipids. The absence these exposure components could be reflected in the increasing levels of PUFAs after smoking cessation.*

- FAs 17:0 and 22:6 were significantly in or decreased over the smoking cessation period with a recovery towards the initial levels.
  *This could be the result of a hyper compensation caused by metabolic adaption processes after smoking cessation*
Summary & outlook

- Clinical study successfully completed
- 39 compliant subjects included
- Untargeted metabolic profiling of plasma, saliva, urine
- Targeted analysis of 44 individual plasma fatty acid species

- Additional targeted methods are currently being developed (Amino acids)
- Urinary analysis of 9 individual eicosanoid species in progress in order to investigate the arachidonic acid pathway

- Publication of the results
Acknowledgement

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Thank you for your attention

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