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

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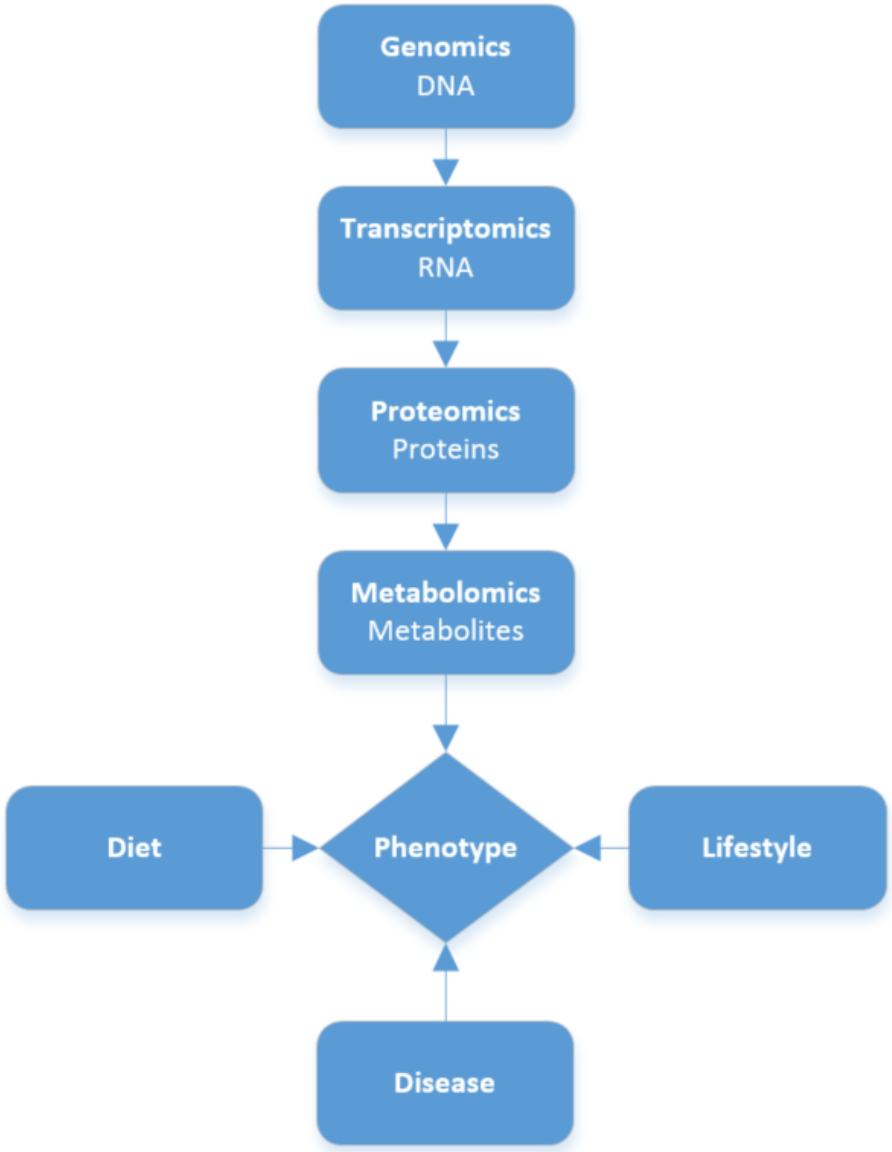
2016 CORESTA CONGRESS
Smoke Science and Product Technology
Berlin, Germany

9-13 October 2016
ST05

Overview

- Introduction to metabolomics
- Smoking cessation study
- Untargeted metabolic profiling of biofluids
- Targeted analysis of plasma fatty acids
- Summary and outlook

Cascade of ‘omics‘



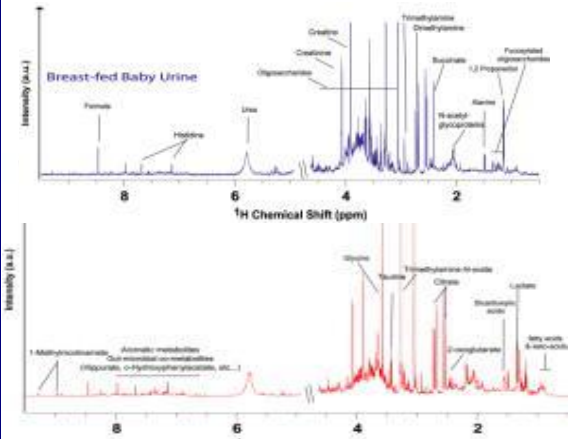
- The **metabolome** represents the entity of all ‘small’ molecules (< 1500 Dalton) and is most predictive of the phenotype of an organism.

- **Metabolomics** is the study of the entire set of small molecules in a biological sample.
  - Identification
  - Quantification
  - Validation

Why metabolomics?

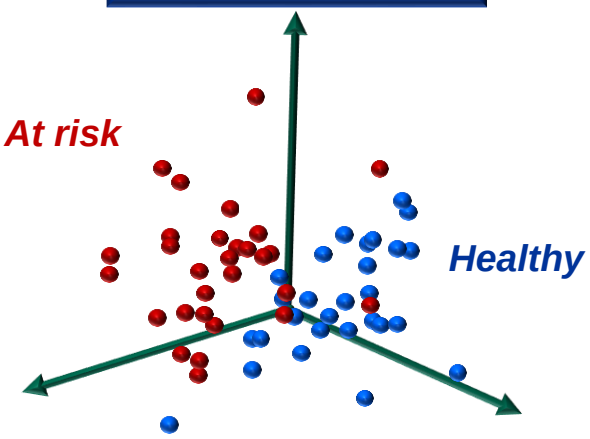


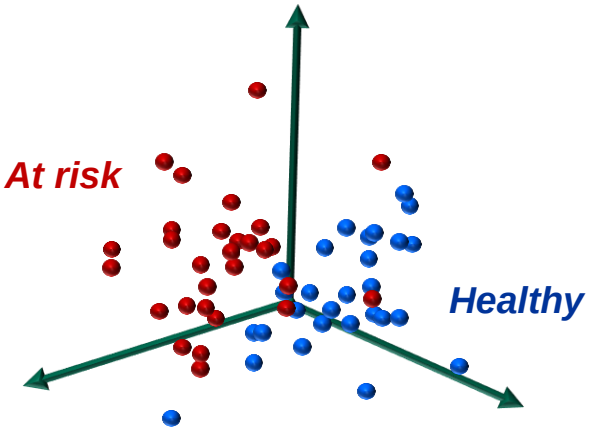


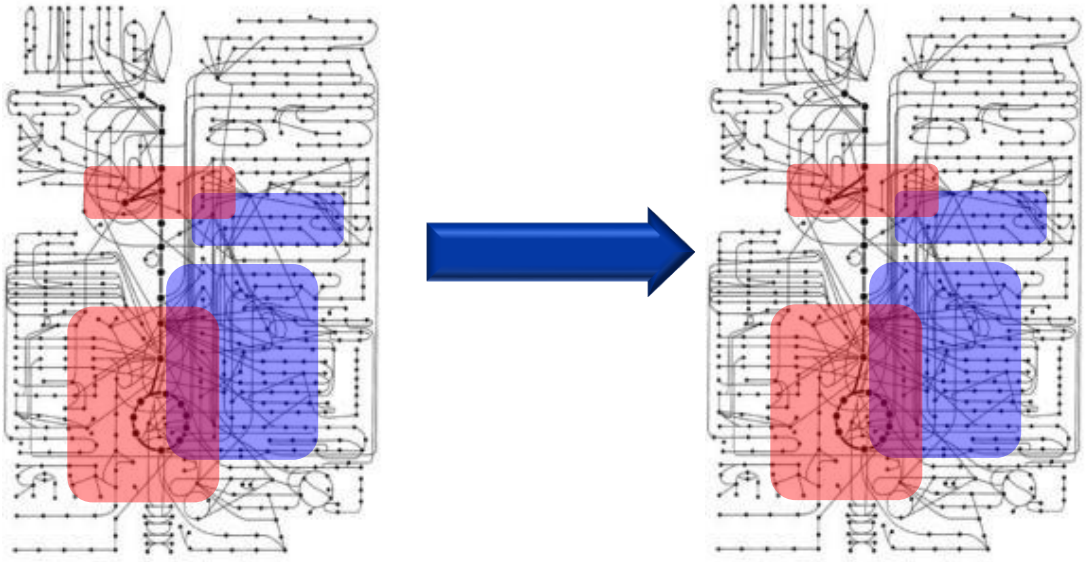




BIOMARKERS







Differences in metabolic pathways

Metabolomic strategies

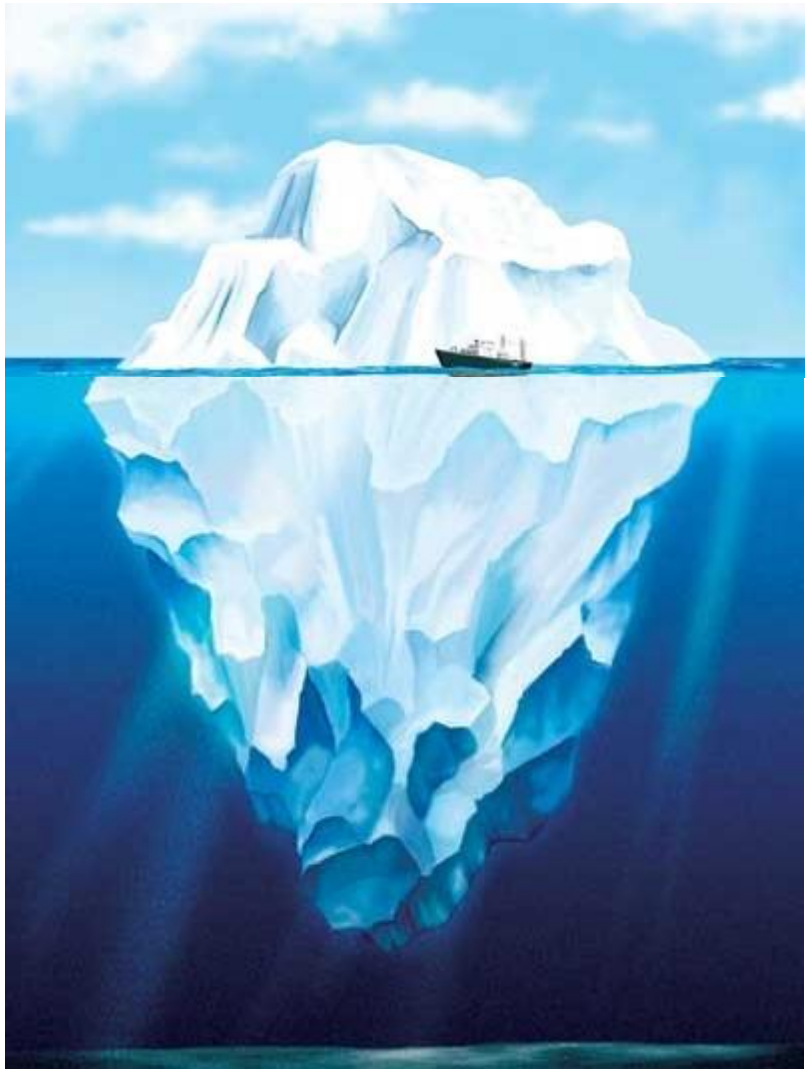
Metabolomic fingerprinting:

- Untargeted screening and identification of as many metabolites in a sample as possible
- ideally identification of the related metabolic pathways.

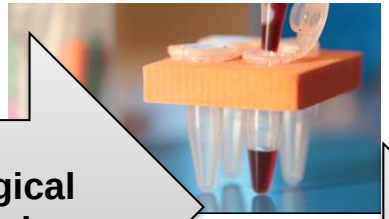
Metabolomic profiling:

- 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

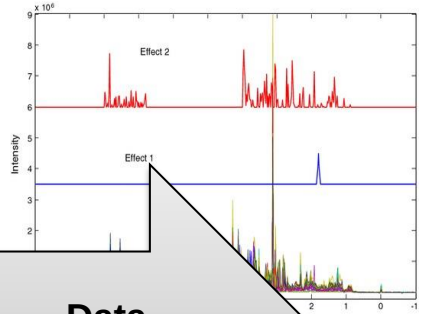
Sample clean-up

- IS-spiking (d_4-*tt*MA)
- Urea digestion (only Urine)
- Protein precipitation
- Methoximation, Silylation



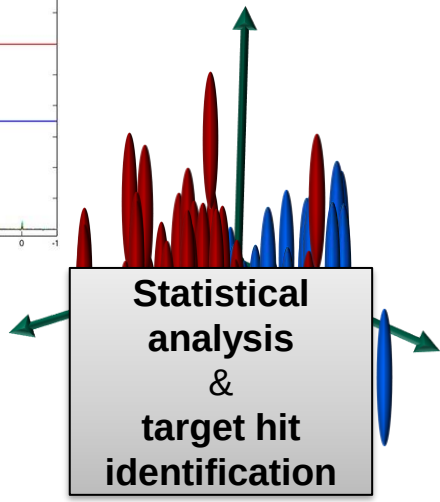
MS Analysis

- 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)*

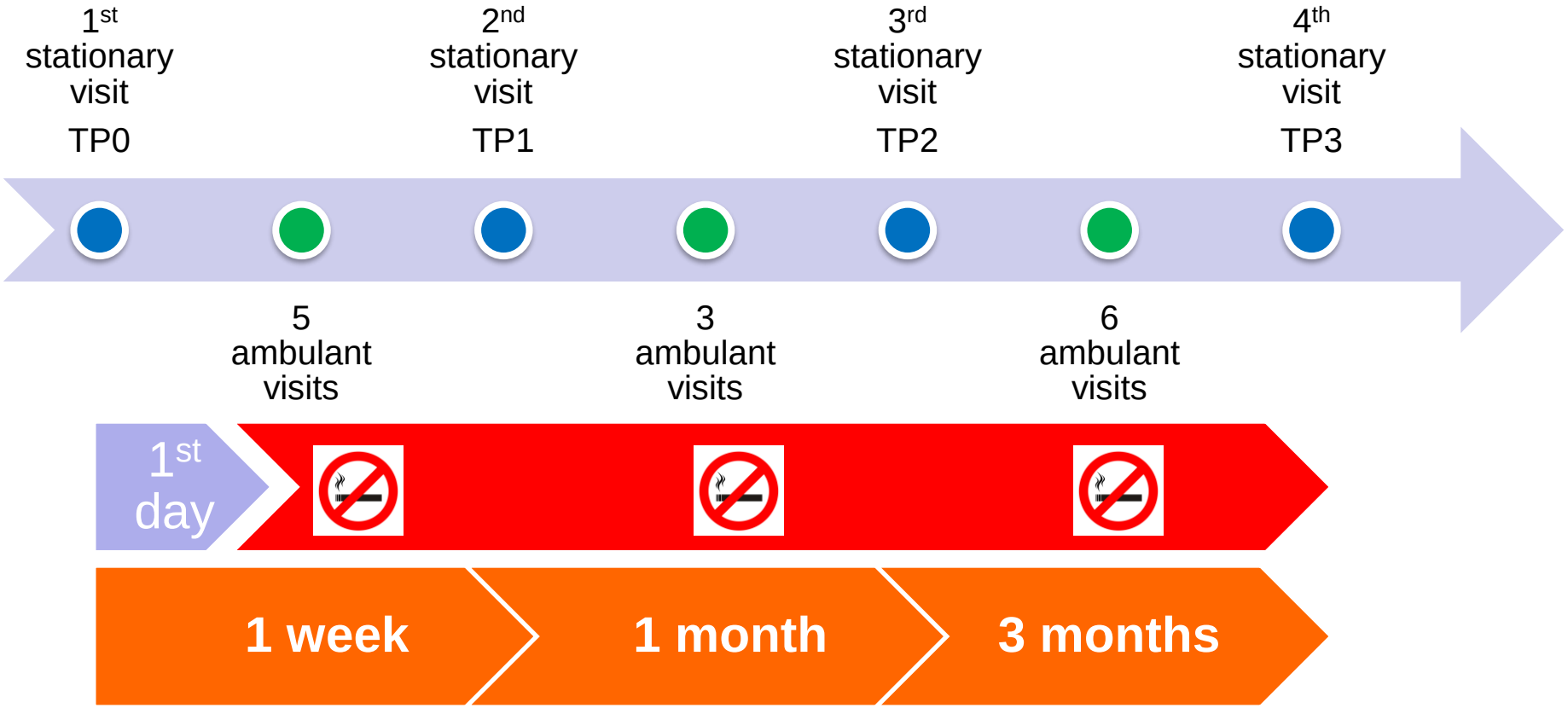
Study design of smoking cessation study

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

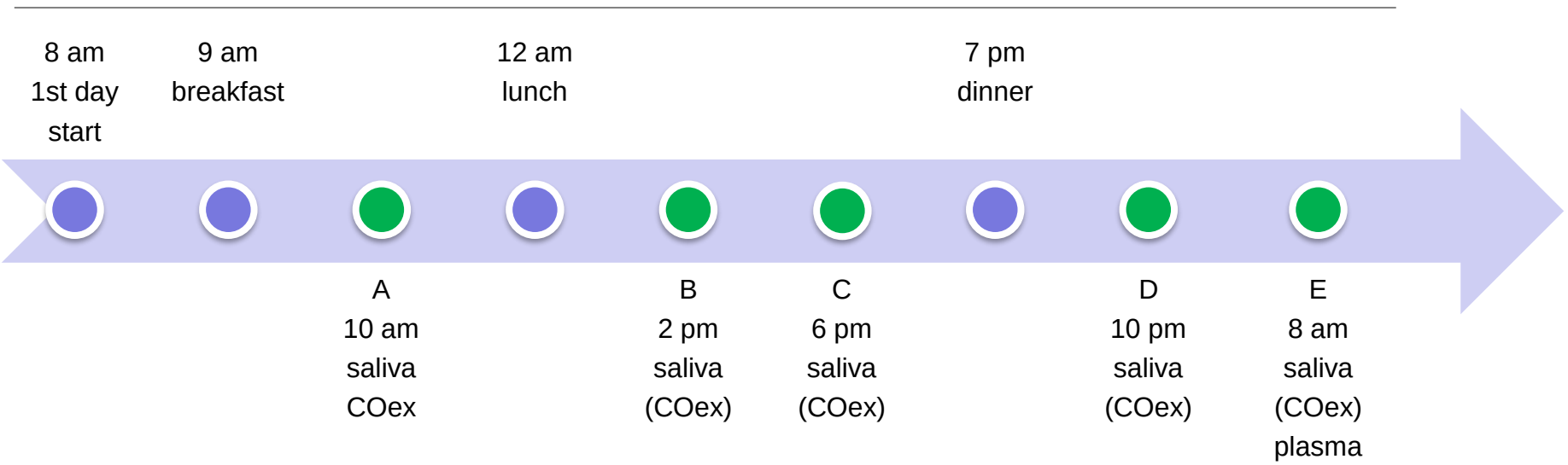
- *3 months:*
  - *Study start (first group in): January 14th 2015*
  - *Study end (last group out): June 23rd, 2015*

- *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



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:	Number
➢ 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 highes 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)

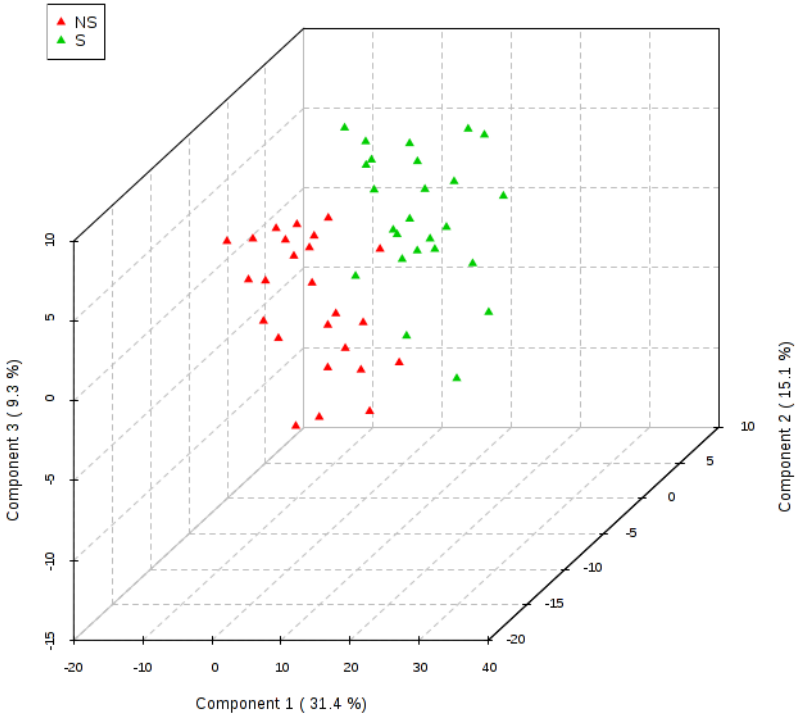
- Tryptophan metabolism
  - Kynurenic acid

General findings

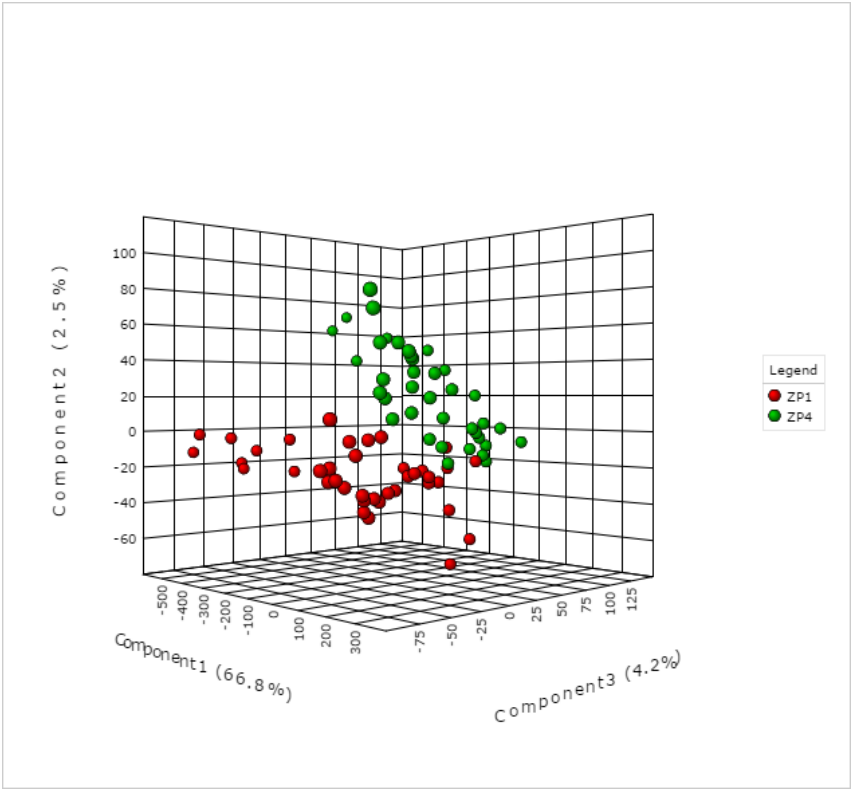
- increasing number of metabolites over time of cessation
- 16 different metabolites over all points in time
- Overall lowest nuber of metabolites

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:

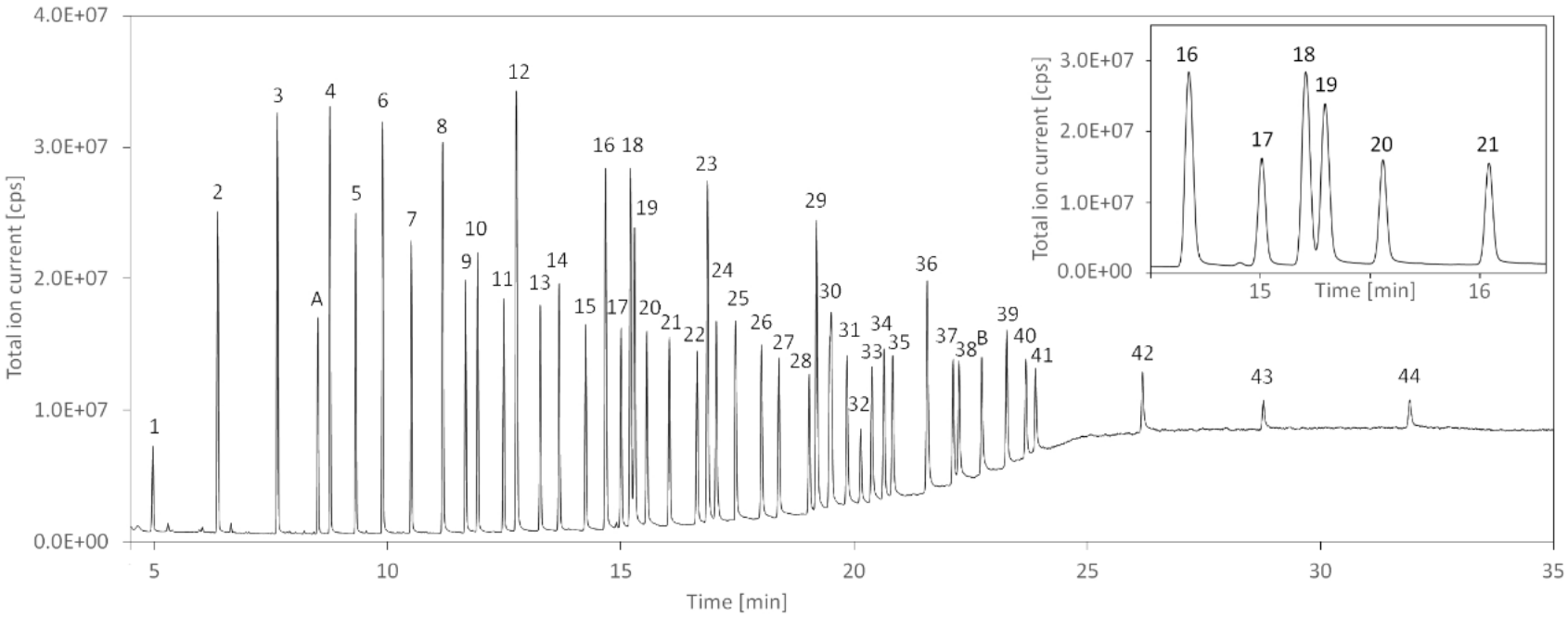
- Overall 102 statistically significant alterations were found thereof 64 different
  - 33 different metabolites in plasma
  - 24 different metabolites in saliva
  - 16 different metabolites in urine

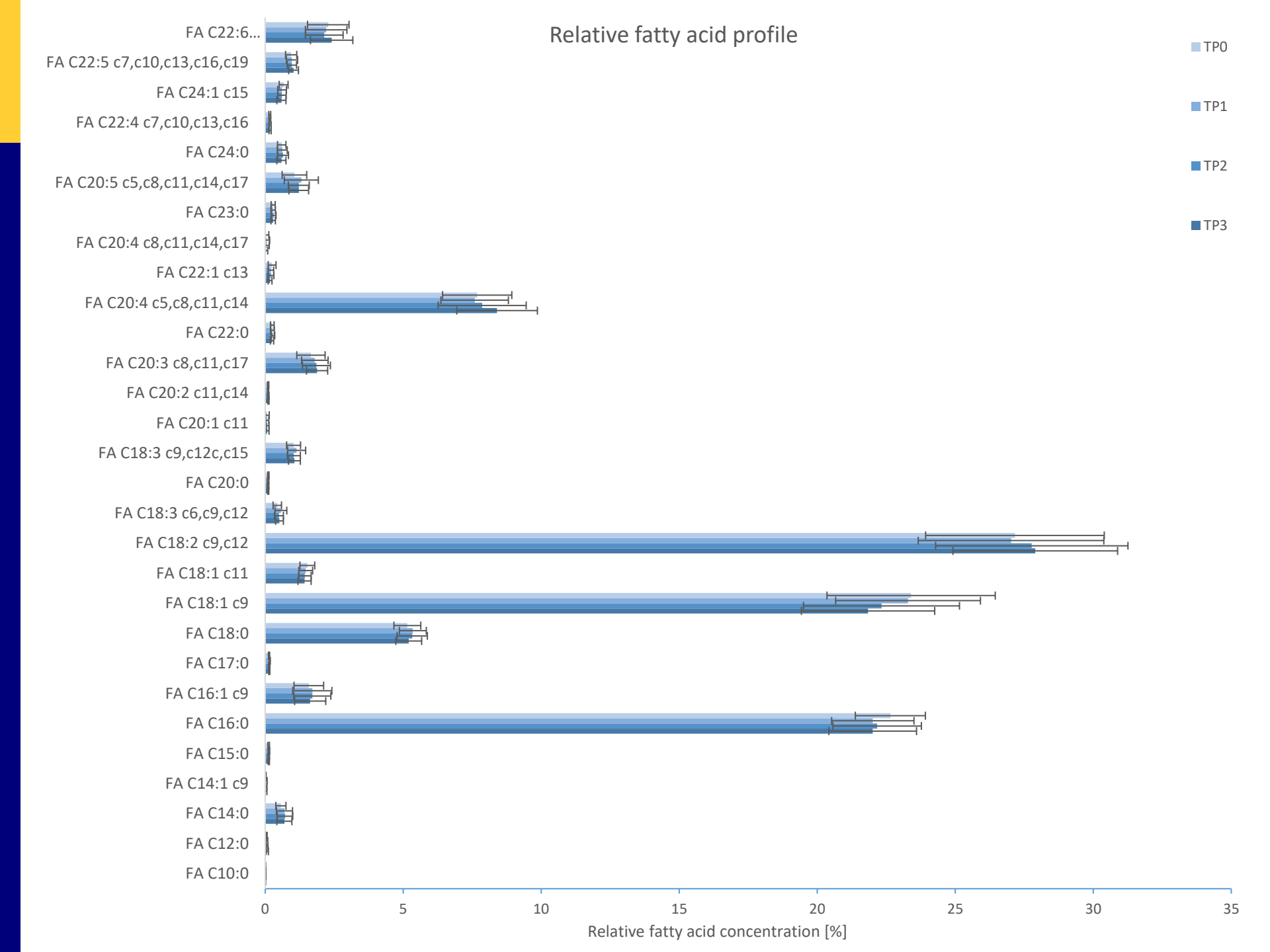
Most interesting finding were alterations in:

- 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))

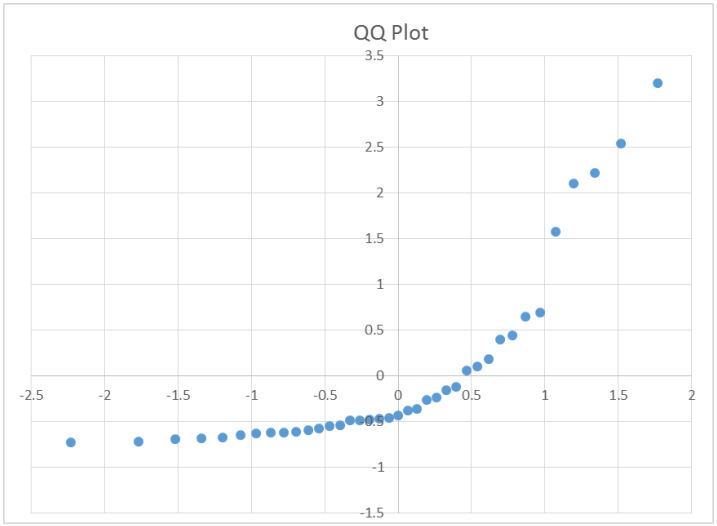



Relative fatty acid profile
TP0
TP1
TP2
TP3
FA C22:6...
FA C22:5 c7,c10,c13,c16,c19
FA C24:1 c15
FA C22:4 c7,c10,c13,c16
FA C24:0
FA C20:5 c5,c8,c11,c14,c17
FA C23:0
FA C20:4 c8,c11,c14,c17
FA C22:1 c13
FA C20:4 c5,c8,c11,c14
FA C22:0
FA C20:3 c8,c11,c17
FA C20:2 c11,c14
FA C20:1 c11
FA C18:3 c9,c12c,c15
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
0
5
10
15
20
25
30
35
Relative fatty acid concentration [%]

Fatty acid method: statistical evaluation

Pre Analysis

- *Gausian 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
    - α = 5 %
- Wilcoxon signed rank test
  - Test for two Groups
    - Timeseries → correction for significance
    - Bonferroni correction α = 5 %→ 1.25 %
    - Paired data
    - No gaussian distribution

Fatty acid method: statistical evaluation

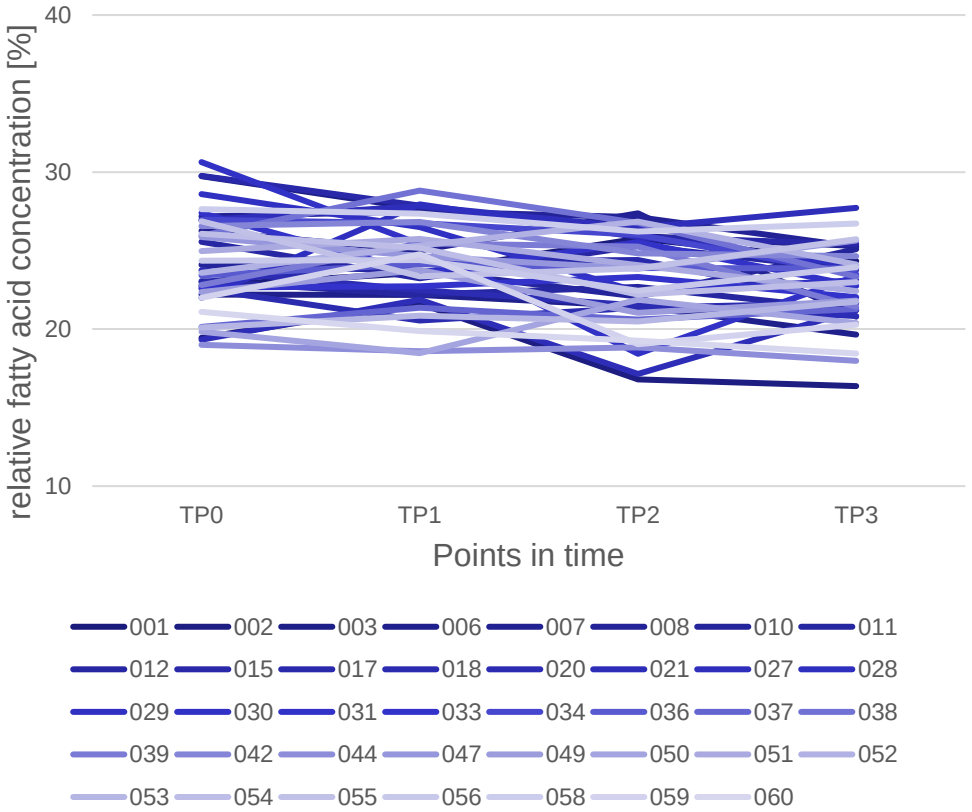
Analyte	Trend	Quade test p-value		Wilcoxon signed rank test p-vlaue				
		TP0-TP1-TP2-TP3		TP0/1	TP0/2	TP0/3	TP1/2	TP2/3
FA C12:0	▲	**0.005214**		**0.009348**	**0.002752**	**0.068240**	**0.451200**	**0.230200**
FA C14:0	▲	**0.000001**		**0.000002**	**0.000001**	**0.000219**	**0.624000**	**0.493900**
FA C16:0	(▼)	0.022240		0.027830	0.017500	0.037230	0.387200	0.735300
FA C17:0	∩	**0.001951**		**0.001468**	**0.538700**	**0.798500**	**0.019710**	**0.485200**
FA C18:1 c9	▼	**0.000059**		**0.714500**	**0.014880**	**0.000129**	**0.012610**	**0.117200**
FA C18:3 c6,c9,c12	▲	**0.000531**		**0.000068**	**0.006540**	**0.004289**	**0.023930**	**0.714500**
FA C18:3 c9,c12c,c15	(∩)	0.049430		0.018950	0.928500	0.538700	0.052570	0.538700
FA C20:3 c8,c11,c17	▲	**0.003908**		**0.038580**	**0.000841**	**0.001246**	**0.084850**	**0.884800**
FA C20:4 c5,c8,c11,c14	▲	**0.000175**		**0.511600**	**0.633800**	**0.000495**	**0.204100**	**0.001635**
FA C22:1 c13	▼	**0.001822**		**0.006844**	**0.052570**	**0.001917**	**0.756200**	**0.224800**
FA C20:5 c5,c8,c11,c14,c17	▲	**0.010380**		**0.008191**	**0.018950**	**0.024860**	**0.357400**	**0.863100**
FA C24:0	(∩)	0.010190		0.418500	0.066100	0.653700	0.050830	0.027830
FA C24:1 c15	▼	**0.003966**		**0.009348**	**0.011590**	**0.005187**	**0.950500**	**0.162500**
FA C22:6 c4,c7,c10,c13,c16,c19	∪	**0.004862**		**0.120600**	**0.017500**	**0.224800**	**0.246900**	**0.000219**

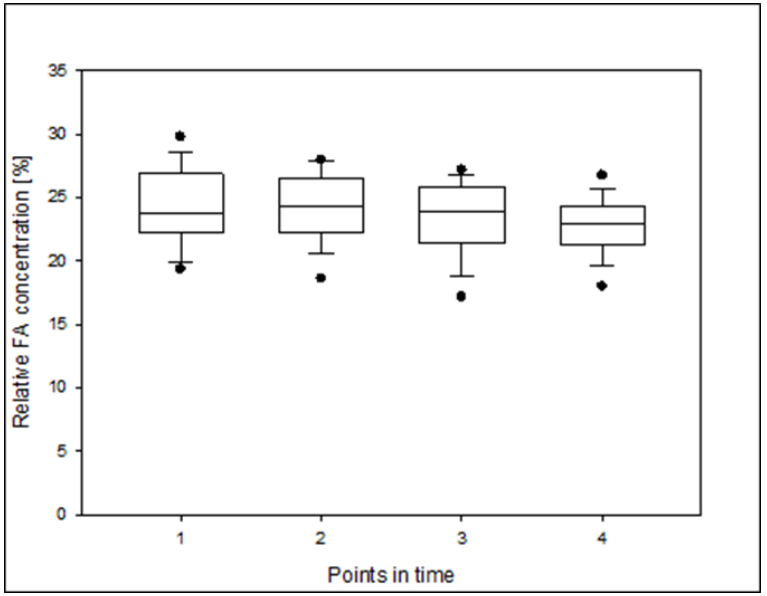
Findings:

- Increased levels in saturated and polyunsaturated fatty acids
- Decreased levels monounsaturated
- Recovery of certain FA after increase or decrease (unspecific)

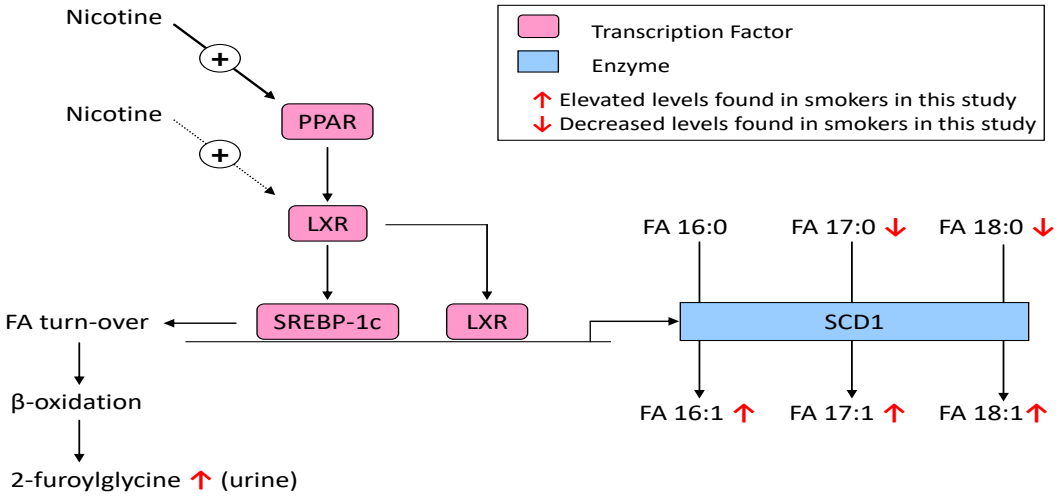
Fatty acid method: statistical evaluation
FA C18:1 c9

	TP0 [%]	TP1 [%]	TP2 [%]	TP3 [%]
Mean	24.27	24.19	23.19	22.71
Median	23.75	24.29	23.84	22.99
SD	3.05	2.61	2.87	2.39





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 succesfully 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 cids)
- ➢ 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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ABF

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- Dr. Daniel Mueller
- ABF Team

Thank you for your attention

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