„Methodology and application of a non-targeted metabolome platform for biological samples of smokers and nonsmokers"

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Overview

Theoretical section

- Introduction to Metabolomics
- Objectives

Experimental section / Results

- Study design
- GC-TOF-MS: Analytical workflow for untargeted biomarker identification
- Fatty acids in plasma of smokers and non-smokers
- Plasma phospholipid analysis by HILIC-MS/MS

Conclusion

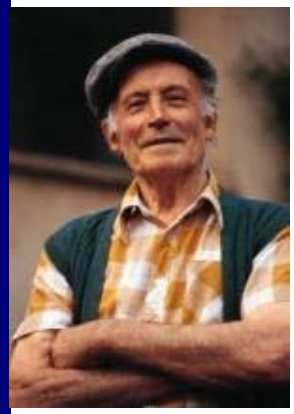
Metabolomics definition

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

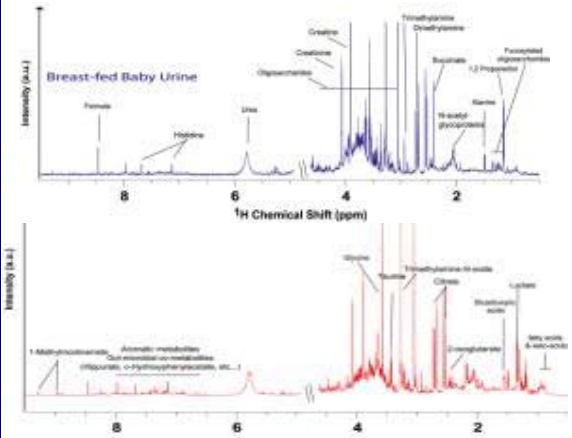
- **Metabolomics** is the study of the entire set of small molecules in a biological sample

    - Identification
    - Quantification
    - Validation

Why metabolomics?

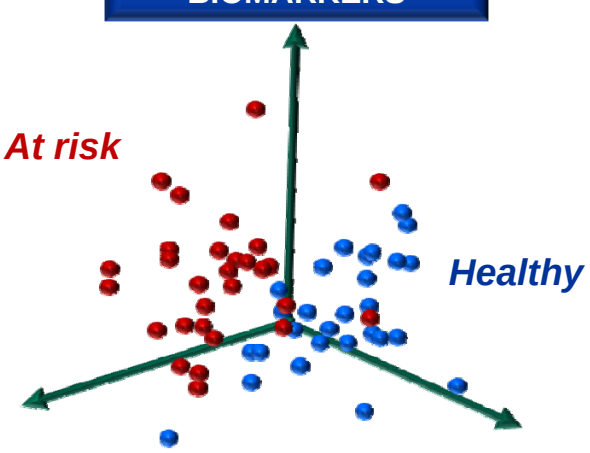


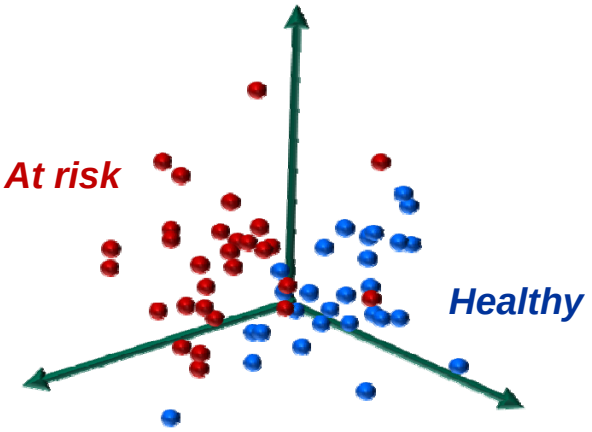


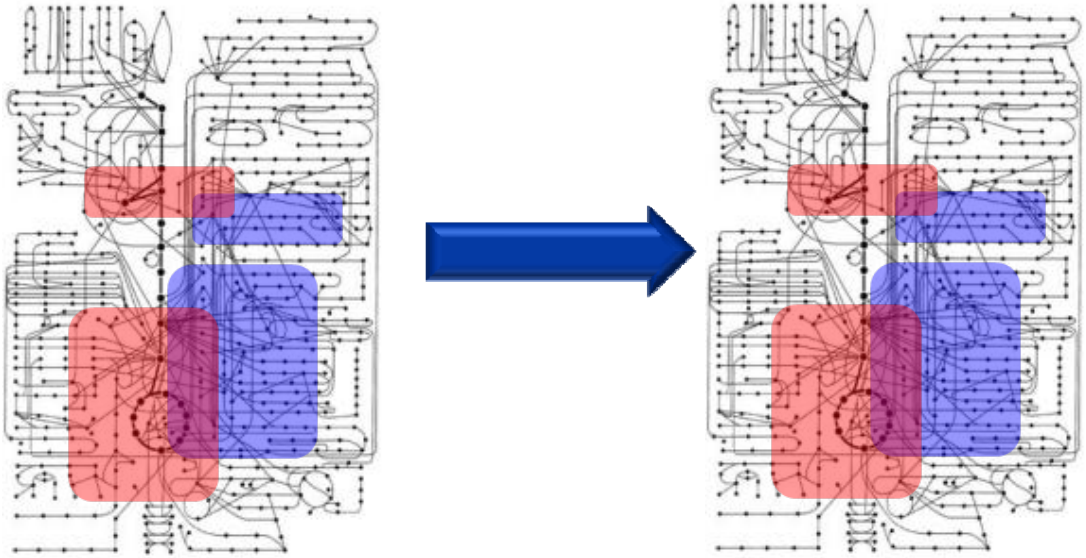




BIOMARKERS







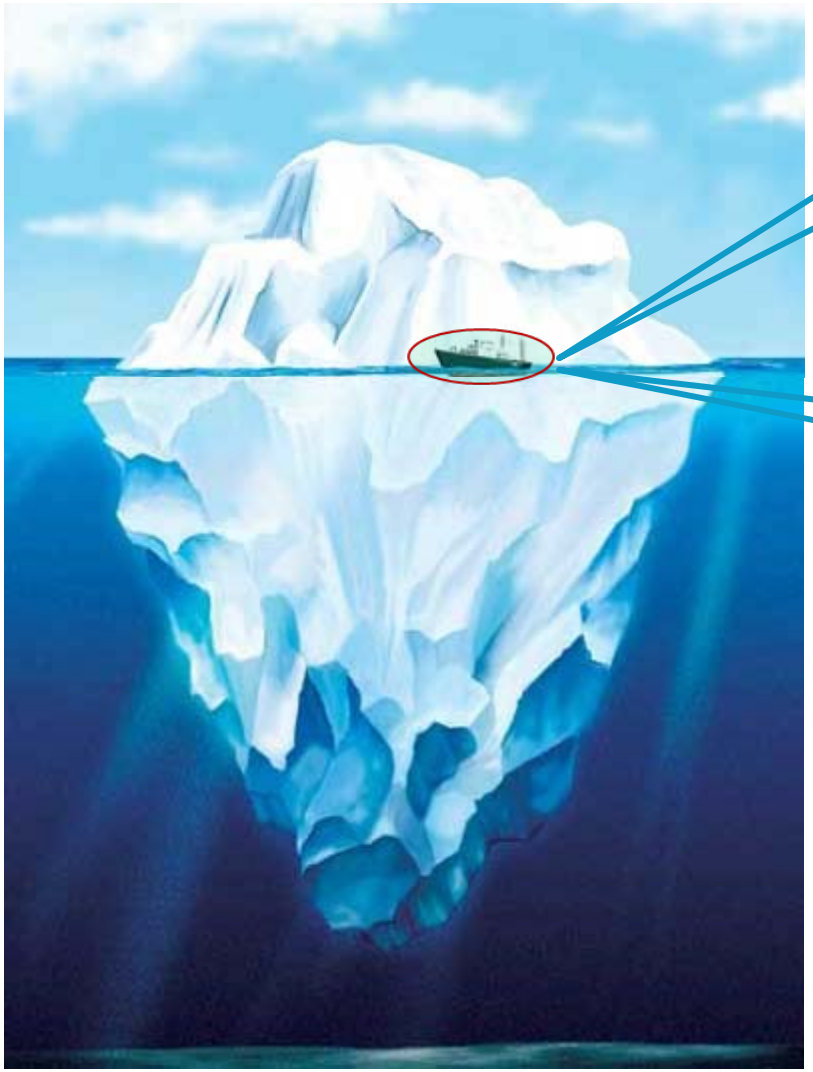
Differences in metabolic pathways

Measuring the metabolic phenotypes: the size of the problem

The Metabolome

Holistic

Targeted



We have a problem !

Can we identify the root cause?

System level

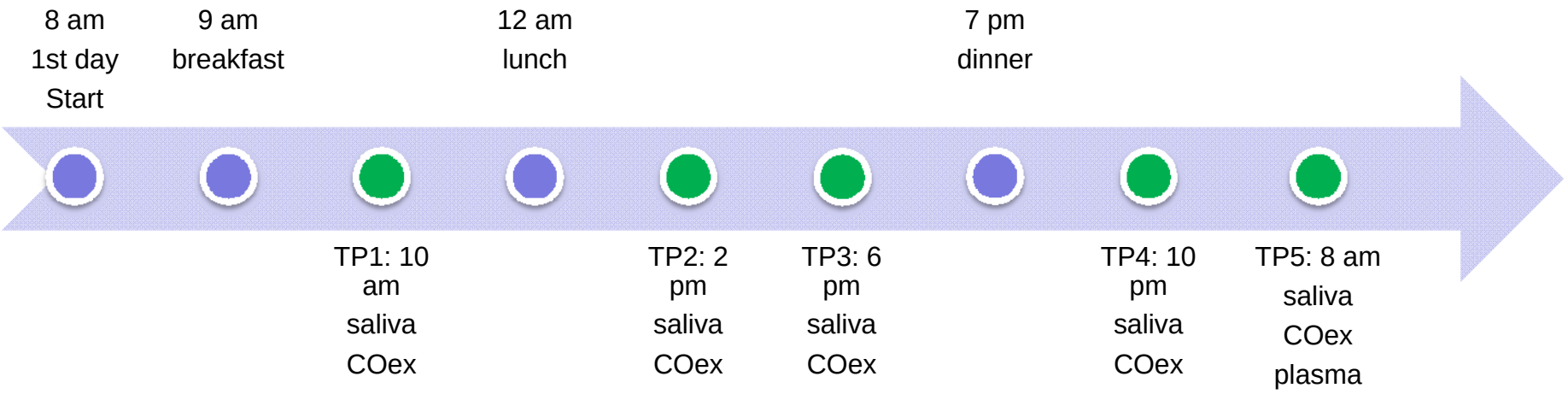
Holistic profile

- *Homeostatic status*
- *Surrogate biomarkers*

Targeted quantitative profiles

- *Lipidomics*

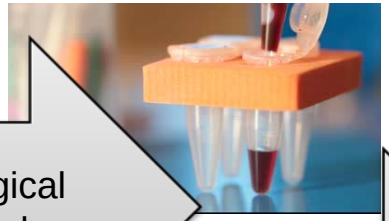
Study design (25 S, 25 NS)





- 24 h-urine sample from each subject was made up from aliquots of 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*

GC-TOF-MS: Analytical workflow for the untargeted biomarker identification



Biological Sample

Urine,
Plasma,
Saliva

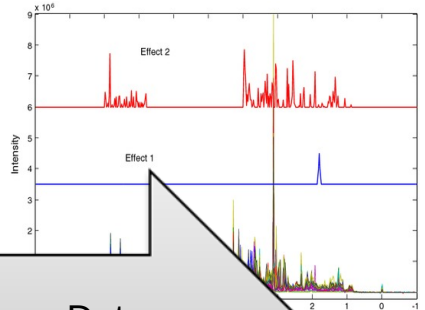
Sample clean-up

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



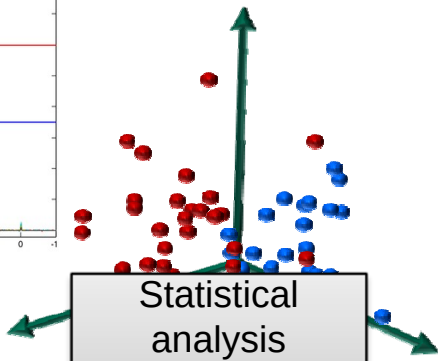
MS Analysis

- 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

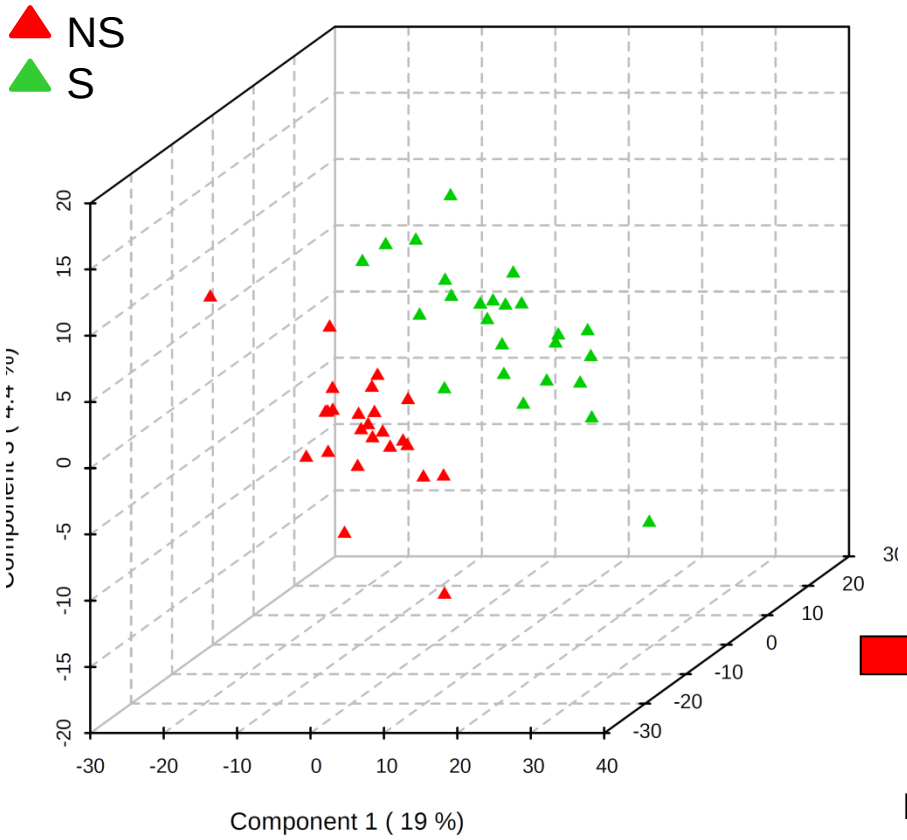


Statistical analysis & Target hit identification

Statistics: PLS-DA
Mann-Whitney –U test, fold change
Identification:
Deconvolution,
Databases (NIST, Golm)
Reference compounds

Metabolic fingerprinting (untargeted)

- Fasted (12h) EDTA-plasma samples were analyzed by untargeted metabolic screening using GC-TOF-MS
- Automated data analysis with Mzmine
- Statisctical analysis by PLS-DA (MetaboAnalyst) and Mann-Whitney U test.

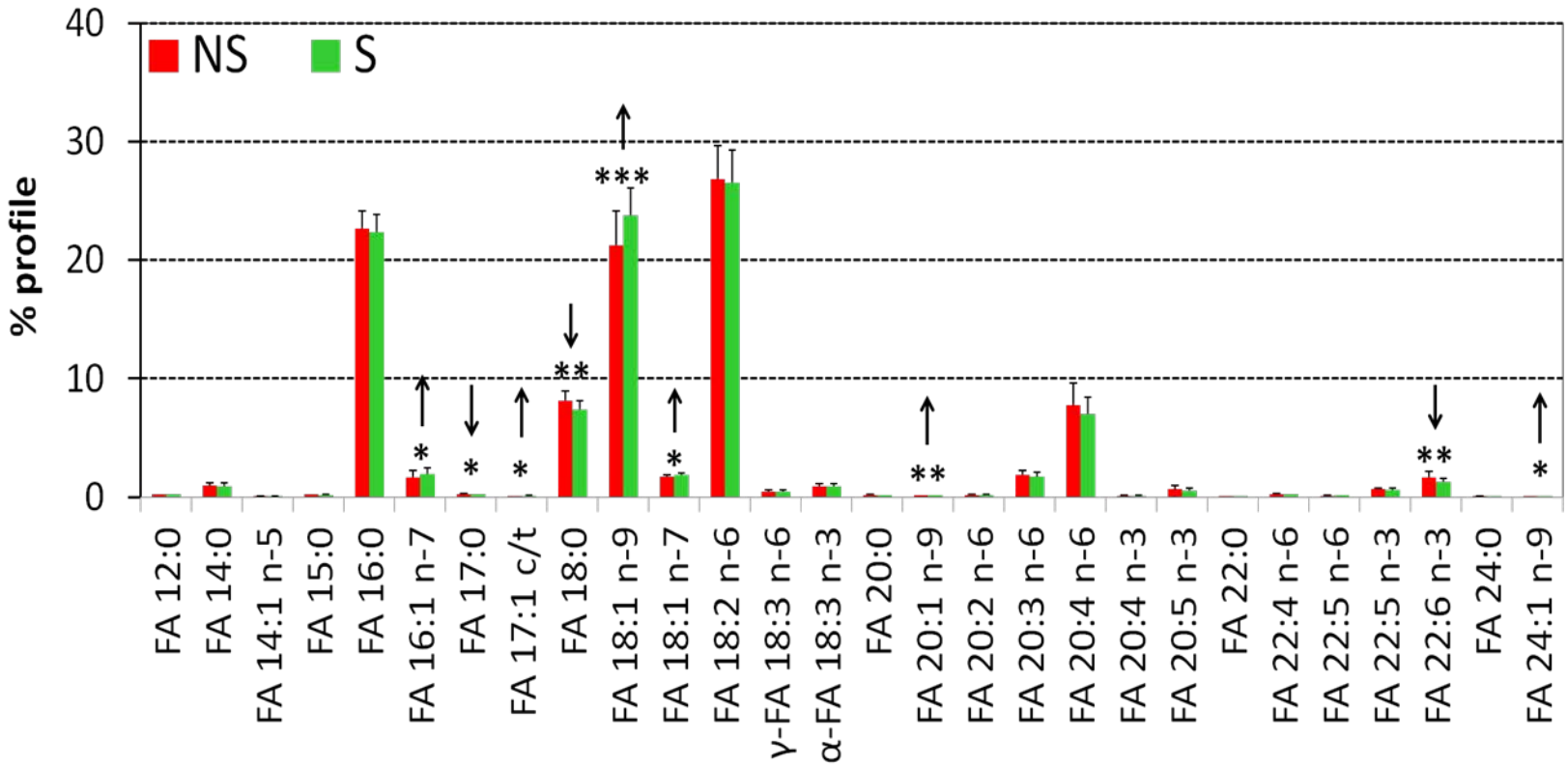


- Clear separation of S and NS due to altered metabolite levels
- 6 significantly altered metabolites in smokers' plasma ($p<0.05$) could be identified
- Palmitoleic acid (16:1) and oleic acid (18:1) were significantly increased in smokers

Fatty acid metabolism seems to be affected by smoking

PLS-DA score plot for plasma of smokers (N=25) and non-smokers (N=25)

Targeted fatty acid analysis by GC-FID

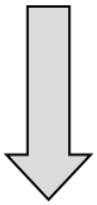


- Results of the untargeted screening were confirmed for palmitoleic acid and oleic acid and showed further alterations in the FA profile
- Mainly MUFAs were increased in smokers, while the saturated fatty acids FA 17:0, FA 18:0 and the FA 22:6 were signifcantly decreased.

Conclusion of the fatty acid analysis

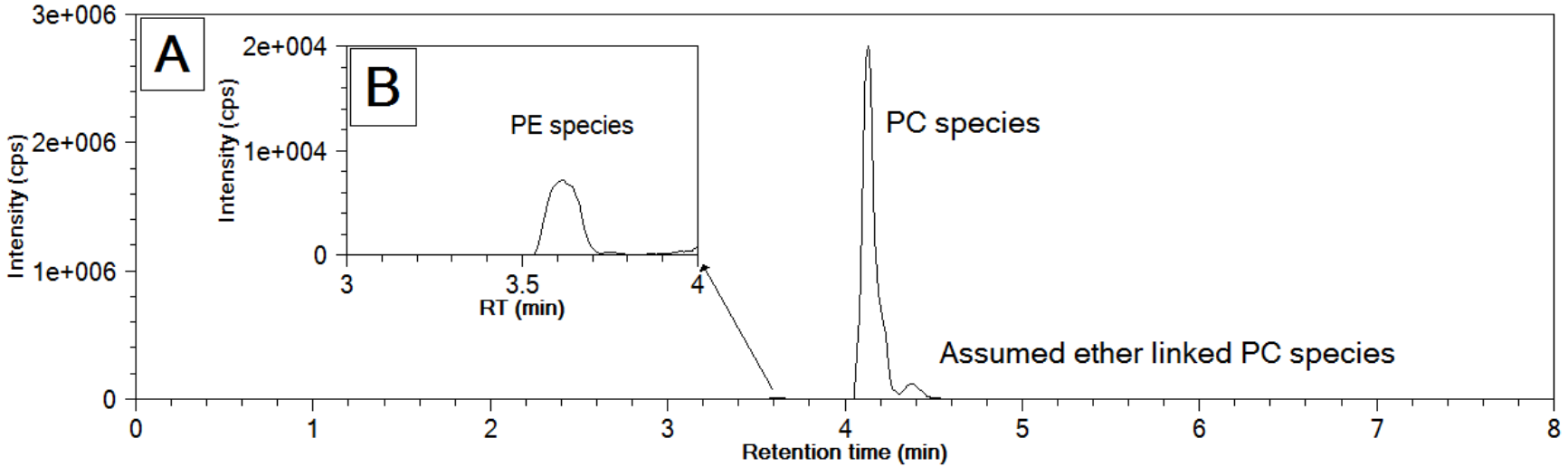
- We found a significant alteration in the fatty acid profile of smokers plasma.
- The proportions of MUFA species, including FA 16:1 n-7, FA 17:1, FA 18:1 n-9, FA 18:1 n-7, FA 20:1 n-9 and FA 24:1 n-9, were significantly increased
- The saturated FAs 17:0 and 18:0 the polyunsaturated fatty acid (PUFA) FA 22:6 n-3 were significantly decreased ($p < 0.031$).

Since FAs are mainly stored as phospholipids in human plasma



Targeted, quantitative analysis of the major phospholipids PC and PE using HILIC-MS/MS

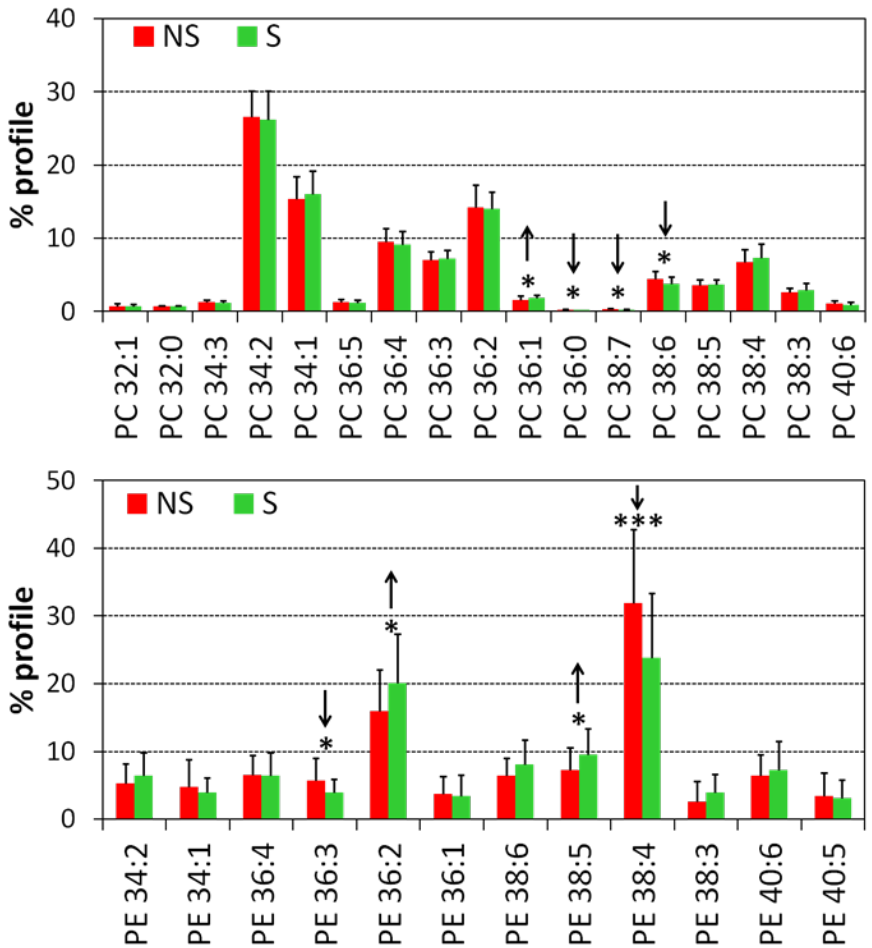
Phospholipid analysis by HILIC-MS/MS



- Development and validation of a HILIC-MS/MS method
- Liquid/liquid extraction with MeOH/CHCl3
- Baseline separation of PE and PC
- Simultaneous quantification of 39 PC and 40 PE species
- Coelution of individual lipid species within each class
- Runtime: 8 min

Robust, fast and sensitive phospholipid profiling using HILIC-MS/MS

Results of the phospholipid analysis

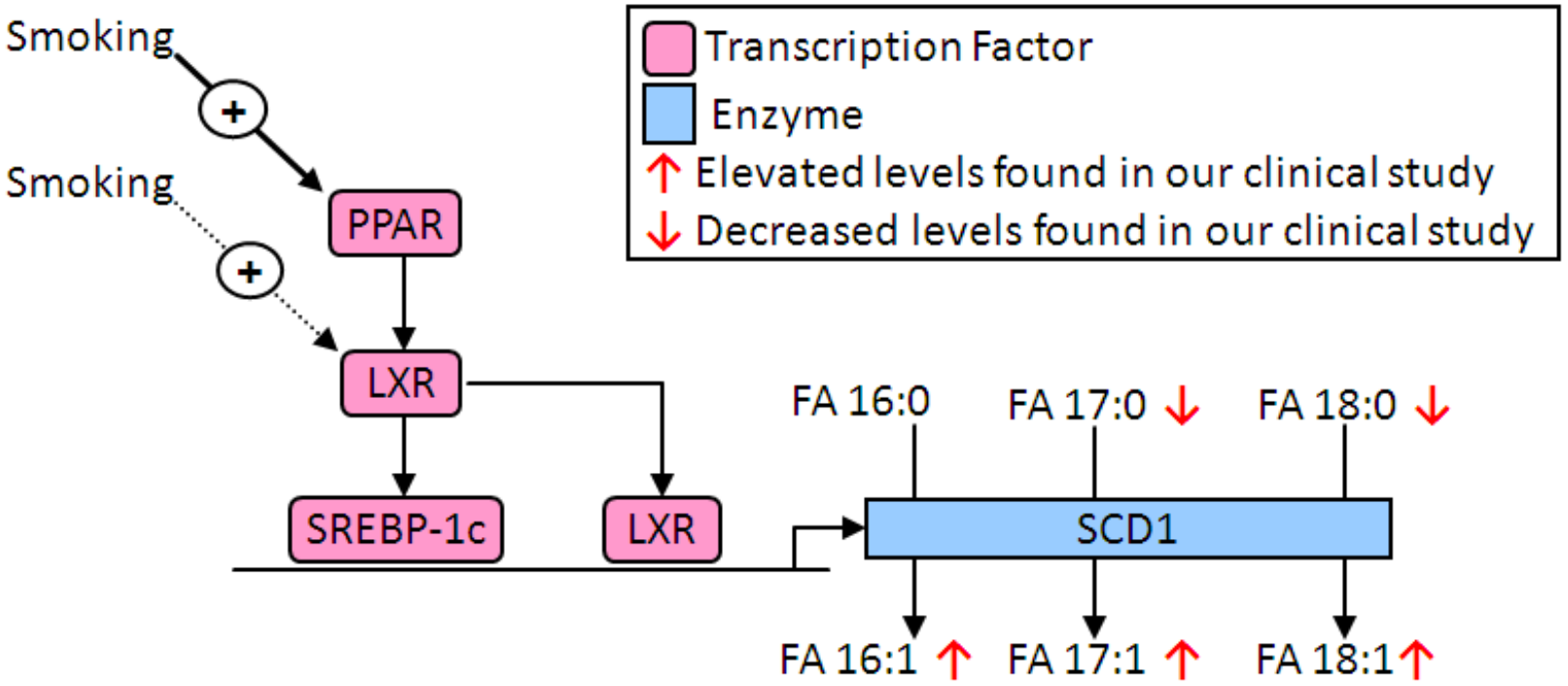


The total relative phospholipid profile revealed that:

Assumed FA composition of significantly altered Phospholipids	
•*Increased*	
PC 36:1	18:1/18:0; 16:0/20:1
PE 36:2	18:1/18:1
PE 38:5	18:1/20:4
Decreased	
PC 36:0	18:0/18:0; 20:0/16:0
PC 38:7	18:1/22:6
PC 38:6	16:0/22:6
PE 38:4	18:0/20:4

- PC 36:1 was significantly increased, whereas PC 36:0, PC 38:7 and PC 38:6 were significantly decreased.
- PE 36:2 and PE 38:5 were significantly increased, whereas PE 36:3 and PE 38:4 were significantly decreased.

Hypothesized mechanism of action

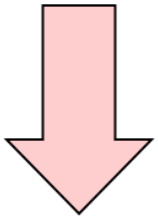


- Smoking triggered activation of the transcription factor peroxysome proliferator activated receptor (PPAR)
- Activation of the transcription factor liver X receptor (LXR) and subsequently the sterol regulatory element binding protein-1c (SREBP-1c)
- Up-regulation of steoryl-Coenzyme A desaturase (SCD1) by LXR could lead to elevated MUFA in smokers plasma

[1] *Yanagita et al., Cell Im, 2012;* [2] *Amoruso et al., Life Sci, 2007*; [3] *Sticozzi et al., Int J Mol Sci, 2010*

Summary and conclusion

- Metabolic fingerprinting revealed altered fatty acids levels in S compared to NS.
- GC-FID FA analysis confirmed these results and showed an increase of additional MUFA.
- These altered FA profiles were also reflected in the PC and PE species profiles, measured by a newly developed and validated HILIC-MS/MS method.



MS-based Metabolomics is particularly well suited to measure endogenous metabolites of physiological regulatory processes of living organisms.

Metabolomics could offer thus the possibility to generate a new generation of biomarkers of health status and smoking effects at the system level

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- Dr. Christian Degen

Sponsor

- Imperial Tobacco



Thank you for your attention

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Backup (1): Quality control samples (QCs)

- Urine/plasma pool spiked with 39 compounds, from different chemical classes, to 50 µM
- 6 QCs were prepared and measured randomly within the batch
- Analysis without IS correction (raw area)

Average % CV of 39/36 components in 6 QC Samples

	% CV	
	Urine	**Plasma**
Mean ± SD	7.3 ± 3.7 (N=39)	10.0 ± 8.5 (N=36)
> 20 %	0	3

Backup(2): Urine Metabolomics

Compound	Significance	Fold change (S/NS)	Identification	Exogenous compound	Endogenous pathway
1,2-Propanediol	**	1.6	Spectrum, RI	TSC	
Glycerol	**	1.4	Standard	TSC	
Levulinic acid	***	2.1	Spectrum, RI	TSC	
Quinic acid	**	2.8	Standard	TSC, pyrolysis of lignin	
3-OH-Pyridine	***	1.9	Standard	TSC, metabolite of pyridin	
Hydroquinone (1	***	1.5	Standard	TSC, Metabolite of benzene, phenol, tyrosine	
Mandelic acid	***	2.2	Standard	TSC, styren metabolite	
Methoxyphenol	***	2.3	Standard	TSC	
4-OH-Cyclohexanecarboxylic acid	**	2.3	Spectrum, RI	TSC?	
2(N-Furoyl)-Glycine	***	2.7	Standard	TSC, metabolite of furfural	Detoxification, lipid metabolism
L-Alanine	**	-1.4	Standard		Detoxification, energy metabolism
p-OH-Phenyl-acetic acid/ Glutamic acid	*	1.6	Standard		Detoxification, energy metabolism
2-Ketoglutaric acid	**	-1.5	Spectrum, RI		Detoxification, energy metabolism
Pyruvat	*	-1.3	Standard		Detoxification, energy metabolism
Uracil	*	-1.3	Spectrum, RI		Detoxification
Glucuronic acid	**	1.3	Standard		Detoxification
Gluconic acid	**	1.4	Spectrum, RI		Oxidative stress
Glucaric acid	*	1.3	Spectrum, RI		Oxidative stress
3,4-Dihydroxybutyric acid	**	1.2	Spectrum, RI		

*p < 0.05, **p < 0.01, ***p < 0.001

1) Lee et.al., J CHROMATOGR, 1993

TSC: Tobacco specific compound

Backup(3): Saliva Metabolomics

Compound	Significance	Fold change (S/NS)	Identification	Exogenous compound	Endogenous pathway
Hexanoic acid	***	1.53	Replib	TSC	
4-Hydroxyphenylethanol	***	4.87	Mainlib	Derivative of phenylethyl alcohol (TSC), diet	
Tyramine	***	22.25	Standard	TSC, diet	Amino acid metabolism
Cadaverine	**	1.89	Mainlib	Diet	Amino acid metabolism
N-Acetylglucosamine	*	1.97	Mainlib		Amino sugar metabolism
Adenine	**	1.65	Golm_quad	Diet	Purine metabolism
Uridine	*	1.51	Mainlib		Pyrimidine metabolism
Adenosine	***	2.36	Standard		Purine metabolism
Guanosine	*	1.86	Mainlib		Purine metabolism
Glucose-6-phosphat	**	-7.69	Mainlib/ Standard		Energy metabolism
Glycerol-3-phosphat	**	-1.82	Replib		Lipid metabolism, Energy metabolism
Malic acid	*	-1.52	Mainlib		Energy metabolism
Benzenepropanoic acid	*	-1.72	Mainlib		Tyrosine metabolism

*p < 0.05, **p < 0.01, ***p < 0.001 Mann-Whitne-U Test

TSC: Tobacco smoke compound

Backup(4): Data processing workflow

