„Untargeted metabolomic profiling in saliva of smokers and non-smokers by a validated GC-TOF-MS method"

Max Scherer, Daniel Mueller, Nikola Pluym, Gerhard Scherer

ABF, Analytisches-Biologisches Forschungslabor GmbH, München, Germany

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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
- Targeted analysis of tyramine in saliva of smokers and non-smokers

Summary and Conclusion

Metabolomics definition

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

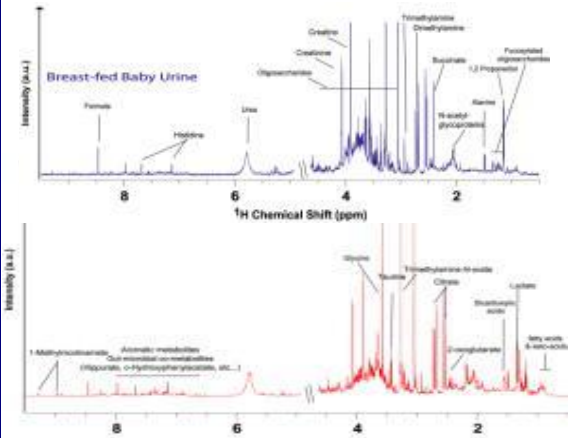
- **Metabolomics** is the study of the entire set of small molecules in a biological sample

    - Identification
    - Quantification
    - Validation

Why metabolomics?

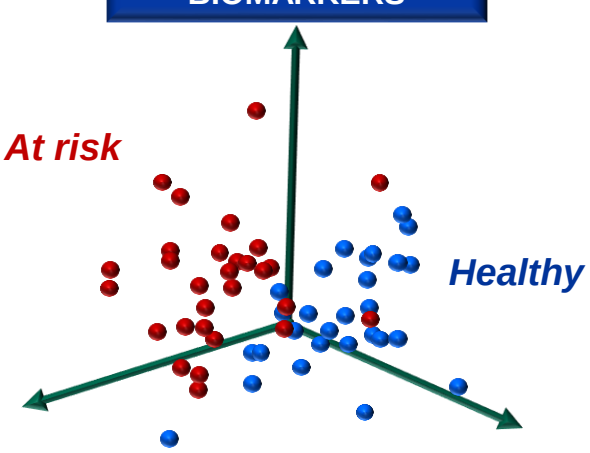


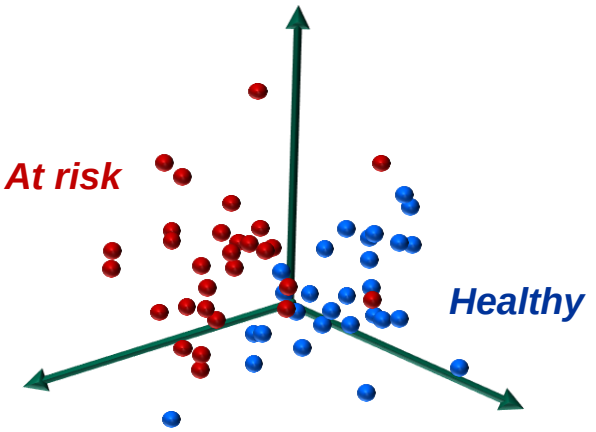


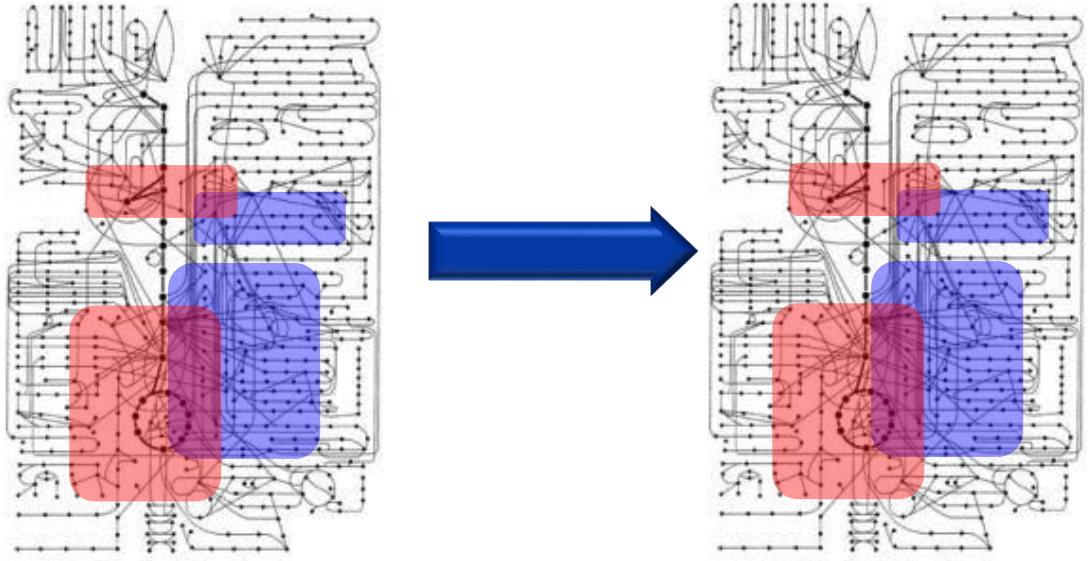




BIOMARKERS







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

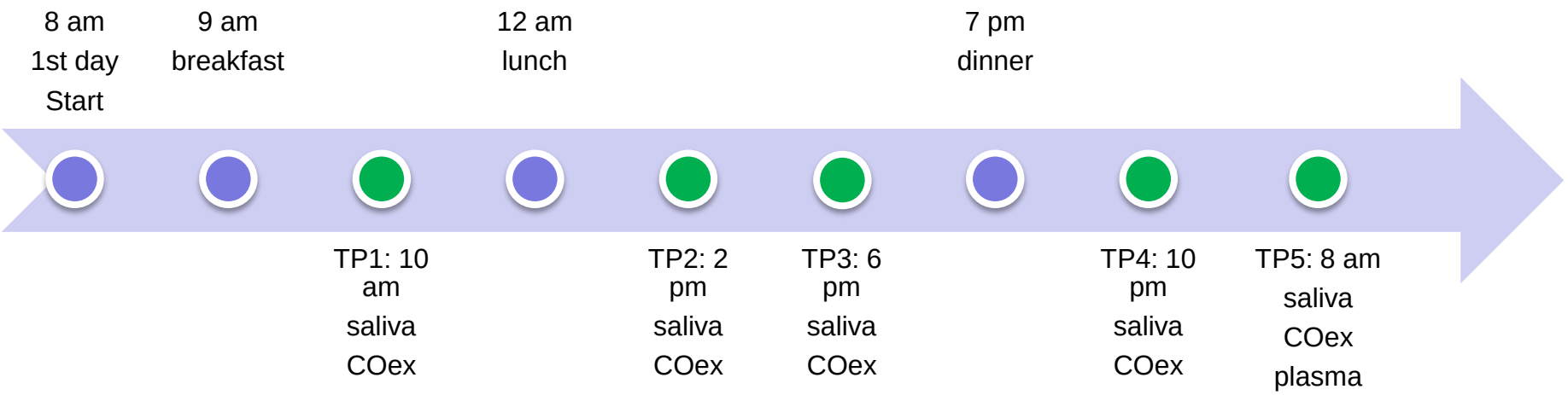
Holistic profile

- *Homeostatic status*
- *Surrogate biomarkers*

Targeted quantitative profiles

- *Lipidomics*

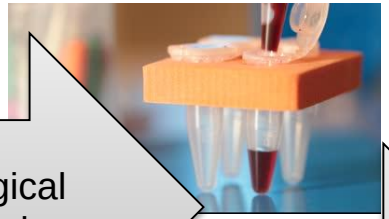
Study design (25 S, 25 NS)





- 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

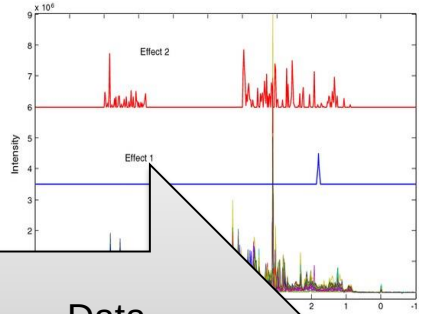
Sample clean-up

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



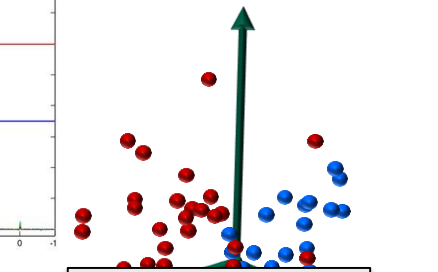
MS Analysis

- 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

Mueller et al. JPR, Dec 2013
Müller et al. JCB, Mar 2014

Saliva – Univariate statistics

- Mann-Whitney-U-Test

	Peaks in peakliste from *MZmine*
sum	14894
p < 0.05	720
p < 0.01	297
p < 0.001	60

	ID (m/z / RT)	p-value (Mann-Whitney-U-Test)	component
1	249.07/17.091	2.3E-10	**3-OH-cotinine**
2	338.13/17.915	4.1E-08	**tyramine**
3	175.09/17.914	1.3E-07	**tyramine**
4	339.14/17.915	1.9E-07	**tyramine**
5	174.09/17.916	2.0E-07	**tyramine**
6	176.08/17.911	2.1E-07	**tyramine**
7	179.07/17.915	3.3E-07	**tyramine**
8	161.55/17.916	5.7E-07	**tyramine**
9	100.03/17.916	5.9E-07	**tyramine**
10	177.08/17.916	7.1E-07	**tyramine**
11	340.13/17.916	9.7E-07	**tyramine**
12	60.01/17.918	1.7E-06	**tyramine**
13	59.01/17.92	2.0E-06	**tyramine**
…			
51	98.03/14.884	6.2E-04	**cotinine**
…			

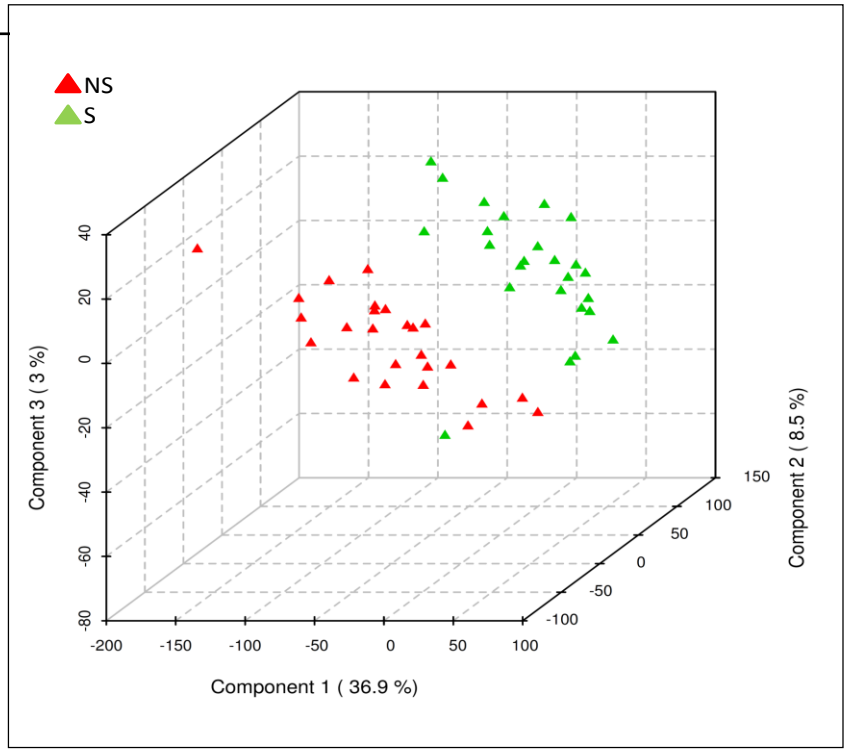
Mueller et al. JPR, Dec 2013

Saliva – Group separation

- Tobacco specific biomarkers like cotinine and 3-OH-cotinine were removed

Rank	ID (m/z / RT)	Compound	P-Value (MWU-Test)
1	174.09/17.916	**tyramine**	***
2	73.03/17.92	**tyramine**	***
3	59.01/17.92	**tyramine**	***
4	86.04/17.923	**tyramine**	***
5	74.03/17.921	**tyramine**	***
6	44.98/17.923	**tyramine**	***
7	75.54/17.343	**cadaverine**	**
8	179.07/14.058	**4-hydroxyphenylethanol**	***
9	75.01/17.924	**tyramine**	***
10	131.05/17.924	**tyramine**	***
…			

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



Mueller et al. JPR, Dec 2013

Saliva – Potential targets

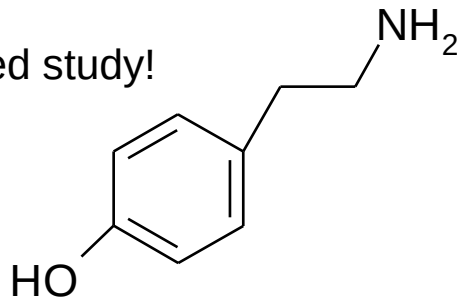
Compound	Significance	Fold change (S/NS)	Identification	Exogenous compound	Endogenous pathway
Hexanoic acid	***	1.53	Replib	TSC	
4-Hydroxyphenylethanol	***	4.87	Mainlib	Derivative of phenylethyl alcohol (TSC), diet	
Tyramine	***	22.25	Standard	TSC, diet	Amino acid metabolism
Cadaverine	**	1.89	Mainlib	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
		(NS/S)			
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

TSC: Tobacco smoke compound

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

Mueller et al. JPR, Dec 2013

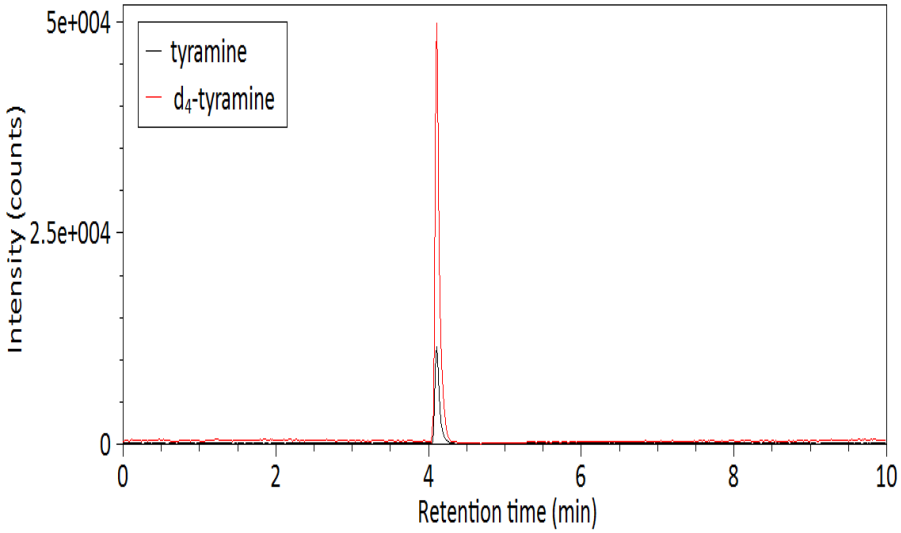
Tyramine

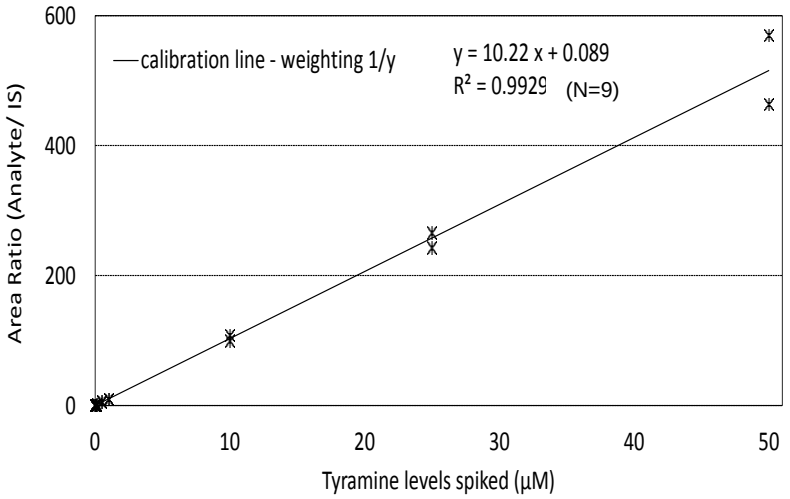


- Dietary component (chocolate and fermented foods) – diet controlled study!
- Tobacco constituent (low concentration)
- Endogenous origin: decarboxylation of tyrosine
- Monoamine oxidase A and B, a enzyme which degradates tyramine, is inhibited by smoking[1]
- Tyramine is an indirect sympathometicum and acts as a releasing agent for catecholamines
  (dopamine, noradrenaline, adrenaline)
- Tyramine can be converted to dopamine by CYP2D6[2]
- Tyramine can cause migraine, hypertension, increased heart rate, elevated blood sugar levels…[2,3,4]

1)Fowler et a., Proc Natl Acad Sci U S A, 1996 *2)Benedetti et al., Fundam Clin Pharm, 2007*
3) Herbert, Br J Pharmacol, 2008 *4) VanDenBerg, J Clin Pharmacol, 2003*

Tyramine analysis by HILIC-MS/MS

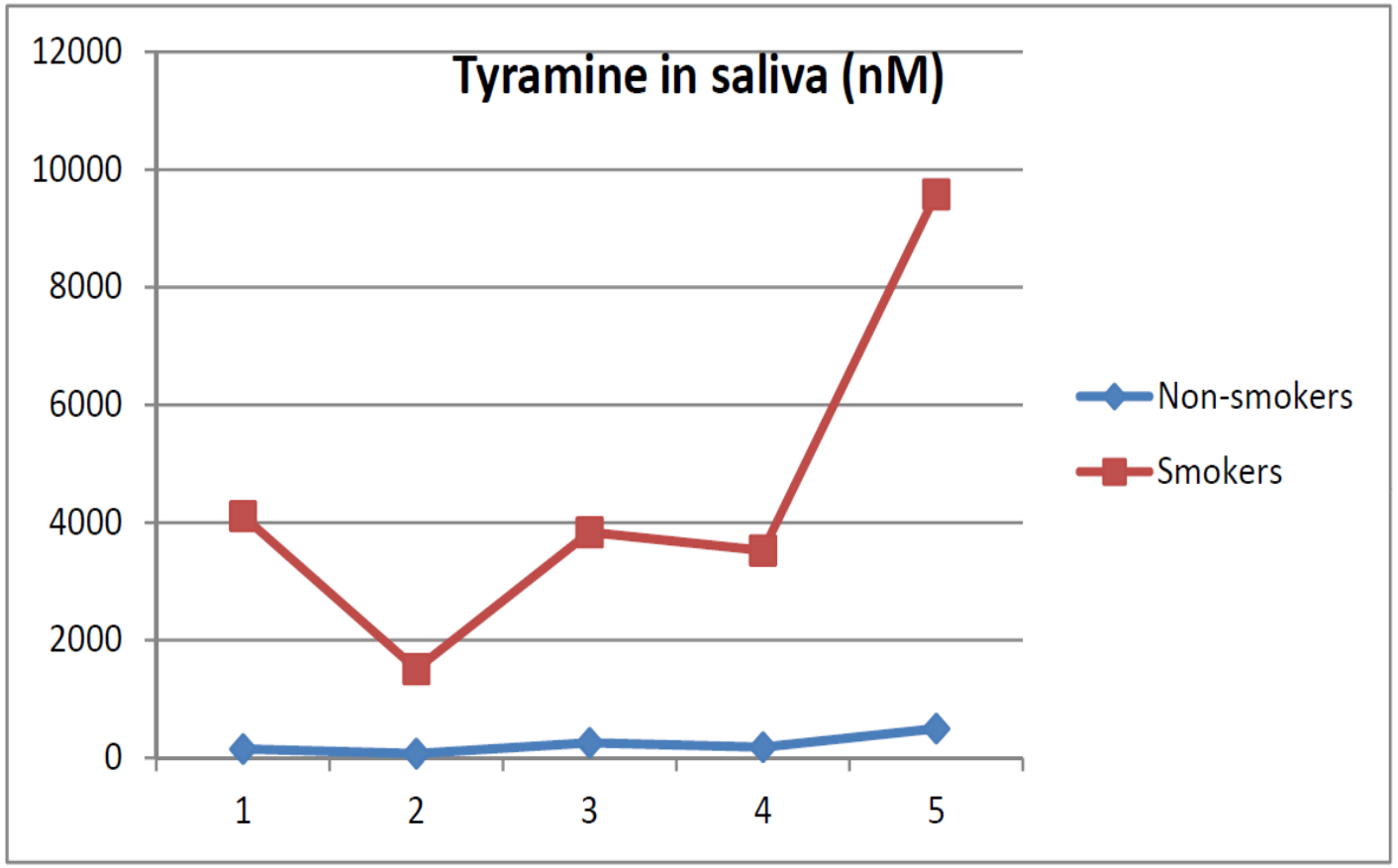




- Development and validation of a targeted HILIC-MS/MS method for the quantification of tyramine in various biological matrices (plasma, urine, Saliva)
- Centrifugation to remove mucins
- Protein precipitation with MeOH
- Centrifugation, supernatant transferred to autosampler vial for LC-MS/MS analysis
- Quantifiaction using an authentic reference standard and d_4-tyramine as IS
- ESI+; injection volume: 2µl; runtime: 10 min (gradient elution)

Results - Tyramine analysis TP1-TP5 (25 S, 25 NS)

Tyrami (p)



8 am
Start

saliva COex

saliva COex

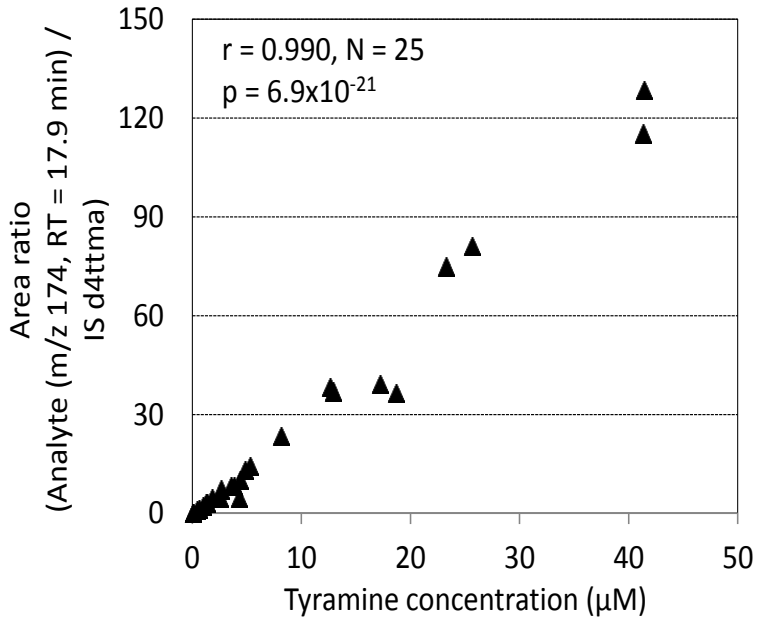
saliva COex

saliva COex

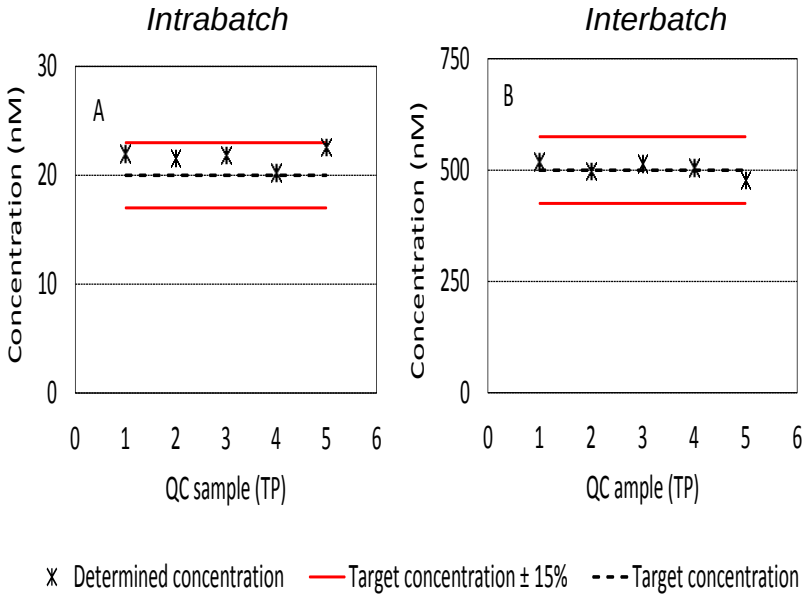
saliva COex plasma

Saliva - Validity check of the results

Correlation of tyramine levels, determined with different methodologies



QC samples for targeted tyramine analysis by HILIC-MS/MS (Intra- and inter-bactch precision)



Summary

- Metabolic fingerprinting revealed altered metabolite levels in S as compared to NS.
- The elevation of some salivary metabolites can be explained by an uptake from tobacco smoke (e.g. hexanoic acid, 4-OH-Ph-ethanol) others not (e.g. adenine, adenosine, cadaverine, tyramine).
- From those, tyramine showed by far the highest significane amongst S and NS (74 to 402-fold elevation).
- The elevation of tyramine levels in S could be confirmed by a targeted LC-MS/MS method.
- An increase of tyramine in S by a factor of 74-400 cannot be explained by inhibition of MAO A in the oral cavity, since TP 5 showed the highest amounts.
- Dietary uptake and uptake from tobacco smoke can aslo be excluded.
- We hypothesize that smoking shifts the oral flora activity to increased protein degradation leading to increased decarboxylation rate in smokers as compared to non-smokers.
- Whether the elevated tyramine levels in smokers have any neuroactive effects remains to be determined

Conclusion

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.

We could demonstrate that saliva, as noninvasively accesible body fluid, is a suitable biological matrix for investigating the human metabolome.

For additional information please check:

Mueller *DC, Piller M, Niessner R, Scherer M, Scherer G. Untargeted metabolomic profiling in saliva of smokers and on*

Acknowledgment

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- Dr. Nikola Pluym

Sponsor

- Imperial Tobacco



Thank you for your attention

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Backup (1): Quality control samples (QCs)

- Saliva 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 components in 6 QC Samples

	% CV
	Sakiva
Mean ± SD	7.3 ± 3.7 (N=39)
> 20 %	0

Backup(2): Studies from the literature (biogenic amines)

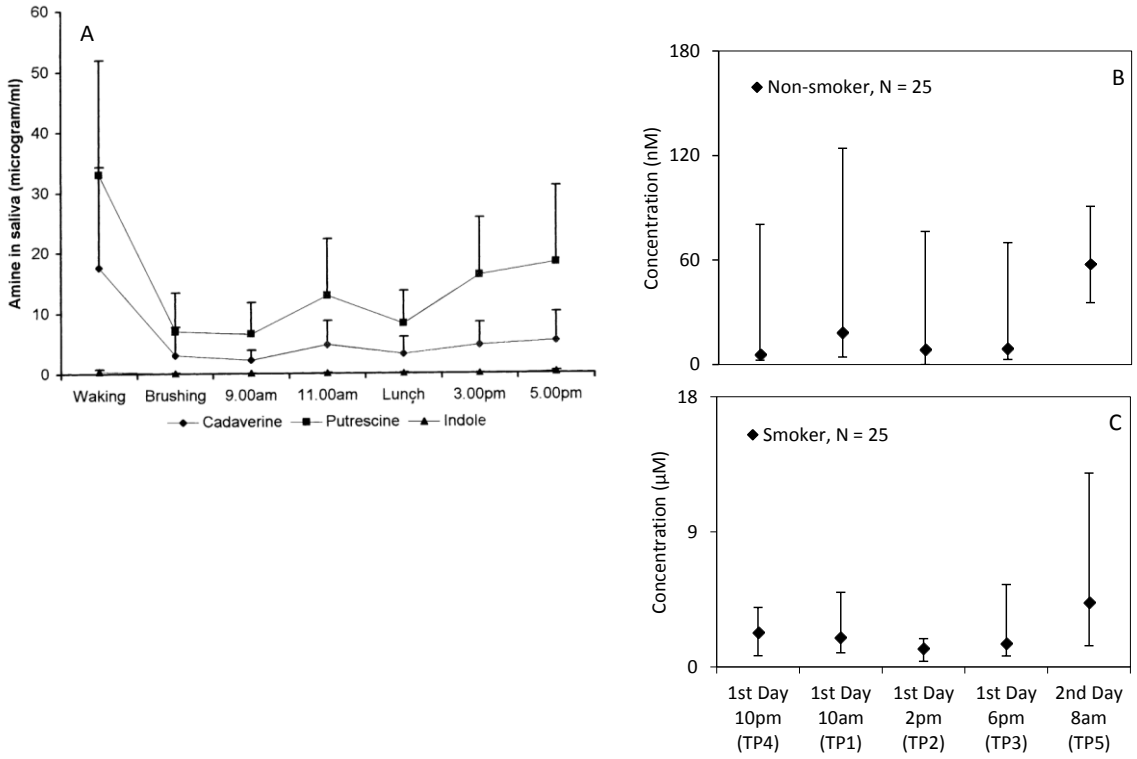


Fig. 50. Time profiles of biogenic amines determined in saliva of healthy human subjects.
A: Biogenic amines in saliva of non-smokers showing the highest levels in the morning before toothbrushing (shown as mean ± SD, taken from Cooke et al. 2003). B and C: Tyramine in smokers and non-smokers showing the highest concentration in the morning of the 2nd study day before toothbrushing (shown as medians with error bars showing 25 and 75 percentile range.
he saliva in the morning of the 1st study day (TP1) was collected after toothbrushing.

3: Data processing workflow

