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Analytical Studies on the Impact of Smoking Cessation on the Metabolic Profile

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Plagiarism Declaration

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I, hereby declare that this Thesis was prepared by me independently only using the references and resources stated. This work has not been part of another examination process. Parts of this work have been published in scientific journals.

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Michael Christoph Göttel

Bad Schussenried, den 26.06.2018

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Abstract

In the present project, a diet controlled clinical study over three months was conducted to investigate the impact of smoking cessation on the metabolic profile of adult men. For this purpose, blood plasma, urine and saliva samples of 60 healthy volunteers were collected prior to and 1 week, 1 and 3 months after smoking cessation. The verification of the subjects' compliance was verified by their carbon monoxide level in exhaled breath as well as their cotinine concentrations in saliva and urine at short intervals during the course of the study. Thirty-nine subjects were found to be compliant over the entire study. Untargeted analysis of plasma, saliva and urine samples of the compliant subjects were performed by means of a GC-TOF-MS platform. In total, 52 metabolites were found to be significantly different prior and post quitting, including 26 in plasma, 20 in saliva and 12 in urine. For most metabolites, alterations upon cessation were in the same direction as differences between smokers and non-smokers observed in a previous study, indicating partial recovery from physiological effects of smoking.

To verify the results from the (untargeted) fingerprinting, targeted methods for the most affected metabolites (fatty acids and amino acids) were developed, validated and applied to the samples of the compliant subjects. The results of the targeted analysis of fatty acids by means of a GC-TOF-MS method were in agreement with the fingerprinting method. Saturated fatty acids and poly-unsaturated fatty acids were found to increase, while monounsaturated fatty acids were observed to decrease after smoking cessation. It is noteworthy that arachidonic acid, a precursor of biological active eicosanoids, was significantly altered in both analytical approaches. Therefore, a targeted method for the quantification of eicosanoids was applied to the samples. A significant decrease was obtained for the prostaglandins $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, thromboxane 2,3-d-TXB_2 and leukotriene E_4 upon quitting smoking, indicating a partial recovery of smoking-related oxidative stress and inflammation.

The targeted and untargeted approach showed that smoking cessation revealed a statistically significant decline of kynurenine, tryptophan and lysine and an elevation of glycine and serine concentrations. Interestingly, these differences were not found between smokers and non-smokers. This observation could indicate that the plasma concentrations of these amino acids are not influenced by tobacco smoke exposure, but factors associated with smoking cessation, e.g. changes in dietary habits. On the other hand, while smokers had increased levels of asymmetric dimethyl-arginine (ADMA), arginine and methionine compared to non-smokers, these metabolites were not observed to change over the three months of smoking cessation period.

Kurzzusammenfassung

In der vorliegenden Arbeit wurde eine klinische Studie mit 60 gesunden Freiwilligen durchgeführt, um zu untersuchen, inwieweit es sich auf das Metabolom von erwachsenen Männern auswirkt, wenn diese aufhören zu rauchen. Hierfür wurden biologische Proben von 60 Probanden gesammelt. Die Compliance wurde mittels der Analyse von Kohlenmonoxid im Exhalat und Cotinin im Speichel und Urin überprüft, was zu einer Verringerung der Probanden auf 39 führte. Die Plasma-, Speichel- und Urinproben der complianten Probanden wurden mittels einer validierten Fingerprintingmethode auf metabolische Veränderungen untersucht. Insgesamt wurden 52 verschiedene statistisch veränderte Metaboliten identifiziert, von denen 26 in Plasma, 20 in Speichel und 12 in Urin gefunden wurden. Die gefundenen Veränderungen indizierten eine teilweise Erholung des Stoffwechsels nach dem Beenden des Rauchens. Um die Ergebnisse zu verifizieren, wurden quantitative Methoden für die am auffälligsten veränderten Stoffwechselwege (Fettsäuren und Aminosäuren) entwickelt, validiert und auf die Proben der complianten Probanden angewendet.

Die Ergebnisse der Fettsäureanalytik bestätigten die des Fingerprintings. Gesättigte und mehrfach ungesättigte Fettsäuren zeigten mehrheitlich eine höhere Konzentration, während einfach ungesättigte Fettsäuren eine erhöhte Konzentration nach dem Beenden des Rauchens aufwiesen. Interessant war die Tatsache, dass Arachidonsäure im Fingerprinting und in der Fettsäureanalytik identifiziert wurde, die als Vorstufe zu vielen Eicosanoiden bekannt ist. Demzufolge wurde eine quantitative Analyse der Eicosanoide durchgeführt. Hierbei wurden statistisch signifikant niedrigere Level der Prostaglandine $PGF_{2\alpha}$ und 8-iso-$PGF_{2\alpha}$ sowie des Thromboxanes 2,3-d-TXB_2 und des Leukotriens E_4 nach dem Aufhören des Rauchens festgestellt. Dies indiziert eine teilweise Erholung der durch das Rauchen induzierten Veränderungen des Eicosanoidhaushaltes, die durch oxidativen Stress und Entzündungsvorgänge hervorgerufen werden.

Für die Analyse der Aminosäuren wurden zusätzlich Proben einer Studie, die Raucher und Nichtraucher vergleicht, mit der hierfür entwickelten LC-MS/MS Methode analysiert. Nach dem Beenden des Rauchens waren die Spiegel von Kynurenin, Tryptophan und Lysin erniedrigt, während die von Glycin und Serin erhöht waren. Diese Veränderungen konnten nicht in den Proben von Rauchern und Nichtrauchern festgestellt werden, weswegen es wahrscheinlich ist, dass diese auf Einflüssen beruhen, die spezifisch auf das Aufhören des Rauchens schließen lassen. Im Vergleich der Raucher mit den Nichtrauchern wurden erhöhte Level von asymmetrischem Dimethyl-Arginin, Arginin und Methionin in Rauchern gefunden. Innerhalb der dreimonatigen Dauer der Rauchentwöhnungsstudie wurden keine Anzeichen für eine Normalisierung dieser Veränderungen gefunden, weswegen davon auszugehen ist, dass diese eine längere Erholungsdauer benötigen, sofern eine solche überhaupt eintritt.

Table of Contents

Glossary

°C	Centigrade Celsius
µg	Microgram
µL	Microliter
µm	Micrometre
µmol	Micromole
11-dh-TXB_2	11-Dehydro Thromboxane B_2
12-HETE	12-Hydroxy Eicosatetraenoic acid
2,3-d-8-iso-$PGF_{2\alpha}$	2,3-Dinor-8-iso Prostaglandin $F_{2\alpha}$
2,3-d-TXB_2	2,3-Dinor Thromboxane B_2
5-HT	Serotonin
8-iso- $PGF_{2\alpha}$	8-Iso-Prostaglandin $F_{2\alpha}$
AA	Amino Acid
ABF	Analytisch Biologisches Forschungsinstitut GmbH
Acn	Acetonitril
ADMA	Asymmetric dimethyl-Arginine
Ala	L-Alanine
amu	Unified Atomic Mass Unit
APCI	Atmospheric Pressure Chemical Ionization
Arg	L-Arginine
Asn	L-Asparagine
Asp	L-Aspartic Acid
Bl	Blood
BMI	Body Mass Index
BSTFA	N,O-bis (trimethylsilyl) trifluoroacetamide
CE	Capillary Electrophoresis
Cit	Citrullin
cm	Centimetre
CO	Carbon Monoxide
CO_{ex}	Carbon Monoxide in Exhaled Breath
CO_{Hb}	Carbon Monoxide in Blood
CRO	Clinical Research Organization
CRS	Clinical Research Services
CV	Coefficient of Variation
CYP2A6	Cytochrome P450 2A6
DIMS	Direct Infusion Mass Spectrometry
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
Eff.	Effect
EI	Electron Impact Ionization
Ex	Exhaled Breath
Exp.	Exposure
FA 10:0 iso	8-Methylnonanoic Acid
FA 10:0	Decanoic Acid
FA 11:0	Undecanoic Acid
FA 12:0	Lauric Acid

FA 13:0	Tridecanoic Acid
FA 14:0	Myristic Acid
FA 14:1 9Z	Myristoleic Acid
FA 15:0	Pentadecanoic Acid
FA 15:1 10Z	cis-10-Pentadecenoic Acid
FA 16:0	Palmitic Acid
FA 16:1 9Z	Palmitoleic Acid
FA 17:0	Heptadecanoic Acid
FA 17:1 10Z	cis-10-Heptadecanoic Acid
FA 18:0	Stearic Acid
FA 18:1 11Z	11-Octadecenoic Acid
FA 18:1 9E	trans-9-Elaidic Acid
FA 18:1 9Z	cis-9-Oleic Acid
FA 18:2 9E, 12E	Linolelaidic Acid
FA 18:2 9Z, 12Z	Linoleic Acid
FA 18:3 6Z, 9Z, 12Z	γ-Linolenic Acid
FA 18:3 9Z, 12Z 15Z	α-Linolenic Acid
FA 20:0	Arachidic Acid
FA 20:1 11Z	11-Eicosenoic Acid
FA 20:2 11Z, 14Z	cis-11,14-Eicosadienoic Acid
FA 20:3 8Z, 11Z 14Z	cis-8,11,14-Eicosatrienoic Acid
FA 20:4 5Z, 8Z, 11Z, 14Z	cis-5,8,11,14-Eicosatetraenoic Acid
FA 20:4 8Z, 11Z, 14Z, 17Z	8,11,14,17-Eicosatetraenoic Acid
FA 20:5 5Z, 8Z, 11Z, 14Z, 17Z	cis-5,8,11,14,17-Eicosapentaenoic Acid
FA 21:0	Heneicosanoic Acid
FA 22:0 13Z, 16Z	cis-13,16-Docosadienoic Acid
FA 22:0	Behenic Acid
FA 22:1 13Z	Erucic Acid
FA 22:4 7Z, 10Z, 13Z, 16Z	Docosatetraenoic Acid
FA 22:5 7Z, 10Z, 13Z, 16Z, 19Z	Docosapentaenoic Acid
FA 22:6 4Z, 7Z, 10Z, 13Z, 16Z, 19Z	cis-4,7,10,13,16,19-Docosahexaenoic Acid
FA 23:0	Tricosanoic Acid
FA 24:0	Tetracosanoic Acid
FA 24:1 15Z	Nervonic Acid
FA 25:0	Pentacosanoic Acid
FA 26:0	Hexacosanoic Acid
FA 28:0	Octaconanoic Acid
FA 30:0	Triacontanoic Acid
FA 32:0	Dotriacontanoic Acid
FA 4:0	Butyric Acid
FA 6:0	Hexanoic Acid
FA 8:0	Octanoic Acid
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FDA	Food and Drug Administration of the USA
FID	Flame Ionization Detector
FMOC	Fluorenylmethoxycarbonyl
g	Gravitational Acceleration

GC	Gas Chromatograph
Gln	L-Glutamine
Glu	L-Glutamic Acid
Gly	Glycine
h	Hour
His	L-Histidine
HMDB	The Human Metabolome Database
HPHCS	Harmful and Potentially Harmful Constituents of Tobacco Smoke
HR-MS	High Resolution Mass Spectrometry
Hyp	trans-4-Hydroxy-L-Proline
Hz	Hertz
IARC	International Agency for Research on Cancer
ID	Inner Diameter
Ile	L-Isoleucine
IS	Internal Standard
kg	Kilogram
kV	Kilovolt
Kyn	Kynurenine
LC	Liquid Chromatography
Leu	L-Leucine
L-N-MMA	L-N-Mono-methyl-Arginine
LOD	Limit of Detection
LOQ	Limit of Quantification
LT	Leukotriene
LTE_4	Leukotriene E_4
LXR	Liver X Receptor
Lys	L-Lysine
m/z	Mass-to-Charge Ratio
ME	Methyl Ester
Met	L-Methionine
Mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mmol	Millimole
mol	Mole
MOX	Methoxyamine
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometer
MS/MS	Tandem Mass Spectrometry
MUFA	Mono-Unsaturated Fatty Acid
ng	Nanogram
nm	Nanometre
NMR	Nuclear Magnetic Resonance Spectroscopy
NPD	Nitrogen Phosphorus Detector
NS	Non-Smoker
PAH	Polycyclic Aromatic Hydrocarbons

PDA	Photodiode Array Detector
PG	Prostaglandin
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
Phe	L-Phenylalanine
PLS-DA	Partial Least Square Discriminant Analysis
PPAR	Peroxisome Proliferator Activated Receptor
ppm	Parts per Million
Pro	L-Proline
psi	Pounds per Square Inch
PUFA	Polyunsaturated Fatty Acid
QC	Quality Control
rpm	Rounds per Minute
RT	Retention Time
S	Smoker
Sa	Saliva
S/N	Signal to Noise Ratio
SCD1	Stearoyl-Coenzyme A Desaturase 1
SD	Standard Deviation
SDMA	Symmetric dimethyl-Arginine
Ser	L-Serine
SFA	Saturated Fatty Acid
Sig.	Significance
SIM	Selected Ion Monitoring
SMRM	Scheduled Multiple Reaction Monitoring
SOP	Standard Operation Procedure
Thr	L-Threonine
TIC	Total Ion Chromatogram
TMCS	Trimethylchlorosilane
TOF	Time of Flight
TP	Point in Time
t-PGDM	Tetranor Prostaglandin D Metabolite
t-PGEM	Tetranor Prostaglandin E Metabolite
Trp	L-Tryptophan
TSC	Tobacco Smoke Component
TtMA	trans,trans-Muconic Acid
TX	Thromboxane
Tyr	L-Tyrosine
ULOQ	Upper Limit of Quantification
UPLC	Ultra-Performance Liquid Chromatography
Ur	Urine
UV	Ultraviolet
UV-VIS	Ultraviolet-visible Spectroscopy
V	Volt
v/v	Volume to Volume
Val	L-Valine
VIP	Variable Importance in Projection

List of Figures

List of Tables

1 Introduction

Smoking is an important risk factor for six out of eight of the leading causes of premature death causing approximately 5 to 6 million deaths per year. [1-3] According to the WHO Report 2015, about 21 percent of the world's population are currently smoking, showing a slight overall decrease of smoking prevalence compared to 2007 with 23 percent of smokers worldwide. [4] Several reasons for the decline in smokers of conventional cigarettes are discussed. A major reason for this decrease of two percent is certainly the fact that many smokers successfully quit smoking. On the other hand, the usage of non-combustible nicotine delivery systems like e-cigarettes is steadily increasing and might present a possible alternative for smokers. [5-8]

Some effects of quitting smoking can directly be realized. After 20 minutes of non-smoking the first physiological effects are already noticeable. The heart rate and blood pressure return to a normal level, consequently the blood circulation enhances and the temperature in the extremities begins to rise. [9] After approximately 12 h, the blood carbon monoxide level drops to normal and a subsequent higher oxygen supply to the body is achieved. [10, 11] Depending on the personal health and smoking behaviour two weeks to three months after having quit smoking, the circulation improves further as does the lung function. [12, 13] One to nine months after smoking cessation coughing and shortness of breath decrease and cilia[1] in the lungs regain normal function which reduces the risk of infections. [12, 14] A year after cessation the risk of coronary heart disease is half that of someone who continued smoking and the risk for heart attacks is already decreased. [15, 16] Five years after smoking cessation the risk of several cancers e.g. cancers of the throat, mouth, oesophagus, and bladder is half that of a smoker, who continues to smoke. [15, 17] After 10 years of non-smoking the risk of larynx cancer drops significantly and the risk of dying from lung cancer is halved compared to the smoking reference. [12, 15] Fifteen years after having quit smoking the risk of coronary heart disease reaches that of a non-smoker. [17] Unfortunately, not all effects of smoking appear to be completely reversible. [18] Even after 30 years never-smokers[2] can be differentiated from former smokers which show an increased risk of lung cancer and adenocarcinoma. [20] An important factor for the degree of recovery is the age when smoking cessation starts. While, the overall mortality rate halves when quitting at the age of fifty years, cessation before the age of thirty years in age leads to almost a total reversion to the mortality rates of never-smokers. [21]

[1] Cilium is an organelle in the lung. The hair like structure helps to remove mucus from the lung.
[2] A never-smoker is a person who has smoked < 19 cigarettes per lifetime. [19]

2 Fundamentals

2.1 Smoking Cessation

In 2010 nearly 70% of all smokers from an representative survey stated that they wanted to quit smoking and more the 50% have reported that they started an attempt to quit smoking during the previous year. [22] Whenever trying to improve the personal health by quitting smoking the weaning of the smoking habit is a difficult step, due to the physical and mental addiction to the consummation of tobacco products. This addiction is primarily based on the dependence on nicotine. [23-27]

Especially but not only, for women the initial weight gain after smoking cessation is an important factor to quit this approach. [28-31] There are also several studies trying to identify the influence of genomic patterns on the successful approach of smoking cessation. [32-34] Cytochrome P450 2A6 (CYP2A6) is the enzyme which catalyses the oxidation of nicotine to cotinine. [35, 36] The polymorphism of the gene of CYP2A6 is of special interest and comprises mainly three core polymorphisms. Literature describes a wild type with normal enzyme activity, a gene expression with total deletion and one with hampered transitional activity. [37] During smoking cessation, the different polymorphism types showed different degrees of withdrawal symptoms. Subjects with a high activity showed severe to moderate withdrawal symptoms while subjects with low activity showed mainly weak and sometimes only moderate symptoms. [37-39] Subsequently, more of the high activity group of CYP2A6 quitted the cessation approach.

The fact that not all persons successfully quit smoking is an especially a challenging problem in smoking cessation studies. Therefore, the study design must include more subjects than required for the projected purpose. Consequently, smoking cessation studies are more cost-intensive due to non-compliance and subsequent dropout of subjects.

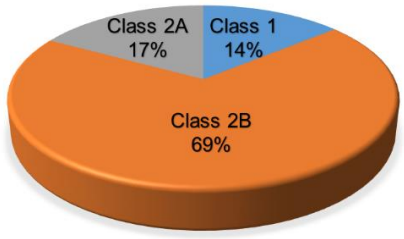
The biochemical validation via the determination of biomarkers of exposure to tobacco smoke such as carbon monoxide or cotinine is important but leads to further difficulties like additional sampling or sampling within planned sampling points when the biomarker has a too short half-life for the planned timeframe. [40] Thinking of large cohort studies with several thousand participants over decades the analytical verification of the compliance would be tremendous on both the analytical perspective as well as on the financial one. Therefore, large scale cohort studies often lack subsequent biochemical validation. It is noteworthy, that the success rate of persons who quit smoking in a clinical trails is higher (30% after the first year) [41] compared to those quitting themselves with programme (15%) and without any advice (7%). [42]

2.2 Perturbations Evoked by Smoking and Smoking Cessation

Tobacco smoke has shown to be a complex matrix with more than 6000 different chemical substances which have been identified in cigarette smoke. [43] Several of the identified compounds are characterized as harmful substances and carcinogens. [15, 44, 45] The International Agency for Research on Cancer (IARC) classified more than 80 carcinogens in cigarette mainstream smoke, including 11 established (Class 1), 14 probable (Class 2A) and 56 possible (Class 2B) human carcinogens. [46]

Table 1: Composition of the IARC as carcinogens classified substances in cigarette mainstream smoke. [46]

IARC Category	Number
Class 1	11
Class 2A	14
Class 2B	56
Sum	81



Those carcinogens are often metabolized to form reactive intermediates inducing oxidative stress and inflammatory responses which cause lipid, protein and DNA damage as observed in malignant tumours. [47] Biological effects caused by oxidative stress or inflammation can be investigated by monitoring suitable endogenous compounds, such as eicosanoids (e.g. prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)), 8-hydroxydesoxyguanosin, or malondialdehyde – all of them are well established biomarkers of effect to determine oxidative stress. [48-54] Elevated levels of the aforementioned biomarkers of effect have been frequently reported in smokers. [52-56] Still, the number of established biomarkers of effect is rather low when it comes to specific smoking-induced effects and especially smoking cessation. Biomarkers of exposure like nicotine and its metabolites are widely known to correlate well with the smoking dose. [57-59] Moreover, several other harmful and potentially harmful constituents of tobacco smoke (HPHCs) have been extensively investigated as suitable biomarkers of tobacco smoke exposure to date, *inter alia* tobacco-specific nitrosamines, aromatic amines, polycyclic aromatic hydrocarbons or mercapturic acids. [60-67] However, changes in physiological and pathophysiological pathways evoked by smoking are still not well understood in particular the recovery after smoking cessation of perturbations evoked by smoking. [15] Analysis based on omics disciplines are a promising approach to help understanding these biochemical processes.

2.3 Metabolomics

Metabolites, “small” molecules (<1500 Dalton) including exogenous and endogenous sources, are constituting the metabolome (the entity of all small molecules in an organism). [68, 69] The human metabolome database (HMDB) contains currently more than 90.000 metabolites determined in humans of which approximately 65.000 are listed as endogenous compounds. [70-72]

Metabolomics, the determination of metabolites, is the fourth omics discipline in the cascade of omics (cf. Figure 1) and therefore the closest point to the phenotype of an organism. [68, 73, 74] While the analysis on the superordinate disciplines genomics, transcriptomics and proteomics is relatively well advanced, metabolomics is far behind but rapidly rising. [68] While the technologies for the analysis of the genome, transcriptome, and proteome have significantly progressed and standards were established, the analysis on the metabolomics level is still emerging. [73, 75] However, the combined analysis of all omics disciplines will lead to a better understanding of the biochemical and biological mechanisms and the response of an organism on alteration, like perturbations induced by smoking.

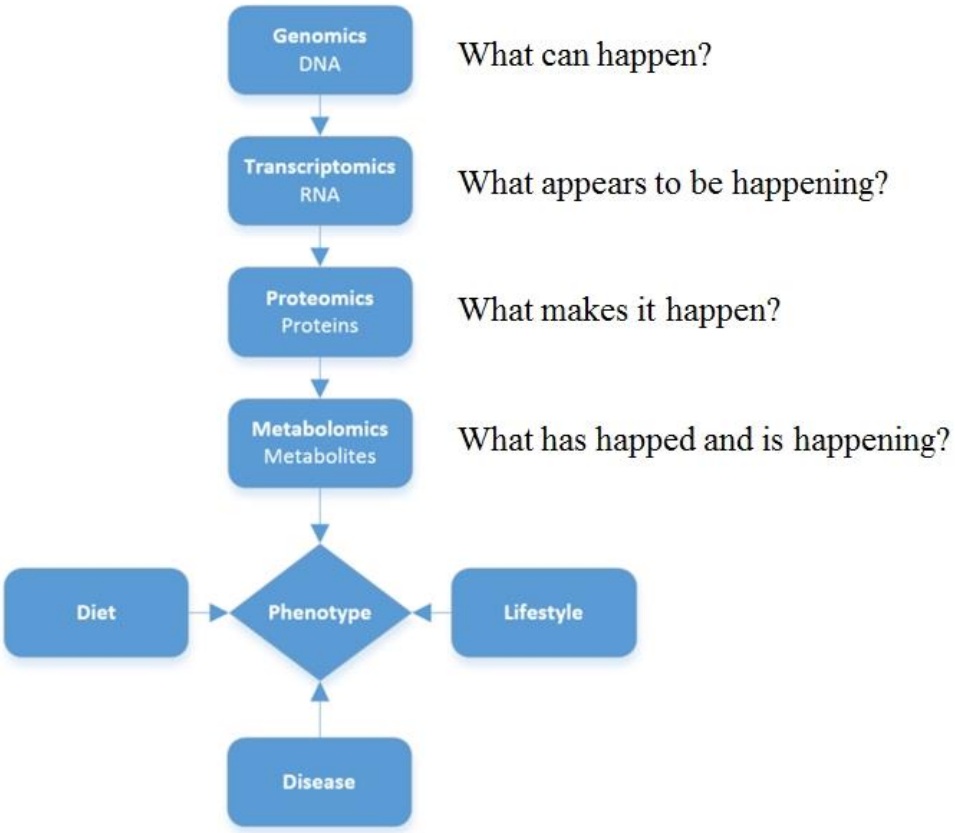


Figure 1: Cascade of omics displaying the influences on the phenotype of an organism by the omics disciplines as well as diet, lifestyle and disease. Adapted from Dettmer et al. [73] and Mueller. [76]

There are several exogenous sources like disease and diet which have obviously an impact on the phenotype of an organism (cf. Figure 1). Furthermore, also lifestyle aspects like smoking and cessation have an important influence on the phenotype and therefore on the metabolite levels. [77]

The analysis of all omics disciplines together is obviously the most comprehensive way, but also the most cost intensive one. A good approach is to start with the basic level and the question "What has happened?" (cf. Figure 1) which is related to the metabolites and changes in metabolic patterns. Currently, two main techniques are used to analyse the metabolome. Those are metabolic fingerprinting and metabolic profiling.[3]

2.3.1 Metabolic Fingerprinting

On the one hand, there is metabolomic fingerprinting. This strategy focusses on the untargeted screening and the identification of as many metabolites in a sample as possible leading to the so called "fingerprint". Ideally, metabolic pathways related to these metabolites can be identified. [68, 69]

This approach is normally selected when the impact, effect or origin of a perturbation is widely unknown. Unfortunately, there is currently no scientific method available to determine all metabolites in a biological sample at once (cf. Figure 2). [78] Due to the large number and different physical, biological and chemical properties of the human metabolites as well as the limited technical methods it is necessary to preselect a fingerprinting platform. The commonly used techniques are nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography (LC) linked to high resolution mass spectrometry (HR-MS), gas chromatography (GC) hyphenated to time of flight (TOF) mass spectrometry (MS) and direct injection mass spectrometry (DIMS). [68, 69, 77, 79, 80] Each technique has advantages and disadvantages. The most important choice is to decide between sensitivity of the method, the ability to identify the metabolites of interest and to which extend the analysis can be automatized so that high throughput screenings are possible (cf. Figure 2). [68, 81]

Fingerprinting methods are further characterized by a typical experimental design including sampling which ideally is specifically designed for metabolic analysis, sample preparation depending on the platform selected, sample analysis via the instrument of choice and data processing. [68] The data processing step is often split into two steps, first the pre-processing step comprising chromatographic evaluation, processing and statistical testing and second the identification step were the metabolites altered are identified.

[3] Up to now metabolomics lacks in fixed definitions so the descriptions presented here are the most commonly used ones.

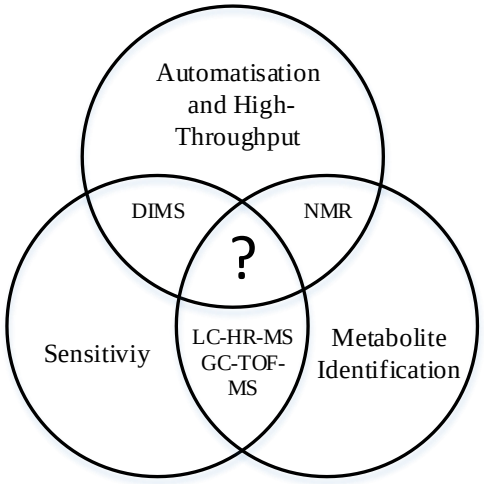


Figure 2: Important aspects (automatization and high throughput, sensitivity and ability of identification of metabolites) for the fingerprinting analysis and the related technologies. The lack of a missing global metabolic fingerprinting method is also indicated. Adapted from Dunn et al. [68]

2.3.2 Metabolic Profiling

On the other hand, there is metabolomic profiling. This strategy concentrates on the quantification of a selected number of predefined metabolites, which are normally related to a specific metabolic pathway like fatty acids or amino acids. [68, 69] Ideally, this step is subsequently performed after a fingerprinting approach to confirm the results generated as the false positive rate of profiling approaches is lower compared to the fingerprinting approaches. [81] Furthermore, profiling approaches are applied when the hypothesis is stated or when the altered pathway is already known. [69]

The analytical methods for metabolic profiling are therefore highly specialised and designed for the pathway of interest. Profiling methods utilize consequently the whole spectrum offered by the instrument-based analytics.

One of the biggest issues with metabolic profiling is the quality of the data produced. Often the methods applied lack in a consequent validation of analytical parameters and sample preparation as well as verification of the stability of raw material and samples.

2.4 Biomarkers

Biomarkers are useful indicators for determination and assessment of the health status or exposure to potentially harmful environmental sources and are therefore determined for biomonitoring. Most screenings for biomarkers are performed in blood plasma and urine as these body fluids are easily accessible. Non-invasive body fluids like urine are preferred for the development of tests as the compliance and willingness for testing is improved. Unfortunately, not all biomarkers can be determined in urine or other non-invasive body fluids. Therefore, blood plasma provides access to a broad range of analytes and is thus preferred for complex tests. Furthermore, blood plasma compared to urine requires no standardisation by an additional factor except the volume analysed.

The metabolites identified in metabolic fingerprinting and profiling approaches can be categorized into two groups of biomarkers. On the one hand, there are biomarkers of exposure and on the other hand there are biomarkers of effect.

2.4.1 Biomarkers of Exposure

Biomarkers of exposure are chemicals or their direct metabolites, determined after an environmental exposure to the substance in the body. The biomonitoring of these biomarkers can give a direct correlation to an exposure to the source. Biomarkers of exposure generally respond quite fast upon exposition. Biomarkers of exposure like nicotine and the originating metabolites like cotinine are widely known to correlate to the smoking dose and are therefore used to verify the compliance in smoking cessation studies. [82-84]

2.4.2 Biomarkers of Effect

Biomarkers of effect originate from endogenous pathways that are altered by an exogenous effect. Compared to biomarkers of exposure the response of biomarkers of effect to an exposure is long.

Endogenous metabolites such as intermediates and end-products of biological processes that are identified as biomarkers of effect potentially serve for the biomonitoring of the physiological status and the determination of alterations, possibly leading to future diseases. [85] When it comes to specific smoking-induced effects and specially to smoking cessation, the number of established biomarkers of effect is rather low. Hence, the focus of this thesis lies within the identification of biomarkers of effect and the identification of the related pathophysiological pathways altered by smoking and cessation, as alterations in endogenous mechanisms evoked by smoking are still not well understood. [15]

2.5 Initial Study Comparing Smokers and Non-Smokers

Fundamental for this thesis was the work of Mueller et al. [76, 86, 87] including a clinical study, the method development of a metabolic fingerprinting and measurement of samples of 25 smokers and 25 non-smokers.

The study design is described in detail in literature. [76, 86, 87] To gain samples for the metabolic measurements a clinical diet controlled study under the Helsinki declaration [88] and German national requirements was conducted. The healthy male subjects stayed at the clinical research organization (CRO) for 24 h. There the smokers consumed on average 13 cigarettes (with a range of 9 to 19 cigarettes) ad libitum. To avoid false positive results the non-smoking subjects were not allowed to enter the smoking areas. For the verification of the compliance with non-smoking the carbon monoxide levels in exhaled breath (CO_{ex}) were monitored during the participation of the course (cf. Figure 3 A-E). The study was designed with 5 sampling points (cf. Figure 3 A-E) for the generation of saliva, a single blood plasma sample after 24 h (sampling point E) at the CRO and a continuous 24-h urine pool collected in three fractions. Meals were served at defined points in time (cf. Figure 3).

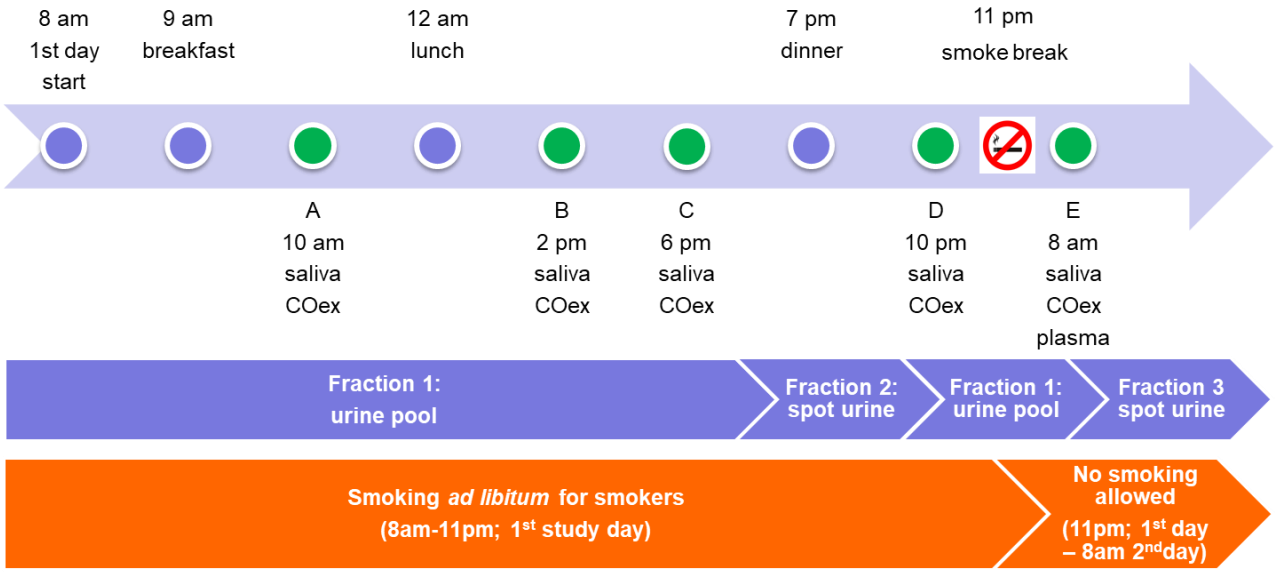


Figure 3: Study design of the study comparing smokers and non-smokers. Adapted from Mueller. [76]

The metabolic fingerprinting method developed and validated by Mueller et al. [76, 86, 87] is based on a two-step extraction of the biological samples and a two-step derivatisation followed by an analysis via GC-TOF-MS and a complex evaluation of the data comprising baseline correction, peak picking, crop filtering, mass detection, chromatogram building, chromatogram deconvolution,

retention time normalization, join alignment, peak finding, duplicate filtering, peak list row filtering and a normalization by the internal standard. For statistical testing two tests were applied: the unpaired, non-parametric Mann-Whitney-U test in combination with the multivariate partial least square discriminant analysis (PLS-DA). Finally, a manual evaluation of the statistical data was performed to identify the metabolites correlated to the statistical results.

The application of the fingerprinting method to the biological samples of the clinical study revealed several alterations in the metabolic profile. In total, 8 statistical significant alterations were determined in blood plasma, 12 in saliva and 18 in urine. Most of the alterations observed was assigned to the fatty acid and lipid metabolism. Furthermore, the amino acid and energy metabolism appeared to be affected by smoking.

To confirm the results of the fingerprinting the fatty acid and phospholipid composition was further analysed in targeted profiling approaches. [76, 86] Therefore, the blood plasma samples were externally analysed for the fatty acid profile. The saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs) were decreased in smokers while the monounsaturated fatty acids (MUFAs) were increased.

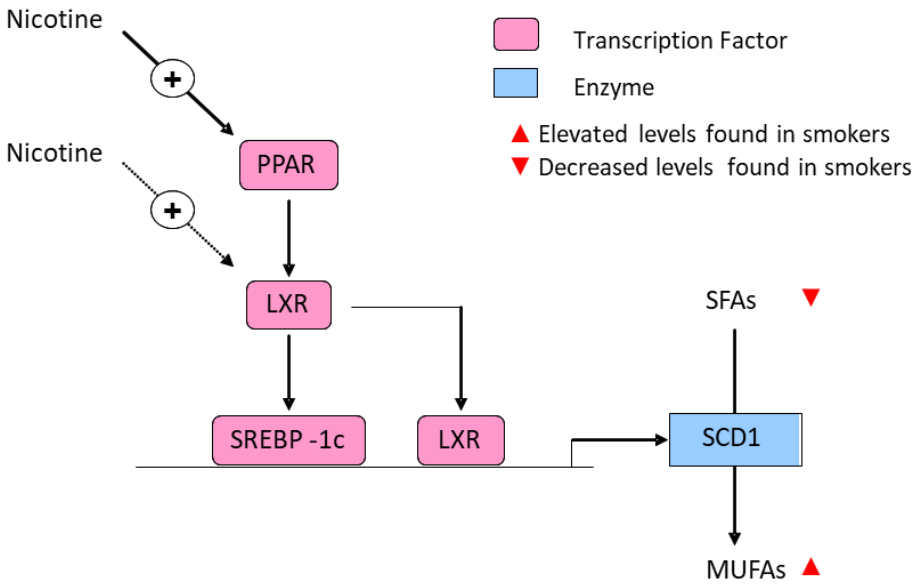


Figure 4: Hypothesis of an altered fatty acid metabolism where nicotine leads to a transcription factor activated increase of the enzyme stearoyl Coenzyme desaturase 1 (SCD1), which catalyses the desaturation of saturated fatty acids to mono unsaturated fatty acids. Adapted from Mueller. [76]

The hypothesis for this alteration in the FA metabolism was an altered FA desaturation caused by an up-regulation of stearoyl-Coenzyme A desaturase 1 (SCD1, cf. Figure 4) as suggested in literature [86, 89-91]. The reports show that nicotine activates the transcription factor peroxisome proliferator activated receptor (PPAR). Subsequently, the transcription factors liver X receptor (LXR) and the sterol regulatory element binding protein-1c (SREBP-1c) are activated. [92, 93] Furthermore, Chu et al. [94] stated that the LXR plays a central role in the upregulation of the enzyme SCD1, which in fact catalyses the desaturation of SFAs to MUFAs. [95] The results of the fatty acid analysis were partially confirmed via a new developed and validated phospholipid profiling method.

2.6 Progress in Analytic and Tobacco Research

While the cigarette was designed in the late 18th century to create a beneficial product for the salvage of the waste from the cigar production, the wide distribution and acceptance of cigarettes grew during the next centuries. Initially smoking was considered to be healthy or uncritical until around 1900, when scientists became sceptic and started research in this field. Gettler and Mattice were the first to identify carbon monoxide as a biomarker for smoking via a gas expansion determination method in 1933. [96] In 1941 German doctors became the first to discover the relation between lung cancer and tobacco smoking. [97] Furthermore, Nazi Germany was the first country with a strong anti-tobacco movement [98], public anti-smoking campaigns, an institute for research on the harms of tobacco [99] and health education [100] on this topic. In the mid-1900s many other countries joined the anti-tobacco movement [101] but the tobacco industry financed research to rebut the emerging scientific results. [102, 103]

The first international breakthrough on the hazardous effects of smoking was in 1950 when five case-control studies on the correlation of smoking and cancer with conform results were published. [101] From this point onwards, the overall trend in smoking was negative in the developed world. While the prevalence to smoking was more than 50% in 1950 in West Germany it dropped to ~23% until 2017 (cf. Figure 5). [104]

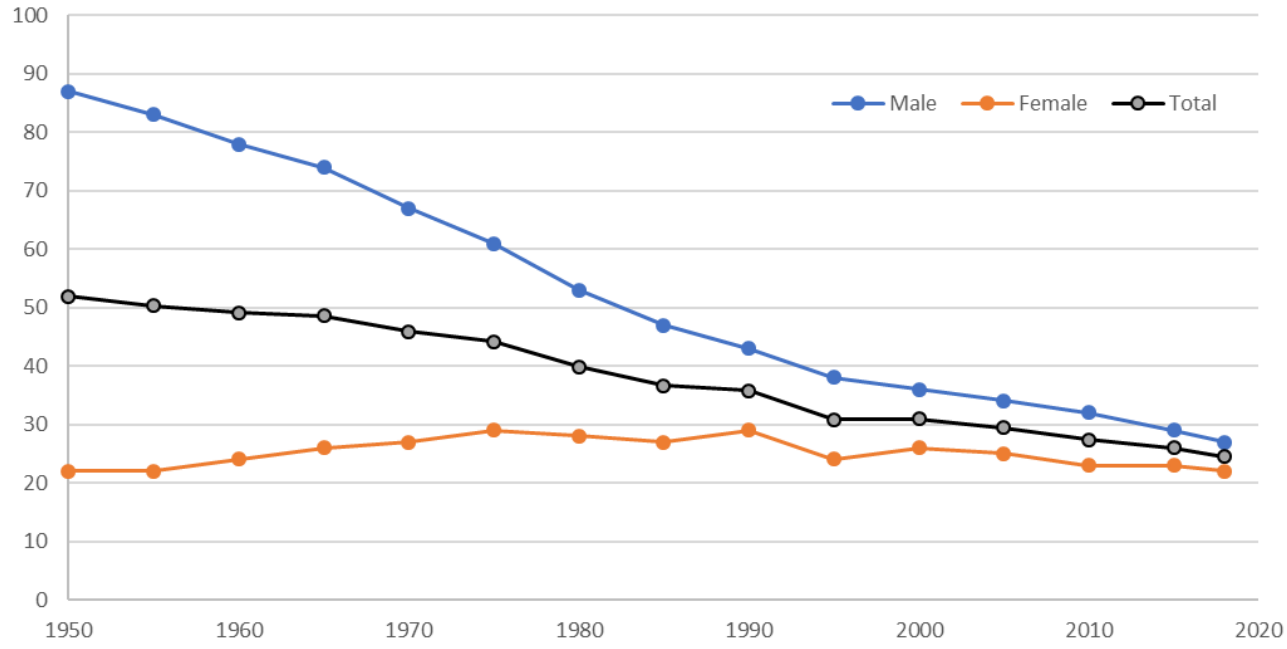


Figure 5: Smoking prevalence in Germany (West Germany before 1990) among adults (>18 years) as a timeline from 1950 to the present. The figure shows the total prevalence (black) and the prevalence among women (red) and men (blue). Adapted from data of the international smoking statistics. [104]

Interestingly, the number of smoking women is relatively stable between 20% and 30% while the number of smoking men has decreased from nearly 90% to below 30%. The low use of tobacco products among women is probably based on the gender patterned use of tobacco during the 19th century where it was socially not well accepted when women smoked and smoking was considered a masculine feature. [105]

While the early tobacco research focused mainly on cancer, the analysis of biomarkers for the exposure to tobacco smoke in human matrices started to emerge in the 1960s and beginning 1970s together with the broader availability of chromatographic instruments like GCs. Consequently, smokers were differentiated from non-smokers and even passive smokers (a terminus already created in Nazi Germany [106]) by applying blood, urine, saliva and breast milk to an analysis method for nicotine via GC-NPD. [107-109] Furthermore, the identification of nicotine metabolites in human matrices like cotinine and hydroxy cotinine in urine could be achieved. [110]

During the 1960s, smoking cessation studies became popular. Focus of these studies was the assistance in smoking cessation and maximisation of success via different methods as for instance medical treatment, instruction, television programmes or hypnosis. [111-113] In conjunction to cessation studies, statistical significant physiological effects and metabolic changes were observed. [114] Quickly weight gain was considered to be smoking cessation related. [115] The need of the utilization of instrumental analysis techniques for the verification of cessation studies was formed in the 1980s and was confirmed by mainly four biomarkers of exposure. [116] For the acute exposure to tobacco smoke carbon monoxide was analysed in exhaled breath (CO_{ex}) using electrochemical gas analysers [117] or blood (CO_{Hb}) via UV-VIS spectrometry. [118] To determine long-time abstinence from smoking, nicotine, cotinine and thiocyanate were determined. The determination of nicotine and cotinine was achieved either by GC-NPD [119] or the HPLC technology hyphenated to a photometric detector, which had come up during the 1960s and 1970s. [120, 121] Thiocyanate in plasma was mainly analysed with UV-VIS photometric techniques. [122]

The accumulation of data on the harmful effects of smoking and passive smoke during the 1970s led in consequence to a substantial increase in tobacco industry financed research against these facts to weaken the upcoming decreasing social acceptability for smoking. [123] During this campaign not only scientific “fake news” were published in scientific journals also pseudo-scientific novels like “smoking is good for you” [124] were created. In consequence research founded by the tobacco industry during the 1990s was banned in several scientific journals, health journals, universities, and research institutes. [102, 123, 125, 126]

The enhanced availability and rapidly improving technology of computers, chromatographic instruments and detectors, especially mass spectrometers, led to a gain in biomarker identification and measurability during the last two decades of the 20th and the first decade of the 21st century (cf. next section 2.6.1 for examples). [127, 128] These advances launched the rapidly growing field of metabolomics (cf. section 2.3) at the beginning of the 21st century, which is mainly MS and NMR based. [73] Consequently, this approach was applied to the field of tobacco research so that studies were specially designed for metabolomic analysis of the smoking behaviour or evaluated on the perspective of smoking. [87, 129-131]

The data on metabolomic studies on the topic of smoking cessation is rather small compared to the direct exposure to tobacco smoke. There is only one source: the smoking cessation specific evaluation of the KORA survey [132] with emphasis on smoking cessation. Due to the fact that this study was not designed for the evaluation of smoking but for long-term epidemiological studies, this study lacks in several important points: the cessation was announced by self-report, the diet was not controlled and the compliance was not consequently verified and had too long intervals due to the great timeframe observed. To date, there are – up to the author's best knowledge – no other metabolomics studies on the acute impact of smoking cessation on metabolites of easily accessible human body fluids. This thesis addresses this gap and tries to contribute to the field of smoking cessation.

2.6.1 Metabolites Related to Smoking

The number of biomarkers for the consume of tobacco cigarettes is rather high compared to those applicable for smoking cessation. Table 2 summarizes among others the most common markers of effect and exposure for smoking, their origin and the currently most common analysis methods applied for the determination. The range of methods for the analysis of these markers covers a wide spectrum. Mainly, liquid and gas chromatography-based methods are applied for the separation of the analytes from the complex human matrices. For the detection of these metabolites mass spectrometry and UV spectroscopy are the most commonly used methodologies.

As a general rule, the matrices applied to analysis are dependent on their accessibility and applicability. In consequence, blood, which is the so called "gold standard" for human metabolome analysis, is often not used as an invasive method is necessary to gain the matrix and therefore the compliance with subjects is not as easy as for the sampling of saliva or urine. The use of saliva is difficult as it lacks a parameter for normalisation and the consumption is dependent on the sampling

method which needs to be standardised. Urine is by far the easiest accessible and correctable body fluid as the standardisation via urinary creatinine or better 24-h-excretion are well accepted in science. [133] These characteristics are also represented in the use of these matrices in Table 2 where urine is the most applied matrix followed by blood and saliva. Exhaled breath and breastmilk play a subordinated role and are only applied for special purposes.

Important for the consideration of Table 2 is the separation into groups and single biomarkers, as the listing of all markers covered within the groups would degrade the readability of the table. These groups commonly cover several markers that were analysed on behalf of the exposure to tobacco smoke and often only a few of them were determined in cessation protocols.

To monitor the acute smoking status and metabolic alterations several exposure and effect markers were identified during the last century so that the most functional and specific markers became clear (highlighted bolt in Table 2). These groups are often analysed in multi-methods covering ideally the complete group with gas or liquid chromatographic methods hyphenated to mass spectrometric detection. For instance, Piller et al. [59] developed a LC-MS method for determination of nicotine and its ten major metabolites in urine and Kavvadias et al. [134] described a method for the quantification of 5 tobacco-specific N-nitrosamines in urine of smokers and non-smokers.

Among the few biomarkers for cessation the major part are markers for the direct exposure like nicotine and its metabolites, polycyclic aromatic hydrocarbons and tobacco specific nitrosamines. Naturally, biomarkers of exposure for tobacco smoking should be applicable for the use as biomarkers for quitting smoking at least up to a certain extend. These markers are important but they indicate just the recent exposure to tobacco smoke. Consequently, a conclusion on a recovery or change of a perturbation in the metabolism evoked by smoking after stopping the exposure cannot be taken.

Comparing the table on behalf of the number of smoking and cessation relevant biomarkers of effect, the need for research on smoking cessation relevant biomarkers is obvious. Only a few eicosanoids and methyl arginines were analysed in terms of cessation studies. This project was realized to survey this deficit and advance research on smoking cessation relevant biomarkers of effect.

Table 2: Overview of biomarkers related to smoking and cessation. The table shows whether or not a biomarker or group of biomarkers is considered to be related to smoking and/or cessation (as exposure (Exp.) or effect (Eff.) marker) and the human matrices (exhaled breath (Ex), urine (Ur), blood (Bl) and saliva (Sa)) as well as the most common analysis platform. The most important markers are highlited in bolt.

Biomarker	**Origin**	**Smoking**		**Cessation**		**Matrix**	**Analysis**	**Literature**
Groups of Analytes		**Exp.**	**Eff.**	**Exp.**	**Eff.**			
Aromatic amines	Aromatic amines	✓				Ur	GC-MS	[135-137]
DNA adducts	PAHs	✓				Ur	LC-MS	[138-140]
Eicosanoids	Oxidative stress		✓		✓	Ur/Bl	LC-MS	[53, 141-144]
Fatty acids	Oxidative stress		✓			Bl	GC-FID	[86]
Haemoglobin adducts	Acrylnitrile, Ethylendioxid	✓		✓		Bl	GC-MS	[145-151]
Mercapturic acids	Acrolein; Benzene, Lipid peroxidation	✓	✓	✓		Ur	LC-MS	[66, 152-157]
Methyl arginines	Inflammatory effects		✓		✓	Bl	LC-MS	[132, 158-161]
Nicotine metabolites	Nicotine	✓		✓		Ur/Bl/Sa	LC-MS	[59, 162]
Phospholipids	Oxidative stress		✓			Bl	LC-MS	[86]
Polycyclic aromatic hydrocarbons	Polycyclic aromatic hydrocarbons	✓		✓		Ur	LC-MS	[62, 163, 164]
Steroids	-		✓			Bl	LC-MS	[165]
Tobacco specific nitrosamines	Nicotine	✓		✓		Ur	LC-MS	[134, 166-169]
Tryptophan metabolites	-		✓			Ur/Bl	LC-UV	[170]
Biomarker	**Origin**	**Smoking**		**Cessation**		**Matrix**	**Analysis**	**Literature**
Single Analytes		**Exp.**	**Eff.**	**Exp.**	**Eff.**		**Groups**	
4-Hydroxy-2-nonenale	Lipid peroxidation		✓		✓	Ur/Bl	LC-MS	[171, 172]
8-Hydroxydeoxy guanosine	Oxidative stress		✓			Ur/Bl	GC-MS	[173]
8-Oxo-2'-deoxyguanosine	Oxidative stress		✓			Ur	LC-MS	[174]
Acetonitrile	Acetonitrile	✓		✓		Ur/Bl/Sa	GC-MS	[175, 176]
Benzene	Benzene	✓				Ur	GC-MS	[177]
CO	CO	✓		✓		Ex	Gas-sensor[4]	[178-180]
Homo cysteine	Inflammatory effects		✓		✓	Bl	LC-UV	[181, 182]
Hydroxy pyrene	PAH	✓				Ur	LC-UV	[183]
Hydroxyproline	-		✓			Ur	LC-UV	[184]
Malondialdehyde	Oxidative stress		✓			Ur	LC-UV	[185, 186]
Serine	-		✓			Bl	LC-UV	[132]
Thiocyanate	Hydrogen cyanide	✓				Ur/Bl/Sa	GC-MS	[187-189]
ttMA	Benzene	✓		✓		Ur/Bl	GC-MS	[190-192]
Vitamines B6, B12	Inflammatory effects		✓			Bl	LC-UV	[181]
β-Carotene	Oxidative stress		✓			Bl	LC-PDA	[193, 194]

[4] The analysis of CO is usually performed in exhaled breath by hand units with an electrochemical gas sensor. [178]

2.7 Analytical Methodologies

During this thesis it was essential to develop targeted analytical methods for the quantification of analyte groups identified in the fingerprinting approach to be altered after smoking cessation. The aim was to verify and expand the results from fingerprinting with validated quantitative analytical methods to achieve reliable results as fingerprinting results are considered to be susceptible to be defective.

2.7.1 Fatty acids

So far, most of the FA methods described in literature target only a subset of individual FA species for example certain disease- or food-related FAs and/or lack of comprehensive validation data. [195-199] For the wide-ranging analysis of FAs within clinical studies it is important to analyse a wide range of FAs including geometric and positional isomers in order to distinguish between exogenous and endogenous FAs as well as short-chain and long-chain FAs, as the mechanism of alteration evoked by smoking is not fully understood, yet. [86, 200] Therefore, further research in this area is required in order to decipher the underlying pathophysiological mechanisms.

There are two techniques mainly used for the profiling of FAs. Gas chromatography in conjunction to either flame ionization detection (FID) or mass spectrometry. [195, 201, 202] While GC-FID is primarily known to be robust and cost efficient, but relatively unspecific, GC-MS offers the capability to distinguish co-eluting analytes by their unique mass spectrum. GC-MS instruments are commonly operated in the electron impact ionization (EI) mode, leading to a reproducible fragmentation pattern [203] with additional structural information. [204] Unfortunately, the most intensive and therefore sensitive fragments occurring applying EI ionisation are parts of all FA fragmentations. [205] In consequence, a good separation of fatty acids must be achieved to neatly quantify using the most sensitive FA fragments. Furthermore, specific qualifiers are inevitable.

GC-MS methods for the analysis of FAs described so far are restricted to quadrupole mass spectrometers in the selected ion monitoring (SIM) mode. [205-207] These methods are hampered by a limited mass resolution owing to the nature of quadrupole instruments. Furthermore, these methods are restricted to targeted analysis of a pre-defined set of analytes, meaning that retrospective analysis of additional FAs is not possible. Furthermore, an identification of probable contaminates is often difficult to identify. Structural information, which is an important advantage of GC-EI-MS as opposed to GC-FID, which is the most commonly used technique for FA analysis, is lost by applying SIM mode to achieve more sensitivity. Therefore, time-of-flight (TOF)

technology instead of quadrupole MS for the simultaneous quantification of the 44 individual FA species was used for the development of the method presented here (cf. section 4.4 and 5.4). To the author's best knowledge, this is the first time that GC-TOF-MS has been used for the targeted quantification of individual FA species in human metabolic studies. By using TOF-MS instruments the total mass spectrum is acquired permanently which offers an advantage in retrospective analysis [208]. The levels of sensitivity were comparable between TOF-MS and quadrupole instruments operated in SIM mode. [209] Moreover, the increased mass resolution of TOF instruments compared to standard quadrupole instruments allows for a better selectivity of the assay. Furthermore, by applying and evaluating two instead of the frequently used single qualifier the identification of possible contaminates is improved without losing sensitivity due to the application of TOF technology.

2.7.2 Amino acids

In order to further verify the results from the fingerprinting, a targeted multi-analyte method for the quantification of AAs has been developed as there is up to the best of the author's knowledge no method available for the simultaneous analysis of all analytes of interest. Several different methods are commonly applied for amino acid analysis utilizing CE, GC-MS, LC-UV, LC-MS/MS, CE-UV or photometry as the AA analyser. [210, 211] The number of different analysis platforms applied, shows the difficulty of the amino acid analysis based on the wide range of polarity and acidity due to the different functional groups of AAs. [212] Depending on the purpose of use, each has its advantages and disadvantages:

The amino acid analyser is often described as the gold standard of AA analysis, but a special instrument is required which is used for the determination of AAs only. This and the high analysis time is obviously the main disadvantage compared to other systems. [213] Likewise, CE is often not available in laboratories which offers the underivatized analysis of AAs when hyphenated to MS, but is limited in sample volume which makes it often necessary to concentrate the analytes. [214] Furthermore, the applicability for routine analysis is not ideal due to shifting retention times. [215]

Utilizing GC-MS methods the best separation of the AAs can be achieved. Unfortunately, a derivatization is required to analyse AAs via GC/MS where some AAs are lost like Cys and Trp through oxidative destruction and Asn and Gln via deamidation to Asp and Glu. [216, 217]

When applying LC-UV a cost efficient and often available platform with reproducible results is

used. The analysis requires a derivatisation and is mainly limited due to the lack of analyte specificity given by optical detection methods. [210] Consequently, higher separation times are required for optical detection compared to MS. [218] Underivatized LC-MS/MS analysis of AAs lacks in sensitivity of analytes and leads into further difficulties like poor separation due to the different chemical and physical properties of the amino acids. [219, 220] Therefore, masking either the amino- or the acid-group enhances the ionisation and consequently the sensitivity. Furthermore, the separation is advanced due to the improved applicability to a specific phase.

In accordance to literature [218], FMOC derivatization in combination with LC-MS/MS analysis was found to be the most robust and efficient analysis method. In contrast to most FMOC-based AA methods described in literature, LC-MS/MS is beneficiary by using multiple reaction monitoring (MRM) mode. Due to the improved specificity, separation of individual AA species is no longer necessarily required except for the isobaric AA species. The most challenging problem by using FMOC-Cl as derivatization reagent is the reaction with water and the resulting FMOC-OH interfering with the detection via UV but not the MS detection. [221]

The intention was to develop and validate a specific AA profiling method, covering the proteinogenic AAs and additional Trp and Arg metabolites which could be of interest in the context of smoking cessation (cf. section 5.5.2). [222, 223] The main goal during method development was to optimize the separation of the isobars, SDMA and ADMA as well as Leu and Ile. For this purpose, several LC columns were tested with the focus on baseline separation of the AAs mentioned afore. It was possible to improve the chromatographic performance, enabling the separation of these AAs, as well as the accuracy and reproducibility of the method up to a satisfying level. The newly developed and validated AA method (cf. section 4.5 and 5.5) was specifically designed to quantify AA in the context of smoking cessation.

3 Research Aims

Smoking causes chronic diseases like cancer [224-226], cardiovascular [227-229] and respiratory [230-232] diseases. It is the most important risk factor for peripheral atherosclerosis, abdominal aortic aneurysms and lung cancer. [2, 233] In order to prevent these diseases, it is of paramount importance to understand the involvement of smoking in the patho-mechanisms of these diseases. For this purpose, it is helpful to identify biomarkers which indicate perturbations in biochemical pathways at very early stages of the diseases. Untargeted metabolic screening has evolved as a promising approach to analyse the entity of small molecules (<1500 Dalton), which are most predictive for the phenotype as well as changes physiological pathways of an organism. [74]

The purpose of this PhD project was to determine changes in the metabolome by analysing body fluids upon smoking cessation. To this end, a clinical study with 60 healthy smokers, who were willing to quit smoking was conducted. The smoking cessation study was projected to run over three months. Biological samples (blood, urine, saliva) were collected under strictly controlled dietary conditions immediately before as well as one week, one month and three months after quitting smoking. Additionally, the compliance was verified measuring smoking specific biomarkers of exposure (cotinine and carbon monoxide) in saliva, urine and breath samples provided on the occasion of ambulatory visits at increasing intervals between the stationary visits to the clinic.

The biological samples collected during the stationary stays (blood, saliva, urine) were analysed with an untargeted fingerprinting GC-TOF-MS method as developed for a previous study by Mueller et al. (cf. section 2.5). [76, 86, 87] As these and other previous studies had shown changes in the fatty acid and amino acid metabolism [76, 86, 87, 132, 200, 234], targeted methods for the quantification of these metabolites were developed, validated and applied to the biofluid samples of the present study. Of special interest was the trend of metabolite levels over time after smoking cessation.

The hypothesis for the outcome of this investigation was that the metabolome of the quitters will approach that of non-smokers over time. Furthermore, additional (endogenous) metabolites were expected to be identified, which are responsible for the difference between the metabolome of subjects when still smoking and after having quit. Another important aspect of this PhD thesis was, whether perturbations evoked by smoking are reversible in the assessed period of three months after smoking cessation.

4 Experimental

4.1 Clinical Study Description

A human study with 60 volunteers was conducted at the Clinical Research Services (CRS, Mönchengladbach, Germany). The study was carried out as described in literature. [77] According to the Helsinki declaration [88] and national requirements, ethical approval was received by the responsible ethical commission Nordrhein (Germany). The subjects of this study were healthy male Caucasian smokers. The body mass index (BMI) range for the study was set to 18 to 29 kg/m^2. Strong smokers were required with a consummation of at minimum 15 cigarettes per day during a period of at least 12 months.[5] All subjects had to have a strong intension to stop smoking, which was encouraged by a financial incentive. The inclusion and exclusion criteria are shown in detail in Table 3 and Table 4.

Table 3: Subject inclusion criteria for smoking cessation study.

Inclusion criteria
• Male • Healthy • Age: 20 - 50 years • BMI: 18-29 kg/m^2 • Strong smokers • Cigarette consummation of at minimum 15 cigarettes per day during the last year • CO_{ex}>10 ppm (during afternoon or evening) • Strong intension to stop smoking

[5] The smoking status behaviour the last year was achieved from self-reports of the study subjects.

Table 4: Subject exclusion criteria for smoking cessation study.

Exclusion criteria
• Female
• Non-smoker
• Aged under 20 or over 50
• BMI under 18 or over 29 kg/m^2
• Nutrition restriction (e.g. vegetarian)
• Chronical diseases (e.g. asthma, hypertension, chronic obstructive lung disease)
• Regular intake of medication
• Infrequent smoker (no daily consummation of at least 15 cigarettes)
• Nicotine substitution (e.g. nicotine plaster, e-cigarettes with nicotine, nicotine gum)
• Current consummation of other tobacco products than cigarettes
• Consummation of less than 15 cigarettes per day during the last year

Prior to study permission, subjects had to pass a preliminary investigation to check their eligibility for the study. There the subjects were informed about the design of the smoking cessation study. Investigations were done including a standard medical examination and a questionnaire about the personal smoking behaviour. Table 5 shows an overview over the conducted investigations during the examination.

Table 5: Overview preliminary investigations for subjects' study permission.

Preliminary investigations
• Interrogation about personal data and behaviour (including smoking behaviour, consummation of nicotine substitution and anamnesis)
• Interrogation about state of health and intake of medication
• Standard medical examination (including body weight and height)
• Determination of CO_{ex}

4.1.1 Study Design

The clinical study was conducted with 12 sub-groups each containing three to six subjects. To handle the different subjects, the sampling points and starting times for meals were adjusted by shifting the starting times for 15 minutes (e.g. subject 1: sampling A at 10 am and lunch at 12 pm; subject 2: sampling A at quarter past 10 am and lunch at quarter past 12 am).

The study timeline is highlighted in Figure 6. At the first point in time (TP 0), when they were still smoking the subjects stayed at the CRS for 24 h (8 a.m. to 8 a.m.). TP 0 was used as baseline and reference TP for the bioanalytical evaluations. During the night of the first stationary visit, the subjects had to quit smoking (11pm; no cigarette, tobacco or nicotine product use in any form during the course of the study). The first stationary visit was followed by five ambulant visits during the first week and led directly into the second stationary visit after one week (TP 2, Figure 6). This resulted in daily supervision during the first nine days. Three ambulant visits were conducted during the following three weeks leading to the third stationary visit (TP3, Figure 6), after a duration of one month. During the next two months 6 more ambulant visits were conducted leading to the final stationary visit (TP 3, Figure 6), after a total duration of three months of the clinical study.

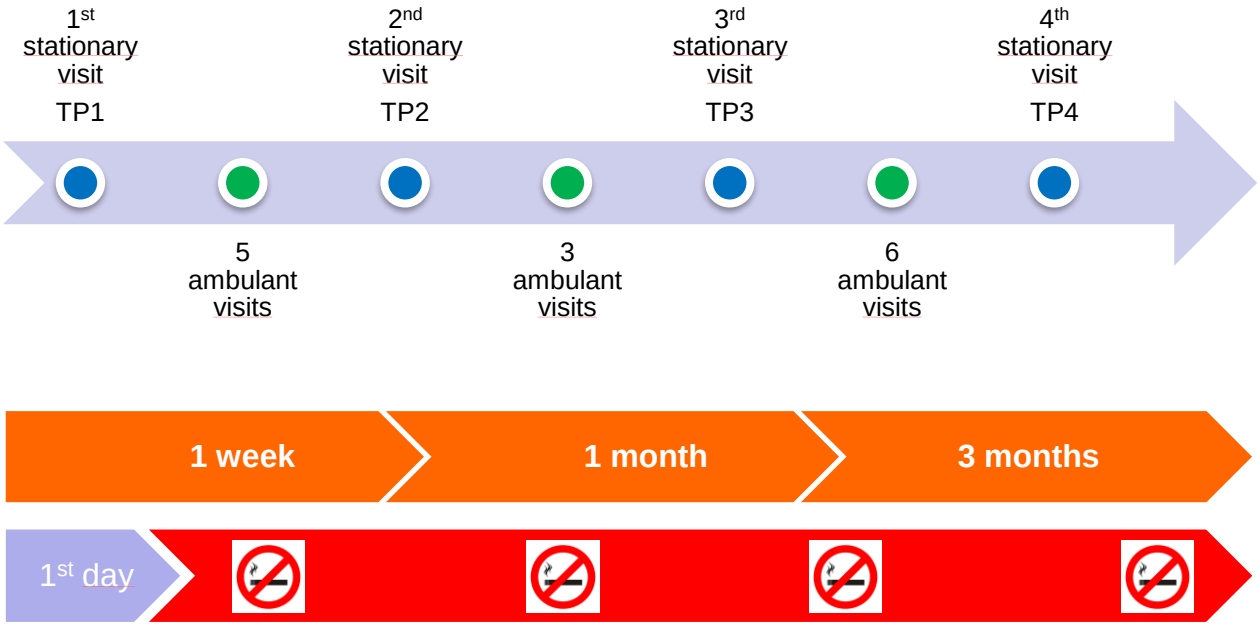
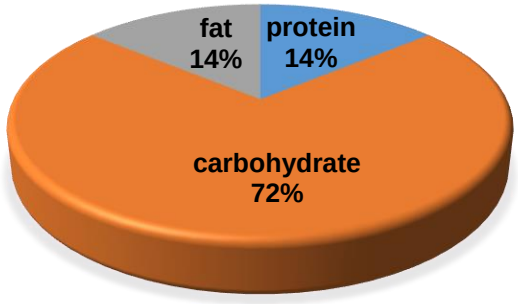


Figure 6: Timeline of clinical smoking cessation study.

4.1.2 Nutrition

To minimize nutritional effects on the metabolic profile, the clinical study was conducted under strictly dietary controlled conditions. 12 h of fasting prior to the stationary visit were compulsory.[6] During stationary visits, three meals were served at defined points in time. Breakfast at 9 am, lunch at 12 am and dinner at 7 pm (see Figure 7). The amount of food was normalized to the weight of the subjects, in five categories (see Table 6). Therefore, the amount of lunch was adjusted while all subjects were served the same breakfast and dinner. Calculation of the caloric intake was according to the Harris-Benedict equation. [235] The nutrition composition was served as defined prior to the study comprising 72% carbohydrates, 14% proteins and 14% fat (Table 6).[7] For standardization purposes the subjects had to consume the complete quantity served. During the stationary visits, the subjects were only allowed to drink still mineral water. The water consummation for the subjects was set to three litres per day. Each subject had to drink the whole amount of water *ad libitum* during the 24 h of a stationary visit. To avoid the uptake of pyrolysis products (e.g. polycyclic aromatic hydrocarbons or nitrosamines) by nutrition, which might interfere with the uptake of these compounds by the exposure to tobacco smoke, grilled, fried or otherwise extensively heated or smoked food was prohibited during the stationary visits.

Table 6: Nutrition composition and body weight categories for stationary visits.

Body weight [kg]	Caloric intake [kJ]	Nutrient composition [%]
55-65	7535	
66-75	8519	
76-85	9368	
86-95	10192	
96-105	10715	

[6] The subjects were only allowed to drink still mineral water during the 12 h prior to a stationary visit.

[7] A detailed nutrition of an exemplary stationary visit is attached in Appendix A.

The design of a stationary visit is presented in Figure 7 designed in maximum analogy to the initial study comparing smokers and non-smokers (cf. chapter 2.5 and literature [76, 86, 87]). At 8 am on the first day, the subjects arrived at the CRS. General and administrational instructions were made and the study design was explained. The subjects were allowed to smoke *ad libitum* until cessation at 11 pm, but had to reach a minimum of 15 cigarettes on their last smoking day at the CRS.

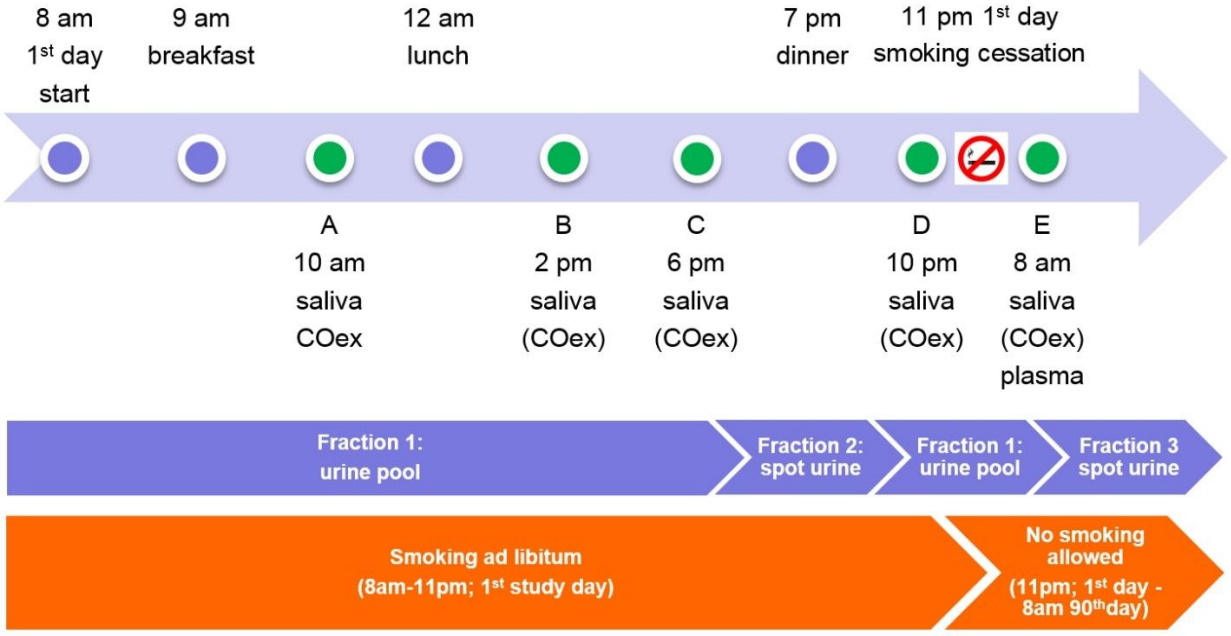


Figure 7: Timeline for a stationary visit. Adapted from Mueller [76].

4.1.3 Sampling

Body fluids (blood plasma, saliva, and urine) were collected for metabolic investigations in adult men. The sampling at each stationary visit was performed with the same timeline and in accordance to the proceeding described by Mueller et al. for the initial metabolome study at ABF. [86, 87] EDTA-plasma samples were taken at 8 am on the second day of each stationary visit under fasting conditions (cf. Figure 7). Sampling was done with cooled vacutainers in sitting position. After collection, the plasma was immediately centrifuged at 4°C, aliquoted, frozen with dry ice and stored at -20°C prior to analysis. The collection of saliva was done at five points in time starting on the first day of each stationary visit at 10 am (A, see Figure 7), 2 pm (B), 6 pm (C), 10 pm (D) and ending on the second day at 8 am (E). Saliva was sampled by a modified unstimulated spitting method described by Navazesh which avoids systematic errors due to the standardized protocol. [236, 237] Twenty minutes prior to the sampling of saliva any oral intake (also no smoking during

the first stationary visit) tooth brushing, chewing gum etc. was prohibited except for drinking of water. To remove any particles accumulated in the oral cavity, the mouth cavity had to be rinsed three times for 20 seconds with water prior to sampling of saliva which was done in sitting position. Saliva was accrued for 30 seconds in the oral cavity and then spitted into a 50-mL sterile polypropylene tube (Greiner Bio-One, Frickenhausen, Germany). The procedure was repeated until approximately two millilitres of saliva were collected. The sampling procedure should not take more than five minutes. If needed, a picture of a citrus fruit was shown as expedient to stimulate saliva production. The samples were frozen at -20°C directly after collection.

The urine collection was conducted in three different fractions. Fraction 1 embodied all urine voids during the 24 h stationary visit except for Fractions 2 and 3 (Figure 7). Fraction 2 was the complete urine voided after 6 pm on the first day of each stationary visit. The urine voided in the morning between 4 am and 8 am on the second day of each stationary visit was collected as Fraction 3. The urine fractions were vortexed directly after collection and aliquots were build which were frozen directly on dry ice and stored at -20°C prior to analysis. During the collection periods the urine fractions were stored in a refrigerator at 4 to 8°C. To create an entire 24-h urine the fractions 1, 2 and 3 were proportionally combined by total weight of the single fractions.

4.1.4 Ambulatory Visits

In order to check the compliance with the (non-)smoking status, subjects visited the CRS at increasing intervals in between the stationary visits. During each of these ambulatory visits a spot urine sample and a saliva sample were collected. The spot urine sample was voided, aliquoted, frozen directly on dry ice and stored at -20°C prior to analysis. Saliva was collected through a Salivette® (Sarstedt AG & Co, Nümbrecht, Germany). The Salivette® was used as specified in the instruction manual except for the fact that the swab was directly dropped into the oral cavity without touching it. After three minutes, the swab was spit back into the centrifugation container of the Salivette®. The Salivette® was frozen and stored prior to analysis at -20°C.

4.2 Compliance Operation Procedure

The compliance of the subjects was verified by measuring carbon monoxide in exhaled breath (CO_{ex}) and cotinine in saliva and urine as described in literature. [77] CO_{ex} was measured at each ambulatory and at the beginning of each stationary visit in order to verify the immediate smoking status. At the first stationary visit, CO_{ex} was additionally determined at each point in time saliva was collected (see sampling point A to E, Figure 7) to monitor the basal level. The dropout cut-off level for CO_{ex} in this study was set to 6 ppm which has been reported as cut-off level to distinguish smokers from non-smokers. [180, 238, 239] Prior to use, the CO analyser was calibrated with a specified CO gas.

The saliva and spot urine samples provided at the ambulatory visits were analysed for cotinine, a primary nicotine metabolite (cf. Figure 6), by means of a validated LC-MS/MS SOP method. [240]

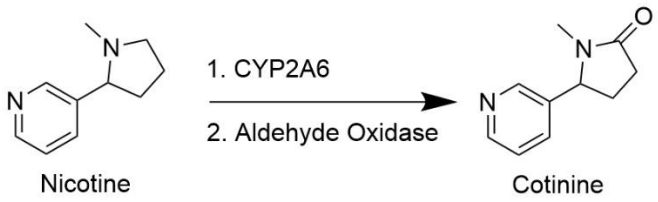


Figure 8: Degradation of nicotine to cotinine by Cytochrome P450 2A6 (CYP2A6) and the aldehyde oxidase.

Furthermore, all "A" saliva samples and randomly selected "B" to "E" samples (Figure 7) collected during the stationary visits were analysed for compliance. Cotinine was chosen for compliance monitoring due to the significantly longer half-life of 16 h to 19 h compared to nicotine. [241, 242] Whereas regular active smokers have cotinine levels >100 ng/mL in saliva, cotinine levels of 2 to 15 ng/mL are reported for passive smokers. [243, 244] Therefore, a cotinine concentration of >15 ng/ml was set as dropout cut-off for cotinine in saliva to distinguish between smokers and non-smokers. Urine samples from ambulatory visits (Figure 6) were analysed additionally to confirm the results obtained for the saliva cotinine determination. The cotinine cut-off in urine was set to 50 ng/mL as suggested in literature. [244]

4.2.1 Chemicals

Chemicals and solutions for the compliance measurements were obtained from the following sources: Water (Optigrade®) and methanol (Picograde®) were purchased from Promochem (Wesel, Germany). Methanol (absolute ULC/MS grade) was from Biosolve BV (Valkenswaard, Nether-

lands). Water with 0.1% ammonium acetate (LC-MS CHROMASOLV® grade) and cotinine solution (drug standard) were supplied by Sigma Aldrich Corporation (St. Louis, Missouri, United States of America). Cotinine-d3 was purchased from Toronto Research Chemicals Inc. (Toronto, Canada)

4.2.2 Instruments

For the compliance verification, the following instruments were used: HP 1100 HPLC (Agilent Technologies, Santa Clara, USA) equipped with PAL auto sampler (CTC Analytics AG, Zwingen, Switzerland) coupled to API 4000 tandem mass spectrometer (AB Sciex Pte. Ltd; Danaher Corporation, Washington D.C., United States of America). A high speed micro centrifuge CT15RE (VWR LLC, Radnor, Pennsylvania, United States of America). The multi tube vortexer VX 2500 (VWR LLC, Radnor, Pennsylvania, United States of America) and single tube vortexer Reax 2000 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). For the CO determination the Micro II Smokerlyzer CO Monitor (Bedfont Scientific Ltd, Harrietsham, Maidstone, Kent, United Kingdom) was used.

4.2.3 Software

For the evaluation of the compliance data the Analyst Software 1.5.2 (AB Sciex, Darmstadt, Germany), Office 2013 and Office 365 (Microsoft, Redmond, USA) were applied.

4.2.4 Calibration Standards

The calibrations of the cotinine analysis were performed using the standard addition method with weighting. For this purpose, increasing amounts of the authentic standard compound were spiked in water for saliva and in an analyte-free matrix pool for urine. The calibration ranges were 2 to 2000 ng/mL for saliva and 2 to 1000 ng/mL for urine.

4.2.5 Quality Control Samples

In addition to the set of study samples, 93 quality control samples for saliva and 54 quality control samples in low, medium and high concentration levels (31 per level for saliva and 18 for urine) were interspersed throughout the entire analytical batch. The QC samples were matrix samples from smokers with a known level of cotinine. The acceptance criteria for QC samples were defined according to FDA guidelines [245] at ±20% for the low level and ±15% for the medium and high level, respectively. At least two-thirds of all QC samples and 50% per level had to meet the acceptance criteria otherwise the batch had to be repeated.

4.2.6 Sample Preparation and Analytical Methodology

Prior to analysis, the samples had to be thawed at room temperature. Salivette® samples were centrifuged after thawing according to the instruction manual.

Saliva and urine samples were prepared according to Mueller et al. [76, 87] with minor modifications. Briefly, 100 µL of saliva or 25 µL of urine were spiked with 25 µL and 10 µL of internal standard solution, respectively. Protein precipitation was conducted by adding 400 µL of methanol in case of saliva and 100 µL in case of urine. The samples were thoroughly vortexed and frozen at -20°C for 1 h to complete the protein precipitation. After thawing the samples at ambient temperature, they were vortexed for another 10 minutes and centrifuged at 15,330 × g (10°C). 200 µL (for analysis of saliva) and 100 µL (for analysis of urine), of the clear supernatant were transferred into an autosampler microvial for LC-MS/MS analysis.

5 µL of the saliva sample and 10 µL of the urine sample were injected in the HPLC system (Agilent 1100 series, Waldbronn, Germany). Separation was performed on a Synergi™ MAX-RP 80, (150 mm × 4.6 mm) column with 4 µm particles (Phenomenex, Torrance, USA). The column temperature was set to 45°C during the isocratic separation which was performed with a mobile phase of 20% water with 10 mM ammonium acetate and 80% methanol. The flow rate was set to 1 mL/min. The tandem mass spectrometry analysis was performed on the triple quadrupole MS (API 4000, AB Sciex, Darmstadt, Germany) in positive ionization mode with an atmospheric pressure chemical ionization (APCI) ionization probe (Turbo V™ Ion Source, AB Sciex, Darmstadt, Germany). The multiple reaction monitoring (MRM) of 177 m/z to 80 m/z for cotinine was normalized to the area of the corresponding signal of the deuterated internal standard cotinine-methyl-d_3 (MRM: 180 m/z to 80 m/z) and then divided by the calibration slope in order to get absolute concentrations.

4.2.7 Data Processing

Evaluation of the chromatographic data was performed using Analyst 1.6.3 (AB Sciex, Darmstadt, Germany) for peak integration and Excel 2013 (Microsoft, Redmond, USA) for calibration and evaluation. The cut-off levels were evaluated in Excel.

4.3 Metabolic Fingerprinting Operation Procedure

The samples (plasma, saliva and urine) from stationary visits of the 39 compliant subjects (for details see section 5.2) were used for analysis by means of the untargeted metabolic fingerprinting method. For this purpose, one sample per matrix (plasma, saliva and urine) and point in time (TP0, TP1, TP2, TP3) was selected resulting in 156 samples per matrix. This included the plasma samples of all points in time, the combined 24-h urine sample and sample E of saliva, which was collected together with the plasma sample under fasting conditions in the morning of the second day of each stationary visit (Figure 7).

4.3.1 Chemicals

Chemicals and solutions for the fingerprinting approach were obtained from the following sources: Water (Optigrade®), methanol (Picograde®), hexane (Picograde®) and acetone (Picograde®) were from Promochem (Wesel, Germany). Methanol (absolute ULC/MS grade) and acetonitrile (ULC/MS grade) were purchased from Biosolve BV (Valkenswaard, Netherlands). Trans, trans-muconic acid-d4 (d4-ttMA) was supplied by Toronto Research Chemicals Inc. (Toronto, Canada).

Methoxyamine HCl (MOX, 98%) and C7-C30 saturated alkanes standard (1000 µg/mL each component in hexane, analytical standard) were obtained from Supelco (Taufkirchen, Germany).

Sigma Aldrich Corporation (St. Louis, Missouri, United States of America) supplied pyridine (anhydrous, 99.8%), sodium sulphate (Puriss p.a. >99%), L-alanine(>98%), D(+) glucose (>99,5%), adenosine (>99%), N,O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA with 1% TMCS, 99%), urease from *Canavalia ensiformis* (Jack bean), sodium phosphate monobasic (anhydrous, purum p.a., ≥99%), L-citrulline (98%), levulinic acid (98%), heptadecanoic acid (≥98%), L-aspartic acid (BioXtra, ≥99%), L-cysteine (≥98%), L-tryptophan (≥98%), L-tyrosine (BioUltra, ≥99.0%), L-histidine(≥98%), uric acid (≥99%), DL-cystine (biochemical reagent), creatinine (anhydrous, ≥98%), palmitic acid (BioXtra, ≥99%), urea (BioReagent), arachidonic acid (from porcine liver, ≥99%), oleic acid (analytical standard, ≥99.0%), octanoic acid (analytical standard, ≥99.5%), cholesterol (Sigma Grade, ≥99%), propylene glycol (≥99.5%), 3-Hydroxybutyric acid (95%), α-ketoglutaric acid (≥99%), glycyl-glycine (≥99%), L-glutamic acid (ReagentPlus®, ≥99%) and sodium hydroxide solution (50% in water).

Merck KGaA (Darmstadt, Germany) delivered trans,trans-muconic acid (ttMA, >98%).

According to the method described by Mueller et al. solutions were prepared for the metabolomic fingerprinting. [7, 8] These were a 1 mmol/L solution of the internal standard d4-ttMA in water

(stored at -20°C prior to use), a 20mg/mL solution of the derivatization reagent methoxyamine-HCl for the methoximation (freshly prepared prior to use) and a 1 unit/mL urease solution in 0.2 M sodium phosphate buffer (set to pH7.0 with NaOH) (freshly prepared prior to use). Also, a methanol/water solution (8/1; v/v) and a methanol/acetonitrile solution (7/3; v/v) were prepared (each week freshly, stored at 4°C). For quality control purposes a mixture of L-alanine, ttMA, D(+)-glucose and adenosine was prepared at a concentration of 1 mmol/L per substance in water (stored at -20°C prior to use). Standards of L-citrulline, levulinic acid, heptadecanoic acid, L-aspartic acid, L-cysteine (≥98%), L-tryptophan, L-tyrosine, L-histidine, uric acid, DL-cystine, creatinine, palmitic acid, urea, arachidonic acid, oleic acid, octanoic acid, cholesterol, propylene glycol, 3-Hydroxybutyric acid, α-ketoglutaric acid, glycyl-glycine and L-glutamic acid for the identification of substances were prepared at a concentration of 2 to 4 mg/mL in methanol. Pyridine was stored over sodium sulphate after opening (stored at room temperature).

4.3.2 Instruments

For the fingerprinting the following instruments were used: an Agilent GC 6890A (Agilent Technologies, Santa Clara, United States of America) equipped with Agilent auto sampler and injector 7683 (Agilent Technologies, Santa Clara, United States of America) coupled to Almsco BenchTOF-dx™ (Almsco, Lantrisant, United Kingdom). The analytical scales Acculab Atilon ATL-124-I (Sartorius AG, Göttingen, Germany) and Sartorius typ1401 (Sartorius AG, Göttingen, Germany). As incubation oven an HP GC 5890 series 2 plus (Hewlett-Packard Company, Palo Alto, California, United States of America) was utilized. A Jouan RC10.22 series vacuum concentrator linked to a Jouan RCT90 cold trap (Societe Jouan, Saint Herblain, France) and an Edwards vacuum pump (Edwards Lifesciences, Irvine, California, United States of America). The vortexer multi tube vortexer VX 2500 (VWR LLC, Radnor, Pennsylvania, United States of America) and single tube vortexer Reax 2000 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). For centrifugation the ROTANTA 460 R centrifuge (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany) and high speed micro centrifuge CT15RE (VWR LLC, Radnor, Pennsylvania, United States of America) were used. The ultrasonic bath Bandelin Sonorex RK 100H (BANDELIN electronic GmbH & Co. KG, Berlin, Germany).

4.3.3 Software

The following software was used for the processing and evaluation of the fingerprinting data: AMDIS 2.71 (Automated Mass Spectral Deconvolution and Identification System) [246, 247]

combined with NIST MS Search 2.0 (National Institute of Standards and Technology, Gaithersburg USA) equipped with the mass spectral databases NIST 05 (replib and mainlib) and Golm Metabolome Database GMD 20111121. [248] Further Chemstation 2.0 (Agilent, Santa Clara, USA) and MassHunter Workstation Software B.07.00 (Agilent, Santa Clara, USA), MetaboAnalyst 3.0 [249-251], MZmine 2.10 [252], Office 2013 and Office 365 (Microsoft, Redmond, USA), ProtoTOF 1.1.1 (Almsco, Llantrisant, United Kingdom), PSPP 0.8.5 [89], R 3.3.0 and RStudio 0.99.902, SigmaPlot 10.0 (Systat Software GmbH, Erkrath, Germany), Viso 2016 (Microsoft, Redmond, USA).

4.3.4 Quality Control Samples

In addition to the set of study samples, blank runs (20 per matrix), reagent blanks (5 per matrix) and quality control (QC) samples (14 per matrix) were interspersed throughout the analytical batch in order to monitor method performance and carry over effects. For the QC samples, a pool of all compliant subjects was prepared for each body fluid and spiked with L-alanine, trans,trans-muconic acid (ttMA), D-glucose and adenosine at 50 µM. The analytical run was accepted if the QC analytes showed reproducibility within a coefficient of variation (CV) of ±20%.

4.3.5 Sample Preparation and Analytical Methodology

The sample preparation of the fingerprinting approach was performed according to the validated method developed by Mueller et al. [87] with minor modifications. Prior to analysis the samples were thawed and aliquots were transferred into safe-seal micro-tubes (Sarstedt AG & Co, Nümbrecht, Germany). 50 µL of plasma and 100 µL of saliva or urine were spiked with 25 µL internal standard (d_4-ttMA). In case of urine, urea was digested by adding 40 units of urease (*Canavalia ensiformis*) followed by incubation for 45 min at 37°C. A two-step extraction and protein precipitation was performed by first adding 150 µL of a methanol/acetonitrile solution (7/3; v/v) to the plasma samples and 500 µL to saliva and urine samples. After vortexing thoroughly, the samples were frozen at -20°C for 1 h to complete the protein precipitation. The samples were centrifuged at 15,330 × g at 4°C for 15 minutes and the clear supernatant was transferred into screw cap glass vials (Ziemer Chromatographie, Langerwehe, Germany). The second step of the extraction and protein precipitation was performed by adding 100 µL of a methanol/water solution (8/1; v/v). This was followed by 30 min of freezing at -20°C and centrifugation at 15,330 × g (4°C) for 15 min. The clear supernatant was combined with the first extract and evaporated to almost dryness in a vacuum concentrator (Jouan RC 10.22, Societé Jouan, Saint Herblain, France). Then, 50 µL of

pyridine was added and the extract was completely dried under a gentle stream of nitrogen. The two-step derivatization was conducted by firstly adding 75 µL of a freshly prepared MOX solution, sonicating for three minutes and incubating at 60°C for 30 min and secondly adding 75 µL of BSTFA with 1% TMCS followed by sonication for three min and incubation at 60°C for 30 min. 2 µL of a C7-C30 saturated alkanes standard were added, the samples were vortexed and centrifuged at 3,300 × g (10°C) for 15 min. 100 µL of the clear supernatant were transferred into auto-sampler microvials.

The different matrices required different split ratios for the injection of 1 µL sample. The split/splitless injector was set to a split ratio of 1/15 for plasma, 1/5 for saliva, and 1/20 for urine. A single taper, deactivated glass-wool liner (Agilent Technologies, Santa Clara, USA) was used and constantly heated at 250°C. The separation was performed on an Agilent GC 6890A (Agilent Technologies, Santa Clara, USA) equipped with an Rxi®-1ms (30 m × 250 µm ID, 0.25 µm film thickness, Restek, Bad Homburg, Germany) fused silica column and the auto-sampler and injector 7683 (Agilent Technologies, Santa Clara, USA) which was set to 10 °C. The helium flow rate was set to 1.1 mL/min. The temperature programme as reported by Mueller *et al.* was used with an initial oven temperature of 50°C for 1 min, an increase of 10°C/min to 300°C which was held for 10 min. [87] The GC was linked to the time-of-flight (TOF) mass spectrometer (BenchTOF-dx™, Almsco, LLantrisant, United Kingdom) by the transfer line constantly heated to 250°C. The TOF-MS operated in electron ionization mode at 70 eV. The mass resolution of the TOF-MS at m/z 219 was found to be 1278 equivalent to 0.17 amu. Data acquisition was performed by scanning from m/z 40 to 650 with a sampling rate of 5 Hz after an initial filament delay of 5.6 min.

4.3.6 Data Processing

Data analysis was performed according to Mueller et al. [87] with minor modifications. The data was baseline corrected via ProtoTOF 1.1.1 (Almsco, LLantrisant, United Kingdom). Peak picking was performed by using MZmine 2.10 [252] including the following steps subsequently: crop filtering, mass detection, chromatogram building, chromatogram deconvolution, retention time normalization, join alignment, peak finding, duplicate filtering, peak list row filtering and export into a .csv file. The peak areas from the peak picking were normalized by the internal standard in Excel. P-values according to the Wilcoxon signed rank test were calculated with R and RStudio Version 1.0.143. A partial least square discriminant analysis (PLS-DA) was performed using the online platform MetaboAnalyst 3.0. [249-251] Significant alterations (variable importance in the projection (VIP) >0.8 of the PLS-DA; P-value <0.05 in the Wilcoxon signed rank test), responsible

for a separation between the different points in time, were subsequently identified by applying the deconvolution software AMDIS 2.71 equipped with the NIST MS search programme 2.0 and mass spectral libraries (NIST 05, GMD 20111121). [246-248] Further, the fold change of the compared groups was calculated as ratio of the mean areas with a cut-off of 5% for both the highest and lowest values to exclude outliers. Prior to statistical evaluation a representative data set was investigated for normal distribution by means of the Shapiro Wilcoxon test. Non-normal distribution was observed as expected based on the experience from our previous study. Correction for multiple testing was performed with the Benjamini-Hochberg procedure. [253]

4.4 Fatty Acid Profiling Operation Procedure

The samples from stationary visits of the 39 compliant subjects (for details see section 5.2) were used for analysis by means of the targeted metabolic profiling of fatty acids. For this purpose, the plasma samples of each point in time (TP0, TP1, TP2, TP3) were selected resulting in 156 samples.

4.4.1 Chemicals

Chemicals and solutions for the fatty acid profiling approach were obtained from the following sources: chloroform (Picograde®), hexane (Picograde®), methanol (Picograde®) and water (Optigrade®) were supplied by Promochem (Wesel, Germany). 8-methylnonanoic acid (FA10:0 *iso*; 99%) was purchased from Ultra Scientific (North Kingstown, United States of America). Pentacosanoic acid (97%) was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Supelco 37 Component FAME Mix (TraceCERT®, containing methyl esters (ME) of the following acids: butyric acid (FA 4:0), hexanoic acid (FA 6:0), octanoic acid (FA 8:0), decanoic acid (FA 10:0), undecanoic acid FA 11:0), lauric acid (FA 12:0), tridecanoic acid (FA 13:0), myristic acid (FA 14:0), myristoleic acid (FA 14:1 9Z), pentadecanoic acid (FA 15:0), cis-10-pentadecenoic acid (FA 15:1 10Z), palmitic acid (FA 16:0), palmitoleic acid (FA 16:1 9Z), heptadecanoic acid (FA 17:0), cis-10-heptadecanoic acid (FA 17:1 10Z), stearic acid (FA 18:0), trans-9-elaidic acid (FA 18:1 9E), cis-9-oleic acid (FA 18:1 9Z), linolelaidic acid (FA18:2 9E, 12E), linoleic acid (FA 18:2 9Z, 12Z), γ-linolenic acid (FA 18:3 6Z, 9Z, 12Z), α-linolenic acid (FA 18:3 9Z, 12Z 15Z), arachidic acid (FA 20:0), 11-eicosenoic acid (FA 20:1 11Z), cis-11,14-eicosadienoic acid (FA 20:2 11Z, 14Z), cis-8,11,14-eicosatrienoic acid (FA 20:3 8Z, 11Z 14Z), cis-11,14,17-eicosatrienoic acid (FA 20:3 11Z, 14Z, 17Z), cis-5,8,11,14-eicosatetraenoic acid (FA 20:4 5Z, 8Z, 11Z, 14Z), cis-5,8,11,14,17-eicosapentaenoic acid (FA 20:5 5Z, 8Z, 11Z, 14Z, 17Z), heneicosanoic acid (FA 21:0), behenic acid (FA 22:0), erucic acid (FA 22:1 13Z), cis-13,16-docosadienoic acid (FA 22:0 13Z, 16Z), cis-4,7,10,13,16,19-docosahexaenoic acid (FA 22:6 4Z, 7Z, 10Z, 13Z, 16Z, 19Z), tricosanoic acid (FA 23:0), tetracosanoic acid (FA 24:0) and nervonic acid (FA 24:1 15Z) at defined levels between 200 to 400 µg/mL) was purchased from Supelco (Taufkirchen, Germany). Octanoic acid (FA 8:0; analytical standard, ≥99.5%); palmitic acid (FA 16:0; analytical standard, ≥99%); oleic acid (FA 18:1 9Z; analytical standard, ≥99%); arachidonic acid (FA 20:4 FA 20:4 5Z, 8Z, 11Z, 14Z; analytical standard, ≥97%); cis-4,7,10,13,16,19-docosahexaenoic acid (FA 22:6 4Z, 7Z, 10Z, 13Z, 16Z, 19Z; analytical standard, ≥98,5%); hexacosanoic acid (FA 26:0; analytical standard, ≥95%); potassium carbonate (anhydrous, puriss p.a., ≥99%) and acetyl chloride (Puriss p.a., ≥99%) were from Sigma Aldrich Corporation (St. Louis, Missouri, United States of America). 8-methylnonanoic

acid methyl ester (FA 10:0 iso; ≥98%); 11-octadecenoic acid methyl ester (FA 18:1 11Z; ≥99%); 8,11,14,17-eicosatetraenoic acid methyl ester (FA 20:4 8Z, 11Z, 14Z, 17Z; 98%); docosatetraenoic acid methyl ester (FA 22:4 7Z, 10Z, 13Z, 16Z; ≥99%); docosapentaenoic acid methyl ester (FA 22:5 7Z, 10Z, 13Z, 16Z, 19Z; ≥99%); pentacosanoic acid methyl ester (FA 25:0; ≥99%); methyl hexacosanoic acid methyl ester (FA 26:0; ≥99%); octaconanoic acid methyl ester (FA 28:0; ≥98%); triacontanoic acid methyl ester (FA 30:0; 98%) and dotriacontanoic acid methyl ester (FA 32:0; ≥98%) were supplied by Larodan AB (Solona, Sweden). The Merck KGaA (Darmstadt, Germany) supplied potassium carbonate (anhydrous, p.a, ≥99%).

The methyl esters were dissolved in hexane for the method development and retention time identification of the substances. Stock solutions at a concentration of 1 mg/mL of FA 10:0 *iso*, FA 8:0, FA 16:0, FA 18:1 9Z and FA 20:4 5Z, 8Z, 11Z, 14Z were prepared in methanol while stock solutions of FA 25:0, FA 22:6 4Z, 7Z, 10Z, 13Z, 16Z, 19Z and FA 26:0 were prepared in methanol/chloroform (9/1; v/v).

4.4.2 Instruments

For the profiling of fatty acids, the following instruments were used: an Agilent GC 6890A (Agilent Technologies, Santa Clara, United States of America) equipped with Agilent auto sampler and injector 7683 (Agilent Technologies, Santa Clara, United States of America) coupled to Almsco BenchTOF-dx™ (Almsco, Lantrisant, United Kingdom). The analytical scales Acculab Atilon ATL-124-I (Sartorius AG, Göttingen, Germany) and Satorius typ1401 (Sartorius AG, Göttingen, Germany), a multi tube vortexer VX 2500 (VWR LLC, Radnor, Pennsylvania, United States of America) and single tube vortexer Reax 2000 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), the ROTANTA 460 R centrifuge (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany), a water bath W760 (Memmert GmbH & Co. KG, Schwabach, Germany).

4.4.3 Software

The following software was used for the processing and evaluation of the fatty acid data: Chemstation 2.0 (Agilent, Santa Clara, USA), MassHunter Workstation Software B.07.00 (Agilent, Santa Clara, USA), Office 2013 and Office 365 (Microsoft, Redmond, USA), ProtoTOF 1.1.1 (Almsco, Llantrisant, United Kingdom and R 3.3.0 and RStudio 0.99.902.

4.4.4 Calibration Standards

Quantification was performed with the standard addition method. A set of 6 representative FAs was selected for calibration and validation experiments comprising various chain lengths and saturation degrees (FA 8:0; FA 16:0; FA 18:1 9Z; FA 20:4 5Z, 8Z, 11Z, 14Z; FA 22:6 4Z, 7Z, 10Z, 13Z, 16Z, 19Z and FA 26:0). Calibration was performed with a set of five to ten calibrators, depending on the target FA and the linear range. Due to the lack of analyte-free matrix samples, the calibration was prepared in water as surrogate matrix. The calibration slopes were compared between plasma and water and revealed similar results. Table 7 shows all analytes and the considered calibrators as well as the internal standards and quantifier and qualifiers.

Table 7: Analytes (numbers cf. Figure 12) and internal standards (characters) of GC-TOF-MS analysis including the appearance in the chromatogram (No.), retention time (RT), quantifier, qualifier and calibration standard.

No	Analyte	RT [min]	Quan [m/z]	Qual 1 [m/z]	Qual 2 [m/z]	Calibration by
A	FA 10:0 ISO	8.42	74	87	143	-
B	FA 25:0	22.66	74	87	396	-
1	FA 4:0	4.90	74	102	87	FA 8:0
2	FA 6:0	6.28	74	87	99	FA 8:0
3	FA 8:0	7.56	74	87	158	FA 8:0
4	FA 10:0	8.68	74	87	186	FA 8:0
5	FA 11:0	9.23	74	87	200	FA 8:0
6	FA 12:0	9.80	74	87	214	FA 16:0
7	FA 13:0	10.42	74	87	228	FA 16:0
8	FA 14:0	11.09	74	87	242	FA 16:0
9	FA 14:1 9Z	11.58	55	69	240	FA 18:1 9Z
10	FA 15:0	11.84	74	87	256	FA 16:0
11	FA 15:1 10Z	12.39	55	69	254	FA 18:1 9Z
12	FA 16:0	12.67	74	87	270	FA 16:0
13	FA 16:1 9Z	13.18	55	69	268	FA 18:1 9Z
14	FA 17:0	13.58	74	87	284	FA 16:0
15	FA 17:1 10Z	14.15	55	69	282	FA 18:1 9Z
16	FA 18:0	14.58	74	87	298	FA 16:0
17	FA 18:1 9E	14.90	55	69	296	FA 18:1 9Z
18	FA 18:1 9Z	15.11	55	69	296	FA 18:1 9Z
19	FA 18:1 11Z	15.20	55	69	296	FA 18:1 9Z
20	FA 18.2 9E,12E	15.46	67	81	294	FA 18:1 9Z
21	FA 18:2 9Z,12Z	15.94	67	81	294	FA 18:1 9Z

No	Analyte	RT [min]	Quan [m/z]	Qual 1 [m/z]	Qual 2 [m/z]	Calibration by
22	FA 18:3 6Z,9Z,12Z	16.53	79	67	292	FA 20:4 5Z,8Z,11Z,14Z
23	FA 20:0	16.76	74	87	326	FA 16:0
24	FA 18:3 9Z,12Z,15Z	16.94	79	67	292	FA 20:4 5Z,8Z,11Z,14Z
25	FA 20:1 11Z	17.35	55	69	324	FA 18:1 9Z
26	FA 21:0	17.91	74	87	340	FA 16:0
27	FA 20:2 11Z,14Z	18.28	67	81	322	FA 18:1 9Z
28	FA 20:3 8Z,11Z,17Z	18.93	79	67	320	FA 20:4 5Z,8Z,11Z,14Z
29	FA 22:0	19.10	74	87	354	FA 16:0
30	FA 20:4 5Z,8Z,11Z,14Z	19.41	79	150	318	FA 20:4 5Z,8Z,11Z,14Z
31	FA 22:1 13Z	19.75	55	69	352	FA 18:1 9Z
32	FA 20:4 8Z,11Z,14Z,17Z	20.04	79	67	318	FA 20:4 5Z,8Z,11Z,14Z
33	FA 23:0	20.29	74	87	368	FA 26:0
34	FA 20:5 5Z,8Z,11Z,14Z,17Z	20.53	79	91	67	FA 22:6 4Z,7Z,10Z,13Z,16Z,19Z
35	FA 22:2 13Z,16Z	20.72	67	81	350	FA 18:1 9Z
36	FA 24:0	21.47	74	87	382	FA 26:0
37	FA 22:4 7Z,10Z,13Z,16Z	22.02	79	67	91	FA 20:4 5Z,8Z,11Z,14Z
38	FA 24:1 15Z	22.15	55	69	348	FA 18:1 9Z
39	FA 22:5 7Z,10Z,13Z,16Z,19Z	23.17	74	91	119	FA 22:6 4Z,7Z,10Z,13Z,16Z,19Z
40	FA 22:6 4Z,7Z,10Z,13Z,16Z,19Z	23.58	79	91	131	FA 22:6 4Z,7Z,10Z,13Z,16Z,19Z
41	FA 26:0	23.81	74	87	410	FA 26:0
42	FA 28:0	26.11	74	87	438	FA 26:0
43	FA 30:0	28.69	74	87	466	FA 26:0
44	FA 32.0	31.83	74	87	494	FA 26:0

4.4.5 Quality Control Samples

In addition to the study samples, a set of quality control (QC) samples at three different concentration levels (low, medium and high) were prepared by spiking a plasma pool with 6 different FAs (see section 4.4.4). For the high-level QC samples, a plasma pool was spiked with these 6 analytes and aliquoted. For the low and medium QCs, the plasma pool was diluted to receive appropriate FA concentrations. QCs were prepared so that the concentrations reflected the expected FA ranges as described in literature [86, 254] as well as from own experiments. The target values were determined in a pre-series (N=10 for each level). 15 quality control samples (5 of each level) were interspersed throughout the analytical batch. The acceptance criteria for QC samples were

defined according to FDA guidelines [245] at ±20% for the low level and ±15% for the medium and high level, respectively. At least two-thirds of all QC samples and 50% per level must meet the acceptance criteria otherwise the batch was repeated.

4.4.6 Sample Preparation and Analytical Methodology

Sample preparation was performed according to a method described by Lepage et al. with minor modifications. [199, 205, 255, 256] Briefly, plasma samples were thawed at room temperature and 50 µL plasma aliquots were spiked with 50 µL of internal standard (FA 10:0 *iso* and FA 25:0 each at 50 µg/mL in methanol) and transferred into a vial. Freshly prepared methanolic acetylchloride (10/1, v/v) was added for derivatization and n-hexane for liquid/liquid extraction of the fatty acid methyl esters (FAMEs). Then, the samples were thoroughly vortexed and incubated in a water bath at 80°C for 2 h. Subsequently, the samples were cooled to room temperature and 5 mL of a potassium carbonate solution (0.43 mol/L) were added. After centrifugation for 15 min at 3,300 × g (10°C), 200 µL of the supernatant was transferred into an autosampler vial and placed in the autosampler at 8°C until analysis.

Gas chromatography was performed using an Agilent GC 6890A equipped with an Agilent autosampler and injector 7683 (Agilent Technologies, Waldbronn, Germany). GC separation was achieved by using an Agilent J&W VF-23ms column 60 m × 250 µm ID fused silica column (Agilent Technologies, Waldbronn, Germany). The column was selected due to extraordinary polarity owing to the cyanopropyl substitution (0.25 µm film thickness) which allows for the separation of geometrical and positional isomers of FAs. Helium served as carrier gas at a constant pressure of 33 cm/s. The split/splitless injector was set to 250°C and equipped with an Agilent deactivated single taper glass wool liner (Agilent Technologies, Santa Clara, United States of America). A split of 1/10 was used for the injection of 1 µL of the sample. The temperature programme of the GC oven was used as follows: The initial oven temperature was set to 50°C for 1 min, then it was raised to 175°C with a rate of 25°C per min. Subsequently the temperature was raised to 250°C with a rate of 4°C per min. This lower heating ramp enables the separation of E- and Z-isomers. The final temperature was held for 11.25 min. The total analysis time was 35 min. The time of flight mass spectrometer (BenchTOF-dx, Almsco, Llantrisant, United Kingdom) operated in electron ionization mode at 70 eV with a filament delay of 230 s. The mass resolution of the TOF MS at m/z 219 was found to be 1278 respectively 0.17 amu. Data acquisition was performed in the full scan mode from 40 to 500 m/z with a sampling rate of 5 Hz.

4.4.7 Validation

Validation was performed according to FDA guidelines [245] in order to determine the analytical performance of the method including accuracy, accuracy after sample dilution, carryover, LOD, LOQ, precision, recovery, reproducibility of re-injections, sample stability and specificity by using 6 representative FA species (cf. section 4.4.4). [245] These 6 individual FA species covered a broad range of polarities from short to long chain FAs including various saturation degrees. For quantification purposes, only the most characteristic fragment ions of each compound were considered (cf. Table 7). Plasma samples for validation were either obtained by spiking a plasma pool or water as surrogate matrix.

4.4.7.1 Accuracy

The accuracy was calculated in water as surrogate matrix due to the lack of analyte-free matrix samples. Analytes were spiked at 3 different concentration levels (low, medium and high), covering the entire calibration range. The levels were analysed in 5 replicates and compared to a calibration prepared with certified reference material.

4.4.7.2 Accuracy after Sample Dilution

Since it was expected that some sample concentrations could exceed the upper limit of quantification (ULOQ), accuracy after sample dilution was assessed. Therefore, three matrix samples were spiked with analyte concentrations above the ULOQ and subsequently used for dilution experiments. Samples were diluted by a factor of 1/1, 1/4 and 1/8 to receive analyte concentrations within the calibration range.

4.4.7.3 Accuracy Carryover

A plasma pool was spiked with the highest calibrator leading to levels above the calibration range. These were injected 5 times in a row, followed by 3 injections of solvent blanks (n-hexane). This procedure was repeated 3 times.

4.4.7.4 Limit of Detection

The limit of detection (LOD), was defined according to IUPAC guidelines [257, 258] and calculated from an blank sample ($\overline{x}_{bl}$) determined in triplicates and the standard deviation (s_{bl}).

$$x_{LOD} = \overline{x}_{bl} + 3\, s_{bl}$$

4.4.7.5 Limit of Quantification

The limit of quantification (LOQ), was defined according to IUPAC guidelines. [257, 258] on the basis of the blank samples ($\bar{x}_{bl}$) and the standard deviation (s_{bl}) analysed for the LOD calculation.

$$x_{LOD} = \bar{x}_{bl} + 10\ s_{bl}$$

4.4.7.6 Precision

Intra- and inter-day precision was determined by analysing samples at 3 different concentration levels (low, medium and high) in 5 independent sample preparations (intra-day, N=5) and on 6 different days (inter-day, N=6), respectively. The concentration levels were prepared by spiking a plasma pool with high concentrations of the analytes. The high pool was further diluted with water to medium and low concentration.

4.4.7.7 Recovery

The extraction efficiency was determined by spiking analyte-free matrix (water) in 6 replicates at 3 different concentration levels (low, medium, high) followed by extraction and derivatization. The results were then compared to the corresponding FA methyl esters in the same molar concentrations directly injected into the GC-TOF-MS instrument. The determined recoveries indicate the sample work-up-related losses of the analyte.

4.4.7.8 Reproducibility of Re-injections

Samples of 2 different concentration levels (low and high) were analysed in triplicates immediately, after 48 h and after 72 h storage in the autosampler.

4.4.7.9 Sample stability

The validation comprised the determination of 4 types of stability parameters:

Short-term stability: Triplicates of spiked matrix samples at 2 different known concentration levels (low and high) were prepared after keeping them at ambient temperature for 24 h.

Post-preparative stability: Stability was determined for 29 days. The spiked plasma samples prepared in advance, were kept at autosampler temperature (8°C) for the time established and analysed with freshly prepared samples serving as reference.

Freeze-thaw stability: Spiked plasma at low and high concentration was stored for 24 h at -20°C, thawed and stored at ambient temperature for 6 h. The freeze-thaw cycle was repeated 6 times before sample analysis by GC-TOF-MS. The concentrations observed were compared against their reference value derived from analysis of fresh samples.

Long-term stability: Long-term stability in matrix was determined in spiked plasma samples (low and high concentration levels) at -20°C. The long-term stability was determined after one month and will further be determined at several points in time (e.g., 6, 12 and 24 months) depending on the stability time required.

4.4.7.10 Selectivity

The selectivity of the method was assessed by comparing retention times (RT) of reference material spiked to water and matrix samples. For each compound three *m/z* values were selected (a quantifier and two qualifiers) that showed no interference at the respective RT. As far as possible 3 ions were taken into account, using the ion with the highest S/N ratio quantifier, followed by the ions with the second and third highest S/N ratio as qualifiers. The selectivity was further verified by analysing the quantifier/qualifier ratios in 6 different matrix samples. Acceptance criteria for the quantifier/qualifier ratio were in the range of ±25% to the target value, which was determined from the corresponding calibration.

4.4.8 Data Processing

The ProtoTOF data files (.HDR; generated by the Almsco TOF-MS software) were converted to Agilent ChemStation files (.d) and loaded into Agilent MassHunter for peak integration. Thresholds were applied at a signal-to-noise ratio of 3/1 and 9/1 for the limit of detection and limit of quantification, respectively. The calculation of concentration was performed with Microsoft Excel. Statistical tests (Quade test and Wilcoxon signed rank test) were calculated using R 3.3.0 and RStudio 0.99.902.

4.5 Amino Acid Profiling Operation Procedure

The samples from stationary visits of the 39 compliant subjects (for details see section 5.2) were used for analysis by means of the targeted metabolic profiling of amino acids. For this purpose, the plasma samples of each point in time (TP0, TP1, TP2, TP3) were selected resulting in 156 samples. Additionally, the samples of the initial study comparing smokers (n=25) and non-smokers (n=25) [76, 86, 87] were included.

4.5.1 Chemicals

Chemicals and solutions for the amino acid profiling approach were obtained from the following sources: acetonitrile (Optigrade®), water (Optigrade®) and methanol (Picograde®) were from Promochem (Wesel, Germany). formic acid (99%), water with 0.1% formic acid (ULC/MS grade) and acetonitrile with 0.1% formic acid (ULC/MS grade) were purchased from Biosolve BV (Valkenswaard, Netherlands). 5-hydroxy-L-tryptophan (98%), L-amino acids kit (analytical standards, containing: L-alanine (99%), L-arginine hydrochloride (100%), L-asparagine (100%), L-aspartic acid (100%), L-cysteine hydrochloride (>98%), L-cystine (>98%), L-glutamic acid (99%), L-glutamine (100%), glycine (100%), L-histidine hydrochloride (100%), trans-4-hydroxy-L-proline (99,8%), L-isoleucine (100%), L-leucine (99%), L-lysine hydrochloride (100%), L-methionine (100%), L-phenylalanine (100%), L-proline (100%), L-serine (>99%), L-threonine (100%), L-tryptophan (100%), L-tyrosine (>98%) and L-valine (100%)), cell free amino acid mixture ^{13}C, ^{15}N,D (containing all 20 standard AAs, enrichment of isotopes ^{13}C 99.6%, ^{15}N 99.0%, non-exchangeable D 97.1%), NG,NG-dimethylarginine dihydrochloride (asymmetric dimethyl-arginine, ≥ 98%), NG,NG′-dimethyl-L-arginine di(p-hydroxyazobenzene-p′-sulfonate) salt (symmetric dimethyl-arginine, ≥ 99%), L-citrulline (≥ 98%) and 9-fluorenylmethyl-chloroformate chloride (FMOC-Cl, 99.8%) were supplied by Sigma Aldrich Corporation (St. Louis, Missouri, USA). Ammonium formate (≥99%), sodium hydroxide solution in water (50%) and sodium tetra-borate decahydrate (99.5%) were obtained from FLUKA (St. Louis, Missouri, USA). Kynurenine (≥98%) and serotonin hydrochloride (≥98%) were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA).

Solutions for the amino acid analysis were prepared as follows. A 100 µmol/L solution of Sodium tetra-borate decahydrate was prepared in water and adjusted to pH 10 with a 0.2 mol/L NaOH (stored at 4°C). For FMOC derivatization a 15 mmol/L solution of FMOC-Cl in acetonitrile was prepared and stored at 4°C prior to analysis. For the calibration, amino acids solutions were prepared as mix-stock solution in 0.1 mol/L HCl.

4.5.2 Instruments

For the profiling of amino acids, the following instruments were used: a Shimadzu Nexera X2 UPLC system including a binary pump, an autosampler, a degasser and a column oven (Shimadzu Corp., Kyoto, Japan). A QTRAP® triple quadrupole mass spectrometer API 6500+ equipped with a Turbo V ion spray source, operating in negative ESI mode, was used for detection (AB Sciex, Darmstadt, Germany). High purity nitrogen was produced by a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany).further Instruments used were the analytical scales Acculab Atilon ATL-124-I (Sartorius AG, Göttingen, Germany) and Sartorius typ1401 (Sartorius AG, Göttingen, Germany), a Jouan RC10.22 series vacuum concentrator linked to a Jouan RCT90 cold trap (Societe Jouan, Saint Herblain, France) and an Edwards vacuum pump (Edwards Lifesciences, Irvine, California, United States of America), the multi tube vortexer VX 2500 (VWR LLC, Radnor, Pennsylvania, United States of America) and single tube vortexer Reax 2000 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) a ROTANTA 460 R centrifuge (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany) and high speed micro centrifuge CT15RE (VWR LLC, Radnor, Pennsylvania, United States of America).

4.5.3 Software

The following software was used for the processing and evaluation of the fatty acid data: Multiquant™ (AB Sciex, Darmstadt, Germany), Office 2013 and Office 365 (Microsoft, Redmond, USA and R 3.3.0 and RStudio 0.99.902.

4.5.4 Calibration Standards

Quantification was performed with the standard addition method. For each analyte (L-alanine (Ala), L-arginine (Arg), asymmetric dimethyl-arginine (ADMA), symmetric dimethyl-arginine (SDMA), L-N-mono-methyl-arginine (L-N-MMA), L-asparagine (Asn), L-aspartic acid (Asp), citrulline (Cit), L-glutamic acid (Glu), L-glutamine (Gln), glycine (Gly), L-histidine (His), trans-4-hydroxy-L-proline (Hyp), L-isoleucine (Ile), L-leucine (Leu), kynurenine (Kyn), L-lysine (Lys), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-serine (Ser), serotonin (5-HT), L-threonine (Thr), L-tryptophan (Trp), L-tyrosine (Tyr) and L-valine (Val)) a separate calibration was provided. Calibration was performed with a set of 6 to 14 calibrators, depending on the target AA and the calibration range. Due to the lack of analyte-free matrix samples, the calibration was prepared in water as surrogate matrix. The calibration slopes were compared between plasma and water and revealed similar results.

4.5.5 Quality Control Samples

In addition to the study samples, a set of quality control (QC) samples at three different concentration levels (low, medium and high) were prepared for all analytes (cf. section 4.5.4). For the high-level QC samples, a plasma pool was spiked with the analytes and aliquoted. The medium level was the plasma pool, not spiked or diluted as all analytes included into the method are present in human plasma. For the low-level QCs, the plasma pool was diluted with water to receive appropriate AA concentrations. QCs were prepared so that the concentrations reflected the expected AA ranges as described in the literature [259] as well as from own experiments and the human metabolome database (HMDB). [70-72] The target values were determined in a pre-series (N=6 for each level). 15 quality control samples (6 of each level) were interspersed throughout the analytical batch. The acceptance criteria for QC samples were defined according to FDA guidelines [245] at ±20% for the low level and ±15% for the medium and high level, respectively. At least two-thirds of all QC samples and 50% per level had to meet the acceptance criteria, otherwise the batch was repeated.

4.5.6 Sample Preparation and Analytical Methodology

Sample preparation was performed as follows. Prior to analysis the samples were thawed and 100-µL aliquots were transferred into safe-seal micro-tubes (Sarstedt AG & Co, Nümbrecht, Germany). 6 µL of a mixture of the 20 standard AAs (500 µmol/L) ^{13}C-, ^{15}N- and D- labelled (cf. Table 8) were added as internal standard and 400 µL of methanol was added to precipitate proteins. The samples were thoroughly vortexed and frozen at -20°C for 1 h to complete the protein precipitation. After thawing the samples at ambient temperature, they were centrifuged for 15 min at 15,330 × g (4 °C). 400 µL of the clear supernatant were transferred into 1.5 mL glass vials (Macherey-Nagel, Düren, Germany) and dried in a vacuum concentrator. The sample was reconstituted by adding 100 µL of a 100 µmol/L borate buffer (pH 10). After vortexing, 100 µL of a 25 mmol/L Fmoc-Cl solution were added to perform the derivatization. The samples were vortexed and incubated for 15 min at ambient temperature. To stop the reaction 4 µL of formic acid were added and the samples were vortexed again. Finally, the samples were centrifuged at 3,300 × g (10°C) for 10 min to remove solid particles and 100 µL of the clear supernatant were transferred into autosampler microvials (BGB Analytik Vertrieb GmbH, Rheinfelden, Germany).

Liquid chromatography was performed with a Shimadzu Nexera X2 UPLC system. A QTRAP® triple quadrupole mass spectrometer API 6500^{+} was used for detection. Chromatographic

separation was achieved by injection of 5 µL sample via gradient elution on a Raptor™ ARC-18 2.7 µm, 3 mm × 100 mm column (Restek GmbH, Bad Homburg, Germany) with 10 mmol/L ammonium formate with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) as mobile phases. The column oven was maintained at 40°C. Chromatographic separation was optimized to obtain good resolution amongst the isobar analytes SDMA and ADMA as well as Leu and Ile. Gradient elution started with 80% A for 0.5 min, followed by a linear decrease to 60% A until 5 min and hold for 3 min, followed by two linear decreases to 40% A (10 min) and 0% A at 13 min.

The column was rinsed for 2 min with 0% A, followed by a re-equilibration at 80% A from 15.1 min until 18 min. The flow rate was set to 800 µl/min throughout the entire run. The turbo spray IonDrive ion source was operated in the negative ionization mode using the following settings: ion spray voltage = -4500 V, ion source heater temperature = 350°C, source gas 1 = 60 psi, source gas 2 = 80 psi, and curtain gas setting = 40 psi. Since numerous mass transitions are required to cover the 26 AAs and their respective IS, analytes were monitored in scheduled multiple reaction monitoring (SMRM) mode, applying a retention time window of ±30 s. Target scan time was set to 0.50 s. Quantifier and qualifier (as far as available) mass transitions and retention times (RT) are shown in Table 8. Quadrupoles Q1 and Q3 were working at unit resolution. Tuning was performed in single substance infusion experiments to achieve the ideal qualifier and quantifies for each substance.

Table 8: Analytes and internal standards LC-MS/MS analysis including the appearance in the chromatogram (No.), retention time (RT), quantifier, qualifier and internal standard (IS) MRM transitions and the correlating IS and labelling.

No	Analyte	RT [min]	Quantifier MRM [m/z → m/z]	Qualifier MRM [m/z → m/z]	IS labelling	IS MRM [m/z → m/z]
1	His	3.67	376.1 → 179.8	376.1 → 153.7	$^{13}C_6$,D_5,$^{15}N_3$-His	390.1 → 193.8
2	Arg	4.31	395.1 → 172.7	395.1 → 198.8	$^{13}C_6$,D_7,$^{15}N_4$-Arg	412.1 → 189.7
3	L-N-MMA	4.56	409.1 → 212.9	409.1 → 186.8	$^{13}C_6$,D_7,$^{15}N_4$-Arg	412.1 → 189.7
4	Asn	4.69	353.1 → 156.9	353.1 → 112.8	$^{13}C_4$,D_3,$^{15}N_2$-Asn	362.1 → 165.9
5	Gln	4.76	367.1 → 170.8	367.1 → 144.9	$^{13}C_5$,D_5,$^{15}N_2$-Gln	379.1 → 182.8
6	ADMA	4.82	423.1 → 227.0	423.1 → 201.0	$^{13}C_6$,D_7,$^{15}N_4$-Arg	412.1 → 189.7
7	Cit	4.86	396.1 → 199.8	396.1 → 173.8	$^{13}C_6$,D_7,$^{15}N_4$-Arg	412.1 → 189.7
8	Ala	4.87	310.1 → 87.9	310.1 → 113.7	$^{13}C_3$,D_4,^{15}N-Ala	318.1 → 95.9
9	SDMA	4.89	423.1 → 227.1	423.1 → 201.0	$^{13}C_6$,D_7,$^{15}N_4$-Arg	412.1 → 189.7
10	Hyp	4.99	352.0 → 129.7	352.0 → 127.8	$^{13}C_5$,D_7,^{15}N-Pro	349.1 → 126.8
11	Ser	5.15	326.0 → 99.9	326.0 → 103.9	$^{13}C_3$,D_3,^{15}N-Ser	333.0 → 103.9
12	Asp	5.26	354.0 → 157.9	354.0 → 114.7	$^{13}C_4$,D_3,^{15}N-Asp	362.0 → 165.9
13	Glu	5.48	368.1 → 171.8	368.1 → 127.9	$^{13}C_5$,D_5,^{15}N-Glu	379.1 → 182.8
14	Thr	5.76	340.1 → 144.1	340.1 → 117.8	$^{13}C_4$,D_5,^{15}N-Thr	350.1 → 154.1
15	Gly	6.02	296.0 → 74.0	296.0 → 99.8	$^{13}C_2$,D_2,^{15}N-Gly	301.0 → 79.0
16	Tyr	7.13	402.1 → 180.0	402.1 → 205.9	$^{13}C_9$,D_7,^{15}N-Tyr	419.1 → 197.0
17	Pro	7.96	336.1 → 113.8	336.1 → 157.8	$^{13}C_5$,D_7,^{15}N-Pro	349.1 → 126.8
18	Met	9.4	370.1 → 173.9	370.1 → 148.0	$^{13}C_5$,D_8,^{15}N-Met	384.1 → 187.9
19	Val	9.67	338.1 → 115.8	338.1 → 141.6	$^{13}C_5$,D_8,^{15}N-Val	352.1 → 129.8
20	Kyn	9.95	429.0 → 207.0	429.0 → 190.0	$^{13}C_{11}$,D_8,$^{15}N_2$-Trp	446.0 → 223.8
21	Ile	10.46	352.1 → 130.0	352.1 → 155.8	$^{13}C_6$,D_{10},^{15}N-Ile	369.1 → 147.0
22	Phe	10.48	386.1 → 163.7	386.1 → 189.7	$^{13}C_9$,D_8,^{15}N-Phe	404.1 → 181.7
23	Leu	10.52	352.1 → 130.0	352.1 → 155.8	$^{13}C_6$,D_{10},^{15}N-Leu	369.1 → 147.0
24	Lys	11.66	589.3 → 144.8	589.3 → 171.0	$^{13}C_6$,D_9,$^{15}N_2$-Lys	606.3 → 161.8
25	Trp	12.23	425.0 → 202.8	425.0 → 229.1	$^{13}C_{11}$,D_8,$^{15}N_2$-Trp	446.0 → 223.8
26	5-HT	12.55	619.1 → 219.1	619.1 → 174.8	$^{13}C_{11}$,D_8,$^{15}N_2$-Trp	446.0 → 223.8

To minimize the contamination of the MS with matrix and to improve the runtime without cleaning a valve was programmed to cut of the first 3.0 min and the rinsing and re-equilibration step after 13.5 min.

4.5.7 Validation

According to FDA Guidelines [245] a detailed analytical validation of the LC-MS/MS method and sample preparation was conducted to determine the analytical performance including accuracy, accuracy after sample dilution, carryover, LOD, LOQ, matrix effects, precision, reproducibility of re-injections, sample stability and specificity across all 26 compounds. These compounds covered a broad range of AAs expected to be part of the human AA profile. For quantification purposes, only the most characteristic MRM transitions for each compound were considered. Samples for validation were either received by spiking a pooled plasma sample of the study or utilizing water as substitute matrix.

4.5.7.1 Accuracy

Assay accuracy was calculated using plasma samples or diluted plasma prepared at 3 different known concentration levels (low, medium and high). Each level was analysed 5 times in row and compared to a calibration prepared with certified reference material.

4.5.7.2 Accuracy after sample dilution

Since it was expected that some sample concentrations could exceed the upper limit of the calibration, a test for the dilution of the sample during the validation procedure was performed. Therefore, 3 matrix samples were spiked with high analyte concentrations and subsequently used for dilution experiments. The dilution was carried out with water by the factors of 1:2, 1:4 and 1:10 to return the analyte concentration back into the calibration range.

4.5.7.3 Carryover

For each analyte the highest calibrator was injected 5 times in a row followed by 1 injection of acetonitrile, which is showing no signal of the analytes. This procedure was repeated 3 times. There should be no interfering signals at the retention time of the analyte with a concentration higher than 20% of the determined limit of quantification.

4.5.7.4 Limit of Detection

The limit of detection (LOD), was defined according to IUPAC guidelines [257, 258] and calculated from an blank sample ($\overline{x}_{bl}$) determined in triplicates and the standard deviation (s_{bl}).

$$x_{LOD} = \overline{x}_{bl} + 3\, s_{bl}$$

4.5.7.5 Limit of Quantification

The limit of quantification (LOQ), was defined according to IUPAC guidelines. [257, 258] on the basis of the blank samples ($\bar{x}_{bl}$) and the standard deviation (s_{bl}) analysed for the LOD calculation.

$$x_{LOD} = \bar{x}_{bl} + 10\ s_{bl}$$

4.5.7.6 Matrix Effects

Matrix effects were qualitatively determined in an infusion experiment. Therefore, 4 different concentrations of standards and internal standards were prepared as infusion solutions. The standards were permanently infused to the capillary connecting HPLC and MS via a T-fitting. Injecting a plasma sample via the LC-system negative or positive matrix effects should be recognized by decrease or increase of the baseline.

4.5.7.7 Precision

Intra- and inter-day precision was determined by analysing samples at 3 different concentration levels (low, medium and high) in five independent sample preparations (intra-day, N=5) and on 6 different days (inter-day, N=6) respectively.

4.5.7.8 Reproducibility of re-injections

To simulate a possible instrument break down, a set of diluted plasma samples (low concentration) and spiked plasma samples (high concentration) has been analysed in triplicates over different points in time. Therefore, the samples have been analysed initially and after 48 h as well as 72 h while they were stored in the auto sampler. The CV of the 3 levels had to be constant (CV <15%; at LOQ <20%).

4.5.7.9 Sample stability

The validation comprises 4 types of stability parameters:

Short-term stability: Triplicates of spiked matrix samples at 2 different known concentration levels (low and high) were prepared after keeping them at ambient temperature for 24 h.

Post-preparative stability: This stability was determined for 4 and 10 days. The prepared spiked plasma samples (ready to inject) were kept at auto sampler temperature (10°C) for the time established and analysed with freshly prepared samples serving as reference.

Freeze-thaw stability: Spiked plasma at low and high concentration was frozen at -20°C, stored there for 12 to 24 h and was then thawed and stored at ambient temperature for 6 h. Up to 6 freeze-thaw cycles were repeated prior to preparation of the samples. The concentrations observed were

compared to their reference value derived from freshly analysed samples.

Post-validation long-term stability: after completion of the validation, long-term stability in matrix for the remaining analytes will be determined by storing a sufficient number of low and high spiked plasma samples at the required long-term storage temperature and analysing them in at least triplicates. The long-term stability was determined after 1 month and will further be determined at several points in time (e.g., 6, 12 and 24 months) depending on the stability time required.

4.5.7.10 Selectivity

The selectivity of the method was analysed by comparing retention times of matrix samples with reference materials in blank samples. For each compound, it was tried to find an MRM transition which shows no interference at the analyte RT. In case of an interfering signal this had to be below 20% of the LOQ. Also, the internal standard was analysed for interfering signals which had to be below 5% of the signal.

4.5.8 Data Processing

Data evaluation (peak integration, calibration, and quantification) was performed using Multiquant™ (AB Sciex, Darmstadt, Germany). Statistical tests (Quade test, Wilcoxon signed rank test and Wilcoxon rank sum test) were calculated using R 3.3.0 and RStudio 0.99.902.

4.6 Eicosanoid Profiling Operation Procedure

The samples from stationary visits of the 39 compliant subjects (for details see section 5.2) were used for analysis by means of the targeted metabolic profiling of eicosanoids. For this purpose, the 24-h urine samples of each point in time (TP0, TP1, TP2, TP3) were selected resulting in 156 samples.

4.6.1 Chemicals

Chemicals and solutions for the eicosanoid profiling approach were obtained from the following sources: acetic acid (≥ 99%), ammonium hydroxide (28% in water), creatinine (anhydrous), formic acid (≥ 95%), hydrochloric acid (~37%), sodium hydroxide (≥ 97%, pellets) were purchased from Sigma-Aldrich (München, Germany). Chloroform (picograde), ethyl acetate (optigrade), methanol (optigrade) and water (optigrade) were obtained from LGC Standards (Wesel, Germany). Tetranor PGE-M (t-PGEM), tetranor PGD-M (t-PGDM), 2,3-dinor-8-iso-$PGF_{2\alpha}$, (2,3-d-8-iso-$PGF_{2\alpha}$) 8-iso-$PGF_{2\alpha}$, 2,3-dinor-TXB_2 (2,3-d-TXB_2), 11-dehydro-TXB_2 (11-dh-TXB_2), LTE_4, 12(S)-HETE, D6-tetranor-PGDM, D6-tetranor-PGEM, D4-8-iso-$PGF_{2\alpha}$, D4-11-dehydro-TXB_2, D5-LTE_4 and D8-12(S)-HETE were purchased from Biomol (Hamburg, Germany) with purities higher than 97%. 0.1% formic acid in water (ULC-MS grade) and 0.1% formic acid in acetonitrile (ULC-MS grade) were supplied by Biosolve BV (Valkenswaard, Netherlands).

4.6.2 Instruments

For the profiling of eicosanoids, the following instruments were used: an API 5000 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) UPLC-MS/MS system, equipped with a 1200 series binary pump (G1312B), a degasser (G1379B) and a column oven (G1316B) (Agilent, Waldbronn, Germany) connected to an HTC Pal autosampler (CTC Analytics, Zwingen, Switzerland) was used for chromatographic separation. Nitrogen for the UPLC-MS/MS system was supplied from a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany). The analytical scales Acculab Atilon ATL-124-I (Sartorius AG, Göttingen, Germany) and Sartorius typ1401 (Sartorius AG, Göttingen, Germany), a Jouan RC10.22 series vacuum concentrator linked to a Jouan RCT90 cold trap (Societe Jouan, Saint Herblain, France) and an Edwards vacuum pump (Edwards Lifesciences, Irvine, California, United States of America), the multi tube vortexer VX 2500 (VWR LLC, Radnor, Pennsylvania, United States of America) and single tube vortexer Reax 2000 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), a ROTANTA 460 R centrifuge (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany) and high speed micro centrifuge CT15RE (VWR LLC, Radnor, Pennsylvania, United States of America).

4.6.3 Software

The following software was used for the processing and evaluation of the eicosanoid data: Analyst 1.6.3 (AB Sciex, Darmstadt, Germany), Office 2013 and Office 365 (Microsoft, Redmond, USA), R (Version 3.3.0) and RStudio (Version 0.99.902).

4.6.4 Calibration Standards

Quantification was performed with the standard addition method. For each analyte, a separate calibration was performed with a set of 8 to 10 calibrators, depending on the target eicosanoid and the calibration range. Calibrators were prepared by spiking non-smoker urine samples with increasing amounts of analytes.

4.6.5 Quality Control Samples

In addition to the set of study samples, 9 quality control samples in low, medium and high concentration levels (3 per level) were interspersed throughout the entire analytical batch. Therefore, urine samples were pooled and partly spiked or diluted to achieve concentrations covering appropriately the calibration range. The acceptance criteria for QC samples were defined according to FDA guidelines [245] at ±20% for the low level and ±15% for the medium and high level respectively. At least 2/3 of all QC samples and 50% per level had to meet the acceptance criteria otherwise the batch had to be repeated.

4.6.6 Sample Preparation and Analytical Methodology

Sample preparation and measurements were performed according to Sterz et al. [53] Instead of the 7 analytes implemented initially tetranor PG E-metabolite (t-PGEM), 8-iso- and 2,3-dinor-8-iso-$PGF_{2\alpha}$ (8-iso-$PGF_{2\alpha}$; 2,3-d-8-iso-$PGF_{2\alpha}$), the thromboxanes (TX) 11-dehydro- and 2,3-dinor-TXB_2 (11-dh-TXB_2, 2,3-d-TXB_2), leukotriene (LT) E_4 (LTE_4) and 12-hydroxyeicosatetraenoic acid (12-HETE), 2 further analytes were implemented namely PG D-metabolite (t-PGDM) and $PGF_{2\alpha}$. The method was successfully revalidated according to FDA guidelines. [245]

All samples were randomized prior to UPLC-MS/MS analysis. Aliquots of 3 ml 24-h urine were used for analysis. According to Sterz et al. [53] 20 µL of acetic acid and 30 µL of an IS-mixture, containing 6 ng D6-t-PGEM, 6 ng D6-t-PGDM, 6 ng D4-8-iso- $PGF_{2\alpha}$, 6 ng D4-11-dh-TXB_2, 1.5 ng D5-LTE_4, and 1.5 ng D8-12-HETE, were added to each sample prior to extraction.

According to Bligh & Dyer [260] with modifications, 11.25 ml B&D solution (methanol:chloroform 2:1 v/v) was added for liquid/liquid extraction to each sample. After vortexing the components

thoroughly, the sample was stored at room temperature for 1 h. Subsequently, 3.75 mL chloroform and 3.75 mL water were added and the sample was vortexed and centrifuged for 10 min at 2500 rpm. The chloroform phase was transferred into a vial and evaporated to dryness in a SpeedVac centrifuge (Thermo Scientific, Dreieich, Germany). The residue was dissolved in 100 µL methanol.

An API 5000 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) UPLC-MS/MS system, equipped with a 1200 series binary pump (G1312B), a degasser (G1379B) and a column oven (G1316B) (Agilent, Waldbronn, Germany) connected to an HTC Pal autosampler (CTC Analytics, Zwingen, Switzerland) was used for chromatographic separation. The Turbo V ion spray source operated in negative electro spray ionization (ESI-) mode (AB Sciex, Darmstadt, Germany). High purity nitrogen was generated by a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany). Chromatographic separation was performed on a Waters (Eschborn, Germany) Acquity ultra performance liquid chromatography (UPLC) BEH C18 column (2.1 × 50 mm) with a 1.7 µm particle size. The column oven was maintained at 30°C and the injection volume was 5 µL. Eluent A consisted of 0.1% formic acid in water, eluent B was 0.1% formic acid in acetonitrile. Gradient elution was performed with 5% B for 1 min, a linear increase to 53% B until 9.5 min, a linear increase to 76% B until 11 min, a step to 100% B until 11.1 min, hold for 1 min at 100% B and re-equilibration from 12.1 min to 14 min with 5% B. The flow rate was set to 600 µL/min. The turbo ion spray source settings were as follows: ion spray voltage = -4 kV, heater temperature = 600°C, source gas 1 = 20 psi, source gas 2 = 5 psi, CAD gas = 5 psi and curtain gas = 40 psi. Analytes were monitored in the multiple reaction monitoring (MRM) mode. The MS 2 stage scans are shown in Figure 2. Quadrupoles were working at unit resolution.

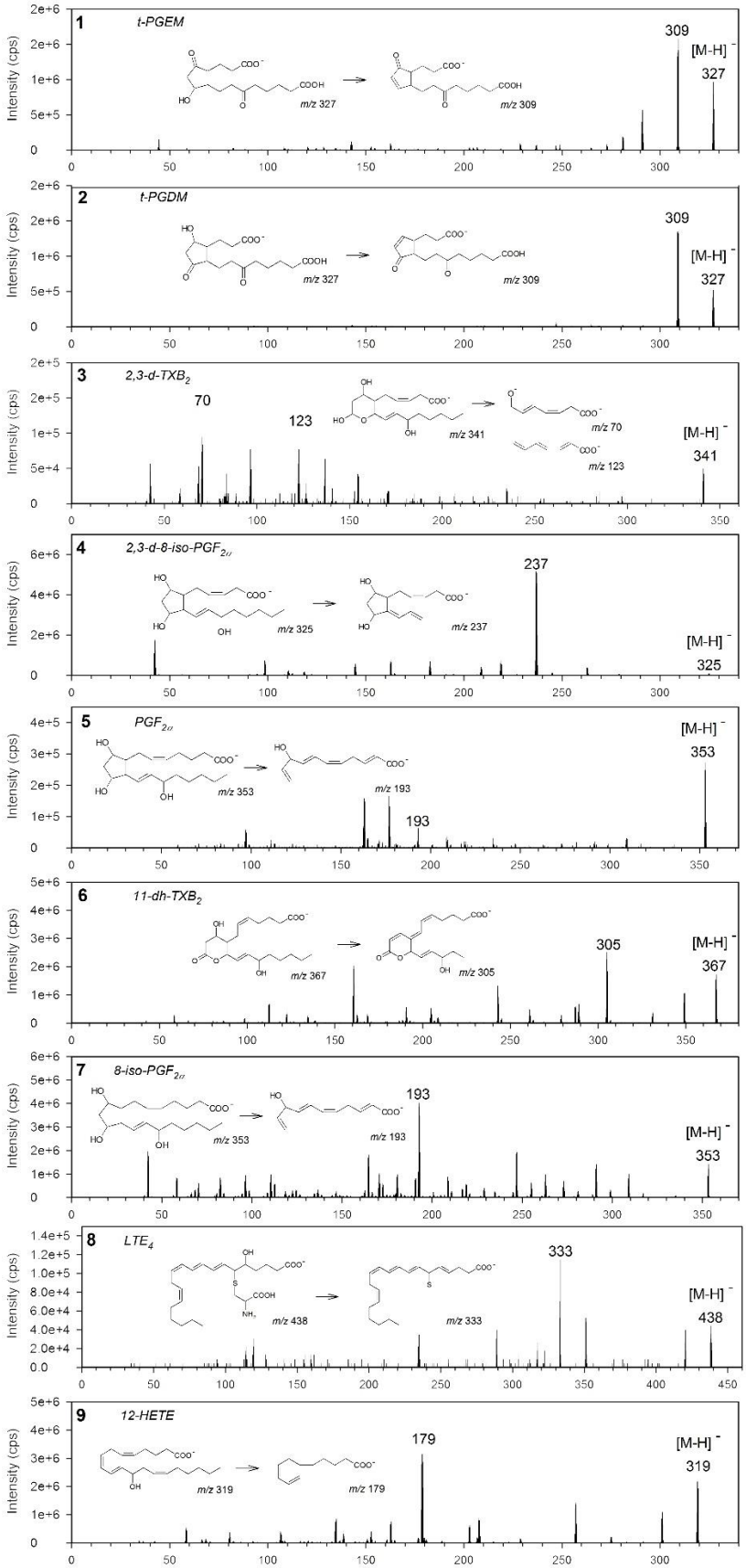


Figure 9: MS2 scan of the eicosanoids. Numbers are assigned to Table 34.

4.6.7 Data Processing

Evaluation of the chromatographic data was performed using Analyst 1.6.3 (AB Sciex, Darmstadt, Germany) and Excel 2013 (Microsoft, Redmond, USA). Different statistical tests were performed applying R (Version 3.3.0) and RStudio (Version 0.99.902) in order to follow alterations in the eicosanoid profile over the course of 3 months of smoking cessation. The Quade test was utilized to identify significant ($\alpha = 0.05$) changes across all points in time. The Wilcoxon signed rank test was applied for cessation-relevant comparisons between 2 points in time (e.g. TP0/TP1) to verify the results of the Quade test. In order to counteract the problem of multiple statistical comparisons, the significance levels were adjusted by the Bonferroni correction. [261, 262]

4.7 Creatinine Determination Operation Procedure

For a potential normalization of urine samples creatinine was determined in the 24-h urine samples. For this purpose, the 24-h urine samples of the 39 compliant subjects (for details see section 5.2) of each point in time (TP0, TP1, TP2, TP3) were selected resulting in 156 samples.

4.7.1 Chemicals

Chemicals and solutions for the creatinine measurement were obtained from the following sources: Creatinine (≥98%, anhydrous) and an aqueous picric acid solution (0.9-1.1%) was supplied by Sigma Aldrich (St. Louis, Missouri, United States of America). Sodium hydroxide solution (5 mol/L water), hydrochloric acid (31.5% in water) and water (Emsure grade) were obtained from Merck KGaA (Darmstadt, Germany).

For sample clean-up, different solutions were prepared. These were a 1 g/L creatinine solution, a 0.1 mol/L hydrochloric acid and a 0.25 mol/L sodium hydroxide solution.

4.7.2 Instruments

For the creatinine quantification, the following instruments were used: an analytical scale Acculab Atilon ATL-124-I (Sartorius AG, Göttingen, Germany), the high-speed micro centrifuge CT15RE (VWR LLC, Radnor, Pennsylvania, United States of America) and the microplate reader Genios (Tecan, Krailsheim, Germany).

4.7.3 Software

The following software was used for the processing and evaluation of the eicosanoid data: Magellan™ data analysis software (Tecan, Krailsheim, Germany), Office 2013 and Office 365 (Microsoft, Redmond, USA)

4.7.4 Calibration Standards

Quantification was performed with the standard addition method. Calibration was performed with a set of /8 calibrators to achieve an appropriate calibration range. Due to the lack of analyte-free matrix samples, the calibration was prepared in water as surrogate matrix. The calibration slopes were compared between plasma and water and revealed similar results. A calibration was prepared on each 96 well plate.

4.7.5 Quality Control Samples

In addition to the set of study samples, quality control samples in low, medium and high concentration levels were measured on each 96-well plate. Therefore, urine samples of known concentrations were aliquoted to achieve concentrations covering appropriately the calibration range. The acceptance criteria for QC samples were defined according to FDA guidelines [245] at ±20% for the low level and ±15% for the medium and high level, respectively. At least 2/3 of all QC samples and 50% per level had to meet the acceptance criteria otherwise the 96-well plate had to be repeated.

4.7.6 Sample Preparation and Analytical Methodology

Sample preparation and measurements were performed according to a validated SOP and DFG method. [263] Briefly, the urine samples were thawed at ambient temperature and 1:51 diluted with water. Therefore, 20 µL urine were aliquoted in Eppendorf cups and 1 mL water was added. Subsequently, the samples were centrifuged at 6000 rpm for 5 min at ambient temperature. All samples were measured in duplicates on 96-well plates, consequently, 50 µL sample were transferred into a well and 200 µL of a freshly prepared 1:1 solution of the sodium hydroxide solution and picric acid solution was added. The 96-well plate was closed with a lid and placed in the plate reader for incubation and measurement. After 45 minutes at 36°C the plates were stirred for 60 s and the absorption measurement at 492 nm with 60 scans was started.

4.7.7 Data Processing

Data calibration and sample evaluation was performed with the Magellan™ software. Correction for the dilution factor was calculated in Excel.

5 Results and Discussion

5.1 Clinical Study

The clinical smoking cessation study was carried out from January to June 2015 as described in section 4.1. The 39 compliant subjects (cf. section 5.2) in this study smoked 15 to 24 cigarettes on the last smoking day at the CRS (Table 9). The self-reported cigarette consumption prior to study start ranged from 16 to 28. The mean age was 30 years with a range of 21 to 50 years. Average body height was 182 cm. The body mass index (BMI) increased over time of smoking cessation (Table 9). This is in accordance with findings reported in literature. [28, 29, 264-267] The gain in BMI of 5.1% after 3 months of cessation is highly significant (Wilcoxon signed rank test $<4\times10^{-7}$; Table 9). A similar increase of the BMI of 2.9% was observed in a large cohort of over 1000 male quitters after 3 months of cessation. [267] The moderately higher weight gain in this study may result from the smaller sample size of 39 compliant subjects which is more affected by few subjects with a rather high weight gain.

Table 9: Descriptive data for the compliant subjects over the four TPs of the cessation study.

	Mean	Min	Max
Age [years]	30	21	50
Body height [cm]	182	168	200
Body weight (TP0) [kg]	81.6	64.2	103.3
Body weight (TP1) [kg]	82.3	64.8	104.5
Body weight (TP2) [kg]	84.3	65.5	108.5
Body weight (TP3) [kg]	85.9	67.0	109.3
BMI TP0 [kg/m2]	24.5	19.5	28.9
BMI TP1 [kg/m2]	24.8	20.4	29.3
BMI TP2 [kg/m2]	25.3	20.6	30.5
BMI TP3 [kg/m2]	25.8	20.8	33.4
Cigarettes smoked on the last day before cessation	17	15	24

5.2 Compliance

In order to verify the compliance of the subjects, saliva and spot urine samples were collected for cotinine measurements during the ambulatory visits (Figure 6) and analysed for their cotinine level as described in section 4.2. In addition, CO_{ex} measurements for immediate check of abstinence from conventional cigarette smoking were performed across all ambulatory visits.

5.2.1 CO_{ex} Measurement

As part of the compliance verification 1195 CO_{ex} measurements were conducted at the stationary and ambulatory visits. The average CO-levels[8] before and after cessation are summarized in Table 10. TP0 was split in TP0 A (subjects smoking) and TP0 B (subjects not smoking for 9 h).

Table 10: Average CO_{ex} [ppm] levels during smoking cessation at the stationary TPs (TP0 - TP3) and the ambulatory visits TPs (TP 0/1, 1/2 and 2/3). TP0 is shown as TP0 A (before smoking cessation) and TP0 B (9 h after smoking cessation).

	TP0 A	TP0 B	TP0/1	TP1	TP1/2	TP2	TP2/3	TP3
Mean	23.5	10.7	1.1	1.5	0.9	1.3	1.1	1.0
Standard deviation	±6.7	±2.8	±1.1	±1.0	±1.0	±1.1	±1.1	±1.1

As expected from literature [179, 180] the CO-level dropped after cessation within 40 h below the exclusion criterion and non-compliant subjects starting smoking could be identified. Subsequently, 4 subjects with CO_{ex} levels exceeding the exclusion criterion (8, 12, 13 and 31 ppm) could be identified and were consequently excluded from the clinical study. The CO_{ex} measurements were confirmed by the analysis of salivary and urinary cotinine.

The identification of 4 non-compliant subjects appeared to be rather low compared with the identification via cotinine (cf. section 5.2.3). However, regarding to the response time and cost efficiency the determination via a CO-analyser was a benefit as subjects could be directly excluded and caused no further study charges. In combination with the cost and time intensive mass spectrometry based cotinine determination which is also time shifted due to the transport time to the laboratory the CO-analysis revealed as powerful extension.

[8] The average CO-level (N=39) was calculated excluding non-compliant subjects.

5.2.2 Quality Control of the Cotinine Determination

The quality control samples (N=93) were interspersed throughout the randomized analytical batches and all acceptance criteria according to the FDA requirements for bioanalytical method validation [245] were fulfilled.

5.2.3 Cotinine Determination

The main instrument for the verification of the compliance with non-smoking was the determination of cotinine in saliva and urine. Consequently, 1933 matrix samples (753 urine samples and 1180 saliva samples) were analysed for their cotinine level. The average levels of compliant subjects are summarized in Table 11.

Table 11: Cotinine concentrations (mean and standard deviation (SD), [µg/mL]) of the LC-MS/MS measurement of compliant subjects' saliva and urine. TP0 A, before cessation; TP0 B, 9 h after cessation; TP0/1A to E, 1st to 5th day after 1st stationary visit; TP1, after 1 week; TP1/2, during 2nd and 4th week; TP2, after 1 month; TP2/3, during 2nd and 3rd month; TP3, after 3 months.

TP	Saliva (mean)	Saliva (SD)	Urine (mean)	Urine (SD)
TP0 A	382.8	114.1	-	-
TP0 B	379.5	113.3	-	-
TP0/1A	91.7	54.9	462.1	239.8
TP0/1 B	34.1	18.5	144.1	80.0
TP0/1 C	15.5	10.1	48.9	24.6
TP0/1 D	8.4	6.9	28.0	14.5
TP0/1 E	4.2	3.2	16.8	8.5
TP1	3.2	1.9	-	-
TP1/2	1.5	2.1	8.1	5.3
TP2	1.7	2.8	-	-
TP2/3	2.2	1.8	6.7	5.6
TP3	1.8	1.8	-	-

During the 1st week after cessation the cotinine values dropped below the exclusion criterion. Until the values dropped below the exclusion criterion, subjects were excluded if apparent increases were determined. Subsequently, 12 non-compliant subjects could be identified with cotinine levels exceeding the exclusion criterion in saliva and urine. Furthermore, the subjects with CO_{ex} values above the exclusion criterion were confirmed by the analysis of urinary and salivary cotinine. Hence,

a total number of 16 subjects showed non-compliance by the analysis of cotinine in body fluids. The decrease of cotinine (cf. Table 11) after cessation in saliva and urine was found to be in accordance to literature. [241, 268] Further, the decrease in saliva and urine showed excellent conformity as far as comparable[9].

5.2.4 Compliance Further Dropouts

Additionally, to the subjects excluded for starting smoking again, 5 further subjects were excluded from the study or quitted themselves. Two of these subjects missed stationary visits and were thus excluded. The other 3 subjects withdrew their study agreement. As it was of interest to know the reason for these dropouts the clinical research institute contacted the subjects. As far as they were available (3 out of 5) they stated that they have no longer interested in the study (N=1) or started smoking again (N=2). The dropout reasons for each subgroup in detail are shown in Table 12. There was no pattern within the subgroups identified.

[9] For the analysis of cotinine only saliva samples from stationary visits were measured, therefore only the values of ambulatory visits could be taken into account for the comparison of both matrices.

Table 12: Dropout statistics of the clinical smoking cessation study with reasons.

Subgroup	Subjects (N)	Dropouts (N)	Reason for dropout	Subjects having successfully completed the study (N)
1	4	1	1 CO_{ex} and cotinine	3
2	5	2	1 cotinine	3
			1 withdrew study agreement	
3	4	1	1 cotinine	3
4	5	2	1 missed stationary visit	3
			1 withdrew study agreement	
5	4	2	2 cotinine	2
6	3	3	1 withdrew study agreement	0
			2 cotinine	
7	6	1	1 cotinine	5
8	6	2	2 cotinine	4
9	5	3	1 missed stationary visit	2
			2 cotinine	
10	6	3	1 cotinine	3
			2 CO_{ex} and cotinine	
11	6	0	-	6
12	6	1	1 CO_{ex} and cotinine	5
∑	60	21		39

5.2.5 Compliance Summary

In total, a dropout rate of 21 of 60 subjects (35%) was obtained, the main reason was the resume to the smoking habit (cf. Table 12). 39 participants were compliant over the course of 3 months. These subjects were selected for the metabolomics analysis and subsequent data evaluation. A dropout rate of approximately 1/3 is in good agreement to other smoking cessation studies which reported dropout rates between 28% and 39%. [41, 269, 270] Noncompliance is a well-known problem in smoking cessation studies. [269] Several approaches try to reduce the dropout rate by contracting the study subjects to procedures like educational sessions during smoking cessation showing negligible effects on the dropout rates. [269] The subjects reported that they felt that quitting smoking is much easier in an extensively supervised environment as given in the current study. Also, they stated that the financial incentive is an inducement to preserve compliance during the course of the study. Noteworthy, the success rate after three months of cessation in our and other clinical studies is three times higher compared to quitting attempts without any assistance as reported by Zhu et al. [42]

5.3 Metabolomic Fingerprinting

Plasma, urine and saliva samples of all compliant participants of the smoking cessation study have been prepared, analysed and evaluated as described in section 4.3 according to the metabolic fingerprinting method developed by Mueller et al. [87] Group differences were determined for each biological matrix comparing TP0 (still smoking) with the different points in time after smoking cessation: TP1 (after 1 week), TP2 (after 1 month) and TP3 (after 3 months of smoking cessation).

As expected, several detectable nicotine metabolites were found to be significantly decreased after 1 week of smoking cessation (Wilcoxon signed rank test, P-value <0.001), e.g., cotinine and trans-3-hydroxycotinine in plasma, saliva, and urine. Additionally, nicotine was found to be decreased in saliva and urine, as well as cotinine glucuronide in urine. The identification of the nicotine metabolites was confirmed by means of authentic reference standards. Moreover, the same pattern of altered nicotine metabolites as compared to the initial study with smokers and non-smokers was identified in this smoking cessation study proving the reliability of the metabolomic fingerprinting method. [87] For plasma, even more nicotine metabolites were identified in the current study, apparently due to the higher sample volume used. Yet, this work focuses on the alterations of endogenous compounds and thus tobacco smoke-related biomarkers of exposure are not within the scope of this thesis.

The results of the metabolic fingerprinting are compiled in Table 14 to Table 16 for plasma, Table 18 to Table 20 for saliva and Table 22 to Table 24 for urine. Three categories of significance levels (Sig.) for the Wilcoxon signed rank test were applied: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. The differences in peak area ratios over time are expressed as fold change. The fold change was determined for all compliant subjects (N=39) for each point in time after cessation compared to baseline (TP1 vs. TP0, TP2 vs. TP0, TP3 vs. TP0) and serves as an indicator, whether a substance has increased or decreased between the corresponding points in time throughout the study. The tables indicate, whether metabolites were identified by using authentic standard compounds or the MS library (NIST 05, GOLM Metabolome Database GMD 20111121). Furthermore, information is provided, whether an identified chemical is a tobacco smoke component (TSC) according to Rodgman et al. [43] and/or a known constituent of the human metabolome database (HMDB). [70-72]

Initially the fingerprinting was evaluated with the unpaired Mann-Whitney-U-test (cf. Appendix B Table 38 to Table 46). The unpaired evaluation shows more metabolites than the paired evaluation nevertheless both evaluations have shown a remarkable conformity. Due to the paired dataset, a paired statistical analysis was chosen for the evaluation.

5.3.1 Plasma

The dataset of plasma comprised approximately 60,000 features (m/z to retention time pairs). Missing values were replaced by half of the minimum of the corresponding feature measured in the respective sample series. The statistical evaluation revealed 230 to 460 significant alterations after Benjamini-Hochberg correction for the different comparisons made (TP1/TP0, TP2/TP0 and TP3/TP0).

5.3.1.1 Quality Control

For quality control purposes, 20 blank runs, 5 reagent blanks and 14 spiked matrix samples (QC samples) were measured and analysed. The quality control substances were selected by their retention time and (bio-)chemical properties to cover prominent metabolic pathways throughout the chromatographic separation. Furthermore, ttMA-d4 was used as internal standard for the normalization of peak areas. Detailed QC plots are shown in Appendix A Figure 16 to Figure 19. The 14 QC samples were interspersed throughout the randomized analytical batch. The coefficients of variation (CV) were within a range of ±20% (cf. Table 13).

Table 13: CV of the quality control samples of plasma investigated (N=14).

Analyte	CV [%]
L-Alanine	11.5
ttMA	13.5
D-Glucose	15.7
Adenosine	19.5

5.3.1.2 Plasma Fingerprint

In plasma, an increasing number of altered metabolites was identified with longer time periods of cessation (cf. Table 14 to Table 16). Several amino acids and their respective metabolites were found to be altered, namely the metabolism of lysine, tryptophan, cysteine and aspartic acid, which showed statistically significant alterations. For kynurenine, a tryptophan metabolite, the lowest p-values for all points in time were observed. Furthermore, levels of the tryptophan metabolite 5-hydroxy-tryptophan as well as tryptophan itself were found to be altered significantly indicating that the tryptophan metabolism is highly affected by smoking cessation.

Moreover, the fatty acid profile showed alterations after 1 month of smoking cessation. After this time period, an increase in various saturated and unsaturated fatty acids was found when compared to baseline (TP0), when the subjects were still smoking. Interestingly, the polyunsaturated arachidonic acid (precursor of several eicosanoid species, which are well described biomarkers of oxidative stress and inflammation) [53] was found to be the only fatty acid to increase upon quitting (Table 15).

Table 14: Plasma fingerprinting: comparison TP1 versus TP0.

Name	Sig.	Fold change TP1/TP0		RT	Standard	HMDB	Origin / Pathway
Morpholine	*	0.83	▼	5.717		✓	TSC
3-Hydroxybutyric acid	**	0.56	▼	8.382	✓	✓	TSC, gluconeogenesis
Lysine	**	0.58	▼	15.440		✓	TSC, lysine metabolism
Glycerol 3-phosphate	**	1.35	▲	16.059			Glycolysis
Kynurenine	***	1.45	▲	20.003		✓	Tryptophan metabolism
5-Hydroxytryptophan	*	0.88	▼	22.012		✓	Tryptophan metabolism

Table 15: Plasma fingerprinting: comparison TP2 versus TP0.

Name	Sig.	Fold change TP2/TP0		RT	Standard	HMDB	Origin / Pathway
Cysteine	**	2.00	▲	13.639	✓	✓	TSC, cysteine metabolism
2,4-dihydroxy-2-(hydroxymethyl)butyric acid	**	0.57	▼	14.783			
Glycerol 3-phosphate	*	1.41	▲	16.059		✓	Glycolysis
Uric acid	*	1.28	▲	19.285	✓	✓	TSC, purine metabolism
FA 17:0	*	0.79	▼	19.567	✓	✓	TSC, fatty acid biosynthesis
Kynurenine	**	1.67	▲	20.003		✓	Tryptophan metabolism
FA18:1 (9Z)	**	0.60	▼	20.172	✓	✓	TSC, fatty acid metabolism
FA 20:4 5Z,8Z,11Z,14Z - Arachidonic acid	*	1.37	▲	21.401	✓	✓	TSC, fatty acid and eicosanoid metabolism
FA 20:1	*	0.37	▼	21.841		✓	TSC, fatty acid metabolism
Lathosterol	***	2.86	▲	27.805		✓	Cholesterol metabolism

Table 16: Plasma fingerprinting: comparison TP3 versus TP0.

Name	Sig.	Fold change TP3/TP0		RT	Standard	HMDB	Origin / Pathway
Aminomalonic acid	*	1.75	▲	12.571		✓	Protein synthesis
Aspartic acid	*	0.61	▼	13.236	✓	✓	TSC, aspartic acid metabolism
α -Ketoglutaric acid	*	1.54	▲	13.665	✓	✓	TSC, Krebs cycle, glutamic acid metabolism
Threonic acid	**	0.38	▼	13.910		✓	ascorbate and aldarate metabolism
Glutamic acid	**	0.66	▼	14.390	✓	✓	TSC, glutamic acid metabolism
Glycerol 3-phosphate	*	1.61	▲	16.059		✓	Glycolysis
FA 14:0	*	0.63	▼	16.731		✓	TSC, fatty acid metabolism
FA 16:1	*	0.58	▼	18.390		✓	TSC, fatty acid metabolism
FA 16:0	***	0.65	▼	18.656	✓	✓	TSC, fatty acid metabolism
Uric acid	*	1.52	▲	19.285	✓	✓	TSC, purine metabolism
Kynurenine	***	1.61	▲	20.003		✓	Tryptophan metabolism
FA 18:2	**	0.55	▼	20.132		✓	TSC, fatty acid metabolism
FA 18:1	**	0.70	▼	20.209		✓	TSC, fatty acid metabolism
Tryptophan	*	2.38	▲	20.240	✓	✓	TSC, Tryptophan metabolism
Cholesterol	*	1.82	▲	27.336	✓	✓	TSC, Cholesterol metabolism

5.3.2 Saliva

The dataset of saliva comprised approximately 100,000 features (m/z to retention time pairs). Missing values were replaced by half of the minimum of the corresponding feature measured in the respective sample series. The statistical evaluation revealed 450 to 650 significant alterations after Benjamini-Hochberg correction for the different comparisons made (TP1/TP0, TP2/TP0 and TP3/TP0).

5.3.2.1 Quality Control

For quality control purposes, 20 blank runs, 5 reagent blanks and 14 spiked matrix samples (QC samples) were measured and analysed. The quality control substances were selected by their retention time and (bio-)chemical properties to cover prominent metabolic pathways throughout the chromatographic separation. Furthermore, ttMA-d4 was used as internal standard for the normalization of peak areas. Detailed QC plots of are shown in Table 17.

Table 17: CV of the quality control samples of Saliva investigated (N=14).

Analyte	CV [%]
L-Alanine	16.9
ttMA	9.7
D-Glucose	19.1
Adenosine	17.7

5.3.2.2 Saliva Fingerprint

In saliva, the highest number of metabolites was identified as compared to plasma and urine. However, in contrast to plasma, the number of identified biomarkers is decreasing over time of smoking cessation (Table 18 to Table 20). Noteworthy, arachidonic acid showed the lowest p-values over all comparisons in saliva ($p < 0.001$).

A few amino acids and their respective metabolites were identified in saliva, e.g. beta-alanine, urocanic acid (histidine metabolite) and tyrosine. However, no consistent pattern in relation to smoking cessation was noticeable. Further metabolites with significant alterations over time in saliva are related to energy metabolism, in particular glycolysis, pentose phosphate pathway and fatty acid metabolism.

Table 18: Saliva fingerprinting: comparison TP1 versus TP0.

Name	Sig.	Fold change TP1/TP0		RT	Standard	HMDB	Origin / Pathway
FA 8:0	*	2.86	▲	9.733	✓	✓	TSC, fatty acid metabolism
beta-Alanine	*	1.89	▲	12.112		✓	TSC, beta-alanine metabolism
Aminomalonic acid	*	1.69	▲	12.552		✓	Protein synthesis
Dihydroxyacetone phosphate	*	1.79	▲	15.713		✓	TSC, lipid metabolism and glycolysis
Desaminotyrosine	*	2.00	▲	15.736		✓	TSC, tyrosine metabolism
Phosphorylethanolamine	*	1.96	▲	16.220		✓	TSC, phospholipid metabolism
Tyrosine	*	1.96	▲	16.994	✓	✓	TSC, tyrosine metabolism
Urocanic acid	*	1.75	▲	17.959		✓	Histidine metabolism
Xanthine	*	2.33	▲	18.259		✓	TSC, purine metabolism
FA 17:0	*	2.86	▲	19.567	✓	✓	TSC, fatty acid metabolism
Ribulose 5-phosphate	*	1.69	▲	19.708		✓	Pentose phosphate pathway
FA 20:4 5Z,8Z,11Z,14Z - Arachidonic acid	***	0.12	▼	21.395	✓	✓	TSC, fatty acid and eicosanoid metabolism
Octadecenamide	*	2.08	▲	21.723		✓	Energy metabolism

Table 19: Saliva fingerprinting: comparison TP2 versus TP0.

Name	Sig.	Fold change TP2/TP0		RT	Standard	HMDB	Origin / Pathway
beta-Alanine	*	2.00	▲	12.112		✓	TSC, beta-Alanine metabolism
L-Threo-2-pentulose	*	3.03	▲	15.327		✓	
Dihydroxyacetone phosphate	*	2.33	▲	15.713		✓	TSC, lipid metabolism and glycolysis
Phosphorylethanolamine	*	2.33	▲	16.220		✓	TSC, phospholipid metabolism
Tyrosine	*	2.00	▲	16.994	✓	✓	TSC, tyrosine metabolism
FA 17:0	*	2.38	▲	19.559	✓	✓	TSC, fatty acid metabolism
Ribulose 5-phosphate	*	2.44	▲	19.708		✓	Pentose phosphate pathway
FA 20:4 5Z,8Z,11Z,14Z - Arachidonic acid	***	0.14	▼	21.395	✓	✓	TSC, fatty acid and eicosanoid metabolism
Pseudouridine	*	2.13	▲	21.506		✓	Pseudouridylation
Octadecenamide	*	2.13	▲	21.723		✓	Energy metabolism
Guanosine	*	0.50	▼	24.729		✓	Nucleoside metabolism

Table 20: Saliva fingerprinting: comparison TP3 versus TP0.

Name	Sig.	Fold change TP3/TP0		RT	Standard	HMDB	Origin /Pathway
Propylene glycol	*	1.20	▲	6.125	✓	✓	TSC
2-Hydroxyisocaproic acid	*	0.47	▼	9.506		✓	TSC
Dihydroxyacetone phosphate	*	2.27	▲	15.713		✓	TSC, lipid metabolism and glycolysis
Phosphorylethanolamine	*	2.63	▲	16.220		✓	TSC, phospholipid metabolism
Cadaverine	*	0.52	▼	16.969		✓	TSC, lysine metabolism
Urocanic acid	*	1.85	▲	17.961		✓	Histidine metabolism
N-Acetylglucosamine	*	4.55	▲	19.324		✓	Amino sugar metabolism
FA 17:0	*	0.56	▼	19.560	✓	✓	TSC, fatty acid metabolism
FA 20:4 5Z,8Z,11Z,14Z - Arachidonic acid	***	0.18	▼	21.395	✓	✓	TSC, fatty acid and eicosanoid metabolism
Guanosine	*	0.59	▼	24.729		✓	Nucleoside metabolism

5.3.3 Urine

The dataset of urine comprised approximately 85,000 features (m/z to retention time pairs). Missing values were replaced by half of the minimum of the corresponding feature measured in the respective sample series. The statistical evaluation revealed 1,000 to 1,400 significant alterations after Benjamini-Hochberg correction for the different comparisons made (TP1/TP0, TP2/TP0 and TP3/TP0).

5.3.3.1 Quality Control

For quality control purposes, 20 blank runs, 5 reagent blanks and 14 spiked matrix samples (QC samples) were measured and analysed. The quality control substances were selected by their retention time and (bio-)chemical properties to cover prominent metabolic pathways throughout the chromatographic separation. Furthermore, ttMA-d4 was used as internal standard for the normalization of peak areas. Detailed QC plots of are shown in

Table 21: CV of the quality control samples of urine investigated (N=14).

Analyte	CV [%]
L-Alanine	8.9
ttMA	9.6
D-Glucose	9.9
Adenosine	46.8

The reason for the high CV for adenosine is probably caused by an interfering matrix peak in urine as the chromatograms of all QC samples and several study samples revealed an interfering signal at the retention time of adenosine in urine. This issue could not be resolved by using a more specific mass fragment due to a lack of sensitivity. As the QC analytes were spiked at low concentrations resembling basal concentration levels, they are more prone to matrix interferences which may cause the variations observed. However, the other three QC substances showed precise and accurate results in urine.

5.3.3.2 Urine Fingerprint

In urine, the number of metabolites was found to increase with the time of smoking abstinence (Table 22 to Table 24), in analogy to plasma fingerprinting. Urine exhibited by far the lowest number of identifiable metabolites as compared to plasma and saliva, respectively, presumably due to strong matrix effects and highly variable individual levels of metabolites in urine. [76] It is noteworthy that a significant increase of the tryptophan metabolite kynurenic acid was observed across all points in time after smoking cessation, reaching the highest significance level after 3 months of cessation. Apparently, tryptophan metabolism seems to be mainly affected as indicated in the observed urine metabolome, in analogy to the results obtained with plasma.

Furthermore, numerous compounds, most likely originating from food or beverages, were identified, such as caffeine and theobromine. However, since these markers showed no consistent trend over time, there seems to be no relation to smoking cessation. Presumably, individual differences in coffee or tea consumption prior to the controlled diet during the stationary visits may have caused the alterations observed.

Table 22: Urine fingerprinting: comparison TP1 versus TP0.

Name	Sig.	Fold change TP1/TP0		RT	Standard	HMDB	Origin / Pathway
Aminomalonic acid	*	0.74	▼	12.555		✓	Protein synthesis
Caffeine	***	0.69	▼	15.999		✓	TSC, nutrition
Kynurenic acid	*	1.30	▲	18.671		✓	TSC, tryptophan metabolism
3-Hydroxysebacic acid	**	0.38	▼	18.929		✓	Fatty acid metabolism

Table 23: Urine fingerprinting: comparison TP2 versus TP0.

Name	Sig.	Fold change TP2/TP0		RT	Standard	HMDB	Origin / Pathway
Acetylglycine	***	0.41	▼	11.067		✓	Protein metabolism
Iso-serine	**	1.82	▲	11.429			
Theobromine	**	2.94	▲	16.102		✓	TSC, nutrition
Kynurenic acid	**	1.64	▲	18.671		✓	TSC, tryptophan metabolism
Hydroxyprolyl-hydroxyproline	*	1.19	▲	22.166		✓	Protein catabolism

Table 24: Urine fingerprinting: comparison TP3 versus TP0.

Name	Sig.	Fold change TP3/TP0		RT	Standard	HMDB	Origin / Pathway
FA 8:0	*	0.65	▼	9.730	✓	✓	TSC, fatty acid metabolism
Acetylglycine	*	0.56	▼	11.067		✓	Protein synthesis, amino acid synthesis
1,2,4-Butanetriol	*	2.63	▲	11.383			TSC
Iso serine	*	2.13	▲	11.429			
Caffeine	***	3.33	▲	15.992		✓	TSC, nutrition
Theobromine	**	2.22	▲	16.113		✓	TSC, nutrition
Kynurenic acid	***	2.08	▲	18.669		✓	TSC, tryptophan metabolism
Cystathionine	*	0.47	▼	19.065		✓	Cysteine metabolism
5-Hydroxyindoleacetic acid	*	0.48	▼	20.067		✓	Serotonin metabolism
Hydroxyprolyl-Hydroxyproline	*	2.17	▲	22.153		✓	Protein catabolism

5.3.4 Fingerprinting Summary

Overall, 84 statistically significant alterations were found across all comparisons evaluated, of which 52 could be assigned to different metabolites. The highest number of metabolites with significant changes was observed in plasma with 26 metabolites, followed by saliva (N=20) and urine (N=12).

In summary, several changes in various metabolic pathways could be identified by using an untargeted metabolic fingerprinting platform applied to samples derived from a smoking cessation study. Most significant alterations were obtained for the fatty acid metabolism, including arachidonic acid which acts as a key precursor in the eicosanoid metabolism and for numerous amino acids, especially the highly affected tryptophan metabolism.

Changes in the fatty acid and amino acid metabolisms are in accordance with data of the KORA cohort study monitoring 140 metabolite concentrations in plasma and applying them to a liquid chromatography and flow injection analysis mass spectrometry. [132] Evaluation of subjects who quit smoking revealed that the effects found in amino acids, ether lipid and glycerophospholipid

metabolism associated with smoking were reversible. [132] This study focuses on long term surveys (years or even decades of quitting are considered) of non-controlled samples in terms of the diet and thus, the data can only be compared to a certain extent with our findings from our diet-controlled study focusing on the first months of smoking cessation. Nevertheless, the main alterations reported [132] are in good agreement and strengthen our observation concerning altered lipid and amino acid metabolic pathways after smoking cessation.

In the initial study [76, 86, 87], alterations in the fatty acid and amino acid metabolism were determined comparing smokers and non-smokers. Eleven of the 38 differing metabolites between smokers and non-smokers were now also identified to be significantly altered after smoking cessation. Additionally, 8 out of these 11 showed the same trend (e.g. guanosine, glutamic acid and oleic acid (FA18:1 9Z)). Hence, some of the differences found in the metabolism of smokers and non-smokers appear to be reversible after 3 months of smoking cessation.

Several analytes related to the fatty acid metabolism could be identified in all matrices. Most of them were fatty acids, predominantly represented in plasma. In analogy to the differences between smokers and non-smokers, monounsaturated fatty acids are increased while smoking and show a decrease after cessation. [86] This is represented by 3 fatty acids, namely palmitoleic acid (FA 16:1 7Z), oleic acid (FA 18:1 9Z) and presumably vaccenic acid (FA18:1 7Z) found in both fingerprinting approaches to be altered in the same manner between smokers/non-smokers and after smoking cessation. Furthermore, a monounsaturated C-20 fatty acid was significantly decreased after cessation. These findings are in accordance with reported data from Cambien et al., who reported increased levels of monounsaturated fatty acids in smokers' plasma. [271] These observations further support the hypothesis that the transcription factor peroxisome proliferator activated receptor (PPAR) is activated by nicotine uptake, which in turn activates the transcription factor liver X receptor (LXR) and subsequently the sterol regulatory element binding protein-1c (SREBP1c) leading to an upregulation of the stearoyl coenzyme A desaturase 1 as previously reported. [86, 89-91] Additionally, the 2 saturated fatty acids tetradecanoic acid (FA 14:0) and heptadecanoic acid (FA 17:0) showed significant alterations after smoking cessation in plasma. Both were found to be increased while the subjects were still smoking. Thus, an opposite trend compared to our previous study showing decreased levels of saturated fatty acids in smokers' compared to non-smokers' plasma was observed. Finally, levels of polyunsaturated fatty acids such as FA 18:2 and especially arachidonic acid (FA 20:4 5Z,8Z,11Z,14Z) change significantly after smoking cessation.

Alterations observed in arachidonic acid levels show contrary trends in saliva and plasma (decrease in saliva; increase in plasma) after smoking cessation. The opposite trends in both matrices may derive from outliers in the fold change calculation, despite a cut-off of 5% of the highest and 5% of the lowest values to eliminate outliers. Nevertheless, changes in the arachidonic acid metabolism may indicate effects in the eicosanoid levels due to smoking cessations. Eicosanoids are well-established biomarkers of effect indicating oxidative stress as well as inflammation and showed a significant increase in smokers as compared to non-smokers. [53] Thus, for a more comprehensive view on the eicosanoid profile, a targeted analysis of the respective eicosanoid species was addressed (cf. section 4.6 section 5.6).

Most alterations could be attributed to amino acids and their respective metabolites covering nearly a quarter of all significant changes observed. Glutamic acid which was increased in smokers compared to non-smokers [76] and decreased after quitting (this study) suggests a reversible effect on its metabolism occurring within 3 months after smoking cessation. For the other amino acids with significant alterations after quitting no differences were observed comparing smokers and non-smokers. Thus, the differences observed in this study appeared to be specifically related to smoking cessation for the majority of amino acids.

As already discussed above, the most striking modifications in amino acid metabolism were contributed to the tryptophan pathway. Alterations found in the tryptophan metabolism represented by the compounds identified in plasma and urine indicate a shift towards the serotonin (5-hydroxytryptamin) biosynthesis while the kynurenine pathway appears to be attenuated in smokers. In addition, the tryptophan level itself is significantly altered after cessation. Hypothetically, these changes may be attributed to up- and down-regulations in the enzyme activities of tryptophan-hydroxylase and tryptophan-2,3-dioxygenase respectively, induced by cessation (Figure 10).

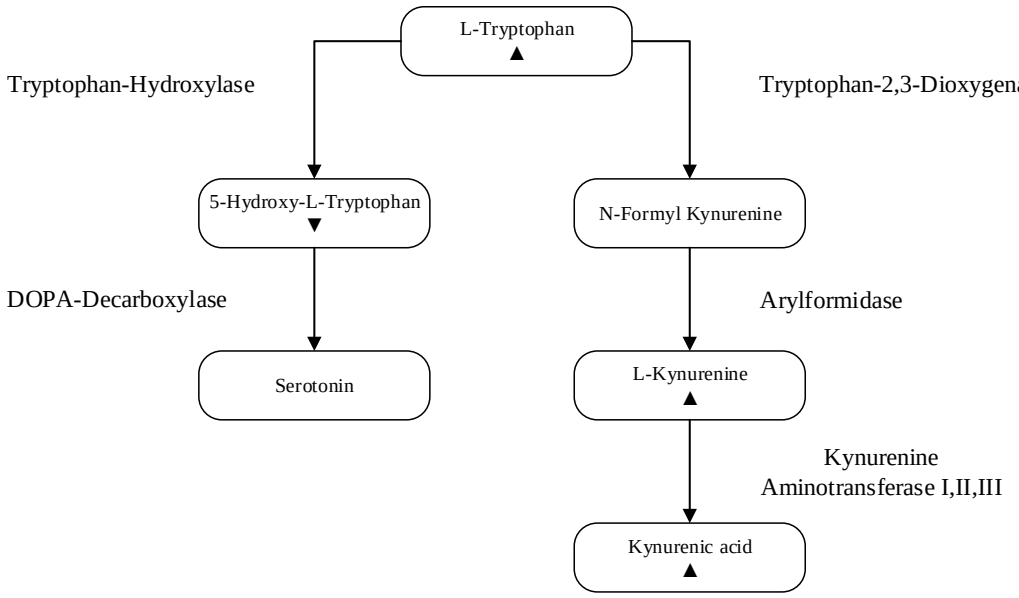


Figure 10: Detail of the tryptophan metabolism showing observed increases (▲) or decreases (▼) after smoking cessation in the serotonin or kynurenine pathway.

Furthermore, indications for an alteration in the glycolysis were found comparing smokers and non-smokers, [76, 87] which were confirmed in the cessation study as reflected in the decreased levels of glycerol-3-phosphate found in both studies suggesting a reversible effect after quitting.

In conclusion, the metabolomic fingerprinting platform revealed several alterations in the metabolome after smoking cessation with numerous endogenous compounds showing reversible changes towards non-smoker profiles after 3 months of smoking cessation, such as fatty acids, glutamic acid, or glycerol-3-phosphate. These classes of compounds may reveal suitable biomarkers for smoking-induced biological effects. Therefore, profiling methods for fatty acids (cf. section 4.4 and 5.4) [272] and amino acids (cf. section 4.5 and 5.5) were developed and applied and a further, already existing method for eicosanoids (cf. section 4.6 and 5.6) was applied.

5.4 Fatty Acid Profiling

Plasma samples of all compliant subjects of the cessation study have been prepared, analysed and evaluated as described in section 4.4. The top-down untargeted metabolic fingerprinting approach followed by the targeted analysis (e.g. FAs) has proven its efficiency. [76, 86, 87] It was feasible to decipher different biochemical pathways, including FAs, which appeared to be affected after 3 months of smoking cessation. [77] This data is in line with a previous clinical study in which the metabolome of smokers and non-smokers were compared under strictly controlled conditions. [76, 86] In order to further verify the results, it was decided to develop a targeted method for the quantification of FAs. The intention was to develop and validate a specific and robust FA profiling method, covering a wide range of FAs (44 individual FA species ranging from C4:0 to C32:0) with varying saturation degrees, including geometrical and positional isomers. The main goal during method development was to optimize the separation of FAs, especially the separation of geometrical and positional isomers, in order to correctly quantify the individual FAs levels. For this purpose, several GC columns were tested with the focus on baseline separation of FA isomers. It was possible to improve the chromatographic performance, enabling the separation of geometrical and positional isomers, as well as the accuracy and reproducibility of the method to a satisfying level.

5.4.1 Quality Control

The area ratio of the two internal standards throughout the entire analytical batch of more than 250 samples showed excellent reproducibility (CV <15%, Figure 11) and therefore proved the robustness of the method even in large sample series. Furthermore, all quality control samples (N=15) met the acceptance criteria which were set in compliance with FDA requirements for bioanalytical method validation [245].

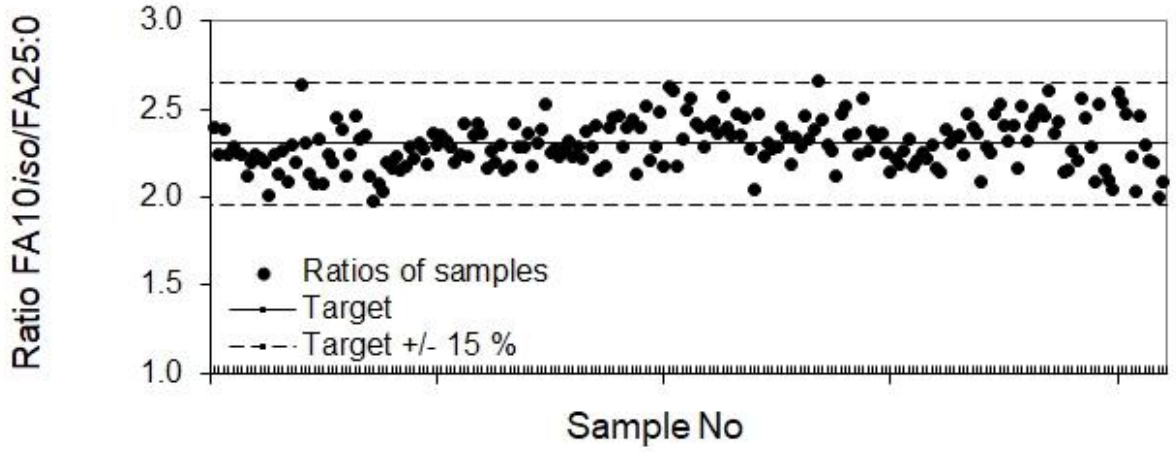


Figure 11: Ratio of the internal standards FA10:0*iso* and FA 25:0 over all samples.

5.4.2 Fatty Acid Profile

The validated GC-TOF-MS method was applied to a set of plasma samples collected during the smoking cessation study (Figure 12). In total, plasma samples derived from 39 compliant subjects over four points in time were analysed, i.e. baseline (TP0), after 1 week (TP1), after 1 month (TP2) and after 3 months (TP3) of smoking cessation. Absolute FA concentrations are expressed as a relative FA profile (Table 25). Generally, a fatty acid profile is more reliable than the individual FA concentrations [273].

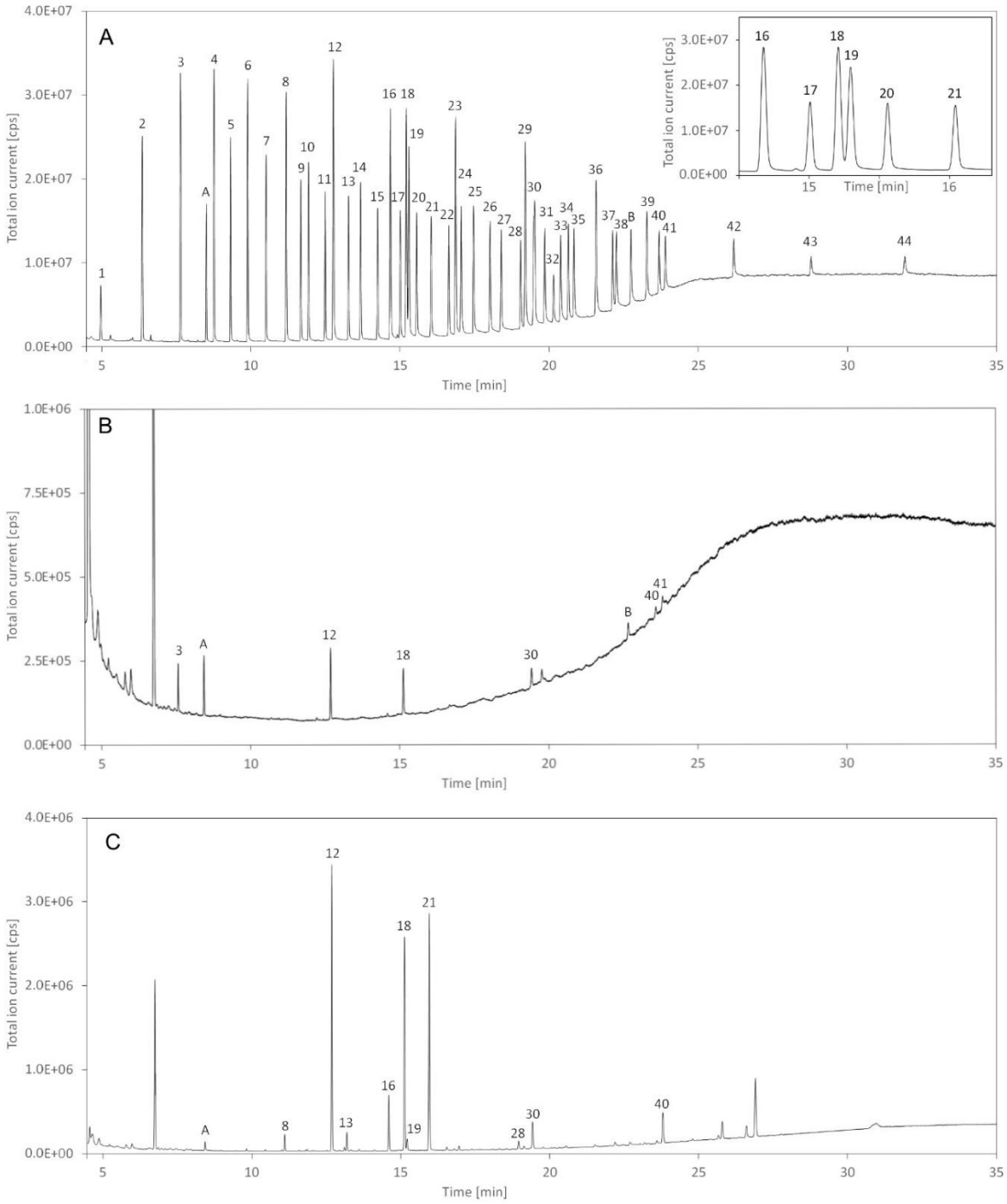


Figure 12: A representative chromatogram (TIC) of a standard mixture containing all 44 individual FA species. Peak assignment is shown in Table 25. The separation of E and Z isomers in particular FA 18:1 9E (17) and FA 18:1 9Z (18) are zoomed. B: Representative chromatogram (TIC) of a calibrator of FA 8:0; FA 16:0; FA 18:1 9Z; FA 20:4 5Z, 8Z, 11Z, 14Z; FA 22:6 4Z, 7Z, 10Z, 13Z, 16Z, 19Z and FA 26:0 at a concentration of 50 µg/mL. C: Representative chromatogram (TIC) of a plasma sample. The most prominent FA species are labelled.

Table 25: Total relative plasma fatty acid profile during the 1st 3 months of smoking cessation. TP0 still smoking, TP1 after 1 week, TP2 after 1 month and TP3 after 3 months of smoking cessation. Calculated as ratio of individual fatty acid levels to the sum of all measured fatty acids. FAs below LOQ were excluded from the calculation of the total FA profile.

No.	Analyte	RT	Mean Value ± Standard Deviation (N=39) [%]			
			TP0	TP1	TP2	TP3
1	FA 4:0	4.90	<LOQ	<LOQ	<LOQ	<LOQ
2	FA 6:0	6.28	<LOQ	<LOQ	<LOQ	<LOQ
3	FA 8:0	7.56	<LOD	<LOQ	<LOQ	<LOD
4	FA 10:0	8.68	0.01 ±0.01	0.01 ±0.01	0.01 ±0.01	0.01 ±0.01
5	FA 11:0	9.23	<LOD	<LOD	<LOD	<LOD
6	FA 12:0[a]	9.80	0.05 ±0.02	0.06 ±0.03[b]	0.07 ±0.03[b]	0.07 ±0.05[b]
7	FA 13:0	10.42	<LOD	<LOD	<LOD	<LOD
8	FA 14:0[a]	11.09	0.57 ±0.19	0.70 ±0.29[b]	0.72 ±0.28[b]	0.70 ±0.27[b]
9	FA 14:1 9Z	11.58	0.01 ±0.03	0.02 ±0.05	0.02 ±0.05	0.02 ±0.04
10	FA 15:0	11.84	0.13 ±0.02	0.14 ±0.03	0.13 ±0.02	0.13 ±0.02
11	FA 15:1 10Z	12.39	<LOD	<LOD	<LOD	<LOD
12	FA 16:0[a]	12.67	22.65 ±1.27	22.01 ±1.49	22.17 ±1.60	22.01 ±1.59
13	FA 16:1 9Z	13.18	1.58 ±0.54	1.71 ±0.71	1.71 ±0.67	1.63 ±0.57
14	FA 17:0[a]	13.58	0.14 ±0.02	0.16 ±0.02[b]	0.15 ±0.02	0.15 ±0.02
15	FA 17:1 10Z	14.15	<LOD	<LOD	<LOD	<LOD
16	FA 18:0	14.58	5.15 ±0.49	5.35 ±0.49	5.33 ±0.55	5.20 ±0.47
17	FA 18:1 9E	14.90	<LOD	<LOD	<LOD	<LOD
18	FA 18:1 9Z[a]	15.11	23.40 ±3.05	23.29 ±2.62	22.33 ±2.83	21.84 ±2.42[b]
19	FA 18:1 11Z	15.20	1.53 ±0.27	1.47 ±0.25	1.44 ±0.23	1.43 ±0.24
20	FA 18.2 9E,12E	15.46	<LOD	<LOD	<LOD	<LOD
21	FA 18:2 9Z,12Z	15.94	27.16 ±3.24	27.02 ±3.36	27.77 ±3.48	27.90 ±2.98
22	FA 18:3 6Z,9Z,12Z[a]	16.53	0.44 ±0.15	0.57 ±0.22[b]	0.50 ±0.16[b]	0.52 ±0.14[b]
23	FA 20:0	16.76	0.12 ±0.02	0.11 ±0.02	0.11 ±0.03	0.11 ±0.02
24	FA 18:3 9Z,12Z,15Z[a]	16.94	1.03 ±0.25	1.14 ±0.33	1.04 ±0.24	1.06 ±0.21
25	FA 20:1 11Z	17.35	0.08 ±0.07	0.07 ±0.06	0.08 ±0.06	0.08 ±0.06
26	FA 21:0	17.91	<LOD	<LOD	<LOD	<LOD
27	FA 20:2 11Z,14Z	18.28	0.11 ±0.03	0.11 ±0.02	0.12 ±0.02	0.12 ±0.02
28	FA 20:3 8Z,11Z,17Z[a]	18.93	1.66 ±0.51	1.80 ±0.48	1.86 ±0.51[b]	1.88 ±0.38[b]
29	FA 22:0	19.10	0.26 ±0.06	0.26 ±0.07	0.28 ±0.07	0.25 ±0.06
30	FA 20:4 5Z,8Z,11Z,14Z[a]	19.41	7.68 ±1.26	7.59 ±1.22	7.86 ±1.59	8.40 ±1.46[b]
31	FA 22:1 13Z[a]	19.75	0.25 ±0.14	0.21 ±0.10[b]	0.21 ±0.11	0.18 ±0.07[b]
32	FA 20:4 8Z,11Z,14Z,17Z	20.04	0.03 ±0.10	0.06 ±0.10	0.05 ±0.10	0.03 ±0.06
33	FA 23:0	20.29	0.29 ±0.07	0.29 ±0.08	0.31 ±0.08	0.29 ±0.08
34	FA 20:5 5Z,8Z,11Z,14Z,17Z[a]	20.53	1.06 ±0.44	1.31 ±0.62[b]	1.22 ±0.38	1.22 ±0.36
35	FA 22:2 13Z,16Z	20.72	<LOD	<LOD	<LOD	<LOD
36	FA 24:0[a]	21.47	0.60 ±0.15	0.62 ±0.18	0.65 ±0.20	0.59 ±0.16
37	FA 22:4 7Z,10Z,13Z,16Z	22.02	0.17 ±0.03	0.17 ±0.03	0.18 ±0.04	0.18 ±0.04
38	FA 24:1 15Z[a]	22.15	0.67 ±0.16	0.61 ±0.16[b]	0.61 ±0.15[b]	0.59 ±0.17[b]
39	FA 22:5 7Z,10Z,13Z,16Z,19Z	23.17	0.94 ±0.20	0.97 ±0.20	0.97 ±0.17	1.03 ±0.18
40	FA 22:6 4Z,7Z,10Z,13Z,16Z,19Z[a]	23.58	2.29 ±0.75	2.21 ±0.75	2.14 ±0.68	2.41 ±0.76
41	FA 26:0	23.81	<LOD	<LOD	<LOD	<LOD
42	FA 28:0	26.11	<LOD	<LOD	<LOD	<LOD
43	FA 30:0	28.69	<LOD	<LOD	<LOD	<LOD
44	FA 32.0	31.83	<LOD	<LOD	<LOD	<LOD

[a]Significant ($p<0.05$) in Quade test, [b]significant vs TP0 ($p<0.0125$) in Wilcoxon signed rank test.

As it was of interest to follow alterations in the FA profile during the course of the smoking cessation study, different statistical tests were performed. The Quade test was utilized in order to identify significant (α=0.05) changes across all points in time (Table 26). Subsequently, the Wilcoxon signed rank test for cessation-relevant comparisons between two points in time was performed (e.g. TP0/TP1, Table 5) in order to verify the results of the Quade test. The results of the Quade test were confirmed by the Wilcoxon signed rank test. As parts of a time series were compared, it was compulsory to counteract the problem of multiple statistical comparisons and consequently applied a correction of the significance level by using the Bonferroni correction [261, 262]: for the Wilcoxon signed rank test p-values <0.0125 were reached by defining α=0.05. For 3 FAs (FA 16:0, FA 18:3 9Z,12Z,15Z, FA 24:0), only the Quade test was found to be significant. In total, 11 FAs showed significant alterations in both tests reflecting either an increase or a decrease over time. Trends (Table 26 and for mean values Table 25) of the significantly altered FAs could be stratified into three groups, namely SFAs, MUFAs and PUFAs.

Table 26: Results of the statistical evaluation using Quades test and Wilcoxon signed rank test. Only analytes with significant changes in at least one test are summarized in this table. Analytes with significant alterations in both tests are highlighted in bold. For both tests the significance level was α=0.05. For the Quades test, p-values of <0.05 (highlighted in bold) and for the Bonferroni-corrected Wilcoxon signed rank test, p-values of <0.0125 (highlighted in bold) were applied. Also given is the trend of the mean values over the course of 3 months of cessation (▲ = increase; ▼ = decrease; ∩ = increase during 1st month and recovery to the initial level after three months of smoking cessation; ∪ = decrease during 1st month and recovery to the initial level after 3 months of smoking cessation; trends in brackets ( ) are not significant in the Wilcoxon signed rank test).

Analyte	Trend	Quade test p-value	Wilcoxon signed rank test p-value				
		TP0-TP1-TP2-TP3	TP0/1	TP0/2	TP0/3	TP1/2	TP2/3
FA 12:0	▲	**0.005214**	**0.009348**	**0.002752**	0.068240	0.451200	0.230200
FA 14:0	▲	**0.000001**	**0.000002**	**0.000001**	**0.000219**	0.624000	0.493900
FA 16:0	(▼)	**0.022240**	0.027830	0.017500	0.037230	0.387200	0.735300
FA 17:0	∩	**0.001951**	**0.001468**	0.538700	0.798500	0.019710	0.485200
FA 18:1 9Z	▼	**0.000059**	0.714500	0.014880	**0.000129**	0.012610	0.117200
FA 18:3 6Z,9Z,12Z	▲	**0.000531**	**0.000068**	**0.006540**	**0.004289**	0.023930	0.714500
FA 18:3 9Z,12Z,15Z	(∩)	**0.049430**	0.018950	0.928500	0.538700	0.052570	0.538700
FA 20:3 8Z,11Z,17Z	▲	**0.003908**	0.038580	**0.000841**	**0.001246**	0.084850	0.884800
FA 20:4 5Z,8Z,11Z,14Z	▲	**0.000175**	0.511600	0.633800	**0.000495**	0.204100	**0.001635**
FA 22:1 13Z	▼	**0.001822**	**0.006844**	0.052570	**0.001917**	0.756200	0.224800
FA 20:5 5Z,8Z,11Z,14Z,17Z	▲	**0.010380**	**0.008191**	0.018950	0.024860	0.357400	0.863100
FA 24:0	(∩)	**0.010190**	0.418500	0.066100	0.653700	0.050830	0.027830
FA 24:1 15Z	▼	**0.003966**	**0.009348**	**0.011590**	**0.005187**	0.950500	0.162500
FA 22:6 4Z,7Z,10Z,13Z,16Z,19Z	∪	**0.004862**	0.120600	0.017500	0.224800	0.246900	**0.000219**

SFAs showed an increase while MUFAs declined over the time of smoking cessation. Moreover, a statistically significant increase during smoking cessation for the PUFAs C18 and C20 was found. Interestingly, the cascade of the 20 carbon chain length FAs (FA 20:3 8Z, 11Z, 17Z, FA 20:4 5Z, 8Z, 11Z, 14Z and FA 20:5 5Z, 8Z, 11Z, 14Z, 17Z) appeared to be affected by smoking cessation.

FA 17:0 and FA 22:6 4Z, 7Z, 10Z, 13Z, 16Z, 19Z were found to be significantly altered, although without showing a clear trend. After an initial increase for FA 17:0 and an initial decrease for FA 22:6 4Z, 7Z, 10Z, 13Z, 16Z, 19Z respectively, both FAs recovered back to the starting level (TP 0).

Fatty acids are important metabolites in various biochemical pathways such as energy storage [274],

cell structure [275] and cell signalling. [276] However, only limited information about the alterations of the FA profile related to smoking has been described in literature. [86, 132, 200, 271, 277-281] Reports on the perturbation of the FA profile after smoking cessation are completely missing.

In this study, increased levels of SFAs were found during the course of smoking cessation, while MUFAs showed a decrease. These results are in accordance with a previous study at ABF conducted by Mueller et al. [86] who observed differences between smokers and non-smokers in FA levels of plasma samples derived from a diet-controlled cross-sectional clinical study. A study reported by Cambien et al. [271] revealed increased MUFA levels in smokers' plasma. These findings support the hypothesis of an altered endogenous FA desaturation in smokers, possibly caused by an upregulation of the stearoyl-Coenzyme A desaturase 1 (cf. section 2.5) during smoking and a partial recovery back to the non-smoker levels upon smoking cessation. [86, 89-91]

Decreased levels of PUFAs of smokers have already been reported by Pawlosky et al. [200] In addition, breath ethane, an indicator for the oxidation of PUFAs was reported to be elevated in smokers. [234] Furthermore, free radicals in cigarette smoke [234, 282] have been shown to interact with various cell components such as lipids [283] and cause a peroxidation of lipids [49, 284] especially PUFAs. [285] The absence of an exposure to these radicals (and possibly other tobacco smoke constituents) could be reflected in the increasing levels of PUFAs after smoking cessation which was also found in this study. Additionally, the alterations of the PUFA levels could also be associated with smoking-related alterations in nutrition intake and uptake of lipids as reported by Thompson et al. [286]

The FAs FA 17:0 and FA 22:6 4Z, 7Z, 10Z, 13Z, 16Z, 19Z showed a significant in- or decrease upon smoking cessation and a recovery towards the initial level after having quit. This could be the result of a hyper compensation caused by metabolic adaption processes after smoking cessation.

With respect to the time course of alterations in the FA profile after smoking cessation, it was found that most of the significant changes were observed after 1 week of cessation, which means that the progress of possible recovery towards a non-smoker profile is triggered immediately upon cessation. It was also found that several FAs did not stabilize on a new level during the course of three months, which indicates that the progress started is not finished after this period of time. However, further research is required, especially with regard to the long-term effects of smoking cessation on the FA profile.

5.4.3 Validation

The accuracy was found to be between 81% and 97% for the samples spiked at low levels (close to the LOQ), 86% to 110% for the medium and 86% to 108% for the high levels (cf. Table 27).

Since the FA levels in plasma samples are expected to exceed the calibration range, accuracy after sample dilution was assessed for water dilutions of 1/1, 1/4 and 1/8 respectively. Accuracies ranged from 90% to 107% for 1/1 dilution, 85% to 112% for the 1/4 and 80% to 106% for a 1/8 dilution.

No carryover was observed between the samples.

The LOD was calculated from blank samples according to IUPAC. [257, 258] Overall, an improvement of the LOD with decreasing number of carbon atoms and increasing saturation degree was observed. The determined LODs were between 0.1 µg/mL and 20.2 µg/mL (Table 27).

The LOQ according to IUPAC was determined in the range of 0.3 µg/mL to 67.0 µg/mL (Table 2).

Table 27: Validation data showing accuracy among the different concentration levels, recovery rate, LOD and LOQ for the selected FAs.

Analyte	Accuracy (N=6) [%]			Recovery rate	LOD	LOQ
	low	medium	high	[%]	[µg/mL]	[µg/mL]
FA 8:0	96.9	110.0	107.7	42	0.3	1
FA 16:0	81.8	89.4	107.8	30	0.1	0.3
FA 18:1 9Z	88.2	87.7	85.6	87	4.3	14.3
FA 20:4 5Z,8Z,11Z,14Z	81.0	88.3	87.5	26	7.3	24.3
FA 22:6 4Z,7Z,10Z,13Z,16Z,19Z	88.3	86.4	85.9	32	17.7	59
FA 26:0	82.6	87.0	96.8	93	20.2	67

Intra- and inter-day precision in matrix were determined in plasma pool samples at 3 different concentration levels: low, medium and high. Precision across all analytes were below 13% CV (Table 28). As expected, the variability expressed as the CV at lower concentration was found to be higher. Also, higher precision was obtained for lower chain lengths due to the higher sensitivities with decreasing number of carbon atoms.

Table 28: Validation data showing the intra- and inter-day precision among the different concentration levels.

Analyte	Intra-day precision (N=5) CV [%]			Inter-day precision (N=6) CV [%]		
	low	medium	high	low	medium	high
FA 8:0	3.8	2.5	3.2	3.8	2.2	3.0
FA 16:0	4.8	3.3	2.7	2.2	2.7	3.0
FA 18:1 9Z	6.4	5.3	2.4	5.9	4.2	4.6
FA 20:4 5Z,8Z,11Z,14Z	6.3	3.6	3.0	6.3	3.9	6.1
FA 22:6 4Z,7Z,10Z,13Z,16Z,19Z	8.9	4.7	2.5	8.6	2.3	8.3
FA 26:0	9.9	11.7	10.0	12.3	9.9	5.3

Since the method covers a wide range of individual FA species with different polarities, the observed recoveries varied between 26% and 93% (Table 27). However, losses during sample clean-up were fully compensated by the IS. Furthermore, recovery rates did not vary with the different concentration levels (CV <15%) and were stable across the triplicates.

The reproducibility of re-injections was observed to be stable for 48 h and 72 h. The CV ranged for low concentrations from 3.5% to 11.9% and for high concentrations from 2.1% to 9.5%.

The stability of analytes in plasma stored at different temperatures, durations and freeze-thaw cycles was characterized. The analytes were found to be stable after storage for 24 h at room temperature. Similarly, post-preparative stability of the extracted plasma samples was proven for 29 days in the autosampler (8°C). Furthermore, 6 freeze-thaw cycles were tested and found to be stable across all analytes. The long-term stability of raw material was confirmed for 1 month and will be further investigated at ABF in the future.

No interfering signals above 20% of the LOQ were detected. Thus, the selectivity of the method is in accordance to the guidelines. [245]

The comprehensive validation experiments according to the FDA guidelines [245], comprising a representative set of 6 FAs, demonstrated acceptable levels for precision, robustness and accuracy. A comparison of the 5 major FAs (FA 16:0, FA 18:0, FA 18:1 9Z, FA 18:2 9Z, 12Z and FA 20:4 5Z, 8Z, 11Z, 14Z), covering approximately 85% of the plasma FA profile, demonstrated excellent analogy between recently published data obtained by a GC-FID profiling approach [86] and the developed GC-TOF-MS method presented here. The main focus as compared to already published methods was a wide analyte coverage, baseline separation of isomers as well as separation of interfering matrix components rather than achieving short analysis times. [205, 287] The performance of the TOF instrument used with regard to analyte calibration was comparable to quadrupole MS instruments. [205, 254] Also, the attained linearity over 4 orders of magnitude for FA 16:0 is comparable to methods reported in literature. [205] Furthermore, the sensitivity of the method was found to be within an acceptable range for the intended purpose.

By using TOF-MS as an uncommon alternative for targeted FA analysis as detection method, it could be demonstrated that the obtained method performance data (Table 28) are comparable to those achieved with commonly used quadrupole instruments. Lagerstedt et al. [254] reported intra- and inter-day precisions in a CV range of 2.5% to 13.2% and Ecker et al. [205] found CVs between 1.8% and 17.7%, which is similar to the method presented here. Therefore, the developed GC-TOF-MS method represents a suitable tool for the quantification of FAs in human plasma samples. The TOF-MS technology offers several advantages over quadrupole instruments, including high mass resolution, yielding better selectivity and complete mass fragment acquisition. Moreover, GC-TOF-MS offers the opportunity of retrospective analysis of metabolites unconsidered hitherto.

A further benefit of the GC-TOF-MS-based FA profiling method is the fact that the untargeted metabolic fingerprinting and the targeted profiling approaches can be performed by using the same analytical instrument, which is cost- and time-efficient for research projects as described here. Furthermore, it was found that the analytical results generated by the TOF-MS instrument are less affected by interference through matrix components and derivatization agents as compared to quadrupole-MS instruments. Finally, it should be mentioned that during this study, no cleaning of the TOF-MS instrument was necessary, even between the fingerprinting approach using silylation agent for derivatization. [76, 87] This benefit should not be underestimated regarding sample throughput.

5.4.4 Fatty Acid Summary

In summary, an accurate, precise and robust method for the quantification of plasma FAs by means of GC-TOF-MS was developed and validated. The current method covers to the best of the authors knowledge the widest range of individual FA species (C4:0 to C32:0). Furthermore, it represents the first FA profiling method for human plasma by using GC-TOF-MS. The method is characterized by baseline separation of positional and geometrical isomers of methylated FAs. Due to the wide range of FAs and the separation of positional and geometrical isomers, the method could serve as a powerful tool for investigating alterations in the FA profile during clinical studies applicable to various areas of research.

Application of the GC-TOF-MS method to samples of the smoking cessation study revealed significant alterations in the FA profile after smoking cessation, indicating a recovery to the FA profiles of non-smokers. These results are in accordance with literature reports and previous studies conducted by Mueller et al. at ABF laboratory. [76, 86, 200, 271]

5.5 Amino Acid Profiling

Plasma samples of all compliant study participants of the smoking cessation trail have been prepared, analysed and evaluated as described in section 4.5. The determination of 26 individual AA species (Figure 13, Table 8) was performed by means of LC-MS/MS operating in ESI-negative ionization mode. Robust quantification was achieved by evaluating a quantifier and a qualifier for each analyte (Table 8). The FMOC-AAs fragmentation pattern was similar to reports from literature. [218] Furthermore, as described by Ziegler and Abel single and double derivatization of some AAs (His, Tyr and Lys) can occur. Additionally, double derivatives for 5-HT and Kyn were observed. For Tyr it was possible to compare the calibration slopes of both, single and double derivatized Tyr, which were found to be similar (within ±15%). For quantification, the single derivatized AA were evaluated except for 5-HT and Lys (see MRM transitions Table 8).

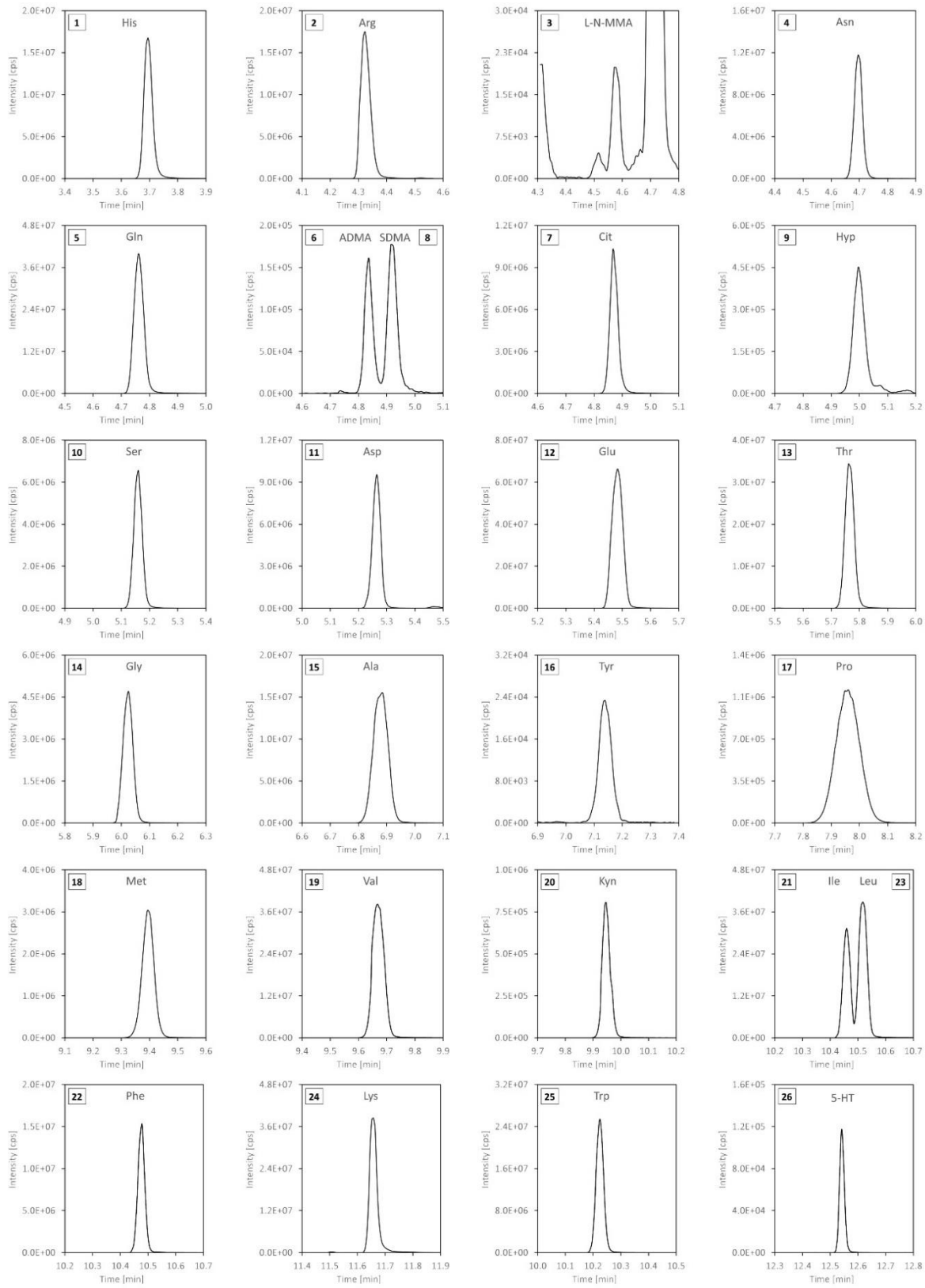


Figure 13: Representative chromatograms 26 amino acids in a plasma sample. Shown is the quantifier mass transition of each analyte. For details cf. Table 8.

Initially, it was tried to include cysteine (Cys), homocysteine (Hcy) and cystine into the method. However, in accordance with reports in literature [288, 289] satisfying stability and precision of the sulphur containing analytes could not be achieved and, consequently, were excluded from the method. Additionally, the sensitivity for 5-hydroxy-tryptophane (5-HTP) was found to be too low. Therefore, 5-HTP was omitted from the multi-method.

Calibration in water as a surrogate matrix revealed similar results as compared to calibration in plasma. The deviations of the calibration slopes were below ±15% except for Kyn, 5-HT, Ile, Met, Trp and Val. For Ile, Tyr and Val the matrix signal was too high and reached the detector limit and therefore no calculation of calibration curves was possible to compare water and plasma for these analytes. The deviation of the calibration slopes of Kyn, 5-HT and Met were >15%, but due to reproducible results in the validation and the number of analytes included into the method, those deviations were found to be within an acceptable range for a multi-analyte method. Furthermore, Kyn and 5-HT were normalized by using non-authentic IS, making higher deviations allegeable. Noteworthy, highly labelled authentic internal standards (Table 8) were included for most of the analytes (19 of 26) making the method more robust and less susceptible for matrix effects.

The dynamic range of the method was found to cover up to 5 orders of magnitude. The calculation of the calibration slope was performed by linear and quadratic regression of the peak area ratios against the concentrations, with application 1/x weighting for most analytes (Table 29). All calibration curves showed correlation coefficients of R^2 >0.98 (N=6, Table 29).

Table 29: Calibration data including regression type, weighting, regression function and coefficient of determination (R^2).

Analyte	Regression	Weighting	Function	R^2(N=6)
His	Linear	1/x	y=0.05581x+-0.01098	1.00
Arg	Linear	1/x	y=0.06375x+0.00362	1.00
L-N-MMA	Linear	1/x	y=0.05937x+5.56507e-6	1.00
Asn	Linear	1/x	y=0.03571x+2.84764e-4	1.00
Gln	Linear	1/x	y=0.03292x+1.98071e-4	1.00
ADMA	Linear	1/x	y=0.06548x+7.22908e-6	1.00
Cit	Linear	1/x	y=0.07340x+9.86590e-4	1.00
SDMA	Linear	1/x	y=0.03085x+8.92715e-6	1.00
Hyp	Linear	1/x	y=0.09129x+4.38436e-4	1.00
Ser	Linear	1/x	y=0.2219x+0.00361	1.00
Asp	Linear	1/x	y=0.01274x+4.488440e-4	1.00
Glu	Quadratic	1/x	y=-1.54094e-5x^2+0.02031x+1.6439e-4	1.00
Thr	Quadratic	1/x	y=-1.34724e-5x^2+0.02016x+6.9023e-4	1.00
Gly	Linear	1/x	y=0.01295x+0.00182	1.00
Ala	Quadratic	1/x	y=-3.56483e-6x^2+0.01043x+6.38550e-4	1.00
Tyr	Linear	1/x	y=0.15487x+0.00700	0.98
Pro	Linear	1/x	y=0.02916x+8.66933e-4	1.00
Met	Linear	1/x	y=0.09190x+2.65926e-4	1.00
Val	Quadratic	-	y=-1.66949e-5x^2+0.01805+0.9698	1.00
Kyn	Linear	-	y=0.05581x+-0.01098	1.00
Ile	Quadratic	-	y=-4.09609e-5x^2+0.02206x+0.05106	1.00
Phe	Quadratic	1/x	y=-1.39761e-4x^2+0.11941x+0.00116	1.00
Leu	Quadratic	1/x	y=-150146e-5x^2+0.02224x+3.59489e-4	1.00
Lys	Quadratic	-	y=-8.51189e-6x^2+0.02983x+0.46372	1.00
Trp	Linear	1/x	y=0.10339x+3.40138e-4	1.00
5-HT	Linear	1/x	y=0.02470x+2.65708e-5	1.00

5.5.1 Quality Control

Calibrators and quality control samples (N=18) for all analytes were interspersed throughout the randomized analytical batch and all acceptance criteria were fulfilled.

5.5.2 Amino Acid Profile

The validated LC-MS/MS method was applied to the plasma samples of both clinical studies (cf. section 4.5). In total, plasma samples derived from 25 S and 25 NS were analysed as well as 39 smokers, who quit smoking at baseline (TP0) and stayed abstinent after 1 week (TP1), 1 month (TP2) and 3 months (TP3). The results of the individual AA species are summarized in Table 30. The determined AA levels were compared to literature [259] as well as data in the HMDB [70] and excellent conformity to our method with the described values was found, except for Glu which showed higher values than expected. Interestingly, the older samples from the initial study comparing S and NS were even more affected (approx. 250% of the highest values described in literature) as compared to the smoking cessation study (approx. 150% of the highest values reported in literature). These findings are highly likely correlated to the stability of Gln discussed in the validation section in detail (cf. section 5.5.3). For the statistical evaluation, the stability should have no impact as all samples of one study are of the same age.

Table 30: Mean concentrations determined of AAs (µmol/L) for S and NS as well as levels at the different points in time (TP0 to TP3) of the smoking cessation study.

Analyte	S	NS	TP0	TP1	TP2	TP3
His	86.5	87.6	88.4	91.6	90.6	90.2
Arg	100.7	88.9	91.7	94.1	87.1	91.1
L-N-MMA	0.078	0.071	0.075	0.078	0.075	0.074
Asn	38.3	37.0	43.7	44.2	43.6	44.0
Gln	367.6	320.9	547.1	565.4	508.8	572.1
ADMA	0.7	0.628	0.690	0.718	0.697	0.685
Cit	42.0	37.8	35.9	37.7	36.4	36.9
SDMA	2.132	2.057	1.966	2.036	1.961	2.012
Hyp	15.6	14.8	13.9	16.0	14.4	15.8
Ser	115.8	112.8	115.0	109.3	109.0	105.6
Asp	23.1	22.1	12.3	12.4	12.9	11.8
Glu	238.7	239.8	153.1	150.1	167.2	136.8
Thr	147.5	146.3	139.4	142.6	136.2	132.1
Gly	211.3	206.5	208.8	205.9	201.3	197.2
Ala	303.0	327.3	276.0	277.3	279.8	280.4
Tyr	59.6	60.0	56.8	59.2	60.4	59.9
Pro	270.2	235.4	251.5	257.5	261.0	258.1
Met	4.2	1.4	18.4	17.4	17.5	19.3
Val	230.5	231.9	233.5	243.1	236.7	236.3
Kyn	2.1	2.1	1.9	2.2	2.2	2.2
Ile	89.6	89.7	82.9	87.3	84.5	87.2
Phe	55.1	57.5	55.6	57.4	56.4	57.2
Leu	103.2	99.2	102.6	102.7	102.5	103.3
Lys	214.7	219.5	209.4	225.3	217.8	223.6
Trp	43.2	44.0	40.4	42.8	41.0	42.0
5-HT	0.111	0.089	0.196	0.158	0.176	0.142

The statistical analysis was performed applying the Wilcoxon rank sum test for the comparison of smokers and non-smokers and the Quade test and Wilcoxon signed rank test in order to follow alterations in the amino acid levels during the 3 months of smoking cessation. The results of the statistical tests are summarized in Table 31.

Table 31: P-values for differences between S and NS as well as baseline and time points after smoking cessation. Significant values are highlighted in bold (▲ = increase; ▼ = decrease).

Analyte	**S/NS**	**Trend**	**TP0-TP3**	**TP1/TP0**	**TP2/TP0**	**TP3/TP0**	**Trend**
	Wilcoxon rank sum test		Quade test	Wilcoxon signed rank test	Wilcoxon signed rank test	Wilcoxon signed rank test	
	p-value	S/NS	p-value	p-value	p-value	p-value	cessation
ADMA	**0.0290**	▲	0.2431	0.1628	0.8890	0.6501	-
Arg	**0.0136**	▲	**0.0186**	0.6240	0.0289	0.4265	-
Gln	0.2548	-	**0.0178**	0.3502	0.0289	0.1894	-
Glu	0.9540	-	**0.0259**	0.8307	0.1240	0.0372	-
Gly	0.9234	-	**0.0035**	0.2829	0.0322	**0.0041**	▼
Kyn	0.5004	-	**3.4E-06**	**1.4E-06**	**4.3E-05**	**0.0001**	▲
Lys	0.4074	-	**0.0004**	**0.0002**	0.0222	**0.0022**	▲
Ser	0.9847	-	**0.0002**	0.0149	**0.0111**	**0.0026**	▼
Thr	0.8325	-	**0.0223**	0.2195	0.5949	0.0459	-
Trp	0.5004	-	**0.0222**	0.0168	0.3796	0.0182	▲
Met	**1.7E-05**	▲	0.3293	0.1015	0.3647	0.4766	-

Due to the different ages of the stored samples (3 years difference), comparisons of AA concentrations between both studies (cf. Table 30) should be avoided, as long as there is no long-term stability approved for the analytes of interest. However, we believe that trends are not influenced by the difference in storage time.

Three AAs (ADMA, Arg and Met) were found to be significantly increased in S as compared to NS. After smoking cessation, several AAs were found to be significantly affected when applying the Quade test. S vs NS differences for Trp were found to be significant only in the Quade test, while the p-values of the Wilcoxon signed rank test were of borderline significance. Furthermore,

Trp showed a slight increase over time after smoking cessation. This observation is in good accordance with the results determined by the untargeted metabolomics fingerprinting approach [77] showing increased Trp values in plasma upon quitting smoking. Additionally, Gly, Ser, Lys and Kyn were found to be significantly altered in both statistical tests. While Gly and Ser showed a decrease upon smoking cessation, Lys and Kyn showed an increase (Table 31, Figure 14).

It is of note that ADMA, Arg, and Met found to be elevated in S[10] were not found to decrease within the first 3 months after smoking cessation. Furthermore, Gly, Kyn, Lys, Ser and Trp observed to be altered after stopping smoking, showed no statistically significant differences between S and NS in the initial study. This observation could indicate that the plasma concentrations of these AAs are not influenced by tobacco smoke exposure but factors associated with smoking cessation, e.g. increase food consumption.

The increased ADMA levels observed in S as compared to NS confirms other reports in literature. [158-160] The same is true for the elevated Arg levels in S compared to NS. [132, 161] The lower levels of L-N-MMA in S reported by Sobczak et al. [161] agrees with Arg metabolite levels approaching NS levels within 7 years. [132] The observation of no alteration within 3 months after smoking cessation is in line with this observation and indicates a slow recovery of the Arg metabolism after quitting smoking. The Arg metabolites are often considered as proteolysis and cardiovascular risk markers [290, 291], which would be in line with smoking as an established risk factor for CVD. [292] Additional, homocysteine (Hcy), another risk marker for cardiovascular disease, is known to be increased in S [181], accordingly the increase of Met, a precursor of Hcy, can be also regarded as indicator for an elevated CVD risk in S.

Lys has not yet been described as potential biomarker in the context of smoking cessation. In the initial fingerprinting approach [77] Lys was also found to be statistically significant altered. Similar to some other analytes the trend was contrariwise likely due to a few outliers with extreme values.

Ser is metabolized to Gly by the Ser-hydroxy-methyl-transferase and both AAs exhibited an elevation upon smoking cessation. Hence, the decreased Ser level could in turn lead to a decreased Gly level. However, it should be mentioned that Gly can also be metabolized to Ser and thus a reduced Gly level leads to a reduced Ser level. [293] In the KORA study, Ser was found to be lower in S. [132] This is in contrast to the results of this study, which shows no difference between S and

[10] Of the samples from the initial study comparing smokers and non-smokers.

NS. Conversely, the increase in Ser observed after smoking cessation in the study supports the finding of the KORA study. Here, the storage conditions of the plasma samples could have an effect on the measured AA levels, as the ideal storage temperature is suggested to be below -70°C [294], whereas our samples were stored at -20°C.

The hypothesis of an alteration of the Trp metabolism by smoking (Figure 10) could be only partially confirmed, as 5-HTP was not included into the method for reasons discussed above. Also 5-HT which could have been affected by the alterations observed in the fingerprinting showed no significant changes after smoking cessation. Nevertheless, elevated Kyn and Trp values (Figure 14) were in good agreement with the results obtained from the untargeted metabolomic fingerprinting analysis. [77] The similar Kyn and Trp levels in S and NS were also described in literature. [170] In contrast to our findings and those of Deac et al., in the KORA study increased Kyn levels in S and no effect of smoking cessation was observed. These changes may be attributed to metabolic adaption processes in the enzyme activities of tryptophan-hydroxylase and tryptophan-2,3-dioxygenase (cf. Figure 10), respectively, induced by smoking cessation.

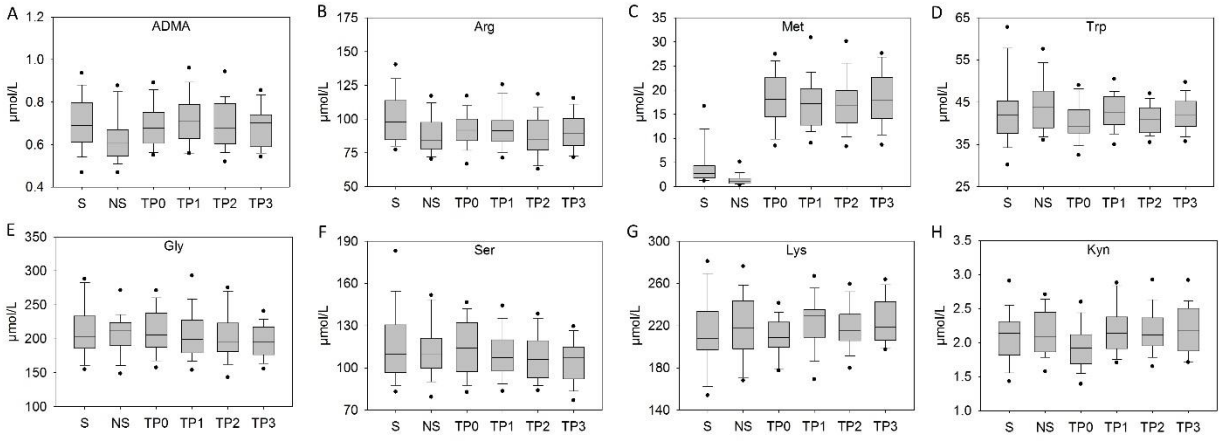


Figure 14: Boxplots of the AA, which show significance between S and NS as well as the points in time TP0–TP3 (A, ADMA; B, Arg; C, Met; D, Trp; E, Gly; F, Ser; G, Lys and F, Kyn.

5.5.3 Validation

The accuracy was found to be between 94% and 101% for the samples spiked at low levels, 99% to 103% for the medium and 98% to 101% for the high levels (Table 32).

Since the AA levels in plasma samples were expected to exceed the calibration range, accuracy after sample dilution was assessed for water dilutions of 1/2, 1/4 and 1/10, respectively. Accuracies ranged from 87% to 110% for 1/2 dilution, 89% to 105% for the 1/4 and 86% to 115% for a 1/10 dilution (Table 32). For 5-HT only a 1/2 dilution showed an acceptable accuracy, however 5-HT

levels >ULOQ are not expected.

In an initial approach, it was tried to develop the method on an Agilent 1100 and 1200 HPLC system with 1100 flow through injector or CTC PAL auto sampler. Due to tremendous carry-over effects on both systems even though multiple washing cycles and programs were tested, the method was developed on the Shimadzu Nexera X2 with SIL-30ACMP auto sampler. This system offers advanced rinsing options including e.g. injection port rinsing which lead to satisfying and reproducible results. For those analytes, for which carry over effects were observed, the blank sample signal after 5 ULOQ injections in a row was compared to the LOD signal of the analyte. No carryover larger than 20% of the LOD signal was observed.

The LOD was calculated according to IUPAC. [257, 258]. The determined LODs were between 0.02 nmol/L and 30 nmol/L (Table 32). Compared to the LC-MS/MS FMOC amino acid method described by Ziegler and Abel [218] with LODs between 1 nmol/L and 15 nmol/L our method shows an improvement for at least some analytes which is likely due to improved sample clean-up and MS technology.

The LOQ according to IUPAC [257, 258] was determined in the range of 0.025 µmol/L to 25 µmol/L (Table 32). LOQs according to IUPAC were between 0.07 nmol/L and 100 nmol/L.

Table 32: Method performance comprising LOD, LOQ, ULOQ, accuracy and accuracy after sample dilution.

Analyte	LOD	LOQ	ULOQ	Accuracy [%]			Accuracy after dilution [%]		
	µmol/L	µmol/L	µmol/L	low	Medium	high	1/10	1/4	1/2
His	0.00002	0.00007	250	100.2	102.0	99.5	98.1	92.0	91.6
Arg	0.0005	0.002	500	97.1	103.2	99.3	102.8	96.5	96.6
L-N-MMA	0.002	0.007	50	100.4	101.5	99.1	96.1	96.6	105.3
Asn	0.0004	0.001	250	100.4	101.8	99.4	107.1	99.7	99.5
Gln	0.004	0.01	1000	99.8	102.3	99.5	109.1	100.6	97.9
ADMA	0.002	0.007	50	100.0	101.8	99.5	89.4	95.7	102.9
Cit	0.0003	0.001	100	100.4	101.9	101.0	85.9	95.4	107.6
SDMA	0.006	0.02	50	100.7	101.4	99.1	90.0	95.2	104.4
Hyp	0.0007	0.002	100	99.2	101.9	99.5	98.5	91.0	95.4
Ser	0.02	0.07	500	99.7	102.4	99.2	109.1	102.4	102.3
Asp	0.002	0.007	500	98.9	100.1	99.7	106.2	98.4	97.6
Glu	0.0007	0.002	500	100.5	101.8	99.0	102.1	97.0	98.0
Thr	0.002	0.007	500	100.5	101.6	99.2	103.2	99.0	98.2
Gly	0.002	0.007	1000	99.7	102.1	99.2	102.1	96.5	95.3
Ala	0.0003	0.001	100	100.2	101.7	99.2	103.2	98.6	98.3
Tyr	0.008	0.03	500	101.0	100.7	100.2	104.5	99.3	100.4
Pro	0.0004	0.001	500	93.7	102.2	99.7	109.4	102.1	99.8
Met	0.0002	0.0007	100	99.7	102.5	99.3	93.8	92.8	101.0
Val	0.03	0.1	500	100.7	102.0	99.0	101.3	99.5	98.7
Kyn	0.0003	0.001	50	100.7	101.1	99.6	115.2	89.3	86.8
Ile	0.0007	0.002	250	99.9	101.7	97.7	100.8	98.7	92.5
Phe	0.002	0.007	250	100.5	102.0	99.3	103.6	97.1	96.9
Leu	0.0003	0.001	500	100.1	102.0	99.5	96.8	95.5	95.0
Lys	0.0004	0.001	1000	100.2	102.3	99.5	86.9	104.9	109.5
Trp	0.001	0.003	250	100.4	101.6	99.4	110.0	101.8	94.1
5-HT	0.0009	0.003	10	98.5	99.4	100.6	-	-	87.8

Intra- and inter-day precision in matrix were determined in plasma pool samples at 3 different concentration levels: low, medium and high. Precision across all analytes were below 15% (CV) (Table 33). These findings are comparable to another method described by Ziegler and Abel et al. (CV = 16.5%). [218]

The reproducibility of re-injections was observed to be stable for 48 h and 72 h, respectively. The CV ranged for low concentrations from 1% to 12% and for high concentrations from 1% to 10% (Table 33).

The stability of analytes in plasma stored at different temperatures, durations and numbers of freeze-thaw cycles was characterized. The analytes except Glu and Gln were found to be stable after storage for 24 h at room temperature. While Gln shows a decrease to 57% and 99% at low and high concentrations, respectively, Glu shows an increase (143% and 106% respectively) over 24 h at ambient temperature. The limited stability and fast deamidiation of Gln to Glu is described in literature [295, 296] leading to incorrect values for both analytes. Consequently, plasma samples should be immediately and rapidly processed in order to minimize the influence of the degradation reactions. In contrast, the FMOC derivatives have shown to be stable at least for 10 days.

Similarly, post-preparative stability of the extracted plasma samples was proven for 10 days in the autosampler at 10°C except for 5-HT, which showed only 4 days of post-preparative stability. This limited stability compared to the other analytes could be due to the different chemical nature of 5-HT as amine compared to all other analytes which are amino acids.

Furthermore, 6 freeze-thaw cycles were tested and found to be stable across all analytes except for 5-HT, Asp, Glu and Gln. Stability for 5-HT showed a decline in concentration after 2 freeze-thaw cycles. For Asp, Glu and Gln decreased levels were observed after 1 freeze/thaw cycle. The limited stability of Asp, Glu and Gln is probably caused by the deamidiation reactions of Gln and Asn to Glu and Asp mentioned above. [295, 296] Additionally, 5-HT showed a limited stability over longer periods, which is in accordance with literature. [297]

The long-term stability of plasma samples was confirmed for 1 month and will be further investigated at ABF in the future.

No interfering signals above 20% of the LOQ were detected. Thus, the selectivity of the method meets the criteria of the FDA guidelines. [245]

Table 33: Method performance comprising intraday precision, interday precision and reproducibility of re-injections. Data are expressed as coefficients of variation (CV).

Analyte	Intraday precision (N=5) [%]			Interday precision (N=6) [%]			Reproducibility (N=3) [%]	
	low	medium	high	low	medium	high	low	high
His	0.9	5.5	1.9	2.0	4.2	1.3	2.2	2.1
Arg	9.2	14.8	2.3	14.1	4.0	2.3	10.4	1.2
L-N-MMA	3.8	7.9	2.3	10.0	10.6	3.5	12.2	2.3
Asn	1.9	5.0	2.7	3.6	4.4	2.4	1.6	3.0
Gln	1.4	7.9	2.3	4.7	5.9	2.6	2.2	2.4
ADMA	2.9	6.4	1.7	7.9	6.5	5.4	3.7	6.4
Cit	2.2	6.5	2.6	7.7	6.0	1.8	3.8	8.4
SDMA	1.6	5.5	2.7	5.4	5.8	2.1	3.2	2.1
Hyp	2.9	8.0	1.4	5.6	4.4	3.2	6.3	4.2
Ser	1.8	8.4	2.5	5.0	5.0	1.1	2.5	1.7
Asp	3.7	9.1	1.0	6.2	5.4	1.4	4.5	1.7
Glu	2.2	5.3	3.9	4.3	4.2	3.6	1.9	3.7
Thr	1.9	4.4	2.6	1.5	4.5	2.0	1.0	3.6
Gly	1.6	6.8	2.4	4.4	4.0	1.1	2.8	3.5
Ala	1.0	5.4	3.4	1.9	5.5	1.3	3.4	3.5
Tyr	4.1	4.2	3.3	4.6	7.4	3.5	3.3	4.1
Pro	2.0	7.4	2.2	4.4	3.9	2.8	2.5	5.4
Met	1.5	9.6	2.0	8.1	4.8	1.2	3.3	2.7
Val	2.9	5.7	5.1	4.4	5.0	2.7	6.3	10.2
Kyn	3.1	4.4	2.3	4.2	5.5	5.5	4.5	5.0
Ile	1.5	5.1	10.0	4.0	6.7	3.2	3.4	7.8
Phe	2.4	5.6	4.5	1.6	5.0	3.6	3.1	5.7
Leu	2.1	5.8	2.2	1.8	3.6	4.4	3.6	9.5
Lys	0.8	7.5	2.9	4.4	4.5	4.7	4.9	6.0
Trp	1.0	4.3	2.7	1.4	4.7	4.5	2.4	8.8
5-HT	3.7	5.3	3.9	8.0	5.9	7.6	6.0	3.5

5.5.4 Amino Acid Summary

In summary, an accurate, precise and robust method for the quantification of 26 plasma AAs by means of an LC-MS/MS method was developed and validated. This method could serve as a powerful tool for the investigation of alterations in the AA profile during clinical studies applicable to various areas of research. Application of the LC-MS/MS method to samples derived from the smoking cessation study and the initial study comparing S and NS revealed statistical significant

alterations in the AA metabolism, which in most cases is presumably caused by exposure to tobacco smoke. While ADMA, Arg and Met were found to be increased in S compared to NS, Trp, Kyn and Lys were found to increase after cessation and Gly and Ser to decrease. The findings of an altered AA metabolism, in particular the Trp metabolism determined by untargeted metabolomics fingerprinting could be confirmed. The fact that none of the analytes increased in the comparison of S and NS shows an alteration after smoking cessation could be due to a slower recovery of these metabolic pathways than the 3 months observed in this study. Furthermore, the finding of several alterations after smoking cessation in non-smoking considered AAs is likely due to adaptation processes after smoking cessation which are not directly related to nicotine uptake as the changes in diet.

5.6 Eicosanoid Profiling

Urine samples of all compliant participants of the smoking cessation study have been prepared, analysed and evaluated as described in section 4.6 according to Sterz et al. [53] The untargeted fingerprinting analysis [77] using the validated GC-TOF-MS method [76, 87] showed changes in the FA, amino acid and energy metabolism, indicating a partial recovery of the smokers' metabolome towards the non-smoker profile upon 3 months of cessation. Subsequently, a targeted GC-TOF-MS method for the quantification of FAs was developed and validated. [272] Several FA species were significantly altered after quitting including saturated as well as mono- and polyunsaturated FAs. Amongst other FAs, arachidonic acid showed a statistically significant increase after 1 month of smoking cessation which prompted us to investigate the eicosanoid profile as arachidonic acid (FA 20:4 5Z, 8Z, 11Z, 14Z) is regarded as a key precursor towards the formation of various eicosanoids. Therefore, an eicosanoid profile was analysed using a validated, targeted UPLC-MS/MS method. [53]

5.6.1 Quality Control

The quality control samples (N=9) fulfilled the acceptance criteria set according to FDA guidelines. [245]

5.6.2 Eicosanoid Profile

The determination of 9 individual eicosanoid species was performed by means of UPLC-MS/MS operating in ESI-negative ionization mode. Table 34 summarizes the results for all 9 eicosanoid species over the 4 points in time of the cessation study (TP0-TP3), given as total amount excreted within 24 h, as well as minimum and maximum values and the trend of the concentration after 3 months of smoking cessation.

Table 34 Results (concentration as median (Med.) and minimum (Min.) and maximum (Max.) range) of the UPLC-MS/MS analysis and trend during the period of three months of smoking cessation.

No.	Analyte	Trend	TP0 (start)		TP1 (1 week)		TP2 (1 month)		TP3 (3 month)	
			Excretion (ng/24 h)		Excretion (ng/24 h)		Excretion (ng/24 h)		Excretion (ng/24 h)	
			Med.	Min-Max	Med.	Min-Max	Med.	Min-Max	Med.	Min-Max
1	t-PGEM	-	4346	1200 - 37596	5099	2554 - 17100	4845	1392 - 16562	4636	1039 - 21638
2	t-PGDM	-	2524	1358 - 4680	2595	1479 - 5197	2532	1106 - 5221	2670	1505 - 5078
3	2,3-d-TXB_2	▼	1450	236 - 3050	997	216 - 3655	702	167 - 2828	928	177 - 4120
4	2,3-d-8-iso-$PGF_{2\alpha}$	-	5139	2057 - 9031	4845	2760 - 9142	4702	1566 - 9999	5211	1798 - 15743
5	$PGF_{2\alpha}$	▼	3235	1199 - 6748	2718	1057 - 21821	2852	1242 - 6487	2984	1481 - 17225
6	11-dh-TXB_2	-	596	153 - 1358	509	185 - 1653	513	155 - 863	526	76 - 5454
7	8-iso-$PGF_{2\alpha}$	▼	595	197 - 1098	510	245 - 1576	486	180 - 1439	473	219 1820
8	LTE_4	▼	130	39 - 355	77	25 - 149	61	24 - 164	67	32 - 157
9	12-HETE	-	20	6 - 320	15	5 - 418	13	3 - 190	16	4 - 185

▼ = decrease; Only trends of in both tests significantly altered analytes are given.

The statistical analysis was performed applying the Quade test and Wilcoxon signed rank test in order to follow statistically significant alterations in the eicosanoid levels during the 3 months of smoking cessation. The results of the Quade test and the Wilcoxon signed rank test are summarized in Table 35.

Table 35: P-values of the statistical evaluation using Quade test and Wilcoxon signed rank test.

Analyte	Quade test	Wilcoxon signed rank test		
	TP0-3	TP0/1	TP0/2	TP0/3
t-PGEM	0.0286	0.0459	0.6637	0.0459
t-PGDM	0.2136	0.3154	0.3721	0.1847
2,3-d-TXB_2	6.7E-06	0.0009	1.2E-06	0.0093
2,3-d-8-iso-$PGF_{2\alpha}$	0.4070	0.7667	0.1311	0.4265
$PGF_{2\alpha}$	0.0386	0.0132	0.0098	0.0197
11-dh-TXB_2	0.1849	0.2041	0.0137	0.1275
8-iso-$PGF_{2\alpha}$	0.0153	0.1501	0.0400	0.0078
LTE_4	2.5E-16	3.6E-12	6.9E-11	3.6E-11
12-HETE	0.2464	0.0222	0.0205	0.2248

For 4 eicosanoids, a significant decrease was observed over time of smoking cessation. $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, 2,3-d-TXB_2 and LTE_4. t-PGEM was identified as significantly altered in the Quade test, while showing no significance in the Wilcoxon signed rank test.

A reduction in urinary concentration of 4 eicosanoids was already obtained after 1 week of smoking cessation (TP1, Figure 15). The most pronounced decrease was observed for LTE_4 and 2,3-d-TXB_2 with a highly significant decline occurring already at TP1, while $PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ showed significant decreases only after 1 month (TP2) and 3 months (TP3) of cessation, respectively.

A decrease of 36% for 2,3-d-TXB_2, 8% for $PGF_{2\alpha}$, 21% for 2,3-d-TXB_2 and 49% for LTE_4 after 3

months of smoking cessation was observed. Sterz et al. [53] determined 80% lower levels for 2,3-d-TXB_2, 27% for 2,3-d-TXB_2 and 32% for LTE_4 comparing smokers and non-smokers with the same UPLC-MS/MS method. Unfortunately, $PGF_{2\alpha}$ was not included to this analysis. Nowak et al. [298] reported 36% lower values in non-smokers 2,3-d-TXB_2 levels, which is in accordance to the differences determined after smoking cessation. The findings are in line with other investigations that also show rather fast alterations in eicosanoid levels after quitting. For example, Riutta *et al*. described decreased levels of TXs, PGs and LTs in their 14-day cessation trail with 30 subjects[299] while Benowitz *et al*. found a significant decrease of 11-dh-TXB_2 in 12 quitters already after 5 days.[144] In a long-term study, decreased levels of F_2-isoprostanes were reported after one year of smoking cessation in 344 quitters.[143] Although, rather high inter-individual variations as determined in this eicosanoids approach were observed in the literature. [53, 298, 300] Reasons for these variations can be both due to endogenous formation/pathways as well as various exogenous sources other than diet.

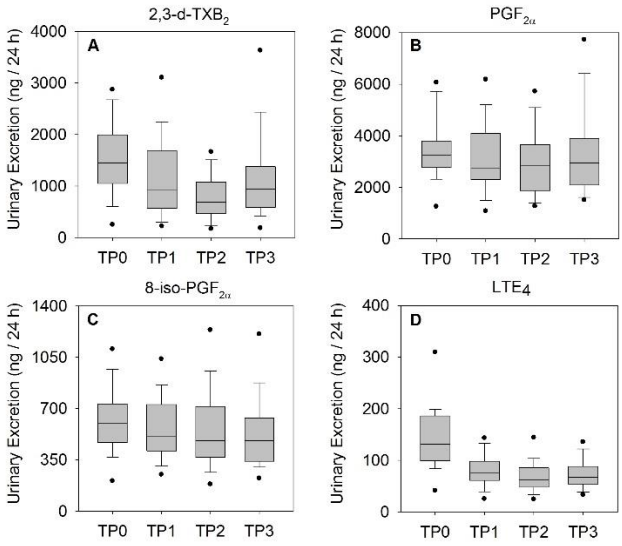


Figure 15: Boxplots of the significantly altered eicosanoids during the four points in time of the study.

Increased levels of eicosanoids may be caused by various disease states like asthma [301, 302], diabetes [303], cancer [303-305] and exogenous sources like smoking. [53, 142, 303] Further, some of the changes may occur relatively small but due to the fact that endogenous biomarkers of effect were monitored, small changes are normal. For example patients with diabetes showed 49% higher values in 8-iso-$PGF_{2\alpha}$ [306] or subjects with a moderate alcohol consumption over 3 weeks [307] showed an 18% increase in 8-iso-$PGF_{2\alpha}$. Furthermore, diabetes patients show 39% higher values in LTE_4 levels than healthy subjects. [308] Thus, the statistically significant alterations determined are plausible. In addition, the number of exogenous sources for changes in eicosanoid levels other than

smoking could be reduced to a minimum due to the study design including only healthy male subjects comprising a strict diet-control. Hence, it is highly likely that the alterations found here are directly linked to smoking cessation.

5.6.3 Eicosanoid Summary

Previous investigations mainly focused on the determination of single eicosanoid species like $PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ or 2,3-d-TXB_2 showing increased concentrations in smokers' urine as compared to non-smokers. [53, 298, 309] Elevated levels of LTE_4 in smokers are also reported in literature by Riutta et al. [310] Hence, the reduced levels of 2,3-d-TXB_2, $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$ and LTE_4 indicate a partial recovery of eicosanoid levels after smoking cessation, which is mainly driven by the reduction of oxidative stress and inflammatory effects after quitting. Interestingly, reductions became already significant after one week of cessation indicating a rather fast response upon quitting especially for LTE_4 and 2,3-d-TXB_2.

5.7 Creatinine Determination

The urinary creatinine values were analysed as described in section 4.7 according to the DFG method described by Blaszkewicz [263] to have the opportunity of a normalisation of analytical data additionally to the normalisation by the excretion over time[11]. The necessity of a normalisation from data of urinary samples is widely known [311-313] due to the different excretion and glomerular filtration rates.

5.7.1 Quality Control

Calibrators and quality control samples for all analytes on all 96 well plates fulfilled the acceptance criteria.

5.7.2 Creatinine Profile

The results of the eicosanoid determination are comprised in Table 36 composed with the weight of the urine fractions. The correlation of both, urinary creatinine and excretion by weight was found to be in an acceptable range (e.g. subject 2 at TP3 increases ~1/3 in creatinine concentration and decreases ~1/3 weight).

[11] The study design was planned to comprise 24 h attention at the stationary visits to have the opportunity of a normalization by daily excretion.

Table 36: Results of the creatinine determination of compliant subjects composed with the urinary excretion at stationary visits.

Subject	Creatinine excretion [mg/mL]				Urine excretion [g/24 h]			
	TP0	TP1	TP2	TP3	TP0	TP1	TP2	TP3
001	381	349	493	430	4021	4407	3453	3677
002	472	698	602	1088	3066	3057	3187	2234
003	716	667	558	616	2049	2844	2656	2223
006	484	517	418	448	3810	3869	3367	4115
007	522	458	1254	642	3148	3252	1416	3172
008	556	457	674	611	3541	3378	2823	2722
010	1504	545	733	769	2114	2852	2274	2310
011	776	581	946	1705	2396	2867	2115	1363
012	582	609	1543	1120	1962	1584	809	1236
015	550	588	672	779	3589	3119	2572	2662
017	1843	970	572	509	1018	2177	2979	3175
018	518	243	496	616	2491	3522	2521	1957
020	644	466	561	583	2886	2964	3069	3029
021	537	459	478	600	3015	3012	2783	2836
027	581	483	443	871	2598	3661	2657	1372
028	448	1106	463	619	2984	1576	2990	3077
029	806	1088	1583	837	2083	1415	956	1646
030	1284	668	1242	1139	1657	3083	1683	2065
031	602	735	727	934	2492	2302	2115	1597
033	751	797	903	876	3219	2212	2327	2264
034	417	553	281	685	2875	2518	2778	2027
036	837	706	826	727	2257	1947	1563	1291
037	373	399	492	735	3317	2206	2630	1980
038	784	890	820	603	2186	2086	1897	2549
039	897	617	919	891	2145	3048	1661	1725
042	567	511	622	440	2893	3134	2332	3620
044	581	931	615	609	3059	1825	2574	1861
047	1168	651	344	793	2373	2698	2810	3362
049	555	601	986	730	3111	2872	2156	2793
050	607	699	723	645	2685	2676	2292	2558
051	513	1009	598	548	3176	2880	2862	2616
052	499	1785	1025	733	3479	2416	2152	3262
053	799	785	925	1076	2783	2721	2796	2296
054	1019	613	608	1733	2292	3216	2253	1606
055	378	474	400	481	4295	3739	3589	4052
056	534	447	403	403	3281	3306	3593	4667
058	465	759	894	826	2820	1994	2000	2949
059	1752	801	775	1286	1094	2379	3023	2012
060	463	390	596	809	4338	3900	2431	3124

5.7.3 Creatinine Summary

The determination of creatinine via UV spectroscopy was performed as this was a fast, convenient and cost-efficient way to get data for the normalization of the metabolic results. However, the determined creatinine values were not further utilized for the metabolic evaluation as the normalization by weight is more precise and no data in the weight documentation of the different urine fractions was missing.

6 Conclusion

A diet-controlled clinical smoking cessation study was successfully conducted including compliance monitoring by analysis of CO_{ex} utilizing a CO analyser as well as salivary and urinary cotinine via LC-MS/MS. 39 out of 60 participants were compliant over the course of 3 months of smoking abstinence. During the clinical study, 3 types of body fluids (plasma, saliva and urine) were collected for metabolomic investigations using a validated untargeted GC-TOF-MS method. Only samples of subjects being compliant over the whole course were taken into account for the metabolic analysis. Significant changes after smoking cessation across all three matrices and for each time span of cessation (1 week, 1 month, and 3 months) were found. Overall, 64 metabolites (excluding nicotine and its metabolites) showing significant alterations in levels after cessation were identified. The majority of altered metabolites were observed in plasma (33 metabolites), followed by 24 in saliva and 16 in urine. Changes were observed to mainly occur in the fatty acid and amino acid metabolism. Especially the tryptophan metabolism appeared to be affected by smoking cessation, which is reflected in increased kynurenine metabolite levels and a decrease in precursors of the serotonin biosynthesis. The fatty acid metabolism also appeared to be significantly affected by smoking cessation, as more than 10% of the identified metabolites could be linked to this pathway.

These classes of compounds may reveal suitable biomarkers for smoking-induced biological effects. Therefore, two new quantitative, multi-analyte methods for fatty acids and amino acids were developed and validated. Furthermore, eicosanoids were determined by making use of a validated method of Sterz et al. for urinary eicosanoids by means of UPLC-MS/MS. [53]

The fatty acid method, which was newly developed and validated for this PhD project, is characterized by baseline separation of positional and geometrical isomers of a wide range of methylated FAs (FA 4.0 to FA 32:0). The uncommon application of GC-TOF-MS for the analysis of FAs revealed sufficient performance and showed that TOF detection is an interesting alternative for the analysis of methylated FAs. The use of two qualifiers for the identification of the analytes was successful and due to the use of TOF technique possible without loss of sensitivity. This is a great advantage as the EI fragmentation of FAs is very similar and the most sensitive fragments are the same among the groups of FAs (MUFAs, PUFAs, SAFAs). Consequently, the most sensitive fragments were utilized for quantification of the FAs and verified by two specific fragments. For the simultaneous quantification of 44 individual FAs a good separation of analytes was necessary which was achieved after testing several columns and developing a specific temperature programme over 35 min on a highly polar cyanopropyl column. Due to these features, the method could serve

as a powerful tool for investigating alterations in the FA profile during clinical studies applicable to various areas of research.

Application of the GC-TOF-MS method for FAs to samples of the smoking cessation study revealed significant alterations in the FA profile after smoking cessation, approaching the FA pattern of non-smokers. SFAs and PUFAs were mainly found to increase, while MUFAs decreased after smoking cessation. These results are in accordance with the untargeted fingerprinting analysis within this project, literature reports and previous studies conducted by Mueller et al. at ABF. [76, 86, 200, 271]

The AA LC-MS/MS method, which was newly developed and validated during this PhD project is characterized by the quick and easy sample preparation. This was achieved by using FMOC derivatization a known robust derivatization for AAs. Further, just a protein precipitation and a concentration step were necessary during sample clean-up leading to a very fast and easy preparation in the context of AA analysis. The number of internal standards covering 19 of 26 analytes with an authentic ^{13}C, ^{15}N and D labelled reverence material is very high but usually very cost intensive. Here, it was possible to use an algae produced mixture of defined composition offering the advantage to have 19 internal standards at the price of a single one. Furthermore, a specific composition of AAs was implemented for the specific sake of smoking cessation relevant observations and verification of alterations observed in the fingerprinting. Due to specific MS/MS fragmentation patterns, only isobaric analytes had to be separated clearly which was achieved by utilizing an RP-UPLC column and developing a specific gradient programme. The validation of the method showed the robustness and accurate reproducibility of the achievable results.

Application of the amino acid LC-MS/MS method to samples derived from the smoking cessation study and the previous study comparing S and NS revealed statistically significant alterations in the AA metabolism. While ADMA, Arg and Met were found to be increased in S compared to NS, Trp, Kyn and Lys were found to increase after cessation and Gly and Ser to decrease. The findings of an altered AA metabolism, in particular the Trp metabolism determined by untargeted metabolomics fingerprinting could be confirmed.

The targeted analysis of eicosanoids was triggered by the finding of an altered arachidonic acid metabolism, which was observed in the untargeted fingerprinting analysis and confirmed in the targeted FA analysis. Eicosanoid analysis was conducted with an already existing and validated method. [53] Statistically significant alterations upon smoking cessation were observed in the concentrations of 4 eicosanoids, namely 2,3-d-TXB_2, $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$ and LTE_4. The decrease in the levels of the 4 analytes found in this study are in accordance with decreased levels reported

for non-smokers [53, 298, 309, 310] and indicate a partial recovery of eicosanoid levels after smoking cessation, which is mainly driven by the reduction of oxidative stress and inflammatory effects after quitting. Interestingly, decreases became already significant after 1 week of cessation, indicating a rather fast response upon quitting especially for LTE_4 and 2,3-d-TXB_2. This is of special interest as there is a lack of biomarkers of effect for smoking cessation responding rather fast upon cessation.

In conclusion, the described untargeted fingerprinting investigations revealed several alterations in the metabolome of persons who quit smoking. The observed results indicate that many metabolic alterations evoked by smoking are reversible in a relatively short time span of 3 months after cessation. The changes in metabolite levels identified in the untargeted analysis could be confirmed by targeted methods.

7 Perspective

This study contributed to the field tobacco research with focus on the impact of smoking cessation on metabolic pathways. The field of tobacco research is important as there are several health risk factors related to smoking. This is of special emphasis as the worldwide prevalence of smoking incorporates a fifth of human population.

There are several questions which could be addressed by making use of the biofluid samples collected in this smoking cessation study as well as the samples from the previous study comparing smokers and non-smokers. First, the analytes identified in the fingerprinting approach to change upon smoking cessation and which have not yet been confirmed by targeted methods could be investigated by targeted methods. A promising approach could be the targeted analysis of energy metabolism related pathways. Second, other platforms for the untargeted metabolic fingerprinting analysis could be considered for analysis. LC-MS, in particular making use of high-resolution MS techniques, could widely extend the range of metabolites to be assessed by an untargeted approach. Finally, the area of analysis could be broadly enlarged by including proteomics, transcriptomics and other omics techniques.

Furthermore, an analytical method specifically designed for the determination of tryptophan metabolites – especially serotonin, which could not be determined due to limited sensitivity – would be of interest. Therefore, the developed AA method could be an appropriate starting point with an extended sample preparation. For instance, concentration steps possibly via ion exchange cartridges could be successful. Additionally, the use of ion trap technology could be beneficial to gain the necessary sensitivity, but goes along with limitations in the number of detectable analytes.

Another interesting point could be the improvement of sensitivity for the super long-chain FAs which were not determined in plasma. For this approach a specific method appears appropriate. The increase of initial heating rates and a shorter high temperature column could lead to satisfying results here, as the peak width of the analytes increased as a result of the long analysis time and achievement of the maximum temperature of the column selected due to the excellent separation characteristics for other purposes.

Both, the improvement of the AA and FA methods for the specific purposes were not developed due to limitations in time and accessibility of the analytical instruments.

As the market for e-cigarettes as a smoking cessation tool and/or a substitute for conventional cigarettes is currently strongly expanding, the health impact of these and other new generation

tobacco/nicotine products could be investigated by similar study designs. Therefore, a study comparing e-cigarette smokers, conventional cigarette smokers and non-smokers to each other would be of interest. Furthermore, a study of subjects who quit e-cigarette smoking and a study of subjects who switch from conventional cigarette smoking to e-cigarettes would expand the knowledge in this discipline. There are therefore still many aspects to be studied in the field of tobacco research that could be investigated and further research should be done. Results from these types of investigations could provide useful information in the public health area of tobacco harm reduction.

8 References

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Appendix A: Clinical Study

Table 37: Exemplary food completion of a stationary visit.

Breakfast	Weight[g]	kcal	Protein [g]	Carbohydrates [g]	Fat [g]
2 Buns	140	352.80	1.96	71.40	1.96
Becel	20	72.00	-	-	8.00
Boiled ham	20	26.90	3.58	0.16	1.40
Jam	20	21.50	2.86	0.13	0.24
Total breakfast		473.20	8.40	71.69	11.60
Lunch					
Stripe cut turkey	200	140.0	22.40	5.40	3.00
Tomato-sauce	50	40.00	0.50	1.30	3.40
Rice (raw-weight)	100	351.70	6.50	78.90	0.50
Salad	100	107.00	1.30	31.00	9.30
Dressing	100	150.00	10.00	40.00	6.00
Plum-cake	80	229.40	2.80	34.20	8.90
Total Lunch		1018.10	43.50	190.80	31.10
Dinner					
3 slices bread	120	265.2	7.32	55.20	1.20
Cold cuts	40	53.76	7.16	0.32	2.80
Cheese	20	70.80	4.80	-	5.10
Becel	20	72.00	-	-	8.00
Jam	25	59.00	0.30	14.40	0.30
Total Dinner		520.76	19.58	69.92	17.40
Total Day		2012.04	71.48	332.41	60.10
Total Day [%]		100	15.40	71.64	12.95

Appendix B: Alternative Evaluation of the Fingerprinting

Table 38: Alternative statistic with the Mann-Whitney-U-test for the plasma fingerprinting: comparison TP1 versus TP0.

Name	Sig.	Fold change TP1/TP0		RT	Standard	HMDB	Origin / Pathway
Morpholine	*	0.83	▼	5.717		✓	TSC
FA 6:0	*	0.81	▼	6.950		✓	TSC, fatty acid metabolism
3-Hydroxybutyric acid	**	0.56	▼	8.382	✓	✓	TSC, gluconeogenesis
Lysine	*	0.58	▼	15.440		✓	TSC, lysine metabolism
Glycerol 3-phosphate	*	1.35	▲	16.059			Glycolysis
1H-Indole-3-propionic acid	*	1.25	▲	18.829		✓	TSC, tryptophan metabolism
Uric acid	*	1.54	▲	19.285	✓	✓	TSC, purine metabolism
Kynurenine	**	1.45	▲	20.003		✓	Tryptophan metabolism
5-Hydroxytryptophan	*	0.88	▼	22.012		✓	Tryptophan metabolism

Table 39: Alternative statistic with the Mann-Whitney-U-test for the plasma fingerprinting: comparison TP2 versus TP0.

Name	Sig.	Fold change TP2/TP0		RT	Standard	HMDB	Origin / Pathway
2-Oxo-3-methylbutanoic acid	*	0.56	▼	7.462		✓	Valine metabolism
Cysteine	*	2.00	▲	13.639	✓	✓	TSC, cysteine metabolism
2,4-dihydroxy-2-(hydroxymethyl)Butyric acid	*	0.57	▼	14.783			
Pyrophosphate	*	0.60	▼	14.812		✓	Energy metabolism
Glycerol 3-phosphate	*	1.41	▲	16.059		✓	Glycolysis
Uric acid	*	1.28	▲	19.285	✓	✓	TSC, purine metabolism
FA 17:0	*	0.79	▼	19.567	✓	✓	TSC, fatty acid biosynthesis
Kynurenine	**	1.67	▲	20.003		✓	Tryptophan metabolism
FA18:1 (9Z)	*	0.60	▼	20.172	✓	✓	TSC, fatty acid metabolism
FA 20:4 5Z,8Z,11Z,14Z - Arachidonic acid	*	1.37	▲	21.401	✓	✓	TSC, fatty acid and eicosanoid metabolism
Pseudouridine	*	1.47	▲	21.511		✓	Pseudouridylation
Octadecenamide	*	0.45	▼	21.723		✓	Energy metabolism
FA 20:1	*	0.37	▼	21.841		✓	TSC, fatty acid metabolism
Lathosterol	**	2.86	▲	27.805		✓	Cholesterol metabolism

Table 40: Alternative statistic with the Mann-Whitney-U-test for the plasma fingerprinting: comparison TP3 versus TP0.

Name	Sig.	Fold change TP3/TP0		RT	Standard	HMDB	Origin / Pathway
Aminomalonic acid	*	1.75	▲	12.571		✓	Protein synthesis
Aspartic acid	**	0.61	▼	13.236	✓	✓	TSC, aspartic acid metabolism
α-Ketoglutaric acid	*	1.54	▲	13.665	✓	✓	TSC, Krebs cycle, glutamic acid metabolism
Threonic acid	**	0.38	▼	13.910		✓	ascorbate and aldarate metabolism
Glutamic acid	**	0.66	▼	14.390	✓	✓	TSC, glutamic acid metabolism
Glycerol 3-phosphate	*	1.61	▲	16.059		✓	Glycolysis
FA 14:0	*	0.63	▼	16.731		✓	TSC, fatty acid metabolism
FA 16:1	*	0.58	▼	18.390		✓	TSC, fatty acid metabolism
FA 16:0	***	0.65	▼	18.656	✓	✓	TSC, fatty acid metabolism
Uric acid	*	1.52	▲	19.285	✓	✓	TSC, purine metabolism
Kynurenine	***	1.61	▲	20.003		✓	Tryptophan metabolism
FA 18:2	**	0.55	▼	20.132		✓	TSC, fatty acid metabolism
FA 18:1	*	0.70	▼	20.209		✓	TSC, fatty acid metabolism
Tryptophan	*	2.38	▲	20.240	✓	✓	TSC, Tryptophan metabolism
Adenosine monophosphate	*	0.68	▼	26.736		✓	TSC, nucleoside metabolism
Cholesterol	*	1.82	▲	27.336	✓	✓	TSC, Cholesterol

Table 41: Alternative statistic with the Mann-Whitney-U-test for the saliva fingerprinting: comparison TP1 versus TP0.

Name	Sig.	Fold change TP1/TP0		RT	Standard	HMDB	Origin / Pathway
FA 8:0	*	2.86	▲	9.733	✓	✓	TSC, fatty acid metabolism
β-Alanine	*	1.89	▲	12.112		✓	TSC, beta-Alanine metabolism
Aminomalonic acid	*	1.69	▲	12.552		✓	Protein synthesis
Tyrosol	*	4.76	▲	13.700		✓	TSC, nutrition
α-Hydroxyglutaric acid	*	1.67	▲	13.899		✓	
Dihydroxyacetone phosphate	*	1.79	▲	15.713		✓	TSC, lipid metabolism and glycolysis
Desaminotyrosine	*	2.00	▲	15.736		✓	TSC, tyrosine metabolism
Phosphorylethanolamine	*	1.96	▲	16.220		✓	TSC, phospholipid metabolism
Tyrosine	*	1.96	▲	16.994	✓	✓	TSC, tyrosine metabolism
Urocanic acid	**	1.75	▲	17.959		✓	Histidine metabolism
Xanthine	*	2.33	▲	18.259		✓	TSC, purine metabolism
FA 17:0	*	2.86	▲	19.567	✓	✓	TSC, fatty acid metabolism
Ribulose 5-phosphate	*	1.69	▲	19.708		✓	Pentose phosphate pathway
FA 20:4 5Z,8Z,11Z,14Z - Arachidonic acid	***	0.12	▼	21.395	✓	✓	TSC, fatty acid and eicosanoid metabolism
Octadecenamide	*	2.08	▲	21.723		✓	Energy metabolism

Table 42: Alternative statistic with the Mann-Whitney-U-test for the saliva fingerprinting: comparison TP2 versus TP0.

Name	Sig.	Fold change TP2/TP0		RT	Standard	HMDB	Origin / Pathway
2-Hydroxyisocaproic acid	*	0.57	▼	9.506		✓	TSC
β-Alanine	*	2.00	▲	12.112		✓	TSC, beta-Alanine metabolism
L-Threo-2-pentulose	*	3.03	▲	15.327		✓	Energy metabolism
Dihydroxyacetone phosphate	*	2.33	▲	15.713		✓	TSC, lipid metabolism and glycolysis
Phosphorylethanolamine	*	2.33	▲	16.220		✓	TSC, phospholipid metabolism
Tyrosine	*	2.00	▲	16.994	✓	✓	TSC, tyrosine metabolism
FA 17:0	*	2.38	▲	19.559	✓	✓	TSC, fatty acid metabolism
Ribulose 5-phosphate	*	2.44	▲	19.708		✓	Pentose phosphate pathway
FA 20:4 5Z,8Z,11Z,14Z - Arachidonic acid	***	0.14	▼	21.395	✓	✓	TSC, fatty acid and eicosanoid metabolism
Pseudouridine	*	2.13	▲	21.506		✓	Pseudouridylation
Octadecenamide	*	2.13	▲	21.723		✓	Energy metabolism
FA 20:1	**	0.58	▼	21.890		✓	TSC, fatty acid metabolism
Guanosine	*	0.50	▼	24.729		✓	Nucleoside metabolism

Table 43: Alternative statistic with the Mann-Whitney-U-test for the saliva fingerprinting: comparison TP3 versus TP0.

Name	Sig.	Fold change TP3/TP0		RT	Standard	HMDB	Origin /Pathway
Propylene glycol	**	1.20	▲	6.125	✓	✓	TSC
2-Hydroxyisocaproic acid	*	0.47	▼	9.506		✓	TSC
FA 8:0	*	2.08	▲	9.733	✓	✓	TSC, fatty acid metabolism
Dihydroxyacetone phosphate	*	2.27	▲	15.713		✓	TSC, lipid metabolism and glycolysis
Phosphorylethanolamine	*	2.63	▲	16.220		✓	TSC, phospholipid metabolism
Glycyl-glycine	*	0.57	▼	16.568	✓	✓	Peptide synthesis
Cadaverine	*	0.52	▼	16.969		✓	TSC, lysine metabolism
Urocanic acid	**	1.85	▲	17.961		✓	Histidine metabolism
N-Acetylglucosamine	*	4.55	▲	19.324		✓	Amino sugar metabolism
FA 17:0	*	0.56	▼	19.560	✓	✓	TSC, fatty acid metabolism
FA 20:4 5Z,8Z,11Z,14Z - Arachidonic acid	***	0.18	▼	21.395	✓	✓	TSC, fatty acid and eicosanoid metabolism
Guanosine	*	0.59	▼	24.729		✓	Nucleoside metabolism

Table 44: Alternative statistic with the Mann-Whitney-U-test for the urine fingerprinting: comparison TP1 versus TP0.

Name	Sig.	Fold change TP1/TP0		RT	Standard	HMDB	Origin / Pathway
Aminomalonic acid	*	0.74	▼	12.555		✓	Protein synthesis
Caffeine	**	0.69	▼	15.999		✓	TSC, nutrition
Kynurenic acid	*	1.30	▲	18.671		✓	TSC, tryptophan metabolism
3-Hydroxysebacic acid	**	0.38	▼	18.929		✓	

Table 45: Alternative statistic with the Mann-Whitney-U-test for the urine fingerprinting: comparison TP2 versus TP0.

Name	Sig.	Fold change TP2/TP0		RT	Standard	HMDB	Origin / Pathway
3-Hydroxy-3-Methylbutanoic acid	*	1.35	▲	9.096		✓	TSC, leucine metabolism
Acetylglycine	**	0.41	▼	11.067		✓	Protein metabolism
iso-Serine	*	1.82	▲	11.429			
Theobromine	**	2.94	▲	16.102		✓	TSC, nutrition
3-(4-Hydroxy-3-methoxyphenyl)propionic acid	**	0.58	▼	17.092			TSC, nutrition
Kynurenic acid	*	1.64	▲	18.671		✓	TSC, tryptophan metabolism
hydroxyprolyl-Hydroxyproline	*	1.19	▲	22.166		✓	Protein catabolism

Table 46: Alternative statistic with the Mann-Whitney-U-test for the urine fingerprinting: comparison TP3 versus TP0.

Name	Sig.	Fold change TP3/TP0		RT	Standard	HMDB	Origin / Pathway
3-hydroxy-3-methylbutanoic acid	*	1.52	▲	9.096		✓	TSC, leucine metabolism
N-hydroxycarbamic acid	*	0.61	▼	9.602			
FA 8:0	*	0.65	▼	9.730	✓	✓	TSC, fatty acid metabolism
Acetylglycine	*	0.56	▼	11.067		✓	Protein synthesis, amino acid synthesis
1,2,4-Butanetriol	*	2.63	▲	11.383			TSC
Iso serine	*	2.13	▲	11.429			
Caffeine	***	3.33	▲	15.992		✓	TSC, nutrition
Theobromine	**	2.22	▲	16.113		✓	TSC, nutrition
Kynurenic acid	***	2.08	▲	18.669		✓	TSC, tryptophan metabolism
Cystathionine	*	0.47	▼	19.065		✓	Cysteine metabolism
3-Hydroxyhippuric acid	**	0.35	▼	19.354		✓	Fatty acid metabolism
5-Hydroxyindoleacetic acid	**	0.48	▼	20.067		✓	Serotonin metabolism
Hydroxyprolyl-Hydroxyproline	*	2.17	▲	22.153		✓	Protein catabolism

Appendix C: Fingerprinting QC Charts

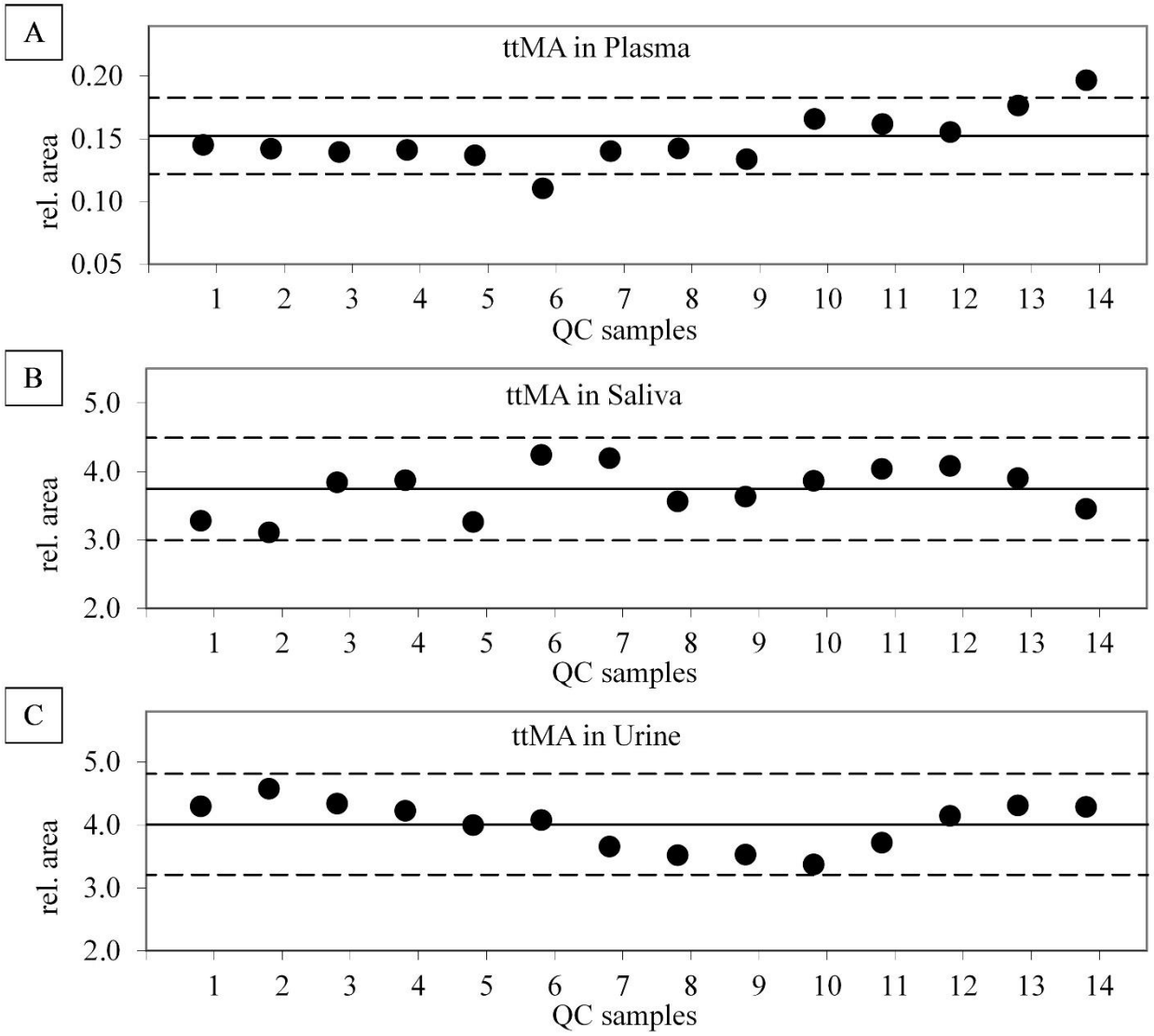

Figure 16: QC-sample charts of trans,trans-muconic acid (ttMA) in plasma (A), saliva (B) and urine (C). QC sample concentrations given as dots (N=14); mean value given as solid line; ±20% difference of mean given as dotted lines.

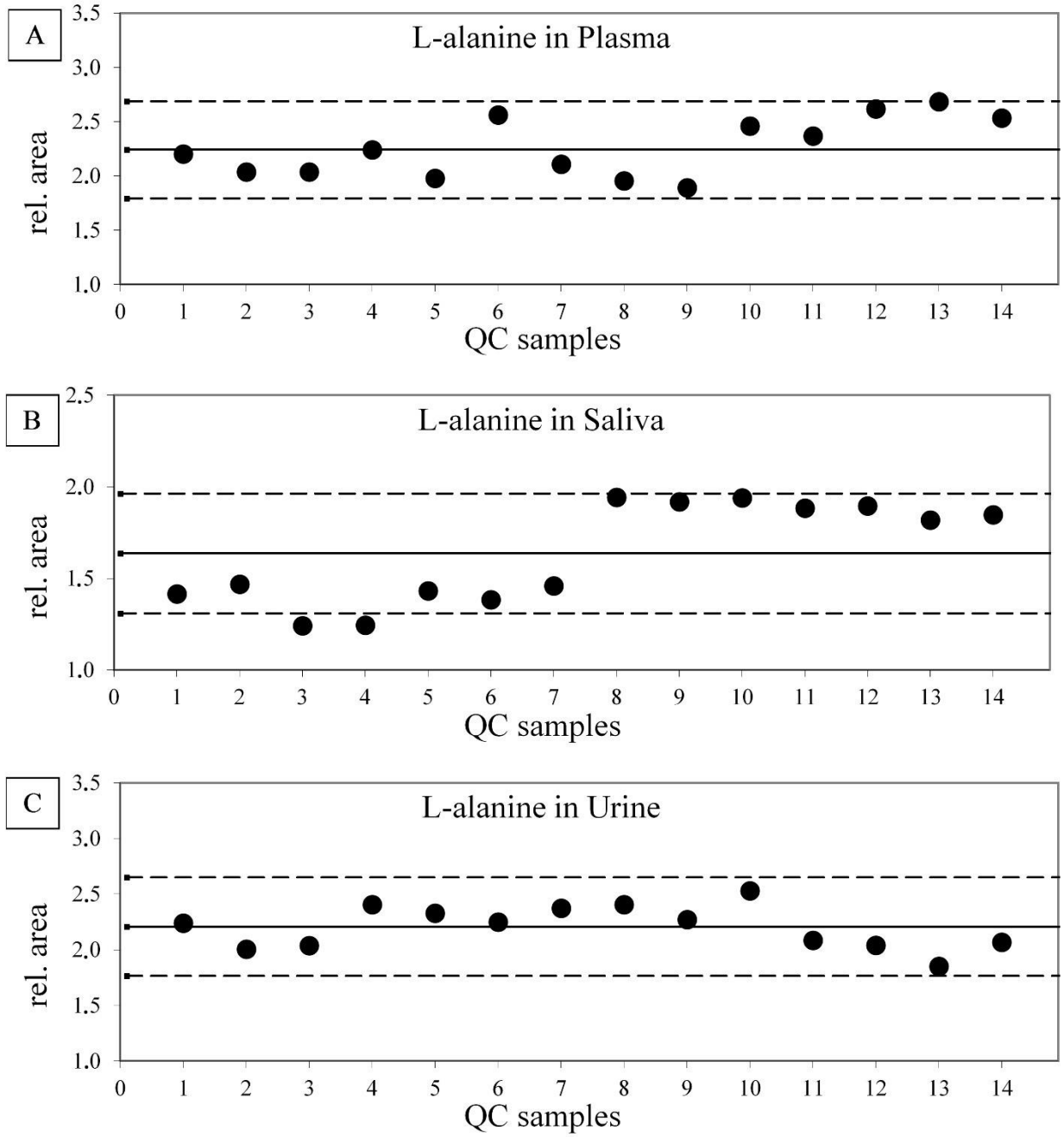

Figure 17: QC-sample charts of L-alanine in plasma (A), saliva (B) and urine (C). Showing the QC samples (N=14), the mean value (line) and ±20% target range (dotted lines).

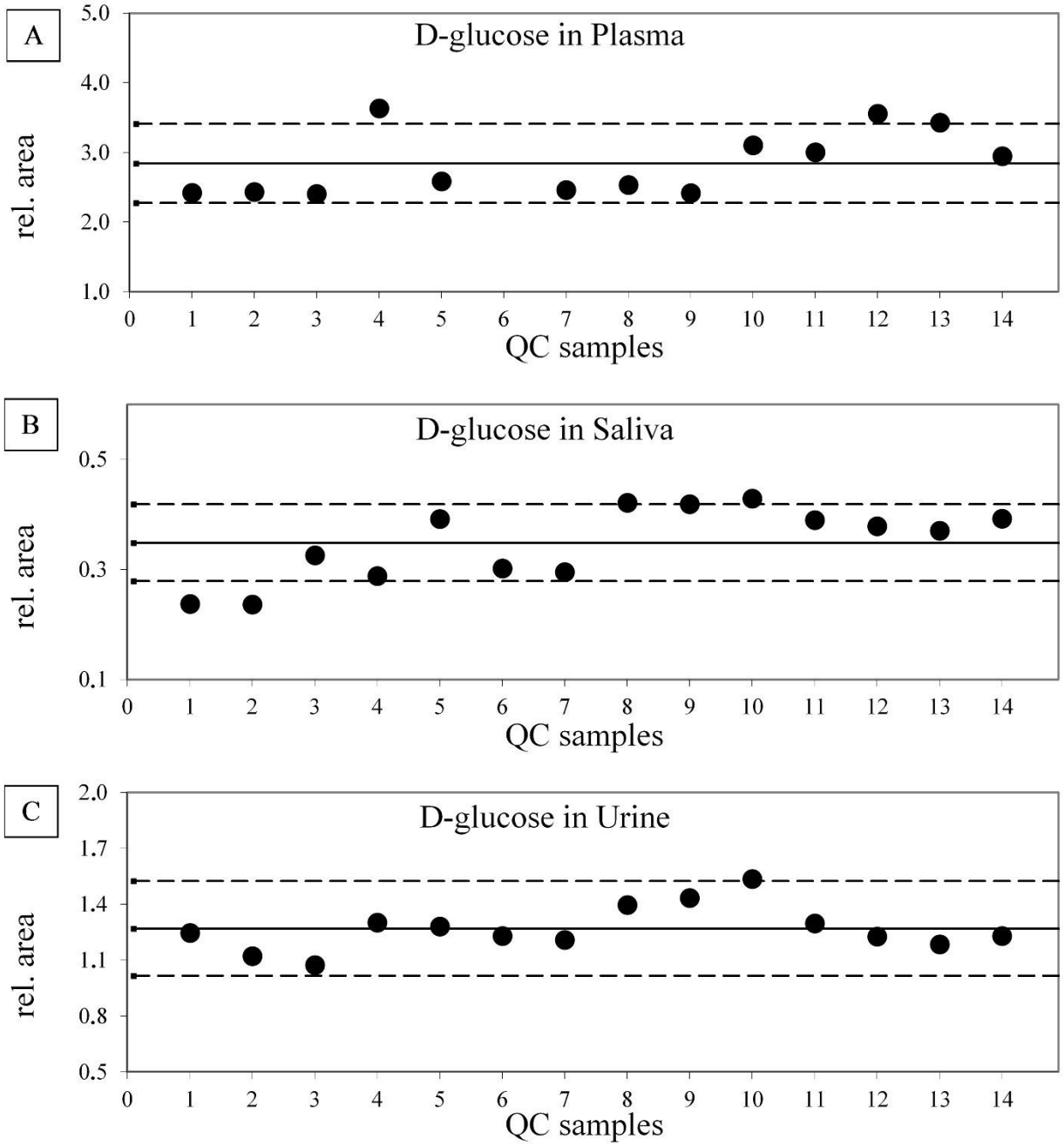

Figure 18: QC-sample charts of D-glucose in plasma (A), saliva (B) and urine (C). Showing the QC samples (N=14), the mean value (line) and ±20% target range (dotted lines).

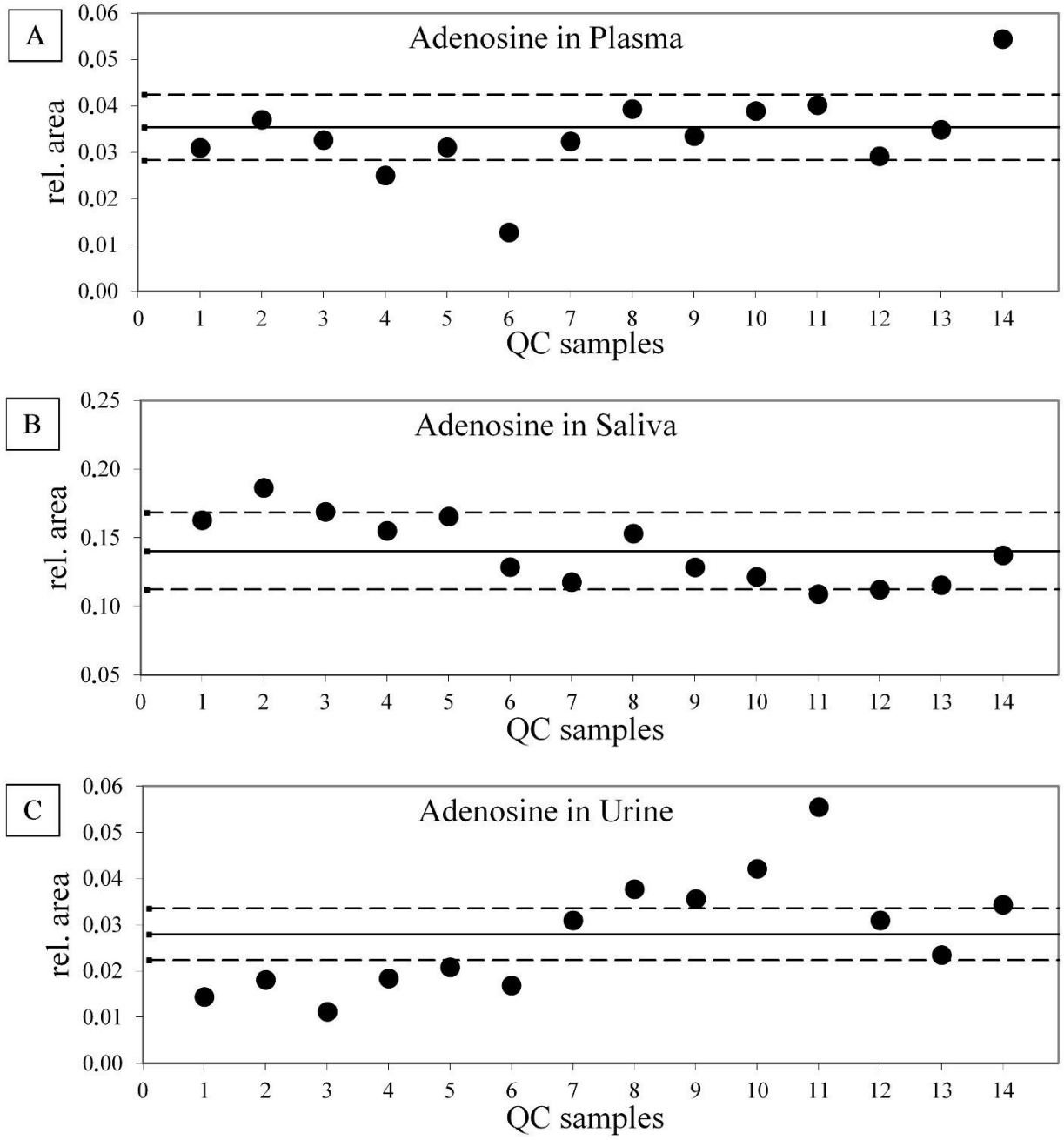

Figure 19: QC-sample charts of adenosine in plasma (A), saliva (B) and urine (C). Showing the QC samples (N=14), the mean value (line) and ±20% target range (dotted lines).

Appendix D: Curriculum Vitae

Personal Data

Name, First Name	Göttel, Michael Christoph
Date of Birth / Place of Birth	08.07.1986 Tettnang, Germany
Personal Status	married
Nationality	German

Ph.D. Studies

Oct. 2014	–	today	Dissertation ABF GmbH, Munich, Germany TU Munich, Chair of Analytical Chemistry Title: *Analytical Studies on the Impact of Smoking Cessation on the Metabolic Profile.*
		Core Areas:	Instrumental Analysis, Method Development, LC-MS/MS, GC-TOF-MS, Method Validation, SOP-Creation, Untargeted Metabolomic Fingerprinting, Supervision of a Clinical Human Study for Smoking Cessation, Compliance Verification, Writing of Scientific Papers

Final Theses

Mar. 2014	–	Aug. 2014	Master Thesis Victoria University of Wellington, Wellington, New Zealand *Extraction, Purification, and Identification of Bioactive Compounds from the New Zealand Marine Sponge Haliclona sp.* PROMOS Scholarship of the DAAD
		Core Areas:	Instrumental Analysis, LC-MS/MS (high resolution), 1D/2D-NMR-Spectroscopy, Preparative Chromatography
Sept. 2012	–	Feb. 2013	Bachelor Thesis Institut für Seenforschung, Langenargen, Germany *Bestimmung ausgewählter endokriner Disruptoren in Oberflächengewässern mittels Gaschromatographie-Massenspektrometrie (GC-MS)*
		Core Areas:	Instrumental Analysis, GC-MS (SBSE), Method Development

Studies

Oct. 2014	–	today	Doctoral Student at Analytisch-Biologisches Forschungslabor GmbH (ABF) in Munich, Germany and at Technischen Universität München at the Chair of Analytical Chemistry (of Prof. Dr. Niessner)
Mar. 2013	–	Sept. 2014	Technische Hochschule Nürnberg Georg Simon Ohm, Germany Course of Studies: Master Applied Chemistry Specialization: Biochemistry Degree: Master of Science
Sept. 2009	–	Feb. 2013	Naturwissenschaftlich-Technische Akademie (nta), Isny im Allgäu, Germany Course of Studies: Bachelor Pharmaceutical Chemistry Degree: Bachelor of Science
			Universität Konstanz, Germany
Oct. 2008	–	Sept. 2009	Course of Studies: Lehramt Chemie, Mathematik
Oct. 2007	–	Sept. 2008	Course of Studies: Bachelor Chemie

Civilian Service

Oct. 2006	–	June 2007	Stiftung Liebenau, Germany

School Education

Sept. 2003	–	Aug. 2006	Edith-Stein-Schule Ravensburg, Germany Ernährungswissenschaftliches Gymnasium Degree: General Qualification for University Entrance
Sept.1997	–	July 2003	Realschule Bodnegg, Germany Degree: General Certificate of Secondary Education
Aug. 1993	–	July 1997	Primary School Grünkraut, Germany

Volunteer Activities

2003	–	2009	Participation in the non-profit association „Weltenwanderer e.V.“
2005	–	2006	Member of the association’s council.
2003	–	2005	Team leader of the „Jungschar Atzenweiler“