The effect of acute and chronic physical activity on metabolite profiles in humans

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“To practice without theory is to sail an uncharted sea;

theory without practice is not to set sail at all.”

(Mervyn Susser, 1968)

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Summary

Next to diet, tobacco use, and alcohol consumption, physical activity belongs to the major modifiable lifestyle factors that influence human health. While insufficient physical activity increases the risk for many chronic diseases, regular physical activity is effective in preventing cardiovascular diseases or metabolic disorders, such as obesity or type 2 diabetes. However, the biological mechanisms involved in the body's adaptation to acute and chronic physical activity are still insufficiently understood. The emerging field of metabolomics represents a promising approach to systematically analyze exercise-related changes in human metabolism, hinting at metabolic pathways that are associated with the performance- or health-enhancing effects of physical activity. Although recent studies have supported the use of metabolomics for discovering biomarkers of an individual's training or fitness status, they show substantial heterogeneity in terms of study designs, study populations, sample types, or applied analytical techniques. Consequently, direct comparisons between findings and the generalizability of results in the young research discipline of exercise metabolomics are currently still impeded.

Intending to address several research gaps and to extend the current state of knowledge, the aim of this cumulative dissertation is to comprehensively investigate the effect of acute and chronic physical activity on metabolite profiles in humans. To reach this aim, metabolomics data from three exercise- or fitness-related (sub-)studies (termed *Study I*, *IIa*/*IIb*) were analyzed. By covering different aspects of physical activity, these studies not only allowed to examine alterations in the metabolome in response to high-intensity interval training (*Study I*) or acute incremental exercise (*Study IIa*), but also to obtain new insights into the relationship between metabolite profiles and the cardiorespiratory fitness as a measure of chronic physical activity (*Study IIa*/*IIb*). Depending on the particular study and available biospecimens, different metabolomics and statistical approaches were utilized. The present thesis includes the study-specific results that have been published in peer-reviewed journals and additionally comprises a functional classification of obtained exercise-/fitness-related metabolite profiles based on a manually conducted metabolite categorization and a web-based pathway analysis.

In the framework of the randomized controlled interventional *Study I*, the effects of a ten-day high-intensity interval training and a subsequent four-day recovery period on the resting urinary metabolome of young active men (n=18) were investigated. Fasting spot urine was

collected before (-1 day) and after (+1 day; +4 days) the training and 64 urinary metabolites were identified by nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectrometry. Despite the high intensity of the training protocol, no overall change in the resting urinary metabolome was observed in the experimental group (n=10). Yet, a significant decrease in urinary hypoxanthine excretion was documented one day after the training. Since hypoxanthine is related to purine degradation, its lower urinary levels may indicate a training-induced adaptation in 'purine metabolism'. To conclude, *Study I* provided the first evidence for urinary hypoxanthine as a possible marker of training adaptation. More research will be needed to examine underlying mechanisms and to prove the utility of hypoxanthine in urine to reflect an athlete's adaptation to training.

Based on *Study IIa*, which comprised the experimental exercise part within the cross-sectional "Karlsruhe Metabolomics and Nutrition" study, the effect of a standardized exercise tolerance test on the urinary metabolome of healthy women and men (n=255) was investigated. Moreover, the relationship between the cardiorespiratory fitness and either single urinary metabolites or metabolite patterns at rest or post-exercise was analyzed. The fitness status was assessed by measuring the peak oxygen uptake during incremental exercise. Spot urine was collected pre- and post-exercise and 47 urinary metabolites were identified by nuclear magnetic resonance spectroscopy. Although principal component analysis did not show a clear separation of the pre- and post-exercise urine samples based on the detected metabolite profile, univariate analysis revealed alterations in 35 metabolites (e.g., lactate, pyruvate, alanine, and acetate). Many of these metabolites belong to 'energy metabolism', 'carbohydrate metabolism', or 'amino acid metabolism'. Thus, *Study IIa* could confirm the ability of urine to capture exercise-induced metabolic alterations and clearly indicated the necessity to control for acute physical activity in urine metabolomics studies. Since only weak associations between the cardiorespiratory fitness and urinary metabolites were observed after adjusting for age, sex, menopausal status, and the lean body mass, *Study IIa* also pointed out the need to control for these confounders in urine metabolomics studies related to physical fitness.

In *Study IIb*, the relationship between the cardiorespiratory fitness and the resting plasma metabolome of healthy women and men of the "Karlsruhe Metabolomics and Nutrition" study (n=252) was systematically examined. Plasma samples were collected in the fasting state and analyzed by nuclear magnetic resonance spectroscopy and mass spectrometry coupled to

one- or two-dimensional gas chromatography or liquid chromatography. Based on this multi-platform metabolomics approach, 427 plasma analytes from various metabolic pathways and chemical classes were detected. Bi- and multivariate association analyses were conducted separately for sexes and adjusted for age and menopausal status (1st step) and further clinical or phenotypical variables (2nd step). In general, *Study IIb* revealed sex-specific associations between the cardiorespiratory fitness and mainly 'lipid metabolism'- or 'amino acid metabolism'-linked plasma metabolites, which, however, were partially influenced by the covariates. Interestingly, sex-specific explanation models for the cardiorespiratory fitness could be improved by including selected plasma analytes in addition to clinical and phenotypical variables. Briefly summarized, *Study IIb* not only proved well-known relationships between the physical fitness and risk factors for cardiometabolic diseases, such as the fat mass, the visceral adipose tissue mass, or blood triglycerides, in metabolically healthy individuals, but also provided evidence of sex-related differences in fitness-associated plasma metabolite patterns. As some fitness-associated metabolites have been inversely linked to the development of cardiometabolic diseases (e.g., phosphatidylcholines) or have been related to exercise-induced adaptations in 'energy metabolism' (e.g., malic acid, succinic acid), *Study IIb* supposed those metabolites to represent potential mediators or markers of the health- or performance-enhancing effects of chronic physical activity. Given the rather exploratory character of *Study IIb*, more research will be required to validate obtained findings and to clarify biological mechanisms underlying the sex-specific differences in fitness-associated metabolite patterns. Finally, *Study IIb* indicated that covariates like sex, age, menopausal status, and the body composition have to be considered if studying blood metabolic markers related to the cardiorespiratory fitness.

In conclusion, the present dissertation can be seen as a valuable contribution to the emerging field of exercise metabolomics. This thesis not only gains deeper knowledge on the effects of acute and chronic physical activity on metabolite profiles in healthy populations, but also provides suggestions for improving future metabolomics studies. Particular urinary or blood metabolites could be highlighted as potential markers for acute physical exercise, training adaptation, or physical fitness and certainly represent a useful starting point for validation studies and mechanistic investigations. As one piece of a big puzzle, the findings from this thesis – together with results from further exercise metabolomics research – might one day be translated into personalized approaches in a sport- or health-related context.

Zusammenfassung

Neben der Ernährung sowie dem Konsum von Tabak und Alkohol gehört körperliche Aktivität zu den wichtigsten modifizierbaren Faktoren des Lebensstils, die Einfluss auf die Gesundheit des Menschen haben. Während unzureichende körperliche Aktivität das Risiko für viele chronische Erkrankungen erhöht, wirkt sich regelmäßige körperliche Aktivität positiv auf die Entstehung von kardiovaskulären Krankheiten oder metabolischen Störungen wie Adipositas oder Typ-2-Diabetes aus. Jedoch sind die biologischen Mechanismen, die den akuten und chronischen Anpassungen des Körpers an Belastung zugrunde liegen, bisher noch nicht hinreichend verstanden. Das junge Forschungsfeld *Metabolomics* stellt einen vielversprechenden Ansatz dar, um belastungsinduzierte Veränderungen im menschlichen Stoffwechsel systematisch zu erforschen und dabei Hinweise auf Stoffwechselwege zu erhalten, welche mit den leistungs- oder gesundheitsfördernden Effekten von körperlicher Aktivität assoziiert sind. Obwohl jüngste Studien den Nutzen von *Metabolomics* für die Entdeckung von Biomarkern des Trainings- oder Fitnessstatus belegen, weisen diese bezüglich Studiendesign/-population, Probenmaterial oder angewandter analytischer Techniken ein hohes Maß an Heterogenität auf. Folglich sind direkte Vergleiche sowie die Generalisierbarkeit der Studienergebnisse in der jungen Forschungsdisziplin *Exercise Metabolomics* gegenwärtig noch erschwert.

Um mehrere Forschungslücken zu schließen und den bisherigen Kenntnisstand zu erweitern, besteht das Ziel dieser kumulativen Dissertation darin, den Einfluss von akuter und chronischer körperlicher Aktivität auf Metabolitenprofile beim Menschen umfassend zu untersuchen. Hierfür wurden *Metabolomics*-Daten dreier belastungs- bzw. fitness-bezogener (Sub-)Studien (bezeichnet als *Studie I*, *IIa*/*IIb*) analysiert. Da diese Studien verschiedene Aspekte der körperlichen Aktivität abdeckten, konnten nicht nur Veränderungen im humanen Metabolom als Antwort auf hochintensives Intervalltraining (*Studie I*) oder akute körperliche Belastung mit ansteigender Intensität (*Studie IIa*) untersucht werden, sondern auch neue Erkenntnisse zum Zusammenhang zwischen Metabolitenprofilen und der kardiorespiratorischen Fitness als Marker für chronische körperliche Aktivität (*Studie IIa*/*IIb*) gewonnen werden. Je nach Studie sowie zur Verfügung stehender biologischer Proben wurden spezielle *Metabolomics*-Ansätze und statistische Verfahren angewandt. Die vorliegende Thesis beinhaltet die studienspezifischen Ergebnisse, welche in Peer-Review-Zeitschriften publiziert

wurden, und enthält überdies eine funktionelle Einordnung der belastungs- bzw. fitness-assoziierten Metabolitenprofile, basierend auf einer manuell durchgeführten Metaboliten-klassifizierung und einer web-basierten *Pathway*-Analyse.

Im Rahmen einer randomisierten kontrollierten Interventionsstudie (*Studie I*) wurde der Einfluss eines zehntägigen hochintensiven Intervalltrainings und einer viertägigen Regenerationsphase auf das Ruhe-Urinmetabolom junger, körperlich aktiver Männer (n=18) untersucht. Hierfür wurden Spontanurinproben im nüchternen Zustand vor (-1 Tag) und nach (+1 Tag; +4 Tage) dem Training gesammelt und 64 Urinmetaboliten mittels Kernspinresonanz-spektroskopie und Flüssigchromatographie mit Massenspektrometrie-Kopplung identifiziert. Trotz der hohen Intensität des Trainingsprotokolls wurden keine generellen Änderungen des Ruhe-Urinmetaboloms in der Experimentalgruppe (n=10) beobachtet. Allerdings wurde ein Tag nach Beendigung des Trainings eine signifikante Abnahme der Hypoxanthinexkretion im Urin dokumentiert. Da Hypoxanthin mit dem Purinabbau in Zusammenhang steht, könnte die verringerte Urinexkretion auf eine trainingsinduzierte Anpassung im ‘Purin-Stoffwechsel‘ hindeuten. Folglich lieferte *Studie I* erste Anhaltspunkte für Hypoxanthin im Urin als möglichen Marker einer Trainingsadaptation. Weitere Studien sind notwendig, um zugrunde liegende Mechanismen aufzuklären und den Nutzen von Hypoxanthin im Urin für die Beurteilung der Trainingsanpassung bei Athleten nachzuweisen.

Basierend auf *Studie IIa*, welche den experimentellen Belastungspart innerhalb der „*Karlsruhe Metabolomics and Nutrition*”-Querschnittsstudie umfasste, wurde der Einfluss eines standardisierten Belastungstoleranztests auf das Urinmetabolom gesunder Frauen und Männer (n=255) untersucht. Überdies wurde der Zusammenhang zwischen der kardio-respiratorischen Fitness und einzelnen Urinmetaboliten oder Urinmetabolitenmustern in Ruhe bzw. nach Belastung analysiert. Der Fitnessstatus wurde durch die Messung der maximal erreichten Sauerstoffaufnahme während des Belastungstests erfasst. Die Spontanurinproben wurden vor und nach Belastung gesammelt und 47 Urinmetaboliten wurden mittels Kernspin-resonanzspektroskopie identifiziert. Obwohl eine Hauptkomponentenanalyse basierend auf dem erfassten Metabolitenprofil keine klare Trennung der Urinproben vor und nach Belastung zeigte, ließen univariate Vergleiche Veränderungen in 35 Metaboliten (z.B. Laktat, Pyruvat, Alanin und Acetat) erkennen. Viele dieser Metaboliten gehören zum ‘Energiestoffwechsel‘, ‘Kohlenhydratstoffwechsel‘ bzw. ‘Aminosäurenstoffwechsel‘. Somit konnte *Studie IIa*

bestätigen, dass Urin für die Erfassung belastungsinduzierter metabolischer Veränderungen geeignet ist. Zudem wurde auf die Notwendigkeit hingewiesen, in zukünftigen Urin-*Metabolomics*-Studien bezüglich akuter körperlicher Aktivität zu kontrollieren. Da zwischen der kardiorespiratorischen Fitness und den Urinmetaboliten lediglich schwache Assoziationen beobachtet wurden, nachdem bezüglich Alter, Geschlecht, Menopausestatus und der fettfreien Körpermasse adjustiert wurde, wies *Studie IIa* zusätzlich darauf hin, dass in zukünftigen Urin-*Metabolomics*-Studien mit Fitnessbezug für diese Kovariaten kontrolliert werden sollte.

In *Studie IIb* wurde der Zusammenhang zwischen der kardiorespiratorischen Fitness und dem Ruhe-Plasmametabolom gesunder Frauen und Männer (n=252) der "*Karlsruhe Metabolomics and Nutrition*"-Studie systematisch untersucht. Die Blutproben wurden im nüchternen Zustand entnommen und mittels Kernspinresonanzspektroskopie und Massenspektrometrie, gekoppelt mit ein- oder zweidimensionaler Gaschromatographie bzw. Flüssigchromatographie, analysiert. Basierend auf diesem Multiplattform-*Metabolomics*-Ansatz wurden 427 Plasmaanalyten aus verschiedenen Stoffwechselwegen und chemischen Klassen detektiert. Bi- und multivariate Assoziationsanalysen wurden geschlechtergetrennt durchgeführt und für Alter und Menopausestatus (1. Schritt) sowie weitere klinische oder phänotypische Variablen (2. Schritt) adjustiert. Insgesamt zeigte *Studie IIb* geschlechterspezifische Zusammenhänge zwischen der kardiorespiratorischen Fitness und v.a. 'Fettstoffwechsel'- bzw. 'Aminosäurenstoffwechsel'-assoziierten Plasmametaboliten, die jedoch teilweise durch die Kovariaten beeinflusst wurden. Interessanterweise konnten geschlechterspezifische Erklärungsmodelle für die kardiorespiratorische Fitness verbessert werden, wenn zusätzlich zu klinischen und phänotypischen Variablen ausgewählte Plasmaanalyten berücksichtigt wurden. Zusammengefasst bestätigte *Studie IIb* nicht nur bekannte Zusammenhänge zwischen der körperlichen Fitness und Risikofaktoren für kardiometabolische Erkrankungen wie z.B. der Fettmasse, dem viszeralen Fettgewebe oder Blut-Triglyceriden in metabolisch gesunden Personen, sondern wies überdies geschlechterbezogene Unterschiede in fitness-assoziierten Plasmametabolitenmustern nach. Da einige fitness-assoziierte Metaboliten bereits einen inversen Zusammenhang mit der Entstehung kardiometabolischer Krankheiten zeigten (z.B. Phosphatidylcholine) bzw. mit belastungsinduzierten Anpassungen im 'Energiestoffwechsel' in Verbindung gebracht wurden (z.B. Malat, Succinat), lieferte *Studie IIb* Hinweise darauf, dass diese Metaboliten potentielle Mediatoren bzw. Marker der gesundheits- oder leistungsfördernden

Effekte von chronischer körperlicher Aktivität darstellen könnten. Angesichts des explorativen Charakters von *Studie IIb* wird zukünftig weitere Forschung benötigt, um die Ergebnisse zu validieren sowie biologische Mechanismen aufzuklären, welche den geschlechterspezifischen Unterschieden in den fitness-assoziierten Metabolitenmustern zugrunde liegen. *Studie IIb* wies außerdem darauf hin, dass Kovariaten wie z.B. Geschlecht, Alter, Menopausestatus und die Körperzusammensetzung berücksichtigt werden sollten, wenn metabolische Marker der kardiorespiratorischen Fitness im Blut untersucht werden.

Zusammenfassend kann die vorliegende Dissertation als wertvoller Beitrag zu dem neuen Forschungsgebiet *Exercise Metabolomics* gesehen werden. Diese Thesis liefert nicht nur vertiefte Erkenntnisse zum Einfluss von akuter sowie chronischer körperlicher Aktivität auf Metabolitenprofile in gesunden Populationen, sondern stellt zugleich Empfehlungen für die Verbesserung zukünftiger *Metabolomics*-Studien bereit. Bestimmte Metaboliten in Urin bzw. Blut konnten als potentielle Marker für akute körperliche Belastung, Trainingsadaptation oder körperliche Fitness hervorgehoben werden und stellen sicherlich einen nützlichen Ausgangspunkt für Validierungsstudien und mechanistische Untersuchungen dar. Als Teil eines großen Puzzles könnten die Ergebnisse dieser Dissertation – zusammen mit Erkenntnissen weiterer *Exercise Metabolomics*-Studien – eines Tages möglicherweise in personalisierte Ansätze im Sport- oder Gesundheitskontext übertragen werden.

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List of Abbreviations

AA	amino acid
ADMA	asymmetric dimethylarginine
ADP	adenosine diphosphate
AEE	activity energy expenditure
ATP	adenosine triphosphate
BCAA	branched-chain amino acid
BIC	Bayesian information criterion
BMC	bone mineral content
BMI	body mass index
BP	blood pressure
CE	capillary electrophoresis
CG	control group
CI	confidence interval
CRF	cardiorespiratory fitness
DXA	dual-energy X-ray absorptiometry
EG	experimental group
FC	fold change
FDR	false discovery rate
FEV1	forced expiratory pressure in one second
FIA	flow injection analysis
FM	fat mass
GC	gas chromatography
GC × GC	two-dimensional gas chromatography
^{1}H	hydrogen-1
Hb	hemoglobin
HDL	high-density lipoprotein
HEI(-NVS)	Healthy Eating Index (modified version)
HGPRT	hypoxanthine guanine phosphoribosyltransferase
HIIT	high-intensity interval training
HMDB	Human Metabolome Database
HR	heart rate

HR_{max}	maximum heart rate
HR_{rest}	resting heart rate
IMP	inosine monophosphate
IPAQ	International Physical Activity Questionnaire
KarMeN	Karlsruhe Metabolomics and Nutrition
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBM	lean body mass
LC	liquid chromatography
LCFA	long-chain fatty acid
LDL	low-density lipoprotein
LLOQ	lower limit of quantification
LysoPC	lyso-phosphatidylcholine
MDBF	*Mehrdimensionaler Befindlichkeitsfragebogen*
MET	metabolic equivalent of task
MRI	Max Rubner-Institut
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSI	Metabolomics Standards Initiative
MS/MS	tandem mass spectrometry
MyoKin	Myokine Kinetics
m/z	mass-to-charge ratio
NCDs	non-communicable diseases
NMR	nuclear magnetic resonance
NVS	*Nationale Verzehrsstudie*
ORA	over-representation analysis
p	*p*-value of context-dependent test
PA	physical activity
PC	principal component (only *Study IIa*); phosphatidylcholine (apart from *Study IIa*)
PCA	principal component analysis
PC aa	diacyl-phosphatidylcholine
PC ae	acyl-alkyl-phosphatidylcholine
PE	physical exercise

PF	physical fitness
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
P_{IAT}	power at the individual anaerobic threshold
PLS	partial least squares
P_{max}	maximal power
PWV	pulse wave velocity
q	quarter
QC	quality control
r	Pearson correlation coefficient
R^2	coefficient of determination
R^2 (adjusted)	adjusted coefficient of determination
RMSE	root mean square error
RPE	rating of perceived exertion
rpm	revolutions per minute
SD	standard deviation
SM	sphingomyelin
SOPs	standard operating procedures
TCA	tricarboxylic acid
TGs	triglycerides
TMAO	trimethylamine N-oxide
T_{max}	time to exhaustion at P_{max}
TRIMP	training impulse
TSP	trimethylsilylpropanoic acid
U	unknown
UPLC	ultra-performance liquid chromatography
V	visit
VATM	visceral adipose tissue mass
VC_{max}	maximal vital capacity
VdW	Van der Waerden
VO_2	oxygen uptake
VO_{2max}	maximal oxygen uptake
VO_{2peak}	peak oxygen uptake
WHO	World Health Organization

1 General Introduction

1.1 Preface

According to the World Health Organization (WHO), insufficient physical activity (PA) represents a major risk factor for global mortality and the development of chronic, non-communicable diseases (NCDs) (WHO, 2009). In fact, approximately 30% of ischemic heart disease burden, 27% of diabetes, and 21-25% of breast and colon cancer can be primarily attributed to physical inactivity (WHO, 2009). Recent estimates furthermore indicate that about 27.5% of the world's population do not meet the WHO global recommendations for PA (Guthold, Stevens, Riley, & Bull, 2018). However, the health benefits of regular PA are well-established. Regular PA does not only contribute to the prevention of important NCD risk factors, such as hypertension, overweight, and obesity (WHO, 2010), it can also delay the onset of dementia (Livingston et al., 2017) and has positive effects on mental health and the quality of life (Das & Horton, 2012; Mammen & Faulkner, 2013). Engaging in regular PA additionally improves the cardiorespiratory fitness (CRF) (McKinney et al., 2016), which has emerged as a strong and independent predictor of all-cause and disease-specific mortality (Harber et al., 2017). Actually, the CRF is inversely associated with the incidence of both cardiovascular and metabolic diseases (Kodama et al., 2009; LaMonte et al., 2005).

Since modern human society is confronted with a pandemic of chronic diseases and conditions characterized by metabolic dysregulation, including obesity, diabetes, or cancer (Newgard, 2017), NCDs represent a major challenge for public health systems in the 21st century (Alberti, 2001; WHO, 2020). Thus, primary prevention is needed at the global level. As an effective and relatively inexpensive strategy for promoting health, PA has gained relevance (WHO, 2019).

Regular PA is known to favorably affect the body composition as well as the skeletal muscle and whole-body physiology and metabolism (Gabriel & Zierath, 2017). However, cellular and molecular mechanisms underlying the exercise-related health improvements have not been fully elucidated until now (Zierath & Wallberg-Henriksson, 2015). Therefore, exercise biology research currently aims to obtain a better understanding of how the adaptive responses of the human organism to physical exercise (PE) are mediated and ultimately linked to the prevention of chronic diseases (Neufer et al., 2015). With regard to the future, it can be

expected that the knowledge gained will allow scientists and health professionals to develop more personalized exercise recommendations and to identify people at risk for cardiovascular or metabolic diseases (Sanford et al., 2020; Zierath & Wallberg-Henriksson, 2015).

In particular, the so-called metabolomics method, i.e., the study of small molecule metabolites in a biological sample (Bujak, Struck-Lewicka, Markuszewski, & Kaliszan, 2015), represents a promising approach to systematically investigate exercise-related metabolic alterations (Heaney, Deighton, & Suzuki, 2017). Due to the continuous advances in analytical techniques and computational technologies, metabolomics is nowadays extensively applied in clinical and biomedical studies aiming to attain an integrated view on human metabolism, its derangements underlying chronic diseases, or its associations with lifestyle factors, such as diet, smoking, or PA (Bujak et al., 2015; German, Hammock, & Watkins, 2005). In the context of PA, metabolomics can provide specific metabolite patterns that closely reflect molecular or cellular responses to acute and chronic exercise (Heaney et al., 2017). Together with the existing knowledge of biochemistry, metabolomics moreover allows to elucidate metabolic pathways which are linked to PA-related adaptations and health benefits (German et al., 2005; Heaney et al., 2017). According to German et al. (2005), the application of metabolomics is therefore a powerful strategy to address current health challenges.

During the past decade, an increasing number of PA-related metabolomics studies have been published. While most experimental studies focused on the effects of acute PE or training interventions on human metabolite concentrations, observational studies mainly examined the relationship between measures of chronic PA, such as the individual PA behavior or physical fitness (PF) status, and metabolite profiles (Daskalaki, Easton, & Watson, 2014; Kelly, Kelly, & Kelly, 2020). According to the current state of research, acute exercise interventions are commonly associated with quantifiable metabolic changes characteristic for an altered lipid, carbohydrate, amino acid (AA), energy, or nucleotide metabolism, whereas higher PA or CRF levels are linked to, e.g., lower circulating phosphatidylcholine (PC) or branched-chain amino acid (BCAA) concentrations (Kelly et al., 2020). However, despite the growing number of PA-related metabolomics studies, their heterogeneity impedes a direct comparison of findings. Furthermore, several study limitations exist. With respect to acute PE intervention studies, the vast majority have been conducted in small, mainly male populations and focused on the analysis of blood metabolites. Thus, more research on the acute metabolic effects of

PE in females is needed. Besides, since initial studies have supported the potential of urine as a non-invasively accessible biofluid to reflect exercise-induced changes in human metabolism (Enea et al., 2010; Muhsen Ali et al., 2016; Mukherjee et al., 2014; Siopi et al., 2017), more investigations evaluating the utility of urine in the field of exercise metabolomics are required. With regard to observational studies focusing on the PF, most metabolomics examinations included rather small sample sizes, were restricted to specific sex or age groups, and only analyzed a limited number of blood metabolites (Floegel et al., 2014; Koh et al., 2018; Kujala et al., 2019; Wientzek et al., 2014). Consequently, in order to yield results which are more applicable to the general population, further cross-sectional studies in larger and more diverse populations are needed. Besides, a combination of different analytical techniques is likely to provide a more comprehensive investigation of PA-related metabolite profiles.

The general aim of the present thesis is to comprehensively analyze the effect of acute and chronic PA on metabolite profiles in humans, thereby intending to address several research gaps and to extend the current state of knowledge in the field of exercise metabolomics. More specifically, this dissertation comprises the investigation of metabolomics data generated in the framework of three exercise- or fitness-related (sub-)studies, which were conducted at or in cooperation with the Max Rubner-Institut (MRI) in Karlsruhe. On the basis of these studies, conclusions on metabolic alterations in response to medium-term high-intensity interval training (HIIT) (*Study I*) or acute incremental exercise (*Study IIa*) shall be drawn. Moreover, the relationship between metabolite profiles and the CRF as a measure of chronic PA shall be systematically analyzed in a large population consisting of both sexes with a wide age range (*Study IIa*/*IIb*). Depending on the particular study, available biological specimens, and advances in analytical techniques, different metabolomics approaches were applied.

As a whole, the three considered (sub-)studies covered different aspects of PA, reaching from acute exercise and training interventions to the assessment of the CRF status. Thus, the results yielded by this dissertation can provide a deeper understanding of acute or chronic metabolic adaptations to PA. Besides, by identifying PE- or PF-associated metabolite profiles in blood or urine, valuable new insights into metabolic pathways that are possibly linked to the beneficial effects of PA are likely to be attained. Finally, findings from this thesis might provide a solid basis for the future selection of exercise-responsive, performance- or health-related biomarkers that allow to draw conclusions on an individual's training, fitness, or health status.

1.2 Outline of the Dissertation

The present dissertation comprises nine main chapters. The relevant theoretical background is presented in Chapter 2, including a brief introduction into exercise metabolism research, the human metabolome, and current metabolomics approaches. Moreover, the application of metabolomics in the field of exercise science is addressed, emphasizing potentials and perspectives of exercise metabolomics and summarizing the current state of research. While Chapter 3 depicts the aims and scope of this thesis, Chapter 4 provides a general description of the main methods utilized in the three included (sub-)studies. Building the core of this cumulative dissertation, Chapters 5 to 7 comprise the specific study findings that have been published in international peer-reviewed journals:

- ***Study I*** **(Chapter 5):**
  Kistner S., Rist M.J., Krüger R., Döring M., Schlechtweg S., Bub A. (2019). High-Intensity Interval Training Decreases Resting Urinary Hypoxanthine Concentration in Young Active Men—A Metabolomic Approach. *Metabolites*, *9*(7), 137. doi:10.3390/metabo9070137.

- ***Study IIa*** **(Chapter 6):**
  Kistner, S., Rist, M. J., Döring, M., Dörr, C., Neumann, R., Härtel, S., Bub, A. (2020). An NMR-Based Approach to Identify Urinary Metabolites Associated with Acute Physical Exercise and Cardiorespiratory Fitness in Healthy Humans—Results of the KarMeN Study. *Metabolites*, *10*(5), 212. doi:10.3390/metabo10050212.

- ***Study IIb*** **(Chapter 7):**
  Kistner, S., Döring, M., Krüger, R., Rist, M. J., Weinert, C. H., Bunzel, D., Merz, B., Radloff, K., Neumann, R., Härtel, S., Bub, A. (2021). Sex-Specific Relationship between the Cardiorespiratory Fitness and Plasma Metabolite Patterns in Healthy Humans—Results of the KarMeN Study. *Metabolites*, *11*(7), 463. doi:10.3390/metabo11070463.

The three research articles are followed by a section that focuses on a functional classification of obtained PE- or PF-related metabolite profiles (Chapter 8). Lastly, Chapter 9 comprises a summary and critical discussion of the study results, depicts strengths as well as limitations of the applied methods, and provides suggestions for future research. As is common in the field of metabolomics, the three publications contain comprehensive supplementary material, e.g., additional figures or tables. To facilitate the understanding of the respective study findings, relevant supplementary information is also provided in the Appendix of this thesis.

2 Theoretical Background

In this theoretical section, relevant information with respect to human exercise metabolism, its principles, and adaptations to chronic PE are given (Chapter 2.1). Then, the concept of the human metabolome, its determinants, as well as the metabolic composition of human blood and urine are described (Chapter 2.2) and the metabolomics method with a special focus on analytical techniques and the consecutive steps of a characteristic metabolomics workflow are introduced (Chapter 2.3). Finally, the application of metabolomics in the field of exercise science is addressed (Chapter 2.4), thereby not only giving insight into potentials and future perspectives of exercise metabolomics research, but also reviewing and summarizing the current state of research of PA-related metabolomics studies.

2.1 Human Exercise Metabolism

Exercise metabolism research investigates how exercise – in its diverse forms – modifies the way the human organism functions at the molecular level, thus intending to understand metabolic processes during and after PE (Hawley, Maughan, & Hargreaves, 2015; Mougios, 2019). Before depicting some inherent principles of exercise metabolism in Chapter 2.1.2, established terms and concepts related to both exercise and metabolism will be defined in the following sub-section.

2.1.1 Exercise and Metabolism – Basic Terminology and Concepts

Physical Activity, Exercise, Training, and Fitness

PA is generally defined as any bodily movement produced by the contraction of skeletal muscles that increases energy expenditure above the resting level (Bouchard, Blair, & Haskell, 2007). This broad concept comprises occupational and leisure-time PA, with the latter describing free time activities such as walking, hiking, or sports (Howley, 2001). Though having been used synonymously with PA, PE essentially represents a sub-category of leisure-time PA (Dasso, 2019). It denotes planned and structured bodily movements to maintain or improve one or more components of the PF (Caspersen, Powell, & Christenson, 1985). When PE is performed repetitively, it is termed exercise training (Bouchard et al., 2007). Concepts for both PE and exercise training typically refer to specific exercise parameters, including exercise type, intensity, duration, and frequency (Howley, 2001). With regard to exercise type, activities can

be broadly classified as endurance- or strength-based. While endurance exercise involves prolonged continuous or intermittent periods of muscular activity against low resistance, resistance exercise is characterized by short bouts of muscular activity against high resistance (Coffey & Hawley, 2017). Another established exercise mode is interval exercise, consisting of repeated intensive exercise bouts interspersed with short recovery periods, such as HIIT (MacInnis & Gibala, 2017). Exercise intensity, in turn, defines the effort associated with PE in either absolute or relative terms. When focusing on endurance exercise, the absolute intensity usually describes the rate of energy expenditure and can be expressed as oxygen uptake (VO_2) per minute, kilocalories per minute, or the metabolic equivalent of task (MET), which is a multiple of the resting VO_2 (Howley, 2001). As opposed, the relative intensity of PE is usually adjusted to maximal physiological responses, being indicated as a percentage of, e.g., maximal oxygen uptake (VO_{2max}) or maximum heart rate (HR_{max}). By using qualitative descriptors (e.g., low, moderate, vigorous, or maximal), exercise intensities can also be subjected to a more general classification. While exercise duration refers to the time spent in an activity, exercise frequency is related to the number of exercise sessions in a given time frame. Depending on whether PA or PE is performed once or repeated over time, it is termed acute or chronic, respectively (Howley, 2001).

In this dissertation, both acute PE and exercise training are focused. With regard to exercise training, a HIIT intervention was conducted (*Study I*). During the last decades, research interest in HIIT as a time-efficient training method has greatly increased (Laursen & Jenkins, 2002) and a number of HIIT protocols have been described in the literature (Cassidy, Thoma, Houghton, & Trenell, 2017). Most HIIT protocols are performed on stationary bicycles or treadmills, being characterized by strenuous exercise intervals, which last between one and four minutes, and recovery periods of low activity or rest. In fact, HIIT is usually performed at intensities > 80-95% of VO_{2max} or > 90% of HR_{max} (Cassidy et al., 2017). With regard to acute PE, a standardized exercise tolerance test was conducted (*Study IIa*). In the literature, various standard protocols for maximal exercise tests on either treadmills or bicycle ergometers exist (Löllgen & Leyk, 2018). These protocols are generally characterized by increasing workloads until individual exhaustion, but can vary with respect to the initial workload, type of workload increases, and total duration. In all cases, the presence of qualified personnel for continuous heart rate (HR), blood pressure (BP), and electrocardiogram monitoring is mandatory (Löllgen & Leyk, 2018).

Whereas PA and PE are behavioral variables, PF can be generally defined as “the ability to carry out daily tasks with vigor and alertness, without undue fatigue and with ample energy to enjoy leisure-time pursuits and to meet unforeseen emergencies” (Manley, 1996, p. 20). In fact, PF depicts a set of attributes including agility, balance, coordination, speed, power, and reaction time, as well as CRF, muscular endurance, muscular strength, body composition, and flexibility (Caspersen et al., 1985). Since these components are of different importance with regard to athletic ability or health, a distinction between performance- and health-related fitness has been proposed (Caspersen et al., 1985; Pate, 1983), see Figure 1.

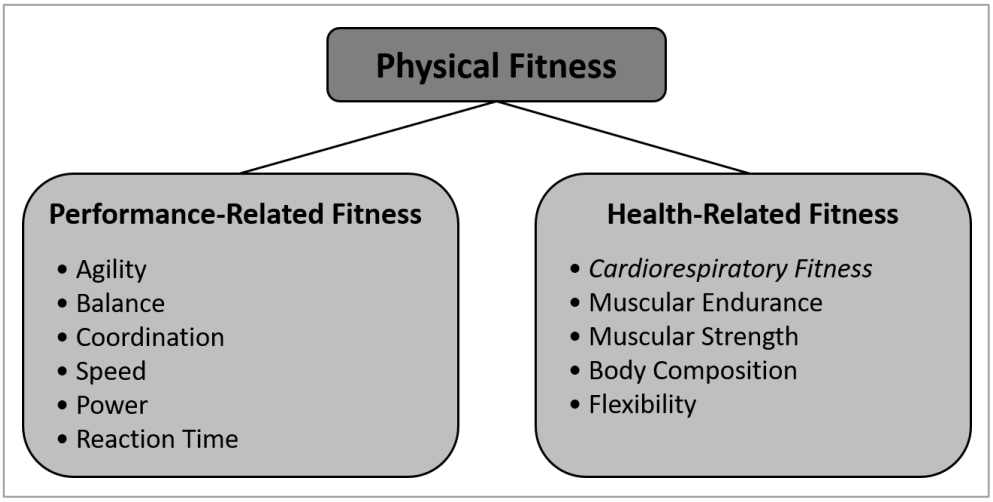

Figure 1. Components of PF. *(Own illustration based on Caspersen et al. (1985)).*

In this dissertation, the health-related fitness, and especially the CRF (*Study IIa/IIb*), is focused because its components are (un)favorably affected by habitual PA behavior and moreover linked to health outcomes (Bouchard et al., 2007). While the body composition, for instance, describes the relative amounts of muscle, fat, or bone in the human organism (Howley, 2001), the CRF reflects the ability of the respiratory and circulatory systems to supply oxygen to muscles during sustained PE (Corbin & Lindsey, 1994; Hill, Long, & Lupton, 1924). The CRF can be assessed by measuring the VO_{2max} during incremental exercise until exhaustion (Löllgen & Leyk, 2018). If it is not possible to determine the VO_{2max}, requiring the presence of a plateau in the VO_2, the peak oxygen uptake (VO_{2peak}) as the highest attained value of VO_2 is commonly utilized instead (Day, Rossiter, Coats, Skasick, & Whipp, 2003). Being not only an important variable that defines the upper limit of endurance performance (Bassett & Howley, 2000) but also an independent predictor of cardiovascular and all-cause mortality (Harber et al., 2017), the CRF is determined by non-modifiable factors, such as age (Laukkanen et al., 2009), sex (Al-

Mallah et al., 2016), or heredity (Bouchard, Boulay, Simoneau, Lortie, & Pérusse, 1988), and modifiable factors like the lean body mass (LBM) (S. Y. S. Wong et al., 2008) or PA behavior (Zeiher et al., 2019). According to McKinney et al. (2016, p. 132), the CRF thus represents an "objective surrogate measure of recent PA patterns". In accordance with this definition, the CRF is considered as a measure of chronic PA in this thesis. Figure 2 provides an overview of known as well as potential correlates and determinants of the CRF.

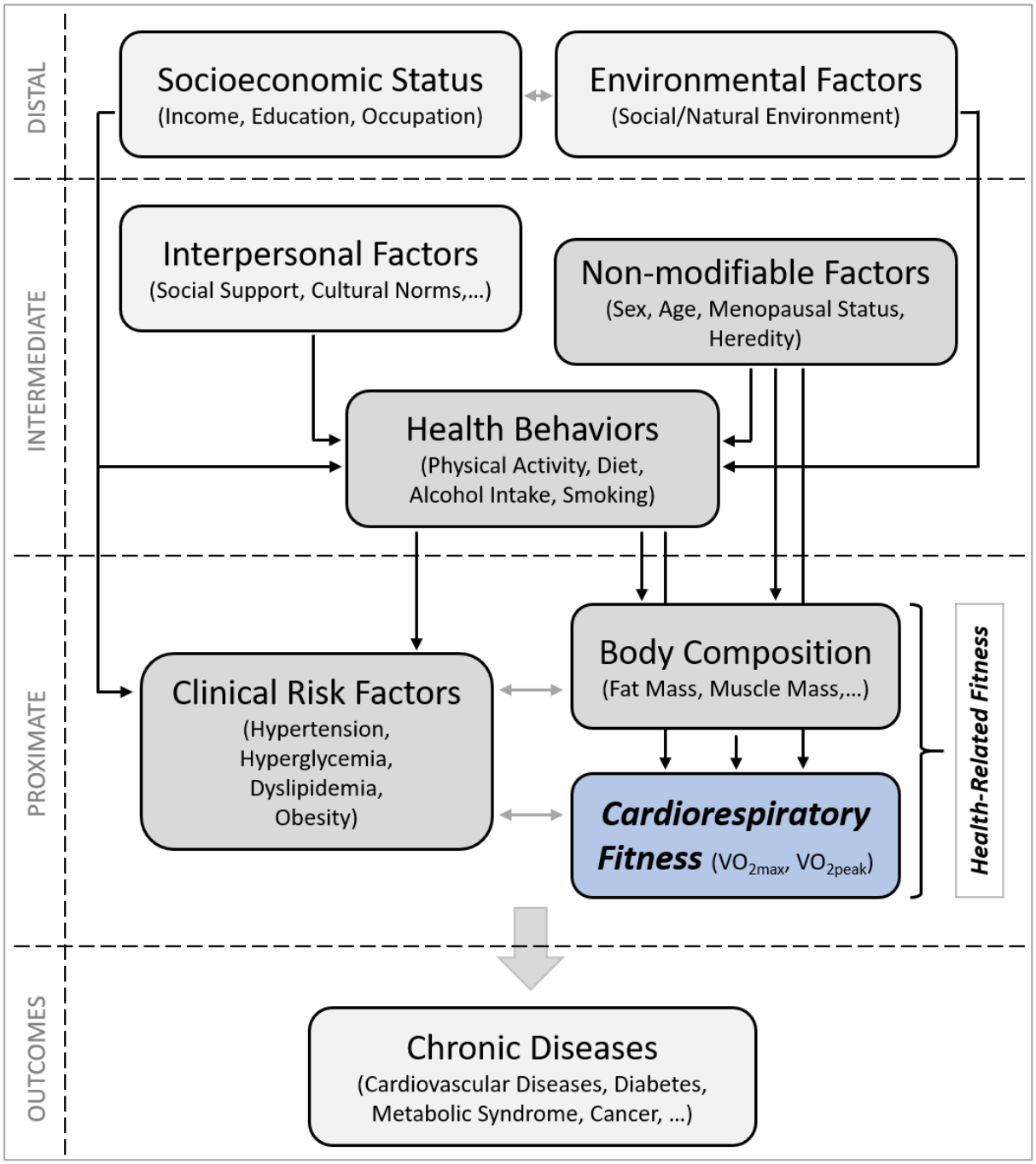

Figure 2. Correlates and determinants of CRF. VO_{2max}: maximal oxygen uptake; VO_{2peak}: peak oxygen uptake. *(Own illustration based on Zeiher et al. (2019)).*

As depicted in Figure 2, further discussed determinants or correlates of the CRF include socioeconomic (e.g., education), environmental (e.g., public green spaces), and interpersonal (e.g., social support) factors that can individually affect health-related behaviors like PA, diet, alcohol intake, or smoking. Representing important components of the health-related fitness, both the body composition and the CRF are linked to traditional clinical risk factors for metabolic or cardiovascular diseases, such as high BP, blood glucose, triglycerides (TGs), low-density lipoprotein (LDL) cholesterol, and obesity (Zeiher et al., 2019).

Metabolism, Metabolic Pathways, Metabolites

Having its origin in the Greek word *metabolé*, meaning transformation or change, the term metabolism describes the sum of all chemical reactions occurring in a living organism (Lal, 2018). While those enzyme-catalyzed reactions are called metabolic reactions and sequences of metabolic reactions constitute metabolic pathways, the intermediates formed in metabolic pathways are referred to as metabolites (Harris, 2017). In general, metabolism comprises both anabolic and catabolic reactions. Anabolism involves energy-consuming biosynthetic processes in which complex molecules are produced from small precursors. Catabolism, on the contrary, includes degradation processes that convert complex biomolecules into simpler molecules, thereby releasing energy (von der Saal, 2020). Adenosine triphosphate (ATP) as a high-energy phosphate compound is mainly responsible for mediating energy transitions in metabolic pathways (Lal, 2018).

Since the human organism continuously exchanges mass and energy with the environment, levels of metabolites in tissues and biofluids rapidly change (Mougios, 2019). Apart from diet or disease, PE represents a major challenge to whole-body and cellular metabolic homeostasis (Hawley, Hargreaves, Joyner, & Zierath, 2014; Suárez, Caimari, del Bas, & Arola, 2017).

2.1.2 Principles of Exercise Metabolism

Acute PE provokes extensive perturbations in numerous organs, tissues, and cells due to the increased metabolic activity of contracting skeletal muscles (Hawley et al., 2014). Actually, the rate of ATP hydrolysis, which is principally needed for powering cross-bridge cycles and, thus, muscle contraction, can increase by more than 100-fold during PE (Gaitanos, Williams, Boobis, & Brooks, 1993). Since the intramuscular stores of ATP are relatively small, several metabolic pathways are activated to regenerate ATP (Baker, McCormick, & Robergs, 2010).

Table 1. Skeletal muscle metabolism during PE in humans. *(Own table based on Hargreaves and Spriet (2020)).*

ATP Hydrolysis	ATP + H_20 ⟶ ADP + P_i + H^+ + energy
ATP Resynthesis	
Substrate-level phosphorylation	
Phosphocreatine degradation	ADP + phosphocreatine + H^+ ⟶ ATP + creatine
Adenylate kinase reaction	2 ADP ⟶ ATP + AMP
Glycolysis	Glucose + 3 ADP ⟶ 2 lactate + 2 H^+ + 3 ATP
Oxidative phosphorylation	
Carbohydrate oxidation	Glucose + 6 O_2 + 36 ADP ⟶ 6 CO_2 + 6 H_20 + 36 ATP
Fatty acid oxidation	Palmitate + 23 O_2 + 130 ADP ⟶ 16 CO_2 + 16 H_20 + 130 ATP

ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate; CO_2: carbon dioxide; H^+: proton; H_20: water; O_2: dioxygen; P_i: inorganic phosphate.

As shown in Table 1, pathways for ATP resynthesis during PE comprise (a) the ATP-phosphagen system, including the degradation of phosphocreatine to creatine and free phosphate, which is transferred to adenosine diphosphate (ADP) to re-form ATP, as well as the adenylate kinase reaction for the formation of ATP and adenosine monophosphate from two ADP molecules; (b) glycolysis, i.e., the degradation of glycogen or glucose to lactate, thereby producing ATP; and (c) processes of complete carbohydrate or fatty acid breakdown coupled to oxidative phosphorylation in the electron-transport chain to yield comparatively high amounts of ATP (Egan & Zierath, 2013; Hargreaves & Spriet, 2020). While the former, “anaerobic” pathways involve substrate-level phosphorylation without any need of oxygen, the latter, “aerobic” pathways are critically reliant on a sufficient oxygen supply to skeletal muscles by the respiratory and cardiovascular system (Hargreaves & Spriet, 2018). Despite being almost concurrently active during PE, the relative contribution of those ATP-generating pathways and, consequently, the use of either intra- or extramuscular energy substrates, are principally determined by the intensity and duration of PE (Egan & Zierath, 2013). During high-intensive and short PE, the major energy-yielding pathways include the degradation of intramuscular phosphocreatine and glycogen while oxidative metabolism usually accounts for 25-30% of energy provision. At low to moderate intensities, the vast majority of ATP is provided by the oxidative breakdown of carbohydrates and lipids, with the latter exhibiting an increased energy contribution during prolonged submaximal PE (Hargreaves & Spriet, 2018). In detail, major substrates for oxidation are muscle glycogen or blood glucose derived from hepatic glycogenolysis, gluconeogenesis, and oral ingestion, as well as fatty acids from either intra-muscular or adipose tissue TGs breakdown (Baker et al., 2010; Hargreaves & Spriet, 2020). Even though several AAs can also be oxidized by contracting skeletal muscles or used for

gluconeogenesis, proteins generally make a minor contribution to energy metabolism during PE (approximately 5-15%), provided that carbohydrate availability is adequate (Baker et al., 2010; van Loon, Greenhaff, Constantin-Teodosiu, Saris, & Wagenmakers, 2001).

A further principle of exercise metabolism refers to the fact that the mainly catabolic nature of acute PE is followed by a largely anabolic post-exercise recovery period, aiming to re-synthesize muscular ATP, phosphocreatine, glycogen, as well as mitochondrial or myofibrillar proteins, myocellular TGs, or liver glycogen (Egan & Zierath, 2013; Mougios, 2019).

To conclude, exercise-induced alterations in metabolism are not solely determined by exercise intensity and duration but also by environmental factors and characteristics of the exercising individuals, such as age, sex, diet, or training status (Hargreaves & Spriet, 2018). How chronic PE provokes long-term metabolic adaptations will be focused in the next sub-section.

2.1.3 Metabolic Adaptations to Chronic PE

Regularly performed PE of adequate intensity and duration leads to morphological, metabolic, as well as functional adaptations that are specific to the exercise mode (Egan & Zierath, 2013). Compared to resistance training, which is known to increase muscle hypertrophy and strength, but elicits only minor adaptations in skeletal muscle metabolism (Deschenes & Kraemer, 2002), both endurance and interval training induce a higher mitochondrial density as well as extensive alterations in substrate metabolism and finally contribute to an improved aerobic performance, CRF, and health status (Egan & Zierath, 2013; Mougios, 2019).

In addition to physiological adaptations, such as an increased cardiac output, blood volume, hemoglobin (Hb) mass, and capillarization, metabolic adaptations like an enhanced oxidative capacity due to a higher muscle mitochondrial content are also concomitant with training-induced improvements in the CRF (Gabriel & Zierath, 2017). Processes underlying these metabolic adaptations usually involve multiple molecular events, including shifts in gene transcription, protein translation, and post-translational modifications (Neufer et al., 2015). As illustrated in Figure 3, early adaptations to acute PE bouts comprise rapid but transient changes in the messenger ribonucleic acid (mRNA) expression of several genes related to muscle function or energy metabolism (Y. Yang, Creer, Jemiolo, & Trappe, 2005). Depending on the particular PE challenge, the exercise-induced increase in mRNA levels typically returns to baseline within 24 hours. Translational processes and elevated protein synthesis rates

subsequently lead to a modest change in the content of specific proteins (Egan & Zierath, 2013). For example, a key regulatory protein of mitochondrial biogenesis, the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α, is known to exhibit an increased expression in the post-exercise period (Baar et al., 2002). Even if each acute exercise session is a necessary stimulus for adaptive responses, PE has to be performed repetitively in order to induce long-term metabolic and functional adaptations. In fact, a constantly increased abundance and/or enzymatic activity of proteins involved in, e.g., glucose or fatty acids transport, tricarboxylic acid (TCA) cycle, mitochondrial ATP production, or glycogen synthesis are reflective of the cumulative effects of acute PE, finally leading to altered exercise-induced responses at the level of substrate metabolism and improvements in submaximal exercise performance (Egan & Zierath, 2013; Neufer et al., 2015).

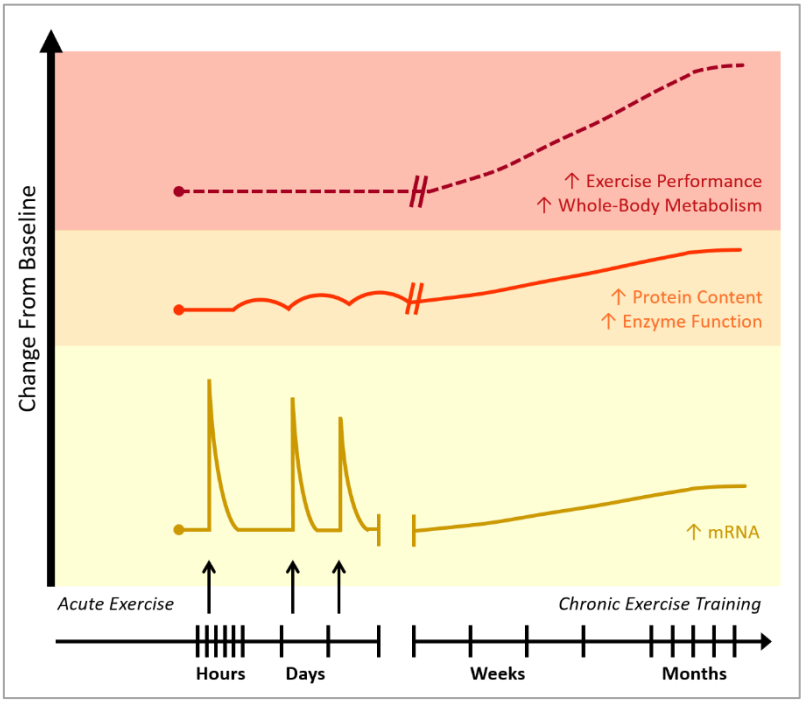

Figure 3. Molecular basis of acute and chronic adaptations to PE. Alterations in mRNA expression (lower panel) and protein content (middle panel) over time as a result of acute exercise and chronic exercise training are illustrated. Repetitive exercise training alters whole-body substrate metabolism, finally leading to an improved exercise performance (upper panel). mRNA: messenger ribonucleic acid. *(Own illustration based on Egan and Zierath (2013)).*

2.1.4 Current Approaches and Challenges in the Study of Exercise Metabolism

Researchers have several methods at their disposal when studying the effects of PE on human metabolism, including indirect calorimetry, metabolic tracer analysis, and blood or muscle tissue sampling (Hargreaves & Spriet, 2018). While most traditional approaches have been

focused on assessing single genes, gene-transcripts, proteins, or metabolites of interest, the recently emerging field of systems biology integrates large datasets from so-called “omics”-technologies, allowing to analyze the sum of genes, transcripts, proteins, or metabolites (i.e., the genome, transcriptome, proteome, or metabolome) in a range of body fluids or tissues as comprehensively as possible (Hawley et al., 2015; N. J. Hoffman, 2017; Nielsen, 2017).

In fact, the molecular mechanisms and metabolic processes underlying the acute and chronic adaptations to PE have not yet been fully elucidated (Neufer et al., 2015). According to Zierath and Wallberg-Henriksson (2015), the application of large-scale genomics, transcriptomics, proteomics, and metabolomics methods is therefore indispensable to receive a preferably global view on how genes, proteins, and metabolism are modulated in response to PE. As depicted in Figure 4, current challenges in the field of exercise biology are related to the integration of an individual’s genetic background with exercise-induced, tissue-specific gene expression and system-wide changes in protein or metabolite profiles. In this context, the application of “omics”-methods is likely to enable an unbiased discovery of PE-responsive biomarkers and signaling pathways, thus filling gaps in the current knowledge on biological processes underlying exercise-related adaptations and health benefits (N. J. Hoffman, 2017; Zierath & Wallberg-Henriksson, 2015).

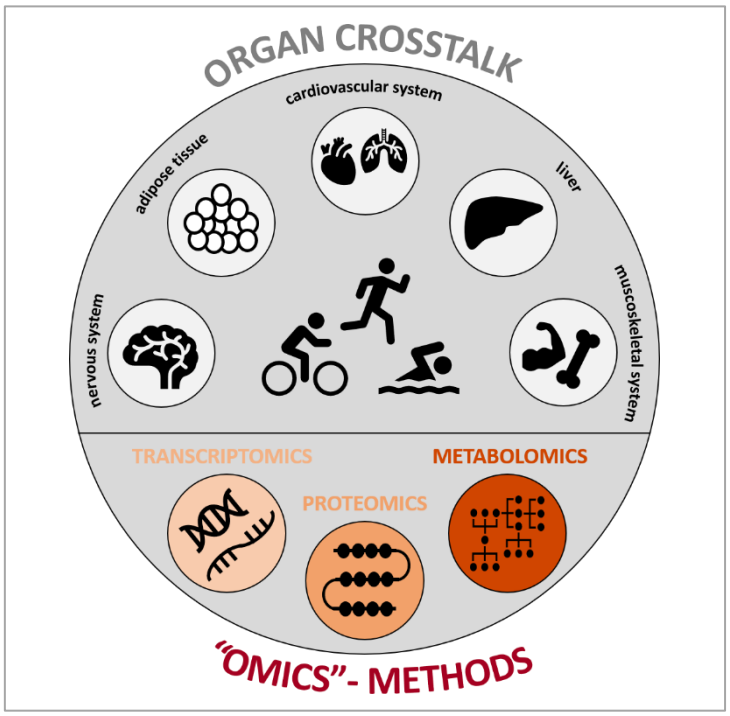

Figure 4. “Omics”-methods for studying the molecular basis of acute and chronic adaptations to PE. *(Own illustration based on Zierath and Wallberg-Henriksson (2015)).*

Since this dissertation focuses on the use of metabolomics as one of the presented "omics"-disciplines for examining how responses to acute and chronic PA in humans are reflected in the metabolome, the next sub-section will give a detailed introduction into the definition and characteristics of the human metabolome.

2.2 The Human Metabolome

In 1998, the term "metabolome" appeared in the literature for the first time (Oliver, Winson, Kell, & Baganz, 1998; Sussulini, 2017). According to Nicholson, Lindon, and Holmes (1999), the metabolome refers to all metabolites present in a given biological system, such as tissues, cells, or fluids. Being defined as small molecules, i.e., < 1,500 Dalton, produced by enzymatic reactions (Bujak et al., 2015; Tebani, Afonso, & Bekri, 2018a), metabolites comprise a wide range of chemical entities like AAs, peptides, nucleosides, sugars, or fatty acids (Wishart, 2008). As illustrated in Figure 5, metabolites represent downstream products of interactions between the genome, transcriptome, and proteome (Bujak et al., 2015; Newgard, 2017).

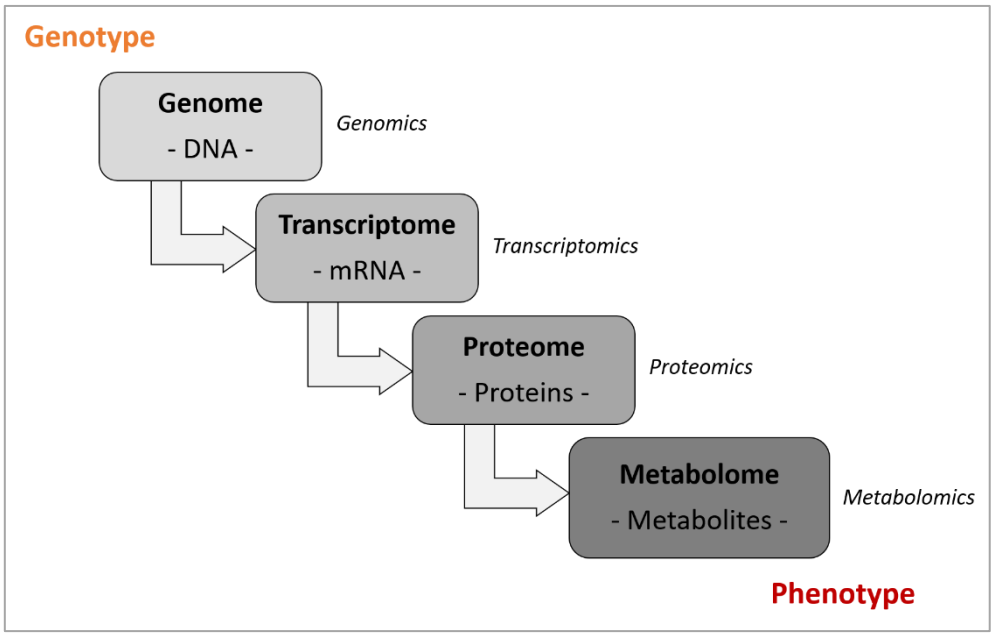

Figure 5. Relationship between the genome, transcriptome, proteome, and metabolome. DNA: deoxyribonucleic acid; mRNA: messenger ribonucleic acid. *(Own illustration based on Sussulini (2017)).*

Due to the fact that the biological information flows from genes to mRNA-transcripts and, finally, from proteins to metabolites, the analysis of the metabolome seems to be a holistic approach for understanding an organism's phenotype (Bouatra et al., 2013; Bujak et al., 2015). While genes and proteins are subject to epigenetic modifications or post-transcriptional

regulations, respectively, metabolites are characterized as direct signatures of cellular metabolism, exhibiting essential functions in, e.g., signal transduction or energy production (Johnson, Ivansisevic, & Siuzdak, 2016). Accordingly, the metabolome can reveal what actually is occurring or has happened in a biological system, whereas the genome, transcriptome, and proteome are restricted to provide insight on what might happen (Riekeberg & Powers, 2017).

The metabolome does not only represent a highly integrated profile of the current biological status in humans (Newgard, 2017), it also incorporates external influences through, e.g., diet, drugs, or PA (A.-H. Emwas et al., 2015; Walsh, 2008). Hence, the metabolome can give an instantaneous snapshot of how human metabolism responds to different physiological challenges or processes (Peng, Li, & Peng, 2015; Suárez et al., 2017).

2.2.1 Influencing Factors and Size of the Human Metabolome

In fact, the human metabolome shows a high degree of physiological variation (Walsh, 2008). As depicted in Figure 6, the metabolome is determined by intrinsic biological factors, such as genotype (Shin et al., 2014), sex and age (Rist et al., 2017; Slupsky et al., 2007), the presence of diseases (Martín-Blázquez et al., 2019; S. J. Yang, Kwak, Jo, Song, & Shin, 2018), the body composition (Bachlechner et al., 2016), a female's menstrual cycle (Wallace et al., 2010), internal circadian rhythms (Grant et al., 2019), or the gut microbiota (Visconti et al., 2019).

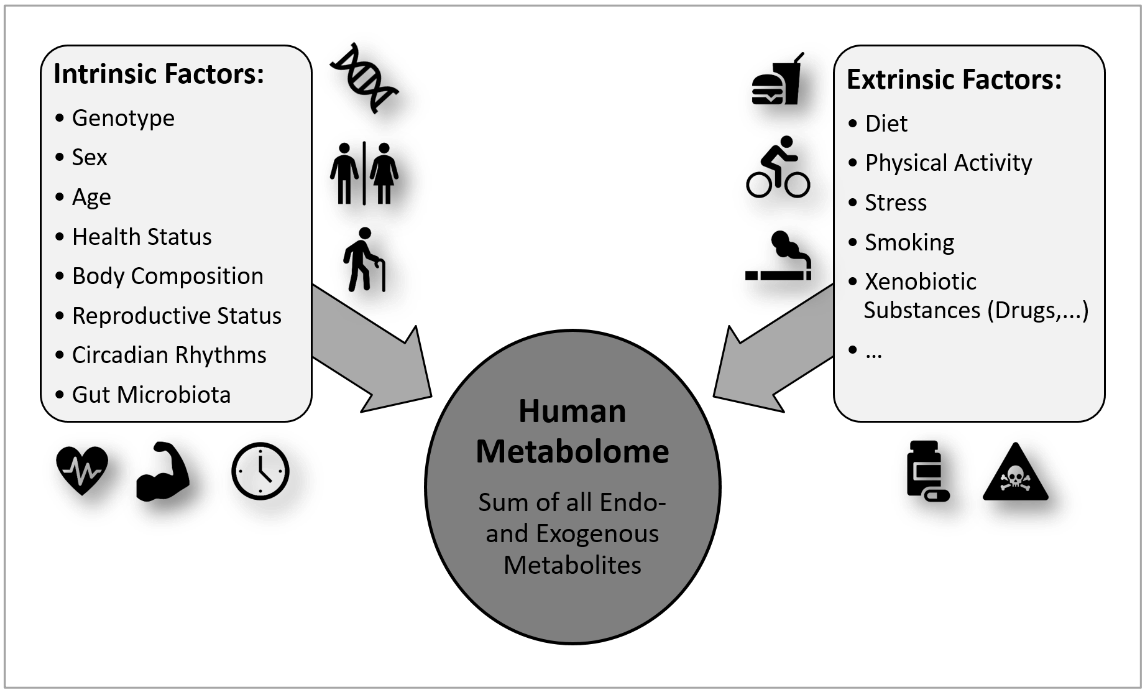

Figure 6. Intrinsic and extrinsic factors influencing the human metabolome. *(Own illustration).*

On the other hand, the human metabolome is affected by a number of lifestyle and environmental factors (Walsh, 2008). The composition of the metabolome has been shown to vary along with dietary habits (Floegel, von Ruesten, et al., 2013), PA behavior (Floegel et al., 2014; Xiao et al., 2016), psychological stress (Sood, Priyadarshini, & Aich, 2013), or cigarette smoking behavior (Gu et al., 2016), and to respond dynamically to nutritional challenges (Krug et al., 2012), acute PE (Lewis et al., 2010; Peake et al., 2014), or drug intake (Fannin et al., 2010).

As a highly variable and dynamic construct, the metabolome comprises not only endogenous but also exogenous metabolites. While the former include intermediates of human metabolic networks, the latter refer to compounds originating from diet, pharmacotherapy, or further environmental exposures (Johnson, Patterson, Idle, & Gonzalez, 2012; Scalbert et al., 2014). As the main entry route for exogenous compounds, diet delivers nutrients to the human body, which are digested, metabolized, and/or previously transformed by the intestinal microbiota (Scalbert et al., 2014; Walsh, 2008). The remaining compounds foreign to the human body are termed xenobiotics (Johnson et al., 2012). Irrespective of being harmless or toxic, most xenobiotics are also substrates of either gut microbial or human metabolism, before being eliminated via urine, bile, or feces (Johnson et al., 2016).

Due to the presence of both endogenous and exogenous metabolites, the exact number of compounds found in the human metabolome is largely unknown (Walsh, 2008). The Human Metabolome Database (HMDB) currently lists a total number of 114,100 metabolites possibly existing in human biofluids, including almost 22,000 detected metabolites and more than 90,000 not directly detected, but expected/predicted metabolites (Wishart et al., 2018).

2.2.2 Composition of the Human Blood and Urine Metabolome

For the assessment of human metabolite profiles or metabolic signatures, which by definition contain sets of various metabolites (Tebani et al., 2018a), the biofluids blood, i.e., plasma or serum, and urine represent the most commonly studied biological matrices – particularly due to their ease of collection and ability to integrate the metabolic composition of several tissues and organs related to multiple biological processes (Dunn & Ellis, 2005; Walsh, 2008).

Blood carries a wealth of metabolic information owing to its role in transporting not only nutrients, electrolytes, dissolved gases, and hormones but also small organic molecules secreted by different tissues (Psychogios et al., 2011; Walsh, 2008). Being a key carrier of

lipoproteins, fats, and hydrophobic compounds, the human blood metabolome is dominated by TGs, phospholipids, fatty acids, steroids, or steroid derivatives, followed by small molecules like AAs, glucose, glycerol, or lactate, and several metabolic waste products, such as urea or creatinine (Psychogios et al., 2011). Since the composition of human blood is well maintained through homeostatic mechanisms, it is a less variable biofluid than, e.g., urine (Walsh, 2008). Psychogios et al. (2011) could show that the known, quantifiable human serum metabolome contains about 4,229 metabolites.

Urine is a sterile, non-invasively obtainable biofluid generated by the kidneys, which remove waste products from the blood. It is typically free from lipids or proteins, mainly containing water, inorganic salts, and a high number of water-soluble metabolites, such as urea, creatinine, ammonia, organic acids, or products from the breakdown of xenobiotic substances (Bouatra et al., 2013). Consequently, the human urine metabolome provides a valuable and rich source of metabolic information (Walsh, 2008). Due to its function in waste removal, the metabolic composition of urine is more susceptible to fluctuations and shows a higher variation than the blood metabolome (Walsh, 2008). Besides, urine dilution and hence the concentration of metabolites can vary to a large extent. Thus, a sample-specific normalization, e.g., to urine volume or osmolality, is needed if examining the urine metabolome (Ulaszewska et al., 2019). By combining data from experimental studies and a literature survey, Bouatra et al. (2013) showed that it is possible to identify and quantify 2,651 metabolite species in human urine with the current technology.

Since the human metabolome is complex, comprising a high number of chemically distinct metabolites with large concentration ranges, different analytical strategies and methodological approaches have been developed in order to allow its systematic investigation (Bujak et al., 2015). The so-called “metabolomics” research method will be described in the following sub-section.

2.3 Metabolomics

As the youngest emerging approach in the systems biology field, metabolomics focuses on the “comprehensive and systematic identification and quantification of small molecule metabolites […] in biological samples at a given point of time” (Bujak et al., 2015, p.109).

2.3.1 Definitions and Current Applications

During the last decades, numerous terminologies to classify metabolomics investigations have appeared in the literature (Sussulini, 2017). While metabolic profiling, for instance, has been utilized to describe metabolomics analyses focusing on a limited number of metabolites, metabolic fingerprinting was linked to the analysis of a preferably high number of metabolites, intending to define a metabolic pattern characteristic for a particular study condition (Bujak et al., 2015). However, since there is still no actual consensus regarding this terminology (Sussulini, 2017), a more general and simplified classification is commonly applied. Based on the fact whether it is a priori known what kind of metabolites shall be assessed, it can be distinguished between targeted and untargeted analyses (Tebani et al., 2018a). A targeted metabolomics approach is defined as a (semi-)quantitative analysis (i.e., relative intensities or absolute concentrations are determined) of a selected group of metabolites, that are possibly related to specific metabolic pathways or chemical classes (Sussulini, 2017). Regarding this strategy, a hypothesis about changes in metabolite profiles is usually given beforehand (Patti, Yanes, & Siuzdak, 2012). In contrast, an untargeted metabolomics approach is characterized by a qualitative or semi-quantitative analysis of as many as possible metabolic compounds belonging to various chemical or biological classes (Sussulini, 2017). Untargeted analyses are mostly not driven by preliminary assumptions (Bujak et al., 2015).

Until recently, metabolomics has mostly been applied in clinical studies aiming to understand pathophysiological mechanisms or to discover disease markers (Bujak et al., 2015; Sussulini, 2017). Though, as one major challenge in metabolomics studies is to overcome inter-individual metabolite variation due to differences in genetics, age, and lifestyle factors, metabolomics is also increasingly utilized for studying changes in human metabolism related to genetic factors, aging, habitual diet, or PA (Johnson et al., 2016). In all metabolomics investigations, the biological complexity of metabolites represents another major challenge. Even though human metabolites are fewer in absolute number than mRNA-transcripts or proteins, they show a broader range of physicochemical properties (German et al., 2005). Thus, multiple analytical techniques have to be combined to assess a preferably high fraction of the metabolome (Tebani et al., 2018a). Analytical technologies currently dominating in metabolomics studies include nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (Bujak et al., 2015). They will be briefly introduced in the next sub-section.

2.3.2 Analytical Techniques

Advanced NMR spectroscopy- and MS-based analytical techniques typically show the ability to detect a wide range of distinct metabolites or metabolite features simultaneously, hence providing a "metabolic snapshot" at a given time point (Beckonert et al., 2007).

NMR spectroscopy

The principle behind NMR spectroscopy is based on measuring the absorption and re-emission of energy by specific atom nuclei owing to variations in an external magnetic field. The mostly used type of nuclei is hydrogen-1 (^{1}H), which is naturally abundant in biospecimens (Tebani et al., 2018a). Thus, NMR is able to profile magnetic resonances arising from hundreds of small molecules within a sample (A.-H. M. Emwas, Salek, Griffin, & Merzaban, 2013). By elucidating molecular structures based on atom-centered nuclear interactions and properties, NMR generates spectral data that finally allow to identify and quantify metabolites (Beckonert et al., 2007). Since the frequency at which ^{1}H absorbs is dependent on its chemical environment in a specific compound, each metabolite produces a specific absorption pattern at distinct frequencies. In an NMR spectrum, so-called chemical shifts, i.e., differences between the resonance frequencies of the compounds of interest and that of a reference substance, are indicated on the frequency axis, permitting metabolite identification (Tebani et al., 2018a). Additionally, as integrals of the respective resonance peaks are directly linked to the number of ^{1}H giving rise to the peak, those areas can be used for the relative or absolute quantification of metabolites (Beckonert et al., 2007). In Figure 7, typical ^{1}H-NMR spectra of either blood or urine are illustrated. While NMR spectra of blood samples are typically characterized by broad bands from high-molecular weight compounds, e.g., lipoproteins, which partly superimpose the sharp peaks resulting from small metabolites, those of urine predominantly contain numerous sharp lines from mostly low-molecular weight compounds (Beckonert et al., 2007).

In general, NMR is suitable for detecting uncharged and polar metabolites as well as relatively small, volatile compounds in the milli- to micromolar range (A.-H. M. Emwas, 2015). Although NMR spectroscopy possesses the advantages of being a relatively reproducible, robust, rapid, quantitative, and non-destructive method with minimal sample manipulation, its main limitations are the comparatively low sensitivity and the spectral complexity with overlapping signals, leading to uncertainties in metabolite assignment (A.-H. M. Emwas, 2015).

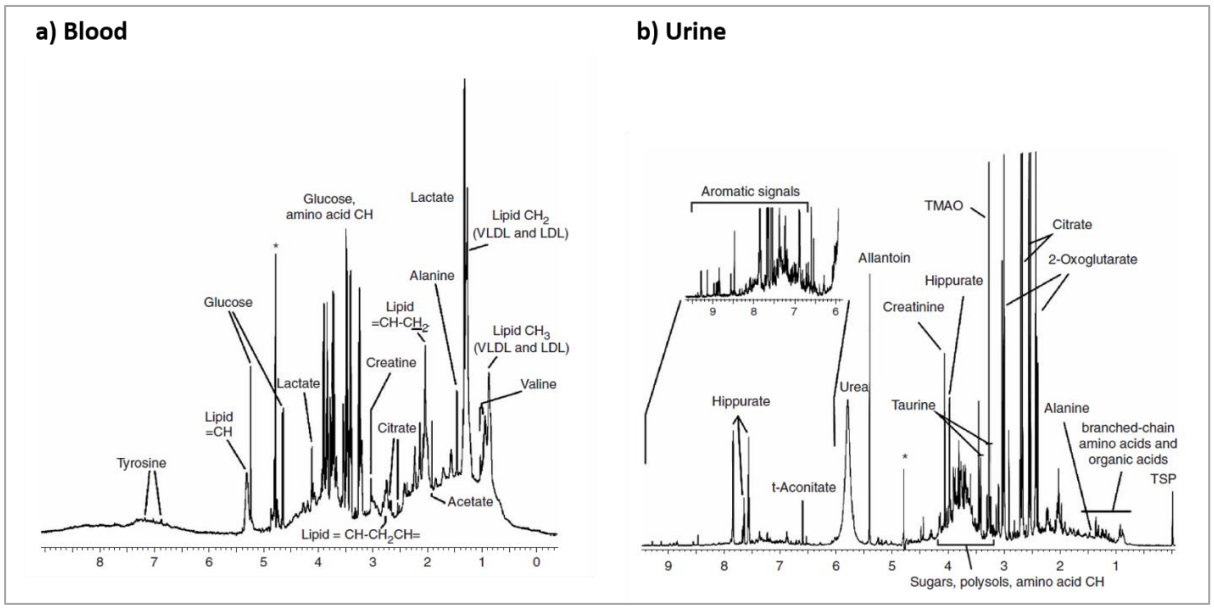

Figure 7. ^{1}H NMR spectra of a) blood and b) urine. *(Adapted from Beckonert et al. (2007)).*

Mass Spectrometry

MS-based platforms have the capability to analyze numerous metabolic compounds according to their molecular weights in a single experiment (German et al., 2005). More precisely, MS measures ionized molecules based on their mass-to-charge ratio (m/z) (Bujak et al., 2015). In a typical MS procedure, a biological sample is introduced into a MS where an ion source causes the ionization and/or fragmentation of contained molecules. Then, a mass analyzer separates the ions according to their m/z, e.g., by determining their deflection in a magnetic field, and a detector records the ions' abundances (Kermit et al., 2013; Tebani et al., 2018a). Results are presented as a mass spectrum, showing a plot of the ions' signal intensities and their m/z, which can be utilized for metabolite identification (Johnson et al., 2016; Tebani et al., 2018a). By including internal standards, quantitative metabolite data can also be obtained (German et al., 2005). To reduce the complexity of a biological sample, MS-based analysis is usually preceded by a separation step using liquid chromatography (LC), gas chromatography (GC), or capillary electrophoresis (CE). As metabolites with distinct physicochemical properties spend a different time in the separation dimension, the detection sensitivity of MS can thus be enhanced (Tebani et al., 2018a).

Compared to NMR spectroscopy, MS-based techniques generally provide a higher sensitivity and versatility (Johnson et al., 2016), allowing to detect a broader range of metabolites even at the pico- to femtomolar level (Lei, Huhman, & Sumner, 2011). However, inherent

limitations of MS are that it is destructive, requiring the separation and/or ionization of the compounds in a sample. MS procedures are also slower, less reproducible, and less quantitative than NMR analyses (Bujak et al., 2015; Tebani et al., 2018a).

In Table 2, the respective (dis)advantages of NMR- and MS-based analyses are summarized.

Table 2. Advantages and limitations of NMR- and MS-based metabolomics analysis. *(Own table).*

	NMR Spectroscopy	Mass Spectrometry
Advantages	• Non-destructive • High-throughput, rapid • Minimal sample manipulation • Higher reproducibility • More quantitative	• Higher sensitivity • Wider detection range • Use of separation techniques
Limitations	• Lower sensitivity • Signal overlapping • Smaller detection range	• Destructive • Slow • Lower reproducibility • Less quantitative (requires internal standards/calibration)

NMR: nuclear magnetic resonance.

2.3.3 A Typical Metabolomics Workflow

Since the data obtained by NMR- or MS-based analyses are considerably complex, requiring extensive data processing and bioinformatic methods to allow biological interpretation, the sequential steps of both targeted and untargeted metabolomics analyses shall be further described. As depicted in Figure 8, a metabolomics workflow usually includes the formulation of a biological question and selection of an experimental design, sample preparation, data acquisition and (pre-)processing, as well as statistical analyses, metabolite identification, and, subsequ ently, the functional interpretation of results (Tebani et al., 2018a).

Biological Question and Experimental Design

The initial step in any metabolomics workflow comprises the clear formulation of a biological problem that should be addressed. Based on the biological question, a suitable metabolomics approach (i.e., targeted or untargeted) has to be selected. Moreover, an appropriate study design (e.g., observational or interventional) with clear inclusion and exclusion criteria for participants (Broadhurst & Kell, 2006) as well as the type of biological samples, their collection and storage conditions have to be defined (Sussulini, 2017).

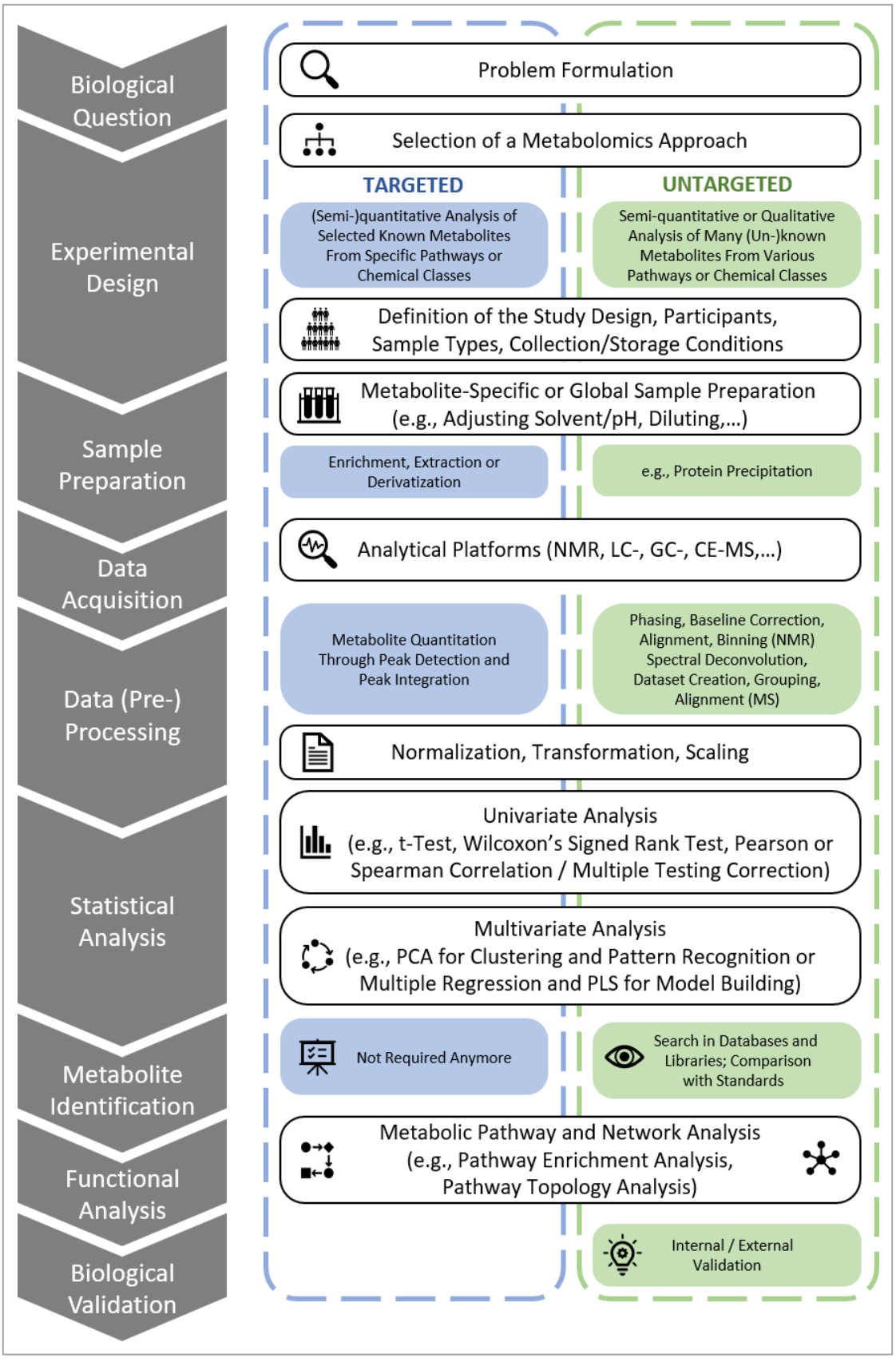

Figure 8. Metabolomics workflow comprising the consecutive steps of both targeted and untargeted analyses. CE: capillary electrophoresis; GC: gas chromatography; LC: liquid chromatography; MS: mass spectrometry; NMR: nuclear magnetic resonance; PCA: principal component analysis; PLS: partial least squares. *(Own illustration based on Sussulini (2017); Tebani et al. (2018a); Tebani, Afonso, and Bekri (2018b)).*

Sample Preparation and Data Acquisition

Once the study has been conducted, pre-analytical sample preparation steps are performed (Y. Wu & Li, 2016). While blood samples are often prepared by protein precipitation (Sussulini, 2017), an established method for urine sample preparation is dilution according to osmolality (Chetwynd, Abdul-Sada, Holt, & Hill, 2016). With regard to data acquisition, several analytical platforms are frequently combined, taking both the chemical diversity of metabolites and their discrepant concentration levels into account (Bowen & Northen, 2010; Sussulini, 2017).

Data (Pre-)Processing and Statistical Analysis

The (pre-)processing of metabolomics data differs between targeted and untargeted analyses. In targeted approaches, no analytical artefacts are carried through to the downstream analysis. Consequently, data processing is primarily focused on the quantification of selected metabolites, involving methods for peak detection and peak integration (Tebani et al., 2018b). In untargeted analyses, the acquired raw data have to be submitted to several pre-processing steps, including spectral deconvolution, dataset creation, grouping, and alignment for MS-based techniques or phasing, baseline correction, alignment, and binning for NMR (Sussulini, 2017). While baseline corrections consider systematic baseline distortions, alignment procedures correct for peak shifts due to pH, salt concentrations, or temperature. Binning reduces the dimension of the spectra by dividing them into segments, so-called “bins”, and assigning a respective value to each bin (Tebani et al., 2018b). As a result, computationally manageable data matrices are obtained (Tebani et al., 2018b). Finally, a standardization (i.e., normalization, transformation, and scaling) of metabolite data is commonly conducted (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006).

With regard to statistical analyses, it can be distinguished between uni-, bi-, and multivariate approaches. Uni- and bivariate analyses usually applied in metabolomics studies include parametric (e.g., t-test, Pearson correlation) or non-parametric (e.g., Wilcoxon’s signed rank test, Spearman correlation) methods that have the potential to detect how a single metabolite is linked to a particular outcome (Antonelli et al., 2019; Tebani et al., 2018b). If the study objective is to analyze how sets of multiple metabolites are associated with an outcome of interest, multivariate methods are preferable (Antonelli et al., 2019). The principal component analysis (PCA) is an example for so-called unsupervised approaches. By reducing the data dimensionality, PCA can reveal patterns or clusters which may underpin relationships between

the analyzed samples (Tebani et al., 2018b). As opposed, supervised methods like the partial least squares (PLS) regression intend to predict a specific outcome based on metabolomics data (Worley & Powers, 2013).

Metabolite Identification, Functional Analysis, and Biological Validation

After revealing the identity of relevant metabolites emanating from untargeted analyses by searching in databases or libraries and confirming it with internal standards (Sussulini, 2017), another key step to successfully comprehend metabolomics data is to link those metabolites to their biological role, particularly by determining their function in metabolic pathways and their interconnectivities (Johnson et al., 2016). Indeed, functional analysis tools like pathway enrichment analyses shift the focus from single metabolites to sets of functionally related metabolites, thus evaluating which pathways are most likely to be affected by the studied conditions. Network topology analyses complete this approach by calculating the impact of an altered metabolite according to its location within a pathway (Chong, Wishart, & Xia, 2019). Finally, the association of relevant metabolites with metabolic pathways can be utilized for providing a rationale to the original biological question (Sussulini, 2017). Also, functional analyses are often a starting point for new hypotheses that can direct future mechanistic investigations (Johnson et al., 2016). In general, metabolomics results should be proven by internal and external validation (Sussulini, 2017).

Moving closer to the focus of this dissertation, the next sub-section will provide insight into the application of metabolomics in the field of exercise science, emphasizing potentials and future perspectives of exercise metabolomics as well as the current state of research.

2.4 The Application of Metabolomics in the Field of Exercise Science

The earliest records for the use of metabolomics in the field of exercise science were made in 2007 and related publications have gradually been growing since then (Duft, Castro, Chacon-Mikahil, & Cavaglieri, 2017). As an emerging research area, exercise metabolomics can provide a global view of the metabolism's response to acute and chronic PA. Hence, its application is not only important for evaluating PA as an interfering factor which can influence the outcome of metabolomics-based studies (Daskalaki et al., 2015; Enea et al., 2010), but also to extend the current knowledge on metabolic adaptations to PE (Zierath & Wallberg-Henriksson, 2015).

2.4.1 Potentials and Perspectives of Exercise Metabolomics

As depicted below and in Figure 9, the possibilities for the application of metabolomics in the field of exercise science are continuously growing (Duft, Castro, Chacon-Mikahil, et al., 2017).

Firstly, given the fact that PE is characterized as an exogenous intervention disturbing the metabolic homeostasis by affecting various biochemical pathways (Egan & Zierath, 2013), a comprehensive characterization of exercise-induced metabolite changes is warranted. This challenge can be met with the use of metabolomics, which allows a systematic investigation of the metabolic response to distinct acute PE interventions or training protocols varying with regard to exercise mode, intensity, and duration in different populations (Duft, Castro, Chacon-Mikahil, et al., 2017). Consequently, a better understanding of metabolic pathways involved in the organism's response to exercise-induced homeostatic disturbances is likely to be obtained (Daskalaki et al., 2014).

Secondly, the application of metabolomics has the potential to investigate to what extent the body's response to PE is reflected in the metabolome of more or less easily collectable biological specimens, such as skeletal muscle tissue, blood, urine, saliva, or sweat (Schranner, Kastenmüller, Schönfelder, Römisch-Margl, & Wackerhage, 2020). Thus, the utility of different matrices for detecting exercise-induced metabolic perturbations can be thoroughly evaluated. In this respect, it is of special interest to explore the limits and advantages of non-invasively accessible samples like urine or saliva for metabolomics studies in order to find promising alternatives to more invasive tissue or blood sampling (Pitti et al., 2019; Sampson, Broadbent, Parker, Upton, & Parker, 2014; Siopi et al., 2017).

Thirdly, metabolomics can facilitate the discovery of PE-responsive biomarkers. As shown by recent studies, metabolomics not only has the ability to reveal metabolic signatures indicative for an increased metabolic demand during PE, but also allows to detect metabolic markers that reflect muscular damage or fatigue, thus providing insight into the pathomechanisms of musculoskeletal disorders or the current physiological state of athletes (Heaney et al., 2017; Manaf et al., 2018; Sampson et al., 2014; Yan et al., 2009). Besides, preliminary evidence that metabolomics might be useful in pinpointing metabolites associated with the training status, i.e., training adaptation or overtraining, has been provided (Enea et al., 2010; Neal et al., 2013). When focusing on elite sports, the detection of metabolic markers reflective of an

athlete's performance ability or trainability could also be facilitated (Schranner et al., 2020; Yan et al., 2009). Additionally, metabolomics opens new perspectives if aiming to identify metabolic markers or metabolite patterns associated with a low PA behavior or PF (Heaney et al., 2017; Xiao et al., 2016), which might also function as early biomarkers of chronic disease risk (Bye et al., 2012; Duft, Castro, Chacon-Mikahil, et al., 2017). Considering the fact that it is not yet fully elucidated how lifestyle factors like PA or health-related phenotypes like the CRF correlate with metabolic pathways (Floegel et al., 2014), metabolomics seems a promising way to enhance the understanding of mechanisms and metabolic pathways mediating the association of a high PA or PF level with low morbidity and mortality (Heaney et al., 2017; Kujala et al., 2019).

Fourthly, metabolomics also permits to evaluate in how far the metabolic response to PE is modulated by characteristics of the exercising individual, including age, sex, health, or the nutritional state. As suggested by Neufer et al. (2015), the so-called "exercise responsome" might provide a benchmark against which those traits could be compared for similarities and specificities. Hence, differences in the metabolic effects of PE between women and men, younger and older, or healthy and diseased individuals are likely to be revealed.

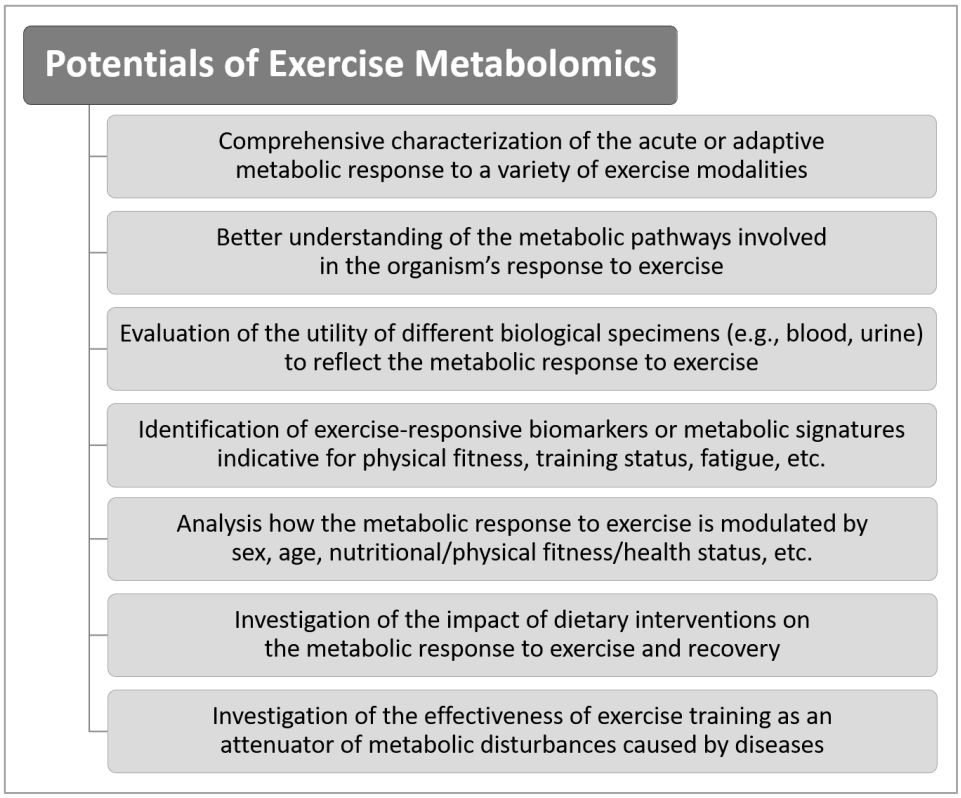

Figure 9. Potentials of metabolomics research in an exercise-related context. *(Own illustration).*

Lastly, metabolomics represents a suitable method to examine the impact of dietary interventions on metabolic perturbations during PE and recovery (Nieman et al., 2012). Furthermore, it has the potential to examine the effectiveness of PE interventions aiming to attenuate metabolic disturbances caused by diseases (Duft, Castro, Chacon-Mikahil, et al., 2017; Grapov et al., 2019).

Before considering future perspectives for the practical application of metabolomics-based findings, it has to be stated that the majority of exercise metabolomics studies still have a rather exploratory and thus hypothesis-generating character (Duft, Castro, Chacon-Mikahil, et al., 2017). Once further studies will have succeeded in confirming hypotheses regarding metabolic processes underlying the health- or performance-enhancing effects of PA and in replicating and validating exercise-responsive biomarkers in different study populations, this information might one day be translated into a sports or exercise science context (Duft, Castro, Chacon-Mikahil, et al., 2017; Heaney et al., 2017). In fact, findings generated by metabolomics studies could be utilized by exercise physiologists, clinicians, or health-care practitioners (Heaney et al., 2017; Zierath & Wallberg-Henriksson, 2015). As summarized in Figure 10, potential applications could reach from the development of more personalized exercise prescription concepts or a better assessment of the efficiency of training regimens to the prediction of the PF status or future events such as the susceptibility to muscular injury or diseases (Bongiovanni et al., 2019; Daskalaki et al., 2014; Heaney et al., 2017).

More specifically, it is believed that a more comprehensive knowledge on the metabolic response to PE might allow clinicians and sport scientists to recommend exercise with greater clarity to expected adaptations (Zafeiridis et al., 2016). Furthermore, it has been suggested that validated biomarkers could offer a means to facilitate the monitoring of an athlete's physiological state, i.e., physical capacity, fatigue, or overtraining (Heaney et al., 2017; Manaf et al., 2018). In this context, there evidently is a growing interest in non-invasively detectable biomarkers. As supposed by Sampson et al. (2014), it is more likely that biomarkers of muscle injury or fatigue will be implemented in (sub-)elite sports if measurements can be conducted non-invasively, for instance in urine, saliva, or sweat samples.

In addition to that, novel diagnostic or prognostic metabolic indicators might be yielded, permitting clinicians to conclude about an individual's CRF status and to identify people at risk

for cardiometabolic diseases (Heaney et al., 2017; Sampson et al., 2014; Zierath & Wallberg-Henriksson, 2015). A further possibility for the application of PA-related markers has been discussed by Daskalaki et al. (2015), who pointed out the difficulty to reveal differences between study groups in human metabolomics studies since they often do not consider the participants' PA levels, that are, however, a frequent cause of inter-individual variability in metabolomics analyses. Thus, it has been proposed that future metabolomics studies could normalize for an individual's PA level by using single or multiple metabolites that were proven to be affected by PA (Daskalaki et al., 2015).

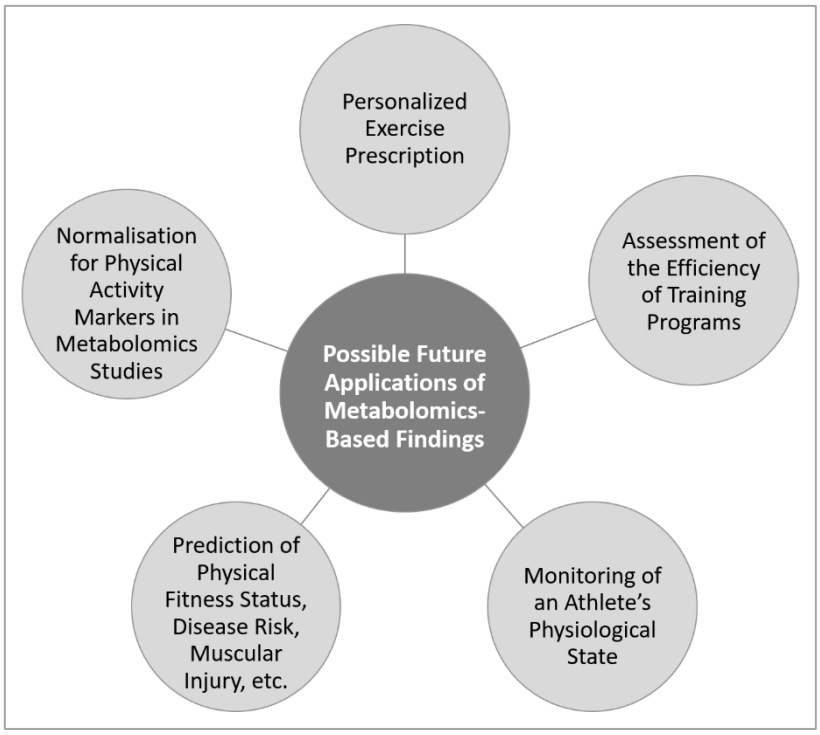

Figure 10. Possible future applications of exercise metabolomics-based findings. *(Own illustration).*

To conclude, the scientific endeavor for studying the metabolic effects of PA by using metabolomics techniques is still emerging (Sakaguchi, Nieman, Signini, Abreu, & Catai, 2019) and especially the practical application of exercise metabolomics-based findings remains to be fully discovered and clearly determined (Duft, Castro, Chacon-Mikahil, et al., 2017; Heaney et al., 2017). Unfortunately, the required knowledge and high costs for complex metabolomics analyses forbid an easy and quick analysis of relevant biomarkers (Heaney et al., 2017). Thus, challenges that have to be met in the future refer, amongst others, to the development of specific technologies for biochemical analyses which can be directly conducted under field conditions, i.e., in a practical sport-related setting (Zielinski & Kusy, 2015a).

In the following sub-section, an overview of publications using metabolomics approaches to examine the effects of PA on the human metabolome will be provided.

2.4.2 Current State of Research

During the past decade, an increasing number of PA-related metabolomics studies have been published (Belhaj, Lawler, & Hoffman, 2021; Kelly et al., 2020). While the primary focus of most interventional studies lay on the response of the human metabolome to acute bouts of different exercise types or, to a lesser extent, medium- (i.e., < 3 months) and long-term (i.e., ≥ 3 months) training protocols, observational studies predominantly aimed to examine the relationship between metabolite profiles and measures of chronic PA, thus revealing metabolic differences between physically active and inactive individuals (Daskalaki et al., 2014; Heaney et al., 2017; Schranner et al., 2020; Tian, Corkum, Moaddel, & Ferrucci, 2021).

With regard to metabolomics studies focusing on acute PE or training interventions, a high percentage was dedicated to the effects of endurance or intermittent exercise. Comparatively little research has so far been addressed to other exercise types, such as team sports (Pitti et al., 2019; Ra, Maeda, Higashino, Imai, & Miyakawa, 2014; Santone et al., 2014) or resistance exercise (Berton et al., 2017; Coelho et al., 2016; Valério et al., 2017; Yde, Ditlev, Reitelseder, & Bertram, 2013). The present thesis comprises the analysis of alterations in the human metabolome in response to a ten-day training intervention consisting of high-intensive interval exercise or a cardiorespiratory endurance test characterized by an acute bout of incremental exercise. Therefore, the current state of research on the effects of both HIIT and acute endurance exercise will particularly be focused in the following.

With respect to metabolomics studies evaluating the chronic effects of PA on the human metabolome, it can be distinguished between studies aiming to reveal how inter-individual differences in metabolite profiles are linked to either habitual PA or PF and studies conducting long-term training regimens in order to analyze how improvements in the individual PF status are associated with training-induced metabolite alterations. The present thesis includes the investigation of the relationship between the resting human metabolome and the CRF as a surrogate measure of chronic PA in a cross-sectional study. Thus, previous studies applying the former approach are focused when depicting the current state of research in the following.

Metabolomics Studies Focusing on HIIT

Considering the current state of research until July 2019 (publication date of *Study I*) on the effects of HIIT on metabolite profiles in humans, it becomes obvious that a rather limited number of studies have investigated HIIT-induced alterations in the human metabolome so far. An overview of metabolomics studies analyzing metabolite alterations in response to acute or medium-term HIIT is provided in Table 3.

The majority of studies have analyzed the effects of single HIIT or sprint interval bouts on metabolite profiles in blood, urine, or muscle tissue, giving insight into the acute response of human metabolism to HIIT. Although different study protocols regarding type, intensity, and duration of HIIT sessions and distinct metabolomics approaches were used in these acute HIIT studies, changes were generally documented for compounds of carbohydrate metabolism (e.g., lactate, pyruvate), TCA cycle intermediates (e.g., citrate, succinate), or AAs. However, studies analyzing medium- or long-term alterations of the human metabolome in response to HIIT are rather scarce. One study investigating the effect of an eight-week sprint interval training on 33 serum metabolites in young males showed a training-induced rise in resting and post-exercise citrate, taurine, and trimethylamine N-oxide (TMAO), as well as lower levels of resting and post-exercise lactate and pyruvate (Pechlivanis et al., 2013). Kuehnbaum, Gillen, Gibala, and Britz-McKibbin (2014) provided the first evidence of metabolic adaptations to a six-week HIIT being reflected in the blood metabolome. Indeed, they showed a HIIT-induced increase in plasma carnitine at rest, hinting at an improved oxidative capacity, and an attenuated post-exercise increase in plasma hypoxanthine after HIIT intervention, pointing to a lower energetic stress in the trained status. Yet, those studies focusing on medium-term HIIT have some limitations. Firstly, results are partly restricted to sedentary, overweight females (Kuehnbaum et al., 2014) and HIIT interventions were performed with a rather moderate frequency (Kuehnbaum et al., 2014; Pechlivanis et al., 2013). Secondly, metabolomics analyses were limited to a comparatively small number of blood metabolites, ranging from 33 (Pechlivanis et al., 2013) to 41 (Kuehnbaum et al., 2014) analytes.

Despite the fact that HIIT has drawn much attention in recent years due to its ability to elicit endurance-like improvements in skeletal muscle metabolism (Cassidy et al., 2017), the application of metabolomics to examine HIIT-induced metabolic alterations has been limited to a few studies. Apart from the need to extend the general knowledge on the effects of HIIT

Table 3. Overview of metabolomics studies focusing on HIIT. *(Own table).*

Nr.	Author (Year)	Subjects		Exercise Intervention			Biological Specimen	Sampling Time	Method (Metabolites)	Main Metabolite Alterations
		n (♂/♀)	State	Type	Duration/Frequency	Intensity				
1	Pechlivanis et al. (2010)	12 (12/0)	Healthy, young, trained	Acute (running sprint intervals)	3 sets of 2x80m runs separated by 10s or 1min with 20min rest between sets	Maximal	Urine	Pre- and 35min post-exercise	NMR (43)	↑: *Lactate, Alanine, Pyruvate, Hypoxanthine, 2-Hydroxyisovalerate, 3-Hydroxyisobutyrate, 2-Oxoisovalerate, 2-Oxoisocaproate, 3-Methyl-2-Oxovalerate, 2-Hydroxybutyrate, 2-Oxoglutarate, Fumarate* ↓: *Citrate, Glycine, Formate, Histidine, Tryptophan, TMAO*
2	Pechlivanis et al. (2013)	14 (14/0)	Healthy, young, trained	Training (running sprint intervals)	8wk à 3 sessions with 2-3 sets of 80m runs	Maximal	Serum	Pre- and 35min post-exercise [before/after training]	NMR (33)	↑ (post-exercise): *Lactate, Pyruvate, Acetoacetate/Acetone* ↓ (post-exercise): *Leucine, Valine, Isoleucine, Arginine/Lysine, Glycine, Methylguanidine* ↑ (post-training, pre-exercise): *Citrate, Taurine, TMAO, Acetoacetate/Acetone, Valine* ↓ (post-training, pre- and post-exercise): *Lactate, Pyruvate*
3	Peake et al. (2014)	10 (10/0)	Healthy, young athletes	Acute (HIIT, bicycle)	10x4min intervals at 80% VO_{2max} with 2min rest at 50w between intervals	Vigorous	Plasma	Pre- and 0min, 1h, and 2h post-exercise	GC-MS (49)	↑: *Citrate, Succinate, Aconitate, Malonate, Alanine, Glutamate, Tyrosine, 4-Methyl-2-Oxopentanoate, 3-Methyl-2-Oxopentanoate, Myristate, Dodecanoate, Decanoate, Palmitoleate, Heptadecenoate, Myristoleate, Oleate* (0min)
4	Danaher et al. (2015)	7 (7/0)	Healthy, young, active	Acute (HIIT, bicycle)	30x20s intervals at 150% VO_{2max} with 40s rest or 30x10s intervals at 300% VO_{2max} with 50s rest	Supra-maximal	Plasma	Pre- and 0min, 0.5h, and 1h post-exercise	GC-MS (55)	↑: *Lactate, Malate, Alanine* (150%, 300% VO_{2max}), *Citrate* (150% VO_{2max}), *Fructose, Sorbose, Cholesterol* (300% VO_{2max}) ↓: *Citrate, Asparagine, Lysine, Octadecanoate, Hexadecanoate, Erythronate, Xylitol, Xylose* (300% VO_{2max})
5	Kuehnbaum et al. (2015)	9 (0/9)	Obese, young, untrained	Training (HIIT, bicycle)	6wk à 3 sessions with 10x1min intervals at 90% HR_{max} and 1min rest at 50w between intervals	Vigorous	Plasma	Pre- and 0min, 20min post-exercise [before /after training]	MSI-CE-MS (41)	↑ (post-exercise): *Carnitine, Acetylcarnitine, Hypoxanthine* ↑ (post-training): *Carnitine* (pre-exercise), *Acetylcarnitine* (post-exercise) ↓ (post-training): *Hypoxanthine* (post-exercise)
6	Pechlivanis et al. (2015)	17 (17/0)	Healthy, young, trained	Acute (running sprint intervals)	3x80m runs with 10 min between the 1st and 2nd run and 10s between the 2nd and 3rd run	Maximal	Urine	Pre- and 1h, 1.5h, and 2h post-exercise	NMR and RP-UPLC-MS (42)	↑: *Hypoxanthine, Inosine* (1h, 1.5h, and 2h), *Lactate, Acetate* (1h and 1.5h), *2-Hydroxyisovalerate, 2-Hydroxybutyrate, 2-Oxoisocaproate, 3-Methyl-2-Oxovalerate, 3-Hydroxyisobutyrate, 2-Oxoisovalerate, 3-Hydroxybutyrate, 2-Hydroxyisobutyrate, Alanine, Pyruvate, Fumarate* (1h) ↓: *Valine, Isoleucine, Succinate, Citrate, Trimethylamine, TMAO, Tyrosine, Formate* (1h and/or 1.5h), *Creatinine, Glycine, Hippurate, 4-Aminohippurate* (1h, 1.5h, and 2h)
7	Zafeiridis et al. (2016)	9 (9/0)	Healthy, young, trained	Acute (HIIT, treadmill)	30s intervals at 110% MAV with 30s rest or 3min intervals at 95% MAV with 3min rest	Supra-maximal/ maximal	Plasma	Pre- and 0-5min post-exercise	NMR (17)	↑: *Glucose, Lactate, Pyruvate, Glycerol, Alanine* (higher after 3min intervals), *Citrate* (higher after 3min intervals), *Succinate, Citrate/Succinate, Lactate/Pyruvate, Lactate/Citrate* ↓: *Glutamine, Proline* (3min intervals)
8	Siopi et al. (2017)	14 (14/0)	Healthy, middle-aged, sedentary	Acute (HIIT, treadmill)	4x4min intervals at 90% HR_{max} with 4min rest at 3km/h	Vigorous	Urine	Pre- and 2h, 4h, and 24h post-exercise	UPLC-MS/MS (64)	↑: *Alanine, Arginine, Asparagine, Betaine, Citrulline, Glutamate, Glutamine, Glycine, Histidine, Homocysteine, Leucine-Isoleucine, Lysine, Methionine, Valine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, γ-Aminobutyrate, Mannitol, Lactate, Pyruvate, Adenosine, Cytosine, Guanine, Thymine, Uracil, 2-Hydroxyisobutyrate, 4-Hydroxyphenyllactate, Acetylcarnitine, Creatine, Hypotaurine, Hypoxanthine, Inosine, Kynurenate, Taurine, TMAO, Xanthine* (2h) ↓: *Citrate, Thiamine, Methylamine* (2h)

Table 3 *(continued)*. Overview of metabolomics studies focusing on HIIT. *(Own table).*

Nr.	Author (Year)	Subjects		Exercise Intervention			Biological Specimen	Sampling Time	Method (Metabolites)	Main Metabolite Alterations
		n (♂/♀)	State	Type	Duration/Frequency	Intensity				
9	Saoi et al. (2019)	7 (7/0)	Healthy, young, active	Acute (HIIT, bicycle)	10x60s intervals at 90% HR_{max} with 60s recovery at 50w	Vigorous	Plasma, muscle	Pre- and 0min, 3h post-exercise	MSI-CE-MS (84)	↑: *Alanine, Lactate, Acetylcarnitine* (plasma/muscle, 0min), *Malate, Urate* (muscle, 0min), *3-Hydroxybutyrate, Succinate, Glutamate, Tryptophan* (plasma, 0min) ↓: *Glutamate* (muscle, 0min)
10	Siopi et al. (2019)	14 (14/0)	Healthy, middle-aged, sedentary	Acute (HIIT, treadmill)	4x4min intervals at 90% HR_{max} with 4min rest at 3km/h	Vigorous	Serum	Pre- and 0min, 1h post-exercise	UPLC-MS/MS (46)	↑: *Alanine, Glutamate, Histidine, γ-Aminobutyrate, Lactate, Pyruvate, Pantothenate, Acetyl-carnitine, Creatine, Cystine, Dimethylamine, Hypoxanthine, Taurine, Xanthine* (1h) ↓: *Guanine, Inosine* (1h)

CE: capillary electrophoresis; GC: gas chromatography; h: hour(s); HIIT: high-intensity interval training; HR_{max}: maximum heart rate; km: kilometer(s); m: meter(s); MAV: maximal aerobic velocity (here: speed at which VO_{2max} was first achieved and maintained for at least one minute); min: minute(s); MS: mass spectrometry; MSI: multi-segment injection; MS/MS: tandem mass spectrometry; n: sample size; NMR: nuclear magnetic resonance; RP: reversed phase; s: second(s); TMAO: trimethylamine N-oxide; UPLC: ultra-performance liquid chromatography; VO_{2max}: maximal oxygen uptake; w: watt; wk: week(s).

interventions on the metabolome of sedentary or recreationally active individuals, an important research gap is linked to the lack of information on alterations in metabolite profiles due to strenuous HIIT programs in already trained persons. Especially in pre-competition phases of elite sports, athletes often complete high-intensive exercise blocks, thus provoking a higher adaptation in performance and an improved resistance against fatigue (Hawley, Myburgh, Noakes, & Dennis, 1997; Laursen & Jenkins, 2002; Tschakert & Hofmann, 2013). In fact, the more intense exercise stimuli were performed with a high frequency, the more intense adaptations, but also overtraining, can be caused (Rodas, Ventura, Cadefau, Cussó, & Parra, 2000). Consequently, a comprehensive assessment of metabolite changes to HIIT protocols usually applied in periodized training schedules is required. By measuring HIIT-induced alterations in the metabolome of individuals with an athletic background, possible biomarkers indicative for either training adaptation or training overload could be identified. Furthermore, metabolomics investigations have the potential to elucidate which metabolic pathways are linked to the phenotypical response of the human organism to strenuous intermittent training. Finally, with regard to future studies, it should be especially evaluated to what extent easily accessible, non-invasive biological matrices like urine or saliva are able to reflect HIIT-related adaptations in human metabolism.

Metabolomics Studies Focusing on Acute Endurance Exercise

Regarding the current state of research until May 2020 (publication date of *Study IIa*) on the effects of endurance exercise on metabolite profiles in humans, the majority of conducted studies investigated how the metabolome responds to single bouts of cycling or running. In contrast, relatively few metabolomics studies have focused on medium- or long-term effects of endurance training (Brennan et al., 2018; Duft, Castro, Bonfante, et al., 2017; Felder et al., 2017; Huffman et al., 2014; Neal et al., 2013; Yan et al., 2009). An overview of those studies analyzing metabolic alterations in response to acute endurance exercise is given in Table 4. Studies were not listed if exercise interventions lasted longer than one day (Karl et al., 2017; Zauber, Mosler, von Hessberg, & Schulze, 2012).

What differs most between the presented studies is the duration and intensity of acute continuous exercise, ranging from 18 minutes to 9 hours or from moderate to maximal, respectively. While a high percentage of the studies used continuous PE protocols of shorter

Table 4. Overview of metabolomics studies focusing on acute endurance exercise. *(Own table).*

Nr.	Author (Year)	Subjects		Exercise Intervention			Biological Specimen	Sampling Time	Method (Metabolites)	Main Metabolite Alterations
		n (♂/♀)	State	Type	Duration	Intensity				
1	Pohjanen et al. (2007)	24 (24/0)	Healthy, young, trained	Acute (continuous, bicycle)	9x10min (2min at 40%, 6min at 60%, 2min at 85% VO_{2peak})	Sub-maximal, varying	Serum	Pre- and 0min post-exercise	GC/TOF-MS (non-targeted)	34 altered metabolites (only *Glycerol* and *Asparagine* were identified).
2	Enea et al. (2010)	22 (0/22)	Healthy, young, untrained (n=10)/trained (n=12)	Acute (continuous, bicycle)	Until exhaustion (26.7 ± 1.8min to 41.1 ± 4.0min) at 75% VO_{max}	Vigorous	Urine	Pre- and 30min post-exercise	NMR (11)	Multivariate analysis did not reveal any distinction of the pre- and post-exercise urinary metabolite profile. No results of univariate analysis provided.
3	Lehmann et al. (2010)	21 (21/0)	Healthy, young	Acute (continuous, treadmill)	60min at 75% VO_{2peak} (n=13)/120min at 55% VO_{2peak} (n=8)	Vigorous/ moderate	Plasma	Pre- and 0h, 3h, 24h post-exercise	LC-MS (non-targeted)	↑: *Medium-chain Acylcarnitines (C6:0, C8:0, C10:0, C10:1, C12:0, C14:2)*
4	Lewis et al. (2010)	70 (60/10)	Middle-aged	Acute (continuous, treadmill)	Until exhaustion	Incre-mental	Plasma	Pre- and 0h, 1h post-exercise	LC-MS (200)	↑: *Lactate, Pyruvate, Alanine, Glycerol, Pantothenate, Methionine, Niacinamide, Glutamine, Inosine, Hypoxanthine, Xanthine*
		25 (19/6)	Amateur runners	Acute (continuous, marathon)	180-300min	Self-paced	Plasma	Pre- and 10min post-exercise	LC-MS (200)	↑: *Lactate, Pyruvate, Glycerol, β-Hydroxybutyrate, Allantoin, Kynurenate* ↓: *Alanine, Threonine, Serine, Proline, Valine, Histidine, Glutamine, Asparagine*
5	Netzer et al. (2011)	30 (22/8)	Healthy, trained	Acute (continuous, bicycle)	Until exhaustion	Incre-mental	Capillary blood	Pre- and 0min post-exercise	MS/MS (60)	↑: *Lactate, Alanine, Short-chain Acylcarnitines (C2, C3)*
6	Chorell et al. (2012)	27 (27/0)	Healthy, young, trained	Acute (continuous, bicycle)	65min (20min at 55%, 25min at 70%, 10min at 55%, 2min at 100%, 8min at 30% VO_{max})	(Sub-) maximal, varying	Plasma	Pre- and post-exercise	GC-TOF-MS (non-targeted, 50 identified)	↑: *Hypoxanthine, Taurine, Ribose, β-D-Methylglucopyranoside, Inositol, Citrate, β-Alanine, Malate* ↓: *Tryptophan, Threonine, Threonate, Valine, Isoleucine*
7	Krug et al. (2012)	15 (15/0)	Healthy, young	Acute (continuous, bicycle)	30min at anaerobic threshold	Vigorous	Plasma	Pre- and 15, 30, 45, 60min post-exercise	MS/MS, NMR (163)	↑: *Lactate, Acylcarnitines* ↓: *Carnitine*
8	Thysell et al. (2012)	24 (24/0)	Healthy, young, trained	Acute (continuous, bicycle)	9x10min (2min at 40%, 6min at 60%, 2min at 85% VO_{2peak})	Sub-maximal, varying	Serum	Pre- and 0min post-exercise	GC/TOF-MS (non-targeted)	↑: *Alanine, Inosine, Fatty Acids* ↓: *Asparagine, Lysine, Serine, Phenylalanine, Methionine, Arginine, Ornithine, Proline, Histidine, Valine, Isoleucine, Allothreonine, Tryptophan*
9	Mukherjee et al. (2014)	17 (17/0)	Healthy, middle-aged, trained (n=9)/ untrained (n=8)	Acute (continuous, bicycle)	45min at 60% WR_{peak}, followed by 90% WR_{peak} until exhaustion	Sub-maximal (moderate to vigorous)	Urine	Pre- and post-exercise (24h-samples)	NMR (32)	↑: *Lactate* (trained < untrained), *Pyruvate, Acetate* (trained < untrained), *Malonate* (trained < untrained), *Fumarate* (trained > untrained), *Hypoxanthine* (trained > untrained), *2-Hydroxybutyrate, 2-Hydroxyisovalerate, 2-Oxoisocaproate, 2-Oxoisovalerate, 3-Hydroxyisobutyrate, Alanine, Inosine* ↓: *3-Hydroxybutyrate, Citrate, Glycine, Formate, Succinate, Hippurate, TMAO, Tryptophan*

Table 4 *(continued)*. Overview of metabolomics studies focusing on acute endurance exercise. *(Own table).*

Nr.	Author (Year)	Subjects		Exercise Intervention			Biological Specimen	Sampling Time	Method (Metabolites)	Main Metabolite Alterations
		n (♂/♀)	State	Type	Duration	Intensity				
10	Nieman et al. (2014)	19 (19/0)	Healthy, trained	Acute (continuous, 75km cycling)	2.71 ± 0.07h	Sub-maximal, self-paced	Plasma	Pre- and 0h, 1.5h, 21h post-exercise	UHPLC-MS/MS, GC-MS (non-targeted, 423)	↑: 80 known metabolites with at least a twofold increase, e.g., *13- and 9-Hydroxy-Octadecadienoate*, *Linoleate*, *Linolenate*, *Arachidonate*, *Adrenate*, *Dicarboxylate and Long-chain Fatty Acids*
11	Peake et al. (2014)	10 (10/0)	Healthy, young athletes	Acute (continuous, bicycle)	61min ± 14s at 65% VO_{2max}	Moderate	Plasma	Pre- and 0min, 1h, and 2h post-exercise	GC-MS (49)	↑: *Succinate, Malonate, Myristic acid, Dodecanoic acid, Decanoic acid, Palmitoleic acid, Heptadecenoic acid, Linolenic acid* ↓: *Leucine, Isoleucine, Valine, Methionine, Alanine, Proline*
12	Breit et al. (2015)	47 (27/20)	Healthy, trained	Acute (continuous, bicycle)	Until exhaustion	Incre-mental	Capillary blood	Pre-, during, and post-exercise	MS/MS (100)	↑: *Acetyl-, Propionyl-, Valeryl-, Butyryl-, Methylmalonyl-, Hydroxyvaleryl-, Octadecadienyl-, Octadecanoyl-, Hexadecanoyl-, Octadecenoylcarnitine, Alanine, Arginine, Glucose, Ornithine, Tryptophan, Methionine, Histidine, Phenylalanine, Citrulline, Glutamate, Tyrosine, Glycine, Lysine, Proline, Serine, Threonine, Valine* ↓: *Aspartate, Carnitine*
13	Muhsen-Ali et al. (2016)	10 (8/2)	Healthy, active	Acute (continuous, bicycle)	45min (5min at 50w, 15min at 40%, 15min at 50%, 10min at 60% WR_{max})	Sub-maximal, varying	Urine	Pre- and 1h, 4h, 7h post-exercise	LC-MS (57)	↑: *Hypoxanthine, Guanine, Urate, 1-Methylurate, Deoxyinosine, Inosine, Xanthosine, Oxobutanoate, 4-Aminobutanoate, Hydroxybutyric acid sulfate, Lactate, 4-Hydroxy-hepantonylglycine, Nonanoyl-, Decanoyl-, Keto-Decanoylcarnitine, Pantothenate, Phenylacetylglycine, Dihydroxyphenyllactate* (1h) ↓: *Glycylproline, Glycylleucine, Xylitol, Rhamnose, Arabinonate, Glucuronate, Propanoylcarnitine, Dihydroxyoxocholanate, Cholate, Threonine, Asparagine, Leucine, Methionine, N-Acetylleucine, N-Acetylglutamine, N-Acetylcitrulline, Riboflavine* (1h)
14	Wallner-Liebmann et al. (2016)	23 (5/18)	Healthy	Acute (continuous, bicycle)	45min between the aerobic and anaerobic threshold	Moderate	Saliva	Pre- and post-exercise	NMR (non-targeted)	Multivariate analysis revealed differences between the saliva samples collected before and after exercise. No single metabolite alterations provided.
15	Zafeiridis et al. (2016)	9 (9/0)	Healthy, young, trained	Acute (continuous, treadmill)	18min at 80% MAV	Vigorous	Plasma	Pre- and 0-5min post-exercise	NMR (17)	↑: *Glucose, Lactate, Pyruvate, Glycerol, Alanine, Citrate, Succinate, Citrate/Succinate, Lactate/Pyruvate, Lactate/Citrate* ↓: *Glutamine, Proline*
16	Nieman et al. (2017)	24 (24/0)	Healthy, trained	Acute (continuous, treadmill)	Until exhaustion (2.26 ± 0.01 h) at 70% VO_{2max}	Vigorous	Plasma	Pre- and 0min post-exercise	UHPLC-MS/MS, GC-MS (non-targeted, 380)	↑: 166 identified metabolites, e.g., *Glycerol*, *Long- and Medium-chain Fatty Acids, Fatty Acid Oxidation Products (Dicarboxylate, Monohydroxy Fatty Acids), Acylcarnitines, Lactate, Succinate, Malate, Hypoxanthine, Ketone Bodies* ↓: 43 identified metabolites, e.g., *Cholate, Glycocholate, Alanine, Asparagine, Proline, Isoleucine, Hydroxyproline, Leucine, Serine, Choline, Betaine*
17	Siopi et al. (2017)	14 (14/0)	Healthy, middle-aged, sedentary	Acute (continuous, treadmill)	36min at 65% HR_{max}	Moderate	Urine	Pre- and 2h, 4h, and 24h post-exercise	UPLC-MS/MS (64)	↑: *Alanine, Arginine, Asparagine, Citrulline, Glutamate, Glutamine, Glycine, Histidine, Homocysteine, Leucine-Isoleucine, Lysine, Methionine, Norvaline-Valine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, γ-Aminobutyrate, Mannitol, Pyruvate, Adenosine, Cytosine, Guanine, Thymine, Uracil, Uridine, Acetylcarnitine, Creatine, Hypotaurine, Hypoxanthine, Inosine, Kynurenate, Taurine, TMAO, Urate, Xanthine* (2h) ↓: *Citrate, Riboflavin* (2h)

Table 4 *(continued)*. Overview of metabolomics studies focusing on acute endurance exercise. *(Own table).*

Nr.	Author (Year)	Subjects		Exercise Intervention			Biological Specimen	Sampling Time	Method (Metabolites)	Main Metabolite Alterations
		n (♂/♀)	State	Type	Duration	Intensity				
18	Davison et al. (2018)	24 (24/0)	Healthy, young, trained	Acute (continuous, treadmill)	60min at 75% VO_{2max}	Vigorous	Serum	Pre- and 0h, 3h post-exercise	LC-MS (non-targeted)	↑: *Propionyl-, Butyryl-, Hexanoyl-, Octanoyl-, Decanoyl-, Undecenoyl-, 4,8-Dimethylnonanoyl-, Dodecenoyl-, Tridecenoyl-, Tetradecadienoyl-, Hexadecatetraenoyl-, Hexadecadienoyl-, Palmitoyl-, Elaidiccarnitine, Hippurate, Myristate, Palmitate, Linolenate, Linoleate, Arachidonate, Adenine, Adenosine*
19	Howe et al. (2018)	9 (9/0)	Healthy, young, trained	Acute (continuous, 80.5km treadmill)	9h 17min ± 1h 18min	Sub-maximal, self-paced	Plasma	Pre- and post-exercise	LC-MS (non-targeted, 446)	↑: e.g., *Long-, Medium-, and Short-chain Acylcarnitines, Fatty Acids, Oxidized Fatty Acids, Acetylcarnitine, Hypoxanthine, Kynurenate* ↓: e.g., *Alanine, Glycine, Serine, Leucine, Isoleucine, Proline, Threonine, Valine, Betaine, Taurine, Arginine, Citrulline, Glycocholate, Taurocholate, Pseudouridine*
20	Manaf et al. (2018)	18 (18/0)	Healthy, young, active	Acute (continuous, bicycle)	Until exhaustion (80.9 ± 13.6min) at an intensity corres-ponding to 3mmol/L blood lactate (110.3 ± 23.0w)	Moderate	Plasma	Pre-exercise, after 10 and 70min of cycling, as well as 0 and 20min post-exercise	LC-MS (80)	Multivariate analysis revealed alterations in the plasma metabolome during exercise. ↑: e.g., *Palmitate, Oleate, Linoleate, Glycerol, Oleoyl-, Palmitoyl-, Acetylcarnitine, 5-Methoxy-3-Indoleacetate, Indole, Indole-3-Lactate, γ-Aminobutyrate, Creatine* ↓: e.g., *Tryptophan, Indole-3-Acetate, Carnitine*
21	Stander et al. (2018)	31 (19/12)	Healthy, trained	Acute (continuous, marathon)	4h 19min ± 49min	Sub-maximal, self-paced	Serum	Pre- and post-exercise	GC × GC-TOF-MS (untargeted, 838)	↑: e.g., *α-Hydroxybutyrate, β-Hydroxybutyrate, β-Hydroxyisobutyrate, Acetoacetate, Citrate, Fumarate, Glycerol, Laurate, Linoleate, Malate, Mannitol, Myristoleate, Oleate, Palmitate, Palmitoleate, Pentadecanoate, Pyruvate, Succinate, Threonate* ↓: e.g., *α-Aminomalonate, Alanine, Aspartate, Glycine, Leucine, Methionine, Phenylalanine, Serine, Tyrosine, Valine*
22	Siopi et al. (2019)	14 (14/0)	Healthy, middle-aged, sedentary	Acute (continuous, treadmill)	36min at 65% HR_{max}	Moderate	Serum	Pre- and 0min, 1h post-exercise	UPLC-MS/MS (46)	↑: *Glutamate, Histidine, Putrescine, Pantothenate, 3-Methylhistidine, Acetylcarnitine, Cystine, Xanthine* (0min), *γ-Aminobutyrate, Guanine, Pyruvate* (1h) ↓: *Tyrosine, Pantothenate, 2-Hydroxyisobutyrate, Dimethylamine, Inosine* (0min)
23	Schader et al. (2020)	76 (76/0)	Amateur runners	Acute (continuous, marathon)	225.1 ± 43.1min	Sub-maximal, self-paced	Plasma	Pre- and 0h, 1d, 3d post-exercise	LC-/FIA-MS/MS (188)	↑: *Acylcarnitines* ↓: *Amino Acids, Phospholipids*
24	Shi et al. (2020)	20 (20/0)	Experienced amateur runners	Acute (continuous, marathon)	< 3h	Sub-maximal, self-paced	Serum	1d pre-exercise, within 1h post-exercise	LC-MS/MS (untargeted)	↑: *Pyruvate, Malate, cis-Aconitate, Galacturonic Acid, Methyl-Fumaric Acid, Alanine, Tyrosine, Phenylalanine, Glycerol, Glyceric Acid, Octanoic Acid, Quinic Acid, Threonate, myo-Inositol, D-Galacturonic Acid, trans-Cinnamate, Vanillin, Ribitol, Hypoxanthine, Adenine, Theophylline, Theobromine* ↓: *Glucosamine, Succinyl-L-Homoserine, Serine, Valine, Asparagine, Inosine*

d: day(s); FIA: flow injection analysis; GC: gas chromatography; GC × GC: two-dimensional gas chromatography; h: hour(s); HR_{max}: maximum heart rate; kg: kilogram(s); km: kilometer(s); LC: liquid chromatography; MAV: maximal aerobic velocity (here: speed at which VO_{2max} was first achieved and maintained for at least one minute); min: minute(s); MS: mass spectrometry; MS/MS: tandem mass spectrometry; n: sample size; NMR: nuclear magnetic resonance; P_{max}: maximal power; RP: reversed phase; s: second(s); TMAO: trimethylamine N-oxide; TOF: time of flight; U(H)PLC: ultra-(high-)performance liquid chromatography; VO_{2max}: maximal oxygen uptake; VO_{2peak}: peak oxygen uptake; w: watt; WR_{max}: maximal work rate; WR_{peak}: peak work rate.

duration (i.e., 18 to 80 minutes) at moderate or vigorous constant intensity, fewer studies investigated the effects of either continuous PE at varying submaximal intensities, maximal incremental exercise tests, or strenuous long-duration (i.e., 2 to 9 hours) PE at self-paced intensities. The mostly analyzed biofluid was blood, followed by urine and saliva.

In the summarized studies, many of the metabolites showing a global increase in response to endurance exercise were related to lipid metabolism, e.g., glycerol, long-chain fatty acids (LCFAs), fatty acids oxidation products, or acylcarnitines. Further exercise-induced alterations have been documented for lactate, pyruvate, TCA cycle intermediates including cis-aconitate, citrate, malate, and succinate, several AAs, as well as ketone bodies like 3-hydroxybutyrate or acetoacetate, reflecting changes in energy-producing metabolic pathways. Since the presented studies vary with regard to the performed exercise protocols, included participants, collected biospecimens, sampling times, analytical techniques, and number of identified metabolites, their results are not directly comparable. However, it can be deduced from the current state of research that acute continuous PE of lower duration and intensity induces rather modest post-exercise shifts in metabolite concentrations compared to more intensive and prolonged continuous PE.

With regard to metabolic alterations in response to maximal incremental exercise, the current state of research is limited to three metabolomics studies. While these studies focused on the effects of a standardized exercise tolerance test on the blood metabolome (Breit et al., 2015; Lewis et al., 2010; Netzer et al., 2011), the response of the urinary metabolome to a stepwise progressive exercise test until exhaustion has not been examined so far. Given that previous studies have revealed that acute endurance exercise causes perturbations in both the blood and urinary metabolome (Siopi et al., 2017; Siopi et al., 2019), the value of urine in the study of human exercise metabolism could be supported. Accordingly, there is a need for further exercise metabolomics studies focusing on urine as a non-invasively collectable biospecimen. Thus, it could be evaluated whether urine is able to reflect exercise-induced adaptations in specific metabolic pathways.

Finally, most studies focusing on acute endurance exercise have been conducted in small, mainly male populations. Hence, more research including larger groups of both women and men will be required to clarify if exercise-induced metabolite alterations differ between sexes.

Metabolomics Studies Focusing on PF or Habitual PA

Regarding the current state of research until July 2021 (publication date of *Study IIb*) on the effects of chronic PA on metabolite profiles in humans, previous work can generally be divided into studies analyzing the relationship between the metabolome and either direct measures of habitual PA, e.g., PA-related energy expenditure, or functional variables like the CRF status, which can be seen as an objective surrogate measure for chronic PA (McKinney et al., 2016). An overview of these mainly observational studies detecting inter-individual differences in the human metabolome related to habitual PA or PF is provided in Table 5.

Approximately half of the listed studies assessed the VO_{2max} (or VO_{2peak}) as a measure of the CRF. Nearly as many studies assessed the PA level of study participants, either objectively by using accelerometers or subjectively by using questionnaires. In two studies, both the CRF and PA were determined in the same cohort, allowing a direct comparison of investigated relationships with the metabolome (Floegel et al., 2014; Wientzek et al., 2014). Further studies measured either the resting heart rate (HR_{rest}) (Lewis et al., 2010), finish time in a marathon (Høeg, Chmiel, Warrick, Taylor, & Weiss, 2020; Lewis et al., 2010; Shi et al., 2020), or blood lactate accumulation (San-Millán et al., 2020) and maximal power output (Castro et al., 2021) during a maximal bicycle test for PF assessment. Moreover, three studies compared the metabolic response to acute PE between more and less fit individuals (Enea et al., 2010; Lewis et al., 2010; Mukherjee et al., 2014). While most of the presented studies focused on blood, only minor metabolomics research has been conducted with urine samples.

Despite different approaches to assess chronic PA, the presented studies provided evidence that both habitual PA and the CRF are associated with specific metabolite patterns that are possibly linked to a better cardiometabolic health. In particular, it was shown that higher levels of PA or PF are associated with higher circulating PC (Bye et al., 2012; Floegel et al., 2014; Høeg et al., 2020; Morris et al., 2015; Wientzek et al., 2014) or lower BCAA concentrations (Fukai et al., 2016; Kujala et al., 2013; Kujala et al., 2019; Morris et al., 2013; Xiao et al., 2016). More ambiguous results were obtained for, e.g., blood lactate, which was either positively (Kujala et al., 2013; Palmnäs et al., 2018) or negatively (Kujala et al., 2019) related to measures of chronic PA. Discrepancies can be explained by distinct characteristics of study participants and the fact that PA or PF measures were either used as continuous variables for correlation and/or regression analyses or considered as categorical variables for group comparisons.

Table 5. Overview of metabolomics studies focusing on PF or habitual PA. *(Own table).*

Nr.	Author (Year)	Subjects		Assessment (Classification) of PF or habitual PA	Biological Specimen	Sampling Time	Method (Metabolites)	Main Metabolite Associations
		n (♂/♀)	State					
1	Enea et al. (2010)	22 (0/22)	Healthy, young, (un)trained	VO_{2max} (high-PF group: 48.6 ± 2.3ml min^{-1} kg^{-1}; low-PF group: 33.8 ± 1.8ml min^{-1} kg^{-1})	Urine	Pre- and 30min post-exercise	NMR (11)	<u>High-PF group:</u> lower increase in *Acetate* in response to a 30s maximal sprint
2	Lewis et al. (2010)	302 (175/127)	Overweight, subcohort of the Framing-ham Heart Study	HR_{rest} (continuous variable)	Plasma	At rest	LC-MS (14)	<u>Positively associated with HR_{rest}:</u> *Glycerol* <u>Negatively associated with HR_{rest}:</u> *Glutamine* [before/after adjusting for age, sex, and BMI]
		70 (60/10)	Middle-aged	VO_{2peak} (high-PF group: ≥ median VO_{2peak}; low-PF group: < median VO_{2peak})	Plasma	Pre- and 0h post-exercise	LC-MS (28)	<u>High-PF group:</u> higher increase in *Glycerol* and *Pantothenate*; lower increase in *Glutamine* and *Methionine* in response to an incremental exercise test
		25 (19/6)	Amateur runners	Finish time in a marathon (fast runners: ≥ median finish time; slow runners: < median finish time)	Plasma	Pre- and 10min post-exercise	LC-MS (28)	<u>Faster runners:</u> higher increase in *Fumarate, Succinate, Malate, Arginosuccinate, Niacinamide*; lower increase in *β-Hydroxybutyrate, Allantoin* in response to a marathon
3	Chorell et al. (2012)	27 (27/0)	Healthy, young, trained	VO_{2max} (high-PF group: VO_{2max} > 60ml min^{-1} kg^{-1}; low-PF group: VO_{2max} < 48ml min^{-1} kg^{-1})	Plasma	At rest	GC-TOF-MS (non-targeted, 50 identified)	<u>High-PF group:</u> ↑ *Docosahexaenoate*, ↓ *Butyrate* and *γ-Tocopherol;* no evident PF-related metabolic pattern detected by multivariate approaches.
4	Bye et al. (2012)	218 (91/127)	Healthy, middle-aged	VO_{2max} (high-/low-PF group; matched for age, fasting time, self-reported PA)	Serum	At rest	NMR (-)	<u>High-PF group:</u> ↑ *PCs, Unsaturated Fatty Acids*, ↓ *Free Choline, Glucose*
5	Kujala et al. (2013)	a) 32 (22/10)/ b) 230 (104/126)/ c) 600 (292/308)/ d) 1244 (660/584)	Healthy adults	Leisure-time PA [MET-h/d]; questionnaire-based (active/inactive groups were matched for sex and age)	Serum	At rest	NMR (> 100)	<u>Active groups:</u> ↑ *HDL Cholesterol* (a,b,c,d), *Omega-3 Fatty Acids* (a), *Docosahexaeonate* (a), *Acetoacetate* (c), *3-Hydroxybutyrate* (d), *Acetate* (c), *Citrate* (b,c,d), *Lactate* (d) ↓ *TGs* (a,b,c,d), *VLDL, LDL, IDL Cholesterol* (a,b,c,d), *Omega-6 Fatty Acids* (c,d), *Omega-7,9 and Saturated Fatty Acids* (a,c,d), *Monounsaturated Fatty Acids* (a,c,d), *Glucose* (a,b,c), *Acetoacetate* (a), *3-Hydroxybutyrate* (a), *Pyruvate* (c,d), *Isoleucine* (a,b,c,d), *Valine* (a), *Phenylalanine* (d), *Tyrosine* (b,d), *α1-acid-Glycoprotein* (a,c,d)
6	Morris et al. (2013)	65 (34/31)	Healthy adults	VO_{2max} (high-PF group: upper VO_{2max} quartile; ♂: > 57ml min^{-1} kg^{-1}/ ♀: > 43ml min^{-1} kg^{-1}) low-PF group: lower VO_{2max} quartile; ♂: < 42ml min^{-1} kg^{-1}/ ♀: < 32ml min^{-1} kg^{-1})	Plasma, urine	At rest	GC-MS (plasma: 29; urine: 26)	<u>High-PF group (plasma, females only):</u> ↑ *Glycine, Creatinine,* ↓ *Alanine, Sarcosine, α-Aminobutyrate, Leucine, Isoleucine, Valine, Serine, Proline, Threonine, Methionine, Hydroxyproline, Phenylalanine, Glutamine, Ornithine, Lysine, 1-Methylhistidine, 3-Methylhistidine, Histidine, Tyrosine, Tryptophan, Cystathionine, Cystine, Asparagine, Aspartate, Cysteine, Citrulline* [after adjusting for age and BMI] <u>High-PF group (urine):</u> ↑ *Creatinine,* ↓ *Cystathionine, Alanine, Sarcosine, Glycine, Ethanolamine, Leucine, Isoleucine, γ-Aminobutyrate, Valine, Proline, Hydroxyproline, Serine, Methionine, Phenylalanine, Lysine, Ornithine, Histidine, Glutamate* [after adjusting for sex, age, and BMI]

Table 5 *(continued)*. Overview of metabolomics studies focusing on PF or habitual PA. *(Own table).*

Nr.	Author (Year)	Subjects		Assessment (Classification) of PF or habitual PA	Biological Specimen	Sampling Time	Method (Metabolites)	Main Metabolite Associations
		n (♂/♀)	State					
7	Lustgarten et al. (2013)	77 (28/49)	Healthy, young	VO_{2max} (continuous variable)	Serum	At rest	UHPLC-MS/MS, GC-MS (non-targeted, 296)	Positively associated with VO_{2max}: *Pyridoxate, 2-Hydroxyisobutyrate, Erythrulose, Tryptophan, Leucylleucine, Glycerate, 4-Ethylphenylsulfate, Pantothenate, 4-Vinylphenolsulfate, Erythronate, N-Acetylornithine, α-Hydroxyisovalerate, Phenyllactate, Phenolsulfate, Indolelactate* [after adjusting for sex] Negatively associated with VO_{2max}: *γ-Tocopherol, 5α-Pregnan-3β,20α-dioldisulfate, Stearoylsphingomyelin, Pentadecanoate* [after adjusting for sex] Stepwise regression analyses for the explanation of VO_{2max}: *Glutamate Oxaloacetate Aminotransferase, 4-Ethylphenylsulfate, Tryptophan, γ-Tocopherol* and *α-Hydroxyisovalerate* [sex-adjusted; R^2 (adjusted)=0.66]. *Piperine, Pyridoxate, Glycerol, 3-Methoxytyrosine, 2-Hydroxyisobutyrate* [in males; R^2 (adjusted)=0.80]. *5-α-Pregnan-3β, 20α-dioldisulfate, Glycerate, Glycerol, N-Acetyl-β-Alanine, Phenolsulfate, 3-Methyl-2-Oxovalerate* [in females; R^2 (adjusted)=0.58].
8	Floegel et al. (2014)	100 (50/50)	Middle-aged, subcohort of the European Prospective Investigation into Cancer and Nutrition Study	a) VO_{2max} b) PAEE [kJ kg^{-1}/d]; accelerometer-measured for 2x4d (continuous variables)	Serum	At rest	FIA-MS/MS (127)	Positively associated with VO_{2max} or PAEE: *Amino Acids* (a,b), *Sphingomyelins* (a), *LysoPCs* (a,b), *Diacyl-PCs* (a) Negatively associated with VO_{2max} or PAEE: *C6 sugars* (a,b)
9	Mukherjee et al. (2014)	17 (17/0)	Healthy, middle-aged, (un)trained	VO_{2peak} (high-PF group: 59.1±5.2ml min^{-1} kg^{-1}; low-PF group: 35.9±9.7ml min^{-1} kg^{-1})	Urine	Pre- and post-exercise 24h-samples	NMR (32)	High-PF group: Higher increase in *Hypoxanthine*, *Fumarate;* Lower increase in *Lactate, Malonate, Acetate* in response to 45min submaximal exercise
10	Wientzek et al. (2014)	100 (50/50)	Middle-aged, subcohort of the European Prospective Investigation into Cancer and Nutrition Study	a) VO_{2max} b) PAEE [kJ kg^{-1}/d] c) vigorous time [h/d] (all continuous variables; b)-c): accelerometer-measured for 2x4d)	Serum	At rest	FIA-MS/MS (127)	Positively associated with VO_{2max}/PAEE/Vigorous Time: *Acyl-Alkyl-PCs (C38:0, 42:3, C42:2, C36:0, C40:6, C40:1, C40:3, C36:1, C34:0, C38:2, C38:6, C44:4, C32:1)*, *Diacyl-PCs (C36:6, C42:1, C36:0, C38:6, C36:5, C32:0, C38:0, C38:5, C40:6, C42:6, C30:3, C32:2, C28:1, C34:4, C34:3)* (a) Negatively associated with VO_{2max}/PAEE/Vigorous Time: *LysoPC C14:0* (b), *Diacyl-PCs C34:4, C32:2, C34:3* (b) [after adjusting for sex, age, measurement occasion, BMI, waist circumference, education, alcohol consumption, smoking status, all other PA/PF measures]
11	Morris et al. (2015)	40 (20/20)	Healthy adults	VO_{2max} (high-PF group: > 43ml min^{-1} kg^{-1}; low-PF group: < 42ml min^{-1} kg^{-1})	Plasma	At rest	ESI-MS/MS (240)	High-PF group: ↑ *PEs C34:3, C36:3, Acyl-Alkyl-PCs C34:2, C34:3, C36:3, C36:4, C36:5, C38:2* [after adjusting for age and BMI]
12	Fukai et al. (2016)	1193 (1193/0)	Healthy adults	Total PA [MET-h/wk]; questionnaire-based (classification in 4 levels)	Plasma	At rest	CE-TOF-MS (-)	Positively associated with total PA: *Pipecolate, HDL Cholesterol* Negatively associated with total PA: *Isoleucine, Proline, Alanine, 4-Methyl-2-Oxopentanoate, TGs* [after adjusting for age, BMI, alcohol intake, smoking status, and energy intake]

Table 5 *(continued)*. Overview of metabolomics studies focusing on PF or habitual PA. *(Own table).*

Nr.	Author (Year)	Subjects		Assessment (Classification) of PF or habitual PA	Biological Specimen	Sampling Time	Method (Metabolites)	Main Metabolite Associations
		n (♂/♀)	State					
13	Muhsen Ali et al. (2016)	10 (8/2)	Healthy, active	VO_{2max} (continuous variable)	Urine	Pre- and 1h, 4h, 7h post-exercise	LC-MS (57)	Positively associated with VO_{2max}: *Oxo-Aminohexanoate* (1h after 45min submaximal exercise)
14	Xiao et al. (2016)	277 (130/147)	Healthy adults from the Shanghai Physical Activity Study	a) PAEE [MET-h/d] b) total PA duration [min/d] c) moderate-to-vigorous PA [min/d] d) light PA [min/d] (all continuous variables; a)-d): accelerometer-measured for 4x7d)	Plasma	At rest	UHPLC, GC-MS (non-targeted, 328)	Positively associated with PAEE: *Betaine, Threonate* Negatively associated with PAEE: *Valine, Isoleucine, Glutamate, 3-Methyloxovalerate, 2-Hydroxybutyrate, α-Hydroxyisovalerate, Glucose, Mannose, γ-Glutamylvaline* [after adjusting for sex, age, BMI, smoking status] Total PA duration and time spent in light PA associated with a similar metabolic pattern.
15	Koh et al. (2018)	141 (82/59)	Healthy, older adults from the Cardiac Aging Study	VO_{2peak} (high-PF group: > mean VO_{2peak}, ♂: > 37ml min^{-1} kg^{-1}/ ♀: > 29ml min^{-1} kg^{-1}; low-PF group: ≤ mean VO_{2peak}, ♂: ≤ 37ml min^{-1} kg^{-1}/ ♂: ≤ 29ml min^{-1} kg^{-1})	Serum	At rest	LC-MS (83)	Negatively associated with VO_{2peak}: *Wide-spectrum Carnitines including Odd Short-chain Carnitines, Alanine, Glutamine/Glutamate* [after adjusting for age, BMI, and diabetes]
16	Palmnäs et al. (2018)	82 (35/47)	Adults with or without symptoms of the metabolic syndrome	a) PAEE [kcal/d or kcal kg^{-1}/d] b) PAL [kcal] c) moderate and vigorous PA [h/d] (high-/low-PA groups based on means; a)-b): doubly labelled water; c): questionnaire-based)	Serum	At rest	NMR (-)	High-PA group: ↑ *Glycine, Lactate* (a,b: in men with symptoms), ↑ *Serine, Arginine* (a,b: in women without symptoms), ↑ *Arginine, Betaine* (a: in women without symptoms), ↑ *Acetoacetate, Creatinine, Serine* (b: in women with symptoms) Positively associated with PAEE/PAL/moderate or vigorous PA: *Lactate* (a,c: in men with symptoms; c: in men without symptoms), *Acetoacetate, Arginine, Creatinine, myo-Inositol, Serine* (a: in women with symptoms)
17	Ding et al. (2019)	5197 (-/-)	Participants of the Nurses' Health Study and the Health Professionals Follow-up Study	Total PA [MET-h/wk]; questionnaire-based (continuous variable)	Plasma	At rest	LC-MS (337)	Positively associated with total PA: *3 Amino Acids (Glycine, Citrulline, Asparagine), 5 Cholesterylesters, 9 PCs and LysoPCs, 5 PEs and LysoPEs* [not adjusted for BMI] *2 Amino Acids (Citrulline, Glycine), 4 Cholesterylesters (C18:2, C18:1, C16:0, C18:3), 5 PCs (C36:4, C34:3, C36:3, C34:2, C36:2), 3 LysoPCs (C18:2, C20:5, C18:1), 2 LysoPEs (C18:2, C18:1), and C38:3 PE Plasmalogen* [additionally adjusted for BMI] Negatively associated with total PA: *Glutamate, 4 TGs, and 5 Diglycerides* [not adjusted for BMI] [after adjusting for age, fasting status, smoking status, body weight, healthy eating index score, total energy intake, alcohol intake, and contributing case-control study (± BMI)]
18	Pang et al. (2019)	4660 (2330/2330)	Adults with or without cardiovascular disease	Total PA [MET-h/d] questionnaire-based (continuous variables)	Plasma	At rest	NMR (225)	Positively associated with total PA: *Large HDL Cholesterol, Glutamine, Docosahexaenoic Acid* Negatively associated with total PA: *VLDL Cholesterol, Alanine, Glucose, Lactate, Acetoacetate, Monounsaturated Fatty Acids* [after adjusting for age, sex, fasting time, region, smoking status, education, income, health, intake of fruit, red meat, sedentary time]

Table 5 *(continued)*. Overview of metabolomics studies focusing on PF or habitual PA. *(Own table).*

Nr.	Author (Year)	Subjects		Assessment (Classification) of PF or habitual PA	Biological Specimen	Sampling Time	Method (Metabolites)	Main Metabolite Associations
		n (♂/♀)	State					
19	Kujala et al. (2019)	580 (580/0)	Healthy, young men	VO_{2max} (high-PF group: 50.7 ± 4.2ml min^{-1} kg^{-1}; low-PF group: 31.8 ± 3.8ml min^{-1} kg^{-1}) (continuous variable)	Serum	At rest	NMR (66)	High-PF group: ↑ *HDL, Glutamine,* ↓ *VLDL, IDL, LDL, TGs, Total and Saturated Fatty Acids, Poly- and Monounsaturated Fatty Acids, ω-6 Fatty Acids, Linoleate, Glycerol, Acetoacetate, 3-Hydroxybutyrate, Lactate, Pyruvate, Isoleucine, Leucine, Phenylalanine, Tyrosine* [after adjusting for age, education, smoking, alcohol, dietary factors] Positively associated with VO_{2max}: *Large HDL Particles, Unsaturated Degree of Fatty Acids* Negatively associated with VO_{2max}: *TGs, Glycerol, Acetoacetate, 3-Hydroxybutyrate, Glycoproteins* [after adjusting for age and body fat (%)]
20	Hoeg et al. (2020)	Phase I: 39 (31/8) Phase II: 28 (24/4) Controls: 14 (12/2)	Healthy, middle-aged runners	Finish time in a 161-km race (10 fastest finishing participants vs. 10 slowest finishing participants)	Plasma	1d pre-exercise, within 1h post-exercise	QTOF-MS/MS (427)	Faster runners: higher increase of *PCs 40:8, 38:5, 32:0, 40:4, 40:5*, 37:4, 38:7, 40:6, 42:5 (post-exercise) Runners (Phase II) vs. controls: 41 (4) PCs significantly higher (lower) in runners
21	San-Millán et al. (2020)	21 (21/0)	Professional male cyclists	Blood lactate at 5 w/kg of a graded maximal bicycle test (gold group: > mean blood lactate; silver group: < mean blood lactate)	Blood	Pre- and post-exercise	UHPLC-MS (355)	Gold group: ↑ *Phenylalanine, Lysine, Asparagine, Serine, Threonine, Valine, Tryptophan, Tyrosine;* higher increase in *Succinate, Fumarate, Malate* and *C3-/C4-Carnitine* post-exercise; higher decrease in *Isoleucine, Leucine* post-exercise
22	Shi et al. (2020)	20 (20/0)	Experienced amateur runners	a) Finish time in a marathon run b) VO_{2max} (continuous variables)	Serum	At rest	LC-MS/MS (untargeted)	Positively associated with finish time, VO_{2max}: *Theobromine* (a), *Cytidine 5′-Monophosphate* (b) Negatively associated with finish time, VO_{2max}: *Beta-Alanine* (a), *cis-Aconitate, Galactonic Acid, Mesaconic Acid* (b)
23	Castro et al. (2021)	70 (70/0)	Sedentary, young men	Maximal power output (continuous variable)	Serum	At rest	NMR (43)	Positively associated with maximal power output: *2-Hydroxyisocaproate, Asparagine, Betaine, Choline, Glutamine, Glycine, Histidine, Ornithine, Proline, Succinate, Threonine, Valine* Negatively associated with maximal power output: *Dimethylamine*
24	Xu et al. (2021)	3802 (1524/2278)	Adults from the Atherosclerosis Risk in Communities Study	Leisure-time PA; questionnaire-based (continuous variable)	Serum	At rest	LC-/GC-MS (245)	Positively associated with leisure-time PA: *Creatinine, Erythronate, Glycerate, Pyridoxate, Threonate, 2-Aminooctanoate, cis-4-Decenoylcarnitine, myo-Inositol, N-Acetylcarnosine, Stachydrine* [after adjusting for age, sex, race, smoking status, BMI, HDL, TGs, total cholesterol, glucose, diabetes status, systolic blood pressure, antihypertensive medication]

BMI: body mass index; CE: capillary electrophoresis; d: day(s); ESI: electrospray ionization; FIA: flow injection analysis; GC: gas chromatography; h: hour(s); HDL: high-density lipoprotein; HR_{rest}: resting heart rate; IDL: intermediate-density lipoprotein; kcal: kilocalorie(s); kg: kilogram(s); kJ: kilojoule(s); km: kilometer(s); LC: liquid chromatography; LDL: low-density lipoprotein; LysoPC: lyso-phosphatidylcholine; LysoPE: lyso-phosphatidylethanolamine; MET: metabolic equivalent of task; min: minute(s); ml: milliliter(s); MS: mass spectrometry; MS/MS: tandem mass spectrometry; n: sample size; NMR: nuclear magnetic resonance; PA: physical activity; PAEE: physical activity energy expenditure; PAL: physical activity level; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PF: physical fitness; QTOF: quadrupole time of flight; R^2 (adjusted): adjusted coefficient of determination; s: second(s); TGs: triglycerides; TOF: time of flight; U(H)PLC: ultra-(high-)performance liquid chromatography; VLDL: very low-density lipoprotein; VO_{2max}: maximal oxygen uptake; VO_{2peak}: peak oxygen uptake; w: watt; wk: week(s).

Indeed, with respect to VO_{2max} (or VO_{2peak}) as a measure of the CRF, the majority of studies analyzed differences in the human metabolome between pre-defined groups of high or low CRF (Bye et al., 2012; Chorell et al., 2012; Enea et al., 2010; Lewis et al., 2010; Morris et al., 2013; Morris et al., 2015; Mukherjee et al., 2014). Comparatively less studies have examined the relationship between the VO_{2max} (or VO_{2peak}) and metabolite profiles across a broad range of CRF levels so far (Floegel et al., 2014; Koh et al., 2018; Lustgarten et al., 2013; Wientzek et al., 2014), with one study having conducted both group comparisons and correlation analyses in the same study population (Kujala et al., 2019). However, limitations of these studies are that results were restricted to either young, middle-aged, or older individuals and therefore not transferable to the general population. Moreover, apart from Kujala et al. (2019), who examined 580 men, all other studies had rather small sample sizes, ranging from 77 to 141 individuals. With regard to metabolomics analyses, only Lustgarten et al. (2013) utilized a combination of different MS-based methods, thereby detecting nearly 300 serum analytes. In contrast to this, a quite limited number of metabolites were analyzed by using single MS- or NMR-based techniques in most remaining studies.

Considering the fact that many of the CRF-associated metabolites reported in the literature have also been linked to other phenotypical variables such as the body composition, it has to be clearly determined whether the observed associations between metabolite profiles and the CRF merely reflect these other phenotypical features or if they can be specifically attributed to the individuals' CRF status (Kelly et al., 2020). Indeed, Kujala et al. (2019) were the first to adjust associations between the CRF and blood metabolites for body fat percentage next to age, smoking, dietary factors, and PA, while previous studies considered the body mass index (BMI) and waist circumference (Koh et al., 2018; Wientzek et al., 2014) as additional confounders. Yet, those studies mainly evaluated associations between the CRF and single blood metabolites instead of taking the combination of various metabolites into account. Although Lustgarten et al. (2013) applied sex-adjusted multivariable regression procedures to identify a set of serum metabolites accounting for the variation inherent in VO_{2max}, they did not control for age, diet, PA, or the body composition.

Taken as a whole, there is a lack of metabolomics research on the relationship between the CRF status and a high number of metabolites in large groups of both healthy women and men with a broad age spectrum. As metabolomics has the potential to comprehensively examine

metabolic signatures related to phenotypical variables like the PF, thus permitting to pinpoint metabolic pathways that may mediate the health effects of chronic PA (Heaney et al., 2017), particularly cross-sectional studies focusing on larger, well-characterized populations and a combination of several analytical techniques should be conducted in the future. One benefit of such large-scale metabolomics studies would be that results allow to draw conclusions across a broad range of PF levels and are therefore more likely to be applicable to the general population. Besides, it could be systematically elucidated to what extent metabolite patterns account for the variation in the individuals' CRF status if simultaneously considering covariates like sex, age, the body composition, and further clinical or functional parameters.

What additionally should be considered is that little research has so far been addressed to the relationship between the CRF and the urinary metabolome. While Morris et al. (2013) have provided evidence that resting urinary metabolites differ according to the individuals' VO_{2max}, three other studies showed that also exercise-induced excursions of urinary metabolites vary between more and less fit individuals (Enea et al., 2010; Muhsen Ali et al., 2016; Mukherjee et al., 2014). However, due to the relatively small number of included study participants and the fact that mainly group comparisons were conducted, there is a need for further studies examining whether urinary metabolites – either in the resting or post-exercise condition – can, equally to blood metabolites, be linked to the CRF in heterogeneous populations.

2.4.3 Synthesis of Research Findings

Regarding the current state of research on the effects of acute and chronic PA on metabolite profiles in humans, it can be summarized that the majority of previously conducted interventional studies focused on acute alterations in the human metabolome in response to endurance exercise or, to a lesser extent, HIIT. Furthermore, observational studies have provided first information on PA- or PF-associated metabolites. However, despite the emerging number of publications, their heterogeneity makes it difficult to draw direct comparisons between findings. Discrepancies between studies were generally observed with respect to included participants (i.e., sex, age, PF, training status, or health status), type of performed exercise, methods for PA or PF assessment, collected biological specimens, time points of sample taking, applied metabolomics techniques, number of identified metabolites, or examination as well as consideration of covariates. As the documented effects of PA on the

human metabolome have consequently been rather diverse until now, more metabolomics studies are needed in order to systematically evaluate how metabolite patterns in a variety of human biomatrices are related to either acute or chronic PA. Based on the current state of knowledge, several research gaps could clearly be defined and implications for future research were deduced:

- Firstly, with regard to metabolomics studies focusing on HIIT, there is a lack of information on the medium-term effects of HIIT interventions lasting several days or weeks on the metabolome of recreationally active individuals or professional athletes. Overcoming this research gap, relevant metabolic markers for either training adaptation or training overload could possibly be detected.

- Secondly, few information is available concerning the acute effects of a standardized incremental exercise test on the human metabolome. Also, it is rather unclear how exercise-induced metabolic perturbations are determined by sex or influenced by an individual's fitness status. Hence, more controlled interventional studies are necessary to distinguish the metabolic response to acute PE between women and men or fit and less fit individuals. Consequently, mechanisms and metabolic pathways underlying the acute and chronic adaptations to PE could potentially be elucidated.

- Thirdly, there is a lack of metabolomics studies examining the relationship between the CRF as a measure of chronic PA and the metabolome in large, heterogeneous, and well-characterized study populations. Thus, more emphasis on cross-sectional studies including both sexes with a broad age and PF spectrum is needed to validate previous findings and to produce results which are more likely to be applicable to the general population. In this respect, an adequate examination and consideration of covariates is mandatory in order to determine metabolite patterns that can be specifically linked to the CRF. Furthermore, as different analytical techniques have scarcely been combined until now, a combination of several metabolomics methods would be helpful to provide a more comprehensive investigation of CRF-associated metabolite patterns. Hence, a profounder knowledge on metabolic markers or mediators of the health- or performance-enhancing effects of chronic PA is likely to be obtained.

One aspect which could consistently be observed across all referenced metabolomics studies is that comparatively little research has been focused on PA-related changes in the urinary metabolome. Nevertheless, initial studies on the acute effects of PE have supported the potential of urine as an easily collectable biofluid for metabolomics analyses (Enea et al., 2010; Muhsen Ali et al., 2016; Mukherjee et al., 2014; Siopi et al., 2017). Therefore, more research evaluating the utility of urine in the field of exercise metabolomics is required. Additionally, since there are only few PA-related metabolomics studies that thoroughly examined the participation and relevance of identified metabolites in pathways of human metabolism, it will be necessary to conduct systematic pathway analyses, thus evaluating which metabolic pathways are most likely to be affected by acute or chronic PA.

A short summary of the findings presented in Chapter 2.4.2 is given in Table 6.

Table 6. Synthesis of research findings. *(Own table).*

	Acute Physical Exercise / Physical Training	**Chronic Physical Activity**
Synthesis of Findings	• Endurance > HIIT > RE > team sports • Blood > urine > saliva ≈ muscle • Males > females • Lack of studies focusing on the effects of a HIIT intervention lasting several days (e.g., in already trained individuals) • Lack of studies focusing on the effects of an acute incremental exercise intervention	• Habitual PA ≈ PF • Blood > urine • Males ≈ females • Lack of cross-sectional studies analyzing the relationship between the CRF and the metabolome in large, heterogeneous, and well-characterized study populations, thereby adequately considering covariates • Lack of studies combining various different metabolomics platforms
	• Lack of systematically conducted pathway analyses	

>: more focused in the literature than; ≈: nearly equally focused/assessed in the literature. HIIT: high-intensity interval training; PA: physical activity; PF: physical fitness; RE: resistance exercise.

3 Aims and Scope of the Dissertation

With the objective of extending the current state of knowledge, the general aim of the present thesis is to comprehensively investigate the effect of acute and chronic PA on metabolite profiles in humans. To address this aim, metabolomics data from three exercise- or fitness-related (sub-)studies (indicated as *Study I* or *Study IIa/IIb*; described in detail in Chapter 4) were analyzed in the framework of this dissertation and the following major purposes were defined:

(1) Investigation of alterations in the human metabolome in response to a medium-term high-intensive intermittent exercise training (*Study I*)
(2) Investigation of alterations in the human metabolome in response to an acute incremental exercise test (*Study IIa*)
(3) Analysis of the relationship between the human metabolome and the CRF (*Study IIa/IIb*)

Based on these three purposes, specific research questions were deduced and separately presented for each of the conducted studies, as depicted below. Altogether, *Study I*, *IIa*, and *IIb* covered different aspects of PA, reaching from a medium-term training (*Study I*) and an acute exercise intervention (*Study IIa*) to the assessment of the CRF status as a measure of chronic PA (*Study IIa*/*IIb*), thus allowing to obtain a comprehensive view of PA-related alterations in the human metabolome. In general, different metabolomics approaches were used, depending on the particular study, available biological specimens, and advances in analytical techniques. With regard to the discussion of all study results, this thesis aimed at a systematic biological interpretation of PE- or PF-related metabolite profiles.

<u>*Study I* (HIIT): Relevance and Research Questions</u>

As outlined in Chapter 2.4.2, previous metabolomics studies focused on the acute effects of single HIIT sessions rather than on the medium- or long-term effects of HIIT interventions lasting several days, weeks, or months. Considering that a non-metabolomics study has shown that a HIIT program characterized by high loads and a daily application resulted in an increased activity of muscle enzymes related to both aerobic and anaerobic metabolism after just two weeks (Rodas et al., 2000), the purpose of *Study I* was to investigate whether the organism's

adaptation to a ten-day HIIT is reflected in the resting urinary metabolome of young active men. Urine as a biological matrix was chosen because it was easily accessible and provided from all study participants before the intervention started as well as after a recovery period of one or four days, respectively. To address several research gaps depicted in Chapters 2.4.2 and 2.4.3, two specific research questions were defined for *Study I*:

- What are the effects of a ten-day HIIT and a subsequent four-day recovery period on the resting urinary metabolome of healthy, young active men?
- Can urinary metabolites as potential biomarkers for either training adaptation or overtraining be identified?

On the one hand, answering these questions might provide evidence if human metabolism was able to regenerate from acute metabolic disturbances due to the daily performed HIIT in a given time frame of one or four recovery days, respectively. On the other hand, the research questions reveal the rather exploratory character of *Study I*, aiming to obtain first information about metabolic markers detectable in urine which could be indicative for an individual's adaptation to a strenuous HIIT protocol. With regard to future investigations or even practical applications intending to facilitate the monitoring of an athlete's training adaptation or overtraining, findings from this study could provide a first informative basis for the selection of possibly relevant metabolites. Besides, knowledge on specific metabolic pathways being altered in response to HIIT could likely be deduced from the results of *Study I*.

<u>*Study IIa* (Acute Incremental Exercise & CRF): Relevance and Research Questions</u>

As outlined in Chapters 2.4.2 and 2.4.3, little research has so far been addressed to the relationship between the CRF as a measure of chronic PA and the resting or post-exercise metabolome in large cross-sectional studies. Especially with regard to the biological matrix urine, only few metabolomics studies characterized by small sample sizes have until now identified single urinary metabolites differing between more and less fit individuals in either the resting state (Morris et al., 2013) or in response to acute PE (Enea et al., 2010; Muhsen Ali et al., 2016; Mukherjee et al., 2014). However, if individuals actually demonstrate a different metabolic profile at rest or a different metabolic response to PE depending on their CRF status, profound knowledge on urinary metabolites reflecting this distinction could provide new insights into biochemical pathways that contribute to the beneficial adaptations of human

metabolism to PA. Therefore, two objectives were pursued with *Study IIa*. Firstly, it should be evaluated if urinary metabolites were able to reflect acute metabolic alterations due to a standardized incremental exercise test, which was performed by 255 healthy women and men with a broad age range. Due to the known effect of sex on human metabolite profiles (Rist et al., 2017), sex-related differences in the response of the urinary metabolome to acute PE should additionally be analyzed. The second aim was to investigate whether either single urinary metabolites or a specific urinary metabolite pattern at rest or in response to PE were associated with the CRF. The biological matrix urine was used for metabolomics analysis as it was provided from all study participants before and after completing the incremental exercise tolerance test. To address the depicted objectives, the following research questions were specified for *Study IIa*:

- What is the effect of a standardized exercise tolerance test until exhaustion on the urinary metabolome in a large cohort of healthy females and males? Are there any sex-related differences in the exercise-induced excursions of urinary metabolites?
- To what extent do single urinary metabolites (at rest/post-exercise) or the exercise-induced metabolite excursions correlate with the CRF status? Do correlations persist when adjusting for covariates?
- Do individuals with a similar CRF status show a similar urinary metabolite profile (at rest/post-exercise) or similar exercise-induced excursions of the urinary metabolite profile? Can a urinary metabolite pattern accounting for the variation in the CRF status be identified when simultaneously considering covariates?

As the metabolomics data were obtained from a comparatively large, heterogeneous, and well-characterized study population, addressing these research questions has the potential to systematically elucidate in how far urinary metabolites at rest as well as post-exercise could help to explain the high variation in the CRF status, when simultaneously considering known covariates like age, sex, menopausal status, and the body composition. Thus, potentially relevant urinary metabolites, which allow to draw conclusions across a broad range of CRF levels in healthy women and men, could be identified. Furthermore, results of *Study IIa* might enable a deeper understanding of metabolic pathways that are associated with both acute PE and the CRF status.

Study IIb (CRF): Relevance and Research Questions

In addition to *Study IIa*, which focused on the relationship between acute PE as well as the CRF and the urinary metabolome, *Study IIb* was addressed to the association between the CRF, selected phenotypical and clinical variables, and the resting plasma metabolome in nearly the same study population consisting of 252 healthy adults with a wide age and PF spectrum. As depicted in Chapter 2.4.2, most previous CRF-related metabolomics studies included rather small sample sizes, were limited to specific age or sex groups, or detected a quite limited number of blood analytes by using a single metabolomics technique (Floegel et al., 2014; Koh et al., 2018; Kujala et al., 2019; Wientzek et al., 2014). Therefore, the purpose of *Study IIb* was to systematically analyze the relationship between the CRF and a comparatively high number of plasma metabolites assessed by multiple analytical platforms in both women and men. Since previous research has largely been restricted to bivariate correlations between the CRF and single blood metabolites, an additional aim of *Study IIb* was to identify plasma metabolite patterns, i.e., sets of plasma analytes that in their relation to each other, are associated with the CRF. The applied multi-platform metabolomics approach had been completed and finally evaluated with regard to the biomatrix plasma but not yet for urine, which was also collected in *Study IIb*. Thus, metabolomics analyses in fasting plasma samples were considered in the framework of this thesis. Since sex, age, and the menopausal status are known determinants of the CRF (Mercuro et al., 2006; Zeiher et al., 2019) and have been linked to a discriminatory plasma metabolite profile in the considered population (Rist et al., 2017), association analyses were conducted in sex-specific sub-groups and adjusted for age and menopausal status. To address the depicted aims, the succeeding research questions were defined for *Study IIb*:

- To what extent do selected phenotypical and clinical variables show age- and menopausal status-adjusted correlations with the CRF in females and males?
- To what extent do single plasma metabolites show age- and menopausal status-adjusted bivariate correlations with the CRF in females and males? Do correlations persist when additionally adjusting for further phenotypical and clinical variables?
- Which plasma metabolites significantly contribute to the age- and menopausal status-adjusted multivariate associations with the CRF? Do multivariate associations persist when additionally adjusting for phenotypical and clinical variables?

- Can sets of phenotypical, clinical, and plasma metabolite variables be identified that account for the variation of the age- and menopausal status-adjusted CRF in females and males? Which plasma metabolites are able to improve initial sex-specific CRF explanation models solely based on phenotypical and clinical variables?

Since *Study IIb* comprised a comparatively large study population consisting of both sexes with a broad age spectrum, answering these questions could lead to the identification of plasma metabolites or metabolite patterns that allow to draw conclusions across a broad range of CRF levels. Indeed, due to the combination of several analytical techniques and a high number of assessed plasma analytes, not only confirmatory data could be provided, but also novel PF-related metabolites might be yielded, possibly leading to new insights on metabolic pathways that are linked to the beneficial effects of a high CRF. Besides, as participants were thoroughly characterized by anthropometric, functional, and clinical examinations, association analyses could be additionally adjusted for parameters related to the body composition, clinical blood biochemistry, lung and arterial function, short-term and habitual PA, or diet, thus determining whether associations can be specifically attributed to the CRF status. With regard to future investigations or even practical applications aiming to conclude about an individual's PF or health status, findings of this systematically conducted study could provide a first informative basis for selecting possibly relevant plasma metabolites that, in combination with clinical or phenotypical variables, explain a preferably high amount of the variation in the CRF.

To reach these aims and to address the research questions, this dissertation comprises the findings of the three mentioned (sub-)studies (Chapters 5 to 7), preceded by a section describing general methods (Chapter 4), and followed by a section focusing on the functional classification of obtained PE- and PF-related metabolite profiles (Chapter 8). A comprehensive summary and discussion of study results, strengths and limitations, as well as conclusions and suggestions for future research will subsequently be provided (Chapter 9). Manuscripts containing the particular methods, results, and discussions have already been published in international journals after peer-reviewing. A schematic overview of the three scientific articles included in this thesis, their main research focuses and classification within the broad research field of exercise biology is provided in Figure 11.

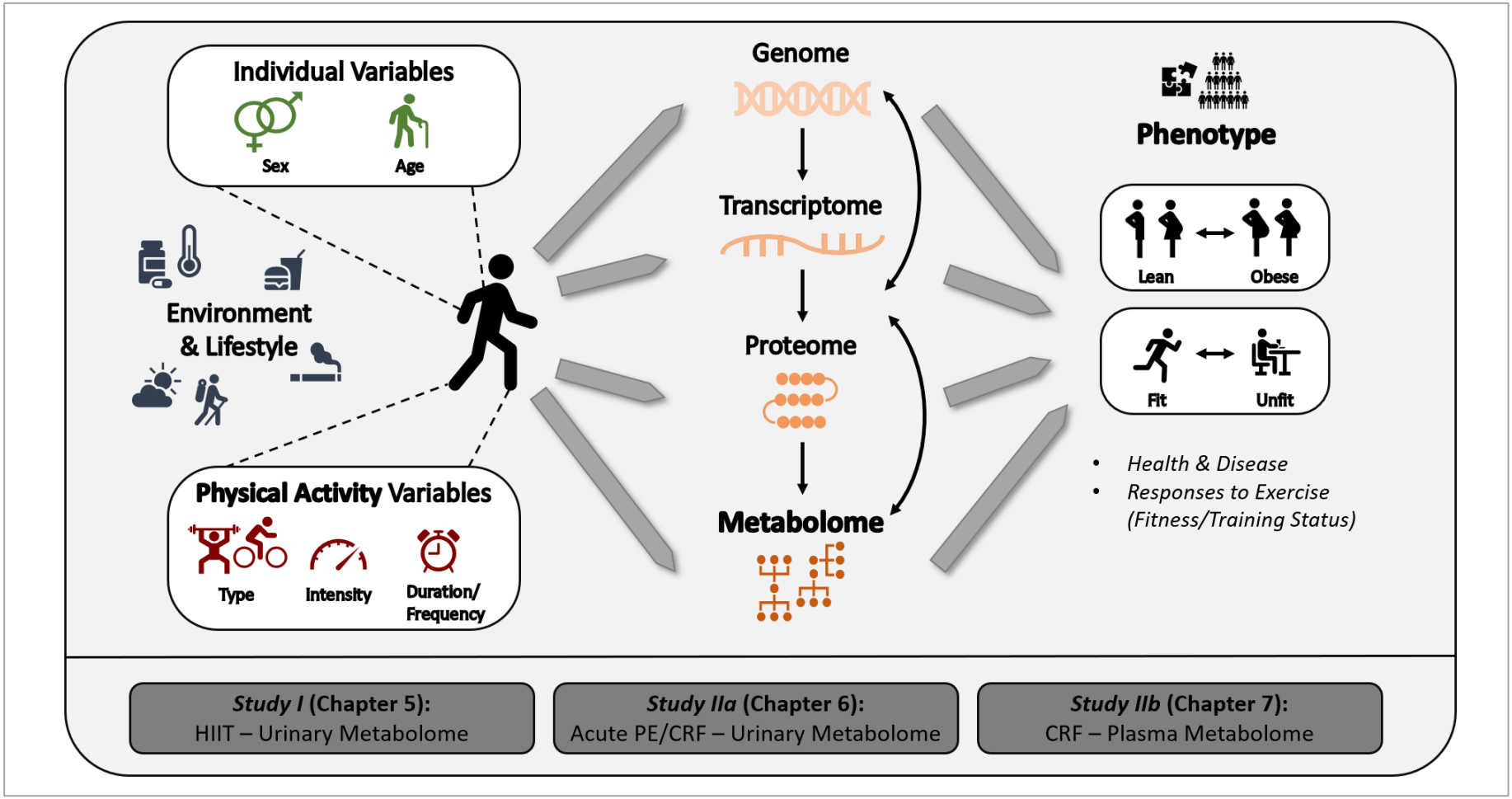

Figure 11. Classification of *Study I*, *IIa*, and *IIb* within the research field of exercise biology. The complex interactions between physical activity, further individual variables (e.g., sex, age, environment, lifestyle), and functional levels in biological systems (i.e., genome, transcriptome, proteome, metabolome) contribute to distinct health- or performance-related phenotypes. By covering different aspects of physical activity, the three research articles included in this thesis aim to extend the current knowledge on alterations in the human metabolome in response to medium-term HIIT (*Study I*) and acute incremental exercise (*Study IIa*), apart from examining the relationship between metabolite profiles and the CRF as a measure of chronic physical activity (*Study IIa*/*IIb*). CRF: cardiorespiratory fitness; HIIT: high-intensity interval training; PE: physical exercise. *(Own illustration based on Belhaj et al. (2021) and Dunn and Ellis (2005)).*

4 General Methods

In this methodological section, a general overview of the three exercise- or fitness-related (sub-)studies whose metabolomics data were analyzed in the framework of this dissertation will be given. Firstly, characteristics of participants and study designs are presented separately for each study (Chapter 4.1). Secondly, all relevant examination methods, i.e., anthropometric and clinical measurements as well as PF assessment are summarized (Chapter 4.2). While the applied metabolomics analyses are described in Chapter 4.3, an overview of data handling and statistical analyses is given in Chapter 4.4. Finally, the assignment of metabolites to pathways of human metabolism for a subsequent functional classification of PE-/PF-related metabolite profiles is depicted (Chapter 4.5). Since the specific methods of the particular studies are precisely described in the publications presented in Chapters 5 to 7, this section focuses on a more general description and a comparative presentation of the main methods used in *Study I*, *IIa*, and *IIb*.

Both *Study I* ("HIIT Study") and *Study II* ("KarMeN Study") were carried out under controlled laboratory conditions. They have focused on different aspects of PA, namely medium-term HIIT (*Study I*), acute PE (*Study IIa)*, as well as the assessment of the CRF as a measure of chronic PA (*Study IIa/IIb*). Compared to *Study I*, which had an experimental study design and whose intervention was performed by young, healthy, and physically active men, *Study II* was a large cross-sectional study including healthy women and men with a broad age and PF range. Although the Karlsruhe Metabolomics and Nutrition (KarMeN) study was mainly addressed to the question how the resting metabolome is affected by sex, age, diet, or the PF, it also comprised an experimental part consisting of a standardized incremental exercise test. In the framework of the present thesis, this experimental part and the subsequent analysis of acute exercise-induced changes in the metabolome are considered as a separate part of the KarMeN study, being indicated as *Study IIa*. For the investigation of the relationship between the CRF and the metabolome in resting conditions, the denomination *Study IIb* is used. While the study design, characteristics of participants, and general investigation methods are described for the whole *Study II* (Chapters 4.1.2 and 4.2), applied metabolomics and statistical approaches are presented separately for *Study IIa* and *Study IIb* (Chapters 4.3 and 4.4).

Figure 12 gives an overview of the three exercise- or fitness-related (sub-)studies whose metabolomics data were generated and/or analyzed in the framework of the present thesis.

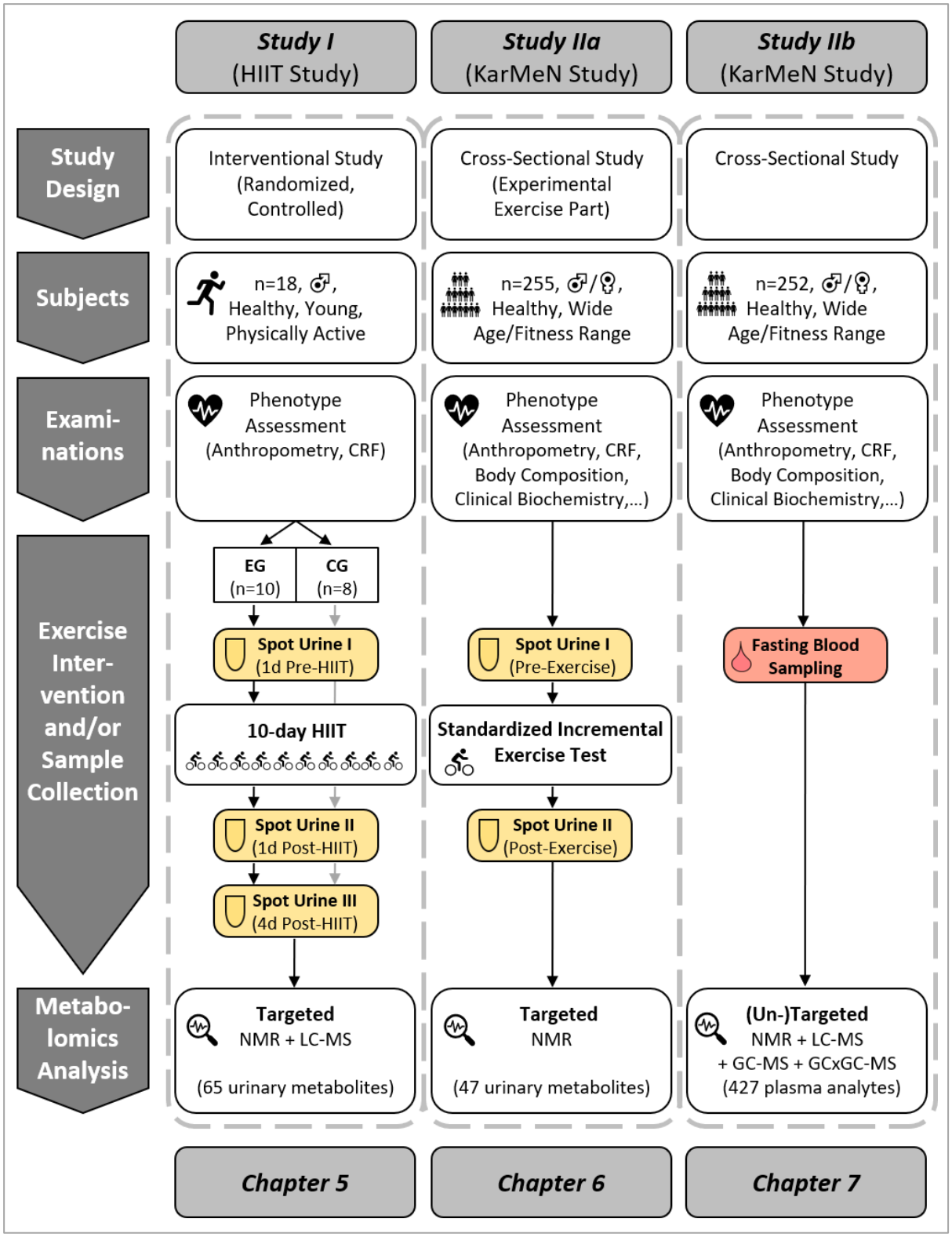

Figure 12. Study designs, examinations, and metabolomics analyses of *Study I*, *IIa*, and *IIb*. CG: control group; CRF: cardiorespiratory fitness; d: day(s); EG: experimental group; GC: gas chromatography; GC × GC: two-dimensional gas chromatography; HIIT: high-intensity interval training; KarMeN: Karlsruhe Metabolomics and Nutrition; LC: liquid chromatography; MS: mass spectrometry; n: sample size; NMR: nuclear magnetic resonance. *(Own illustration).*

4.1 Subjects and Study Designs

4.1.1 Study I (HIIT Study)

The randomized controlled interventional study was conducted at the Department of Sport and Exercise Science at the University of Stuttgart, Germany. Twenty healthy, active men between 20 and 50 years were included in *Study I*. Detailed inclusion and exclusion criteria are listed in Table A-1 (Appendix A1.1). As described in detail in Chapter 5.3.1, two subjects dropped out of the study and the final sample size of *Study I* was n=18.

After preliminary examinations, the participants were randomly assigned to an experimental group (EG) or a control group (CG). For ten days in a row, the EG performed a daily HIIT on a bicycle ergometer, consisting of eight sets at the individual maximal power (P_{max}), with a duration of 60% of the previously determined time to exhaustion at P_{max} (T_{max}), and a passive recovery period until the HR decreased to 65% of the HR_{max} reached in the preliminary incremental exercise test (see Chapter 4.2.2). As described in Chapter 5.3.3, various control parameters like the HR, capillary blood lactate, training impulse (TRIMP), rating of perceived exertion (RPE), and subjective well-being were assessed during or after the daily HIIT. Apart from the pre-testing and the ten study days, the participants of both the EG and CG visited the study center one day before the training started (Visit 1, V1), one day after the last training session (Visit 2, V2), and after four days of recovery (Visit 3, V3). At all visits, the participants provided fasting spot urine samples for subsequent metabolomics analyses.

An overview of the study design including urine sample collection is provided in Figure 13.

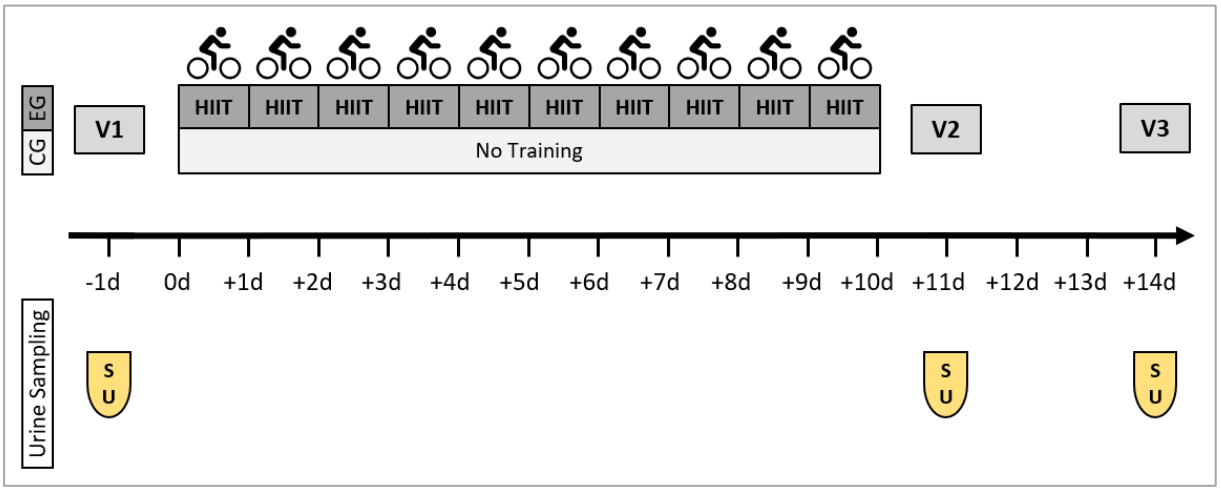

Figure 13. Examination schedule and urine sample collection in *Study I*. CG: control group; d: day(s); EG: experimental group; HIIT: high-intensity interval training; SU: spot urine; V: visit. *(Own illustration).*

4.1.2 Study II (KarMeN Study)

The cross-sectional KarMeN study was conducted at the Division of Human Studies of the MRI in Karlsruhe, Germany. 301 healthy women and men between 18 and 80 years participated in *Study II*. Detailed inclusion and exclusion criteria are listed in Table A-4 (Appendix A2.1). Additionally, a flowchart depicting the general recruitment process of *Study II* as well as the inclusion of subjects to metabolomics data analysis conducted in the framework of this thesis is provided in Figure A-4 (Appendix A2.2). As described in detail in Chapter 6.3.5, 46 individuals were recorded as dropouts in *Study IIa* and the final sample size was n=255 (148 men, 107 women). Regarding *Study IIb*, 49 individuals were excluded from data analysis, resulting in a final sample size of n=252 (150 men, 102 women), see Chapter 7.3.7.

During the study period, all participants visited the study center three times and completed a standardized examination schedule. Figure 14 provides a schematic overview of the visits, examinations, and sample collections. In general, standard operating procedures (SOPs) were applied for all processes related to examinations, measurements, and handling of biological samples. All subjects were characterized by various anthropometric and clinical investigations, which are further described in Chapter 4.2.

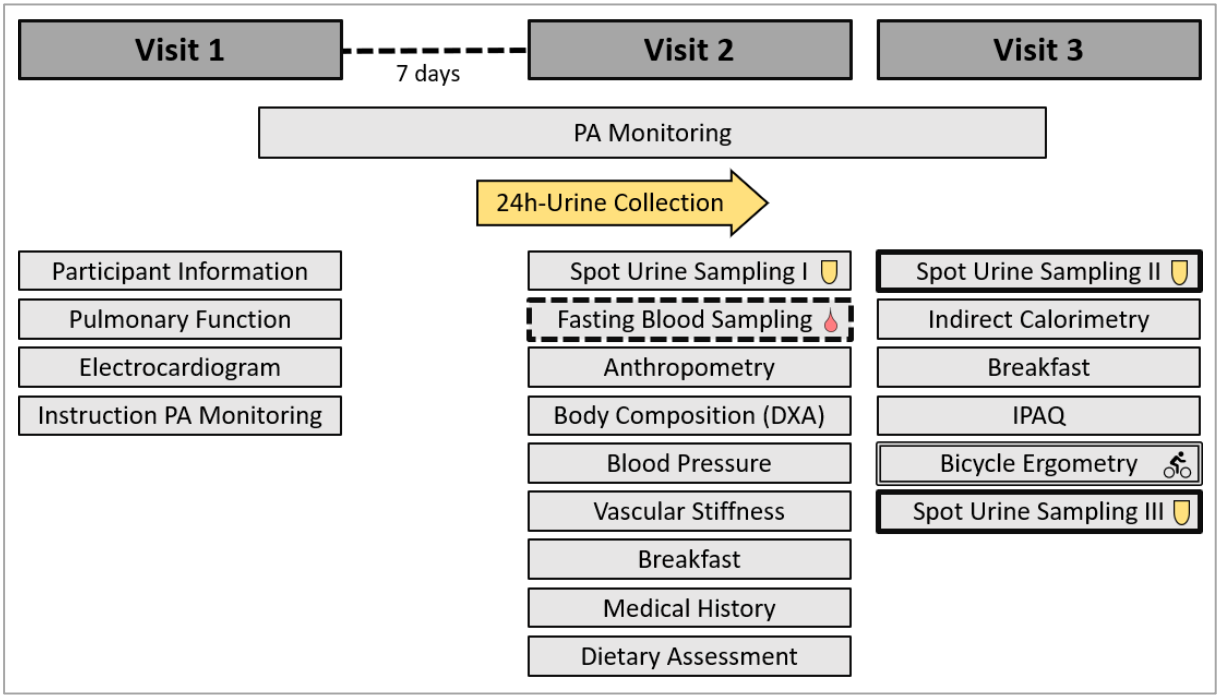

Figure 14. Visits, examinations, and sample collections in *Study II*. Continuous/dashed bold lines: urine or blood samples relevant for metabolomics analyses in *Study IIa* or *IIb*, respectively. Doubled line: experimental part consisting of a standardized incremental exercise test on a bicycle ergometer for determining the cardiorespiratory fitness, relevant for both *Study IIa* and *IIb*. DXA: dual-energy X-ray absorptiometry; h: hour(s); IPAQ: International Physical Activity Questionnaire; PA: physical activity. *(Own illustration based on Bub et al. (2016)).*

Biological samples for metabolomics analyses were collected on the second (Visit 2, V2) and third study day (Visit 3, V3). While fasting blood samples obtained at V2 were used in the framework of *Study IIb*, spot urine samples collected at V3 were relevant for the metabolomics investigations in *Study IIa*. Those spot urine samples were provided in the resting state or after a standardized incremental exercise test on a bicycle ergometer until exhaustion, respectively.

An overview of the examination schedule and spot urine sampling at V3 is given in Figure 15. All subjects entered the study center in the morning after an overnight fast and provided a spot urine sample. This was about 1.5 hours before the bicycle ergometry started. The exercise test, which is described in more detail in Chapter 4.2.2, lasted between 10 and 36 minutes, according to the ability of participants to tolerate incremental workloads. Approximately 15 to 30 minutes post-exercise, the participants provided another spot urine sample.

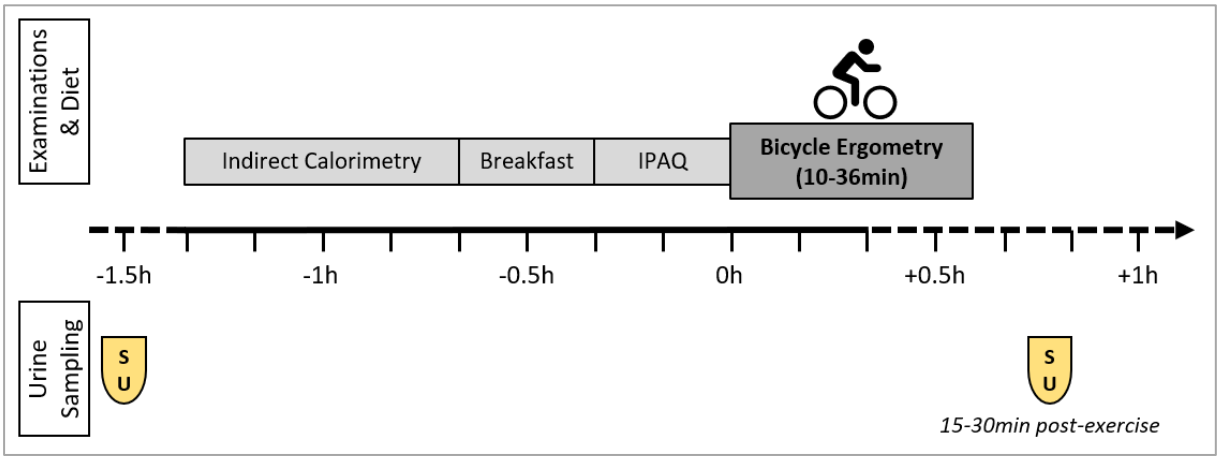

Figure 15. Examination schedule and urine sample collection at visit 3 in *Study II*. h: hour(s); IPAQ: International Physical Activity Questionnaire; min: minute(s); SU: spot urine. *(Own illustration).*

4.2 Methods of Investigation

4.2.1 Anthropometric, Body Composition, and Clinical Examinations

Within *Study I* and *Study II*, participants were subjected to different baseline investigations, including anthropometric, body composition, or clinical measurements. In both studies, body weight and height were measured and the BMI was calculated. In *Study II*, the body composition was additionally assessed by dual-energy X-ray absorptiometry (DXA; Lunar iDXA, GE Healthcare, München, Germany) and LBM, fat mass (FM), or bone mass were calculated. As clinical parameters, the HR_{rest} and BP (Boso Carat Professional, Bosch + Sohn, Jungingen, Germany) were assessed and physiological measurements such as the examination of arterial

stiffness (ArterioGraph, Medexpert, Budapest, Hungary) or pulmonary function (FlowScreen, CareFusion, Hoechberg, Germany) were conducted in *Study II*. Information on short-term and habitual PA as well as regular diet was also obtained from all participants of *Study II*. Moreover, blood samples collected in *Study II* were used for standard clinical biochemistry.

4.2.2 PF Assessment and Further Exercise-Related Preliminary Tests

In *Study I* and *Study II*, all subjects performed a standardized incremental exercise protocol on a bicycle ergometer in order to assess the CRF by measuring the VO_{2max} (*Study I*) or the VO_{2peak} (*Study II*). During the tests, the HR was continuously recorded and respiratory gas exchange was measured breath-by-breath. An overview of the applied exercise tests is given in Table 7.

Table 7. Overview of the standardized incremental exercise tests in *Study I* and *II. (Own table).*

	Study I	***Study II***
Protocol	Start: 100 watt + 15 watt every 30 seconds	Start: 25 watt + 25 watt every two minutes
Ergometer	Excalibur Sport, Lode B.V., Groningen, Netherlands	Ergobike medical, Daum, Fürth, Germany
Endpoints	VO_{2max}, P_{max}, HR_{max}	VO_{2peak}

HR_{max}: maximum heart rate; P_{max}: maximal power; VO_{2max}: maximal oxygen uptake; VO_{2peak}: peak oxygen uptake.

In *Study I*, participants started pedaling at 100 watt and workload was then increased by 15 watt every 30 seconds until volitional fatigue. As described in Chapter 5.3.2, particular exhaustion criteria had to be fulfilled to ensure an individual's maximal effort. Based on the presence of a plateau in the VO_2, the VO_{2max} was determined and subsequently used as a criterion for randomization. A further endpoint of the incremental test was P_{max}, which was used to define the individual workloads at which HIIT sets were performed. Based on the reached HR_{max}, the duration of the passive recovery period between HIIT sets was determined. To define the duration of HIIT sets, the EG additionally conducted a sprint cycling test at the predetermined P_{max} until exhaustion. Endpoint of this test procedure was T_{max}, i.e., the time individuals were able to perform at P_{max}. In *Study II*, the standardized exercise tolerance test began at 25 watt and workload was then increased by 25 watt every two minutes until individual exhaustion. Since the exhaustion criteria of a plateau in the VO_2 was not applied in *Study II*, the VO_{2max} could not be determined with certainty. Thus, the VO_{2peak} as the highest reached VO_2 during the test was utilized instead. As described in Chapters 6.3.2, specific termination criteria, such as the appearance of acute hypertension, were applied.

4.3 Metabolomics Analyses

4.3.1 Sample and Quality Control Preparation

All urine and plasma samples were centrifuged at 1850 g at 4°C for 10 minutes and aliquoted into small portions, before being stored at −80°C (*Study I*) or cryopreserved at −196°C (*Study II*) until metabolomics analyses. Internal quality control (QC) samples were prepared by pooling the respective samples from each study. These QC samples were used for all metabolomics methods in order to assess their reproducibility and precision and to correct for systematic measurement bias.

4.3.2 Overview of Applied Analytical Methods

In Study *I*, *IIa*, and *IIb*, different (non-)targeted analytical methods were applied. An overview of the particular metabolomics techniques and the number of detected analytes is provided in Table 8. Since the specific metabolomics approaches and the respective preparation steps are described in detail in the publications presented in Chapters 5 to 7, the next section only gives a short summary of all applied analytical methods.

Table 8. Overview of samples, metabolomics methods, and number of detected analytes in *Study I*, *IIa*, and *IIb. (Own table).*

	Study I	***Study IIa***	***Study IIb***
Biological Samples			
Urine	x	x	
Plasma			x
Applied Analytical Methods			
NMR (targeted)	x	x	
NMR (non-targeted)			x
LC-MS (targeted, methylated amines)	x		x
LC-MS (targeted, bile acids)			x
LC-/FIA-MS (targeted, Absolute IDQ™ p180 kit)			x
GC-MS (targeted, fatty acids)			x
GC × GC-MS (non-targeted)			x
Total Number of Detected Analytes	**74**	**47**	**659**

FIA: flow injection analysis; GC: gas chromatography; GC × GC: two-dimensional gas chromatography; LC: liquid chromatography; MS: mass spectrometry; NMR: nuclear magnetic resonance.

Targeted NMR Analysis (*Study I, IIa*):

Urine samples from *Study I* and *Study IIa* were analyzed by 1D-^{1}H-NMR spectroscopy. After pre-processing of NMR spectra, metabolite identification was conducted with Chenomx NMR Suite 8.1 (*Study I*) or 8.4 (*Study IIa*) (Chenomx, Edmonton, AB, Canada). As an internal reference for urinary metabolite quantification, the trimethylsilylpropanoic acid (TSP) signal was used. By comparing the respective signals in experimental ^{1}H-NMR spectra to a library of spectral signatures contained in the Chenomx software, 47 urinary metabolites could be identified and quantified with sufficient certainty in both studies. The detected metabolites covered various chemical classes, such as organic acids, AAs, amines, or sugar alcohols (see Chapters 5.3.5 and 6.3.4). In Figure 16, the identification of the purine base hypoxanthine in the urine NMR spectrum of a single participant is exemplified.

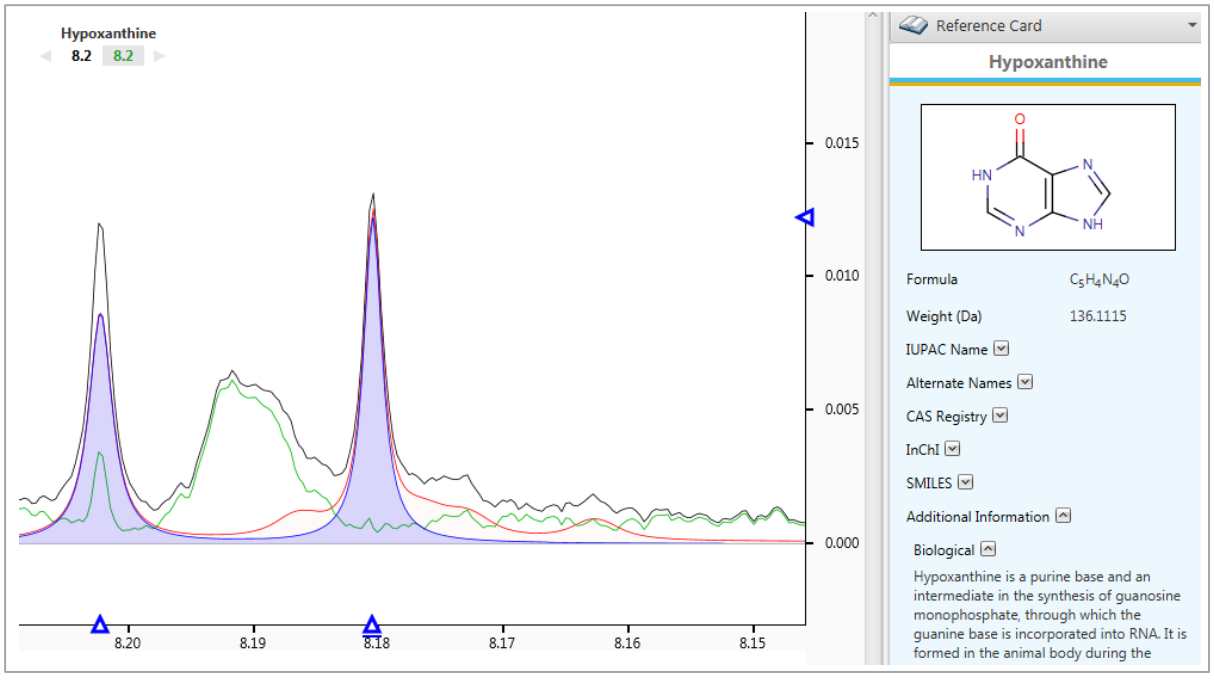

Figure 16. Identification of urinary metabolites (here: hypoxanthine) in ^{1}H-NMR spectra by using Chenomx. *(Own illustration).*

Non-Targeted NMR Analysis (*Study IIb*):

Plasma samples from *Study IIb* were analyzed by 1D-^{1}H-NMR spectroscopy. Pre-processed NMR spectra were bucketed graphically, such that buckets contained only one signal or group of signals and no peaks were split between buckets, if possible. Buckets were related to a previously known, identified analyte or registered as unknown, i.e., that they contained either unspecific signals or overlapped peaks. The identification of metabolites was carried out by using Chenomx NMR Suite 8.1 (Chenomx, Edmonton, AB, Canada) (see Chapter 7.3.6).

Targeted LC-MS Analysis of Methylated Amino Compounds (*Study I, IIb*):

Urine samples from *Study I* and plasma samples from *Study IIb* were also analyzed by ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS). As described in Krüger et al. (2017), the quantification of six methylated amino compounds in plasma was established in *Study II* (see Chapter 7.3.6). In *Study I*, this method was adapted to urine and extended to 27 analytes, including arginine, carnosine, π-, and τ-methylhistidine (see Chapter 5.3.5).

Targeted LC-MS Analysis of Bile Acids (*Study IIb*):

In plasma samples from *Study IIb*, several bile acids were quantified using an LC-MS stable isotope dilution assay, as described in Frommherz et al. (2016) (see Chapter 7.3.6).

Targeted LC-/FIA-MS Metabolite Profiling Using the Absolute IDQ™ p180 kit (*Study IIb*):

Plasma samples from *Study IIb* were also utilized for a targeted metabolite profiling using the Absolute IDQ™ p180 kit developed by Biocrates AG (Innsbruck, Austria). With this method, several acylcarnitines, AAs, PCs, and sphingomyelins (SMs) were detected (see Chapter 7.3.6).

Targeted GC-MS Analysis of Fatty Acids (*Study IIb*):

In plasma samples from *Study IIb*, 48 fatty acids were determined in a quantitative manner by using a GC-MS method, which has been previously described by Ecker, Scherer, Schmitz, and Liebisch (2012) and was applied with minor modifications (see Chapter 7.3.6).

Non-Targeted GC × GC-MS Analysis (*Study IIb*):

Plasma samples from *Study IIb* were also analyzed by a non-targeted GC × GC-MS-based approach, as previously described by Weinert, Egert, and Kulling (2015). With this method, a broad range of metabolites, e.g., AAs, amines, organic acids, sugars, sugar alcohols, or polyols, but also unknown analytes, were detected (see Chapter 7.3.6).

Lists of all analytes measured by the described (non-)targeted metabolomics approaches are provided in the Appendices A3.1 to A3.7, along with their assignment to the four levels of metabolite identification proposed by the Metabolomics Standards Initiative (MSI), namely ID level 1: identified compound; ID level 2: putatively annotated compound; ID level 3: putatively annotated compound class; ID level 4: unknown compound (Sumner et al., 2007).

4.4 Data Handling and Statistical Analyses

Data handling and statistical analyses differed between the included studies according to the particular research questions, examined biospecimens, and applied analytical methods.

With respect to participants, a final number of n=18 (*Study I*), n=255 (*Study IIa*), and n=252 (*Study IIb*) were included into data analyses. Since urinary metabolite data were known to be affected by differences in urine dilution (Y. Wu & Li, 2016), metabolite concentrations were normalized to either urinary creatinine (*Study I*) or osmolality (*Study IIa*). Within each study, the metabolite data from the different analytical platforms were combined into a common data matrix and clearly defined procedures for the elimination of metabolites that were either measured by more than one technique or had a low detection frequency were applied. In *Study I*, nine of the 74 detected urinary metabolites were quantified by both targeted NMR- and LC-MS-based analysis. Only those values obtained by LC-MS analysis, which is the more sensitive of both methods (A.-H. M. Emwas, 2015), were considered for statistical analyses. Due to the normalization to NMR-measured creatinine in *Study I*, 64 urinary metabolites were finally included into data analysis. In *Study IIa*, 47 metabolites were quantified in all urine samples and thus included into data analysis. In *Study IIb*, plasma analytes were considered for statistical analysis if they had a detected frequency ≥ 20% in the female and male sub-group. If identified plasma analytes were assessed by more than one technique, those analytes detected by the less quantitative method were excluded. From an initial number of 659 analytes, 427 were included into data analysis. While no metabolite data transformation was applied in *Study I*, metabolite data were transformed into Van der Waerden (VdW) scores in *Study IIa* and *Study IIb*. By this rank based inverse normal transformation, the issue of values below the limit of detection was taken into account and a uniform scale for all analytes was obtained, i.e., they were finally comparable between analytical platforms.

As listed in Table 9, different uni-, bi-, and multivariate statistical approaches were applied in order to analyze the effects of acute and chronic PA on plasma or urinary metabolite profiles. They are not depicted further in this section, but are described in detail in the respective publications (Chapters 5.3.6, 6.3.5, and 7.3.7). Statistical analyses were conducted with SAS JMP 11.0.0 (SAS Institute Inc. 2013, Cary, NC, USA) and the software R (Version 3.4.2 (*Study I*); Version 3.6.0 (*Study IIa*); Version 4.0.0 (*Study IIb*); https://www.R-project.org). Figures were generated in Excel 2016 or R.

Table 9. Overview of data handling and statistical analyses in *Study I*, *IIa*, and *IIb. (Own table).*

	Study I	***Study IIa***	***Study IIb***
Data Handling			
Final sample size [dropouts]	n=18 [exclusion of n=2 due to illness or technical measurement errors]	n=255 [exclusion of n=46 due to missing spiroergometry data, implausible HR_{rest} data, or outlying metabolite data]	n=252 [exclusion of n=49 due to missing spiroergometry data, implausible HR_{rest} data, errors during analyses, or missing plasma sample]
Sex-separated analysis	- (only males)	Partly	Yes
Final number of metabolites [excluded metabolites]	64 urinary metabolites [exclusion of n=10 due to multiple analyses or use for normalization purposes]	47 urinary metabolites [-]	427 plasma analytes [exclusion of n=232 due to low detection frequency or multiple analyses]
Normalization of metabolite concentrations	To urinary creatinine	To urinary osmolality	-
Metabolite data transformation	-	VdW scores	VdW scores
Univariate Statistical Analysis – Comparison Pre- vs. Post-Exercise Metabolite Concentrations			
Method	Mixed effect model	Wilcoxon's signed rank test	-
Correction for multiple testing	Partly	FDR	-
Bivariate Statistical Analysis – Relationship Between <u>Single</u> Metabolites and the CRF			
Method	-	(Partial) Pearson correlation (^{v})	Partial Pearson correlation (^{v})
Considered confounding factors	-	None (1^{st}) Age, sex, menopausal status, LBM (2^{nd})	Age, menopausal status (1^{st}) (+ 21 phenotypical/ clinical variables (2^{nd}))
Multivariate Statistical Analysis – Comparison Pre- vs. Post-Exercise Metabolite Profiles			
Method	-	PCA (^{v})	-
Multivariate Statistical Analysis – Relationship Between <u>Multiple</u> Metabolites and the CRF			
Method	-	Stepwise multiple linear regression (^{v})	Stepwise multiple linear regression (^{v})
Considered confounding factors	-	Age, sex, menopausal status, LBM	Age, menopausal status (+ selected pheno-typical/clinical variables included in the model)
Multivariate Statistical Analysis – Relationship Between <u>All</u> Metabolites and the CRF			
Method	-	Multiple linear regression (^{v}), PCA (^{v})	PLS regression (^{v})
Considered confounding factors	-	Age, sex, menopausal status, LBM	Age, menopausal status (1^{st}) (+ 21 phenotypical/ clinical variables (2^{nd}))
	Chapter 5	***Chapter 6***	***Chapter 7***

^{v}: using VdW-transformed data; CRF: cardiorespiratory fitness; FDR: false discovery rate; HR_{rest}: resting heart rate; LBM: lean body mass; n: sample size; PCA: principal component analysis; PLS: partial least squares; VdW: Van der Waerden.

4.5 Functional Classification of PE- or PF-Related Metabolite Profiles

4.5.1 Manual Categorization of Identified Metabolites to Metabolic Pathways

Based on the information contained in the HMDB, Version 4 (https://hmdb.ca) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY database (https://www.genome.jp/kegg/pathway.html), all metabolites with the MSI ID levels 1 and 2 in *Study I, IIa*, and *IIb* were assigned to pathways of human metabolism or chemical classes, thus providing a first basis for the functional classification of PE- or PF-related metabolite profiles.

The HMDB is a database providing information about metabolites found in human organism, especially with regard to their origin and biological function, physiological concentrations in biological samples, and associated metabolic pathways (Wishart et al., 2018). The KEGG PATHWAY database serves as a collection of pathway diagrams, depicting molecular interactions between metabolites in cellular processes (Aoki & Kanehisa, 2005). By using the metabolite description contained in the HMDB and the detailed view on metabolites in biological networks provided by the KEGG PATHWAY database, the (putatively) identified metabolites were manually assigned to 8 major pathways and 38 sub-pathways or chemical classes, respectively. An overview of the classification scheme is presented in Table 10.

While the majority of metabolites (e.g., AAs, fatty acids, lipids) could easily be related to major and specific pathways, the grouping of metabolites with various origins or participation in different metabolic pathways was comparatively complex. Especially with regard to sugar-related metabolites, a clear procedure for metabolite classification had to be applied. If sugars or sugar derivatives show an exogenous origin and are not known to be produced in human metabolism (e.g., mannitol), they were assigned to the major pathway ‘xenobiotics and related metabolism’ and the sub-pathway ‘sugars, sugar substitutes and sugar derivatives’. Compounds that can be endogenously produced in human metabolism (e.g., acetate) were classified to the major pathway ‘carbohydrate metabolism’ and the respective sub-pathway. Sugar metabolites were classified to the major pathway ‘carbohydrate metabolism’ and the sub-pathway ‘miscellaneous (unclassified sugar acids/polyols)’ if their origin and participation in human metabolism was largely unknown (e.g, arabitol). Further, non-sugar metabolites showing both an endo- and exogenous origin (e.g., citrate and malate; TCA cycle intermediates and food constituents) were equally classified according to their participation in human

metabolism and not according to their exogenous origin. If exogenous substances are known to be pre-metabolized by the intestinal microbiota (e.g., trimethylamines or polyphenols), they were classified to the major pathway 'mammalian-microbial cometabolism'.

Table 10. Metabolite classification scheme. *(Own table).*

	Major Pathway	Sub-Pathway or Chemical Class
1	**Amino acid metabolism**	Alanine and aspartate metabolism Arginine and proline metabolism; urea cycle Creatine metabolism Cysteine, methionine and taurine metabolism Glutamate metabolism Glycine, serine and threonine metabolism Histidine metabolism Isoleucine, leucine and valine metabolism Lysine metabolism Phenylalanine and tyrosine metabolism Tryptophan metabolism
2	**Carbohydrate metabolism**	Glucose and pyruvate metabolism Inositol phosphate metabolism Miscellaneous (unclassified sugar acids/polyols) Propanoate metabolism
3	**Cofactors and vitamins metabolism**	Glutathione metabolism Nicotinate and nicotinamide metabolism
4	**Energy metabolism**	TCA cycle intermediates
5	**Lipid metabolism**	Bile acid metabolism Carnitine metabolism Glycerolipid metabolism Glycerophospholipid metabolism Ketone body metabolism Long-chain fatty acid metabolism Medium-chain fatty acid metabolism Sphingolipid metabolism
6	**Mammalian-microbial cometabolism**	One carbon metabolism Phenylalanine and tyrosine metabolism Polyphenolic compounds metabolism Trimethylamines metabolism Tryptophan metabolism
7	**Nucleotide metabolism**	Purine metabolism Pyrimidine metabolism
8	**Xenobiotics and related metabolism**	Chemicals Detoxification metabolism Food or plant constituents Sugars, sugar substitutes and sugar derivatives Xanthine metabolism

TCA: tricarboxylic acid.

Choline, a metabolite that participates in multiple human pathways, was classified to the major pathway 'AA metabolism' and the sub-pathway 'glycine, serine and threonine metabolism' owing to its function as a methyl donor via the formation of betaine (Zeisel & da Costa, 2009). Nevertheless, its role as a precursor of PCs or acetylcholine has also to be kept in mind when finally interpreting choline-related results.

Lists of all detected metabolites and their classification to major metabolic pathways, sub-pathways, or chemical classes as well as their specific HMDB ID and KEGG ID are provided in the Appendices A3.1 to A3.7, according to each applied metabolomics method. The specific categorization of (putatively) identified metabolites that were analyzed in the framework of either *Study I, IIa*, *or IIb* is depicted in the following. Pie charts were generated in Excel 2016.

Study-Specific Categorization of Identified Metabolites to Metabolic Pathways

In *Study I*, 64 urinary metabolites were detected by using targeted NMR- and LC-MS-based approaches. Their classification to major metabolic pathways is depicted in Figure 17. As illustrated, most metabolites belonged to 'AA metabolism' (31/64) and 'xenobiotics-related metabolism' (13/64), followed by 'mammalian-microbial cometabolism' (8/64), 'nucleotide metabolism', 'energy metabolism' or 'carbohydrate metabolism' (each 3/64), 'lipid metabolism' (2/64), and 'cofactors and vitamins metabolism' (1/64). The grouping of metabolites to sub-pathways is provided in Figure A-20 (Appendix A4.1).

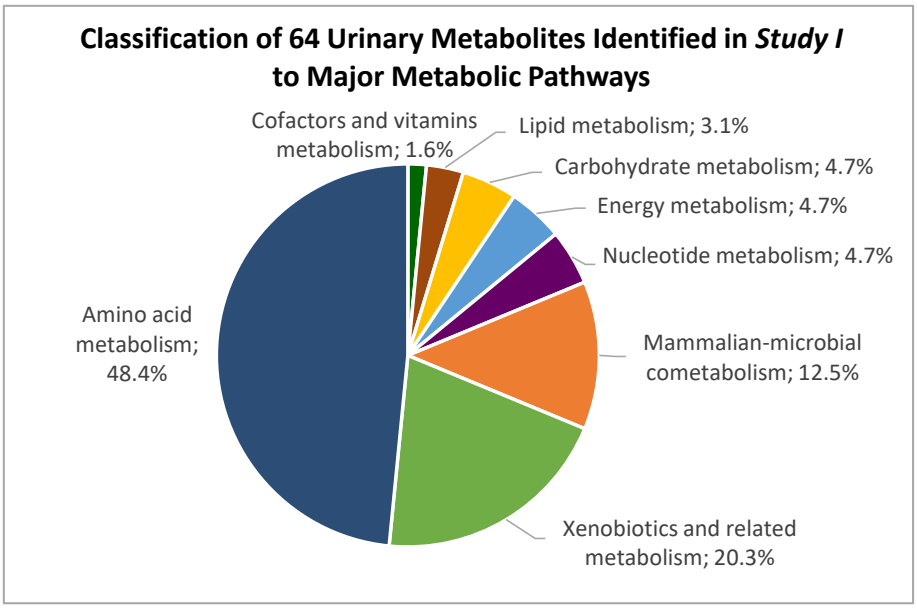

Figure 17. Classification of metabolites detected by targeted NMR- and LC-MS-based analyses in *Study I*. *(Own illustration).*

In *Study IIa*, 47 urinary metabolites were identified by targeted NMR-based analysis. The classification of those metabolites to major metabolic pathways is shown in Figure 18. While the majority of the 47 metabolites were related to 'AA metabolism' (19/47), the remaining metabolites belonged to 'xenobiotics-related metabolism' (10/47), 'mammalian-microbial cometabolism' (6/47), 'nucleotide metabolism', 'energy metabolism' or 'carbohydrate metabolism' (each 3/47), 'lipid metabolism' (2/47), and 'cofactors and vitamins metabolism' (1/47). The further categorization of metabolites to sub-pathways is illustrated in Figure A-21 (Appendix A4.2).

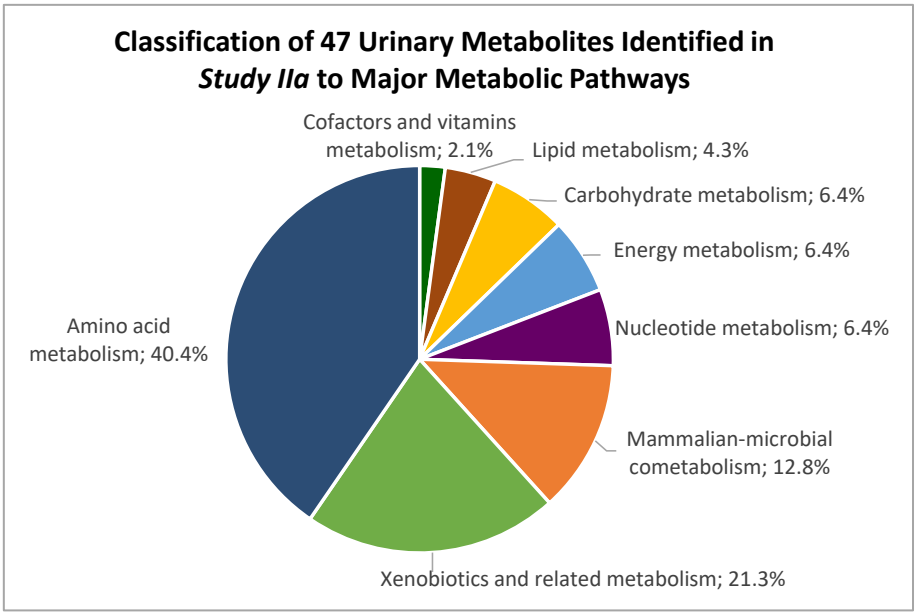

Figure 18. Classification of metabolites detected by targeted NMR-based analysis in *Study IIa*. *(Own illustration).*

In *Study IIb*, 427 plasma metabolites were detected by several (un-)targeted MS- and NMR-based analyses and the identity of 236 metabolites could be determined. The classification of the identified metabolites to major metabolic pathways is illustrated in Figure 19. As depicted, most metabolites belonged to 'lipid metabolism' (164/236) and 'AA metabolism' (41/236), followed by 'xenobiotics-related metabolism' (12/236), 'mammalian-microbial cometabolism' (7/236), 'carbohydrate metabolism' or 'energy metabolism' (each 5/236), and 'nucleotide metabolism' or 'cofactors and vitamins metabolism' (each 1/236). The assignment of metabolites to sub-pathways is provided in Figure A-22 (Appendix A4.3).

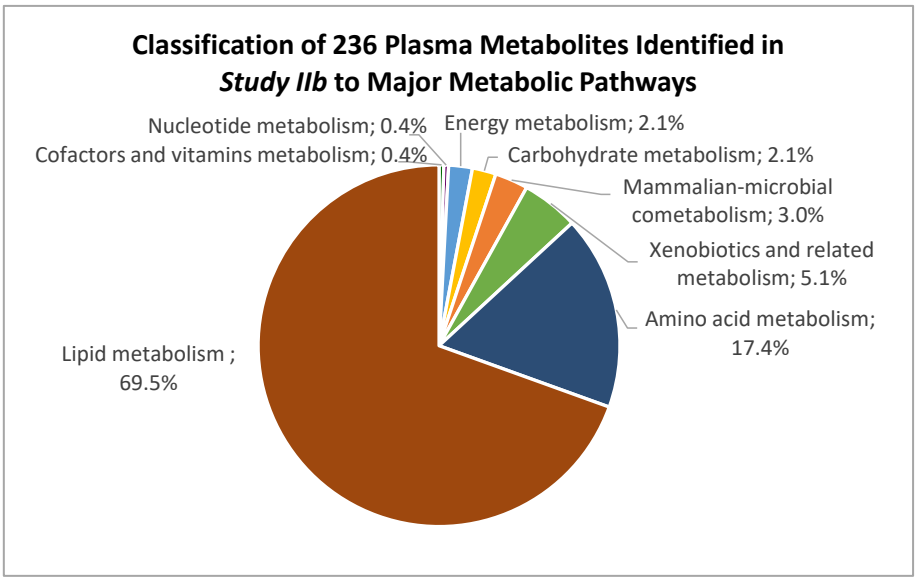

Figure 19. Classification of metabolites detected by (un-)targeted MS- or NMR-based analyses in *Study IIb*. *(Own illustration).*

The presented metabolite classification scheme should not only provide information about metabolic pathways to which the metabolites identified in the framework of either *Study I, IIa*, or *IIb* belonged. Based on this manually conducted metabolite categorization, PE- or PF-related metabolite profiles observed in those studies could furthermore be discussed in a biological context, i.e., it could be estimated which major and specific pathways of human metabolism were affected by medium-term HIIT (*Study I*) or acute PE (*Study IIa*), or associated with the CRF (*Study IIa/IIb*). Apart from the interpretation of PE- or PF-related metabolite profiles included in the particular publications (Chapters 5 to 7), an additional overview and visualization of the classification of PE- or PF-related metabolites to major and specific pathways is provided in Chapter 8. In the respective papers, results from uni-, bi-, or multivariate statistical approaches were presented. Yet, to ensure a certain comparability between studies and to facilitate interpretation, the classification of PE- or PF-related metabolites to metabolic pathways was restricted to the findings from univariate analyses of exercise-induced metabolite changes or bivariate analyses of PF-correlated metabolites, respectively.

Despite the fact that some metabolites participate in several metabolic pathways, this manual classification scheme only allowed to relate a given metabolite to one specific pathway. Consequently, in order to take the belonging of metabolites to various pathways adequately into account, a web-based pathway analysis that considers the interconnectivity between metabolites and thus the interdependence among pathways was additionally conducted.

4.5.2 Pathway Analysis Based on PE- or PF-Related Metabolite Profiles

So-called pathway analyses do not focus on single metabolites but rather on their interaction, revealing metabolic pathways which are most likely to be affected by a specific metabolite profile of interest (Chong et al., 2019). By using the pathway analysis module provided by MetaboAnalyst, Version 5 (https://www.metaboanalyst.ca), the PE- or PF-related metabolites identified in *Study I, IIa*, or *IIb* were mapped to biochemical pathways contained in the KEGG database (Chong et al., 2019). For all analyses, the KEGG pathway data library from October 2019 was selected as a background set, containing 84 metabolite sets that belong to human metabolic pathways.

In detail, the pathway analysis represents a combination of pathway enrichment analysis and pathway topology analysis. While the enrichment analysis investigates if functionally related metabolites are significantly enriched compared to random hits, the topology analysis takes the general pathway structure and the centrality of metabolites in related pathways into account (Chong et al., 2019). With regard to enrichment analysis, an over-representation analysis (ORA) was conducted. Based on a hypergeometric test, ORA evaluates if a particular set of metabolites is represented more than expected by change within a given compound list (J. Xia & Wishart, 2010b). The compound lists from *Study I, IIa*, and *IIb* comprised the particular PE- or PF-related metabolites of interest, which have been obtained by study-specific selection methods that ranked all analyzed metabolites and selected those scoring above or below a certain threshold. Finally, the *p*-values obtained from ORA indicate the probability of seeing a particular number of metabolites from a certain metabolite set, i.e., compounds from a specific pathway, in a given compound list (J. Xia & Wishart, 2010b). If specific pathways showed p-values < 0.05, metabolites involved in those pathways were significantly enriched in the compound lists from *Study I, IIa*, or *IIb*. To account for problems related to multiple comparisons, false discovery rate (FDR) adjustments were conducted. A schematic overview of the ORA approach is provided in Figure 20 (left).

As it is well-known that changes in more important positions of a metabolic network have a stronger impact on a pathway than changes in marginal positions (J. Xia & Wishart, 2010a), ORA was combined with a topology analysis. By using two node centrality measures, namely the “degree centrality” measure (defined as the number of connections a metabolite node has to other metabolite nodes within a pathway) and the “betweenness centrality” measure

(defined as the number of shortest paths from different pathways going through a metabolite node), the potential importance of a given metabolite was evaluated based on its position within a metabolic network (Chong et al., 2019). Finally, to compare the importance of those pathways to which the PE- or PF-related metabolites identified in *Study I, IIa*, *or IIb* were mapped, so-called pathway impact values were determined based on the centrality measures of all metabolites assigned to a particular pathway. In detail, the sum of the node importance measures of the matched metabolites was normalized by the sum of the importance measures of all metabolites in this pathway. While the total importance of each pathway is 1, the importance measure of each metabolite node represents a certain percentage. Accordingly, pathway impact values obtained by topology analysis refer to the cumulative percentage from matched metabolite nodes (J. Xia & Wishart, 2010a). For all analyses, the impact value threshold calculated from pathway topology analysis was set at 0.1. A schematic overview of the pathway topology approach is provided in Figure 20 (right).

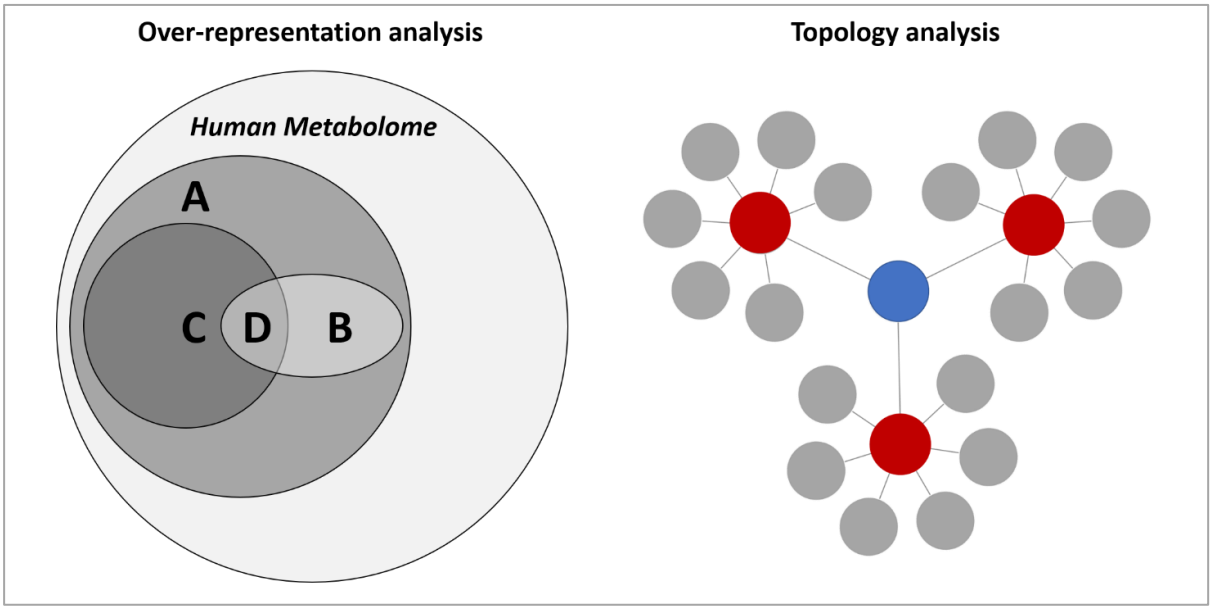

Figure 20. Schematic illustration of ORA and topology analysis. A: compounds covered by the selected background set; B: compounds of a specific pathway; C: compounds of interest; D: overlap between the compounds of interest and compounds of a specific pathway; blue: high betweenness centrality; red: high degree centrality. *(Own illustration based on Chong et al. (2019) and MetaboAnalyst 5.0 (https://www.metaboanalyst.ca)).*

Results are presented in overview graphs containing all matched pathways arranged by log-transformed *p*-values from ORA on the Y-axis and impact values from topology analysis on the X-axis (Chapter 8). Pathways in the top right corner indicate that metabolites involved in those pathways are significantly enriched in the respective compound lists, and that they are likely to have significant impacts on the pathways based on their positions (Chong et al., 2019).

5 Study I

High-Intensity Interval Training Decreases Resting Urinary Hypoxanthine Concentration in Young Active Men—A Metabolomic Approach

Slightly modified version of the published article (10 July 2019).

Kistner S., Rist M.J., Krüger R., Döring M., Schlechtweg S., Bub A. (2019). High-Intensity Interval Training Decreases Resting Urinary Hypoxanthine Concentration in Young Active Men—A Metabolomic Approach. *Metabolites*, *9*(7), 137. doi:10.3390/metabo9070137.

For a better comprehensibility of results, the supplementary material of this article is provided in the Appendices A1.2 to A1.5. Datasets are available under the following link: https://doi.org/10.3390/metabo9070137.

5.1 Abstract

High-intensity interval training (HIIT) is known to improve performance and skeletal muscle energy metabolism. However, whether the body's adaptation to an exhausting short-term HIIT is reflected in the resting human metabolome has not been examined so far. Therefore, a randomized controlled intervention study was performed to investigate the effect of a ten-day HIIT on the resting urinary metabolome of young active men. Fasting spot urine was collected before (−1 day) and after (+1 day; +4 days) the training intervention and 65 urinary metabolites were identified by liquid chromatography (LC)-mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Metabolite concentrations were normalized to urinary creatinine and subjected to univariate statistical analysis. One day after HIIT, no overall change in the resting urinary metabolome, except a significant difference with decreasing means in urinary hypoxanthine concentration, was documented in the experimental group (EG). As hypoxanthine is related to purine degradation, lower resting urinary hypoxanthine levels may indicate a training-induced adaptation in 'purine metabolism'.

Keywords: metabolomics; urinary metabolome; hypoxanthine; high-intensity interval training; NMR spectroscopy; LC-MS.

5.2 Introduction

Over the past decade, research interest in HIIT as a time-efficient method for inducing health benefits has greatly increased (Laursen & Jenkins, 2002). A growing body of evidence supports the potential of HIIT to cause similar or even superior improvements in skeletal muscle energy metabolism and cardiometabolic health as compared to moderate-intensity continuous exercise (Cassidy et al., 2017; Gibala, Little, Macdonald, & Hawley, 2012). In order to allow physicians and sports scientists to recommend more specific exercise programs or training strategies, it is crucial to know more about the cellular mechanisms and metabolic alterations underlying the whole-body and skeletal muscle adaptation to HIIT. On a molecular level, there is already strong evidence that higher exercise intensities lead to a stronger expression of peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α (Egan et al., 2010; Little, Safdar, Bishop, Tarnopolsky, & Gibala, 2011), which is regarded as a major regulator of mitochondrial biogenesis in human skeletal muscle (Spiegelman, 2007). Given the positive effects that seem to be associated with an increase in muscle PGC-1α, e.g., an increase in skeletal muscle oxidative capacity or a greater reliance on fat oxidation (Benton et al., 2008), the elevated activity of PGC-1α following HIIT underpins the potential of HIIT to stimulate long-term metabolic changes that promote widespread health benefits (Gibala et al., 2012; MacInnis & Gibala, 2017). However, until recently, the majority of studies have focused on the investigation of a small number of key molecules or selected metabolites in order to elucidate metabolic adaptations to HIIT (Astorino & Schubert, 2017; Burgomaster et al., 2008; Granata, Oliveira, Little, Renner, & Bishop, 2016; Little et al., 2011). Since several biochemical pathways are interacting with each other, capturing a broader view of changes in metabolism is required.

The emerging metabolomics method offers a more comprehensive approach to simultaneously analyze a wide range of metabolites. Metabolomics facilitates the systematic identification and quantification of a large number of metabolites, defined as low-molecular weight compounds, by using analytical techniques like NMR spectroscopy, LC-MS, or gas chromatography (GC)-MS (Bujak et al., 2015). The complete set of endogenous metabolites like sugars, amino acids (AAs), amines, alcohols, steroids, or nucleosides as well as metabolites of exogenous origin in a given biological system, e.g., biofluids like blood or urine, represent the so-called “metabolome”. It is determined by age and sex (Rist et al., 2017) and can be

influenced by external factors like nutrition (Floegel, von Ruesten, et al., 2013) or physical activity (PA) (Chorell et al., 2012; Kujala et al., 2013). Assessing the metabolome as comprehensively as possible, metabolomics can provide valuable information on human metabolism and its response to physiological challenges like PE (Suárez et al., 2017). Thus, metabolomics contributes to the identification of exercise-responsive biomarkers, which could act as predictors of exercise-specific changes in metabolism. Metabolomics can also give insight into exercise-induced alterations in metabolite profiles, which are possibly associated with particular physiological processes or metabolic pathways.

Until now, a few studies have investigated the HIIT-induced modifications of the human metabolome by using metabolomics. The majority of studies have analyzed the acute metabolic effects of a single HIIT or sprint interval session (Danaher et al., 2015; Peake et al., 2014; Pechlivanis et al., 2010; Pechlivanis et al., 2013; Pechlivanis et al., 2015; Saoi et al., 2019; Siopi et al., 2017; Zafeiridis et al., 2016), documenting higher concentrations of lactate and pyruvate, which reflect a higher reliance on anaerobic energy production (Danaher et al., 2015; Pechlivanis et al., 2010; Pechlivanis et al., 2015; Saoi et al., 2019; Siopi et al., 2017; Zafeiridis et al., 2016), and/or hypoxanthine, which is known to indicate a high rate of adenosine triphosphate (ATP) turnover (Danaher et al., 2015; Pechlivanis et al., 2010; Siopi et al., 2017). However, the medium- and long-term effects of HIIT on the metabolome have rarely been examined so far. The limitations of existing studies are that training interventions were partly restricted to overweight individuals (Kuehnbaum et al., 2014; Kuehnbaum et al., 2015) and performed with a moderate frequency (Kuehnbaum et al., 2014; Kuehnbaum et al., 2015; Pechlivanis et al., 2013). Furthermore, metabolic alterations were only investigated in blood by using either NMR (Pechlivanis et al., 2013) or multi-segment injection capillary electrophoresis-MS (Kuehnbaum et al., 2014; Kuehnbaum et al., 2015). Nevertheless, these studies have provided the first evidence that high-intensity intermittent training is able to induce adaptive metabolic responses. Amongst others, this was shown by lower levels of lactate and pyruvate in both resting and post-exercise serum samples (Pechlivanis et al., 2013) or an attenuated exercise-induced increase in plasma hypoxanthine levels after HIIT (Kuehnbaum et al., 2015), hinting at a lower energetic stress in the trained status.

However, to the best of our knowledge, no other study has investigated to what extent the body's adaptation to an extremely demanding HIIT intervention is reflected in the urinary

metabolome. Especially in elite sports, athletes are often confronted with exhausting training protocols consisting of high-intensity exercise blocks, provoking a higher adaptation in performance and an improved fatigue resistance (Hawley et al., 1997; Laursen & Jenkins, 2002). Therefore, the purpose of this study was to investigate the effect of a ten-day HIIT, which is comparable to a pre-competition preparation phase in a periodized training schedule, on the resting urinary metabolome of young active men. This is the first randomized controlled intervention study using a combined NMR- and LC-MS-based metabolomic approach to assess changes in urinary metabolite profiles, thereby aiming to identify possible biomarkers for either training overload or training adaptation. The detected urinary metabolites represent a broad spectrum of organic compounds found in the human urine metabolome (Bouatra et al., 2013), ranging from AAs to alcohols, keto acids to purine derivatives and methylated amines. Urine as a biological matrix was chosen as it is easily accessible, stable, and under no homeostatic regulation like other biofluids (Bouatra et al., 2013; J. Wu & Gao, 2015). As the urinary metabolome was captured in the resting state, i.e., in the morning before and after the ten-day HIIT period, this study was particularly suitable to analyze if human metabolism is able to restore its disturbed homeostasis, which could be documented in response to acute HIIT (Danaher et al., 2015; Peake et al., 2014; Pechlivanis et al., 2010; Pechlivanis et al., 2013; Pechlivanis et al., 2015; Siopi et al., 2017; Zafeiridis et al., 2016), and can also be assumed due to an exhausting, daily performed HIIT like in this study.

5.3 Materials and Methods

5.3.1 Subjects and Study Design

Twenty healthy men volunteered to take part in this study. All participants were regularly active, competition-experienced, and participated in training at least three times a week. Further inclusion criteria were age between 20 and 50 years and body mass index (BMI) ≤ 30 kg/m^2. Participants were excluded if they used any medication, or if they suffered from musculoskeletal injuries within the preceding twelve months or from chronic diseases. The study consisted of a randomized controlled trial in which the participants were firstly subjected to standardized preliminary tests. Participants were then randomly assigned to either the EG or control group (CG) balanced to their age and training status. Subjects of the EG took part in a ten-day HIIT intervention, whereas subjects of the CG were told to refrain

from heavy physical exercise (PE) during the intervention period. In total, two subjects of the CG dropped out of the study due to illness or technical measurement errors in blood counts, respectively. The study was approved by the ethics committee of the State Medical Chamber of Baden-Württemberg, Stuttgart, Germany (DRKS-ID: DRKS00010841) and was conducted in accordance with the declaration of Helsinki. Written informed consent was obtained from all participants prior to entering the study.

5.3.2 Preliminary Testing

Before the onset of the study, participants were subjected to a preliminary examination that included anthropometric measurements (body weight (in kg), body height (in cm), and length of the lower limbs (in cm)) and the completion of a medical history form. BMI was calculated by dividing the body weight (in kg) by the height (in meters) squared.

In order to establish an individual training protocol and as a criterion for the randomization process, maximal oxygen uptake (VO_{2max}) was measured using an incremental protocol (see Laursen, Shing, Peake, Coombes, and Jenkins (2002)) on a bicycle ergometer (Excalibur Sport Ergometer, Lode B.V., Groningen, Netherlands). Briefly, each participant pedaled at a self-selected speed for five minutes, workload was then augmented to 100 watt and finally increased by 15 watt every 30 seconds until volitional exhaustion, i.e., when a pedal frequency of 60 revolutions per minute (rpm) could no longer be maintained. Respiratory gas exchange was measured breath-by-breath and the heart rate (HR) was recorded. The incremental test was considered valid when the following criteria were fulfilled: (1) an initial linear increase and a subsequent plateau in oxygen consumption, with an increment of less than 2 mL kg^{-1} min^{-1} between the two final measurements (despite the increase in workload), (2) the achievement of 90% of the age-predicted maximum heart rate (HR_{max}), (3) a respiratory exchange ratio >1.10. Endpoints of the incremental test were the VO_{2max} and the maximal power (P_{max}), which was defined as the lowest power output at maximal oxygen consumption.

One day after the incremental test, subjects of the EG performed a sprint cycling test (Laursen et al., 2002) in order to determine the duration of individual training protocols. Each participant pedaled at a self-selected speed for five minutes, before workload was augmented to predetermined P_{max} until exhaustion, i.e., when pedal frequency fell below 60 rpm. Endpoint of this test was T_{max}, which was defined as the time to exhaustion at P_{max}. Based on

the pre-tests, individual training protocols were created (see Table 11), which consisted of eight sets at the intensity P_{max}, a duration of 60% T_{max}, and a relative recovery period defined by the time needed until the HR decreased to 65% of HR_{max} measured in the incremental test.

Table 11. Training protocol. *(Adapted from Kistner et al. (2019)).*

Sets	Intensity	Duration	Rest
8	P_{max}	60% T_{max}	65% HR_{max}

HR_{max}: maximum heart rate; P_{max}: maximal power, T_{max}: time to exhaustion at P_{max}.

5.3.3 Experimental Protocol

The training protocol was completed daily for ten days in a row by the subjects of the EG. After a 5-minutes warm-up at 75 watt, participants performed their individual protocol on a bicycle ergometer (Excalibur Sport Ergometer, Lode B.V., Groningen, Netherlands). Pre-training (after warm-up) and post-training capillary blood was drawn to measure lactate concentrations. To assess individual physiological and psychological stress and as a control for the training process, several tests were performed by the EG. During each training session, the HR was continuously recorded (Polar RS800CX) to calculate the training impulse (TRIMP), following the instructions from Banister et al. (1991). The TRIMP is an objective tool to assess physical effort, considering the individual HR responses to training and the duration of performed exercise. The TRIMP allows the evaluation of the accumulated time in different HR zones based on <60%, 60-70%, 70-80%, 80-90%, and >90% of HR_{max}, as suggested by Edwards et al. (1993). Immediately post-training, the rating of perceived exertion (RPE) was asked, following the instructions from Foster et al. (2001). The RPE represents a subjective but valid method for quantifying exercise intensity. The RPE scale goes from zero to ten, while ten stands for maximal exertion (Foster et al., 2001). Additionally, the EG and CG daily answered a multi-dimensional questionnaire for subjective well-being (MDBF, German: *Mehrdimensionaler Befindlichkeitsfragebogen*), following the instructions from Steyer, Schwenkmezger, Notz and Eid (1997).

All participants were asked to refrain from alcohol, performance enhancing supplements, and drugs during the whole intervention. Besides, they were instructed not to change their eating habits during the study period. Evening meals the days before urine collection were standardized, insofar that participants were asked to consume pasta with tomato sauce.

5.3.4 Urine Sample Collection

Fasting spot urine from all participants was collected into polypropylene collection cups the day before the training started (Visit 1, V1), the day after the last training session (Visit 2, V2), as well as after four days of recovery (Visit 3, V3). All samples were taken between 9:00 and 10:00 a.m., centrifuged at 1850 × g at 4 °C for 10 min, separated into aliquots, and stored at −80 °C until analysis. In order to minimize the risk of bias through exercise, V1 was scheduled at least three days after the pre-tests. Additionally, participants were instructed to refrain from PE 48 hours before urine collection (except participants of the EG at V2).

5.3.5 Metabolomic Analyses

A combined NMR- and LC-MS-based, targeted metabolomic approach was applied to identify and quantify a broad range of metabolites, thus making it possible to assess changes in the urinary metabolome following intervention. A short overview of both analytical methods will be given in the next section; more details are available in the supplement of Rist et al. (2017).

NMR:

All urine samples were analyzed by 1D-^{1}H-NMR spectroscopy, as previously described (Rist et al., 2017). Briefly, urine samples were centrifuged, and supernatants mixed with 10% of a buffer containing 1.5 M KH_2PO_4, 2 mM NaN_3, and 0.58 mM trimethylsilylpropanoic acid (TSP) in D_2O at pH 7.4. Samples were measured at 300 K on a Bruker 600 MHz spectrometer (AVANCE II with 1H-BBI room temperature probe (Bruker BioSpin GmbH, Rheinstetten, Germany)) equipped with a BACS sample changer. Quality control (QC) samples were prepared by pooling fasting urine samples from all participants. At least one QC sample was analyzed per 24 study samples. The identification and quantitative analysis of 47 urinary metabolites, including organic acids, AAs, amines, sugars, sugar alcohols, and others, was carried out with Chenomx NMR Suite 8.1 (Chenomx, Edmonton, AB, Canada). Imprecision was generally <10% for most analytes, except for succinate.

LC-MS:

In addition to NMR analysis, a targeted ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) analysis of methylated amino compounds was conducted using an Acquity H-Class UPLC coupled to a Xevo TQD triple quadrupole MS (both from Waters, Eschborn, Germany). The method was originally developed for the analysis of

trimethylamine N-oxide (TMAO) and five related compounds in plasma samples (Krüger et al., 2017; Rist et al., 2017), but was adapted to urine and extended to 27 analytes in this study.

Briefly, urine samples were diluted 25 times with eluent A (1:1 acetonitrile/aqueous 50 mM ammonium formate) and separated by an inverse acetonitrile gradient on a polar hydrophilic interaction liquid chromatography column (Acquity BEH Amide, 100 × 2.1 mm, 1.7 µm, Waters, Eschborn, Germany). Analytes were detected by positive electron spray ionization and timed multiple reaction monitoring. Matrix-matched calibrators and controls (3 levels), dedicated isotope-labelled internal standards, and standard addition were used for quantification. Imprecision was generally <15% for most analytes and levels, with few exceptions. 13 of the detected metabolites showed concentrations below the lower limit of quantification (LLOQ), which was defined by the following criteria: Signal to noise ratio 10:1, imprecision and bias <30%. However, all values (including values below LLOQ) were considered for statistical calculations, since the occurrence of such values is a property of the population. Although it is known that the single values below the LLOQ are more inaccurate, such values contain valuable information for the description of the whole sample. The use of the values below the LLOQ is, with respect to the statistical properties, better than any artificial replacement.

Nine urinary metabolites (β-aminoisobutyrate, betaine, carnitine, creatine, dimethylamine, N,N-dimethylglycine, histidine, trigonelline, and TMAO) were accessible by both NMR and LC-MS. Analyte concentrations obtained by NMR and LC-MS were compared by linear regression. Results are presented in Table A-2 and Figure A-1 (Appendix A1.2). In brief, the correlations were strong in all cases with $R^2 > 0.87$. Absolute values did also match closely, reflected by slopes near 1 and small intercepts. The only exceptions were TMAO and dimethylamine, where significant deviations between LC-MS and NMR were observed. The reason remains unclear, but is likely related to different standardization and should be considered when comparing results from studies using different methods. Overall, we observed a high correlation between NMR and LC-MS data in our study. LC-MS analysis generally offers a higher sensitivity than NMR (A.-H. M. Emwas, 2015) and peak assignment and integration in NMR is sometimes critical and pH-dependent. Therefore, further data handling and statistical analysis for the named metabolites were carried out with the values obtained by LC-MS.

5.3.6 Data Handling and Statistical Analysis

Metabolite concentrations were normalized to urinary creatinine, thus controlling for variations in urine dilution. Urinary creatinine concentrations were obtained through ^{1}H-NMR spectroscopy and verified by the creatinine concentrations measured using a photometric assay based on the Jaffe reaction (DetectX® Urinary Creatinine Detection Kit, K002-H5, Arbor Assays, Ann Arbor, MI, USA). Differences between groups in anthropometric characteristics, fitness parameters, and baseline metabolite concentrations were examined by Welch's t-test. In order to investigate differences in urinary concentrations for each metabolite, a mixed effect model with treatment (EG, CG), time (V1, V2, V3), and treatment-time interaction as fixed factors were applied. To take account of the repeated measurements and varying baseline values, the random factors were modelled by a random intercept and a general correlation structure of the error terms. Tukey type contrasts were tested for by multiple testing adjusted t-tests. As the applied t-tests were considered robust to deviations from normality assumption and due to a moderate sample size, parametric statistical analysis was used. No kind of multivariate statistical analysis for the consideration of all metabolites simultaneously, like false discovery corrections, were performed due to the moderate sample size. Data are presented as mean ± standard deviation (SD). The level of statistical significance for all analyses was set at $\alpha = 0.05$. Statistical analysis was performed using SAS JMP 11.0.0 (SAS Institute Inc., Cary, NC, USA, 2013) and the software R version 3.4.2 (R Core Team, 2017) using the packages nlme (Pinheiro, Bates, DebRoy, & Sarkar, 2017) and lsmeans (Lenth, 2016). Sample size can partly differ due to missing data, e.g., when technical measurement errors occurred or because measured values were excluded due to biological implausibility.

5.4 Results

Descriptive characteristics of the participants and training protocols are summarized by means and SDs in Table 12. There were no significant differences between the EG and the CG with regard to age, anthropometric parameters, and PF at baseline. Parameters used to create individual training protocols are only presented for the EG. During daily training sessions, subjects of the EG performed eight cycling bursts at an average intensity of 348 watt (=P_{max}) and for a mean duration of 97 seconds (=60% T_{max}), interrupted by a rest period until the HR decreased to an average of 124 beats per minute (=65% of HR_{max}).

Table 12. Descriptive characteristics of participants and training protocols. *(Adapted from Kistner et al. (2019)).*

	Total (n=18)	EG (n=10)	CG (n=8)
Age (years)	30.2 ± 7.6	30.0 ± 8.0	30.4 ± 8.0
Height (cm)	182 ± 7	181 ± 7	184 ± 7
Weight (kg)	79.7 ± 7.6	77.1 ± 8.3	82.9 ± 5.5
BMI (kg $(m^2)^{-1}$)	24.0 ± 2.2	23.6 ± 1.6	24.6 ± 2.8
VO_{2max} (mL kg^{-1} min^{-1})	54.1 ± 8.2	53.0 ± 6.1	55.5 ± 10.6
P_{max} (w)	351 ± 30	348 ± 31	355 ± 30
HR_{max} (bpm)	182 ± 11	181 ± 13	185 ± 8
65% HR_{max} (bpm)	– – –	124 ± 5	– – –
T_{max} (s)	– – –	162 ± 23	– – –
60% T_{max} (s)	– – –	97 ± 14	– – –

All values in mean ± SD. BMI: body mass index; CG: control group; EG: experimental group; HR_{max}: maximum heart rate; n: sample size; P_{max}: maximal power; SD: standard deviation; T_{max}: time to exhaustion at P_{max}; VO_{2max}: maximal oxygen uptake.

5.4.1 Training Parameters

Training parameters demonstrated a high intensity of the daily training, see Figure A-2 (Appendix A1.3). In the EG, an increase in blood lactate concentration was documented after each training session and the post-exercise increase in lactate was 14.2 ± 2.7 mmol/L on the first training day and 16.3 ± 2.7 mmol/L on the last training day. The average TRIMP, which is considered as an objective tool to assess physical effort, increased continuously over the intervention period (from 56.2 ± 13.4 (day 1) to 96.6 ± 30.0 (day 10)). The HR zone scaling shows that the majority of the EG performed in HR zones 3-5, which means that they largely exercised at 70-100% of HR_{max}. The RPE, which is a subjective tool to evaluate exercise intensity, was continuously at a high level in the EG and did not differ between the training days (mean ± SD (day 1 to 10): 9.6 ± 0.5; inter-subject mean ± SD: 9.6 ± 0.2, values are scores in the RPE scale). Regarding the values of the multidimensional questionnaire for subjective well-being, the mean score from the EG immediately decreased after the first training session and remained lower than pre-training during the whole training intervention. In the CG, no changes in MDBF values were observed during the study period.

5.4.2 Urinary Metabolites

A total of 65 urinary metabolites were identified and quantified by NMR spectroscopy and LC-MS, including creatinine for standardization purposes. Normalization of urinary metabolite concentrations to urinary creatinine was conducted with creatinine values obtained by NMR. The NMR signal intensity of creatinine and creatinine concentrations measured with a photometric assay were highly correlated (Pearson's correlation: $r = 0.99$, $p < 0.001$), verifying the validity of the quantitative NMR data.

Spot urine samples were collected one day before intervention started (V1) as well as one and four days after intervention (V2 and V3, respectively). The normalized urinary metabolite concentrations before and after the 10-day HIIT are presented in Table A-3 (Appendix A1.4) and in Figure A-3 (Appendix A1.5), separated for the EG and the CG. Regarding pre-training urinary metabolite concentrations, no statistically significant differences between the EG and the CG could be documented. At V2, hypoxanthine (p = 0.0210) showed significantly different urinary concentrations between the EG and the CG, whereas at V3, urinary concentrations of betaine (p = 0.0055), hypoxanthine (p = 0.0006), and isoleucine (p = 0.0285) significantly differed between both groups. With respect to metabolite alterations within the EG, three metabolites significantly changed over time. Firstly, urinary hypoxanthine concentration showed a significant difference with decreasing means between V1 and V2 (p = 0.0270). Secondly, urinary taurine concentration significantly differed with decreasing means between V1 and V3 (p = 0.0031), as well as urinary asymmetric dimethylarginine (ADMA) concentration (p = 0.0380). Within the CG, urinary concentration of hypoxanthine significantly changed with increasing means between V1 and V3 (p = 0.0437) and V2 and V3 (p = 0.0442). Furthermore, urinary concentrations of citrulline (p = 0.0205), N-methylarginine (p = 0.0134), methyl-succinate (p = 0.0357), and urea (p = 0.0159) showed a significant difference with increasing means between V1 and V3, while no significant changes in urinary metabolites were demonstrated between V1 and V2. Boxplots of top-ranked metabolites, i.e., with at least one p-value < 0.01, are shown in Figure 21.

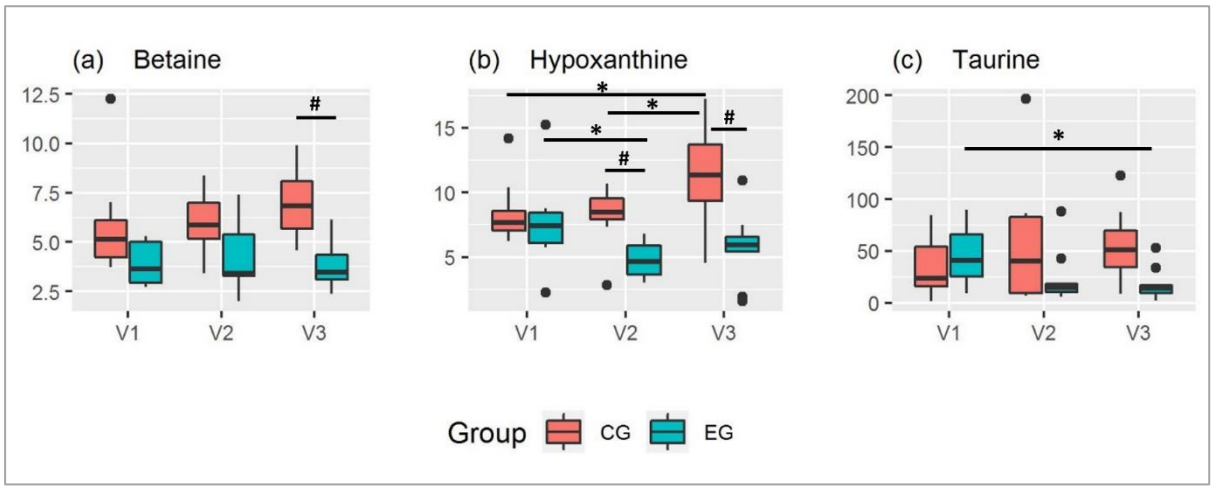

Figure 21. Boxplots of top-ranked metabolites. (a) Betaine (EG: n=9, CG: n=7); (b) hypoxanthine (EG: n=10, CG: n=8); (c) taurine (EG: n=10, CG: n=7); concentrations in mmol/mol creatinine. *: p-value < 0.05 for within-group comparisons; #: p-value < 0.05 for between-group comparisons; CG: control group; EG: experimental group; n: sample size; V1: visit 1 (day before the training started); V2: visit 2 (day after the the last training session); V3: visit 3 (after four days of recovery). Metabolites were chosen as top-ranked if at least one p-value was < 0.01. *(Adapted from Kistner et al. (2019)).*

5.5 Discussion

The purpose of this study was to investigate the effect of a ten-day HIIT on the resting urinary metabolome of young active men, thus analyzing if human metabolism is able to recuperate from an extremely demanding exercise intervention. The main finding of this study is that no overall change in the resting urinary metabolome was observed in the EG, except for a significant difference with decreasing means in urinary hypoxanthine concentration between V1 and V2 and a significant difference with decreasing means in urinary taurine and ADMA concentrations between V1 and V3. Assuming that acute alterations in urinary metabolite concentrations have occurred in response to the daily HIIT sessions – as shown by previous studies with similar high-intensity exercise protocols but with immediate sample taking (Pechlivanis et al., 2010; Pechlivanis et al., 2013; Pechlivanis et al., 2015; Siopi et al., 2017) – the almost unchanged urinary metabolome indicates that metabolism was largely able to restore its disturbed homeostasis in the given time frame of one or four recovery days, respectively. Although urine as a biological matrix has not been widely used in exercise metabolomics studies, the ability of urine to reflect acute exercise-induced metabolic changes occurring in muscle was recently demonstrated (Pechlivanis et al., 2010; Pechlivanis et al., 2015; Siopi et al., 2017). Consequently, the fact that no longer-lasting, overall metabolic alterations in urine have been documented in our study leads to the assumption that skeletal muscle metabolism had already regenerated from acute disturbances due to HIIT intervention.

Acute metabolic changes in response to a single HIIT or comparable sprint interval training session are particularly characterized by elevations in plasma or urinary lactate (Danaher et al., 2015; Gerber, Borg, Hayes, & Stathis, 2014; Pechlivanis et al., 2010; Pechlivanis et al., 2015; Zafeiridis et al., 2016), confirming that a high percentage of the energy required in muscle is provided by the anaerobic energy system (Gastin, 2001). Furthermore, acute elevations in plasma or urinary hypoxanthine were detected (Gerber et al., 2014; Pechlivanis et al., 2010; Pechlivanis et al., 2015; Siopi et al., 2017). The purine derivative hypoxanthine represents the final ATP breakdown product in muscle and was proposed as a metabolic indicator of exercise-induced energetic stress (Sahlin, Tonkonogi, & Söderlund, 1999; Zielinski & Kusy, 2015b). If during high-intensive exercise the ATP degradation rate in muscle is higher than the ATP resynthesis rate, accumulated hypoxanthine can be partly reconverted to inosine

monophosphate (IMP) by the purine salvage enzyme hypoxanthine guanine phosphoribosyl-transferase (HGPRT), thus contributing to ATP restoration, or it leaks out to the blood stream where a further degradation to uric acid or a direct elimination from body via urine occurs (Manfredi & Holmes, 1985; Sutton, Toews, Ward, & Fox, 1980; Zhao, Snow, Stathis, Febbraio, & Carey, 2000; Zielinski & Kusy, 2015b). An elevated urinary elimination of hypoxanthine and its downstream metabolite, uric acid, are seen as the endpoint of 'purine metabolism', representing an indirect marker of the exercise-induced net ATP loss from muscle to plasma (Stathis, Carey, & Snow, 2005; Stathis, Zhao, Carey, & Snow, 1999).

However, despite the high intensity of the training protocol used in our study, we did not observe an increase in urinary hypoxanthine concentration one day after the last training session. This observation is in line with results of Stathis et al. (1999), who showed that urinary hypoxanthine excretion rate was only significantly elevated in the first 2 hours of recovery, whereas 2 to 24 hours post-exercise no increased urinary hypoxanthine concentration was documented any more. As even lower post-training urinary hypoxanthine levels were demonstrated in our study (see Figure 21 (b)), the question arises if adaptations in 'purine metabolism' as a response to the exhausting HIIT have occurred, resulting in a reduced muscular hypoxanthine loss into blood even in resting conditions, finally leading to a decreased delivery of hypoxanthine to the kidneys and consequently to a lower hypoxanthine excretion via urine. Actually, this is the first study showing a decrease in resting urinary hypoxanthine levels after a short-term, but high-intensive interval training. A previous study focusing on training-induced alterations in 'purine metabolism' observed changes in resting as well as post-exercise plasma hypoxanthine concentrations in elite athletes during a one-year training cycle (Zielinski & Kusy, 2012, 2015a). Indeed, it was shown that resting (and post-exercise) plasma hypoxanthine decreased from the general to the competition phase, along with the increasing contribution of high-intensity anaerobic training loads, whereas in the detraining phase, resting (and post-exercise) plasma hypoxanthine concentration rose and returned to high levels (Zielinski & Kusy, 2012, 2015a). According to Zielinski and Kusy (2015a), plasma hypoxanthine concentrations at rest and after standard exercise can be seen as sensitive metabolic indicators of training status, providing valuable information about either training adaptation or overtraining. In contrast to this, another study showed that a seven-day intermittent sprint training on a bicycle ergometer did not change resting urinary

hypoxanthine concentration in active men (Stathis, Carey, Hayes, Garnham, & Snow, 2006). As compared to our study, subjects were untrained and performed a slightly shorter and less intensive training intervention, which could explain the discrepant results. However, several studies documented an attenuated exercise-induced accumulation of plasma or urinary hypoxanthine when an acute maximal activity was performed after repeated high-intensity training compared to pre-training, suggesting a decrease in muscular purine nucleotide loss due to an improved balance between the rates of ATP degradation and its resynthesis (Hellsten-Westing, Balsom, Norman, & Sjodin, 1993; Hellsten-Westing, Norman, Balsom, & Sjodin, 1993; Kuehnbaum et al., 2015; Spencer, Bishop, & Lawrence, 2004; Stathis et al., 2006; Stathis, Febbraio, Carey, & Snow, 1994). Hellsten-Westing, Balsom, et al. (1993) demonstrated that the attenuation in hypoxanthine efflux after a six-week HIIT in habitually active men was associated with a training-induced, elevated activity of the purine salvage enzyme HGPRT in muscle. As HGPRT catalyzes the reconversion of hypoxanthine to IMP (Manfredi & Holmes, 1985), its intensified activity following HIIT was interpreted as a training-induced adaptation to enhance the conservation of muscle purine nucleotides (Hellsten-Westing, Balsom, et al., 1993). Such an adaptation seems advantageous, because a lower loss of purines results in a lower reliance on the comparably slow and metabolically expensive replacement of adenine nucleotides by the purine de novo synthesis pathway (Stathis et al., 2006).

Unfortunately, neither muscular HGPRT nor acute post-exercise alterations in hypoxanthine concentration have been analyzed in the present study. Therefore, it only can be speculated that the decreased resting urinary hypoxanthine levels are evidence of an increased ability of the body to conserve or restore the purine nucleotide pool. A previous study has shown a decrease in resting muscular adenine nucleotide levels after one week of high-intensity intermittent training (Hellsten-Westing, Norman, et al., 1993). Although not analyzed in the present study, a depletion of muscular adenine nucleotide content in the course of the 10-day HIIT is also presumable due to the high intensity and frequency of training. As one possible metabolic adaptation to the short-term HIIT, an elevated HGPRT activity in skeletal muscle could be suggested. However, if muscular HGPRT activity actually increased after ten days of HIIT, it is still questionable to what extent a more efficient salvage in muscle is likely to explain the decrease in urinary hypoxanthine excretion at rest. Indeed, urinary hypoxanthine levels not only depend on muscular hypoxanthine release, but also on its uptake by red blood cells

(Giacomello & Salerno, 1979) or the liver and its further degradation to uric acid (Sahlin et al., 1999; Zielinski & Kusy, 2015b). Thus, it cannot be ruled out that an increased hypoxanthine uptake by other, non-muscle tissues underlay the observed decrease in resting urinary hypoxanthine concentration. As recently shown, not only HGPRT activity in skeletal muscle but also HGPRT activity in erythrocytes is exercise- and training-dependent (Dudzinska et al., 2018; Zielinski, Kusy, & Rychlewski, 2011; Zielinski, Rychlewski, Kusy, Domaszewska, & Laurentowska, 2009). Dudzinska et al. (2018) demonstrated that trained subjects had a significantly higher HGPRT activity in erythrocytes at rest, which might contribute to the reduction of resting plasma as well as urinary hypoxanthine levels by utilizing more hypoxanthine for IMP formation.

Since resting urinary hypoxanthine concentration in the EG was still lower at V3 than pre-training and not significantly different from V2 (see Figure 21 (b)), the possibility exists that the performed training induced an increase in purine salvage efficiency, however, it is still unclear in which tissues this adaptation occurred. Providing detailed information about underlying molecular mechanisms of adaptation to HIIT is out of the scope of this study. It is obvious that subjects of the EG were exposed to a remarkable metabolic and psychological stress, as indicated by an increase in post-exercise blood lactate and a decrease in MDBF scores during intervention, which reflect subjective well-being (Steyer et al., 1997). Nevertheless, adaptations to the exhaustive HIIT intervention were also demonstrated. The TRIMP as a marker of training load continuously increased over the 10-day intervention period in the EG, see Figure A-2 (b) (Appendix A1.3), showing that participants were able to realize a higher physical effort in the course of the intervention. Regarding this and the fact that urinary hypoxanthine levels in the EG significantly changed post-training, being significantly different from urinary hypoxanthine levels in the CG at V2 and V3, alterations in ‘purine metabolism’ could provide one possible mechanism of the body’s adaptation to HIIT. Unfortunately, we are not able to explain the significant differences with increasing means in urinary hypoxanthine levels in the CG between V1 and V3 or V2 and V3, respectively. It is obvious that the CG showed a higher variation in urinary hypoxanthine concentrations at V3 when compared with V1 and V2, see Figure 21 (b) and Table A-3 (Appendix A1.4). We assume that the documented differences are due to a natural variation, which could not be controlled in our study.

The biological relevance of the change in resting urinary taurine levels between V1 and V3 in the EG, see Figure 21 (c) and Table A-3 (Appendix A1.4), has also to be interpreted with caution, since it is known that the urinary excretion of this semi-essential AA varies along with dietary taurine intake (Lambert, Kristensen, Holm, & Mortensen, 2015). Although urine samples were collected after an overnight fast and after the intake of a standardized evening meal, it cannot be excluded that participants consumed different amounts of taurine-containing food like meat, fish, shellfish, or energy drinks (Lambert et al., 2015; Peacock, Martin, & Carr, 2013) the days before urine collection. However, regarding the decrease in urinary taurine within the EG, it could be hypothesized that more taurine was utilized during HIIT intervention. Taurine is highly concentrated in the muscles (Seidel, Huebbe, & Rimbach, 2018) and involved in cell volume regulation, calcium homeostasis, membrane stabilization, and antioxidant activities (Lambert et al., 2015), all important with respect to PE. In a previous study, it was postulated that taurine is released by contracting muscles during exercise due to osmoregulatory processes, thus leading to an acute increase in plasma taurine and, when individuals were sufficiently hydrated, a higher urinary taurine clearance (Cuisinier et al., 2002). As taurine cannot be metabolized by humans, it is either excreted via urine or feces (Lambert et al., 2015). To our knowledge, there is a lack of studies investigating the effect of HIIT on taurine metabolism. A previous study on endurance exercise showed that urinary taurine immediately increased post-exercise, whereas after 24 hours, the urinary taurine excretion declined to slightly lower levels than baseline (Cuisinier, Ward, Francaux, Sturbois, & de Witte, 2001). Assuming that in our study muscular taurine also was acutely released during HIIT intervention, it can be speculated that the lower urinary taurine levels at V3 might be a result of “restoring” the pre-exercise muscular taurine content. Since the reabsorption of taurine in the kidneys is variable, ranging from 40% to 99% (Han, Patters, Jones, Zelikovic, & Chesney, 2006), the possibility exists that in the regeneration phase more taurine was retained in the body, being reflected in a lower urinary taurine excretion. Unfortunately, physiological processes underlying the changes in urinary taurine in the EG remain to be elucidated.

Similar to urinary taurine levels, a decrease in urinary levels of ADMA between V1 and V3 was documented in the EG. ADMA is endogenously produced from the turnover of arginine-methylated proteins and can either be metabolized to citrulline and dimethylamine or excreted via urine (Teerlink, 2005). As ADMA functions as an inhibitor of nitric oxide synthase,

high plasma ADMA levels are considered as a cardiovascular risk factor (Schlesinger, Sonntag, Lieb, & Maas, 2016) and an increased urinary excretion seems to be one of the main mechanisms lowering ADMA plasma levels (Nijveldt et al., 2002). Recently, it was shown that PE decreases ADMA levels in plasma (Riccioni et al., 2015). However, the effect of PE on urinary ADMA excretion in healthy individuals has rarely been examined so far. Despite the statistically significant change in urinary ADMA between V1 and V3 in our study, the absolute difference was marginal, see Table A-3 (Appendix A1.4) and Figure A-3 (Appendix A1.5). Regarding this and the fact that we did not observe a significant difference between V1 and V2 within the EG, we assume that the change in urinary ADMA levels has no biological relevance with respect to HIIT intervention.

The significant differences with increasing means in urinary concentrations of citrulline, N-methylarginine, methylsuccinate, and urea between V1 and V3 within the CG can hardly be explained and might be due to uncontrolled variation. For example, the documented increase in urinary urea levels could likely be an effect of diet, e.g., an increased protein intake, as urea represents the terminal product of protein catabolism, being eliminated in urine (Wang, Ran, & Jiang, 2014). Equally, we hypothesize that the difference between groups in urinary levels of isoleucine at V3 can be seen as an evidence of natural variation in the CG, leading to a higher isoleucine excretion at V3 when compared with the EG. Differences between groups in urinary betaine concentration at V3 could also be due to uncontrolled variation in the CG. However, as betaine functions as an organic osmolyte and methyl donor (Lever & Slow, 2010), its importance for exercise performance has recently been discussed (J. R. Hoffman, Ratamess, Kang, Rashti, & Faigenbaum, 2009). With regard to this and the fact that we could observe a slight, but not significant increase of urinary betaine levels in the CG (see Figure 21 (a)), it can be speculated that the CG, which had to refrain from their usual daily training during the study period, demonstrated a minor use of betaine for exercise-related physiological processes, thus leading to a higher urinary betaine excretion in the inactive state. However, as the urinary betaine levels already differed slightly at V1 between both groups, it could be also suggested that diet might account for the higher urinary betaine excretion in the CG. Differences in urinary betaine could be explained by a different dietary intake of betaine-containing food like wheat, shellfish, or spinach (Craig, 2004; Garg et al., 2016).

A clear limitation of the present study is that the participants of the CG were instructed to refrain from heavy exercise, however, their actual PA behavior was not recorded. Similarly, the daily diet of study participants was not controlled. This has to be taken into account when interpreting the results, as it is known that the urinary excretion of some metabolites can be affected by diet. Another limitation of our study is that we conducted a targeted metabolomic approach, which was limited to a specific number of known compounds, and the lack of a comparative metabolomic analysis in blood, which would have been of great interest in order to facilitate the interpretation of documented metabolite alterations. Furthermore, as the present study was a follow up investigation of a study focusing on a univariate and less varying endpoint, there is a lack of statistical power regarding the analyses of urinary metabolites which show a comparatively higher biological variance. Therefore, the obtained results need to be evaluated cautiously and can only be interpreted for each single metabolite. As expected with respect to the moderate sample size, the observed changes in the metabolite concentrations are marginal when considering multiple hypotheses, which take all metabolites simultaneously into account. Consequently, the present study has to be considered as a pilot study, providing first hints about the possible effects of HIIT on urinary metabolites. More studies have to be done to confirm the observed HIIT-induced metabolic changes and to extend the obtained results to a broader population, i.e., not only to young, trained men but also to untrained individuals and females. With regard to future studies, it would be appropriate to investigate the effects of a short-term HIIT not only on resting urinary as well as blood metabolite levels but also on acute post-exercise metabolic changes in the course of intervention. Thus, more reliable conclusions about HIIT-induced adaptations in metabolism could be drawn.

5.6 Conclusions

We investigated the effect of a ten-day HIIT on 64 urinary metabolites in young active men. To our knowledge, this was the first study using a combined NMR- and LC-MS-based metabolomic approach to assess changes in the resting urinary metabolome, which possibly are related to the body's adaptation to a HIIT protocol comparable to pre-competition training schedules. Our findings show that no overall change in the resting urinary metabolome, except a decrease in the urinary hypoxanthine concentration, was caused in the EG one day after the HIIT intervention. This result indicates that the metabolism was able to quickly regenerate

from acute metabolic disturbances due to the exhausting HIIT. However, as resting urinary hypoxanthine levels were lower and significantly different following HIIT, training-induced adaptations in ‘purine metabolism’ can be suggested. To reveal underlying mechanisms, further studies are necessary.

6 Study IIa

An NMR-Based Approach to Identify Urinary Metabolites Associated with Acute Physical Exercise and Cardiorespiratory Fitness in Healthy Humans—Results of the KarMeN Study

Slightly modified version of the published article (21 May 2020).

Kistner, S., Rist, M. J., Döring, M., Dörr, C., Neumann, R., Härtel, S., Bub, A. (2020). An NMR-Based Approach to Identify Urinary Metabolites Associated with Acute Physical Exercise and Cardiorespiratory Fitness in Healthy Humans—Results of the KarMeN Study. *Metabolites*, *10*(5), 212. doi:10.3390/metabo10050212.

For a better comprehensibility of results, the supplementary material of this article is provided in the Appendices A2.3 to A2.8. Datasets, R-scripts for figure generation, as well as overview tables with computed values are available under the following link: https://doi.org/10.3390/metabo10050212.

6.1 Abstract

Knowledge on metabolites distinguishing the metabolic response to acute physical exercise (PE) between fit and less fit individuals could clarify mechanisms and metabolic pathways contributing to the beneficial adaptations to exercise. By analyzing data from the cross-sectional Karlsruhe Metabolomics and Nutrition (KarMeN) study, we characterized the acute effects of a standardized exercise tolerance test on urinary metabolites of 255 healthy women and men. In a second step, we aimed to detect a urinary metabolite pattern associated with the cardiorespiratory fitness (CRF), which was determined by measuring the peak oxygen uptake (VO_{2peak}) during incremental exercise. Spot urine samples were collected pre- and post-exercise and 47 urinary metabolites were identified by nuclear magnetic resonance (NMR) spectroscopy. While the univariate analysis of pre- to post-exercise differences revealed significant alterations in 37 urinary metabolites, principal component analysis (PCA) did not show a clear separation of the pre- and post-exercise urine samples. Moreover, both bivariate correlation and multiple linear regression analyses revealed only weak relationships between

the VO_{2peak} and single urinary metabolites or urinary metabolic pattern, when adjusting for covariates like age, sex, menopausal status, and lean body mass (LBM). Taken as a whole, our results show that several urinary metabolites (e.g., lactate, pyruvate, alanine, and acetate) reflect acute exercise-induced alterations in the human metabolism. However, as neither pre- and post-exercise levels nor the fold changes (FCs) of urinary metabolites substantially accounted for the variation of the covariate-adjusted VO_{2peak}, our results furthermore indicate that the urinary metabolites identified in this study do not allow to draw conclusions on the individual's physical fitness (PF) status. Studies investigating the relationship between the human metabolome and functional variables like the CRF should adjust for confounders like age, sex, menopausal status, and LBM.

Keywords: metabolomics; urinary metabolome; urinary metabolites; NMR spectroscopy; acute physical exercise; cardiorespiratory fitness; VO_{2peak}; exercise metabolomics.

6.2 Introduction

Over the past decades, a growing body of evidence has demonstrated the inverse relationship between the CRF and all-cause and disease-specific mortality (Blair et al., 1995; Harber et al., 2017). As one of the most widely investigated physiological parameters (Harber et al., 2017), the CRF is assessed by measuring the maximal oxygen uptake (VO_{2max}) during an incremental exercise test until exhaustion (Löllgen & Leyk, 2018). If the VO_{2max} cannot be determined with certainty, requiring the presence of a plateau in oxygen uptake (VO_2), the VO_{2peak} as the highest value of VO_2 attained during an incremental test is frequently used instead (Day et al., 2003). Representing an individual's ability to take up, transport, and utilize oxygen during sustained PE (Hill et al., 1924), the CRF is influenced by a variety of factors, such as age (Laukkanen et al., 2009; Zeiher et al., 2019), sex (Al-Mallah et al., 2016; Zeiher et al., 2019), LBM (S. Y. S. Wong et al., 2008), or heredity (Bouchard et al., 1988). Furthermore, behavioral factors like diet, smoking, alcohol intake, and particularly physical activity (PA) are associated with the CRF (McKinney et al., 2016; Zeiher et al., 2019). Regular PE is known to induce physiological adaptations like an increased cardiac output or skeletal muscle capillarization as well as an augmented activity of mitochondrial enzymes, resulting in a higher aerobic capacity (Gabriel & Zierath, 2017). These alterations are accompanied by a change in skeletal muscle fuel selection during acute exercise, being reflected in an increased ratio of fat to

carbohydrate oxidation (Egan & Zierath, 2013). However, the molecular mechanisms and metabolic pathways underlying the whole-body and skeletal muscle adaptation to PE still remain to be completely elucidated (Zierath & Wallberg-Henriksson, 2015).

The emerging field of metabolomics has the potential to simultaneously analyze a wide range of metabolites (Bujak et al., 2015), thereby facilitating a more comprehensive characterization of exercise-induced changes in human metabolism. As the human metabolome reflects the end-product of interactions between genes, proteins, and the cellular environment (Bujak et al., 2015), investigating the impact of acute or chronic PA on a high number and variety of metabolites can provide novel insights into the underlying biochemistry of exercise, possibly hinting at specific metabolic pathways related to the phenotypical response of the human organism to exercise (Daskalaki et al., 2014; Sakaguchi et al., 2019). Thus, metabolomics might contribute to the identification of exercise-responsive biomarkers, which are reflective of an individual's PF status. Particularly with regard to the health benefits associated with a high CRF, the investigation of metabolic differences between more and less fit individuals is of great interest not only for sport scientists but also for physicians in the context of exercise prescription and health examination (Heaney et al., 2017).

Until now, several studies have examined both the acute (Breit et al., 2015; Enea et al., 2010; Lewis et al., 2010; Muhsen Ali et al., 2016; Mukherjee et al., 2014; Netzer et al., 2011; Pechlivanis et al., 2010; Pechlivanis et al., 2015; Siopi et al., 2017; Siopi et al., 2019) and chronic (Bye et al., 2012; Chorell et al., 2012; Floegel et al., 2014; Koh et al., 2018; Lustgarten et al., 2013; Morris et al., 2013) effects of PA on the human metabolome by using metabolomics. Although blood represents the most widely used biofluid in metabolomics research (Bujak et al., 2015), recent publications have demonstrated the ability of urine as a non-invasively collectable biological material to reflect acute exercise-induced perturbations in metabolism, e.g., after short-term exercise at maximal intensities (Enea et al., 2010; Pechlivanis et al., 2010; Pechlivanis et al., 2015) or submaximal endurance exercise (Mukherjee et al., 2014; Siopi et al., 2017). However, to the best of our knowledge, no previous study has investigated to what extent the urinary metabolome responds to a standard exercise tolerance test, during which individuals perform a stepwise progressive exercise program until exhaustion.

A few metabolomics studies have provided evidence of changes in the blood metabolome due to maximal incremental exercise testing on either bicycle ergometer (Breit et al., 2015; Lewis

et al., 2010; Netzer et al., 2011) or treadmill (Lewis et al., 2010). Interestingly, Lewis et al. (2010) demonstrated that the exercise-induced excursions of some plasma metabolites differentiated between more and less fit individuals, pointing out the possibility that these metabolites could represent important mediators of the salutary effects of PE and potential biomarkers of PF. With regard to the relationship between acute exercise-related changes in urinary metabolites and the PF status, research is rather scarce. Three studies investigating the alterations in the urinary metabolome due to submaximal endurance exercise (Muhsen Ali et al., 2016; Mukherjee et al., 2014) or short-term intensive exercise (Enea et al., 2010) either compared the exercise-induced changes in urinary metabolites between groups of different training status (Enea et al., 2010; Mukherjee et al., 2014) or aimed to predict the VO_{2max} with the post-exercise urinary metabolite pattern (Muhsen Ali et al., 2016). However, limitations of existing studies are the relatively small sample sizes, ranging from 10 (Muhsen Ali et al., 2016) to 22 (Enea et al., 2010) participants, and the moderate number of quantified analytes (11 (Enea et al., 2010) or 27 (Mukherjee et al., 2014) metabolites, respectively) in two of the mentioned studies. Besides, results are partly restricted to young women (Enea et al., 2010) or middle-aged men (Mukherjee et al., 2014) and therefore hardly transferable to the general population.

Based on the limited data available on exercise-induced excursions of urinary metabolites being associated with PF and due to the fact that no previous study has investigated to what extent the body's response to a standardized incremental exercise is reflected in the urinary metabolome, we pursued two major objectives with our study. Firstly, we aimed to characterize acute alterations in urinary metabolites due to a standardized exercise tolerance test on a bicycle ergometer until individual exhaustion, which was performed by 255 healthy women and men of the cross-sectional KarMeN study. The second aim was to investigate if either the urinary metabolites at rest, post-exercise, or the ratio of post- to pre-exercise metabolite concentrations are associated with the CRF, which was determined by measuring the VO_{2peak}. As the metabolomics data were obtained from a comparatively large and heterogeneous population, this study was particularly suitable to analyze in how far urinary metabolite patterns can account for the variation in the VO_{2peak}, when simultaneously considering covariates like age, sex, menopausal status, and LBM − all factors determining both the PF (Mercuro et al., 2006; Zeiher et al., 2019) and the human metabolome

(Armbruster et al., 2018; Jourdan et al., 2012; Korostishevsky et al., 2016; Rist et al., 2017; Stretch et al., 2011). A targeted NMR-based approach was applied to quantify the pre- and post-exercise urinary levels of 47 metabolites. Ranging from organic acids, keto acids, alcohols to purine derivatives and amino acids (AAs), the detected metabolites represent a relatively broad spectrum of organic compounds found in the human urine metabolome (Bouatra et al., 2013). Urine as a biological specimen was chosen because it is easily accessible, stable, and under less homeostatic regulation than other biofluids (Bouatra et al., 2013; J. Wu & Gao, 2015).

6.3 Materials and Methods

6.3.1 Subjects and Study Design

The KarMeN study is a cross-sectional study which was performed between March 2012 and July 2013 at the Division of Human Studies of the Max Rubner-Institut (MRI) in Karlsruhe, Germany. The main objective of the KarMeN study was to investigate the impact of sex, age, body composition, and major lifestyle factors like diet and PA on the metabolome of healthy women and men. Detailed information about inclusion and exclusion criteria as well as a detailed description of the study design were provided in Bub et al. (2016).

Briefly, 301 healthy, non-smoking individuals (172 men, 129 women) between 18 and 80 years were included. Volunteers were thoroughly characterized by anthropometric and clinical examinations. The body composition was assessed by dual-energy X-ray absorptiometry (DXA; Lunar iDXA, GE Healthcare, München, Germany) and the LBM and fat mass (FM) were calculated with the supplementary software enCOREv16. Blood hemoglobin (Hb) was measured by a certified clinical chemistry laboratory (MVZ Labor PD Dr. Volkmann, Karlsruhe, Germany). Besides, resting energy expenditure and CRF were assessed and data on regular PA and menopausal status in women were collected. As the menstrual cycle is known to influence metabolite profiles (Wallace et al., 2010), all premenopausal women were scheduled for examinations within their luteal phase. The study was approved by the ethics committee of the State Medical Chamber of Baden-Württemberg, Stuttgart, Germany and was conducted in accordance with the declaration of Helsinki. The study was registered at the German Clinical Trials Register (DRKS00004890). The WHO universal trial number is U1111-1141-7051. Written informed consent was obtained from all participants prior to entering the study.

6.3.2 Exercise Examination Day and Urine Sample Collection

On the exercise examination day, participants entered the study center early in the morning. Before the bicycle ergometry started, volunteers passed a resting phase for the indirect calorimetry measurement (40 min), consumed a standardized breakfast consisting of two slices of wheat bread with butter and either cheese, ham, or jam (20 min), and had a second resting phase for answering the International Physical Activity Questionnaire (IPAQ) (20 min). Immediately after entering the study center, all participants provided fasting spot urine into 100 mL polypropylene collection cups (Sarstedt, Nümbrecht, Germany). This was approximately 90 minutes before the bicycle ergometry started. In addition to that, the first available spot urine after the completion of the PE test was obtained from all participants approximately 15 to 30 minutes post-exercise.

Participants performed a standardized exercise test on a bicycle ergometer (Ergobike medical, Daum, Fürth, Germany) until individual maximal performance was reached, see Biniaminov et al. (2018). Briefly, according to the WHO-loading protocol (Fletcher et al., 2013), each participant started pedaling at 25 watt and workload was then augmented by 25 watt every two minutes until individual exhaustion. Respiratory gas exchange was measured breath-by-breath by using a cardiopulmonary exercise testing system (MetaMax 3B, Cortex, Leipzig, Germany). As a measure of PF, the VO_{2peak} was determined. The VO_{2peak} was defined as the highest achieved VO_2 during the test. It is either expressed as an absolute value in L min^{-1} or relative to the body weight in mL kg^{-1} min^{-1}. Further important endpoints of the incremental test were the maximal power (P_{max}), maximum heart rate (HR_{max}), and the power at the individual anaerobic threshold (P_{IAT}). The P_{IAT} was determined by the Ergonizer software (Ergonizer, Version 4, Freiburg, Germany) after having measured lactate in capillary blood samples taken from an individual's earlobe before, during, and after the test. Throughout the entire procedure, the heart rate (HR) was recorded by a HR monitor (T31 coded, Polar Electro GmbH Deutschland, Büttelborn, Germany). Additionally, a continuous hemodynamic monitoring was conducted by running a 12-channel electrocardiogram (CardioDirect 12, DelMar Reynolds GmbH, Feucht, Germany) and by measuring the blood pressure (BP) every 2-3 minutes on the right upper arm (Boso Carat Professional, Bosch + Sohn, Jungingen, Germany). Break-off criteria for the maximal exercise test were predefined according to national ergometry standards and listed in the following: ST-segment depression > 3 mm, ST-

segment elevation > 1 mm, acute hypertension with systolic BP > 230 mmHg or diastolic BP > 115 mmHg, appearance of angina pectoris symptoms or severe dyspnea.

6.3.3 Urine Sample Preparation

After urine sample collections, all urine specimens were centrifuged at 1850 g at 4 °C for 10 minutes and transferred into prechilled cryovials. They were initially frozen at −20 °C for one day and then cryopreserved at −196 °C until analysis. As previously shown, this procedure does not affect metabolomics results (Rist et al., 2013).

6.3.4 ^{1}H-NMR Analysis

In order to identify and quantify a relatively broad range of urinary metabolites before and after the cycle ergometry, all urine samples were analyzed by 1D-^{1}H-NMR spectroscopy. As previously described (Rist et al., 2017), urine samples were centrifuged and supernatants were mixed with 10% of a buffer containing 1.5 M KH_2PO_4, 2 mM NaN_3, and 5.8 mM trimethylsilylpropanoic acid (TSP) in D_2O at pH 7.4 in 5 mm NMR tubes (Duran, purchased from Roth GmbH & Co KG, Karlsruhe, Germany). Samples were measured at 300 K on a Bruker 600 MHz spectrometer (either AVANCE III equipped with a ^{1}H,^{13}C,^{15}N-TCI inversely detected cryoprobe or AVANCE II with 1H-BBI room temperature probe (Bruker BioSpin GmbH, Rheinstetten, Germany)) equipped with either SampleXpress or BACS sample changer using a 1D nuclear overhauser enhancement spectroscopy experiment with presaturation for water suppression. A prescan delay of 4 seconds was used together with a mixing time of 10 ms. Pulse lengths were determined automatically by the Bruker AU program, pulsecal. 64 k complex data points corresponding to a sweep width of 20 ppm were recorded. All spectra were treated identically using an exponential apodization function, introducing an additional linewidth of 0.3 Hz and automated phasing, baseline correction, and referencing using the Bruker macro, apk0.noe. Quality control (QC) samples were prepared by pooling urine samples from all participants. On each measurement day, at least three QC samples were analyzed along with approximately 70 urine study samples, ensuring the comparability of the spectra over the time. Since alignment of metabolites in urine spectra for non-targeted analysis is still very challenging, a targeted NMR-based metabolomics approach was applied. The identification and quantification of 47 urinary metabolites, including organic acids, AAs, amines, sugars, sugar alcohols, and others was carried out with the Chenomx NMR Suite 8.4 (Chenomx, Edmonton,

AB, Canada). Further metabolites could either not be detected in each urine sample or not identified and/or quantified with sufficient confidence. Imprecision was generally <15%, with few exceptions. No systematic variation between the spectrometers was observed. Metabolite concentrations were normalized to osmolality, thus controlling for variations in urine dilution (Chetwynd et al., 2016), and results are given in µmol/L per mOsm/kg urine. The osmolality of urine samples was determined by freezing point depression using an Advanced Instrument Micro-Osmometer model 3MO (Norwood, MA, USA).

6.3.5 Data Handling and Statistical Analysis

We excluded 46 individuals due to missing spirometry data (n=40), outlying urinary metabolite concentrations (n=5), and implausible data on resting heart rate (HR_{rest}) (n=1). Thus, data from 255 individuals were included into the following analyses. Descriptive characteristics of the study participants are presented as mean and standard deviation (SD) for the total study sample and separately for women and men. Sex differences in basic characteristics were examined by Welch's t-test.

For each of the 47 metabolites, the fold change (FC) between the normalized post- and pre-exercise concentrations was calculated per participant and data were shown as median and percentiles (25^{th}, 75^{th}). Compared to absolute differences in metabolite concentrations, FCs are unitless and therefore easier to interpret, allowing the direct comparison of exercise-induced excursions between different metabolites. As the urinary metabolite concentrations did not follow a normal distribution, the data were subjected to nonparametric univariate statistical analysis. In order to identify urinary metabolites that were significantly different between pre- and post-exercise, Wilcoxon's signed-rank tests were used. The false discovery rate (FDR) was applied to correct the obtained *p*-values for multiple hypothesis testing, using the method of Benjamini and Hochberg with an extension to the corresponding simultaneous tests. Adjusted *p*-values were compared to the level of statistical significance, which was set at $\alpha = 0.05$. Metabolites with FDR-corrected *p*-values < 0.05 and median FCs $\leq \frac{1}{1.1}$ or ≥ 1.1 were considered as exercise-responsive metabolites. A volcano plot was provided to visualize the magnitude (FCs) and significance (FDR-corrected *p*-values) of differences in urinary metabolites. To compare the metabolite FCs between women and men, the Wilcoxon rank-sum test was used and FDR-corrected *p*-values were reported.

Due to the non-normal data distribution and the occurrence of outliers in the urinary metabolite data, all variables were transformed into Van der Waerden (VdW) scores prior to the following analyses. In detail, by using this rank based inverse normal transformation, the data were converted into ranks, transformed to a scale between 0 and 1 and then, the corresponding standard normal quantiles were calculated. To investigate the relationships between the FCs of urinary metabolites, Pearson correlation coefficients (*r*) with 95% confidence intervals (CIs) were calculated. Furthermore, PCA was conducted on the transformed metabolite data, which were centered and scaled to unit variance, in order to visualize the main variability on a reduced dimensionality. Two different approaches were applied. The first approach was based on a data matrix of 2 × 255 participants from the pre- and post-exercise condition and 47 metabolites. The second approach dealt with a data matrix of 255 participants and 2 × 47 metabolites from the pre- and post-exercise condition. Relationships between the VO_{2peak} and either pre- and post-exercise urinary metabolite concentrations or urinary metabolite FCs were analyzed by Pearson correlation. To remove any confounding association, we also performed partial correlation analyses by using the residuals from linear regressions, where both the transformed VO_{2peak} and the transformed metabolite variables were regressed on age, sex, menopausal status, and LBM.

In a next step, multiple linear regression procedures were conducted to analyze the relationship between the VO_{2peak} and all urinary metabolite variables simultaneously. Three different models were calculated with the previously obtained residuals of VO_{2peak} as the dependent variable and the residuals of (a) pre-exercise urinary metabolites, (b) post-exercise urinary metabolites, and (c) urinary metabolite FCs, respectively, entering as independent variables. By this construction, the analyzed relationships were confounder-adjusted and hence uncorrelated with known determinants of both the VO_{2peak} and the urinary metabolome. In more detail, two stepwise regression models with either forward or backward elimination were performed for each (a), (b), and (c) to obtain a ranking of the adjusted metabolite variables according to their contribution for explaining the adjusted VO_{2peak}. For the selection and elimination processes, the Bayesian information criterion (BIC) was used. Finally, based on the results of the stepwise regression procedures, the models with the minimum BIC were chosen in order to select those metabolite variables which should enter into a suitable final model. The adjusted coefficient of determination (R^2 (adjusted)) was used

to compare the three obtained final models with respect to their potential in explaining the variation of the adjusted VO_{2peak}. Statistical analysis was performed using SAS JMP 11.0.0 (SAS Institute Inc., Cary, NC, USA, 2013) and the software R Version 3.6.0 (R Core Team, 2019), using the packages XLConnect (Mirai Solutions GmbH, 2020), ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2019), corrplot (Wei, 2017), and ggrepel (Slowikowski, 2020a).

6.4 Results

6.4.1 Basic Characteristics of Study Participants

Basic characteristics of the KarMeN study participants who were included in the present data analysis are summarized by means and SDs in Table 13. Our study sample consisted of 255 healthy individuals, 148 men (58%) and 107 women (42%) with a mean age of 46.1 years.

Table 13. Basic characteristics of study participants, total and stratified by sex. *(Adapted from Kistner et al. (2020)).*

Characteristics of Participants	Total (n=255)	Men (n=148)	Women (n=107) [1]
Age (years)	46.1 ± 16.9 *	42.8 ± 17.6	50.7 ± 14.7
Body weight (kg)	72.5 ± 11.6 *	78.5 ± 10.0	64.2 ± 8.1
Height (cm)	174.6 ± 9.6 *	180.1 ± 7.5	166.9 ± 6.5
BMI (kg $(m^2)^{-1}$)	23.7 ± 2.8 *	24.2 ± 2.7	23.1 ± 2.9
LBM (kg)	50.6 ± 10.4 *	58.0 ± 6.6	40.3 ± 3.8
FM (%)	27.9 ± 8.7 *	23.2 ± 6.5	34.5 ± 6.7
Hb (g dL^{-1}) [2]	14.4 ± 1.1 *	15.0 ± 0.9	13.5 ± 0.8
BP systolic (mmHg)	123.6 ± 15.4 *	127.2 ± 13.6	118.6 ± 16.4
BP diasystolic (mmHg)	83.1 ± 10.5	83.9 ± 10.2	82.1 ± 10.9
HR_{rest} (bpm)	62.4 ± 9.4 *	59.9 ± 9.3	65.8 ± 8.6
VO_{2peak}, absolute (L min^{-1})	2.83 ± 1.00 *	3.46 ± 0.81	1.96 ± 0.42
VO_{2peak}, relative (mL kg^{-1} min^{-1})	38.8 ± 11.6 *	44.5 ± 10.7	30.9 ± 7.4
P_{IAT} (watt)	144.5 ± 46.2 *	170.5 ± 41.1	108.4 ± 22.6
P_{max} (watt)	215.2 ± 69.1 *	257.0 ± 56.0	157.5 ± 36.0
HR_{max} (bpm)	170.8 ± 16.8 *	174.1 ± 16.1	166.2 ± 16.8

All values in mean ± SD; [1] n=49 in the pre- and n=58 in the post-menopausal state on the examination day; [2] n=254 (total), n=106 (women); * $p < 0.05$: significant difference between women and men by Welch's t-test; BP: blood pressure; BMI: body mass index; FM: fat mass; Hb: hemoglobin; HR_{max}: maximum heart rate; HR_{rest}: resting heart rate; LBM: lean body mass; n: sample size; P_{IAT}: power at the individual anaerobic threshold; P_{max}: maximal power; SD: standard deviation; VO_{2peak}: peak oxygen uptake.

During the incremental exercise test, participants reached a mean absolute VO_{2peak} of 2.83 L min^{-1}. As the VO_{2peak} was strongly associated with sex and menopausal status ($R^2 = 0.59$), age ($R^2 = 0.35$), and LBM ($R^2 = 0.69$), subsequent correlation and regression analyses were adjusted for these factors.

6.4.2 Alterations of Urinary Metabolites in Response to a Standardized Exercise Test

Uni- and Bivariate Analysis

By using an NMR-based approach, a total of 47 urinary metabolites were quantified in spot urine samples collected before and after a standardized incremental exercise test and measured concentrations were normalized to the osmolality of urine samples. To describe and compare the metabolite excursions, FCs between the normalized post- and pre-exercise urinary metabolite concentrations were calculated per participant. In Table 14, alterations in the urinary metabolite levels are summarized, with metabolites being ranked according to their median FC. The normalized absolute urinary metabolite concentrations before and after the bicycle ergometer test are presented as boxplots in Figure A-5 (Appendix A2.3). Boxplots of the FCs of urinary metabolites are provided in Figure A-6 (Appendix A2.4).

Univariate analysis revealed that 37 out of the 47 measured metabolites showed a significantly different urinary excretion post-exercise when compared to pre-exercise. While six of these metabolites (lactate, mannitol, trans-aconitate, alanine, carnitine, and acetate) demonstrated a median FC ≥ 1.5, 17 metabolites showed a median FC between 1.2 and 1.5, 10 metabolites exhibited a median FC between 1.0 and 1.2, two metabolites demonstrated a median FC between 0.8 and 1.0, and two metabolites showed a median FC ≤ 0.8. To facilitate the detection of exercise-responsive metabolites, which were defined as metabolites with FDR-adjusted *p*-values < 0.05 and median FCs $\leq \frac{1}{1.1}$ or ≥ 1.1, respectively, the significance and magnitude of the post- to pre-exercise differences are visualized in a volcano plot, see Figure A-7 (Appendix A2.5). Due to the relatively high inter-individual variation observed in the metabolite FC data and due to the known effect of sex on human metabolite profiles, we also investigated if there were any sex-related differences in urinary metabolite FCs. However, with the exception of citrate and trans-aconitate, women and men did not show any further significant difference in metabolite FCs. Results of the sex-stratified analysis of pre- to post-exercise urinary metabolite changes are presented in Table A-5 and Table A-6 (Appendix A2.6).

Especially in the exercise-induced excursions of the urinary lactate excretion, a high variance between individuals was detected, see Table 14 and Figure A-6 (Appendix A2.4). When analyzing the associations between the FCs of lactate as the main exercise-responsive metabolite and the FCs of all other metabolites, it was shown that the exercise-related change

in the urinary lactate excretion was most closely linked to alterations in the urinary excretion of pyruvate (r = 0.76), alanine (r = 0.62), methylsuccinate (r = 0.57), acetate (r = 0.56), and hypoxanthine (r = 0.56).

Table 14. Changes in urinary metabolites after a standardized exercise test. *(Adapted from Kistner et al. (2020)).*

Nr.	Metabolite (Abbreviation)	Median FC (25^{th}, 75^{th} percentiles)		FDR-corrected *p*-value
1	*Lactate (Lac)*	4.70	(1.64, 32.98)	<0.0001
2	*Mannitol (Man)*	2.34	(1.30, 4.29)	<0.0001
3	*trans-Aconitate (t-Aco)*	1.96	(1.05, 3.49)	<0.0001
4	*Alanine (Ala)*	1.74	(1.38, 2.20)	<0.0001
5	*Carnitine (Car)*	1.68	(1.24, 2.37)	<0.0001
6	*Acetate (Acet)*	1.55	(1.14, 2.68)	<0.0001
7	*Taurine (Tau)*	1.49	(1.19, 2.02)	<0.0001
8	*Pyruvate (Pyr)*	1.48	(0.88, 3.57)	<0.0001
9	*Threonine (Thr)*	1.40	(1.12, 1.86)	<0.0001
10	*Guanidoacetate (Gua)*	1.39	(1.14, 1.71)	<0.0001
11	*N,N-Dimethylglycine (DMG)*	1.38	(1.13, 1.68)	<0.0001
12	*Betaine (Bet)*	1.32	(1.12, 1.58)	<0.0001
13	*Glycine (Gly)*	1.32	(1.13, 1.63)	<0.0001
14	*Histidine (His)*	1.30	(1.07, 1.62)	<0.0001
15	*Succinate (Suc)*	1.30	(0.92, 1.76)	<0.0001
16	*cis-Aconitate (c-Aco)*	1.29	(1.08, 1.74)	<0.0001
17	*Methylsuccinate (MSuc)*	1.28	(1.12, 1.54)	<0.0001
18	*Leucine (Leu)*	1.28	(1.05, 1.71)	<0.0001
19	*Acetone (Ace)*	1.28	(0.85, 1.99)	<0.0001
20	*Creatine (Cre)*	1.26	(0.85, 1.91)	<0.0001
21	*Citrate (Cit)*	1.24	(1.05, 1.48)	<0.0001
22	*2-Hydroxyisobutyrate (2-OH-Isob)*	1.24	(1.07, 1.43)	<0.0001
23	*Isoleucine (Ile)*	1.21	(0.94, 1.49)	<0.0001
24	*4-Hydroxyphenylacetate (4-OH-Phe)*	1.19	(0.97, 1.69)	<0.0001
25	*Formate (For)*	1.16	(0.96, 1.36)	<0.0001
26	*3-Aminoisobutyrate (BAIBA)*	1.15	(0.94, 1.40)	<0.0001
27	*Valine (Val)*	1.14	(0.97, 1.39)	<0.0001
28	*3-Hydroxyisovalerate (3-OH-Isov)*	1.13	(1.02, 1.28)	<0.0001
29	*Gluconate (Glu)*	1.13	(0.92, 1.44)	<0.0001
30	*Tyrosine (Tyr)*	1.12	(0.97, 1.41)	<0.0001
31	Tartrate (Tar)	1.12	(0.74, 1.51)	0.8643
32	*Methylamine (MA)*	1.10	(0.93, 1.41)	<0.0001
33	Dimethylsulfone (DMS)	1.09	(0.87, 1.43)	0.0013
34	Glycolate (Glyc)	1.07	(0.86, 1.27)	0.0066
35	Methanol (Met)	1.07	(0.71, 1.72)	0.1777
36	Urea (Urea)	1.03	(0.87, 1.20)	0.0715
37	Pseudouridine (Pse)	1.03	(0.88, 1.20)	0.1605
38	Dimethylamine (DMA)	1.02	(0.86, 1.21)	0.2783
39	Hypoxanthine (Hyp)	1.00	(0.67, 1.65)	0.4068
40	Uracil (Ura)	0.98	(0.79, 1.22)	0.2208
41	Creatinine (Crea)	0.98	(0.84, 1.18)	0.7036
42	1-Methylnicotinamide (MNA)	0.97	(0.78, 1.24)	0.3221
43	Trimethylamine N-oxide (TMAO)	0.97	(0.79, 1.19)	0.0778
44	*3-Methylxanthine (3-MXan)*	0.90	(0.70, 1.16)	<0.0001
45	*3-Indoxylsulfate (3-Ind)*	0.87	(0.68, 1.04)	<0.0001
46	*Trigonelline (Tri)*	0.73	(0.63, 0.89)	<0.0001
47	*Hippurate (Hip)*	0.70	(0.54, 0.91)	<0.0001

For each metabolite, FCs between normalized post- and pre-exercise concentrations were calculated per participant and presented as median, 25^{th}, and 75^{th} percentiles. Significant pre- to post-exercise differences are shown by FDR-corrected *p*-values obtained by Wilcoxon's signed-rank test. Metabolites are sorted from high to low median FCs. Exercise-responsive metabolites (i.e., metabolites with FDR-adjusted *p*-values < 0.05 and median FCs $\leq \frac{1}{1.1}$ or ≥ 1.1, respectively) are indicated in italics. FC: fold change; FDR: false discovery rate.

Multivariate Analysis

PCA was applied as an unsupervised method to describe the differentiation in the metabolite profile between pre- and post-exercise urine samples. Two different approaches were conducted to compare either the participants in the pre- and post-exercise condition (based on a data matrix of 2 × 255 participants and 47 metabolites) or the pre- and post-exercise urinary metabolite profile (based on a data matrix of 2 × 47 metabolites and 255 participants). With regard to the first approach, no clear separation of the participants to either the pre- or post-exercise state was visible in the score plots of the first, second, and third principal component (PC), see Figure 22 and Figure A-8 (Appendix A2.7). However, with respect to the second approach, a partial separation between the pre- and post-exercise metabolite profile could be observed in the loading plots of the first three PCs. The metabolite with the greatest change in correlation to the first PC between the pre- and post-exercise condition was lactate. The separation of the other metabolites was mainly detectable in the second and third PC, see Figure 23 and Figure A-9 (Appendix A2.7).

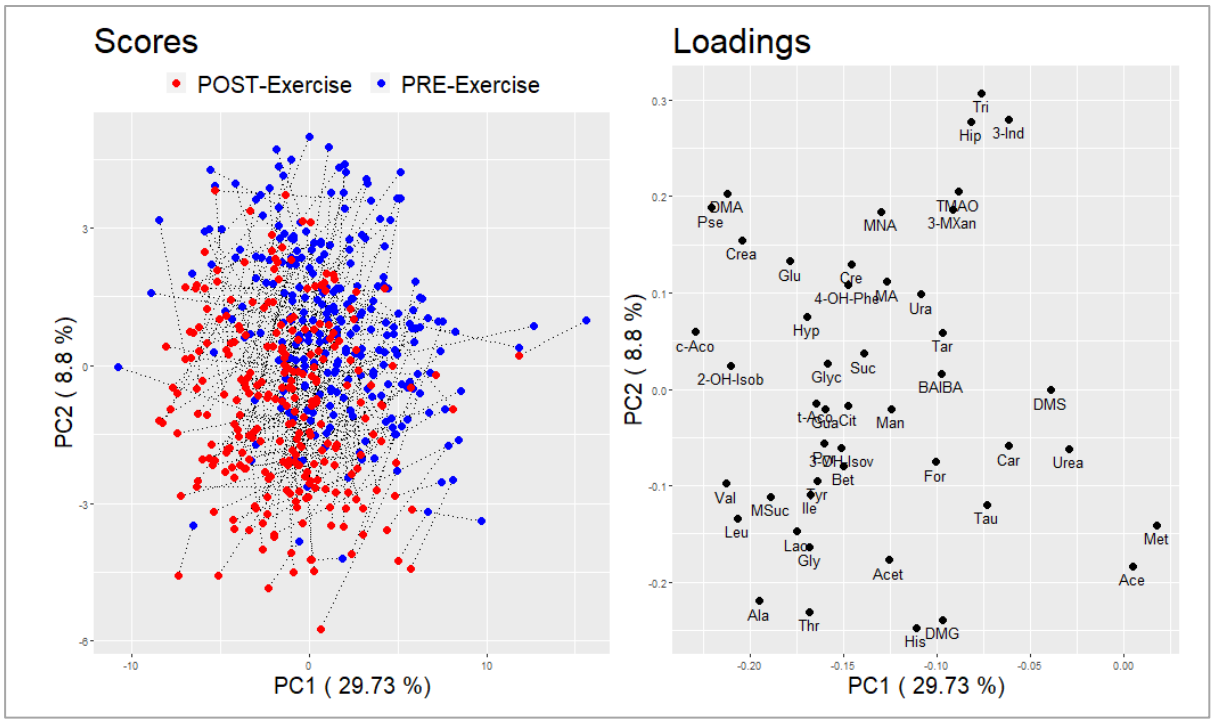

Figure 22. PCA score and loading plot of a combined pre- and post-exercise urinary metabolite data matrix containing 2 × 255 participants and 47 metabolites. The first two principal components are visualized; left: score plot, data points stand for participants and are color-coded according to the pre- or post-exercise state; right: loading plot, data points stand for metabolites. PC: principal component; PCA: principal component analysis. *(Reprinted from Kistner et al. (2020)).*

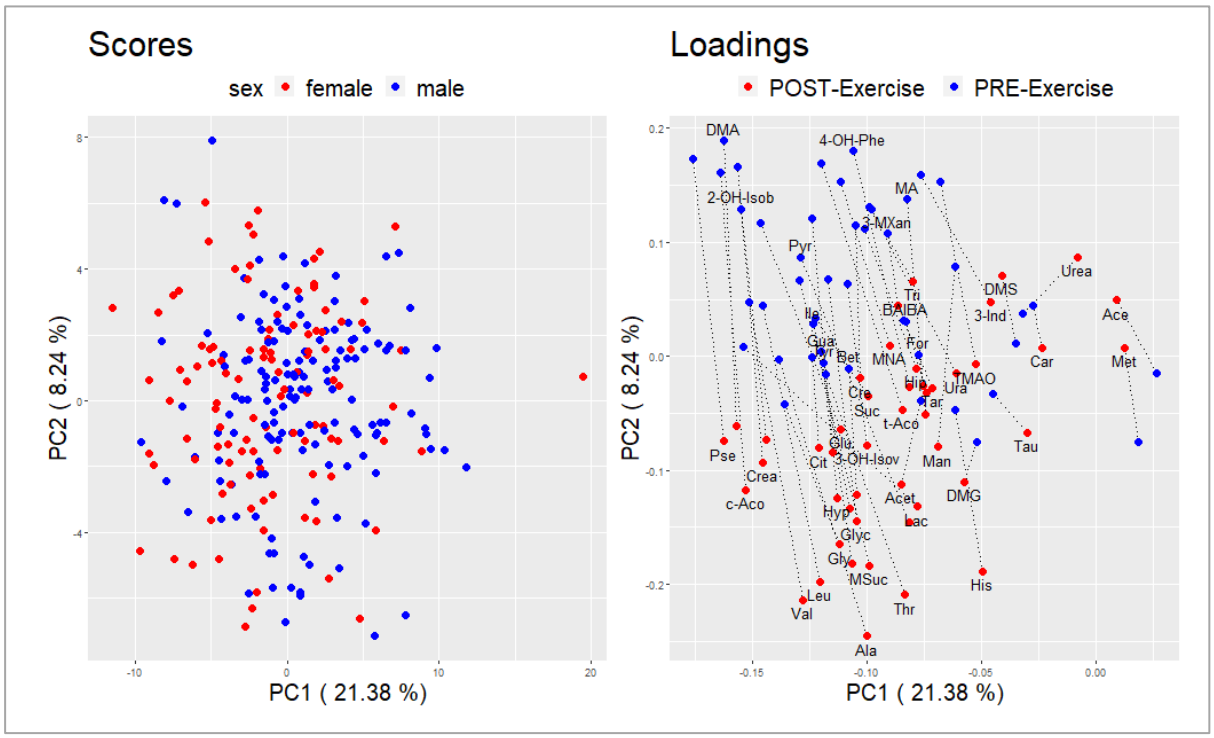

Figure 23. PCA score and loading plot of a combined pre- and post-exercise urinary metabolite data matrix containing 255 participants and 2 × 47 metabolites. The first two principal components are visualized; left: score plot, data points stand for participants and are color-coded according to the sex of the participants; right: loading plot, data points stand for metabolites and are color-coded according to the pre- or post-exercise state. PC: principal component; PCA: principal component analysis. *(Reprinted from Kistner et al. (2020)).*

6.4.3 Relationship between the CRF and Urinary Metabolites

Different exploratory approaches were applied to investigate the relationship between urinary metabolite measures and the CRF status. Firstly, we focused on bivariate associations between the VO_{2peak} and single metabolite measures, followed by partial associations independent of phenotypical variables known to determine both the VO_{2peak} and the metabolome. Secondly, multiple linear regression analyses were conducted to examine the relationship between the VO_{2peak} and all urinary metabolites simultaneously. To assess which of the metabolite measures (pre-exercise, post-exercise, or the post- to pre-exercise ratio, i.e., FC) correlates best with the VO_{2peak}, all analyses were performed separately for each of the three metabolite parameters.

Bivariate Analyses

Pearson correlation coefficients and partial correlations adjusted for age, sex, menopausal status, and LBM were calculated for the associations of the VO_{2peak} with urinary metabolite measures and are presented in Table A-7 (Appendix A2.8). Correlations were considered

statistically significant when the 95% CIs did not include zero. A visual comparison of the pairwise associations is additionally provided in correlation heat maps, see Figure 24 for the pre-exercise measures, Figure 25 for the post-exercise measures, and Figure 26 for the metabolite FCs.

Results of the unadjusted correlations revealed that 22 pre-exercise urinary metabolites, 24 post-exercise urinary metabolites, and seven FCs of urinary metabolites showed a correlation with the VO_{2peak} which was significantly different from zero. With regard to the pre-exercise metabolite measures, the strongest correlations were observed for citrate (r = −0.46), guanidoacetate (r = −0.39), lactate (r = −0.38), trigonelline (r = −0.35), and succinate (r = −0.32), see Figure 24 (right/up; 1st row). For the post-exercise metabolite measures, guanidoacetate (r = −0.41), citrate (r = −0.39), gluconate (r = −0.36), trigonelline (r = −0.36), creatine (r = −0.35), hippurate (r = −0.34), and succinate (r = −0.31) were most strongly associated with the VO_{2peak}, see Figure 25 (right/up; 1st row). When regarding the FCs of urinary metabolites, correlations with the VO_{2peak} were comparatively low with $r \leq 0.3$ or ≥ -0.3, respectively, see Figure 26 (right/up; 1st row).

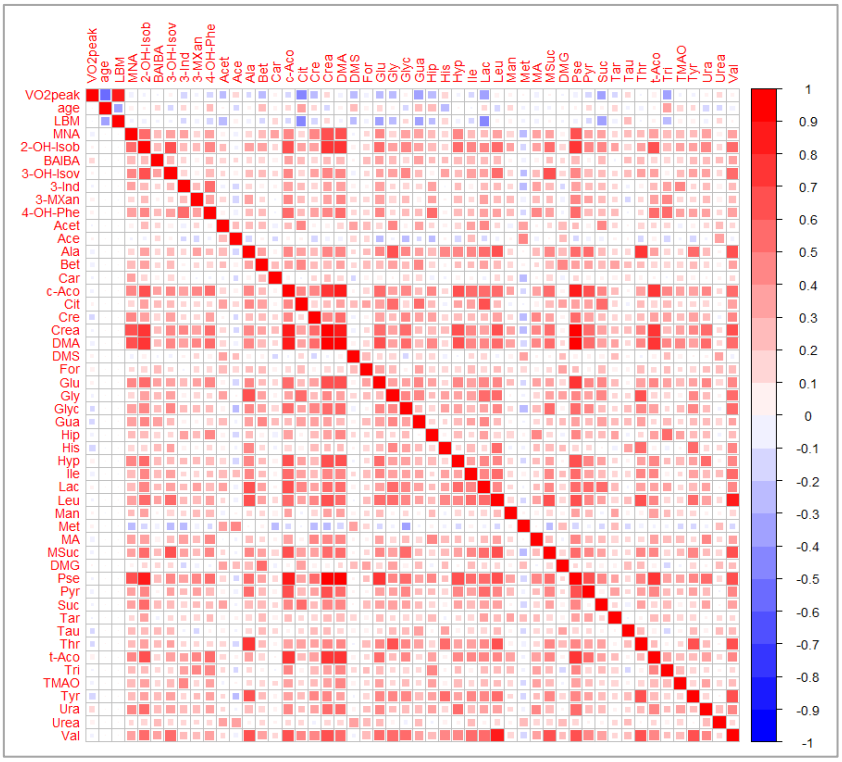

Figure 24. Heat map of correlations between pre-exercise urinary metabolites, VO_{2peak}, age, and LBM. Right/up: unadjusted bivariate associations; left/low: partial associations adjusted for age, sex, menopausal status, and LBM. Pearson correlations were performed on VdW-transformed data. LBM: lean body mass; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2020)).*

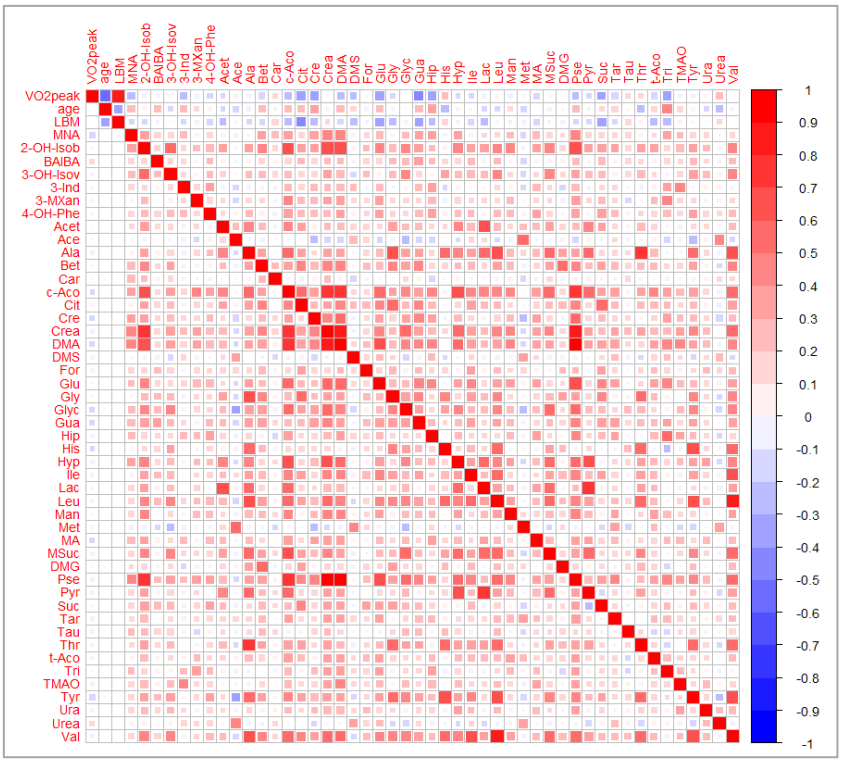

Figure 25. Heat map of correlations between post-exercise urinary metabolites, VO_{2peak}, age, and LBM. Right/up: unadjusted bivariate associations; left/low: partial associations adjusted for age, sex, menopausal status, and LBM. Pearson correlations were performed on VdW-transformed data. LBM: lean body mass; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2020)).*

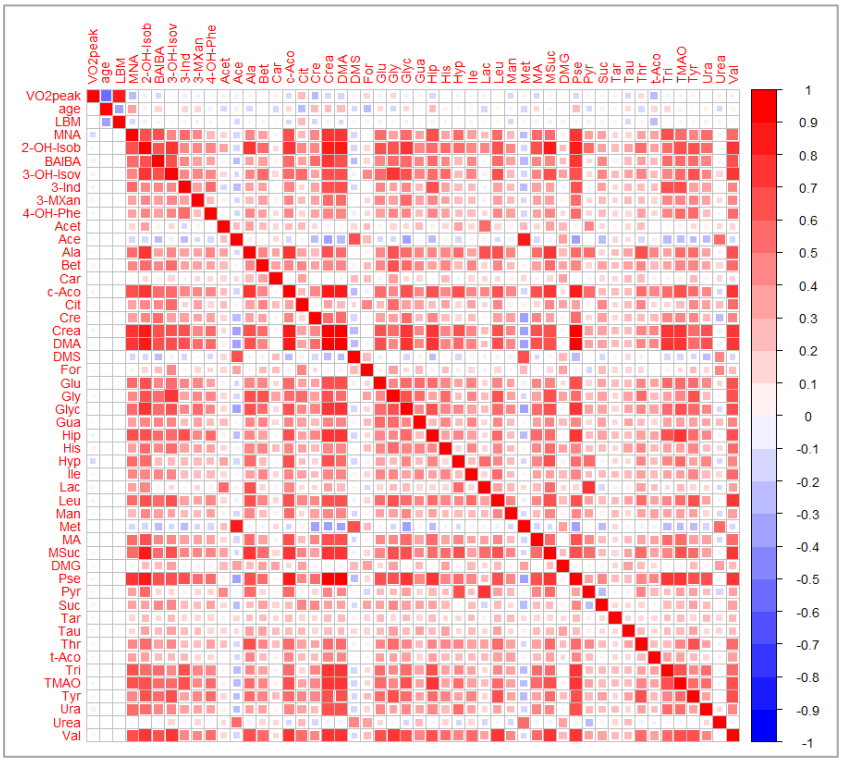

Figure 26. Heat map of correlations between urinary metabolite FCs, VO_{2peak}, age, and LBM. Right/up: unadjusted bivariate associations; left/low: partial associations adjusted for age, sex, menopausal status, and LBM. Pearson correlations were performed on VdW-transformed data. FCs: fold changes; LBM: lean body mass; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2020)).*

To examine if the described correlations were dependent on certain influencing factors such as age, sex, menopausal status, and LBM, both the VO_{2peak} and the urinary metabolite measures were adjusted for these factors and correlation analyses were performed on the corresponding residuals. Results of the partial correlation analyses showed that only three pre-exercise urinary metabolites, three post-exercise urinary metabolites, and two FCs of urinary metabolites exhibited a partial correlation with the VO_{2peak} which was significantly different from zero. After adjusting for confounding factors, the most correlated urinary metabolite measures included pre-exercise histidine (r = −0.17), tyrosine (r = −0.16), and uracil (r = 0.16), post-exercise tyrosine (r = −0.18), 1-methylnicotinamide (r = −0.15), and guanidoacetate (r = −0.12), as well as the FCs of urinary 1-methylnicotinamide (r = −0.13) and hypoxanthine (r = −0.13), see Table A-7 (Appendix A2.8) and Figure 24, Figure 25, or Figure 26, respectively (left/down; 1st column). In addition, bivariate correlations of age and LBM with the VO_{2peak} and the urinary metabolite measures confirmed that these factors were strongly correlated with the VO_{2peak} and weakly to moderately associated with some urinary metabolite parameters, see 2nd and 3rd row in Figure 24, Figure 25, or Figure 26, respectively.

Multivariate Analyses

To analyze the relationship between the VO_{2peak} and all urinary metabolites at once, several multiple linear regression procedures were conducted. By adjusting both the VO_{2peak} as the dependent variable and the urinary metabolite measures as the independent variables for age, sex, menopausal status, and LBM, the investigated associations were uncorrelated with these phenotypical variables. When all 47 urinary metabolite measures were included into a multiple linear regression model, the adjusted pre-exercise urinary metabolites explained the variation in the adjusted VO_{2peak} to 29.5% (R^2 = 0.295, R^2 (adjusted) = 0.135). While the adjusted post-exercise urinary metabolites similarly accounted for up to 29.6% (R^2 = 0.296, R^2 (adjusted) = 0.137) of the variation in the adjusted VO_{2peak}, the inclusion of all 47 adjusted FCs of urinary metabolites resulted in a comparatively lower proportion of explained variance in the adjusted VO_{2peak} (R^2 = 0.141, R^2 (adjusted) = −0.054). In a next step, we aimed to select the best set of urinary metabolite variables describing the adjusted VO_{2peak}. Therefore, two stepwise regression models with either forward or backward selection were performed for each metabolite measure (i.e., pre-exercise, post-exercise and FC). As a criterion for the selection of urinary metabolite variables, the BIC was used. For each metabolite measure, the

model with the lowest BIC was chosen and the respective metabolite variables were entered into a final multiple linear model. The included urinary metabolite variables as well as criteria for the evaluation and comparison of the three final models are summarized in Table 15.

Table 15. Final multiple linear models for the adjusted VO_{2peak}. *(Adapted from Kistner et al. (2020)).*

Model	R^2	R^2 (adjusted)	BIC	Std.-Beta	95% CI (lower)	95% CI (upper)
Pre-Exercise	**0.176**	**0.153**	**300.9**			
cis-Aconitate				−0.441	−0.291	−0.106
3-Aminoisobutyrate				0.232	0.047	0.163
trans-Aconitate				0.295	0.054	0.212
Tyrosine				−0.213	−0.157	−0.038
Guanidoacetate				−0.208	−0.165	−0.038
Uracil				0.206	0.033	0.156
Lactate				0.230	0.041	0.218
Post-Exercise	**0.081**	**0.070**	**306.6**			
Tyrosine				−0.197	−0.145	−0.034
3-Aminoisobutyrate				0.182	0.028	0.141
1-Methylnicotinamide				−0.149	−0.126	−0.013
FCs	**0.000**	**0.000**	**311.4**			
(intercept-only model)						

Metabolite variables were selected based on the results of the stepwise regression analyses. All variables were VdW-transformed prior to analysis and adjusted for age, sex, menopausal status, and LBM. BIC: Bayesian information criterion; CI: confidence interval (of the corresponding regression coefficient in the considered linear model); FCs: fold changes; LBM: lean body mass; R^2 (adjusted): (adjusted) coefficient of determination; Std.-Beta: Standard-Beta (corresponding regression coefficient using only standardized variables); VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

With regard to the pre-exercise metabolite measures, seven urinary metabolite variables were included into the final model, resulting in an R^2 (adjusted) of 0.153 and a BIC of 300.9. For the post-exercise metabolite measures, three urinary metabolite variables were selected for the final model, which showed a comparatively lower R^2 (adjusted) of 0.070 and a higher BIC of 306.6. When regarding the FCs of urinary metabolites, the model with the minimal BIC did not contain any metabolite variables. Thus, the intercept-only model was obtained for the explanation of the adjusted VO_{2peak} based on the urinary metabolite FCs.

Apart from the multiple regression analyses, PCA was performed using either the adjusted pre- or post-exercise urinary metabolite data or the adjusted data on urinary metabolite FCs, in order to detect clusters of participants potentially related to their PF status. The first, second, and third PC were visualized in score and loading plots, see Figure A-10, Figure A-11, and Figure A-12 (Appendix 2.7). In the score plots, the data points were color-coded according to the individual VO_{2peak} of the participants. No evident cluster of participants with a similar PF level could be detected based on the pre- or post-exercise urinary metabolite profile, respectively. Nor did we observe a grouping of participants with a similar PF level when conducting the PCA on urinary metabolite FCs.

6.5 Discussion

The main finding of this study is that 37 out of 47 measured metabolites showed a significantly different urinary concentration post-exercise when compared to pre-exercise. While the strongest increase was observed for urinary lactate, mannitol, trans-aconitate, alanine, carnitine, and acetate (all demonstrating a median FC > 1.5), the strongest decrease was observed for urinary hippurate and trigonelline (both demonstrating a median FC < 0.8). However, PCA did not permit a clear separation of the pre- and post-exercise urine samples. With regard to the investigated relationship between the VO_{2peak} and urinary metabolites, both bivariate correlation and multiple linear regression analyses revealed only weak associations when adjusting for confounding covariates like age, sex, menopausal status, and LBM. In the following sub-sections, the post-exercise alterations in urinary metabolites as well as their relation to the CRF status of the KarMeN study participants are discussed.

6.5.1 Post-Exercise Alterations in Urinary Metabolites Are Partly Reflective of Energy Metabolism

The pre- to post-exercise comparison of urinary metabolite concentrations confirmed some well-established exercise-induced changes in pathways related to ‘energy metabolism’. However, some novel urinary metabolites being altered in response to PE were also revealed. A summary of the origin and pathways of the identified metabolites and the documented effect of acute PE on urinary metabolite levels is provided in Figure 27.

The highest post-exercise increase was observed for urinary lactate (median FC = 4.70), which can be explained by an enhanced carbohydrate catabolism in the exercising muscles. During an incremental exercise test, individuals continuously utilize an increased amount of adenosine triphosphate (ATP). To maintain the muscular ATP resynthesis rate, the required energy is initially provided by mainly aerobic processes and then, with augmented intensity, to an increased degree by the anaerobic energy system (Bertuzzi, Nascimento, Urso, Damasceno, & Lima-Silva, 2013). As the end-product of anaerobic glucose breakdown, lactate is released into the blood stream and partly excreted via urine (Adeva-Andany et al., 2014). The documented increase in urinary lactate elimination is in accordance with the results of other exercise metabolomics studies (Enea et al., 2010; Muhsen Ali et al., 2016; Mukherjee et al., 2014; Pechlivanis et al., 2010; Pechlivanis et al., 2015; Siopi et al., 2017), even though the

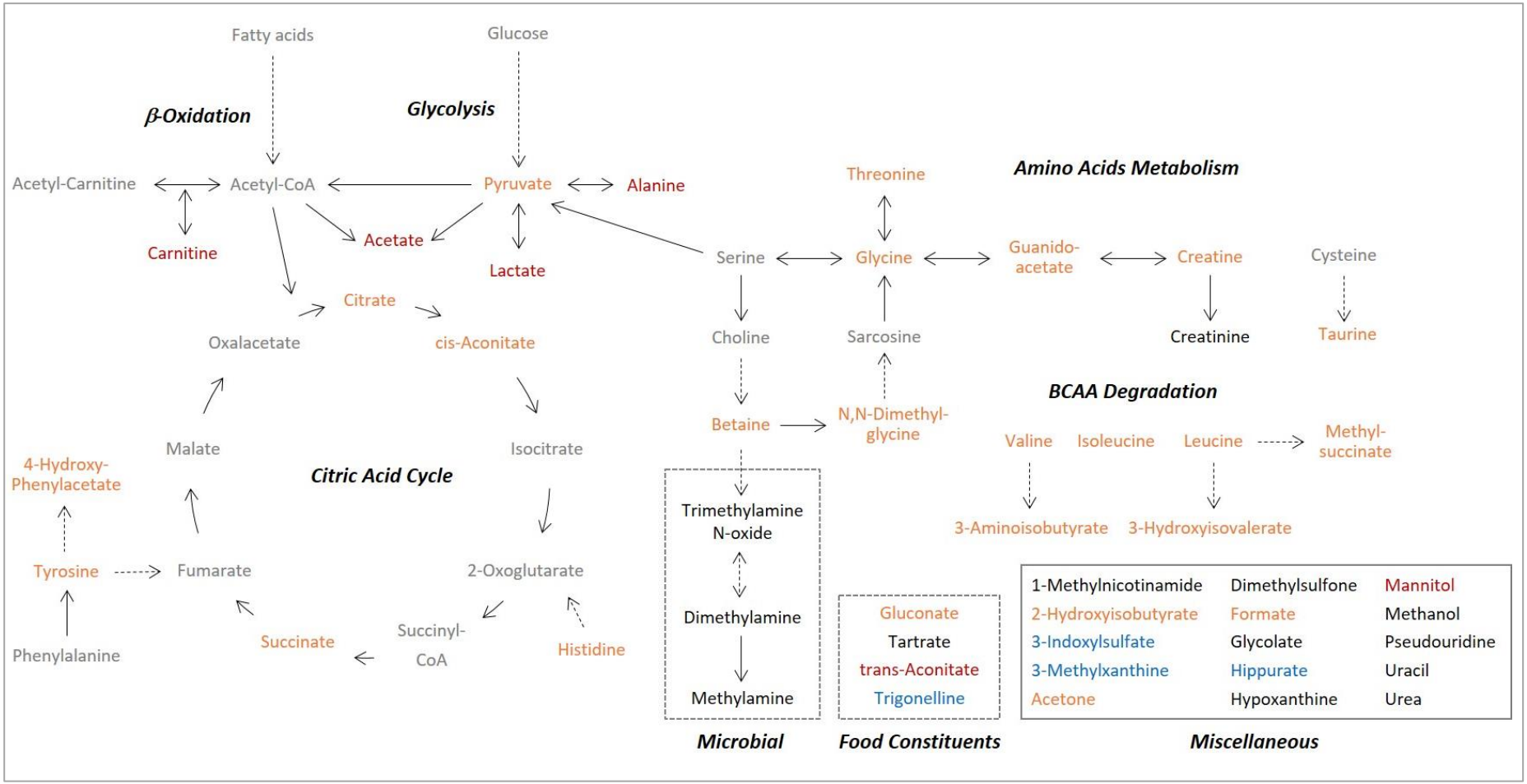

Figure 27. Pathways and origin of identified metabolites. The post-exercise changes of the urinary metabolites are color-coded as follows: red, FC ≥ 1.5 and FDR-corrected $p < 0.05$; orange: 1.5 > FC ≥ 1.1 and FDR-corrected $p < 0.05$; black: 1.1 > FC > $\frac{1}{1.1}$ or FDR-corrected $p \geq 0.05$; blue: FC ≤ $\frac{1}{1.1}$ and FDR-corrected $p < 0.05$; grey: undetected metabolites. BCAA: branched-chain amino acid; FC: fold change between normalized post- and pre-exercise urinary metabolite concentrations; FDR: false discovery rate; *p*: *p*-value obtained by Wilcoxon's signed-rank test to analyze significant pre- to post-exercise differences. *(Reprinted from Kistner et al. (2020)).*

magnitude of urinary lactate excretion was generally greater in response to maximal exercise protocols (Pechlivanis et al., 2010; Pechlivanis et al., 2015; Siopi et al., 2017). When compared with other metabolites, we observed a remarkably high inter-individual variation in the post-exercise urinary lactate concentrations. Unfortunately, we were unable to explain this variation by any of the measured physiological parameters and assume it to be a result of the heterogeneous study population and further influencing factors (e.g., the maximally reached exercise intensity, the anaerobic lactic capacity, or the elimination rate of lactate from muscle via blood to urine). However, despite the high variation in the lactate FCs, we noticed that those persons with a high increase in urinary lactate also showed a high increase in urinary pyruvate, alanine, and acetate – all metabolites resulting from interconnected metabolic pathways, see Figure 27. For instance, the post-exercise increase of urinary pyruvate (median FC = 1.48) can equally be seen as a result of an increased glycolysis. When the pyruvate production rate during exercise is higher than the capacity of the mitochondria to take up pyruvate, the skeletal muscle has to remove it from the cytosol (Baker et al., 2010). Thus, pyruvate is either directly released into the blood or previously converted to lactate via lactate dehydrogenase reaction (Baker et al., 2010) or to alanine via transamination (Adeva-Andany et al., 2014), thereby providing substrates for gluconeogenesis in the liver (Adeva-Andany et al., 2014; G. Wu, 2009). Our results demonstrated a median 1.74-fold increase in the urinary alanine excretion after exercise, which is in line with previous work (Enea et al., 2010; Mukherjee et al., 2014; Pechlivanis et al., 2015; Siopi et al., 2017). As the alanine flux into the bloodstream during exercise was shown to be higher than the alanine uptake by the liver (Felig & Wahren, 1971), it can be assumed that this AA accumulated in blood and was therefore excreted via urine after the incremental test. Next to pyruvate and alanine, the post-exercise increase in urinary lactate was accompanied by a median 1.55-fold increase in urinary acetate, confirming the results of previous studies investigating the effect of acute PE on the urinary metabolome (Enea et al., 2010; Mukherjee et al., 2014; Pechlivanis et al., 2015). Two mechanisms for explaining this observation are discussed in the literature. Firstly, the rise in urinary acetate is likely to be the consequence of an increased hydrolysis of acetyl-CoA, which was produced from pyruvate but could not entirely enter into the tricarboxylic acid (TCA) cycle and was therefore released into the blood (Knowles, Jarrett, Filsell, & Ballard, 1974; Pechlivanis et al., 2015). Secondly, acetate might be directly produced in a radical-removing

reaction from pyruvate, which seems able to convert reactive oxygen species into carbon dioxide and acetate (Bassenge, Sommer, Schwemmer, & Bünger, 2000; Liu et al., 2018).

With regard to the remaining AAs (Gly, His, Ile, Leu, Tau, Thr, Tyr, Val), we observed a post-exercise increase in their urinary excretion, reaching from a median 1.12-fold (Tyr) to a median 1.49-fold (Tau) rise. This observation points to higher circulating AA concentrations, but it remains speculative if this was due to an increased exercise-induced protein degradation or a higher AAs availability owing to the breakfast participants consumed after the first spot urine collection. Although the rise in urinary alanine was consistent across several exercise-related metabolomics studies, this global increase in AAs has not been detected in previous work. While Siopi et al. (2017) could confirm a higher urinary excretion of glycine, histidine, taurine, tyrosine, and threonine two hours after high-intensity interval and continuous moderate exercise, other studies documented an unchanged urinary excretion of taurine (Pechlivanis et al., 2010; Pechlivanis et al., 2015), isoleucine, leucine, and valine (Pechlivanis et al., 2010) or a decreased level in urinary glycine (Mukherjee et al., 2014; Pechlivanis et al., 2010; Pechlivanis et al., 2015), leucine (Muhsen Ali et al., 2016), histidine (Pechlivanis et al., 2010), threonine (Muhsen Ali et al., 2016), and tyrosine (Pechlivanis et al., 2015), respectively, in response to the different exercise protocols. With respect to the increase in urinary taurine, a semi-essential AA highly concentrated in human muscles (Seidel et al., 2018), it can be supposed that the acute PE resulted in an increased muscular taurine release. Consistent with this explanation, previous work has demonstrated an exercise-induced release of taurine from contracting muscles due to osmoregulatory processes, leading to a higher taurine level in both blood and urine (Cuisinier et al., 2002). However, as the urinary excretion of taurine furthermore depends on the dietary taurine intake (Lambert et al., 2015), it cannot be completely excluded that the intake of taurine-containing food like cheese at breakfast led to the observed increase in urinary taurine excretion.

Four further metabolites related to ‘AA metabolism’ showed a post-exercise increase in their urinary level, namely methylsuccinate (median FC = 1.28) and 3-hydroxyisovalerate (median FC = 1.13), both byproducts of the leucine degradation pathway (Dercksen et al., 2013; Duran, Walther, Bruinvis, & Wadman, 1983), 4-hydroxyphenylacetate (median FC = 1.19), a degradation product of tyrosine, and 3-aminoisobutyrate (median FC = 1.15), a valine degradation product and recently discovered myokine (Tanianskii, Jarzebska, Birkenfeld,

O'Sullivan, & Rodionov, 2019). The AA derivative N,N-dimethylglycine (median FC = 1.38) and guanidoacetate (median FC = 1.39), an intermediate in the metabolic pathway of several AAs, were also reported to be elevated in the post-exercise urine samples. To our knowledge, no earlier metabolomics studies have reported exercise-related changes in the urinary concentration of these metabolites.

With regard to metabolites of the TCA cycle, i.e., citrate, cis-aconitate, and succinate, a median 1.24- to 1.30-fold increase in their urinary levels was observed post-exercise. A significant rise in urinary succinate has previously been documented in response to a 30 seconds maximal sprint (Enea et al., 2010), whereas a decrease in both urinary succinate (Mukherjee et al., 2014; Pechlivanis et al., 2015) and citrate (Mukherjee et al., 2014; Pechlivanis et al., 2010; Pechlivanis et al., 2015; Siopi et al., 2017) was reported after intermittent (Pechlivanis et al., 2010; Pechlivanis et al., 2015; Siopi et al., 2017) or submaximal exercise (Mukherjee et al., 2014; Siopi et al., 2017). Compared to this, other studies showed that acute exercise was followed by an increase in plasma TCA cycle intermediates, suggesting that these compounds spilled over from muscle into the circulation due to an increased TCA cycle flux (Peake et al., 2014; Zafeiridis et al., 2016). However, whether the increased post-exercise urinary excretion of TCA cycle metabolites in our study can also be traced back to this explanation, remains unclear. Interestingly, we observed that women had a lower FC of urinary citrate than men (median 1.16- vs. 1.29-FC). This might hint at sex-specific differences in exercise-related citrate turnover.

To our knowledge, this is the first study reporting a post-exercise increase in the urinary excretion of carnitine (median FC = 1.68). Carnitine, which can either be synthesized from the AA lysine or ingested through diet, is largely stored in the skeletal muscle, where it is involved in the translocation of long-chain fatty acids (LCFAs) into the mitochondrial matrix for subsequent β-oxidation and the buffering of accumulating acetyl-CoA (Stephens, 2018). Previous work has shown that acute PE results in a higher muscle and plasma concentration of acetylcarnitines (reflecting an enhanced pyruvate and fatty acids oxidation) or long-chain acylcarnitines (reflecting an increased mobilization of free fatty acids) (Arenas et al., 1991; Breit et al., 2015; Carlin, Reddan, Sanjak, & Hodach, 1986; Lehmann et al., 2010; Zhang et al., 2017), accompanied by a decrease in muscular (Carlin et al., 1986; Hiatt, Regensteiner, Wolfel, Ruff, & Brass, 1989) and blood (Arenas et al., 1991; Carlin et al., 1986) free carnitine. However,

neither a decreased nor an increased urinary excretion of free carnitine has been noticed so far (Hiatt et al., 1989; Pechlivanis et al., 2010; Pechlivanis et al., 2015).

In addition to carnitine, urinary mannitol and trans-aconitate demonstrated a more than 1.9-fold increase in response to the incremental exercise test. Mannitol is a polyol found in many foods and used as an artificial sweetener. It is produced by microorganisms (Feng et al., 2016) and cannot be metabolized by humans (Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2002). Although an exercise-related increase of urinary mannitol was also shown by Siopi et al. (2017), the reasons for this alteration are currently unclear. As mannitol in urine seems to show diurnal variation (Slupsky et al., 2007), an effect of time on the mannitol excretion cannot be excluded. Trans-aconitate is an organic acid present in plants like wheat or soybean and seems not to be degraded in human metabolism (Yuhara, Yonehara, Hattori, Kobayashi, & Kirimura, 2015). Since participants of this study consumed wheat bread before exercising, it is presumable that the increase in urinary trans-aconitate was due to its dietary intake and not owing to the acute PE. That women demonstrated a higher increase in urinary trans-aconitate than men (median 2.33- vs. 1.53-FC) could indicate sex-related differences in the biokinetics of absorbed trans-aconitate.

With regard to betaine (median FC = 1.32), an increased urinary excretion has previously been documented in response to intermittent and submaximal endurance exercise (Siopi et al., 2017). Betaine functions as an organic osmolyte and methyl donor; it is endogenously produced or ingested through foods like wheat or spinach (Ueland, Holm, & Hustad, 2005). However, as the urinary betaine excretion seems to be relatively stable, not being considerably affected by food intake or hydration status (Lever & Slow, 2010), we are currently unable to explain the observed increase in urinary betaine. Similarly, it is unclear why the level of urinary acetone (median FC = 1.28) increased after exercise. The ketone body is produced in the liver when there is a shortage of carbohydrates, e.g., during prolonged exercise or in the fasting state (Evans, Cogan, & Egan, 2017). Concerning creatine, there is one study confirming the post-exercise increase in its urinary excretion (Siopi et al., 2017) and one study showing opposite results (Muhsen Ali et al., 2016). Altogether, the observed increases in urinary creatine, formate, gluconate, and methylamine can hardly be explained and might be due to uncontrolled variation. However, one interesting finding is the post-exercise rise in the urinary excretion of 2-hydroxyisobutyrate (median FC = 1.24), which has already been shown

in previous exercise metabolomics studies (Pechlivanis et al., 2015; Siopi et al., 2017). Urinary 2-hydroxyisobutyrate is mainly known as a degradation product of gasoline additives entering the body through inhalational exposure (Amberg, Rosner, & Dekant, 1999). Recently, it was also suggested to be a gut microbial metabolite (Yap et al., 2010) and an indicator of the increased lactate production due to alcohol consumption (Irwin et al., 2018). As the same carrier protein seems to be responsible for the (re)absorption of both lactate and 2-hydroxy-isobutyrate in the kidney, it was assumed that elevated lactate levels inhibit the 2-hydroxy-isobutyrate reabsorption rate, thus contributing to a rise in its urinary excretion (Irwin et al., 2018). However, it remains largely speculative if this mechanism also underlay the exercise-related increase of urinary 2-hydroxyisobutyrate in this study.

The four metabolites showing a decrease in the post-exercise urine samples were identified as either microbial cometabolites, namely 3-indoxylsulfate (Behr et al., 2017) and hippurate (Lees, Swann, Wilson, Nicholson, & Holmes, 2013), or diet-related metabolites, namely 3-methylxanthine (Geraets, Moonen, Wouters, Bast, & Hageman, 2006) and trigonelline (Madrid-Gambin et al., 2016); both markers of coffee consumption. As we could not find a relevant link between these metabolites and acute exercise, we assume that the observed decline in their urinary levels was due to uncontrolled variation.

Rather unexpectedly, no change was documented in the urinary excretion of hypoxanthine, the final ATP degradation product, which is known to be released during PE from muscles to blood and excreted via urine (Zielinski & Kusy, 2015b). In agreement with its proposed function as an indicator of exercise-related energetic stress (Sahlin et al., 1999; Zielinski & Kusy, 2015b), hypoxanthine was increased in the post-exercise urine samples of several metabolomics studies (Enea et al., 2010; Muhsen Ali et al., 2016; Mukherjee et al., 2014; Pechlivanis et al., 2010; Pechlivanis et al., 2015; Siopi et al., 2017). The discrepant results could be explained by a comparatively lower exercise intensity or duration in the KarMeN study or a time point of sample taking that was too early to capture the post-exercise rise of hypoxanthine in urine.

With regard to our multivariate approaches, we could not observe a clear distinction between the pre- and post-exercise urine samples, see Figure 22 and Figure A-8 (Appendix A2.7), suggesting that the incremental exercise test did not cause a substantial variation in the

overall urinary metabolite profile. Conversely, the PCA loading plots in Figure 23 and Figure A-9 (Appendix A2.7) showed a partial separation between the pre- and post-exercise urinary metabolites, which was predominantly detectable in the second and third PC. These results indicate that there was a variation in the urinary metabolite profile which, however, could not primarily and solely be attributed to the performed exercise. Although the results of our univariate analysis revealed that 37 out of 47 urinary metabolites significantly changed in response to exercise, it was also apparent that the degree of changes in urinary metabolites was less profound than in other exercise-related metabolomics studies (Enea et al., 2010; Pechlivanis et al., 2010; Pechlivanis et al., 2015; Siopi et al., 2017). Furthermore, we generally observed a high inter-individual variability in urinary metabolite concentrations and calculated FCs, which was probably due to the heterogeneous study population and the fact that the dietary intake on the day before the bicycle ergometry was not standardized. Taken together, discrepancies regarding the type, duration, and intensity of exercise as well as the time point of sample taking and the selected study population only allowed a limited comparison of our results to other studies. What additionally has to be taken into account when interpreting our findings is that large sample sizes like in this study can amplify the detection of statistically significant metabolite changes, which are, however, not necessarily biologically relevant (Faber & Fonseca, 2014). Nevertheless, as we could detect known exercise-related metabolite changes being reflected in the urinary metabolome, we conclude that metabolomics studies focusing on urine need to control for PA.

6.5.2 Urinary Metabolites at Rest and after Exercise Are Not Substantially Related to PF

Despite a high inter-individual variance in both the analyzed urinary metabolites and the VO_{2peak}, we revealed only weak to moderate associations between the CRF and the pre- and post-exercise urinary metabolite profile or the exercise-related metabolite FCs, respectively. When comparing the results of the single and partial correlation analyses, it became obvious that the associations between the VO_{2peak} and the urinary metabolite variables were strongly influenced by age, sex, menopausal status, and LBM. After adjusting for these covariates, only few single urinary metabolites showed weak correlations with the VO_{2peak} which were significantly different from zero, namely pre-exercise urinary histidine (r = −0.17), tyrosine (r = −0.16), and uracil (r = 0.16), post-exercise urinary 1-methylnicotinamide (r = −0.15),

guanidoacetate (r = −0.12), and tyrosine (r = −0.18), and FCs of urinary 1-methylnicotinamide (r = −0.13) and hypoxanthine (r = −0.13). Even if no reliable urinary marker for the VO_{2peak} could be detected in this study, our results hint at a few potentially interesting urinary metabolites that are independently of known covariates linked to the VO_{2peak}. For example, the slight negative association between pre-exercise urinary histidine and the VO_{2peak} is in line with a previous study documenting a significant lower urinary histidine excretion in a high-PF group compared to a low-PF group (Morris et al., 2013). As opposed to this, the weak negative association between the FC of urinary hypoxanthine and the VO_{2peak} does not confirm previous results from Mukherjee et al. (2014) who showed that more fit individuals demonstrated a higher increase in urinary hypoxanthine after a submaximal endurance exercise than less fit participants. The discrepant results could be explained by distinct approaches to detect differences in post-exercise metabolite alterations or by different study populations. We used correlation and regression analyses instead of group comparisons to be able to draw conclusions across a broad range of PF levels in healthy women and men. Previous studies characterizing the human urinary metabolome in relation to acute PE and the PF status mainly observed fitness-associated differences in exercise-induced metabolite changes between pre-defined groups of either athletes or untrained persons (Enea et al., 2010; Mukherjee et al., 2014). We suppose that maybe an athletic background with several years of training and possibly a distinct genetic background is necessary to substantially alter metabolic pathways.

Our multivariate approaches, which analyzed the relationship between the adjusted VO_{2peak} and all urinary metabolites at once, showed that there was no evident metabolic pattern related to the PF status in neither the pre- nor the post-exercise condition. Thus, it can be summarized that the 47 analyzed metabolites do not strongly account for the variation in the CRF after having adjusted for age, sex, menopausal status, and LBM. When furthermore comparing the ability of selected urinary metabolite variables to explain the variation in the adjusted VO_{2peak}, we noticed that the adjusted pre-exercise urinary metabolites resulted in a comparatively better model in the sense of BIC than the adjusted post-exercise urinary metabolites or respective FCs. This observation indicates that acute changes in urinary metabolites might primarily be driven by the energy requirements due to the performed exercise and less reflective of an individual's PF status.

6.5.3 Strengths and Limitations

The present study has several strengths and limitations. Firstly, a high number of healthy women and men with a wide age range were included in this study, which was characterized by highly standardized clinical and physiological examinations and a strictly scheduled experimental setting. Thus, we were able to investigate how urinary metabolites are associated with both acute PE and the CRF status in a comparatively large population. Additionally, owing to the comprehensive characterization of study participants, potential confounders like age, sex, LBM, and menopausal status could be considered. To furthermore minimize the variability in metabolomics measurements, pre-menopausal women were scheduled for the bicycle ergometry within the luteal phase of their menstrual cycle. One limitation of this study is that the time point of post-exercise urine sampling was not strictly controlled. Participants were told to collect their first available spot urine after having completed the incremental test, which was approximately 15 to 30 minutes post-exercise. Besides, as the participants collected the pre-exercise spot urine samples before breakfast, it has to be taken into account that the urinary metabolite changes could be related to both the post-exercise and the post-prandial state. Additionally, no causal relationships between the individuals' PF status and the metabolome can be proven due to the cross-sectional study design. Even though our NMR-based analysis had the advantage to allow the absolute quantification of urinary metabolites with known identity and of different chemical classes, this targeted approach was limited to a comparatively small selection of urinary compounds. In future studies, it would be appropriate to analyze the acute and chronic effects of PE on a broader spectrum of metabolites in both blood and urine.

6.6 Conclusions

We investigated the effect of a standardized exercise tolerance test on 47 urinary metabolites of 255 healthy women and men. Besides, we analyzed whether the VO_{2peak} measured in the incremental test is associated with exercise-related metabolite excursions and pre- or post-exercise urinary metabolite patterns. Although PCA did not show a clear separation of the pre- and post-exercise urine samples, univariate analysis revealed significant pre- to post-exercise alterations in the urinary excretion of 37 metabolites – with the strongest increase being observed for lactate, mannitol, trans-aconitate, alanine, carnitine, and acetate. However, only

weak relationships between the VO_{2peak} and single urinary metabolites or urinary metabolite patterns, respectively, could be revealed after adjusting for covariates like age, sex, menopausal status, and LBM. Our findings indicate that the analyzed urinary metabolites partly reflect acute exercise-related changes in the human metabolism, but do not allow to conclude about the individuals' PF status. We recommend future urinary metabolomics studies to control for acute PE and to consider the mentioned confounders if investigating functional variables like the VO_{2peak}.

7 Study IIb

Sex-Specific Relationship Between the Cardiorespiratory Fitness and Plasma Metabolite Patterns in Healthy Humans—Results of the KarMeN Study

Slightly modified version of the published article (17 July 2021).

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For a better comprehensibility of results, the supplementary material of this article is provided in the Appendices A2.9 to A2.15. Datasets, R-scripts for calculation of results and figure generation, as well as overview tables with computed values are available under the following link: https://doi.org/10.3390/metabo11070463.

7.1 Abstract

The cardiorespiratory fitness (CRF) represents a strong predictor of all-cause mortality and is strongly influenced by regular physical activity (PA). However, the biological mechanisms involved in the body's adaptation to PA remain to be fully elucidated. The aim of this study was to systematically examine the relationship between the CRF and plasma metabolite patterns in 252 healthy adults from the cross-sectional Karlsruhe Metabolomics and Nutrition (KarMeN) study. The CRF was determined by measuring the peak oxygen uptake during incremental exercise. Fasting plasma samples were analyzed by nuclear magnetic resonance spectroscopy and mass spectrometry coupled to one- or two-dimensional gas chromatography or liquid chromatography. Based on this multi-platform metabolomics approach, 427 plasma analytes were detected. Bi- and multivariate association analyses, adjusted for age and menopausal status, showed that the CRF was linked to specific sets of metabolites primarily indicative of 'lipid metabolism'. However, CRF-related metabolite patterns largely differed between sexes. While several phosphatidylcholines were mainly linked to the CRF in females, single lyso-phosphatidylcholines and sphingomyelins were associated with the CRF in males.

When controlling for further assessed clinical and phenotypical parameters, the sex-specific CRF tended to be correlated with a smaller number of metabolites linked to 'lipid metabolism', 'amino acid metabolism', or 'xenobiotics-related metabolism'. Interestingly, sex-specific CRF explanation models could be improved when including selected plasma analytes in addition to clinical and phenotypical variables. In summary, this study revealed sex-related differences in CRF-associated plasma metabolite patterns and proved known associations between the CRF and risk factors for cardiometabolic diseases such as fat mass, visceral adipose tissue mass, or blood triglycerides in metabolically healthy individuals. Our findings indicate that covariates like sex and, especially, the body composition have to be considered when studying blood metabolic markers related to the CRF.

Keywords: cardiorespiratory fitness; physical fitness; metabolomics; plasma metabolome; plasma metabolite patterns; metabolite profiles.

7.2 Introduction

The CRF is a health-related component of physical fitness (PF) (Bouchard et al., 2007), reflecting the ability of the circulatory, respiratory, and muscular systems to take up, transport, and utilize oxygen during sustained physical exercise (PE) (Hill et al., 1924). It is affected by sex (Al-Mallah et al., 2016; Zeiher et al., 2019), age (Laukkanen et al., 2009; Zeiher et al., 2019), lean body mass (LBM) (S. Y. S. Wong et al., 2008), heredity (Bouchard et al., 1988), and behavioral factors like diet, smoking, or PA (Zeiher et al., 2019). Actually, the CRF is not only an objective measure of regular PA (McKinney et al., 2016), but has also emerged as a strong predictor of all-cause and disease-specific mortality (Harber et al., 2017). Representing the main modifiable determinant of the CRF, regular PA is known to favorably influence body composition and glucose-insulin homeostasis, as well as the lipoprotein profile (McKinney et al., 2016). At the muscular level, beneficial adaptations to PA comprise an increased capillarization, a higher mitochondrial density, and enhanced oxidative metabolism, finally leading to an improved endurance capacity (Egan & Zierath, 2013). However, despite profound knowledge on the health-promoting benefits of PA, the molecular mechanisms and metabolic pathways involved in the whole-body and skeletal muscle adaptation to PA are still insufficiently understood (Zierath & Wallberg-Henriksson, 2015).

The emerging field of metabolomics is a promising approach to systematically investigating exercise-induced changes in human metabolism related to performance and health (Heaney et al., 2017). By applying nuclear magnetic resonance (NMR) spectroscopy- or mass spectrometry (MS)-based techniques, metabolomics permits the simultaneous analysis of a high number and variety of metabolites, i.e., low-molecular weight compounds, that represent the end-products of interactions between genes, proteins, and the cellular environment (Bujak et al., 2015). Thus, metabolomics can help to identify PA- or PF-associated metabolite profiles, possibly hinting at metabolic pathways that are linked to the well-known effects of exercise (Heaney et al., 2017).

Interestingly, recent metabolomics studies have provided the first evidence that higher levels of PA or PF are linked to lower circulating branched-chain amino acid (BCAA) (Kujala et al., 2013; Kujala et al., 2019; Morris et al., 2013; Xiao et al., 2016) and higher circulating phosphatidylcholine (PC) concentrations (Bye et al., 2012; Floegel et al., 2014; Morris et al., 2015; Wientzek et al., 2014). However, research on the relationship between the CRF and the blood metabolome in large populations, including both sexes with a broad age spectrum, is rather scarce. In fact, only half of the studies that assessed the maximal oxygen uptake (VO_{2max}) as the gold standard of aerobic fitness conducted correlation or regression analyses (Floegel et al., 2014; Koh et al., 2018; Kujala et al., 2019; Lustgarten et al., 2013; Wientzek et al., 2014), while the other half examined differences between groups with a high or low CRF (Bye et al., 2012; Chorell et al., 2012; Morris et al., 2013; Morris et al., 2015). Limitations of the former studies are that the results were restricted to young (Kujala et al., 2019; Lustgarten et al., 2013), middle-aged (Floegel et al., 2014; Wientzek et al., 2014), or older (Koh et al., 2018) individuals, being thus hardly transferable to the general population. Besides, studies either had rather small sample sizes (Koh et al., 2018; Lustgarten et al., 2013) or solely included male subjects (Kujala et al., 2019). Apart from Lustgarten et al. (2013), who detected nearly 300 serum analytes, the remaining studies focused on a limited number of metabolites. Since several CRF-associated metabolites that were reported in the literature have also been linked to other phenotypical variables such as body composition, adjustments for potential confounders are decisive to determine if correlations can be specifically attributed to the CRF (Kelly et al., 2020).

As a way of overcoming those limitations, we applied a multi-platform metabolomics approach and exploratory bi- and multivariate statistical procedures to systematically analyze the relationship between the CRF and 427 plasma metabolites in 252 healthy women and men from the cross-sectional KarMeN study. Participants had a wide age range and were thoroughly characterized by anthropometric, functional, and clinical examinations (Bub et al., 2016). Therefore, we were able to take a variety of known and potential confounders into account. Since it has already been shown that sex, age, and menopausal status are determinants of the CRF (Mercuro et al., 2006; Zeiher et al., 2019) and are also linked to a discriminatory plasma metabolite profile in the KarMeN population (Rist et al., 2017), all analyses were conducted in sex-specific sub-groups and adjusted for age and menopausal status. Firstly, both bi- and multivariate associations between the CRF and metabolites were calculated, using bivariate correlation or partial least squares (PLS) regression analyses, respectively. Secondly, to identify associations that were independent of other phenotypical or clinical variables, correlation analyses and PLS models were additionally adjusted for parameters related to body composition, clinical blood biochemistry, lung and arterial function, short-term and habitual PA, or diet. Thirdly, cross-validated stepwise regression procedures were conducted, thus selecting sets of phenotypical, clinical, and plasma metabolite variables that contribute to a preferably good explanation of the CRF.

7.3 Materials and Methods

7.3.1 Subjects and Study Design

The cross-sectional KarMeN study was conducted between March 2012 and July 2013 at the Division of Human Studies of the Max Rubner-Institut (MRI) in Karlsruhe, Germany. Details on inclusion and exclusion criteria, as well as a comprehensive description of the study design and examination procedures, have already been published (Bub et al., 2016). Briefly, 301 healthy, non-smoking individuals (172 men, 129 women) between 18 and 80 years of age were included. All subjects visited the study center three times and were thoroughly characterized by anthropometric, clinical, and functional examinations. Moreover, data on PA, diet, and the menopausal status of female subjects were collected. Since the menstrual cycle is known to affect metabolite profiles (Wallace et al., 2010), all premenopausal women were scheduled for examinations within their luteal phase. On the morning of the second study day, fasting

plasma samples were collected using 9 mL EDTA plasma tubes (S-Monovette, Sarstedt, Nümbrecht, Germany). The plasma samples were immediately centrifuged at 1850 × g at 4 °C, aliquoted into small portions, and cryopreserved at −196 °C until metabolomics analyses. Serum samples (S-Monovette Z-gel, Sarstedt, Nümbrecht, Germany) were collected for standard clinical biochemistry analyses.

7.3.2 Anthropometry and Body Composition Assessment

Body weight and height were measured in underwear and without shoes (Seca 285, Hamburg, Germany), and the body mass index (BMI) was calculated by dividing the body weight in kilograms by the height in meters squared. The body composition was assessed by dual-energy X-ray absorptiometry (DXA; Lunar iDXA, GE Healthcare, München, Germany) and the LBM, fat mass (FM), visceral adipose tissue mass (VATM), as well as the bone mineral content (BMC) were determined with the supplementary software enCOREv16. The FM (%) was calculated by dividing the total FM by the total body weight. Approval for DXA measurements was received from the Federal Office for Radiation Protection (Z5-22462/2-2011-043).

7.3.3 PF and PA Assessment

As a measure of PF, the CRF was assessed by a standardized incremental exercise test on a bicycle ergometer (Ergobike medical, Daum, Fürth, Germany). All participants started pedaling at 25 watt and the workload was then augmented by 25 watt every 2 minutes until individual exhaustion, as previously described (Biniaminov et al., 2018; Kistner et al., 2020). The respiratory gas exchange was measured breath-by-breath by using a cardiopulmonary exercise testing system (MetaMax 3B, Cortex, Leipzig, Germany). Since the VO_{2max} could not be determined with certainty, as it requires the presence of a plateau in oxygen uptake, the peak oxygen uptake (VO_{2peak}), i.e., the highest attained oxygen uptake during the test, was assessed and expressed relative to the body weight in mL kg^{-1} min^{-1}. During the entire procedure, the heart rate (HR) was recorded (T31 coded, Polar Electro GmbH Deutschland, Büttelborn, Germany). In addition, continuous hemodynamic monitoring was conducted by running a 12-channel electrocardiogram (CardioDirect 12, DelMar Reynolds GmbH, Feucht, Germany) and by measuring the blood pressure (BP) every 2-3 minutes on the right upper arm (Boso Carat Professional, Bosch + Sohn, Jungingen, Germany). As measures of PA, both short-term and habitual PA were determined (Biniaminov et al., 2018). Briefly, the level of short-

term PA was assessed during a period of seven consecutive days by combined accelerometry and HR measurements (Actiheart, CamNtech, Cambridge, UK). The average activity energy expenditure (AEE) during the study week was finally calculated by the supplied software (Version 4.0.103) and given in kcal/day. To obtain the habitual PA for the last three months, participants filled out the standardized International Physical Activity Questionnaire (IPAQ). The average weekly PA was calculated and expressed in metabolic equivalent of task (MET)-min/week.

7.3.4 Dietary Assessment

Food consumption for the day prior to blood sampling was assessed by conducting a 24-hour recall in a personal interview, using the software EPIC-Soft, as described in detail elsewhere (Bub et al., 2016; Merz et al., 2018). In order to evaluate the diet quality of participants, a modified version of the Healthy Eating Index (HEI) was calculated, which was initially applied in the second German National Nutrition Survey ("*Nationale Verzehrsstudie* (NVS) II") (Wittig et al., 2010) and adapted with minor modifications in the KarMeN study. The so-called HEI-NVS evaluates the overall diet quality, with scores ranging from 0 (low quality) to 110 (high quality).

7.3.5 Clinical Examinations

Clinical parameters like the resting heart rate (HR_{rest}), as well as systolic and diastolic BP, were measured after a resting period of at least five minutes in a sitting position (Boso Carat Professional, Bosch + Sohn, Jungingen, Germany). The pulmonary function was assessed by spirometry (FlowScreen, CareFusion, Hoechberg, Germany) and the maximal vital capacity (VC_{max}), as well as the forced expiratory pressure in one second (FEV1), were recorded. Moreover, arterial stiffness was determined (ArterioGraph, Medexpert, Budapest, Hungary) and the pulse wave velocity (PWV) was calculated. Standard clinical biochemistry analyses in fasting serum samples (e.g., hemoglobin (Hb), glucose, HbA1c, triglycerides (TGs), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol) were carried out by the certified medical laboratory MVZ Labor PD Dr. Volkmann (Karlsruhe, Germany). Insulin concentrations were determined with an enzyme-linked immunosorbent assay (ME E-0900, LDN, Nordhorn, Germany).

7.3.6 Metabolomics Analyses

To obtain a broad coverage of the plasma metabolome, a number of complementary (non-) targeted analytical techniques were applied. Quality control (QC) samples prepared by pooling plasma samples from all KarMeN participants were analyzed along with study samples in all applied methods. The following section provides a brief summary of the different methods; further details are available in the supplement of Rist et al. (2017).

Non-targeted two-dimensional gas chromatography (GC × GC)-MS analysis. Plasma samples were analyzed by a non-targeted GC × GC-MS-based approach using a Shimadzu GCMS QP2010 Ultra instrument equipped with a ZOEX ZX2 modulator (Weinert et al., 2015). The GC × GC-MS raw data files were then processed by non-targeted alignment using in-house developed R-modules (Egert, Weinert, & Kulling, 2015). By means of regularly injected QC samples, signal intensity drift, i.e., intra- and interbatch effects occurring during the measurement period, were corrected. With this method, a wide range of metabolites, e.g., amino acids (AAs), amines, organic acids, sugars, sugar alcohols, or polyols, could be detected.

Targeted gas chromatography (GC)-MS analysis of fatty acids. The chromatographic separation of plasma fatty acids usually requires the application of specialized polar columns and can thus not be conducted adequately by using a standard apolar × medium-polar GC × GC column setup. Therefore, a previously described method by Ecker et al. (2012) to detect plasma fatty acids as methyl esters was applied with minor modifications. By using a GC single quadrupole instrument (Shimadzu GCMS QP2010 Ultra) and a BPX90 column (Trajan Scientific), 48 fatty acids were finally determined in a quantitative manner.

Liquid chromatography (LC)-MS metabolite profiling using the Absolute IDQ™ p180 kit. Plasma samples were also utilized for targeted metabolite profiling using the Absolute IDQ™ p180 kit developed by Biocrates AG (Innsbruck, Austria). The general preparation and quantification procedure has already been described (Römisch-Margl et al., 2012). For the chromatographic separation of AAs and biogenic amines, a Zorbax Eclipse XDB-C18 column (3 × 100 mm, 3.5 µm; Agilent, Waldbronn, Germany) equipped with a SecurityGuard™ column (C18, 4.0 × 3.0 mm; Phenomenex, Aschaffenburg, Germany) was used. PC and sphingomyelin (SM) species were analyzed by flow injection analysis into the analytical system, comprising a Nexera UHPLC system (Shimadzu) coupled to an API QTRAP15500 mass spectrometer (AB

Sciex, Darmstadt, Germany). With this method, a variety of acylcarnitines, AAs, biogenic amines, SMs, and PCs were detected. While lyso-phosphatidylcholine (lysoPC) species always have one acyl-bound fatty acid, other included PC species are characterized by two acyl-bounds (PC aa) or one acyl- and one alkyl-bound (PC ae) fatty acid, respectively. In general, each analyzed PC represents a sum parameter of different PC species with identical residue sums (e.g., PC ae C32:1 may consist of PC ae C16:0/C16:1, PC ae C18:0/C14:1, etc.).

Targeted LC-MS analysis of methylated amino compounds. The quantification of six amino compounds in plasma was conducted by ultra-performance liquid chromatography-tandem MS, using an Acquity H-Class UPLC coupled to a Xevo TQD triple quadrupole MS (both from Waters, Eschborn, Germany), as previously established (Weinert et al., 2017). Plasma samples were diluted with acetonitrile after protein precipitation and separated by an inverse acetonitrile gradient on a polar hydrophilic interaction liquid chromatography column (Acquity BEH Amide, Waters, Eschborn, Germany). Target analytes, as well as deuterated internal standards, were monitored by positive electrospray ionization in multiple reaction monitoring mode.

Targeted LC-MS analysis of bile acids. 14 bile acids in plasma samples were quantified using a 1200 series HPLC system (Agilent, Waldbronn, Germany) coupled to a Q-Trap 3200 MS (AB Sciex, Darmstadt, Germany), as described in Frommherz et al. (2016).

Non-targeted NMR analysis. Plasma samples were analyzed by 1D-^{1}H-NMR spectroscopy. Briefly, they were measured at 310 K on an AVANCE II 600 MHz NMR spectrometer equipped with a 1H-BBI probe head and a BACS sample changer (Bruker BioSpin GmbH, Rheinstetten, Germany). All obtained plasma spectra were automatically phased with the Bruker AU program apk0.noe. Using the program AMIX 3.9.14 (Bruker BioSpin GmbH, Rheinstetten, Germany), they were then referenced to the EDTA signal and bucketed graphically, so that buckets contained only one signal or group of signals and no peaks were split between buckets, whenever possible. Buckets were either annotated to a previously known and identified analyte, or registered as unknown. The identification of metabolites was carried out with Chenomx NMR Suite 8.1 (Chenomx, Edmonton, Canada). The detected analytes included organic acids, AAs, amines, and sugar alcohols.

7.3.7 Data Handling and Statistical Analysis

The data from the different analytical platforms were integrated into a common data matrix, consisting of 301 samples and 657 plasma analytes. With respect to the study participants, we excluded 49 individuals due to missing spiroergometry data (n=40), technical errors during analyses (n=7), implausibly low HR_{rest} data (n=1), and a missing plasma sample (n=1). If identified metabolites were measured by more than one of the analytical platforms, those metabolites that were detected by the less quantitative method were excluded (n=149). Further analytes were deleted if they had a detected frequency lower than 20% in either the female or male sub-group (n=81). Thus, the final data matrix contained 252 individuals and 427 plasma analytes. Prior to statistical analyses, metabolite data were transformed into Van der Waerden (VdW) scores. By using this rank-based inverse normal transformation, the data were converted into ranks, transformed to a scale between 0 and 1, and then, the corresponding standard normal quantiles were calculated. This transformation took the issue of values below the limit of detection into account and led to a uniform scale for all analytes, i.e., they were finally comparable between analytical platforms.

Based on sex-specific VO_{2peak} quartiles, the sex-specific VO_{2peak} data were divided into four quarters (q), and differences in basic characteristics between the sub-groups of the corresponding participants of the quarters were examined by Welch ANOVA (chi-squared test) for numeric (categorical) variables. All subsequent statistical analyses were conducted separately for sexes and the non-modifiable factors age (and menopausal status in females) were treated as confounders. Random forest regression algorithms considering age, phenotypical, and clinical variables were applied to impute missing values for AEE and PWV. Similar to the metabolite data, both the VO_{2peak} and considered phenotypical and clinical parameters were transformed into VdW scores prior to statistical analyses. To examine the sex-specific relationship between the VO_{2peak} and selected phenotypical and clinical parameters, Pearson correlations adjusted for age (and menopausal status in females) were calculated. Correlations were considered statistically significant when the 95% confidence intervals (CIs) did not include zero.

Regarding metabolomics data analysis in the sex-specific sub-groups, three major aims were pursued: (i) to investigate the relationship between the VO_{2peak} and single plasma metabolites

(bivariate association analyses), (ii) to determine the relationship between the VO_{2peak} and all plasma metabolites simultaneously (multivariate association analyses), and (iii) to identify a set of plasma metabolites possibly improving the explanation of the VO_{2peak} in the presence of phenotypical and clinical data (multiple regression analyses).

(i) Bivariate association analyses

To examine the sex-specific relationship between the VO_{2peak} and single plasma metabolites, adjusted for age and menopausal status, partial Pearson correlation coefficients (*r*) with 95% CIs were calculated. In a second step, correlations independent of further phenotypical and clinical variables were assessed. More specifically, we performed sex-specific correlation analyses by adjusting not only for the above-mentioned confounders but also for the following phenotypical and clinical parameters: LBM, FM (%), VATM, BMC, height, Hb, glucose, insulin, HbA1c, TGs, HDL and LDL cholesterol, HR_{rest}, systolic and diastolic BP, PWV, VC_{max}, FEV1, AEE, total MET, and HEI-NVS.

(ii) Multivariate association analyses

To analyze the relationship between the VO_{2peak} and all 427 plasma metabolite variables simultaneously, PLS regression analyses using nested cross-validation were conducted separately for women and men. The PLS analyses were either applied on confounder-adjusted metabolite variables or on metabolite variables additionally adjusted for the above-listed phenotypical and clinical parameters. The outer loop contained 20 random splits in a calibration dataset (containing 80% of all samples) and a test dataset (containing the remaining 20% of all samples). The data were preprocessed, including the formation of VdW scores, respective adjustments, and unit variance scaling based on the calibration data. As the inner loop, a single random eight-fold cross-validation was used to tune the PLS model, based on the root mean square error (RMSE). Thereby, the number of predictive components was restricted to being at most ten. A rank for the obtained PLS regression models was assigned to each metabolite variable according to the negative absolute value of its regression coefficient. By calculating the geometric means of the ranks across the 20 random splits, a final rank product for each metabolite variable was obtained. The model performance was evaluated by the mean of RMSEs on the test samples across the 20 random splits. Moreover, 2500 permutations of the VO_{2peak} values were run, and the relative frequency of permutation-

obtained rank products below the previously calculated rank products was assessed. If the relative frequency was < 0.05, the contribution of a metabolite variable to the multivariate association with the VO_{2peak} was considered significant.

(iii) Multiple linear regression analyses

To assess the relationship between the VO_{2peak} and sets of phenotypical, clinical, and metabolite variables, three different exploratory multiple linear regression models were calculated for each sex, with the confounder-adjusted VO_{2peak} as the dependent variable, and with stepwise forward-selected confounder-adjusted clinical, phenotype, and metabolite variables as independent variables. In detail, the following approaches were applied:

- Approach 1: Only phenotypical/clinical variables (n=21) were stepwise selected.
- Approach 2: All phenotypical/clinical variables (n=21) were included and only plasma metabolite variables (n=427) were stepwise selected.
- Approach 3: Phenotypical/clinical variables (n=21) as well as plasma metabolite variables (n=427) were stepwise selected.

While in approach 2, all phenotypical/clinical variables entered the model as fixed variables before considering the plasma metabolites, in approach 3 all variables entered the model in a competing manner. To obtain a ranking of confounder-adjusted phenotypical, clinical, or metabolite variables according to their contribution for explaining the adjusted VO_{2peak}, the models were built by maximizing the coefficients of determination (R^2).

In addition to the ranking of variables, a single linear multiple regression model was calculated for each of the approaches 1 to 3 in order to obtain a manageable number of variables that, in combination, explained the CRF. For variable selection, the previously described stepwise multiple linear regression analyses were performed on a calibration dataset (containing 80% of the samples) and the predictive accuracy of each step was assessed on the test dataset (containing the remaining 20% of the samples). The selection was stopped if the predictive accuracy decreased for the first time. In total, the analysis was repeated 1000 times with random assignments of samples into calibration and test datasets. Finally, the number of times each variable was present in those cross-validated stepwise regression models was counted (a relative frequency of 1 means that the variable was always considered in stepwise variable selection). All variables with a relative frequency ≥ 0.05 were then included in a final

model with respect to approaches 1 to 3. The obtained final models were described by the adjusted coefficient of determination (R^2 (adjusted)) and compared within each sub-population. Statistical analysis was performed by using SAS JMP 11.0.0 (SAS Institute Inc. 2013, Cary, NC, USA) and the software R Version 4.0.0 (R Core Team, 2020), using the packages named caret (Kuhn, 2020), openxlsx (Schauberger & Walker, 2020), and missForest (Stekhoven, 2013). Figures were generated in Excel 2016 or R, using the packages named ggplot2 (Wickham, 2016), ggrepel (Slowikowski, 2020b), and ggpubr (Kassambara, 2020).

7.3.8 Metabolite Classification

For the biological interpretation of CRF-related metabolite profiles, identified and putatively annotated metabolites, i.e., compounds with the Metabolomics Standards Initiative (MSI)-level 1 or 2 (Sumner et al., 2007), respectively, were manually assigned to 8 major and 32 specific pathways of human metabolism based on the information provided by the Human Metabolome Database (HMDB), Version 4.0 (Wishart et al., 2018) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY database (Aoki & Kanehisa, 2005).

Annotation of analytes detected using untargeted approaches, showing relevant associations with the CRF, was performed as follows: spectra of GC × GC-MS analytes were matched against an in-house spectral library as well as against the FiehnLib and the NIST17 libraries. If matching was unsuccessful, structural hypotheses were derived depending on the presence of known diagnostic fragments (see Table S9.2 in Ulaszewska et al. (2019)) and additionally, especially in the case of sugars and sugar-like compounds, based on the compound's position in the two-dimensional chromatogram. Unfortunately, the spectra of CRF-related unknown analytes were mostly unspecific, which hampered structural elucidation. In the case of NMR, the identification of CRF-associated unknown analytes was not possible because the particular buckets contained either unspecific signals or overlapped peaks.

7.4 Results

7.4.1 Metabolomics Data

427 plasma analytes were included in the final data analysis. Untargeted methods yielded 234 analytes, of which 43 (18.4%) could be identified with sufficient certainty. 193 analytes were derived from targeted analyses and were thus known a priori. Of the 236 identified

metabolites, most belonged to 'lipid metabolism' (69.5%) and 'AA metabolism' (17.4%), followed by 'xenobiotics-related metabolism' (5.1%), 'mammalian-microbial cometabolism' (3.0%), 'carbohydrate metabolism' or 'energy metabolism' (each 2.1%), and 'nucleotide metabolism' or 'cofactors and vitamins metabolism' (each 0.4%).

7.4.2 Basic Characteristics of Study Participants

The study sample consisted of 252 healthy individuals, 150 males (M, 59.5%) and 102 females (F, 40.5%), with a mean age of 45.9 ± 17.1 years and a mean VO_{2peak} of 38.9 ± 11.7 mL kg^{-1} min^{-1}. The characteristics of participants are presented according to sex-specific VO_{2peak} quarters, see Figure 28. In the radar plots, the respective means of the lowest VO_{2peak} quarter (1st q) were used as a reference value and the means of the other quarters (2nd q, 3rd q, 4th q) were related to the means of the first quarter. The absolute means, standard deviations, and respective units are provided in Table A-8 and Table A-9 (Appendix A2.9).

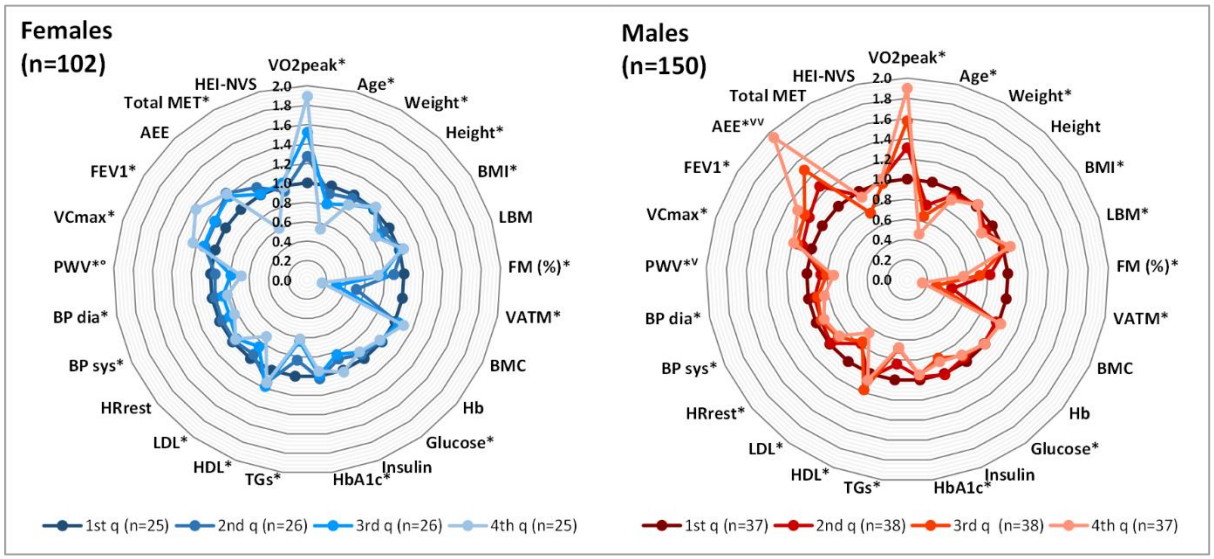


Figure 28. Radar plots visualizing the basic characteristics of KarMeN participants according to sex-specific VO_{2peak} quarters. The means of the 1st q were used as reference values to the means of the 2nd, 3rd, and 4th q. *: significant differences between quarters according to Welch ANOVA. °: n=23 (1st q), n=25 (2nd q); v: n=36 (1st q); vv: n=35 (1st q), n=37 (2nd q). AEE: activity energy expenditure; BMC: bone mineral content; BMI: body mass index; BP: blood pressure; dia: diastolic; FEV1: forced expiratory pressure in one second; FM: fat mass; Hb: hemoglobin; HDL: high-density lipoprotein; HEI-NVS: Healthy Eating Index (modified version); HR_{rest}: resting heart rate; LBM: lean body mass; LDL: low-density lipoprotein; MET: metabolic equivalent of task; n: sample size; PWV: pulse wave velocity; q: quarter; sys: systolic; TGs: triglycerides; VATM: visceral adipose tissue mass; VC_{max}: maximal vital capacity; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2021))*.

In both females and males, there were statistically significant differences between VO_{2peak}-related quarters with regard to age, weight, BMI, FM (%), and VATM. Furthermore, differences for clinical parameters like fasting blood glucose, HbA1c, TGs, HDL and LDL cholesterol, systolic and diastolic BP, PWV, VC_{max}, and FEV1 were observed across the quarters of the sex-specific

VO_{2peak}. In females, additional differences between VO_{2peak}-related quarters could be observed for height and total MET, while in males, differences were detected for LBM, HR_{rest}, and AEE. The menopausal status in women also significantly differed between VO_{2peak} quarters, with increasing ratios of pre- to post-menopausal women from the 1st q to the 4th q, see Table A-8 (Appendix A2.9). As the non-modifiable factors age and menopausal status were associated with the sex-specific VO_{2peak}, and since they have already been shown to determine plasma metabolite patterns in KarMeN subjects (Rist et al., 2017), these variables were treated as confounders in all subsequent analyses.

7.4.3 Sex-Specific Relationship between the CRF and Phenotypical/Clinical Variables

To examine sex-specific relations between the VO_{2peak} and selected phenotypical as well as clinical variables, correlations adjusted for age (and menopausal status in females) were calculated. A visual comparison of the pairwise correlations in women and men is provided in Figure 29. After adjustments for the above-mentioned confounding factors, the VO_{2peak} in females showed positive correlations with HDL cholesterol (r = 0.43) and negative correlations with the FM (%) (r = −0.61), VATM (r = −0.44), PWV (r = −0.38), and TGs (r = −0.33), that were

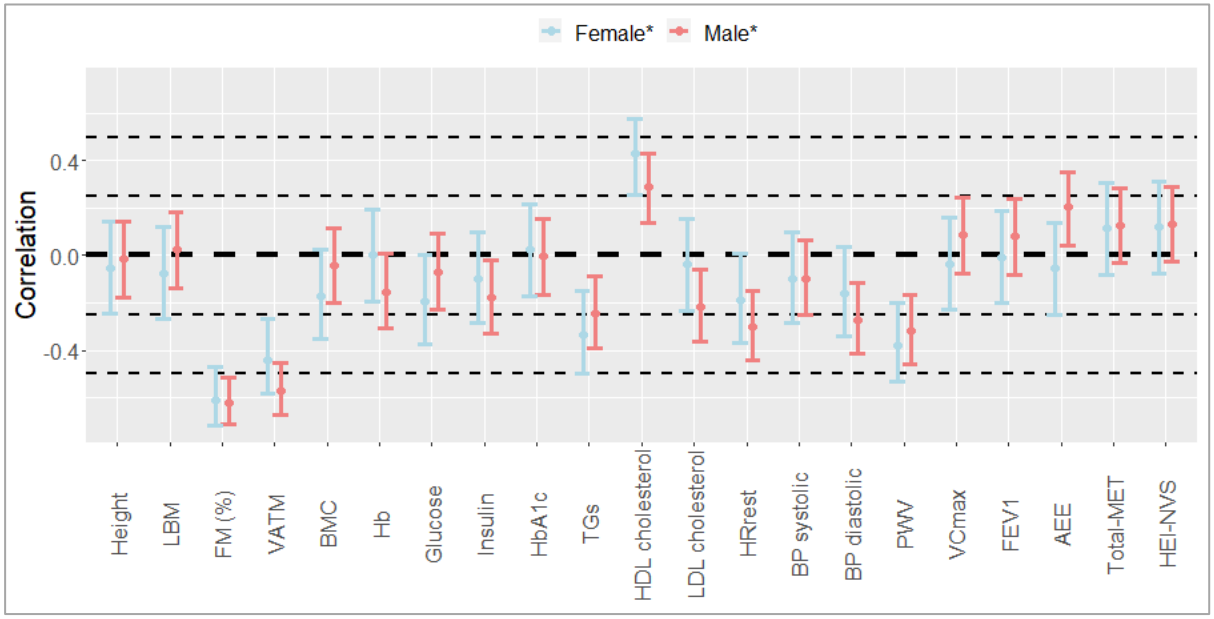


Figure 29. Confounder-adjusted sex-specific correlations between the VO_{2peak} and phenotypical/clinical variables. * Pearson correlations were performed on VdW-transformed data adjusted for age (and menopausal status in females) and respective correlation coefficients (r; dots) and 95% confidence intervals (CIs; bars) are illustrated. AEE: activity energy expenditure; BMC: bone mineral content; BP: blood pressure; FEV1: forced expiratory pressure in one second; FM: fat mass; Hb: hemoglobin; HDL: high-density lipoprotein; HEI-NVS: Healthy Eating Index (modified version); HR_{rest}: resting heart rate; LBM: lean body mass; LDL: low-density lipoprotein; MET: metabolic equivalent of task; PWV: pulse wave velocity; TGs: triglycerides; VATM: visceral adipose tissue mass; VC_{max}: maximal vital capacity; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2021))*.

all significantly different from zero. In males, not only HDL cholesterol (r = 0.29) but also the AEE (r = 0.20) showed positive correlations with the VO_{2peak}. Compared to females, negative correlations to the VO_{2peak} that were significantly different from zero were not only detected for FM (%) (r = −0.62), VATM (r = −0.57), PWV (r = −0.32), and TGs (r = −0.25) but additionally for HR_{rest} (r = −0.30), diastolic BP (r = −0.27), LDL cholesterol (r = −0.22), and insulin (r = −0.18).

7.4.4 Sex-Specific Relationship between the CRF and Plasma Metabolites

Bivariate Association Analyses

Correlation coefficients were calculated for the associations between the VO_{2peak} and plasma metabolites, with adjustments for known confounders, i.e., age and menopausal status (*), and additionally for phenotypical and clinical variables (**). While a graphical overview of all sex-specific bivariate correlations is provided in Figure A-13 (Appendix A2.10), correlation coefficients and the upper and lower levels of 95% CIs of significantly correlating metabolites are provided in Table A-10 to Table A-13 (Appendix A2.11). In Table 16, the number of metabolites with significant correlations to the VO_{2peak} is shown, along with their categorization to major metabolic pathways. The classification of VO_{2peak}-correlated plasma metabolites to both major and specific metabolic pathways is moreover visualized in pie charts, see Figure A-14 to Figure A-17 (Appendix A2.12) for confounder-adjusted findings and Figure 36, Figure 37, Figure 39, and Figure 40 (Chapter 8.3) for confounder- and phenotypical/ clinical variables-adjusted findings.

Table 16. Number of detected plasma metabolites, according to major metabolic pathways, and number of metabolites significantly correlating with the VO_{2peak}, shown separately by sex. *(Adapted from Kistner et al. (2021))*.

Metabolic Pathway	Total Number of Plasma Metabolites	Number of Plasma Metabolites Correlating with the VO_{2peak}			
		Females (n=102)		Males (n=150)	
		*	**	*	**
All	427	125	59	112	24
Lipid metabolism	164	63	27	36	8
Amino acid metabolism	41	4	6	8	2
Xenobiotics and related metabolism	12	2	2	4	1
Mammalian-microbial cometabolism	7	2	0	1	0
Carbohydrate metabolism	5	2	1	1	0
Energy metabolism	5	3	1	2	0
Cofactors and vitamins metabolism	1	1	0	0	0
Nucleotide metabolism	1	0	0	0	0
Unknown	191	48	22	60	13

* confounder (age/menopausal status)-adjusted; ** additionally adjusted for 21 phenotypical/clinical variables; n: sample size; VO_{2peak}: peak oxygen uptake.

Confounder-adjusted correlation analyses revealed that 125 metabolites in females and 112 metabolites in males showed correlations with the VO_{2peak} that were significantly different from zero. Overall, only a limited number of common correlations were observed between sexes, and generally stronger correlations could be found in the female sub-group. In women, 79 plasma metabolites were positively correlated with the VO_{2peak}, among them 21 acyl-alkyl-phosphatidylcholine (PC ae) species (C44:3, C34:3, C42:4, C42:3, C42:2, C34:2, C40:3, C36:2, C44:6, C42:5, C36:3, C44:4, C42:1, C44:5, C40:5, C38:2, C40:4, C32:1, C32:2, C30:0, C40:1), 11 diacyl-phosphatidylcholine (PC aa) species (C42:2, C34:2, C36:2, C40:2, C42:0, C40:3, C42:4, C42:1, C28:1, C42:5, C36:3), SM C16:0, lysoPC C18:2, two acylcarnitines (C14:2, C10:2), the long-chain fatty acid (LCFA) C24:0, citrate, glyceric acid, acetate, and two unknown analytes, all demonstrating an $r \geq 0.25$. The majority of the 46 negatively correlated metabolites in females were unknown, except for two LCFAs (C16:1 9cis, C18:1 11cis) with an $r \leq -0.25$. In males, 33 plasma metabolites showed positive correlations with the VO_{2peak}, including three lysoPCs (C18:2, C18:1, C17:0) and three unknown analytes with an $r \geq 0.25$. Similar to females, most of the 79 negatively correlated metabolites in males remained unknown. Only two SMs (C18:0, C18:1) and diacyl-PC C40:6 with an $r \leq -0.25$ could be identified. Overall, PCs largely showed weak to moderate positive correlations in females, whereas most PCs in males were either not or slightly negatively linked to the VO_{2peak}. Significant bivariate correlations with the same directions in both sexes were observed for lysoPC C18:2 (F: $r = 0.30$; M: $r = 0.34$), glyceric acid (F: $r = 0.29$; M: $r = 0.21$), acetate (F: $r = 0.26$; M: $r = 0.19$), succinic acid (F: $r = 0.24$; M: $r = 0.20$), malic acid (F: $r = 0.21$; M: $r = 0.22$), and the LCFA C16:1 9cis (F: $r = -0.32$; M: $r = -0.20$), in addition to several unknown analytes with mainly negative correlations to the VO_{2peak} in both women and men, see Figure A-13 (Appendix A2.10).

After additionally adjusting for phenotypical and clinical variables, 59 metabolites ($0.20 \leq |r| \leq 0.39$) in females and 24 metabolites ($0.16 \leq |r| \leq 0.25$) in males still exhibited weak to moderate correlations with the VO_{2peak} that were significantly different from zero. The majority of the VO_{2peak}-related plasma metabolites in both females and males belonged to ‘lipid metabolism’, followed by ‘AA metabolism’ and ‘xenobiotics and related metabolism’. However, only a few correlations with the same directions in both sexes were detected (e.g., U1.156). The top 10 of sex-specific positive and negative partial correlations between the VO_{2peak} and plasma metabolites are summarized in Table 17.

Table 17. Top 10 of sex-specific partial correlations between the VO_{2peak} and plasma metabolites. *(Adapted from Kistner et al. (2021)).*

	Positive Correlations		Negative Correlations	
	Metabolites	***r* (95% CIs)**	**Metabolites**	***r* (95% CIs)**
Females				
1	PC ae C40:3	**0.37 (0.19; 0.53)**	U3.961	**−0.39 (−0.55; −0.22)**
2	PC ae C42:4	**0.31 (0.13; 0.48)**	U3.956	**−0.32 (−0.49; −0.14)**
3	C5-carnitine	**0.31 (0.12; 0.47)**	U3.950	**−0.31 (−0.48; −0.12)**
4	PC ae C38:3	**0.28 (0.09; 0.45)**	U4.252	**−0.31 (−0.47; −0.12)**
5	Choline	**0.27 (0.08; 0.44)**	U3.971	**−0.31 (−0.47; −0.12)**
6	Glyceric acid	**0.27 (0.08; 0.44)**	U0978	**−0.30 (−0.46; −0.11)**
7	U0856	**0.27 (0.08; 0.44)**	U0975	**−0.28 (−0.45; −0.09)**
8	PC ae C36:2	**0.26 (0.07; 0.44)**	U2.656	**−0.28 (−0.45; −0.09)**
9	Acetylornithine	**0.26 (0.07; 0.43)**	U1.156	**−0.28 (−0.45; −0.09)**
10	PC ae C44:3	**0.26 (0.07; 0.43)**	U3.060	**−0.26 (−0.43; −0.07)**
Males				
1	U0130	0.19 (0.03; 0.34)	U2.250	**−0.25 (−0.39; −0.09)**
2	Alanine	0.18 (0.02; 0.33)	U2.822	−0.21 (−0.36; −0.05)
3	C6 (C4:1-DC)-carnitine	0.18 (0.02; 0.33)	U(Sugar-like 4)	−0.21 (−0.36; −0.05)
4	U2.910	0.18 (0.02; 0.33)	U1331	−0.21 (−0.36; −0.05)
5	PC aa C36:3	0.18 (0.02; 0.33)	Tartaric acid	−0.21 (−0.36; −0.05)
6	U3.385	0.17 (0.01; 0.32)	U1.156	−0.20 (−0.35; −0.04)
7	Glutamate	0.17 (0.01; 0.32)	U0.936	−0.19 (−0.34; −0.03)
8	-	-	U1.159	−0.19 (−0.34; −0.03)
9	-	-	U1.166	−0.19 (−0.34; −0.03)
10	-	-	PC ae C38:6	−0.18 (−0.33; −0.02)

Pearson correlations were performed on VdW-transformed data adjusted for age, menopausal status, and phenotypical/ clinical variables. Results of partial correlations are presented as Pearson correlation coefficients (*r*) and the lower and upper limit of the 95% confidence intervals (CIs), rounded to two decimal places. $|r| \geq 0.25$ are indicated in bold. PC aa: diacyl-phosphatidylcholine; PC ae: acyl-alkyl-phosphatidylcholine; U: unknown; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake. For unknown NMR-analytes, the chemical shift of the lower bucket border is indicated in ppm.

In females, the top 10 positively correlated plasma metabolites included five acyl-alkyl-PCs (C40:3, C42:4, C38:3, C36:2, C44:3), acylcarnitine C5, choline, glyceric acid, and acetylornithine, while the top 10 negatively correlated plasma metabolites were unknown analytes. In males, only seven plasma metabolites were positively correlated with the VO_{2peak}, namely two AAs (alanine, glutamate), acylcarnitine C6 (C4:1-DC), diacyl-PC C36:3, and three unknown analytes. The top 10 negatively correlated plasma metabolites in males comprised eight unknown analytes, the xenobiotic tartaric acid, and acyl-alkyl-PC C38:6. Sex-related differences in the direction of the relations were obvious for tartaric acid (F: $r = 0.23$; M: $r = -0.21$), diacyl-PC C42:1 (F: $r = 0.23$; M: $r = -0.18$), U2.250 (F: $r = 0.11$; M: $r = -0.25$), C5-carnitine (F: $r = 0.31$; M: $r = -0.05$), and alanine (F: $r = -0.19$; M: $r = 0.18$). While several PCs still showed weak to moderate positive correlations in females, single PCs in males tended to be slightly negatively linked to the VO_{2peak}, see Figure A-13 (Appendix A2.10).

Multivariate Association Analyses

The multivariate association between the VO_{2peak} and all 427 plasma analytes was assessed based on rank products obtained in cross-validated PLS models. Rank products were calculated by the geometric mean of the ranks of the regression coefficients of each metabolite in PLS models, across 20 random splits. For each metabolite variable, the importance of its contribution to the multivariate association was determined by permutation tests. A graphical overview of all sex-specific associations is provided in Figure A-13 (Appendix A2.10). Regarding females, PLS analysis showed that metabolites highly contributing to the confounder-adjusted multivariate association with the CRF included several diacyl- and acyl-alkyl-PCs, the LCFA C16:1 9cis, as well as a number of unknowns. In males, metabolites with high contributions to the multivariate association comprised specific lysoPCs (C18:2, C18:1, C17:0), SMs (C18:0, C18:1), and diacyl-PC C40:6 next to unidentified analytes, when adjusting for age. As opposed to confounder-adjusted multivariate association analyses, the mean of the RMSEs based on the test samples was not always higher in the permutations than by using the original data, if additionally controlling for phenotypical and clinical variables, see Figure A-18 (Appendix A2.13). Consequently, the multivariate relationship between the CRF and all 427 plasma analytes lost importance after applying additional adjustments.

To visualize the sex-specific association patterns of plasma metabolites, the results of both bi- and multivariate association analyses were combined in a volcano plot, see Figure 30 for confounder-adjusted findings (*) and Figure A-19 (Appendix 2.14) for confounder- and phenotypical/clinical variables-adjusted findings (**)). In the upper right and left corners, metabolites with moderate ($|r| \geq 0.25$) bivariate correlations and significant contributions to multivariate associations are detectable. Metabolites in the upper middle region showed weak ($|r| \leq 0.25$) bivariate correlations but significant contributions to multivariate associations, i.e., their relationship with the VO_{2peak} depended on all other considered analytes. In contrast, the lower right and left corners include metabolites with moderate bivariate correlations but no relevant contributions to multivariate associations, i.e., their relationship with the VO_{2peak} lost relevance if other metabolite variables with possibly redundant information were taken into account. For a subsequent metabolic interpretation of CRF-related metabolite patterns, plasma metabolites with relevant bivariate correlations ($|r| \geq 0.25$) or significant contributions to multivariate associations were considered.

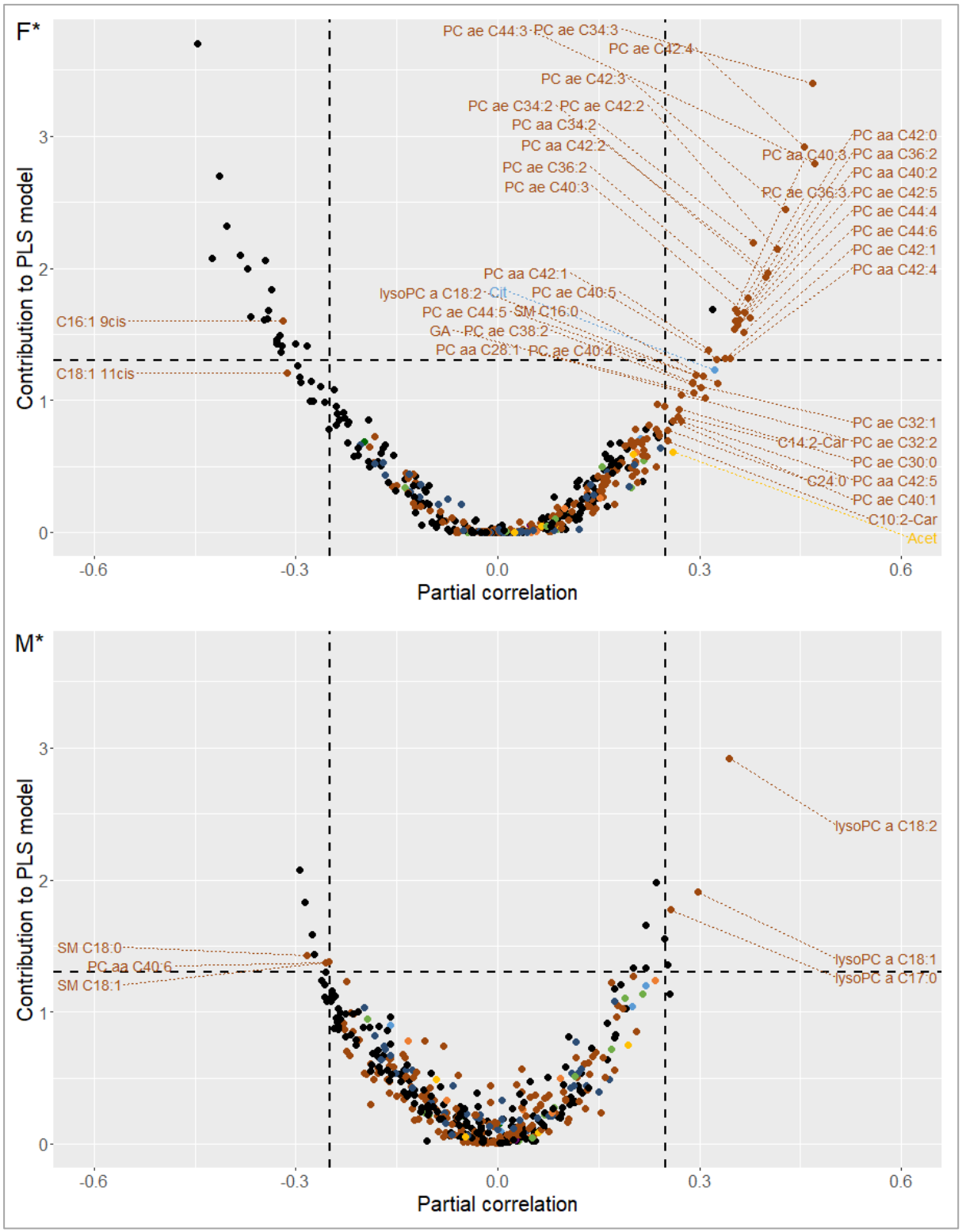


Figure 30. Volcano plots illustrating sex-specific plasma metabolite patterns associated with the VO_{2peak}. F: females; M: males; * confounder (age/menopausal status)-adjusted; Y-axis: significance of contribution of each metabolite variable to the PLS model, expressed as the negative logarithm of the relative frequencies of permutation-obtained rank products below measured rank products; X-axis: direction and strength of partial correlations between the VO_{2peak} and metabolite variables, expressed as Pearson correlation coefficients (*r*) of VdW-transformed variables. The classification of metabolites to metabolic pathways is color-coded as follows: 'amino acid metabolism' (dark blue); 'carbohydrate metabolism' (yellow); 'cofactors and vitamins metabolism' (dark green); 'energy metabolism' (light blue); 'lipid metabolism' (brown); 'mammalian-microbial cometabolism' (orange); 'nucleotide metabolism' (purple); 'xenobiotics and related metabolism' (light green); 'unknown' (black). PLS: partial least squares; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake. *(Adapted from Kistner et al. (2021))*.

Multiple Regression Analyses

To additionally investigate the sex-specific relationship between the VO_{2peak} and selected metabolite variables in the presence of phenotypical and clinical data, multiple linear regression analyses were performed. By adjusting all included variables for age and menopausal status, the examined associations were independent of these non-modifiable variables. As described in the methods section, sex-specific models were calculated based on three different sets of phenotypical, clinical, and metabolite variables. Results of the cross-validated stepwise regression analyses are presented in Table A-14 to Table A-19 (Appendix A2.15).

Finally, the most suitable combination of phenotypical, clinical, and metabolite variables for a preferably good explanation of the VO_{2peak} was selected. The included variables ("approach 1–3 selection") and R^2 (adjusted) for the evaluation of the sex-specific final models are summarized in Table 18. With regard to approach 1 selection, seven phenotypical or clinical variables were selected for the final model of females, resulting in an R^2 (adjusted) of 0.40. For the final model of males, six phenotypical or clinical variables were selected, leading to an R^2 (adjusted) of 0.43. When including all 21 phenotypical and clinical variables, an R^2 (adjusted) of 0.36 for women and an R^2 (adjusted) of 0.39 for men was obtained, see Table A-14 or Table A-15 (Appendix A2.15), respectively.

Table 18. Summary of sex-specific final models for the confounder-adjusted VO_{2peak}. *(Adapted from Kistner et al. (2021))*.

Model	Females (n=102)	Males (n=150)
Approach 1 Selection	**R^2 (adjusted) = 0.40** FM (%), HDL cholesterol, LBM, PWV, Hb, BP systolic, BP diastolic	**R^2 (adjusted) = 0.43** FM (%), HDL cholesterol, BMC, AEE, TGs, LDL cholesterol
Approach 2 Selection	**R^2 (adjusted) = 0.72** All phenotypical/clinical variables + *PC ae C40:3, U3.961, S-Methylcysteine, Tartaric acid, U1.148, Serine, C24:0, Kynurenine, U0992*	**R^2 (adjusted) = 0.62** All phenotypical/clinical variables + *PC aa C36:3, U0130, Tartaric acid, C6 (C4:1-DC)-Carnitine, C14:1-OH-carnitine, U2.250, Malic acid, Glutamate, C24:0, U1.226*
Approach 3 Selection	**R^2 (adjusted) = 0.68** FM (%), *PC ae C40:3, myo-Inositol, U0975, U3.961, U7.294, Glycine, U2.313, Lysine, C18:1-carnitine*	**R^2 (adjusted) = 0.59** FM (%), *Malic acid, Taurocholate, PC aa C36:3, U0130, PC aa C36:6, Glutamate, U(Similar to Uracil), U1.226*

Variables were selected based on the results of the stepwise regression analyses and included in sex-specific final models. All variables were VdW-transformed and adjusted for age (and menopausal status in females). Selected metabolite variables are indicated in italics. Approach 1: only phenotypical/clinical variables (n=21) were stepwise selected; approach 2: all phenotypical/clinical variables (n=21) were included and only plasma metabolite variables (n=427) were stepwise selected; approach 3: phenotypical/clinical variables (n=21) as well as plasma metabolite variables (n=427) were stepwise selected. AEE: activity energy expenditure; BMC: bone mineral content; BP: blood pressure; FM: fat mass; Hb: hemoglobin; HDL: high-density lipoprotein; LBM: lean body mass; LDL: low-density lipoprotein; n: sample size; PC aa: diacyl-phosphatidylcholine; PC ae: acyl-alkyl-phosphatidylcholine; PWV: pulse wave velocity; R^2 (adjusted): adjusted coefficient of determination; TGs: triglycerides; U: unknown; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

With approach 2, the added value of plasma metabolites for explaining the confounder-adjusted VO_{2peak} in the presence of all 21 phenotypical and clinical variables was considered. Nine or ten metabolites, respectively, were additionally selected for the final models, leading to a comparatively higher performance in both sexes (F: R^2 (adjusted) = 0.72; M: R^2 (adjusted) = 0.62). Contrary to approach 2, phenotypical and clinical parameters as well as metabolite variables entered the model in a competing stepwise manner in approach 3. Actually, FM (%) was the only phenotypical/clinical variable being included in the final models of both sexes. In females, nine plasma analytes were additionally selected, resulting in an R^2 (adjusted) of 0.68. In males, eight plasma analytes completed the model, which finally showed an R^2 (adjusted) of 0.59. In summary, approach 2 as well as approach 3 selection demonstrated an improved performance in both sexes, compared to the initial models solely based on phenotypical and clinical variables. While the acyl-alkyl-PC C40:3 was present in the females' final models for both approaches 2 and 3, diacyl-PC C36:3, malic acid, as well as glutamate, were selected for the final models of males.

7.5 Discussion

The major finding of our systematic association analyses is that the VO_{2peak} was related to sex-specific sets of plasma metabolites that primarily belong to 'lipid metabolism'. However, the observed correlations were rather moderate, and, independently of other clinical or phenotypical variables considered, only a small number of metabolites were significantly correlated with the CRF. Multiple regression analyses revealed that models explaining the sex-specific VO_{2peak} could be improved when including selected plasma metabolites in addition to clinical and phenotypical parameters. For a metabolic interpretation of CRF-related metabolite patterns, a graphical overview of identified metabolites with relevant bivariate correlations ($|r| \geq 0.25$) or significant contributions to multivariate associations with the CRF and their pathway classification is provided in Figure 31.

Apart from detecting bi- and multivariate associations between the CRF and plasma metabolites, which are discussed in the following sub-sections, we were also able to prove well-known relationships between the CRF and several health-related clinical or phenotypical variables (Zeiher et al., 2019) in the KarMeN population. After correcting for age and menopausal status, we confirmed previous studies showing that the CRF correlated negatively

with the FM (%) (Tobita, Kusaka, Ohtaki, & Hashizume, 2003), VATM (S. L. Wong et al., 2004), PWV (Fernberg, Fernström, & Hurtig-Wennlöf, 2017), and TGs (Carnethon, Gulati, & Greenland, 2005; S. Lee et al., 2005) and positively with HDL cholesterol (Carnethon et al., 2005; S. Lee et al., 2005) in both females and males. Equally consistent with the literature, but only present in men, were negative correlations of the age-adjusted VO_{2peak} with the HR_{rest} (Laukkanen et al., 2009), diastolic BP (Carnethon et al., 2005; Tobita et al., 2003), LDL cholesterol (Lakoski et al., 2011), and insulin (Laukkanen et al., 2009). In summary, our data provide evidence that even in a study sample consisting of metabolically healthy individuals and independent of age, individuals with a higher CRF generally demonstrated lower values of clinical parameters, some of which are recognized as traditional risk factors for chronic metabolic or cardiovascular diseases.

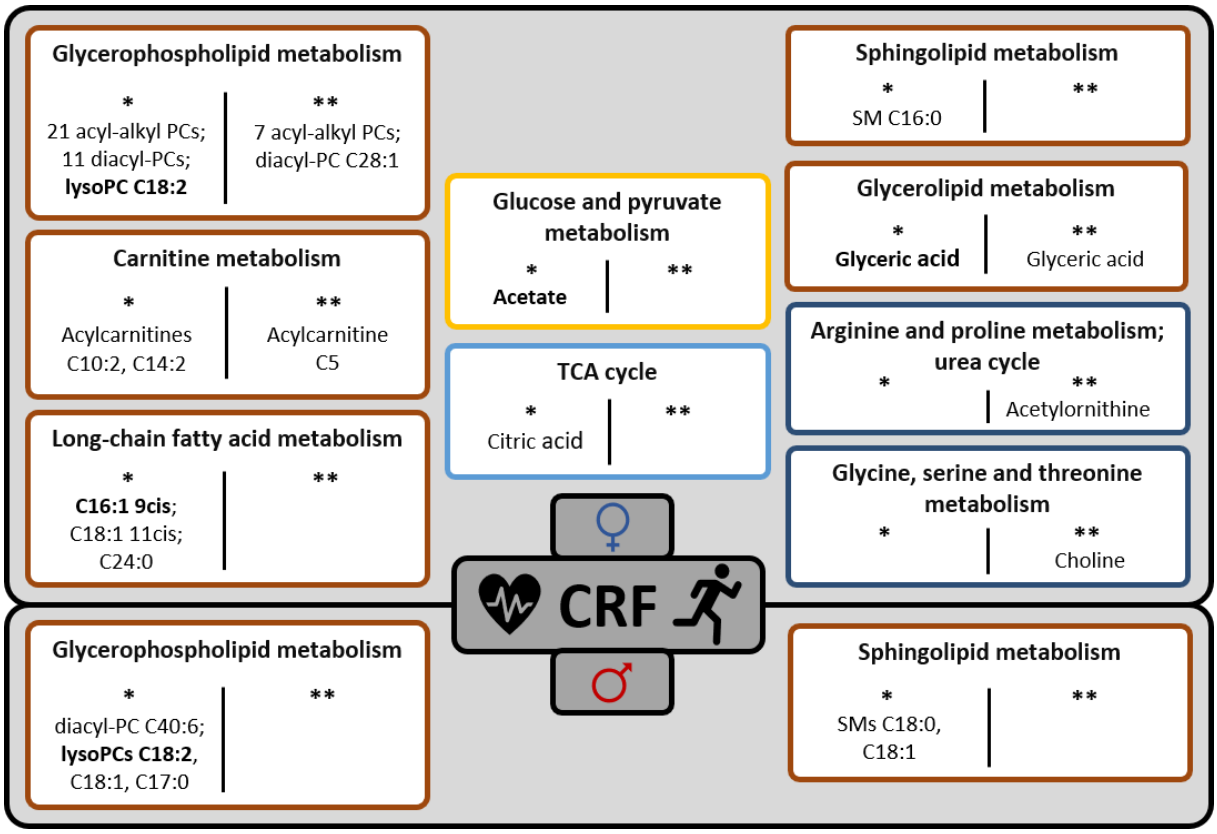


Figure 31. Classification of relevant CRF-associated plasma metabolites to major and specific metabolic pathways. Top: females; bottom: males. Major metabolic pathways are color-coded as follows: 'amino acid metabolism' (dark blue); 'carbohydrate metabolism' (yellow); 'energy metabolism' (light blue); 'lipid metabolism' (brown). * findings from confounder (age/menopausal status)-adjusted bi-/multivariate association analyses; ** findings from bivariate correlation analyses additionally adjusted for phenotypical/clinical variables. CRF: cardiorespiratory fitness; PC: phosphatidylcholine; SM: sphingomyelin; TCA: tricarboxylic acid. Metabolites with significant bivariate correlations to the CRF in both females and males are indicated in bold. *(Reprinted from Kistner et al. (2021))*.

7.5.1 Sex-Specific Plasma Metabolite Patterns Related to the CRF

Age- and Menopausal Status-Adjusted Findings

As shown by the results of age- and menopausal status-adjusted (*) correlation and PLS regression analyses, the CRF-related plasma metabolite pattern in females mainly comprised PCs, all of which were individually positively correlated with the VO_{2peak}. PCs represent the most abundant phospholipid component in cellular membranes and plasma lipoproteins, being important for cell integrity or the assembly and stability of lipoproteins (Cole, Vance, & Vance, 2011). Besides, acyl-alkyl-PCs were proposed as antioxidants preventing lipoprotein oxidation (Wallner & Schmitz, 2011). Previous studies reported lower levels of acyl-alkyl-PCs in obese or insulin-resistant individuals (Pietiläinen et al., 2007; Wallner & Schmitz, 2011) and showed that specific acyl-alkyl-PCs were related to higher HDL cholesterol and a lower risk for type 2 diabetes (Floegel, Stefan, et al., 2013). In line with our results, Wientzek et al. (2014) demonstrated age- and sex-adjusted positive associations between the CRF and several serum PCs in middle-aged adults. Likewise, higher plasma levels of four acyl-alkyl-PCs were observed in adults with a high CRF compared to less fit adults, when controlling for age and BMI (Morris et al., 2015). While those relationships seemed to be independent of sex, associations between the CRF and specific PCs in the KarMeN population were more pronounced in women.

With regard to men, age-adjusted association analyses revealed that the CRF-related plasma metabolite pattern was dominated by three lysoPCs (C18:2, C18:1, C17:0), all of which were positively linked to the VO_{2peak}, and two SMs (C18:0, C18:1), which were negatively linked to the VO_{2peak}. In fact, lysoPC C18:2 also showed a relevant positive correlation with the CRF in females. LysoPCs represent hydrolysis products from PCs, with relevant roles for cell signaling. As a major component of oxidized LDL, lysoPCs are also supposed to regulate the pathophysiological processes underlying atherosclerosis (Schmitz & Ruebsaamen, 2010). It is noteworthy that saturated lysoPCs are assumed to exert pro-inflammatory effects, whereas polyunsaturated lysoPCs such as C18:2 do not seem to possess inflammatory properties (Hung, Sok, & Kim, 2012). Actually, circulating lysoPCs were found to be reduced in obese individuals (Barber et al., 2012) and, especially, lysoPC C18:2 has been linked to a lower risk for type 2 diabetes (Floegel, Stefan, et al., 2013) or cardiovascular disease (Y. K. Lee et al., 2013). As is consistent with our findings, Wientzek et al. (2014) showed positive correlations

between the CRF and serum lysoPCs C18:2 and C18:1, after controlling for sex and age. Thus, it is possible that lysoPC C18:2, in particular, might provide a potential link between the CRF and its protective effects on chronic diseases. Our data moreover suggest a sex-dependent regulation of 'glycerophospholipid metabolism' in relation to the CRF status. Although PC hydrolysis and lysoPC formation have been shown to be generally higher in men than in women, possibly due to differences in enzymatic activities, body composition, and/or hormonal or lifestyle factors (Beyene et al., 2020), the exact mechanisms underlying sex-related differences in CRF-associated phospholipids are largely speculative. However, we assume sex to be an important factor when studying how PC metabolism is linked to the health-beneficial effects of a high CRF. In addition to lysoPCs, SMs are present in cell membranes or linked to lipoproteins (Iqbal, Walsh, Hammad, & Hussain, 2017). Higher plasma SMs have been proposed as independent risk factors for cardiovascular diseases (Jiang et al., 2000). In particular, SMs with saturated acyl chains (C18:0 to C24:0) were closely correlated with parameters of obesity or insulin resistance (Hanamatsu et al., 2014). As is consistent with our results, previous studies reported negative correlations between the CRF and blood SM C18:0 (Lustgarten et al., 2013; Saleem et al., 2020) or SM C18:1 (Saleem et al., 2020; Wientzek et al., 2014) in young (Lustgarten et al., 2013) and middle-aged (Wientzek et al., 2014) adults or patients with coronary artery disease (Saleem et al., 2020). Despite sex-related differences, our findings indicate that even in healthy individuals and across a broad age range, a higher CRF tends to be associated with lower values of potential novel blood biomarkers of the pathophysiological processes underlying cardiometabolic diseases.

Further relevant CRF-related plasma metabolites in females were two acylcarnitines (C14:2, C10:2), the LCFA C24:0, glyceric acid, acetate, and citric acid, all of which were positively linked to the VO_{2peak}, and two LCFAs (C16:1 9cis, C18:1 11cis), which showed negative correlations with the VO_{2peak}. Palmitoleic acid C16:1 9cis also significantly contributed to the multivariate association with the CRF. C16:1 9cis is an abundant fatty acid in human blood and adipose tissue. It can be ingested through diet or endogenously produced, and is assumed to act as a beneficial lipokine that prevents the negative effects of adiposity on insulin sensitivity (Tricò et al., 2020). Since circulating C16:1 9cis has been shown to be proportional to FM (Tricò et al., 2020), the negative correlation between the CRF and C16:1 9cis might be explained by the generally lower FM (%) in fitter females. Acylcarnitines are intermediates in the transport of

LCFAs to mitochondria or byproducts of β-oxidation (McCann, George De la Rosa, Rosania, & Stringer, 2021). Although blood acylcarnitines have been identified as markers of insulin resistance, mitochondrial overload, and incomplete fat oxidation (Koves et al., 2008), they are also physiologically elevated in conditions with high lipolytic rates (Soeters et al., 2009). Recently, it has been shown that a training-induced rise in fasting levels of muscular long- and medium-chain acylcarnitines (e.g., C14:2 and C10:2) were related to an improved CRF and potentially reflective of a more robust carnitine buffering system (Huffman et al., 2014). Glyceric acid is a sugar acid that connects several pathways, e.g., 'glycerolipid metabolism' and 'glycolysis/gluconeogenesis'. Even if its biological relevance with regard to PE has, to our knowledge, not yet been described, Lustgarten et al. (2013) also revealed a positive association between the CRF and circulating glyceric acid in healthy females. Equally in line with our results, blood acetate was shown to be higher in physically active adults (Kujala et al., 2013). Acetate is either directly formed from pyruvate, providing a source for acetyl-coenzyme A (Liu et al., 2018), or can be produced by the intestinal microbiota, finally entering circulation (Frampton, Murphy, Frost, & Chambers, 2020). It has been suggested that a higher PF is linked to a greater abundance of gut bacteria with positive health effects, being reflected in the release of fermentation metabolites like acetate (Antunes et al., 2020; Bressa et al., 2017). In addition to its anti-inflammatory and vasodilatory properties (Frampton et al., 2020), acetate has been proposed as an important energy substrate during endurance exercise in mice (Okamoto et al., 2019). However, the extent to which the microbiome indeed affects the PE capacity in humans, and whether resting blood acetate might mirror this association, requires further investigation. While the tricarboxylic acid (TCA) cycle intermediate citric acid only showed a relevant positive correlation with the VO_{2peak} in females, malic acid and succinic acid tended to be positively linked to the CRF in both sexes. Previous studies revealed a rise in fasting plasma malic acid after weight loss and PE intervention in obese women (Campbell et al., 2014), a training-induced increase in muscular succinic acid in subjects at risk of metabolic disease (Huffman et al., 2014), or a slightly positive correlation between the CRF and serum succinic acid in healthy young men (Castro et al., 2021). Despite weak bivariate correlations, our results support the suggestion that specific TCA cycle intermediates might be possibly interesting blood markers of the beneficial effects of chronic PA occurring at a muscular level, such as an increased mitochondrial density or TCA cycle capacity.

To conclude, we provided evidence of sex-specific CRF-associated plasma metabolite patterns, after adjusting for age and menopausal status. Sex-related differences were especially observed for 'lipid metabolism'-related PCs, which generally showed relevant associations with the VO_{2peak} in females but not in males. However, weak to moderate correlations between the CRF and lysoPC C18:2, LCFA C16:1 9cis, glyceric acid, as well as the 'energy metabolism'- or 'carbohydrate metabolism'-related succinic acid, malic acid, or acetate, were observed in both sexes. Accordingly, our results suggest that CRF-related adaptations in 'glycerophospholipid metabolism' might vary between sexes, whereas the consequences of a high CRF on, e.g., the TCA cycle, seem to be present in females and males.

Age-, Menopausal Status-, and Phenotypical/Clinical Variables-Adjusted Findings

A higher CRF is generally concomitant with a healthier body composition, adaptations in heart and arterial function, or cardiometabolic risk parameters. Hence, we next aimed to identify metabolite patterns that were independent of further assessed phenotypical and clinical variables associated with the CRF. When adjusting for potential covariates (**), a comparatively smaller number of metabolites were correlated with the VO_{2peak} in both sexes. Thus, it can be suggested that many of the observed associations cannot be primarily and specifically attributed to the CRF. As no relevant CRF-related information seemed to remain in the overall plasma metabolite profile, findings from PLS regression analyses are not further discussed.

After applying additional adjustments, seven acyl-alkyl-PCs, C5-carnitine, choline, glyceric acid, acetylornithine, and diacyl-PC C28:1 still showed moderate positive correlations with the VO_{2peak} in females. Two of these acyl-alkyl-PCs (C40:3, C44:3) were also present in a cluster of 19 PCs that were related to the CRF in the study of Wientzek et al. (2014), when controlling for sex, age, BMI, and waist circumference, among others. The fact that we adjusted for more precise body composition measures and parameters of lipoprotein metabolism, which are known to be linked to both the CRF (Parto, Lavie, Swift, & Sui, 2015) and PCs (Cole et al., 2011; Floegel, Stefan, et al., 2013), could explain the study-specific results. Choline participates in multiple pathways of 'lipid metabolism' or 'AA metabolism', serving as a precursor for PC species, the neurotransmitter acetylcholine, or betaine. Circulating choline can result from diet or PC breakdown (Zeisel & da Costa, 2009) and was shown to be either lower (Bye et al., 2012) or higher (Castro et al., 2021; Høeg et al., 2020) in fitter individuals. Even though a more

efficient conversion of PCs to choline in trained individuals was assumed by Høeg et al. (2020), it cannot be excluded that female KarMeN subjects with a high CRF were also characterized by a higher dietary choline intake.

In summary, sex-specific differences in CRF-related plasma metabolites were still detectable, e.g., for some PCs that remained positively correlated with the female CRF but were not or even slightly negatively linked to the male CRF. Moreover, the xenobiotic tartaric acid showed sex-specific correlations with the CRF. This might be due to differences in the dietary intake of wine, vinegar, or grapes (Regueiro, Vallverdu-Queralt, Simal-Gandara, Estruch, & Lamuela-Raventos, 2014; T. Xia, Zhang, Duan, Zhang, & Wang, 2020) in more or less fit women or men. The AA alanine showed a slightly negative correlation to the female VO_{2peak} but a slightly positive correlation to the male VO_{2peak}. In the literature, circulating alanine was mostly negatively linked to the CRF status (Koh et al., 2018; Morris et al., 2013).

7.5.2 Sex-Specific CRF Explanation Models

Contrary to a previous attempt to explain the variability of the CRF in the KarMeN population based on urinary metabolites (Kistner et al., 2020), we were now able to identify sets of plasma metabolites that, together with clinical and phenotypical variables, contributed to a relatively good explanation of the VO_{2peak} in both sexes, after adjusting for age and menopausal status. In all approaches, the FM (%) entered the models as the first variable, already explaining 33.5% or 42.3% of the confounder-adjusted VO_{2peak} in females or males, respectively. Six (females) or five (males) further phenotypical or clinical parameters slightly improved the models (approach 1 selection). However, when phenotypical, clinical as well as metabolite variables entered the models in a competing manner, plasma analytes led to a fairly improved performance in both sexes, as demonstrated by an R^2 (adjusted) of 0.68 for females and 0.59 for males (approach 3 selection). Regarding females, acyl-alkyl-PC C40:3 was the second most important determinant of the VO_{2peak}. Interestingly, this PC sum parameter also showed relevant bi- and multivariate associations with the CRF. As the relationship between acyl-alkyl-PC C40:3 and the VO_{2peak} did not appear to be influenced by other assessed plasma analytes and as the bivariate correlation persisted independently of adjustments, the ability of acyl-alkyl-PC C40:3 to predict the CRF in females should be given special consideration in further studies. Regarding males, the TCA cycle intermediate malic acid was

the second variable being included in the model after the FM (%) (approach 3 selection). In a study by Lustgarten et al. (2013), blood metabolites-based CRF explanation models were also sex-dependent. Seven serum metabolites in females and five serum metabolites in males explained 58 or 80% of the CRF variability, respectively. However, as regression models were not adjusted for age, body composition, diet, or PA, it is uncertain if those metabolites are specifically indicative of the CRF. As demonstrated by our stepwise regression models, the VO_{2peak} was largely determined by the FM (%) in the KarMeN study, supporting the assumption that some associations between the CRF and plasma metabolites might be mechanistically linked to clinical or phenotypical traits that were also influenced by chronic PA. In fact, Kujala et al. (2019) have shown that comparatively few blood metabolites remained significantly associated with the CRF, after adjusting for the body fat content in healthy young men. Nevertheless, our findings could emphasize the additional value of metabolomics data for explaining the variability inherent in the VO_{2peak}, hinting at some possibly relevant plasma metabolites that could help to infer an individual's CRF status. Further studies will be needed to verify if those metabolites are indeed specific for the CRF, and to what extent they are influenced by other functional or morphological characteristics of the human organism.

7.5.3 Strengths and Limitations

The major strength of this study is that it provides a systematic overview of the relationship between the CRF and the plasma metabolome in a relatively large population consisting of both women and men with a wide age range. The KarMeN study was characterized by highly standardized anthropometric and clinical examinations, as well as a strictly controlled procedure for blood collection, CRF assessment, and metabolomics analyses. To minimize the variability in metabolomics measurements, plasma samples were collected in the fasting state, and pre-menopausal women were examined within the luteal phase of their menstrual cycle. As the KarMeN study focused on healthy, non-smoking subjects with a normal to moderately high weight, excluding individuals with supplement use or hormonal treatment, the metabolic variation related to diseases, medication, or metabolic disorders was also markedly reduced. To control for known confounders from the very beginning, we conducted sex-specific analyses adjusted for age and menopausal status. Owing to the comprehensive characterization of study participants, further potential confounding factors related to body

composition, clinical blood biochemistry, lung and arterial function, short-term and habitual PA, as well as diet, could be considered. Another strength of this study is the applied multi-platform metabolomics approach, allowing the detection of a large number of plasma analytes from a broad range of biochemical classes and pathways. Limitations of the study include the cross-sectional design, as it does not allow the deriving of causal relationships. Furthermore, some of the plasma analytes showing relevant associations with the CRF could not be identified with sufficient certainty and thus, regrettably, remained unknown.

7.6 Conclusions

In summary, our findings demonstrated sex-dependent relationships between the CRF and plasma metabolites in the KarMeN population. Apart from proving well-known associations between the CRF and further, partly health-related phenotypical or clinical variables in both sexes, we could identify a number of PCs, lysoPCs, and SMs as being associated with the VO_{2peak} in either females or males, when controlling for age and menopausal status. However, independently of selected clinical or phenotypical variables, the sex-specific CRF tended to be correlated with a rather small number of plasma metabolites primarily related to 'lipid metabolism', 'AA metabolism', or 'xenobiotics-related metabolism'. Hence, many of the observed associations between the CRF and metabolites were likely to be mediated by the considered clinical or phenotypical parameters. Although the variability of the CRF was largely determined by the FM (%) in both sexes, our stepwise regression analyses revealed certain sets of plasma metabolites able to improve sex-specific VO_{2peak} explanation models. In particular, acyl-alkyl-PC C40:3 could be identified as a possibly interesting metabolite parameter for conclusions on the CRF status in healthy females. Remarkably, CRF-associated metabolites have already been discussed as being reflective of exercise-induced adaptations in muscular 'energy metabolism' (e.g., malic acid, succinic acid, acylcarnitines) or inversely linked to the development of cardio-metabolic diseases (e.g., PCs, lysoPCs). Thus, those metabolites might represent potential mediators of the performance- or health-enhancing effects of chronic PA. However, more research is needed to clarify the mechanisms and metabolic pathways underlying sex-specific differences in CRF-associated metabolite profiles. Finally, we recommend future studies on blood metabolic markers related to the CRF to conduct sex-separated analyses and to consider age, menopausal status, body composition, and other health-related variables as covariates.

8 Functional Classification of PE- or PF-Related Metabolite Profiles

In addition to the detailed interpretation of PE- or PF-related metabolite profiles provided in the published articles, this chapter gives a comparative overview of the categorization of metabolites associated with medium-term HIIT (*Study I*), acute incremental PE (*Study IIa*), or the CRF status (*Study IIb*) to major and specific metabolic pathways based on a manually conducted metabolite classification procedure, as described in Chapter 4.5.1. Furthermore, the results of a web-based pathway analysis tool depicted in Chapter 4.5.2 are presented. Thus, comprehensive information on metabolic pathways which are most likely to be affected by PE or related to the PF status shall be provided.

8.1 Study I

In *Study I*, three urinary metabolites with a significantly different excretion either one day or four days post-HIIT compared to one day pre-HIIT could be identified, see Chapter 5.4.2. The manual classification of those metabolites to major metabolic pathways is visualized in Figure 32.

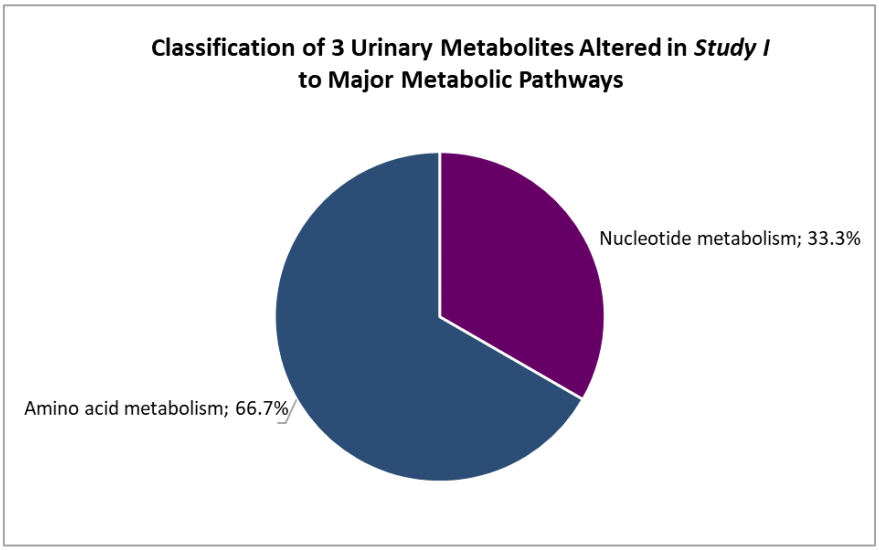


Figure 32. Classification of altered metabolites to major metabolic pathways (*Study I*). Three urinary metabolites changing in response to a ten-day HIIT were detected. While hypoxanthine as the only urinary metabolite with a significantly lower concentration one day post-HIIT belonged to 'nucleotide metabolism', taurine and asymmetric dimethylarginine as the only urinary metabolites with a significant decrease four days post-HIIT belonged to 'amino acid metabolism'. HIIT: high-intensity interval training. *(Own illustration).*

As only three metabolites were altered in response to HIIT, the categorization to sub-pathways is not further illustrated graphically. Briefly, hypoxanthine as the only HIIT-related metabolite

associated with ‘nucleotide metabolism' belonged to the sub-pathway ‘purine metabolism’. While the AA taurine was related to the sub-pathway ‘cysteine, methionine and taurine metabolism’, ADMA was associated with the sub-pathway ‘arginine and proline metabolism; urea cycle’.

By conducting a pathway analysis, two of the three urinary metabolites changing in response to HIIT, namely hypoxanthine and taurine, were assigned to three pathways contained in the KEGG database. A complete list of those pathways and matched metabolites is given in Table A-27 (Appendix A4.4). Since ‘taurine and hypotaurine metabolism’ was the only pathway with a *p*-value < 0.05 and a pathway impact value > 0.1, no graphical overview of the pathway analysis results from *Study I* is provided. In fact, ‘taurine and hypotaurine metabolism’ did not remain significantly enriched after FDR-correction.

8.2 Study IIa

In *Study IIa*, 35 metabolites with a significantly different urinary excretion post-exercise and median FCs $\leq \frac{1}{1.1}$ or ≥ 1.1 could be identified, see Chapter 6.4.2. While the results of the manual classification of those exercise-responsive metabolites to major metabolic pathways are visualized in Figure 33, the further categorization to sub-pathways is illustrated in Figure 34.

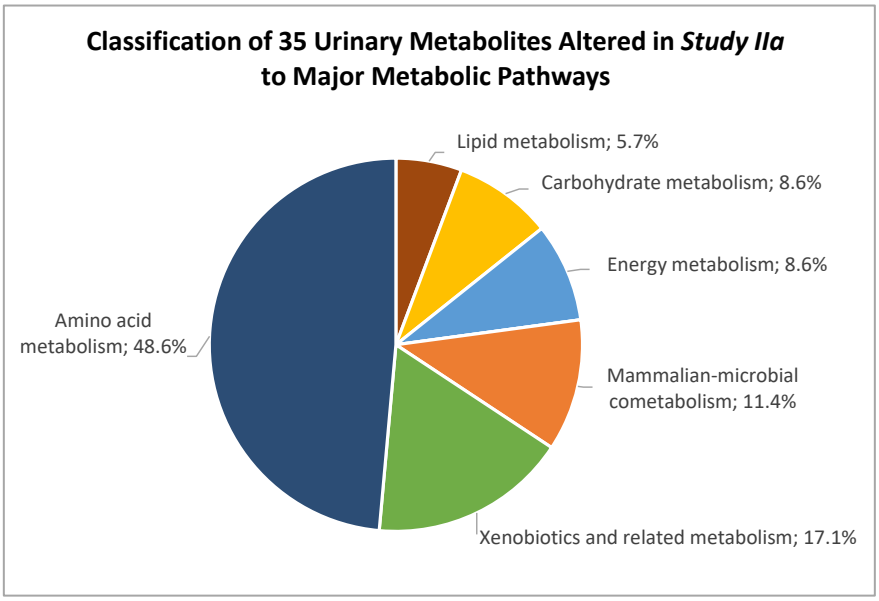


Figure 33. Classification of altered metabolites to major metabolic pathways (*Study IIa*). 35 urinary metabolites changing in response to an incremental exercise test were detected in Study *IIa*. Most of them belonged to ‘amino acid metabolism’ (17/35) and ‘xenobiotics-related metabolism’ (6/35), followed by ‘mammalian-microbial cometabolism’ (4/35), ‘energy metabolism’ or ‘carbohydrate metabolism’ (each 3/35), and ‘lipid metabolism’ (2/35). *(Own illustration).*

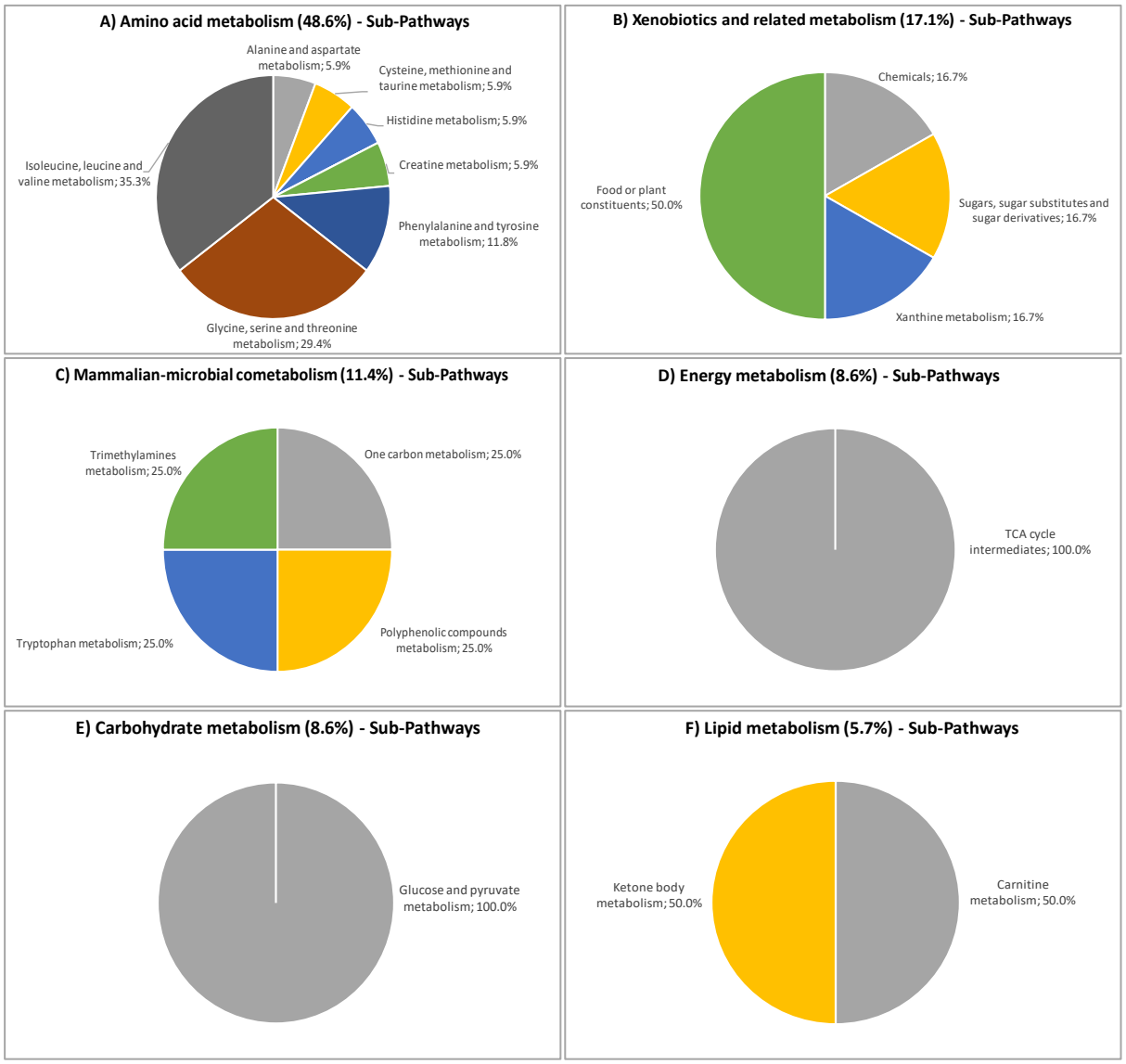


Figure 34. Classification of altered metabolites to sub-pathways (*Study IIa*). The 35 exercise-responsive metabolites detected in *Study IIa* were further classified to 19 sub-pathways. *(Own illustration).*

Briefly, the classification of the 35 exercise-responsive metabolites to major and specific pathways revealed that most of the 17 metabolites belonging to 'AA metabolism' were associated with 'isoleucine, leucine and valine metabolism' (35.3 %), 'glycine, serine and threonine metabolism' (29.4 %), or 'phenylalanine and tyrosine metabolism' (11.8 %), see Figure 34. While the six xenobiotics-related metabolites were classified as 'food or plant constituents' (50.0 %), 'chemicals', 'sugars, sugar substitutes or sugar derivatives', and 'xanthine metabolites' (each 16.7 %), the four mammalian-microbial cometabolites belonged to four sub-pathways, namely 'trimethylamines metabolism', 'tryptophan metabolism', 'polyphenolic compounds metabolism', and 'one carbon metabolism' (each 25.0 %). The three metabolites belonging to either 'energy metabolism' or 'carbohydrate metabolism' were

classified to only one sub-pathway, namely 'TCA cycle intermediates' or 'glucose and pyruvate metabolism', respectively. The two 'lipid metabolism'-related compounds were either linked to 'ketone body metabolism' (50.0%) or 'carnitine metabolism' (50.0%).

Apart from the manual classification, a pathway analysis was conducted on the 35 exercise-responsive metabolites. 23 metabolites were assigned to 26 pathways contained in the KEGG database. A graphical overview of the results is presented in Figure 35 and a complete list of those pathways the metabolites were mapped to is provided in Table A-28 (Appendix A4.5).

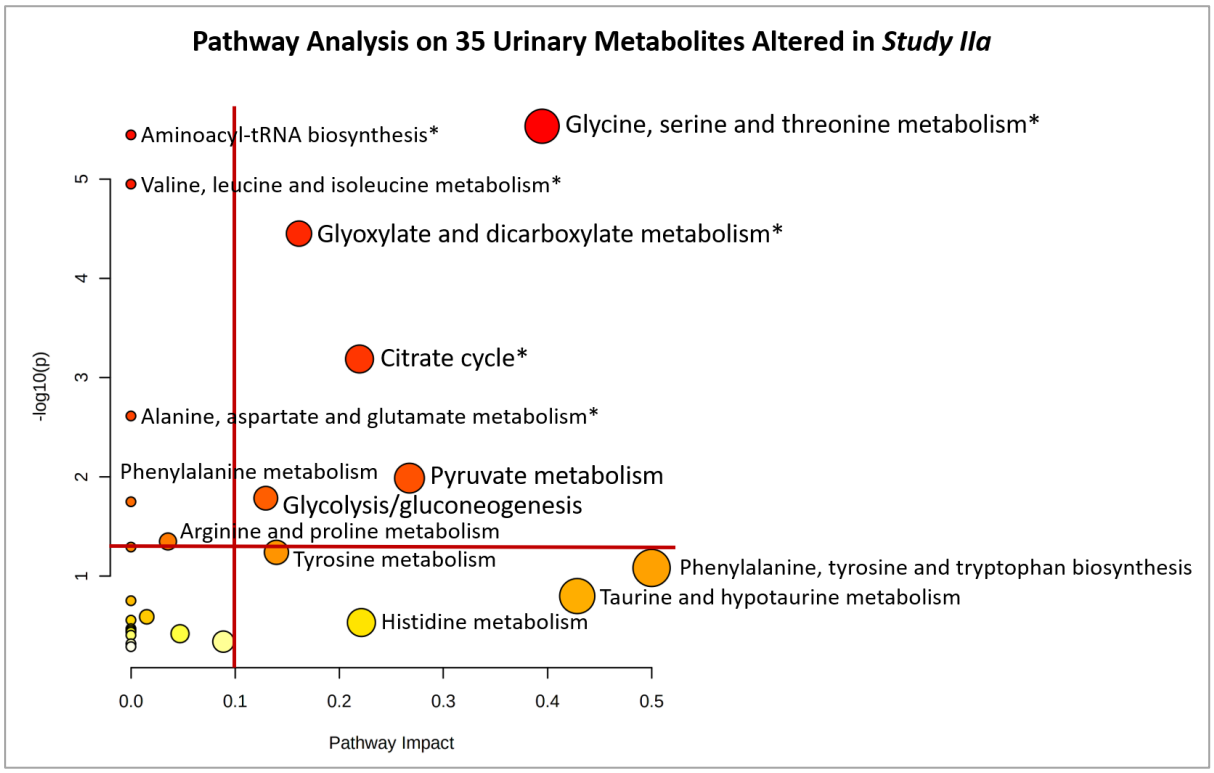


Figure 35. Results of pathway analysis on 35 altered metabolites (*Study IIa*). In the overview graph, the 26 matched pathways are arranged by log-transformed *p*-values from over-representation analysis (Y-axis) and pathway impact values from topology analysis (X-axis). While the node color is based on the *p*-value, the node radius is determined by the pathway impact value. The impact value threshold was set at 0.10 (vertical red line) and the significance level at α=0.05 (horizontal red line). *: still significant after false discovery rate correction. *(Own illustration based on graphs provided by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca)).*

Briefly, 'glycine, serine and threonine metabolism', 'glyoxylate and dicarboxylate metabolism', 'citrate cycle', 'pyruvate metabolism', and 'glycolysis/gluconeogenesis' could be identified as those pathways that seemed most affected by the acute exercise test conducted in *Study IIa*. As shown in Figure 35, they all demonstrated *p*-values < 0.05 and impact values > 0.1. While seven metabolites (betaine, creatine, guanidinoacetate, glycine, N,N-dimethylglycine, pyruvate, threonine) were related to 'glycine, serine and threonine metabolism', six

metabolites (acetate, cis-aconitate, citrate, formate, glycine, pyruvate) were assigned to 'glyoxylate and dicarboxylate metabolism'. The exercise-related metabolites categorized under 'citrate cycle' were cis-aconitate, citrate, pyruvate, and succinate and those being mapped to both 'pyruvate metabolism' and 'glycolysis/gluconeogenesis' were acetate, lactate, and pyruvate, see Table A-28 (Appendix A4.5). As the most altered pathways share several metabolites, a certain interconnectivity between those pathways could be assumed.

In *Study IIa*, almost no relevant correlations between urinary metabolites and the CRF could be observed, after controlling for covariates. Therefore, the manual classification and pathway analysis were restricted to the urinary metabolites being altered in response to acute PE.

8.3 Study IIb

In *Study IIb*, the CRF showed significant correlations with 59 plasma analytes in females and 24 analytes in males, after adjusting for age, menopausal status, and further phenotypical and clinical variables, see Chapter 7.4.4 and Table A-11 or Table A-13 (Appendix A2.11). As a first step for the functional interpretation of CRF-related metabolites, all identified plasma metabolites with significant bivariate correlations to the CRF were classified to major and specific metabolic pathways. While a classification of age- and menopausal status-adjusted CRF-correlated metabolites has already been conducted in the framework of the published manuscript, see Figure A-14 to Figure A-17 (Appendix 2.12), only those plasma analytes with significant bivariate correlations that were independent of further phenotypical and clinical variables were considered for a manual classification to metabolic pathways and a web-based pathway analysis in this sub-section. Both approaches were conducted separately for sexes:

<u>Female sub-group</u>

In females, 37 of 59 CRF-correlated plasma analytes were of known identity and could hence be considered for the manual classification to major and specific metabolic pathways. As shown in Figure 36, 27 CRF-correlated metabolites belonged to 'lipid metabolism'. They were mainly linked to 'glycerophospholipid metabolism' (70.4 %), 'LCFA metabolism' (14.8 %), and 'carnitine metabolism' (7.4 %), see Figure 37. Besides, the six metabolites belonging to 'AA metabolism' were associated with 'arginine and proline metabolism; urea cycle' and 'glycine, serine and threonine metabolism' (each 33.3%) or 'tryptophan metabolism' and 'isoleucine, leucine and valine metabolism' (each 16.7%). While the two xenobiotics-related metabolites

were classified as ‘food or plant constituents’, the single metabolites belonging to either ‘energy metabolism’ or ‘carbohydrate metabolism’ were a ‘TCA cycle’ intermediate or associated with ‘inositol phosphate metabolism’, respectively.

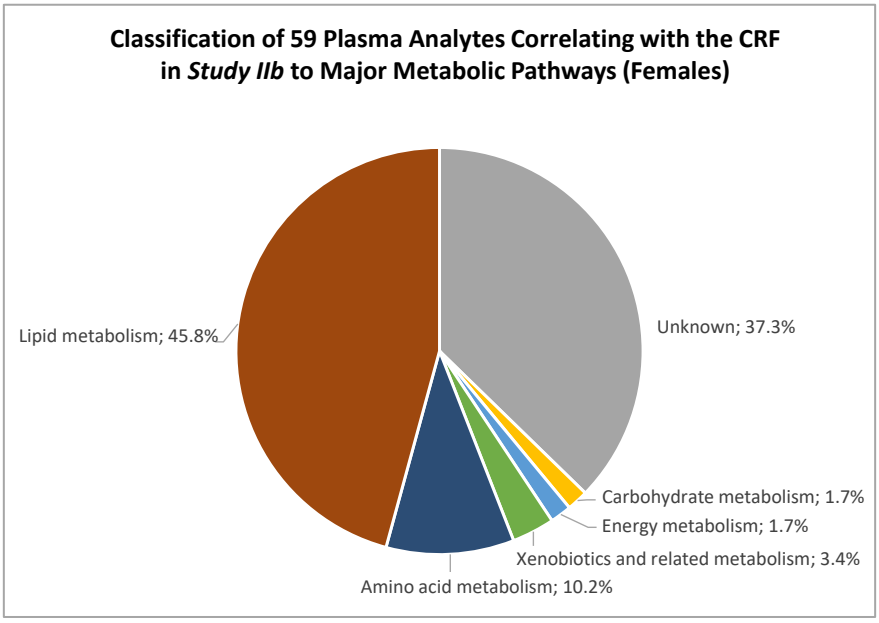
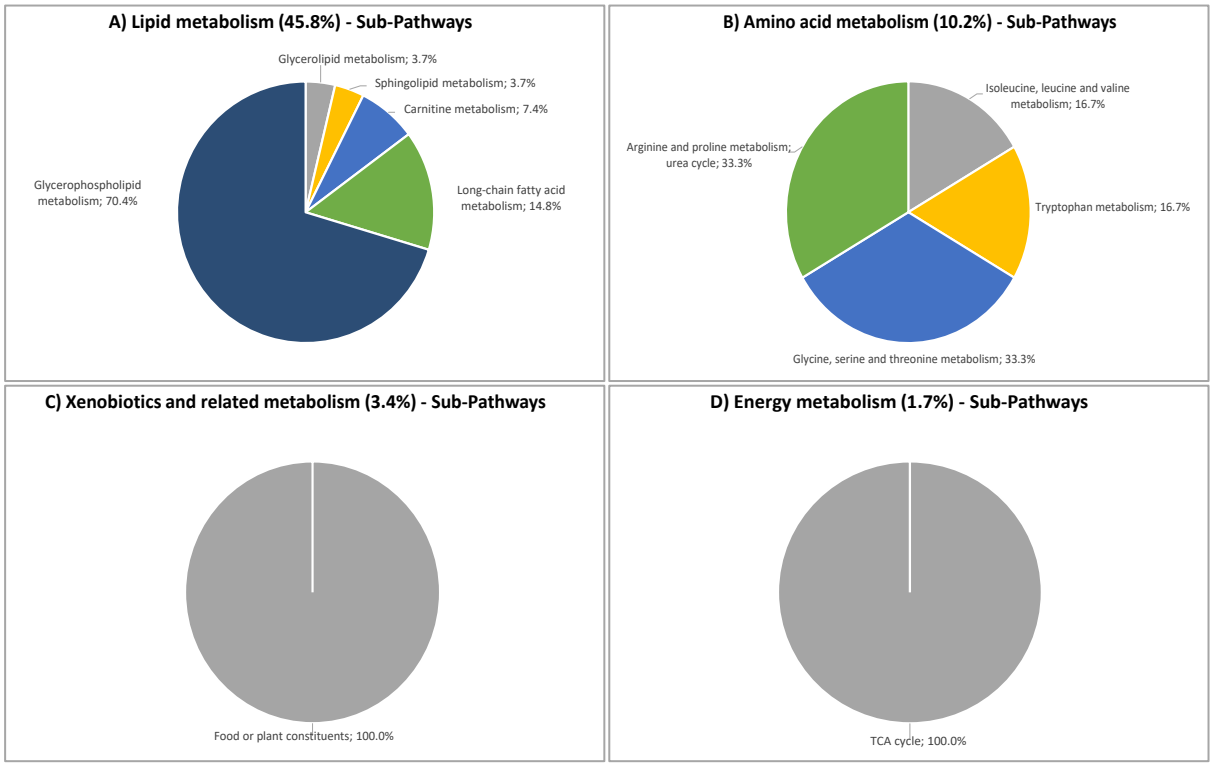


Figure 36. Classification of CRF-correlated analytes in females to major metabolic pathways (*Study IIb*). 59 plasma analytes showed significant bivariate correlations with the CRF, after adjusting for age, menopausal status, and 21 phenotypical/ clinical variables. Most of them belonged to ‘lipid metabolism’ (27/59) and ‘amino acid metabolism’ (6/59), followed by ‘xenobiotics and related metabolism’ (2/59), ‘energy metabolism’, or ‘carbohydrate metabolism’ (each 1/59). 22 plasma analytes were unknown. CRF: cardiorespiratory fitness. *(Own illustration).*



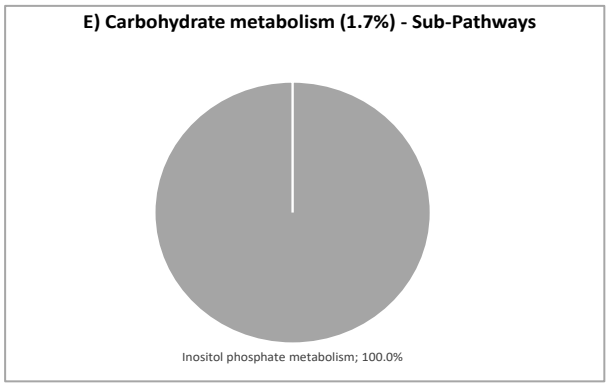


Figure 37. Classification of CRF-correlated analytes in females to sub-pathways (*Study IIb*). The 37 CRF-correlated plasma metabolites with known identity were further classified to 12 sub-pathways. CRF: cardiorespiratory fitness. *(Own illustration).*

Additionally, a pathway analysis was conducted on CRF-correlated plasma metabolites. Since 22 analytes were of unknown identity and no HMDB IDs were available for two further metabolites, only 35 metabolites could be included into analysis. From these metabolites, 10 were assigned to 20 KEGG pathways. In Figure 38, a graphical overview of the results is presented and a complete list of mapped pathways is provided in Table A-29 (Appendix A4.6).

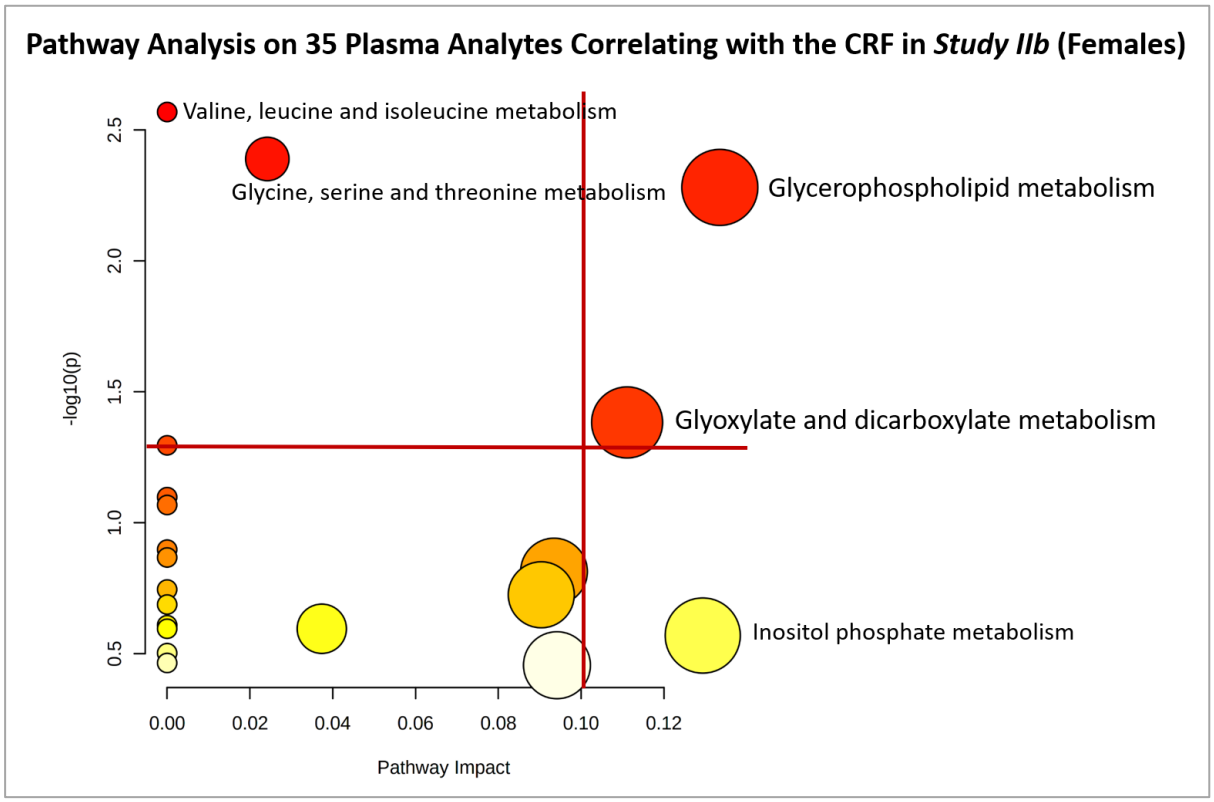


Figure 38. Results of pathway analysis on 35 CRF-correlated, identified analytes in females (*Study IIb*). In the overview graph, the 20 matched pathways are arranged by log-transformed *p*-values from over-representation analysis (Y-axis) and pathway impact values from topology analysis (X-axis). While the node color is based on the *p*-value, the node radius is determined by the pathway impact value. The impact value threshold was set at 0.10 (vertical red line) and the significance level at α=0.05 (horizontal red line). CRF: cardiorespiratory fitness. *(Own illustration based on graphs provided by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca)).*

According to the pathway analysis, the most relevant metabolic pathways linked to the CRF of females in *Study IIb* were 'glycerophospholipid metabolism' and 'glyoxylate and dicarboxylate metabolism', both showing *p*-values < 0.05 and impact values > 0.1. While three CRF-correlated metabolites (phosphatidylcholine, choline, ethanolamine) were mapped to 'glycero-phospholipid metabolism', two metabolites (citrate, glycerate) were categorized under 'glyoxylate and dicarboxylate metabolism', see Table A-29 (Appendix A4.6). Further relevant pathways included 'valine, leucine and isoleucine metabolism' (threonine, valine), 'glycine, serine and threonine metabolism' (choline, threonine, glycerate), and 'inositol phosphate metabolism' (myo-inositol). Yet, the former two only demonstrated *p*-values < 0.05 but pathway impact values < 0.1 and the latter only showed a pathway impact value > 0.1 but a *p*-value > 0.05. Finally, no metabolic pathway remained significant after FDR-correction.

Male sub-group

With regard to males, 11 of 24 CRF-correlated plasma analytes were of known identity and thus considered for the manual classification to major and specific metabolic pathways. As illustrated in Figure 39, eight CRF-correlated metabolites belonged to 'lipid metabolism'. In detail, they were linked to 'glycerophospholipid metabolism' (50.0 %), 'bile acid metabolism' (25.0 %), 'LCFA metabolism' and 'carnitine metabolism' (each 12.5 %), see Figure 40.

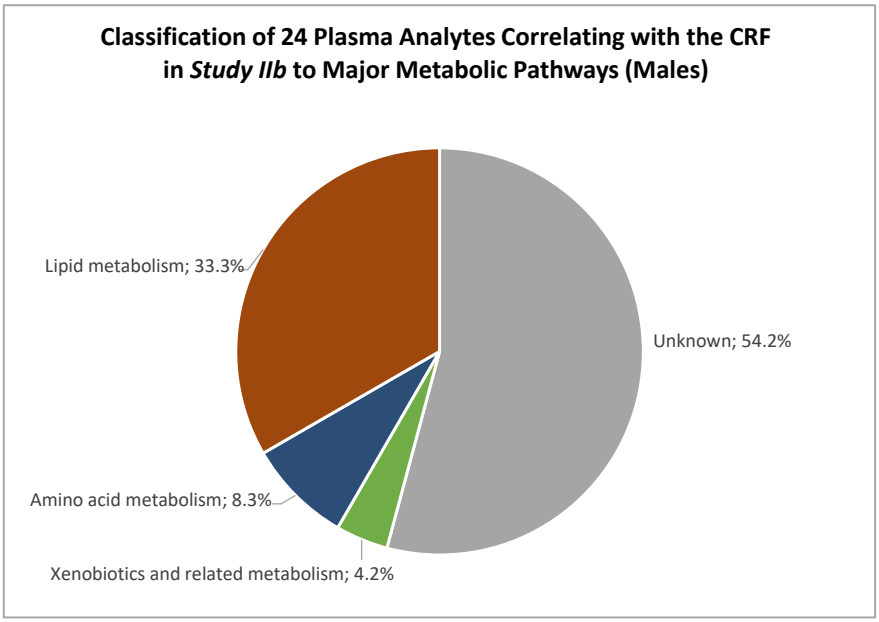


Figure 39. Classification of CRF-correlated analytes in males to major metabolic pathways (*Study IIb*). 24 plasma analytes showed significant bivariate correlations with the CRF, after adjusting for age and 21 phenotypical/clinical variables. Most of them belonged to 'lipid metabolism' (8/24) and 'amino acid metabolism' (2/24), followed by 'xenobiotics and related metabolism' (1/24). 13 plasma analytes were unknown. CRF: cardiorespiratory fitness. *(Own illustration).*

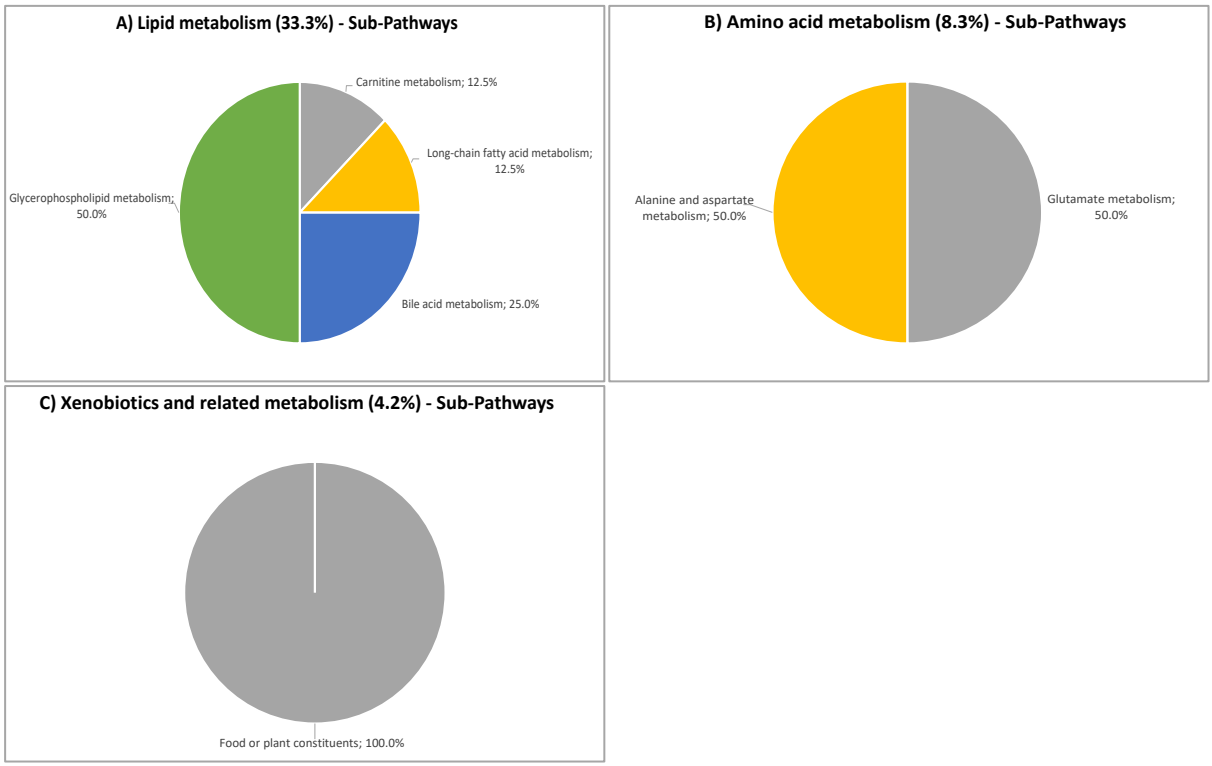


Figure 40. Classification of CRF-correlated analytes in males to sub-pathways (*Study IIb*). The 11 CRF-correlated plasma metabolites with known identity were further classified to 7 sub-pathways. CRF: cardiorespiratory fitness. *(Own illustration).*

The two metabolites belonging to 'AA metabolism' were either associated with 'alanine and aspartate metabolism' or 'glutamate metabolism', while the only xenobiotics-related metabolite was classified as a 'food or plant constituent'.

Moreover, a pathway analysis was conducted on CRF-correlated plasma analytes. Since 13 analytes were of unknown identity, only 11 metabolites could be included into analysis. Of those metabolites, four were assigned to 17 pathways contained in the KEGG database. A graphical overview of the results is presented in Figure 41 and a complete list of mapped pathways is provided in Table A-30 (Appendix A4.6). The most relevant metabolic pathways linked to the CRF of males in *Study IIb* included 'alanine, aspartate and glutamate metabolism' and 'D-glutamine and D-glutamate metabolism' (p-value < 0.05; impact value > 0.1). While the CRF-correlated metabolites categorized under 'alanine, aspartate and glutamate metabolism' were alanine and glutamate, the only metabolite being mapped to 'D-glutamine and D-glutamate metabolism' was glutamate, see Table A-30 (Appendix A4.6). Further relevant metabolic pathways included 'aminoacyl tRNA biosynthesis' (alanine, glutamate), 'linoleic acid metabolism' (phosphatidylcholine), 'nitrogen metabolism' (glutamate) and 'arginine

biosynthesis' (glutamate). However, they either demonstrated a *p*-value > 0.05 or a pathway impact value < 0.1. After FDR-correction, no metabolic pathway remained significant.

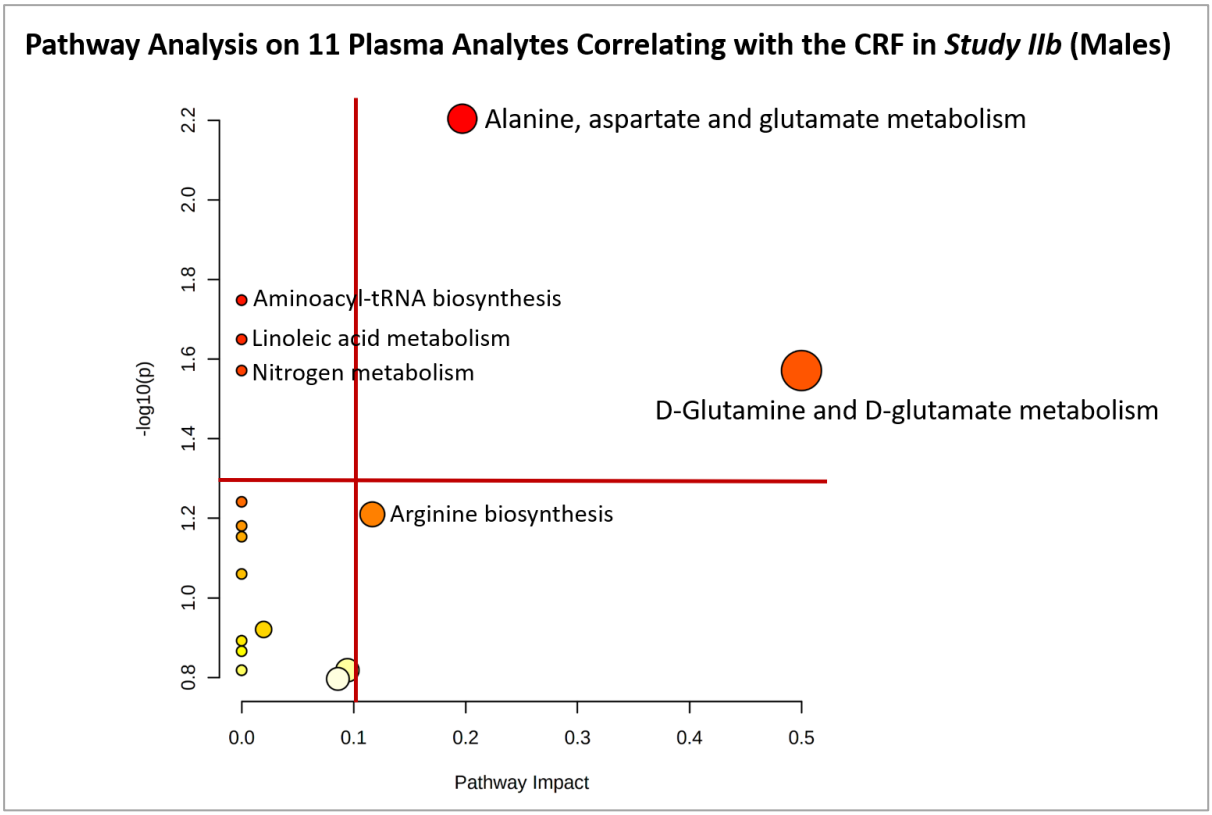


Figure 41. Results of pathway analysis on 11 CRF-correlated, identified analytes in males (*Study IIb*). In the overview graph, the 17 matched pathways are arranged by log-transformed *p*-values from over-representation analysis (Y-axis) and pathway impact values from topology analysis (X-axis). While the node color is based on the *p*-value, the node radius is determined by the pathway impact value. The impact value threshold was set at 0.10 (vertical red line) and the significance level at α=0.05 (horizontal red line). CRF: cardiorespiratory fitness. *(Own illustration based on graphs provided by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca)).*

9 General Discussion

Metabolomics is a powerful tool to assess PA-related alterations in human metabolism. By allowing a comprehensive investigation of PE- or PF-associated metabolite profiles in easily obtainable biofluids like blood or urine, metabolomics can provide valuable new insights into metabolic pathways that are linked to the health-beneficial or performance-enhancing effects of PA (Kelly et al., 2020). With regard to future applications, metabolomics research can facilitate the discovery of novel exercise-responsive biomarkers, which are likely to reflect an individual's training, fitness, or health status (Daskalaki et al., 2014). However, the application of metabolomics in the field of exercise science is still in its early stages (Heaney et al., 2017). As described in Chapter 3, the general aim of the present thesis was to comprehensively analyze the effect of acute and chronic PA on metabolite profiles in humans. Several clearly defined research gaps and deficiencies of previous studies should thereby be addressed and the current state of knowledge in the field of exercise metabolomics should be extended. To reach these purposes, metabolomics data from three exercise- or fitness-related (sub-)studies (indicated as *Study I* or *Study IIa*/*IIb*, see Chapter 4) were analyzed. The particular study results, all of which have been published in international peer-reviewed journals, were presented in Chapters 5 to 7, and a functional classification of obtained PE- or PF-related metabolite profiles was provided in Chapter 8. This section summarizes the main findings of the respective (sub-)studies and discusses them in a wider and specific context (Chapter 9.1). Finally, strengths and limitations of the conducted research are addressed (Chapter 9.2) and general conclusions and suggestions for future research are provided (Chapter 9.3).

9.1 Main Findings

Summarizing the overall findings from *Study I*, *IIa*, and *IIb*, specific PE- and PF-related human metabolite profiles could be detected and finally be associated with particular metabolic pathways. The ten-day HIIT intervention was linked to slight alterations in the major pathways 'nucleotide metabolism' and 'AA metabolism' (*Study I*), whereas the acute incremental exercise test mainly led to variations in the major pathways 'AA metabolism', 'xenobiotics and related metabolism', 'mammalian-microbial cometabolism', 'energy metabolism', 'carbohydrate metabolism', and 'lipid metabolism' (*Study IIa*). In contrast to this, the CRF as a surrogate measure of chronic PA was principally associated with the major pathway 'lipid

metabolism', followed by 'AA metabolism' and 'xenobiotics and related metabolism', when controlling for age, menopausal status, and further phenotypical and clinical variables (*Study IIb*). Figure 42 provides a compact summary of the three studies, its research focuses, and major metabolic pathways that were linked to either PE or PF. A more detailed discussion of the study-specific findings will be given in the following sub-sections.

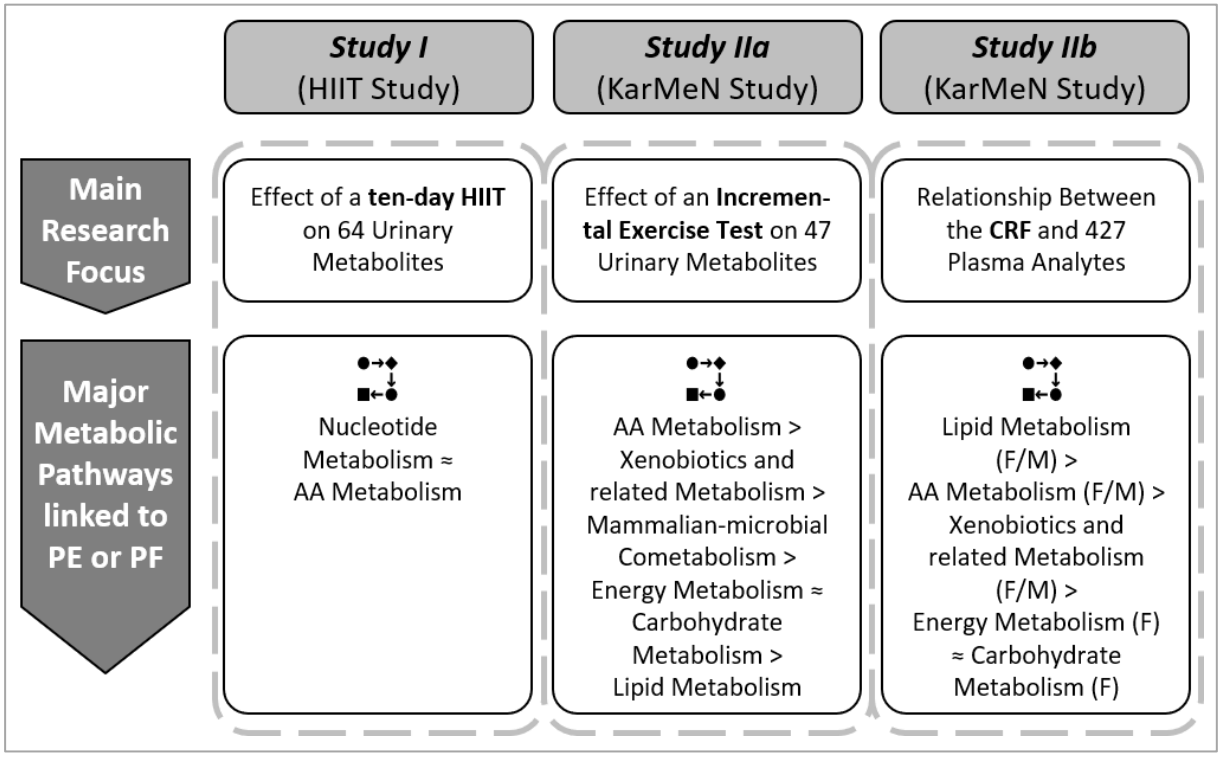


Figure 42. Summary of the main research focuses and major metabolic pathways linked to PE or PF in *Study I*, *IIa*, and *IIb*. >: more related to PE or PF than; ≈ equally related to PE or PF. AA: amino acid; CRF: cardiorespiratory fitness; F: females; HIIT: high-intensity interval training; KarMeN: Karlsruhe Metabolomics and Nutrition; M: males; PE: physical exercise; PF: physical fitness. *(Own illustration).*

9.1.1 Study I

In the framework of the randomized controlled interventional *Study I* ("HIIT Study"), medium-term changes in the metabolome in response to high-intensive intermittent exercise training were investigated. More specifically, the purpose of this study was to examine the effects of a ten-day HIIT and a subsequent four-day recovery period on the resting urinary metabolome of ten young active men, who constituted the EG of *Study I*. Due to the reliable identification and quantification of 64 urinary metabolites by NMR-/LC-MS-based metabolomics analyses, *Study I* allowed to obtain first information about urinary metabolic markers which might be indicative for an individual's adaptation to HIIT. Moreover, a deeper knowledge on altered metabolic pathways was intended to be deduced from the results of *Study I*.

The main findings from *Study I* can be summarized as follows:

1. Despite the high intensity of the ten-day training protocol, there was no overall change in the resting urinary metabolome in the EG.
2. One day after HIIT intervention, a significantly lower resting urinary hypoxanthine concentration than pre-training was detected in the EG.
3. Four days after HIIT intervention, significantly lower resting urinary taurine and ADMA concentrations than pre-training were detected in the EG.
4. Between the EG and CG, differences in single urinary metabolites were only observed post-training. One day after HIIT intervention, urinary hypoxanthine was significantly lower in the EG. Four days after HIIT intervention, urinary betaine, hypoxanthine, and isoleucine significantly differed between both groups.

Regarding the first finding and the fact that alterations in urinary metabolite concentrations were known to occur acutely in response to single high-intensity exercise bouts (Pechlivanis et al., 2013; Pechlivanis et al., 2015; Siopi et al., 2017), it can be supposed that the participants' metabolism was able to largely regenerate from acute metabolic disturbances due to the exhausting HIIT in a given time frame of one or four recovery days, respectively. However, since resting urinary levels of the purine derivative hypoxanthine were significantly lower in the EG one day after HIIT intervention, training-induced adaptations in 'purine metabolism' might be suggested. During high-intensive exercise, hypoxanthine represents the final ATP breakdown product in skeletal muscles, from where it can leak out to the blood (Zielinski & Kusy, 2015b), see Figure 43. In fact, circulating hypoxanthine has not only been proposed as a metabolic indicator of exercise-induced, acute energetic stress (Sahlin et al., 1999), but has also emerged as a potential biomarker for an athlete's training status (Mahanty & Xi, 2020; Zielinski & Kusy, 2015a). As recently shown, exercise training can lead to an increased enzymatic activity in the purine salvage pathway in skeletal muscles (Hellsten-Westing, Balsom, et al., 1993) and erythrocytes (Dudzinska et al., 2018; Pospieszna et al., 2020), being partly accompanied by reduced blood hypoxanthine (Pospieszna et al., 2020). Actually, lower circulating hypoxanthine at rest and post-exercise is likely to reflect an increased capacity of trained individuals to reduce purine loss and to restore the purine nucleotide pool in either muscles or erythrocytes (Pospieszna et al., 2020; Zielinski & Kusy, 2015a).

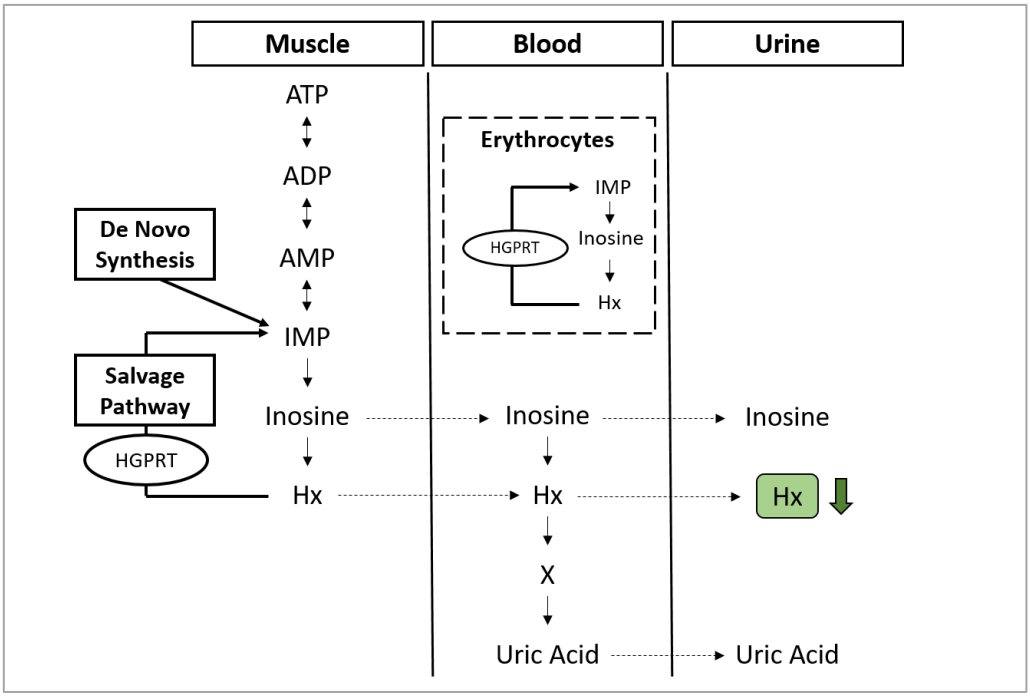


Figure 43. Pathways of purine nucleotide degradation during and after exercise. During high-intensive exercise, muscular ATP degradation rate is higher than ATP resynthesis rate, being reflected in a higher accumulation of IMP, inosine, and Hx. Hx can be reconverted to IMP by the purine salvage enzyme HGPRT, contributing to ATP restoration, or it can leak out to the blood, where it is either degraded to uric acid or directly eliminated from the body via urine. HGPRT activity in muscles and erythrocytes is likely to increase in response to training. The accompanying reduction in blood Hx seems to indicate an increased capacity to reduce purine loss and to recover muscular or erythrocyte purine pools. In *Study I*, lower resting urinary Hx was documented after a ten-day HIIT (green box). Hence, HIIT-induced adaptations in 'purine metabolism' might be assumed. ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate; HGPRT: hypoxanthine guanine phosphoribosyltransferase; HIIT: high-intensity interval training; Hx: hypoxanthine; IMP: inosine monophosphate; X: xanthine. *(Own illustration based on Zielinski and Kusy (2015b)).*

Since *Study I* was the first to demonstrate a training-related decrease in the urinary excretion of hypoxanthine, two assumptions can be stated: Firstly, it might be possible that also lower resting urinary hypoxanthine levels are indicative of an increased purine salvage efficiency. Secondly, it could be speculated that adaptations in 'purine metabolism' already emerged in response to the daily conducted, ten-day HIIT. Unfortunately, it was outside the scope of this study to provide detailed information about molecular mechanisms underlying the metabolic adaptations to HIIT. Likewise, it is still unclear in which tissues the proposed alterations in 'purine metabolism' occurred. As a consequence, further research is necessary to reveal the exact mechanisms contributing to the suggested HIIT-induced adaptations in 'purine metabolism'. Besides, the utility of urinary hypoxanthine as a reliable biomarker for an athlete's training adaptation has to be evaluated in future studies.

To sum up, the present and previous findings suggest the capability of hypoxanthine to indicate an athlete's reaction to training regimens that are typically used in pre-competition

preparation phases (Zielinski & Kusy, 2015a). Regarding future applications, the analysis of hypoxanthine might possibly help to monitor successful training adaptation (as reflected in a lower post-exercise increase in blood (or urinary) hypoxanthine or maybe even lower resting hypoxanthine levels). Given the fact that existing studies were mainly restricted to amateur or professional male athletes, more research is needed to evaluate whether the obtained results can be extended to a broader population consisting of both (un)trained women and men. If hypoxanthine can indeed be seen as a metabolic marker reflecting an individual's training status, exercise scientists would perhaps be allowed to develop more specific training strategies based on initial hypoxanthine concentrations or to modify exercise programs based on training-induced adaptations in 'purine metabolism'.

Apart from hypoxanthine, which was classified to the major pathway 'nucleotide metabolism' (Chapter 8.1), the 'AA metabolism'-related taurine and ADMA showed significantly lower urinary levels in the EG after four days of recovery compared to pre-training. As discussed in Chapter 5.5, it might be assumed that muscular taurine was utilized during HIIT intervention due to its roles in cell volume regulation, skeletal muscle calcium homeostasis, and membrane stabilization (Lambert et al., 2015). Thus, the lower urinary taurine excretion post-training could be interpreted as a result of restoring the pre-training muscular taurine content. Yet, as the urinary taurine excretion is also known to vary along with dietary taurine intake (Lambert et al., 2015), the physiological processes underlying the changes in urinary taurine within the EG could hardly be determined. Regarding ADMA, the absolute difference in its urinary excretion between pre- and four days post-HIIT was rather marginal. Hence, no biological relevance with respect to HIIT intervention could be assumed. Similarly, it was supposed that the differences in single urinary metabolites between the EG and CG four days post-HIIT largely appeared due to uncontrolled variation.

In conclusion, the main finding of *Study I* was that the purine derivative hypoxanthine emerged as the only urinary metabolite with a significant change from pre-HIIT to one day post-HIIT in the EG, as well as significant differences between the EG and CG one and four days post-HIIT. This observation complements other HIIT-related metabolomics studies that documented acute elevations in blood or urinary hypoxanthine in response to a single HIIT session (Gerber et al., 2014; Siopi et al., 2017; Siopi et al., 2019) or a reduced post-exercise increase in plasma hypoxanthine after a six-week HIIT intervention (Kuehnbaum et al., 2015).

Broadly speaking, *Study I* could confirm changes in 'purine metabolism' in response to HIIT. What is new is that HIIT-induced adaptations seemed to be reflected in resting urinary hypoxanthine levels. Providing the first evidence that also urinary hypoxanthine might be a possibly interesting marker for concluding about an individual's training adaptation, the results of *Study I* should definitely serve as a starting point for further research. In the future, it would be appropriate to comprehensively analyze the effects of HIIT on not only resting urinary and blood levels of hypoxanthine as well as other purine derivatives, but also on acute post-exercise metabolite changes in the course of a training intervention. Consequently, more reliable conclusions on HIIT-induced alterations in 'purine metabolism' could be drawn.

Aside from examining the effects of specific training interventions on the human metabolome, metabolomics generally permits to study the acute metabolic effects of single exercise bouts, as well as their dependency on individual factors such as sex or the PF status. However, only few and rather small metabolomics studies have until now been focused on the question if urine is able to reflect acute exercise-induced alterations in human metabolism and whether those changes differ between women and men or more and less fit persons. These research gaps were addressed with *Study IIa*, whose findings will be discussed in the next sub-section.

9.1.2 Study IIa

On the basis of *Study IIa*, which comprised the experimental exercise part within the cross-sectional KarMeN study, alterations in the human metabolome in response to acute incremental exercise were investigated. In more detail, the aim of this study was to examine the effect of a standardized exercise tolerance test on the urinary metabolome of 255 healthy women and men with a broad age range, thereby evaluating sex-related differences in exercise-induced metabolite excursions. Additionally, *Study IIa* aimed to analyze if single urinary metabolites or a specific urinary metabolite pattern at rest or in response to PE were linked to the CRF. By applying an NMR-based approach, 47 urinary metabolites were identified and quantified. Since the metabolomics data were obtained from a large, well-characterized study population, *Study IIa* permitted to detect urinary metabolites which, independently of covariates like age, sex, menopausal status, and body composition, allow to draw conclusions across a broad range of CRF levels. Finally, a deeper understanding of metabolic pathways linked to both acute PE and PF should be deduced from the results of *Study IIa*.

The main findings from *Study IIa* can be summarized as follows:

1. After the standardized incremental exercise test, relevant alterations in the urinary excretion of 35 metabolites (31↑; 4↓) were detected. Except for two metabolites (citrate; trans-aconitate), no sex-related differences in pre- to post-exercise urinary metabolite changes were observed.
2. Despite a high inter-individual variation in both urinary metabolites and the CRF, only weak to moderate correlations between the CRF and the pre- or post-exercise urinary metabolites or exercise-related urinary metabolite excursions were revealed. After controlling for the covariates age, sex, menopausal status, and the LBM, most of the detected bivariate correlations did not persist.
3. There was no evident urinary metabolite pattern in neither the pre- nor the post-exercise condition that strongly accounted for the variation of the CRF, after adjusting for covariates.

Based on the first finding, i.e., the detection of 35 exercise-responsive urinary metabolites, *Study IIa* could confirm the ability of the urinary metabolome to mirror acute exercise-related changes in human metabolism, which has also been observed in preceding metabolomics studies (Enea et al., 2010; Muhsen Ali et al., 2016; Pechlivanis et al., 2010; Siopi et al., 2017). While multivariate PCA did not show a clear separation between the pre- and post-exercise urine samples in the first three PCs, univariate analysis revealed high post-exercise increases for, e.g., lactate, mannitol, trans-aconitate, alanine, carnitine, acetate, taurine, and pyruvate, as well as decreases for hippurate and trigonelline. Thus, even if the inter-individual variability in the urinary metabolite profile seemed to exceed its systematic variation in response to the acute PE, the pre- to post-exercise differences of single urinary metabolites (e.g., lactate, pyruvate, acetate, succinate, cis-aconitate, or citrate) were likely to reflect exercise-induced alterations in energy-producing pathways (e.g., ‘glycolysis’ or ‘TCA cycle’) in both sexes, as shown in Figure 27 (Chapter 6.5.1). Some novel exercise-responsive metabolites with still unknown functions (e.g., mannitol, hippurate, or 2-hydroxyisobutyrate) were also detected.

More specifically, the functional classification of PE-related metabolite profiles revealed that the exercise-responsive urinary metabolites primarily belonged to ‘glycine, serine and threonine metabolism’, ‘glyoxylate and dicarboxylate metabolism’, ‘citrate cycle’, and

'pyruvate metabolism'. As depicted in Chapter 8.2, a certain interconnectivity between those pathways could be observed. Actually, several metabolic pathways were indicative for an increased ATP turnover in response to the incremental exercise test. Rather unexpectedly, however, no post-exercise increase in the urinary excretion of the final ATP degradation product hypoxanthine has been documented. This could be explained by a comparatively lower intensity or duration of the acute PE than in previous studies or a urine sample collection time point that was too early to capture the post-exercise rise in urinary hypoxanthine.

As described by the second and third finding, no single urinary metabolite or metabolite sets that could help to infer an individual's CRF status were identified in *Study IIa*. Until now, metabolomics studies have mainly observed differences in urinary metabolites between pre-defined groups with a different PF status (Enea et al., 2010; Morris et al., 2013; Mukherjee et al., 2014) or have identified a potential predictive urinary marker for the CRF (Muhsen Ali et al., 2016). However, since those studies focused on rather small sample sizes or specific sub-groups, e.g., young women (Enea et al., 2010) or middle-aged men (Mukherjee et al., 2014), their results were hardly transferable to the general population. By examining the relationship between the CRF and urinary metabolites in the large, well-characterized KarMeN cohort, *Study IIa* led to the assumption that the analyzed urinary metabolites were rather neglectable if aiming to conclude about the CRF in a heterogeneous population. In fact, based on the findings of *Study IIa*, it could be supposed that many of the observed, weak to moderate correlations between the CRF and urinary metabolites were likely to be mediated by the covariates sex, age, menopausal status, and LBM. Bearing in mind that sex-related differences in CRF-associated plasma metabolite patterns have been documented in the KarMeN population within *Study IIb* (Chapter 7.4.4), which covered a higher number of metabolites than *Study IIa*, also future studies on PF-related urinary metabolites should possibly be recommended to conduct statistical analyses separately for women and men instead of using sex as a covariate. Whereas the latter approach bears the risk of masking sex-dependent biological processes (Barupal et al., 2019), sex-specific analyses could facilitate to identify metabolic pathways which might be differentially regulated with regard to the PF status in women and men.

While *Study IIa* could not detect relevant relations between the PF and post-exercise urinary metabolites in a mixed study population, the utility of metabolomics measurements before

and after a graded exercise test to reveal metabolic adaptations occurring along with an improved endurance capacity has recently been demonstrated by San-Millán et al. (2020). In fact, professional cyclists with lower levels of lactate accumulation at a given exercise intensity also showed higher increases in circulating TCA cycle metabolites, suggesting a link between improved mitochondrial networks and the capacity to sustain higher workloads. With regard to future research, standardized incremental exercise protocols to assess the CRF or blood lactate in both the general and specific athletic population should be increasingly combined with metabolomics analyses. Once further PF-related markers in easily accessible biomatrices such as urine or blood have been revealed and validated, they might improve the monitoring of the training or fitness status as well as the prediction of physical performance (San-Millán et al., 2020).

Briefly summarized, *Study IIa* provided evidence of the utility of urine in human metabolomics studies to capture exercise-induced metabolic alterations. By reporting quantifiable changes in urinary metabolites that could be linked to particular pathways indicative for an increased ATP turnover or changes in 'AA metabolism', *Study IIa* completed and validated previous research on the acute effects of PE on metabolite profiles in humans (Kelly et al., 2020). Also, despite the obvious disadvantage that only a single post-exercise time point for urine sampling existed, *Study IIa* could hint at previously unnoticed but potentially interesting exercise-responsive metabolites, which should be focused in further studies. Finally, the clear recommendation to control for acute PA in future urine metabolomics studies could be deduced from the present and existing findings. Although the urinary metabolites were partly reflective of acute exercise-induced changes in human metabolism, they were not strongly related to the CRF in *Study IIa*. Undeniably, the conclusions drawn from this study depended on the number of analyzed urinary metabolites. Given that the applied NMR-based targeted analysis was limited to a rather small selection of metabolic compounds, more research is needed in order to extend the current knowledge on a broader variety of urinary metabolites. Nevertheless, what could definitively be deduced from the findings of *Study IIa* is the necessity to take the previously mentioned covariates adequately into account when examining the relationship between the human metabolome and functional variables like the CRF. Thus, it could be proven whether observed associations were indeed specific for the individuals' CRF and furthermore different between sexes.

Apart from focusing on a more comprehensive investigation of CRF-related urinary metabolite profiles in the future, a better understanding of the relationship between the CRF and blood metabolite profiles should also be pursued. In fact, as described for urine, blood metabolomics studies have so far been restricted to specific age or sex groups, included a limited number of participants, or detected rather few metabolites (Floegel et al., 2014; Koh et al., 2018; Kujala et al., 2019; Lustgarten et al., 2013; Wientzek et al., 2014). Those study deficiencies have been addressed with *Study IIb* and obtained results will be discussed in the next sub-section.

9.1.3 Study IIb

In the framework of the cross-sectional *Study IIb* ("KarMeN Study"), the relationship between the CRF and the resting plasma metabolome was systematically investigated. More precisely, the aim of *Study IIb* was to analyze how either single plasma metabolites or metabolite patterns were associated with the CRF status in 252 healthy adults. In addition to that, confirmatory data regarding the relationship between the CRF and several health-related phenotypical and clinical parameters should be provided. In total, 427 plasma analytes from various biochemical classes and pathways were detected. Since the non-modifiable factors sex, age, and menopausal status represent known determinants of the CRF (Mercuro et al., 2006; Zeiher et al., 2019) and have already been linked to a discriminatory plasma metabolite profile in the KarMeN population (Rist et al., 2017), association analyses were conducted separately for sexes and adjusted for age and menopausal status. Hence, *Study IIb* not only permitted the detection of plasma metabolites or metabolite patterns that, independently of known covariates, allow to draw conclusions across a broad range of CRF levels, but also enabled an evaluation of sex-related differences in CRF-associated metabolite profiles. Moreover, to determine whether observed relationships can indeed be specifically attributed to the CRF status, association analyses were additionally adjusted for further assessed parameters related to the body composition, clinical blood biochemistry, lung and arterial function, short-term and habitual PA, or diet. As a last purpose, sex-specific sets of phenotypical, clinical, and plasma metabolite variables for a preferably good explanation of the CRF should be selected. Finally, a profounder knowledge on metabolic pathways that might be linked to the beneficial effects of chronic PA was intended to be obtained based on the CRF-associated plasma metabolite patterns identified in *Study IIb*.

The main findings from *Study IIb* can be summarized as follows:

1. Well-known relationships between the CRF and health-related phenotypical or clinical variables, e.g., FM (%), VATM, TGs, and HDL cholesterol, were confirmed in both sexes, after adjusting for age and menopausal status.
2. Based on bi- and multivariate association analyses, CRF-related plasma metabolite patterns primarily indicative of 'lipid metabolism' were identified in both sexes, after adjusting for age and menopausal status. Yet, the specific CRF-related metabolites largely differed between sexes. While several PCs were predominantly linked to the CRF in females, only single lysoPCs and SMs were associated with the CRF in males. However, bivariate correlations between the CRF and succinic acid, malic acid, acetate, glyceric acid, lysoPC C18:2, as well as the LCFA C16:1 9cis were similar in both sexes.
3. After additionally adjusting for further assessed clinical and phenotypical variables, the sex-specific multivariate relationship between all 427 plasma metabolites and the CRF lost its significance. Besides, the number of plasma metabolites with relevant bivariate correlations to the CRF was reduced in both sexes. Even if both the females' and males' CRF remained correlated with certain plasma metabolites of 'lipid metabolism', 'AA metabolism', and 'xenobiotics-related metabolism', sex-related differences were still apparent.
4. Sex-specific CRF explanation models could be improved by including selected plasma metabolites in addition to clinical and phenotypical variables. In both sexes, the FM (%) was the main determinant of the CRF. If selecting clinical, phenotypical, and metabolite variables in a competing manner, acyl-alkyl-PC C40:3 was the second most important variable in the females' model, whereas it was malic acid in the males' model.

Based on the first finding, *Study IIb* could provide evidence that even in a study population consisting of metabolically healthy adults, the sex-specific CRF is inversely correlated with traditional risk factors for chronic metabolic or cardiovascular diseases (e.g., FM (%), VATM, TGs) and positively linked to the cardioprotective HDL cholesterol. Actually, *Study IIb* was not only able to replicate well-known relationships between the CRF as a recognized predictor of adverse health outcomes (Zeiher et al., 2020) and several health-related variables, but also to show the independency of these observations from the non-modifiable factors sex, age, and menopausal status. Despite the cross-sectional design of *Study IIb*, these results underline the

assumption that the health effects of a high CRF might be partly mediated by reduced overall and abdominal adiposity (S. L. Wong et al., 2004), lower blood TGs, as well as higher circulating HDL cholesterol (Carnethon et al., 2005; D.-C. Lee, Artero, Sui, & Blair, 2010). Equally in line with the literature, but only present in men, were slightly negative correlations between the CRF and further health-related variables, namely HR_{rest} (Laukkanen et al., 2009), diastolic BP (Tobita et al., 2003), LDL cholesterol (Lakoski et al., 2011), and insulin (Laukkanen et al., 2009). The reason why these relationships were not detectable in women could not be determined with certainty, but might be due to the smaller sample size of the female sub-group.

Apart from confirming well-known correlations between the CRF and several recognized risk factors for cardiometabolic diseases in women and men, *Study IIb* could demonstrate a sex-dependent relationship between the CRF and plasma metabolite patterns in the KarMeN population, as summarized by the second finding. In fact, both bi- and multivariate association analyses showed that numerous 'lipid metabolism'-related PC species were linked to the females' CRF, whereas only few 'lipid metabolism'-related lysoPCs and SMs characterized the CRF-associated metabolite pattern in males. These observations were independent of age and menopausal status, partially confirming results from Wientzek et al. (2014), who showed age- and sex-adjusted positive associations between the CRF and serum PCs and lysoPCs in middle-aged adults. Yet, while those relationships seemed to be unaffected by sex, *Study IIb* provided the first evidence of sex-specific differences in CRF-related plasma metabolite patterns, especially with regard to representatives of 'glycerophospholipid metabolism'. Thus, more research is needed to clarify underlying biological mechanisms and molecular pathways. While the findings from *Study IIb* proposed that CRF-related modifications in 'glycerophospholipid metabolism' might vary between women and men, slightly positive correlations between the CRF and 'energy metabolism'-related succinic acid and malic acid were observable in both sexes, potentially hinting at an augmented TCA cycle capacity at rest in persons with a higher CRF, which might be independent of sex.

Remarkably, several CRF-associated metabolites have already been inversely linked to the development of cardiometabolic diseases (e.g., PCs, lysoPCs (Floegel, Stefan, et al., 2013)) or were discussed as markers of exercise-induced adaptations in muscular 'energy metabolism' (e.g., succinic acid (Huffman et al., 2014) or malic acid (Campbell et al., 2014)). In particular, succinic acid has recently emerged as a "myo-metabokine", that might play an important role

in skeletal muscle adaptation to exercise training (Maurer, Hoene, & Weigert, 2021). Apart from its role as a TCA cycle intermediate, succinic acid seems to mediate muscle fiber type switch, angiogenesis-related processes, as well as an increased mitochondrial content in mice and has been positively linked to improvements in insulin sensitivity in humans (Maurer et al., 2021). Together with these current findings, *Study IIb* points out the possibility that the mentioned metabolites might reflect, or even underlie, the health- or performance-enhancing effects of chronic PA.

With respect to the third finding, *Study IIb* led to the suggestion that many of the observed associations between the CRF and plasma metabolites are mediated by the considered clinical or phenotypical covariates and can thus not be primarily and specifically attributed to the CRF. In fact, independently of clinical and phenotypical variables, a rather small number of CRF-related plasma metabolites remained, and adjusted sex-specific bivariate correlations were quite moderate. However, the functional classification of PF-related metabolite profiles revealed that the CRF was still linked to 'glycerophospholipid metabolism' in both sexes (Chapter 8.3), as reflected by a sub-group of acyl-alkyl- and diacyl-PCs in women or single diacyl-PCs in men. Rather unexpectedly, *Study IIb* could not confirm the negative relationship between measures of chronic PA and circulating BCAAs, which has previously been described in the literature (Fukai et al., 2016; Morris et al., 2013; Xiao et al., 2016). Reasons for the discrepant findings could be that the referenced studies referred to questionnaire- or accelerometer-based PA measures (Fukai et al., 2016; Xiao et al., 2016) instead of PF measures, that they conducted group comparisons (Morris et al., 2013), or that they only controlled for age and BMI (Fukai et al., 2016; Morris et al., 2013; Xiao et al., 2016). Actually, in the latest publication from Kujala et al. (2019), the negative associations between the PF and serum isoleucine and leucine in young men did not persist after adjusting for the FM (%). This observation indicates that the relationship between BCAAs and the PF might be mechanistically linked to the FM, which is – equally to the CRF – influenced by chronic PA.

Indeed, as indicated by the first finding and the literature, a better CRF is concomitant with a healthier body composition, adaptations in heart function, and changes in cardiometabolic risk blood parameters (D.-C. Lee et al., 2010). Due to the inter-relationship of different health-related clinical or phenotypical variables (Kelly et al., 2020), more large-scale metabolomics studies should be conducted to systematically elucidate and disentangle their effects on the

human metabolome. Especially well-conducted prospective studies might be helpful to relate lifestyle behaviors, such as PA or diet, and phenotypical features like the CRF or the body composition to specific metabolite profiles and, subsequently, the incidence of chronic diseases. By clarifying which lifestyle and phenotypical factors influence the metabolome in such a way that it is consistent with a good health status, recommendations for the prevention of chronic diseases could be deduced in the future (German, Watkins, & Fay, 2005). Apart from studying the independent effects of several health-related variables on metabolite profiles, it might also be helpful to consider the combined effect of, e.g., a high PA or CRF, low obesity, low BP, absence of smoking and alcohol intake, as well as a desirable eating behavior, on the metabolome in healthy individuals. Consequently, a benchmark for defining a "healthy metabolome" could be provided and metabolite profiles associated with single health-related lifestyle or phenotypical variables might be compared for similarities and specificities.

With regard to the fourth finding, *Study IIb* could demonstrate the ability of selected plasma metabolites to improve sex-specific, age- and menopausal status-adjusted CRF explanation models based on phenotypical and clinical variables. In contrast to *Study IIa*, which could hardly explain the variability of the CRF in the KarMeN population with urinary metabolites, the findings from *Study IIb* hinted at possibly important plasma metabolites which might help to conclude about an individual's CRF status. As one interesting example, acyl-alkyl-PC C40:3 should be mentioned. Firstly, this phospholipid parameter showed significant correlations with the females' CRF, which persisted independently of conducted adjustments. Secondly, acyl-alkyl-PC C40:3 represented the second most chosen variable after the FM (%) when including both phenotypical, clinical, and plasma metabolite variables in explanation models for the females' CRF, resulting in an R^2 (adjusted) of 0.41, see Table A-18 (Appendix 2.15). In contrast, HDL cholesterol was the second most important determinant of the CRF in women when only considering phenotypical and clinical variables, leading to a comparatively lower R^2 (adjusted) of 0.35, see Table A-14 (Appendix 2.15). Accordingly, S*tudy IIb* led to the assumption that acyl-alkyl-PC C40:3 might help to predict the CRF in females together with the FM (%) and emphasized the need for validation studies. Although the exact biological role of acyl-alkyl-PC C40:3 still remains to be elucidated, the findings from *Study IIb* could support the importance of expanding lipid measurements traditionally limited to TGs or HDL and LDL cholesterol to a detailed lipid analysis at the molecular species level, especially in the context

of cardiometabolic risk evaluation in clinical research and practice, as suggested by Carrard et al. (2021). Considering that different lipid species within the same subclass can be classified as either cardiometabolic favorable or deleterious (Chew et al., 2019; Hung et al., 2012), associations with health-related variables are most likely to become obvious when focusing on lipid species (Carrard et al., 2021). Unfortunately, the biological relevance of most glycerophospholipid or sphingolipid species is far from being entirely understood. Nevertheless, by proving sex-specific associations between the CRF as a recognized health parameter (Harber et al., 2017) and plasma PCs, lysoPCs, or SMs, *Study IIb* could emphasize the potential importance of specific circulating lipid species for performance or health maintenance.

Briefly summarized, *Study IIb* could provide evidence of sex-related differences in CRF-related plasma metabolite patterns. Apart from confirming some previously detected PF-associated blood metabolites in a large and heterogeneous population, *Study IIb* additionally hinted at several novel, partly sex-specific CRF-related plasma metabolites and respective pathways with possibly important roles concerning the health- or performance-enhancing effects of chronic PA. Though, to what extent (changes in) phenotypical features like the CRF or body composition, which are both influenced by chronic PA, causally determine human metabolite profiles, has to be elucidated in further, mainly interventional metabolomics studies. Given the rather exploratory character of *Study IIb*, obtained results should be regarded as a starting point for future research and mechanistic investigations. Due to the fact that some plasma analytes showing associations with the CRF in *Study IIb* could not be identified with sufficient certainty, further attempts for a reliable identification of still unknown CRF-associated analytes will be required. Nevertheless, what could undeniably be deduced from *Study IIb* is the necessity to conduct sex-separated analyses and to adjust for further health-related variables (e.g., age, body composition, etc.) if examining relationships between the CRF and blood metabolites. On the other hand, since correlations between the CRF and certain plasma metabolites remained independently of considered covariates, the need to assess and control for PF in future blood metabolomics studies could also be deduced from *Study IIb*.

In summary, the findings from *Study I*, *IIa*, and *IIb* could provide evidence of metabolites or metabolite patterns being associated with acute PE, medium-term HIIT, or the CRF status. Based on the particular results, directions for future studies were proposed. Figure 44 gives a compact overview of the main study findings and suggestions for future research.

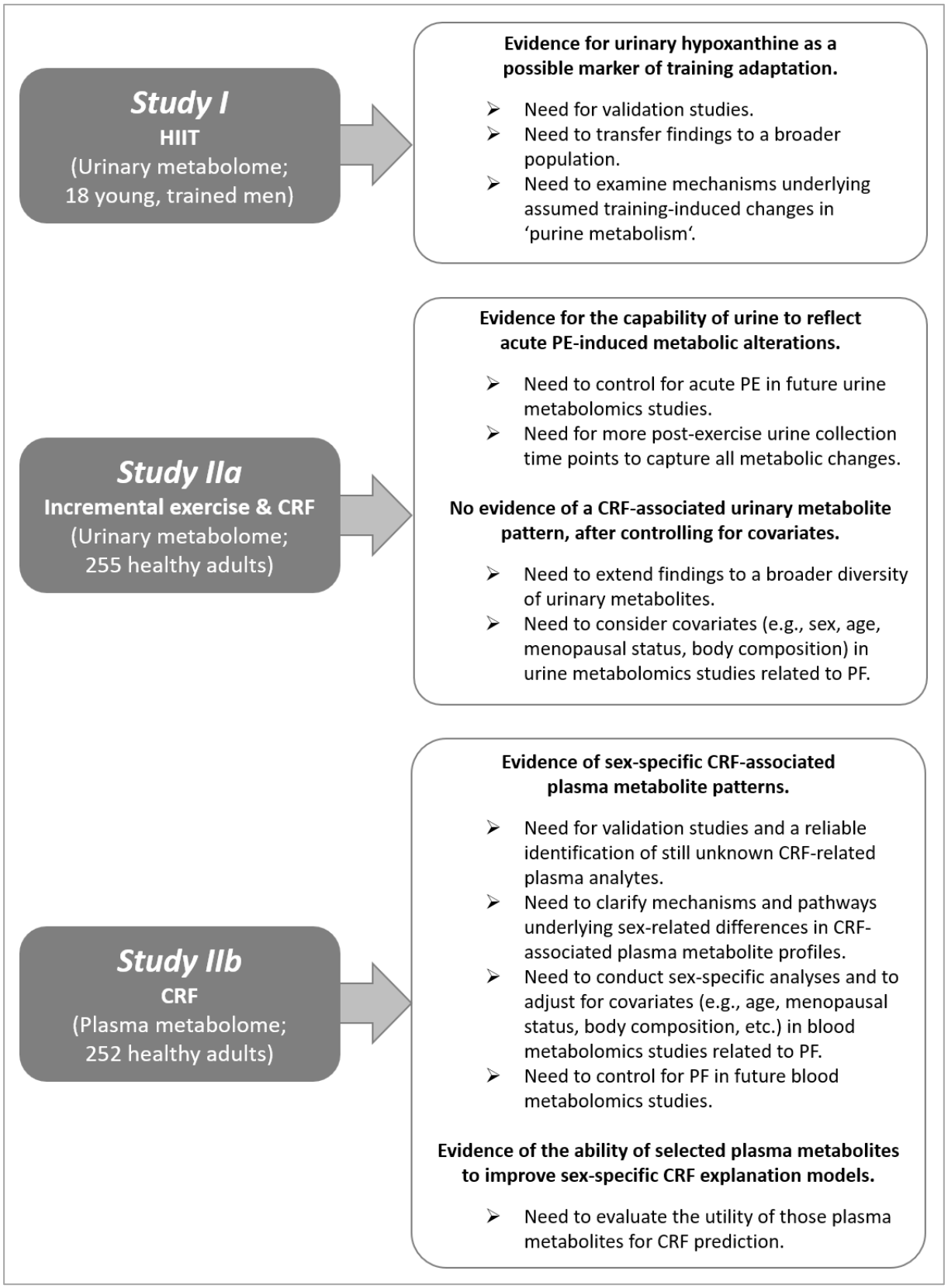


Figure 44. Suggestions for future research based on findings from *Study I*, *IIa*, and *IIb*. CRF: cardiorespiratory fitness; HIIT: high-intensity interval training; PE: physical exercise; PF: physical fitness. *(Own illustration).*

9.2 Strengths and Limitations

In general, the considered studies covered different aspects of PA, reaching from a medium-term HIIT (*Study I*) and an acute exercise intervention (*Study IIa*) to the assessment of the CRF as a measure of chronic PA (*Study IIa*/*IIb*). Therefore, a comprehensive and differentiated view on PA-related metabolic alterations could be obtained in the framework of the present thesis. Of course, the results from *Study I*, *IIa*, and *IIb* should not be interpreted irrespective of the particular study designs, examination methods, as well as applied metabolomics, statistical, or metabolic pathway analyses. As described in detail in the specific publications, study limitations exist due to constraints on research designs or methodology. In this sub-section, a comparative overview of the respective study strengths and limitations is provided.

9.2.1 Study Designs, Study Populations, and Methods of Investigation

Strengths

A clear strength of the three particular (sub-)studies is their study design. Both the randomized controlled interventional *Study I* and the cross-sectional *Study II* had clearly defined inclusion and exclusion criteria. Regarding recruitment processes, only healthy subjects with a normal to moderately high weight were included, thus reducing the metabolic variation related to diseases or medication. As previous PA-related metabolomics studies principally focused on male participants (Kelly et al., 2020), the inclusion of both sexes can be seen as an advantage of *Study II*. To reduce the variability in metabolomics analyses in *Study II* further, all pre-menopausal women were examined within the luteal phase of their menstrual cycle.

Besides, both *Study I* and *Study II* were characterized by a strictly scheduled experimental setting and highly standardized experimental procedures. More specifically, participants of *Study I* followed an individually standardized training protocol and were told to refrain from alcohol and drugs, not to change their dietary habits, and to eat a standardized dinner the days before urine collection. With respect to *Study II*, SOPs were established for all relevant steps, including recruitment, examinations, and preanalytical sample handling (Bub et al., 2016). Owing to the comprehensive characterization of subjects in *Study II* based on various clinical examinations, many potential confounding factors could be considered when studying CRF-related metabolite profiles. In particular, the accurate assessment of body composition (e.g., LBM, FM, and VATM) by DXA represents a strength of *Study II*. Compared to other

methods like computed tomography or magnetic resonance imaging, DXA has the advantage of low X-ray exposure, short scanning times, and minor costs (Kaul et al., 2012; Neeland, Grundy, Li, Adams-Huet, & Vega, 2016). Also, it has been shown that DXA measures the VATM precisely in both sexes and across a broad BMI range (Kaul et al., 2012). In *Study II*, it was therefore not required to measure the less accurate waist circumference as a surrogate marker for abdominal obesity, which has been considered as a covariate in previous PA- or PF-related metabolomics studies (Floegel et al., 2014; Wientzek et al., 2014). Since diet and short-term as well as habitual PA behavior were additionally assessed, they could also be included as covariates in the systematic analysis of CRF-related metabolite patterns within *Study IIb*.

A further strength of both *Study I* and *Study II* is that the CRF assessment was based on highly standardized incremental exercise protocols until individual exhaustion. In fact, the direct measurement of the VO_{2max} by breath gas analysis during maximal exercise represents the gold standard for evaluating the CRF status (Tran, 2018). While in *Study I* all participants met the traditional criteria for VO_{2max}, namely a plateau in VO_2, the VO_{2max} could not be determined with certainty in *Study II*. Therefore, the VO_{2peak} as the highest attained VO_2 during the test was utilized instead. However, since VO_{2max} and VO_{2peak} are often the same in healthy subjects (Day et al., 2003; Tran, 2018), the direct assessment of the CRF in both studies represents a clear advantage over studies using either rough VO_{2max} estimations or subjectively assessed, questionnaire-based measures for regular PA (Brandes, 2012; Finger et al., 2013).

Limitations

One limitation of the included studies is that their results cannot be transferred to the general healthy population. While *Study I* was restricted to few young active men, *Study II* comprised a large population consisting of both sexes with a broad age range – however, healthy obese persons and smokers were not included. Moreover, due to the cross-sectional design of *Study II*, no causal relationships between the CRF and the metabolome could be proven.

Further weaknesses within the research designs refer to the not completely controlled eating behavior (*Study I, IIa*) or considered dietary intake (*Study IIb*). In *Study I*, the daily diet during HIIT intervention was not strictly controlled. In *Study IIa*, the pre-exercise breakfast was only semi-standardized, as subjects consumed the same amount of bread with different toppings. Hence, an effect of diet on the assessed urinary metabolites could not be entirely excluded.

Regarding *Study IIb*, the HEI-score as a measure of diet quality was calculated based on the food consumption on the day before blood sampling. Although the HEI was considered as a confounder in the analysis of CRF-associated metabolite patterns, the effects of specific foods or nutrients on the relationship between the CRF and the metabolome could not be excluded. However, dietary factors have also rarely been adequately considered in other PE- or PF-related metabolomics studies so far (Kelly et al., 2020; Schranner et al., 2020). Thus, a better control and/or consideration of diet should be focused in future investigations.

With respect to PA or CRF assessment, single limitations are observable. Although the subjects of the CG in *Study I* were instructed to refrain from exercise, their actual PA behavior was not recorded. In *Study II*, the initial sample size decreased due to missing spiroergometry data. Unfortunately, 40 subjects had to be excluded from the final analyses because they did not reach their maximal effort, either due to a lack of motivation or since predefined break-off criteria (e.g., acute hypertension) have been met (n=23), no ergometry was conducted (n=9), or technical measurement errors occurred (n=8). Moreover, *Study II* focused on subjective rather than on objective exhaustion criteria for CRF assessment. Hence, the achievement of maximal exhaustion could not be determined with absolute certainty.

9.2.2 Biological Samples, Collection Time Points, and Metabolomics Analyses

Strengths

Especially in *Study II*, SOPs were applied for the participants' characterization as well as for the collection, preparation, and storage of biological samples, i.e., urine (*Study IIa*) and plasma (*Study IIb*). Due to the identical treatment of the specimens, the non-biological variability in subsequent metabolomics analyses could be minimized (Bub et al., 2016). Regarding *Study IIb*, a clear strength is that plasma samples were collected in the fasting state. Hence, the circadian and diet-related variation of the metabolome could be kept as low as possible. Moreover, the pre-analytical sample handling and metabolomics analyses were conducted under highly controlled conditions in both *Study I* and *Study II*. By analyzing QC samples along with plasma or urine samples in all applied analytical methods, the repeatability and precision of analyses could be ensured. An additional strength of *Study I* is that it demonstrated the comparability of the urinary concentrations of nine specific metabolites measured by both targeted NMR and LC-MS, thus supporting the validity of these quantitative approaches.

While clear advantages of *Study I* and *Study IIa* are the absolute quantification of metabolites with known identities and thus the possibility to classify them to specific biochemical classes or pathways, one major strength of *Study IIb* is its multi-platform metabolomics approach, which allowed to obtain a broader coverage of the human metabolome than it would have been possible by one analytical technique alone. In fact, more than 600 (un)known analytes were registered in plasma samples from *Study IIb* with (un)targeted methods. Therefore, the multi-platform approach offered the ability to detect novel CRF-related blood metabolites. According to Wishart (2019), a central benefit of untargeted analyses is that the importance of previously unknown analytes can be recognized. Due to the presence of several still unidentified variables, *Study IIb* will certainly provide a starting point for future research, pinpointing those unknown CRF-associated plasma analytes which are worth to be identified.

Limitations

Regarding the availability and collection of biological specimens, certain limitations can be documented. In both *Study I* and *Study IIa*, only spot urine samples were available for targeted metabolomics analyses, while the multi-platform metabolomics approach of *Study IIb* had so far been completed and finally evaluated for the biological matrix plasma but not yet for urine. Accordingly, the three (sub-)studies were restricted to either urine or plasma. In *Study I* and *Study IIa*, only two post-training or one post-exercise time point(s) for sample collection existed, respectively. With regard to *Study IIa*, the post-exercise urine sampling was not strictly controlled, occurring approximately 15-30 minutes after the acute PE. To facilitate the analysis and interpretation of PE-related metabolite changes, future studies should focus on a more controlled collection of multiple post-exercise or post-training urine (and blood) samples.

While a further restriction of *Study I* and *Study IIa* refers to the targeted metabolomics analyses, which were limited to a comparatively small number of 64 or 47 metabolites, respectively, it can be regarded as a disadvantage of *Study IIb* that not all considered plasma analytes could be identified with sufficient certainty. Hence, more attempts to reliably identify still unknown CRF-associated plasma analytes will be required. Unfortunately, seven subjects were excluded from the final analyses in *Study IIb* due to technical errors during GC × GC-MS analyses. In future studies, such dropouts could be prevented by repeating inaccurate measurements.

9.2.3 Data Handling and Statistical Approaches

Strengths

Regarding metabolomics data handling, clearly defined and thus reproducible procedures for eliminating metabolites that were either measured by more than one technique or had a low detection frequency were applied in all three (sub-)studies. More precisely, if analytes from different platforms could be identified as the same metabolite, those analytes detected by the less quantitative method were excluded in *Study I* and *Study IIb*. Accordingly, it could be ensured that the identical biological information contained in these specific analytes was only uniquely considered in subsequent statistical analyses.

A further strength of the present thesis refers to the combination of descriptive, inferential, and exploratory statistical procedures as well as uni-, bi-, and multivariate approaches, wherever applicable. As described in Chapter 2.3.3, metabolomics studies characteristically combine the advantages of uni- and bivariate procedures, such as the ease of application and interpretation, with the benefits of multivariate procedures, such as the capability to consider relations between different metabolites and their orchestrated or complementary behavior with respect to biological processes (Saccenti, Hoefsloot, Smilde, Westerhuis, & Hendriks, 2013). Given the mainly exploratory character of *Study IIa* and *Study IIb* when examining the relationship between the CRF and the metabolome, the applied data analysis strategy was composed of different types of association analyses, namely bi- and multivariate association analyses as well as multiple linear regression analyses with variable selection. Hence, a systematic overview of CRF-associated metabolite profiles could be obtained.

Since multivariate modelling approaches often have the problem of overfitting, i.e., when a model lacks generalizability, a broad consensus on the necessity to properly validate multivariate models exists (Broadhurst & Kell, 2006; Gertsman & Barshop, 2018; Saccenti et al., 2013). Thus, both the cross-validated stepwise multiple linear regression and PLS regression using a nested cross-validation in combination with permutation tests can be seen as a strength of the data analysis strategy in *Study IIb*. While internal cross-validation decreases the level of overfitting by dividing the data into a training and test set and then calculates the predictive ability of the model, permutation tests evaluate the actual model performance by comparing it to a model constructed using randomly permuted data (Westerhuis et al., 2008).

Additionally, owing to the removal of signal-free regions in NMR spectra and highly correlating analytes which have been identified as the same metabolite, multivariate models were less likely to be distracted from the noise contained in the respective data (Worley & Powers, 2013).

Finally, the sophisticated data analysis strategy applied in *Study IIb* seems to represent an appropriate approach which could be relatively easily transferred to further investigations, getting comprehensive insights into the relationship between the human metabolome and other lifestyle or phenotypical variables of interest. In the framework of the KarMeN study, it might be possible to focus on more PF- or PA-related variables, e.g., the P_{IAT}, the accelerometry-assessed AEE, or the questionnaire-assessed MET, as well as on further health-related variables like the PWV as a measure of arterial stiffness.

Limitations

Unfortunately, neither corrections for multiple testing nor multivariate statistical procedures were applicable in *Study I* due to the small sample size (EG: n=10; CG: n=8) in comparison with the number of investigated variables (64 urinary metabolites). Hence, the obtained results needed to be evaluated cautiously and could only be interpreted for each single metabolite.

In *Study IIb*, not all CRF-associated plasma analytes could be precisely identified. Most of the relevant GC × GC-MS analytes turned out to be trace analytes with a low detection frequency in the study sample and CRF-related NMR buckets contained either unspecific signals or overlapped peaks. Also, the presence of multiple variable collinearities, e.g., due to NMR signals arising from the same metabolite, could not be excluded. Hence, the question arises whether or not it would be useful to disregard the respective data in the future when applying multivariate statistical approaches that are likely to be influenced by unidentified features from untargeted metabolomics analyses (Gertsman & Barshop, 2018; Saccenti et al., 2013).

9.2.4 Metabolite Classification and Pathway Analysis

Strengths

The biological interpretation represents an essential step in metabolomics studies (Sussulini, 2017). By combining a manual classification of identified metabolites to metabolic pathways with a web-based pathway analysis, a comprehensive examination of PE- or PF-associated metabolic pathways could be ensured. Besides, the strengths of one approach were able to

compensate the weaknesses related to the other. A clear advantage of the manual approach is that each PE- or PF-related metabolite was assigned to one major and one specific pathway, thereby also considering metabolites of exogenous origin or metabolite structures which do not yet exist in the KEGG database, e.g., specific PCs, LCFAs, or acylcarnitines. In contrast, the pathway analysis has the strength to take not only the interaction between metabolites but also the centrality of metabolites in respective pathways into account (Chong et al., 2019), and allows to map a single metabolite to various sub-pathways. Despite different approaches and the fact that obtained results were not directly comparable, the majority of pathways with obviously relevant associations to PE- or PF-related metabolite profiles could be detected by both methods.

Limitations

A limitation of both the manual classification and the web-based pathway analysis is that the magnitude of PE-related metabolite changes or metabolite associations with the PF could not be directly considered, i.e., metabolites with less relevant observations were treated like metabolites with more relevant observations. Additionally, both approaches were dependent on the compound coverage of the analytical techniques and required the identification of metabolites. Thus, a certain bias was likely to be introduced. Another disadvantage of the manual classification is that the participation of metabolites in several sub-pathways could not be considered because a given metabolite was only related to one specific pathway. In contrast, the pathway analysis has the restriction that not each PE- or PF-linked metabolite was actually mapped to one or more specific metabolic pathways. Hence, both approaches led to a certain information loss. A main limitation of the web-based tool is that lipids were not adequately covered by the KEGG database. Since this database only considers functional groups, but does not distinguish between acyl side chains, lipid species such as PCs were only included once for pathway analysis.

In Table 19, a comparative summary of the described strengths and limitations is provided.

Table 19. Summary of strengths and limitations of *Study I*, *IIa*, and *IIb*. *(Own table).*

	Strengths	Limitations
Study Designs, Study Populations, Investigation Methods	• Clearly defined inclusion and exclusion criteria (*Study I, II*) • Inclusion of both sexes (*Study II*) • Strictly scheduled experimental procedures (*Study I, II*); SOPs for the recruitment and all examinations (*Study II*) • Comprehensive clinical examinations, e.g., CRF assessment (*Study I, II*), accurate body composition assessment by DXA (*Study II*), assessment of habitual PA and diet (*Study II*) → Consideration of many confounding factors (*Study II*)	• Limited generalizability (*Study I, II*) and causality (*Study II*) of results • Incomplete control of PA behavior (*Study I*) or dietary factors (*Study I, II)* • Missing spiroergometry data for forty participants (*Study II*) • Application of subjective rather than objective exhaustion criteria for CRF assessment (*Study II*)
Biological Samples, Collection Time Points, Metabolomics Analyses	• SOPs for the collection, preparation, and storage of samples (*Study II*) • Collection of blood samples in the fasting state (*Study IIb*) • Standardized metabolomics analyses with QC samples ensuring the precision and repeatability of analyses (*Study I, II*) • Identification and absolute quantification of metabolites (*Study I, IIa*) • Assessment of the validity of targeted NMR and LC-MS data regarding nine urinary metabolites (*Study I*) • Comprehensive analysis of metabolic compounds by a multi-platform metabolomics approach (*Study IIb*)	• Restricted availability of biological samples → Focus on either spot urine (*Study I, IIa*) or plasma (*Study IIb*) • Comparatively few post-training (*Study I*) or post-exercise (*Study IIa*) sampling time points • Comparatively small number of analyzed metabolites (*Study I, IIa*) • Limited identification of CRF-related analytes (*Study IIb*) • Missing GC × GC-MS data for seven participants (*Study IIb*)
Data Handling, Statistical Approaches	• Clearly defined procedures for metabolomics data handling (*Study I, IIa, IIb*) • Combination of bi- and multivariate methods for a systematic analysis of CRF-associated metabolite profiles (*Study IIa, IIb*) • Sophisticated multivariate data analysis strategy with strict variable selection and validation procedures (*Study IIb*)	• Lack of corrections for multiple testing or the application of multivariate methods due to inadequate sample size (*Study I*) • Unidentified variables might influence multivariate approaches due to noise or multiple variable collinearities (*Study IIb*)
Metabolite Classification, Pathway Analysis	• Combination of two different approaches for a comprehensive analysis of PE- or PF-related metabolic pathways (*Study I, IIa, IIb*)	• No differentiation between more or less relevant PE- or PF-related metabolites (*Study I, IIa, IIb*) • Dependency of mapped metabolic pathways on the metabolite coverage and a clear metabolite identification (*Study I, IIa, IIb*)

CRF: cardiorespiratory fitness; DXA: dual-energy X-ray absorptiometry; GC × GC: two-dimensional gas chromatography; LC: liquid chromatography; MS: mass spectrometry; NMR: nuclear magnetic resonance; PA: physical activity; PE: physical exercise; PF: physical fitness; QC: quality control; SOPs: standard operating procedures.

9.3 Conclusions and Future Research

Taken together, the present dissertation could add valuable findings on the effects of acute and chronic PA on human metabolite profiles to the literature. Apart from substantiating several previous findings, the three research articles included in this thesis could extend the current state of knowledge on alterations in the human metabolome in response to medium-term HIIT (*Study I*) or acute incremental exercise (*Study IIa*) and provided new insights into the relationship between the metabolome and the CRF as a measure of chronic PA (*Study IIa*/*IIb*). Based on different metabolomics approaches, study-specific statistical procedures, and the subsequent functional classification of PE-/PF-related metabolite profiles, the findings yielded by this thesis showed that the particular training or exercise interventions as well as the CRF status were associated with specific pathways of human metabolism.

Briefly summarized, *Study I* demonstrated HIIT-related changes in 'purine metabolism' and assumed urinary hypoxanthine to be a possibly interesting metabolic marker for training adaptation. With regard to future studies, the utility of urinary hypoxanthine as a reliable and specific biomarker for an athlete's adaptation to training has to be validated. Also, the exact mechanisms underlying the supposed training-induced modifications in 'purine metabolism' will deserve further investigation. In *Study IIa*, the ability of urine to reflect acute exercise-induced metabolic alterations could be confirmed by registering well-established changes in energy-producing pathways, such as 'glycolysis' and 'TCA cycle', or 'AA metabolism'. While the urinary metabolites identified in *Study IIa* did not allow to draw conclusions on the CRF status, *Study IIb* could provide evidence of CRF-associated plasma metabolite patterns. In fact, *Study IIb* revealed sex-specific relationships between the CRF and mainly 'lipid metabolism'- or 'AA metabolism'-linked plasma metabolites which, however, seemed to be partially mediated by clinical or phenotypical covariates. Nevertheless, as some CRF-associated metabolites have already been inversely linked to the development of cardiometabolic diseases (e.g., PCs, lysoPCs) or are likely to reflect exercise-induced adaptations in 'energy metabolism' (e.g., malic acid, succinic acid), *Study IIb* supposed those metabolites to represent potential mediators or markers of the health- or performance-enhancing effects of chronic PA. Given the rather exploratory character of *Study IIb*, more research will be needed to clarify biological mechanisms and pathways underlying the sex-specific differences in CRF-associated metabolite profiles.

Undeniably, metabolomics is a new innovative technology that opens promising avenues to elucidate the complex and interconnected metabolic networks underlying the adaptive responses of the human organism to PA (Zierath & Wallberg-Henriksson, 2015). However, as also demonstrated by the three studies included in the present thesis, main challenges still encountered in human exercise metabolomics research refer to an inadequate metabolite identification, an incomplete standardization of experimental procedures, the dependency of findings on included study participants, and, hence, a limited ability to directly compare results to findings from other studies (Belhaj et al., 2021; Kelly et al., 2020). In addition, the high inter-individual variability in metabolomics measurements owing to differences in sex, age, body composition, lifestyle, nutritional status, or training status represents another major challenge in PE- or PF-related investigations. To overcome this challenge and to account for potential confounding factors, large-scale human metabolomics studies that screen and control for covariates in an experimental setting will be required in the future (Belhaj et al., 2021; Broadhurst & Kell, 2006; Sanford et al., 2020).

Based on the findings from *Study IIa* and *IIb*, the importance to statistically adjust for a variety of assessed phenotypical and clinical covariates in heterogeneous study populations could be deduced. Thus, it can be determined if observed relationships can be specifically attributed to the CRF status. Vice versa, *Study IIa* and *IIb* also clearly indicated the general need to control for acute PE and an individual's PF status in non-exercise-related metabolomics studies. With regard to the acute PE intervention in *Study IIa*, a high inter-individual variance in post-exercise urinary metabolite excursions was observed. To attenuate variations in exercise-related metabolite alterations due to differences in dietary behavior or not strictly controlled time points for sample taking, future research should include standardized dietary plans for the day before and during the exercise trial as well as more controlled collections of multiple biological samples during and after the exercise intervention. In fact, these recommendations have already been adequately considered in the framework of the so-called "Myokine Kinetics (MyoKin)" study – a randomized crossover human exercise intervention study conducted at the MRI in Karlsruhe. As the MyoKin participants followed a prescribed standardized diet and provided blood as well as urine samples at strictly scheduled time points before, during, and after two PE interventions at either moderate or vigorous intensity, this study will permit to advance further in the examination of acute exercise-related changes in the metabolome.

To conclude, the present dissertation can be seen as a valuable contribution to the emerging research field of exercise metabolomics, not only gaining deeper insights into the effects of acute and chronic PA on metabolite profiles in specific healthy populations, but also providing conceptual and methodological suggestions for improving future metabolomics investigations. Actually, the three included studies were mainly explorative with a hypothesis-generating character. Therefore, the molecular mechanisms that might explain the observed metabolite differences between pre- or post-exercise conditions or between more or less fit individuals could not be elucidated in the framework of this thesis. Even though the applied (un)targeted approaches led to the discovery of PE- or PF-related changes in the human metabolome, which is generally considered as the closest reflection of an organism's phenotype (Bujak et al., 2015), metabolomics alone only provides a snapshot of the occurred metabolic processes and will thus not be sufficient to decipher the complex molecular basis of exercise biology or metabolic health and disease (Belhaj et al., 2021). Indeed, there are several future directions to complement human exercise metabolomics research, such as the application of "multi-omics" approaches, "fluxomics" analyses, or animal model systems:

Firstly, to fully understand the interconnected nature of genetic, transcriptional, and post-translational networks underlying the metabolic adaptations to PA, the integration of metabolomics with other "omics"-technologies will be required in the future (N. J. Hoffman, 2017; Kelly et al., 2020). Secondly, more mechanistic insights into the dynamics of metabolic reactions might be yielded by the application of metabolic flux analyses, i.e., a method that combines stable isotope tracing of metabolites with MS or NMR spectroscopy (Johnson et al., 2016). In so-called "fluxomics" analyses, the flux of metabolites through different metabolic pathways can be measured by monitoring heavy atoms from labelled tracer substrates, e.g., glucose or AAs, in particular downstream metabolic products (Wilkinson, Brook, Smith, & Atherton, 2017). Accordingly, detailed information on intracellular metabolic rates and relative pathway activities with respect to particular phenotypes or experimental conditions can be attained (Johnson et al., 2016). Thirdly, a complementary approach to identify the tissue-specific origin of PA-related metabolite changes might be the implementation of animal model systems. In fact, future animal studies could provide access to certain tissues which are relatively inaccessible in human exercise studies, such as skeletal muscle, liver, or fat tissue (Belhaj et al., 2021).

Finally, although it was outside the scope of *Study I*, *IIa*, and *IIb* to clarify the exact molecular mechanisms contributing to the observed PE- or PF-related metabolite profiles, the three (sub-)studies included in this thesis clearly emphasized the potential for metabolomics to detect metabolic alterations related to either acute or chronic PA in easily obtainable human biospecimens. Specific urinary or blood metabolites were highlighted as potential markers for PE or PF and certainly represent an interesting starting point for future mechanistic investigations and validation studies. Obviously, especially observational investigations like *Study II* can help to explore associations between the human metabolome and health- or performance-related variables such as the CRF. Yet, they do not allow to prove cause- and effect-relationships. Therefore, to determine what actually might have caused changes in human metabolite profiles and whether the marker metabolites are merely correlates of the PF status or actually causative for an improved aerobic capacity or health status will continue to be a major challenge.

Nevertheless, as one piece of a big puzzle, the findings obtained in the present thesis – together with results from further metabolomics research – might one day be translated into a sport- or health-related context, serving as a basis for precision medicine strategies, such as the assessment of the individual training, fitness, and cardiometabolic health status or the development of personalized exercise prescription concepts (Heaney et al., 2017; Trivedi, Hollywood, & Goodacre, 2017).

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Appendix

A1 Study I – Supplementary Material

A1.1 Inclusion and Exclusion Criteria (*Study I*)

Table A-1. Inclusion and exclusion criteria for participation in *Study I*. *(Own table).*

Inclusion Criteria	Exclusion Criteria
• Healthy men • Age between 20 and 50 years • Volunteers with at least three years of training and competition experience • Volunteers willing to conduct all examinations and exercise tests • Participants giving their written and informed consent	• Volunteers with cardiovascular or endocrine diseases • Volunteers with acute or chronic musculoskeletal injuries • Volunteers on regular medication • Volunteers with a BMI > 30

BMI: body mass index.

A1.2 Comparison of LC-MS and NMR Metabolite Data (*Study I*)

Table A-2. Linear regression analysis for comparing the urinary concentration of nine metabolites measured with both LC-MS and NMR. *(Adapted from Kistner et al. (2019)).*

	β-Amino-isobutyrate	Betaine	Carnitine	Creatine	Dimethyl-amine	N,N-Dimethyl-glycine	Histidine	TMAO	Trigonelline
r	0.99	0.95	1.00	0.93	0.97	0.98	0.99	1.00	0.99
R^2	0.97	0.90	0.99	0.87	0.94	0.96	0.98	0.99	0.99
intercept	−13.38	−1.07	−1.14	−6.46	21.12	−5.12	50.50	−17.85	−3.21
slope	0.99	0.94	1.18	0.81	0.64	1.25	1.11	1.46	0.94

LC: liquid chromatography; MS: mass spectrometry; NMR: nuclear magnetic resonance; *r*: Pearson correlation coefficient; R^2: coefficient of determination; TMAO: trimethylamine N-oxide.

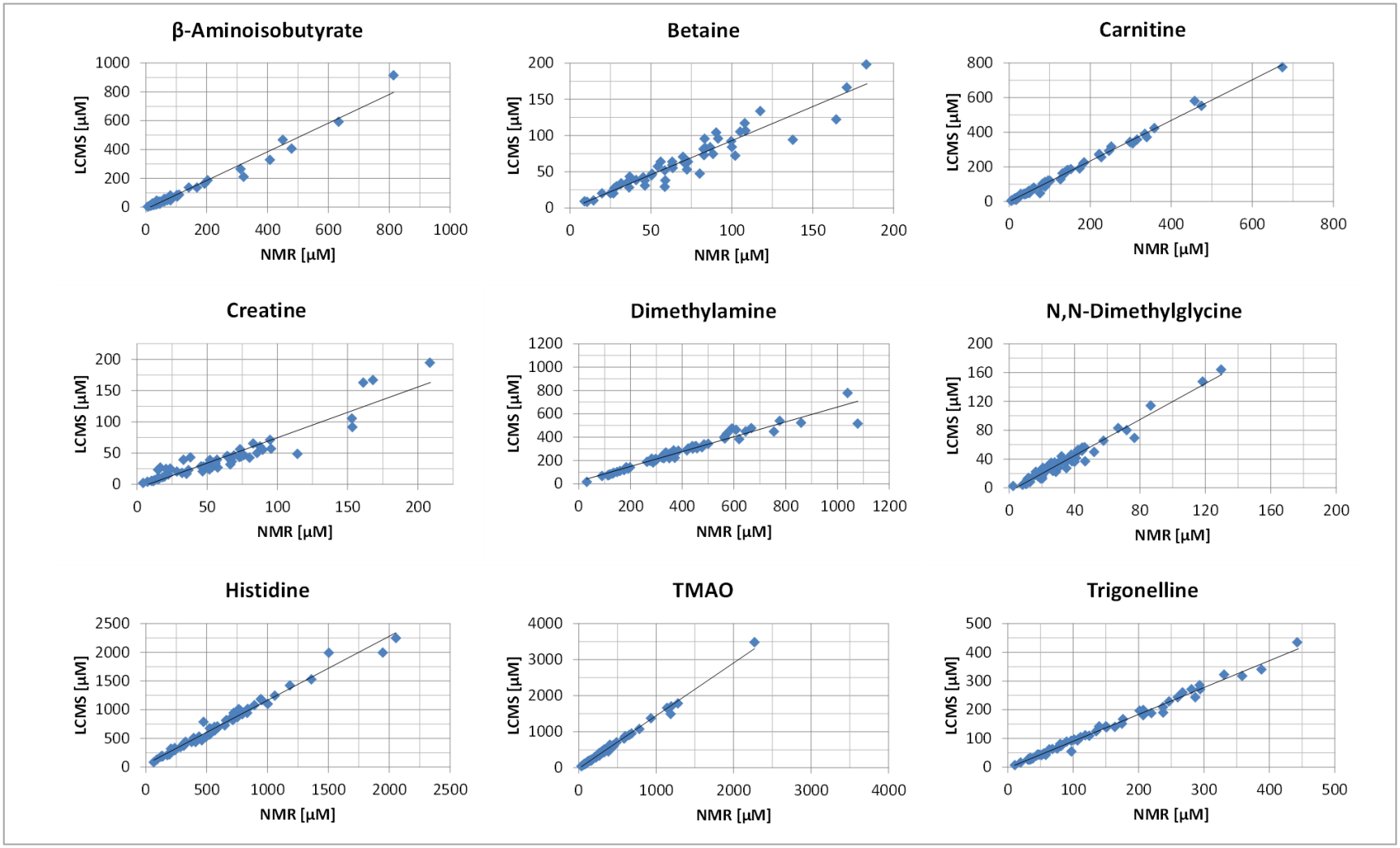


Figure A-1. Comparison of the urinary concentration of nine metabolites measured with both LC-MS and NMR by linear regression. LC: liquid chromatography; MS: mass spectrometry; NMR: nuclear magnetic resonance. *(Reprinted from Kistner et al. (2019)).*

A1.3 Training Parameters (*Study I*)

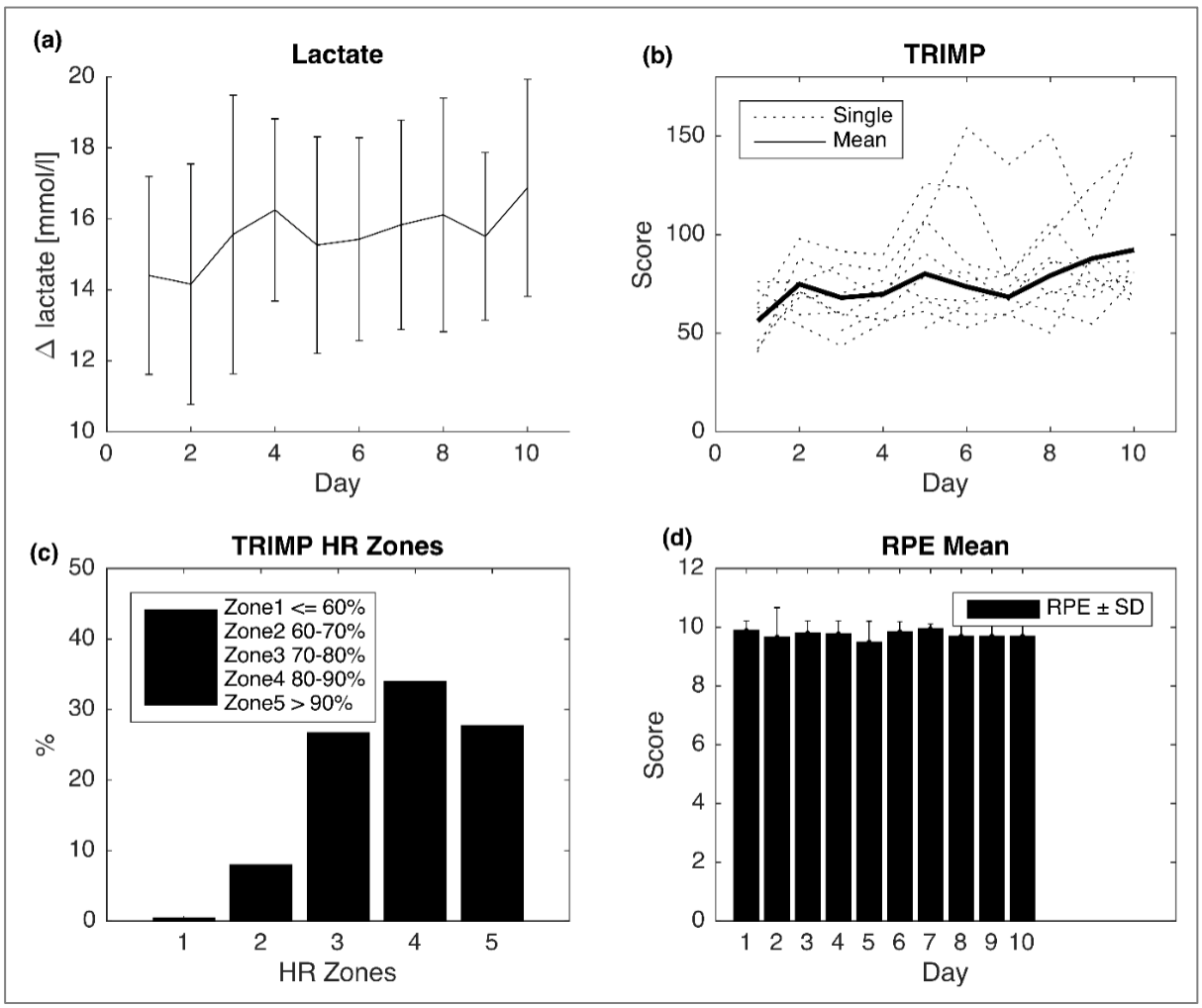


Figure A-2. Training parameters. (a) Differences in post- to pre-exercise blood lactate concentrations in the EG on each study day (mean ± SD); (b) Mean and single TRIMP score(s) of the EG on each study day; (c) HR zone scaling (% of time which the participants of the EG spent in each HR zone); (d) RPE score of the EG on each study day (mean ± SD). EG: experimental group; HR: heart rate; RPE: rating of perceived exertion; SD: standard deviation; TRIMP: training impulse. *(Reprinted from Kistner et al. (2019)).*

A1.4 Table of Urinary Metabolite Concentrations (*Study I*)

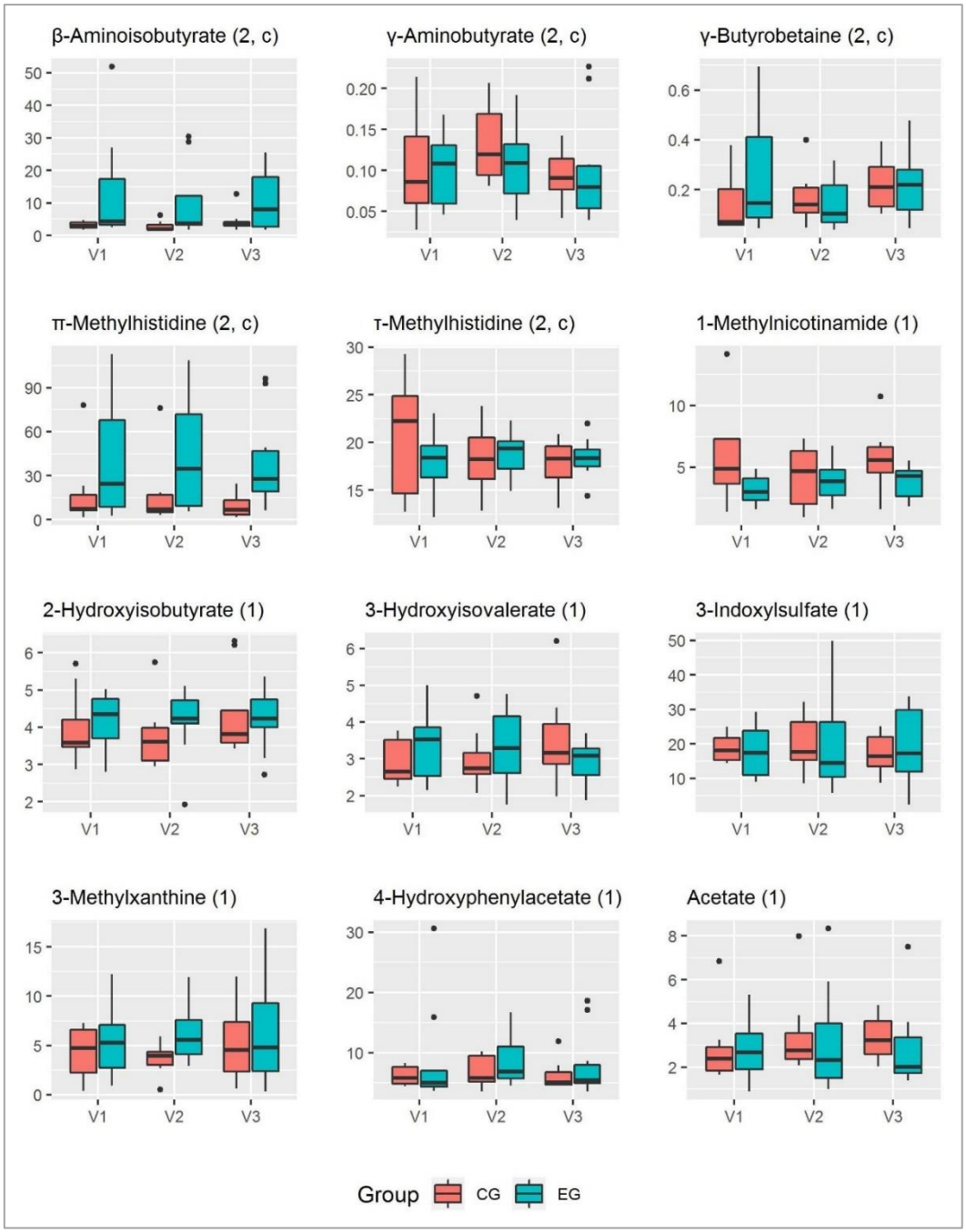
Table A-3. Normalized urinary metabolite concentrations at V1, V2, and V3 in the EG and CG. *(Adapted from Kistner et al. (2019)).*

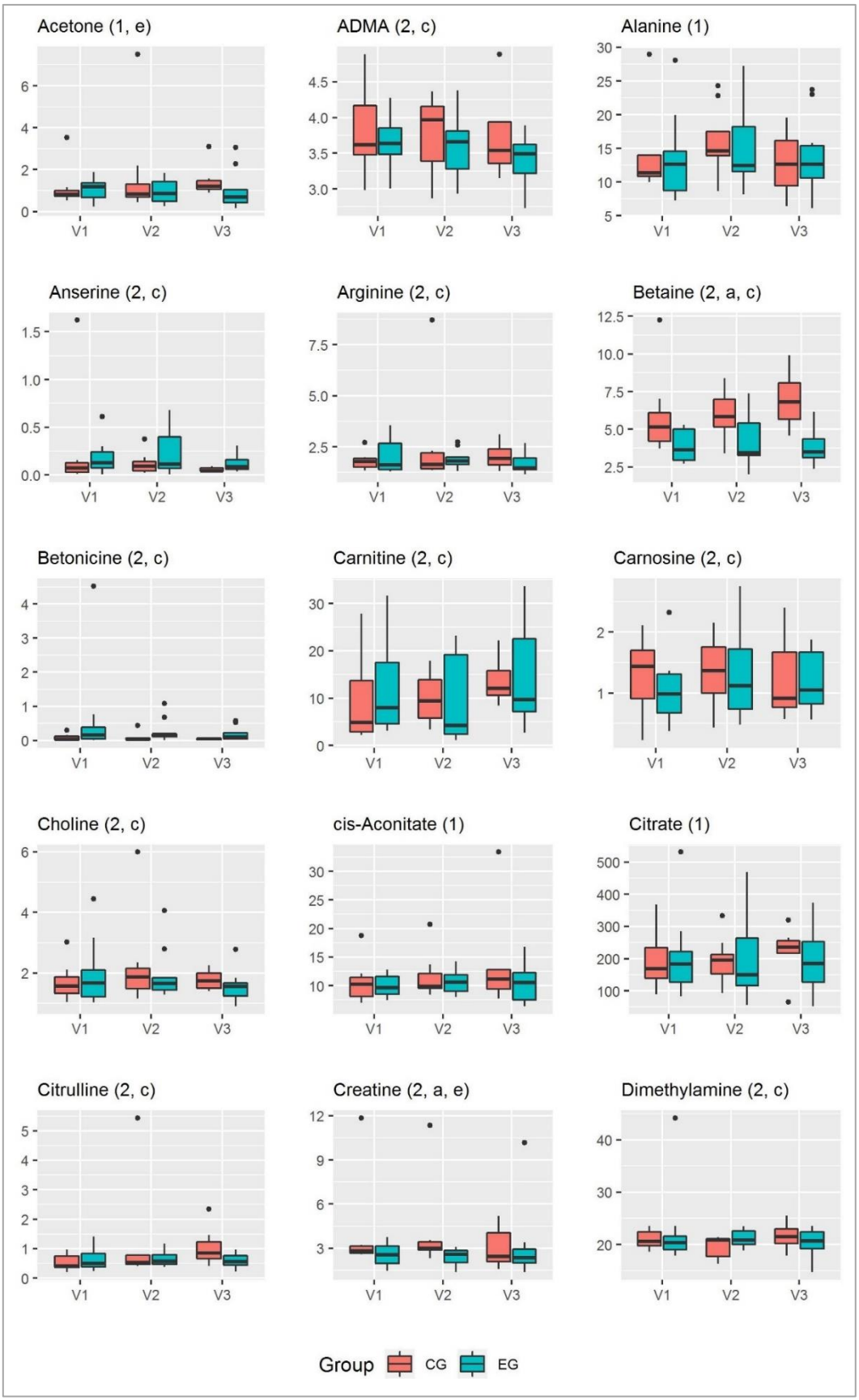
Metabolites	EG (n=10)			CG (n=8)		
	V1	V2	V3	V1	V2	V3
β-Aminoisobutyrate [2, c)]	13 ± 16	10 ± 11	10 ± 9	3.2 ± 1.1	2.9 ± 1.7	4.6 ± 3.7
γ-Aminobutyrate [2, c)]	0.10 ± 0.04	0.11 ± 0.05	0.10 ± 0.07	0.10 ± 0.07	0.13 ± 0.05	0.09 ± 0.03
γ-Butyrobetaine [2, c)]	0.25 ± 0.22	0.14 ± 0.10	0.22 ± 0.14	0.15 ± 0.12	0.18 ± 0.11	0.22 ± 0.12
π-Methylhistidine [2, c)]	42 ± 40	45 ± 40	39 ± 32	19 ± 27	19 ± 26	10 ± 8
τ-Methylhistidine [2, c)]	18 ± 3	19 ± 2	18 ± 2	21 ± 7	18 ± 4	18 ± 3
1-Methylnicotinamide [1]	3.1 ± 1.1	3.8 ± 1.6	3.9 ± 1.3	5.9 ± 3.9	4.3 ± 2.6	5.7 ± 2.7
2-Hydroxyisobutyrate [1]	4.2 ± 0.7	4.2 ± 0.9	4.2 ± 0.8	4.0 ± 1.0	3.8 ± 0.9	4.3 ± 1.2
3-Hydroxyisovalerate [1]	3.4 ± 1.0	3.4 ± 1.1	2.9 ± 0.6	2.9 ± 0.6	3.0 ± 0.8	3.5 ± 1.3
3-Indoxylsulfate [1]	18 ± 8	21 ± 16	20 ± 11	19 ± 4	20 ± 8	17 ± 6
3-Methylxanthine [1]	5.2 ± 3.5	6.4 ± 3.1	6.3 ± 5.4	4.3 ± 2.6	3.6 ± 1.6	5.5 ± 4.3
4-Hydroxyphenylacetate [1]	8.7 ± 8.5	8.5 ± 4.0	7.9 ± 5.5	6.2 ± 1.6	6.9 ± 2.6	6.3 ± 2.5
Acetate [1]	2.8 ± 1.4	3.2 ± 2.4	2.9 ± 1.9	2.9 ± 1.7	3.5 ± 2.0	3.3 ± 1.0
Acetone [1, e)]	1.1 ± 0.5	1.0 ± 0.6	1.0 ± 0.9	1.2 ± 1.0	1.8 ± 2.4	1.5 ± 0.8
ADMA [2, c)]	3.7 ± 0.4 [†b]	3.6 ± 0.5	3.4 ± 0.4 [†b]	3.8 ± 0.7	3.8 ± 0.6	3.7 ± 0.6
Alanine [1]	14 ± 6	15 ± 6	14 ± 6	14 ± 6	16 ± 5	13 ± 5
Anserine [2, c)]	0.18 ± 0.18	0.23 ± 0.23	0.13 ± 0.10	0.29 ± 0.59	0.12 ± 0.12	0.06 ± 0.02
Arginine [2, c)]	2.1 ± 0.9	1.9 ± 0.5	1.7 ± 0.6	1.8 ± 0.5	2.7 ± 2.7	2.1 ± 0.6
Betaine [2, a) c)]	3.9 ± 1.1	4.3 ± 1.8	3.9 ± 1.3 [*]	6.0 ± 3.0	6.0 ± 1.7	7.0 ± 1.9 [*]
Betonicine [2, c)]	0.64 ± 1.38	0.28 ± 0.34	0.19 ± 0.21	0.09 ± 0.10	0.10 ± 0.15	0.04 ± 0.03
Carnitine [2, c)]	12 ± 10	10 ± 9	15 ± 11	10 ± 10	10 ± 6	15 ± 5
Carnosine [2, c)]	1.0 ± 0.6	1.3 ± 0.7	1.2 ± 0.5	1.3 ± 0.7	1.4 ± 0.6	1.3 ± 0.7
Choline [2, c)]	2.0 ± 1.1	1.9 ± 0.9	1.6 ± 0.5	1.7 ± 0.7	2.3 ± 1.7	1.8 ± 0.3
cis-Aconitate [1]	10 ± 2	11 ± 2	11 ± 3	11 ± 4	12 ± 4	13 ± 8
Citrate [1]	200 ± 130	200 ± 130	190 ± 100	200 ± 100	200 ± 70	230 ± 70
Citrulline [2, c)]	0.7 ± 0.4	0.6 ± 0.3	0.6 ± 0.3	0.5 ± 0.3 [†b]	1.3 ± 1.8	1.1 ± 0.7 [†b]
Creatine [2, a) c)]	2.6 ± 0.8	2.4 ± 0.6	3.2 ± 2.7	4.1 ± 3.4	4.2 ± 3.2	3.1 ± 1.4
Dimethylamine [2, c)]	23 ± 7.8	21 ± 2	20 ± 3	21 ± 2	19 ± 2	22 ± 3
N,N-Dimethylglycine [2, a) c)]	2.3 ± 0.8	2.7 ± 1.3	2.2 ± 0.9	2.5 ± 1.1	2.7 ± 0.7	2.8 ± 0.8
Dimethylsulfone [1]	4.2 ± 3.6	3.9 ± 2.8	3.9 ± 3.1	3.3 ± 1.4	4.5 ± 4.4	4.3 ± 2.2
Formate [1]	11 ± 5	13 ± 9	13 ± 7	13 ± 5	15 ± 9	16 ± 5
Gluconate [1]	24 ± 8	26 ± 6	24 ± 5	24 ± 5	23 ± 7	2 ± 7

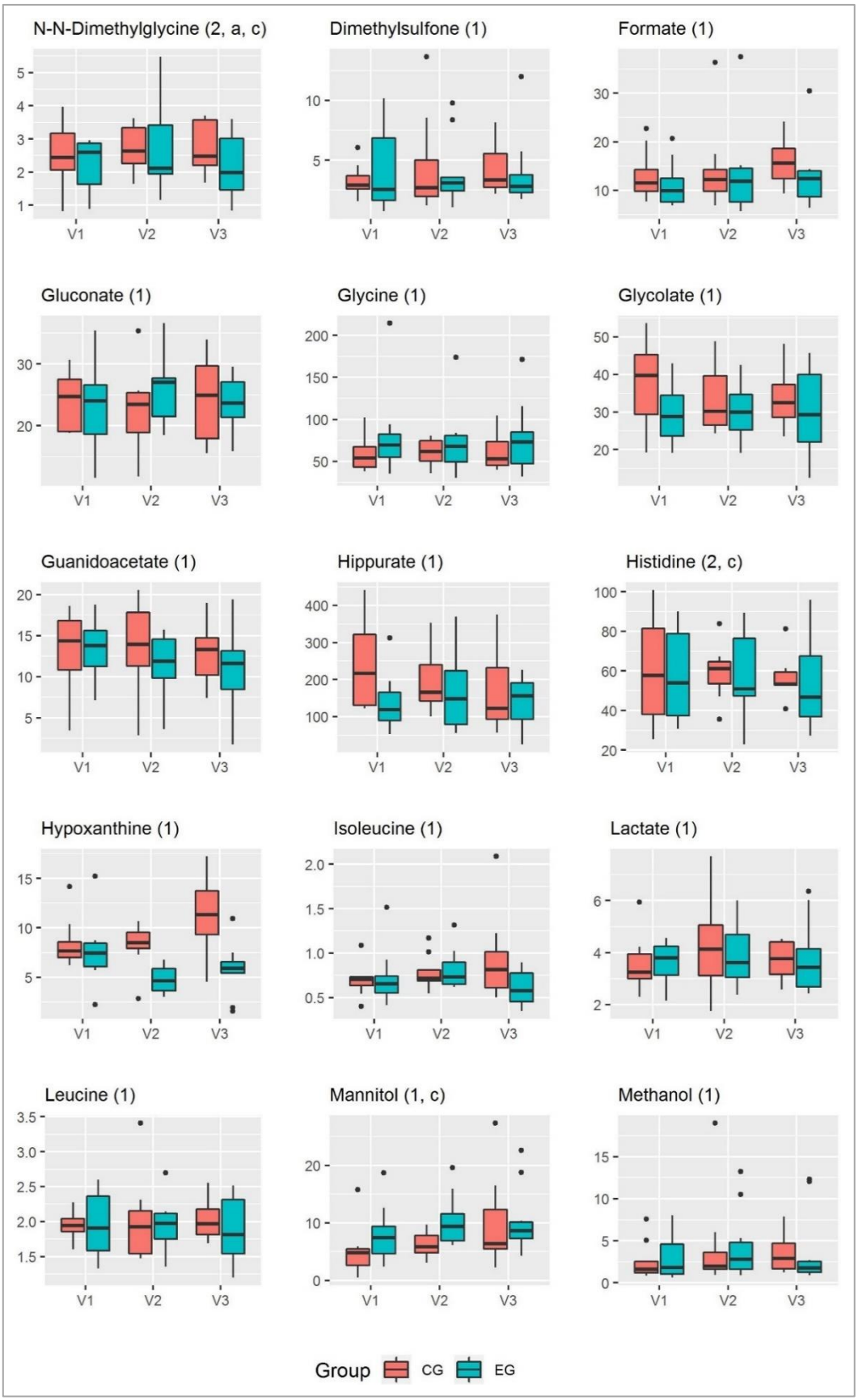
Glycine [1]	80	±	50	72	±	40	78	±	41	60	±	22	61	±	16	62	±	24
Glycolate [1]	29	±	8	31	±	8	30	±	11	37	±	13	34	±	9	34	±	8
Guanidoacetate [1]	13	±	4	11	±	4	11	±	5	13	±	5	14	±	6	13	±	4
Hippurate [1]	140	±	80	170	±	110	140	±	70	250	±	130	190	±	80	170	±	110
Histidine [2, c)]	58	±	22	59	±	21	55	±	25	60	±	28	60	±	15	57	±	12
Hypoxanthine [1]	7.6	±	3.3 †a	4.8	±	1.4 * †a	5.8	±	2.6 *	8.5	±	2.7 †b	8.2	±	2.4 * †c	11.4	±	3.9 * †b †c
Isoleucine [1]	0.72	±	0.32	0.81	±	0.22	0.61	±	0.19 *	0.70	±	0.20	0.78	±	0.20	0.95	±	0.51 *
Lactate [1]	3.6	±	0.8	3.8	±	1.2	3.8	±	1.4	3.6	±	1.1	4.3	±	1.9	3.7	±	0.8
Leucine [1]	2.0	±	0.5	2.0	±	0.4	1.9	±	0.5	1.9	±	0.2	2.0	±	0.6	2.0	±	0.3
Mannitol [1, c)]	8.1	±	4.8	10.4	±	4.4	10.5	±	5.7	5.3	±	5.1	6.3	±	2.5	10.2	±	8.8
Methanol [1]	2.9	±	2.5	4.4	±	4.2	3.7	±	4.5	2.6	±	2.4	4.5	±	6.1	3.6	±	2.5
Methylamine [1, b)]	2.9	±	1.6	2.4	±	1.2	2.3	±	1.0	2.3	±	0.8	2.4	±	0.7	2.2	±	0.7
N-Methylarginine [2, c)]	0.03	±	0.03	0.02	±	0.01	0.02	±	0.02	0.01	±	0.01 †b	0.04	±	0.06	0.04	±	0.03 †b
N-Methylproline [2, a) c)]	0.08	±	0.07	0.20	±	0.31	0.09	±	0.04	0.25	±	0.34	0.15	±	0.15	0.19	±	0.16
Methylsuccinate [1]	5.4	±	1.4	6.1	±	2.3	5.7	±	1.4	6.1	±	2.4 †b	6.2	±	1.1	7.4	±	3.4 †b
Proline [2, a) c)]	0.61	±	0.28	0.56	±	0.15	0.53	±	0.07	0.67	±	0.46	1.10	±	1.17	0.91	±	0.43
Pseudouridine [1]	11	±	1	11	±	1	11	±	1	11	±	1	11	±	1	11	±	1
Pyruvate [1]	2.2	±	0.8	2.2	±	0.7	2.0	±	0.5	2.0	±	0.6	2.6	±	1.3	1.8	±	0.5
Sarcosine [2, a) c)]	0.05	±	0.02	0.05	±	0.02	0.06	±	0.05	0.09	±	0.06	0.11	±	0.11	0.07	±	0.06
SDMA [2, c)]	33	±	4	35	±	4	33	±	3	34	±	2	31	±	1	33	±	6
Stachydrine [2, c)]	15	±	19	28	±	30	17	±	12	17	±	16	11	±	11	12	±	11
Succinate [1]	1.4	±	0.7	2.1	±	1.4	2.0	±	1.1	2.5	±	1.3	2.3	±	1.8	2.3	±	0.8
Tartrate [1, b) d)]	1.8	±	1.4	2.9	±	3.0	2.1	±	1.8	1.6	±	1.1	1.9	±	1.4	1.3	±	0.5
Taurine [1, c)]	44	±	28 †b	23	±	25	18	±	15 †b	36	±	32	61	±	68	56	±	38
Threonine [1]	6.8	±	2.2	6.8	±	1.8	7.2	±	2.9	7.6	±	2.4	7.6	±	2.7	7.2	±	3.4
trans-Aconitate [1]	3.2	±	0.7	3.9	±	1.4	3.5	±	0.7	3.1	±	0.6	2.9	±	1.7	3.0	±	0.8
Trigonelline [2, c)]	15	±	15	12	±	10	14	±	11	12	±	8	11	±	7	11	±	10
Trimethylamine [2, c)]	0.28	±	0.17	0.25	±	0.10	0.20	±	0.07	0.25	±	0.09	0.31	±	0.19	0.20	±	0.06
TMAO [2, c)]	56	±	55	45	±	28	37	±	23	36	±	17	30	±	12	58	±	42
Tyrosine [1]	7.4	±	2.6	7.5	±	2.4	7.1	±	2.5	8.0	±	2.7	8.6	±	3.7	8.1	±	2.6
Uracil [1]	7.2	±	2.8	6.9	±	3.7	6.8	±	3.1	6.0	±	1.4	5.9	±	2.5	6.0	±	1.1
Urea [1]	2400	±	1100	2700	±	1100	2300	±	800	2100	±	500 †b	2600	±	1400	2900	±	700 †b
Valine [1]	2.3	±	0.7	2.5	±	0.6	2.4	±	0.6	2.6	±	0.4	2.6	±	0.4	2.6	±	0.5

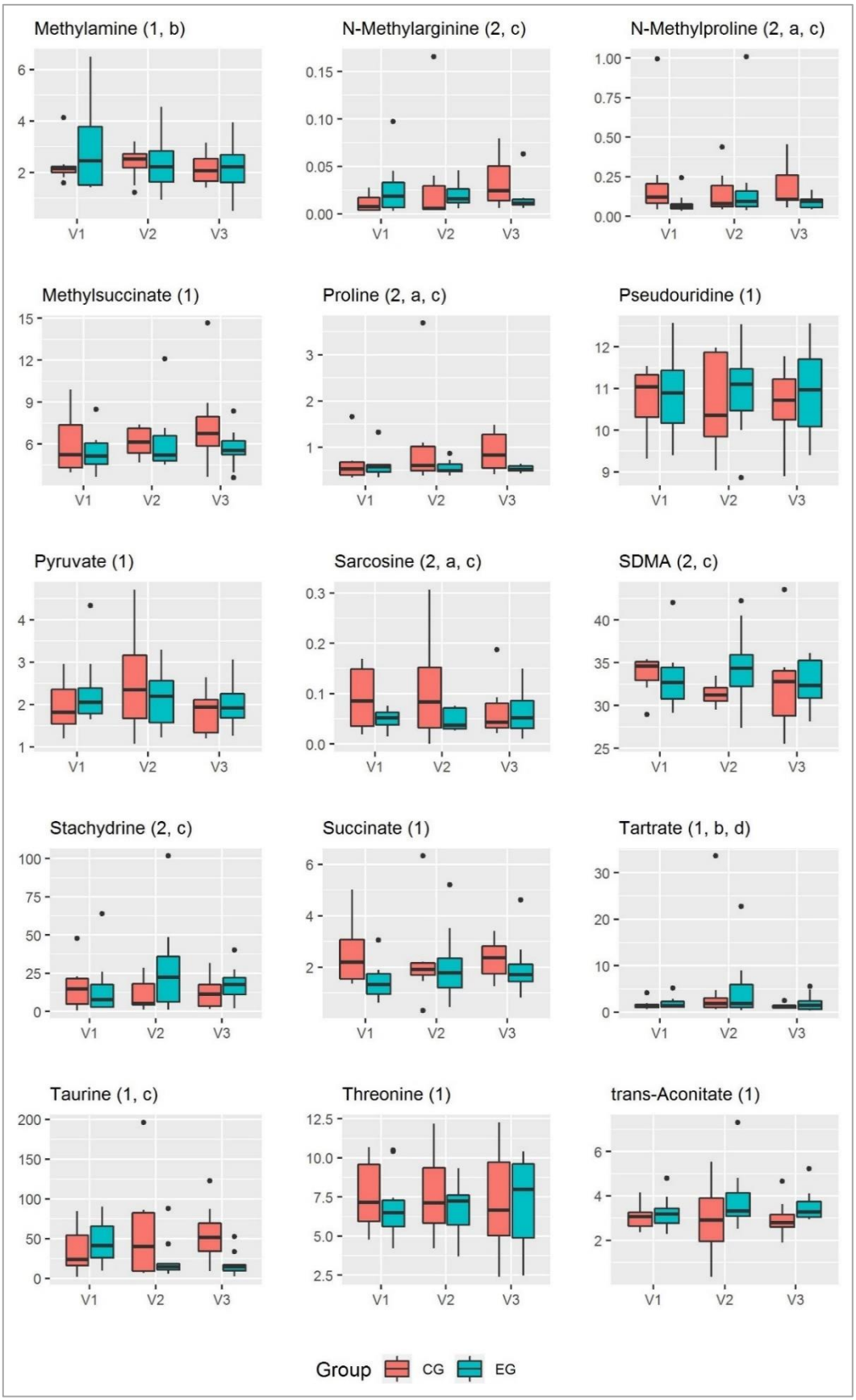
All values in mean ± SD (mmol/mol creatinine). [1] NMR-detected; [2] LC-MS-detected; [a)] n=9 for EG; [b)] n=9 for EG only at V2; [c)] n=7 for CG; [d)] n=7 for CG only at V2; [e)] n=7 for CG only at V3; * $p \leq 0.05$: significant difference between groups; †[a] $p \leq 0.05$: significant difference between V1 and V2 within one group; †[b] $p \leq 0.05$: significant difference between V1 and V3 within one group; †[c] $p \leq 0.05$: significant difference between V2 and V3 within one group. ADMA: asymmetric dimethylarginine; CG: control group; EG: experimental group; n: sample size; *p*: *p*-value based on t-test; SDMA: symmetric dimethylarginine; TMAO: trimethylamine N-oxide; V1: Visit 1; V2: Visit 2; V3: Visit 3.

A1.5 Boxplots of Urinary Metabolite Concentrations (*Study I*)




Acetone (1, e)
ADMA (2, c)
Alanine (1)
Anserine (2, c)
Arginine (2, c)
Betaine (2, a, c)
Betonicine (2, c)
Carnitine (2, c)
Carnosine (2, c)
Choline (2, c)
cis-Aconitate (1)
Citrate (1)
Citrulline (2, c)
Creatine (2, a, e)
Dimethylamine (2, c)
V1
V2
V3
Group
CG
EG


N-N-Dimethylglycine (2, a, c)
Dimethylsulfone (1)
Formate (1)
Gluconate (1)
Glycine (1)
Glycolate (1)
Guanidoacetate (1)
Hippurate (1)
Histidine (2, c)
Hypoxanthine (1)
Isoleucine (1)
Lactate (1)
Leucine (1)
Mannitol (1, c)
Methanol (1)
V1
V2
V3
Group
CG
EG


Methylamine (1, b)
N-Methylarginine (2, c)
N-Methylproline (2, a, c)
Methylsuccinate (1)
Proline (2, a, c)
Pseudouridine (1)
Pyruvate (1)
Sarcosine (2, a, c)
SDMA (2, c)
Stachydrine (2, c)
Succinate (1)
Tartrate (1, b, d)
Taurine (1, c)
Threonine (1)
trans-Aconitate (1)
V1
V2
V3
Group
CG
EG

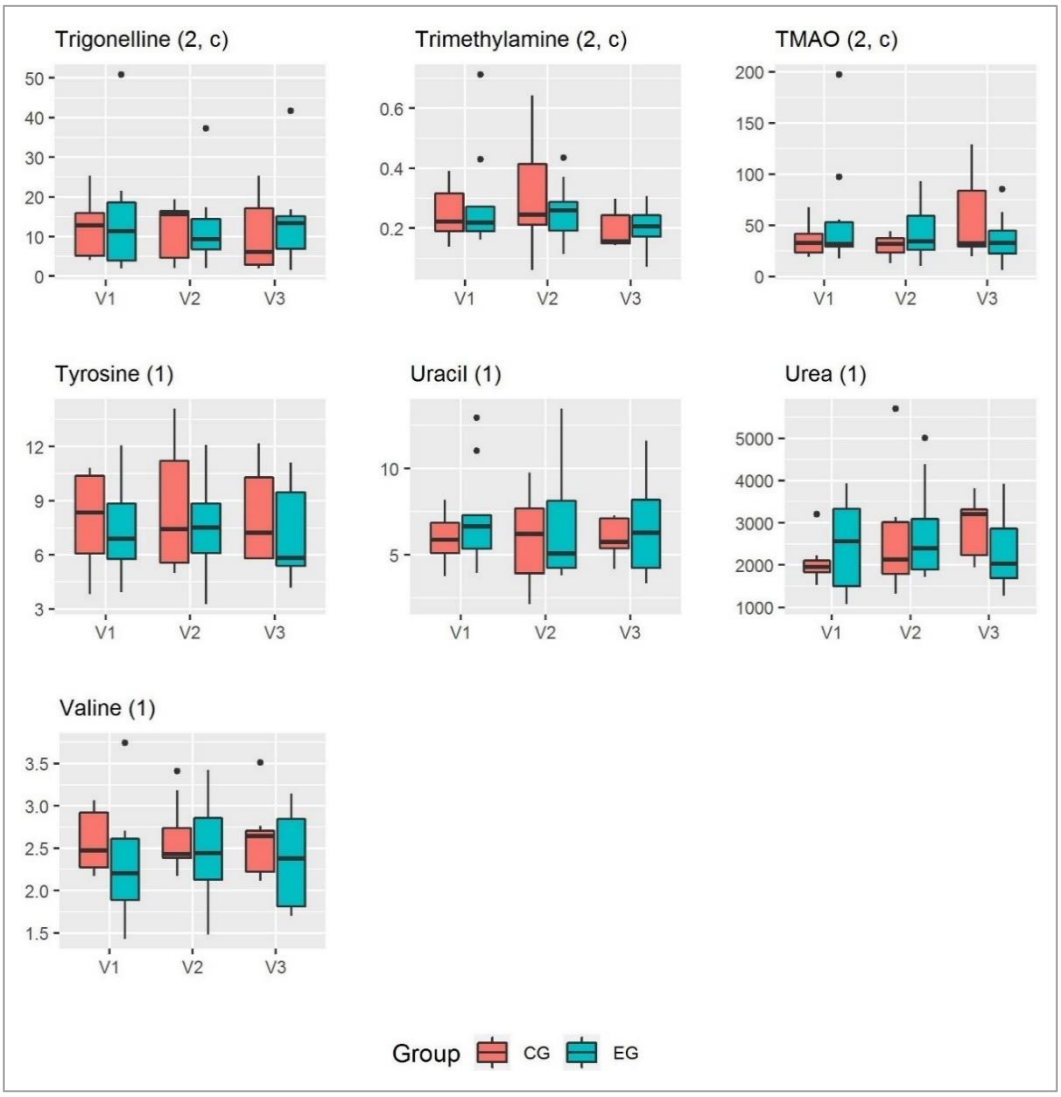


Figure A-3. Boxplots of urinary metabolite concentrations at V1, V2, and V3. (1): NMR-detected, (2): LC-MS-detected; concentrations in mmol/mol creatinine; n=10 (EG) and n=8 (CG), except: a) n=9 (EG); b) n=9 (EG; only at V2); c) n=7 (CG); d) n=7 (CG; only at V2); e) n=7 (CG; only at V3). ADMA: asymmetric dimethylarginine; CG: control group; EG: experimental group; LC: liquid chromatography; MS: mass spectrometry; n: sample size; NMR: nuclear magnetic resonance; SDMA: symmetric dimethylarginine; TMAO: trimethylamine N-oxide; V1: Visit 1; V2: Visit 2; V3: Visit 3. *(Reprinted from Kistner et al. (2019)).*

A2 Study II – Supplementary Material

A2.1 Inclusion and Exclusion Criteria (*Study II*)

Table A-4. Inclusion and exclusion criteria for participation in *Study II*. *(Adapted from Bub et al. (2016)).*

Inclusion Criteria	Exclusion Criteria
• Healthy men and women • Age of 18 years or older • Non-smokers • Volunteers willing to conduct all examinations and tests • Participants giving their written and informed consent	• Smokers • Volunteers on regular medication • Volunteers taking supplements • Women using hormonal contraceptives • Pregnant or breastfeeding women • Volunteers with diseases of the cardio-vascular system, gastrointestinal tract, metabolism, nervous system, lungs, skin, viscera, and infectious or immunological diseases in therapeutic need • Volunteers with tumors, acute or chronic infectious diseases • Volunteers with known allergy against para-aminobenzoic acid or intolerance against Finalgon • Volunteers with drug or alcohol abuse • Volunteers who may not adhere to the study protocol • Volunteers who gave no written consent • Institutionalized patients in psychiatric hospitals

A2.2 Flowchart: From the Recruitment Process to Data Analysis (*Study II*)

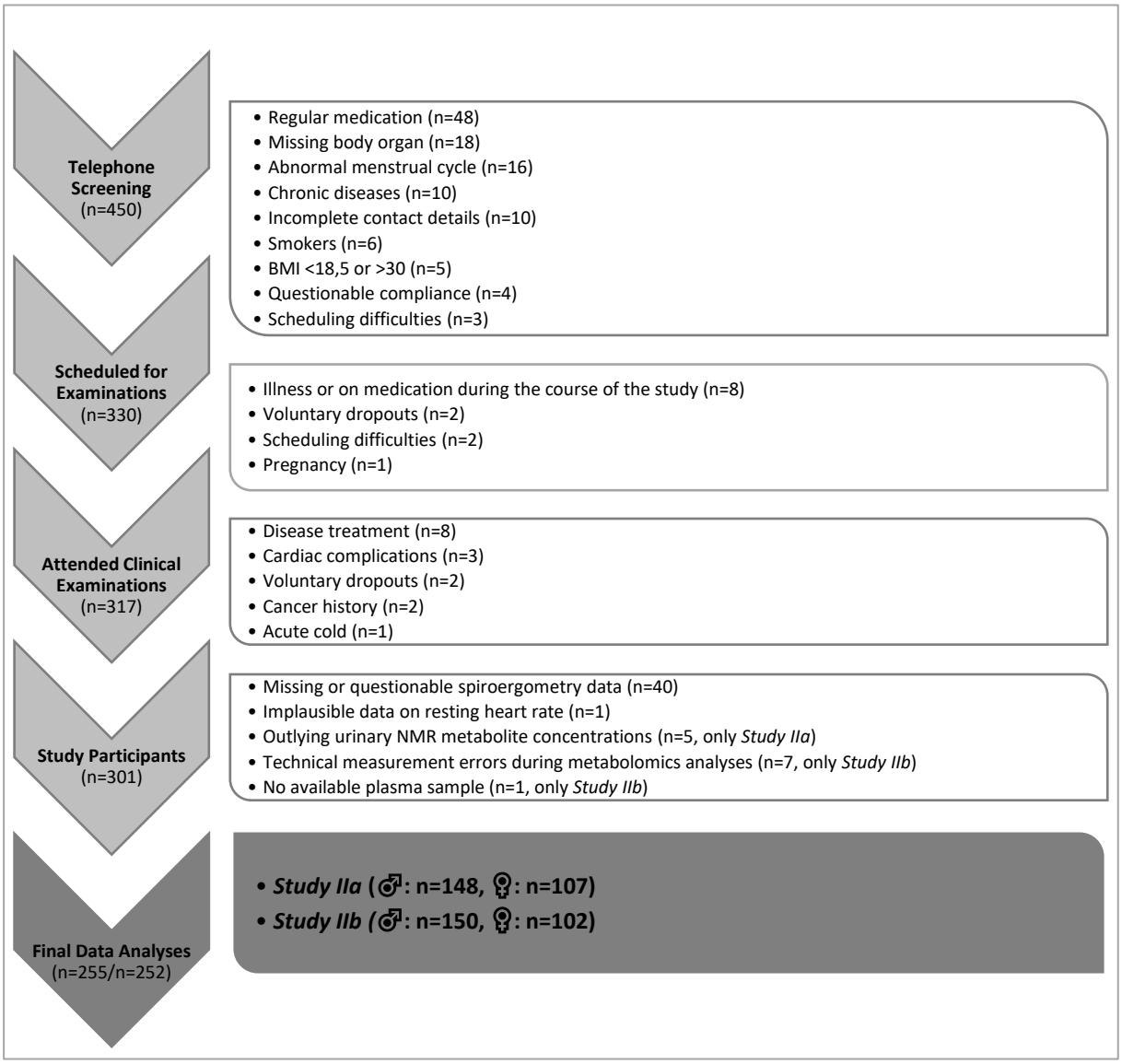
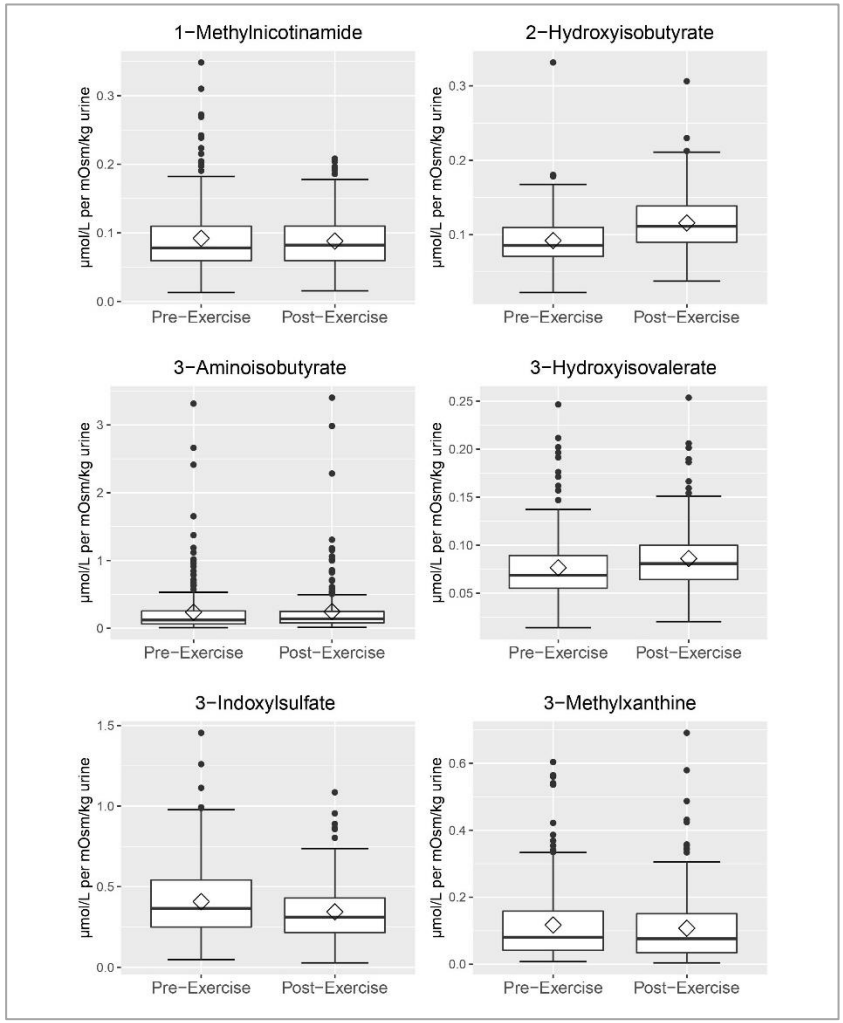
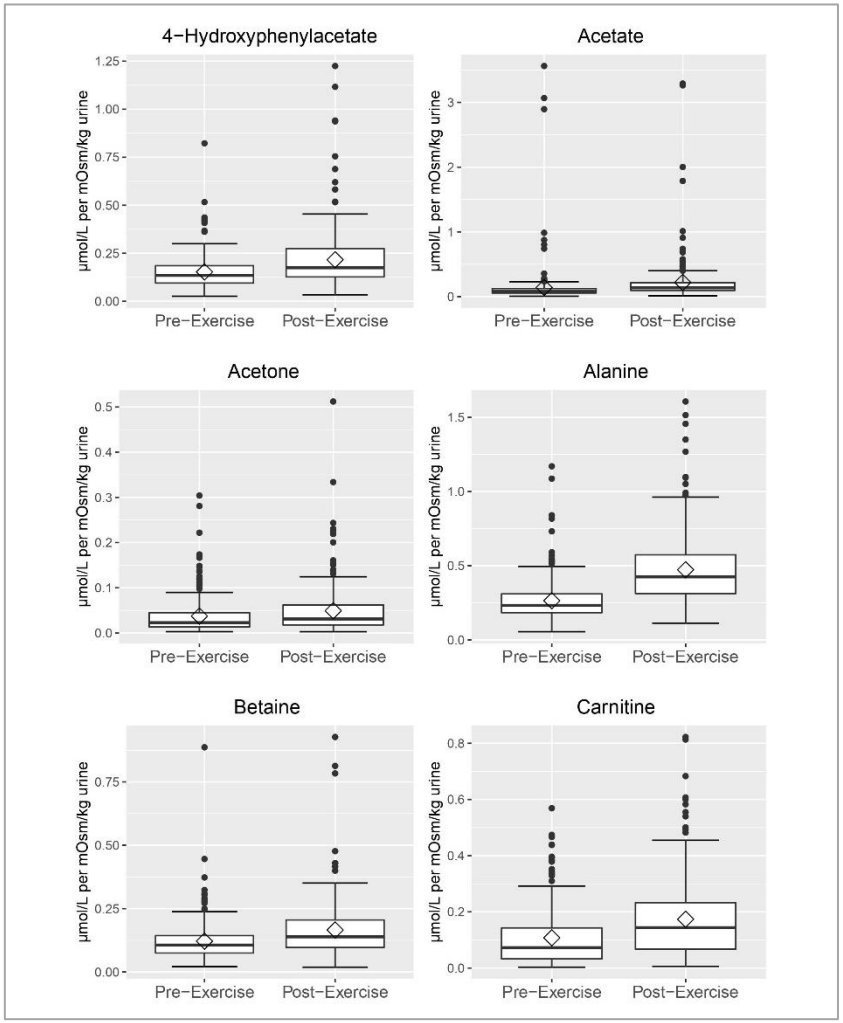
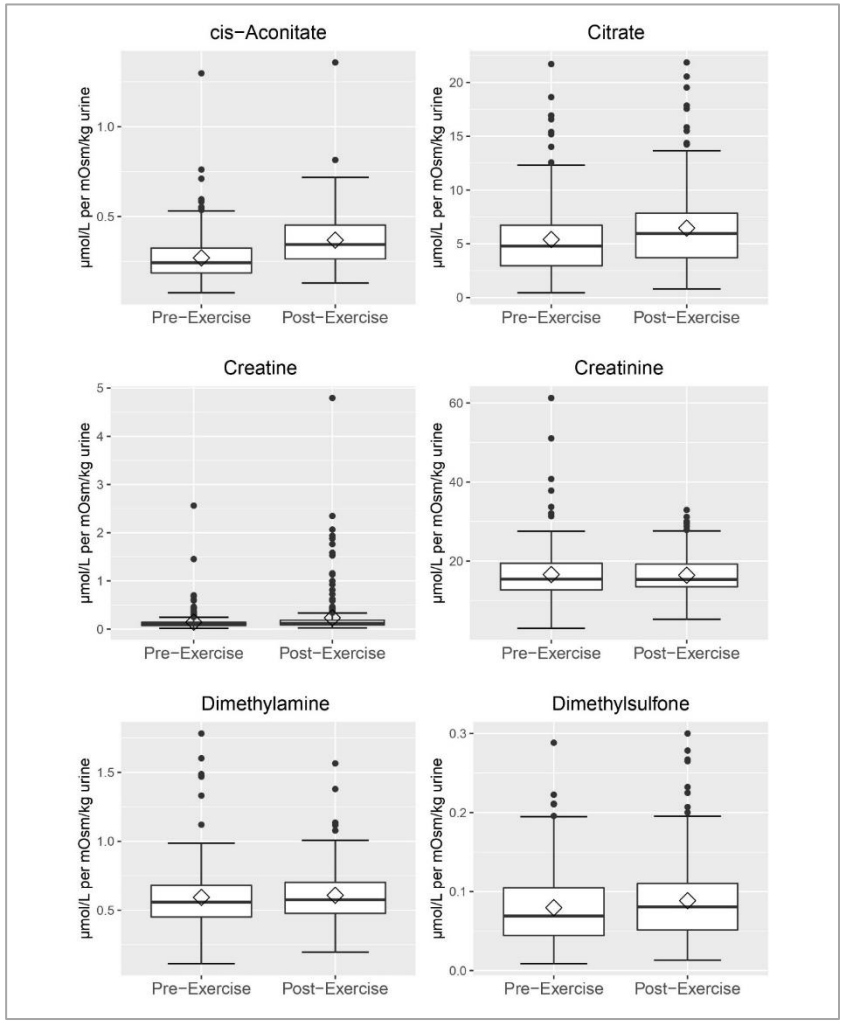


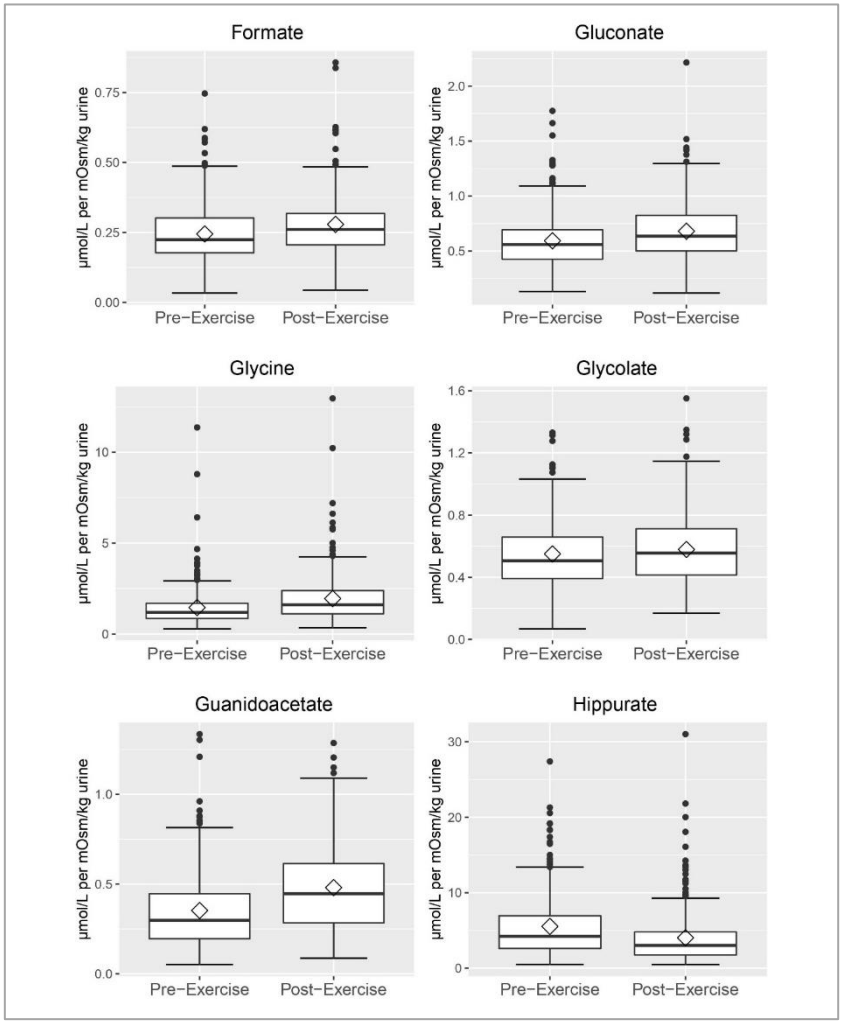
Figure A-4. Flowchart of *Study II*: From the recruitment process to data analysis. On the right side, reasons for the exclusion of participants are presented. BMI: body mass index; n: sample size; NMR: nuclear magnetic resonance. *(Own illustration based on Bub et al. (2016)).*

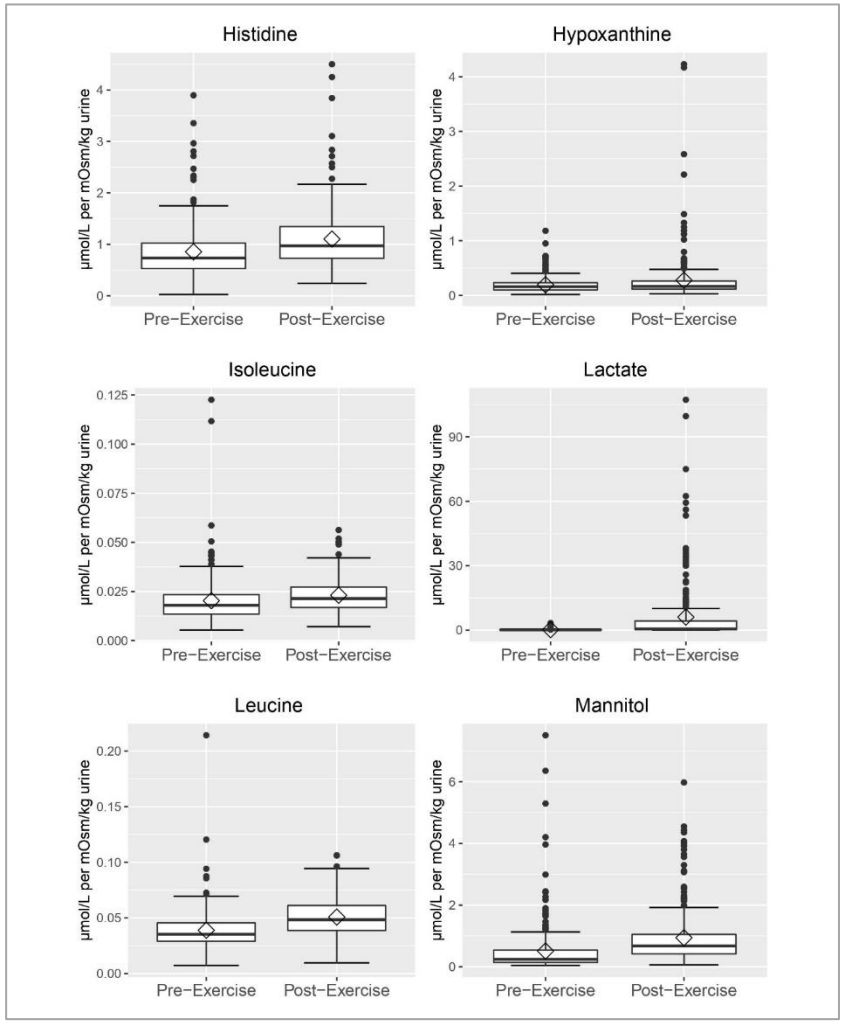
A2.3 Boxplots of Urinary Metabolite Concentrations Pre- vs. Post-Exercise (*Study IIa*)

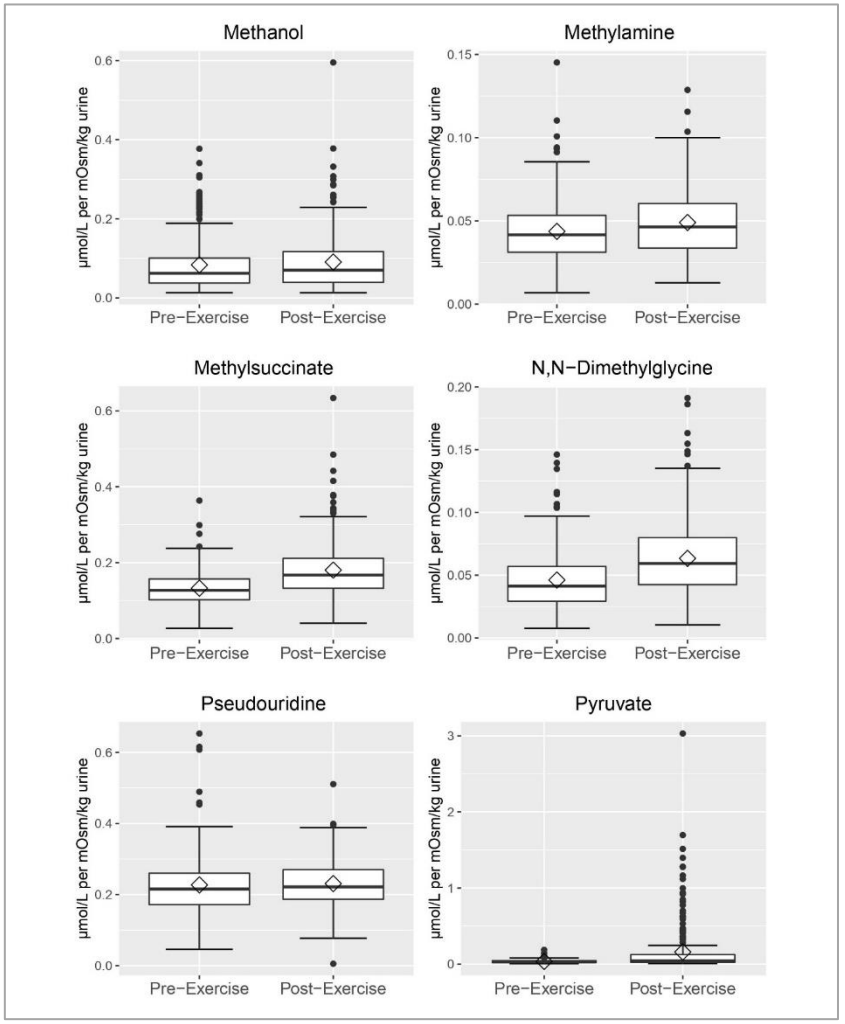





cis−Aconitate
Citrate
Creatine
Creatinine
Dimethylamine
Dimethylsulfone
µmol/L per mOsm/kg urine
Pre−Exercise
Post−Exercise


Formate
Gluconate
Glycine
Glycolate
Guanidoacetate
Hippurate
µmol/L per mOsm/kg urine
Pre−Exercise
Post−Exercise


Histidine
Hypoxanthine
Isoleucine
Lactate
Leucine
Mannitol
µmol/L per mOsm/kg urine
Pre−Exercise
Post−Exercise


Methanol
Methylamine
Methylsuccinate
N,N−Dimethylglycine
Pseudouridine
Pyruvate
µmol/L per mOsm/kg urine
Pre−Exercise
Post−Exercise

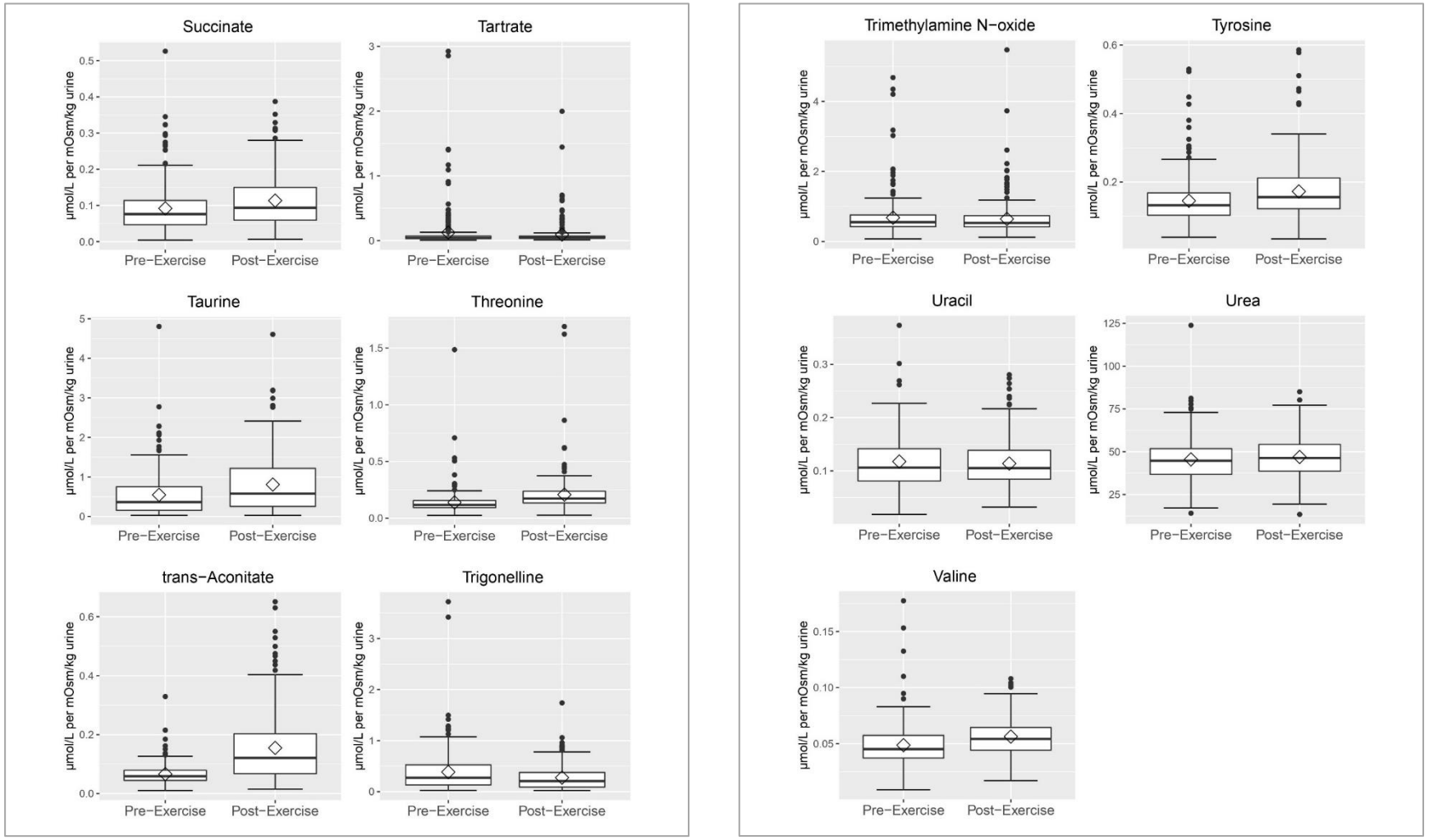
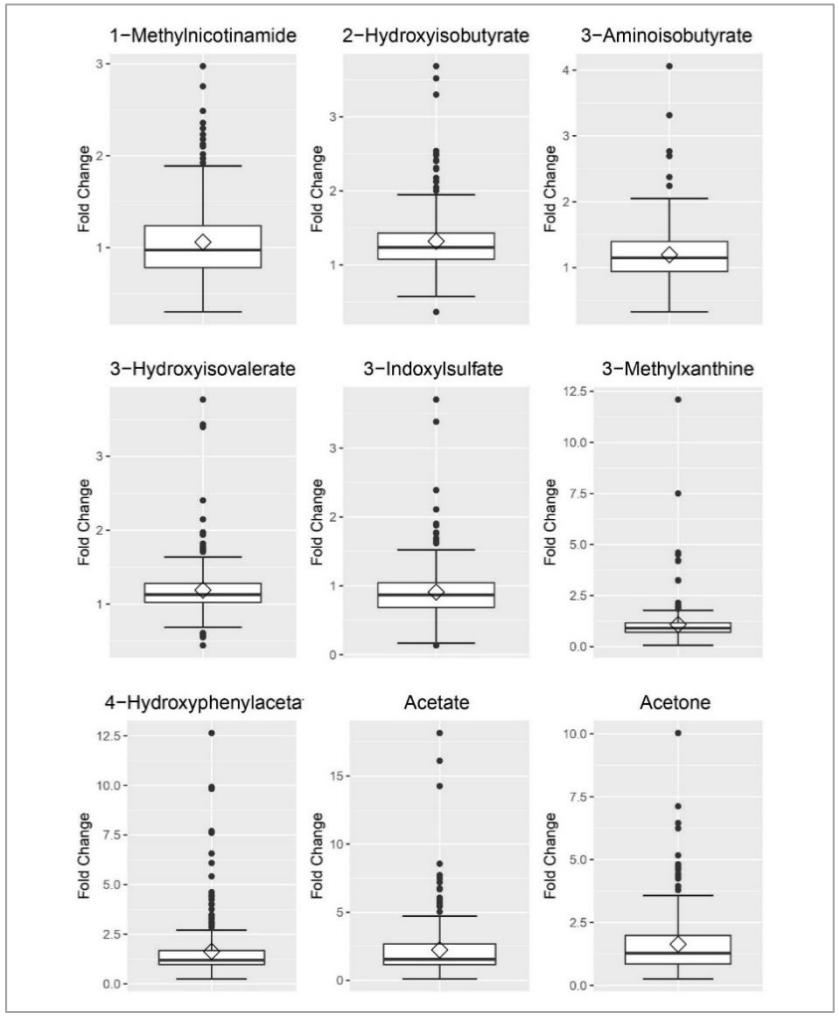
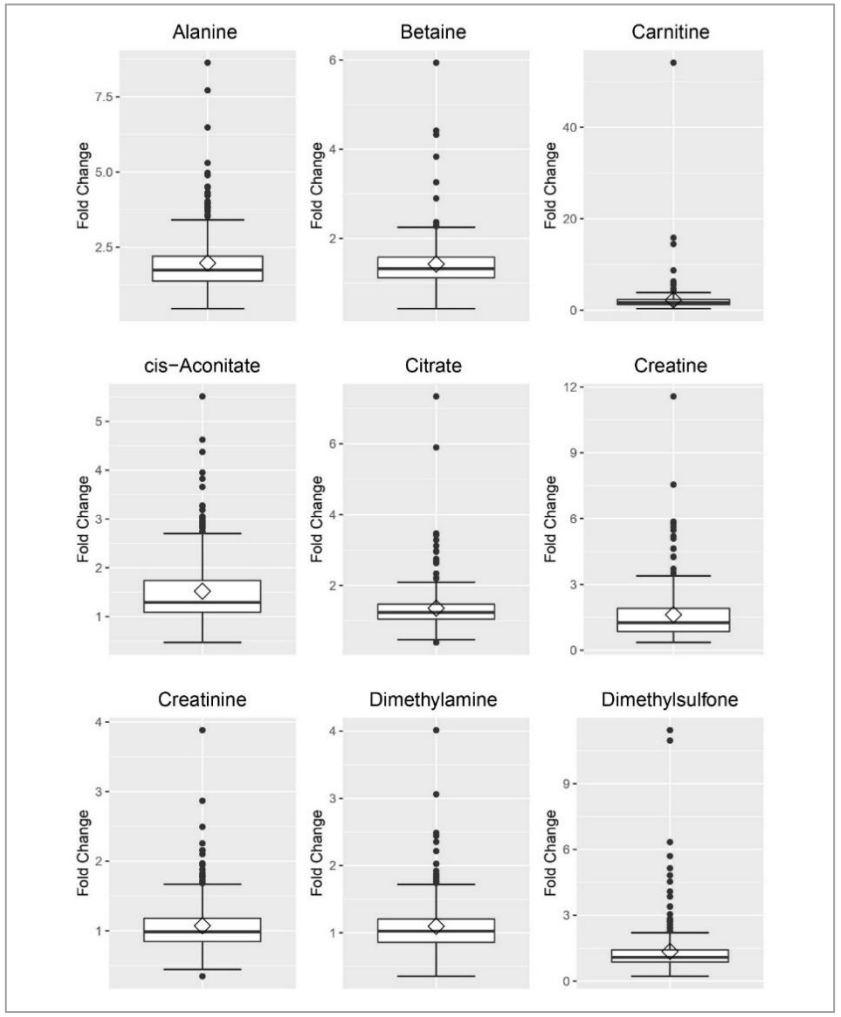
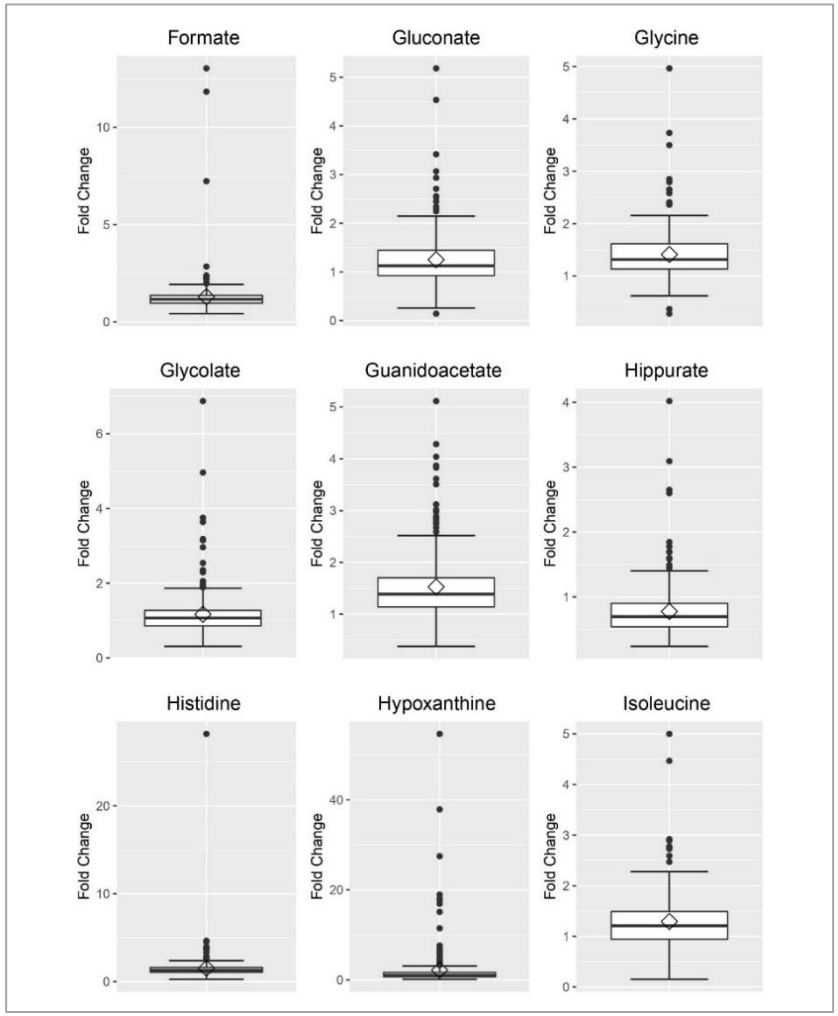


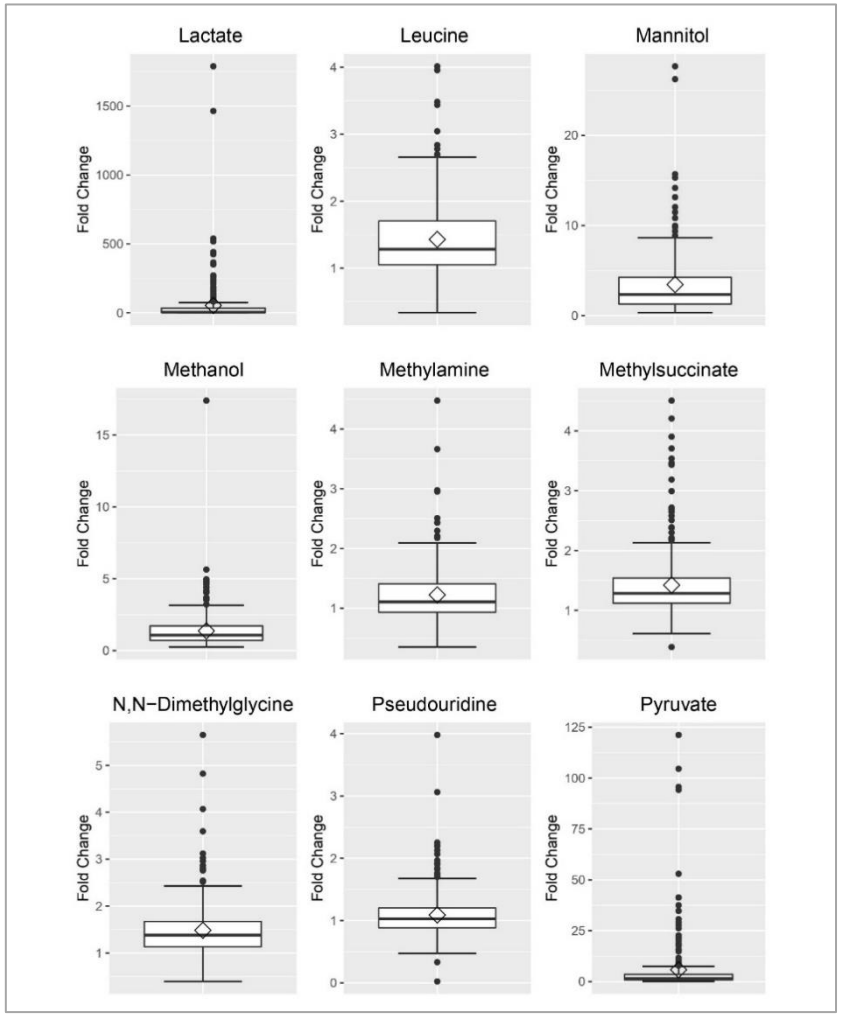
Figure A-5. Boxplots of urinary metabolite concentrations pre- vs. post-exercise. ◊: mean. *(Reprinted from Kistner et al. (2020)).*

A2.4 Boxplots of Urinary Metabolite FCs (*Study IIa*)






Formate
Gluconate
Glycine
Glycolate
Guanidoacetate
Hippurate
Histidine
Hypoxanthine
Isoleucine
Fold Change


Lactate
Leucine
Mannitol
Methanol
Methylamine
Methylsuccinate
N,N−Dimethylglycine
Pseudouridine
Pyruvate
Fold Change

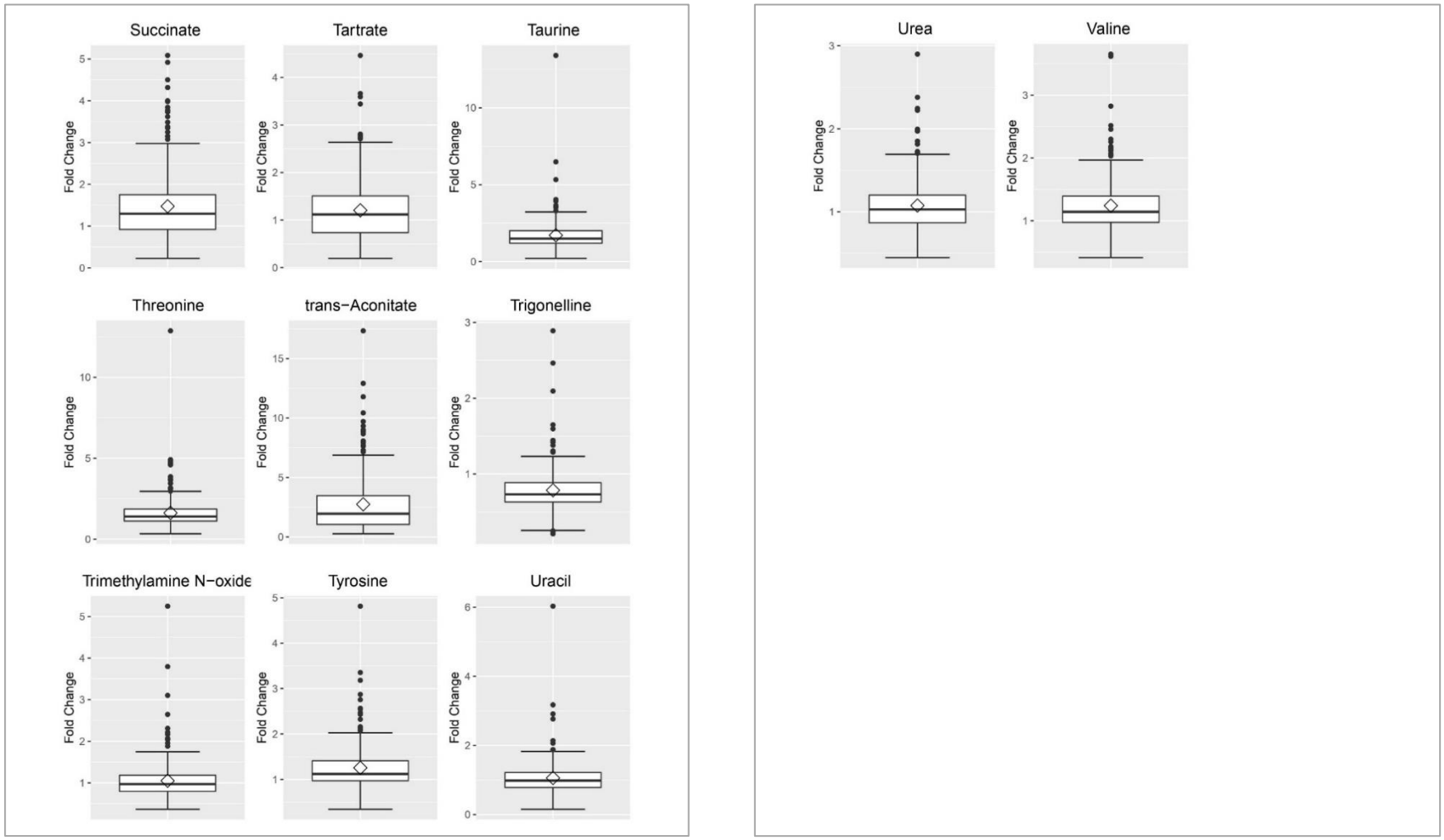


Figure A-6. Boxplots of urinary metabolite FCs. ◊: mean; FCs: fold changes. *(Reprinted from Kistner et al. (2020)).*

A2.5 Volcano Plot of Exercise-Related Changes in Urinary Metabolites (*Study IIa*)

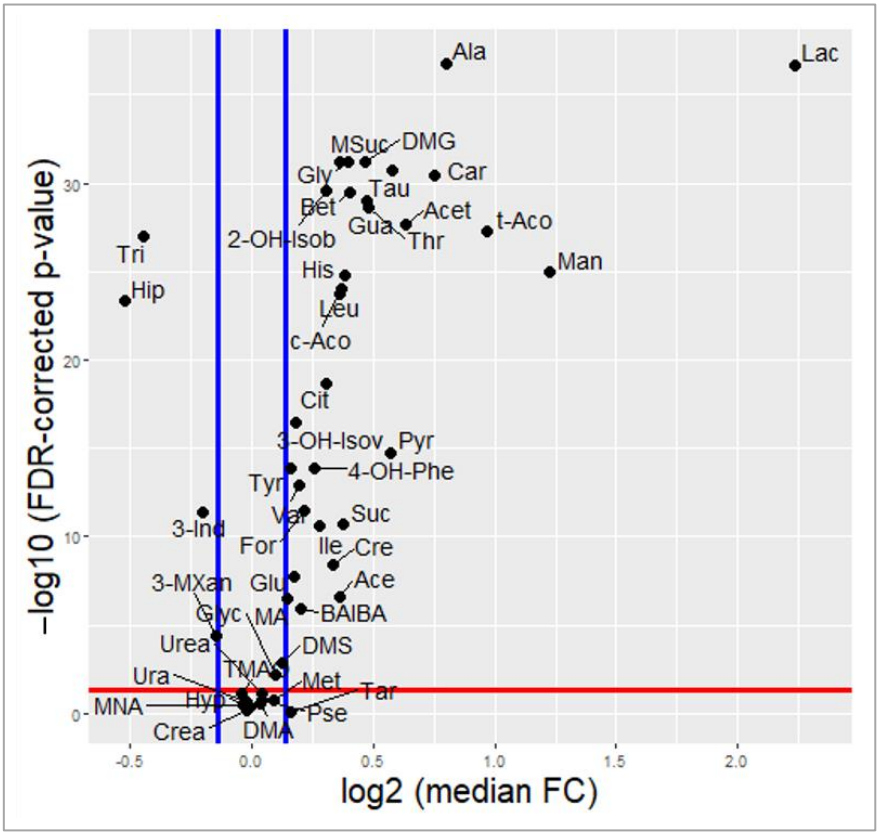


Figure A-7. Volcano plot of exercise-related changes in urinary metabolites. Y-axis: represents the statistical significance of metabolite changes based on *p*-values obtained from Wilcoxon's signed-rank tests and subsequent FDR corrections (red line: α=0.05); X-axis: represents the magnitude of metabolite changes based on median FCs (blue lines: FC of 1.1 or $\frac{1}{1.1}$, respectively). Metabolites located in the upper left and upper right part were defined as exercise-responsive metabolites in this study. FC: fold change; FDR: false discovery rate. *(Reprinted from Kistner et al. (2020)).*

A2.6 Comparison of Urinary Metabolite FCs between Sexes (*Study IIa*)

Table A-5. Urinary metabolite FCs, stratified by sex. *(Adapted from Kistner et al. (2020)).*

Metabolites	Men (n=148)		Women (n=107)		*p*-values
	Median FC (25th, 75th percentiles)		Median FC (25th, 75th percentiles)		(FDR-corrected)
1-Methylnicotinamide	0.94	(0.74, 1.20)	1.02	(0.81, 1.30)	0.4065
2-Hydroxyisobutyrate	1.23	(1.07, 1.36)	1.25	(1.10, 1.52)	0.5860
3-Aminoisobutyrate	1.17	(0.92, 1.41)	1.14	(0.94, 1.39)	0.8956
3-Hydroxyisovalerate	1.13	(1.03, 1.32)	1.13	(0.99, 1.26)	0.8272
3-Indoxylsulfate	0.90	(0.68, 1.02)	0.84	(0.68, 1.08)	0.8956
3-Methylxanthine	0.90	(0.70, 1.10)	0.90	(0.70, 1.20)	0.8272
4-Hydroxyphenylacetate	1.18	(0.96, 1.56)	1.24	(0.97, 1.94)	0.8272
Acetate	1.56	(1.16, 2.70)	1.51	(1.11, 2.65)	0.8272
Acetone	1.15	(0.80, 1.80)	1.47	(0.96, 2.38)	0.0504
Alanine	1.73	(1.40, 2.14)	1.76	(1.36, 2.41)	0.8956
Betaine	1.31	(1.10, 1.55)	1.32	(1.12, 1.62)	0.8272
Carnitine	1.67	(1.31, 2.27)	1.69	(1.20, 2.45)	0.9035
cis-Aconitate	1.25	(1.08, 1.58)	1.37	(1.11, 1.92)	0.4065
Citrate	**1.29**	**(1.10, 1.54)**	**1.16**	**(0.96, 1.34)**	**0.0129**
Creatine	1.16	(0.74, 1.86)	1.42	(1.00, 2.21)	0.1340
Creatinine	0.97	(0.84, 1.14)	0.99	(0.85, 1.23)	0.8272
Dimethylamine	1.02	(0.85, 1.14)	1.04	(0.88, 1.30)	0.4081
Dimethylsulfone	1.05	(0.88, 1.36)	1.10	(0.85, 1.49)	0.8272
Formate	1.19	(1.03, 1.38)	1.08	(0.93, 1.31)	0.2582
Gluconate	1.13	(0.94, 1.38)	1.12	(0.86, 1.55)	0.9035
Glycine	1.31	(1.15, 1.68)	1.32	(1.07, 1.58)	0.4506
Glycolate	1.05	(0.86, 1.26)	1.08	(0.91, 1.30)	0.7141
Guanidoacetate	1.42	(1.13, 1.74)	1.36	(1.16, 1.63)	0.8272
Hippurate	0.69	(0.55, 0.84)	0.71	(0.53, 0.99)	0.8272
Histidine	1.25	(1.07, 1.61)	1.36	(1.11, 1.65)	0.8272
Hypoxanthine	1.03	(0.67, 1.61)	0.96	(0.69, 1.67)	0.8899
Isoleucine	1.21	(0.95, 1.48)	1.23	(0.94, 1.60)	0.8272
Lactate	3.54	(1.47, 53.87)	5.94	(1.93, 20.31)	0.8377
Leucine	1.32	(1.07, 1.67)	1.27	(1.02, 1.77)	0.8272
Mannitol	2.04	(1.21, 3.76)	2.96	(1.57, 5.63)	0.0610
Methanol	1.04	(0.71, 1.54)	1.09	(0.67, 1.84)	0.8272
Methylamine	1.08	(0.89, 1.37)	1.18	(0.97, 1.44)	0.4065
Methylsuccinate	1.26	(1.11, 1.54)	1.30	(1.12, 1.59)	0.8272
N,N-Dimethylglycine	1.36	(1.13, 1.68)	1.40	(1.15, 1.64)	0.8956
Pseudouridine	1.03	(0.88, 1.17)	1.02	(0.90, 1.24)	0.8956
Pyruvate	1.34	(0.85, 3.43)	1.69	(0.92, 5.87)	0.4508
Succinate	1.36	(0.97, 1.78)	1.19	(0.87, 1.74)	0.4065
Tartrate	1.09	(0.74, 1.49)	1.14	(0.72, 1.56)	0.8272
Taurine	1.44	(1.18, 1.93)	1.68	(1.24, 2.15)	0.4065
Threonine	1.42	(1.17, 1.85)	1.36	(1.04, 1.90)	0.5011
trans-Aconitate	**1.53**	**(0.95, 2.82)**	**2.33**	**(1.47, 4.44)**	**0.0097**
Trigonelline	0.74	(0.65, 0.90)	0.73	(0.60, 0.88)	0.8272
Trimethylamine N-oxide	0.98	(0.77, 1.18)	0.96	(0.80, 1.20)	0.9035
Tyrosine	1.12	(0.98, 1.37)	1.11	(0.96, 1.45)	0.8956
Uracil	0.99	(0.84, 1.26)	0.95	(0.74, 1.16)	0.4065
Urea	1.04	(0.89, 1.19)	1.00	(0.85, 1.23)	0.8272
Valine	1.17	(0.97, 1.38)	1.13	(0.97, 1.47)	0.8956

For each metabolite, the FC between normalized post- and pre-exercise concentrations was calculated per participant and the data were presented as median and 25th and 75th percentiles, stratified by sex. Sex-differences in metabolite FCs were analyzed by Wilcoxon rank-sum test and obtained *p*-values were FDR-adjusted. Bold: significantly different metabolite FCs between men and women. FC: fold change; FDR: false discovery rate; n: sample size.

Table A-6. Ranking of metabolite FCs, stratified by sex. *(Adapted from Kistner et al. (2020)).*

Men (n=148)		Women (n=107)	
Metabolite	**Median FC**	**Metabolite**	**Median FC**
Lactate	3.54	Lactate	5.94
Mannitol	2.04	Mannitol	2.96
Alanine	1.73	**trans-Aconitate**	**2.33**
Carnitine	1.67	Alanine	1.76
Acetate	1.56	Carnitine	1.69
trans-Aconitate	**1.53**	Pyruvate	1.69
Taurine	1.44	Taurine	1.68
Threonine	1.42	Acetate	1.51
Guanidoacetate	1.42	Acetone	1.47
Succinate	1.36	Creatine	1.42
N,N-Dimethylglycine	1.36	N,N-Dimethylglycine	1.40
Pyruvate	1.34	cis-Aconitate	1.37
Leucine	1.32	Guanidoacetate	1.36
Glycine	1.31	Histidine	1.36
Betaine	1.31	Threonine	1.36
Citrate	**1.29**	Glycine	1.32
Methylsuccinate	1.26	Betaine	1.32
cis-Aconitate	1.25	Methylsuccinate	1.30
Histidine	1.25	Leucine	1.27
2-Hydroxyisobutyrate	1.23	2-Hydroxyisobutyrate	1.25
Isoleucine	1.21	4-Hydroxyphenylacetate	1.24
Formate	1.19	Isoleucine	1.23
4-Hydroxyphenylacetate	1.18	Succinate	1.19
3-Aminoisobutyrate	1.17	Methylamine	1.18
Valine	1.17	**Citrate**	**1.16**
Creatine	1.16	Tartrate	1.14
Acetone	1.15	3-Aminoisobutyrate	1.14
Gluconate	1.13	3-Hydroxyisovalerate	1.13
3-Hydroxyisovalerate	1.13	Valine	1.13
Tyrosine	1.12	Gluconate	1.12
Tartrate	1.09	Tyrosine	1.11
Methylamine	1.08	Dimethylsulfone	1.10
Dimethylsulfone	1.05	Methanol	1.09
Glycolate	1.05	Formate	1.08
Methanol	1.04	Glycolate	1.08
Urea	1.04	Dimethylamine	1.04
Hypoxanthine	1.03	1-Methylnicotinamide	1.02
Pseudouridine	1.03	Pseudouridine	1.02
Dimethylamine	1.02	Urea	1.00
Uracil	0.99	Creatinine	0.99
Trimethylamine N-oxide	0.98	Hypoxanthine	0.96
Creatinine	0.97	Trimethylamine N-oxide	0.96
1-Methylnicotinamide	0.94	Uracil	0.95
3-Methylxanthine	0.90	3-Methylxanthine	0.90
3-Indoxylsulfate	0.90	3-Indoxylsulfate	0.84
Trigonelline	0.74	Trigonelline	0.73
Hippurate	0.69	Hippurate	0.71

Metabolites are sorted from high to low median FCs. Bold: significantly different metabolite FCs between sexes. FC: fold change; n: sample size.

A2.7 Score and Loading Plots of PCA (*Study IIa*)

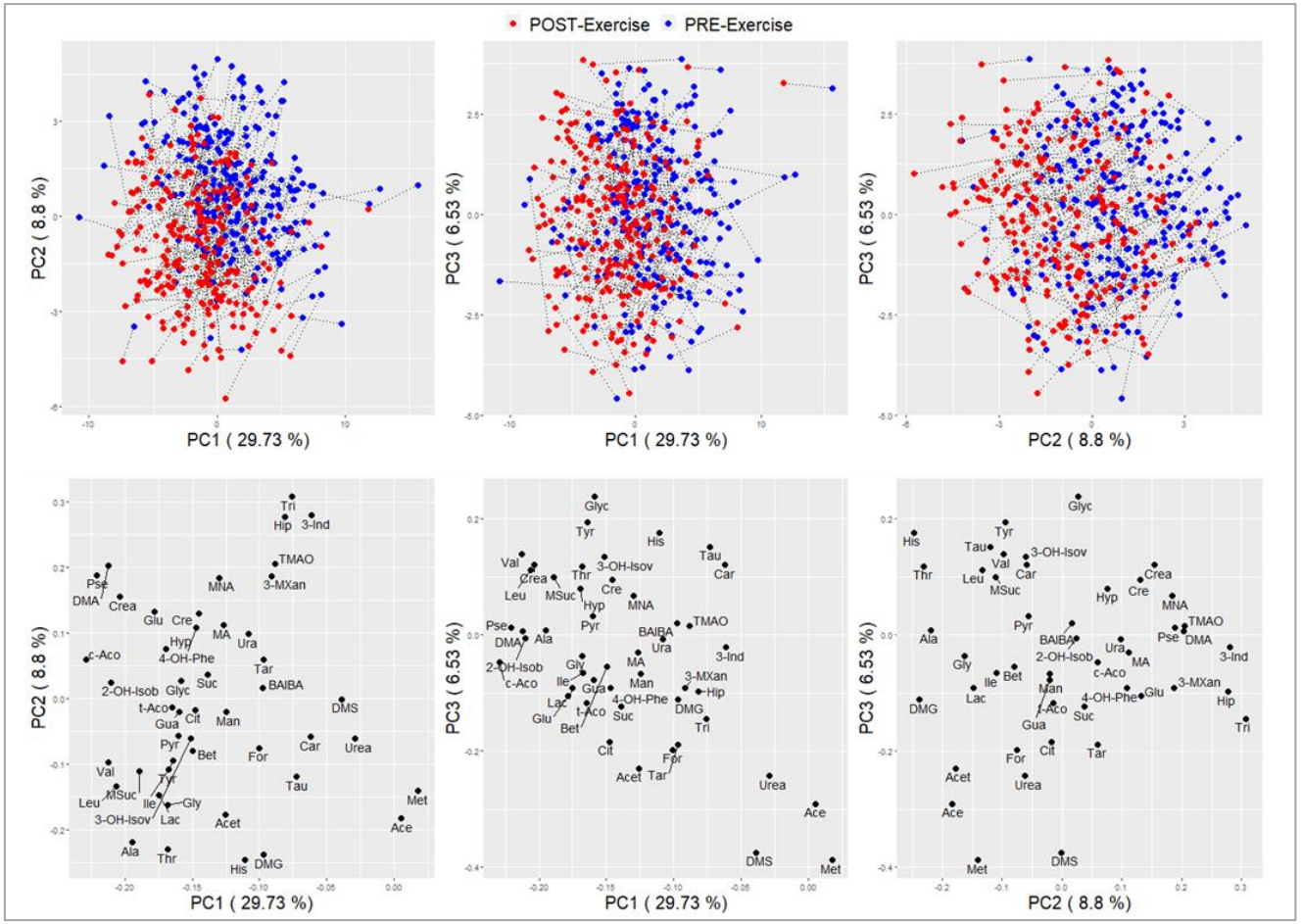


Figure A-8. PCA score and loading plots of a combined pre- and post-exercise urinary metabolite data matrix containing 2 × 255 participants and 47 metabolites. The first three principal components are visualized; top: score plots, data points stand for participants and are color-coded according to the pre- or post-exercise state; bottom: loading plots, data points stand for metabolites. PC: principal component; PCA: principal component analysis. *(Reprinted from Kistner et al. (2020)).*

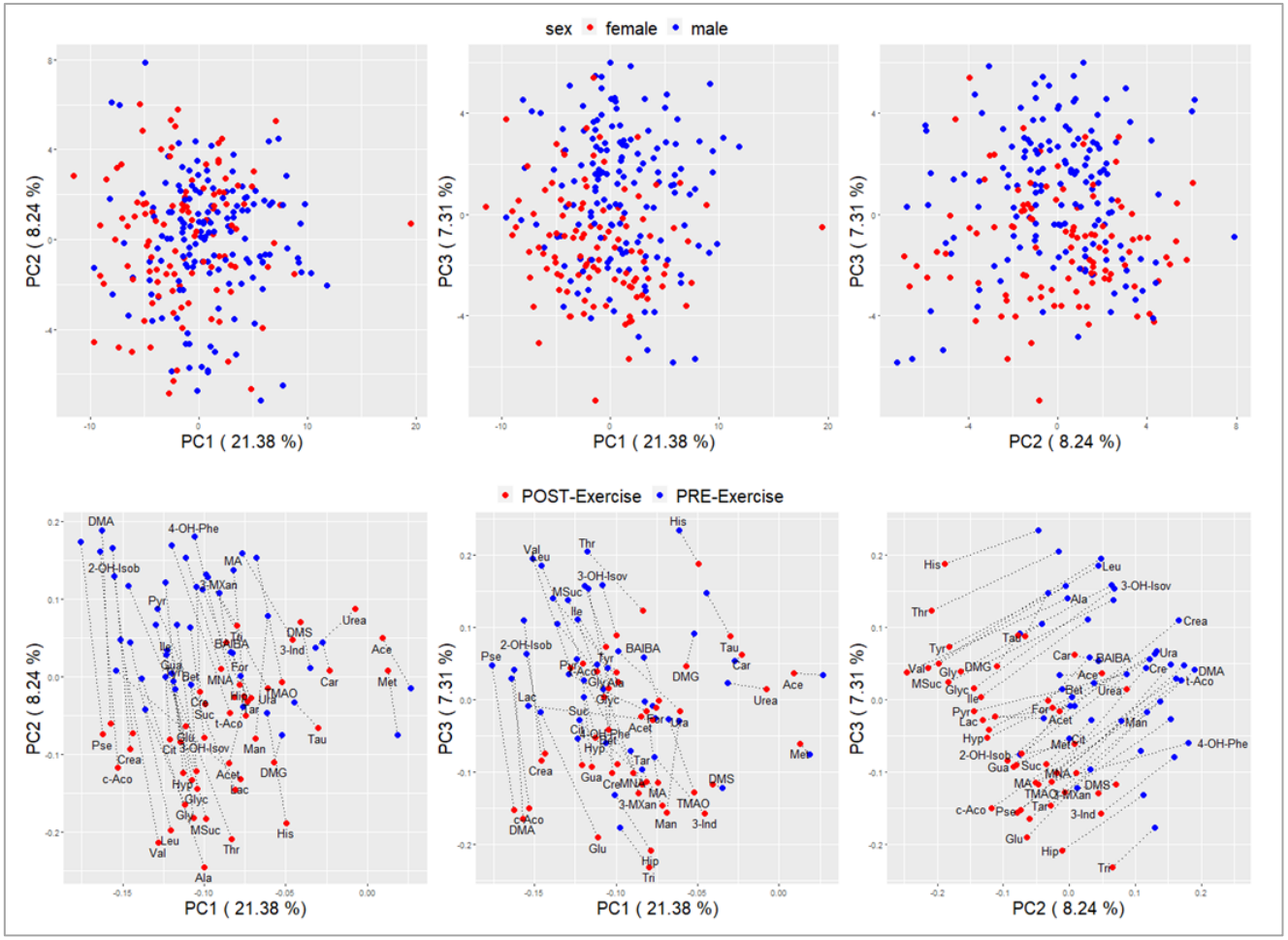


Figure A-9. PCA score and loading plots of a combined pre- and post-exercise urinary metabolite data matrix containing 255 participants and 2 × 47 metabolites. The first three principal components are visualized; top: score plots, data points stand for participants and are color-coded according to sex; bottom: loading plots, data points stand for metabolites and are color-coded according to the pre- or post-exercise state. PC: principal component; PCA: principal component analysis. *(Reprinted from Kistner et al. (2020)).*

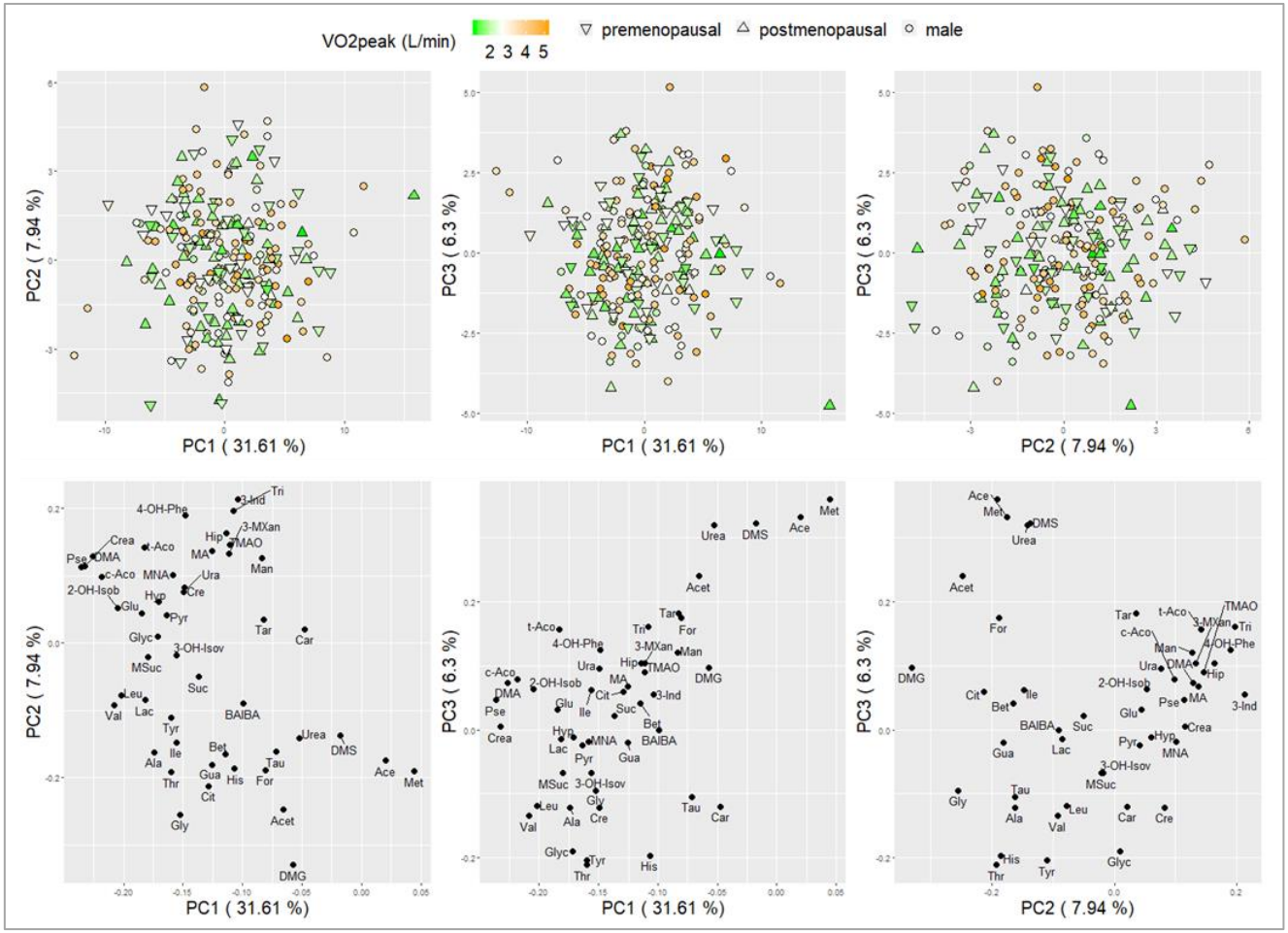


Figure A-10. PCA score and loading plots of the adjusted pre-exercise urinary metabolite data. The first three principal components are visualized; top: score plots, data points stand for participants and are color-coded according to the VO_{2peak}; bottom: loading plots, data points stand for metabolites. PC: principal component; PCA: principal component analysis; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2020)).*

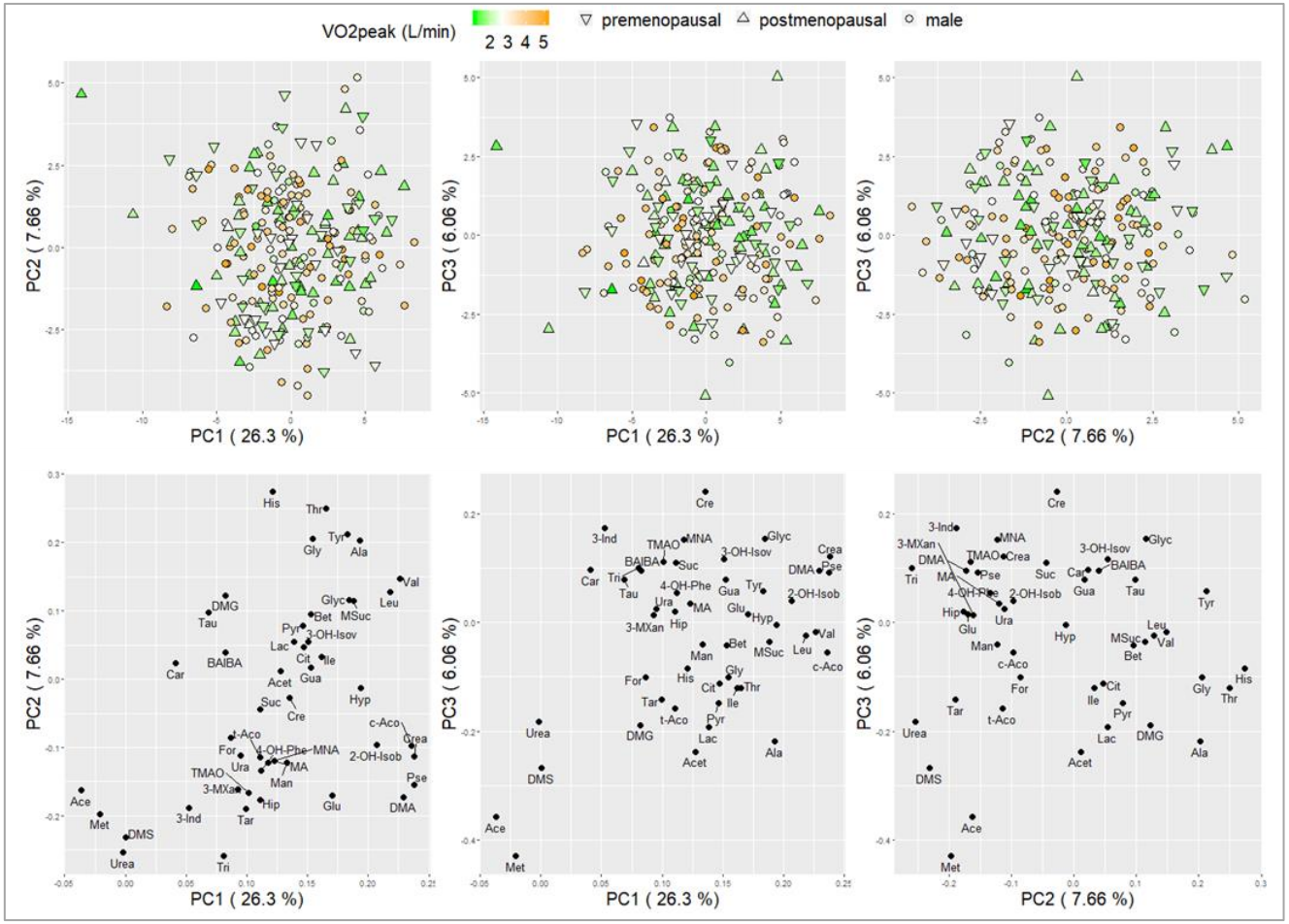


Figure A-11. PCA score and loading plots of the adjusted post-exercise urinary metabolite data. The first three principal components are visualized; top: score plots, data points stand for participants and are color-coded according to the VO_{2peak}; bottom: loading plots, data points stand for metabolites. PC: principal component; PCA: principal component analysis; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2020)).*

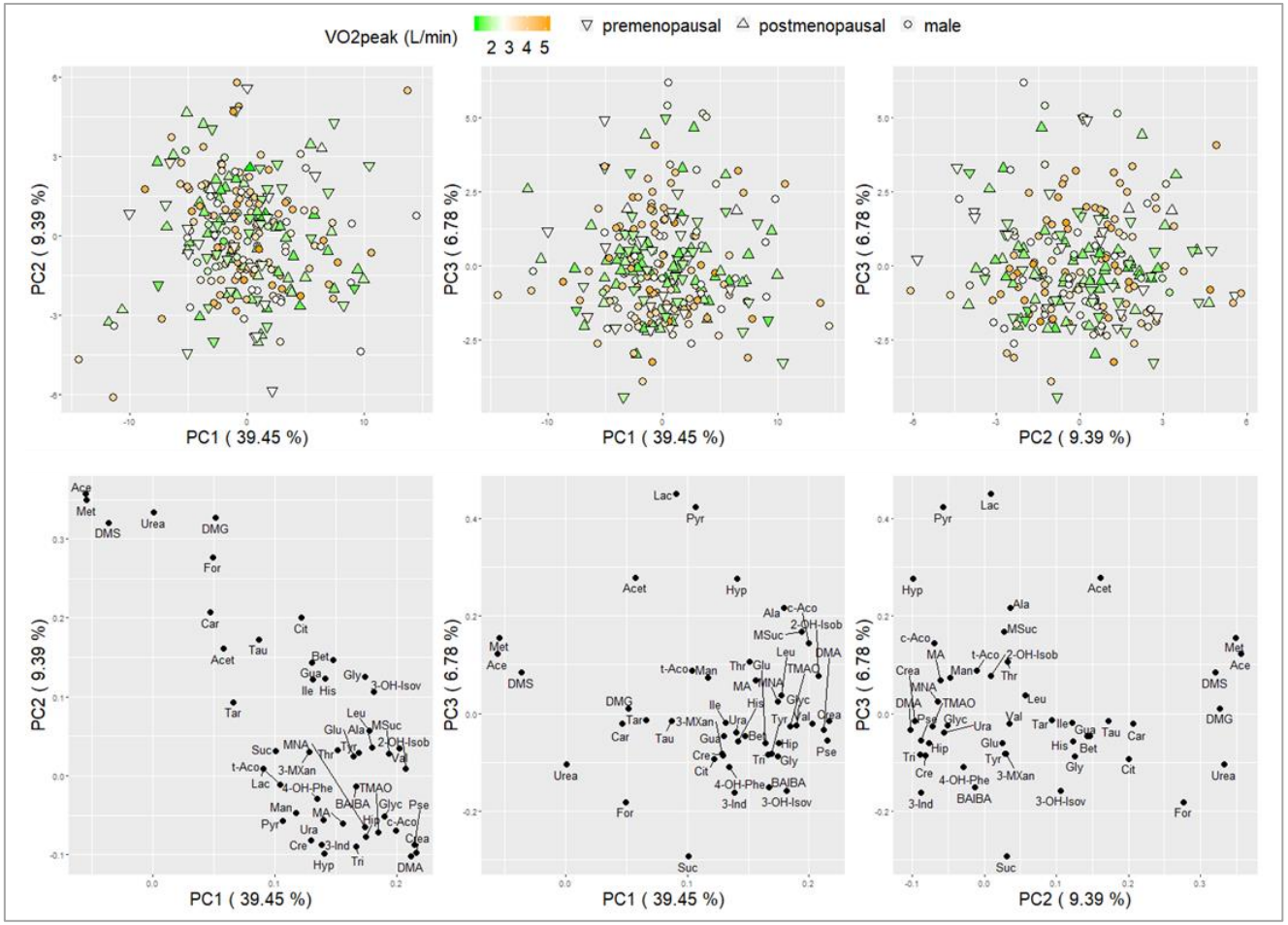


Figure A-12. PCA score and loading plots of the adjusted data on urinary metabolite FCs. The first three principal components are visualized; top: score plots, data points stand for participants and are color-coded according to the VO_{2peak}; bottom: loading plots, data points stand for metabolites. FCs: fold changes; PC: principal component; PCA: principal component analysis; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2020)).*

A2.8 Correlation Between the VO_{2peak} and Urinary Metabolites (*Study IIa*)

Table A-7. Pearson correlation coefficients for the association between VO_{2peak} and pre- and post-exercise urinary metabolite concentrations as well as exercise-induced metabolite FCs. *(Adapted from Kistner et al. (2020)).*

Metabolites	Pre−Exercise		Post−Exercise		FCs	
	r (unadjusted)	*r* (adjusted*)	*r* (unadjusted)	*r* (adjusted*)	*r* (unadjusted)	*r* (adjusted*)
1−Methylnicotinamide	−0.10	−0.06	**−0.27**	**−0.15**	**−0.25**	**−0.13**
2−Hydroxyisobutyrate	−0.03	0.05	−0.09	0.03	−0.09	−0.04
3−Aminoisobutyrate	−0.01	0.12	−0.04	0.12	−0.12	−0.07
3−Hydroxyisovalerate	0.05	0.01	0.06	−0.05	0.00	−0.04
3−Indoxylsulfate	**−0.18**	−0.02	**−0.22**	−0.04	−0.09	−0.04
3−Methylxanthine	−0.06	0.08	−0.10	0.08	−0.08	0.04
4−Hydroxyphenylacetate	**−0.17**	0.06	**−0.16**	−0.03	−0.01	−0.05
Acetate	**−0.20**	0.02	−0.12	−0.01	0.08	−0.03
Acetone	**0.14**	0.01	0.08	−0.01	−0.09	−0.02
Alanine	−0.10	−0.08	−0.07	−0.09	0.05	−0.01
Betaine	**−0.21**	−0.06	**−0.20**	−0.03	0.01	0.04
Carnitine	0.07	−0.04	**0.15**	−0.02	0.08	0.04
cis−Aconitate	**−0.15**	−0.04	**−0.29**	−0.10	−0.13	−0.05
Citrate	**−0.46**	0.03	**−0.39**	0.06	**0.26**	0.02
Creatine	**−0.24**	−0.10	**−0.35**	−0.11	**−0.21**	−0.02
Creatinine	**0.13**	−0.02	0.02	−0.09	−0.14	−0.06
Dimethylamine	−0.08	−0.04	**−0.25**	−0.10	**−0.18**	−0.04
Dimethylsulfone	**−0.23**	0.05	**−0.22**	0.09	0.02	0.01
Formate	−0.08	0.09	0.02	0.07	**0.16**	0.01
Gluconate	**−0.30**	0.05	**−0.36**	0.04	−0.06	0.02
Glycine	**−0.23**	−0.03	**−0.17**	−0.03	0.12	0.00
Glycolate	0.06	−0.10	−0.01	−0.12	−0.09	0.00
Guanidoacetate	**−0.39**	−0.10	**−0.41**	**−0.12**	0.02	−0.01
Hippurate	**−0.30**	−0.03	**−0.34**	−0.05	−0.14	−0.05
Histidine	**0.17**	**−0.17**	**0.20**	−0.11	0.00	0.06
Hypoxanthine	−0.04	0.07	−0.11	−0.09	−0.06	**−0.13**
Isoleucine	−0.12	−0.10	**−0.14**	−0.05	−0.02	0.05
Lactate	**−0.38**	0.04	−0.09	0.04	0.09	0.04
Leucine	0.08	−0.02	0.08	−0.05	0.00	−0.04
Mannitol	0.03	0.03	−0.09	0.03	−0.10	−0.01
Methanol	0.01	0.08	0.04	0.09	0.04	0.00
Methylamine	−0.10	−0.08	**−0.19**	−0.12	−0.11	−0.02
Methylsuccinate	0.02	−0.06	−0.02	−0.10	−0.04	−0.05
N,N−Dimethylglycine	−0.01	0.03	−0.01	0.04	0.00	0.02
Pseudouridine	−0.10	0.00	**−0.22**	−0.05	−0.12	−0.04

Pyruvate	**−0.16**	−0.06	**−0.12**	−0.11	−0.03	−0.05
Succinate	**−0.32**	−0.02	**−0.31**	0.02	0.06	0.05
Tartrate	**−0.13**	0.02	**−0.20**	−0.08	−0.03	−0.05
Taurine	**0.15**	−0.12	0.10	−0.09	−0.11	0.04
Threonine	−0.01	−0.10	0.10	−0.04	**0.14**	0.03
trans−Aconitate	0.09	0.11	**−0.20**	0.07	**−0.23**	−0.02
Trigonelline	**−0.35**	0.04	**−0.36**	0.05	−0.06	−0.01
Trimethylamine N−oxide	−0.03	−0.03	−0.06	−0.07	−0.05	−0.04
Tyrosine	−0.04	**−0.16**	−0.06	**−0.18**	−0.02	−0.04
Uracil	0.06	**0.16**	0.06	0.07	0.00	−0.10
Urea	**0.15**	0.10	**0.23**	0.10	0.08	−0.01
Valine	0.02	−0.05	−0.01	−0.07	−0.03	−0.02

Correlations were conducted on VdW scores of the analyzed variables. * correlations were adjusted for sex, menopausal status, age, and lean body mass; bold: 95% confidence intervals do not include zero; FCs: fold changes; *r*: Pearson correlation coefficient; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

A2.9 Basic Characteristics of KarMeN Subjects According to VO_{2peak} Quarters (*Study IIb*)

Table A-8. Characteristics of female KarMeN participants (n=102) according to VO_{2peak} quarters. *(Adapted from Kistner et al. (2021)).*

Characteristics	VO_{2peak} Quarters				*p*
	1st q (n=25)	2nd q (n=26)	3rd q (n=26)	4th q (n=25)	
VO_{2peak} (mL kg^{-1} min^{-1})	21.6 ± 2.1	27.5 ± 1.7	32.9 ± 1.5	40.9 ± 2.9	**<.0001**
Age (years)	62.3 ± 9.6	57.1 ± 11.1	50.3 ± 10.9	33.7 ± 9.6	**<.0001**
Pre-/Post-Menopausal State$^{\Delta}$	2/23	7/19	15/11	22/3	**<.0001**
Weight (kg)	68.9 ± 9.1	66.2 ± 7.2	61.0 ± 6.2	60.9 ± 7.0	**0.0008**
Height (cm)	164.2 ± 5.9	166.7 ± 6.6	166.8 ± 5.2	169.6 ± 7.2	**0.0484**
BMI (kg/m²)	25.6 ± 3.4	23.8 ± 1.9	21.9 ± 2.0	21.2 ± 1.8	**<.0001**
LBM (kg)	39.4 ± 4.3	40.6 ± 3.5	40.2 ± 3.1	41.1 ± 4.5	0.5504
FM (%)	39.2 ± 5.2	35.1 ± 4.8	30.3 ± 4.9	28.7 ± 4.3	**<.0001**
VATM (kg)	0.81 ± 0.48	0.42 ± 0.25	0.25 ± 0.22	0.12 ± 0.10	**<.0001**
BMC (kg)	2.24 ± 0.24	2.31 ± 0.38	2.34 ± 0.25	2.46 ± 0.30	0.0567
Hb (g dL^{-1})	13.7 ± 0.8	13.5 ± 0.7	13.5 ± 0.6	13.3 ± 0.9	0.408
Glucose (mg dL^{-1})	89.6 ± 8.1	85.3 ± 7.6	82.4 ± 6.5	82.0 ± 7.3	**0.0034**
Insulin (µlU mL^{-1})	10.3 ± 4.5	9.0 ± 3.0	8.5 ± 3.4	10.5 ± 4.5	0.2199
HbA1c (%)	5.47 ± 0.30	5.61 ± 0.37	5.42 ± 0.29	5.19 ± 0.44	**0.0065**
TGs (mg dL^{-1})	102.2 ± 35.4	85.0 ± 32.9	65.0 ± 18.1	62.7 ± 15.0	**<.0001**
HDL cholesterol (mg dL^{-1})	69.7 ± 13.0	79.2 ± 17.2	82.2 ± 18.8	79.4 ± 13.8	**0.0198**
LDL cholesterol (mg dL^{-1})	145.0 ± 30.4	137.4 ± 32.9	123.1 ± 32.0	104.2 ± 27.5	**<.0001**
HR_{rest} (1 min^{-1})	67.6 ± 7.8	66.2 ± 7.6	64.2 ± 10.1	64.8 ± 8.6	0.5298
BP systolic (mmHg)	128.4 ± 16.6	124.9 ± 16.9	113.5 ± 12.9	106.7 ± 7.2	**<.0001**
BP diastolic (mmHg)	88.7 ± 9.2	86.0 ± 8.5	78.7 ± 10.5	74.5 ± 8.2	**<.0001**
PWV (m s^{-1})°	9.73 ± 1.76	9.28 ± 2.20	7.69 ± 1.16	6.64 ± 0.81	**<.0001**
VC_{max} (L)	3.25 ± 0.46	3.61 ± 0.54	3.66 ± 0.42	4.04 ± 0.68	**<.0001**
FEV1 (L)	2.47 ± 0.40	2.75 ± 0.40	2.79 ± 0.42	3.36 ± 0.63	**<.0001**
AEE (kcal d^{-1})	639.4 ± 199.7	782.8 ± 296.7	754.4 ± 185.4	777.5 ± 257.4	0.0814
Total MET (MET-min week^{-1})	6470 ± 4066	7033 ± 4451	6518 ± 3338	3920 ± 2099	**0.0008**
HEI-NVS	73.9 ± 9.6	69.8 ± 10.0	76.2 ± 10.4	73.2 ± 8.4	0.1805

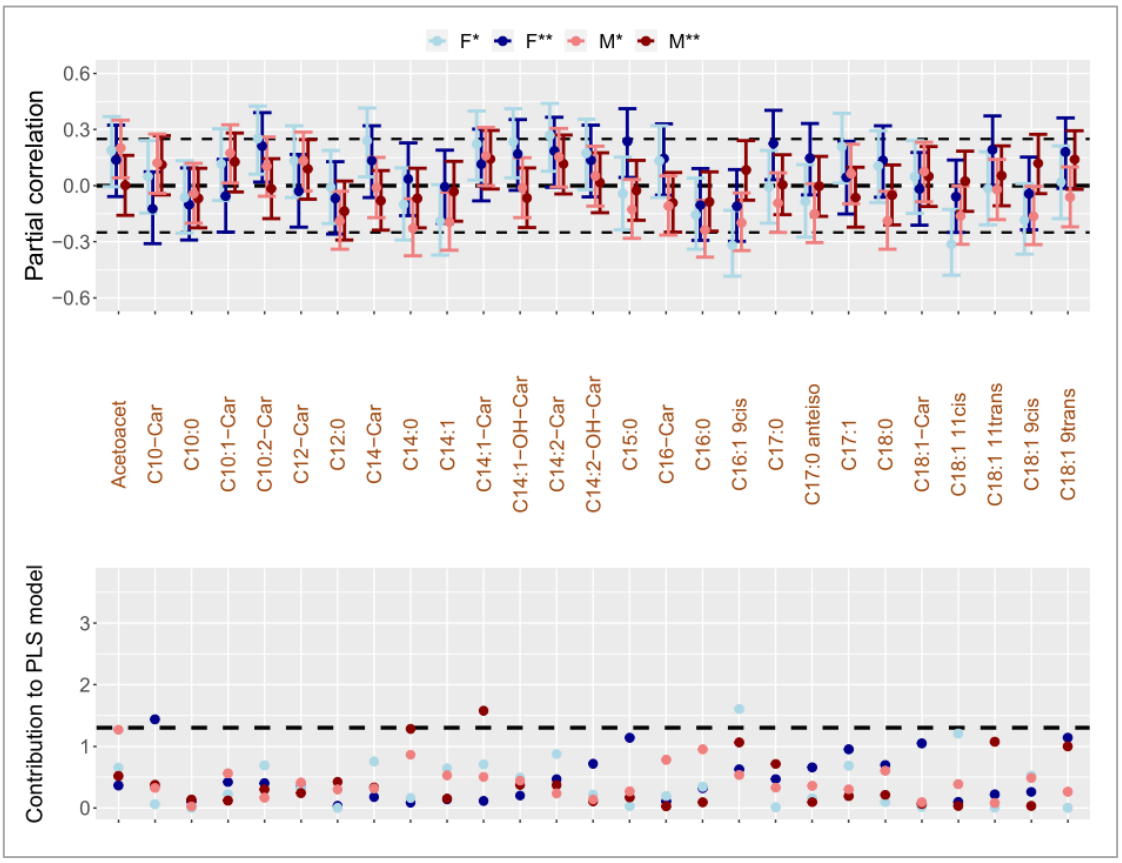
Based on the VO_{2peak} quartiles in the female sub-group, the VO_{2peak} data were divided into four quarters (q) and basic characteristics of the sub-groups of the corresponding females of the quarters are presented. Data are given in mean ± SD; $^{\Delta}$: number of female participants in the pre-/post-menopausal state; ° n=23 (1st q), n=25 (2nd q); bold *p*-value: significant differences between quarters according to Welch ANOVA (Chi² test) for numeric (categorical) variables. AEE: activity energy expenditure; BMC: bone mineral content; BMI: body mass index; BP: blood pressure; FEV1: forced expiratory pressure in one second; FM: fat mass; Hb: hemoglobin; HDL: high-density lipoprotein; HEI-NVS: Healthy Eating Index (modified version); HR_{rest}: resting heart rate; KarMeN: Karlsruhe Metabolomics and Nutrition; LBM: lean body mass; LDL: low-density lipoprotein; MET: metabolic equivalent of task; n: sample size; PWV: pulse wave velocity; TGs: triglycerides; VATM: visceral adipose tissue mass; VC_{max}: maximal vital capacity; VO_{2peak}: peak oxygen uptake.

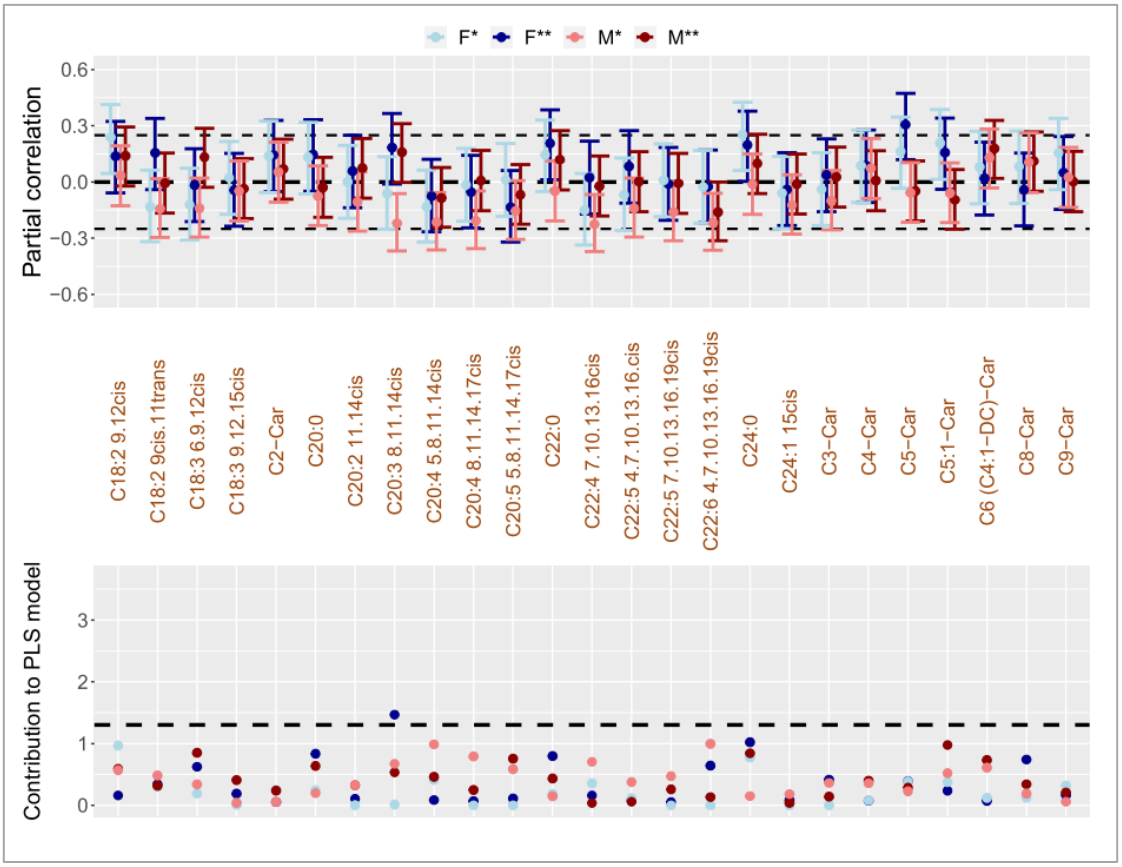
Table A-9. Characteristics of male KarMeN participants (n=150) according to VO_{2peak} quarters. *(Adapted from Kistner et al. (2021)).*

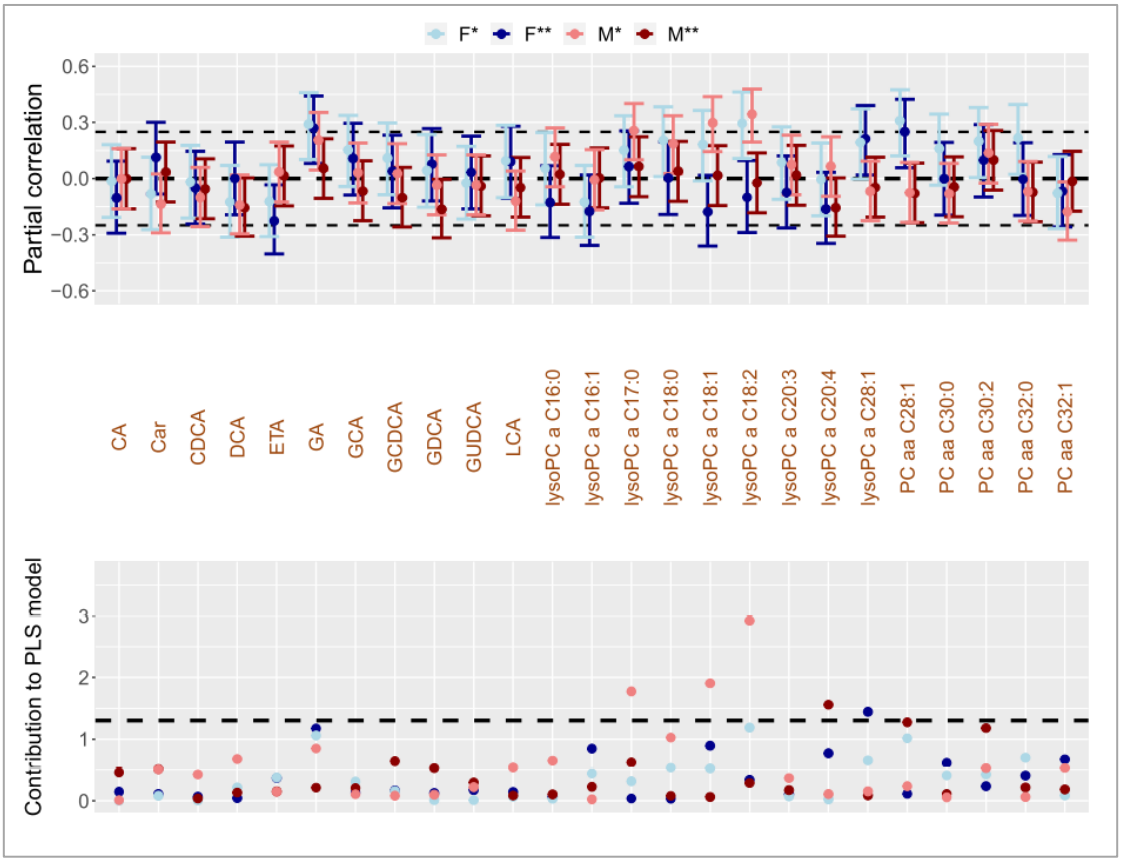
Characteristics	VO_{2peak} Quarters				*p*
	1st q (n=37)	2nd q (n=38)	3rd q (n=38)	4th q (n=37)	
VO_{2peak} (mL kg^{-1} min^{-1})	30.8 ± 4.0	40.3 ± 2.2	48.5 ± 2.3	58.5 ± 4.7	**<.0001**
Age (years)	59.0 ± 14.4	45.1 ± 17.3	38.4 ± 13.7	27.6 ± 7.8	**<.0001**
Weight (kg)	83.2 ± 10.4	79.4 ± 10.5	76.6 ± 9.9	75.2 ± 7.6	**0.0025**
Height (cm)	177.4 ± 7.2	180.7 ± 6.2	181.5 ± 9.0	180.9 ± 6.3	**0.0809**
BMI (kg/m²)	26.4 ± 2.6	24.3 ± 2.6	23.2 ± 2.1	23.0 ± 2.2	**<.0001**
LBM (kg)	56.1 ± 6.0	57.7 ± 6.6	57.7 ± 6.9	60.5 ± 6.8	**0.0449**
FM (%)	28.8 ± 3.9	23.7 ± 5.2	20.8 ± 4.7	16.1 ± 4.2	**<.0001**
VATM (kg)	1.70 ± 0.74	0.77 ± 0.55	0.50 ± 0.44	0.26 ± 0.15	**<.0001**
BMC (kg)	3.19 ± 0.41	3.11 ± 0.42	3.27 ± 0.45	3.27 ± 0.44	0.3633
Hb (g dL^{-1})	15.1 ± 1.2	15.0 ± 0.8	15.0 ± 0.9	15.1 ± 0.8	0.8748
Glucose (mg dL^{-1})	90.6 ± 9.7	86.6 ± 8.0	84.5 ± 6.6	83.4 ± 7.5	**0.004**
Insulin (µlU mL^{-1})	10.9 ± 4.4	11.0 ± 6.5	9.1 ± 3.1	9.5 ± 2.9	0.1293
HbA1c (%)	5.54 ± 0.34	5.37 ± 0.33	5.26 ± 0.28	5.25 ± 0.32	**0.0012**
TGs (mg dL^{-1})	119.9 ± 45.8	101.0 ± 60.2	82.1 ± 37.5	81.1 ± 29.3	**0.0002**
HDL cholesterol (mg dL^{-1})	58.5 ± 13.5	61.3 ± 13.7	68.6 ± 16.0	62.7 ± 11.4	**0.0376**
LDL cholesterol (mg dL^{-1})	150.7 ± 44.0	117.7 ± 41.0	114.2 ± 33.5	97.7 ± 28.1	**<.0001**
HR_{rest} (1 min^{-1})	64.1 ± 10.4	63.3 ± 7.8	57.2 ± 7.6	56.0 ± 8.2	**<.0001**
BP systolic (mmHg)	133.4 ± 14.8	126.3 ± 14.4	126.1 ± 11.2	122.0 ± 11.0	**0.0044**
BP diastolic (mmHg)	91.0 ± 9.6	85.2 ± 9.2	82.5 ± 7.2	76.3 ± 8.6	**<.0001**
PWV (m s^{-1})^{v}	8.39 ± 1.49	7.23 ± 1.30	6.74 ± 0.85	6.17 ± 0.64	**<.0001**
VC_{max} (L)	4.89 ± 0.90	5.35 ± 0.87	5.61 ± 0.85	5.80 ± 0.72	**<.0001**
FEV1 (L)	3.63 ± 0.75	4.20 ± 0.80	4.39 ± 0.76	4.68 ± 0.66	**<.0001**
AEE (kcal d^{-1})^{vv}	798.7 ± 499.3	1017.0 ± 562.3	1193.3 ± 483.3	1546.9 ± 625.7	**<.0001**
Total MET (MET-min week^{-1})	6323 ± 5690	5986 ± 5073	4769 ± 3599	5922 ± 3938	0.3929
HEI-NVS	70.1 ± 11.0	72.2 ± 9.8	69.1 ± 8.8	72.4 ± 10.0	0.3828

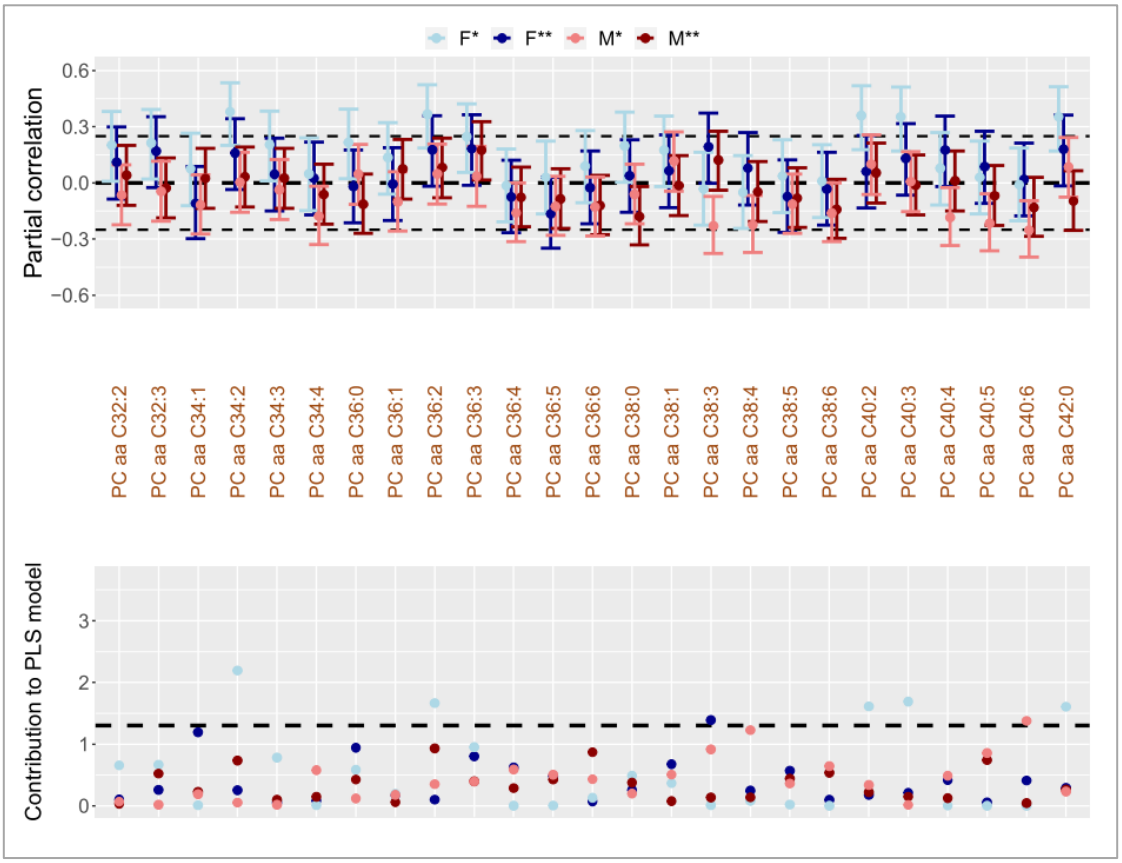
Based on the VO_{2peak} quartiles in the male sub-group, the VO_{2peak} data were divided into four quarters (q) and basic characteristics of the sub-groups of the corresponding males of the quarters are presented. Data are given in mean ± SD; ^{v} n=36 (1st q); ^{vv} n=35 (1st q), n=37 (2nd q); bold *p*-value: significant differences between quarters according to Welch ANOVA. AEE: activity energy expenditure; BMC: bone mineral content; BMI: body mass index; BP: blood pressure; FEV1: forced expiratory pressure in one second; FM: fat mass; Hb: hemoglobin; HDL: high-density lipoprotein; HEI-NVS: Healthy Eating Index (modified version); HR_{rest}: resting heart rate; KarMeN: Karlsruhe Metabolomics and Nutrition; LBM: lean body mass; LDL: low-density lipoprotein; MET: metabolic equivalent of task; n: sample size; PWV: pulse wave velocity; TGs: triglycerides; VATM: visceral adipose tissue mass; VC_{max}: maximal vital capacity; VO_{2peak}: peak oxygen uptake.

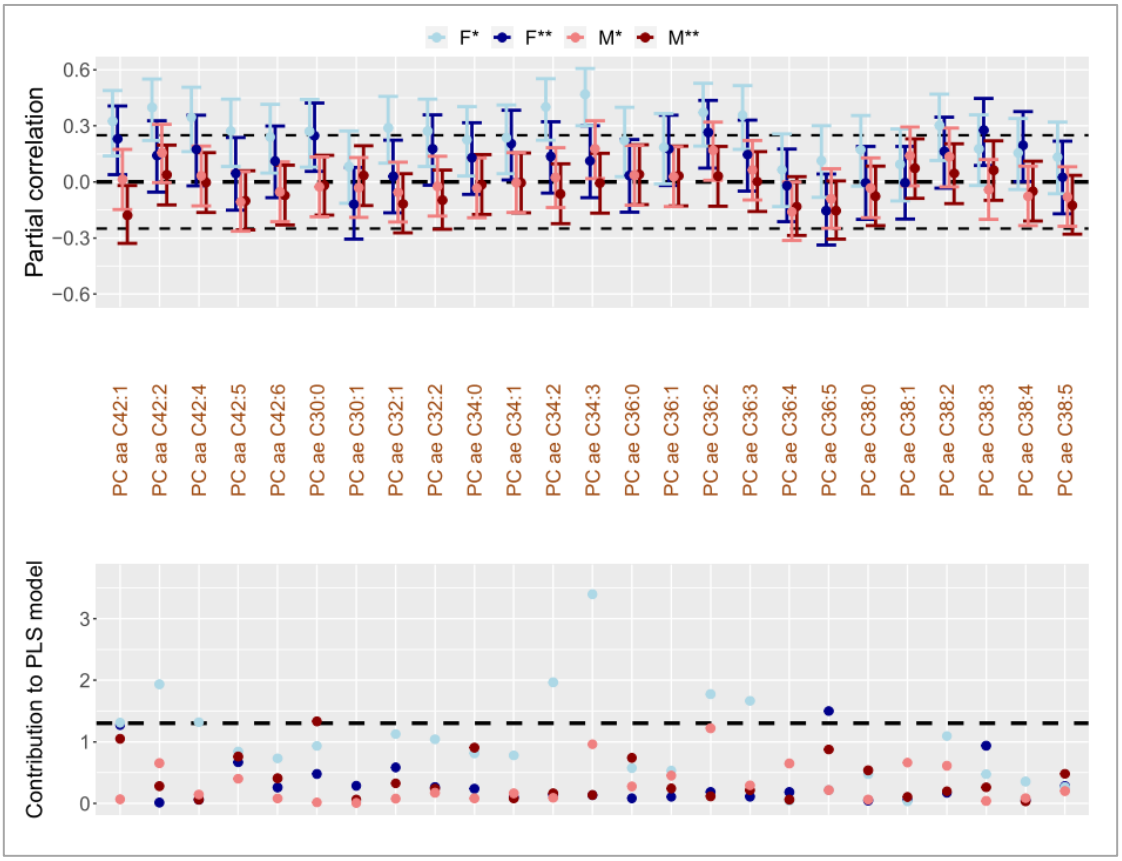
A2.10 Bi- and Multivariate Associations between the VO_{2peak} and Plasma Analytes (*Study IIb*)

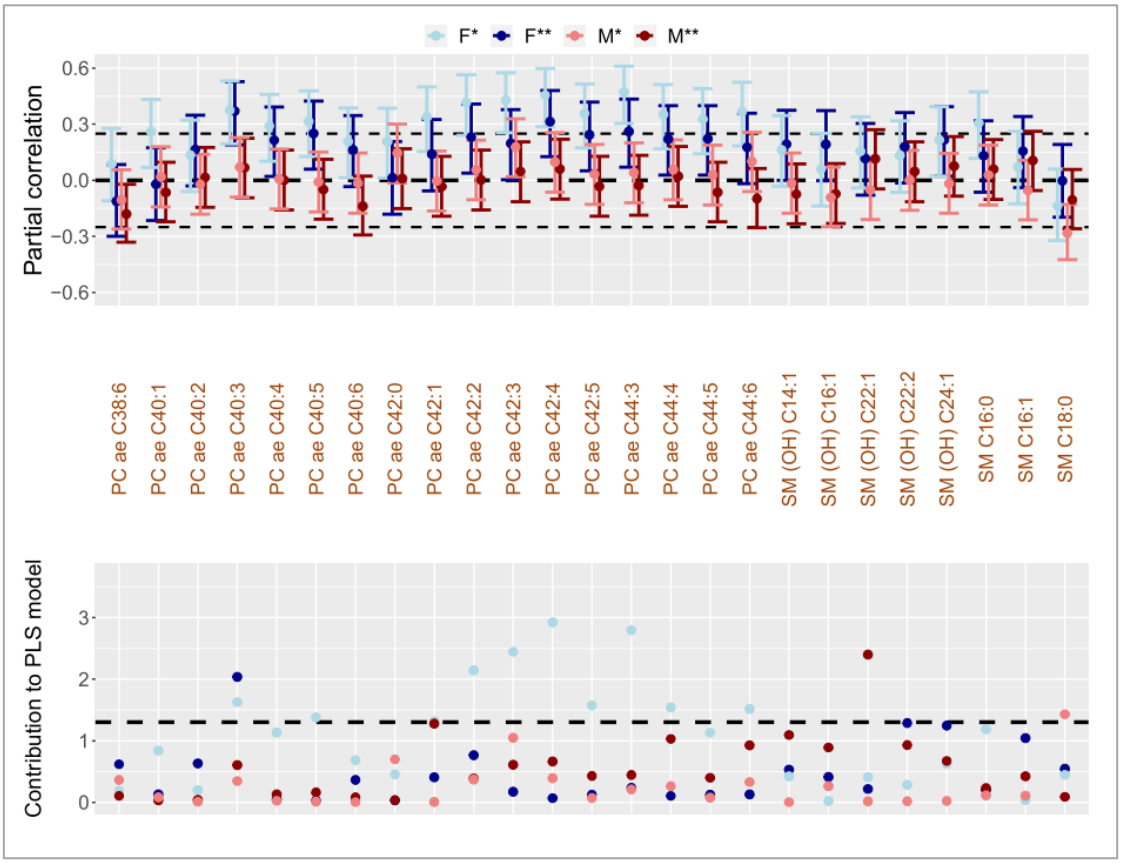


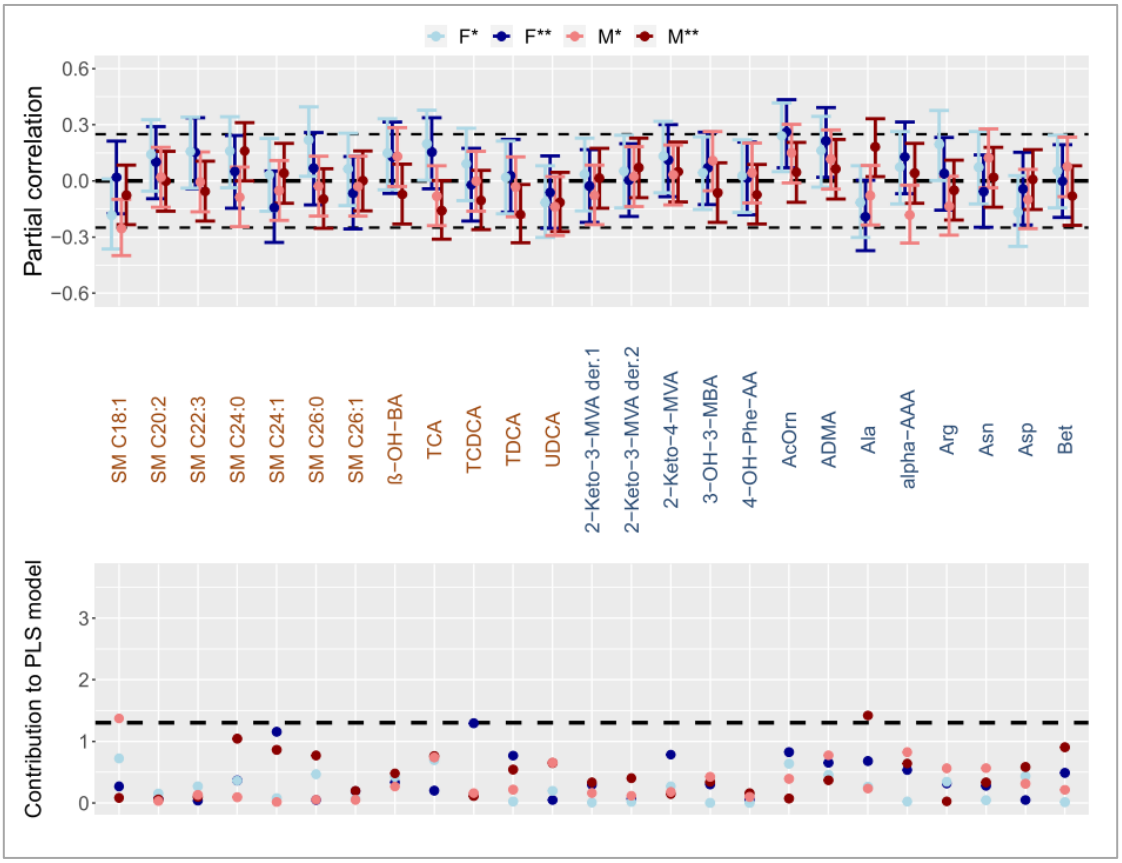


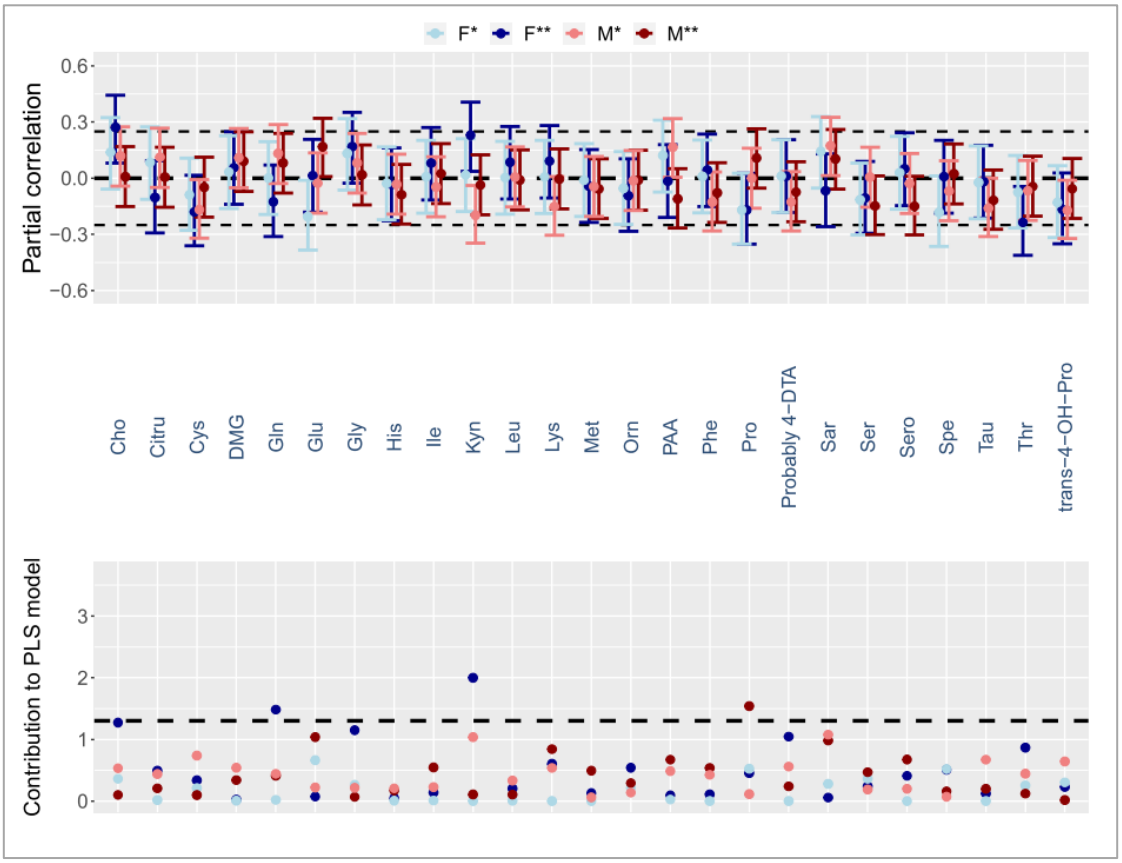

F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
CA
Car
CDCA
DCA
ETA
GA
GCA
GCDCA
GDCA
GUDCA
LCA
lysoPC a C16:0
lysoPC a C16:1
lysoPC a C17:0
lysoPC a C18:0
lysoPC a C18:1
lysoPC a C18:2
lysoPC a C20:3
lysoPC a C20:4
lysoPC a C28:1
PC aa C28:1
PC aa C30:0
PC aa C30:2
PC aa C32:0
PC aa C32:1
Contribution to PLS model
3
2
1
0

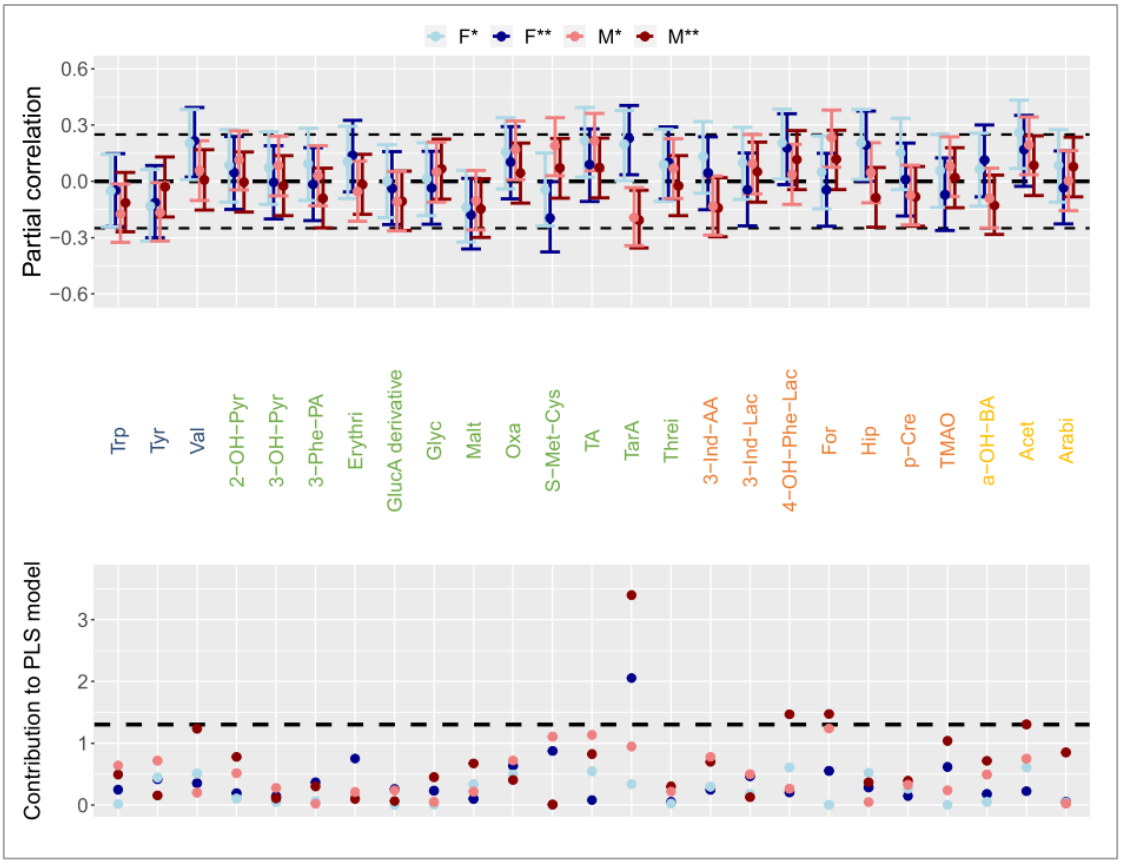

F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
PC aa C32:2
PC aa C32:3
PC aa C34:1
PC aa C34:2
PC aa C34:3
PC aa C34:4
PC aa C36:0
PC aa C36:1
PC aa C36:2
PC aa C36:3
PC aa C36:4
PC aa C36:5
PC aa C36:6
PC aa C38:0
PC aa C38:1
PC aa C38:3
PC aa C38:4
PC aa C38:5
PC aa C38:6
PC aa C40:2
PC aa C40:3
PC aa C40:4
PC aa C40:5
PC aa C40:6
PC aa C42:0
Contribution to PLS model
3
2
1
0

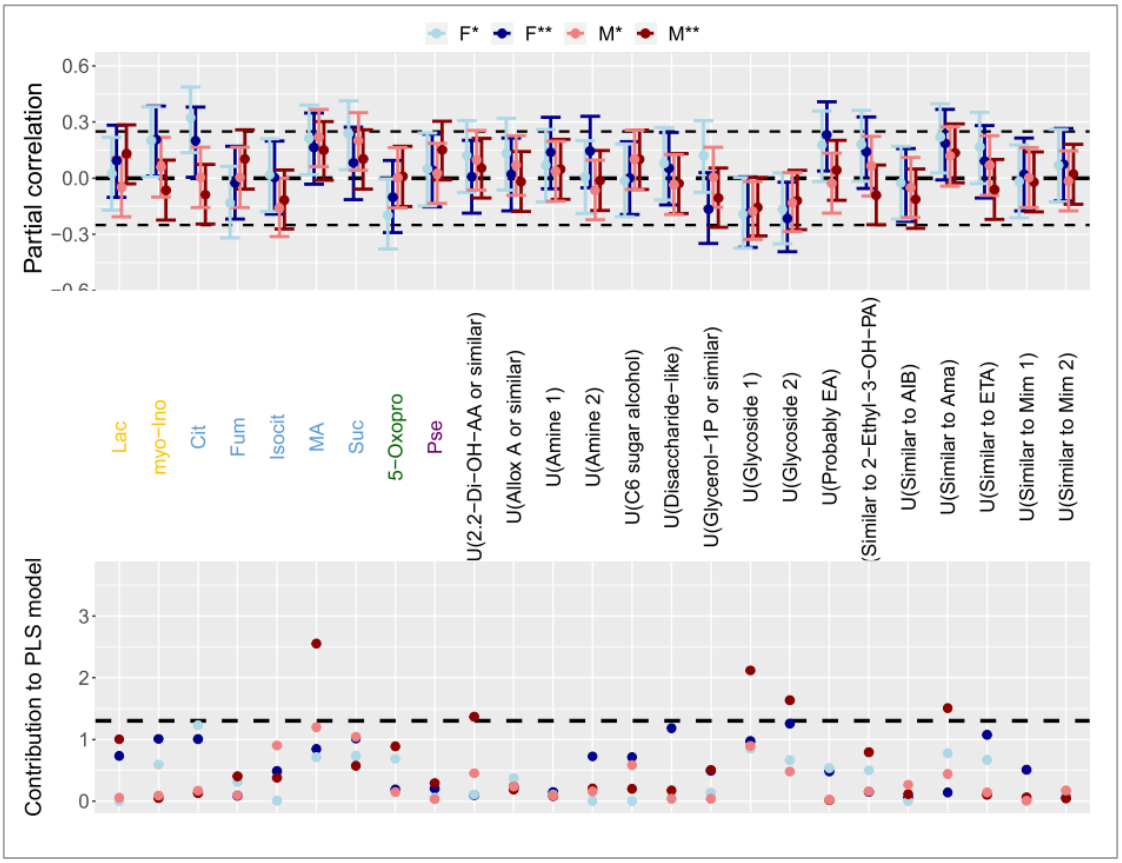

F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
PC aa C42:1
PC aa C42:2
PC aa C42:4
PC aa C42:5
PC aa C42:6
PC ae C30:0
PC ae C30:1
PC ae C32:1
PC ae C32:2
PC ae C34:0
PC ae C34:1
PC ae C34:2
PC ae C34:3
PC ae C36:0
PC ae C36:1
PC ae C36:2
PC ae C36:3
PC ae C36:4
PC ae C36:5
PC ae C38:0
PC ae C38:1
PC ae C38:2
PC ae C38:3
PC ae C38:4
PC ae C38:5
Contribution to PLS model
3
2
1
0

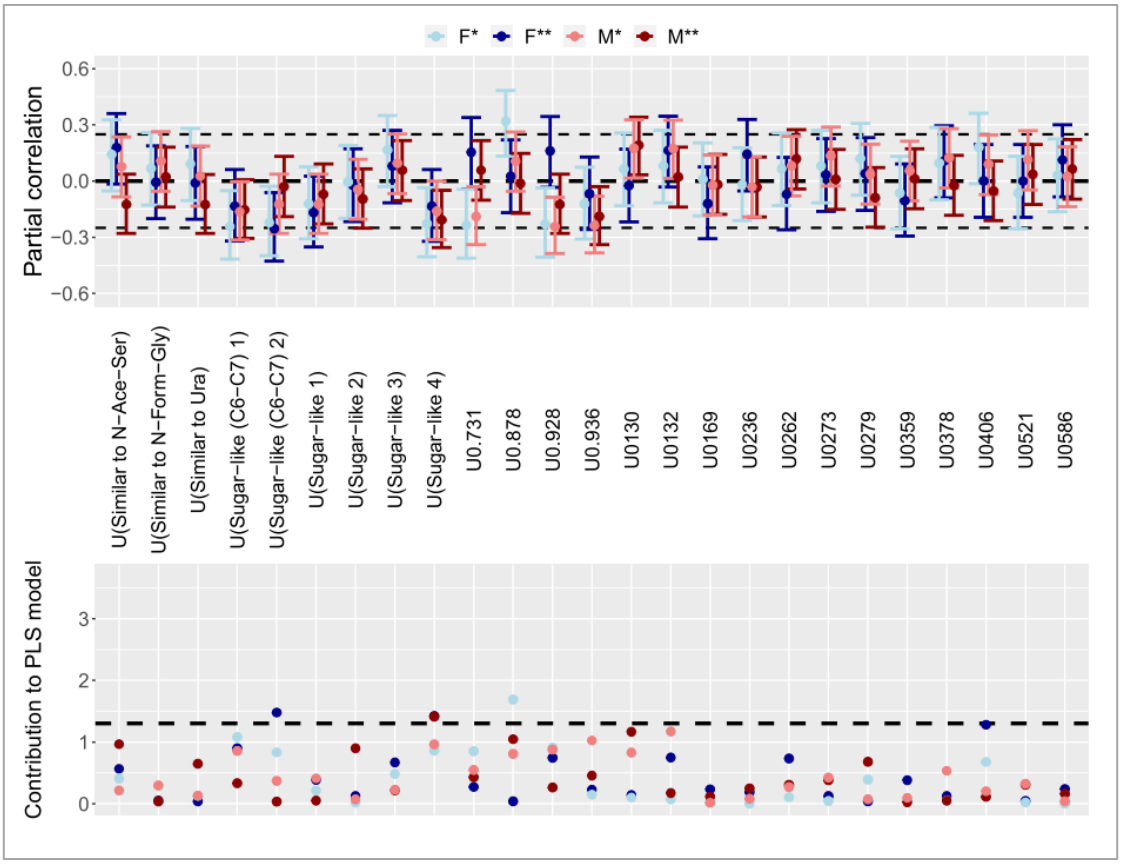

F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
PC ae C38:6
PC ae C40:1
PC ae C40:2
PC ae C40:3
PC ae C40:4
PC ae C40:5
PC ae C40:6
PC ae C42:0
PC ae C42:1
PC ae C42:2
PC ae C42:3
PC ae C42:4
PC ae C42:5
PC ae C44:3
PC ae C44:4
PC ae C44:5
PC ae C44:6
SM (OH) C14:1
SM (OH) C16:1
SM (OH) C22:1
SM (OH) C22:2
SM (OH) C24:1
SM C16:0
SM C16:1
SM C18:0
Contribution to PLS model
3
2
1
0

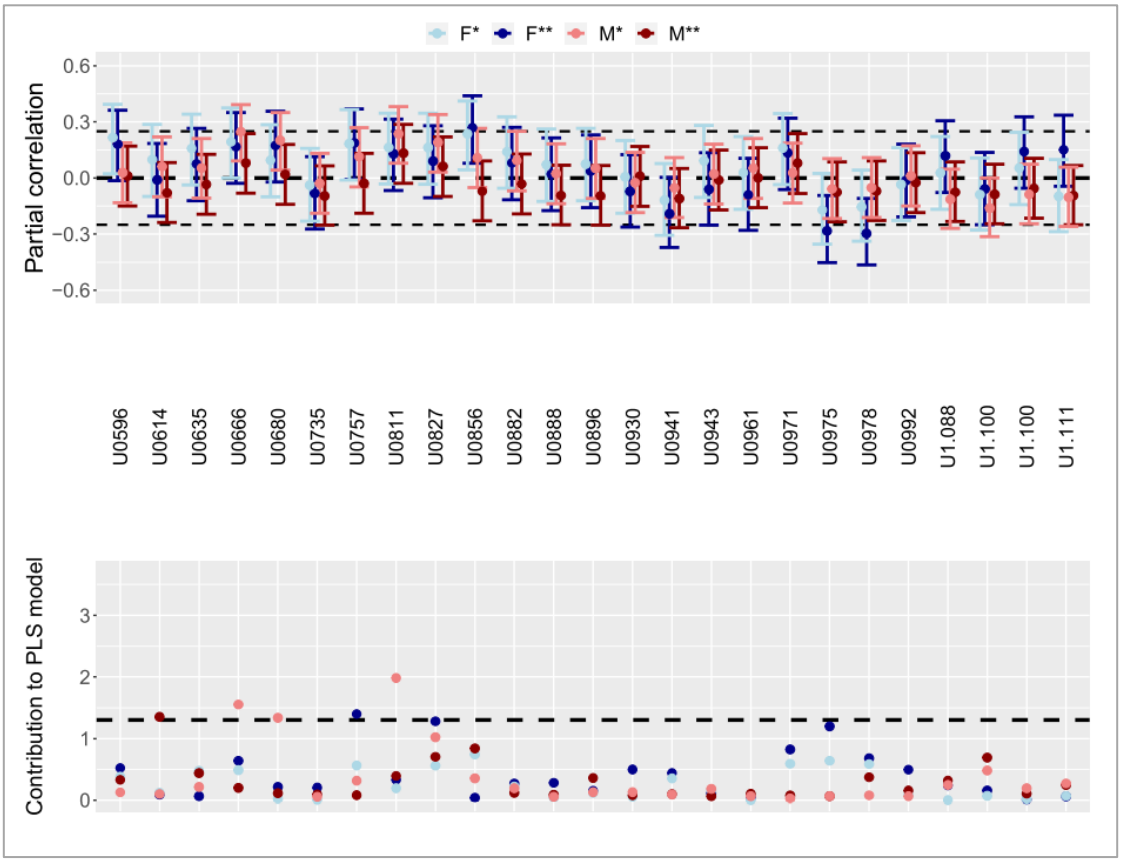

F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
SM C18:1
SM C20:2
SM C22:3
SM C24:0
SM C24:1
SM C26:0
SM C26:1
ß−OH−BA
TCA
TCDCA
TDCA
UDCA
2−Keto−3−MVA der.1
2−Keto−3−MVA der.2
2−Keto−4−MVA
3−OH−3−MBA
4−OH−Phe−AA
AcOrn
ADMA
Ala
alpha−AAA
Arg
Asn
Asp
Bet
Contribution to PLS model
3
2
1
0

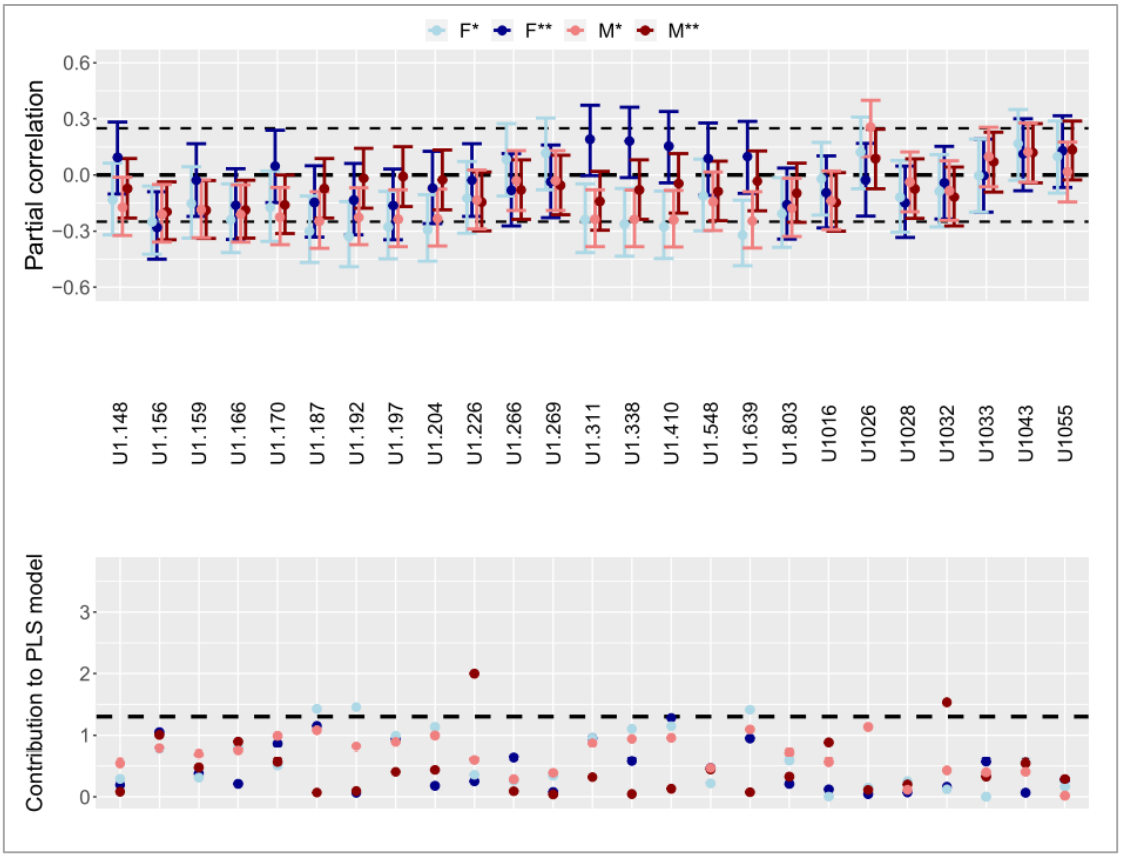

F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
Cho
Citru
Cys
DMG
Gln
Glu
Gly
His
Ile
Kyn
Leu
Lys
Met
Orn
PAA
Phe
Pro
Probably 4−DTA
Sar
Ser
Sero
Spe
Tau
Thr
trans−4−OH−Pro
Contribution to PLS model
3
2
1
0

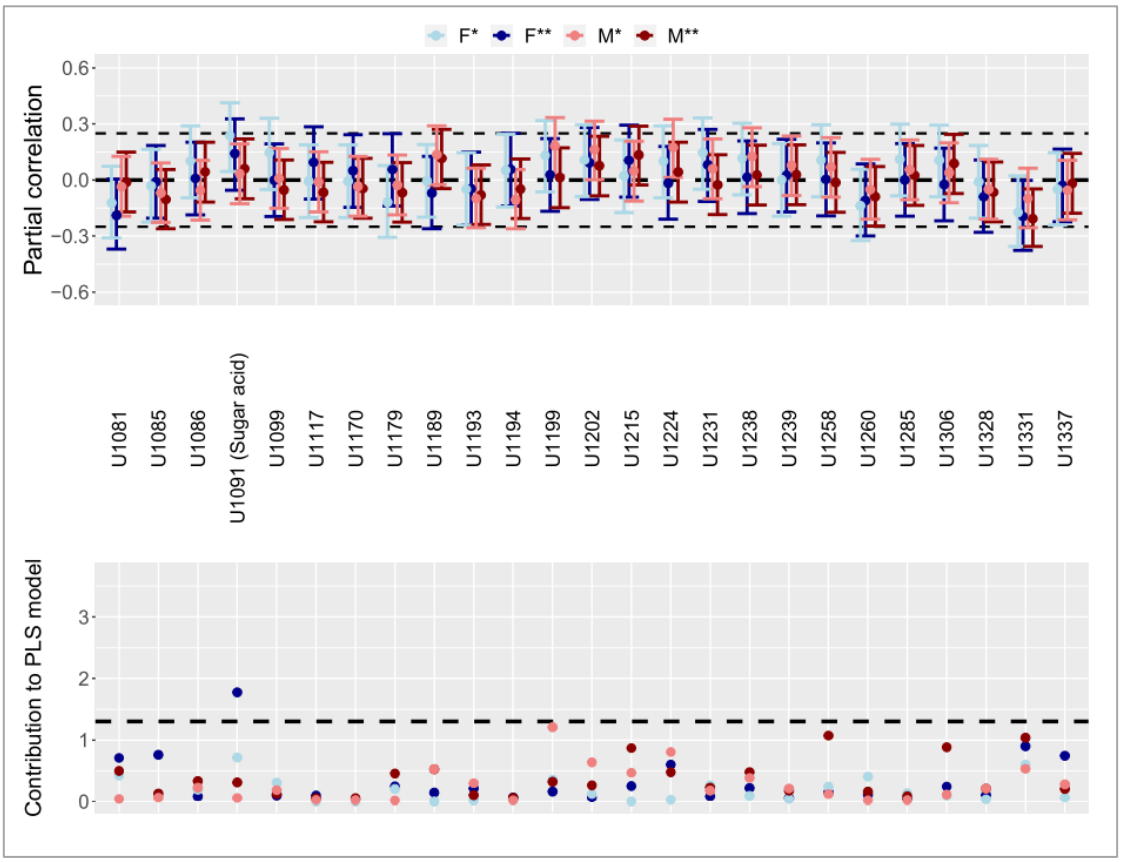

F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
Trp
Tyr
Val
2−OH−Pyr
3−OH−Pyr
3−Phe−PA
Erythri
GlucA derivative
Glyc
Malt
Oxa
S−Met−Cys
TA
TarA
Threi
3−Ind−AA
3−Ind−Lac
4−OH−Phe−Lac
For
Hip
p−Cre
TMAO
a−OH−BA
Acet
Arabi
Contribution to PLS model
3
2
1
0

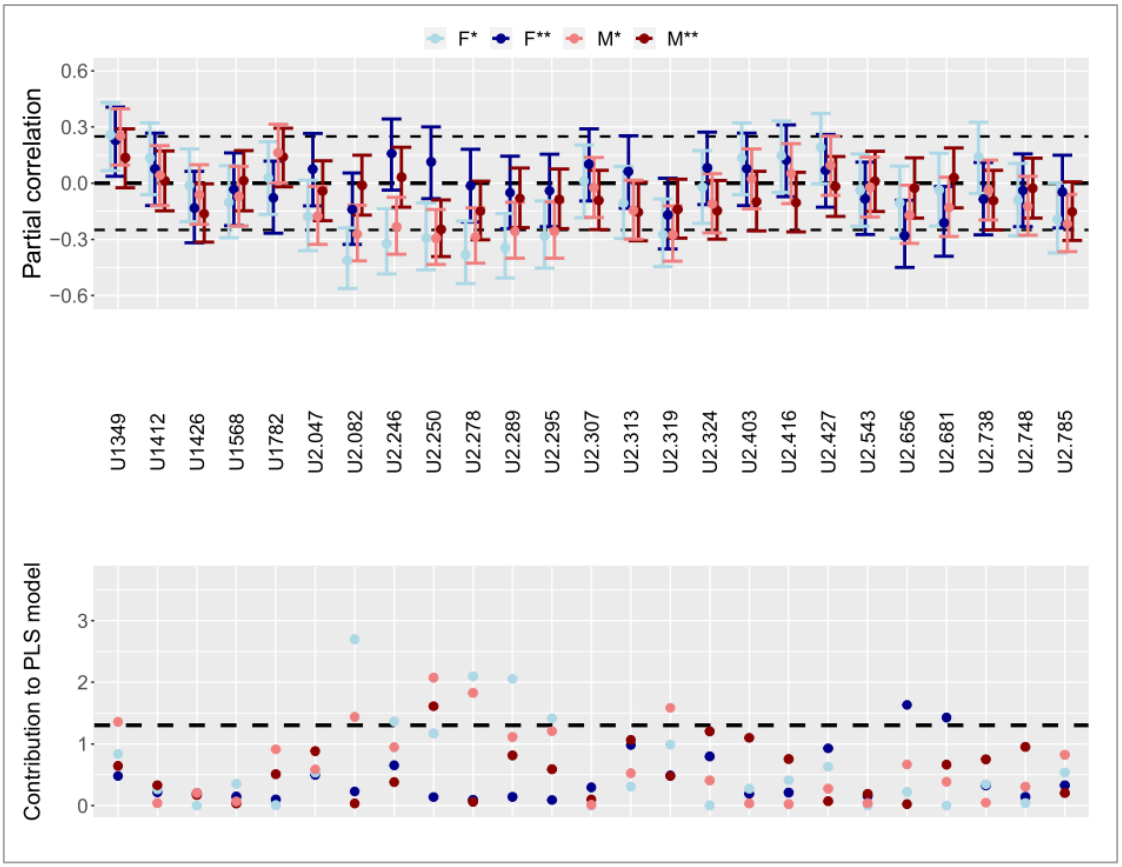

F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
Lac
myo−Ino
Cit
Fum
Isocit
MA
Suc
5−Oxopro
Pse
U(2.2−Di−OH−AA or similar)
U(Allox A or similar)
U(Amine 1)
U(Amine 2)
U(C6 sugar alcohol)
U(Disaccharide−like)
U(Glycerol−1P or similar)
U(Glycoside 1)
U(Glycoside 2)
U(Probably EA)
(Similar to 2−Ethyl−3−OH−PA)
U(Similar to AIB)
U(Similar to Ama)
U(Similar to ETA)
U(Similar to Mim 1)
U(Similar to Mim 2)
Contribution to PLS model
3
2
1
0

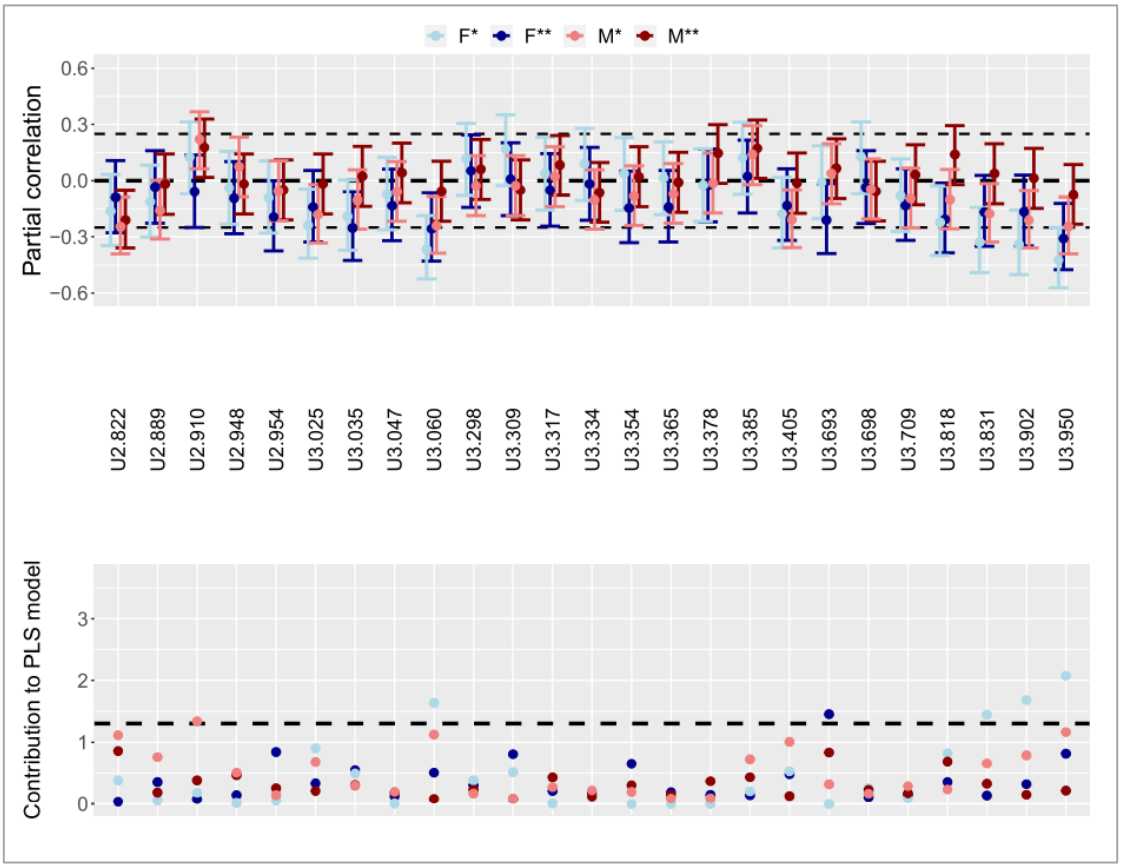

F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
U(Similar to N−Ace−Ser)
U(Similar to N−Form−Gly)
U(Similar to Ura)
U(Sugar−like (C6−C7) 1)
U(Sugar−like (C6−C7) 2)
U(Sugar−like 1)
U(Sugar−like 2)
U(Sugar−like 3)
U(Sugar−like 4)
U0.731
U0.878
U0.928
U0.936
U0130
U0132
U0169
U0236
U0262
U0273
U0279
U0359
U0378
U0406
U0521
U0586
Contribution to PLS model
3
2
1
0


F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
U0596
U0614
U0635
U0666
U0680
U0735
U0757
U0811
U0827
U0856
U0882
U0888
U0896
U0930
U0941
U0943
U0961
U0971
U0975
U0978
U0992
U1.088
U1.100
U1.100
U1.111
Contribution to PLS model
3
2
1
0


F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
U1.148
U1.156
U1.159
U1.166
U1.170
U1.187
U1.192
U1.197
U1.204
U1.226
U1.266
U1.269
U1.311
U1.338
U1.410
U1.548
U1.639
U1.803
U1016
U1026
U1028
U1032
U1033
U1043
U1055
Contribution to PLS model
3
2
1
0


F*
F**
M*
M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
U1081
U1085
U1086
U1091 (Sugar acid)
U1099
U1117
U1170
U1179
U1189
U1193
U1194
U1199
U1202
U1215
U1224
U1231
U1238
U1239
U1258
U1260
U1285
U1306
U1328
U1331
U1337
Contribution to PLS model
3
2
1
0


F* F** M* M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
U1349
U1412
U1426
U1568
U1782
U2.047
U2.082
U2.246
U2.250
U2.278
U2.289
U2.295
U2.307
U2.313
U2.319
U2.324
U2.403
U2.416
U2.427
U2.543
U2.656
U2.681
U2.738
U2.748
U2.785
Contribution to PLS model
3
2
1
0


F* F** M* M**
Partial correlation
0.6
0.3
0.0
−0.3
−0.6
U2.822
U2.889
U2.910
U2.948
U2.954
U3.025
U3.035
U3.047
U3.060
U3.298
U3.309
U3.317
U3.334
U3.354
U3.365
U3.378
U3.385
U3.405
U3.693
U3.698
U3.709
U3.818
U3.831
U3.902
U3.950
Contribution to PLS model
3
2
1
0

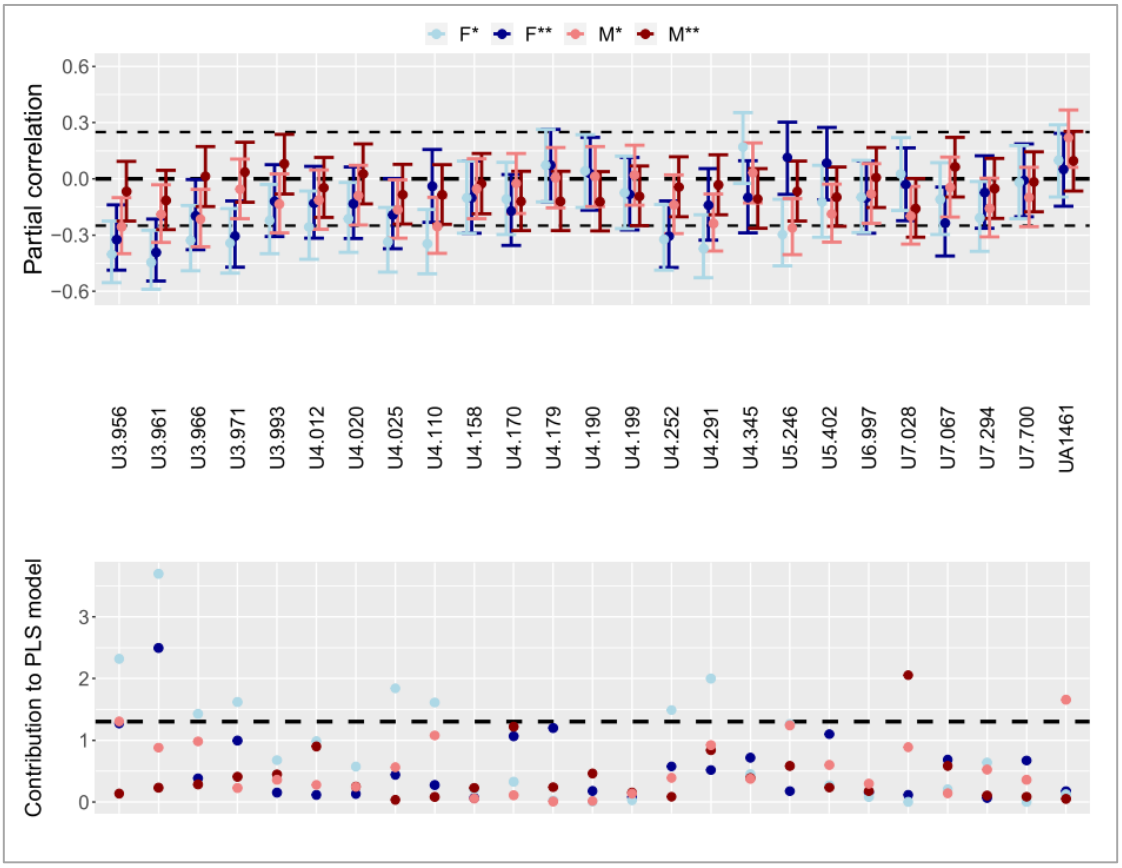


Figure A-13. Graphical overview of bi- and multivariate associations between the VO_{2peak} and plasma metabolites. F: females; M: males; * confounder (age/menopausal status)-adjusted; ** additionally adjusted for clinical and phenotypical variables. Associations were performed on VdW scores of the analyzed variables. Above: Partial correlations, expressed as Pearson correlation coefficients (dots) with 95% confidence intervals (bars). Below: Significance of contribution of each metabolite variable to the obtained PLS models for the VO_{2peak} based on rank products measured by permutation tests. The classification of metabolites to major metabolic pathways is color-coded as follows: 'amino acid metabolism' (dark blue); 'carbohydrate metabolism' (yellow); 'cofactors and vitamins metabolism' (dark green); 'energy metabolism' (light blue); 'lipid metabolism' (brown); 'mammalian-microbial cometabolism' (orange); 'nucleotide metabolism' (purple); 'xenobiotics and related metabolism' (light green); 'unknown' (black). PLS: partial least squares; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2021)).*

A2.11 Significant Correlations Between the VO_{2peak} and Plasma Analytes (*Study IIb*)

Table A-10. Plasma metabolites with significant correlations to the VO_{2peak} in females, after adjusting for age and menopausal status. *(Adapted from Kistner et al. (2021)).*

Metabolites	*r*	95% CI (lower limit)	95% CI (upper limit)
Phosphatidylcholine acyl-alkyl C44:3	**0.47**	**0.30**	**0.61**
Phosphatidylcholine acyl-alkyl C34:3	**0.47**	**0.30**	**0.61**
Phosphatidylcholine acyl-alkyl C42:4	**0.46**	**0.29**	**0.60**
Phosphatidylcholine acyl-alkyl C42:3	**0.43**	**0.25**	**0.57**
Phosphatidylcholine acyl-alkyl C42:2	**0.42**	**0.24**	**0.56**
Phosphatidylcholine acyl-alkyl C34:2	**0.40**	**0.22**	**0.55**
Phosphatidylcholine diacyl C42:2	**0.40**	**0.22**	**0.55**
Phosphatidylcholine diacyl C34:2	**0.38**	**0.20**	**0.53**
Phosphatidylcholine acyl-alkyl C40:3	**0.37**	**0.19**	**0.53**
Phosphatidylcholine acyl-alkyl C36:2	**0.37**	**0.19**	**0.53**
Phosphatidylcholine diacyl C36:2	**0.37**	**0.19**	**0.52**
Phosphatidylcholine acyl-alkyl C44:6	**0.37**	**0.18**	**0.52**
Phosphatidylcholine diacyl C40:2	**0.36**	**0.18**	**0.52**
Phosphatidylcholine acyl-alkyl C42:5	**0.36**	**0.17**	**0.52**
Phosphatidylcholine acyl-alkyl C36:3	**0.36**	**0.17**	**0.52**
Phosphatidylcholine diacyl C42:0	**0.35**	**0.17**	**0.51**
Phosphatidylcholine diacyl C40:3	**0.35**	**0.17**	**0.51**
Phosphatidylcholine acyl-alkyl C44:4	**0.35**	**0.17**	**0.51**
Phosphatidylcholine diacyl C42:4	**0.35**	**0.16**	**0.51**
Phosphatidylcholine acyl-alkyl C42:1	**0.34**	**0.15**	**0.50**
Phosphatidylcholine acyl-alkyl C44:5	**0.33**	**0.14**	**0.49**
Phosphatidylcholine diacyl C42:1	**0.32**	**0.14**	**0.49**
Citric acid	**0.32**	**0.14**	**0.49**
Unknown 0.878	**0.32**	**0.13**	**0.48**
Phosphatidylcholine acyl-alkyl C40:5	**0.31**	**0.13**	**0.48**
Phosphatidylcholine diacyl C28:1	**0.31**	**0.12**	**0.47**
Sphingomyelin C16:0	**0.31**	**0.12**	**0.47**
Phosphatidylcholine acyl-alkyl C38:2	**0.30**	**0.11**	**0.47**
Lyso-phosphatidylcholine acyl C18:2	**0.30**	**0.11**	**0.46**
Glyceric acid	**0.29**	**0.10**	**0.46**
Phosphatidylcholine acyl-alkyl C40:4	**0.29**	**0.10**	**0.46**
Phosphatidylcholine acyl-alkyl C32:1	**0.29**	**0.10**	**0.46**
Phosphatidylcholine diacyl C42:5	**0.27**	**0.08**	**0.44**
Phosphatidylcholine acyl-alkyl C32:2	**0.27**	**0.08**	**0.44**
Phosphatidylcholine acyl-alkyl C30:0	**0.27**	**0.08**	**0.44**
Tetradecadienylcarnitine	**0.27**	**0.08**	**0.44**
Acetate	**0.26**	**0.07**	**0.43**
Phosphatidylcholine acyl-alkyl C40:1	**0.26**	**0.07**	**0.43**
Unknown 1349	**0.26**	**0.07**	**0.43**
Decadienylcarnitine	**0.25**	**0.06**	**0.43**
Tetracosanoic acid	**0.25**	**0.06**	**0.43**
Phosphatidylcholine diacyl C36:3	**0.25**	**0.06**	**0.42**
Acetylornithine	0.24	0.05	0.42
Phosphatidylcholine diacyl C42:6	0.24	0.05	0.41
Tetradecanoylcarnitine	0.24	0.05	0.41
Unknown 1091 (Sugar acid)	0.24	0.05	0.41
all-cis-9.12-Octadecadienoic acid	0.24	0.05	0.41
Succinic acid	0.24	0.05	0.41
Phosphatidylcholine acyl-alkyl C34:1	0.24	0.04	0.41
Unknown 0856	0.24	0.04	0.41
Hydroxytetradecenoylcarnitine	0.23	0.04	0.41
Phosphatidylcholine acyl-alkyl C34:0	0.23	0.03	0.40
Tetradecenoylcarnitine	0.22	0.03	0.40
Phosphatidylcholine acyl-alkyl C36:0	0.22	0.03	0.40
Unknown (Similar to Aminomalonic acid)	0.22	0.03	0.40
Sphingomyelin C26:0	0.22	0.02	0.40
Phosphatidylcholine diacyl C32:0	0.22	0.02	0.39
Threonic acid	0.22	0.02	0.39
Hydroxysphingomyelin C24:1	0.22	0.02	0.39
Unknown 0596	0.22	0.02	0.39

Phosphatidylcholine diacyl C36:0	0.22	0.02	0.39
Phosphatidylcholine diacyl C32:3	0.21	0.02	0.39
Malic acid	0.21	0.02	0.39
cis-10-Heptadecenoic acid	0.21	0.01	0.39
Tiglylcarnitine	0.21	0.01	0.39
Phosphatidylcholine acyl-alkyl C40:6	0.21	0.01	0.39
Phosphatidylcholine acyl-alkyl C42:0	0.21	0.01	0.39
4-Hydroxyphenyllactic acid	0.21	0.01	0.38
Hippuric acid	0.20	0.01	0.38
Phosphatidylcholine diacyl C34:3	0.20	0.01	0.38
Lyso-phosphatidylcholine acyl C18:0	0.20	0.01	0.38
Valine	0.20	0.01	0.38
Phosphatidylcholine diacyl C32:2	0.20	0.01	0.38
myo-Inositol	0.20	0.01	0.38
Phosphatidylcholine diacyl C30:2	0.20	0.01	0.38
Tartaric acid	0.20	0.00	0.38
Taurocholate	0.20	0.00	0.38
Phosphatidylcholine diacyl C38:0	0.20	0.00	0.38
Arginine	0.20	0.00	0.38
5-Oxoproline	−0.20	−0.38	0.00
Glutamate	−0.20	−0.38	−0.01
Unknown 7.294	−0.21	−0.39	−0.01
Unknown 4.020	−0.21	−0.39	−0.02
Unknown (Sugar or sugar-like (C6-C7) 2)	−0.22	−0.40	−0.03
Unknown 3.818	−0.22	−0.40	−0.03
Unknown 3.993	−0.22	−0.40	−0.03
Unknown (Sugar or sugar-like 4)	−0.23	−0.40	−0.03
Unknown 0.928	−0.23	−0.41	−0.04
Unknown 0.731	−0.24	−0.41	−0.04
Unknown 3.025	−0.24	−0.41	−0.05
Unknown 1.166	−0.24	−0.42	−0.05
Unknown 1.311	−0.24	−0.42	−0.05
Unknown (Sugar or sugar-like (C6-C7) 1)	−0.24	−0.42	−0.05
Unknown 1.156	**−0.25**	**−0.42**	**−0.06**
Unknown 4.012	**−0.26**	**−0.43**	**−0.07**
Unknown 1.338	**−0.26**	**−0.43**	**−0.07**
Unknown 2.319	**−0.28**	**−0.45**	**−0.09**
Unknown 1.410	**−0.28**	**−0.45**	**−0.09**
Unknown 1.197	**−0.28**	**−0.45**	**−0.09**
Unknown 2.295	**−0.28**	**−0.45**	**−0.09**
Unknown 1.204	**−0.29**	**−0.46**	**−0.10**
Unknown 2.250	**−0.29**	**−0.46**	**−0.11**
Unknown 5.246	**−0.30**	**−0.47**	**−0.11**
Unknown 1.187	**−0.30**	**−0.47**	**−0.11**
cis-11-Octadecenoic acid	**−0.31**	**−0.48**	**−0.13**
cis-9-Hexadecenoic acid	**−0.32**	**−0.48**	**−0.13**
Unknown 1.639	**−0.32**	**−0.49**	**−0.14**
Unknown 2.246	**−0.32**	**−0.49**	**−0.14**
Unknown 4.252	**−0.32**	**−0.49**	**−0.14**
Unknown 3.831	**−0.33**	**−0.49**	**−0.14**
Unknown 1.192	**−0.33**	**−0.49**	**−0.14**
Unknown 3.966	**−0.33**	**−0.49**	**−0.14**
Unknown 4.025	**−0.34**	**−0.50**	**−0.15**
Unknown 3.902	**−0.34**	**−0.50**	**−0.16**
Unknown 3.971	**−0.34**	**−0.50**	**−0.16**
Unknown 2.289	**−0.35**	**−0.51**	**−0.16**
Unknown 4.110	**−0.35**	**−0.51**	**−0.16**
Unknown 3.060	**−0.37**	**−0.52**	**−0.19**
Unknown 4.291	**−0.37**	**−0.53**	**−0.19**
Unknown 2.278	**−0.38**	**−0.54**	**−0.20**
Unknown 3.956	**−0.40**	**−0.55**	**−0.23**
Unknown 2.082	**−0.41**	**−0.56**	**−0.24**
Unknown 3.950	**−0.42**	**−0.57**	**−0.25**
Unknown 3.961	**−0.45**	**-0.59**	**-0.28**

Correlations were conducted on VdW scores of the analyzed variables. bold: $|r| \geq 0.25$. CI: confidence interval; *r*: Pearson correlation coefficient; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

Table A-11. Plasma metabolites with significant correlations to the VO_{2peak} in females, after adjusting for age, menopausal status, and phenotypical/clinical variables. *(Adapted from Kistner et al. (2021)).*

Metabolites	*r*	95% CI (lower limit)	95% CI (upper limit)
Phosphatidylcholine acyl-alkyl C40:3	**0.37**	**0.19**	**0.53**
Phosphatidylcholine acyl-alkyl C42:4	**0.31**	**0.13**	**0.48**
Valerylcarnitine	**0.31**	**0.12**	**0.47**
Phosphatidylcholine acyl-alkyl C38:3	**0.28**	**0.09**	**0.45**
Choline	**0.27**	**0.08**	**0.44**
Glyceric acid	**0.27**	**0.08**	**0.44**
Unknown 0856	**0.27**	**0.08**	**0.44**
Phosphatidylcholine acyl-alkyl C36:2	**0.26**	**0.07**	**0.44**
Acetylornithine	**0.26**	**0.07**	**0.43**
Phosphatidylcholine acyl-alkyl C44:3	**0.26**	**0.07**	**0.43**
Phosphatidylcholine acyl-alkyl C40:5	**0.25**	**0.06**	**0.42**
Phosphatidylcholine diacyl C28:1	**0.25**	**0.06**	**0.42**
Phosphatidylcholine acyl-alkyl C30:0	**0.25**	**0.06**	**0.42**
Phosphatidylcholine acyl-alkyl C42:5	0.24	0.05	0.42
Pentadecanoic acid	0.24	0.04	0.41
Unknown (Probably Erythronic acid)	0.23	0.04	0.41
Phosphatidylcholine acyl-alkyl C42:2	0.23	0.04	0.41
Phosphatidylcholine diacyl C42:1	0.23	0.04	0.41
Kynurenine	0.23	0.04	0.41
Unknown 1349	0.23	0.04	0.41
Tartaric acid	0.23	0.04	0.40
Heptadecanoic acid	0.23	0.03	0.40
Phosphatidylcholine acyl-alkyl C44:5	0.22	0.03	0.40
Phosphatidylcholine acyl-alkyl C44:4	0.22	0.03	0.40
Valine	0.22	0.02	0.39
Hydroxysphingomyelin C24:1	0.22	0.02	0.39
Phosphatidylcholine acyl-alkyl C40:4	0.21	0.02	0.39
Asymmetric Dimethylarginine	0.21	0.02	0.39
Lyso-phosphatidylcholine acyl C28:1	0.21	0.02	0.39
Decadienylcarnitine	0.21	0.02	0.39
myo-Inositol	0.21	0.01	0.39
Docosanoic acid	0.21	0.01	0.38
Phosphatidylcholine acyl-alkyl C34:1	0.21	0.01	0.38
Citric acid	0.20	0.01	0.38
Phosphatidylcholine acyl-alkyl C42:3	0.20	0.00	0.38
Tetracosanoic acid	0.20	0.00	0.38
Phosphatidylcholine acyl-alkyl C38:4	0.20	0.00	0.38
U1331	−0.20	−0.38	0.00
S-Methylcysteine	−0.20	−0.38	0.00
U3.966	−0.20	−0.38	0.00
U3.818	−0.21	−0.39	−0.01
U3.693	−0.21	−0.39	−0.02
U2.681	−0.21	−0.39	−0.02
Unknown(Glycoside 2)	−0.22	−0.39	−0.02
Ethanolamine	−0.23	−0.40	−0.03
U7.067	−0.24	−0.41	−0.04
Threonine	−0.24	−0.41	−0.04
Unknown 3.035	**−0.25**	**−0.43**	**−0.06**
Unknown (Sugar-like (C6-C7) 2)	**−0.25**	**−0.43**	**−0.06**
Unknown 3.060	**−0.26**	**−0.43**	**−0.07**
Unknown 1.156	**−0.28**	**−0.45**	**−0.09**
Unknown 2.656	**−0.28**	**−0.45**	**−0.09**
Unknown 0975	**−0.28**	**−0.45**	**−0.09**
Unknown 0978	**−0.30**	**−0.46**	**−0.11**
Unknown 3.971	**−0.31**	**−0.47**	**−0.12**
Unknown 4.252	**−0.31**	**−0.47**	**−0.12**
Unknown 3.950	**−0.31**	**−0.48**	**−0.12**
Unknown 3.956	**−0.32**	**−0.49**	**−0.14**
Unknown 3.961	**−0.39**	**−0.55**	**−0.22**

Correlations were conducted on VdW scores of the analyzed variables. bold: $|r| \geq 0.25$. CI: confidence interval; *r*: Pearson correlation coefficient; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

Table A-12. Plasma metabolites with significant correlations to the VO_{2peak} in males, after adjusting for age. *(Adapted from Kistner et al. (2021)).*

Metabolites	*r*	95% CI (lower limit)	95% CI (upper limit)
Lyso-phosphatidylcholine acyl C18:2	**0.34**	**0.19**	**0.48**
Lyso-phosphatidylcholine acyl C18:1	**0.30**	**0.14**	**0.44**
Lyso-phosphatidylcholine acyl C17:0	**0.26**	**0.10**	**0.40**
Unknown 1026	**0.26**	**0.10**	**0.40**
Unknown 1349	**0.25**	**0.10**	**0.40**
Unknown 0666	**0.25**	**0.09**	**0.39**
Unknown 0811	0.24	0.08	0.38
Formate	0.23	0.08	0.38
Unknown 2.910	0.22	0.06	0.37
Unknown A1461	0.22	0.06	0.37
Malic acid	0.22	0.06	0.37
Threonic acid	0.21	0.06	0.36
Glyceric acid	0.21	0.05	0.35
Unknown 0680	0.20	0.04	0.35
Acetoacetate derivative	0.20	0.04	0.35
Succinic acid	0.20	0.04	0.35
Acetate	0.19	0.03	0.34
Unknown 0827	0.19	0.03	0.34
S-Methylcysteine	0.19	0.03	0.34
Lyso-phosphatidylcholine acyl C18:0	0.19	0.03	0.34
Unknown 1199	0.18	0.02	0.33
Phosphatidylcholine acyl-alkyl C42:3	0.18	0.02	0.33
Phosphatidylcholine acyl-alkyl C34:3	0.18	0.02	0.33
Unknown 0130	0.18	0.02	0.33
Decenoylcarnitine	0.17	0.01	0.33
Unknown 0132	0.17	0.01	0.32
Unknown 1224	0.17	0.01	0.32
Sarcosine	0.17	0.01	0.32
Oxalic acid	0.17	0.01	0.32
Phosphatidylcholine acyl-alkyl C36:2	0.17	0.01	0.32
Phenylacetic acid	0.17	0.01	0.32
Unknown 1782	0.16	0.00	0.31
Unknown 1202	0.16	0.00	0.31
Phosphatidylcholine acyl-alkyl C36:4	−0.16	−0.31	0.00
all-cis-7.10.13.16.19-Docosapentaenoic acid	−0.16	−0.31	0.00
Phosphatidylcholine diacyl C36:4	−0.16	−0.31	0.00
Unknown 1.100	−0.16	−0.31	0.00
Phosphatidylcholine diacyl C38:6	−0.16	−0.31	0.00
cis-11-Octadecenoic acid	−0.16	−0.31	0.00
Unknown (Sugar or sugar-like (C6-C7) 1)	−0.16	−0.32	0.00
cis-9-Octadecenoic acid	−0.16	−0.32	0.00
Unknown 4.025	−0.16	−0.32	0.00
Tyrosine	−0.17	−0.32	−0.01
Cysteine	−0.17	−0.32	−0.01
Unknown 2.656	−0.17	−0.32	−0.01
trans-4-Hydroxyproline	−0.17	−0.32	−0.01
Unknown 1.148	−0.17	−0.32	−0.01
Tryptophan	−0.17	−0.33	−0.01
Unknown 3.831	−0.18	−0.33	−0.02
Unknown 2.047	−0.18	−0.33	−0.02
Unknown (Glycoside 1)	−0.18	−0.33	−0.02
Phosphatidylcholine diacyl C32:1	−0.18	−0.33	−0.02
Phosphatidylcholine diacyl C34:4	−0.18	−0.33	−0.02
Unknown 1.803	−0.18	−0.33	−0.02
Unknown 3.025	−0.18	−0.33	−0.02
alpha-Aminoadipate	−0.18	−0.33	−0.02
Phosphatidylcholine diacyl C40:4	−0.18	−0.33	−0.02
Unknown 1.159	−0.19	−0.34	−0.03
Unknown 5.402	−0.19	−0.34	−0.03
Octadecanoic acid	−0.19	−0.34	−0.03
Unknown 0.731	−0.19	−0.34	−0.03
Dodecanoic acid	−0.19	−0.34	−0.03

Unknown 3.961	−0.19	−0.34	−0.03
Tartaric acid	−0.19	−0.34	−0.03
cis-9-Tetradecenoic acid	−0.20	−0.34	−0.04
Kynurenine	−0.20	−0.35	−0.04
cis-9-Hexadecenoic acid	−0.20	−0.35	−0.04
Unknown 7.028	−0.20	−0.35	−0.04
all cis-8.11.14.17-Eicosatetraenoic acid	−0.21	−0.36	−0.05
Unknown 3.405	−0.21	−0.36	−0.05
Unknown 1.156	−0.21	−0.36	−0.05
Unknown 3.902	−0.21	−0.36	−0.05
Unknown 1.166	−0.21	−0.36	−0.05
all-cis-5.8.11.14-Eicosatetraenoic acid	−0.21	−0.36	−0.06
Unknown 3.966	−0.22	−0.36	−0.06
Phosphatidylcholine diacyl C40:5	−0.22	−0.36	−0.06
all cis-4.7.10.13.16.19-Docosahexaenoic acid	−0.22	−0.37	−0.06
Unknown 2.785	−0.22	−0.37	−0.06
all-cis-8.11.14-Eicosatrienoic acid	−0.22	−0.37	−0.06
Phosphatidylcholine diacyl C38:4	−0.22	−0.37	−0.07
all-cis-7.10.13.16-Docosatetraenoic acid	−0.23	−0.37	−0.07
Unknown 1.170	−0.23	−0.37	−0.07
Unknown 1.192	−0.23	−0.37	−0.07
Tetradecanoic acid	−0.23	−0.37	−0.07
Phosphatidylcholine diacyl C38:3	−0.23	−0.38	−0.07
Unknown 2.246	−0.23	−0.38	−0.08
Unknown 1.204	−0.23	−0.38	−0.08
Hexadecanoic acid	−0.24	−0.38	−0.08
Unknown 1.311	−0.24	−0.38	−0.08
Unknown 1.197	−0.24	−0.38	−0.08
Unknown 1.338	−0.24	−0.38	−0.08
Unknown 0.936	−0.24	−0.38	−0.08
Unknown 4.291	−0.24	−0.38	−0.08
Unknown 1.410	−0.24	−0.39	−0.08
Unknown 3.060	−0.24	−0.39	−0.08
Unknown 0.928	−0.24	−0.39	−0.09
Unknown 2.822	**−0.25**	**−0.39**	**−0.09**
Unknown 3.950	**−0.25**	**−0.39**	**−0.09**
Unknown 1.639	**−0.25**	**−0.39**	**−0.09**
Unknown 1.187	**−0.25**	**−0.39**	**−0.09**
Phosphatidylcholine diacyl C40:6	**−0.25**	**−0.40**	**−0.10**
Unknown 4.110	**−0.25**	**−0.40**	**−0.10**
Sphingomyelin C18:1	**−0.26**	**−0.40**	**−0.10**
Unknown 3.956	**−0.26**	**−0.40**	**−0.10**
Unknown 2.295	**−0.26**	**−0.40**	**−0.10**
Unknown 2.289	**−0.26**	**−0.40**	**−0.10**
Unknown 5.246	**−0.26**	**−0.41**	**−0.11**
Unknown 2.082	**−0.27**	**−0.41**	**−0.12**
Unknown 2.319	**−0.28**	**−0.42**	**−0.12**
Sphingomyelin C18:0	**−0.28**	**−0.42**	**−0.13**
Unknown 2.278	**−0.29**	**−0.43**	**−0.13**
Unknown 2.250	**−0.29**	**−0.43**	**−0.14**

Correlations were conducted on VdW scores of the analyzed variables. bold: $|r| \geq 0.25$. CI: confidence interval; *r*: Pearson correlation coefficient; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

Table A-13. Plasma metabolites with significant correlations to the VO_{2peak} in males, after adjusting for age and phenotypical/ clinical variables. *(Adapted from Kistner et al. (2021)).*

Metabolites	*r*	95% CI (lower limit)	95% CI (upper limit)
Unknown 0130	0.19	0.03	0.34
Alanine	0.18	0.02	0.33
Hexanoylcarnitine (Fumaryl-L-carnitine)	0.18	0.02	0.33
Unknown 2.910	0.18	0.02	0.33
Phosphatidylcholine diacyl C36:3	0.18	0.02	0.33
Unknown 3.385	0.17	0.01	0.32
Glutamate	0.17	0.01	0.32
Unknown 1.170	−0.16	−0.31	0.00
all cis-4.7.10.13.16.19-Docosahexaenoic acid	−0.16	−0.31	0.00
Unknown 1426	−0.16	−0.32	0.00
Glycodeoxycholate	−0.16	−0.32	0.00
Phosphatidylcholine diacyl C42:1	−0.18	−0.33	−0.02
Taurodeoxycholate	−0.18	−0.33	−0.02
Phosphatidylcholine diacyl C38:0	−0.18	−0.33	−0.02
Phosphatidylcholine acyl-alkyl C38:6	−0.18	−0.33	−0.02
Unknown 1.166	−0.19	−0.34	−0.03
Unknown 1.159	−0.19	−0.34	−0.03
Unknown 0.936	−0.19	−0.34	−0.03
Unknown 1.156	−0.20	−0.35	−0.04
Tartaric acid	−0.21	−0.36	−0.05
Unknown 1331	−0.21	−0.36	−0.05
Unknown (Sugar or sugar-like 4)	−0.21	−0.36	−0.05
Unknown 2.822	−0.21	−0.36	−0.05
Unknown 2.250	**−0.25**	**−0.39**	**−0.09**

Correlations were conducted on VdW scores of the analyzed variables. bold: $|r| \geq 0.25$. CI: confidence interval; *r*: Pearson correlation coefficient; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

A2.12 Classification of VO_{2peak}-correlated Plasma Analytes to Metabolic Pathways (*Study IIb*)

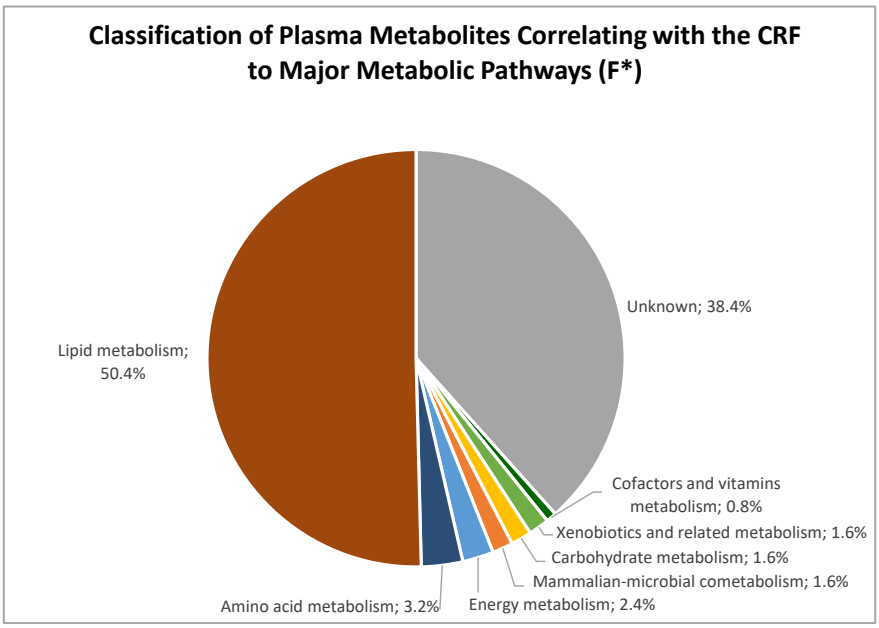


Figure A-14. Classification of CRF-correlated metabolites in females to major metabolic pathways. 125 plasma metabolites showed significant bivariate correlations with the CRF in females, after adjusting for age and menopausal status (*). Most of them belonged to 'lipid metabolism' (63/125) and 'amino acid metabolism' (4/125), followed by 'energy metabolism' (3/125), 'mammalian-microbial cometabolism', 'carbohydrate metabolism' or 'xenobiotics-related metabolism' (each 2/125), and 'cofactors and vitamins metabolism' (1/125). 48 plasma analytes were unknown. CRF: cardiorespiratory fitness; F: females. *(Reprinted from Kistner et al. (2021)).*

Figure A-15. Classification of CRF-correlated analytes in females to sub-pathways. The 77 CRF-correlated plasma metabolites with known identity were further classified to 15 sub-pathways. CRF: cardiorespiratory fitness. *(Reprinted from Kistner et al. (2021)).*

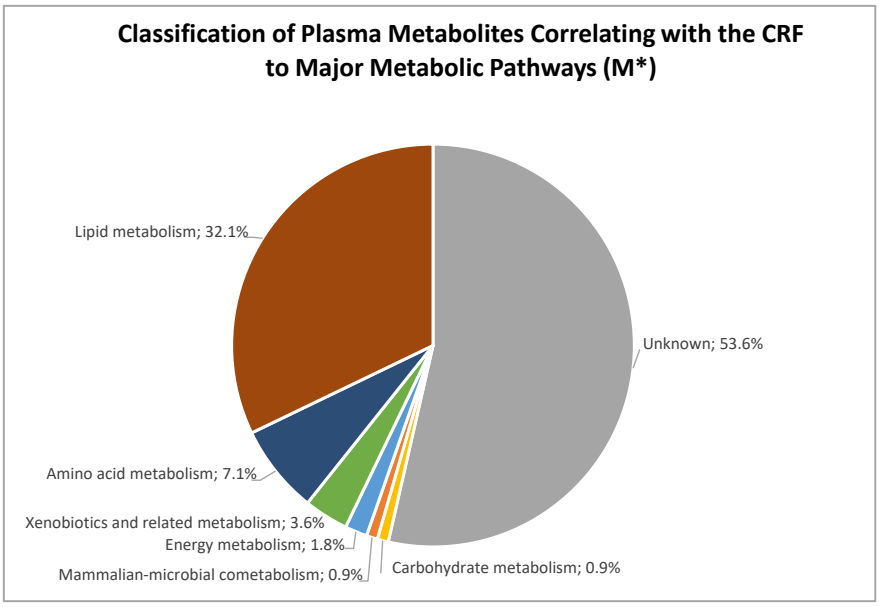


Figure A-16. Classification of CRF-correlated metabolites in males to major metabolic pathways. 112 plasma metabolites showed significant bivariate correlations with the CRF in males, after adjusting for age (*). Most of them belonged to 'lipid metabolism' (36/112) and 'amino acid metabolism' (8/112), followed by 'xenobiotics and related metabolism' (4/112), 'energy metabolism' (2/112), 'carbohydrate metabolism' and 'mammalian-microbial cometabolism' (each 1/112). 60 plasma analytes were unknown. CRF: cardiorespiratory fitness; M: males. *(Reprinted from Kistner et al. (2021)).*

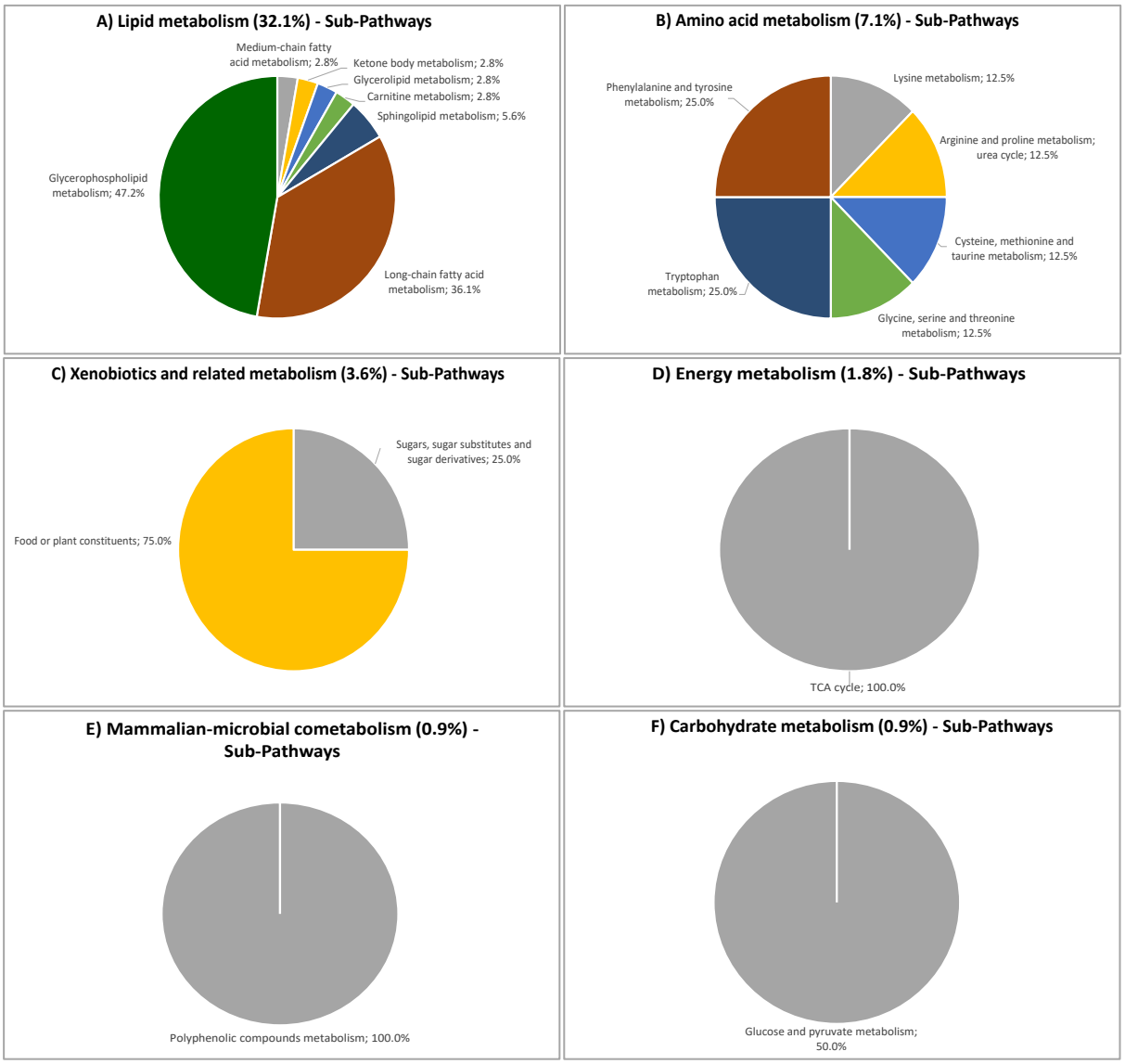


Figure A-17. Classification of CRF-correlated analytes in males to sub-pathways. The 52 CRF-correlated plasma metabolites with known identity were further classified to 18 sub-pathways. CRF: cardiorespiratory fitness. *(Reprinted from Kistner et al. (2021)).*

A2.13 Evaluation of PLS approaches (*Study IIb*)

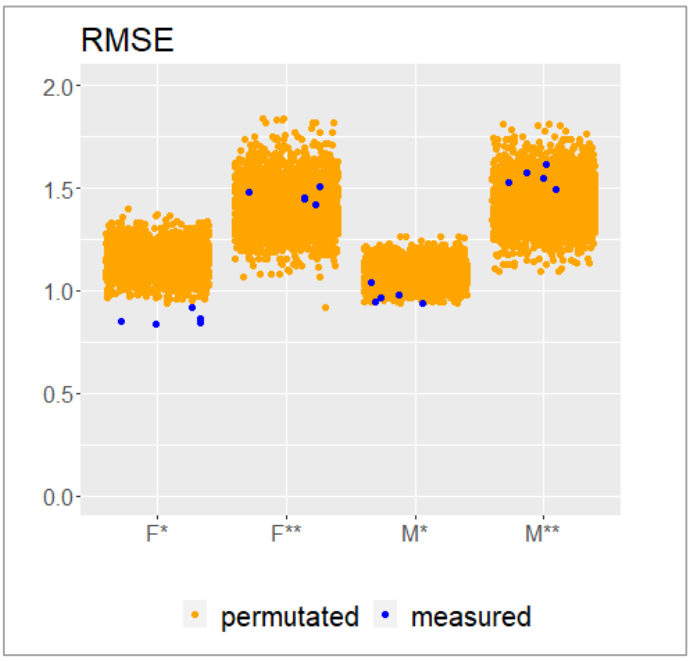


Figure A-18. Mean of the RMSEs on the test samples across the 20 repetitions of the PLS approaches. F: females; M: males; *: confounder (age/menopausal status)-adjusted; **: additionally adjusted for clinical and phenotypical variables; blue: 5 repetitions for each sub-group using the original data; orange: 2500 permutation for each sub-group. Since the mean RMSE based on the test samples was not always higher in the permutations for F** and M**, the respective results from multivariate association analyses have to be interpreted with caution. PLS: partial least squares; RMSE: root mean square error. *(Reprinted from Kistner et al. (2021)).*

A2.14 Volcano Plots of VO_{2peak}-associated plasma metabolite patterns (*Study IIb*)

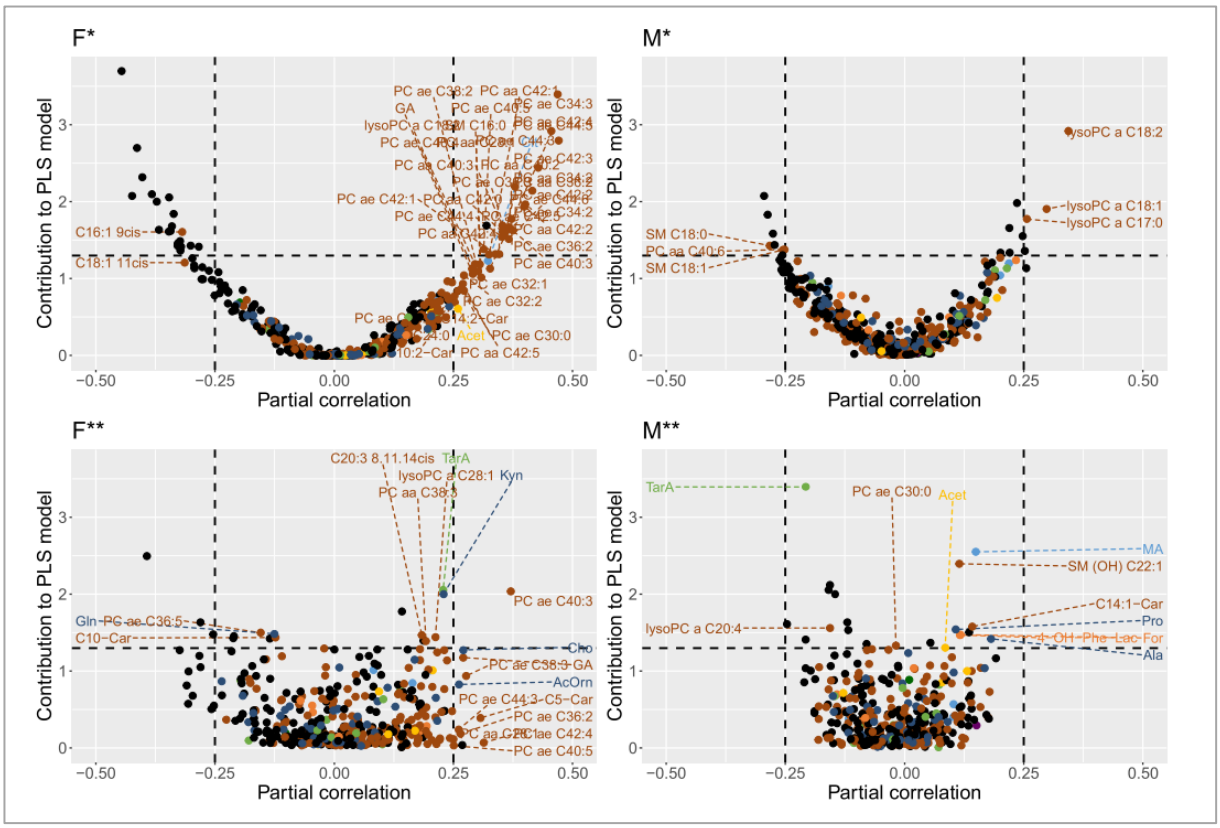


Figure A-19. Volcano plots illustrating sex-specific plasma metabolite patterns associated with the VO_{2peak} for each sub-group. F: females; M: males; *: confounder (age/menopausal status)-adjusted, **: additionally adjusted for clinical and phenotypical variables. Y-axis: significance of contribution of each metabolite variable to the PLS model, expressed as the negative logarithm of the relative frequencies of permutation-obtained rank products below measured rank products. X-axis: direction and strength of partial correlations between the VO_{2peak} and metabolite variables, expressed as Pearson correlation coefficients (*r*) of VdW-transformed variables. The classification of metabolites to metabolic pathways is color-coded as follows: 'amino acid metabolism' (dark blue); 'carbohydrate metabolism' (yellow); 'cofactors and vitamins metabolism' (dark green); 'energy metabolism' (light blue); 'lipid metabolism' (brown); 'mammalian-microbial cometabolism' (orange); 'nucleotide metabolism' (purple); 'xenobiotics and related metabolism' (light green); 'unknown' (black). The mean of the RMSEs based on the test samples was not always higher in the permutations when additionally controlling for phenotypical and clinical variables. Therefore, the respective results from multivariate association analyses (F**/M**) have to be interpreted with caution. PLS: partial least squares; RMSE: root mean square error; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake. *(Reprinted from Kistner et al. (2021)).*

A2.15 Multiple Linear Regression Analyses (*Study IIb*)

Table A-14. Stepwise regression analysis for VO_{2peak} explanation (approach 1, females). *(Adapted from Kistner et al. (2021)).*

Step	Variable	R^2	R^2 (adjusted)	Selection Frequency[V]
1	FM (%)	0.33	0.33	1
2	HDL cholesterol	0.36	0.35	0.650
3	LBM	0.39	0.38	0.435
4	PWV	0.41	0.39	0.309
5	Hb	0.42	0.39	0.205
6	BP systolic	0.43	0.39	0.116
7	BP diasystolic	0.44	0.40	0.059
8	HR_{rest}	0.45	0.40	0.032
9	Insulin	0.46	0.41	0.016
10	Total MET	0.47	0.41	0.005
11	TGs	0.48	0.41	0.003
12	LDL cholesterol	0.48	0.41	0.001
13	HbA1c	0.48	0.41	0
14	FEV1	0.49	0.40	0
15	VC_{max}	0.49	0.40	0
16	VATM	0.49	0.40	0
17	HEI-NVS	0.49	0.39	0
18	AEE	0.49	0.39	0
19	Height	0.50	0.38	0
20	BMC	0.50	0.37	0
21	Glucose	0.50	0.36	0

Approach 1: Only phenotypical/clinical variables (n=21) were stepwise selected in the female sub-group (n=102). All variables were transformed into VdW scores and adjusted for age and menopausal status prior to analysis. [V]: relative frequency the variable was present in cross-validated stepwise regression models (regarding 1000 iterations). AEE: activity energy expenditure; BMC: bone mineral content; BP: blood pressure; FEV1: forced expiratory pressure in one second; FM: fat mass; Hb: hemoglobin; HDL: high-density lipoprotein; HEI-NVS: Healthy Eating Index (modified version); HR_{rest}: resting heart rate; LBM: lean body mass; LDL: low-density lipoprotein; MET: metabolic equivalent of task; PWV: pulse wave velocity; TGs: triglycerides; VATM: visceral adipose tissue mass; VC_{max}: maximal vital capacity; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

Table A-15. Stepwise regression analysis for VO_{2peak} explanation (approach 1, males). *(Adapted from Kistner et al. (2021)).*

Step	Variable	R^2	R^2 (adjusted)	Selection Frequency[V]
1	FM (%)	0.42	0.42	1
2	HDL cholesterol	0.43	0.42	0.632
3	BMC	0.44	0.43	0.391
4	AEE	0.45	0.43	0.212
5	TGs	0.45	0.43	0.136
6	LDL cholesterol	0.46	0.43	0.066
7	HR_{rest}	0.46	0.44	0.038
8	FEV1	0.46	0.43	0.019
9	Height	0.47	0.43	0.012
10	PWV	0.47	0.43	0.006
11	LBM	0.47	0.43	0.004
12	VATM	0.47	0.43	0.003
13	Hb	0.47	0.42	0
14	VC_{max}	0.48	0.42	0
15	Total MET	0.48	0.42	0
16	HEI-NVS	0.48	0.42	0
17	BP diasystolic	0.48	0.41	0
18	Glucose	0.48	0.41	0
19	Insulin	0.48	0.40	0
20	HbA1c	0.48	0.40	0
21	BP systolic	0.48	0.39	0

Approach 1: Only phenotypical/clinical variables (n=21) were stepwise selected in the male sub-group (n=150). All variables were transformed into VdW scores and adjusted for age prior to analysis. [V]: relative frequency the variable was present in cross-validated stepwise regression models (regarding 1000 iterations). AEE: activity energy expenditure; BMC: bone mineral content; BP: blood pressure; FEV1: forced expiratory pressure in one second; FM: fat mass; Hb: hemoglobin; HDL: high-density lipoprotein; HEI-NVS: Healthy Eating Index (modified version); HR_{rest}: resting heart rate; LBM: lean body mass; LDL: low-density lipoprotein; MET: metabolic equivalent of task; PWV: pulse wave velocity; TGs: triglycerides; VATM: visceral adipose tissue mass; VC_{max}: maximal vital capacity; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

Table A-16. Stepwise regression analysis for VO_{2peak} explanation (approach 2, females). *(Adapted from Kistner et al. (2021)).*

Step	Variable	R^2	R^2 (adjusted)	Selection Frequency[V]
1	FM (%)	0.33	0.33	fixed
2	HDL cholesterol	0.36	0.35	fixed
3	LBM	0.39	0.38	fixed
4	PWV	0.41	0.39	fixed
5	Hb	0.42	0.39	fixed
6	BP systolic	0.43	0.39	fixed
7	BP diasystolic	0.44	0.40	fixed
8	HR_{rest}	0.45	0.40	fixed
9	Insulin	0.46	0.41	fixed
10	Total MET	0.47	0.41	fixed
11	TGs	0.48	0.41	fixed
12	LDL cholesterol	0.48	0.41	fixed
13	HbA1c	0.48	0.41	fixed
14	FEV1	0.49	0.40	fixed
15	VC_{max}	0.49	0.40	fixed
16	VATM	0.49	0.40	fixed
17	HEI-NVS	0.49	0.39	fixed
18	AEE	0.49	0.39	fixed
19	Height	0.50	0.38	fixed
20	BMC	0.50	0.37	fixed
21	Glucose	0.50	0.36	fixed
22	*Phosphatidylcholine acyl-alkyl C40:3*	0.58	0.46	1
23	*Unknown 3.961*	0.62	0.51	0.838
24	*S-Methylcysteine*	0.66	0.55	0.606
25	*Tartaric acid*	0.70	0.60	0.452
26	*Unknown 1.148*	0.73	0.64	0.366
27	*Serine*	0.76	0.67	0.226
28	*Tetracosanoic acid*	0.78	0.70	0.173
29	*Kynurenine*	0.79	0.71	0.108
30	*Unknown 0992*	0.81	0.72	0.060
31	*Unknown 1.266*	0.82	0.74	0.049
32	*myo-Inositol*	0.83	0.75	0.025
33	*Ethanolamine*	0.84	0.76	0.011
34	*Unknown 7.700*	0.85	0.78	0.008
35	*Unknown 0757*	0.86	0.79	0.007
36	*Glucuronic acid derivative*	0.87	0.80	0.004
37	*Phosphatidylcholine acyl-alkyl C34:0*	0.88	0.82	0.003
38	*Unknown 2.681*	0.90	0.83	0.002
39	*trans-11-Octadecenoic acid*	0.90	0.84	0.001
40	*Glycine*	0.91	0.85	0
41	*Lysine*	0.92	0.86	0
42	*Unknown 1043*	0.93	0.87	0
43	*Unknown 3.971*	0.93	0.88	0
44	*Probably 4-deoxythreonic acid*	0.94	0.89	0
45	*Unknown 0978*	0.94	0.90	0
46	*Choline*	0.95	0.90	0
47	*Unknown (Similar to Uracil)*	0.95	0.91	0
48	*Asymmetric Dimethylarginine*	0.96	0.92	0
49	*Phosphatidylcholine diacyl C34:3*	0.96	0.93	0
50	*Sarcosine*	0.97	0.93	0

Approach 1: All phenotypical/clinical variables (n=21) were included and only plasma metabolite variables (n=427) were stepwise selected in the female sub-group (n=102). All variables were transformed into VdW scores and adjusted for age and menopausal status prior to analysis. [V]: relative frequency the variable was present in cross-validated stepwise regression models (regarding 1000 iterations). Metabolite variables are indicated in italics. AEE: activity energy expenditure; BMC: bone mineral content; BP: blood pressure; FEV1: forced expiratory pressure in one second; FM: fat mass; Hb: hemoglobin; HDL: high-density lipoprotein; HEI-NVS: Healthy Eating Index (modified version); HR_{rest}: resting heart rate; LBM: lean body mass; LDL: low-density lipoprotein; MET: metabolic equivalent of task; PWV: pulse wave velocity; TGs: triglycerides; VATM: visceral adipose tissue mass; VC_{max}: maximal vital capacity; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

Table A-17. Stepwise regression analysis for VO_{2peak} explanation (approach 2, males). *(Adapted from Kistner et al. (2021)).*

Step	Variable	R^2	R^2 (adjusted)	Selection Frequency[V]
1	FM (%)	0.42	0.42	fixed
2	HDL cholesterol	0.43	0.42	fixed
3	BMC	0.44	0.43	fixed
4	AEE	0.45	0.43	fixed
5	TGs	0.45	0.43	fixed
6	LDL cholesterol	0.46	0.43	fixed
7	HR_{rest}	0.46	0.44	fixed
8	FEV1	0.46	0.43	fixed
9	Height	0.47	0.43	fixed
10	PWV	0.47	0.43	fixed
11	LBM	0.47	0.43	fixed
12	VATM	0.47	0.43	fixed
13	Hb	0.47	0.42	fixed
14	VC_{max}	0.48	0.42	fixed
15	Total MET	0.48	0.42	fixed
16	HEI-NVS	0.48	0.42	fixed
17	BP diasystolic	0.48	0.41	fixed
18	Glucose	0.48	0.41	fixed
19	Insulin	0.48	0.40	fixed
20	HbA1c	0.48	0.40	fixed
21	BP systolic	0.48	0.39	fixed
22	*Phosphatidylcholine diacyl C36:3*	0.51	0.43	1
23	*Unknown 0130*	0.54	0.45	0.719
24	*Tartaric acid*	0.56	0.48	0.555
25	*Hexanoylcarnitine (Fumaryl-L-carnitine)*	0.58	0.50	0.403
26	*Hydroxytetradecenoylcarnitine*	0.61	0.52	0.284
27	*Unknown 2.250*	0.62	0.54	0.199
28	*Malic acid*	0.65	0.56	0.155
29	*Glutamate*	0.67	0.59	0.119
30	*Tetracosanoic acid*	0.69	0.61	0.080
31	*Unknown 1.226*	0.70	0.62	0.059
32	*Unknown 3.993*	0.72	0.64	0.036
33	*Unknown 1331*	0.73	0.66	0.026
34	*5-Oxoproline*	0.75	0.67	0.018
35	*Glycodeoxycholate*	0.76	0.69	0.010
36	*cis-9-Octadecenoic acid*	0.77	0.70	0.009
37	*Phosphatidylcholine diacyl C28:1*	0.78	0.71	0.006
38	*Fumaric acid*	0.79	0.72	0.004
39	*Threonic acid*	0.80	0.72	0.002
40	*Unknown 1.100*	0.80	0.73	0.001
41	*Serine*	0.81	0.74	0.001
42	*Hydroxysphingomyelin C22:1*	0.82	0.75	0
43	*Unknown 1179*	0.82	0.75	0
44	*Unknown 1026*	0.83	0.76	0
45	*Phenylalanine*	0.84	0.77	0
46	*Valine*	0.85	0.78	0
47	*Arabitol*	0.85	0.78	0
48	*Unknown 1.187*	0.86	0.79	0
49	*Unknown 1258*	0.87	0.80	0
50	*Unknown (2.2-Dihydroxyacetic acid or similar)*	0.87	0.81	0

Approach 1: All phenotypical/clinical variables (n=21) were included and only plasma metabolite variables (n=427) were stepwise selected in the male sub-group (n=150). All variables were transformed into VdW scores and adjusted for age prior to analysis. [V]: relative frequency the variable was present in cross-validated stepwise regression models (regarding 1000 iterations). Metabolite variables are indicated in italics. AEE: activity energy expenditure; BMC: bone mineral content; BP: blood pressure; FEV1: forced expiratory pressure in one second; FM: fat mass; Hb: hemoglobin; HDL: high-density lipoprotein; HEI-NVS: Healthy Eating Index (modified version); HR_{rest}: resting heart rate; LBM: lean body mass; LDL: low-density lipoprotein; MET: metabolic equivalent of task; PWV: pulse wave velocity; TGs: triglycerides; VATM: visceral adipose tissue mass; VC_{max}: maximal vital capacity; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

Table A-18. Stepwise regression analysis for VO_{2peak} explanation (approach 3, females). *(Adapted from Kistner et al. (2021)).*

Step	Variable	R^2	R^2 (adjusted)	Selection Frequency[V]
1	FM (%)	0.33	0.33	1
2	*Phosphatidylcholine acyl-alkyl C40:3*	0.42	0.41	0.823
3	*myo-Inositol*	0.49	0.48	0.632
4	*Unknown 0975*	0.57	0.55	0.493
5	*Unknown 3.961*	0.59	0.57	0.316
6	*Unknown 7.294*	0.64	0.61	0.242
7	*Glycine*	0.66	0.64	0.183
8	*Unknown 2.313*	0.69	0.66	0.138
9	*Lysine*	0.70	0.67	0.079
10	*Octadecenoylcarnitine*	0.71	0.68	0.057
11	*Malic acid*	0.73	0.69	0.047
12	*Decanoic acid*	0.75	0.72	0.04
13	*Tetracosanoic acid*	0.77	0.73	0.035
14	*Unknown 2.681*	0.78	0.75	0.028
15	BP systolic	0.80	0.76	0.018
16	*Unknown 1091 (Sugar acid)*	0.81	0.78	0.011
17	*Pseudouridine*	0.83	0.79	0.005
18	*Tetradecenoylcarnitine*	0.84	0.80	0.004
19	*Unknown 0992*	0.85	0.82	0.004
20	*Taurine*	0.86	0.83	0.002
21	*Unknown (Similar to Mimosine 1)*	0.87	0.83	0.002
22	*Unknown 0971*	0.88	0.84	0.002
23	VATM	0.89	0.85	0.002
24	*Phosphatidylcholine diacyl C28:1*	0.90	0.87	0.001
25	*Unknown 7.028*	0.91	0.88	0
26	*Glucuronic acid derivative*	0.92	0.89	0
27	*Unknown 1224*	0.92	0.90	0
28	*Hydroxytetradecenoylcarnitine*	0.93	0.90	0
29	*Phosphatidylcholine acyl-alkyl C38:1*	0.93	0.91	0
30	*Unknown 1199*	0.94	0.91	0
31	*Threitol*	0.94	0.92	0
32	*Unknown 1.803*	0.95	0.93	0
33	*Unknown A1461*	0.95	0.93	0
34	*Unknown 7.067*	0.96	0.94	0
35	*Lyso-phosphatidylcholine acyl C20:4*	0.96	0.94	0
36	*Unknown (Similar to 2-Ethyl-3-hydroxypropionic acid)*	0.97	0.95	0
37	*Unknown 3.956*	0.97	0.95	0
38	*Threonic acid*	0.97	0.95	0
39	*Unknown 3.035*	0.97	0.96	0
40	*all-cis-9.12-Octadecadienoic acid*	0.98	0.96	0
41	*Tartaric acid*	0.98	0.96	0
42	*Betaine*	0.98	0.96	0
43	*Unknown (Similar to 2-Aminoisobutyric acid)*	0.98	0.97	0
44	*Unknown (Sugar or sugar-like 2)*	0.98	0.97	0
45	*Unknown 1.159*	0.99	0.97	0
46	*3-Indoleacetic acid*	0.99	0.98	0
47	Glucose	0.99	0.98	0
48	*Unknown 1239*	0.99	0.98	0
49	*Unknown 1.269*	0.99	0.98	0
50	*Unknown 0586*	0.99	0.99	0

Approach 3: Phenotypical/clinical variables (n=21) as well as plasma metabolite variables (n=427) were stepwise selected in the female sub-group (n=102). All variables were transformed into VdW scores and adjusted for age and menopausal status prior to analysis. [V]: relative frequency the variable was present in cross-validated stepwise regression models (regarding 1000 iterations). Metabolite variables are indicated in italics. BP: blood pressure; FM: fat mass; VATM: visceral adipose tissue mass; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

Table A-19. Stepwise regression analysis for VO_{2peak} explanation (approach 3, males). *(Adapted from Kistner et al. (2021)).*

Step	Variable	R^2	R^2 (adjusted)	Selection Frequency[V]
1	FM (%)	0.42	0.42	1
2	*Malic acid*	0.46	0.45	0.742
3	*Taurocholate*	0.48	0.47	0.503
4	*Phosphatidylcholine diacyl C36:3*	0.51	0.50	0.366
5	*Unknown 0130*	0.54	0.52	0.293
6	*Phosphatidylcholine diacyl C36:6*	0.56	0.54	0.223
7	*Glutamate*	0.59	0.56	0.150
8	*Unknown (Similar to Uracil)*	0.60	0.58	0.088
9	*Unknown 1.226*	0.61	0.59	0.055
10	*Unknown 3.693*	0.64	0.61	0.042
11	*all cis-5.8.11.14.17-Eicosapentaenoic acid*	0.65	0.63	0.029
12	*Phosphatidylcholine diacyl C42:2*	0.67	0.64	0.014
13	*cis-9-Octadecenoic acid*	0.68	0.65	0.012
14	*Unknown 1331*	0.70	0.66	0.008
15	*Arabitol*	0.71	0.68	0.006
16	*Unknown 0132*	0.72	0.68	0.003
17	*Tetracosanoic acid*	0.73	0.70	0.003
18	*Unknown 2.738*	0.74	0.71	0.003
19	*Tetradecanoic acid*	0.75	0.72	0.003
20	*Unknown 1026*	0.76	0.73	0.002
21	*Tartaric acid*	0.77	0.74	0.001
22	*Unknown 2.403*	0.78	0.75	0.001
23	*Unknown 2.307*	0.79	0.76	0
24	*Unknown (Similar to N-Acetylserine)*	0.80	0.76	0
25	*Unknown (Glycoside 1)*	0.81	0.77	0
26	*Unknown 1.803*	0.82	0.78	0
27	*Phosphatidylcholine diacyl C42:4*	0.83	0.79	0
28	*Glutamine*	0.83	0.80	0
29	*Unknown (Glycerol-1-phosphate or similar)*	0.84	0.80	0
30	*Phosphatidylcholine diacyl C34:3*	0.85	0.81	0
31	Height	0.85	0.82	0
32	*Unknown 1258*	0.86	0.82	0
33	*Taurine*	0.86	0.83	0
34	*cis-9.trans-11-Octadecadienoic acid*	0.87	0.83	0
35	*Sphingomyelin C24:0*	0.87	0.84	0
36	*Glycolic acid*	0.88	0.84	0
37	*Unknown 0.878*	0.88	0.85	0
38	*cis-10-Heptadecenoic acid*	0.89	0.85	0
39	AEE	0.90	0.86	0
40	*Erythritol*	0.90	0.86	0
41	*Unknown 1117*	0.90	0.87	0
42	*Tiglylcarnitine*	0.91	0.87	0
43	*Tetradecadienylcarnitine*	0.91	0.88	0
44	*Oxalic acid*	0.92	0.88	0
45	*Valine*	0.92	0.88	0
46	*Methionine*	0.92	0.89	0
47	VC_{max}	0.93	0.89	0
48	*Unknown 7.028*	0.93	0.90	0
49	*Acetoacetate derivative*	0.93	0.90	0
50	*Proline*	0.94	0.90	0

Approach 3: Phenotypical/clinical variables (n=21) as well as plasma metabolite variables (n=427) were stepwise selected in the male sub-group (n=150). All variables were transformed into VdW scores and adjusted for age prior to analysis. [V]: relative frequency the variable was present in cross-validated stepwise regression models (regarding 1000 iterations). Metabolite variables are indicated in italics. AEE: activity energy expenditure; FM: fat mass; VC_{max}: maximal vital capacity; VdW: Van der Waerden; VO_{2peak}: peak oxygen uptake.

A3 Metabolomics Analyses

A3.1 List of Metabolites Identified by Targeted NMR-Based Analysis (*Study I, IIa*)

Table A-20. Metabolites identified by targeted NMR-based analysis and included in *Study I* and *IIa*. *(Own table).*

Abbreviation	Metabolite Name	MSI ID Level	Unit	Major Pathway	Sub-Pathway	HMDB ID	KEGG ID
BAIBA	β-Aminoisobutyrate	2	mmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0003911	C05145
MNA	1-Methylnicotinamide	2	mmol/L	Cofactors and vitamins metabolism	Nicotinate and nicotinamide metabolism	HMDB0000699	C02918
2-OH-Isob	2-Hydroxyisobutyrate	2	mmol/L	Xenobiotics and related metabolism	Chemicals	HMDB0000729	-
3-OH-Isov	3-Hydroxyisovalerate	2	mmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000754	C20827
3-Ind	3-Indoxylsulfate	2	mmol/L	Mammalian-microbial cometabolism	Tryptophan metabolism	HMDB0000682	-
3-MXan	3-Methylxanthine	2	mmol/L	Xenobiotics and related metabolism	Xanthine metabolism	HMDB0001886	C16357
4-OH-Phe	4-Hydroxyphenylacetate	2	mmol/L	Amino acid metabolism	Phenylalanine and tyrosine metabolism	HMDB0000020	C00642
Acet	Acetate	2	mmol/L	Carbohydrate metabolism	Glucose and pyruvate metabolism	HMDB0000042	C00033
Ace	Acetone	2	mmol/L	Lipid metabolism	Ketone body metabolism	HMDB0001659	C00207
Ala	Alanine	2	mmol/L	Amino acid metabolism	Alanine and aspartate metabolism	HMDB0000161	C09885
Bet	Betaine	2	mmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000043	C00719
Car	Carnitine	2	mmol/L	Lipid metabolism	Carnitine metabolism	HMDB0000062	C00318
c-Aco	cis-Aconitate	2	mmol/L	Energy metabolism	TCA cycle intermediates	HMDB0000072	C00417
Cit	Citrate	2	mmol/L	Energy metabolism	TCA cycle intermediates	HMDB0000094	C00158
Cre	Creatine	2	mmol/L	Amino acid metabolism	Creatine metabolism	HMDB0000064	C00300
Crea	Creatinine	2	mmol/L	Amino acid metabolism	Creatine metabolism	HMDB0000562	C00791
DMA	Dimethylamine	2	mmol/L	Mammalian-microbial cometabolism	Trimethylamines metabolism	HMDB0000087	C00543
DMS	Dimethylsulfone	2	mmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0004983	C11142
For	Formate	2	mmol/L	Mammalian-microbial cometabolism	One carbon metabolism	HMDB0000142	C00058
Glu	Gluconate	2	mmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0000625	C00257
Gly	Glycine	2	mmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000123	C00037
Glyc	Glycolate	2	mmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0000115	C00160
Gua	Guanidoacetate	2	mmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000128	C00581
Hip	Hippurate	2	mmol/L	Mammalian-microbial cometabolism	Polyphenolic compounds metabolism	HMDB0000714	C01586
His	Histidine	2	mmol/L	Amino acid metabolism	Histidine metabolism	HMDB0000177	C00135
Hyp	Hypoxanthine	2	mmol/L	Nucleotide metabolism	Purine metabolism	HMDB0000157	C00262
Ile	Isoleucine	2	mmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000172	C00407
Lac	Lactate	2	mmol/L	Carbohydrate metabolism	Glucose and pyruvate metabolism	HMDB0000190	C00256
Leu	Leucine	2	mmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000687	C00123
Man	Mannitol	2	mmol/L	Xenobiotics and related metabolism	Sugars, sugar substitutes and sugar derivatives	HMDB0000765	C00392
Met	Methanol	2	mmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0001875	C00132
MA	Methylamine	2	mmol/L	Mammalian-microbial cometabolism	Trimethylamines metabolism	HMDB0000164	C00218

MSuc	Methylsuccinate	2	mmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0001844	C08645
DMG	N,N-Dimethylglycine	2	mmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000092	C01026
Pse	Pseudouridine	2	mmol/L	Nucleotide metabolism	Pyrimidine metabolism	HMDB0000767	C02067
Pyr	Pyruvate	2	mmol/L	Carbohydrate metabolism	Glucose and pyruvate metabolism	HMDB0000243	C00022
Suc	Succinate	2	mmol/L	Energy metabolism	TCA cycle intermediates	HMDB0000254	C00042
Tar	Tartrate	2	mmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0000956	C00898
Tau	Taurine	2	mmol/L	Amino acid metabolism	Cysteine, methionine and taurine metabolism	HMDB0000251	C00245
Thr	Threonine	2	mmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000167	C00188
t-Aco	trans-Aconitate	2	mmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0000958	C02341
Tri	Trigonelline	2	mmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0000875	C01004
TMAO	Trimethylamine N-oxide	2	mmol/L	Mammalian-microbial cometabolism	Trimethylamines metabolism	HMDB0000925	C01104
Tyr	Tyrosine	2	mmol/L	Amino acid metabolism	Phenylalanine and tyrosine metabolism	HMDB0000158	C00082
Ura	Uracil	2	mmol/L	Nucleotide metabolism	Pyrimidine metabolism	HMDB0000300	C00106
Urea	Urea	2	mmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0000294	C00086
Val	Valine	2	mmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000883	C00183

HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; MSI: Metabolomics Standards Initiative; MSI ID: 1=identified compound; TCA: tricarboxylic acid.

A3.2 List of Analytes Detected by Untargeted NMR-Based Analysis (*Study IIb*)

Table A-21. Analytes detected by untargeted NMR-based analysis and included in *Study IIb*. *(Own table).*

Abbreviation	Metabolite Name	MSI ID Level	Unit	Major Pathway	Sub-Pathway	HMDB ID	KEGG ID
P_x0.7314_0.6973	U0.731	4	Bucket Integral	-	-	-	-
P_x0.8785_0.8170	U0.878	4	Bucket Integral	-	-	-	-
P_x0.9285_0.8785	U0.928	4	Bucket Integral	-	-	-	-
P_x0.9360_0.9285	U0.936	4	Bucket Integral	-	-	-	-
P_x1.0885_1.0824	U1.088	4	Bucket Integral	-	-	-	-
P_x1.1003_1.0945	U1.100	4	Bucket Integral	-	-	-	-
P_x1.1114_1.1064	U1.111	4	Bucket Integral	-	-	-	-
P_x1.1488_1.1309	U1.148	4	Bucket Integral	-	-	-	-
P_x1.1560_1.1527	U1.156	4	Bucket Integral	-	-	-	-
P_x1.1595_1.1560	U1.159	4	Bucket Integral	-	-	-	-
P_x1.1667_1.1617	U1.166	4	Bucket Integral	-	-	-	-
P_x1.1701_1.1667	U1.170	4	Bucket Integral	-	-	-	-
P_x1.1875_1.1843	U1.187	4	Bucket Integral	-	-	-	-
P_x1.1927_1.1876	U1.192	4	Bucket Integral	-	-	-	-
P_x1.1979_1.1946	U1.197	4	Bucket Integral	-	-	-	-
P_x1.2043_1.2010	U1.204	4	Bucket Integral	-	-	-	-
P_x1.2260_1.2107	U1.226	4	Bucket Integral	-	-	-	-
P_x1.2660_1.2260	U1.266	4	Bucket Integral	-	-	-	-

P_x1.2695_1.2660	U1.269	4	Bucket Integral	-	-	-	-
P_x1.3110_1.2695	U1.311	4	Bucket Integral	-	-	-	-
P_x1.3383_1.3110	U1.338	4	Bucket Integral	-	-	-	-
P_x1.5481_1.5406	U1.548	4	Bucket Integral	-	-	-	-
P_x1.6396_1.5481	U1.639	4	Bucket Integral	-	-	-	-
P_x1.8038_1.7844	U1.803	4	Bucket Integral	-	-	-	-
P_x1.9400_1.9341	Acetate	2	Bucket Integral	Carbohydrate metabolism	Glucose and pyruvate metabolism	HMDB0000042	C00033
P_x2.0478_1.9697	U2.047	4	Bucket Integral	-	-	-	-
P_x2.0826_2.0478	U2.082	4	Bucket Integral	-	-	-	-
P_x2.2466_2.2197	U2.246	4	Bucket Integral	-	-	-	-
P_x2.2503_2.2466	U2.250	4	Bucket Integral	-	-	-	-
P_x2.2787_2.2608	U2.278	4	Bucket Integral	-	-	-	-
P_x2.2894_2.2787	U2.289	4	Bucket Integral	-	-	-	-
P_x2.2959_2.2894	U2.295	4	Bucket Integral	-	-	-	-
P_x2.3074_2.2959	U2.307	4	Bucket Integral	-	-	-	-
P_x2.3136_2.3074	U2.313	4	Bucket Integral	-	-	-	-
P_x2.3193_2.3136	U2.319	4	Bucket Integral	-	-	-	-
P_x2.3246_2.3193	U2.324	4	Bucket Integral	-	-	-	-
P_x2.4038_2.3963	U2.403	4	Bucket Integral	-	-	-	-
P_x2.4161_2.4099	U2.416	4	Bucket Integral	-	-	-	-
P_x2.4278_2.4207	U2.427	4	Bucket Integral	-	-	-	-
P_x2.5439_2.5232	U2.543	4	Bucket Integral	-	-	-	-
P_x2.6565_2.6479	U2.656	4	Bucket Integral	-	-	-	-
P_x2.6813_2.6766	U2.681	4	Bucket Integral	-	-	-	-
P_x2.7381_2.7343	U2.738	4	Bucket Integral	-	-	-	-
P_x2.7484_2.7439	U2.748	4	Bucket Integral	-	-	-	-
P_x2.7856_2.7484	U2.785	4	Bucket Integral	-	-	-	-
P_x2.8223_2.7856	U2.822	4	Bucket Integral	-	-	-	-
P_x2.8896_2.8828	U2.889	4	Bucket Integral	-	-	-	-
P_x2.9107_2.9072	U2.910	4	Bucket Integral	-	-	-	-
P_x2.9486_2.9405	U2.948	4	Bucket Integral	-	-	-	-
P_x2.9548_2.9486	U2.954	4	Bucket Integral	-	-	-	-
P_x3.0251_3.0120	U3.025	4	Bucket Integral	-	-	-	-
P_x3.0359_3.0251	U3.035	4	Bucket Integral	-	-	-	-
P_x3.0477_3.0415	U3.047	4	Bucket Integral	-	-	-	-
P_x3.0609_3.0554	U3.060	4	Bucket Integral	-	-	-	-
P_x3.2989_3.2925	U3.298	4	Bucket Integral	-	-	-	-
P_x3.3097_3.3029	U3.309	4	Bucket Integral	-	-	-	-
P_x3.3177_3.3097	U3.317	4	Bucket Integral	-	-	-	-
P_x3.3345_3.3178	U3.334	4	Bucket Integral	-	-	-	-

P_x3.3543_3.3346	U3.354	4	Bucket Integral	-	-	-	-
P_x3.3650_3.3543	U3.365	4	Bucket Integral	-	-	-	-
P_x3.3785_3.3650	U3.378	4	Bucket Integral	-	-	-	-
P_x3.3851_3.3785	U3.385	4	Bucket Integral	-	-	-	-
P_x3.4055_3.3851	U3.405	4	Bucket Integral	-	-	-	-
P_x3.6935_3.6685	U3.693	4	Bucket Integral	-	-	-	-
P_x3.6988_3.6935	U3.698	4	Bucket Integral	-	-	-	-
P_x3.7090_3.6988	U3.709	4	Bucket Integral	-	-	-	-
P_x3.8188_3.8077	U3.818	4	Bucket Integral	-	-	-	-
P_x3.8315_3.8188	U3.831	4	Bucket Integral	-	-	-	-
P_x3.9025_3.8832	U3.902	4	Bucket Integral	-	-	-	-
P_x3.9500_3.9448	U3.950	4	Bucket Integral	-	-	-	-
P_x3.9560_3.9500	U3.956	4	Bucket Integral	-	-	-	-
P_x3.9614_3.9560	U3.961	4	Bucket Integral	-	-	-	-
P_x3.9664_3.9614	U3.966	4	Bucket Integral	-	-	-	-
P_x3.9713_3.9664	U3.971	4	Bucket Integral	-	-	-	-
P_x3.9934_3.9856	U3.993	4	Bucket Integral	-	-	-	-
P_x4.0126_3.9996	U4.012	4	Bucket Integral	-	-	-	-
P_x4.0200_4.0126	U4.020	4	Bucket Integral	-	-	-	-
P_x4.0254_4.0200	U4.025	4	Bucket Integral	-	-	-	-
P_x4.1102_4.0758	U4.110	4	Bucket Integral	-	-	-	-
P_x4.1582_4.1509	U4.158	4	Bucket Integral	-	-	-	-
P_x4.1700_4.1588	U4.170	4	Bucket Integral	-	-	-	-
P_x4.1799_4.1705	U4.179	4	Bucket Integral	-	-	-	-
P_x4.1900_4.1828	U4.190	4	Bucket Integral	-	-	-	-
P_x4.1999_4.1950	U4.199	4	Bucket Integral	-	-	-	-
P_x4.2525_4.2460	U4.252	4	Bucket Integral	-	-	-	-
P_x4.2910_4.2630	U4.291	4	Bucket Integral	-	-	-	-
P_x4.3457_4.3115	U4.345	4	Bucket Integral	-	-	-	-
P_x5.2462_5.1841	U5.246	4	Bucket Integral	-	-	-	-
P_x5.4020_5.2679	U5.402	4	Bucket Integral	-	-	-	-
P_x6.9974_6.9516	U6.997	4	Bucket Integral	-	-	-	-
P_x7.0289_6.9974	U7.028	4	Bucket Integral	-	-	-	-
P_x7.0670_7.0473	U7.067	4	Bucket Integral	-	-	-	-
P_x7.2941_7.2293	U7.294	4	Bucket Integral	-	-	-	-
P_x7.7003_7.6591	U7.700	4	Bucket Integral	-	-	-	-
P_x8.4809_8.4763	Formate	2	Bucket Integral	Mammalian-microbial cometabolism	One carbon metabolism	HMDB0000142	C00058

HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; MSI: Metabolomics Standards Initiative; MSI ID: 2= putatively annotated compound; 4=unknown compound.

A3.3 List of Metabolites Identified by Targeted LC-MS-Based Analysis (*Study I, IIb*)

Table A- 22. Metabolites identified by targeted LC-MS-based analysis and included in *Study I* and *IIb*. *(Own table).*

Abbreviation	Metabolite Name	MSI ID Level	Unit	Major Pathway	Sub-Pathway	HMDB ID	KEGG ID
BAIBA	β-Aminoisobutyrate	1	µmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0003911	C05145
GABA	γ-Aminobutyrate	1	µmol/L	Amino acid metabolism	Glutamate metabolism	HMDB0000112	C00334
GBB	γ-Butyrobetaine	1	µmol/L	Mammalian-microbial cometabolism	Trimethylamines metabolism	HMDB0001161	C01181
Ans	Anserine	1	µmol/L	Amino acid metabolism	Histidine metabolism	HMDB0000194	C01262
Arg	Arginine	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0000517	C00062
ADMA	Asymmetric Dimethylarginine	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0001539	C03626
Bet	Betaine [a]	1	µmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000043	C00719
Beto	Betonicine	1	µmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0029412	C08269
Car	Carnitine [a]	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0000062	C00318
Carn	Carnosine	1	µmol/L	Amino acid metabolism	Histidine metabolism	HMDB0000033	C00386
Cho	Choline [a]	1	µmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000097	C00114
Citr	Citrulline	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0000904	C00327
Cre	Creatine	1	µmol/L	Amino acid metabolism	Creatine metabolism	HMDB0000064	C00300
DMA	Dimethylamine	1	µmol/L	Mammalian-microbial cometabolism	Trimethylamines metabolism	HMDB0000087	C00543
His	Histidine	1	µmol/L	Amino acid metabolism	Histidine metabolism	HMDB0000177	C00135
OH-Pro	Hydroxyproline	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0240251	C01015
DMG	N,N-Dimethylglycine [a]	1	µmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000092	C01026
Marg	N-Methylarginine	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0029416	C03884
Mpro	N-Methylproline	1	µmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0094696	-
p-Mhis	π-Methylhistidine	1	µmol/L	Amino acid metabolism	Histidine metabolism	HMDB0000001	C01152
Pro	Proline	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0000162	C00148
Sar	Sarcosine [a]	1	µmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000271	C00213
Sta	Stachydrine	1	µmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0004827	C10172
SDMA	Symmetric Dimethylarginine	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0003334	-
t-Mhis	τ-Methylhistidine	1	µmol/L	Amino acid metabolism	Histidine metabolism	HMDB0000479	C01152
Tri	Trigonelline	1	µmol/L	Xenobiotics and related metabolism	Food or plant constituents	HMDB0000875	C01004
TMA	Trimethylamine	1	µmol/L	Mammalian-microbial cometabolism	Trimethylamines metabolism	HMDB0000906	C00565
TMAO	Trimethylamine N-oxide [a]	1	µmol/L	Mammalian-microbial cometabolism	Trimethylamines metabolism	HMDB0000925	C01104

Metabolites detected in urine samples from *Study I* (all) or in plasma samples from *Study IIb (ᵃ)*. HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; MSI: Metabolomics Standards Initiative; MSI ID: 1=identified compound.

A3.4 List of Metabolites Identified by Targeted LC-MS-Based Analysis (*Study IIb*)

Table A-23. Metabolites identified by targeted LC-MS-based analysis and included in *Study IIb*. *(Own table).*

Abbreviation	Metabolite Name	MSI ID Level	Unit	Major Pathway	Sub-Pathway	HMDB ID	KEGG ID
CA	Cholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000619	C00695
CDCA	Chenodeoxycholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000518	C02528
DCA	Deoxycholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000626	C04483
GCA	Glycocholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000138	C01921
GCDCA	Glycochenodeoxycholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000637	C05466
GDCA	Glycodeoxycholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000631	C05464
GUDCA	Glycoursodeoxycholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000708	-
LCA	Lithocholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000761	C03990
TCA	Taurocholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000036	C05122
TCDCA	Taurochenodeoxycholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000951	C05465
TDCA	Taurodeoxycholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000896	C05463
UDCA	Ursodeoxycholate	1	nmol/L	Lipid metabolism	Bile acid metabolism	HMDB0000946	C07880

HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; MSI: Metabolomics Standards Initiative; MSI ID: 1=identified compound.

A3.5 List of Metabolites Identified by Targeted LC-/FIA-MS-Based Analysis Using the Biocrates Absolute IDQ™ p180 kit (*Study IIb*)

Table A-24. Metabolites identified by targeted LC-/FIA-MS-based analysis using the Biocrates Absolute IDQ™ p180 kit and included in *Study IIb*. *(Own table).*

Abbreviation	Metabolite Name	MSI ID Level	Unit	Major Pathway	Sub-Pathway	HMDB ID	KEGG ID
AcOrn	Acetylornithine	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0003357	C00437
ADMA	Asymmetric Dimethylarginine	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0001539	C03626
Ala	Alanine	1	µmol/L	Amino acid metabolism	Alanine and aspartate metabolism	HMDB0000161	C09885
alpha-AAA	alpha-Aminoadipate	1	µmol/L	Amino acid metabolism	Lysine metabolism	HMDB0000510	C00956
Arg	Arginine	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0000517	C00062
Asn	Asparagine	1	µmol/L	Amino acid metabolism	Alanine and aspartate metabolism	HMDB0000168	C00152
Asp	Aspartate	1	µmol/L	Amino acid metabolism	Alanine and aspartate metabolism	HMDB0000191	C00049
C10:1-Car	Decenoylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	-	-
C10:2-Car	Decadienylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0006469	-
C10-Car	Decanoylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0000651	-
C12-Car	Dodecanoylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0002250	-
C14:1-Car	Tetradecenoylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0013329	-
C14:1-OH-Car	Hydroxytetradecenoylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	-	-
C14:2-Car	Tetradecadienylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	-	-
C14:2-OH-Car	Hydroxytetradecadienylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	-	-

C14-Car	Tetradecanoylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0005066	-
C16-Car	Hexadecanoylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0000222	C02990
C18:1-Car	Octadecenoylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0094687	-
C2-Car	Acetylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0000201	C02571
C3-Car	Propionylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0000824	C03017
C4-Car	Butyrylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0002013	C02862
C5:1-Car	Tiglylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0002366	-
C5-Car	Valerylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0013128	-
C6 (C4:1-DC)-Car	Hexanoylcarnitine (Fumaryl-L-carnitine)	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0000705	-
C8-Car	Octanoylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0000791	C02838
C9-Car	NoNAylcarnitine	1	µmol/L	Lipid metabolism	Carnitine metabolism	HMDB0013288	
Citru	Citrulline	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0000904	C00327
Gln	Glutamine	1	µmol/L	Amino acid metabolism	Glutamate metabolism	HMDB0000641	C00064
Glu	Glutamate	1	µmol/L	Amino acid metabolism	Glutamate metabolism	HMDB0000148	C00025
Gly	Glycine	1	µmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000123	C00037
His	Histidine	1	µmol/L	Amino acid metabolism	Histidine metabolism	HMDB0000177	C00135
Ile	Isoleucine	1	µmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000172	C00407
Kyr	Kynurenine	1	µmol/L	Amino acid metabolism	Tryptophan metabolism	HMDB0000684	C00328
Leu	Leucine	1	µmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000687	C00123
Lys	Lysine	1	µmol/L	Amino acid metabolism	Lysine metabolism	HMDB0000182	C00047
lysoPC a C16:0	Lyso-phosphatidylcholine acyl C16:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0010382	-
lysoPC a C16:1	Lyso-phosphatidylcholine acyl C16:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0010383	-
lysoPC a C17:0	Lyso-phosphatidylcholine acyl C17:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0012108	-
lysoPC a C18:0	Lyso-phosphatidylcholine acyl C18:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0010384	-
lysoPC a C18:1	Lyso-phosphatidylcholine acyl C18:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0002815	-
lysoPC a C18:2	Lyso-phosphatidylcholine acyl C18:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	-	-
lysoPC a C20:3	Lyso-phosphatidylcholine acyl C20:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0010393	-
lysoPC a C20:4	Lyso-phosphatidylcholine acyl C20:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0010395	-
lysoPC a C28:1	Lyso-phosphatidylcholine acyl C28:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0029221	-
Met	Methionine	1	µmol/L	Amino acid metabolism	Cysteine, methionine and taurine metabolism	HMDB0000696	C00073
Orn	Ornithine	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0000214	C00077
PC aa C28:1	Phosphatidylcholine diacyl C28:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	-	-
PC aa C30:0	Phosphatidylcholine diacyl C30:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0011203	-
PC aa C30:2	Phosphatidylcholine diacyl C30:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	-	-
PC aa C32:0	Phosphatidylcholine diacyl C32:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0000564	-
PC aa C32:1	Phosphatidylcholine diacyl C32:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007897	-
PC aa C32:2	Phosphatidylcholine diacyl C32:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008002	-
PC aa C32:3	Phosphatidylcholine diacyl C32:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	-	-
PC aa C34:1	Phosphatidylcholine diacyl C34:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007879	-
PC aa C34:2	Phosphatidylcholine diacyl C34:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007880	-

PC aa C34:3	Phosphatidylcholine diacyl C34:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007882	-
PC aa C34:4	Phosphatidylcholine diacyl C34:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007884	-
PC aa C36:0	Phosphatidylcholine diacyl C36:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007886	-
PC aa C36:1	Phosphatidylcholine diacyl C36:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007887	-
PC aa C36:2	Phosphatidylcholine diacyl C36:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0000593	-
PC aa C36:3	Phosphatidylcholine diacyl C36:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007981	-
PC aa C36:4	Phosphatidylcholine diacyl C36:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007982	-
PC aa C36:5	Phosphatidylcholine diacyl C36:5	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007984	-
PC aa C36:6	Phosphatidylcholine diacyl C36:6	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008657	-
PC aa C38:0	Phosphatidylcholine diacyl C38:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0007985	-
PC aa C38:1	Phosphatidylcholine diacyl C38:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008269	-
PC aa C38:3	Phosphatidylcholine diacyl C38:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008271	-
PC aa C38:4	Phosphatidylcholine diacyl C38:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008145	-
PC aa C38:5	Phosphatidylcholine diacyl C38:5	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008306	-
PC aa C38:6	Phosphatidylcholine diacyl C38:6	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008147	-
PC aa C40:2	Phosphatidylcholine diacyl C40:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008308	-
PC aa C40:3	Phosphatidylcholine diacyl C40:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008119	-
PC aa C40:4	Phosphatidylcholine diacyl C40:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008536	-
PC aa C40:5	Phosphatidylcholine diacyl C40:5	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	-	-
PC aa C40:6	Phosphatidylcholine diacyl C40:6	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008121	-
PC aa C42:0	Phosphatidylcholine diacyl C42:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008537	-
PC aa C42:1	Phosphatidylcholine diacyl C42:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008091	-
PC aa C42:2	Phosphatidylcholine diacyl C42:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008092	-
PC aa C42:4	Phosphatidylcholine diacyl C42:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008191	-
PC aa C42:5	Phosphatidylcholine diacyl C42:5	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008415	-
PC aa C42:6	Phosphatidylcholine diacyl C42:6	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0008607	-
PC ae C30:0	Phosphatidylcholine acyl-alkyl C30:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013341	-
PC ae C30:1	Phosphatidylcholine acyl-alkyl C30:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013402	-
PC ae C32:1	Phosphatidylcholine acyl-alkyl C32:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013404	-
PC ae C32:2	Phosphatidylcholine acyl-alkyl C32:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013411	-
PC ae C34:0	Phosphatidylcholine acyl-alkyl C34:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013405	-
PC ae C34:1	Phosphatidylcholine acyl-alkyl C34:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013412	-
PC ae C34:2	Phosphatidylcholine acyl-alkyl C34:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0011151	-
PC ae C34:3	Phosphatidylcholine acyl-alkyl C34:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013413	-
PC ae C36:0	Phosphatidylcholine acyl-alkyl C36:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013406	-
PC ae C36:1	Phosphatidylcholine acyl-alkyl C36:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013414	-
PC ae C36:2	Phosphatidylcholine acyl-alkyl C36:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013418	-
PC ae C36:3	Phosphatidylcholine acyl-alkyl C36:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013425	-
PC ae C36:4	Phosphatidylcholine acyl-alkyl C36:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013407	-
PC ae C36:5	Phosphatidylcholine acyl-alkyl C36:5	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013415	-

PC ae C38:0	Phosphatidylcholine acyl-alkyl C38:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013408	-
PC ae C38:1	Phosphatidylcholine acyl-alkyl C38:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013416	-
PC ae C38:2	Phosphatidylcholine acyl-alkyl C38:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013431	-
PC ae C38:3	Phosphatidylcholine acyl-alkyl C38:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013439	-
PC ae C38:4	Phosphatidylcholine acyl-alkyl C38:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013420	-
PC ae C38:5	Phosphatidylcholine acyl-alkyl C38:5	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013432	-
PC ae C38:6	Phosphatidylcholine acyl-alkyl C38:6	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013409	-
PC ae C40:1	Phosphatidylcholine acyl-alkyl C40:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013433	-
PC ae C40:2	Phosphatidylcholine acyl-alkyl C40:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013437	-
PC ae C40:3	Phosphatidylcholine acyl-alkyl C40:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013445	-
PC ae C40:4	Phosphatidylcholine acyl-alkyl C40:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013442	-
PC ae C40:5	Phosphatidylcholine acyl-alkyl C40:5	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013444	-
PC ae C40:6	Phosphatidylcholine acyl-alkyl C40:6	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013422	-
PC ae C42:0	Phosphatidylcholine acyl-alkyl C42:0	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013423	-
PC ae C42:1	Phosphatidylcholine acyl-alkyl C42:1	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013434	-
PC ae C42:2	Phosphatidylcholine acyl-alkyl C42:2	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013438	-
PC ae C42:3	Phosphatidylcholine acyl-alkyl C42:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013458	-
PC ae C42:4	Phosphatidylcholine acyl-alkyl C42:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013448	-
PC ae C42:5	Phosphatidylcholine acyl-alkyl C42:5	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013451	-
PC ae C44:3	Phosphatidylcholine acyl-alkyl C44:3	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013449	-
PC ae C44:4	Phosphatidylcholine acyl-alkyl C44:4	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013453	-
PC ae C44:5	Phosphatidylcholine acyl-alkyl C44:5	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013456	-
PC ae C44:6	Phosphatidylcholine acyl-alkyl C44:6	1	µmol/L	Lipid metabolism	Glycerophospholipid metabolism	HMDB0013450	-
Phe	Phenylalanine	1	µmol/L	Amino acid metabolism	Phenylalanine and tyrosine metabolism	HMDB0000159	C00079
Pro	Proline	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0000162	C00148
Ser	Serine	1	µmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000187	C00065
Sero	Serotonin	1	µmol/L	Amino acid metabolism	Tryptophan metabolism	HMDB0000259	C00780
SM (OH) C14:1	Hydroxysphingomyelin C14:1	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM (OH) C16:1	Hydroxysphingomyelin C16:1	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	HMDB0029216	-
SM (OH) C22:1	Hydroxysphingomyelin C22:1	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM (OH) C22:2	Hydroxysphingomyelin C22:2	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM (OH) C24:1	Hydroxysphingomyelin C24:1	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM C16:0	Sphingomyelin C16:0	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM C16:1	Sphingomyelin C16:1	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM C18:0	Sphingomyelin C18:0	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM C18:1	Sphingomyelin C18:1	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM C20:2	Sphingomyelin C20:2	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM C22:3	Sphingomyelin C22:3	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM C24:0	Sphingomyelin C24:0	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM C24:1	Sphingomyelin C24:1	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	HMDB0012107	-

SM C26:0	Sphingomyelin C26:0	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	-	-
SM C26:1	Sphingomyelin C26:1	1	µmol/L	Lipid metabolism	Sphingolipid metabolism	HMDB0013461	-
Spe	Spermidine	1	µmol/L	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0001257	C00315
Tau	Taurine	1	µmol/L	Amino acid metabolism	Cysteine, methionine and taurine metabolism	HMDB0000251	C00245
Thr	Threonine	1	µmol/L	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0000167	C00188
Trp	Tryptophan	1	µmol/L	Amino acid metabolism	Tryptophan metabolism	HMDB0000929	C00078
Tyr	Tyrosine	1	µmol/L	Amino acid metabolism	Phenylalanine and tyrosine metabolism	HMDB0000158	C00082
Val	Valine	1	µmol/L	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000883	C00183

HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; MSI: Metabolomics Standards Initiative; MSI ID: 1=identified compound.

A3.6 List of Metabolites Identified by Targeted GC-MS-Based Analysis (*Study IIb*)

Table A-25. Metabolites identified by targeted GC-MS-based analysis and included in *Study IIb*. *(Own table).*

Abbreviation	Metabolite Name	MSI ID Level	Unit	Major Pathway	Sub-Pathway	HMDB ID	KEGG ID
C10:0	Decanoic acid	1	mg/L	Lipid metabolism	Medium-chain fatty acid metabolism	HMDB0000511	C01571
C12:0	Dodecanoic acid	1	mg/L	Lipid metabolism	Medium-chain fatty acid metabolism	HMDB0000638	C02679
C14:0	Tetradecanoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0000806	C06424
C14:1	cis-9-Tetradecenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0002000	C08322
C15:0	Pentadecanoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0000826	C16537
C16:0	Hexadecanoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0000220	C00249
C16:1 9cis	cis-9-Hexadecenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0003229	C08362
C17:0	Heptadecanoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0002259	-
C17:0 anteiso	14-Methylhexadecanoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0031067	-
C17:1	cis-10-Heptadecenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0060038	-
C18:0	Octadecanoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0000827	C01530
C18:1 11cis	cis-11-Octadecenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0240219	-
C18:1 11trans	trans-11-Octadecenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0003231	C08367
C18:1 9cis	cis-9-Octadecenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0000207	C00712
C18:1 9trans	trans-9-Octadecenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0000573	C01712
C18:2 9.12cis	all-cis-9.12-Octadecadienoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0000673	C01595
C18:2 9cis.11trans	cis-9.trans-11-Octadecadienoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0003797	C04056
C18:3 6.9.12cis	all-cis-6.9.12-Octadecatrienoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0003073	C06426
C18:3 9.12.15cis	all-cis-9.12.15-Octadecatrienoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0001388	C06427
C20:0	Eicosanoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0002212	C06425
C20:2 11.14cis	cis-11.14-Eicosadienoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0005060	C16525
C20:3 8.11.14cis	all-cis-8.11.14-Eicosatrienoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0002925	C03242
C20:4 5.8.11.14cis	all-cis-5.8.11.14-Eicosatetraenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0001043	C00219
C20:4 8.11.14.17cis	all cis-8.11.14.17-Eicosatetraenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0002177	-
C20:5 5.8.11.14.17cis	all cis-5.8.11.14.17-Eicosapentaenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0001999	C06428

C22:0	Docosanoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0000944	C08281
C22:4 7.10.13.16cis	all-cis-7.10.13.16-Docosatetraenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0002226	C16527
C22:5 4.7.10.13.16.cis	all-cis-4.7.10.13.16-Docosapentaenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0001976	-
C22:5 7.10.13.16.19cis	all-cis-7.10.13.16.19-Docosapentaenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0006528	C16513
C22:6 4.7.10.13.16.19cis	All cis-4.7.10.13.16.19-Docosahexaenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0002183	C06429
C24:0	Tetracosanoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0002003	C08320
C24:1 15cis	cis-15-Tetracosenoic acid	1	mg/L	Lipid metabolism	LCFA metabolism	HMDB0002368	C08323

HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; LCFA: long-chain fatty acid; MSI: Metabolomics Standards Initiative; MSI ID: 1=identified compound.

A3.7 List of Analytes Detected by Untargeted GC × GC-MS-Based Analysis (*Study IIb*)

Table A-26. Analytes detected by untargeted GC × GC-MS-based analysis and included in *Study IIb*. *(Own table).*

Abbreviation	Metabolite Name	MSI ID Level	Unit	Major Pathway	Sub-Pathway	HMDB ID	KEGG ID
P_A0031	2-Hydroxypyridine	2	Intensity	Xenobiotics and related metabolism	Chemicals	HMDB0013751	C02502
P_A0044	Lactic acid	1	Intensity	Carbohydrate metabolism	Glucose and pyruvate metabolism	HMDB0000186	C00243
P_A0052	Glycolic acid	1	Intensity	Xenobiotics and related metabolism	Food or plant constituents	HMDB0000115	C03547
P_A0088	Acetoacetate derivative	2	Intensity	Lipid metabolism	Ketone body metabolism	-	-
P_A0096	alpha-Hydroxybutyric acid	1	Intensity	Carbohydrate metabolism	Propanoate metabolism	HMDB0000008	C05984
P_A0099	Oxalic acid	1	Intensity	Xenobiotics and related metabolism	Food or plant constituents	HMDB0002329	C00209
P_A0114	3-Hydroxypyridine	2	Intensity	Xenobiotics and related metabolism	Chemicals	-	-
P_A0130	Unknown 0130	4	Intensity	-	-	-	-
P_A0132	Unknown 0132	4	Intensity	-	-	-	-
P_A0145	p-Cresol	1	Intensity	Mammalian-microbial cometabolism	Phenylalanine and tyrosine metabolism	HMDB0001858	C01468
P_A0155	beta-Hydroxybutyric acid	1	Intensity	Lipid metabolism	Ketone body metabolism	HMDB0000357	C01089
P_A0164	Unknown (Similar to 2-Aminoisobutyric acid)	3	Intensity	-	-	-	-
P_A0169	Unknown 0169	4	Intensity	-	-	-	-
P_A0170	2-Keto-3-methylvaleric acid derivative	1	Intensity	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000491	-
P_A0207	Unknown (Similar to Mimosine 1)	3	Intensity	-	-	-	-
P_A0209	2-Keto-3-methylvaleric acid derivative 2	1	Intensity	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000491	C03465
P_A0220	3-Hydroxy-3-methylbutyric acid	1	Intensity	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000754	C20827
P_A0222	2-Keto-4-methylvaleric acid	1	Intensity	Amino acid metabolism	Isoleucine, leucine and valine metabolism	HMDB0000695	C00233
P_A0236	Unknown 0236	4	Intensity	-	-	-	-
P_A0248	Unknown (Similar to 2-Ethyl-3-hydroxypropionic acid)	3	Intensity	-	-	-	-
P_A0249	Unknown (Similar to Ethanolamine)	3	Intensity	-	-	-	-
P_A0262	Unknown 0262	4	Intensity	-	-	-	-
P_A0273	Unknown 0273	NA	Intensity	-	-	-	-

P_A0277	Unknown (2.2-Dihydroxyacetic acid or similar)	3	Intensity	-	-	-	-
P_A0279	Unknown 0279	4	Intensity	-	-	-	-
P_A0306	Ethanolamine	1	Intensity	Lipid metabolism	Glycerophospholipid metabolism	HMDB0000149	C00189
P_A0346	Unknown (Similar to Uracil)	3	Intensity	-	-	-	-
P_A0359	Unknown 0359	4	Intensity	-	-	-	-
P_A0364	Phenylacetic acid	1	Intensity	Amino acid metabolism	Phenylalanine and tyrosine metabolism	HMDB0000209	C07086
P_A0378	Unknown 0378	4	Intensity	-	-	-	-
P_A0382	Succinic acid	1	Intensity	Energy metabolism	TCA cycle	HMDB0000254	C00042
P_A0406	Unknown 0406	4	Intensity	-	-	-	-
P_A0412	Glyceric acid	1	Intensity	Lipid metabolism	Glycerolipid metabolism	HMDB0000139	C00258
P_A0421	Probably 4-deoxythreonic acid	2	Intensity	Amino acid metabolism	Glycine, serine and threonine metabolism	HMDB0002453	-
P_A0440	Fumaric acid	1	Intensity	Energy metabolism	TCA cycle	HMDB0000134	C00122
P_A0478	Unknown (Similar to N-Formylglycine)	3	Intensity	-	-	-	-
P_A0521	Unknown 0521	4	Intensity	-	-	-	-
P_A0560	3-Phenylpropionic acid	1	Intensity	Xenobiotics and related metabolism	Chemicals	HMDB0000764	C05629
P_A0563	S-Methylcysteine	1	Intensity	Xenobiotics and related metabolism	Food or plant constituents	HMDB0002108	C22040
P_A0567	Unknown (Similar to Aminomalonic acid)	3	Intensity	-	-	-	-
P_A0586	Unknown 0586	4	Intensity	-	-	-	-
P_A0596	Unknown 0596	NA	Intensity	-	-	-	-
P_A0614	Unknown 0614	4	Intensity	-	-	-	-
P_A0622	Unknown (Similar to Mimosine 2)	3	Intensity	-	-	-	-
P_A0635	Unknown 0635	NA	Intensity	-	-	-	-
P_A0666	Unknown 0666	4	Intensity	-	-	-	-
P_A0680	Unknown 0680	4	Intensity	-	-	-	-
P_A0684	Malic acid	1	Intensity	Energy metabolism	TCA cycle	HMDB0000156	C00149
P_A0710	Threitol	1	Intensity	Xenobiotics and related metabolism	Sugars, sugar substitutes and sugar derivatives	HMDB0004136	C16884
P_A0710b	Erythritol	1	Intensity	Xenobiotics and related metabolism	Sugars, sugar substitutes and sugar derivatives	HMDB0002994	C16884
P_A0730	5-Oxoproline	1	Intensity	Cofactors and vitamins metabolism	Glutathione metabolism	HMDB0000267	C01879
P_A0735	Unknown 0735	NA	Intensity	-	-	-	-
P_A0739	trans-4-Hydroxyproline	1	Intensity	Amino acid metabolism	Arginine and proline metabolism; urea cycle	HMDB0000725	C01157
P_A0757	Unknown 0757	4	Intensity	-	-	-	-
P_A0766	Unknown (Probably Erythronic acid)	3	Intensity	-	-	-	-
P_A0778	Cysteine	1	Intensity	Amino acid metabolism	Cysteine, methionine and taurine metabolism	HMDB0000574	C00097
P_A0779	Unknown (Similar to N-Acetylserine)	3	Intensity	-	-	-	-
P_A0798	Threonic acid	1	Intensity	Xenobiotics and related metabolism	Sugars, sugar substitutes and sugar derivatives	HMDB0000943	C01620
P_A0811	Unknown 0811	NA	Intensity	-	-	-	-
P_A0827	Unknown 0827	4	Intensity	-	-	-	-
P_A0856	Unknown 0856	4	Intensity	-	-	-	-

P_A0882	Unknown 0882	NA	Intensity	-	-	-	-
P_A0888	Unknown 0888	4	Intensity	-	-	-	-
P_A0896	Unknown 0896	4	Intensity	-	-	-	-
P_A0899	4-Hydroxyphenylacetic acid	1	Intensity	Amino acid metabolism	Phenylalanine and tyrosine metabolism	HMDB0000020	C00642
P_A0905	Tartaric acid	1	Intensity	Xenobiotics and related metabolism	Food or plant constituents	HMDB0000956	C00898
P_A0930	Unknown 0930	NA	Intensity	-	-	-	-
P_A0941	Unknown 0941	4	Intensity	-	-	-	-
P_A0943	Unknown 0943	4	Intensity	-	-	-	-
P_A0961	Unknown 0961	4	Intensity	-	-	-	-
P_A0971	Unknown 0971	NA	Intensity	-	-	-	-
P_A0975	Unknown 0975	4	Intensity	-	-	-	-
P_A0978	Unknown 0978	4	Intensity	-	-	-	-
P_A0992	Unknown 0992	NA	Intensity	-	-	-	-
P_A1013	Arabitol	1	Intensity	Carbohydrate metabolism	Miscellaneous (unclassified sugar acids/polyols)	HMDB0000568	C01904
P_A1016	Unknown 1016	NA	Intensity	-	-	-	-
P_A1025	Unknown (Alloxanic acid or similar)	3	Intensity	-	-	-	-
P_A1026	Unknown 1026	NA	Intensity	-	-	-	-
P_A1028	Unknown 1028	4	Intensity	-	-	-	-
P_A1032	Unknown 1032	NA	Intensity	-	-	-	-
P_A1033	Unknown 1033	NA	Intensity	-	-	-	-
P_A1043	Unknown 1043	NA	Intensity	-	-	-	-
P_A1054	Unknown (Sugar or sugar-like 1)	3	Intensity	-	-	-	-
P_A1055	Unknown 1055	NA	Intensity	-	-	-	-
P_A1072	Unknown (Glycerol-1-phosphate or similar)	3	Intensity	-	-	-	-
P_A1081	Unknown 1081	4	Intensity	-	-	-	-
P_A1085	Unknown 1085	NA	Intensity	-	-	-	-
P_A1086	Unknown 1086	NA	Intensity	-	-	-	-
P_A1091	Unknown 1091 (Sugar acid)	3	Intensity	-	-	-	-
P_A1099	Unknown 1099	NA	Intensity	-	-	-	-
P_A1117	Unknown 1117	NA	Intensity	-	-	-	-
P_A1130	Citric acid	1	Intensity	Energy metabolism	TCA cycle	HMDB0000094	C00158
P_A1135	Isocitric acid	1	Intensity	Energy metabolism	TCA cycle	HMDB0000193	C00311
P_A1148	Hippuric acid	1	Intensity	Mammalian-microbial cometabolism	Polyphenolic compounds metabolism	HMDB0000714	C01586
P_A1170	Unknown 1170	NA	Intensity	-	-	-	-
P_A1179	Unknown 1179	NA	Intensity	-	-	-	-
P_A1189	Unknown 1189	NA	Intensity	-	-	-	-
P_A1193	Unknown 1193	NA	Intensity	-	-	-	-
P_A1194	Unknown 1194	NA	Intensity	-	-	-	-
P_A1199	Unknown 1199	NA	Intensity	-	-	-	-

P_A1202	Unknown 1202	NA	Intensity	-	-	-	-
P_A1215	Unknown 1215	NA	Intensity	-	-	-	-
P_A1224	Unknown 1224	NA	Intensity	-	-	-	-
P_A1225	4-Hydroxyphenyllactic acid	1	Intensity	Mammalian-microbial cometabolism	Polyphenolic compounds metabolism	HMDB0000755	C03672
P_A1231	Unknown 1231	NA	Intensity	-	-	-	-
P_A1238	Unknown 1238	NA	Intensity	-	-	-	-
P_A1239	Unknown 1239	NA	Intensity	-	-	-	-
P_A1243	Unknown (Sugar or sugar-like 2)	3	Intensity	-	-	-	-
P_A1258	Unknown 1258	NA	Intensity	-	-	-	-
P_A1260	Unknown 1260	4	Intensity	-	-	-	-
P_A1267	C6 sugar alcohol	3	Intensity	-	-	-	-
P_A1268	Glucuronic acid derivative	1	Intensity	Xenobiotics and related metabolism	Detoxification metabolism	HMDB0000127	C00191
P_A1272	Unknown (Sugar or sugar-like 3)	3	Intensity	-	-	-	-
P_A1285	Unknown 1285	NA	Intensity	-	-	-	-
P_A1289	3-Indoleacetic acid	1	Intensity	Mammalian-microbial cometabolism	Tryptophan metabolism	HMDB0000197	C00954
P_A1300	Unknown (Glycoside 1)	3	Intensity	-	-	-	-
P_A1306	Unknown 1306	NA	Intensity	-	-	-	-
P_A1316	Unknown (Glycoside 2)	3	Intensity	-	-	-	-
P_A1328	Unknown 1328	4	Intensity	-	-	-	-
P_A1331	Unknown 1331	4	Intensity	-	-	-	-
P_A1337	Unknown 1337	4	Intensity	-	-	-	-
P_A1349	Unknown 1349	4	Intensity	-	-	-	-
P_A1364	Unknown (Sugar or sugar-like 4)	3	Intensity	-	-	-	-
P_A1412	Unknown 1412	NA	Intensity	-	-	-	-
P_A1414	myo-Inositol	1	Intensity	Carbohydrate metabolism	Inositol phosphate metabolism	HMDB0000211	C09654
P_A1416	Unknown (Sugar or sugar-like 5)	3	Intensity	-	-	-	-
P_A1426	Unknown 1426	NA	Intensity	-	-	-	-
P_A1437	Unknown (Sugar or sugar-like 6)	NA	Intensity	-	-	-	-
P_A1446	Unknown (Amine 1)	3	Intensity	-	-	-	-
P_A1461	Unknown A1461	NA	Intensity	-	-	-	-
P_A1483	3-Indolelactic acid	1	Intensity	Mammalian-microbial cometabolism	Tryptophan metabolism	HMDB0000671	C02043
P_A1498	Unknown (Amine 2)	3	Intensity	-	-	-	-
P_A1568	Unknown 1568	NA	Intensity	-	-	-	-
P_A1626	Pseudouridine	2	Intensity	Nucleotide metabolism	Pyrimidine metabolism	HMDB0000767	C02067
P_A1766	Unknown (Disaccharide-like)	NA	Intensity	-	-	-	-
P_A1774	Maltose	1	Intensity	Xenobiotics and related metabolism	Sugars, sugar substitutes and sugar derivatives	HMDB0000163	C00208
P_A1782	Unknown 1782	4	Intensity	-	-	-	-

HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; MSI: Metabolomics Standards Initiative; MSI ID: 1=identified compound; 2=putatively annotated compound; 3=putatively annotated compound class; 4=unknown compound. TCA: tricarboxylic acid.

A4 Metabolic Pathway Analyses

A4.1 Classification of Metabolites Identified in *Study I* to Sub-Pathways

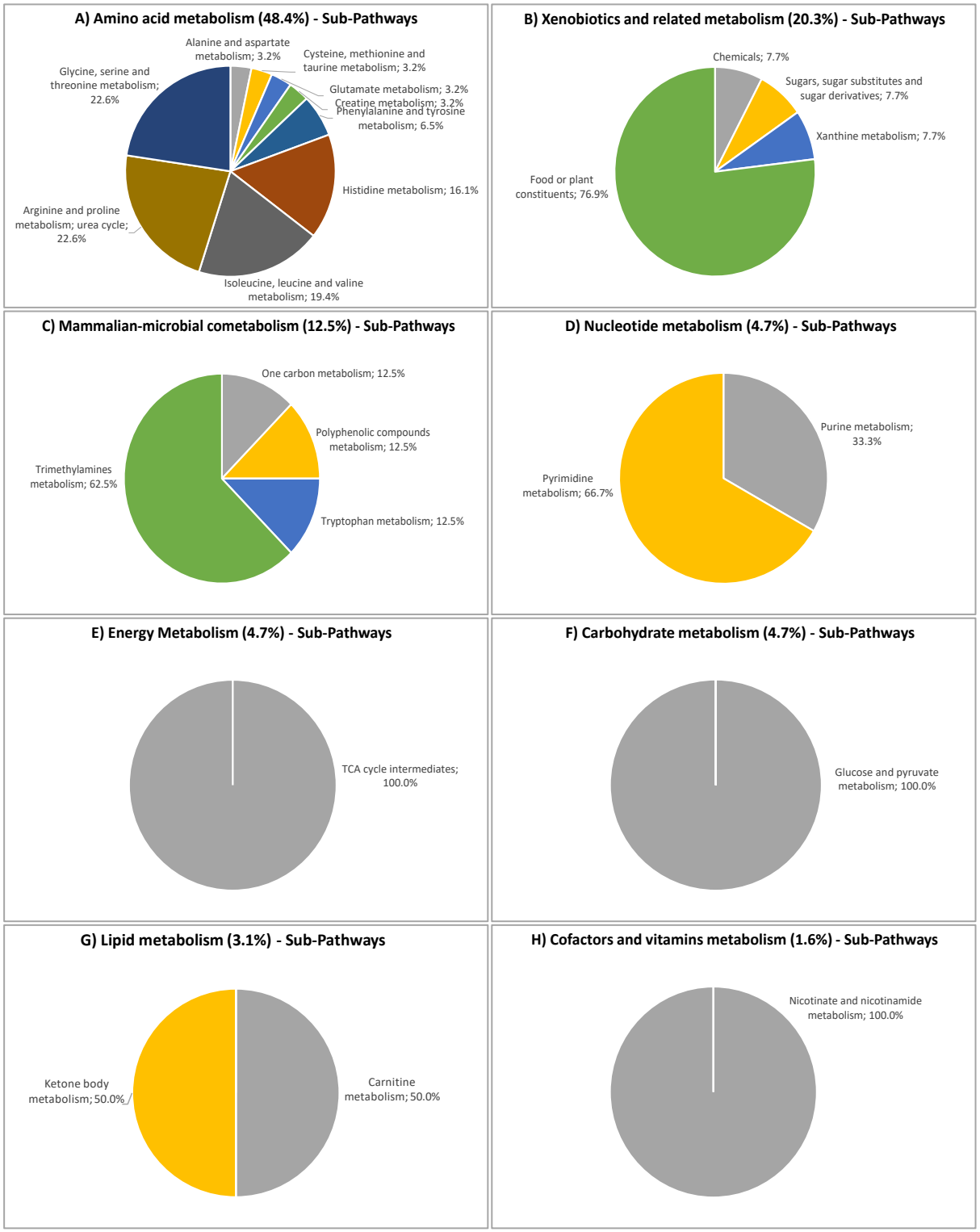


Figure A-20. Classification of the 64 metabolites identified in *Study I* to sub-pathways. TCA: tricarboxylic acid**.** *(Own illustration).*

A4.2 Classification of Metabolites Identified in *Study IIa* to Sub-Pathways

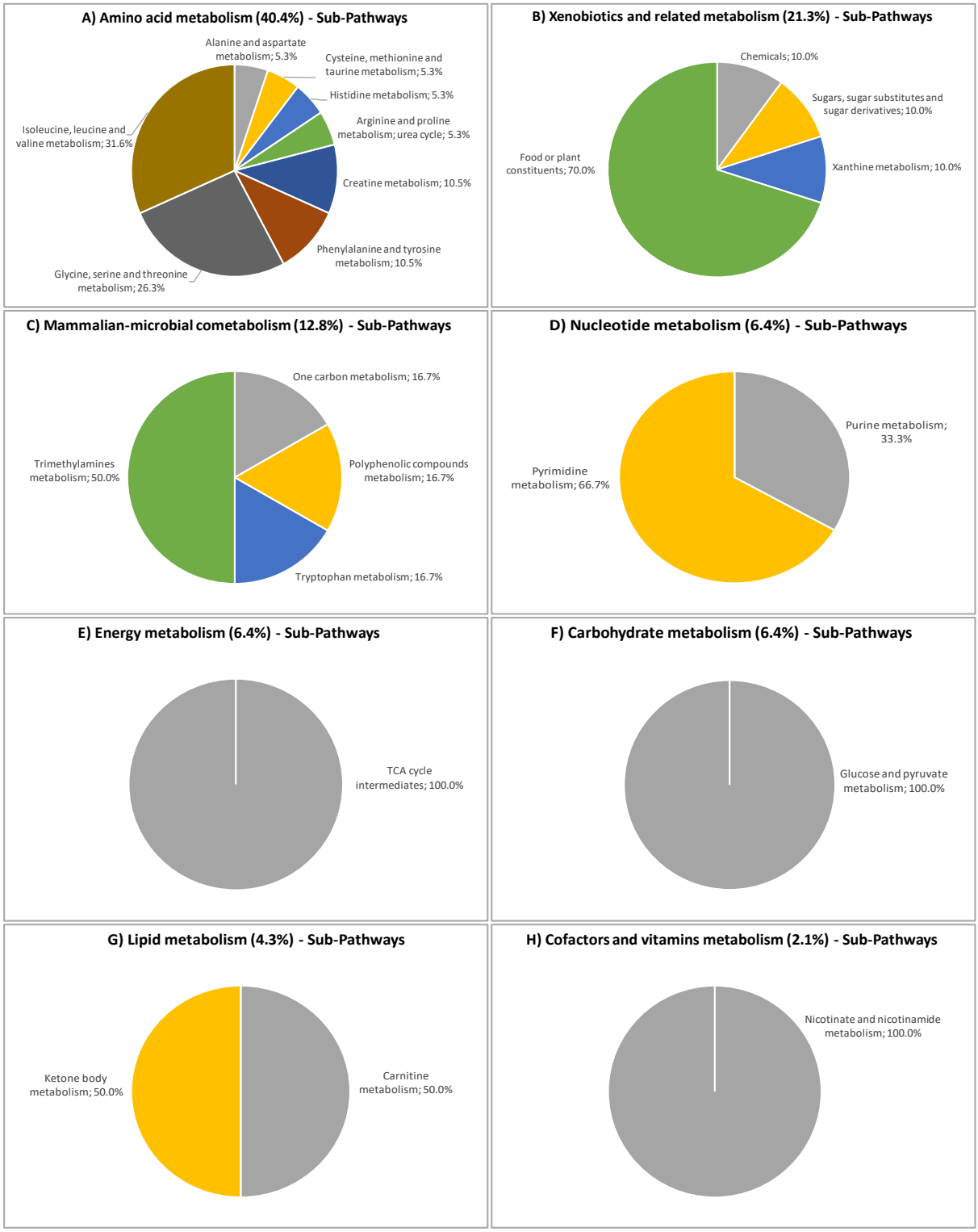


Figure A-21. Classification of the 47 metabolites identified in *Study IIa* to sub-pathways. TCA: tricarboxylic acid**.** *(Own illustration).*

A4.3 Classification of Metabolites Identified in *Study IIb* to Sub-Pathways

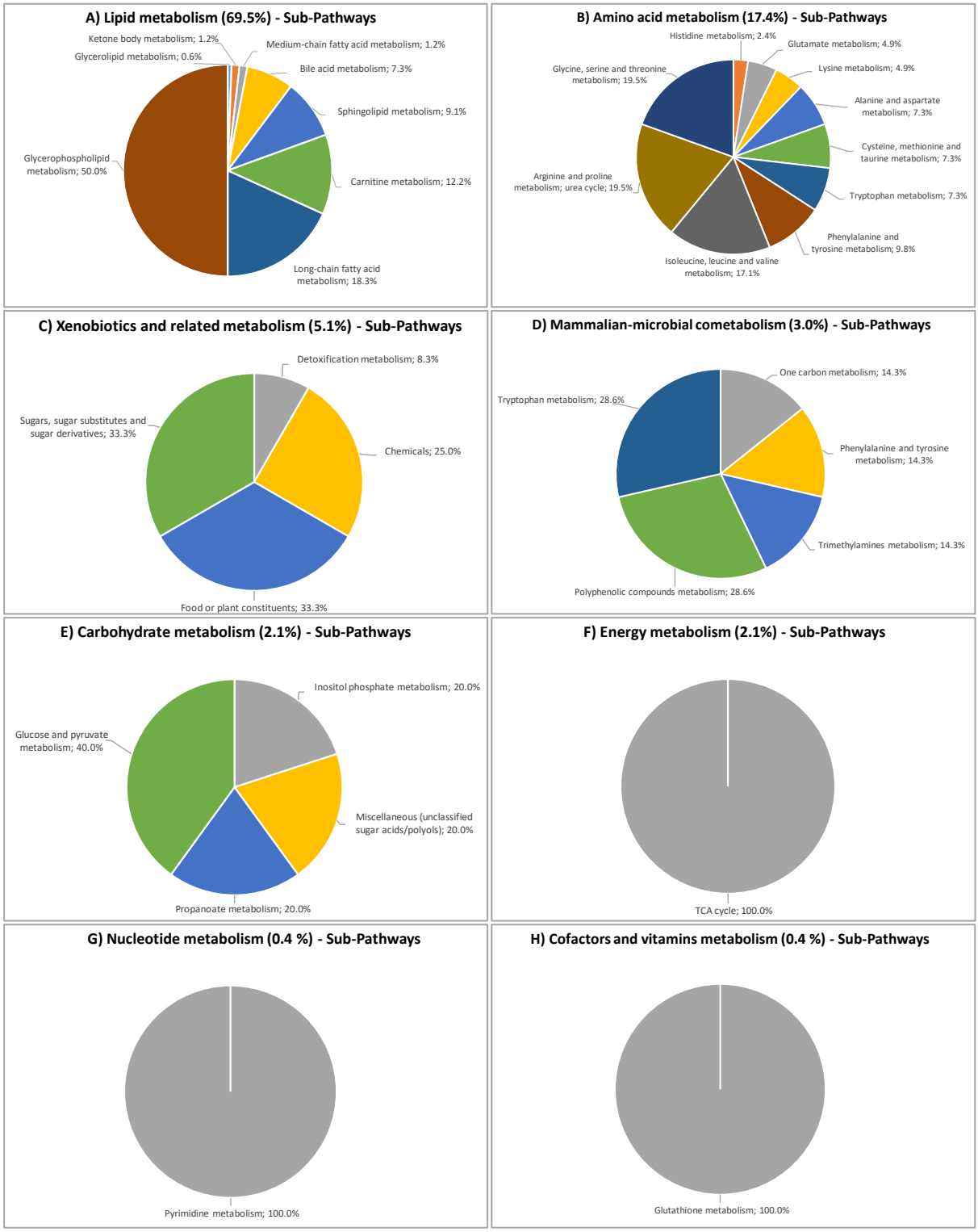


Figure A-22. Classification of the 236 metabolites identified in *Study IIb* to sub-pathways. TCA: tricarboxylic acid**.** *(Own illustration).*

A4.4 Results of Pathway Analysis (*Study I*)

Table A-27. Results of pathway analysis based on three urinary metabolites altered in *Study I. (Own table based on data provided by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca)).*

Nr.	Pathway Name	Match Status	-log(*p*)	FDR *p*	Impact	Matched Metabolites
1	Taurine and hypotaurine metabolism	1 of 8	**1.81E+00**	1.00E+00	0.43	Taurine
2	Primary bile acid biosynthesis	1 of 46	1.06E+00	1.00E+00	0.01	Taurine
3	Purine metabolism	1 of 65	9.18E-01	1.00E+00	0.02	Hypoxanthine

-log(*p*): logarithmic *p*-value from over-representation analysis; FDR *p*: false discovery rate-corrected *p*-value; Impact: pathway impact value calculated from pathway topology analysis. bold: *p*-values < 0.05.

A4.5 Results of Pathway Analysis (*Study IIa)*

Table A-28. Results of pathway analysis based on 35 urinary metabolites altered in *Study IIa. (Own table based on data provided by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca)).*

Nr.	Pathway Name	Match Status	-log(*p*)	FDR *p*	Impact	Matched Metabolites
1	Glycine, serine and threonine metabolism	7 of 33	**5.53E+00**	**1.51E-04**	0.39	Betaine; Guanidinoacetate; N,N-Dimethylglycine; Glycine; L-Threonine; Creatine; Pyruvate
2	Aminoacyl-tRNA biosynthesis	8 of 48	**5.44E+00**	**1.51E-04**	0.00	L-Histidine; Glycine; L-Valine; L-Alanine; L-Isoleucine; L-Leucine; L-Threonine; L-Tyrosine
3	Valine, leucine and isoleucine biosynthesis	4 of 8	**4.95E+00**	**3.15E-04**	0.00	L-Threonine; L-Leucine; L-Isoleucine; L-Valine
4	Glyoxylate and dicarboxylate metabolism	6 of 32	**4.45E+00**	**7.45E-04**	0.16	cis-Aconitate; Citrate; Glycine; Acetate; Pyruvate; Formate
5	Citrate cycle (TCA cycle)	4 of 20	**3.19E+00**	**1.09E-02**	0.22	Succinate; cis-Aconitate; Citrate; Pyruvate
6	Alanine, aspartate and glutamate metabolism	4 of 28	**2.61E+00**	**3.41E-02**	0.00	L-Alanine; Citrate; Pyruvate; Succinate
7	Pyruvate metabolism	3 of 22	**1.99E+00**	1.23E-01	0.27	Pyruvate; Lactate; Acetate
8	Glycolysis / Gluconeogenesis	3 of 26	**1.79E+00**	1.66E-01	0.13	Pyruvate; Lactate; Acetate
9	Phenylalanine metabolism	2 of 10	**1.75E+00**	1.66E-01	0.00	Hippurate; L-Tyrosine
10	Arginine and proline metabolism	3 of 38	**1.35E+00**	3.75E-01	0.04	Guanidinoacetate; Creatine; Pyruvate
11	Valine, leucine and isoleucine degradation	3 of 40	1.29E+00	3.88E-01	0.00	L-Valine; L-Isoleucine; L-Leucine
12	Tyrosine metabolism	3 of 42	1.24E+00	4.02E-01	0.14	L-Tyrosine; Pyruvate; 4-Hydroxy-phenylacetate
13	Phenylalanine, tyrosine and tryptophan biosynthesis	1 of 4	1.08E+00	5.33E-01	0.50	L-Tyrosine
14	Taurine and hypotaurine metabolism	1 of 8	8.00E-01	9.51E-01	0.43	Taurine
15	Ubiquinone and other terpenoid-quinone biosynthesis	1 of 9	7.53E-01	9.88E-01	0.00	L-Tyrosine
16	Primary bile acid biosynthesis	2 of 46	5.91E-01	1.00E+00	0.02	Glycine; Taurine
17	Butanoate metabolism	1 of 15	5.58E-01	1.00E+00	0.00	Succinate
18	Histidine metabolism	1 of 16	5.34E-01	1.00E+00	0.22	L-Histidine
19	Pantothenate and CoA biosynthesis	1 of 19	4.72E-01	1.00E+00	0.00	L-Valine
20	Selenocompound metabolism	1 of 20	4.54E-01	1.00E+00	0.00	L-Alanine
21	beta-Alanine metabolism	1 of 21	4.37E-01	1.00E+00	0.00	L-Histidine
22	Pentose phosphate pathway	1 of 22	4.21E-01	1.00E+00	0.05	D-Gluconic acid
23	Propanoate metabolism	1 of 23	4.06E-01	1.00E+00	0.00	Succinate
24	Glutathione metabolism	1 of 28	3.41E-01	1.00E+00	0.09	Glycine
25	Porphyrin and chlorophyll metabolism	1 of 30	3.20E-01	1.00E+00	0.00	Glycine
26	Cysteine and methionine metabolism	1 of 33	2.91E-01	1.00E+00	0.00	Pyruvate

-log(*p*): logarithmic *p*-value from over-representation analysis; FDR *p*: false discovery rate-corrected *p*-value; Impact: pathway impact value calculated from pathway topology analysis. bold: *p*-values < 0.05. TCA: tricarboxylic acid.

A4.6 Results of Pathway Analysis (*Study IIb*)

Table A-29. Results of pathway analysis based on 35 identified plasma analytes correlating with the CRF in females (after adjusting for confounders and phenotypical/clinical variables) in *Study IIb*. *(Own table based on data provided by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca)).*

Nr.	Pathway Name	Match Status	-log(*p*)	FDR *p*	Impact	Matched Metabolites
1	Valine, leucine and isoleucine metabolism	2 of 8	**2.57E+00**	1.47E-01	0.00	L-Threonine; L-Valine
2	Glycine, serine and threonine metabolism	3 of 33	**2.39E+00**	1.47E-01	0.02	Choline; L-Threonine; D-Glycerate
3	Glycerophospholipid metabolism	3 of 36	**2.28E+00**	1.47E-01	0.13	Phosphatidylcholine; Choline; Ethanolamine
4	Glyoxylate and dicarboxylate metabolism	2 of 32	**1.38E+00**	8.50E-01	0.11	Citrate; D-Glycerate
5	Linoleic acid metabolism	1 of 5	1.30E+00	8.50E-01	0.00	Phosphatidylcholine
6	Ascorbate and aldarate metabolism	1 of 8	1.10E+00	1.00E+00	0.00	myo-Inositol
7	Aminoacyl-tRNA biosynthesis	2 of 48	1.07E+00	1.00E+00	0.00	L-Valine; L-Threonine
8	alpha-Linolenic acid metabolism	1 of 13	8.97E-01	1.00E+00	0.00	Phosphatidylcholine
9	Arginine biosynthesis	1 of 14	8.67E-01	1.00E+00	0.00	N-Acetylornithine
10	Glycerolipid metabolism	1 of 16	8.13E-01	1.00E+00	0.09	D-Glycerate
11	Pantothenate and CoA biosynthesis	1 of 19	7.45E-01	1.00E+00	0.00	L-Valine
12	Citrate cycle (TCA cycle)	1 of 20	7.25E-01	1.00E+00	0.09	Citrate
13	Pentose phosphate pathway	1 of 22	6.88E-01	1.00E+00	0.00	D-Glycerate
14	Galactose metabolism	1 of 27	6.09E-01	1.00E+00	0.00	myo-Inositol
15	Alanine, aspartate and glutamate metabolism	1 of 28	5.95E-01	1.00E+00	0.00	Citrate
16	Phosphatidylinositol signaling system	1 of 28	5.95E-01	1.00E+00	0.04	myo-Inositol
17	Inositol phosphate metabolism	1 of 30	5.69E-01	1.00E+00	0.13	myo-Inositol
18	Arachidonic acid metabolism	1 of 36	5.02E-01	1.00E+00	0.00	Phosphatidylcholine
19	Valine, leucine and isoleucine degradation	1 of 40	4.64E-01	1.00E+00	0.00	L-Valine
20	Tryptophan metabolism	1 of 41	4.56E-01	1.00E+00	0.09	L-Kynurenine

-log(*p*): logarithmic *p*-value from over-representation analysis; FDR *p*: false discovery rate-corrected *p*-value; Impact: pathway impact value calculated from pathway topology analysis. bold: *p*-values < 0.05. TCA: tricarboxylic acid.

Table A-30. Results of pathway analysis based on 11 identified plasma analytes correlating with the CRF in males (after adjusting for confounders and phenotypical/clinical variables) in *Study IIb*. *(Own table based on data provided by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca)).*

Nr.	Pathway Name	Match Status	-log(*p*)	FDR *p*	Impact	Matched Metabolites
1	Alanine, aspartate and glutamate metabolism	2 of 28	**2.20E+00**	4.51E-01	0.20	L-Alanine; L-Glutamate
2	Aminoacyl-tRNA biosynthesis	2 of 48	**1.75E+00**	4.51E-01	0.00	L-Alanine; L-Glutamate
3	Linoleic acid metabolism	1 of 5	**1.65E+00**	4.51E-01	0.00	Phosphatidylcholine
4	Nitrogen metabolism	1 of 6	**1.57E+00**	4.51E-01	0.00	L-Glutamate
5	D-Glutamine and D-glutamate metabolism	1 of 6	**1.57E+00**	4.51E-01	0.50	L-Glutamate
6	alpha-Linolenic acid metabolism	1 of 13	1.24E+00	6.55E-01	0.00	Phosphatidylcholine
7	Arginine biosynthesis	1 of 14	1.21E+00	6.55E-01	0.12	L-Glutamate
8	Butanoate metabolism	1 of 15	1.18E+00	6.55E-01	0.00	L-Glutamate
9	Histidine metabolism	1 of 16	1.15E+00	6.55E-01	0.00	L-Glutamate
10	Selenocompound metabolism	1 of 20	1.06E+00	7.31E-01	0.00	L-Alanine
11	Glutathione metabolism	1 of 28	9.21E-01	7.90E-01	0.02	L-Glutamate
12	Porphyrin and chlorophyll metabolism	1 of 30	8.92E-01	7.90E-01	0.00	L-Glutamate
13	Glyoxylate and dicarboxylate metabolism	1 of 32	8.66E-01	7.90E-01	0.00	L-Glutamate
14	Arachidonic acid metabolism	1 of 36	8.18E-01	7.90E-01	0.00	Phosphatidylcholine
15	Biosynthesis of unsaturated fatty acids	1 of 36	8.18E-01	7.90E-01	0.00	(4Z,7Z,10Z,13Z,16Z,19Z)-Docosahexaenoic acid
16	Glycerophospholipid metabolism	1 of 36	8.18E-01	7.90E-01	0.09	Phosphatidylcholine
17	Arginine and proline metabolism	1 of 38	7.96E-01	7.90E-01	0.09	L-Glutamate

-log(*p*): logarithmic *p*-value from over-representation analysis; FDR *p*: false discovery rate-corrected *p*-value; Impact: pathway impact value calculated from pathway topology analysis. bold: *p*-values < 0.05.