universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Gender-specific differences in the metabolome of older adults“

verfasst von / submitted by

Sabine Trettenhahn, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2022 / Vienna 2022

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 838
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Ernährungswissenschaften
Betreut von / Supervisor:	Univ.-Prof. Mag. Dr. Karl-Heinz Wagner
Mitbetreut von / Co-Supervisor:	Mag. Dr. Bernhard Franzke, Bakk.

Affirmation

I hereby declare that the master's thesis submitted was in all parts exclusively fabricated on my own, and that other sources or aids than those explicitly referred to have not been utilised. All passages quoted from publications or paraphrased from these sources are properly cited and attributed. The thesis was not previously submitted in same or similar form to another examination committee and was not published elsewhere.

Vienna, July 2022

Sabine Trettenhahn

Signature

Acknowledgement

First the project partners of the NutriAging study should be mentioned, namely the University of Vienna and the Comenius University Bratislava. Funding for the project was provided by Interreg Slovakia - Austria (European Regional Development Fund).

The pre-test, the planning, the practical work in the laboratory as well as the processing and analysis of the data were carried out in collaboration with Setka Nadine, BSc. Setka Nadine proved to be an excellent and motivated lab partner and it was a pleasure to work with her. In terms of data evaluation and analysis, the present work focuses on the sex difference, while Setka Nadine has a focus on the pathways, in particular citrate cycle and the urea cycle in her thesis “Effects of increased protein intake and resistance training on metabolites of the citrate cycle and urea cycle in older adults.”

We owe the realization of the project to our supervisors and study leaders Univ.-Prof. Mag. Dr. Karl-Heinz Wagner (Department of Nutritional Sciences), Mag. Dr. Bernhard Franzke, Bakk. (Department of Nutritional Sciences) and Univ.-Prof. Dr. Wolfram Weckwerth (Functional and Evolutionary Ecology).

Furthermore, I wish to thank the entire Department of Molecular Systems Biology at the University of Vienna (mosys) for providing us assistance and support, and for letting us operate in their laboratory. During the laboratory work and evaluation, we received support from Mag. Lena Fragner (Functional and Evolutionary Ecology) and Martin Brenner, BSc MSc. (Functional and Evolutionary Ecology, Department of Pharmaceutical Sciences).

I also want to thank Dr. Ammar Tahir, MSc, who explained further basics for the analysis of metabolomics data.

I would like to sincerely thank all the supervisors and contributors for their guidance and patience and for the opportunity to work on this project.

Contents

List of Figures

List of Tables

List of abbreviations

AA	Amino acid
ASM	Appendicular skeletal muscle mass
BMI	Body mass index
BW	Body weight
BCAAs	Branched-chain amino acids
EDTA	Ethylenediaminetetraacetic acid
FFM	Fat-Free Mass
HPLC	High-performance liquid chromatography
IS	Internal standard
LC-MS	Liquid chromatography-mass spectrometry
LMI	Lean Mass Index
GC-MS	Gas chromatography-mass spectrometry
PE	Pentaerythritol
PGP	Phenyl-ß-D-glucopyranoside
QC	Quality Control
TMS	Tetramethylsilane
SMI	Skeletal Muscle Mass Index
SNP	Single-nucleotide polymorphism

Intervention and Testings

CON/I1	Control group = intervention group 1
RP+T/I2	Recommended protein plus resistance training group = intervention group 2
HP+T/I3	High protein plus resistance training group = intervention group 3
T1	Testing day 1
T2	Testing day 2
T3	Testing day 3

Metabolites

ALA	Alanine	ASP	Aspartic acid
ARG	Arginine	α-TOC	Alpha-Tocopherol
ASN	Asparagine	β-ALA	Beta-Alanine

CIT	Citric acid	LEU	Leucine
CRE	Creatinine	LYS	Lysine
CYS	Cysteine	MAL	Malic acid
CYT	Cystine	MET	Methionine
FUM	Fumaric acid	OAA	Oxaloacetic acid
GABA	4-Amino-butyrate	OMV	2-Oxo-3 Methyl-valerate
GLN	Glutamine	ORN	Ornithine
GLU	Glutamic acid	OXA	Oxalic acid
GLY	Glycine	OXO	2-Oxoglutaric acid
GLYC	Glycolic acid	PHE	Phenylalanine
GRI	Glyceric acid	PRO	Proline
HIS	Histidine	PYR	Pyruvic acid
HYP	4-Hydroxyproline	SER	Serine
IAA	Indole-3-Acetate	SUC	Succinic acid
ILE	Isoleucine	THR	Threonine
K-LEU	Keto-Leucine	TRA	Threonic acid
KYN	Kynurenine	TRP	Tryptophan
KYNA	Kynurenic acid	TYR	Tyrosine
LAC	Lactic acid	VAL	Valine

1. Introduction

1.1. Scientific background

1.1.1. Metabolomics

Metabolomics is the study of all low-molecular weight substances (<1000-1500 Da) within a biological system. Excluded are biological polymers like proteins or nucleic acids. The metabolome encompasses the activity of small molecules, as well as metabolic pathways, intermediates, and end products (Audano, et al., 2018; Castelli, et al., 2022).

The metabolome is not a purely endogenous phenomenon. Metabolites are produced by cell metabolism itself as an outcome of the cascade of gene, mRNA and protein expression, but also originate from the microbiota, food and drinks, drugs and the environment (Rist, et al., 2017).

According to previous studies endogenous factors such as age, sex, body mass index (BMI) seem to have the greatest impact (Rist, et al., 2017). Furthermore, there are exogenous factors such as diet, medication, physical activity, psychological stress, and other environmental, socioeconomic and geographical influences, that can have a strong influence on the human metabolome. Since many metabolites have not yet been discovered or characterized, and metabolism in general is not fully understood, the exact molecular mechanisms behind the influencing factors are also still unclear in many ways (Auer, et al., 2016; Castelli, et al., 2022; Kelly, et al., 2020; Rist, et al., 2017).

The present work addresses the gender-specific disparities in the metabolome of older adults, with a particular focus on the influence of differences in body composition.

1.1.2. Sex

It has been proven that there are molecular-biological discrepancies between the sexes. These include inter alia sexual dimorphisms, sex-specific gene expression profiles and polymorphisms, and hormonal differences. Several metabolomics studies

have shown that baseline levels of metabolites such as amino acids, lipids and sugars in serum differ between men and women, leading to the assumption that the metabolites measured are differentially regulated between men and women. Molecular-biological disparities, the gut microbiome as well as behavioral or psychosocial distinctions between the sexes seem to have a significant influence on the metabolism and therefore the metabolome (Auer, et al., 2016; Kelly, et al., 2020; Krumsiek, et al., 2015; Rist, et al., 2017).

1.1.3. Age

Commonly, the term "older adults" is used for people who are 65 years and older. People aged 65 to 74 are referred to as "early older adults" and people aged 75 and older are referred to as "late older adults" (Orimo, et al., 2006). The exact origin of this classification into age groups is not known, but it is used as a categorical classification in several publications.

At the beginning of 2022, 19.5 % of the total Austrian population was aged 65 and older. In 2018, the year in which the data for the present thesis was collected, the ratio was 18.7 % (statista, 2022). In 2021, the quota of women in Austria amounted to 50.8 %, among the age group 60 and older even to 55.3 %. In 2021, life expectancy for women was 84.2 years, thus 4.7 years higher than for men (Bundeskanzleramt, 2022).

Aging itself is a universal, natural and multifactorial process associated with an organism-wide loss of homeostasis and regenerative capacity. Ageing is accompanied by a multitude of biological, physiological and neurological processes and their increasing loss of function and dysregulation (Adav & Wang, 2021).

Since the human metabolome is a response to various intrinsic and extrinsic factors, it changes throughout the entire life cycle (Adav & Wang, 2021; Vignoli, et al., 2018). As the influences accumulate throughout life, the heterogenity between individuals also increases with age, making it challenging to produce representable data regarding the human metabolome of older adults (Vignoli, et al., 2018). This heterogeneity in the human population is probably accountable for the heterogeneity in the results of studies on metabolic biomarkers of ageing. For example, Vignoli et al. (2018) described creatine, creatine, aromatic amino acids, glycine and glutamate, as

metabolites associated with age. Jové et al. (2016) discovered the metabolites pyruvate, α-keto acids, α-hydroxy acids, lactate, niacin, choline, lysine, glucose and phosphatidycholines as biomarkers for a longer life span (Jové, et al., 2016; Vignoli, et al., 2018).

Nevertheless, patterns can be found across several studies. Overall, it can be concluded that ageing affects amino acid metabolism, citrate cycle intermediates, oxidative stress markers, nucleic acid metabolism and lipid metabolism and therefore the metabolome (Adav & Wang, 2021; Jové, et al., 2016; Vignoli, et al., 2018).

In terms of ageing and metabolism, women's metabolism is particularly worth to mention, as it has been found that the menstrual cycle, menopause, the post-menopausal period and the associated fluctuations and changes in hormone levels have a significant impact on metabolism throughout the entire life cycle (Chella Krishnan et al., 2018; Vignoli, et al., 2018).

1.1.4. Body composition

1.1.4.1. Body fat

Most of the adipose tissue in mammals consists of white adipose tissue, either under the skin as subcutaneous adipose tissue or in the deep abdominal regions as visceral adipose tissue. Under normal physiological conditions, women have on average twice the body fat percentage of men (Lundsgaard & Kiens, 2014; Chella Krishnan, et al., 2018). The causes for this can be manifold. Possible biological explanations would be the different lipoprotein lipase (LPL) activity in the sexes as well as the adipose-mediated sex hormone metabolism, especially the different distribution of adrenergic receptors and oestrogen receptors (Chella Krishnan, et al., 2018).

Men tend to accumulate visceral fat in the abdominal region (android fat distribution), while premenopausal women tend to have subcutaneous fat on the hips and thighs (gynoid fat distribution). In women, a redistribution of fat tissue from gynoid fat distribution to android fat distribution can be observed in age after menopause (Chella Krishnan, et al., 2018).

The adipose tissue plays an important role in the regulation of metabolism and energy homeostasis, due to the release of various adipokines and cytokines

(especially leptin, adiponectin, tumor necrosis factor (TNF)-α, and interleukin (IL)-6) (Chella Krishnan, et al., 2018).

Since android fat distribution is associated with an increase in metabolic disturbances and diseases such as cardiovascular events and diabetes mellitus, (Chella Krishnan, et al., 2018; Kang, et al., 2011), it can be assumed that changes in fat distribution in women as they age also have an impact on the metabolome.

Several studies conclude that there is an association between increased body weight and the composition of the metabolome (Kučera, et al., 2018; Oberbach, et al., 2011). For example, in the study by Oberbach et al. (2011), 12 of 163 metabolites were significantly associated with increased fat content, including glycine and glutamine, which are also part of the present work (Oberbach, et al., 2011).

1.1.4.2. Lean Mass

50 % of the total amount of free amino acids in the body can be found in muscle mass (Auer, et al., 2016). Skeletal muscle mass and muscle quality decrease with age, accompanied by physical disability and loss of independence, thus reducing life quality. Physical activity and optimisation of the diet can slow down this process (Hansen, 2018). Protein intake influences digestive hormones as well as circulating amino acids and their metabolites, so physiological studies suggest that older adults need among others an increased protein intake to maintain muscle protein synthesis and counteract age-related functional decline (Martin, et al., 2019).

On average, women have two-thirds the lean mass of men (Lundsgaard & Kiens, 2014; Chella Krishnan, et al., 2018). These discrepancies in the proportion of muscle mass between the sexes can be attributed to gender-specific regulations of molecular metabolism and dimorphisms in the intrinsic properties of skeletal muscle. There is evidence that gender has an even greater influence on gene expression in muscle tissue than age and physical activity levels (Lundsgaard & Kiens, 2014).

It is known that sex hormones, especially testosterone, regulate the turnover of body and muscle proteins. Especially testosterone acts as an important determinant of the amino acid concentration in the blood. Among other functions, testosterone increases protein synthesis and the reutilisation of intracellular amino acids in the skeletal muscles (Auer, et al., 2016; Ferrando, et al., 1998).

Testosterone levels in men are 10-15 times higher than in women. At young age, there are no significant differences between the sexes in muscle protein breakdown or muscle loss due to lack of physical activity. Therefore, it is likely that other stimulating factors compensate for the lower testosterone level in women. For example, the female hormone oestrogen, that might increase the energy expenditure rate (Hansen, 2018).

In women, the loss of muscle mass increases during menopause and flattens after. There is an upregulation of protein degradation in skeletal muscle and an upregulation of catabolic genes (e.g., MuRF1 and FOXO3 mRNA expression) in muscle tissue. In addition, the levels of sex hormones such as oestrogen and progesterone decrease after menopause. The loss of oestrogen can lead to less sensitivity to anabolic stimuli and thus accelerated muscle breakdown. These could be possible reasons why women tend to a higher loss of muscle mass in old age than men (Hansen, 2018; Chella Krishnan, et al., 2018).

2. Material and Methods

2.1. Study Design

The present study was designed randomised, controlled, and observer blind. The participants were randomly divided into three intervention groups: control group (CON/I1), recommended protein plus resistance training group (RP+T/I2) and high protein plus resistance training group (HP+T/I3).

The NutriAging Protein Study started in July 2018 with a baseline of 155 male and female participants aged 65 to 84 (85 years at the end of the intervention). 116 (86.5 %; 54 % females, 72.9±4.8 years) finished their assigned intervention five months later in December 2018.

2.1.1. Intervention

In the first 6 weeks of the intervention, subjects in the recommended protein group consumed the recommended protein intake of 1 g protein/kgBW/day and subjects in the high protein group 2 g protein/kg body weight/day. From week 8, the subjects of groups RP and HP carried out resistance training twice a week for a further 8 weeks in addition to the increased protein intake. Intervention group 1 served as a control group. During the time of the intervention, the subjects in this group consumed 0.8-1 g protein per kg body weight/day without additional training. Figure 1 shows the procedure of the study.

Inclusion and exclusion criteria as well as further information on the nutritional aspect, the training and the course of the study in general can be found in the paper by Unterberger et al. (2021) "Effects of an increased habitual dietary protein intake followed by resistance training on fitness, muscle quality and body composition of seniors: A randomised controlled trial" (Unterberger, et al., 2022).

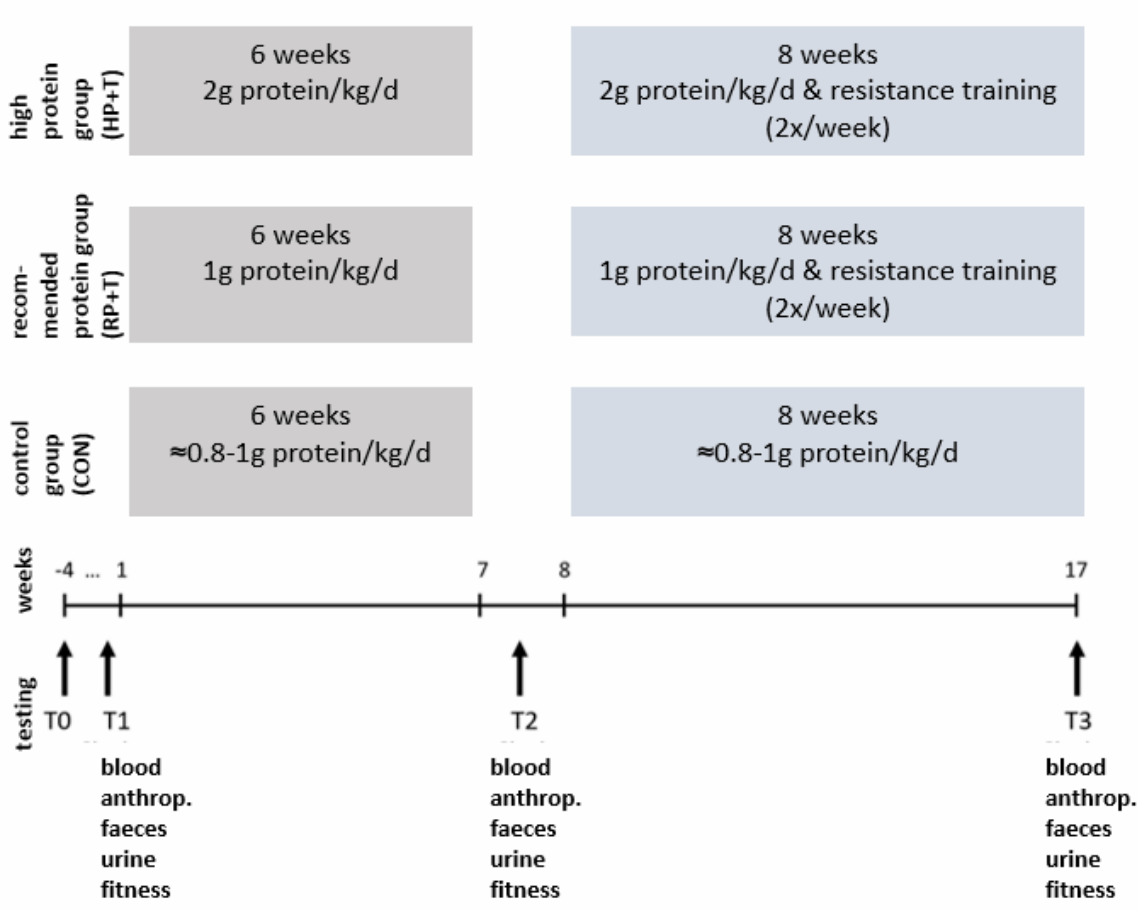

Figure 1 Study Design

Samples were drawn at three time points during the study. The collected data included blood, urine, and faeces collections, anthropometric measurements, and quantitative fitness measurements (including parameters indicative for muscle mass, strength, and function). On testing day 1 (T1) the first samples were taken. These form the baseline. The second testing (T2) occurred 6 weeks after the start of the intervention, after the increased protein intake of groups I2 and I3 and before the start of resistance training. The third and last collecting of data took place on testing day 3 (T3) at the end of the intervention, 8 weeks after the second measurement.

2.1.2. Participants

Out of 155 participants, the blood plasma of 142, i.e., 384 plasma samples were examined. The samples from 13 participants were missing due to premature termination of the study. After measurement only those subjects were included in the statistical analysis who had participated until the end of the study. 25 subjects, i.e., 35

plasma samples, did not meet this requirement and were excluded. 1 participant, i.e., 3 samples were retroactively excluded, because she/he didn´t fulfil the inclusion criteria. In our evaluation we examined plasma samples which were prepared with heparin as a coagulant. 6 plasma samples were prepared with Ethylenediaminetetraacetic acid (EDTA); therefore 18 samples were excluded. 1 sample showed a deviation from the mean of -46.39 % for the internal standard PGP (Phenyl-ß-D-glucopyranoside). This participant, i.e. further 3 samples, was excluded. From each of two test subjects 1 sample was missing, so those, i.e., 4 further samples were excluded.

Out of 142 Participants and 384 measured samples, 107 participants (CON: n = 40; RP: n = 31; HP: n = 36) and 321 measured samples remained for the statistical analysis. Among the 107 subjects there were 54 women and 53 men, which corresponds to a proportion of 50.5 % women. Compared to the study from Unterberger et al. (2021), which was published first, we analysed 9 participants (CON: n = 1; RP: n = 4; HP: n = 4) less (Unterberger, et al., 2022).

The detailed breakdown of the participants, as well as the quantity and reasons for exclusion, are illustrated in Figure 2.

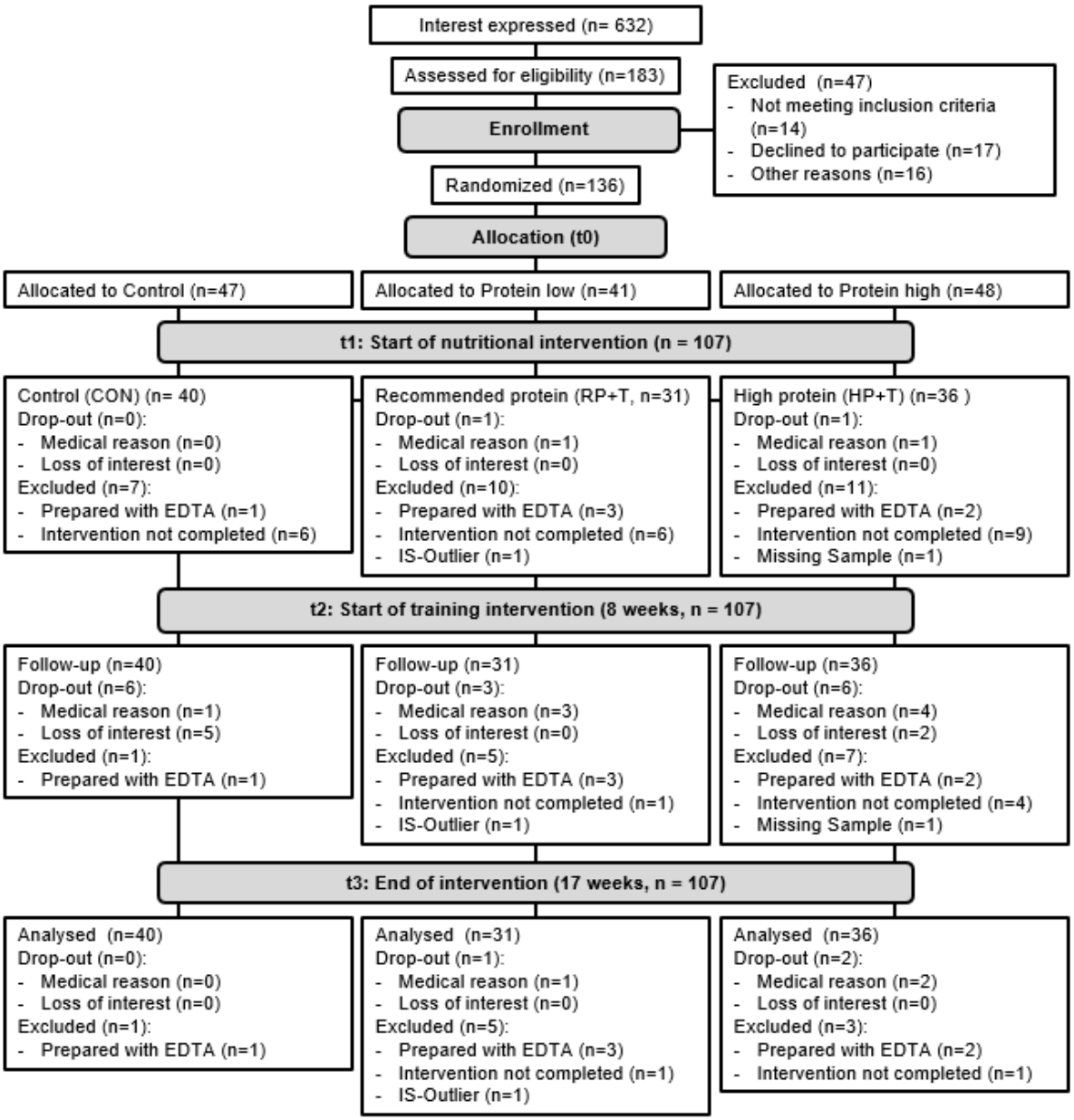

Figure 2 Flow-chart of the participants

2.2. Materials

2.2.1. Biological Samples

For our analysis, we used human plasma from the NutriAging study 2018 (Unterberger, et al., 2022), which had already been separated from the whole blood in our laboratory and treated with heparin as an anticoagulant. The samples were kept at -80°C for further clinical analysis.

2.2.2. Plasma Pool

The pool was created to establish the most suitable method in the preliminary study and as a quality control in the main study. Previously prepared human plasma samples (Plasma separated from fresh blood, heparin as an anticoagulant, kept in -80°C) from the ActiveAging-study (Franzke, et al., 2018) were thawed, transferred into a 50 mL tube, and vigorously vortexed for 10 seconds. The end-pool consisted of approximately 33 mL plasma. The pool was divided into aliquots of 200 µL per Safe-Lock tubes (Eppendorf, Hamburg, Germany) and stored at -80°C. The samples were kept on ice during each transfer.

2.2.3. Internal Standards

The internal standard consisted of PE (Pentaerythritol 98 % purity; 136.15 g/mol), PGP (Phenyl-ß-D-glucopyranoside 97 % purity; 256.25 g/mol) and raffinose (D-(+)-Raffinose Pentahydrate 98 % purity; 594.51 g/mol). To achieve a final concentration of 25 mM, 18.5 mg PE was dissolved in 5.326 mL H_2O. For 125 mM PGP, 163.6 mg PGP was dissolved in 4.954 mL H_2O and to attain 50 mM raffinose, 60.2 mg raffinose was dissolved in 5.282 mL H_2O. The stocks were stored at -20°C.

2.2.4. Quality control mix

The quality control (QC) mix was designed to help annotate a selection of metabolites. In addition, the selected metabolites in plasma can be quantified absolutely (calculation of the actual concentration) with the QC.

Table 1 and Table 2 list all components which were mixed in our QC. The concentrations of the QC-substances were adjusted to the mean concentrations of the metabolites in adult human blood plasma.

Table 1 GC-MS Human Quality Control Composition

Components	**c [mM]**
Amino Acids	
Asparagine	0.45
Glutamine	6
Tryptophan	0.48
Commercial Mix	1
Ornithine	0.6
b-Alanine	0.03
Polyamines	
Spermidine	0.05
Extra	
Hypoxanthine	0.1
Urea	1
Sugar & Alcohols	
Sucrose	0.02
Glucose	0.8
Fructose	0.1
Threitol	0.02
myo-Inositol	0.3
Galactose	0.56

Components	**c [mM]**
Organic Acids	
Citric acid	2
Fumaric acid	0.02
Gluconic acid	0.075
Malic acid	0.04
Pyruvic acid	1
Succinic acid	0.25
2-Oxoglutaric acid	0.1
Lactic acid	1
Oxaloacetic acid	0.1
Glycolic acid	0.1
Pantothenic acid	0.04
GABA	0.008

Table 2 Commercial mix: Amino acid Standard Solution for calibrating amino acid analyzers, Stock No. AA-S-18 (Sigma-Aldrich, Inc.)

Components	c [mM]	Components	c [mM]
Amino acids		*Amino Acids*	
L-Alanine	2.5	L-Leucine	2.5
Ammonium Chloride	2.5	L-Lysine	2.5
L-Arginine	2.5	L-Methionine	2.5
L-Aspartic acid	2.5	L-Phenylalanine	2.5
Cystine	2.5	L-Proline	2.5
L-Glutamic acid	2.5	L-Serine	2.5
Glycine	2.5	L-Threonine	2.5
L-Histidine	2.5	L-Tyrosine	2.5
L-Isoleucine	2.5	L-Valine	2.5

Since metabolism differs from person to person, the concentrations of the individual metabolites also vary. To optimally annotate both high- and low-concentration metabolites, we created a dilution series. The dilution series of the individual measuring batches contained the concentrations 0 µM, 1 µM, 3 µM, 8 µM, 16 µM and 40 µM QC-Mix. The optimal dilution factors were tested and determined in the preliminary study. As dilution solvent we used distilled Milli-Q® H_2O.

In addition, we added the same concentrations of the internal standards to the QC as in the samples: 25 mM PE, 125 mM PGP and 50 mM raffinose.

The QCs were vacuum dried in a SpeedVac centrifugation with the setting “quick gradient”. This gradient reached 0 bar faster than the “standard gradient” we used for the plasma samples, plasma pools, and blanks and had a duration of about two hours (depending on how many samples were in the SpeedVac at the same time). The dried QC-Mixes were stored at -20°C and derivatized like the human plasma samples.

2.3. Methods

2.3.1. Preliminary experiments

Our preliminary test began in October 2020. After that we conducted our main analysis of samples from March 2021 to June 2021.

To determine the most suitable method, we performed several tests using plasma from older people from a previous study. We tested different amounts of plasma (20 µL, 50 µL, and 100 µL plasma) and two different solvents for the extraction (methanol, chloroform, and methanol + chloroform). For the measurement using GC-MS, we tested the three different dilutions (split rates): the split rates splitless (without dilution), split 2 (a dilution of 1:2) and split 5 (a dilution of 1:5). We adapted the quality control mix to the human metabolism, added relevant metabolites, and tested different dilution factors. We also calculated and tested the optimal amount for the internal standards PE, PGP and raffinose.

We discovered that a plasma level of 20 µL resulted in a loss of metabolites that are present in human blood only in small amounts (e.g., 2-oxoglutaric acid and oxaloacetic acid). A volume of 100 µL resulted in an overload of metabolites that occur in larger quantities in the blood (e.g., urea and glucose), which led to surrounding metabolites being masked and no longer annotatable.

Since the main factors in our study were increased protein intake and exercise, and the focus was on muscle and energy metabolism, changes in the polar metabolome were more likely to be expected. Accordingly, apolar metabolites, such as the lipidome, were filtered out. To determine the optimal solvent, 2 different solvents were tested: the extraction with methanol, that with methanol and chloroform and an extraction with chloroform only to see which metabolites dissolve in chloroform. The highest concentrations could be observed when extracting only with methanol. We also discovered that the additional extraction with chloroform had an impact on the internal standards PGP and raffinose.

For the methanol extraction method, the internal standard PGP proved to be the most stable. For the PE and raffinose standards a larger variability between samples, QCs, and controls (plasma pool and blanks) was observed.

Similar to the plasma volume of 100 µL, the splitless and split 2 split rates resulted in an overload of metabolites with higher concentrations, making it difficult or impossible to annotate surrounding metabolites.

In conjunction with the plasma volume of 50 µL and the split-5 dilution rate of the GC measurement, stronger dilution rates also proved to be optimal for QC. Accordingly, we chose dilutions of 1:40, 3:40, 8:40, and 16:40 for QC.

2.3.2. Main laboratory analysis

Based on our preliminary experiment, we concluded that the plasma volume of 50 µL, the extraction with methanol, and the split rate 5 provided the best results for our study. As internal standard we utilized all three solvents (PE, PGP, and raffinose).

2.3.2.1. Extraction

Frozen raw plasma samples were slowly thawed on ice for 30 minutes and vortexed vigorously for 10 seconds every 10 minutes until completely thawed.

The extraction was carried out with methanol for HPLC LC-MS grade previously stored at -20°C. The IS were added to methanol. 5 mL methanol contained 2.5 µL of the solubilized PE stock, 2.5 µL of the solubilized PGP stock and 2.5 µL of the solubilized raffinose stock.

50 µL plasma of each sample was transferred into 2 mL Safe-Lock tubes (Eppendorf, Hamburg, Germany), treated with 300 µL of the ice-cold methanol/IS-mix and vortexed immediately for 10 seconds. The samples were placed in a thermal shaker for 15 minutes at 4°C for incubation and then centrifuged at 18,000 x g for 4 minutes at 4°C. Each step was carried out on ice and for each transfer we used RPT Graduated Filter sterile tips.

The supernatant was transferred into new 2 mL Safe-Lock tubes and frozen at -20°C for 17-25 hours. The samples were dried with SpeedVac vacuum centrifugation for 5 hours using the method “standard gradient”. The dried samples were stored at -20°C until derivatization.

For each extraction batch we applied the same extracting method on a blank value (50 µL Milli-Q® H_2O) and a plasma pool (50 µL plasma from our preliminary study).

2.3.2.2. Derivatisation

For derivatization, 40 mg/mL MEOX (methylamine hydrochloride; 98%) was dissolved in pyridine (pyridine anhydrous; 99.8%). 20 µL of the mixture was added to each dried sample, pool, blank, and QC and vortexed for 90 minutes at 700 rpm and 30°C. Afterwards 80 µL MSTFA (N-Methyl-N-(trimethylsilyl)trifluoracetamid) per sample were added and the mixture was again vortexed for 30 minutes at 750 rpm and 37°C. After 4 minutes of centrifugation at 14,000 x g and 24° C, the supernatant was removed, transferred to GC-MS vials with an insert and accurately sealed.

The measurement of the metabolites in the prepared human plasma occurred via Gas Chromatography Time-of-Flight Mass Spectrometer (GC-MS), LECO Pegasus BT. We used helium as a carrier gas. Before each measurement, relevant external parts of the device were cleaned with isopropanol. Pyridine for HPLC (≥99.9 %) was used as a washing solution. As liner we used an Agilent 5183-4647 Split Liner 4 mm, which was replaced before each measurement process. As previously mentioned, a dilution of 1:5 was used for the flow-through (split rate 5).

A measure process consisted mainly of 2 washings with pyridine, 1 alkane standard mixture for functional tests of GC systems (C10 - C40, 50 mg/L each), 1 QC dilution series, 15 samples, 2 blanks, 4 plasma pools and 2 repetitions of the QC8 from the dilution series. The measurement of one run took about 24 hours. A total of 25 batches were measured.

The data was achieved and processed using the software's ChromaTOF for BT (version 5.31.16) and MS-Dial (version 4.48). To prevent overloading of our data with pyridine, the measurement started after 276 seconds. Next time it would be advisable to start the measurement at 270 seconds, as the alkane C10 of the measured alkane standard series was not detected after 276 seconds.

2.4. Evaluation and Statistical Analysis

2.4.1. Preparation of data

After annotation, the peak area of each metabolite was delineated using the MS-Dial program. In the final analysis, the data came from the MS-Dial program; ChromaTOF was used only for annotation.

2.4.2. Metabolite selection

To narrow down the selection, we have restricted ourselves to the metabolites of the citric acid cycle and the urea cycle as well as a selection of metabolites relevant to muscle metabolism. Those metabolites from the metabolic pathways that are not listed could not be annotated with certainty and were therefore not further addressed.

In total, 44 metabolites were annotated, 33 of them were present in the QC and 11 untargeted.

The derivatives of 4-aminobutyric acid (GABA) 3TMS and 4-hydroxyproline 3TMS have similar quantum masses, so we could not distinguish them with certainty even after comparison with our QC. In the parallel analysis by Setka Nadine (2022), the results of GABA were found to be similar to those of 4-hydroxyproline. Therefore, it can be assumed that this derivative is 4-hydroxyproline 3TMS. The other 4-hydroxyproline listed is the trans-2TMS derivative.

Urea was found in such large quantities in the plasma of some subjects that the peak was overloaded. The area indicates differences in overall urea concentration but could not be used for absolute quantification because of the overload. In addition, the overload of urea caused the nearby serine derivative 2TMS to be masked in some subjects and therefore had to be excluded from the analysis. To determine serine concentration only the derivative 2TMS was used.

Table 3 shows the metabolites that were annotated, divided into those that were added to the QC mix and those that were additionally annotated and Figure 3 shows the selected metabolites in form of their main pathways. The metabolites highlighted in grey are those that were also added to the QC mix, those in white were additionally annotated (untargeted). The metabolites completely shaded in grey

(Succinyl-CoA, Isocitrate, Arginino-Succinate and Citrulline) were only added to the graph for the sake of completeness and were not included in our analysis.

For detection and annotation, we examined the retention index, spectrum, and quantum masses characteristic of the metabolites. As reference, we used the Fiehn Laboratory for Untargeted Metabolomics, the Human Metabolome Database (HMDB), PubChem, NIST MS Search (version 2.3), and validated data and libraries from the MOSYS laboratory. 33 of the selected metabolites were also added to the quality control mix and thus annotated. All others (11, untargeted) were only annotated using the above references.

Indole-3-acetate and kynurenine showed slight discrepancies between the spectra and quantum masses we observed and the references. Kynurenic acid could be confidently annotated with MS-Dial, but the annotation could not be confirmed with ChromaTOF. The annotation of these 3 metabolites could not be completely dismissed, therefore we included them in our statistical analysis despite the uncertainty.

Table 3 Metabolites included in the statistical analysis

Metabolites present in QC

Abbreviation	Metabolite	Abbreviation	Metabolite
ALA	Alanine	LEU	Leucine
ARG	Arginine	LYS	Lysine
ASN	Asparagine	MAL	Malic acid
ASP	Aspartic acid	MET	Methionine
β-ALA	Beta-Alanine	OAA	Oxaloacetic acid
CIT	Citric acid	ORN	Ornithine
CYT	Cystine	OXO	2- Oxoglutaric acid
FUM	Fumaric acid	PHE	Phenylalanine
GABA	4-Amino-butyrate	PRO	Proline
GLN	Glutamine	PYR	Pyruvic acid
GLU	Glutamic acid	SER	Serine
GLY	Glycine	SUC	Succinic acid
GLYC	Glycolic acid	THR	Threonine
HIS	Histidine	TRP	Tryptophane
ILE	Isoleucine	TYR	Tyrosine
LAC	Lactic acid	VAL	Valine

Untargeted Metabolites

Abbreviation	Metabolite	Abbreviation	Metabolite
OMV-	2-Oxo-3 Methyl-valerate	IAA	Indole-3-Acetate
HYP	4-Hydroxyproline	K-LEU	Keto-Leucine
α-TOC	Alpha-Tocopherol	KYNA	Kynurenic acid
CRE	Creatinine	KYN	Kynurenine
CYS	Cysteine	OXA	Oxalic acid
GRI	Glyceric acid	TRA	Threonic acid

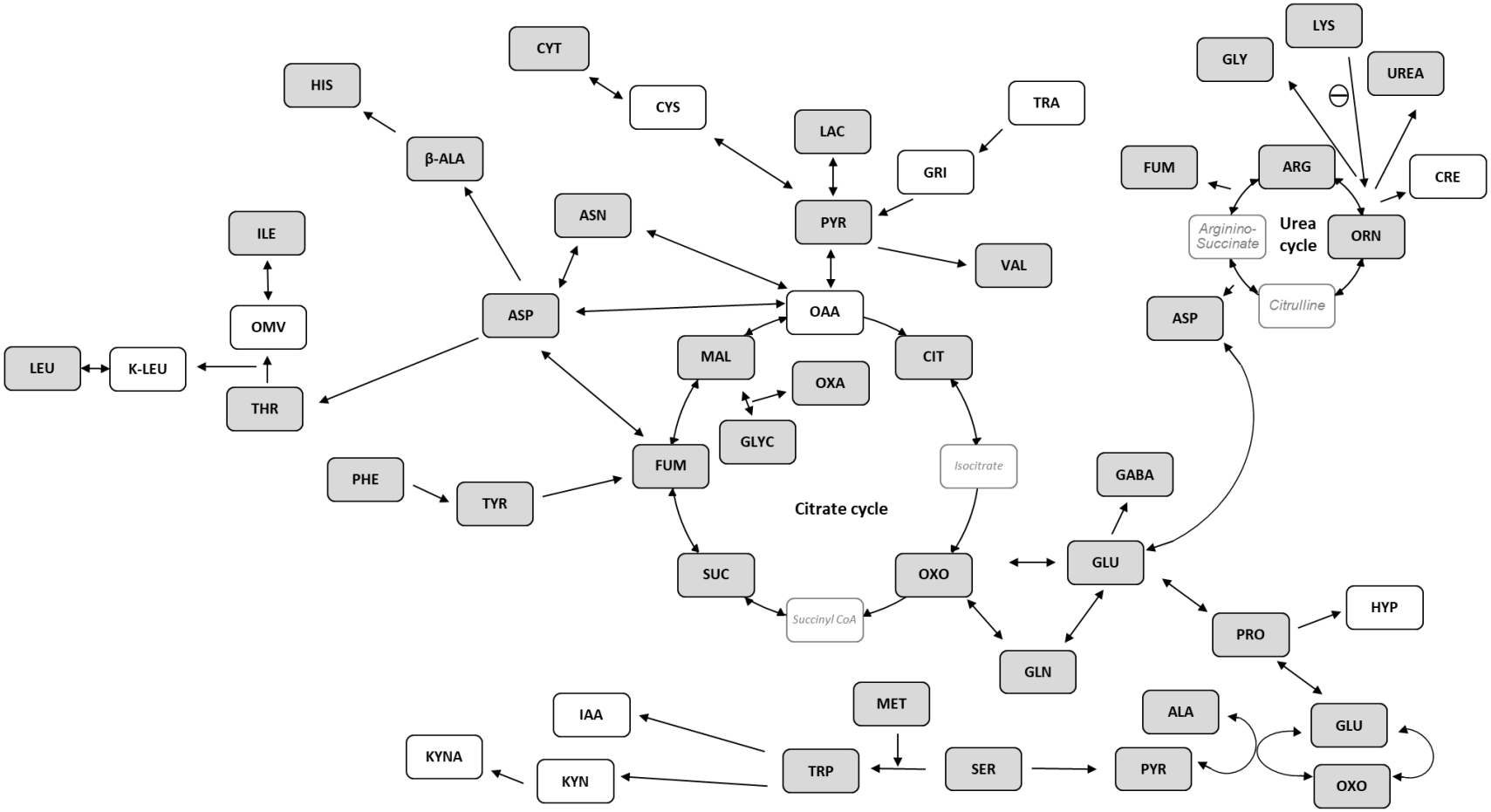

Figure 3 Annotated and quantified metabolites. Grey: Metabolites added in the QC-Mix, White: Untargeted Metabolites. Light tinted: not included in the analysis

2.4.3. Statistical analysis

Data preparation and statistical analysis were performed using IBM SPSS statistics software (version 28.0.0.0) and Excel.

The chromatographic peak areas of the TMS derivatives were added and the area of the PGP standard was subtracted from each sum. This served to minimize batch differences after derivatization and measurement. The PE and raffinose standards were excluded from the analysis due to high variability.

Those subjects with an area of the internal standard PGP that deviated more than 16 % from the mean were excluded from the statistical analysis (N = 1).

Our detected metabolites were semi-quantified (relative quantification) and provided as chromatographic peak areas. Based on the area alone, no statement can be made about the absolute quantity, but differences between the subjects were discernible. In addition, it would be possible to absolutely quantify our target metabolites using the co-measured QC.

The focus of the present analysis was on the baseline data of the participants. The other two measurement days as well as the various intervention groups were therefore not considered in the statistical evaluation of this master thesis.

For determination of significant differences, the general linear model One-way ANOVA was used. To determine correlations, the bivariate correlation with confidence interval estimation according to Fisher was used. The analysis was conducted with standardised means (95 % confidence interval), missing values were excluded. As significance level, $\alpha = 0.05$ was set.

3. Results and Discussion

3.1. Sex

25 out of 45 selected metabolites showed significant differences between males and females.

23 metabolites were in females significant lower than in males: beta-alanine (-28.77 %), ketoleucine (-23.25 %), isoleucine (-23.12 %), 3-methyl-2-oxopentanoic acid (-21.65 %), malic acid (-20.17 %), leucine (-20.02 %), 2-oxoglutaric acid (-19.46 %), proline (-18.54 %), methionine (-17.21 %), fumaric acid (-15.68 %), phenylalanine (-14.45 %), Urea (-13.59 %), valine (-11.61 %), tryptophan (-11.31 %), asparagine (-9.57 %), ornithine (-9.38 %), glycolic acid (-8.74 %), cysteine (-8.43 %), alanine (-8.15 %), tyrosine (-7.37 %), glutamine (-7.42 %), lactic acid (-7.28 %), and lysine (-6.11 %).

Among the 45 metabolites, the concentrations of only 6 were higher in women than in men, 2 of them significantly: threonic acid (+16.56 %) and glycine (+8.72 %).

The details of the mean standardised areas and standard deviations as well as differences [%] between males and females and the significance levels are shown in Table 4.

The results of the present study suggest that gender affected the whole metabolome. As in previous studies, significant differences were found between the sexes for most amino acid concentrations. Our observation that amino acid concentrations were generally higher in men than in women could also be confirmed in various previous studies (Bell, et al., 2021; Darst, et al., 2019; Mittelstrass, et al., 2011; Ruoppolo, et al., 2014; Vignoli, et al., 2018).

Table 4 Areas of metabolites of all participants and separated by gender

Metabolite	Total (N=107)	Female (N=54)	Male (N=53)	w to m [%]	p-value
2-Oxoglutaric acid	0.0203 ± 0.0091	0.0181 ± 0.0078	0.0225 ± 0.0098	-19.46	**0.01**
3-Methyl-2-oxopentanoic acid	0.2119 ± 0.0519	0.1864 ± 0.0414	0.2379 ± 0.0487	-21.65	**0.00**
4-Hydroxyproline	0.0390 ± 0.0188	0.0369 ± 0.0204	0.0412 ± 0.0169	-10.46	0.24
Alanine	8.7067 ± 1.8782	8.3402 ± 1.9028	9.0802 ± 1.7940	-8.15	**0.04**
alpha-Tocopherol	0.2900 ± 0.0661	0.3082 ± 0.0641	0.2713 ± 0.0633	+13.6	**0.00**
Arginine	0.0441 ± 0.0084	0.0438 ± 0.0083	0.0444 ± 0.0086	-1.27	0.73
Asparagine	0.1642 ± 0.0315	0.1560 ± 0.0274	0.1726 ± 0.0334	-9.57	**0.01**
Aspartic acid	0.0037 ± 0.0029	0.0032 ± 0.0010	0.0041 ± 0.0040	-22.94	0.10
beta-Alanine	0.0226 ± 0.0105	0.0189 ± 0.0095	0.0265 ± 0.0102	-28.77	**0.00**
Citric acid	0.5434 ± 0.1456	0.5264 ± 0.1547	0.5607 ± 0.1349	-6.12	0.22
Creatinine	0.3170 ± 0.0726	0.3145 ± 0.0801	0.3195 ± 0.0648	-1.56	0.72
Cysteine	0.1382 ± 0.0219	0.1322 ± 0.0205	0.1443 ± 0.0218	-8.43	**0.00**
Cystine	0.4032 ± 0.1307	0.4091 ± 0.1322	0.3971 ± 0.1301	+3.03	0.64
Fumaric acid	0.0178 ± 0.0036	0.0163 ± 0.0030	0.0194 ± 0.0036	-15.68	**0.00**
GABA/ 4-Hydroxyproline	0.0041 ± 0.0024	0.0037 ± 0.0025	0.0044 ± 0.0023	-16.23	0.12
Glutamic acid	0.3971 ± 0.1259	0.3767 ± 0.1208	0.4180 ± 0.1287	-9.88	0.09
Glutamine	2.8601 ± 0.4474	2.7509 ± 0.3545	2.9713 ± 0.5048	-7.42	**0.01**
Glyceric acid	0.0311 ± 0.0062	0.0302 ± 0.0057	0.0320 ± 0.0066	-5.45	0.15
Glycine	5.1342 ± 1.0652	5.3465 ± 1.1669	4.9178 ± 0.9114	+8.72	**0.04**
Glycolic acid	0.0188 ± 0.0042	0.0179 ± 0.0045	0.0197 ± 0.0036	-8.74	**0.03**
Histidine	0.7126 ± 0.1468	0.7012 ± 0.1339	0.7241 ± 0.1594	-3.17	0.42
Indole 3-acetate	0.0771 ± 0.0513	0.0683 ± 0.0332	0.0861 ± 0.0639	-20.74	0.07
Isoleucine	1.9629 ± 0.5358	1.7084 ± 0.5056	2.2223 ± 0.4344	-23.12	**0.00**
Ketoleucine	0.0930 ± 0.0263	0.0808 ± 0.0189	0.1053 ± 0.0271	-23.25	**0.00**
Kynurenic acid	0.0020 ± 0.0013	0.0020 ± 0.0012	0.0020 ± 0.0013	-0.25	0.98
Kynurenine	0.0020 ± 0.0006	0.0019 ± 0.0006	0.0020 ± 0.0006	-7.04	0.22
Lactic acid	17.1570 ± 2.3701	16.5056 ± 2.2629	17.8208 ± 2.3112	-7.38	**0.00**
Leucine	3.5065 ± 0.8088	3.1196 ± 0.7248	3.9007 ± 0.6960	-20.02	**0.00**
Lysine	1.2257 ± 0.1717	1.1874 ± 0.1557	1.2647 ± 0.1798	-6.11	**0.02**
Malic acid	0.0093 ± 0.0030	0.0083 ± 0.0026	0.0104 ± 0.0030	-20.17	**0.00**
Methionine	0.2168 ± 0.0425	0.1966 ± 0.0328	0.2375 ± 0.0416	-17.21	**0.00**
Ornithine	1.3542 ± 0.3520	1.2881 ± 0.3314	1.4215 ± 0.3625	-9.38	**0.05**
Oxalic acid	0.0679 ± 0.0273	0.0665 ± 0.0141	0.0692 ± 0.0362	-3.98	0.60
Oxaloacetic acid	0.0016 ± 0.0011	0.0015 ± 0.0007	0.0017 ± 0.0014	-9.64	0.46
Phenylalanine	0.6794 ± 0.1141	0.6269 ± 0.0928	0.7328 ± 0.1095	-14.45	**0.00**
Proline	6.4922 ± 2.5092	5.8344 ± 2.2901	7.1624 ± 2.5655	-18.54	**0.01**
Pyruvic acid	0.5972 ± 0.2305	0.5571 ± 0.2019	0.6380 ± 0.2517	-12.67	0.07
Serine	0.7746 ± 0.2075	0.7748 ± 0.2112	0.7745 ± 0.2057	+0.05	0.99
Succinic acid	0.0631 ± 0.0111	0.0632 ± 0.0114	0.0629 ± 0.0110	+0.41	0.91
Threonic acid	0.2053 ± 0.0746	0.2208 ± 0.0820	0.1894 ± 0.0632	+16.57	**0.03**
Threonine	0.5655 ± 0.1163	0.5516 ± 0.1219	0.5797 ± 0.1096	-4.84	0.21
Tryptophan	2.1754 ± 0.4922	2.0462 ± 0.4346	2.3070 ± 0.5160	-11.31	**0.01**
Tyrosine	2.5395 ± 0.4537	2.4432 ± 0.4468	2.6375 ± 0.4434	-7.37	**0.03**
Urea	23.3521 ± 6.7010	21.6645 ± 6.3724	25.0717 ± 6.6462	-13.59	**0.01**
Valine	8.1010 ± 1.5152	7.6060 ± 1.5801	8.6054 ± 1.2733	-11.61	**0.00**

Values of normalised areas (IS PGP) are shown as mean±stdv (95% Confidence Interval). The variable "w to m" indicates the difference between women and men in percent. Colours: red = higher in women, blue = higher in men. p-values refer to differences between women and men. Significant effect $p < 0.05$ (generel linear model One-way ANOVA)

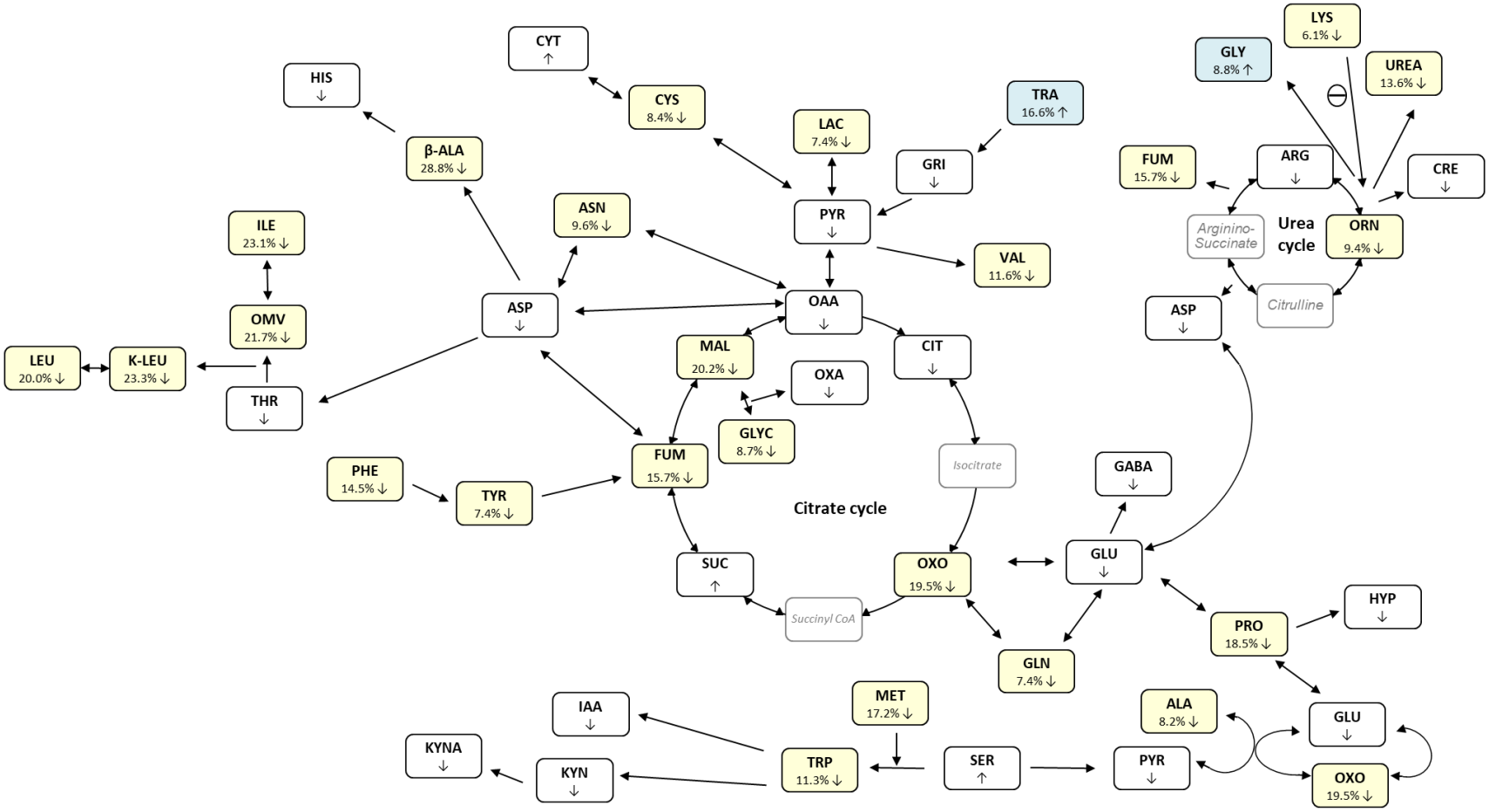

Figure 4 Difference in metabolite concentrations between men and women. Metabolites that differed significantly between the sexes are expressed in percentages. The differences (percentage and arrows) refer to women in relation to men. Colours: blue = area was significantly higher in women, yellow = area was significantly higher in men.

3.2. Baseline characteristics

Increased protein intake in men as well as the different body composition of men and women are some of the central explanations for the differences in the metabolome between men and women in many studies (Chella Krishnan, et al., 2018; Oberbach, et al., 2011; Vignoli, et al., 2018).

Since the present analysis also revealed significant differences in the metabolome between men and women, the question arose whether the differences in the metabolome could be explained by protein intake and body composition. Therefore, body composition, body fat percentage, Lean Mass Index (LMI) and Skeletal Muscle Mass Index (SMI) were chosen to be linked to metabolites.

Table 5 shows the baseline data for sex [f/m], age [years], body mass [kg], height [m], BMI [kg/m²], body fat [kg], body fat [%], lean body mass [kg], LMI [kg/m²], SMI [%], and protein intake [g/kgBW/d]. Significant differences between the sexes were observed for the parameters body mass ($p < 0.001$), height ($p < 0.001$), body fat ($p = 0.004$), body fat percentage ($p < 0.001$), lean body mass ($p < 0.001$), LMI ($p < 0.001$), and SMI ($p < 0.001$).

Table 5 Baseline characteristics of older men and women

	N	Total	N	Females	N	Males	w to m [%]	p-value
Sex [f/m], (% females)]		107		54 (50.5%)		53 (49.5%)		
Age [years]	107	72.81 ± 4.69	54	73.21 ± 4.47	53	72.40 ± 4.91	1.11	0.38
Body mass [kg]	107	74.49 ± 13.72	54	67.46 ± 12.51	53	81.65 ± 10.99	-17.37	**0.00**
Height [m]	107	1.69 ± 0.10	54	1.62 ± 0.06	53	1.76 ± 0.07	-8.14	**0.00**
BMI [kg/m²]	107	26.03 ± 3.99	54	25.71 ± 4.34	53	26.35 ± 3.62	-2.41	0.41
Body fat [kg]	107	17.66 ± 7.29	54	19.60 ± 7.94	53	15.46 ± 5.80	26.77	**0.00**
Body fat [%]	100	23.81 ± 7.49	54	28.11 ± 6.55	46	18.76 ± 4.95	49.82	**0.00**
Lean body mass [kg]	102	56.75 ± 11.63	54	47.86 ± 6.00	48	66.75 ± 1.09	-28.31	**0.00**
Lean Mass Index [kg/m²]	100	20.52 ± 2.75	54	19.40 ± 2.14	46	21.83 ± 2.83	-11.10	**0.00**
Skeletal Muscle Mass Index [%]	100	34.41 ± 5.83	54	30.10 ± 3.47	46	39.47 ± 3.48	-23.75	**0.00**
Protein intake [g/kgBW/d]	107	0.85 ± 0.34	54	0.80 ± 0.37	53	0.90 ± 0.30	-10.96	0.14

Values of body fat [%] are shown as mean±stdv (95% Confidence Interval). Missing values were excluded from the analysis. The variable "w to m" indicates the difference between women and men in percent. p-Values refer to differences between women and men. Colours: red = higher in women, blue = higher in men. Significant effect $p < 0.05$ (generel linear model One-way ANOVA), BMI (body mass index).

Tables 6-9 show the correlation coefficients between metabolite areas and the selected parameters representative for body composition.

Table 6 Corelations between metabolite concentrations and protein intake [g/kgBW/d] of older men and women

Metabolite	Total (N=102)	Females (N=54)	Males (N=48)
Correlation	R (95% CI)	R (95% CI)	R (95% CI)
2-Oxoglutaric acid	0.12 (-0.07,0.3)	0.15 (-0.12,0.4)	0.03 (-0.25,0.29)
3-Methyl-2-oxopentanoic acid	-0.12 (-0.3,0.07)	-0.23 (-0.47,0.04)	-0.22 (-0.46,0.05)
4-Hydroxyproline	0.15 (-0.05,0.33)	0.06 (-0.21,0.32)	0.25 (-0.02,0.49)
Alanine	-0.10 (-0.29,0.09)	-0.06 (-0.32,0.21)	-0.23 (-0.47,0.04)
alpha-Tocopherol	0.09 (-0.1,0.28)	0.08 (-0.19,0.34)	0.21 (-0.06,0.46)
Arginine	0.05 (-0.14,0.24)	0.09 (-0.18,0.35)	-0.02 (-0.29,0.25)
Asparagine	0.16 (-0.04,0.34)	0.17 (-0.1,0.42)	0.08 (-0.19,0.34)
Aspartic acid	-0.09 (-0.27,0.1)	-0.05 (-0.32,0.22)	-0.17 (-0.42,0.1)
beta-Alanine	0.05 (-0.14,0.24)	-0.11 (-0.37,0.16)	0.13 (-0.15,0.38)
Citric acid	-0.12 (-0.3,0.08)	-0.20 (-0.45,0.07)	-0.04 (-0.3,0.24)
Creatinine	-0.06 (-0.25,0.13)	-0.14 (-0.39,0.13)	0.05 (-0.23,0.31)
Cysteine	-0.16 (-0.34,0.03)	-0.25 (-0.48,0.02)	-0.18 (-0.43,0.1)
Cystine	-0.07 (-0.26,0.12)	-0.08 (-0.34,0.19)	-0.04 (-0.31,0.23)
Fumaric acid	-0.04 (-0.23,0.15)	-0.12 (-0.37,0.15)	-0.11 (-0.37,0.16)
GABA/ 4-Hydroxyproline	0.09 (-0.1,0.27)	-0.02 (-0.29,0.25)	0.19 (-0.09,0.44)
Glutamic acid	-0.10 (-0.28,0.09)	-0.14 (-0.39,0.13)	-0.11 (-0.37,0.16)
Glutamine	0.00 (-0.19,0.19)	0.15 (-0.12,0.4)	-0.21 (-0.45,0.07)
Glyceric acid	0.18 (-0.01,0.36)	0.17 (-0.1,0.42)	0.17 (-0.1,0.42)
Glycine	-0.02 (-0.21,0.17)	0.07 (-0.2,0.34)	-0.10 (-0.36,0.17)
Glycolic acid	0.04 (-0.15,0.23)	0.03 (-0.24,0.29)	-0.02 (-0.29,0.25)
Histidine	0.09 (-0.1,0.28)	0.13 (-0.14,0.39)	0.03 (-0.24,0.3)
Indole 3-acetate	-0.08 (-0.27,0.11)	-0.05 (-0.31,0.22)	-0.17 (-0.42,0.11)
Isoleucine	0.00 (-0.19,0.19)	-0.07 (-0.33,0.2)	-0.09 (-0.35,0.19)
Ketoleucine	-0.09 (-0.27,0.1)	-0.23 (-0.47,0.04)	-0.15 (-0.4,0.12)
Kynurenic acid	-0.07 (-0.26,0.12)	-0.06 (-0.33,0.21)	-0.09 (-0.35,0.19)
Kynurenine	-0.07 (-0.26,0.12)	-0.15 (-0.4,0.12)	0.00 (-0.27,0.27)
Lactic acid	-0.03 (-0.22,0.16)	0.00 (-0.27,0.27)	-0.17 (-0.42,0.1)
Leucine	0.03 (-0.16,0.22)	-0.03 (-0.3,0.24)	-0.07 (-0.33,0.21)
Lysine	0.09 (-0.1,0.28)	0.06 (-0.22,0.32)	0.08 (-0.2,0.34)
Malic acid	-0.15 (-0.33,0.04)	-0.19 (-0.44,0.08)	-0.25 (-0.48,0.03)
Methionine	0.13 (-0.06,0.32)	0.15 (-0.13,0.4)	0.01 (-0.27,0.27)
Ornithine	0.42 (0.25,0.56)	0.47 (0.24,0.66)	0.33 (0.07,0.55)
Oxalic acid	0.05 (-0.14,0.23)	-0.06 (-0.32,0.21)	0.10 (-0.18,0.36)
Oxaloacetic acid	0.06 (-0.13,0.25)	0.10 (-0.17,0.36)	0.03 (-0.24,0.3)
Phenylalanine	0.02 (-0.17,0.21)	0.06 (-0.21,0.32)	-0.17 (-0.42,0.1)
Proline	-0.04 (-0.23,0.15)	-0.16 (-0.41,0.11)	0.01 (-0.27,0.28)
Pyruvic acid	-0.17 (-0.35,0.02)	-0.23 (-0.47,0.037)	-0.18 (-0.43,0.09)
Serine	0.15 (-0.04,0.33)	0.19 (-0.08,0.44)	0.11 (-0.16,0.37)
Succinic acid	0.03 (-0.16,0.22)	0.10 (-0.17,0.36)	-0.06 (-0.32,0.21)
Threonic acid	0.09 (-0.11,0.27)	0.20 (-0.07,0.44)	-0.01 (-0.28,0.26)
Threonine	0.17 (-0.02,0.35)	0.12 (-0.15,0.38)	0.21 (-0.07,0.45)
Tryptophan	-0.02 (-0.21,0.17)	-0.06 (-0.32,0.21)	-0.06 (-0.33,0.21)
Tyrosine	0.02 (-0.18,0.2)	0.04 (-0.23,0.31)	-0.09 (-0.35,0.18)
Urea	0.10 (-0.09,0.29)	-0.10 (-0.35,0.18)	0.27 (0,0.5)
Valine	-0.05 (-0.24,0.14)	-0.14 (-0.39,0.14)	-0.06 (-0.32,0.22)

Bivariate correlations between metabolites and protein intake in gram per kilogram body weight per day. Colours: red = positive correlation, blue = negative correlation. Missing values handling: pairwise, exclude.

Table 7 Corelations between metabolite concentrations and body fat [%] of older men and women

Metabolite	Total (N=102)	Females (N=54)	Males (N=48)
Correlation	R (95% CI)	R (95% CI)	R (95% CI)
2-Oxoglutaric acid	-0.16 (-0.34,0.04)	-0.08 (-0.34,0.19)	0.03 (-0.25,0.32)
3-Methyl-2-oxopentanoic acid	-0.22 (-0.4,-0.03)	0.27 (0,0.5)	0.01 (-0.27,0.29)
4-Hydroxyproline	0.01 (-0.19,0.2)	0.19 (-0.08,0.44)	-0.01 (-0.29,0.28)
Alanine	-0.01 (-0.2,0.19)	0.17 (-0.11,0.42)	0.26 (-0.03,0.51)
alpha-Tocopherol	0.22 (0.03,0.4)	0.03 (-0.24,0.3)	0.09 (-0.2,0.36)
Arginine	0.00 (-0.2,0.19)	0.10 (-0.18,0.35)	-0.06 (-0.33,0.23)
Asparagine	-0.16 (-0.35,0.03)	-0.01 (-0.28,0.25)	0.04 (-0.25,0.32)
Aspartic acid	-0.17 (-0.36,0.02)	0.20 (-0.07,0.44)	-0.21 (-0.47,0.08)
beta-Alanine	-0.10 (-0.29,0.1)	0.30 (0.04,0.53)	0.08 (-0.21,0.36)
Citric acid	-0.23 (-0.41,-0.04)	-0.14 (-0.39,0.13)	-0.32 (-0.55,-0.04)
Creatinine	0.19 (0,0.37)	0.30 (0.04,0.53)	0.21 (-0.08,0.47)
Cysteine	-0.11 (-0.3,0.08)	0.15 (-0.12,0.4)	0.06 (-0.23,0.34)
Cystine	0.12 (-0.08,0.3)	0.17 (-0.1,0.42)	0.02 (-0.27,0.3)
Fumaric acid	-0.30 (-0.47,-0.11)	-0.02 (-0.29,0.25)	-0.06 (-0.34,0.23)
GABA/ 4-Hydroxyproline	0.02 (-0.17,0.22)	0.24 (-0.03,0.48)	0.06 (-0.23,0.34)
Glutamic acid	0.16 (-0.04,0.34)	0.39 (0.13,0.59)	0.35 (0.07,0.57)
Glutamine	-0.12 (-0.31,0.08)	0.04 (-0.23,0.31)	0.07 (-0.22,0.35)
Glyceric acid	-0.13 (-0.32,0.07)	-0.16 (-0.41,0.11)	0.10 (-0.19,0.37)
Glycine	-0.01 (-0.21,0.18)	-0.22 (-0.46,0.05)	-0.10 (-0.37,0.19)
Glycolic acid	-0.03 (-0.22,0.16)	0.15 (-0.12,0.41)	0.11 (-0.18,0.38)
Histidine	0.06 (-0.13,0.25)	0.23 (-0.04,0.47)	0.06 (-0.23,0.33)
Indole 3-acetate	-0.09 (-0.28,0.11)	0.04 (-0.23,0.31)	0.02 (-0.27,0.3)
Isoleucine	-0.20 (-0.38,0)	0.34 (0.08,0.56)	-0.09 (-0.37,0.2)
Ketoleucine	-0.23 (-0.41,-0.04)	0.20 (-0.07,0.44)	-0.01 (-0.29,0.28)
Kynurenic acid	-0.04 (-0.23,0.16)	-0.04 (-0.31,0.23)	-0.21 (-0.46,0.08)
Kynurenine	0.03 (-0.16,0.23)	0.26 (-0.01,0.49)	0.01 (-0.27,0.3)
Lactic acid	-0.09 (-0.28,0.11)	-0.06 (-0.32,0.21)	0.39 (0.12,0.61)
Leucine	-0.20 (-0.38,0)	0.34 (0.08,0.56)	-0.02 (-0.3,0.27)
Lysine	-0.08 (-0.27,0.11)	-0.01 (-0.28,0.25)	0.22 (-0.07,0.48)
Malic acid	-0.27 (-0.44,-0.08)	-0.03 (-0.3,0.24)	-0.10 (-0.37,0.19)
Methionine	-0.23 (-0.41,-0.04)	0.17 (-0.1,0.42)	0.10 (-0.19,0.37)
Ornithine	-0.13 (-0.32,0.07)	0.04 (-0.23,0.3)	-0.05 (-0.33,0.24)
Oxalic acid	0.02 (-0.17,0.22)	0.05 (-0.22,0.31)	0.10 (-0.19,0.37)
Oxaloacetic acid	0.00 (-0.19,0.2)	0.01 (-0.25,0.28)	0.02 (-0.26,0.3)
Phenylalanine	-0.12 (-0.3,0.08)	0.31 (0.04,0.53)	0.25 (-0.04,0.5)
Proline	-0.13 (-0.32,0.06)	0.04 (-0.23,0.3)	0.09 (-0.2,0.37)
Pyruvic acid	0.04 (-0.16,0.23)	0.16 (-0.11,0.41)	0.29 (0,0.53)
Serine	-0.02 (-0.21,0.18)	0.09 (-0.18,0.35)	-0.14 (-0.41,0.15)
Succinic acid	-0.04 (-0.23,0.15)	-0.09 (-0.35,0.18)	0.00 (-0.28,0.29)
Threonic acid	0.04 (-0.16,0.23)	-0.20 (-0.45,0.07)	0.06 (-0.23,0.34)
Threonine	0.00 (-0.19,0.19)	0.17 (-0.1,0.42)	0.04 (-0.24,0.32)
Tryptophan	-0.04 (-0.24,0.15)	0.17 (-0.1,0.42)	0.17 (-0.12,0.43)
Tyrosine	0.07 (-0.12,0.26)	0.20 (-0.07,0.44)	0.41 (0.15,0.63)
Urea	0.00 (-0.19,0.2)	0.28 (0.01,0.51)	0.15 (-0.14,0.42)
Valine	0.00 (-0.19,0.19)	0.43 (0.18,0.62)	0.10 (-0.19,0.37)

Bivariate correlations between metabolites and body fat in percent. Colours: red = positive correlation, blue = negative correlation. Missing values handling: pairwise, exclude.

Table 8 Corelations between metabolite concentrations and the Lean Mass Index [kg/m²] of older men and women

Metabolite	Total (N=102)	Females (N=54)	Males (N=48)
Correlation	R (95% CI)	R (95% CI)	R (95% CI)
2-Oxoglutaric acid	0.00 (-0.2,0.19)	-0.12 (-0.37,0.16)	-0.11 (-0.38,0.18)
3-Methyl-2-oxopentanoic acid	0.40 (0.23,0.55)	0.21 (-0.07,0.45)	0.25 (-0.03,0.50)
4-Hydroxyproline	0.13 (-0.07,0.31)	0.28 (0.01,0.51)	-0.12 (-0.39,0.17)
Alanine	0.23 (0.03,0.4)	0.22 (-0.06,0.46)	0.06 (-0.23,0.34)
alpha-Tocopherol	-0.29 (-0.46,-0.1)	0.02 (-0.25,0.29)	-0.37 (-0.59,-0.10)
Arginine	-0.19 (-0.37,0.01)	0.00 (-0.27,0.27)	-0.41 (-0.62,-0.14)
Asparagine	-0.02 (-0.22,0.17)	-0.10 (-0.36,0.17)	-0.20 (-0.46,0.09)
Aspartic acid	0.12 (-0.08,0.3)	0.29 (0.02,0.52)	-0.01 (-0.29,0.28)
beta-Alanine	0.29 (0.1,0.46)	0.22 (-0.05,0.46)	0.08 (-0.20,0.36)
Citric acid	0.06 (-0.14,0.25)	-0.09 (-0.35,0.18)	0.10 (-0.19,0.38)
Creatinine	0.19 (-0.01,0.37)	0.29 (0.03,0.52)	0.09 (-0.20,0.36)
Cysteine	0.37 (0.19,0.53)	0.52 (0.3,0.69)	0.09 (-0.20,0.37)
Cystine	0.08 (-0.11,0.27)	0.11 (-0.16,0.37)	0.13 (-0.16,0.40)
Fumaric acid	0.31 (0.13,0.48)	-0.12 (-0.38,0.15)	0.34 (0.07,0.57)
GABA/ 4-Hydroxyproline	0.17 (-0.03,0.35)	0.30 (0.04,0.53)	-0.06 (-0.34,0.22)
Glutamic acid	0.33 (0.15,0.49)	0.38 (0.13,0.59)	0.20 (-0.09,0.46)
Glutamine	-0.04 (-0.23,0.16)	-0.03 (-0.29,0.24)	-0.26 (-0.51,0.025)
Glyceric acid	-0.09 (-0.28,0.11)	-0.25 (-0.49,0.02)	-0.12 (-0.39,0.17)
Glycine	-0.25 (-0.42,-0.06)	-0.06 (-0.33,0.21)	-0.33 (-0.56,-0.05)
Glycolic acid	0.18 (-0.02,0.36)	0.14 (-0.13,0.39)	0.05 (-0.24,0.33)
Histidine	-0.18 (-0.36,0.02)	0.00 (-0.27,0.27)	-0.41 (-0.62,-0.14)
Indole 3-acetate	0.32 (0.14,0.49)	0.27 (0,0.5)	0.29 (0.01,0.53)
Isoleucine	0.36 (0.18,0.52)	0.26 (-0.01,0.49)	0.10 (-0.18,0.38)
Ketoleucine	0.32 (0.13,0.48)	0.07 (-0.2,0.33)	0.19 (-0.10,0.45)
Kynurenic acid	-0.07 (-0.27,0.12)	-0.05 (-0.32,0.22)	-0.06 (-0.34,0.22)
Kynurenine	0.07 (-0.13,0.26)	0.13 (-0.14,0.38)	-0.12 (-0.39,0.17)
Lactic acid	0.18 (-0.01,0.36)	0.01 (-0.26,0.27)	0.12 (-0.17,0.39)
Leucine	0.31 (0.12,0.48)	0.26 (0,0.5)	-0.03 (-0.31,0.26)
Lysine	-0.01 (-0.2,0.19)	0.01 (-0.26,0.27)	-0.23 (-0.48,0.06)
Malic acid	0.30 (0.11,0.47)	-0.19 (-0.43,0.09)	0.42 (0.15,0.63)
Methionine	0.14 (-0.06,0.33)	0.08 (-0.19,0.34)	-0.23 (-0.49,0.05)
Ornithine	-0.20 (-0.38,-0.01)	-0.17 (-0.42,0.1)	-0.46 (-0.66,-0.20)
Oxalic acid	-0.02 (-0.22,0.17)	-0.12 (-0.37,0.16)	-0.03 (-0.31,0.26)
Oxaloacetic acid	-0.01 (-0.21,0.18)	-0.06 (-0.32,0.21)	0.00 (-0.28,0.29)
Phenylalanine	0.33 (0.15,0.49)	0.27 (0,0.5)	0.07 (-0.21,0.35)
Proline	0.27 (0.08,0.44)	0.16 (-0.11,0.41)	0.17 (-0.12,0.43)
Pyruvic acid	0.30 (0.11,0.47)	0.29 (0.03,0.52)	0.21 (-0.08,0.47)
Serine	-0.11 (-0.3,0.08)	0.00 (-0.27,0.27)	-0.27 (-0.51,0.02)
Succinic acid	-0.10 (-0.29,0.09)	-0.21 (-0.46,0.06)	-0.03 (-0.32,0.25)
Threonic acid	-0.40 (-0.55,-0.22)	-0.42 (-0.62,-0.17)	-0.31 (-0.54,-0.02)
Threonine	-0.01 (-0.21,0.18)	0.16 (-0.12,0.41)	-0.32 (-0.55,-0.04)
Tryptophan	-0.03 (-0.22,0.17)	0.09 (-0.18,0.35)	-0.35 (-0.57,-0.07)
Tyrosine	0.00 (-0.2,0.19)	0.09 (-0.18,0.35)	-0.29 (-0.53,-0.01)
Urea	0.33 (0.14,0.49)	0.33 (0.07,0.55)	0.18 (-0.11,0.44)
Valine	0.28 (0.09,0.45)	0.36 (0.1,0.57)	-0.08 (-0.35,0.21)

Bivariate correlations between metabolites and Lean Mass Index in kilograms per square metre. Colours: red = positive correlation, blue = negative correlation. Missing values handling: pairwise, exclude.

Table 9 Corelations between metabolite concentrations and the Skeletal Muscle Mass Index [%] of older men and women

Metabolite	Total (N=102)	Females (N=54)	Males (N=48)
Correlation	R (95% CI)	R (95% CI)	R (95% CI)
2-Oxoglutaric acid	0.16 (-0.04,0.34)	-0.03 (-0.3,0.24)	-0.03 (-0.32,0.25)
3-Methyl-2-oxopentanoic acid	0.36 (0.18,0.52)	-0.16 (-0.41,0.12)	-0.02 (-0.31,0.26)
4-Hydroxyproline	0.08 (-0.12,0.27)	-0.10 (-0.36,0.17)	0.02 (-0.27,0.3)
Alanine	0.08 (-0.11,0.27)	-0.14 (-0.4,0.13)	-0.28 (-0.53,0)
alpha-Tocopherol	-0.27 (-0.44,-0.08)	-0.08 (-0.34,0.19)	-0.07 (-0.34,0.22)
Arginine	0.03 (-0.17,0.22)	-0.05 (-0.32,0.22)	0.05 (-0.24,0.33)
Asparagine	0.20 (0,0.38)	-0.01 (-0.27,0.26)	-0.05 (-0.33,0.23)
Aspartic acid	0.20 (0,0.38)	-0.12 (-0.38,0.15)	0.17 (-0.12,0.43)
beta-Alanine	0.22 (0.03,0.4)	-0.24 (-0.48,0.03)	-0.08 (-0.36,0.2)
Citric acid	0.21 (0.02,0.39)	0.10 (-0.17,0.36)	0.32 (0.04,0.55)
Creatinine	-0.12 (-0.31,0.08)	-0.25 (-0.49,0.02)	-0.24 (-0.49,0.05)
Cysteine	0.22 (0.02,0.39)	-0.02 (-0.28,0.25)	-0.09 (-0.36,0.2)
Cystine	-0.08 (-0.27,0.11)	-0.12 (-0.38,0.15)	-0.02 (-0.3,0.27)
Fumaric acid	0.35 (0.17,0.51)	-0.05 (-0.31,0.23)	0.06 (-0.23,0.33)
GABA/ 4-Hydroxyproline	0.07 (-0.13,0.26)	-0.15 (-0.4,0.12)	-0.06 (-0.33,0.23)
Glutamic acid	-0.03 (-0.22,0.17)	-0.27 (-0.5,-0.01)	-0.34 (-0.57,-0.06)
Glutamine	0.15 (-0.04,0.34)	-0.07 (-0.33,0.2)	-0.10 (-0.37,0.19)
Glyceric acid	0.10 (-0.1,0.29)	0.03 (-0.24,0.3)	-0.11 (-0.39,0.18)
Glycine	-0.08 (-0.27,0.12)	0.18 (-0.09,0.43)	0.08 (-0.21,0.35)
Glycolic acid	0.09 (-0.1,0.28)	-0.15 (-0.41,0.12)	-0.13 (-0.4,0.16)
Histidine	-0.01 (-0.2,0.19)	-0.20 (-0.45,0.07)	-0.05 (-0.33,0.24)
Indole 3-acetate	0.15 (-0.05,0.34)	0.07 (-0.2,0.33)	0.00 (-0.29,0.28)
Isoleucine	0.36 (0.18,0.52)	-0.22 (-0.46,0.06)	0.08 (-0.21,0.35)
Ketoleucine	0.35 (0.17,0.51)	-0.11 (-0.37,0.16)	-0.01 (-0.29,0.28)
Kynurenic acid	0.02 (-0.17,0.22)	0.07 (-0.2,0.33)	0.20 (-0.08,0.46)
Kynurenine	0.04 (-0.16,0.23)	-0.22 (-0.46,0.05)	-0.03 (-0.31,0.26)
Lactic acid	0.11 (-0.09,0.3)	-0.05 (-0.31,0.22)	-0.40 (-0.61,-0.13)
Leucine	0.36 (0.18,0.52)	-0.21 (-0.45,0.06)	0.01 (-0.27,0.29)
Lysine	0.15 (-0.05,0.33)	0.08 (-0.19,0.34)	-0.22 (-0.47,0.07)
Malic acid	0.31 (0.12,0.47)	-0.04 (-0.3,0.23)	0.09 (-0.2,0.36)
Methionine	0.35 (0.16,0.51)	-0.14 (-0.4,0.13)	-0.11 (-0.38,0.18)
Ornithine	0.16 (-0.04,0.34)	-0.08 (-0.34,0.19)	0.07 (-0.22,0.35)
Oxalic acid	0.00 (-0.2,0.19)	-0.04 (-0.3,0.23)	-0.09 (-0.37,0.2)
Oxaloacetic acid	0.00 (-0.19,0.2)	0.03 (-0.24,0.29)	-0.04 (-0.32,0.25)
Phenylalanine	0.27 (0.08,0.44)	-0.18 (-0.43,0.09)	-0.26 (-0.51,0.02)
Proline	0.20 (0,0.38)	-0.01 (-0.27,0.26)	-0.10 (-0.38,0.19)
Pyruvic acid	0.03 (-0.17,0.22)	-0.12 (-0.37,0.16)	-0.30 (-0.54,-0.02)
Serine	0.04 (-0.16,0.23)	-0.06 (-0.32,0.21)	0.12 (-0.17,0.39)
Succinic acid	0.00 (-0.19,0.19)	0.00 (-0.27,0.27)	-0.01 (-0.3,0.27)
Threonic acid	-0.15 (-0.34,0.04)	0.04 (-0.23,0.3)	-0.06 (-0.34,0.23)
Threonine	0.06 (-0.13,0.26)	-0.11 (-0.37,0.16)	-0.07 (-0.34,0.22)
Tryptophan	0.14 (-0.06,0.32)	-0.10 (-0.36,0.17)	-0.17 (-0.43,0.12)
Tyrosine	0.03 (-0.16,0.22)	-0.12 (-0.38,0.15)	-0.41 (-0.62,-0.14)
Urea	0.11 (-0.08,0.3)	-0.16 (-0.41,0.11)	-0.17 (-0.43,0.12)
Valine	0.18 (-0.01,0.36)	-0.25 (-0.49,0.02)	-0.11 (-0.39,0.18)

Bivariate correlations between metabolites and Skeletal Muscle Mass Index in percent. Colours: red = positive correlation, blue = negative correlation. Missing values handling: pairwise, exclude.

3.2.1. Protein intake

The first evaluation of dietary intake revealed that women consumed 10.96 % less protein with an average intake of 0.8±0.37 g/kgBW/d than men with 0.90±0.30 g/kgBW/d. However, this difference was not significant.

The correlation analyses between protein intake and metabolite areas showed a weak positive association for one metabolite with a correlation coefficient above 0.4 (ornithine (r = 0.47)) and a weak negative association for 5 metabolites in women.

In men, a weak positive association with protein intake was found for 5 metabolites and a weak negative association for 4 metabolites.

It should be noted that the first protein intake was measured with two 24-h-recalls in most cases, whereas the protein intake at the other two test time points was generally measured with about four 24-hour recalls. Due to this lower number of recalls the observed increased protein intake in men a possible explanation for the metabolic differences between the sexes must be seen with caution.

3.2.2. Body fat

The body fat in percent differed significantly between men and women. Women had with an average body fat content of 28.11±6.55 % 49.82 % more body fat than men with an average fat content of 18.76±4.95 %.

In relation to the metabolites, in females a weak positive association between metabolite areas and body fat percentage could be observed for 12 metabolites and a weak negative association for 2 metabolites.

In men, a weak positive correlation between metabolite areas and percentage body fat was found for 8 metabolites, including 1 metabolite with a correlation coefficient above 0.4 (valine (r = 0.43)) and a weak negative correlation for 3 metabolites.

In quantitative terms, body fat percentage seemed to have a greater association to the metabolome in women than in men. However, this could also be due to the higher body fat percentage in women.

3.2.3. Lean Mass Index

The LMI was calculated with the following formula (Schutz, et al., 2002):

FFM [kg]= BW [kg] * [1- (body fat [%]/ 100)]

LMI [kg/m²] = FFM [kg]/ (height [m])²

Normalized LMI [kg/m²] = LMI [kg/m²] + 6.1 * (1.8 – height [m])

The following classifications were used for the LMI: <18 % = “below average”; 18-20 % = “average”; 20-22 % = “above average”; 22-23 % = “excellent”; 23-26 % = “superior”; 26-28 % = “suspicion of steroid use”; >28 % = “steroid usage likely” (Schutz, et al., 2002).

The LMI differed significantly between the sexes. The mean LMI value of women, 19.40±2.14 kg/m², was 11.10 % lower than the value of men, 21.83±2.83 kg/m². According to the classification, the mean LMI of women was in “average” range, while that of men was “above average”.

Among the selected metabolites, a distinct positive association with LMI could be observed for cysteine (r = 0.52) in females, 15 metabolites showed a weak positive association, and 3 metabolites showed a weak negative association with LMI, including 1 metabolite with a correlation coefficient below -0.4 (threonic acid (r = -0.42)).

In males, a weak negative association between LMI and metabolite areas could be observed for 6 metabolites, including 1 metabolite with a correlation coefficient above 0.4 (malic acid (r = 0,42)), and a weak negative correlation could be observed for 14 metabolites, including 3 metabolites with a correlation coefficient below -0.4 (arginine (r = -0.41), histidine (r = -0.41) and ornithine (r = -0.46)).

3.2.4. Skeletal Muscle Index

The SMI value was calculated using the following formula (Moon, et al., 2018):

SMI [%] = ASM [kg]/ BW [kg] * 100)

Appendicular skeletal muscle mass (ASM) in [kg] for SMI is usually measured using Dual Energy X-ray Absorptiometry (DXA). As the SMI takes the limbs into

account, it is a suitable tool to reflect the skeletal muscle mass of the extremities and the BMI, thus contributing to the diagnosis of possible muscle atrophy, such as occurs in sarcopenia (Moon, et al., 2018).

The SMI differed significantly between males and females. Women had an average SMI of 30.10±3.47 %, which was significantly lower by 23.75 % than that of men with a mean value of 39.47±3.48 %.

With respect to the selected metabolites, no positive correlation between SMI and the metabolites were found for any metabolite in females and a weak negative association for 8 metabolites.

In men, a weak positive association between SMI and metabolite areas was found for 2 metabolites and a weak negative association for 8 metabolites, of which 2 metabolites had a correlation coefficient of less than 0.4 (tyrosine ($r = -0.41$), lactic acid ($r = -0.40$)).

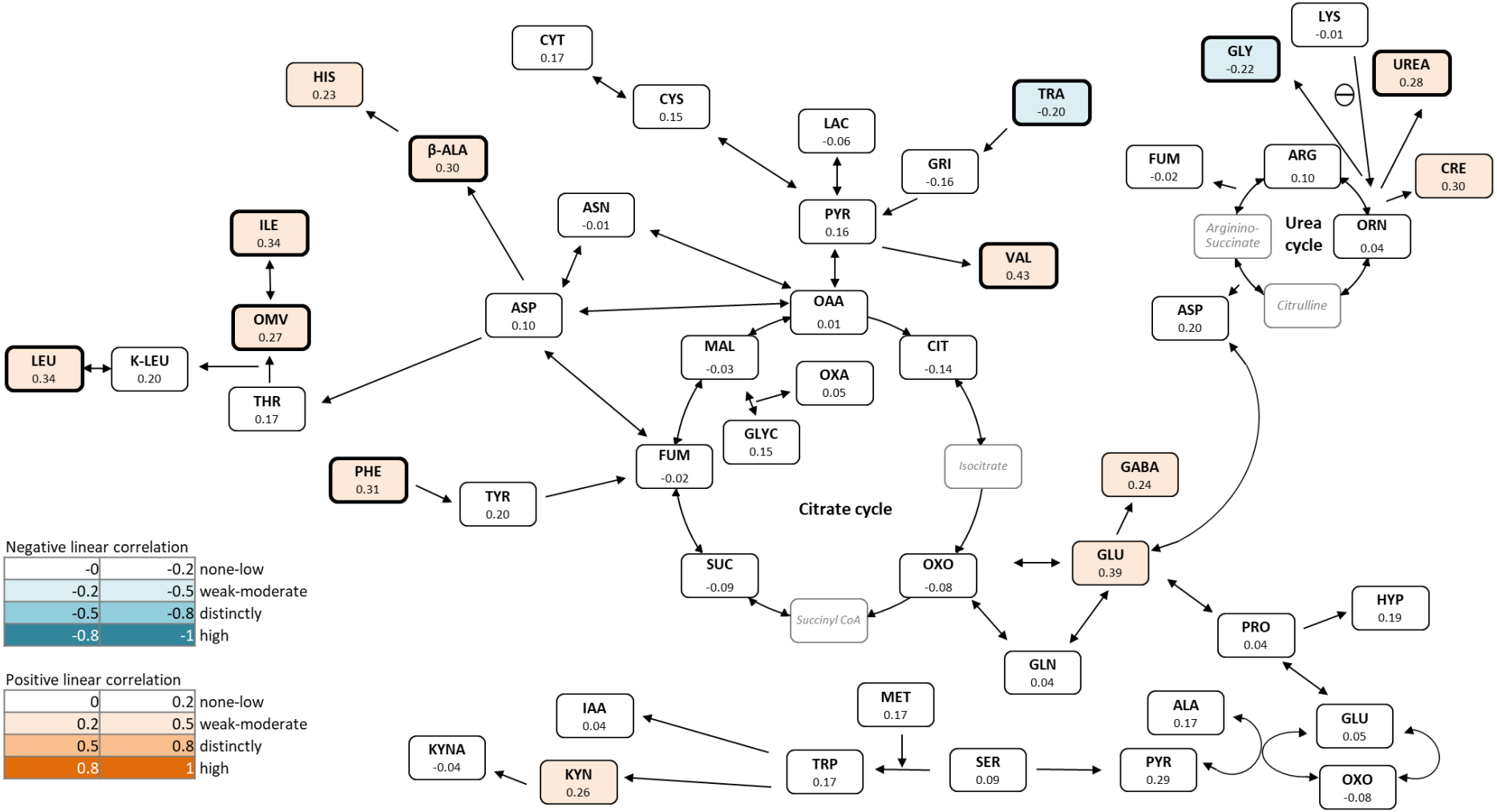

Figure 5 Correlations of metabolites and body fat [%] in women. Metabolites outlined in thick: significant difference between men and women

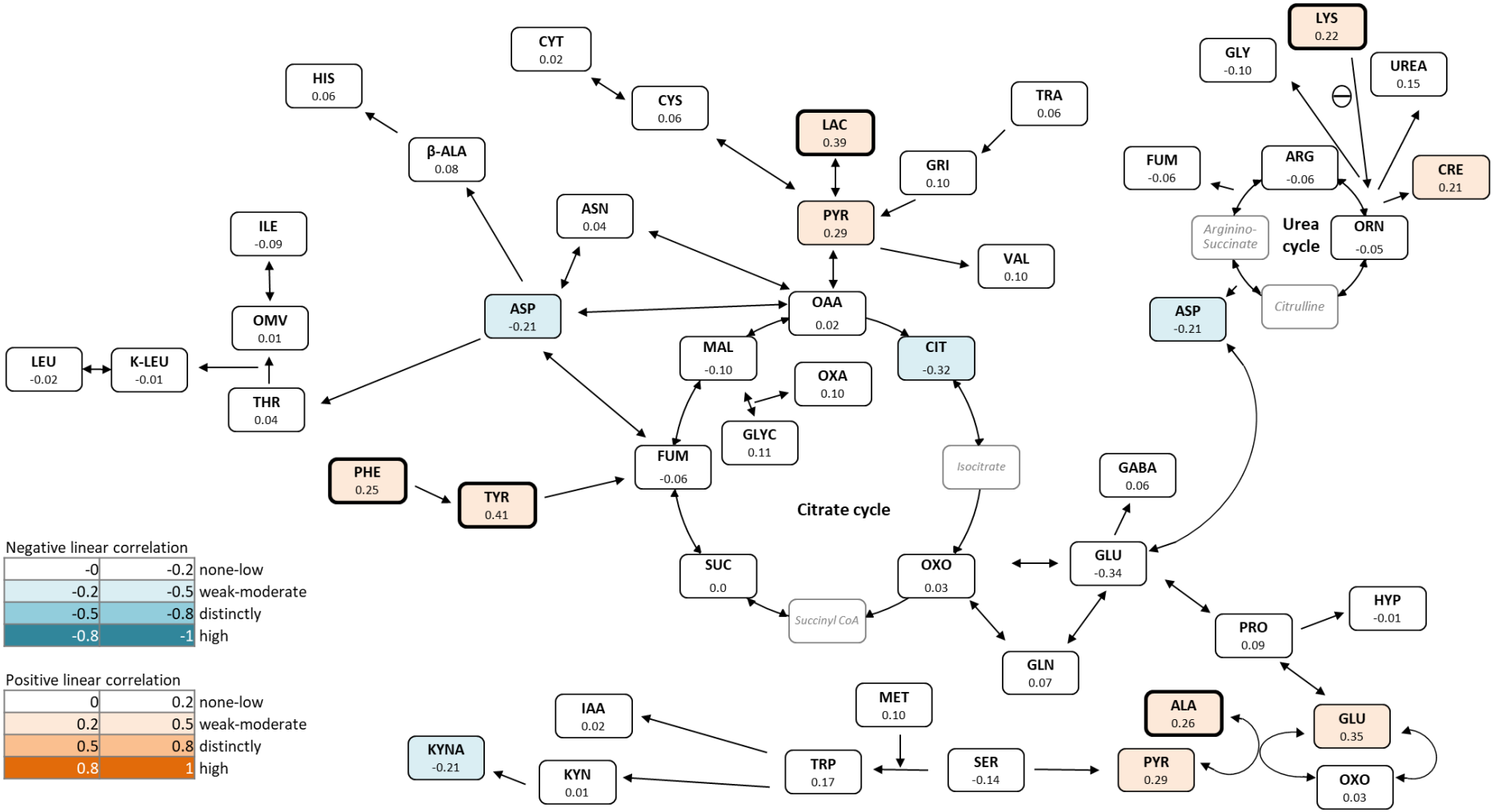

Figure 6 Correlations of metabolites and body fat [%] in men. Metabolites outlined in thick: significant difference between men and women

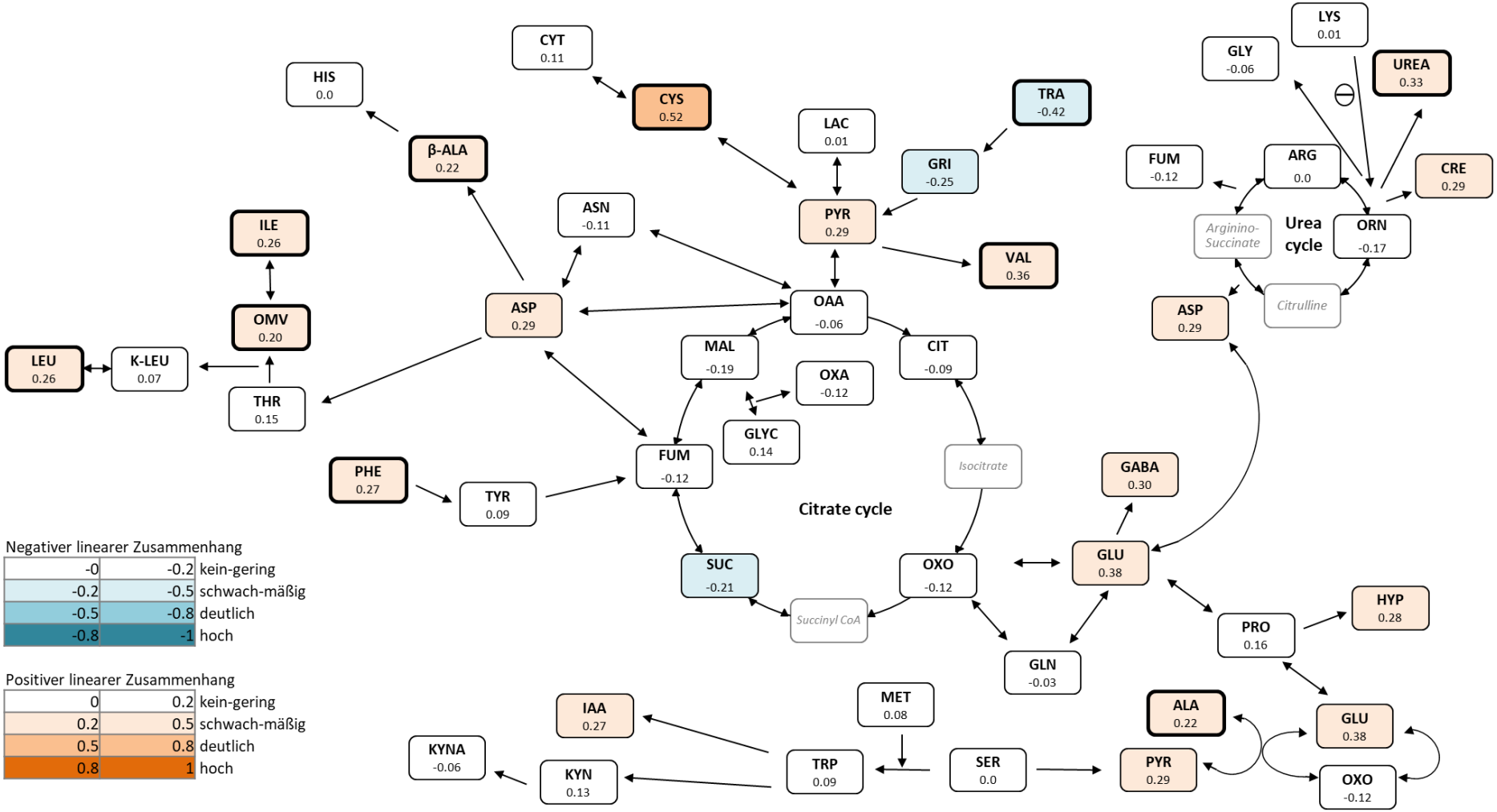

Figure 7 Correlations of metabolites and Lean Mass Index [kg/m²] in women. Metabolites outlined in thick: significant difference between men and women

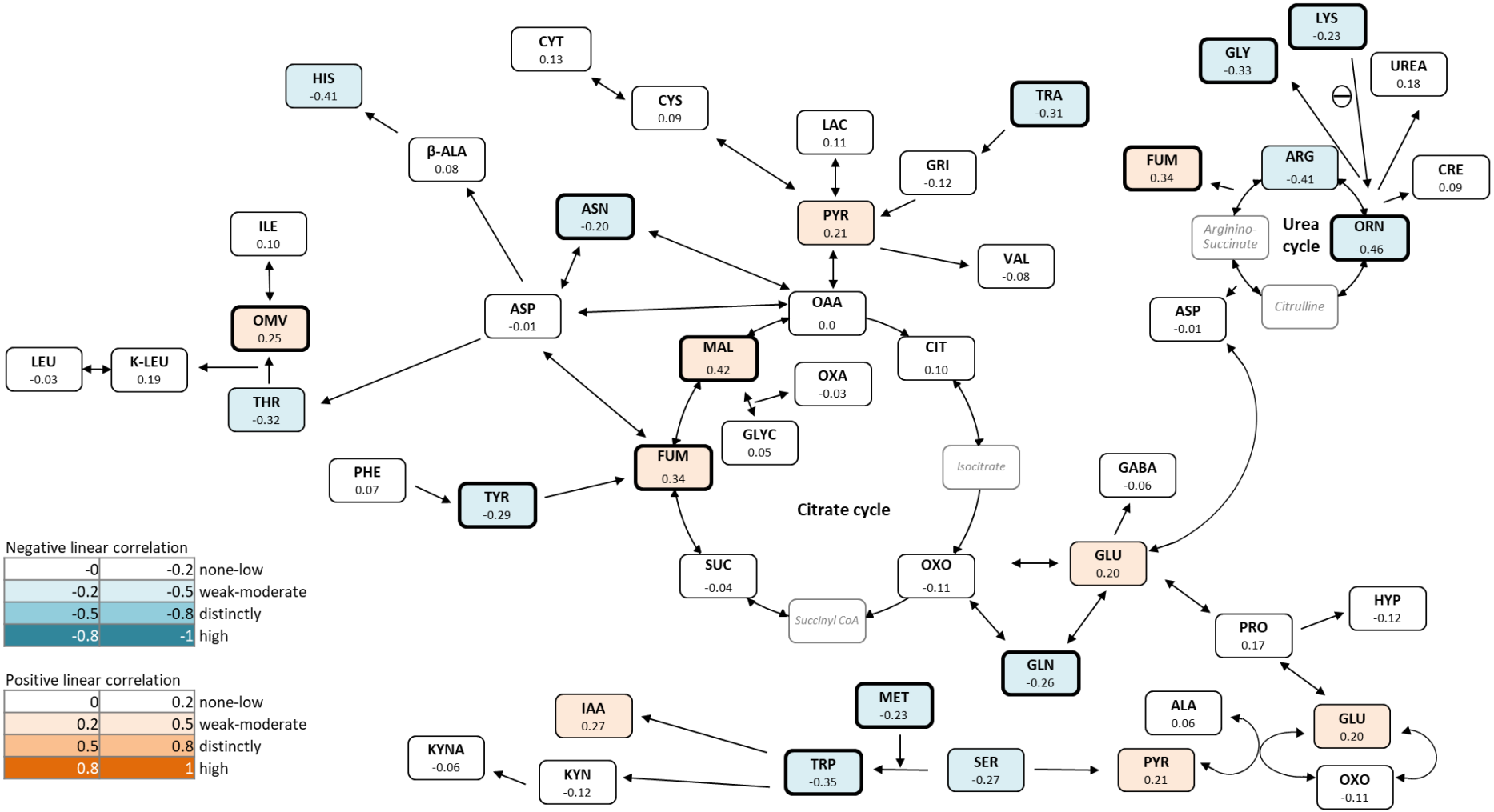

Figure 8 Correlations of metabolites and Lean Mass Index [kg/m²] in men. Metabolites outlined in thick: significant difference between men and women

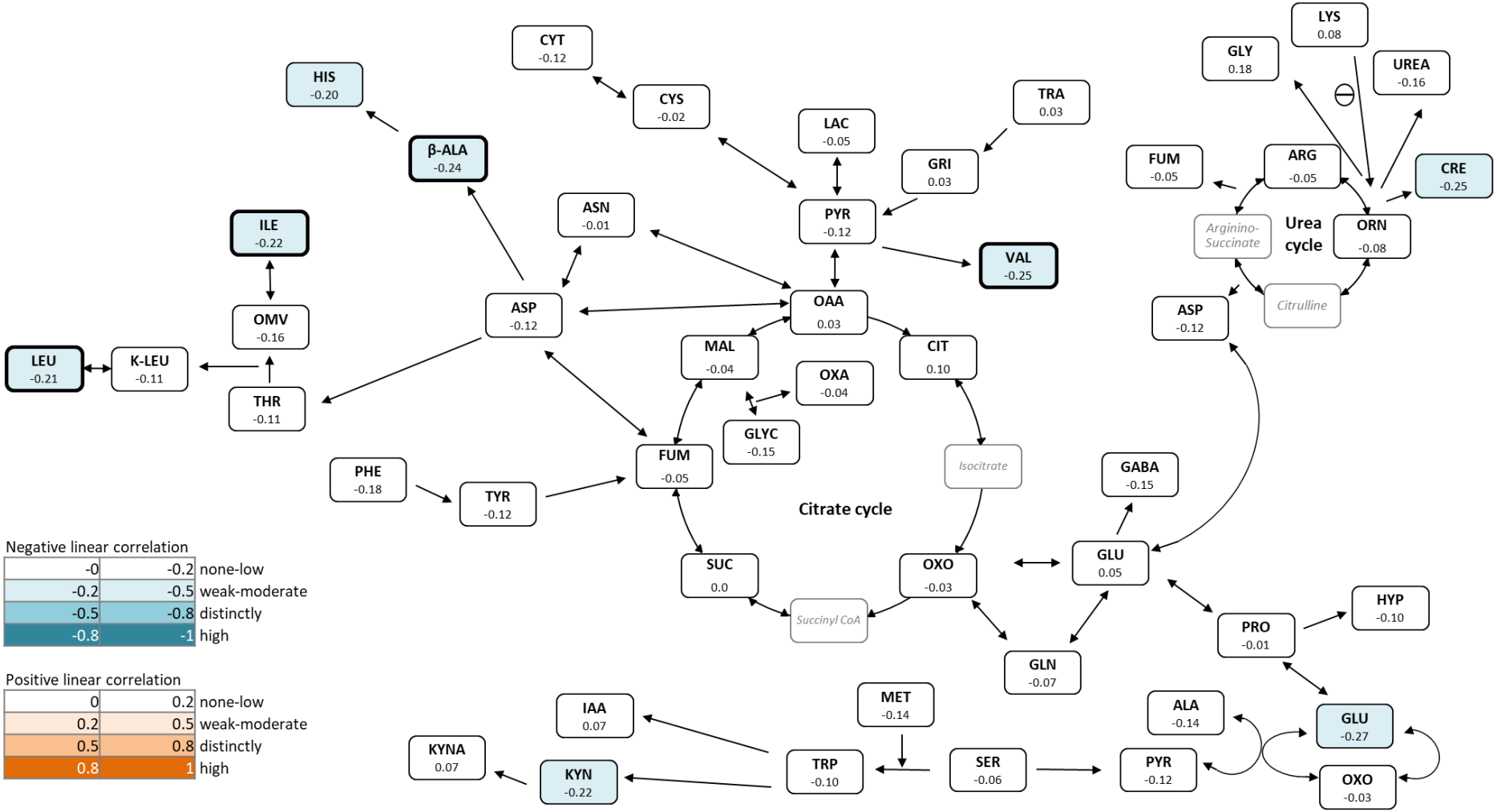

Figure 9 Correlations of metabolites and Skeletal Muscle Mass Index [%] in women. Metabolites outlined in thick: significant difference between men and women

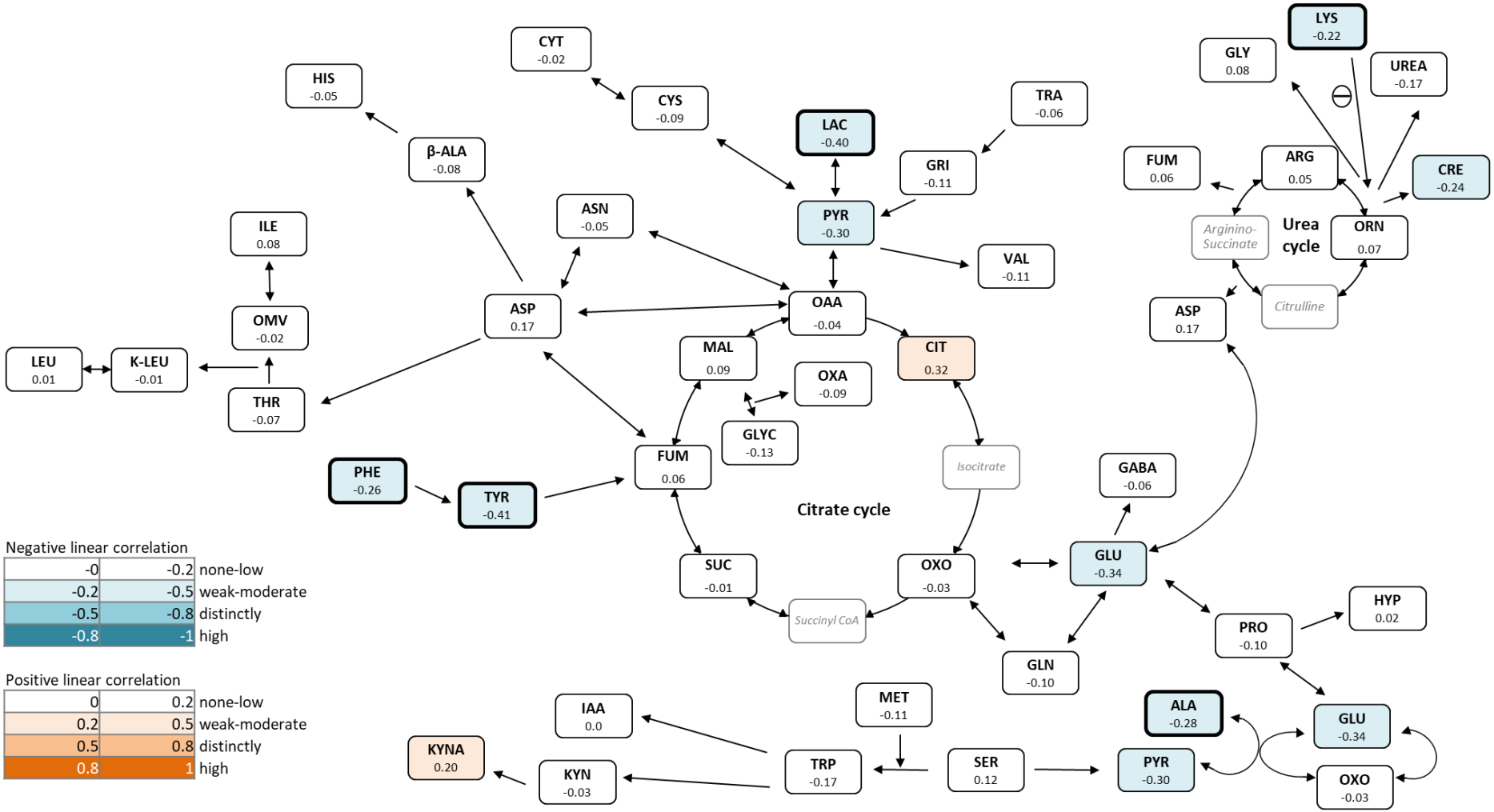

Figure 10 Correlations of metabolites and Skeletal Muscle Mass Index [%] in men. Metabolites outlined in thick: significant difference between men and women

3.3. Body Composition

Studies show that differences in body composition, particularly greater muscle mass in men and greater fat mass in women, have a significant impact on the metabolome and may be responsible for the differences between the sexes (Auer, et al., 2016; Ferrando, et al., 1998; Lundsgaard & Kiens, 2014).

In the present thesis, there were no strong correlations between the selected metabolites and the body composition parameters body fat, LMI and SMI. However, trends could be observed, and most of these trends consisted between the metabolites and LMI, suggesting that LMI had the greatest influence on the concentration of the selected metabolites. In addition, slight differences in the trends of the associations between the metabolites and the parameters could be observed between the sexes.

Glycine and threonic acid were the only two metabolites that differed significantly between the sexes and were significantly higher in women.

In our analysis, glycine was significantly higher in women than in men (by 8.72 %). The correlation analyses with the selected parameters for body composition (body fat percentage, LMI and SMI) did not show any notable associations, so it can be assumed that the significant difference between males and females was of other origin. Gender differences in certain SNPs (single-nucleotide polymorphism) would be a possible explanation for our observation. Mittelstrass et al. (2011) reported a 14 % higher glycine concentration in women in their analysis. They found that genome-wide the SNP effect of eight SNPs on chromosome 2 associated with glycine were significantly higher in women than in men (Mittelstrass, et al., 2011).

Threonic acid was 16.56 % higher in women than in men. We also found a weak negative association between the amount of threonic acid and the LMI in women with a correlation coefficient of $r = -0.42$. However, a strong deviation in the values could also be observed in both sexes. Unfortunately, no suitable studies on threonic acid could be found to discuss the significant difference between males and females.

Beta-alanine areas differed most between the sexes, with women having a 28.77 % lower amount. Women had a 23.75 % lower SMI and an 11.10 % lower LMI than men. The correlation tests between the metabolite's beta-alanine, histidine and

the parameters SMI and LMI showed slight differences between males and females. The difference in muscle mass could explain why beta-alanine and histidine were higher in men, although only beta-alanine was significantly higher ($p < 0.001$). Beta-alanine and histidine synthesise the dipeptide carnosine, which has the highest concentration in the muscle mass (Stout, et al., 2008).

In men and women, different metabolites were associated to varying degrees with body composition parameters, suggesting that body composition has a diverse link to the metabolome depending on gender. However, it should also be noted that other factors such as diet in general, hormonal balance, lifestyle and socioeconomic factors, genetic dimorphisms, the microbiome and other molecular biological factors also play an important role in gender-specific metabolism and influence the metabolome (Auer, et al., 2016; Kelly, et al., 2020; Krumsiek, et al., 2015; Rist, et al., 2017).

3.3.1. Branched-Chain Amino Acids

Valine, together with leucine and isoleucine, belongs to the branched-chain amino acids (BCAAs). An increased circulating amount of BCAAs in human blood is associated with obesity, as well as with lean mass (Mikkola, et al., 2020).

BCAAs were significantly lower in women than in men: Isoleucine (Δ 23.12 %), Leucine (Δ 20.02 %) and Valine (Δ 11.61 %). Among many others, the study by Rist et al. (2017) also found significant lower BCAA concentrations in women compared to men. The reason for this is assumed to be a higher muscle mass or an increased protein intake in men (Rist, et al., 2017).

In the present analysis, we found that protein intake did not differ significantly between males and females at baseline. There were significant differences between the sexes in terms of body fat percentage, LMI and SMI. For each of these three parameters, a weak correlation with BCAAs was observed in women, while no correlation was observed in men. The lower concentration of BCAAs in women would be a possible explanation for the observed effect.

4. Conclusion

The metabolome differed significantly between males and females. The concentrations of the metabolites were predominantly higher in men than in women. The body composition differed significantly between the sexes. The analysis showed no correlations above r = 0.60 between protein intake and the selected metabolites as well as the selected body composition parameters body fat percentage, LMI and SMI and metabolite levels.

The correlation coefficients were too low to make definite statements, but a tendency was recognisable for some metabolites. Many of the metabolites that correlated weakly to moderately with the parameters differed significantly between the sexes. There were tendencies for body composition to influence the metabolome differently depending on sex. It was noticeable that body composition seemed to have a greater link on BCAAs in women than in men. That might be attributed to the significant lower of BCAA levels in women.

In the analysis of the baseline data, protein intake showed no impact on the gender differences in the metabolome. Setka Nadine (2022) describes in her master's thesis a tendential effect of increased protein intake on the metabolome and the metabolic pathways citrate cycle and urea cycle, however no gender difference. In addition, other dietary parameters such as fat, carbohydrate and energy intake also play an important role in metabolism and could therefore be included in the analysis.

In our analysis, we investigated 45 metabolites. To confirm these results, it would be useful to analyse a number of metabolites and their correlations with body composition parameters. In addition, the difference between the sexes in other parameters such as energy expenditure, intracellular and extracellular mass, physical fitness parameters and serological parameters such as inflammatory status, blood lipid levels and blood composition could be further explored.

Setka Nadine (2022) observed a possible effect of resistance training on some of the selected metabolites, so it can be assumed that the fitness level of the test persons also had an influence on the metabolome.

The data used comes from only one measurement. Since, as already described, factors such as diet and lifestyle have a significant influence on the metabolome, this could be a limitation. To validate the results, the data from the control group of the other two test days could be used.

The present thesis shows gender-specific differences in the metabolome, but still leaves many questions unanswered and provides the basis for further research.

5. Fazit

Das Metabolom unterschied sich signifikant zwischen den Geschlechtern. Die Konzentrationen der Metaboliten waren bei Männern überwiegend höher als bei Frauen. Die Körperzusammensetzung unterschied sich signifikant zwischen den Geschlechtern. Die Analyse ergab keine Korrelationen über r = 0,60 zwischen der Proteinzufuhr und den ausgewählten Metaboliten sowie den ausgewählten Körperzusammensetzungsparametern Körperfettanteil, LMI und SMI und der Quantität der Metaboliten.

Die Korrelationskoeffizienten waren zu niedrig, um eindeutige Aussagen zu treffen, aber für einige Metaboliten war eine Tendenz erkennbar. Viele der Metabolite, die schwach bis mäßig mit den Parametern korrelierten, unterschieden sich signifikant zwischen den Geschlechtern. Weiters zeigten sich Tendenzen, dass die Körperzusammensetzung das Metabolom je nach Geschlecht unterschiedlich beeinflusst. Auffällig war, dass die Körperzusammensetzung bei Frauen einen größeren Zusammenhang mit der BCAA-Konzentration zu haben schien als bei Männern. Dies könnte auf den signifikant niedrigeren BCAA-Spiegel bei Frauen zurückzuführen sein.

Bei der Analyse der Basisdaten zeigte die Proteinzufuhr keinen Einfluss auf die Geschlechtsunterschiede im Metabolom. Setka Nadine (2022) beobachtete in ihrer Masterarbeit einen Effekt erhöhter Proteinzufuhr auf das Metabolom und die Stoffwechselwege Citratzyklus und Harnstoffzyklus, trennte aber nicht nach Geschlecht. Die Daten von Setka Nadine (2022) zeigen, dass die Proteinzufuhr möglicherweise einen Einfluss auf ein paar der ausgewählten Metabolite hatte. Darüber hinaus spielen auch andere Ernährungsparameter wie die Fett-, Kohlenhydrat- und Energiezufuhr eine wichtige Rolle für den Stoffwechsel und könnten daher in die Analyse einbezogen werden.

In unserer Analyse haben wir 45 Metaboliten untersucht. Um die Ergebnisse zu bestätigen, wäre es sinnvoll, weitere Metabolite und ihre Korrelation mit Parametern der Körperzusammensetzung zu analysieren. Darüber hinaus könnte der Unterschied zwischen den Geschlechtern bei anderen Parametern wie Energieverbrauch, intrazelluläre und extrazelluläre Masse, körperliche Fitnessparameter und

serologische Parameter wie Entzündungsstatus, Blutfettwerte und Blutzusammensetzung weiter untersucht werden.

Setka Nadine (2022) beobachtete in ihrer Masterarbeit einen möglichen Effekt des Krafttrainings auf manche der ausgewählten Metabolite, so dass davon auszugehen ist, dass auch das Fitnessniveau der Probanden einen Einfluss auf das Metabolom hatte.

Die verwendeten Daten stammten aus nur einer Messung. Da, wie bereits beschrieben, Faktoren wie Ernährung und Lebensstil einen erheblichen Einfluss auf das Metabolom haben, könnte dies eine Einschränkung darstellen. Zur Validierung der Ergebnisse könnten die Daten der Kontrollgruppe der anderen beiden Testtage herangezogen werden.

Die vorliegende Arbeit zeigt geschlechtsspezifische Unterschiede im Metabolom, lässt aber noch viele Fragen offen und bietet die Grundlage für weitere Forschung.

6. Summary

There are differences in molecular biological and physiological parameters between the sexes. These gender-specific variations affect general metabolism and thus the individual metabolites. The aim of the present thesis is to investigate the intergender distinctions in the metabolome in older adults.

The NutriAging study of 2018 involved 155 healthy subjects aged 65 to 84 years. Of these subjects the data from 107 people were analysed, including 54 women and 53 men. To detect amino acids and polar metabolites from 50 µL of human blood plasma by GC-MS, we used a targeted and an untargeted approach. 45 of the detected metabolites were annonated and investigated in detail.

25 of the 45 metabolites differed significantly between the sexes, with the concentrations of 23 metabolites being significantly higher in men than in women. Furthermore, there were significant differences in body fat percentage, Lean Mass Index and Skeletal Muscle Mass Index between the sexes. The correlation analysis with the parameters protein intake, body fat percentage, Lean Mass Index and Skeletal Muscle Mass Index showed that none of the selected metabolites correlate strongly with these parameters. However, trends have been observed suggesting that gender differences in the metabolome may be related to differences in body composition.

However, further research would be needed to better explain the differences in the metabolome between the sexes, particularily investigating older adults.

Key words: Metabolomics, Older Adults, Gender, Fat Mass, Muscle Mass, BCAAs

7. Zusammenfassung

Es gibt Unterschiede zwischen den Geschlechtern in molekularbiologischen und physiologischen Parametern. Diese geschlechtsspezifischen Variationen wirken sich generell auf den Stoffwechsel und damit auf die spezifischen Metaboliten aus. Das Ziel der vorliegenden Arbeit war es, die geschlechtsspezifischen Unterschiede im Metabolom von älteren Personen zu untersuchen.

An der NutriAging-Studie von 2018 nahmen 155 gesunde Probanden im Alter von 65 bis 84 Jahren teil. Von diesen Probanden wurden die Daten von 107 Personen ausgewertet, darunter 54 Frauen und 53 Männer. Zum Nachweis von Aminosäuren und polaren Metaboliten aus 50 µL menschlichem Blutplasma mittels GC-MS wurde ein targeted und einen untargeted Ansatz angewandt. 45 der nachgewiesenen Metaboliten wurden annotiert und im Detail untersucht.

25 der 45 Metaboliten unterschieden sich signifikant zwischen den Geschlechtern, wobei die quantitativen Mengen von 23 Metaboliten bei Männern signifikant höher waren als bei Frauen. Darüber hinaus konnten signifikante Unterschiede im Körperfettanteil, im Lean Mass Index und im Skelettmuskelmasseindex zwischen den Geschlechtern festgestellt werden. Die Korrelationsanalyse mit den Parametern Proteinzufuhr, Körperfettanteil, Lean Mass Index und Skelettmuskelmasseindex zeigte keine Signifikanzen. Es wurden jedoch Tendenzen beobachtet, die darauf hindeuten, dass geschlechtsspezifische Unterschiede im Metabolom möglicherweise mit den Unterschieden in der Körperzusammensetzung zusammenhängen könnten.

Weitere Untersuchungen wären erforderlich, um die signifikanten Unterschiede im Metabolom zwischen den Geschlechtern vor allem bei älteren Personen besser erklären zu können.

Schlüsselwörter: Metabolomics, Ältere Personen, Geschlecht, Fettmasse, Muskelmasse, BCAAs

8. References

Adav, S., & Wang, Y. (2021). Metabolomics Signatures of Aging: Recent Advances. *Aging and disease, 12*(2), 646-661. doi:https://doi.org/10.14336/AD.2020.0909

Audano, M., Maldini, M., De Fabiani, E., Mitro, N., & Caruso, D. (30. 4 2018). Gender-related metabolomics and lipidomics: From experimental animal models to clinical evidence. *Journal of Proteomics*(178), S. 82-91. doi:https://doi.org/10.1016/j.jprot.2017.11.001

Auer, M., Cecil, A., Roepke, Y., Bultynck, C., Pas, C., Fuss, C., . . . T'Sjoen, G. (2016). 12-months metabolic changes among gender dysphoric individuals under cross-sex hormone treatment: a targeted metabolomics study. *Scientiific Reborts, 6, 37005*. doi:https://doi.org/10.1038/srep37005

Bell, J., Santos Terreira, D., Fraser, A., Soares, A., Howe, L., Lawlor, D., . . . O'Keeffe, L. (24. 2 2021). Sex differences in systemic metabolites at four life stages: cohort study with repeated metabolomics. *BMC Med*, S. 19: 58. doi:10.1186/s12916-021-01929-2

Bundeskanzleramt. (20. 4 2022). Von Frauen und Männer in Österreich, Zahlen, Daten, Fakten 2020: https://www.bundeskanzleramt.gv.at/dam/jcr:8ca56dbf-aa9f-4ac2-935e-99564faa9aa0/gender-index-2020.pdf abgerufen

Castelli, F., Rosati, G., Moguet, C., Fuentes, C., Marrugo-Ramírez, J., Lefebvre, T., . . . Junot, C. (1 2022). Metabolomics for personalized medicine: the input of analytical chemistry from biomarker discovery to point-of-care tests. *Anal Bioanal Chem, 414*(2), S. 759–789. doi:10.1007/s00216-021-03586-z

Chella Krishnan, K., Mehrabian, M., & Lusis, A. (October 2018). Sex differences in metabolism and cardiometabolic disorders. *Curr Opin Lipidol*, S. 29(5):404-410. doi:10.1097/MOL.0000000000000536

Darst, B., Koscik, R., Hogan, K., Johnson, S., & Engelmann, C. (24. 2 2019). Longitudinal plasma metabolomics of aging and sex. *Aging (Albany NY), 11*(4), S. 1262–1282. doi:10.18632/aging.101837

Ferrando, A., Tiption, K., Doyle, D., Phillips, S., Cortiella, J., & Wolfe, R. (1998). Testosterone injection stimulates net protein synthesis but not tissue amino acid

transport. *Endocrinology and Metabolism, 275*(5), S. E864-E871. doi:https://doi.org/10.1152/ajpendo.1998.275.5.E864

Franzke, B., Schober-Hapler, B., Hofmann, M., Oesen, S., Tosevska, A., Henriksen, T., . . . Wagner, K. (24. 4 2018). Age and the effect of exercise, nutrition and cognitive training on oxidative stress – The Vienna Active Aging Study (VAAS), a randomized controlled trial. *Free Radical Biology and Medicine, 121*, S. 69-77. Von https://doi.org/10.1016/j.freeradbiomed.2018.04.565 abgerufen

Hansen, M. (2018). Female hormones: Do they influence muscle and tendon protein metabolism? *Proceedings of the Nutrition Society, 77*(1), S. 32-41. doi:10.1017/S0029665117001951

Jové, M., Maté, I., Naudí, A., Mota-Martorell, N., Portero-Otín, M., De la Fuente, M., & Pamplona, R. (May 2016). Human Aging Is a Metabolome-related Matter of Gender. *The Journals of Gerontology: Series A, 71*(5), S. 578–585. doi:https://doi.org/10.1093/gerona/glv074

Kang, S., Yoon, J., Ahn, H., Kim, S., Lee, K., Shin, H., . . . Lim, S. (11. 11 2011). Android Fat Depot Is More Closely Associated with Metabolic Syndrome than Abdominal Visceral Fat in Elderly People. *PLoS One, 6*(11), S. e27694. doi:10.1371/journal.pone.0027694

Kelly, R., Kelly, M., & Kelly, P. (1. 12 2020). Metabolomics, physical activity, exercise and health: A review of the current evidence. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1866*(12), S. 165936. doi:https://doi.org/10.1016/j.bbadis.2020.165936

Krumsiek, J., Mittelstrass, K., Do, K., Stückler, F., Ried, J., Adamski, J., . . . Kastenmüller, G. (4. 8 2015). Gender-specific pathway differences in the human serum metabolome. *Metabolomics, 11*(6), S. 1815-1833. doi:10.1007/s11306-015-0829-0

Kučera, J., Spáčil, Z., Friedecký, D., Novák, J., Pekař, M., & Bienertová-Vašků, J. (28. 10 2018). Human White Adipose Tissue Metabolome: Current Perspective. *Obesity, 26*(12), S. 1870-1878. doi:https://doi.org/10.1002/oby.22336

Lundsgaard, A., & Kiens, B. (30. 9 2014). Gender Differences in Skeletal Muscle Substrate Metabolism – Molecular Mechanisms and Insulin Sensitivity. *Front Endocrinol (Lausanne), 5*, S. 195. doi:10.3389/fendo.2014.00195

Martin, K., Pencharz, P., Rafii, M., Ball, R., Szwiega, S., Elango, R., & Courtney-Martin, G. (10 2019). The Phenylalanine Requirement of Elderly Men and Women Measured by Direct 13C Carbon Oxidation Method Is Similar to That of Young Adults. *The Journal of Nutrition, 149*(10), S. 1776–1784. doi:https://doi.org/10.1093/jn/nxz137

Mikkola, T., Salonen, M., Kajantie, E., Kautiainen, H., & Eriksson, J. (5 2020). Associations of Fat and Lean Body Mass with Circulating Amino Acids in Older Men and Women. *The Journals of Gerontology: Series A, 75*(5), S. 885–891. doi:https://doi.org/10.1093/gerona/glz126

Mittelstrass, K., Ried, J., Yu, Z., Krumsiek, J., Gieger, C., Prehn, C., . . . Illig, T. (11. 8 2011). Discovery of Sexual Dimorphisms in Metabolic and Genetic Biomarkers. *PLoS Genet, 7*(8), S. e1002215. doi:10.1371/journal.pgen.1002215

Moon, J., Park, S., Ryu, S., & Park, C. (2 2018). New Skeletal Muscle Mass Index in Diagnosis of Sarcopenia. *J Bone Metab., 25*(1), S. 15-21. doi:10.11005/jbm.2018.25.1.15

Oberbach, A., Blüher, M., Wirth, H., Till, H., Kovac, P., Kullnick, Y., . . . von Bergen, M. (9. 8 2011). Combined Proteomic and Metabolomic Profiling of Serum Reveals Association of the Complement System with Obesity and Identifies Novel Markers of Body Fat Mass Changes. *J. Proteome Res., 10*(10), S. 4769–4788. doi:https://doi.org/10.1021/pr2005555

Orimo, H., Ito, H., Suzuki, T., Araki, A., Hosoi, T., & Sawabe, M. (2006). Reviewing the definition of "elderly". *Geriatr Gerontol Int, 6*(3), S. 149-158. doi:10.1111/j.1447-0594.2006.00341.x

Rist, M., Roth, A., Frommherz, L., Weinert, C., Krüger, R., Merz, B., . . . Watzl, B. (16. 8 2017). Metabolite patterns predicting sex and age in participants of the Karlruhe Metabolomics and Nutrition (KarMeN) study. *PLoS ONE, 12*(8), S. e0183228. doi:https://doi.org/10.1371/journal.pone.0183228

Ruoppolo, M., Campesi, I., Scolamiero, E., Pecce, R., Caterino, M., Cherchi, S., . . . Franconi, F. (11. 10 2014). Serum metabolomic profiles suggest influence of sex and oral contraceptive use. *Am J Transl Res, 6*(5), S. 614-624. doi:PMID: 25360225; PMCID: PMC4212935

Schutz, Y., Kyle, U., & Pichard, C. (7 2002). Fat-free mass index and fat mass index percentiles in Caucasians aged 18-98 y. *Int J Obes Relat Metab Disord, 26*(7), S. 953-60. doi:10.1038/sj.ijo.0802037

statista. (15. 2 2022). Von Altersstruktur in Österreich von 2012 bis 2022: https://de.statista.com/statistik/daten/studie/217431/umfrage/altersstruktur-in-oesterreich/ abgerufen

Stout, J., Graves, B., Smith, A., Hartman, M., Cramer, J., Beck, T., & Harris, R. (7. 11 2008). The effect of beta-alanine supplementation on neuromuscular fatigue in elderly (55–92 Years): a double-blind randomized study. *Int Soc Sports Nutr, 5*(21). doi:https://doi.org/10.1186/1550-2783-5-21

Unterberger, S., Aschauer, R., Zöhrer, P., Draxler, A., Franzke, B., Strasser, E., . . . Wessner, B. (5 2022). Effects of increased habitual dietary protein intake and resistance training on physical performance, muscle quality and body composition of older adults: a randomised controlled trial. *Clin Nutr, 41*(5), S. 1034-1045. doi:10.1016/j.clnu.2022.02.017

Vignoli, A., Tenori, L., Luchinat, C., & Saccenti, E. (17. 1 2018). Age and Sex Effects on Plasma Metabolite Association Networks in Healthy Subjects. *Journal of Proteome Research*, S. 97-107. doi:https://doi.org/10.1021/acs.jproteome.7b00404

9. Appendix

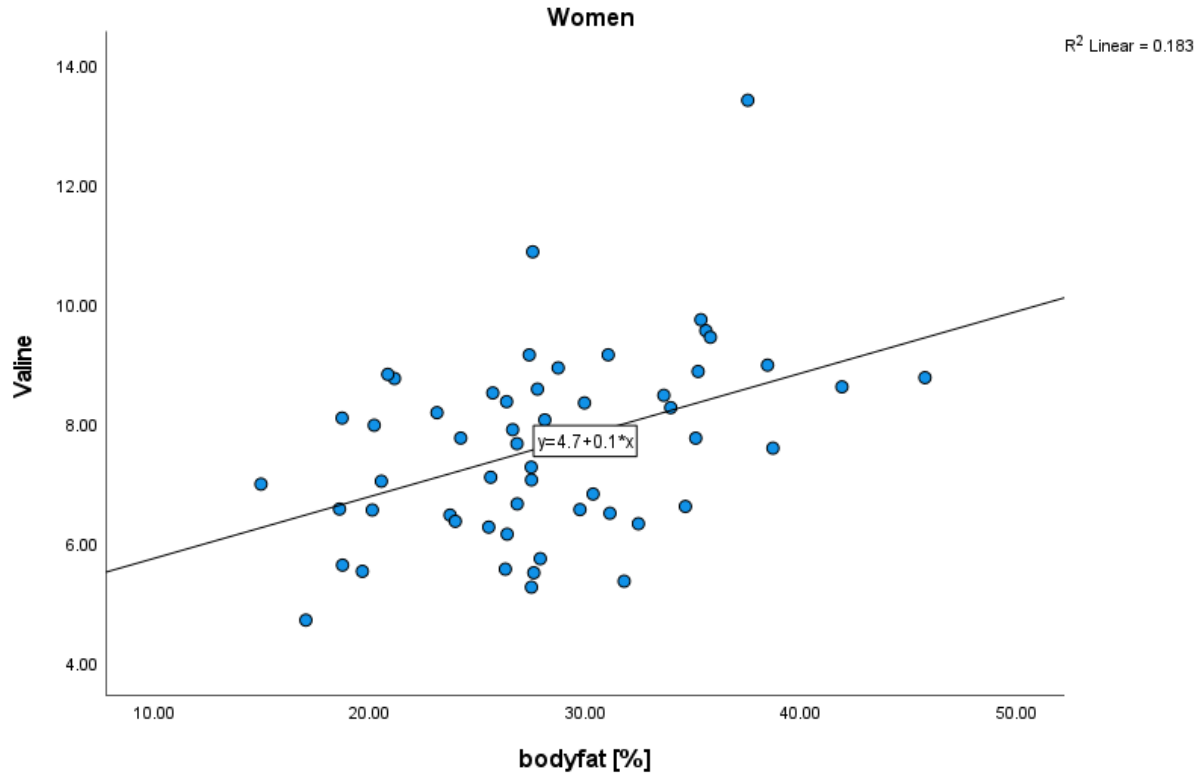

Figure 11 Scatter plot of valine in relation to body fat in percent in women

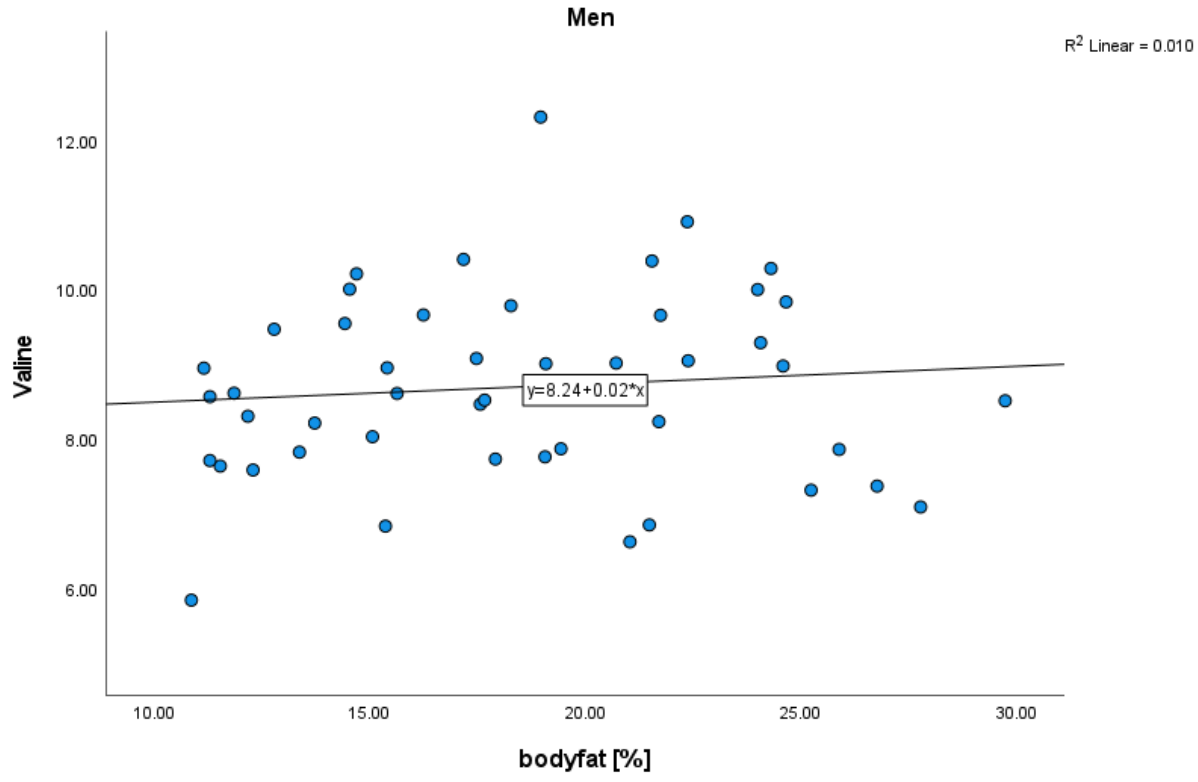

Figure 12 Scatter plot of valine in relation to body fat in percent in men

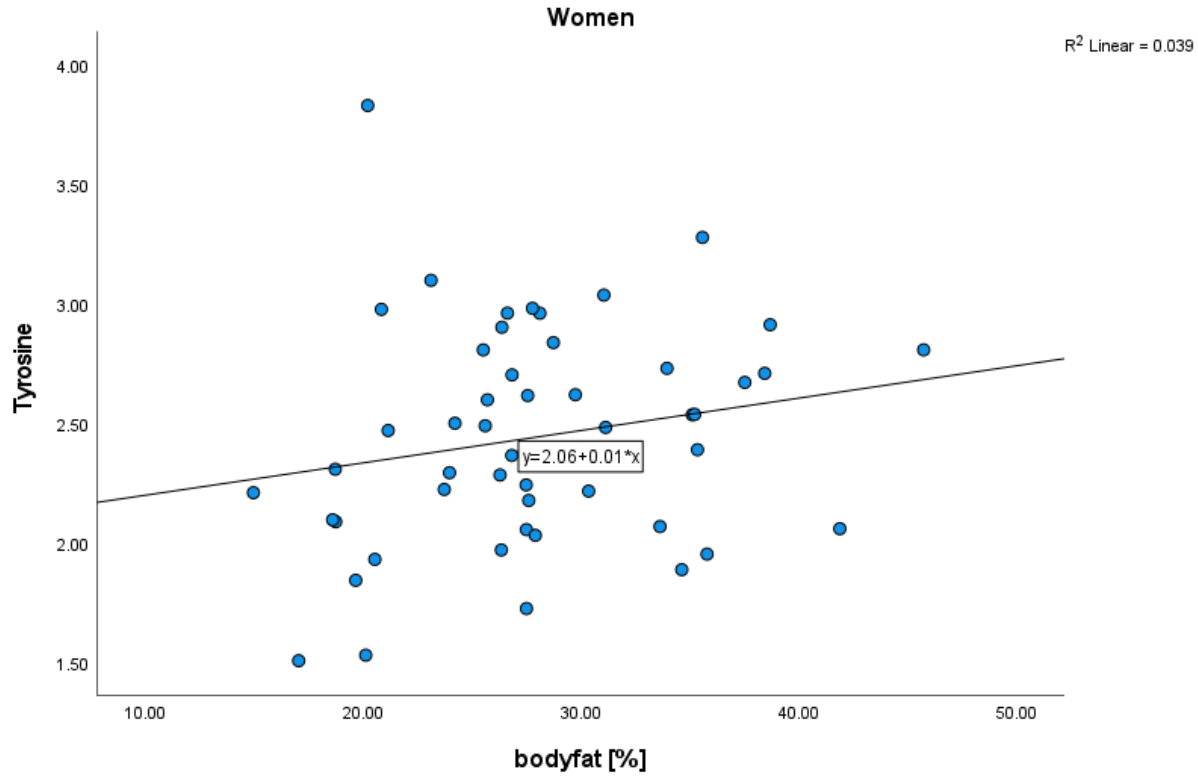

Figure 13 Scatter plot of tyrosine in relation to body fat in percent in women

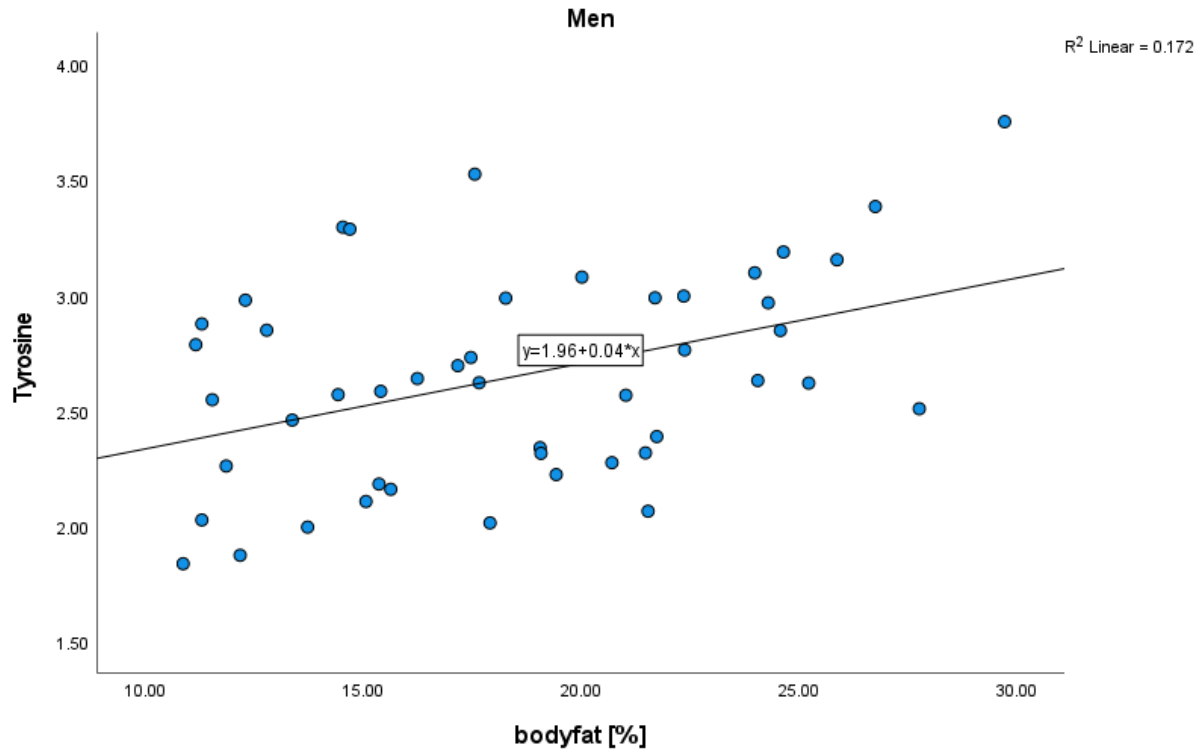

Figure 14 Scatter plot of tyrosine in relation to body fat in percent in men

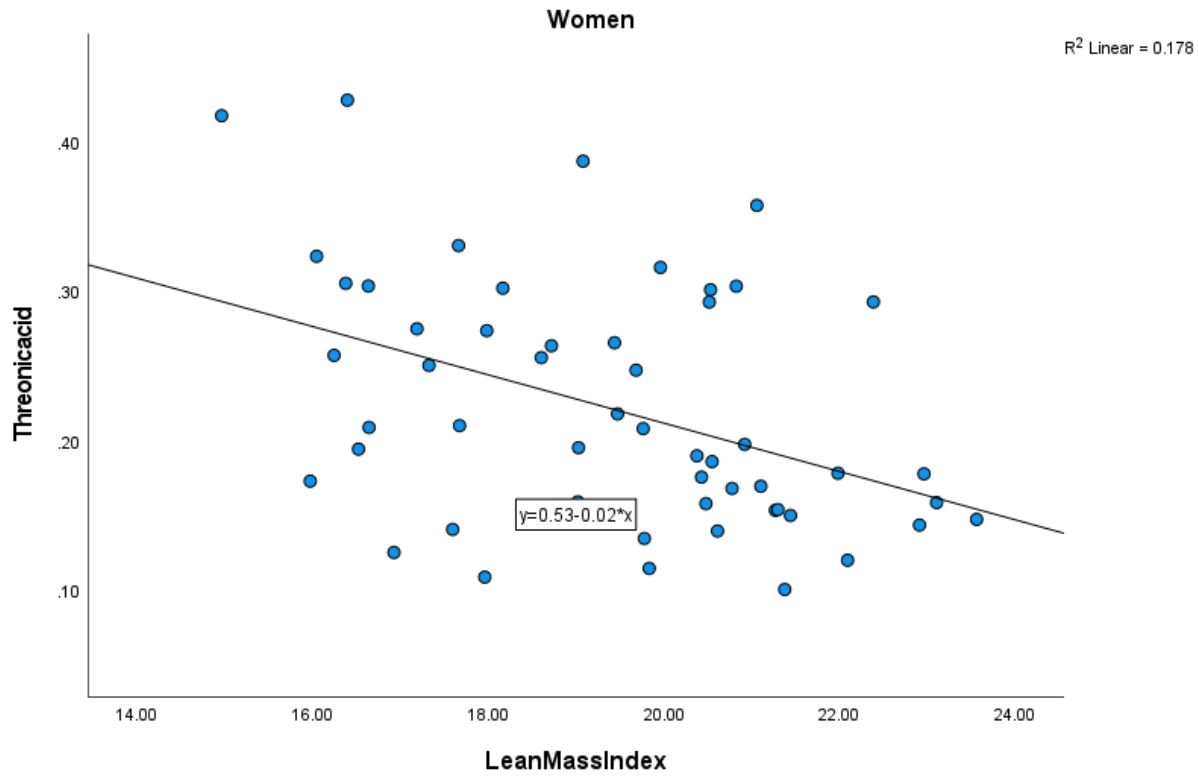

Figure 15 Scatter plot of threonic acid in relation to Lean Mass Index in women

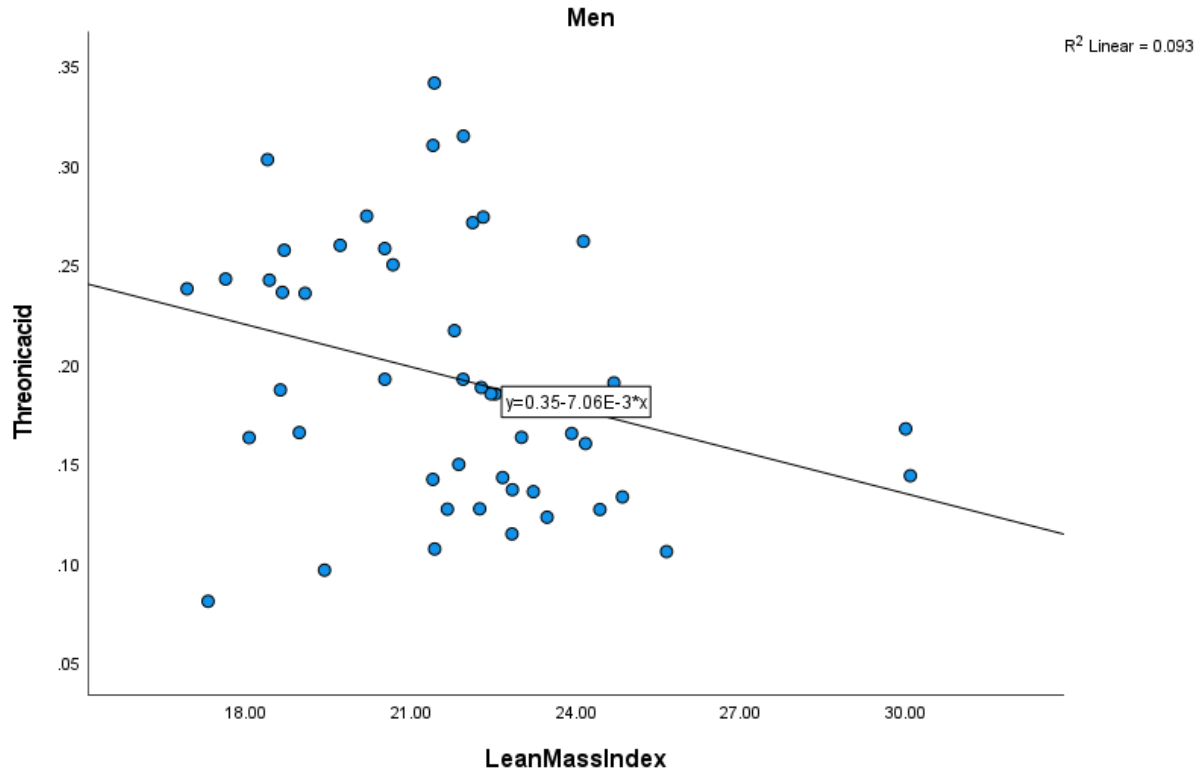

Figure 16 Scatter plot of threonic acid in relation to Lean Mass Index in men

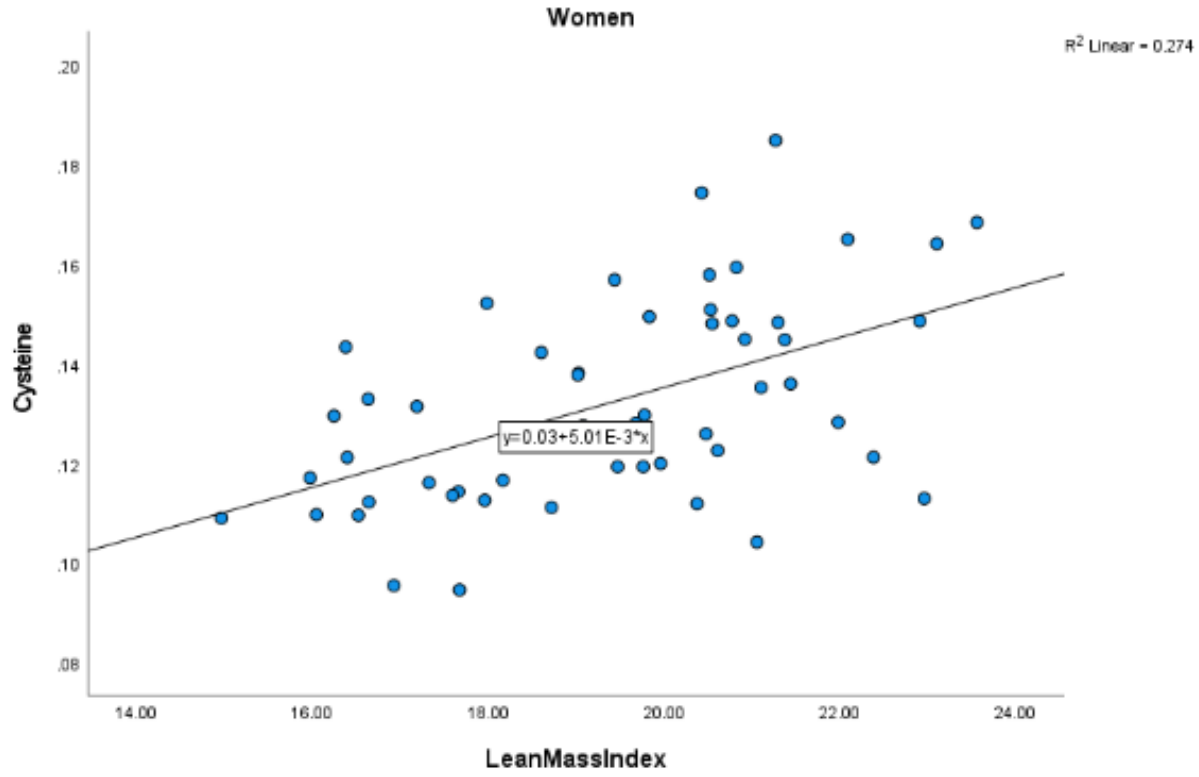

Figure 17 Scatter plot of cysteine in relation to Lean Mass Index in women

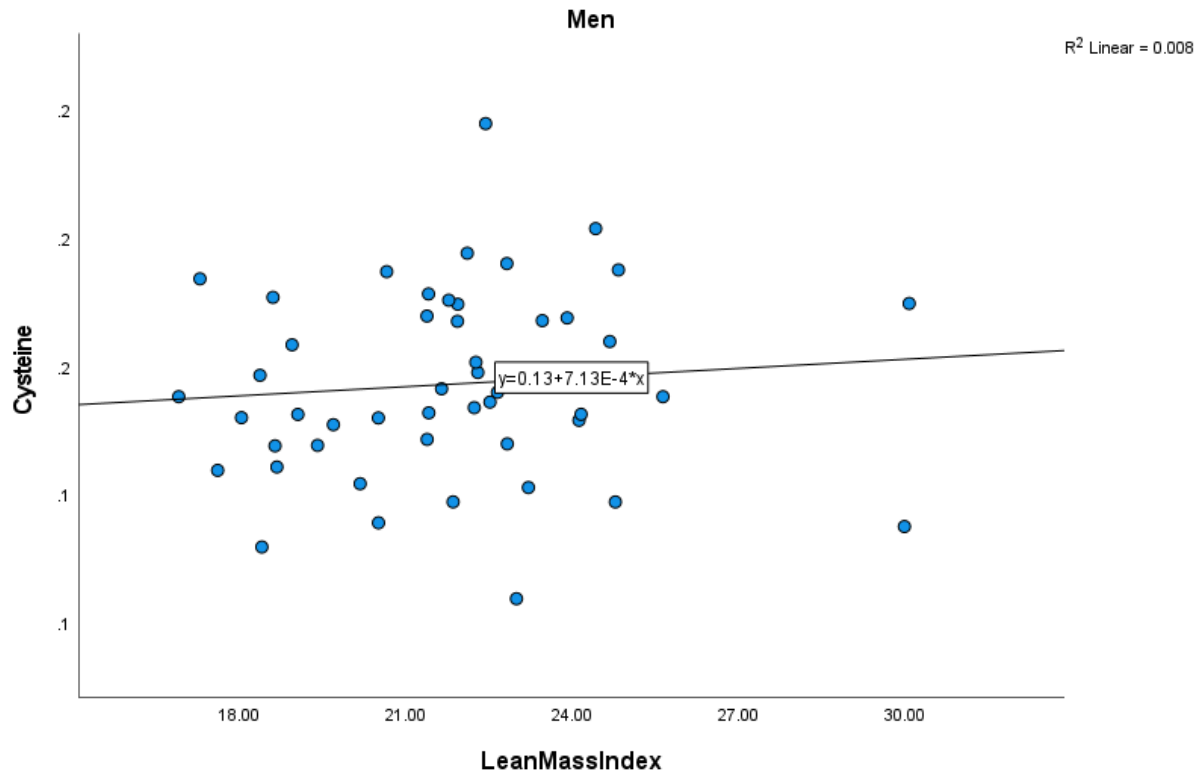

Figure 18 Scatter plot of cysteine in relation to Lean Mass Index in men

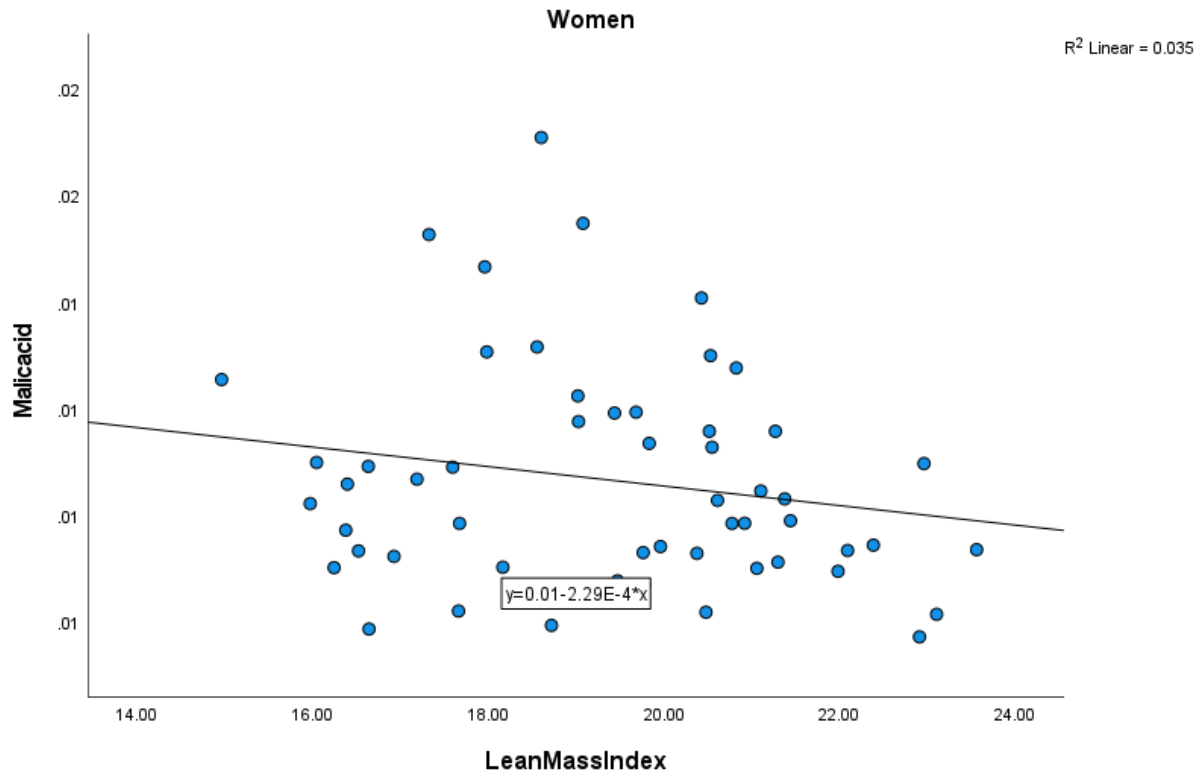

Figure 19 Scatter plot of malic acid in relation to Lean Mass Index in women

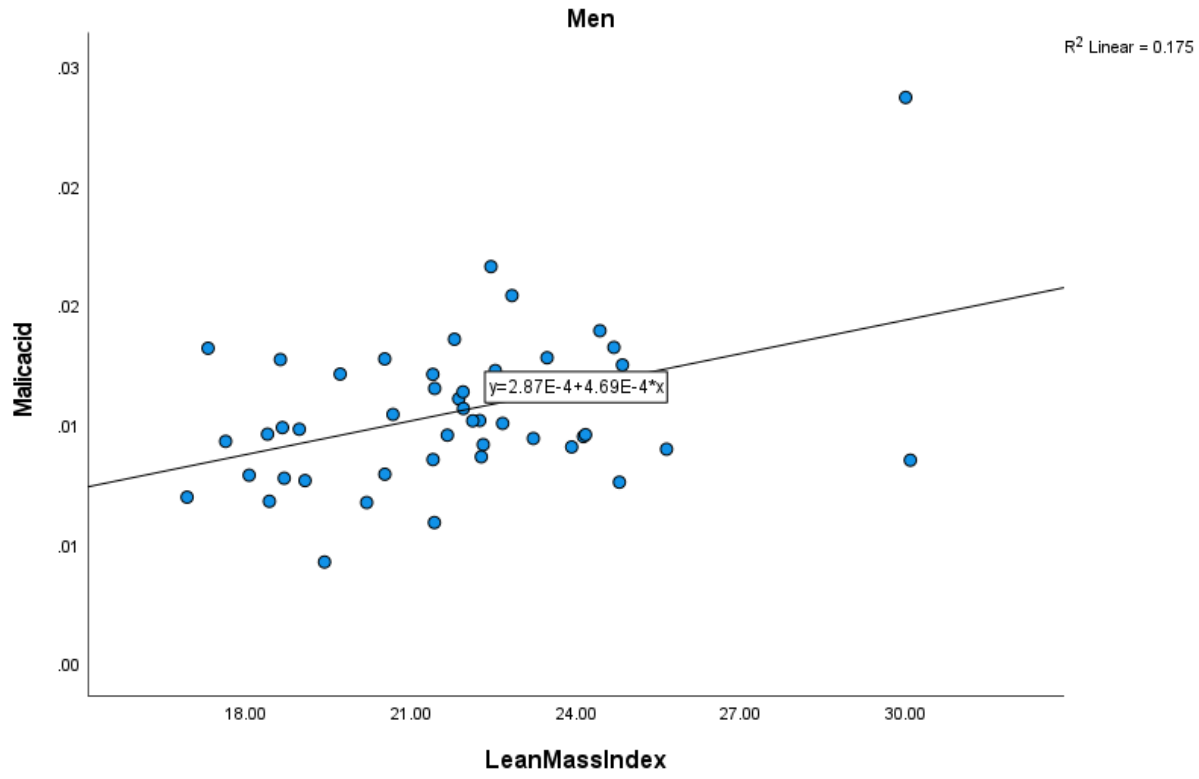

Figure 20 Scatter plot of malic acid in relation to Lean Mass Index in men

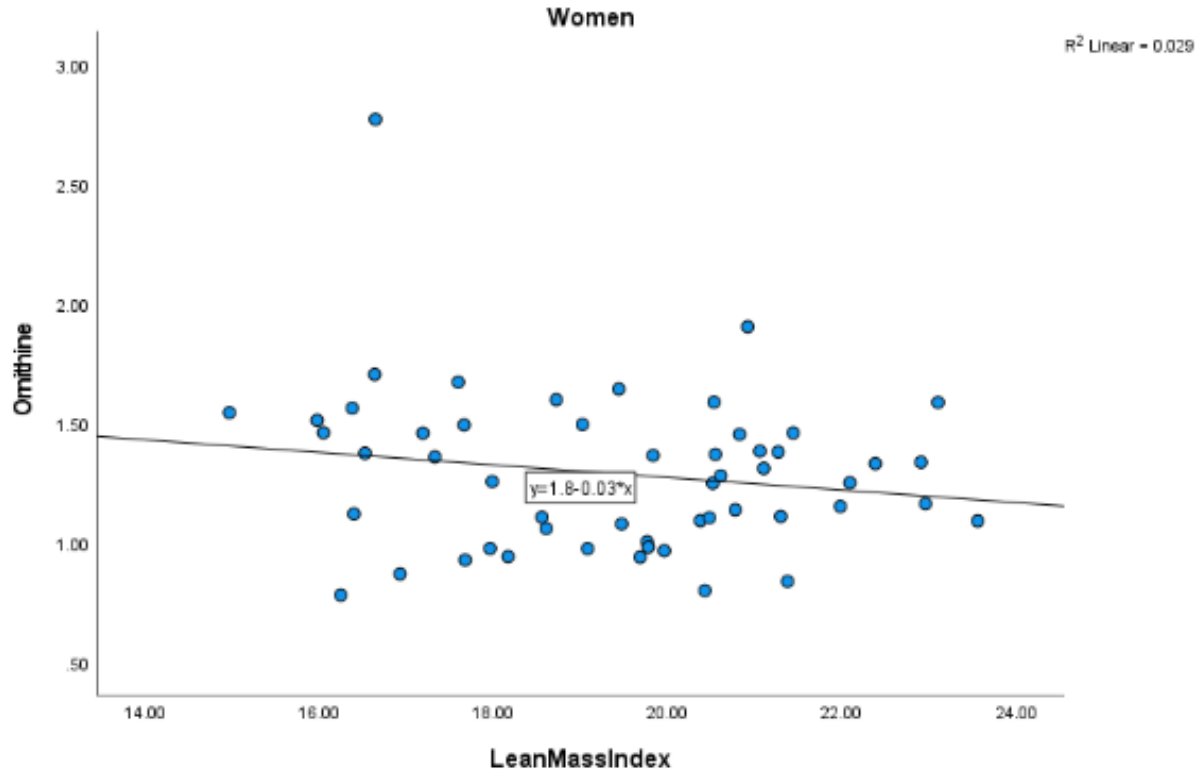

Figure 21 Scatter plot of ornithine in relation to Lean Mass Index in women

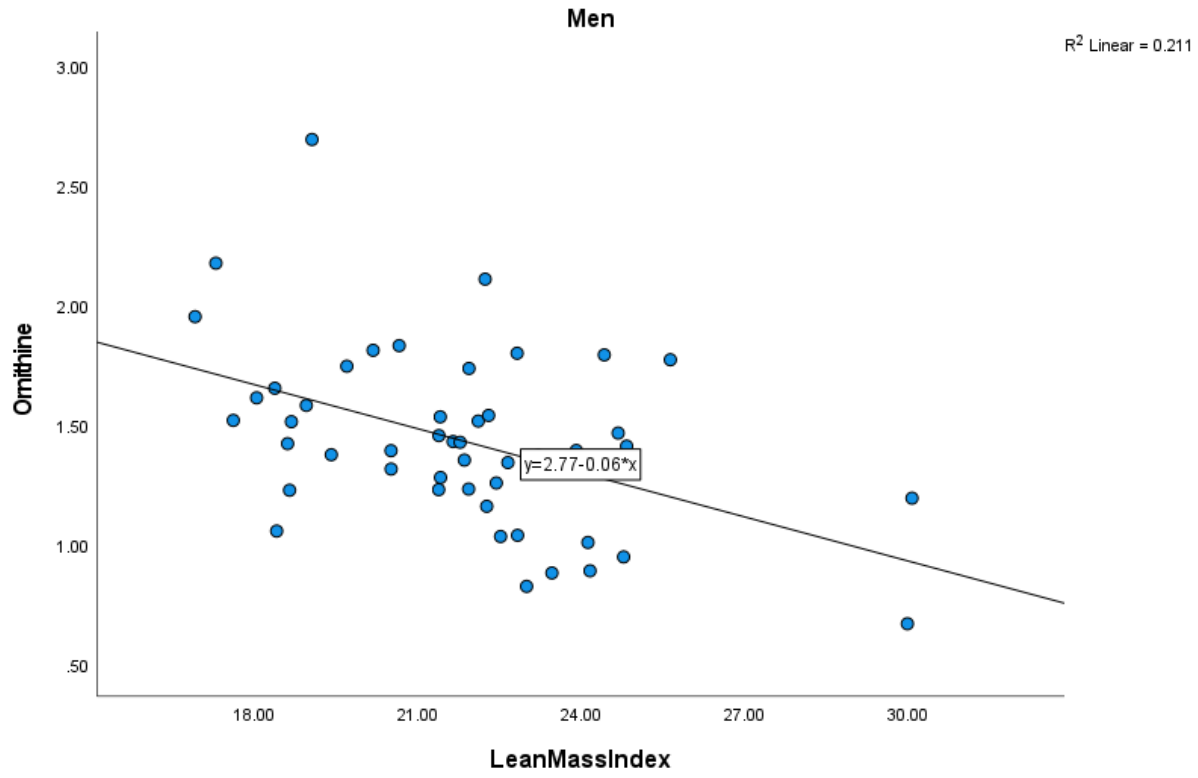

Figure 22 Scatter plot of ornithine in relation to Lean Mass Index in men

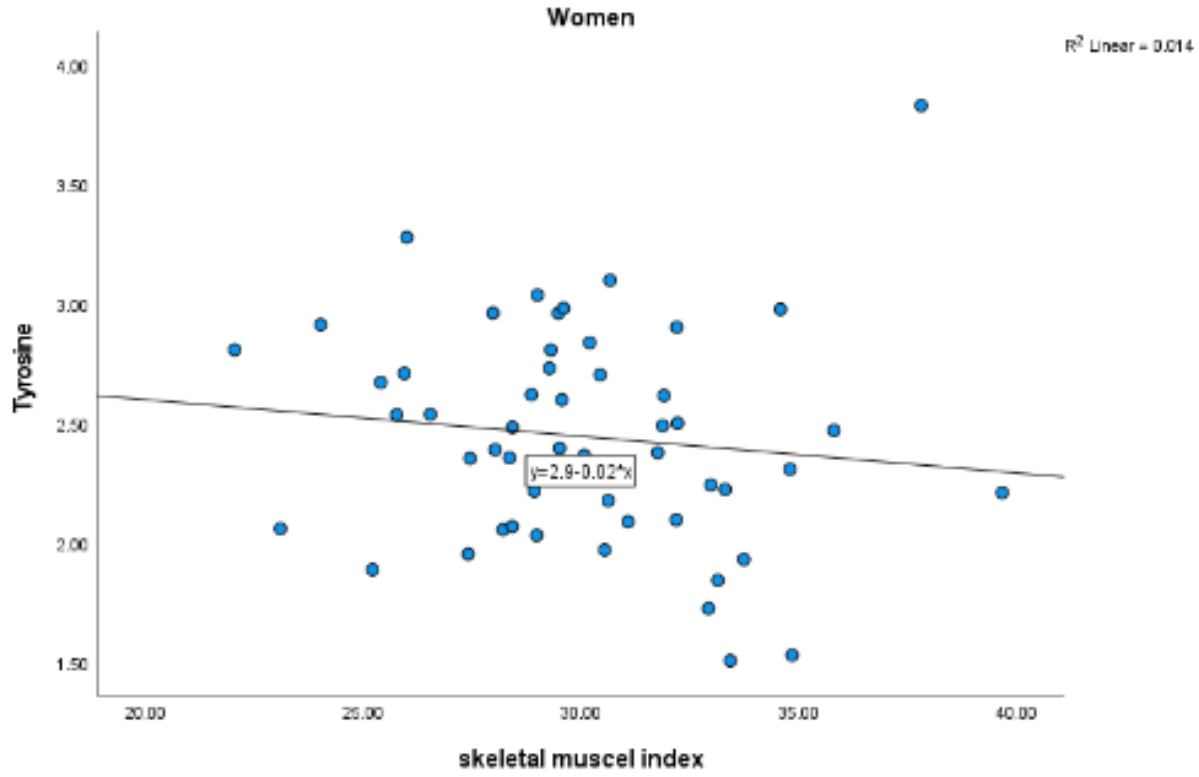

Figure 23 Scatter plot of tyrosine in relation to Skeletal Muscle Index in women

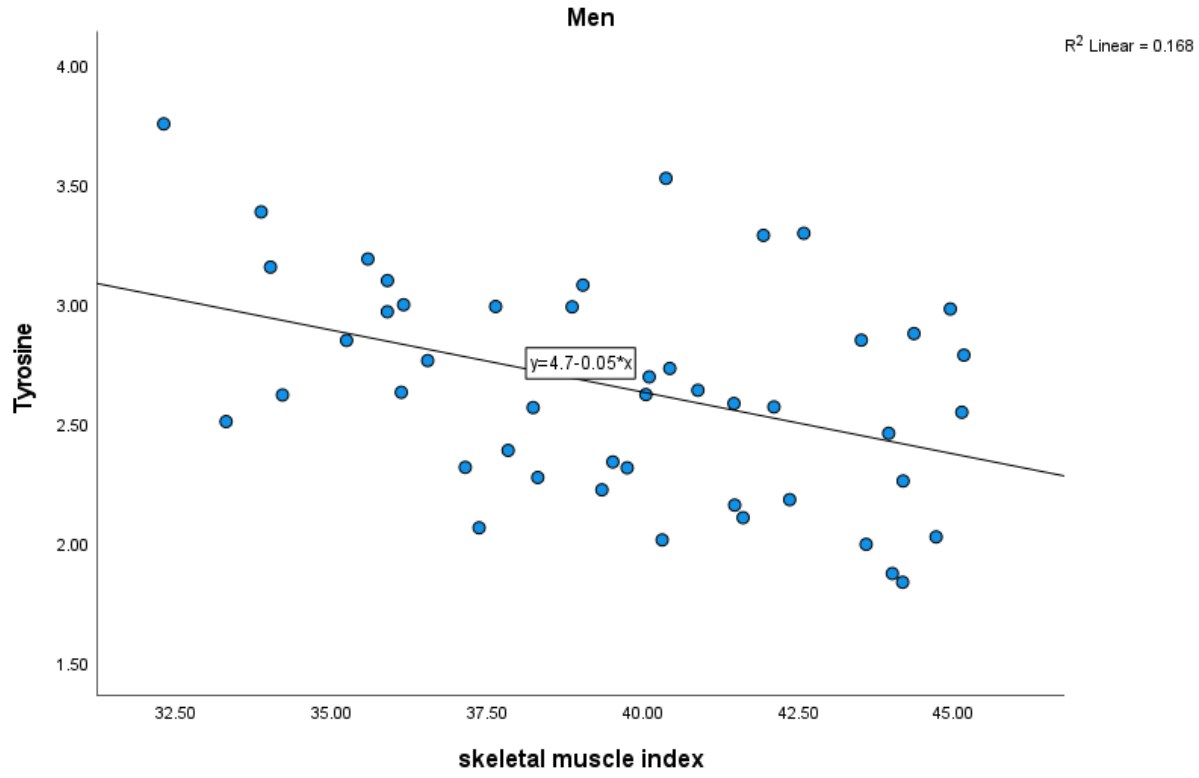

Figure 24 Scatter plot of tyrosine in relation to Skeletal Muscle Index in men

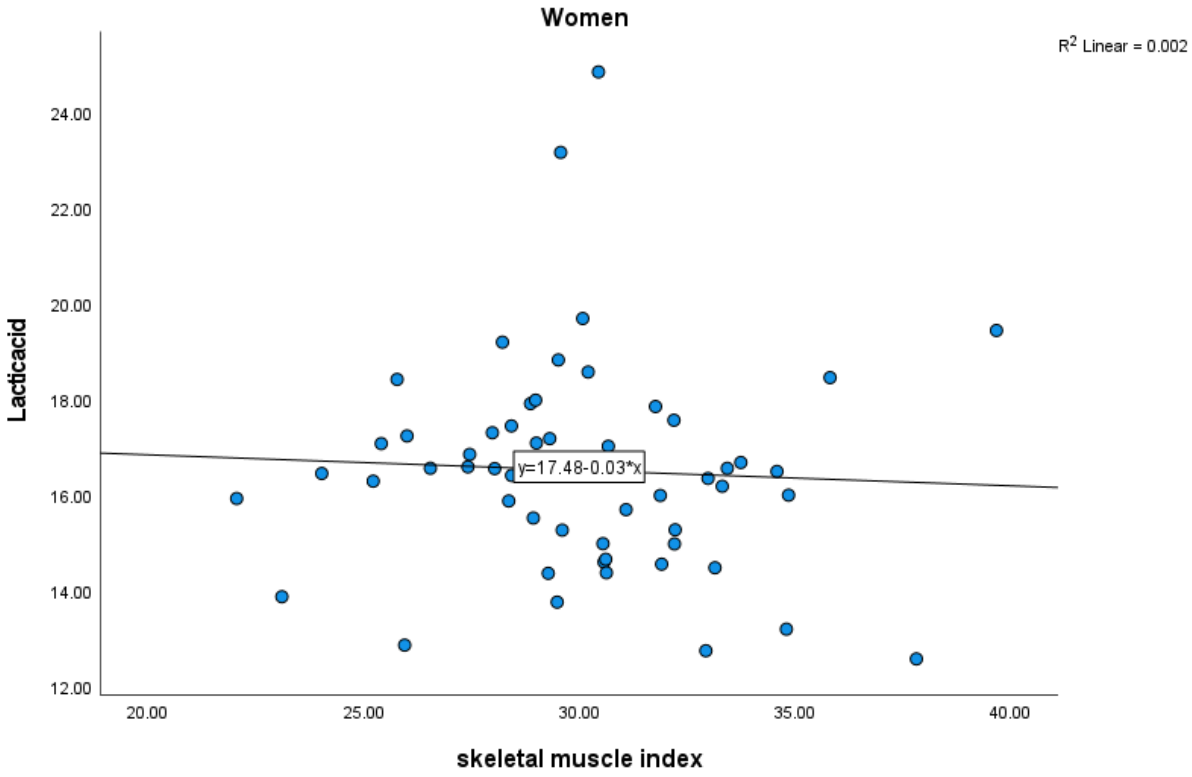

Figure 25 Scatter plot of lactic acid in relation to Skeletal Muscle Index in women

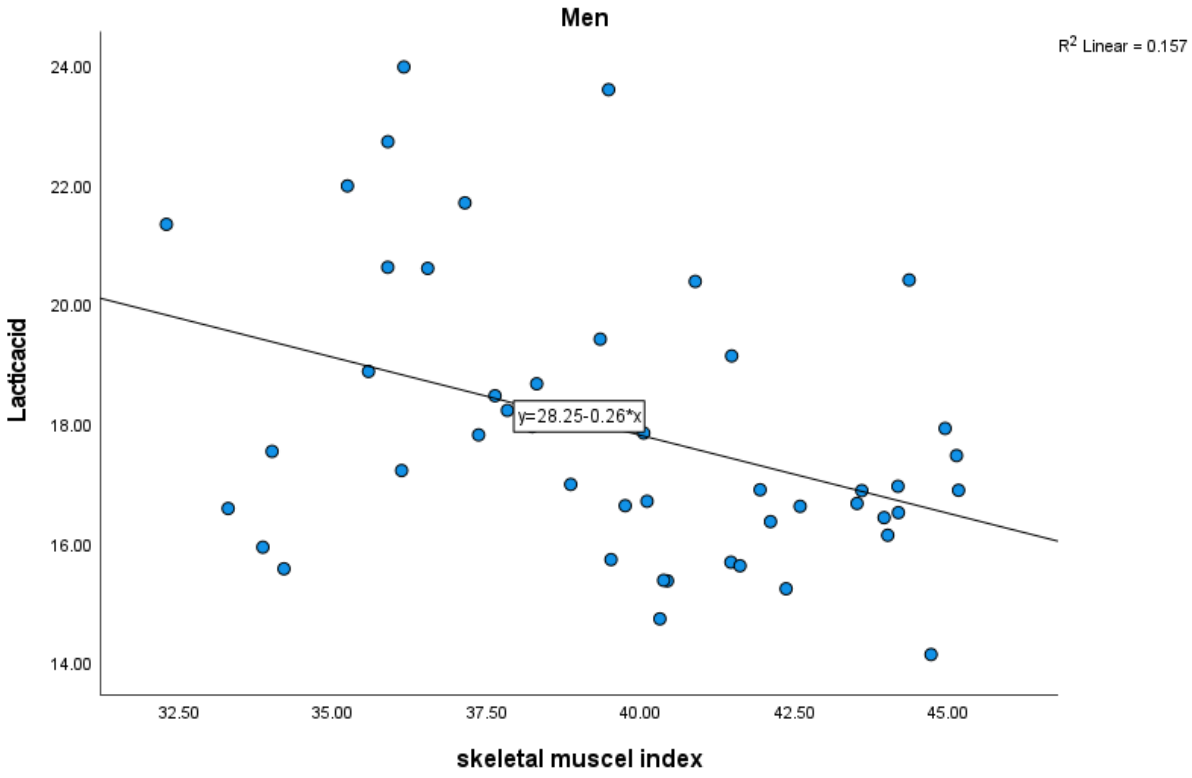

Figure 26 Scatter plot of lactic acid in relation to Skeletal Muscle Index in men