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Metabolomics for monitoring of pathological mechanisms and response to therapeutic interventions

Dissertation

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List of abbreviations

CD – Crohn's Disease

EEN – Exclusive Enteral Nutrition

HMDB – Human Metabolome Database

HPLC - High-performance liquid chromatography

IGF - Insulin-like growth factor

IGFBP - Insulin-like growth factor protein binding

LC – Liquid chromatography

MS – Mass spectrometry

PLSDA - Partial least squares-discriminant analysis

QQQ – Triple quadrupole

TOF – Time of flight

VLCFA – Very long chain fatty acid

List of publications

This dissertation is based on the following publications:

Publication I: Marques JG, Shokry E, Frivolt K, Werkstetter KJ, Brückner A, Schwerd T, et al. Metabolomic Signatures in Pediatric Crohn's Disease Patients with Mild or Quiescent Disease Treated with Partial Enteral Nutrition: A Feasibility Study. SLAS Technol. 2021;26(2):165-77.

Publication II: Marques JG, Schwerd T, Bufler P, Koletzko S, Koletzko B. Metabolic changes during exclusive enteral nutrition in pediatric Crohn's disease patients. Metabolomics. 2022;18(12):96.

Publication III: Marques J, Shokry E, Uhl O, Baber L, Hofmeister F, Jarmusch S, et al. Sarcopenia: investigation of metabolic changes and its associated mechanisms. Skeletal Muscle. 2023;13(1):2.

1. Contribution to the publications

1.1 Contribution to paper I

For the first paper, I have conducted the analytical procedure from samples preparation, LC-MS method development, data acquisition, and data processing. Data cleaning and statistical analysis were done in collaboration with Dr. Shokry, as well the writing and editing the first draft of this manuscript. Therefore, we decided to share the first authorship of it.

1.2 Contribution to paper II

On this paper I have performed all the pre-analytical procedure of sample preparation, data acquisition, data processing, and statistical analysis. I have written the first draft of the manuscript and submitted it.

1.3 Contribution to paper III

For the third paper, I have developed and validated the method for quantification of bile acids in plasma samples, prepare the samples, acquired the LC-MS data for bile acids, processed the data. Data cleaning and statistical analysis were done in collaboration with Dr. Shokry, as well the writing and editing the first draft of this manuscript. Thus, we decided to share the first authorship of it.

2. Introductory summary

Here I present the application of mass spectrometry-based metabolomics in different contexts. In the first two papers, high-resolution mass spectrometry was used to explore the metabolome of children with Crohn's disease under nutritional intervention in a non-targeted fashion. In the third paper, targeted metabolomics was used to access metabolic differences in patient suffering from sarcopenia.

2.1 Mass spectrometry-based metabolomics

The human metabolome is a complex collection of small molecules with diverse physicochemical properties and function. Up to date, the size of the small molecules pool in the human organism is unknow, and the number of molecules identified grows every year. The most comprehensive data base for metabolome is the Human Metabolome Database (HMDB) (Wishart et al., 2021), and up to now, the number of metabolite entries in the data base is 248047. To access such complexity within biological systems, many analytical techniques has been explored. Regular increase in instrument capabilities, such as sensitivity and acquisition speed rate, has been pushing the boundaries of mass spectrometry, reflecting in the increase of metabolites included in public data bases such as HMDB. Due to the complexity of the biological matrix used for metabolomics, techniques for separation are often used prior mass spectrometry analysis. Gas and liquid chromatography are the most popular and flexible techniques covering a wide range of physicochemical properties. Capillary electrophoresis is also used in metabolomics for more specialized metabolites due its performance in resolving polar and charged compounds (Zhang & Ramautar, 2021).

2.1.1 Untargeted Metabolomics

Mass spectrometry based untargeted metabolomics is a powerful analytical technique for the comprehensive analysis of the metabolic composition of biological samples. One of the main advantages of this technique is its ability to identify and quantify many metabolites simultaneously. The large amount of data generated is also complex, and demand high performance bioinformatic tools (Alonso, Marsal, & Julià, 2015).

Unlike targeted metabolomics, where only a limited number of known metabolites are analyzed, untargeted metabolomics provides a global view of the metabolic state of a sample. This allows researchers to uncover previously unknown metabolites and metabolic pathways, providing insights into biological processes and mechanisms.

Another advantage of mass spectrometry based untargeted metabolomics is its high sensitivity and specificity. Mass spectrometry can detect and quantify a wide range of metabolites, including small molecules, peptides, lipids, and even post-translational modifications, at low concentrations. This makes it possible to detect subtle changes in the metabolic profile of a sample, even in a complex matrix in the presence of other compounds. This sensitivity is particularly useful in the analysis of complex biological samples, such as tissues and fluids, where the metabolic composition can be influenced by a variety of factors, such as disease, genetic variation, or environmental factors.

Untargeted metabolomics therefore is used as a discovery-based platform that allows hypothesis generation based on global detection of metabolites and relative quantification. Identification is achieved using databases/libraries to correlate accurate mass, fragmentation pattern and retention times.

2.1.2 Targeted Metabolomics

Targeted metabolomics focuses on the quantification of specific, pre-determined metabolites in a biological sample. The goal of targeted metabolomics is to measure the concentrations of specific metabolites of interest, such as amino acids, fatty acids, or other small molecules. Unlike untargeted metabolomics, which aims to analyze the entire metabolome of a sample, targeted metabolomics is focused on a specific set of metabolites.

One of the main advantages of this technique is its high sensitivity and specificity, allowing for the detection and quantification of even low-abundance metabolites (Xiao, Zhou, & Ressom, 2012). This is particularly useful in situations where small changes in the levels of certain metabolites can have significant biological implications, such as in disease diagnosis or drug development. Mass spectrometry targeted metabolomics can also provide information on the structure and chemical identity of metabolites, which can be useful in determining their biological function.

Focusing on specific metabolites, allows for a more in-depth and precise analysis of biological processes, helping researchers to gain a better understanding of metabolic pathways and biological functions. It is also useful in detecting small changes in the levels of specific metabolites, which can have significant biological implications.

2.1.3 Metabolomics in medical research

Metabolomics is a rapidly growing field of medical research. It provides a comprehensive picture of the metabolic state of a biological system, including changes in the levels of metabolites in response to genetic and environmental factors, such as disease and drugs. By analyzing the metabolic profile of a sample, the biological process underlying diseases and response to treatment can be investigated for to new therapeutic targets, and discovery of biomarkers (Clish, 2015).

In recent years, advances in technology have allowed for high-throughput and large-scale analysis of metabolites, enabling researchers to investigate complex metabolic networks and understand the interactions between different metabolic pathways. Metabolomics has been applied in a variety of areas, including cancer research (Beger, 2013; McCartney et al., 2018), where it has been used to develop biomarkers for early detection (Barbosa et al., 2019) and prognosis (Hoang, Udupa, & Le, 2019). Additionally, metabolomics has been used to study the metabolic changes that occur in response to different diets (Laurens et al., 2020), to investigate the effects of environmental toxins (Zhao et al., 2022), and to understand the complex interplay between genetics and metabolism in diseases such as obesity and diabetes (Libert, Nowacki, & Natowicz, 2018).

3. Summary of analysis and results

In this section, the scope of the studies that comprise this dissertation will be presented.

3.1 Publication I and II

In the first two publications we investigated metabolic alterations in children with Crohn's disease during nutrition-based therapies.

3.1.1 Introduction

3.1.1.1 Crohn's disease – Disease and therapy

Crohn's disease (CD) is a chronic inflammatory bowel disease that affects the digestive system, causing a range of symptoms such as abdominal pain, diarrhea, weight loss, and malnutrition. The exact cause of Crohn's disease is unknown, but it is thought result from a combination of genetic and environmental factors (Fiocchi, 2015). There is currently no cure for Crohn's disease, but various treatment options are available to manage symptoms and improve quality of life. These include medications such as anti-inflammatory drugs and immunosuppressants, as well as surgical intervention in selected cases. The goal of therapy for Crohn's disease is to reduce inflammation and prevent complications, allowing individuals to lead a full and active life.

3.1.1.2 Exclusive Enteral Nutrition – First line treatment for paediatric CD

Exclusive enteral nutrition (EEN) is a therapeutic approach that involves providing all the patient's nutritional requirements through a liquid formula, excluding all other food. EEN has been used as a treatment for CD and is effective in inducing remission and improving symptoms in children and adolescents, making it a first-line treatment option for this population (Ashton, Gavin, & Beattie, 2019) .

The mechanism by which EEN induces remission is not fully understood, but evidence indicates that it works by reducing exposure of the inflamed gut to various stimuli, including food antigens, bacteria, and bile acids, which can worsen symptoms. EEN is also thought to modulate the gut microbiome and improve gut barrier function, thereby reducing inflammation and promoting healing (Day & Lopez, 2015).

3.1.1.3 Partial Enteral Nutrition

Partial enteral nutrition (PEN) is a therapeutic approach that involves providing some, but not all, of a patient's calories intake through a liquid formula, allowing for the consumption of solid foods. PEN is less restrictive than exclusive enteral nutrition (EEN) allowing some degree of dietary freedom, however showing lower efficacy for inducing remission (Johnson, Macdonald, Hill, Thomas, & Murphy, 2006).

3.1.2 Methods

3.1.2.1 Samples

Plasma samples are suitable to detect systemic variation in the human organism and provide a reliable reading of metabolic changes due to environmental exposure and nutrition (Sotelo-Orozco, Chen, Hertz-Picciotto, & Slupsky, 2021). We used plasma samples drawn for prospective studies evaluating the impact of PEN and EEN in children with CD.

3.1.2.2 Analytical method

To profile the metabolome, plasma samples where extract using methanol, analysed by High Performance Liquid Chromatography (HPLC) coupled with a hybrid Quadrupole Time of Flight mass spectrometry (qTOF). After data processing, 318 features were annotated by in silico matching using accurate mass and fragmentation pattern.

3.1.3 Results

3.1.3.1 Publication I

Results from the pairwise comparison after 12 months on PEN showed substantial difference between children with Crohn's disease treated with PEN and non-PEN groups in the metabolome of CD patients in remission or with mild disease activity. Inflammatory markers were associated with individual metabolites and chemical classes such as isoprenoids and phospholipids. Identified compounds comprise metabolites produced by human or bacterial metabolism, as well as xenobiotics recognized as flavoring agents and environmental contaminants and their biotransformation products.

3.1.3.2 Publication II

Plasma samples from 14 pediatric Crohn's Disease patients before and after 3–4 weeks on exclusive enteral nutrition were analyzed. Among the 13 compounds which decreased during exclusive enteral nutrition, most are related to diet, while one is a bacterial metabolite.

Food additives and other phytochemicals were the major metabolites, which decreased following the exclusion of a regular diet during exclusive enteral nutrition. An alteration in bacterial biomarkers may reflect changes in intestinal microbiota composition and metabolism

3.2 Publication III

In this study we explore targeted metabolomics to investigate metabolic changes and mechanism underling sarcopenia.

3.2.1 Introduction

Sarcopenia is defined as a progressive and generalized reduction in muscle mass and function, accompanied by a decline in muscle strength, power, and endurance, leading to physical frailty. The exact causes of sarcopenia are not well understood, but a combination of aging-related physiological changes, including changes in hormone levels, reduced physical activity, and oxidative stress, has been implicated.

3.2.2 Methods

3.2.2.1 Samples

Samples from 22 patients, (17 men) hip fracture patients undergoing surgery (8 sarcopenic, age 81.4+6.3, and 14 non-sarcopenic, age 78.4±8.1) were used in this study.

3.2.2.2 Analytical method

For this study, targeted metabolomics was used and a panel of metabolites were measured, including the following:

- Amino acids;
- Acyl carnitines;
- Free fatty acids;
- Bile acids.

Data were acquired using HPLC coupled to triple quadrupole mass spectrometers (QQQ).

In addition to the metabolomics data, IGF-I and IGFBP3 were measured using an automated chemiluminescent assay system.

3.2.3 Results

Citrulline was the only metabolite found significantly different between patients with sarcopenia and controls by using univariate analysis, after correcting for multiple testing. Multivariate modeling allowed the detection of the non-esterified fatty acid 26:2, and decanedioyl carnitine as the top three metabolites.

These findings point to a potential link between sarcopenia and mitochondrial dysfunction and portraits several possible biochemical pathways which might be involved in the disease pathogenesis.

4. General Conclusions

4.1 Crohn's disease

The first two publications investigating the metabolic response to nutrition therapies in children with Crohn's disease are descriptive and present a readout on metabolic alterations. This untargeted approach provided valuable insights on the etiopathogenesis of CD, however it shows association between treatment outcome and metabolic phenotype and not a causal relationship. The association between xenobiotics and inflammation status in both studies reinforce the relevance of the environmental factors as important players in the pathogenesis of CD, however, the mechanism of their involvement is unclear. In the second publication, the decrease in Theophylline/Theobromine and Bacteriohopane-32,33,34,35-tetrol, lead us to hypothesize the role of xenobiotics in modulating microbiota composition, by triggering and/or sustaining outgrowth of harmful bacterial species associated with epithelial invasion. On a clinical setting, exclusive enteral nutrition seems to be effective by depleting the ingestion of exogenous compounds on a comprehensive approach, however it would hardly impact the quality of life of children if sustained for long periods. Therefore, the identification of chemical compounds associated in triggering and/or onset CD is of high importance. The putative identification of Theophylline/Theobromine, a compound present in chocolate and its derivatives, should therefore be validated, and, in case of positive findings, recommended to be excluded from the diet of children with CD. Another recurrent finding, is the association of lipids and disease status and response to treatment. Although both studies found lipids associated with CD, the methods used were not suitable for a comprehensive lipidome readout, therefore, further studies are warranted to better understand the role of lipids in CD.

4.2 Sarcopenia

The use of a targeted platform on samples from sarcopenic patients allowed to formulate a mechanistic theory underlying muscle loss in elderly's patients. The association of increased VLC-FA with sarcopenia z-score may strength the hypothesis of mitochondrial disfunction causing accumulation of these species. This study may be translated for clinical practice by recommending, on top of muscle exercise, evaluating the use of natural antioxidants, such as selenium containing food, or oral supplementation to improve mitochondrial health and respiration capacity, in addition with a low carbohydrate diet aiming reducing insulin levels promoting a "free path" for lipids oxidation.

5. Paper I

The following pages present the publication: Marques JG, Shokry E, Frivolt K, Werkstetter KJ, Brückner A, Schwerd T, et al. Metabolomic Signatures in Pediatric Crohn's Disease Patients with Mild or Quiescent Disease Treated with Partial Enteral Nutrition: A Feasibility Study. SLAS Technol. 2021;26(2):165-77.

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Metabolomic Signatures in Pediatric Crohn's Disease Patients with Mild or Quiescent Disease Treated with Partial Enteral Nutrition: A Feasibility Study

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Abstract

Little is known about the metabolic response of pediatric Crohn's disease (CD) patients to partial enteral nutrition (PEN) therapy and the impact of disease activity and inflammation. We analyzed plasma samples from a nonrandomized controlled intervention study investigating the effect of partial enteral nutrition (PEN) on bone health and growth throughout one year with untargeted metabolomics using high-performance liquid chromatography (HPLC) coupled with high-resolution mass spectrometry (HRMS). Thirty-four paired samples from two time points (baseline and 12 months) were analyzed. Patients (median age: 13.9 years, range: 7–18.9 years, 44% females) were in remission or had mild disease activity. The intervention group received a casein-based formula for 12 months, providing ~25% of estimated daily energy requirements. Sparse partial least squares discriminant analysis (splsda) was applied for group discrimination and identifying sources of variation to identify the impact of PEN. We also investigated the correlation of metabolites with inflammation markers, including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and fecal calprotectin. After 12 months, our results show substantial difference between PEN and non-PEN groups in the metabolome of CD patients in remission or with mild disease activity. Inflammatory markers were associated with individual compounds and chemical classes such as isoprenoids and phospholipids. Identified compounds comprise metabolites produced by human or bacterial metabolism, as well as xenobiotics recognized as flavoring agents and environmental contaminants and their biotransformation products. Further longitudinal studies that also include patients with higher disease activity are warranted to evaluate the suitability of these metabolic biomarkers for predicting disease activity.

Keywords

enteral nutrition, inflammatory markers, pediatric inflammatory bowel disease, untargeted metabolomics, xenobiotics

Introduction

Enteral nutrition (EN) therapy is effective in pediatric Crohn's disease (CD) patients. Most published studies investigated exclusive enteral nutrition (EEN) or, more recently, specific exclusion diets.[1] Partial enteral nutrition (PEN), or nutritional supplementation with liquid formulas that provides 35–50% of habitual caloric intake with continued consumption of a normal diet, has also been applied as a dietary therapy for improving the nutritional status of children with CD.[2–4] Although PEN was not effective in inducing disease remission, it can help to maintain remission in patients who were initially treated with EEN.[3,5–8] Data on the long-term efficacy of PEN for maintenance of remission in pediatric CD patients are inconclusive, and PEN's mechanisms of action are not understood.[9] Hypotheses were generated on the impact of EN intervention on the gut microbiome,

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mucosal integrity, and the immune system, the three components of the current inflammatory bowel disease (IBD) paradigm.[10] Some studies investigated dietary effects on the intestinal microbiota by tracing changes in the levels of some bacterial metabolites, while others investigated the potential impact on gene regulation for cell growth and inflammatory pathways.[10–17] Studying the effects on the metabolome, characterizing small molecules that are intermediate or end products of biochemical reactions, may provide insights on an individual's genome and its interactions with environmental exposures that affect cellular metabolism and functions.[18,19] We have previously applied targeted metabolomics to investigate the impact of PEN therapy on the metabolic profile of pediatric CD patients in remission.[20] In the present work, we assess the feasibility of liquid chromatography–quadrupole-time-of-flight tandem mass spectrometry (LC-QTOF-MS/MS) to explore a more global picture of impacts of PEN therapy and to investigate the potential of the technique in CD biomarker research, especially in patients in remission.

While targeted metabolomics quantifies a defined set of metabolites of interest, untargeted mass spectrometry (MS)-based metabolomics enables comprehensive profiling of up to 4000 distinct molecules involved in different metabolic pathways in a few microliters of plasma.[21] The untargeted approach may be applied in the study of conditions when the affected metabolic pathways are unknown.[21,22] A few studies[23–26] applied targeted and untargeted metabolomics to identify signatures for CD; however, none has been conducted to evaluate the impact of PEN adjunctive therapy on the metabolic profiles of pediatric CD in remission or mild disease activity. We also investigated associations between metabolome and markers reflecting the degree of inflammation.

Materials and Methods

Patient Cohort and Samples

This study was performed with samples obtained in a prospective nonrandomized controlled intervention trial[20] to assess the effect of PEN on bone–muscle geometry in CD patients aged 6–19 years with quiescent or mild disease based on the mathematically weighted pediatric CD activity index (wPCDAI).[27] Patients in the intervention group received a casein-based complete liquid formula (Modulen IBD, Nestlé, Frankfurt/Main, Germany), providing for a duration of 12 months ~25% of daily energy requirements, estimated based on reference values from the German, Austrian, and Swiss nutrition societies for children, adolescents, and adults.[28] Patients who did not agree to PEN treatment were assigned to the control group and followed by the same protocol. Both groups continued their medical maintenance treatment. Disease activity, biochemical parameters, and ethylenediaminetetraacetic acid (EDTA) plasma were collected at baseline and then every 3 months for up to one-year follow-up. Forty-two patients were recruited between February 2016 and March 2017 in the Department of Pediatrics of the two university hospitals in Munich (LMU Munich and Technical University Munich). Samples from baseline (t0) and after 12 months of follow-up (t12) were selected for analysis. Paired samples were available from 34 CD patients. CRP, ESR, fecal calprotectin, and leucocyte count [white blood cells (WBCs)] were determined as part of routine clinical care.

Sample Preparation

Blood was drawn in EDTA–blood collection tubes (S Monovette, Sarstedt, Nümbrecht, Germany), and tubes were placed immediately on crushed ice. Within 30 min, plasma was separated from cells in a precooled centrifuge, aliquoted in cryotubes, and stored at −80 °C until further analysis. Preanalytical handling of samples was kept identical throughout the study period. On the day of analysis, samples were thawed on ice. For protein precipitation and sample extraction, 100 µL of plasma were transferred into a 1.5 mL Eppendorf tube, and 900 µL of ice-cold high-performance liquid chromatography (HPLC)-grade methanol was added, vortexed for 30 s, and set on freezer at −20 °C for 30 min. The tubes were centrifuged at 4000×g for 10 min, and the supernatant was filtered using a polytetrafluoroethylene (PTFE) 45 µm 96-well filter plate. The filtrate was transferred to vials and kept at −80 °C prior to analysis. To prepare a quality control (QC) sample, 100 µL of each sample extract was pooled together for analysis. Plasma QC samples were used to provide a representative sample containing all plasma samples. QC sample was injected in the beginning of the analysis and then after every fifth study sample to guarantee reproducibility.

Analytical Method: LC-QTOF-MS(/MS)

The LC-QTOF-MS(/MS) experiments were performed on a 1290 Infinity II HPLC system coupled to a 6545 Q-TOF (both from Agilent, Santa Clara, CA). Chromatographic separation was achieved using a Poroshell 120 EC-C18 (2.1× 150 mm, 2.7 µm) column (Agilent). The column oven was set to 40 °C; the autosampler was set to 10 °C. Analysis was performed with the instrument in the 2 GHz, extended dynamic range in the positive (POS) and negative (NEG) ionization modes using an Agilent Jet Stream (AJS) electrospray ionization (ESI) ion source, using the following operation parameters: capillary voltage: 3500 V (POS) / 4000 V (NEG); nozzle voltage 0 V (POS) / 500 V (NEG); nebulizer pressure: 40 psi (POS and NEG); gas temperature: 290 °C (POS and NEG); sheath gas flow: 12 L/min (POS and NEG); sheath gas temperature: 380 °C (POS and

NEG); fragmentor voltage: 170 V (POS and NEG); skimmer voltage: 65 V; and octupole radio frequency (RF): 750 V (POS and NEG). Data were acquired using MassHunter Acquisition B.09.00 software (Agilent) by injecting 6 µL of extract in a 16 min run at a flow rate of 0.4 mL/min using a gradient with the following mobile phases: A, water (0.1% formic acid); and B, methanol (0.1% formic acid). Gradient elution was performed with an initial mixture of 5% B and 95% A, then increased to 60% B throughout 4 min, to 99% B at 12 min, held until 14 min, returned to 5% B at 15.1 min, and held to 16 min.

LC-QTOF-MS Analysis

The QC samples were injected in triplicate in the full-scan MS acquiring mode from 100 to 1050 m/z, in the POS and NEG ion modes, to create an inclusion list to be used in the auto MS/MS mode. The data obtained in the MS experiment from the QC samples were extracted using the batch recursive feature extraction algorithm in MassHunter Profinder B.08.00 software (Agilent), then the features were evaluated individually among the replicates to ensure reproducibility and exported as CEF (Cluster Exchange Format) files. Mass Profiler software (Agilent) was used for alignment of features using retention time (RT) tolerance of up to 0.3 min and mass tolerance of ±15 ppm. Features with 100% occurrence in the replicates were used to create a target MS/MS inclusion list.

LC-QTOF-MS/MS Analysis

Data were acquired using data-dependent acquisition (DDA) (auto MS/MS mode) using the features present in the target MS/MS inclusion list as preferred for fragmentation, using a delta m/z of 15 ppm and delta RT of 0.15 min. A collision cell operates with fixed collision energies of 10, 20, and 40 eV, using nitrogen as collision gas. The acquisition mass ranges used were 100 to 1050 m/z at 4 spectra/s in the MS and 50–800 m/z at 3 spectra/s in the time of flight (TOF) for the fragments generated in the collision cell (MS/MS). The precursor threshold was set as 1000 counts (absolute) and 0.01% (relative) with active exclusion of two spectra for 0.2 min and five maximum precursors per cycle.

Data Processing

Samples acquired on auto MS/MS acquisition mode were extracted using the batch recursive feature extraction algorithm in Profinder B.08.00. The features were evaluated individually, and data were exported as CEF files. Mass Profiler Professional (Agilent) was used for alignment, normalization, and quality control. Raw data were normalized using a quantile algorithm, and features with less than 100% occurrence between the QCs and with a coefficient of variance higher than 25% were excluded. Principal component analysis was used to perform a QC on samples to exclude any outlier by visual inspection. Identification of features was performed by library search using Mass Hunter METLIN Personal Compound Database and Library (PCDL) (Agilent) at the MS/MS level. The features that did not match with the library compounds had their formulas generated by a molecular formula generator (MFG) algorithm. Analysis of the QC samples in the full-scan MS acquisition mode resulted in the detection of 2599 features in the negative-ion mode and 2074 features in the positive-ion mode. A preferred precursor mass list was built from these features that was used in the auto MS/MS acquisition mode for the study samples. After QC, a total of 322/140 features was putatively identified using MS/MS data, and 52 and 85 features had the formula generated in the negative and positive ion modes, respectively. The data were then exported in CSV (comma-separated values) format for statistical analysis.

Statistical Analysis

Statistical analysis was performed independently on the generated datasets comprising 374 and 225 annotated compound peak lists in NEG and POS modes, respectively. Each dataset was composed of paired sample data for control (Non-PEN) and intervention (PEN) groups at two time points (baseline, and after 12 months of treatment with or without PEN). As a first step, sparse partial least squares discriminant analysis (splsda) analyses were performed using the absolute concentrations at the two time points to visualize the group separation. Then, to eliminate observed baseline differences, relative concentrations were used to build the model, which were calculated by dividing the metabolites' concentration data for the second time point (12 months) by the respective baseline concentrations. For model optimization, a tuning process using M-fold cross validation (CV) was carried out for the selection of parameters giving the best model performance [number of principal components (PCs) and key metabolites to keep in each PC (keep X)]. Accordingly, three PCs were selected for the POS and NEG ion mode datasets with a keep X of (30, 9, 8) and (24, 34, 34) key metabolites for the two PCs, respectively.

Another aspect of the analysis was to investigate which metabolites correlated most with markers used in assessing clinical and biochemical disease activity, comprising CRP, ESR, WBCs, and fecal calprotectin. For this purpose, an integrative analysis [sparse partial least squares (spls)] model was applied on the within-group matrices of the matched datasets. Respectively, multilevel spls models were built for both positive and negative modes, and tuning was performed for selection of the optimum features giving the best model performance in terms of regression coefficient (R^2), mean squared error of prediction (MSEP), root

Table 1. Baseline Patient Characteristics: Subcohort of a Nonrandomized Intervention Trial on PEN in Pediatric CD Patients.[20]

Patient Characteristics		Non-PEN (n= 18)	PEN (n= 16)	p Value
Gender (male/female)		11/7	8/8	n/a
Age at diagnosis (years)		8.7 ± 3.6	10.8 ± 2.6	0.06
Age at study inclusion (years)		12.9 ± 3.2	14.6 ± 1.9	0.07
Time (years) of IBD until study inclusion		4.2 ± 2.8	3.7 ± 2.5	0.65
Positive family history		5/18	6/16	n/a
Extra-intestinal involvement		4/18	5/16	n/a
Disease location	L1 Terminal ileum	2/18	1/16	n/a
	L2 Colon	7/18	4/16	n/a
	L3 Ileocolonic	9/18	11/16	n/a
	+L4 (Upper GI tract)	16/18	8/16	n/a
Disease behavior	B1 Nonstricturing, nonpenetrating	17/18	14/16	n/a
	B2 Stricturing	1/18	2/16	n/a
	B3 Penetrating	0/18	0/16	n/a
	Perianal involvement	7/18	3/16	n/a
Therapy at baseline	Azathioprine	10/18	7/16	n/a
	5-Aminosalicylates	3/18	4/16	n/a
	Infliximab	12/18	11/16	n/a
	Methotrexate	2/18	2/16	n/a
	Adalimumab	1/18	0/16	n/a
Disease activity	Remission (wPCDAI < 12.5)	16/18	14/16	n/a
	Mild disease (wPCDAI ≥ 12.5 ≤ 40)	2/18	2/16	n/a

CD: Crohn's disease; GI: gastrointestinal; IBD: inflammatory bowel disease; PEN: partial enteral nutrition; wPCDAI: weighted pediatric CD activity index.
IBD phenotype was determined according to disease activity according to wPCDAI.[27]

mean squared error of prediction (RMSEP), and prediction residual error sum of squares (PRESS). Then, correlations between the metabolome and the inflammation markers were visualized using a clustered image map (CIM) and correlation circles plots. The entire data analysis was performed in R using the mixOmics package (http://cran.r-project.org).[29]

Ethics

Ethical Committees of LMU Munich (no. 690-15) and Technical University Munich (no. 316/16 S) reviewed the study protocol. Written informed consents of adult patients and of minor patients' parents or caregivers, and age-appropriate assent of patients, have been obtained. The study was registered at the German Clinical Trials Registry (no. DRKS00010278).

Results

Impact of Partial Enteral Nutrition

We analyzed 34 paired samples obtained at baseline and after 12 months from the control (Non-PEN, n= 18) and intervention (PEN, n= 16) groups, respectively. Patient details are shown in **Table 1**. Results of the splsda analyses using the absolute concentrations at the two time points showed significant baseline differences between the non-randomized control (Non-PEN) and intervention (PEN) groups, which were evident in group separation, especially using the POS data (**Suppl. Fig. S1**). Differences at baseline make it difficult to determine whether the separation in the subsequent time point was due to the treatment or just an extension of the encountered baseline differences (e.g., age difference before initiation of treatment). Similar to our previous approach using targeted metabolomics,[28] we used relative concentrations to build the splsda models, as previously described in the Materials and Methods section. By applying relative concentration data, almost complete separation was obtained between the treatment groups at t12, which was evident in the score plot (**Fig. 1**). Using the POS-mode data, all samples of the two groups (Non-PEN and PEN) were completely separated on the first PC (PC1) except two samples, one of which was lying exactly on PC1 (**Fig. 1A**). In contrast, a less efficient separation was obtained using the NEG-mode data, in which six out of 34 samples were not separated on PC1 and were located with the other treatment group on the same side of PC1 (**Fig. 1B**). Thus, the models were able to accurately separate 94% and 82% of samples using the POS and NEG modes,

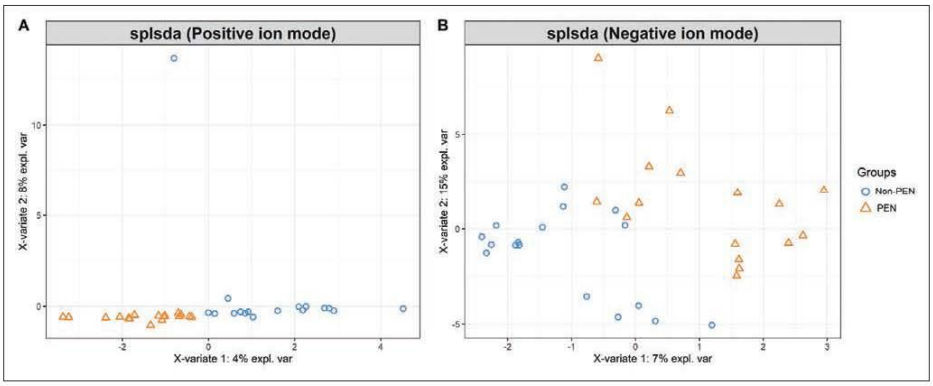

Figure 1. Sparse partial least squares discriminant analysis (splsda) score plot of the relative concentration data of the plasma metabolome of patient samples for the control [Non-PEN (partial enteral nutrition)] and intervention (PEN) groups in the (A) positive-ion (POS) and (B) negative-ion (NEG) modes at 12 months post intervention (t12).

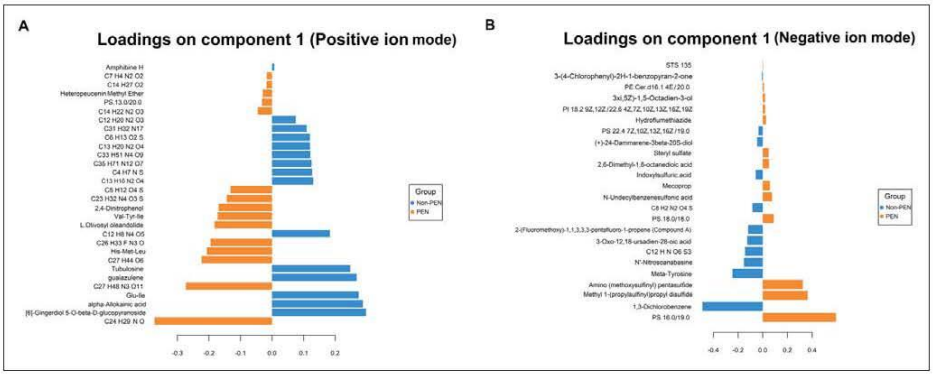

Figure 2. The loading plot represents the key features selected for the first principal component (PC1) of the sparse partial least squares discriminant analysis (splsda) models in the (A) positive-ion (POS) and (B) negative-ion (NEG) modes. Colors indicate the group in which the mean concentrations of the metabolite are maximal.

respectively. The selected features (key metabolites) for PC1 using both POS- and NEG-mode data are shown in **Figure 2**. In the POS mode, more than half of the key metabolites driving the group separation were unidentifed, including the metabolite showing the highest variable importance (**Fig. 2A**). Among the key features showing the highest importance, however, are [6]-gingerdiol 5-O-beta-D-glucopyranoside, alpha-allokainic acid, guaiazulene, tubulosine, L-olivosyl oleandolide, 2,4-dinitrophenol, and three peptides (Glu-Ile, His-Met-Leu, and Val-Tyr-Ile). The control (Non-PEN) group showed higher levels of the first five compounds and the peptide Glu-Ile, while the intervention group (PEN) showed higher levels of L-olivosyl oleandolide, 2,4-dinitrophenol, and the other two peptides (His-Met-Leu and Val-Tyr-Ile). In the NEG data, the five major metabolites driving the group separation were PS 16.0/19.0, 1,3-dichlorobenzene, methyl 1-(propylsulfinyl) propyl disulfide, amino (methoxysulfinyl) pentasulfide, and meta-tyrosine (**Fig. 2B**). The intervention group showed higher levels of principally PS 16.0/19.0 in addition to

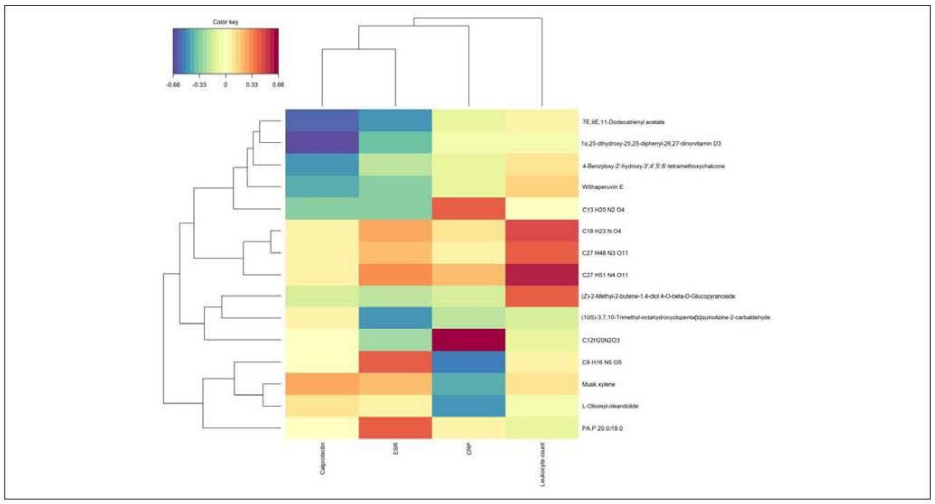

Figure 3. Clustered image map (CIM) obtained by a sparse partial least squares (spls) model using metabolomics data in positive-ion mode. The plot shows pairwise correlations between the metabolite species and biochemical markers, applying a threshold value of 0.4. The red and blue colors indicate positive and negative correlations, respectively, whereas yellow indicates small correlation values. The metabolites and the biochemical markers are clustered on the left and the top sides of the CIM, respectively.

methyl 1-(propylsulfinyl)propyl disulfide and amino (methoxysulfinyl) pentasulfide. Other phospholipids (PLs) were found among the discriminating components between the control and intervention groups, such as PS(18:0/18:0), PI[18:2(9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)], PE-Cer[d16:1(4E)/20:0], and PS[22:4(7Z,10Z,13Z,16Z)/19:0], which showed higher levels in the intervention group (PEN) relative to the control group (Non-PEN) in all compounds except for PS[22:4(7Z,10Z,13Z,16Z)/19:0]. The control group showed higher levels of 1,3-dichlorobenzene.

Associations of Inflammatory Biomarkers and the Metabolome Scanned in Positive-and Negative-Ion Modes

Based on the clinical disease activity score of wPCDAI,[27] 30 patients were in remission, and four patients had mild disease (**Table 1**). Accordingly, elevated levels of systemic inflammation markers, such as CRP (≥1 mg/dL),[30] ESR (>15 mm/h for boys and >20 mm/h for girls),[31] and WBC count ($>11\times10^9$/L),[32] were found in only single patients, but more patients showed increased levels of fecal calprotectin (>50 mg/L),[33] a sensitive biomarker of intestinal inflammation (**Suppl. Fig. S4**).

Positive-Ion Mode. Using the POS-mode data, a spls model was built after thoroughly selecting the number of PCs and features, which yielded R^2 values of 0.58, 0.52, 0.40, and 0.54 for CRP, ESR, WBC count, and calprotectin, respectively. Considering the obtained R^2 values, the models were considered of moderate to high predictive accuracy for all inflammation markers.[34] Selection of the optimal model dimensions and features comprising each dimension was tuned to obtain the best model performance assessed in terms of highest R^2 and lowest MSEP and RMSEP.

The CIM plot shows three major clusters, the first of which comprises the WBCs, while the second comprises CRP, and the third comprises ESR and calprotectin (**Suppl. Fig. S2**). To facilitate the interpretation, we investigated the obtained correlations exceeding an arbitrary threshold of 0.4, as shown in **Figure 3**.

We observed a cluster of metabolites all negatively associated with both ESR and fecal calprotectin; however, a higher correlation was obtained with the latter. This cluster comprised five metabolites, one of them not recognized by matching with the PCDL library search (C13H20N2O4); the remaining four metabolites were denoted as 7E,9E 11-dodecatrienyl acetate, 1α,25-dihydroxy-25,25-diphenyl-26,27-dinorvitaminD3, 4-benzyl-oxy-2′-hydroxy-3′,4′,5′,6′-tetramethoxychalcone, and withaperuvin E. These four metabolites were also negatively associated with CRP, albeit to a lesser extent. Generally, a global view of the CIM plot shows that only ESR and calprotectin majorly shared the same directions of associations with the key metabolites

selected by the model, except for two metabolites: (10S)-3,7,10-trimethyl-1,3a,4,8,9,9a,10,10a-octahydrocyclopenta[b] quinolizine-2-carbaldehyde and an unidentified compound (C12H20N2O3).

CRP is associated negatively with a metabolite cluster comprising L-olivosyl oleandolide, musk xylene, and an unknown metabolite (C9H16N5O5), which were, however, positively associated with fecal calprotectin and ESR. Another metabolite cluster of three unannotated compounds (C18H23NO4, C27H48N3O11, and C27H51N4O11) positively associated with all the investigated inflammatory markers, particularly the WBC count, which showed the closest positive association. Apart from the clusters discussed, we detected high positive correlations of an unidentified compound (C12H20N2O3) with CRP. The lowest inverse correlation was found between calprotectin and 1α,25-dihydroxy-25,25-diphenyl-26,27-dinorvitamin D3.

Negative-Ion Mode. Using the NEG-mode data, the investigated inflammatory markers showed the same clustering behavior as that seen using the POS-mode data, in which ESR and calprotectin formed one cluster, which together with CRP gave rise to another cluster, while the WBC count clustered separately (**Suppl. Fig. S3**). To focus on only the higher meaningful correlations, we applied a correlation threshold value of 0.4, and then we replotted the CIM (**Fig. 4**).

A cluster of lipid metabolites comprising long-chain fatty acids with four PLs (PS 21:0/18:0, PS 22:2/19:1, PS 18:4/20:0, and PE 22:6/21:0) and one 2-hydroxy ceramide (Cer t18:0/24:0, 2-OH) showed a close inverse correlation with ESR and CRP, and to a lesser extent with calprotectin, but almost no correlation for WBC count except for PE 22:6/21:0. ESR, CRP, and calprotectin also showed similar patterns of correlation with 1-chloro-2,2-bis(4′-chlorophenyl)ethylene and an unannotated compound (C16H4N2O9S2). The greatest discrepancies between CRP and ESR were inverse correlations of CRP to saccharin and 4-dodecyl benzene sulfonic acid, while these correlated positively to ESR. Similar to ESR, calprotectin correlated positively with 4-dodecyl benzene sulfonic acid, albeit with a much closer correlation. Calprotectin showed a close inverse association with a cluster of three metabolites—1-chloro-2,2-bis(4′-chlorophenyl)ethylene, 3-methylbutyl-3-oxobutanoate, and 1-(9H-pyrido[3,4-b]indol-1-yl)-1,4-butanediol—which were also negatively correlated with CRP but to a lesser extent. Apart from the previously mentioned clusters, we detected close positive and negative correlations of WBC count with 1,3-dichloropropanol (1,3-DCP) and acetyl-N-formyl-5-methoxykynurenamine (AFMK), respectively. The number of PCs and features used in building the spls model between the metabolome in the NEG mode and the inflammation markers was optimized using the previously mentioned model performance parameters. The model could predict all the inflammatory markers with high accuracy, with R^2 values of 0.56, 0.73, 0.45, and 0.76 for CRP, ESR, WBC count, and calprotectin, respectively.

Discussion

Impact of PEN Treatment

Our results show a remarkable effect of 12 months of PEN treatment, compared to a normal self-selected diet, on the metabolome of pediatric CD patients in remission or with mild disease activity. This was evident in the substantial separation between the PEN and non-PEN groups, especially using the POS-mode relative concentration data. These results agree with our previous findings in the same patients using targeted metabolomics,[20] in which we detected significant differences during the one year of nutritional intervention, especially after 3 months of PEN treatment. In the present work, however, only samples from baseline and t12 were analyzed.

In the POS mode, more than half of the compounds responsible for the separation were unidentified, including the most important one (**Fig. 2A**). The remaining metabolites were mostly single species rather than metabolite groups, which could be food constituents present as additives, spices, or contaminants such as [6]-gingerdiol 5-O-beta-D-glucopyranoside,[35] guaiazulene,[36] tubulosine,[37] 1,4-dinitrophenol,[38] or bacterial metabolites such as L-olivosyl oleandolide.[39] There were, however, a number of peptides comprising essential amino acids (Glu-Ile, His-Met-Leu, and Val-Tyr-Ile), whose difference between the groups (Non-PEN and PEN) could be relevant to the consumption of the formula. Modulen IBD is a whole-protein casein-based formula; therefore, an impact on patients' protein and peptide levels may be expected in patients receiving the formula.

In the NEG-mode data, we observed that the metabolite most driving the group separation was PS 16.0/19.0, a phosphatidyl serine comprising palmitic acid. This finding supports our previous findings using the targeted metabolomics, in which the wide majority of the metabolites driving the group separation in targeted analysis were PCs, mostly comprising palmitic acid, the major fatty acid (FA) representing >30% of the total FA in the formula provided to the PEN patients.[40] As shown in the loadings plot (**Fig. 2B**), more PL species showed higher levels in the intervention group, which indicates a possible increase in PL synthesis with the formula consumption. Similar to the POS data, some of the key metabolites for the group discrimination were single species like 1,3-dichlorobenzene, which is the second most important loading. 1,3-Dichlorobenzene is used in herbicides, insecticides, medicine, and dyes, and therefore it can be a potential environmental contaminant from different sources such as food, water, or air pollution.[41] Two compounds,

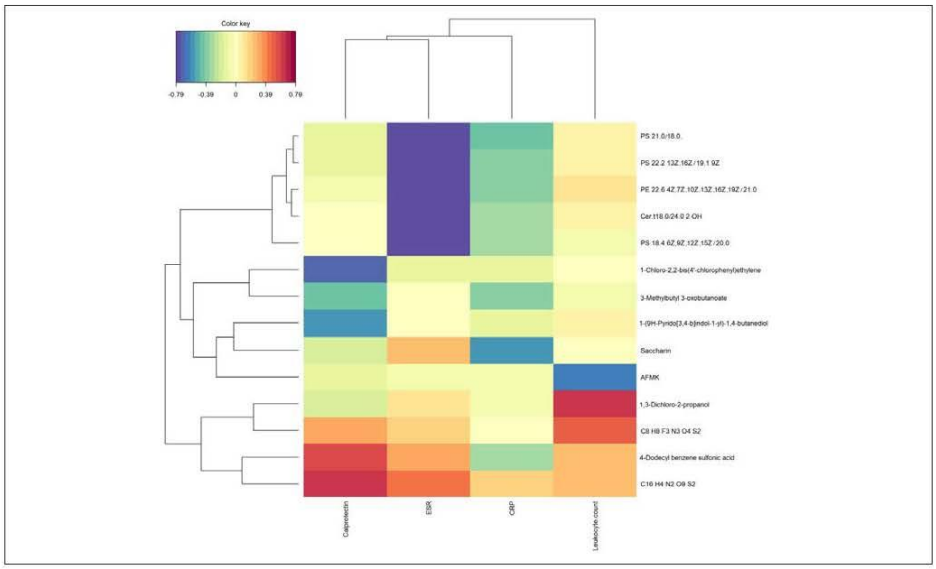

Figure 4. Clustered image map (CIM) obtained using a sparse partial least squares (spls) model using metabolomics data in negative-ion mode, showing pairwise correlations between the metabolite species and biochemical markers applying a threshold value of 0.4. The red and blue colors indicate positive and negative correlations, respectively, whereas yellow indicates small correlation values. The metabolites and the biochemical markers are clustered on the left and the top sides of the CIM, respectively.

methyl 1-(propylsulfinyl)propyl disulfide and amino (methoxysulfinyl) pentasulfide, are also recognized as food constituents and used as potential biomarkers for the consumption of specific foods.[42,43] In contrast, m-Tyr is a product of nonenzymatic free radical oxidative modification of phenylalanine (Phe) residues. It is a rare metabolite present in low concentrations under physiologic circumstances, and its elevated levels were linked to protein damage.[44] m-Tyr was identified among the most discriminating metabolites between the Non-PEN and PEN groups post intervention, with higher levels in the control group. This could imply the amelioration of the protein damage in subjects receiving the formula, relative to subjects in the control group. Clinically, PEN therapy was found to improve growth in a PEN subgroup of pre- and early pubertal patients with growth potential, as previously reported by our group.[20] In this sense, the present work using the untargeted approach confirmed the results obtained by the targeted approach and was able to detect more metabolic differences associated with the consumption of the formula. These findings support the combined use of targeted and untargeted metabolomics as complementary strategies to maximize information on patient samples.

Associations of Inflammatory Biomarkers and the Metabolome

The patients included in the cohort were clinically stable, either in remission or with low wPCDAI scores (**Suppl. Fig. S4**). Accordingly, levels of inflammatory markers were generally low and in the normal range for the majority of the subjects. Nonetheless, we analyzed the association of clinical markers with metabolites as a hypothesis-free explorative study on potential metabolic alterations at a relatively low inflammatory state.

Positive-Ion Mode

Our results show associations between inflammatory markers and clusters of metabolites, as well as single metabolite species related to inflammation. One major metabolite cluster was composed of chemically and structurally related compounds comprising one or more isoprenoid units, which showed similar inverse correlation patterns with ESR and fecal calprotectin. The members of this cluster were dodecatrienyl acetate (sesquiterpenoid), 25-dihydroxy-25,25-diphenyl-26,27-dinorvitamin D3 (triterpenoid), withaperuvin

E (sesquiterpene lactone), and 4-benzyl-oxy-2′-hydroxy-3′,4′,5′,6′-tetramethoxychalcone (flavone derivative). Isoprenoids have been previously related to treatment and prevention of autoimmune diseases by acting as reduced nicotinamide adenine dinucleotide phosphate (NADPH) activators, thus providing normal or increased levels of NADPH oxidase, while decreased NADPH oxidase activity has been related to disease progression.[45] In other reports, diterpenoids and triterpenoids were reported at significantly lower levels in CD patients than in healthy controls.[46] Among this cluster, 25-dihydroxy-25,25-diphenyl-26,27-dinorvitamin D3, a derivative of vitamin D3, showed the highest inverse correlation with fecal calprotectin. Vitamin D deficiency was repeatedly reported in patients with IBD; however, it is controversial whether it is merely a consequence of the disease or contributes to the inflammatory condition.[47] Decreased vitamin D3 levels may be attributed to less outdoor activity and sunshine exposure due to lower general well-being and possibly also to reduced lipid absorption, both associated with chronic intestinal disease. Withaperuvin E is a plant-derived steroid[48] that belongs to a family of compounds with potential anti-inflammatory activity mediated by inhibition of tumor necrosis factor alpha (TNF-α)-induced nuclear factor kappa B (NF-κB) activity.[49] 4-Benzyl-oxy-2′-hydroxy-3′,4′,5′,6′-tetrametho xychalcone is a flavone derivative with activity against ulcers, gastritis, and IBD.[50]

Another cluster comprising L-olivosyl oleandolide (a bacterial metabolite),[39] musk xylene, and an unknown metabolite (C9H16N5O5) was inversely correlated with CRP, and alternatively positively correlated with fecal calprotectin and ESR. There were no reports linking either of these compounds to CD; however, concerns were raised regarding the toxic and carcinogenic effects of musk xylene, a synthetic fragrance used as a fixative in cosmetics and perfumes and a potential water contaminant.[51] In the POS-mode data, some of the strong correlations could not be interpreted due to the unknown identities of the compounds, including the C12H20N2O3 correlated with CRP and the cluster of metabolites closely correlated with the WBC count, of which three members were unannotated.

Negative-Ion Mode

Inflammatory markers were associated with a lipid cluster comprising phospholipids and ceramides, with xenobiotics comprising sweeteners, flavors, food additives, and contaminants. This reflects that inflammation in CD may be triggered or modulated by exogenous and endogenous factors.

Similar to the results obtained using data in the POS mode, a group of structurally related metabolites comprising phospholipids containing long-chain fatty acids (LCFAs) and ceramides clustered together, showing high negative correlations with ESR and CRP, the two most recognized inflammatory markers in IBD. Calprotectin showed inverse or no correlation with the same cluster. Other studies have linked CD to disturbed lipid metabolism, especially regarding sphingolipids and phospholipids.[52,53] These compounds play crucial roles in maintaining barrier function as well as modulating inflammation and immunity.[54] Moreover, ceramides decrease the release of TNF and induce autophagy, a process strongly implicated in the pathogenesis of CD, in addition to their impact on cell signaling.[55–57] Elevated levels of phospholipase A2 (PLA2) were reported in serum and colonic mucosa of IBD patients.[58] PLA2 are enzymes hydrolyzing phospholipids into lysophospholipids, which were proposed to be involved in the inflammatory process and pathogenesis of IBD.[59,60] ESR, CRP, and calprotectin correlated negatively with 1-chloro-2,2-bis(4′-chlorophenyl)ethylene; however, we found no previous reports linking 1-chloro-2,2-bis chlorophenyl ethylene to IBD to provide an explanation for this finding. The artificial sweetener saccharin was positively associated with ESR. Artificial sweeteners, like saccharin and sucralose, were previously linked to increased IBD risk, with a proposed inhibition of gut bacteria and digestive proteases, and thus enhanced digestion of the mucus layer and gut barrier considered the "bacteria-protease-mucus-barrier hypothesis."[61,62] It is difficult, however, to understand why saccharin correlates in different directions with CRP and ESR, respectively. Inconsistency in the inflammatory markers' response to inflammation and their lack of accuracy have been reported frequently.[63,64] Similar to saccharin, 4-dodecyl benzene sulfonic acid was positively associated with ESR but negatively associated with CRP. It was also positively correlated with calprotectin and the WBC count. 4-Dodecyl benzene sulfonic acid is a xenobiotic belonging to the family of benzenesulfonic acids and derivatives, with increased levels previously linked to IBD.[46,65] 4-Dodecylbenzenesulfonic acid is a major compound in laundry detergents,[66] and its sodium salt is applied in antimicrobial formulations for treating organic vegetables and fruits.[67]

A cluster of three metabolites—1-chloro-2,2-bis(4′-chlorophenyl)ethylene, 3-methylbutyl 3-oxobutanoate, and 1-(9H-pyrido[3,4-b]indol-1-yl)-1,4-butanediol—showed a close inverse association with calprotectin, principally the first of these three. Although information on the source of 1-chloro-2,2-bis(4′-chlorophenyl)ethylene is lacking in the published literature, the most usual way for exposure to the other two species is dietary intake. 3-Methylbutyl 3-oxobutanoate is a sweet ethereal flavoring agent,[68] while 1-(9H-pyrido[3,4-b]indol-1-yl)-1,4-butanediol is a natural β-carboline indole alkaloid derived from plant origins.[69] β-Carboline alkaloids may occur in many plant-derived foods, thermally processed protein-rich foods, and beverages,[70] and were demonstrated to exhibit anti-inflammatory activity via potent inhibition of nitric oxide (NO), TNFα, and interleukin-6 (IL6).[71,72]

1,3-DCP, an unannotated compound (C8H8F3N3O4S2), and AFMK were the three main compounds showing high correlations with the WBC count. The first two compounds were positively correlated with WBCs, while AFMK was inversely correlated with WBCs. 1,3-DCP is an organochlorine compound that may occur as a food and water contaminant.[73,74] 1,3-DCP can be metabolized either by cytochrome P450 2E1 (CYP2E1) into cytotoxic compounds, or by bacterial enzymes into epichlorohydrin, 3-MCPD, glycidol, and glycerol, which exert their toxicity by cellular glutathione depletion through conjugation with glutathione and may also induce a loss of mitochondrial function.[75] AFMK acts as an antioxidant and was reported to have anti-inflammatory effects mediated by the decrease in TNFα and interleukin-8 (IL8) production by monocytes.[76] As an oxidation product of melatonin, it could be endogenously produced or be derived from exogenous melatonin found in some plant- and animal-derived foods. Melatonin was reported to have beneficial effects in inflammatory and enteroimmune diseases.[77,78]

Strengths and Limitations

The strengths of our study are the use of a powerful analytical platform and a longitudinal observation throughout a period of one year following a standardized study design. The limitations are the limited sample size and a nonrandomized assignment of patients to the study arms, which was chosen since a randomized approach was not feasible in the children and adolescents studied.[20] The inclusion of only patients in remission with a relatively low level of inflammation may have decreased the likelihood of identifying metabolic responses that are altered by higher disease activity or that may serve as a trigger for inflammation. Around 25% of the markers detected in this study remain uncharacterized, of which some were closely correlated with inflammatory markers or key metabolites in studying the impact of PEN therapy; thus, the findings could not be fully interpreted.

Conclusions

It appears worthwhile to explore the combination of targeted and untargeted approaches in monitoring treatment effects in CD. The diversity of the metabolites detected by the untargeted technique, comprising products of human or bacterial metabolism, xenobiotics recognized as flavoring agents, environmental contaminants, and their biotransformation products, found associated with inflammatory markers in pediatric CD patients points to further opportunities for applying metabolomics in biomarker research and in understanding mechanistic pathways involved in the disease. Further studies are warranted in patients with a broader range of disease activity and abnormal inflammatory markers to explore whether the present findings can be replicated, identify the unknown compounds, and address their possible contribution to the etiopathogenesis of CD.

Acknowledgments

Dr. Klara Frivolt, a young pediatrician in training at the University of Bratislava, died on August 5, 2017, in Hungary while pregnant with her second child. Klara had spent two research periods of 18 months each at the Division of Gastroenterology and Hepatology at the Dr. von Hauner Children's Hospital, LMU Munich, Germany. She had received several research awards, including the Nestlé Nutrition Award from the European Crohn and Colitis Organization (ECCO) in 2014, which she used to conduct this study. In a very enthusiastic way, she supervised this study as a pediatric scientist. On July 4, 2017, she successfully defended her PhD thesis. Klara Frivolt was an extremely talented young physician, scientist, and mother, and a wonderful person. We are in deep grief.

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Declaration of Conflicting Interests

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6. Paper II

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ORIGINAL ARTICLE

Metabolic changes during exclusive enteral nutrition in pediatric Crohn's disease patients

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Abstract

Background and aims Exclusive enteral nutrition is recommended as a first-line treatment in active pediatric Crohn's Disease, but its mechanism of action is still not clear. We aimed to assess alterations in the metabolic profile of newly diagnosed pediatric Crohn's Disease patients before and during exclusive enteral nutrition therapy.

Methods Plasma samples from 14 pediatric Crohn's Disease patients before and after 3–4 weeks on exclusive enteral nutrition were analyzed using mass spectrometry. T-test, fold change and orthogonal partial least squares discriminant analysis were used for mining significant features. Correlation analysis was performed between the annotated features and the weighted pediatric Crohn's disease activity index using Pearson r distance.

Results Among the 13 compounds which decreased during exclusive enteral nutrition, most are related to diet, while one is a bacterial metabolite, Bacteriohopane-32,33,34,35-tetrol. The phosphatidic acid metabolite PA(15:1/18:0) was significantly reduced and correlated with the weighted pediatric Crohn's disease activity index. Lipids increased during exclusive enteral nutrition therapy included phosphatidylethanolamines; PE(24:1/24:1), PE(17:2/20:2) and one lactosylceramide; LacCer(d18:1/14:0).

Conclusion Food additives and other phytochemicals were the major metabolites, which decreased following the exclusion of a regular diet during exclusive enteral nutrition. An alteration in bacterial biomarkers may reflect changes in intestinal microbiota composition and metabolism. Thus, metabolomics provides an opportunity to characterize the molecular mechanisms of dietary factors triggering Crohn's Disease activity, and the mechanisms of action of exclusive enteral nutrition, thereby providing the basis for the development and evaluation of improved intervention strategies for prevention and treatment.

Keywords Metabolomics · Biomarkers · Crohn's disease · Exclusive Enteral Nutrition

1 Introduction

Genetic and environmental factors, including the gut microbiota, play an important role in the pathophysiology of Crohn's disease (CD) [1]. CD has been attributed to complex mechanisms triggering an dysregulated immune

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response to commensal gut microbiota [2]. Exclusive enteral nutrition (EEN) is an established therapy to induce remission in pediatric CD patients with efficacy in up to 80% of individuals [3], and it is recommended as first-line therapy in European guidelines [4–6]. A meta-analysis based on 18 studies concluded that there is no significant difference in efficacy using EEN or corticosteroids treatment to induce remission in pediatric CD, however, EEN seems to be superior in promoting mucosal healing and faster reduction of PCDAI (pediatric Chrohn's disease activity index) [7]. The benefits of using EEN in pediatric patients extend beyond promoting mucosal healing, contributing to the improvement of nutritional status [8], bone metabolism, and muscle mass [9]. However, the mechanism of EEN action is still unclear. Several studies have focused on investigating how EEN may affect the microbiome, and most report an overall decrease in microbiome diversity during EEN therapy [10–17]. However, the use of different techniques to assess taxonomic shifts and the high diversity of microorganisms present in the human microbiota and its interindividual variation generates heterogenous results, especially at taxonomic resolution lower than phylum-level [18]. In addition to changes in microbiota composition, CD-associated dysbiosis affects microbial metabolic functions. Previous research demonstrated alterations in microbial functions with a shift in genetic abundance related to oxidative stress pathways, carbohydrate metabolism and amino acid biosynthesis, which was considered more disturbed than microbiota composition shifts [19]. In another observational study, the authors reported a reduction in metabolic activity of the intestinal microbiome during enteral feeding for two weeks, based on analyzing exhaled breath and fecal samples using gas chromatography/mass spectrometry [20]. Recently, Diederen et al. reported a reduction in microbiome diversity and changes in the fecal metabolome during EEN in pediatric CD patients, with alterations in amino acids, cadaverine, trimethylamine, and bile acids levels [21].

Over the past years, untargeted metabolomics using mass spectrometry has been applied as powerful tool for identification and tracking of biomarkers which help in understanding the system biology and treatment outcomes [22]. Metabolomics have provided new insights into metabolic alterations in CD patients versus healthy subjects using both serum and fecal samples [23,24]. However, only few studies were performed on pediatric population [25] and could not detect the differences induced by EEN therapy, which is one of the points of strength of our study.

2 Methods

2.1 Plasma samples

Plasma samples from 14 pediatric CD patients (age (mean ± SD) 13.5 ± 2.2 years, 8 boys, 12 newly diagnosed), before and after 25 ± 5 days on EEN treatment were analyzed. Thirteen patients received Modulen® IBD (Nestlé Nutrition) and one patient was treated with Neocate Junior® (Nutricia). Samples were obtained from a previously published study [15].

The sample preparation procedure was previously described 26. Briefly, after samples were thawed on ice, 100 µL of plasma were precipitated with the addition of 900 µL of ice-cold high-performance liquid chromatography (HPLC)-grade methanol in a 1.5 mL Eppendorf tube, vortexed and rested at -20 °C for 30 min. The tubes were then centrifuged, and the supernatant was filtered in a polytetrafluoroethylene (PTFE) 45 µm 96-well filter plate. Samples were kept at − 80 °C before analysis. Quality control (QC) samples were created by pooling aliquots from the study samples and used to create an inclusion list in the method development and to ensure reproducibility in the analysis.

2.2 LC-QTOF-MS(/MS)

The analysis was conducted on a 1290 Infinity II HPLC system using a Poroshell 120 EC-C18 column (2.1 × 150 mm, 2.7 µm) coupled to a 6545 Q-TOF (both from Agilent, Santa Clara, CA), as previously described [26]. Briefly, 6 µL of the quality control sample were injected in triplicate in full scan (MS) acquisition mode. Data from the MS experiment was then used to create an acquisition list to be used in the auto MS/MS acquisition mode. The analysis was performed with the instrument in the 2 GHz, extended dynamic range in the negative ionization (NEG) mode using an Agilent Jet Stream (AJS) electrospray ionization (ESI) ion source. Operation parameters were set as follows: capillary voltage: − 4000 V; nozzle voltage − 500 V; nebulizer pressure: 40 psi; gas temperature: 290 °C; sheath gas flow: 12 L/min; sheath gas temperature: 380 °C; fragmentor voltage: 170 V; and skimmer voltage: 65 V. The instrument was operated using MassHunter Acquisition B.09.00 software (Agilent). Chromatographic separation was achieved in 16 min run time using a mobile phase A, water (0.1% formic acid); and B, methanol (0.1% formic acid) at a flow rate of 0.4 mL/min. Gradient elution was performed with an initial mixture of 5% B and 95% A, then increased to 60% B throughout 4 min, to 99% B at 12 min, held until 14 min, returned to 5% B at 15.1 min, and held to 16 min.

2.2.1 LC-QTOF-MS analysis

The full-scan analysis was performed in triplicate using the QC samples acquired over the range of 100 to 1050 m/z in the NEG mode to create an inclusion list to further create the auto MS/MS acquisition mode. Data were extracted using batch recursive feature extraction algorithm in MassHunter Profinder B.08.00 software (Agilent) and after evaluation exported as CEF (Cluster Exchange Format) files. The features were aligned on Mass Profiler software (Agilent) using retention time (RT) tolerance of up to 0.3 min and mass tolerance of ± 15 ppm. Features with a 100% occurrence in the replicates were used to create a target MS/MS inclusion list.

2.2.2 LC-QTOF-MS/MS analysis

Analysis of the study samples was performed using data-dependent acquisition (DDA) (auto MS/MS) acquisition mode using the inclusion list as preferred ions for fragmentation, using delta m/z of 15 ppm and delta retention time (RT) of 0.15 min. The collision cell operates with fixed collision energies of 10, 20, and 40 eV using nitrogen (N2) as the collision gas. The acquisition parameters were set as follows: acquisition mass range: 100 to 1050 m/z at 4 spectra/s in the MS, and 50–800 m/z at 3 spectra/s in the time of flight (TOF).

2.3 Data processing and statistical analysis

Samples from 14 pediatric CD patients, from before and after at least 19 days of EEN were analyzed pairwise. Data were processed as described in our previous work [26]. Briefly, quantile normalization was applied on raw data for features filtered based on QC procedure, where features with less than 100% occurrence between the QC and with coefficient of variance higher than 25% were excluded. Principal component analysis was used to perform a QC on samples to exclude any outlier by visual inspection. Identification of features was performed by library search using Mass Hunter METLIN Personal Compound Database and Library (PCDL) (Agilent Technologies, Santa Clara, USA) at MS/MS level.

Then, Principal component analysis (PCA) was used to evaluate reproducibility across measurements by checking the location of the QC samples on the PCA plots. After quality control, annotated compounds were used in the statistical analysis using MetaboAnalyst 4.0 [27]. Fold change and t-test, using false discovery rate (FDR) to correct for multiple testing, were performed to detect significant changes in certain metabolites between the pairwise samples over time. Orthogonal partial least squares discriminant analysis (OPLS-DA) model was built, and significant metabolites related to the differences between the pairwise samples were identified using the S-plot, comprising the combination of magnitude (covariance), with the effect and reliability (correlation) for the model variables concerning model component scores. Correlation analysis was performed using the difference between the values of the annotated features and the weighted pediatric Crohn's disease activity index wPCDAI [28] scores (after EEN - before EEN) using Pearson r distance measurement.

3 Results

An average of 8000 features were detected per sample from the total of samples analyzed, 6000–7000 features had formulas generated and 3000–4000 were putatively annotated. However, after a strict QC, 318 features were filtered with match score higher than 70 and are shown in the supplementary material containing their respective retention time and m/z (S1). PCA performed on those data showed clustering of all QCs together on obtained PCA models (Data not shown) which indicates the system's stability and consistent performance throughout the analysis.

A volcano plot (Fig. 1) was built presenting the compounds filtered by pvalues and fold change (FC) and shows eight compounds found significant on the t-test with a p-value < 0.05 (Table 1). Albeit, after FDR correction, only 4 features remained significant at $p < 0.05$. Considering FC, with arbitrary cut-off of 1.3, two compounds (Bacteriohopane-32,33,34,35-tetrol, and PA(15:1/18:0)) were filtered as significant. Orthogonal PLS-DA model provided complete separation between the samples before and after EEN treatment. Figure 2 depicts the scores plot and the s-plot with features of importance showing the most significant compounds ordered by the covariance loading values obtained using an arbitrary cut-off value of 3.6, resulting in 10 annotated compounds with increased concentration after treatment and 13 compounds which decreased in concentration (Table 2).

As previously reported [15], disease activity measured by wPCDAI decreased significantly ($p < 0.001$) over the course of EEN treatment. The average score (± SD) was 48.8 ± 18.6 before start of EEN and decreased to 16.4 ± 10.1 after 3–4 weeks on EEN therapy. wPCDAI was developed as a score to stratify the severity of Crohn's disease in pediatric patients with ranges between 0 to 100 with higher scores signifying more active disease. A score of < 12,5 is consistent remission, > 40 indicates moderate disease, and > 57,5 severe disease. A 17.5-point decrease in PCDAI is taken as evidence of small improvement and 37,5 as moderate improvement [28]. Correlation analysis was performed between the annotated compounds and the wPCDAI scores. The most

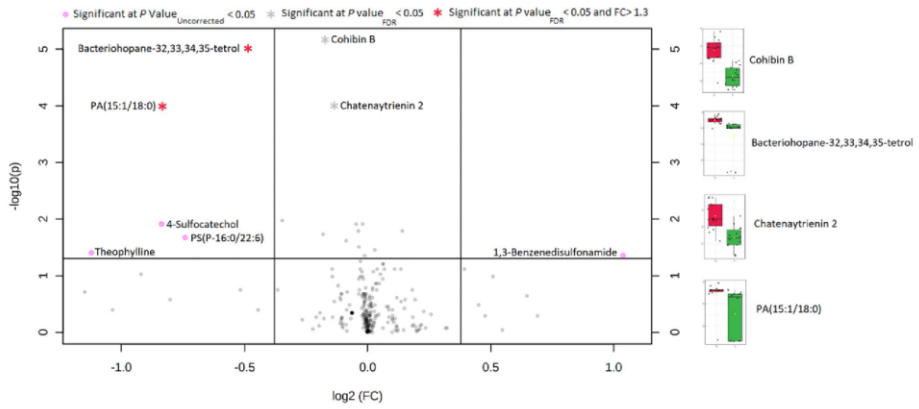

Fig. 1 Volcano plot with log2 fold change (FC) in the x-axis and –log10 of p values on the y axis. The lines indicate FC > 1.3. Box whisker plot showing the interquartile range of the significant metabolites before (red) and after EEN treatment (green)
* Entities significant after FDR correction for multiple testing

Table 1 Results of univariate analysis using t-test

Feature	Change post-EEN therapy	p-value	p-value (FDR)
Bacteriohopane-32,33,34,35-tetrol	-	*	*
Cohibin B	-	*	*
PA(15:1/18:0)	-	*	*
Theophylline/Theobromine	-	*	na
4-Sulfocatechol	-	*	na
PS(P-16:0/22:6)	-	*	na
Chatenaytrienin 2	-	*	*
1,3-Benzenedisulfonamide	+	*	na

significant compounds with R values < -0.5 for the negative correlation and > 0.3 for the positive are shown in Fig. 3. The following compounds were found negatively correlated with wPCDAI scores: 1-Phosphatidyl-1D-myo-inositol 3-phosphate; Erythromycin ethylsuccinate; 1,2,4,5,7-Pentathiocane; dTDP / Thymidine 5'-diphosphate; PS(22:4/19:0); Medicagenic acid beta-maltoside; Phe Arg Val; Anisole; and PS(18:2/21:0) with R values of -0.71, -0.67, -0.66, -0.64, -0.58, -0.55, -0.53, -0.52, -0.52, respectively). Conversely, we filtered 9 compounds positively correlated with wPCDAI: CDP-DG(16:0/20:4), 1,2-bis(Chloromethoxy)ethane; Theophylline/Theobromine; LMST03020510; Navalioside; 2α-Fluoro-19-nor-22-oxa-1α,25-dihydroxyvitamin D3; PA(15:1/18:0); 3-Acetylthiophene; and LMST01080090 (R = 0.30, 0.31, 0.35, 0.35, 0.35, 0.43, 0.50, 0.54, and 0.67, respectively).

4 Discussion

The use of EEN therapy clearly shows an impact on the plasma metabolome, with complete separation by orthogonal PLS-DA. Some features with a higher importance in the model appear related to the exclusion of a regular diet and they decreased most during EEN treatment. The compound annotated as Theophylline, for instance, presented the same match score as Theobromine, natural methylxanthines present in chocolate derivatives and beverages that are highly consumed by children and teenagers [29]. Other compounds related to diet that decreased during EEN therapy included: the food additive (+/-)-3-[(2-Methyl-3-furyl)thio]-2-Butanone [30]; organic compounds found in fruits: 3-(3,4-Dihydroxyphenyl)-1-Propanol 3'-Glucoside [31] and 2-Phenylethyl beta-D-glucopyranoside [32]; in tomatoes: N-Nitrosotomatidine [33] and other phytochemicals naturally occurring in fats and oils, green vegetables, herbs, and spices: (Methoxysulfinyl) Pentasulfide [34] and 4-(3-Hydroxy-7-phenyl-6-heptenyl)-1,2-benzenediol [35]. Violacene is a polyhalogenated monocyclic monoterpene [36] which is produced by diverse genera of bacterial strains which were isolated from various marine to freshwater and soil environments as well as marine algae and could probably reflect cessation of food intake [37]. A slight reduction shift was detected in Prostaglandin D2-1-glyceryl ester, a bioactive lipid involved in the endocannabinoid system with potential anti-inflammatory properties in vivo [38].

The compounds found elevated during EEN treatment in the multivariate analysis present two features annotated

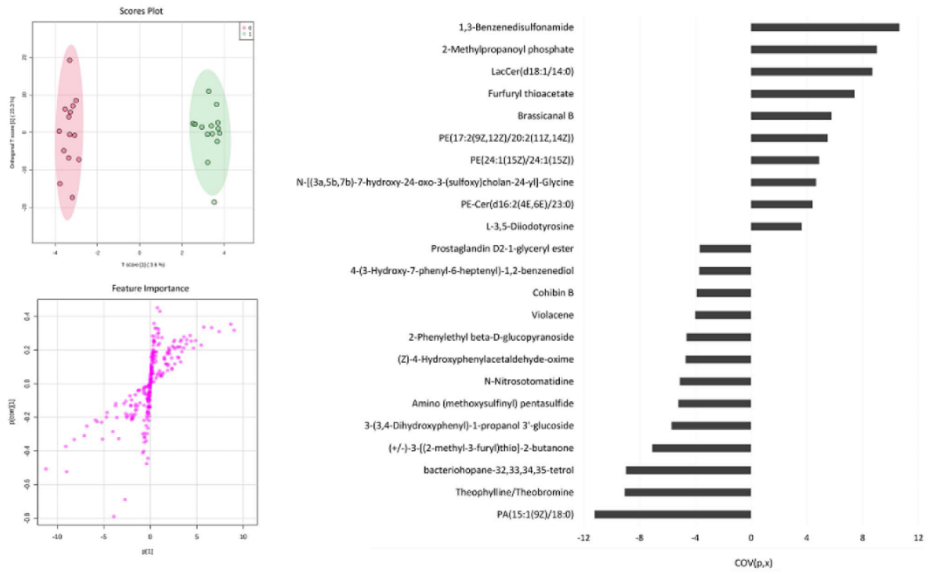

Fig. 2 Scores plot and Splot from the Ortogonal partial least square discriminant analysis (OPLS DA) (Left) and bar chart with of the most important features in the model with cut off of 3.6

Table 2 List of the significant compounds using orthogonal PLS-DA ordered by the covariance loading values which increased or decreased post EEN therapy

Increased after EEN therapy	Decreased after EEN therapy
1,3-Benzenedisulfonamide	PA(15:1/18:0
2-Methylpropanoyl phosphate	Theophylline/Theobromine
LacCer(d18:1/14:0)	Bacteriohopane-32,33,34,35-tetrol
Furfuryl thioacetate	(+/-)-3-[(2-methyl-3-furyl) thio]-2-butanone
Brassicanal B	3-(3,4-Dihydroxyphenyl)-1-Propanol 3'-Glucoside
PE(17:2/20:2)	Amino (methoxysulfinyl) pentasulfide
PE(24:1/24:1)	N-Nitrosotomatidine
N-[(3a,5b,7b)-7-hydroxy-24-oxo-3-(sulfoxy) cholan-24-yl]-Glycine	(Z)-4-Hydroxyphenylacetaldehyde-oxime
PE-Cer(d16:2/23:0)	2-Phenylethyl beta-D-glucopyranoside
L-3,5-Diiodotyrosine	Violacene
	Cohibin B
	4-(3-Hydroxy-7-phenyl-6-heptenyl)-1,2-benzenediol
	Prostaglandin D2-1-glyceryl ester)

as phosphatidylethanolamine (PE), PE(24:1/24:1), and PE(17:2/20:2) and one ceramide phosphoethanolamine (PE-Cer): PE-Cer(d16:2/23:0). PE are estimated to comprise 15–25% of the total lipid content in mammalian cells and exert remarkable bio-activities [39]. In Gram-negative bacteria, PE comprises around 75% of the phospholipid cell envelop and are dynamic key compounds modulating metabolic activities [40].

Other elevated compounds were 3,5-Diiodo-L-tyrosine, involved in thyroid hormone synthesis [41] and LacCer(d18:1/14:0). Lactosylceramide (LacCer) was previously related with CD, however the role in the pathogenesis was unclear [42]. Another study indicated the potential of Lactosylceramide as a potential biomarker of inflammatory bowel disease in children [43]. LacCer is highly expressed in phagocytes and epithelial cells and may play an essential role in the human innate immune system, binding pathogenic microorganisms [44]. Furfuryl thioacetate, a naturally occurring aroma compound [45], and Furfuryl B, a secondary metabolite with antimicrobial activity produced by many plants source of edible vegetable oils [46] were also elevated. Two chemical entities appear with weighing importance in the OPLS-DA model, 2-Methylpropanoyl phosphate and 1,3-Benzenedisulfonamide; however, neither of them has been previously reported in the human metabolome. Interestingly, compounds containing the

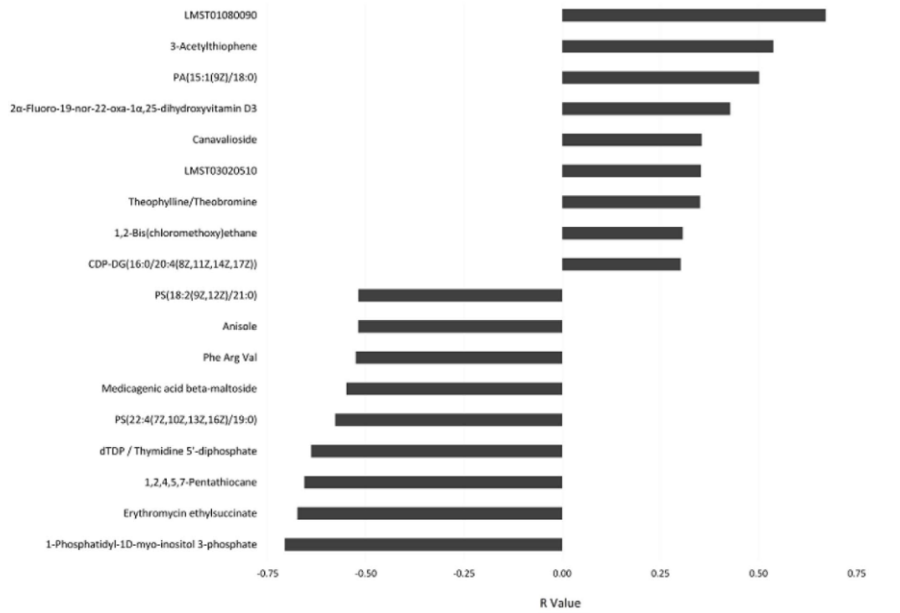

Fig. 3 Bar chart showing features correlated with wPCDAI scores with Pearson correlation coefficient cut off of -0.5 for the negatively correlated features and 0.3 for the positive

sulphonamide moiety present potential biological activities, such as carbonic anhydrase and COX-1/2 inhibition, as well as anti-inflammatory, and antitumor activities [47].

Some of the features with high importance in the multivariate model were found significant in the t-test and fold change, concomitantly. Bacteriohopane-32,33,34,35-tetrol, a bacterial metabolite, and biomarker of *Burkholderia, Pseudomonas, and Ralstonia spp* [48[, decreased during treatment. *Burkholderia spp* is a *Proteobacteria* known for causing dysfunction of GALT and gut microbiota in IBD [49], with the potential to invade intestinal epithelial cells [50]. An increase in the abundance of proteobacteria has been reported in IBD patients [51], in active or aggressive Crohn's disease [52]. A decrease of Bacteriohopane-32,33,34,35-tetrol concentration after EEN treatment may indicate an effect on the gut microbiome with a decrease in *Burkholderia* and/or *Pseudomonas* population. Thus, the presence of elevated PE in plasma could be a marker of gram-negative bacterial cell membranes that underwent cell death.

Two other features annotated as Cohibin B and Chatenaytrienin 2 were significantly ($p < 0.05$) decreased with EEN treatment. Both compounds belong to the class of organic compounds known as annonaceous acetogenins, a class of natural compounds with a wide variety of biological activities and are powerful inhibitors of complex I (NADH : ubiquinone oxidoreductase) in mammalian and insect mitochondrial electron transport systems [53].

The most important metabolite in the OPLS-DA model was PA(15:1/18:0), also found significantly decreased in the t-test and fold change. PA is the simplest class of glycerophospholipids (GPL) present in virtually all organisms, from bacteria to higher plants. It is an intermediate in lipid membrane synthesis and storage and is also involved in many eukaryotic processes. Besides, PA influences membrane structure and interacts with diverse proteins due to its unique physicochemical properties in comparison to other GPL, thus acting as a lipid mediator in various signaling and cellular processes 54.

We correlated the metabolome with the patients' wPCDAI scores to investigate what metabolites were associated with clinical improvement. The results show two metabolites in common with the list of features related to EEN treatment. There was a parallel decrease of PA(15:1/18:0),

which correlated with wPCDAI scores, indicating that this metabolite could play a role in the CD pathophysiology. Another compound showing a similar trend was Theophylline/Theobromine reflecting exclusion of chocolate derivatives and beverages. These products were previously shown to modify the intestinal microbiome in a rat model, with a decrease in *E. coli; Bifidobacterium spp.; Streptococcus spp.; and Clostridium histolyticum-C* [55].

4.1 Strengths and limitations

The strengths of our study are the use of a novel sensitive analytical methodology with the capability of detection of a wide variety of endogenous and exogenous compounds and inclusion of mostly treatment naïve patients. The limitations are the putative annotation of compounds, the small sample size, a limited number of two samples per patient, and the absence of dietary data at baseline, and the associations with hypothesis raising nature without providing evidence on causality.

5 Conclusion

We report remarkable alterations in the plasma metabolic profile of pediatric CD patients treated with EEN. Markers reflecting the change of a mixed regular to a formula diet showed marked changes, including xenobiotics, such as food additives, other phytochemicals, and Theophylline/Theobromine which also correlated with a CD activity index. The decrease in the concentration of a biomarker of proteobacteria (Bacteriohopane-32,33,34,35-tetrol) and the increase in (PE) concentration, the main compound in cell membrane composition of gram-negative bacteria, together may indicate an alteration in potential pathogenic bacteria populations and metabolism. Thus, metabolomics provides an opportunity to characterize the molecular mechanisms of dietary factors triggering CD activity and unravel the mechanisms of action of EEN.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11306-022-01953-0.

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Author contributions TS, PB, SK, and BK coordinated the project and critically revised the manuscript; TS and PB recruited patients and collected clinical data; JM acquired mass spectrometry data, performed the statistical analysis, data interpretation, and drafted the manuscript; All co-authors reviewed and approved the final manuscript.

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Data Availability The data underlying this article will be shared at reasonable request to the corresponding author.

Declarations

Conflict of interest JGM has nothing to declare. SK reports personal fees from Nestle, Danone, Biocodex, Shire, Abbvie, Pharmacosmos, Janssen, Pfizer, Takeda, Mead-Johnson, and research grants paid to the institution from Mead Johnson outside the submitted work. BK benefitted from support for scientific or educational activities from Barilla, Bayer, Cheplapharm, Cogitando, Danone, DSM, DGC, Hipp, Nestlé, and Reckitt Benkiser. TS received speaker's fees from MSD and Nutricia. PB received personal fees (lectures, advisory board) from AbbVie, Orphalan, Mirum, Amgen, Nestlé Nutrition Institute, Nutricia, and Albireo with no activities connected to the presented work.

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7. Paper III

The following pages present the publication:

Marques J, Shokry E, Uhl O, Baber L, Hofmeister F, Jarmusch S, et al. Sarcopenia: investigation of metabolic changes and its associated mechanisms. Skeletal Muscle. 2023;13(1):2.

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Skeletal Muscle

RESEARCH

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Sarcopenia: investigation of metabolic changes and its associated mechanisms

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Abstract

Background Sarcopenia is one of the most predominant musculoskeletal diseases of the elderly, defined as age-related progressive and generalized loss of muscle mass with a simultaneous reduction in muscle strength and/or function. Using metabolomics, we aimed to examine the association between sarcopenia and the plasma metabolic profile of sarcopenic patients, measured using a targeted HPLC-MS/MS platform.

Methods Plasma samples from 22 (17 men) hip fracture patients undergoing surgery (8 sarcopenic, age 81.4+6.3, and 14 non-sarcopenic, age 78.4±8.1) were analyzed. *T* test, fold change, orthogonal partial least squares discriminant analysis, and sparse partial least squares discriminant analysis were used for mining significant features. Metabolite set enrichment analysis and mediation analysis by PLSSEM were thereafter performed.

Results Using a univariate analysis for sarcopenia *z* score, the amino acid citrulline was the only metabolite with a significant group difference after FDR correction. Positive trends were observed between the sarcopenia *z* score and very long-chain fatty acids as well as dicarboxylic acid carnitines. Multivariate analysis showed citrulline, non-esterified fatty acid 26:2, and decanedioyl carnitine as the top three metabolites according to the variable importance in projection using oPLS-DA and loadings weight by sPLS-DA. Metabolite set enrichment analysis showed carnitine palmitoyltransferase deficiency (II) as the highest condition related to the metabolome.

Conclusions We observed a difference in the plasma metabolic profile in association with different measures of sarcopenia, which identifies very long-chain fatty acids, Carn.DC and citrulline as key variables associated with the disease severity. These findings point to a potential link between sarcopenia and mitochondrial dysfunction and portraits a number of possible biochemical pathways which might be involved in the disease pathogenesis.

Keywords Sarcopenia, Metabolomics, Energy metabolism, Mitochondrial metabolism, Fatty acid metabolism, Lipid metabolism, Fatty acid oxidation

Background

Sarcopenia is one of the most predominant musculoskeletal diseases of the elderly, defined as age-related progressive and generalized loss of muscle mass with a simultaneous reduction in muscle strength and/or function [1, 2]. Sarcopenia not only poses a risk for loss of activity of daily living, negatively impacting the quality of life, but also is associated with a high risk of falls, fractures, hospitalization, and mortality [3, 4]. Thus, it can be related to serious social and economic implications, reflected in the high health care costs [5, 6]. A better

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understanding of the factors underlying this condition may offer subsidies for treatment strategies. However, this can be complicated as sarcopenia is a multifactorial disease, which can be attributed to several factors and the elemental biological mechanisms are yet not clearly elucidated. Metabolomics is an omics science based on identifying and measuring the small molecule substrates, intermediates, and products of cell metabolism, globally known as "the metabolome." It is a powerful approach, since the metabolome directly reflects the underlying biochemical activity and the physiological state, thus best representing the molecular phenotype. Therefore, metabolomics can be a valuable tool for understanding the metabolic disruptions associated with sarcopenia and can be also correlated with the severity of the disease.

In 2019, The European Working Group on Sarcopenia in Older People (EWGSOP2) published the revised consensus on the use of low muscle strength as the primary criterion for the identification of sarcopenia, with the diagnosis further confirmed by the presence of low muscle quantity or quality and physical performance as an indicator of severity [7–9]. Based on these criteria, sarcopenia was defined as the combination of reduced handgrip strength and loss of skeletal muscle mass, calculated as skeletal muscle index (SMI) [7, 9]. A z-transformation of both values represents the degree of sarcopenia on a metric scale.

The aim of this study was to examine the association between sarcopenia, defined as reduced handgrip strength and loss of skeletal muscle mass as the primary criterion of sarcopenia, as well as the SMI, with the plasma metabolic profile of sarcopenic patients, measured using a targeted metabolomics platform. Data were also provided on somatotropic axis parameters and relevant associations with the metabolic profile have been additionally investigated. The resulting associations were then used to draw a picture of the biochemical pathways involved in the disease pathogenesis with a potential link between sarcopenia and mitochondrial dysfunction.

Methods

Patient recruitment

This metabolomics study was performed as a secondary analysis on plasma samples obtained from 22 hip fracture patients of both sexes undergoing surgery. Briefly, patients aged over 70 years with a proximal hip fracture of the femur undergoing surgery were recruited from November 2017 to March 2019. Patients were excluded if they suffered from specific neuromuscular diseases (myasthenia gravis, muscular dystrophy, ALS, polio), severe dementia, chronic inflammatory disease (e.g., Crohn's disease, ulcerative colitis, rheumatoid arthritis with systemic anti-inflammatory therapy), or have been subject to systemic corticosteroid therapy (above 7.5mg per day), or cancer therapy in the last 5 years. All participants provided written informed consent before enrolment. Informed consent was taken before surgery with enough time to think about participation. In case of concerns of the patient, the patient was not included.

Patient data

The collected data includes information on the demographic, family, and socioeconomic characteristics, alcohol intake, smoking, and comorbidities as well as anthropometry (weight, height, BMI, fat mass (FM), and fat mass index (FMI). The anthropometric measurements were obtained by physical examination of the study participants by trained study personnel. Bio-Impedance Analysis (BIA; BIA 101, Akern, Florence, Italy) was performed after surgery and used for measuring lean mass. Measurements were taken under standard conditions, with the patient in a supine position and surface electrodes placed on the wrist and ankle contralateral to the side of the fracture. Appendicular lean mass (aLM) was estimated using the equation developed by Sergi et al. [10]. The skeletal muscle index [SMI, (kg/m^2)] was calculated by dividing aLM by body height squared. Assigned cutoffs of 7 kg/m^2 in men and 5.5 kg/m^2 in women were used to define low SMI. Handgrip strength was assessed with a Saehan DHD-1 Digital Hand Dynamometer, with the patient lying supine. The maximal value of three consecutive measurements of both hands was used for the analysis. Similar to SMI, handgrip strength was defined as low, if below 27 kg and 16 kg, in men and women, respectively [7]. A z score combining handgrip strength and muscle mass was calculated separately for men [z score $\text{sarcopenia}_{\text{men}}$ = [(27−handgrip strength)/SD (handgrip strength)] + [(7.0−SMI)/SD (SMI)] and women [z score $\text{sarcopenia}_{\text{women}}$ = [(16−handgrip strength)/SD (handgrip strength)] + [(5.5−SMI)/SD (SMI)]. Z-transformation of both values represents the degree of sarcopenia on a metric scale. The higher the z-score, the more sarcopenic the patient. Data were provided on the insulin growth factor (IGF) axis parameters including IGF-1, insulin growth factor binding protein 3 (IGFBP3) and IGF1/IGFBP3 ratio.

IGF-I and IGFBP3 measurement

Blood samples for measurement of serum concentrations of IGF-I and IGFBP3 were centrifuged and serum was stored at −80°C until analysis. Serum hormone concentrations (ng/ml) of IGF-I and IGFBP3 were measured at the Endocrine Laboratory of the University Hospital Munich using the IDS-iSYS automated chemiluminescent assay system (Immunodiagnostic System Ltd., Boldon, England, UK). Validation data for all assays and

reference intervals have been published elsewhere [11, 12]. The assays are calibrated against the latest recombinant standards (02/254 for IGF-I).

Metabolomic measurements

For the metabolomic measurements, plasma samples were obtained after centrifugation of blood samples collected from patients in EDTA tubes, then stored at −80°C until analysis. Overall, a targeted metabolomics approach was applied for measuring a total of 300 metabolites in the patients' samples at the Dept. of Paediatrics, LMU Munich. Concentrations were calculated in µmol/l. The measured metabolites belonged to the following classes:

Amino acids

Twenty-two amino acids including alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), valine (Val), citrulline (Cit), ornithine (Orn), and proline (Pro) were analyzed in plasma samples obtained from patients by ion-pair liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) as previously described by Harder et al. [13].

Acyl carnitines

Carnitines (free carnitine (Carn) and acylcarnitine (Carn.a)) were analyzed using a modified method from Giesbertz et al. [14]. Briefly, proteins of 50 µL plasma samples were precipitated by a tenfold amount of methanol including isotopic labeled internal standards D3-Carnitine C2 (DLM-754-PK, Cambridge Isotope Laboratories), D3Carnitine C8 (DLM-755-0.01, Cambridge Isotope Laboratories), and D3-Carnitine C16 (DLM-1263-0.01, Cambridge Isotope Laboratories). After centrifugation, 50 µL of the supernatant was evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residuals were re-dissolved in 50 µL hydrogen chloride-1-butanol solution, and derivatization was conducted at 60 °C for 10 min shaking at 600 rpm. Thereafter, the hydrogen chloride-1-butanol solution was evaporated to dryness and the residuals re-dissolved in 50 µL methanol. The butylated acylcarnitines were separated on a 1200-SL HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, pump, autosampler, column oven, and a 150 × 2.1 mm Kinetex® reversed-phase column with 2.6 µm particles (Phenomenex, Torrance, USA). Mobile phase A consisted of 5mM ammonium acetate in water and mobile phase B consisted of 333 µL 7.5 M ammonia acetate in 1 L methanol/ acetonitrile/isopropanol (1:4:5). The mass spectrometric detection was performed on a hybrid triple quadrupole mass spectrometer (4000 QTRAP, AB Sciex, Darmstadt, Germany) with a Turbo Ion source operating in negative ESI mode.

Non-esterified fatty acids (NEFA)

Sixty-three non-esterified fatty acids were measured in patients' samples, using HPLC-MS/MS run in negative ESI mode as described previously by Hellmuth et al. [15]. The same formula CX:Y was used to indicate the chain length as well as the number of double bonds.

Bile acids

A new method for bile acids analysis was developed and validated using HPLC-MS/MS. The method description is presented in the supplementary material (Supplementary M1). Briefly, seventeen bile acids were measured, including cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), glycocholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycolithocholic acid (GLCA), taurolithocholic acid (T LCA), taurocholic acid 3-sulfate (TCA-3S), and taurolithocholic acid 3-sulfate (TLCA-3S).

Quality control

Due to the limited number of samples, only one batch was used in all analyses and quality control samples (QC) were used to check the within-batch variations (intra-batch CV% = 20%). Six QC prepared by pooling aliquots of all available study samples were consistently measured at regular intervals within the batch at the beginning, middle, and end of the batch. Measurements greater than 1.5 standard deviations (SD) away from the next closest measurement were considered as outliers and subsequently set to NA (not available). Measurements with >50% missing values were excluded.

Statistical analysis

Descriptive statistics

The demographic and phenotypic characteristics of the study participants, including age, sex, BMI, comorbidities (diabetes mellitus, rheumatoid arthritis, thyroid, and parathyroid dysfunction, spine diseases, chronic lung diseases, kidney diseases, cancer, diarrhea intolerance), smoking, alcohol and drug intake (proton pump inhibitors (PPI), corticosteroids, anti-estrogenic therapy, tranquilizers), mobility problems, dizziness, stumbling, falls during the preceding year to the study, and activities as sports, daily outdoor activities, were summarized as mean (SD) and proportions for continuous and categorical variables, respectively. Wilcoxon rank-sum test and

Fisher's exact test were used to investigate the differences between groups for numerical and categorical variables, respectively. Results are shown in Table 1.

Data analysis

For the metabolomics data, after normalization and scaling, linear regression models were used to study the associations between plasma metabolite levels and different measures of sarcopenia (sarcopenia *z* scores and SMI) using the sarcopenia measures as the outcome and the plasma metabolites as the independent variables. Models were initially adjusted using potential confounders including age, sex, BMI, smoking, alcohol intake, and comorbidities such as cancer; however, it was noticed that the associations between the metabolite levels and the sarcopenia measures were not appreciably influenced by the inclusion of these confounders, hence they were not included in the models, especially considering the small sample size. Volcano plots were used to depict the results of the models with β on *x*-axis and |log10 (*P*)| values on *y*-axis indicating the sign, magnitude, and strength of the association, respectively. False discovery rate (FDR) [16] was used to minimize the occurrence of false positives, a common issue in multiple testing. Nevertheless, we also inspected associations with uncorrected *P* values for further interpretation, because of the exploratory nature of the analysis and regarded some as potentially meaningful differences, principally if they are common among the same metabolite class or subclass and share the same tendencies. These associations were referred to as trends albeit not significant after FDR correction due to the low statistical power. The cutoff for uncorrected *P* values was depicted as a red dotted line in the volcano plot. Additionally, sarcopenic and non-sarcopenic patient groups were defined using the sarcopenia *z* score cutoff values. To explore the group differences, unadjusted comparisons using multiple univariate tests were performed within the Metaboanalyst 5.0 software which includes fold change (FC) analysis and Wilcoxon rank-sum test. Then, a combination of both tests was used to produce a volcano plot using a cut-off of 1.5 and 0.05 for FC and *P* value, respectively [17]. Concomitantly, for class discrimination and identification of metabolites responsible for group separation, multivariate analyses were also conducted including principal component analysis (PCA), partial least squares–discriminant analysis (PLS-DA), and orthogonal partial least squares–discriminant analysis (oPLS-DA).

Metabolite set enrichment analysis (MSEA) was also performed using the metabolomics data sets, and the pathway was considered significantly enriched if *P* values were smaller than 0.05 and those significant after FDR correction were inspected. Both multivariate analysis and pathway enrichment analysis were carried out also using Metaboanalyst 5.0. Causal effect relationships involved in sarcopenia were investigated using Mediation Analysis by PLSSEM [18–20] using SmartPLS software. First, factor analysis was performed for the selection of indicator variables most associated with the relevant latent variables for each of the metabolite class (AA, NEFA, BA, Carn.a, TCA). Then bias-corrected and accelerated bootstrap was conducted to test the statistical significance of the investigated pathways using 0.05 as a significance level and the total effects, total indirect effects, and specific indirect effects were calculated.

Results

The demographic and phenotypic characteristics of the study participants are described in detail in Table 1. Overall, the 22 patients included 17 men and 5 women aged 79.5±7.5 years. Eight patients were found to suffer sarcopenia as per the EWGSOP2 guidelines, while the remaining 14 subjects were non-sarcopenic. Regarding the definition of sarcopenia, which is the combination of low handgrip strength and low muscle mass, handgrip strength contributes to a higher degree than muscle mass in the patients investigated. This is also reflected in a greater sarcopenia *z* score. The sarcopenic group was aged 81.4±6.3 years while the non-sarcopenic group was aged 78.4±8.1 y. We noticed lower levels of insulin growth factor (IGF-I) and IGF-I/insulin growth factor binding protein 3 (IGFBP3) in the sarcopenic group relative to the non-sarcopenic group.

Association between measures of sarcopenia and the plasma metabolite levels

Linear regression models were used to investigate the associations between the sarcopenia *z* score SMI, and maximum handgrip strength as measures of sarcopenia and the plasma metabolite levels. For sarcopenia *z* score, the amino acid citrulline (Cit) was the only metabolite found significant after false discovery rate (FDR) correction, which stood out as highly significant ($p<0.001$). Positive trends (not significant after FDR correction) were observed between the sarcopenia *z* scores and long as well as very long-chain non-esterified fatty acid (VLC-NEFA), namely NEFA 16:3, NEFA 24:2, NEFA 26:1, and NEFA 26:2) as well as dicarboxylic acid carnitines (Carn-DC) (Supplementary Table 1). Volcano plot for the group differences between the sarcopenic and non-sarcopenic groups using FC analysis and Wilcoxon rank-sum test identified Cit, 4 Carn-DC, namely Carn.3.0.DC, Carn.6.0.DC, Carn.8.0.DC, Carn.10.0.DC in addition to Carn.4.1, Carn.8.1, and Carn.6.OH as well as NEFA 26:2, as shown in the volcano plot (Fig. 1) of which only Cit remained significant after FDR correction ($P<0.01$). For

Table 1 Study population characteristics

Characteristics	Non-sarcopenic *(n=14)*	Sarcopenic *(n=8)*	*P* value
Age	78.4±8.1	81.4+6.3	ns
Sex			
Male	11 (79%)	6 (25%)	ns
Female	3 (21%)	2 (75%)	
Body measures			
Body weight (kg)	76.1±15.8	64.6±15.2	ns
Body height (cm)	167.6+8.5	170.5±10.1	ns
Body mass index (BMI)	26.9±4.2	22.2±4.5	0.02
Fat mass (kg)	27.0±10.7	17.3+6.5	ns
Fat mass percent (FM%)	33.5±6.0	26.2±7.4	ns
Free fat mass (kg)	51.5±10.3	47.3±10.7	ns
Free fat mass percent (FFM%)	66.5±6.0	73.8±7.4	ns
Measures of sarcopenia			
Grip strength	27.3±8.4	18.1±4.9	0.01
Appendicular lean mass (aLM)	18.8±4.3	18.4±4.8	ns
Skeletal muscle index (SMI)	6.6±0.9	6.3±1.3	ns
Sarcopenia *z* score	−1.67±1.21	0.89±1.06	<0.001
Co-morbidities			
Smoking	2 (14%)	1 (13%)	ns
Chronic lung disease	0 (0%)	1(13%)	ns
Kidney disease	2 (14%)	2 (25.0%)	ns
Rheumatoid arthritis (without systemic anti-inflammatory therapy)	3 (21%)	1 (12.5%)	ns
Cancer (> 5 years in their anamnesis)	3 (21%)	4 (50%)	ns
Parathyroid gland dysfunction	0 (0%)	1 (13%)	ns
Thyroid gland dysfunction	2 (14%)	1 (13%)	ns
Diabetes mellitus (DM)	2 (14%)	2 (25%)	ns
Spine disease	1 (7%)	0 (0%)	ns
Diarrhea	0 (0%)	3 (38%)	<0.05
Medication			
Regular drug intake	13 (93%)	7 (88%)	ns
Corticosteroids (<7.5mg per day)	4 (29%)	2 (25%)	ns
DM drug treatment	1 (7%)	0 (0%)	ns
Proton pump inhibitors (PPI)	7 (50%)	4 (50%)	ns
Tranquilizers	3 (21%)	2 (25%)	ns
Mobility/balance problems			
Mobility problems	0 (0%)	3 (38%)	<0.05
Stumbling	3 (21%)	2 (25%)	ns
Dizziness	5 (36%)	3 (38%)	ns
Walking aid	2 (14%)	6 (75%)	<0.01
Household independent	3 (21%)	4 (50%)	ns
Needs help in shopping	1 (7%)	5 (63%)	<0.05
Number of falls in the preceding year	2 (14%)	6 (75%)	<0.01
Activities			
Daily outside activity	1 (7%)	3 (38%)	ns
Regular sports	9 (64%)	2 (25%)	ns
Surgeries	0 (0%)	2 (12.5%)	ns
IGF axis parameters			
IGF1	68.4±19.0*	53.8+28.5	ns
IGFBP3	1588±430*	1419±567	ns
IGF1/IGFBP3	16.9±5.0	14.2+4.1	ns

*Values are expressed in "mean± SD range" or "absolute number (percentage)"

ns not significant at $P<0.05$

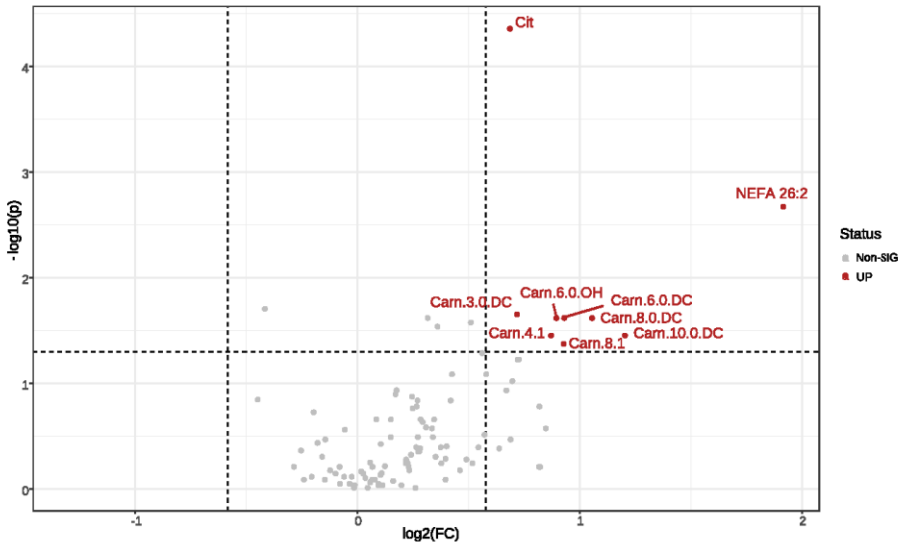

Fig. 1 Volcano plot depicting significant metabolites between the sarcopenic and non-sarcopenic groups using a fold change (FC) threshold of 1.5 and *t* test threshold of 0.05. The log-transformed FC and *P* values are represented on *x*- and *y*-axes, respectively. The red circles represent features above the selected thresholders

the SMI, we found a negative association between SMI and mid to long-chain acylcarnitines (Carn.a) as well as mid-chain NEFA. Additionally, negative trends (not significant after FDR correction) were found with primary bile acids (BA): cholic acid (CA), and chenodeoxycholic acid (CDCA) and a tertiary BA: ursodeoxycholic acid (UDCA) while positive trends with secondary BA taurolithocholic acid (TLCA) and glycolithocholic acid GLCA and one primary BA: taurocholic acid (TCA) and the two amino acids (AA): His and Val (Supplementary Table 1). None of these associations were significant after FDR correction (Supplementary Table 1). Similar to the SMI, negative association between the maximum handgrip strength and mid to long-chain Carn.a (Carn.12.0, Carn.12.1, Carn.14.1, Carn.14.2, and Carn.16.1). However, the most striking observation was the positive trends observed between the handgrip strength and several long and very long-chain NEFA (VLC-NEFA), represented in 22 NEFA species as shown in Supplementary Table 1 albeit not significant after FDR correction. Similar to the SMI, positive associations were found between the TCA, TLCA, and the maximum handgrip strength. Additionally, TCA-3S and TCDCA were also positively associated.

Multivariate analysis

Multivariate models were applied to evaluate the separation between sarcopenic and non-sarcopenic patient groups. Among the different approaches described in the methods section, orthogonal partial least square discriminant analysis (oPLS-DA) and sparse partial least square discriminant analysis (sPLS-DA) were found to be the most efficient models. Complete group separation was obtained using oPLS-DA (Fig. 2A). Cit, NEFA 26:2, and Carn 10.DC were recognized as the top metabolites according to the variable importance in projection (VIP), driving the separation using this model (Fig. 2C). sPLS-DA was found to separate the two groups to a great extent on the first principal compound (PC1) (Fig. 2B). Similar to oPLS-DA, Cit, NEFA 26:2, and Carn 10.DC were recognized as metabolites with the highest loading weights in the model, in addition to other relevant metabolites, such as other Carn. DC (Carn 8.DC and Carn 6.DC) and very long-chain fatty acids (VLC-FA) as NEFA 24:2 (Fig. 2D).

Metabolite set enrichment analysis

Quantitative enrichment analysis (QEA) (Fig. 3) showed that the top enriched metabolite sets identified

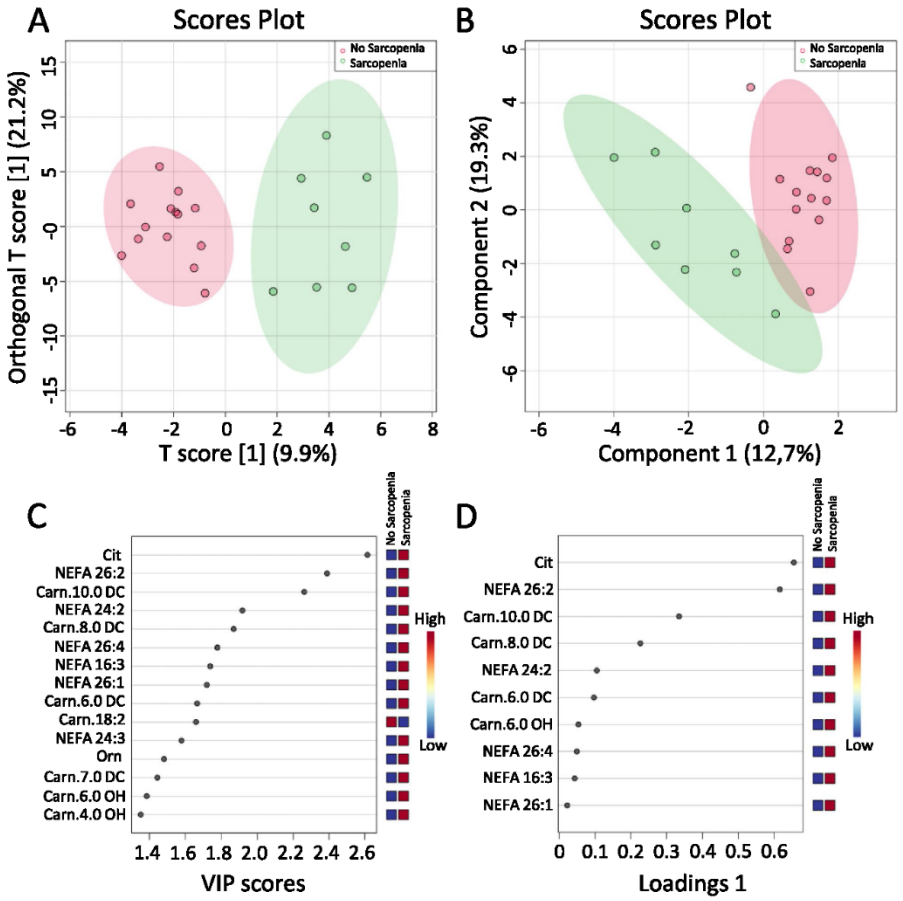

Fig. 2 **A** Score plot from the orthogonal partial least square discriminant analysis (oPLS-DA) showing complete group separation from non-sarcopenic (red circles) and sarcopenic (green circles) subjects (95% confidence ellipses are shaded around each group). **B** Score plot from the sparse partial least square discriminant analysis (sPLS-DA) depicting group separation to a great extent on PC 1 (x-axis) and to a large extent using PC1 and PC2 from non-sarcopenic (red circles) and sarcopenic (green circles) subjects (95% confidence ellipses are shaded around each group). **C** Variable importance in projection (VIP) scores plot from the orthogonal partial least square discriminant analysis (oPLS-DA) presenting the metabolites driving the separation between the non-sarcopenic group and the sarcopenic group. **D** Loadings plot from the sparse partial least square discriminant analysis (sPLS-DA) ranking the metabolites with the highest loadings weight in the model responsible for driving the separation between the non-sarcopenic group (0) and the sarcopenic group [1]

by the difference in the metabolic profiles between the sarcopenic and the non-sarcopenic groups were carnitine palmitoyltransferase deficiency (II), long-chain -3-hydroxy acyl-coA dehydrogenase deficiency (LCHAD), carnitine palmitoyltransferase deficiency (I), very long-chain acyl co-A dehydrogenase deficiency (VLCAD), and Pearson syndrome (uncorrected $P<0.05$). All the conditions related to these metabolite sets share the common feature of being mitochondrial respiratory chain disorders (Supplementary Table 2).

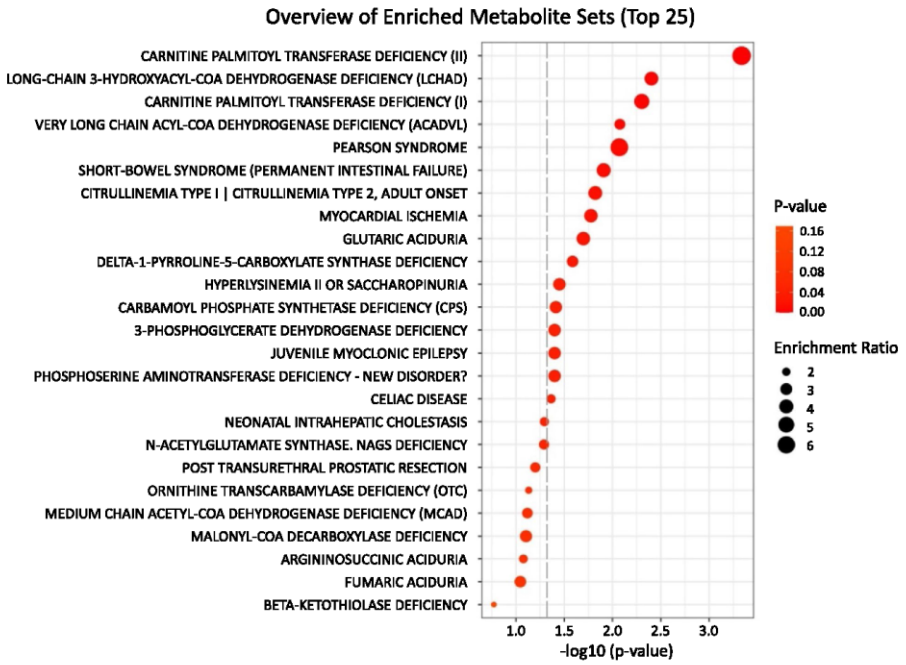

Fig. 3 Quantitative enrichment analysis (QEA) overview presenting the top 25 related metabolic pathways ranked according to the *P* value. Enrichment ratio is computed by hits/expected, where hits = observed hits; expected = expected hits. Dashed line indicates *P*=0.05 (-log10 (*P* value)= 1.3

Associations between the somatotropic axis parameters and the metabolome

Negative trends were found between IGF-I and three VLC-FA species (NEFA 26:2, NEFA24:4, NEFA 24:2), and Cit. Fewer associations were found between the metabolome and IGFBP3, where it was only negatively associated with two metabolites: Carn.a 20:0 and CA. As for the ratio IGF-I/IGFBP3, it was the most associated with the metabolome, especially the NEFA. This was evident in the multiple negative trends observed between the NEFA, specifically the LC- and the VLC-NEFA and the ratio IGF-I/IGFBP3 including NEFA 13:0, NEFA 17:1, NEFA 16:1, NEFA 14:1, NEFA 12:1, NEFA 19:1, NEFA 16:3, NEFA 16:0, NEFA 22:2, NEFA 18:2, NEFA 14:2, NEFA 24:4, NEFA 22:4, NEFA 14:0, NEFA 24:3, NEFA 22:3, and NEFA 24:2. This in addition to Carn.a 3:0 and a BCKA, 3-methyl-2-oxo butanoic acid which were also negatively associated. None of the abovementioned associations between the somatotropic axis parameters and the metabolome were found significant after correction for FDR (Table 2).

Mediation analysis by PLSSEM

Given that specific metabolite species were commonly associated with both, IGF-I and sarcopenia, we hypothesized that a somatotropic axis may impact sarcopenia mediated by metabolic changes. To test this hypothesis, PLSSEM was used using each of the metabolite class sets as mediators (NEFA, BA, AA, and Carn.a). We noticed that none of the direct or the indirect effects from IGF-I to sarcopenia was significant using any of the metabolite classes except VLC-FA. The specific indirect effects from IGF-I to sarcopenia mediated by VLC-FA were found significant ($P<0.05$) indicating the role of the somatotropic axis shares with VLC-FA in sarcopenia. A diagram depicting the path model from IGF-I to sarcopenia mediated by NEFA and the results of pathways significance (path coefficients, specific indirect effects, and total

Table 2 Results from the linear models for the associations between the plasma metabolites with (A) insulin-like growth factor I (IGF-I) and (B) IGF-I/insulin-like growth factor-binding protein (IGFBP3) in the study population

Class	Metabolite	Regression coefficient (*β*)	*P* value	*P* value (after FDR correction)
A. Insulin-like growth factor I (IGF-I)				
AA	Cit	−0.024	0.016	ns
NEFA	NEFA.26:2	−0.021	0.043	ns
	NEFA.24:4	−0.02	0.045	ns
	NEFA.24:2	−0.02	0.05	ns
B. IGF-I/insulin-like growth factor BP3				
NEFA	NEFA.13:0	−0.181	0.001	ns
	NEFA.17:1	−0.165	0.004	ns
	NEFA.16:1	−0.164	0.004	ns
	NEFA.14:1	−0.163	0.005	ns
	NEFA.12:1	−0.16	0.006	ns
	NEFA.19:1	−0.156	0.007	ns
	NEFA.16:3	−0.15	0.011	ns
	NEFA.16:0	−0.149	0.011	ns
	NEFA.22:2	−0.143	0.015	ns
	NEFA.18:2	−0.143	0.016	ns
	NEFA.14:2	−0.141	0.017	ns
	NEFA.24:4	−0.138	0.02	ns
	NEFA.22:4	−0.136	0.022	ns
	NEFA.14:0	−0.133	0.026	ns
	NEFA.24:3	−0.127	0.034	ns
	NEFA.22:3	−0.121	0.047	ns
	NEFA.24:2	−0.127	0.034	ns
Carn.a	Carn.3.0	0.125	0.038	ns

AA amino acids, *Carn.a* acylcarnitines, *NEFA* non-esterified fatty acids. *P* values as *ns* non-significant

indirect effects) are depicted in Supplementary Figure 1 and Supplementary Table 3, respectively.

Discussion

A peculiar profile to this study was the higher levels of circulating LC- and VLC-FA, and their downstream metabolites (Carn D.C., especially the mid-chain ones) along with the remarkably higher levels of the Cit in patients with sarcopenia relative to non-sarcopenic ones (Supplementary Table 1). This was evident in the trends for higher levels of these metabolites in association with sarcopenia *z* score either using the linear regression models (where the sarcopenia *z* score is treated as a continuous variable) or using univariate/multivariate methods employing OPLS-DA/SPLS-DA, where the patients were divided into sarcopenic and non-sarcopenic groups. The most characteristic compounds detected as top significant metabolites were Cit, NEFA 26:2, Carn 10. DC in addition to other VLC-NEFA and Carn.DC as previously discussed in the results section (Figs. 1 and 2).

The mechanism behind the accumulation of Carn.DC is probably relevant to dicarboxylic acids produced by the ω-oxidation of long-chain fatty acid (LC-FA) and VLC-FA [21]. The corresponding activated CoA esters undergo some cycles of β-oxidation in the peroxisome up to a certain point, probably C10-dicarboxylyl-CoA, which is transported to the mitochondria as Carn. 10.DC [22]. Therefore, it would be fair to hypothesize that the elevation in levels of Carn.DC is a consequence of the elevated levels of VLC-NEFA. Complementary to this picture, we observed a trend for higher levels of mid to LC- NEFA and LC- Carn.a in association with SMI despite not being significant after FDR correction. This metabolic picture is common in mitochondrial long-chain LC-FA oxidation disorders, as carnitine-acylcarnitine translocase deficiency, which involves a transport defect of LC-FA across the mitochondrial membrane, causing an elevation in the levels of plasma LC-FA and monocarboxylic LC-Carn.a [23] [22]. The excess of LC-FA and VLC-FA (C20 or more) synthesized by the elongation of precursor LC-FA via reactions catalyzed by microsomal elongation enzymes is the first subject to ω-oxidation and producing dicarboxylyl-CoA esters which then undergo β-oxidation (chain shortening) in peroxisomes but only to a limited extent, producing C10-dicarboxylyl-CoA, which cannot enter the mitochondrion, leading to its accumulation, in accordance with our results [22]. Our hypothesis is supported by the results of the pathway enrichment analysis, showing that the top enriched pathways identified between the sarcopenic and the non-sarcopenic groups are related to mitochondrial disorders. Even though the exact mechanisms underlying sarcopenia are not fully understood, there is evidence that the accumulation of damaged mitochondria could trigger motor neuron and muscle fiber death [24]. Furthermore, studies have identified mitochondrial as one of the central players contributing to the pathogenicity of the disease [25, 26].

Previous studies showed that mitochondrial dysfunction arising from the abnormal accumulation of mitochondrial DNA induced the early appearance of several age-related phenotypes, including sarcopenia, in mice [27–29]. Several reasons can contribute to mitochondrial dysfunction/damage including ROS-induced damage. ROS production increases with aging due to the decreased levels of antioxidant enzymes and might be secondary to age muscle denervation [24].

Another observation that stood out was the significantly higher levels of Cit in sarcopenic relative to non-sarcopenic subjects, which was the only metabolite found significant after FDR correction. Cit was

previously linked to muscle wasting through NO-induced stress being a precursor in nitric oxide (NO) metabolism [30]. iNOS converts L-arginine to Cit, releasing NO, which reacts with the reactive oxygen species (ROS) (superoxide anions O_2-) forming the toxic molecule peroxynitrite (ONOO-), leading to oxidative stress and muscle fiber loss, a mechanism triggered by tumor necrosis factor-alpha (TNFα) [31, 32]. The implication of the iNOS/NO pathway in TNFα-induced muscle atrophy was previously reported in the literature despite that the detailed mechanism remains unclear [31, 33, 34]. Some studies demonstrated that NO and peroxynitrite levels were found to decrease levels of transcription factors involved in myogenesis and skeletal muscle health [31, 35].

Our findings are strengthened by the consistent results obtained when using either univariate or multivariate analysis, which both showed VLC-FA and Carn. DC as well as Cit as key variables associated with sarcopenia as well as the complementary findings from SMI and handgrip strength which further supported the findings. Although SMI and maximum handgrip strength did not show associations with the exact same set of metabolites, the findings complement those obtained using sarcopenia *z* score whether by univariate or multivariate analysis, such as the negative association between SMI and mid to long-chain acylcarnitines (Carn.a) and mid- and LC- NEFA. This supports the assumption of an impaired mitochondrial function affecting the β-oxidation of the fatty acids and leading to the accumulation of LC- Carn.a and mid-chain NEFA, thus suggesting incomplete oxidation (Supplementary Table 1). Another class that popped up with SMI and maximum handgrip strength are bile acids, with two common findings which are the positive associations between TCA and TLCA and both measures of sarcopenia (Supplementary Table 1). Other bile acids were uniquely associated either to SMI or maximum handgrip strength. For SMI, primary bile acids (CA, CDCA) and tertiary (UDCA) showed negative trends, in contrast with conjugated bile acids (TLCA, GLCA) while positive trends were found between TCA-3S and TCDCA, and maximum handgrip strength. For the sarcopenia z-score, the only bile acid associated was CA which was positively associated with. A recent study demonstrated the atrophic effects of the two bile acids (DCA and CA) on skeletal muscle fibers through TGR5, a plasma membrane G-protein-coupled receptor, in association with increased levels of oxidative stress and protein catabolic pathways [36]. These findings are in line with previous studies on the role of DCA in protein catabolism and energy consumption through TGR5 activation, thus suggesting it as a potential biomarker of sarcopenia, arising in patients with advanced non-alcoholic fatty liver disease (NAFLD) [37]. Another study reported an association between serum bile acids and skeletal muscle volume (SMV) in NAFLD patients [38]. In this study, DCA levels were negatively correlated with SMV of the upper and lower limbs and total SMV while CDCA levels were positively correlated with an increased SMV of the lower limbs. Hepatocytes exposed to high levels of bile acids were shown to exhibit changes in mitochondrial function, including reduced electron transport, impaired mitochondrial respiration, mitochondrial swelling, and outer membrane permeabilization, known as mitochondrial permeability transition (MPT). All these events can eventually cause cell death [39–41]. Oxidative stress has been recognized as a primary factor in bile acid-induced MPT which can be responsive to antioxidant treatment [42]. We speculate that the opposite directions of associations between unconjugated and conjugated bile acids with SMI may reflect reduced conjugation of BA in the peroxisomes, which might be secondary to the change in the mitochondrial function affecting the peroxisomes. Evidence has been provided, that peroxisomes and mitochondria exhibit a close functional interplay and coordinated biogenesis to address certain conditions and demands [43].

Regarding the associations between IGF-I and the metabolome, we observed the involvement of the same metabolite classes, VLC-FA (NEFA 26:2, NEFA24:4, NEFA 24:2) and Cit (Table 2). As for the ratio IGF-I/IGFBP3, it was the most associated with several LC- and the VLC-NEFA (Table 2) which might suggest the implication of IGF-I in the mechanisms related to sarcopenia. We tried to test this hypothesis through PLSSEM. PLSSEM is regarded as an effective tool for conducting exploratory research to develop or extend theory and is particularly useful with small sample sizes which makes a good choice in the current analysis [20]. Despite the small number of samples, we observed that specific indirect effects from IGF-I to sarcopenia mediated by VLC-FA were found significant ($P<0.05$), which further supports our hypothesis.

Lower levels of IGF-I have been linked to mitochondrial dysfunction in aging rats characterized by permeabilization, loss of membrane potential, increased proton leak rates, intramitochondrial free radical production, and a reduction of ATPase and complex IV activities which were ameliorated by exogenous administration of IGF-I in those aging rats [44, 45]. IGF-I therapy was found to improve the oxidative stress damage observed in aging mice with mitochondrial dysfunction [46] and normalize the antioxidant enzyme activities [44, 45]. Thus, these findings suggest that IGF-I has a cytoprotective effect closely related to mitochondrial protection, decreasing free radical production, oxidative damage, and apoptosis, and an increase of ATP

production [45]. Impaired insulin action was linked to dysregulation of mitochondrial function, considering that insulin signaling is a prerequisite for mitochondrial DNA and protein synthesis and thus stimulating the mitochondrial oxidative capacity and ATP production [47, 48]. In line with this, deletion of insulin receptor (IR) and IGF-I receptor (IGF-IR) in the heart, was associated with the downregulation of genes of the mitochondrial electron transport chain and thus mitochondrial fatty acid β-oxidation in the heart [49, 50]. On the other hand, other studies demonstrated that IGF-I has no direct effect on lipid oxidation as a growth hormone which can directly stimulate fatty acid oxidation in an action not mediated by insulin-like growth factor-I [51]. We also tested this hypothesis but none of the direct or the indirect effects of GH on the lipid oxidation was found significant (results not shown). Therefore, our hypothesis that reduced levels of IGF-I might play a role in mitochondrial dysfunction is reflected in the elevation in VLC-FA and Carn.DC profiles which may play a role in sarcopenia. Our hypothesis is supported by previous reports on the decreases in growth hormone and plasma IGF-I with aging both in humans and animal models [52–54]. However, further studies are required to confirm these findings using large cohorts with a bigger sample size.

Strengths and limitations

Based on our findings, we have drawn a picture on the biochemical pathways implicated in sarcopenia which may contribute to the disease pathogenesis and could potentially be regarded as biomarkers correlated with the severity of the disease. However, this study has some limitations which worth mentioning such as the small sample size, lack of information on the diet, and education, in addition to bias in subject selection. The study also applies a targeted metabolomics approach which may have missed some relevant metabolites. Accordingly, it can be considered as a proof-of-principle, model building pilot study for future larger studies targeting metabolic changes related to sarcopenia.

Conclusions

In conclusion, we observed a difference in the plasma metabolic profile in association with different measures of sarcopenia, which identifies VLC-FA and Carn.DC as well as Cit as key variables associated with the disease severity. These findings point to a potential link between sarcopenia and mitochondrial dysfunction and portraits a number of possible biochemical pathways which might be involved in the disease pathogenesis. Large-scale studies can be used in the future to confirm the findings.

Abbreviations

AA	Amino acids
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BMI	Body mass index
CA	Cholic acid
Carn	Free carnitine
Carn.a	Acylcarnitine
Carn-DC	Dicarboxylic acid carnitines
CDCA	Chenodeoxycholic acid
Cit	Citrulline
DCA	Deoxycholic acid
EWGSOP2	The European Working Group on Sarcopenia in Older People
FC	Fold change
FDR	False discovery rate
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycocholic acid
GLCA	Glycolithocholic acid
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
IGF	Insulin growth factor
IGFBP3	Insulin growth factor-binding protein 3
IGF-IR	Insulin growth factor—insulin receptor
Ile	Isoleucine
IR	Insulin receptor
LCA	Lithocholic acid
LC-FA	Long-chain fatty acid
Leu	Leucine
Lys	Lysine
Met	Methionine
MSEA	Metabolite set enrichment analysis
NEFA	Non-esterified fatty acid
NO	Nitric oxide
ONOO-	Peroxynitrite
oPLS-DA	Orthogonal partial least squares–discriminant analysis
Orn	Ornithine
PC	Principal compound
PCA	Principal component analysis
Phe	Phenylalanine
PLS-DA	Partial least squares discriminant analysis
Pro	Proline
QC	Quality control
QEA	Quantitative enrichment analysis
ROS	Reactive oxygen species
Ser	Serine
SMI	Skeleton muscle index
sPLSDA	Sparse least square discriminant analysis
TCA	Taurocholic acid
TCA-3S	Taurocholic acid 3-sulfate
TCDCA	Taurochenodeoxycolic acid
TDCA	Taurodeoxycholic acid
Thr	Threonine
TLCA	Taurolithocholic acid
TLCA-3S	Taurolithocholic acid 3-sulfate
TNFα	Tumor necrosis factor-alpha
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
VIP	Variable importance in projection
VLC-FA	Very long-chain fatty acids
VLC-NEFA	Very long-chain NEFA

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13395-022-00312-w.

Additional file 1: Supplementary Table 1. Chromatography and mass spectrometry parameters for the HPLC-MS/MS analysis of bile acids.

Additional file 2: Supplementary Table 1. Association of the metabolome with sarcopenia z-score SMI, and maximum grip strength.

Additional file 3: Supplementary Table 2. Result from Quantitative Enrichment Analysis.

Additional file 4: Supplementary Table 3. Results of pathway significance using mediation analysis by partial least squares-structural equation modeling (PLSSEM).

Additional file 5: Supplementary Figure 1. Path model by PLSSEM showing causal effect relationship from IGF-I to sarcopenia mediated by NEFA.

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Authors' contributions
Berthold Koletzko: investigation, resources, supervision, and writing—reviewing and editing; Engy Shokry: conceptualization, formal analysis, visualization, and writing—original draft; Fabian Hofmeister: methodology and data curation; Jair Marques: methodology, conceptualization, data curation, visualization, and writing—original draft; Lisa Baber: methodology and data curation; Martin Bidlingmaier: methodology and data curation; Michael Drey: project administration, investigation, resources, supervision, funding acquisition, and writing—reviewing and editing; Olaf Uhl: methodology, data curation, and writing—reviewing and editing; Stefanie Jarmusch: methodology and data curation; Uta Ferrari: supervision, investigation, and writing—reviewing and editing. The authors read and approved the final manuscript.

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Availability of data and materials
The data underlying this article will be shared at reasonable request to the corresponding author.

Declarations

Ethics approval and consent to participate
The study abides by the Declaration of Helsinki principles and the protocol was approved by the Ethics Committee of LMU Munich (IRB No. 328-15).

Competing interests
The authors declare that they have no competing interests.

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Master's Degree in Analytical Chemistry

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