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Original article

A ketogenic diet substantially reshapes the human metabolome

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SUMMARY

Background: Western dietary habits (WD) have been shown to promote chronic inflammation, which favors the development of many of today's non-communicable diseases. Recently, ketogenic diets (KD) have emerged as an immune-regulating countermeasure for WD-induced metaflammation. To date, beneficial effects of KD have been solely attributed to the production and metabolism of ketone bodies. Given the drastic change in nutrient composition during KD, it is reasonable to assume that there are widespread changes in the human metabolome also contributing to the impact of KD on human immunity. The current study was conducted to gain insight into the changes of the human metabolic fingerprint associated with KD. This could allow to identify metabolites that may contribute to the overall positive effects on human immunity, but also help to recognize potential health risks of KD.

Methods: We conducted a prospective nutritional intervention study enrolling 40 healthy volunteers to perform a three-week ad-libitum KD. Prior to the start and at the end of the nutritional intervention serum metabolites were quantified, untargeted mass spectrometric metabolome analyses and urine analyses of the tryptophan pathway were performed.

Results: KD led to a marked reduction of insulin (−21.45% ± 6.44%, $p = 0.0038$) and c-peptide levels (−19.29% ± 5.45%, $p = 0.0002$) without compromising fasting blood glucose. Serum triglyceride concentration decreased accordingly (−13.67% ± 5.77%, $p = 0.0247$), whereas cholesterol parameters remained unchanged. LC-MS/MS-based untargeted metabolomic analyses revealed a profound shift of the human metabolism towards mitochondrial fatty acid oxidation, comprising highly elevated levels of free fatty acids and acylcarnitines. The serum amino acid (AA) composition was rearranged with lower abundance of glucogenic AA and an increase of BCAA. Furthermore, an increase of anti-inflammatory fatty acids eicosatetraenoic acid ($p < 0.0001$) and docosahexaenoic acid ($p = 0.0002$) was detected. Urine analyses confirmed higher utilization of carnitines, indicated by lower carnitine excretion (−62.61% ± 18.11%, $p = 0.0047$) and revealed changes to the tryptophan pathway depicting reduced quinolinic acid (−13.46% ± 6.12%, $p = 0.0478$) and elevated kynurenic acid concentrations (+10.70% ± 4.25%, $p = 0.0269$).

Data described in the manuscript will be made available upon reasonable request pending application and approval. Untargeted mass spectrometric metabolome analysis data is uploaded to https://zenodo.org/record/7100510#.YyrxXLTP1GM and will be made accessible upon publication.

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Conclusions: A KD fundamentally changes the human metabolome even after a short period of only three weeks. Besides a rapid metabolic switch to ketone body production and utilization, improved insulin and triglyceride levels and an increase in metabolites that mediate anti-inflammation and mitochondrial protection occurred. Importantly, no metabolic risk factors were identified. Thus, a ketogenic diet could be considered as a safe preventive and therapeutic immunometabolic tool in modern medicine.
Trial registration: German Clinical Trials Register; DRKS-ID: DRKS00027992 (www.drks.de).

Abbreviations

KD	Ketogenic diet
LC-MS/MS	Liquid chromatography tandem mass spectrometry
TOF-MS	Time-of-flight mass spectrometry
AA	Amino acid
BCAA	Branched chain amino acid
WS	Western diet
BHB	β-hydroxybutyrate
OXPHOS	Oxidative phosphorylation
IDO	Indoleamine 2,3-dioxygenase
KAT	Kynurenine aminotransferase
KMO	Kynurenine-3-monooxygenase
ETA	Eicosatetraenoic acid
DHA	Docosahexaenoic acid
T0	prior to KD
T1	after KD

Introduction

The “Western Diet” (WD) is characterized by an excessive consumption of ultra-processed food, predominantly containing high quantities of sugar and refined carbohydrates. This dietary pattern is closely linked to the development of “lifestyle diseases” such as obesity, diabetes, cardiovascular diseases, and autoimmune disorders [1,2]. Experimental evidence indicates that the increased ingestion of carbohydrates - entailing elevated plasma levels of glucose and consequently insulin - triggers aberrant activation of the NLRP3 inflammasome with subsequently elevated secretion of pro-inflammatory cytokines such as IL-1β, and furthermore reprograms immune cells toward inflammatory phenotypes. This harmful chronic immune activation is increasingly recognized as a major driver of most of today's non-communicable diseases [1,2]. Clinical data, however, are conflicting [3,4].

Nutritional interventions restricting carbohydrates could have the potential to counteract the deleterious effects of WD on human immunity and thus might serve as both preventive and complementary therapeutic measures. Ketogenic diets (KD) have increasingly gained attention in this context [5,6]. By a very limited uptake of carbohydrates, a KD induces the hepatic synthesis of ketone bodies, mainly β-hydroxybutyrate (BHB), providing a potent alternate source of energy to peripheral tissues [7]. BHB not only serves as a substrate for mitochondrial oxidative phosphorylation (OXPHOS) but is increasingly known to exert pleiotropic effects as a vital metabolic and signaling mediator [7,8].

We recently provided the first in-vitro and in-vivo evidence that a KD markedly boosts human CD4 and CD8 T-cell immune capacity and reinforces differentiation of regulatory and memory T cells [9]. These beneficial changes are due to a fundamental immunometabolic reprogramming towards mitochondrial OXPHOS on a KD. So far, these effects were only attributed to ketone bodies. However, it could be assumed that the drastic modification of nutrient composition during the dietary intervention might evoke profound changes to the human serum metabolite composition, which could also contribute to the overall impact of KD on human immunity [10]. Recent studies have already shown that macronutrient composition directly affects systemic metabolic processes [11,12], and changes in circulating metabolomes are inextricably linked to the differentiation and functionality of particular T-cell subsets [13,14].

However, the global effects of limited carbohydrate intake on human metabolic profiles have not yet been elucidated. Here, we present the first prospective nutritional intervention study deciphering the metabolic fingerprint of healthy humans following a KD. Through evaluation of untargeted serum metabolomics combined with blood insulin and lipid profiles as well as urinary analyses, we provide a holistic understanding of metabolic changes on a KD that might improve future clinical applications of very-low-carbohydrate dietary interventions.

Material and methods

In vivo study design

The effect of a KD on the human serum metabolite profile was investigated in a prospective nutritional intervention study. The data presented here is part of a nutritional intervention study for which changes in mRNA expression of IFNγ in T cells was set as the primary endpoint. Secondary endpoints included serum metabolite profile analysis and analysis of urinary amino acid metabolic pathways. Analyses not prespecified were considered exploratory. Prior to (T0) and after (T1) the dietary intervention blood was withdrawn at our laboratory facilities. 40 healthy subjects were included, aged over 18 years (subject characteristics are depicted in Table 1). Women during pregnancy and lactation and volunteers with current intake of glucocorticoids or with intake of glucocorticoids within the last seven days were excluded, as well as subjects with severe metabolic disorders (e.g., diabetes mellitus), autoimmune, hematological, or immunological diseases. All participants were nonsmokers and not suspected of suffering from any other acute or chronic diseases. To verify inclusion and exclusion criteria, a comprehensive and structured medical history interview and physical examination were performed by board-certified physicians prior to enrollment. Research was performed according to the Declaration of Helsinki (ethical principles for medical research involving human subjects). Informed consent was obtained from all volunteers. The study design and the study protocol were approved by the Institutional Ethics Committee of the Ludwig-Maximilian-University Munich, Germany (No. 19-523). The study was registered at the DKRS (German Clinical Trials Register; DRKS-ID: DRKS00027992).

At the start of the diet, all volunteers attended nutritional counseling by board-certified nutritional physicians and

Table 1
Characteristics of study subjects.

	T0	T1
Mean age [years]	42.35	
Age distribution [years]	20–74	
Sex (male/female/diverse)	16/24/0	
Body weight (mean; range) [kg]	75.06 [48.00–110.00]	72.34 [48.00–106.00]
BMI (mean; range) [kg/m^2]	23.79 (17.84–34.71)	23.10 (17.84–33.46)

nutritionists. Prior to enrollment volunteers conducted a typical Western diet (average macronutrient composition before intervention: 55–60% carbohydrates, 25–30% fat, 10–15% protein, Supporting information Fig. S1). During KD, a maximum of 10% of the daily caloric intake was provided by carbohydrates, with increased proportions of proteins and fats (average macronutrient composition on KD: 10% carbohydrates, 60–70% fat, 20–30% protein, Supporting information Fig. S1). The precise amount of carbohydrates permitted per day was adjusted individually to the respective body weight and the corresponding energy requirement. Besides these preconditions, all subjects were permitted to conduct their individual KD ad libitum for three weeks. Adherence to the dietary intervention was closely monitored throughout the study by assessing blood ketone body concentrations on a regular basis using point-of-care testing (Glucomen Aero 2K; Berlin Chemie AG, Berlin, Germany). Study flow is depicted in Supporting information Fig. S2a. All subjects completed the study and achieved sufficient levels of ketone bodies throughout the course of the dietary intervention (Supporting information Fig. S2b).

Anthropometric assessment

For calculation of body mass index (BMI), weight measurements were performed using a Tanita HD-395 Body Weight Scale according to the manufacturer's instructions. Body height was determined using a Soehnle Professional 5003 according to the manufacturer's protocol.

Blood sampling

Blood sampling was performed prior to the start (T0) and on the last day of the KD (T1). Blood was withdrawn at a fasting state. S-Monovette® Serum tubes (Cat.# 02.226.160, Sarstedt) were stored at an upright position for 30 min at room temperature to allow coagulation. Serum was collected at 15 °C, 2750 g/min for 10 min, immediately frozen and stored at −80 °C until analysis.

Serum metabolite quantification

Urea and glucose concentrations were measured by kinetic colorimetric assays using the UREAL (Roche Diagnostics, Cat.# 05171873 190) and GLUC3 kit (Roche Diagnostics, Cat.# 05168791 190). Cholesterol, triglycerides and lipoprotein concentrations were measured via enzymatic color assays using the CHOL2 (Roche Diagnostics, Cat.# 05168538 190), TRIGL (Roche Diagnostics, Cat.# 05171407 190), HDLC4 kit (Roche Diagnostics, Cat.# 07528582 190) and LDLC3 kit (Roche Diagnostics, Cat.# 07005768 190). These analyses were performed on a Cobas 8000/c702 system (Roche Diagnostics) according to the instructions of the manufacturer. Cortisol, insulin and c-peptide concentrations were measured via electrochemiluminescence immunoassays (ECLIA) using the Cortisol II (Roche Diagnostics, Cat.# 07027150 190), Insulin (Roche Diagnostics, Cat.# 07027559 190) and C-Peptide kit (Roche Diagnostics, Cat.# 07027168 190) on a Cobas 8000/e801 system (Roche Diagnostics) according to the instructions of the manufacturer.

LC-MS/MS-based untargeted metabolomic analyses

Untargeted metabolome analyses were performed to assess the metabolic profile in the serum of healthy volunteers performing three weeks KD. Untargeted analysis was carried out on a Nexera UHPLC system connected to a Q-TOF mass spectrometer (TripleTOF 6600, AB Sciex). Chromatographic separation was achieved by using a HILIC UPLC BEH Amide 2.1 × 100, 1.7 μm column with 0.4 ml/min flow rate. The mobile phase consisted of 5 mM ammonium acetate in water (eluent A) and 5 mM ammonium acetate in acetonitrile/water (95/5, v/v) (eluent B). The following gradient profile was used: 100% B from 0 to 1.5 min, 60% B at 8 min and 20% B at 10–11.5 min and 100% B at 12–15 min. Aliquots of 5 μl per sample were injected into the UHPLC-TOF-MS. The autosampler was cooled to 10 °C and the column oven heated to 40 °C. A quality control (QC) sample was pooled from all samples and injected after every 10 samples. MS settings in the positive mode were as follows: Gas 1 55, Gas 2 65, Curtain gas 35, Temperature 500 °C, Ion Spray Voltage 5500, declustering potential 80. The mass range of the TOF-MS scans were 50–2000 *m/z* and the collision energy was ramped from 15 to 55 V. MS settings in the negative mode were as follows: Gas 1 55, Gas 2 65, Cur 35, Temperature 500 °C, Ion Spray Voltage −4500, declustering potential −80. The mass range of the TOF-MS scans were 50–2000 *m/z* and the collision energy was ramped from −15 to −55 V.

The “msconvert” from ProteoWizard [15] was used to convert raw files to mzXML (de-noised by centroid peaks). The bioconductor/R package xcms [16] was used for data processing and feature identification. More specifically, the matchedFilter algorithm was used to identify peaks (full width at half maximum set to 7.5 s). Then the peaks were grouped into features using the “peak density” method [16]. The area under the peak was integrated to represent the abundance of features. The retention time was adjusted based on the peak groups presented in most samples. To annotate features with names of metabolites, the exact mass and MS2 fragmentation pattern of the measured features were compared to the records in HMBD [17] and the public MS/MS spectra in MSDIAL [18], referred to as MS1 and MS2 annotation, respectively. In this experiment, all samples were pooled to generate a reference sample, which was measured seven times throughout the measurements. The reference samples were used to control the drifting effect of the mass spectrometer's signal. A method adapted from EigenMS [19] was used for this purpose. In this process, we used singular value decomposition of the reference measurements to estimate the variance contributed by the artifact reasons, which were further removed from all measurements to get the normalized metabolite intensity matrix. Missing values were imputed with half of the limit of detection (LOD) methods, i.e., for very features, the missing values were replaced with half of the minimal measured value of that feature in all measurements. Paired t-test was used to compare the features’ intensity from T0 to T1. Significant enrichment of the metabolites in the groups was

defined as a mean difference (mean diff.) >1.5 with a false-discovery-rate FDR<0.05. When MS2 spectra were acquired in our experiments, we manually reviewed our MS2 fragmentation pattern and compared it with records in the public database or previously measured reference standards to evaluate the correctness of the annotation. Subsequent relative quantification of tryptophan was performed using the untargeted data set and MultiQuant 3.0.3 (Ab Sciex). For fatty acid analysis, no saponification was performed beforehand, allowing assessment of non-esterified fatty acids and intact lipids.

Urine analyses

Second morning urine samples were collected from participants in 10 ml Tubes and stabilized with 30 μl hydrochloric acid. Prior to sample collection: fish and seafood, bananas, cheese, almonds, nuts, green, black tea, vanilla, and vanilla containing products, cocoa, chocolate, alcohol, coffee, caffeinated drinks, energy drinks, and nicotine were avoided. Liquid Chromatography (LC) system consisted of an Inifinity 1290 LC with autosampler (Agilent Technologies Inc.). A Restek Raptor ARC-18 column (2.7 μm, 100 × 2.1 mm) was used for chromatographic separation. Injection volume was 8 μl. Solvent A consisted of water with 0,15% formic acid and 0,01% Trifluoracetic acid (TFA). Solvent B consisted of Methanol with 0,15% formic acid and 0,01% TFA. A gradient elution was performed at a flow rate of 0,4 ml/min with the following conditions: 97% A (0 min–0,65 min) → 85% A (0,65–1,3 min) → 35% A (1,3–1,9 min) → 35% A (1,9–3 min) → 20% A (3,01–5 min) → 97% A (5–7,5 min). A SCIEX TripleQuad 5500+ System (SCIEX) with multiple reaction monitoring (MRM) in scheduled mode was used. All analytes were ionised with positive electrospray ionisation (ESI+) except quinolinic acid (and quinolinic acid-d3) with negative electrospray ionisation (ESI-). Source conditions were (ESI+): Curtain gas 35; Collision gas 8; IonSpray Voltage 5500; Temperature 500; Gas1 62; Gas2 60. (ESI-): Curtain gas 35; Collision gas 8; IonSpray Voltage - 4500; Temperature 500; Gas1 62; Gas2 60. +MRM (Q1-Mass/Q3-Mass/DeclusteringPotential/CollisionEnergy/RetentionTime): Tryptophan (205.2/188.1/72/31/3.12); Tryptophan-d5 (210.2/193.1/92/31/3.12); L-kynurenine (209.1/192.1/80/13/2.85); L-kynurenine-d3 (213.1/196.1/80/13/2.85); 3-hydroxykynurenine (225.1/110/87/27/1.45); 3-hydroxykynurenine-d4 (229.1/110/72/27/1.45); Kynurenic acid (190.2/144.1/101/50/3.38); Kynurenic acid-d5 (195.1/149.1/100/45/3.38); Serotonin (177.1/160.1/68/24/2.9); Serotonin-d4 (181.1/164.1/68/24/2.9); Nicotinamide (123.05/85/100/30/1.15); Nicotinamide-13C6 (129/85/100/30/1.15); NAD+ (664.3/136.1/145/50/1.52); (NAD+)-13C5 (669.4/136.1/145/52/1.52). -MRM: Quinolinic acid (166.1/78.1/-55/-20/1.03); Quinolinic acid-d3 (169.1/81.1/-55/-20/1.03) Urine samples were assayed using a non-derivatized dilute and shoot LC/MS-MS method. All analytes had specific isotope labelled internal standards to prevent ion suppression effects. Internal validation of the assay was performed according to CLSI (clinical and laboratory standards institute). A calibration master mix was generated out of stock solutions (1000 ng/ml) of each analyte in solvent A. Master mix and stock solutions were stored at −80 °C until analysis. The analyte concentration in the master mix was adjusted to the expected concentration range in urine and varies between 0,5 and 200 μmol/l. A 4-point calibration (0, 1:100; 1:10, 1) was made from this master mix in solvent A. Out of specific isotope labelled stock solutions (100 − 1000 ng/ml) a internal standard mix was generated in solvent A and stored at −80 °C until analysis. Quality controls were used (Recipe, ClinCheck urine control, biogenic amines L1 & L2). For all analytes for which no certified QC material was available, own target values and target ranges were defined in the above QC samples by spiking and dilution with calculating of the recovery rate. 25 μl Urine (Cal, QC) was spiked with 20 μl internal standard mix and diluted with 250 μl solvent A in 1,5 ml microtubes. After vortexing (60 s) and centrifugation (10 min, 4 °C, 5000 g) 100 μl supernatant was transferred into autosampler vials for analysis. Data acquisition and analysis was carried out with Analyst 1.7 (SCIEX). Linear regression lines were determined from 4-point calibrations. The specific internal standards were used to normalize the measured values and to suppress potential matrix effects.

Statistical analyses

If not stated otherwise, statistical analysis was performed using GraphPad Prism 9.2 (GraphPad Software, Inc., United States). All data sets were analyzed for normal distribution using the Kolmogorov–Smirnov and D'Agostino & Pearson tests. Paired t-test or Wilcoxon matched-pairs signed rank test, as appropriate, served for comparisons. Data are presented as mean ± standard error of the mean (SEM) or as box plots with median, 25th and 75th percentiles and range, unless described otherwise. Statistical significance was assumed at a p-value of <0.05, with *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. For statistical analysis of untargeted LC-MS/MS metabolome and urine analyses, please refer to the respective method section.

Results

All healthy volunteers adhered to the KD and successfully generated ketone bodies over the course of the three-week diet (Fig. 1a, Supporting information Fig. S2b). Neither unintended effects nor adverse events were reported. All participants slightly lost weight during the course of the diet. Median BMI reduction was −0.69 kg/m^2, representing a reduction of 2.9% (detailed weight and BMI changes are depicted in Table 1).

Ketogenic diet improves blood lipid- and insulin-profile

Laboratory analyses of serum samples were performed prior to (T0) and after (T1) a three-week ketogenic diet. Limiting total carbohydrate intake to less than 30 g per day led to significantly reduced serum concentration of insulin ($p = 0.0038$) and c-peptide ($p = 0.0002$). Of note, fasting glucose levels remained stable within the physiological range throughout the study (Fig. 1b-d). Consequently, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was significantly reduced on a KD ($p = 0.0164$, Supporting information Fig. S2c).

Total blood cholesterol concentrations as well as levels of LDL, non-HDL and HDL remained unaltered (Fig. 1e, Supporting information Fig. S3). Triglycerides were markedly reduced after three weeks of KD ($p = 0.0247$, Fig. 1f). Serum urea concentration was elevated under KD ($p = 0.0093$, Fig. 1g). These changes were not related to weight loss, as correlation analysis revealed no significant associations (Supporting information Table S1).

KD markedly impacts on the human metabolome

To investigate the impact of a three-week ad libitum KD on serum metabolites, we performed LC-MS/MS-based untargeted metabolomic analyses (TOF-MS) on human sera prior to the start (T0) and at the end (T1) of the nutritional intervention. Principal component analysis including all detected features was able to separate T1 and T0 samples only on the basis of the metabolic fingerprint (Fig. 2a). Further analysis revealed a major impact of KD on serum metabolite levels with a total of 95 significantly regulated metabolites, of which 49 metabolites showed higher

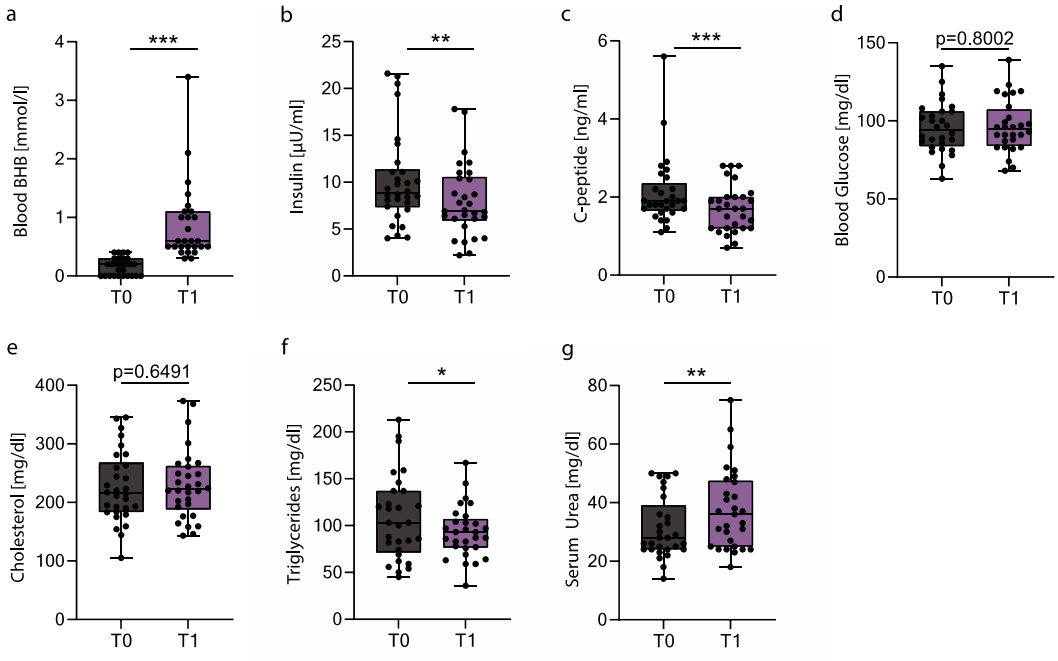

Fig. 1. Laboratory analysis of serum metabolites prior to (T0) and after (T1) healthy participants conducted a three-week ad libitum diet. **a-g** Fasting serum concentrations as indicated, n = 30, *p < 0.05, **p < 0.01, ***p < 0.001.

concentrations at T1, whereas 46 metabolites exhibited lower abundance at T1 (Fig. 2b/c).

To test if we were able to separate the two time points more clearly by combining multiple metabolites, we next trained random forest models. These trained models performed well with area under the receiver curve (AUC) > 0.98. Several major contributing factors could be detected, with a group of 14 features representing >50% of MeanDecreaseGini (Supporting information Fig. S4). The features identified mainly hint to metabolic processes of fatty acids, amino acids, and ketones, reflecting the major changes in macronutrient composition on a KD (Supporting information Table S2). These calculations confirm a distinct serum metabolite profile of participants on a KD.

Upregulation of fatty acid metabolism on a KD

To further decipher the effect of KD on the human serum metabolome, relative quantification of metabolites in the sera of study participants was performed, displaying a marked change in serum levels of various metabolites. As expected, β-hydroxybutyrate was significantly upregulated ($p < 0.0001$), whereas reduced serum levels of hexoses ($p = 0.002$) could be detected (Fig. 3a/b). On a KD, the amount of fat being consumed increases, which was reflected in elevated concentrations of fatty acids comprising unsaturated/saturated as well as short/medium and long chain fatty acids (Fig. 3c-f, Supporting information Fig. S5a–e).

Carnitines are pivotal for fatty acid transport to the mitochondria. Accordingly, an increase in numerous acylcarnitines was shown on a KD (Fig. 3g/h, Supporting information Fig. S5f-m). This phenomenon also expanded to phosphocholines (Supporting information Fig. S5n/o), whereas phosphatidylcholines were diminished at T1 (Supporting information Fig. S5p-r).

Omega-3 (ω-3) and omega-6 (ω-6) fatty acids are known to contribute to pro- and anti-inflammatory pathways, especially via prostaglandin and leukotriene synthesis. In this regard, for both linoleic acid ($p = 0.01$) and linolenic acid ($p = 0.002$), higher abundance on a KD could be detected (Fig. 3i/j). Eicosatetraenoic acid (ETA, $p < 0.0001$) and docosahexaenoic acid (DHA, $p = 0.0002$) - at the endpoint of the anti-inflammatory pathway - displayed elevated concentrations at T1 (Fig. 3k/l). Additionally, for platelet activating factor - known as pro-inflammatory mediator - significantly lower levels could be detected at T1 ($p < 0.0001$, Supporting information Fig. S5s).

Amino acids display complex alterations on a KD. Glucogenic amino acids alanine ($p < 0.0001$), glutamine ($p = 0.002$) and proline ($p = 0.001$) showed lower abundance on a KD (Fig. 3m-o). Essential branched-chain amino acids (BCAA) valine ($p < 0.0001$) and isoleucine/leucine ($p < 0.0001$), however, exhibited higher concentrations on a KD (Fig. 3p/q). In summary, we here provide evidence for a significant change to the human serum metabolome on a KD.

Since a three-week ketogenic diet resulted in significant changes in serum metabolites, urine analyses were carried out to confirm and substantiate the metabolic changes caused by KD. The profile of endogenous metabolites in urine not only provides information about renal function, but also about systemic metabolic processes [20,21].

The increase in carnitines required for enhanced fatty acid metabolism - detected in the serum metabolome on a KD - was also evident in urine metabolite analysis: Carnitine concentrations were significantly reduced ($p = 0.0047$), reflecting decreased excretion due to increased metabolic demand for these transport molecules (Fig. 4a).

Tryptophan metabolism is rearranged towards mitoprotection on a KD

Profound changes were also found regarding the tryptophan metabolism. KD significantly induced the activity of indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme catalyzing the first reaction of the kynurenine pathway by catabolizing tryptophan to N-formylkynurenine ($p = 0.0009$, Fig. 4b) [22]. Consistent with

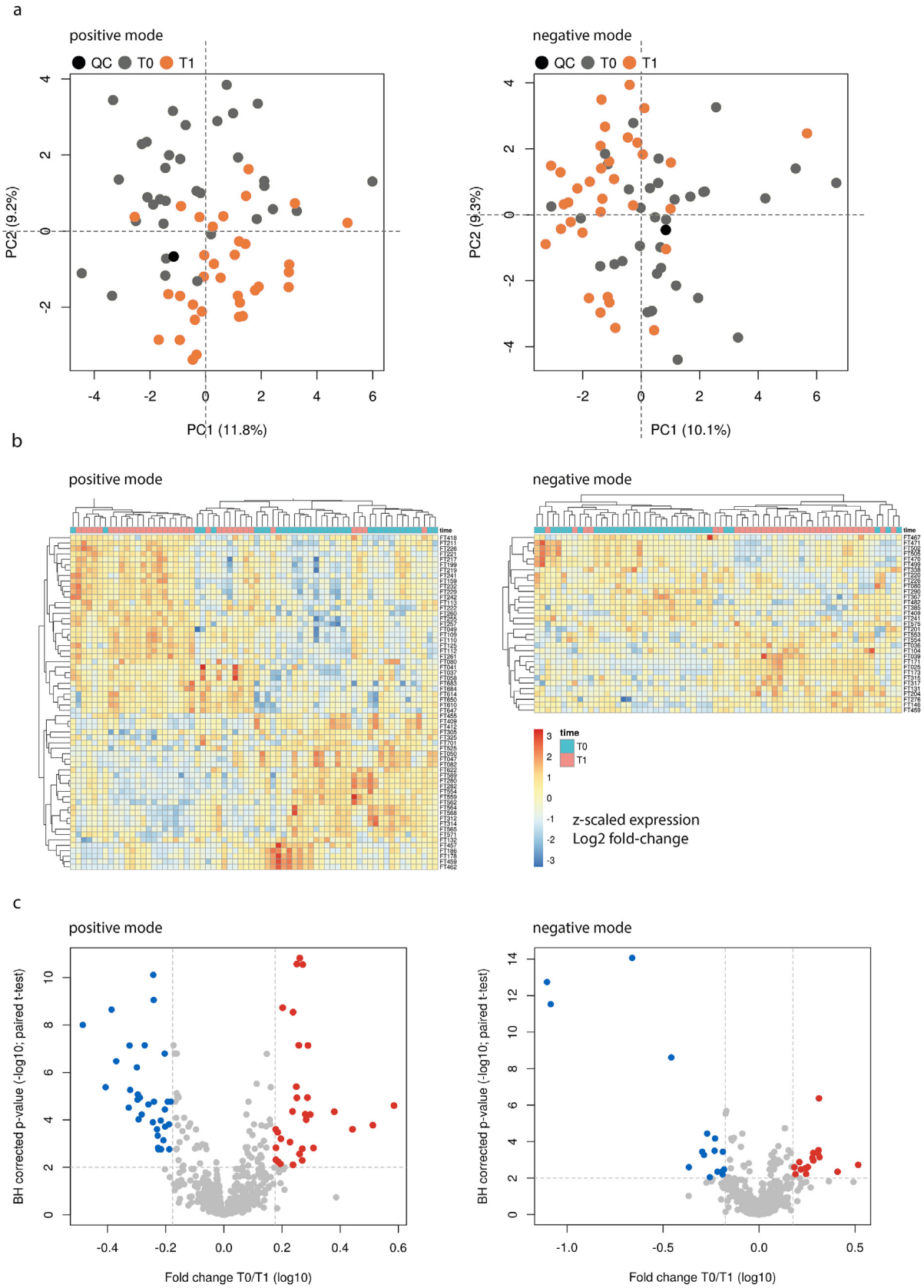

Fig. 2. Untargeted mass spectrometric metabolome analysis of metabolites in the serum of healthy volunteers performing a three-week KD via TOF mass spectrometry (LC-MS/MS). Shown are negative and positive mode results. **a** Heatmaps of differentially regulated serum metabolites in response to KD. The colour and intensity of the boxes illustrate upregulation (red) or downregulation (blue) of metabolites. **b** Representation of T1/T0 differential regulated serum metabolites using Volcano plots. Each point represents a metabolite as a function of fold change (Log 2 fold-change, x-axis) and statistical significance (Log 10 FDR-corrected p-values, y-axis). Red highlights significantly upregulated, blue the significantly downregulated metabolites. **c** Principal component analysis depicts metabolic profiling results prior (T0) and after (T1) KD. n = 40.

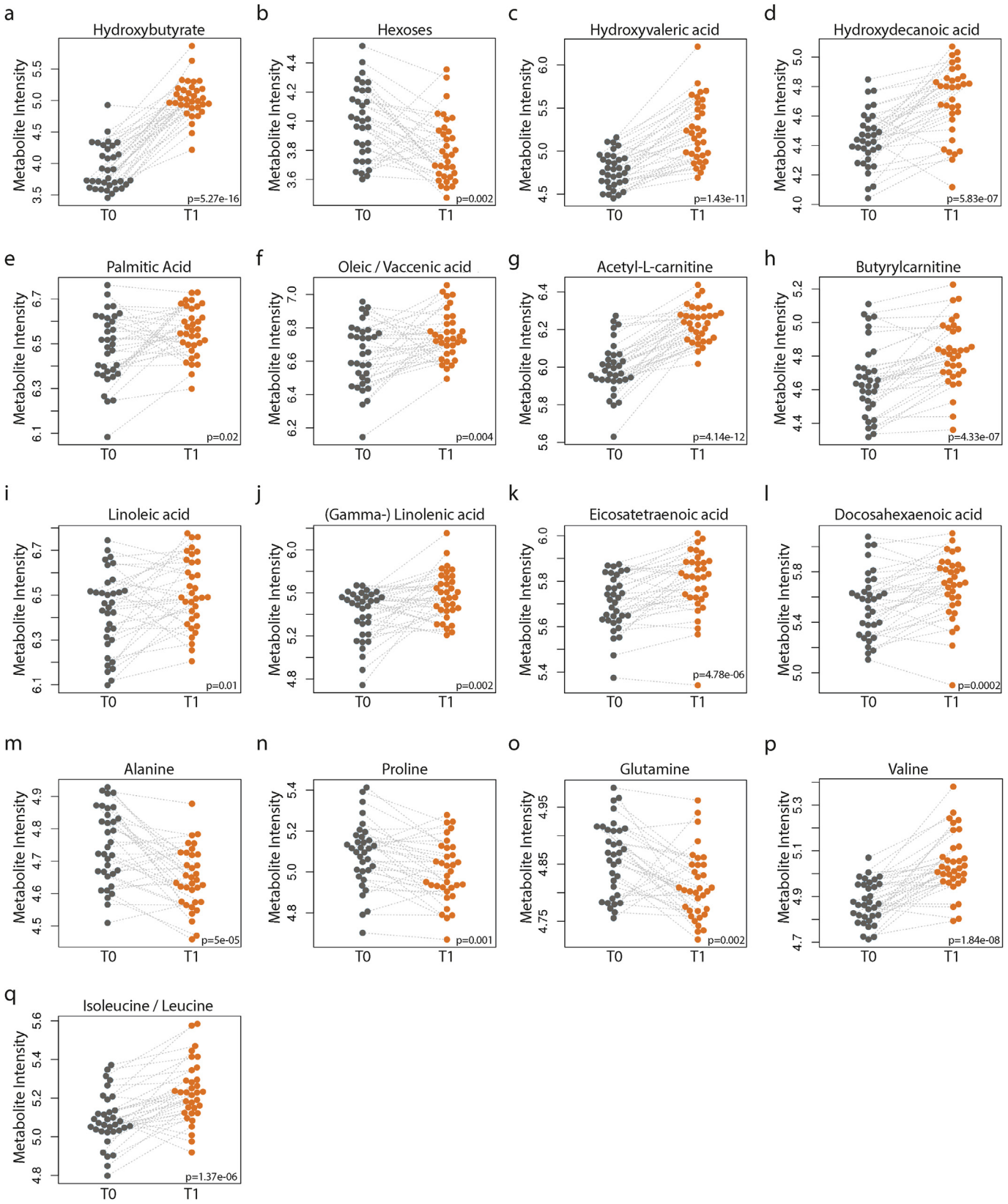

Fig. 3. Mass spectrometric non-targeted serum metabolome analyses before (T0) and after (T1) KD. Relative quantification (normalized metabolite intensity, log 10, arbitrary units) of (a/b) β-hydroxybutyrate and hexoses, (c–f) fatty acids, (g/h) carnitines, (i–l) omega-3/6 polyunsaturated fatty acids and (m–q) amino acids. *P*-values as indicated, n = 40.

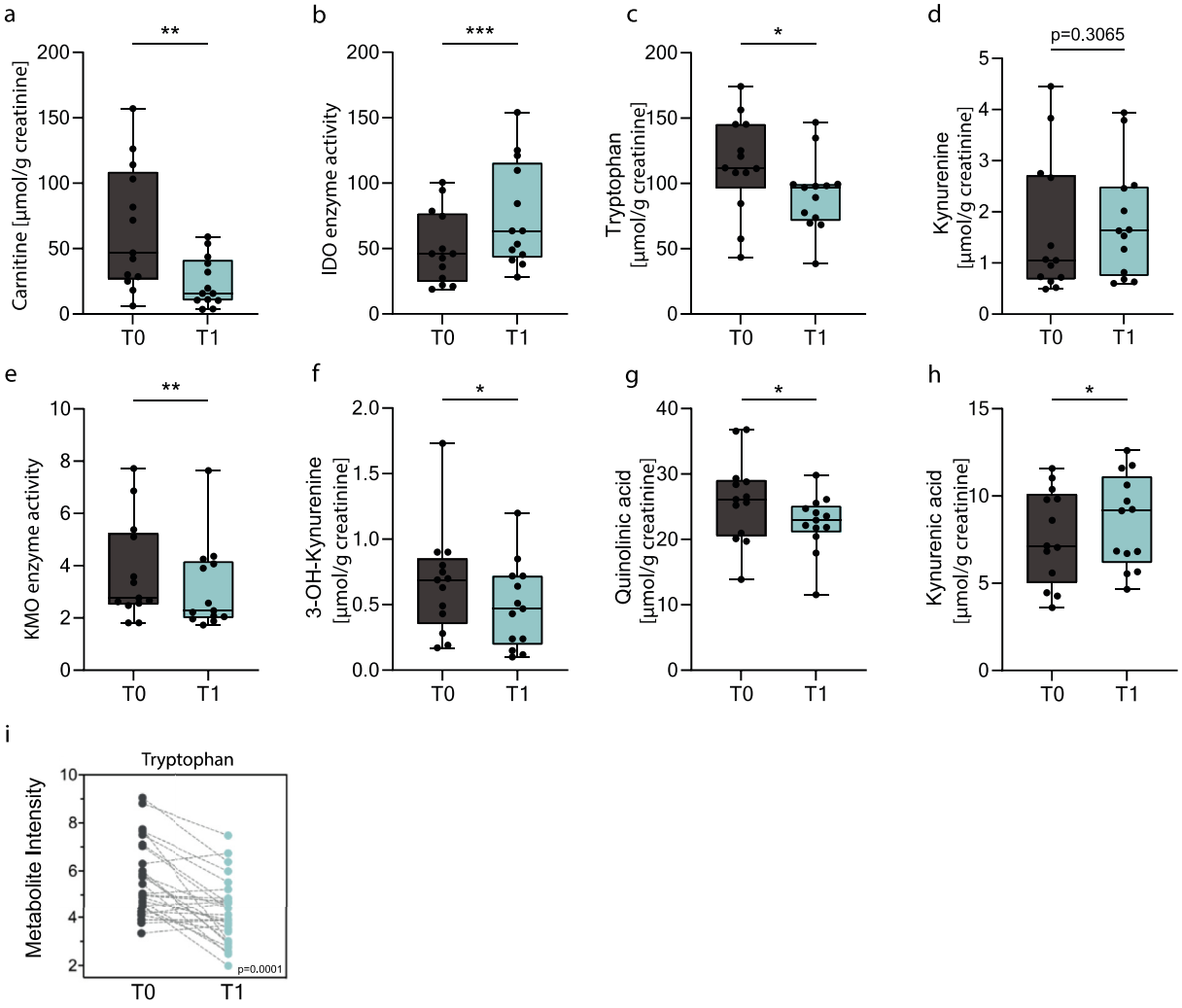

Fig. 4. Analysis of urine samples prior to (T0) and after (T1) healthy participants conducted a three-week ad libitum diet. **a-h** Urinary concentrations and calculated enzyme activities as indicated, n = 13. IDO enzyme activity = Kynurenine [µmol/g creatinine]/Tryptophan [µmol/g creatinine] × 1000, KMO enzyme activity = quinolinic acid/kynurenic acid. KMO=Kynurenine-3-monooxygenase. **i** Mass spectrometric non-targeted serum metabolome analyses before (T0) and after (T1) KD, relative quantification (normalized metabolite intensity, log 10, arbitrary units) of tryptophan. *p < 0.05, **p < 0.01, ***p < 0.001.

these results, tryptophan levels were significantly decreased ($p = 0.0103$), while an increase of kynurenine was observed (Fig. 4c/d). Physiologically, kynurenine is further metabolized to kynurenic acid via the kynurenine aminotransferase (KAT) or hydroxylated by kynurenine-3-monooxygenase (KMO) which finally leads to the production of quinolinic acid [22]. KMO activity was significantly attenuated on a KD ($p = 0.0081$, Fig. 4e). Consequently, concentrations of both 3-hydroxy-L-kynurenine ($p = 0.0407$), and quinolinic acid ($p = 0.0478$) were reduced (Fig. 4f/g) while secretion of kynurenic acid was markedly enhanced ($p = 0.0269$, Fig. 4h). Of note, analysis of mass spectrometric metabolome data corroborated a decrease of tryptophan ($p = 0.0001$, Fig. 4i).

Discussion

Very low-carbohydrate diets, based on the endogenous production of ketones as alternative energy substrates, have emerged as a possible countermeasure to nutrition-induced "metaflammation" [23]. The ketone BHB has been shown to put the brake on overactive innate immunity by inhibition of the NLRP3 inflammasome [24,25]. Moreover, we could recently demonstrate a strong positive influence of KD on human adaptive immunity by improving T-cell functions and reinforcing differentiation of regulatory and memory T cells [9]. While the production and metabolic utilization of ketone bodies is seen as being mainly responsible for these beneficial effects, the role of other metabolites in this setting has not yet been investigated. It is important, however, to gain insight into the changes to the human metabolic fingerprint caused by KD, not only to identify metabolites that contribute to the positive overall influence on human immunity, but also to identify potential health risks of KD.

We here show that even a short-term ketogenic diet of three weeks fundamentally reshapes the human metabolome. We found an astonishingly fast readjustment of the metabolic steady state towards production and utilization of ketone bodies with improved insulin and triglyceride levels, and an augmentation of metabolites mediating anti-inflammation and mitochondrial protection.

Reducing carbohydrates improves insulin levels and modulates amino acids closely linked to T-cell differentiation

Sharp reduction of carbohydrate intake, as performed by our study participants, led to a clear decrease of both insulin and c-

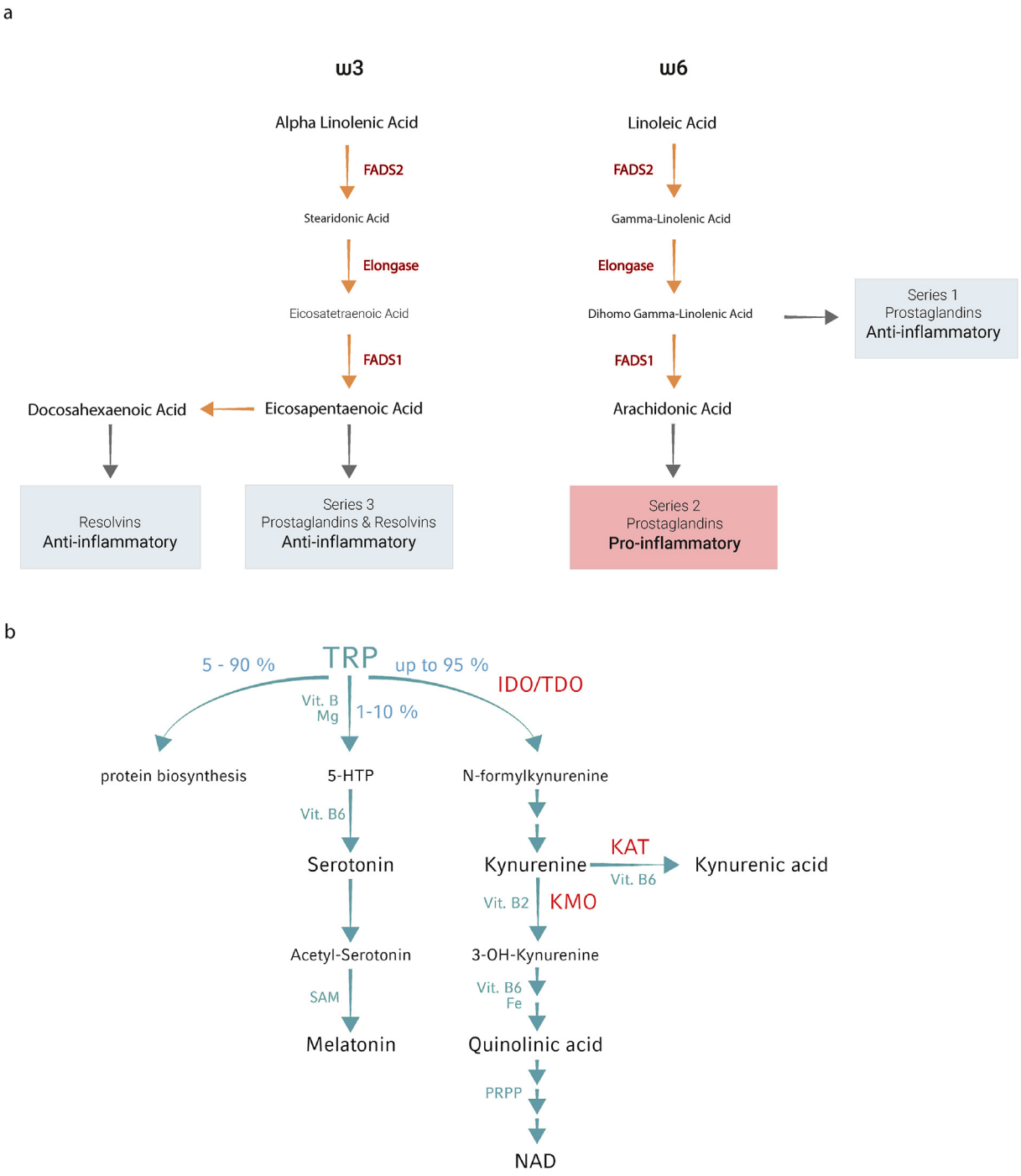

Fig. 5. **a**Schematic diagram of the omega-3 (left) and omega-6 (right) polyunsaturated fatty acid pathway with resulting pro- and anti-inflammatory lipid mediators. FAD=Fatty acid desaturase. **b**Schematic diagram of tryptophan metabolism. TRP = Tryptophan, IDO=Indoleamine 2,3-dioxygenase, KAT=Kynurenine aminotransferase, KMO=Kynurenine-3-monooxygenase. NAD=Nicotinamide adenine dinucleotide. Modified according to [38].

peptide levels. This could explain the moderate weight loss that occurred throughout the entire study population even without any caloric restriction [26,27]. Our data shows no evidence that the extent of weight loss correlates to the changes of serum metabolic parameters, pointing to a more comprehensive effect of ketone bodies and nutrient composition.

Importantly, fasting serum glucose concentration remained stable, indicating an unimpaired physiological function of hepatic gluconeogenesis on a KD [9]. The increased urea concentration was presumably due to an enhanced protein metabolism, as participants following a KD generally consume more protein than those on conventional diets. Concentrations of the glucogenic AA proline, alanine, and glutamine were markedly reduced, which might be the result of elevated consumption due to gluconeogenesis. Conversely, BCAA valine, leucine and isoleucine showed higher abundances, most likely resulting from increases in protein uptake. These changes of the AA profile could also contribute to the previously reported redirection of the CD4 T-cell fate on a KD [9]. BCAA

are essential for Treg expansion and function, whereas glutamine is intimately linked to the differentiation of pro-inflammatory Th1 and Th17 lineages while attenuating the development of Tregs [28–31].

Ketogenic diet shifts metabolism towards fatty acid utilization and improves lipid profile

Disproving the common assumption that a KD negatively affects lipid metabolism, increased fat consumption did not deteriorate but rather improved serum lipid profiles of the participants. All cholesterol parameters remained unaltered, highlighting the significance of endogenous synthesis - particularly under the influence of insulin - rather than nutritive uptake [32]. Fasting triglyceride levels significantly decreased, which indicates increased lipolysis due to lower blood insulin and use of triglycerides for ketone body synthesis. Given the marked reduction in carbohydrate intake, the reduced TAG may also reflect reduced de novo lipogenesis [26]. Of note, a profound upregulation of both free fatty acids and acylcarnitines was detected. It has previously been shown that serum acylcarnitines adequately reflect tissue acylcarnitine content [33,34]. Elevated acylcarnitines on a KD represent the increased demand for long-chain fatty acids as substrates of β-oxidation, that must be transported into the mitochondrial matrix using carnitine as a transport shuttle system [35]. Thus, metabolomics corroborates profound nutritional changes with increased ingestion of fat and indicates a substantial augmentation of fatty acids and fatty acid transport into mitochondria to support energy supply through ATP-generation via tricarboxylic acid cycle on a KD [36,37]. Interestingly, we further found a shift in fatty acid serum profiles: While levels of both essential fatty acids, linoleic acid (ω-6) and linolenic acid (ω-3), were increased on a KD, particularly anti-inflammatory eicosatetraenoic acid (ETA) and docosahexaenoic acid (DHA), both end products of (ω-3) α-linolenic acid metabolism, were significantly elevated. Although it cannot be deciphered whether endogenous synthesis based on linolenic acid or altered nutritional uptake account for the increase of ETA and DHA, the observed shift may contribute to the dampening of innate inflammation (Fig. 5a).

Ketogenic diet modulates tryptophan metabolism increasing immunomodulatory and mitoprotective metabolites

Augmented T-cell function on a KD is causally linked to increased mitochondrial energy production [39], which requires optimal metabolic support of mitochondrial fitness [40]. In this context, urine analyses of the tryptophan/kynurenine/quinolinic acid pathway may yield additional information. We could show that a period of KD markedly shifts the metabolism of tryptophan towards kynurenine and kynurenic acid while attenuating synthesis of quinolinic acid. We were also able to confirm these findings via mass spectrometric metabolome analysis. Kynurenic acid is assumed to exert protective effects in mitochondrial respiration, whereas accumulation of quinolinic acid is associated with mitochondrial dysfunction [41,42]. Thus, increased oxidative phosphorylation is supported by improved mitochondrial protection. Metabolites from tryptophan metabolism have also been discussed to impact human Treg/Th17 balance. Kynurenic acid was shown to attenuate the Th17/IL17 axis while promoting generation of tolerogenic Treg [43,44], and kynurenine has been proven to enhance Treg expansion and suppressive function [45,46]. The increased abundance of kynurenine and kynurenic acid on a KD could thus influence the balance of T-cell subsets in favor of anti-inflammation (Fig. 5b).

Conclusion

Overall, we demonstrate that even after a relatively short period of KD, profound changes in metabolite composition occurred that could have a positive impact on both metabolic programming and immune cell fate, beyond ketone effects. It is important to point out that no metabolic risk factors have been identified in this setting. A ketogenic diet thus appears to be a useful preventive and therapeutic immunometabolic tool at least in short- and medium-term approaches. Future studies are needed to address whether long-term KD produces similar beneficial effects and whether the implementation of short-term KD into the usual diet might provide lasting metabolic benefits.

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Author contributions

David Effinger and Simon Hirschberger contributed equally to Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing original and revised draft. **Polina Yoncheva:** Formal analysis, Investigation. **Annika Schmid:** Investigation. **Till Heine:** Investigation. **Patrick Newels:** Formal analysis, Investigation, Visualization. **Burkhard Schütz:** Investigation, Methodology, Resources. **Chen Meng:** Formal analysis, Investigation, Visualization. **Michael Gigl:** Investigation. **Karin Kleigrewe:** Formal analysis, Investigation, Methodology, Resources. **Lesca-Miriam Holdt:** Investigation. **Daniel Teupser:** Resources. **Simone Kreth:** Conceptualization, Supervision, Writing original and revised draft. All authors contributed to the interpretation of the data and approved the final version of the manuscript.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2023.04.027.

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