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Exploring the non-targeted metabolomic landscape in endurance-trained runners following 10 weeks of different dietary patterns and concomitant training

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ABSTRACT

Introduction: Established guidelines recommend carbohydrate-rich diets to optimize performance in endurance sports. However, alternative dietary strategies, such as the low-arbohydrate and high-fat (LCHF) diet, have gained increasing attention due to their potential to influence metabolic flexibility and endurance performance outcomes. In this study, we aim to investigate the combined effects of a LCHF diet, along with low glycemic index (LOW-GI) and high glycemic index (HIGH-GI) diets, in conjunction with regular endurance exercise, on the metabolomic profiles of recreational runners. The classification into LOW-GI and HIGH-GI groups is based on the premise that metabolic regulation, particularly insulin response and glucose metabolism, differs significantly between the consumption of high-glycemic and low-glycemic foods.

Methods: The participants ($n = 49$, 28 ± 4 years, BMI: 24.2 ± 2.8 kg/m2, VO2 peak: 56 ± 8 ml/min/kg) were randomly allocated to a LOW-GI ($n = 16$), a HIGH-GI ($n = 16$) or a LCHF ($n = 17$) diet for 10 weeks and the same endurance training intervention. Fasting plasma samples were collected both pre- and post-intervention and were prepared for non-targeted metabolomic analysis using liquid chromatography coupled to high-resolution mass spectrometry.

Results: The LCHF diet had a considerable impact on plasma lipids, whereas the respective effects in the LOW-GI and HIGH-GI groups were less pronounced. Specifically, 179 up- or down-regulated metabolites were identified in the LCHF group, 111 in the LOW-GI group, and 139 in the HIGH-GI group. Phospholipids and sphingolipids were found to be the most prominent metabolites in the samples. Furthermore, the regulation of glycerolipids, carnitine, amino acids, and carbon acids exhibited differential patterns across the groups.

Discussion: There is evidence to suggest that the LCHF diet enhances fat metabolism, as indicated by increased levels of carnitine and ketone bodies, as well as a downregulation of amino acids. Conversely, the presence of specific carbon acids might diminish carbohydrate metabolism and impair endurance performance. In contrast, the LOW-GI group may have demonstrated augmented metabolic flexibility due to the upregulations of both carnitines and carbon acids in the samples. The elevated glycerolipids content in the HIGH-GI group suggests a potential reduction in fatty acid oxidation due to hyperinsulinemia.

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Metabolism; carbohydrate intake; glycemic index; non-targeted metabolomics; phospholipids

1. Introduction

Nutrition and exercise trigger a network of metabolic interactions and adaptations. To assess these interactions, the use of omics technologies is increasingly applied in both sports and nutrition science [1]. Metabolomics cover the study of metabolites in biological systems, such as plasma, serum, or urine [2]. In the context of sport and exercise science, non-targeted metabolomic approaches – where the analytes of

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interest are not predetermined – hold significant potential for generating new hypotheses and deepening our understanding of health and disease [3,4]. When integrated with dietary factors, such as macronutrient composition and glycemic index (GI), metabolomics can provide valuable insights into how different nutritional strategies modulate these mechanisms and enhances our understanding of the interactions between diet, exercise, and metabolism, offering new perspectives on improving performance and long-term health outcomes [5].

The interplay between daily diet, exercise, and metabolism is complex [6] and subject of ongoing research [7,8]. Among the various metabolites discussed are phospholipids, sphingolipids, carnitines, and glycerolipids, which might have the potential to influence performance and substrate metabolism. Phospholipids, which are ubiquitous in nature, are not only indispensable constituents of cellular membranes [9], they might also have the potential to influence endurance performance by affecting the levels of free choline, a precursor for acetylcholine [10,11]. Additionally, studies have shown that ceramides, which are hydrolyzed from sphingolipids [12], have the potential to impede glucose uptake [13] and thereby suppress AMPK activity and impede fatty acid catabolism [14]. Carnitines have been demonstrated to exert a direct influence on the intermediate metabolism of glucose and the beta-oxidation of long-chain fatty acids [15,16] via the carnitine palmitoyl transferase system [17,18]. Moreover, glycerolipids have been speculated to play a role in lipogenesis and glycolysis [19,20].

In the field of sports nutrition, the efficacy of a low-carbohydrate, high-fat (LCHF) diet for increasing fat oxidation has been a topic of recent research [21–24]. However, findings have also indicated that, from a performance perspective, such a diet may not be advisable without caution due to potential impairment of carbohydrate (CHO) metabolism [25]. One potential alternative is the consideration of the glycemic index (GI) of the daily diet [26,27]. The GI reflects the insulinemic response after the ingestion of CHO. Insulin, an anabolic hormone, is capable of suppressing fat oxidation and promoting glucose oxidation via different pathways [28–31]. Consequently, following the ingestion of a high GI CHO, not only blood glucose and insulin are increased, but also fat oxidation is limited and CHO oxidation increased. However, there is currently limited knowledge about the effects of the GI on substrate metabolism. Therefore, the non-targeted metabolomics approach we have employed enables a profiling of metabolites, using spectral libraries for identification. This approach is expected to deepen the understanding of metabolic adaptations resulting from the interplay between exercise and nutritional intervention, and further support previously reported results [27]. By distinguishing the metabolomic profile of athletes following different nutritional patterns, which differ in their carbohydrate content and glycemic index, this study may offer valuable insights into biological processes associated with improved metabolism and performance in the short term and maybe also enhanced health in the long term.

2. Materials and methods

2.1. Ethics approval

The study was conducted in the compliance with the declaration of Helsinki, approved by the Ethics Committee of the Medical University of Vienna (Nr. 1307/2023) and registered at Clinical Trials (NCT05241730). Written informed consent was obtained from all participants. As this study is part of a larger project, the methodology of the whole project will only be described briefly. Therefore, a more detailed description of the methodology of the study and the results of the performance tests and blood marker analysis has been published elsewhere [27,32]. The methodology employed in this study will be elucidated in detail. For the purposes of this analysis, a random sample was selected from the initial project population.

2.2. Study design

The study was designed as a randomized parallel group study (Figure 1) and study duration was 10 weeks. After initial medical screening, participants underwent anthropometric measurements and different performance assessments. Subjects were randomized into one of the three experimental groups according to their

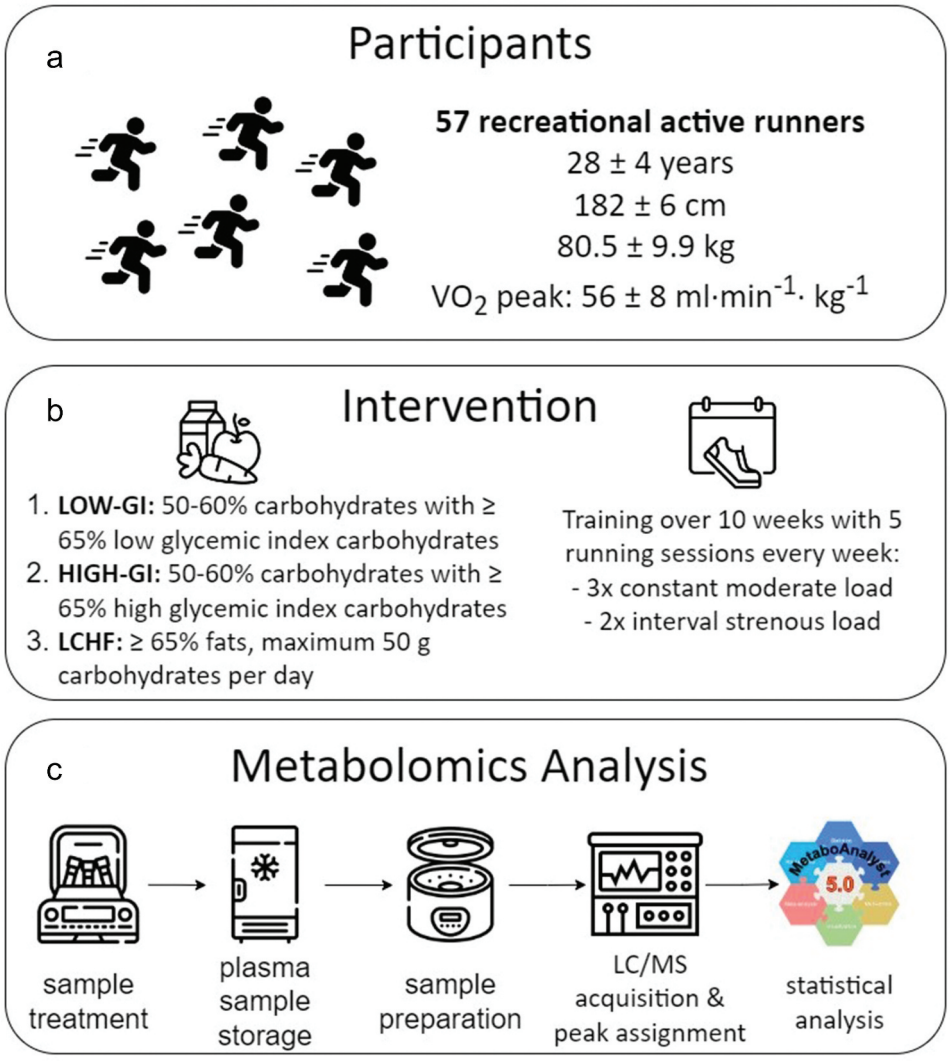

Figure 1. Schematic overview of the study design. (a) Fifty-seven recreational active male runners were recruited. (b) Subjects were randomly assigned to one of the three nutritional regimes. Training intervention was the same for all test subjects. (c) Sample treatment and preparation were performed according to previous described methods. Data treatment and statistical analysis were performed using MetaboAnalyst 5.0.

respective peak oxygen uptake (VO_2 peak) measured in a graded exercise test via breath-by-breath gas analysis (MetaLyzer 3B, Cortex Biophysik GmbH, Leipzig, Germany).

2.3. Participants

After giving written informed consent, 57 healthy males involved in endurance training on a regular basis (2–3 training sessions per week), with a mean (± standard deviation) age of 28 ± 4 years, height 182 ± 6 cm, weight 80.5 ± 9.9 kg, and with an average VO_2 peak of 56 ± 8 ml·min^{-1}· kg^{-1}, volunteered to participate in the study.

2.4. Nutritional intervention

Participants were randomly allocated to one of the three experimental groups as previously described in Moitzi et al. [27]:

(1) **LOW-GI**: 50–60% of the daily energy intake should derive from carbohydrates. More than 65% of these carbohydrates should be low glycemic. Examples of such carbohydrates are whole meal bread, apples, or low-fat yogurt.
(2) **HIGH-GI**: 50–60% of the daily energy intake should derive from carbohydrates. More than 65% of these carbohydrates should be high glycemic. Examples of such carbohydrates are white bread, white rice, or bananas.
(3) **LCHF**: More than 65% of the daily energy intake should derive from fats. The daily carbohydrate intake should not exceed 50 g. Meat, fish, low-starch vegetables, such as cabbage, dietary products with low carbohydrate content and eggs were allowed without any restriction.

After detailed dietary training, the interventions were carried out as free-living intervention. Participants were not restricted in their daily energy intake and prepared meals on their own following the respective guidelines of the assigned group. The completion of 24-h-recalls twice a week for the period of the intervention ensured that the subjects adhered to their assigned diet. Additionally, food frequency questionnaires were completed at the beginning, at mid time and at the end of the intervention.

2.5. Endurance training intervention

The endurance training plan was the same for all groups and the training zones were adjusted individually for each subject based on the results of the graded exercise test. The plan consisted of five running sessions per week, three of which were steady state (intensity around lactate threshold) and two of which were interval sessions (intensity around deviation maximum according to Cheng et al. [33]). The mean total duration of training was 230 min per week. Sessions were carried out individually by the subjects using a heat rate belt (Polar H10, Polar Electro Oy, Kempele, Finland). Compliance to the training plan was checked via the training records on a Polar sport watch (Polar Vantage M, Polar Electro Oy, Kempele, Finland). An example week of the training intervention can be found in the supplementary material of a previously published paper [27].

2.6. Sample collection, storage, and preparation

Blood samples were obtained following an antecubital vein puncture before and after the 10-week intervention in a seated position after an overnight fast. Directly after collection, the tube (BD Vacutainer Tube K2 Edta, Becton Dickinson AG, Allschwil, Schwitzerland) was prepared by centrifugation at 1500 rpm for 20 min at room temperature (EBA 270, Hettich Zentrifugen, Tuttlingen, Germany). After separation, supernatant plasma was instantly collected and stored in 100 µl-aliquots and stored at −20°C until further analysis.

For plasma preparation, the protocol according to Dunn, Broadhurst, Begley, et al. [34] and Jiye et al. [35] was followed. For the quality control (QC), an aliquot of the same plasma sample and one blank were used per run. First, one aliquot per subject and the QCs were homogenized at room temperature. Subsequently, the sample was mixed with 500 µl of 95% methanol, briefly vortexed and then homogenized in a cooled ultrasonic bath. After a three-hour incubation period at −20°C and 20 min in the 4°C cooled centrifuge at 15.000 rpm, a protein pellet precipitated. The supernatant was decanted and dried in a vacuum concentrator (Savant SpeedVac SPD1030–230, Thermo Fisher Scientific, Massachusetts, US). The dried samples were stored at −20°C until measurement. For the measurement, the samples were mixed with 300 µl of a methanol-formic acid solvent and homogenized in an ultrasonic bath.

2.7. Metabolomics measurements

The samples were analyzed using LC-MS. The EXIONLC AD SYSTEM liquid chromatography system (AB Sciex, Darmstadt, Germany) was coupled with an ESI X500 QTOF mass spectrometer (AB Sciex, Darmstadt, Germany). Separation was achieved on a reversed-phase C18 column (Kinetex; 2.1 mm × 10 cm, 2.6 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) using a binary gradient. The mobile phases consisted of mobile phase A (Water/Formic Acid, 100:0.02) and mobile phase B (Acetonitrile/Formic Acid, 100:0.02). The gradient profile was as follows: 0–0.5 min at 2% mobile phase B; 0.5–2.5 min transitioning from 2% to 75%

mobile phase B; 2.6–4 min at 95% mobile phase B; and 4–5 min for re-equilibration at 2% mobile phase B, with a flow rate of 400 µL/min, column temperature maintained at 40°C, and an injection volume of 5 µL for each sample. For mass spectrometric detection, the heater temperature was set to 500°C, with ion source gas 1 and ion source gas 2 at 30 psi, curtain gas at 45 psi, and spray voltages of −4.5 kV for negative ion mode and +5.0 kV for positive ion mode. The TOFMS and TOF/MSMS scan ranges were set from 50 to 1500 m/z, with an accumulation time of 0.1 ms, decluttering potential at −80 V, collision energy at 45 V, and voltage spread at 15 V. The metabolome and lipidome were profiled using an in-house workflow previously established and described in Tahir et al. [36]. Briefly, lipid identification was conducted using MSDIAL ver. 4.9.221218 Windows x64 via the integrated Lipidblast package, ensuring selection of the appropriate modifier type and all possible adducts in the MSDIAL Lipidblast MSP file tab. Metabolite identification was also performed using MSDIAL ver. 4.9.221218 Windows x64, employing the spectral database package "ESI (+)-MS/MS from standards + bio + in silico (16,995 unique compounds), last edited on 21 August 2022. In instances where peak annotation could not be achieved with the included spectra library, HMDB and METLIN Gen235 (purchased on 20 January 2023) were utilized.

2.8. Statistical analyses

Statistical analyses were performed using the software Statistical Package for Social Sciences (SPSS Version 28.0). Data are expressed as mean ± standard deviation. When analyzing the differences between the three experimental groups on either timepoint an analysis of variances (ANOVA) with Tukey post hoc analyses was used. If no normal distribution could be assumed, the non-parametric procedure (Kruskal-Wallis-Test) was used. Significance was accepted at $p < 0.05$. The effect sizes for one-way ANOVA (ηp^2) are displayed for significant results.

For the analysis of the measured metabolites, Metaboanalyst 6.0 (https://www.metaboanalyst.ca/) was used. To analyze changes per group, a paired analysis was carried out with the pre and post values. Features were filtered based on interquartile range with a cutoff of 40%. All samples were normalized by genistein, log transformed, and auto scaled. For the volcano plot, the analyses were carried out as paired t-tests per group with a fold change threshold of 1.5 and a p-value threshold of 0.05 using the raw data from the pre and post samples. For the one-way ANOVA of the post samples, a threshold of 0.05 and Tukey's HSD was used. To compare different metabolites between the groups after the intervention, K-means clustering analysis was done using two cluster numbers and only samples from after the intervention.

3. Results

3.1. Participants

Figure 2 shows the participant flow. A total of eight dropouts resulted in 16 subjects each in LOW-GI and HIGH-GI and 17 subjects in LCHF being included in the analysis. The premature termination of the study was due to personal reasons or an infection with Covid-19, which made it impossible to carry out the training intervention.

Baseline characteristics of subjects, such as age, anthropometric, and endurance performance measures, showed no difference between groups as displayed in Table 1.

3.2. Nutritional intervention

Before the intervention, no significant differences in total energy intake, absolute or relative carbohydrate intake, protein intake, or fat intake (resp., $p > 0.05$, Table 2) were observed. Additionally, the glycemic index of the supplied carbohydrates was not different between groups ($p > 0.05$).

Total energy intake during the intervention (LOW-GI: 1519 ± 215 kcal, HIGH-GI: 2052 ± 425 kcal, LCHF: 1721 ± 446 kcal) differed significantly between groups ($p = 0.003$, $\eta 2 = 0.215$). Post hoc analysis revealed that the energy intake was significantly different between LOW-GI and HIGH-GI ($p < 0.001$) and HIGH-GI and LCHF ($p = 0.012$). Further, intake of macronutrients differed significant between groups ($p < 0.001$ for all analysis, Table 2). Pairwise comparisons between absolute carbohydrate and fat intake were significant ($p < 0.05$). For

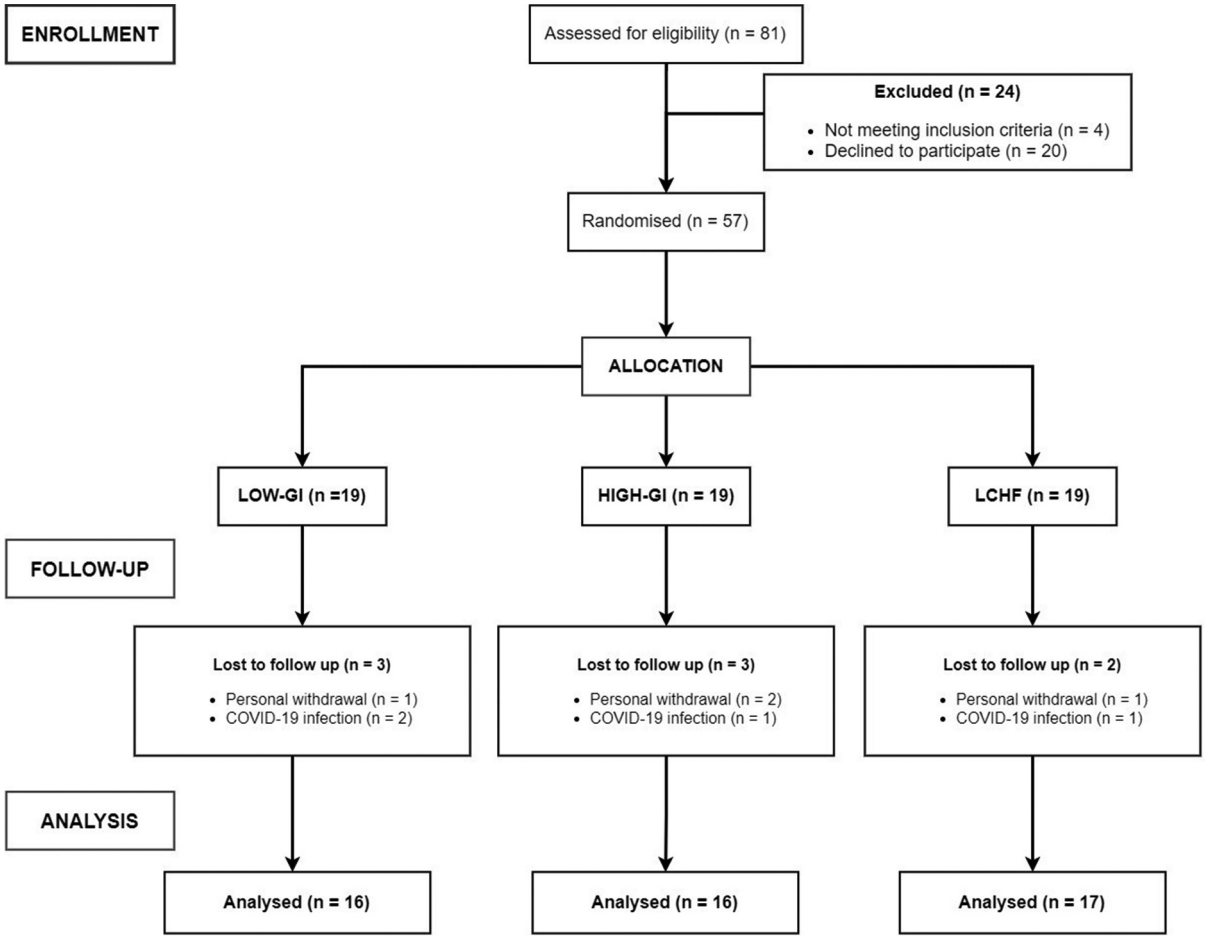

Figure 2. Study flow chart.

Table 1. Subject's characteristics.

	LOW-GI	HIGH-GI	LCHF	*p*-value
Age [years]	30 ± 4	28 ± 3	27 ± 5	.185*
Height [cm]	182 ± 6	182 ± 7	182 ± 7	.991
Weight [kg]	79.1 ± 9.6	80.8 ± 12.1	79.9 ± 7.3	.916*
BMI [kg·min^{-2}]	24.0 ± 2.9	24.4 ± 3.0	24.2 ± 2.2	.833*
Active days per week	3 ± 1	3 ± 1	3 ± 1	.823
VO_2 peak [mL·min^{-1}·kg^{-1}]	56 ± 9	57 ± 7	55 ± 8	.818

*p-value obtained from non-parametric test.

Table 2. Energy and macronutrient intake before and during the intervention.

	LOW-GI		HIGH-GI		LCHF		
	Before	During	Before	During	Before	During	*p*-value
Energy intake [kcal]	1986 ± 454	1519 ± 215^{b}	2068 ± 620	2052 ± 425^{a,c}	2087 ± 686	1721 ± 446^{b}	**0.003***
Carbohydrate intake [g]	249.0 ± 54.4	194.3 ± 33.9^{b,c}	251.1 ± 80.5	266.4 ± 68.5^{a,c}	244.7 ± 78.5	43.1 ± 13.8^{a,b}	**<0.001**
Protein intake [g]	74.2 ± 20.6	71.3 ± 12.7^{c}	82.3 ± 33.1	76.9 ± 20.9^{c}	86.5 ± 38.1	114.7 ± 36.2^{a,b}	**<0.001***
Fat intake [g]	67.3 ± 24.2	42.7 ± 8.6^{b,c}	68.8 ± 23.8	62.6 ± 12.7^{a,c}	76.3 ± 33.0	116.1 ± 31.9^{a,b}	**<0.001**
Carbohydrate intake [%]	51.8 ± 6.7	54.1 ± 3.9^{c}	50.8 ± 5.0	54.1 ± 5.6^{c}	48.9 ± 7.3	11.7 ± 4.0^{a,b}	**<0.001**
Protein intake [%]	15.4 ± 2.5	19.2 ± 2.7^{b,c}	16.3 ± 2.3	15.5 ± 2.7^{a,c}	16.8 ± 2.4	27.8 ± 3.1^{a,b}	**<0.001**
Fat intake [%]	30.9 ± 6.0	25.0 ± 2.8^{c}	30.1 ± 5.4	27.7 ± 4.0^{c}	33.0 ± 6.5	61.1 ± 4.4^{a,b}	**<0.001**
Glycemic index	62 ± 9	42 ± 4	56 ± 9	64 ± 3			**<0.001#**

Bold numbers represent a significant difference between the groups during the intervention. **p*-value obtained from non-parametric test. #*p*-value obtained from unpaired t-test. [a, b, c]indicate significant difference between groups: [a]compared to LOW-GI, [b]compared to HIGH-GI, [c]compared to LCHF.

absolute protein intake, the intake in LCHF differed significantly from LOW-GI ($p < 0.001$) and HIGH-GI group ($p < 0.001$). Relative protein intake differed significantly in all groups (all comparisons $p < 0.05$). For relative fat and carbohydrate intake, the comparison between LOW-GI and LCHF and HIGH-GI and LCHF was significant. Regarding the glycemic index, subjects adhered to the diet and a statistically significant higher GI was found in the HIGH-GI group (LOW-GI: 42 ± 4, HIGH-GI: 64 ± 3, $p < 0.001$).

3.3. Exercise intervention

No differences in training minutes were found neither in total (LOW-GI: 2060 ± 310 min, HIGH-GI: 1869 ± 444 min, LCHF: 1961 ± 378 min, $p > 0.05$), nor when splitting up the minutes in steady-state (LOW-GI: 1434 ± 200 min, HIGH-GI: 1286 ± 330 min, LCHF: 1362 ± 278 min, $p > 0.05$) and interval (LOW-GI: 626 ± 126 min, HIGH-GI: 583 ± 121 min, LCHF: 599 ± 115 min, $p > 0.05$) sessions (Figure 3).

3.4. Metabolomics analysis

A comprehensive master file containing all identified peaks across the various groups is included in the supplementary materials (https://phaidra.univie.ac.at/o:2107160). Additionally, an excel-file details the measured peaks along with their expression levels in each of the intervention groups. This file is formatted to be opened and analyzed in Metaboanalyst without any further manipulation. From the original 3833 measured metabolites, 1902 were removed, because it was not possible to annotate them accordingly. Another 408 duplicates were removed, resulting in a total of 1523 metabolites used for the analysis in Metaboanalyst.

All statistical analyses were conducted using raw p-values to preserve sensitivity in detecting metabolic shifts induced by dietary interventions. Given the exploratory nature of this study, we prioritized biological interpretation and fold-change analysis over strict multiple-testing correction. While false discovery rate (FDR) adjustments are commonly applied in targeted metabolomics, they are often relaxed in untargeted metabolomics to avoid excessive false negatives, which could obscure meaningful trends. Our results are supported by their consistency with established metabolic pathways, reinforcing their biological relevance. To ensure full transparency, the complete dataset, including effect sizes, raw p-values, and statistical outcomes, is provided in the supplementary materials.

3.4.1. Baseline validation

All groups were checked for baseline similarity, to ensure that any observed effects are attributable to the intervention rather than preexisting differences. To assess this, a principal component analysis (PCA) for the baseline groups was performed. The analysis revealed no discernible differences between the groups at baseline (Figure 4).

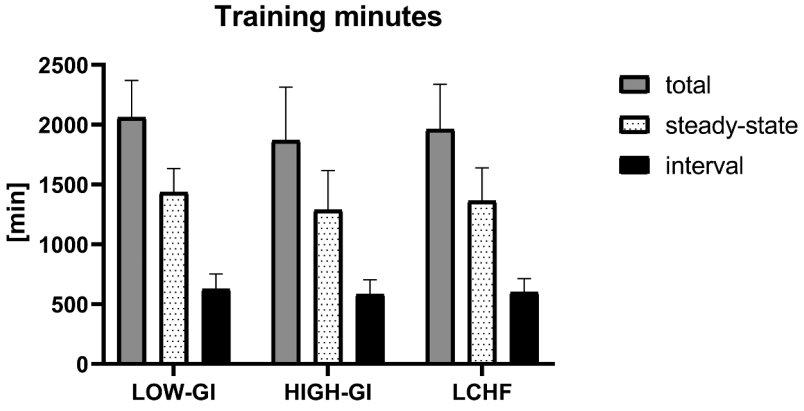

Figure 3. Training minutes in total and divided into in basic and interval sessions over the study period for the three groups.

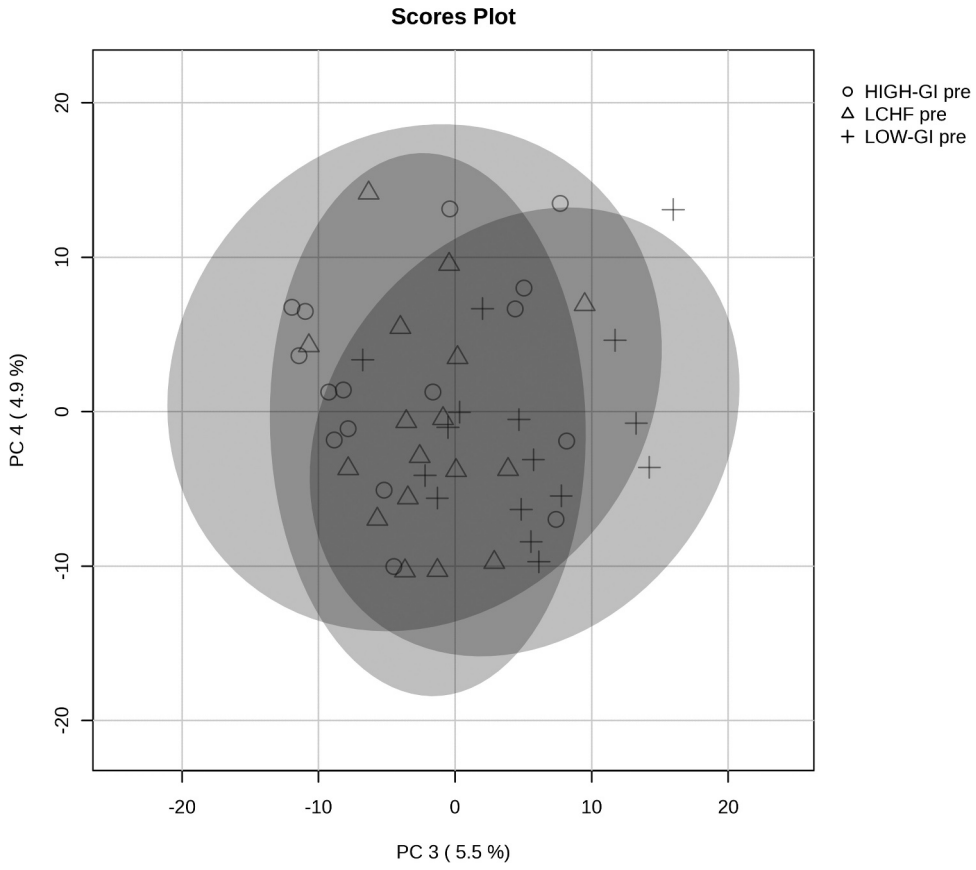

Figure 4. Principal component analysis (PCA) scores plot demonstrating the distribution of samples across PC3 and PC4. PC3 accounts for 5.5% of the total variance, while PC2 explains 4.9%. The clustering of data points indicates that there are no significant differences between the groups at baseline, confirming their similarity prior to the intervention.

Table 3. Top-5 upregulated metabolites for each group.

	Family	FC	log2(FC)	raw p-value	log10(p)
LOW-GI					
Glucocochlearin	Natural product	5.37	2.43	.002	2.64
N-Acetylgalactosamine 6-sulfate	Amino acid	5.51	2.46	.013	1.89
Euparin	Polyphenol	6.07	2.60	<.001	3.86
L-HISTIDINE	Amino acid	6.21	2.63	.008	2.11
PI 38:6	Phoshpolipid	9.20	3.20	.001	3.22
HIGH-GI					
VAE 9:0	Fatty acid	4.60	2.20	.011	1.94
DG 25:1	Glycerolipid	4.75	2.25	.004	2.35
SM 35:8;O2(FA 22:4)	Sphingomyeline	7.41	2.89	.001	3.03
NAOrn 22:2	Amino acid	10.31	3.37	<.001	4.91
SM 12:0;O2/8:0	Sphingomyeline	11.26	3.49	<.001	4.65
LCHF					
DG 36:3|DG 18:1_18:2	Glycerolipid	5.58	2.48	.005	2.33
ST 29:2;O	Sterol lipid	5.87	2.55	.001	3.20
PC O-40:8|PC O-18:2_22:6	Phoshpolipid	6.74	2.75	<.001	3.86
PC O-36:9|PC O-18:5_18:4	Phoshpolipid	7.59	2.92	.001	2.91
PC O-9:0_24:6	Phoshpolipid	8.63	3.11	.005	2.31

3.4.2. Changes during the intervention

By implementing the non-targeted metabolomics workflow, we were able to identify distinct patterns with significantly (up- and down) regulated metabolites in all groups. The volcano plot revealed 179 metabolites significantly regulated in the LCHF group, 139 in the HIGH-GI group and 111 in the LOW-GI group. The analysis of the significantly related metabolites revealed that phospholipids (187 hits across all groups) were the substance class most represented, followed by

glycerolipids (50 hits across all groups) and natural products (41 hits across all groups), such as piperine or retinoids.

Table 3 presents the five most upregulated metabolites according to their fold change (FC) from each group, accompanied by their corresponding FC and raw p-value. Of the 57 metabolites that were upregulated in the LCHF group, the most prominent upregulated substance classes were phospholipids ($n = 25$), carnitines ($n = 5$) and bile acids, carbon acids, glycerolipids, and phosphinic acids ($n = 3$, respectively). In contrast, the LOW-GI group demonstrated a significant upregulation of 47 metabolites when comparing the sample from before the intervention with samples after the intervention. The most prevalent metabolites were glycerolipids ($n = 9$), natural products ($n = 9$), amino acids ($n = 7$), and phospholipids ($n = 7$). In the HIGH-GI group, 14 upregulated metabolites were identified. The most prominent substance classes were sphingolipids ($n = 3$), glycerolipids ($n = 3$), phospholipids ($n = 2$), and fatty acids ($n = 2$).

A closer examination of the downregulated metabolites revealed that 121 were identified in the LCHF group, 64 in the LOW-GI group, and 125 in the HIGH-GI group (Table 4). In the LCHF group, the top three substance classes were phospholipids ($n = 55$), glycerolipids ($n = 16$) and natural products ($n = 15$). Additionally, the LOW-GI group exhibited the greatest abundance of phospholipids ($n = 41$), glycerolipids ($n = 5$), and natural products ($n = 5$) among the top three substance classes. In contrast, the HIGH-GI group demonstrated the highest representation of phospholipids ($n = 57$), sphingolipids ($n = 15$), and glycerolipids ($n = 14$).

3.4.3. Differences after the intervention

The PCA as well as the K-means clustering analysis revealed the presence of two clusters. PC1 accounts for 15% of the variance and PC2 for 10%. Both of them show the LCHF group being distinct from the other two groups (Figure 5).

Similar results were found, when carrying out the K-means clustering. In the first cluster, only samples from the LCHF group were included. In the second cluster, samples from both the LOW-GI and HIGH-GI groups were present (Figure 6).

A comparison of the metabolites via a two-sample t-test of LOW-GI and LCHF samples after the intervention revealed 395 significant differences between the two groups. Similarly, 342 significant differences were observed between the HIGH-GI and LCHF groups. As also demonstrated by the K-means clustering, only 199 significant differences were identified between LOW-GI and HIGH-GI. After the intervention, 365 features were found to be significantly different between the groups. The top 10 features with the lowest p-value are shown in Table 5.

A closer examination of the post-hoc analysis revealed that 10 metabolites exhibited significant differences between all groups, predominantly comprising amino acids such as taurine, histidine, and

Table 4. Top-5 upregulated metabolites for each group.

	Family	FC	log2(FC)	raw p-value	log10(p)
LOW-GI					
PC O-40:5|PC O-16:2_24:3	Phoshpolipid	0.08	−3.59	<.001	3.90
NAGlySer 13:1;O	Fatty acid amide	0.12	−3.10	<.001	4.24
PA(18:1(9Z)-O(12,13)/8:0)	Phosphatidic acid	0.15	−2.70	.001	2.85
Tetrathionic Acid	Thiosulphuric acids	0.16	−2.68	.028	1.55
PC O-42:9	Phoshpolipid	0.16	−2.68	.001	3.23
HIGH-GI					
NAGly 16:2	Fatty acid amide	0.06	−4.03	<.001	5.76
DG 36:6	Glycerolipid	0.09	−3.48	<.001	3.66
VAE 14:1	Fatty acid	0.09	−3.41	<.001	4.96
Cer 12:1;O3/19:0(2OH)	Ceramide	0.11	−3.24	<.001	4.35
PC 37:10	Phoshpolipid	0.12	−3.11	<.001	4.21
LCHF					
2-[(4S,5S,5aS,9aS)-4-methoxy-6,6,9a-trimethyl-5-[(2E,4E,6E)-octa-2,4,6-trienoyl]oxy-1-oxo-4,5,5a,7,8,9-hexahydro-3 H-benzo[e]isoindol-2-yl]pentanedioic acid	Natural product	0.07	−3.78	<.001	3.81
Phenylalanine	Amino acid	0.11	−3.21	.002	2.61
HILIC-L-(-)-Phenylalanine	Amino acid	0.11	−3.20	.001	2.83
DG(18:3(9,11,15)-OH(13)/0:0/i-15:0)	Glycerolipid	0.11	−3.18	<.001	3.57
HILIC-Homoproline methyl ester	Amino acid	0.11	−3.16	.001	2.94

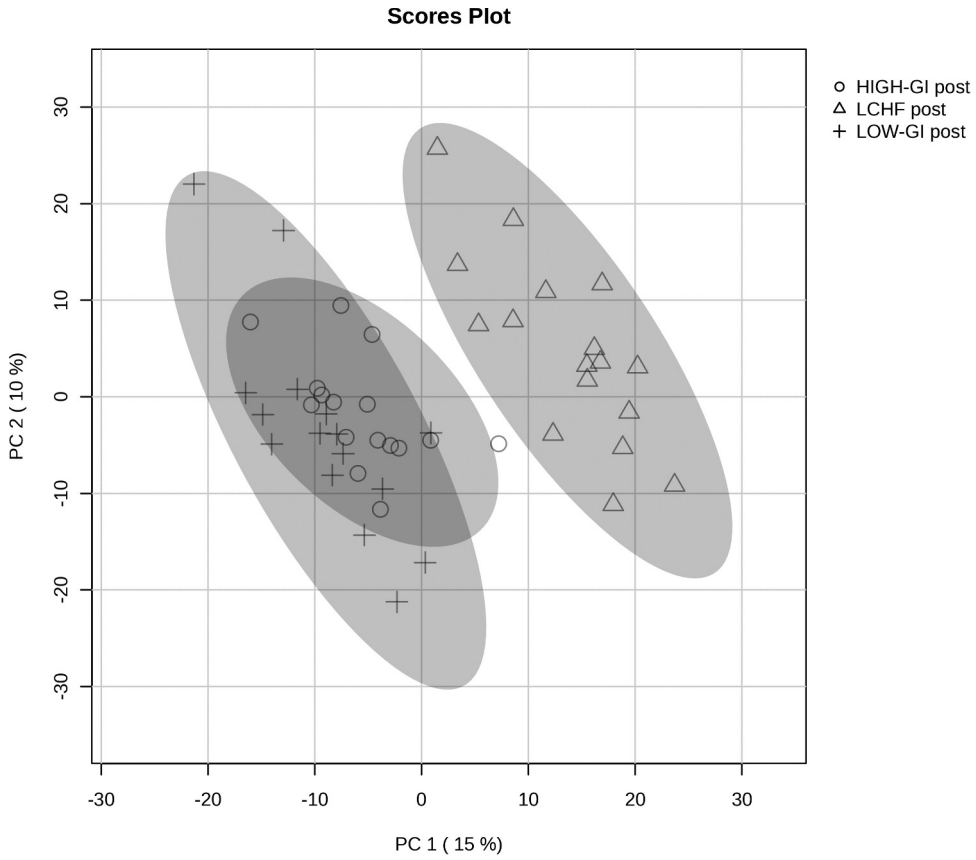

Figure 5. Principal component analysis (PCA) scores plot demonstrating the distribution of samples across the first two principal components (PC1 and PC2). PC1 accounts for 15% of the total variance, while PC2 explains 10%.

N-acetyltaurine. A comparison between LCHF and LOW-GI revealed the presence of 39 metabolites exhibiting notable differences. The majority of the metabolites that exhibited significant differences between the two groups were phospholipids, along with a few glycerolipids and amino acids. The comparison between the LCHF and HIGH-GI groups identified 31 metabolites with notable discrepancies, the majority of which were phospholipids and carnitines. In contrast, the comparison between the two carbohydrate groups revealed only 16 metabolites with significant differences, the majority of which were also phospho- and sphingolipids, along with two glycerolipids. A comparison of the LCHF group with both CHO groups revealed 185 significant metabolites, while a comparison of the LOW-GI group with the other groups yielded 59 metabolites and a comparison of the HIGH-GI group with the other groups yielded 25 metabolites. In addition to phospho- and sphingolipids, carnitine, glycerolipids, and amino acids exhibited notable differences when the LCHF group was compared to the CHO groups. Notably, glycerolipids and fatty acids exhibited differential regulation between the LOW-GI and the other groups. Carbon acids exhibited significant differences between the HIGH-GI and LOW-GI or LCHF groups.

4. Discussion

This 10-week lasting interventional study focused on the metabolic landscape of recreational active runners, which followed a prescribed training and nutritional intervention. Participants were similar at baseline, but during the intervention period the dietary pattern differed significantly in their energy and CHO intake and GI, while the training intervention remained consistent for all participants. Non-targeted metabolomic analysis revealed a distinct difference between the LCHF diet and the carbohydrate-rich diets. The volcano plot indicated that the LCHF diet exhibited the greatest change, with 179 metabolites exhibiting significant regulation, followed by the HIGH-GI group, which exhibited 139 regulated metabolites. In contrast, the metabolomic landscape of the LOW-GI group was the least affected, with only 111 metabolites exhibiting

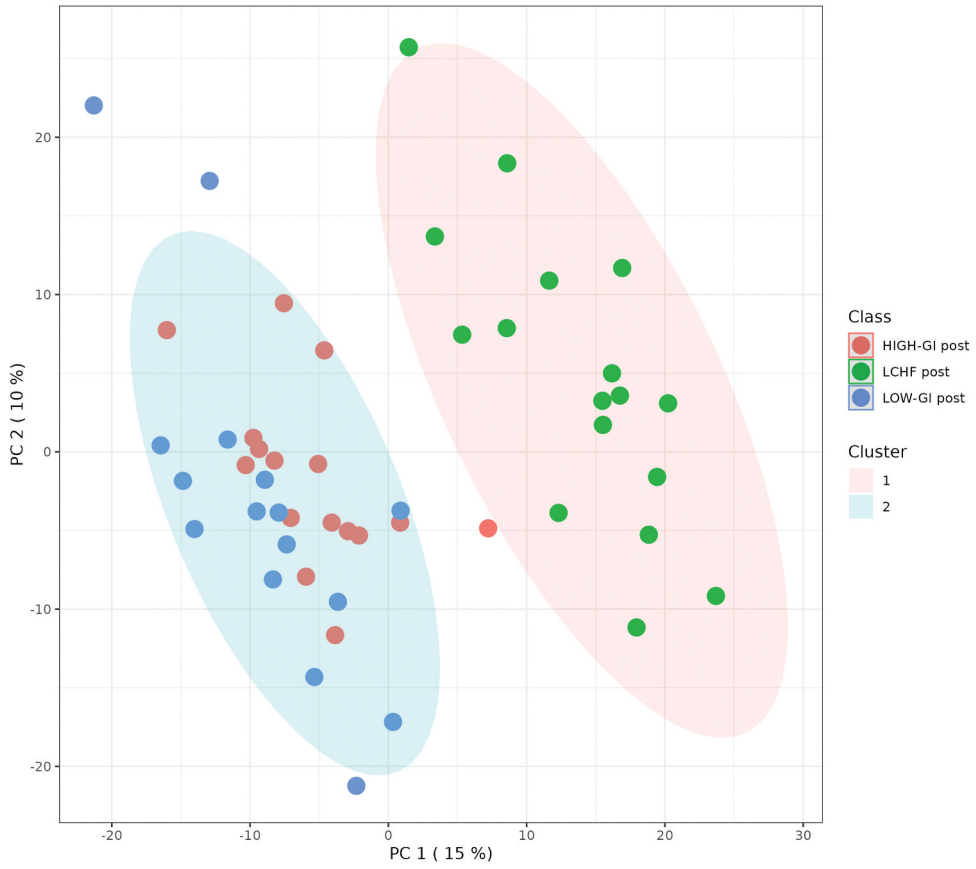

Figure 6. K-means clustering after the intervention.

Table 5. Top-10 features of one-way ANOVA.

	Family	p-value	log10(p-value)	Post-hoc tests
Phosphoric acid	Inorganic aicd	<.001	48.9	LOW-GI vs. other
Taurochenodeoxycholate-7-sulfate	Bile Acid	<.001	35.3	LCHF vs. other
Isothiocyanic Acid	Sulfonic acid	<.001	32.2	LCHF vs. other
Para-cresol	Amino acid	<.001	22.4	LCHF vs. other
LysoPC(20:4(8Z,11Z,14Z,17Z)/0:0)	Phospholipid	<.001	22.0	LCHF vs. other
D-Glucose	Monosaccharid	<.001	22.2	LCHF vs. other
Taurine	Amino acid	<.001	19.5	all
Cyclocytidine	Nucleoside	<.001	18.6	LCHF vs. other
Gluconic acid	Carbon acid	<.001	18.4	LCHF vs. other
Furaquinocin C	Natural product	<.001	18.3	LCHF vs. other

significant regulation throughout the intervention. The primary aim of this study was to provide a descriptive overview of changes in the metabolomic landscape following the 10-week nutritional- and training intervention period. Therefore, the observed differences should be used to raise new hypothesis for future investigations. The following section will present and discuss several new hypotheses.

In the present study, the LCHF group demonstrated the greatest number of upregulated phospholipids. This may be attributable to the fact that, phospholipids are present in a variety of dietary sources, including milk, egg yolk, and soy bean [37]. The low phospholipid content of fruits, grains, and vegetables [10] explains why they are the most prominent family being downregulated in the carbohydrate groups. While phospholipids do not directly influence ATP production and thus the energy supply, their presence affects energy metabolism by supporting and regulating cell structure and signal transmission [38]. Given the significant influence of energy metabolism in the LCHF group, with an increased fat oxidation and decreased carbohydrate oxidation [27], the extent to which phospholipids regulate energy metabolism needs further investigation. Furthermore, the impact of phospholipids on performance during prolonged running is a subject of ongoing debate [10,39]. Our findings from this

observation published in Moitzi et al. [27] appear to contradict those reported by Høeg et al. [39], which indicated that ultramarathon-runners with faster finishing times exhibited higher concentrations of phospholipids. From our data, the LOW-GI group demonstrated the most significant enhancements in both the graded exercise test and the 5 km time trial [27]. Nevertheless, it should be noted that comparisons between the two studies are challenging due to differences in the respective study populations (professional vs. nonprofessional athletes).

Furthermore, sphingolipids were dominantly identified in the HIGH-GI group, and some sphingolipids were found to exhibit notable differences between the groups following the 10-week intervention which align with previous reports after a very low-carbohydrate, high-fat diet, either alone or combined with high-intensity interval training [16]. Derived primarily from milk and dairy products, sphingolipids are increasingly recognized as bioactive lipids and the changed intake due to the nutritional intervention might explain the highest regulation of sphingolipids in the HIGH-GI group. It appears that ceramides, which are hydrolyzed from sphingolipids, exert a significant influence over the insulin signaling cascade [13,14]. Nevertheless, the precise underlying mechanisms remain unclear, particularly given the significant downregulation of sphingolipids observed in the HIGH-GI group with the highest intake of refined sugars. This is despite the fact that the literature would suggest that insulin signaling was hypothesized to be the most upregulated in this group when compared to those following LOW-GI or LCHF diets [40].

Upregulated metabolites especially in the LCHF group included phosphocholine, carnitine, and other fatty acids. These findings align with those of a study by Effinger et al. [41]. The increased fat intake during an LCHF diet was found to be associated with an increase in serum fatty acids and acylcarnitine, while glucogenic amino acids, including alanine, glutamine, and proline, experienced a reduction, as observed in our data. The lower insulin levels during a LCHF diet, but also during the low GI nutrition, influence energy metabolism. Carnitine and its derivates have a direct influence on the intermediate metabolism of glucose and beta-oxidation of long-chain fatty acids [15,16] via the carnitine palmitoyl transferase system [18]. Thereby, carnitine plays an indispensable role in the oxidation of fatty acids due to its function in facilitating the transfer of fatty acids into the mitochondria. The hypothesis that a low GI nutrition may influence substrate metabolism toward a higher fat oxidation, as observed in previous studies on a LCHF diet [22,24], is supported by the measured carnitine levels in the samples. A total of six carnitines were identified in the HIGH-GI group, all of which demonstrated downregulation, indicating a reduction in fatty acid oxidation. In contrast, the LOW-GI group exhibited upregulation of a single carnitine. The LCHF group exhibited seven carnitines, five of which were upregulated and two downregulated. These observations may be indicative of an increased fatty acid oxidation in the LCHF group, with the LOW-GI group displaying indications of enhanced fat oxidation and the HIGH-GI group exhibiting a restricted fatty acid metabolism.

Due to the increased concentration of free fatty acids following a LCHF diet, an increased concentration of different lipids was measured in this group. Blood ketone body concentrations were measured after the 10-week intervention and were around 0.8 ± 0.4 mmol/l, indicating that ketosis was ongoing. This indicates a successful metabolic adaptation to a ketogenic diet, where the body shifts from glucose to fat metabolism as a primary source of energy. This shift could be also perceived in the observed downregulation of the amino acids not only in the LCHF group, but also after the low GI nutrition [42]. The downregulation of amino acids may indicate that the body may be optimizing its metabolic pathways, utilizing ketones rather than amino acids for energy, hereby preserving muscle mass and minimizing protein breakdown [43]. On the other hand, the upregulation in the phosphocholines may indicate an enhanced membrane synthesis and repair, which is particularly important in a metabolic state that prioritizes fat utilization [44]. Taken together, these observations may indicate a positive metabolic state involving efficient use of energy and cellular housekeeping and may rise new hypothesis to what extend the carbohydrate content and GI in the daily diet play a role in improving metabolic health and reducing amino acid catabolism. However, from the metabolomic measurements, carbon acids such as lactic acid or glutamic acid may be indicative of reduced carbohydrate oxidation in the LCHF group [45].

Moreover, the function of glycerolipids in the metabolomic profile will now be shortly discussed. They play a pivotal role in energy storage and provision, as fatty acids are synthesized to ATP via beta-oxidation, whereas the released glycerol can be used for energy production via glycolysis. Additionally, glycerolipids are involved in lipogenesis, the conversion of excess carbohydrates into fats, which occurs when there is a high carbohydrate intake [20]. The majority of upregulated glycerolipids

were identified in the HIGH-GI group (three out of 14 metabolites), followed by the LOW-GI group (nine out of 47 metabolites) and the LCHF group (three out of 54 metabolites). Consequently, there is evidence to suggest that glycerolipids may be linked to a reduction in fat oxidation and insulin sensitivity in the HIGH-GI group [46]. In contrast, among the downregulated metabolites in the LCHF and LOW-GI groups, approximately 13% and 12%, respectively, were glycerolipids, suggesting an elevated rate of fat oxidation. One potential explanation for this observation is the chronic hyperinsulinemia that occurs during the high-GI diet. Elevated insulin levels exert a substantial impact on fatty acid oxidation, primarily through the inhibition of fat oxidation and the promotion of glucose utilization [30]. However, also in the LOW-GI group, upregulation of glycerolipids was found, indicating a need for further data to elucidate the role of insulin, glycerolipids, and the regulation of energy metabolism.

A reduction in energy intake was observed in the LOW-GI and LCHF groups. At the outset of the study, participants were instructed to refrain from restricting their energy intake and to consume food until they feel full. Accordingly, the intervention was conceptualized as a free-living intervention, whereby subjects were instructed to prepare their own food in accordance with the respective group. This design was selected to ensure the greatest possible resemblance to real-life conditions. It is possible that the decrease observed in the LCHF group is attributable to the increased satiety that has been documented following such a diet [47]. In the LOW-GI group, the increased fiber intake may have regulated the feeling of hunger, and due to even insulin and glucose curves, cravings decreased [48]. However, although subjects received detailed nutritional training for their respective group, food selection was most regulated in these two groups. Yet, no distinct metabolomic landscape, which might reflect the differences in energy intake, was observed. Consequently, the extent to which reduced energy intake may have influenced the metabolomic landscape remains uncertain, but it could be speculated that some upregulated lipids in the LCHF and LOW-GI group are due to the reduced energy intake compared to the HIGH-GI group.

Recent research on non-targeted metabolomics analysis in the context of dietary patterns combined with endurance exercise has elucidated the complex interrelationships between nutrition, metabolism, and athletic performance. As evidenced by the presented data, the type of diet consumed can significantly influence the metabolic adaptations that occur during prolonged exercise. This suggests the possibility of a synergistic effect, whereby both dietary strategies and exercise modalities work together to optimize metabolic outcomes. It has recently been demonstrated that the metabolic profiling of an athlete's blood samples, which are influenced by their specific training regimes and dietary habits, can reveal distinct patterns [49]. A closer examination of the ANOVA, the PCA and cluster analysis conducted on the samples following the intervention might at the first glance not corroborate our hypothesis that the magnitude of the GI influences metabolism [26]. The LCHF group showed a distinctive metabolic profile, while no such clear distinction was observed between the two CHO-rich groups, which only differed in GI.

The limitations of the study design have been discussed previously [27,32]. Specifically, to these results, it remains unclear how the exact nutritional intake or physical activity on the day before the measurement might influence results, as this has not been evaluated. However, it can be excluded that changes in body composition or weight loss might have influenced the results because changes were similar in all three groups. While our method allowed us to describe the metabolomic landscape, the non-targeted approach does not allow precise ordering of species at the "molecular species level" or "full structure level." Without this level of detail, drawing conclusions about potential biomarkers and pathway analyses becomes challenging. Therefore, it is essential to take these identified species and reanalyze them using a targeted metabolomics approach, validating the targets with internal standards. Therefore, the study's results were not used to discuss mechanism or draw definite conclusions, but rather to describe observed differences between nutritional regimes, and raise new hypothesis for future investigations.

5. Conclusion

The present study investigated the impact of low-glycemic index, high-glycemic index, and low-carbohydrate, high-fat diets on the metabolomic profiles of recreational active runners. According to a principal component analysis, no distinct differences were observed between the high GI and low GI

diets, whereas the LCHF group exhibited a unique metabolic signature. The most prevalent lipid families among all groups were identified as phospholipids, glycerolipids, and sphingolipids.

The LCHF diet resulted in a distinct shift toward fat metabolism, as evidenced by an increased carnitine content, elevated ketone bodies, and the downregulation of amino acids, which indicates a protein-saving effect. Nevertheless, the presence of certain carbon acids may suggest a reduction in carbohydrate metabolism, which could have a detrimental impact on overall performance. Furthermore, in the LOW-GI group, an increase in carnitine and carbon acids accompanied by a downregulation of amino acids may be indicative of enhanced metabolic flexibility. Moreover, the upregulation of glycerolipids indicates an impaired fat oxidation following the high GI nutrition.

In conclusion, new hypotheses regarding the influence of different diets have been proposed. Further investigation with a more precise methodology and comprehensive analysis of targeted metabolites playing a role in energy metabolism would facilitate a deeper understanding of long-term effects of these dietary interventions on physically active individuals.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article or its supplementary materials (https://phaidra.univie.ac.at/o:2107160).

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