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Original article

Combined mediterranean diet-based sustainable healthy diet and multicomponent training intervention impact on plasma biomarkers and metabolome in older adults

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SUMMARY

Background and aims: Healthy dietary patterns and exercise practices have been associated with improved metabolic and inflammatory profiles. However, studies regarding the combined effect of these interventions on plasma biomarkers and metabolome in older adults are sparser. The primary aim of this study was to investigate the impact of a combined Mediterranean Diet-based Sustainable Healthy Diet (SHD) and Multicomponent Training (MT) intervention on the plasma biomarkers and metabolome and how dietary intake and exercise could modulate these effects.

Methods: SHD intervention included a weekly supply of Mediterranean Diet-based SHD food and four nutrition sessions involving a Mediterranean-Diet culinary workshop, and the exercise program included 50-min MT group sessions, held three times a week, lasting both 12 weeks. Plasma biomarkers were obtained through standard biochemical analysis. A proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy-based metabolomics approach was used to study the metabolome in blood plasma. Repeated measures ANOVA were performed and adjusted for confounders.

Results: SHD + MT intervention significantly decreased HDL-C and calcium. SHD + MT showed some changes in common with the SHD and MT group, namely a significant decrease in citrate levels ($p = 0.009$ for SHD + MT; $p = 0.037$ for SHDT) and an increase in pyruvate ($p < 0.001$ for MT and SHD + MT). The SHD + MT group also revealed specific changes in the levels of some amino acids (decrease in alanine, glutamine and lysine: $p = 0.026$; $p < 0.001$; $p = 0.038$, respectively). Increases in formate ($p = 0.025$) and unsaturated lipids ($p = 0.011$) are consistent with changes in energy and lipoprotein metabolism.

Conclusion: Our data show that a combined lifestyle intervention program, including a Mediterranean Diet-based SHD and MT, could modulate biomarker and metabolome and there seems to be a metabolic path associated to these interventions in older adults. Due to its wide-ranging relevance, it is pertinent to assess to what extent combined SHD and MT can contribute to better clinical profiles.

1. Background

Sustainable healthy dietary patterns (SHD), richer in local and traditional plant-based foods - such as the Mediterranean Diet [1] -

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have been not only associated with lesser environmental impact but also with nutritional adequacy, longevity, and health outcomes, claimed by their synergic antioxidant and anti-inflammatory properties [2–4]. SHD could improve the lipid (lowering low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglycerides (TG)) and the inflammatory profile and are associated with a reduction in C-reactive protein (CRP) [5–8]. Mediterranean Diet has also shown an impact on the levels of several metabolites in blood plasma, such as amino acids, triglycerides, phospholipids, cholesterol esters, and fatty acids [9]. Mediterranean Diet metabolic profile has been associated with lower cardiovascular disease risk [10]. Some plasma metabolites have been linked with the intake of particular foods included in a Mediterranean Diet-based Sustainable Diet, namely betaines with whole grains, n−3 polyunsaturated fatty acids (PUFA) with fish, and tocopherol and linolenic acid with walnuts [7].

Limited research exists on the impact of multicomponent training (MT) - combining aerobic, resistance, flexibility, and balance exercises - on plasma biomarkers, with most studies focusing on either aerobic or resistance training and showing a decrease in LDL-C, TC, CRP, insulin, and glucose, and an increase in high-density lipoprotein cholesterol (HDL-C) [11,12]. Previous studies on MT programs have supported the hypothesis that they can effectively improve the lipid profile by reducing TG levels and increasing HDL-C levels [13–16]. Some studies have also shown that levels of lactate, pyruvate, fatty acids, and ketone bodies generally rise after exercise [17–19]. Furthermore, research on MT has revealed significant changes in metabolites, including increased levels of several amino acids such as alanine, histidine, glycine, creatine, isoleucine, leucine, threonine, and valine [17,18].

Studies regarding the effect of combined practical diet (focusing on a dietary pattern instead of a specific food group/nutrient) and exercise interventions in this population age range (older adults) are sparser, and the few existing studies suggest an intensification of the effects of isolated interventions with additional beneficial health outcomes [20]. Therefore, the primary aim of this study was to investigate how a combined Mediterranean Diet-based SHD and MT intervention affects plasma biomarkers and metabolome, and to assess the potential influence of dietary intake and exercise on this relationship.

2. Methods

2.1. Study design and participants

The present study is based on the “Multidimensional Health Impact of Multicomponent Exercise and Sustainable Healthy Diet Interventions in the Elderly (MED-E)” project, a quasi-experimental, 12-week, four-armed intervention trial. This project's data collection was conducted between November 2021 and December 2022 in the Oporto Metropolitan Area, North of Portugal.

The study included individuals aged 65 years or older who were living in the community, had independent mobility and had no previous medical history of neuropsychiatric, musculoskeletal, or cardiovascular conditions that contraindicate their participation in moderate exercise and testing. The exclusion criteria included individuals already engaged in regular exercise training and/or followed by a nutritionist.

The study protocol was approved by the Ethical Committee of the Faculty of Food and Nutrition Sciences of the University of Porto (ref. 17/2021/CEFCNAUP/2021, 10th of March 2021) and the Ethics Committee of Northern Region Health Administration (report CE/2022/71, 7th of July 2022). Before data collection, informed consent was obtained from all participants. Data coding, security, and storage procedures followed the guidelines and was approved by the Data Protection Unit of the University of Porto (ref. P-9/2022, 18th of November 2022). Information regarding confidentiality and the transfer of data obtained within the scope of the MED-E project was present in the study information document for participants, attached to the informed consent.

The sample size was estimated based on previous literature and the ANOVA to analyse the differences between and within groups [21]. A sample size calculation was conducted using G*Power 3.1.3 (Universität Düsseldorf, Düsseldorf, Germany) [22]. Based on a moderate effect size of 0.40, an alpha risk of 0.05, and a beta risk of 0.20 in a two-sided test, we predicted the inclusion of 25 participants per group. This corresponds to a total of at least 100 community-dwelling older adults, both men and women, accounting for an anticipated dropout rate of 20%. Additional details regarding the study protocol for the MED-E project are available elsewhere [23].

Participants included in the study were assigned into a 12-week control group (CG), MT group, SHD group, or combined SHD and MT (SHD + MT) group.

2.2. Sustainable healthy diet (SHD) and multicomponent training (MT) interventions

Participants in the Mediterranean Diet-based SHD intervention (SHD and SHD + MT groups) were supplied weekly with a SHD food basket. This food basket, which prioritized traditional and locally sourced foods, featured an abundance of planted-based foods such as walnuts and pulses (ranging from chickpeas to different types of beans), extra-virgin olive oil and oily fish, along all the 12-week intervention period. The program was possible through the support of a national food retailer chain. In accordance with the Mediterranean Diet and the latest EAT-Lancet recommendations [2], the weekly individual food supply comprised 125 g of walnuts (considering a daily serving of 25 g); 350 g of dry pulses (based on a daily serving of 50 g); 280 g of extra-virgin olive oil (one bottle of olive oil was provided per month); and two portions of oily fish (sardine fillets and tuna, 100 g per serving). The intervention also included four sessions: three group sessions (including a culinary workshop focused in a Mediterranean Diet-based SHD recipes) and one telephone interview, conducted by a qualified nutritionist, ranging from 30 to 60 min.

The MT exercise program included 50-min group sessions three times a week on non-consecutive days. The sessions were divided into three key components: 8–10 min of warm-up (including walking, postural, mobility, and stretching exercises), 30–35 min of specific training (including balance, coordination, strength, and aerobic conditioning), and 5 min cooldown (breathing and stretching exercises).

Participants in the CG engaged in a monthly 50-min social activity session, with up to 25 individuals, featuring low-intensity physical activity and sessions providing nutrition-related information as an adjunct to standard care. CG participants were contacted weekly via phone calls to ensure retention and motivation.

Specialized and well-trained nutrition and exercise researchers conducted all the evaluations. Participants underwent testing on two different occasions: the first assessment was conducted prior to the beginning of the training – baseline/pre-intervention assessment (T1), while the second evaluation occurred after a 12-week intervention – post-intervention assessment (T2).

2.3. Blood collection

Venous blood samples were collected at the beginning and after the intervention. All the biochemical analyses were performed in a certified laboratory.

After an overnight fast of at least 8 h, venous blood samples from the antecubital vein were collected by a competent professional. Blood samples were collected in a room specially designated and equipped for this purpose and separated into serum and plasma.

The blood volume collected was 10–30 mL, 16 mL transferred into gel tubes (8 mL per tube, 2 Vacumed® gel separator + clot activator yellow cap tubes, FLMedical, reference 44,728) and 9 mL of blood into sodium heparin tube (1 Vacumed® sodium heparin tube, FLMedical, reference 44,359). From collection to sample processing, there was a period of 20–30 min of resting until centrifugation. The gel tubes were centrifuged for 10 min, at 18 °C, and 3500 rpm and the sodium heparin tube at 4 °C, with the same time and force parameters. Separation occurred after 10 min of centrifugation.

Serum (supernatant from gel tubes) was stored in four eppendorfs of 2 mL each and plasma (supernatant from the sodium heparin tube) stored in 2 eppendorfs of 1 mL each.

Aliquots were stored on the day of blood collection, after separation, in a first phase at −20 °C (for no more than 2 h) and in a second phase at −80 °C. Storage at −80 °C was guaranteed until separation for dosing and consequent analysis.

2.4. Measurement of plasma biomarkers

The following nutritional and age-related plasma biomarkers were analyzed in serum blood samples: glucose, fructosamine, insulin, cortisol, high sensitivity C-reactive protein (hs-CRP), lipid profile: total cholesterol, LDL-C, HDL-C, and triglycerides, iron, phosphorus, calcium, magnesium, vitamin B12, and osmolality. All analysis were performed with the ALINITY c® equipment, except for the measurement of insulin and cortisol that were performed by a chemiluminescence microparticle immunoassay (CLIA) with the ALINITY i® equipment. Traditional biochemical parameters, such as glucose, magnesium, and the lipidic profile, were enzymatically measured [24]. The LDL-C was computed using the Friedewald formula (only valid for TG < 350 mg/dL), which estimates the levels of LDL-C by subtracting HDL-C and one fifth of the TG value from the TC level [25]. Frutosamine, iron, phosphorus, and calcium were analysed through colorimetric techniques. CRP analysis was performed using immunoturbidimetry techniques. Urea and sodium were measured enzymatically and by indirect potentiometry, respectively, for the calculation of osmolality. Plasma osmolality was computed using the following formula: 2*[sodium] + [urea nitrogen]/3 + [glucose]/20 [26,27].

2.5. Plasma metabolic profiling by NMR spectroscopy

Plasma samples were immediately frozen at −80 °C until analysis by an untargeted nuclear magnetic resonance (NMR)-based metabolomics approach. The NMR metabolic profiling of plasma samples was performed at the Nuclear Magnetic Resonance Laboratory of the Materials Centre of the University of Porto (CEMUP). Plasma samples for NMR analysis were prepared according to the procedures recommended by Beckonert and colleagues [28].

Briefly, 500 μL of saline solution (NaCl 0.9%, 10% deuterium oxide) was added to 250 μL of plasma, followed by centrifugation (4500 g, 5 min, 4 °C), and 600 μL of supernatant was transferred to 5 mm NMR tubes. The NMR analysis was performed in a Bruker Ascend 600 14.1 T spectrometer operating at 600 MHz, equipped with a 3-channel digital AQS/2 Bruker Avance III HD console, a 5 mm BBO Cryogenic Prodigy Probe (298 K), and a SampleXpress autosampler. All plasma samples were acquired with a Carr–Purcell–Meiboom–Gill (CPMG or T2-edited) pulse sequence with water presaturation (cpmgpr1d in Bruker library) [29].

The acquisition parameters were 64 k data points, 12,019.23 spectral width, 2.73 s (sec) acquisition time, 4 s relaxation delay, 0.0015 s fixed echo time, 20 loops for T2 filter, and 256 scans. For one representative plasma sample, two-dimensional (2D) spectra (heteronuclear single quantum coherence (HSQC) and total correlation spectroscopy (TOCSY)) were acquired to aid in metabolite annotation. The NMR spectra of the standard compounds in databases, such as the Biological Magnetic Resonance Data Bank (BMRB) [30], the Human Metabolome Database (HMDB) [31], and the Chenomx NMR Suite (version 8.4, Chenomx Inc., Alberta, Canada), were also considered for metabolite annotation.

These comprehensive resources were complemented by information from previous literature [32–34]. The annotated metabolites and lipid resonances considered for subsequent statistical analysis (Table S1) are shown in Supplementary material. The peak area of each metabolite or lipid resonance was integrated with Amix (version 3.9.14, Bruker Biospin, Rheinstetten, Germany) and normalized to the total spectral area (excluding the water resonance). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database [35] was used to interpret the metabolic pathways in which the significantly altered metabolites participate.

2.5.1. Dietary intake assessment and mediterranean diet adherence

Dietary intake was assessed through two repeated 24-h dietary recall methods, in non-consecutive days, following European Food Safety Authority recommendations [36]. Energy and nutritional intake, based on the 24-h recalled food data, were estimated using the nutritional analysis module eAT24 (an electronic assessment tool for 24-h recall that uses the FoodEx2 classification system [37]). The Mediterranean Diet Adherence Screener (MEDAS) was used to assess participants' adherence to the Mediterranean Diet, ranging the total score between 1 and 14, in which scores greater than 9 indicate high adherence to the Mediterranean Diet [38,39].

2.5.2. Physical activity, body mass index and sociodemographic assessments

Data was collected through a structured questionnaire on sociodemographic and economic status, clinical history, tobacco use, medication, cognition [40,41], and quality of life [42,43].

Daily physical activity (PA) was assessed through the short form of the International Physical Activity Questionnaire (IPAQ-SF) [44], validated for Portuguese populations [45]. Data collected with IPAQ-SV was converted as median MET-minutes/week of vigorous physical activity (VPA), moderate physical activity (MPA) and walking, according to the guidelines [46]. Then, the total physical activity variable was computed (Total MET-minutes/week).

Height and body mass were recorded using a portable stadiometer and balance weighing scales, respectively. Body mass index (BMI) was calculated using the standard formula: mass (kg)/height^2 (m). Body composition and anthropometric assessments data collection are mentioned and detailed elsewhere [23].

2.6. Statistical analysis

Descriptive measures were calculated for all variables. Measures of central tendency and dispersion, presented as mean and standard deviation (SD) and frequencies (percentages), were used as appropriate to describe participants' characteristics. Data were verified for normality of distribution. The distribution of the data was verified by analysing symmetry and flatness ranges. Variables that did not meet the assumption of normality were analysed with non-parametric statistics.

Pearson's Chi–Square test, One-way analysis of variance (ANOVA), and the corresponding non-parametric test (Kruskal–Wallis) were used to analyse statistical differences in the

participants baseline characteristics. Possible confounding variables (e.g., BMI, total energy intake, daily physical activity …) were then entered as covariates in the analysis of variance model as indicated.

Paired Samples T-student Tests were used to compare means of plasma biomarkers, metabolome, and nutritional intake at baseline (T1) and post-intervention (T2) period in each intervention group; when the variables departed from normality, the Related-Samples Wilcoxon Signed Rank Test was used instead.

A multivariate factorial ANOVA, considering time as a within-subjects factor and group as a between-subjects factor, with repeated measures on one factor (time), was performed for differences in main effects (p(group) and p(time)) and time by group interactions (p(time*group)) for each dependent variable of interest. The analysis included normal, log-transformed, or ranked data, depending on the distribution. The association models are presented with adjusted data, considering sex, age, total energy intake, total physical activity, and BMI as covariates in the repeat-measures ANOVA model.

A significance level of $\alpha = 0.05$ was established. All statistical procedures were performed with Statistical Package for the Social Sciences (SPSS)® software, version 29.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Participants' baseline characteristics

The baseline sociodemographic and clinical characteristics of participants are shown in Table 1. No significant differences were found among the four intervention groups in these characteristics, except for the BMI and usual PA. Even though the average BMI for all the groups falls into the pre-obesity category (in SHD − obesity class I), the participants in the SHD + MT group displayed the lowest BMI values, combined with the highest levels of regular PA. These variables were used as covariates in the association models. Also, despite no significant differences were observed in energy and macronutrients intake (except for saturated fat), SHD had a higher intake of added sugars and CG had a higher intake of total and saturated fat. All groups demonstrated a remarkable commitment to following the Mediterranean Diet (high adherence), except for the CG group, which displayed a slightly less rigorous adherence level.

Out of the 87 participants who initially underwent assessment, three individuals from the control group dropped out due to illness, resulting in incomplete post-intervention evaluations. Furthermore, 11 participants refuse to provide blood samples. Hence, the association models included 73 participants. There were no statistically significant differences in any descriptive parameters between the dropout participants and the other older adults.

3.2. Plasma biomarkers, metabolome, and nutritional data

Table 2 shows the impact of all the intervention groups on the plasma biomarkers. The combined SHD + MT intervention displays a statistically significant impact on lipid and calcium biomarkers. HDL-C and calcium showed significant decreases from baseline to post-intervention in SHD + MT and MT. Osmolality reduced significantly in MT post-intervention ($p < 0.001$). In the final adjusted model, insulin presented significant differences between assessment moments. Only fructosamine, calcium, and HDL-C remained significantly different for the time*group interactions. Osmolality changes lost significance along the intervention period - p (time) −, when compared to the unadjusted model, but remained between groups and for the time*group interactions ($p < 0.001$).

Considering the plasma metabolome in Fig. 1 and Table 3, the SHD + MT group shows some changes in common with MT and SHD groups. In the SHD + MT group, only pyruvate ($p < 0.001$) increased significantly, whereas a significant increase in lactate ($p = 0.009$ and $p = 0.008$) and pyruvate ($p < 0.001$) levels was observed in the MT and SHD groups, respectively. In addition to the change in pyruvate levels, only a significant decrease of citrate was observed in common in the SHD + MT ($p = 0.009$) and SHD ($p = 0.037$). The SHD + MT group revealed specific changes in the levels of amino acids (decrease in alanine, glutamine and lysine: $p = 0.026$; $p < 0.001$; $p = 0.038$, respectively), formate ($p = 0.025$) and unsaturated lipids ($p = 0.011$), consistent with changes in energy, amino acid and lipoprotein metabolism. The MT has significant changes in a greater number of metabolites. Specific changes were observed, namely a significant increase in the levels of essential and non-essential amino acids, acetate, glycerol, and a decrease in glucose. The SHD also had significant increases in alanine, leucine, tyrosine, and methanol and a decrease in acetone. In the final adjusted association model (Table 3), the changes that remained significant were for acetate ($p = 0.049$) and threonine ($p = 0.002$) between intervention assessment periods and for betaine ($p = 0.017$) and lysine ($p = 0.045$) between groups, and lactate ($p = 0.027$), pyruvate ($p = 0.002$), phenylalanine ($p = 0.008$), proline ($p = 0.023$), and tyrosine ($p = 0.021$), when considering the time*group interactions.

In the supplementary material, Table S2 presents macro and micronutrient differences between intervention groups. Total energy and protein intake statistically decrease in CG and MT. Total fat ($p = 0.017$), saturated fatty acids (SFA, $p = 0.023$) and mono (MUFA, $p = 0.015$) and polyunsaturated fatty acids (PUFA, $p = 0.006$) significantly decreased in CG; polyunsaturated fatty acids increased only in SHD + MT participants. The intake of carbohydrates decreased significantly in exercise intervention ($p = 0.003$). Water intake increased for both MT and SHD + MT participants.

Notably, the groups showed significant variation specifically in total PUFA ($p = 0.020$) − Table S3. Significant differences were also observed in PUFA, carbohydrates and fiber intake over the intervention period, as revealed by the repeated measures ANOVA (Table S3).

4. Discussion

Our data shows that a 12-week combined Mediterranean Diet-based SHD and MT intervention significantly impacted plasma biomarkers and metabolome in community-dwelling older adults.

The combined SHD + MT intervention significantly decreased HDL-C and calcium and a decrease in TC and LDL-C was also observed in diet groups. Despite being within the recommended range and not clinically significant, the unexpected decrease in HDL-C might be explained by the fact that significant changes in HDL-C may require a combination of high-levels and high-intensity exercise [47], which may not be achieved with the type of exercise program implemented in this study and considering the sample's age range (≥65 years old). It is notable that only the groups with diet interventions improved the lipid profile (TC and LDL-C), despite not reaching statistical significance due to high standard deviation and considering that participants had normal baseline and post-intervention levels. Studies with combined approaches state complementary effects: diet programs commonly lower TC [48,49] and LDL-C concentrations, whereas exercise interventions increase HDL-C and reduce TG levels [11,12,50]. Our study partly corroborates these findings, particularly regarding the diet effects in lipid profile.

Literature has demonstrated that higher serum calcium levels are observed in active individuals [51], which aligns with our

Table 1
Baseline sample characteristics.

Characteristics	Total (n = 87)	CG (n = 19)	MT (n = 20)	SHD (n = 26)	SHD + MT (n = 22)	p-value
Age (years), mean (SD)[a]	72 ± 5	71 ± 4	75 ± 5	72 ± 5	71 ± 5	0.083
Age, range	63–88	65–79	66–82	64–88	63–85	
Sex (female), n (%)[c]	64 (73.6)	13 (68.4)	16 (80.0)	16 (61.5)	19 (86.4)	0.215
Civil Status, n (%)[c]						
Single	5 (5.7)	3 (15.8)	–	–	2 (9.1)	0.169
Married	51 (58.6)	11 (57.9)	13 (65.0)	16 (61.5)	11 (50.0)	
Divorced/Separated	10 (11.5)	3 (15.8)	–	3 (11.5)	4 (18.2)	
Widowed	21 (24.1)	2 (10.5)	7 (35.0)	7 (26.9)	5 (22.7)	
Years of formal education, mean (SD)[b]	9 ± 4	11 ± 5	9 ± 4	8 ± 3	9 ± 5	0.320
Education levels, n (%)[c]						
≤3 years	3 (3.4)	1 (5.3)	–	–	2 (9.1)	0.372
4–6 years	32 (36.8)	4 (21.1)	9 (45.0)	11 (42.3)	8 (36.4)	
≥7 years	52 (59.8)	14 (73.7)	11 (55.0)	15 (57.7)	12 (54.5)	
Work condition (retired), n (%)[c]	85 (97.7)	18 (94.7)	20 (100.0)	26 (100.0)	21 (95.5)	0.509
Current smoker (no), n (%)[c]	86 (98.9)	18 (94.7)	20 (100.0)	26 (100.0)	22 (100.0)	0.305
BMI (kg/m^2), mean (SD)[a]	28.8 ± 4.3	28.2 ± 4.0	28.5 ± 4.2	30.9 ± 3.9	27.0 ± 4.1	**0.012**
Total energy intake (kcal/day), mean (SD)[b]	1726 ± 570	1907 ± 675	1693 ± 552	1715 ± 579	1612 ± 468	0.690
Macronutrients intake (% TEV), mean (SD)						
Protein[b]	17 ± 4	16 ± 3	18 ± 3	17 ± 5	15 ± 3	0.108
Carbohydrates[a]	54 ± 8	53 ± 7	53 ± 7	54 ± 9	56 ± 8	0.456
Added sugars[b]	6 ± 5	6 ± 5	6 ± 5	7 ± 4	5 ± 5	0.334
Lipids[a]	29 ± 7	31 ± 6	29 ± 6	29 ± 8	28 ± 8	0.756
MUFA[a]	12 ± 3	12 ± 3	12 ± 4	12 ± 3	12 ± 3	0.977
PUFA[b]	5 ± 2	5 ± 2	5 ± 2	5 ± 2	6 ± 3	0.675
SFA[a]	9 ± 3	10 ± 3	9 ± 3	8 ± 3	8 ± 3	**0.046**
MEDAS score, mean (SD)[b]	9 ± 2	9 ± 2	9 ± 2	10 ± 2	10 ± 1	0.185
Daily physical activity (MET-min/week), mean (SD)[b]						
Total physical activity	2322 ± 2753	1227 ± 1151	1957 ± 1115	1577 ± 2120	4479 ± 4080	**<0.001**
Vigorous physical activity (VPA)	945 ± 2148	152 ± 438	926 ± 550	594 ± 2048	2064 ± 3380	**<0.001**
Moderate physical activity (MPA)	756 ± 1187	212 ± 473	769 ± 1115	509 ± 673	1505 ± 1727	**<0.001**
Walking	621 ± 792	864 ± 1136	262 ± 392	473 ± 485	910 ± 867	**0.002**
WHOQOL-OLD score, mean (SD)[a]	101 ± 11	101 ± 12	103 ± 11	99 ± 12	102 ± 10	0.594
MoCA score, mean (SD)[a]	20 ± 4	21 ± 4	20 ± 5	21 ± 4	20 ± 4	0.602

BMI: Body Mass Index; MEDAS: The Mediterranean Diet Adherence Screener Questionnaire; WHOQOL-OLD: The World Health Organization Quality of Life for Old Module; MoCA: Montreal Cognitive Assessment; % TEV: percentage of total energetic value; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids.
[a] Listed p values for One-way ANOVA test.
[b] Listed p values for Independent Samples Kruskal–Wallis test.
[c] Listed p values for χ^2: Pearson's Chi–Square test; Bolded p-values are significant at the 0.05 level.

Table 2
Intervention impact on plasma biomarkers at baseline (T1) and post-intervention (T2), unadjusted and adjusted models.

Plasma biomarkers, mean (SD)		Reference values	Mean difference (T2-T1)				Adjusted model•		
			CG	MT	SHD	SHD + MT	p (time)	p (group)	p (time*group)
Energetic pathways biomarkers	Glucose (mg/dl)	70–110	2 ± 8	−3 ± 6	−10 ± 39	3 ± 11	0.556	0.272	0.101
	Fructosamine (μmol/l)	205–285	8 ± 19	−4 ± 11	−8 ± 19 **0.021**[b]	−3 ± 15	0.582	0.194	**0.049**
Hormonal biomarkers	Insulin (μU/ml)	3.0–25.0	0.7 ± 3.0	0.2 ± 1.9	0.2 ± 3.9	0.8 ± 2.6	**0.032**	0.126	0.966
	Cortisol (μg/dl)	3.7–19.4	0.6 ± 2.4	1.0 ± 2.8	0.1 ± 2.8	0.9 ± 2.2	0.446	0.614	0.525
Inflammatory biomarker	hs-CRP (mg/dl)	<1.0	1.2 ± 3.1	−0.7 ± 2.0	1.8 ± 9.7	0.8 ± 5.9	0.967	0.679	0.156
Lipid metabolism biomarkers	TC (mg/dl)	<190	11 ± 37	1 ± 18	−1 ± 24	−4 ± 32	0.331	0.671	0.254
	LDL-C (mg/dl)	<130	7 ± 33	3 ± 19	−2 ± 20	−1 ± 28	0.167	0.634	0.521
	HDL-C (mg/dl)	>45	3 ± 8	−4 ± 6 **0.016**[a]	1 ± 6	−4 ± 6 **0.022**[a]	0.093	0.640	**0.003**
	TG (mg/dl)	<150	6 ± 29	7 ± 40	−2 ± 43	6 ± 34	0.489	0.084	0.960
Vitamins and minerals	Iron (μg/dl)	♂: 65-75 ♀: 50-170	7 ± 20	−10 ± 31	−4 ± 29	1 ± 25	0.369	0.584	0.352
	Calcium (mg/dl)	♂: 8.8–10.0 ♀: 8.4–10.2	0.3 ± 0.7	−0.2 ± 0.3 **0.039**[b]	−0.1 ± 0.5	−0.2 ± 0.3 **0.017**[b]	0.421	0.961	**0.006**
	Phosphorus (mg/dl)	2.5–4.5	0.0 ± 0.6	0.0 ± 0.3	−0.1 ± 0.4	0.0 ± 0.3	0.857	0.817	0.232
	Magnesium (mg/dl)	1.6–2.6	0.1 ± 0.2	0.0 ± 0.1	0.0 ± 0.1	0.1 ± 0.1	0.813	0.584	0.141
	Vitamin B12 (ng/l)	187–883	4 ± 76	53 ± 130	0 ± 58	45 ± 122	0.130	0.374	0.430
Hydration status biomarker	Osmolality (mOsm/kg)	275–295	2 ± 6	−12 ± 5 **<0.001**[b]	−1 ± 4	−2 ± 7	0.714	**<0.001**	**<0.001**

T1: first moment of evaluation – baseline/pre-intervention assessment; T2: second moment of evaluation – post-intervention assessment.
[a] Listed p values for Paired Samples T-student Test.
[b] Listed p values for Related-Samples Wilcoxon Signed Rank Test; •Listed p values for within and between-subjects effects assessed by repeated measures ANOVA, adjusted model for baseline age (years), sex, baseline energy intake (kcal/day), total physical activity (MET-min/week), and BMI (kg/m^2); Bolded p-values are significant at the 0.05 level.

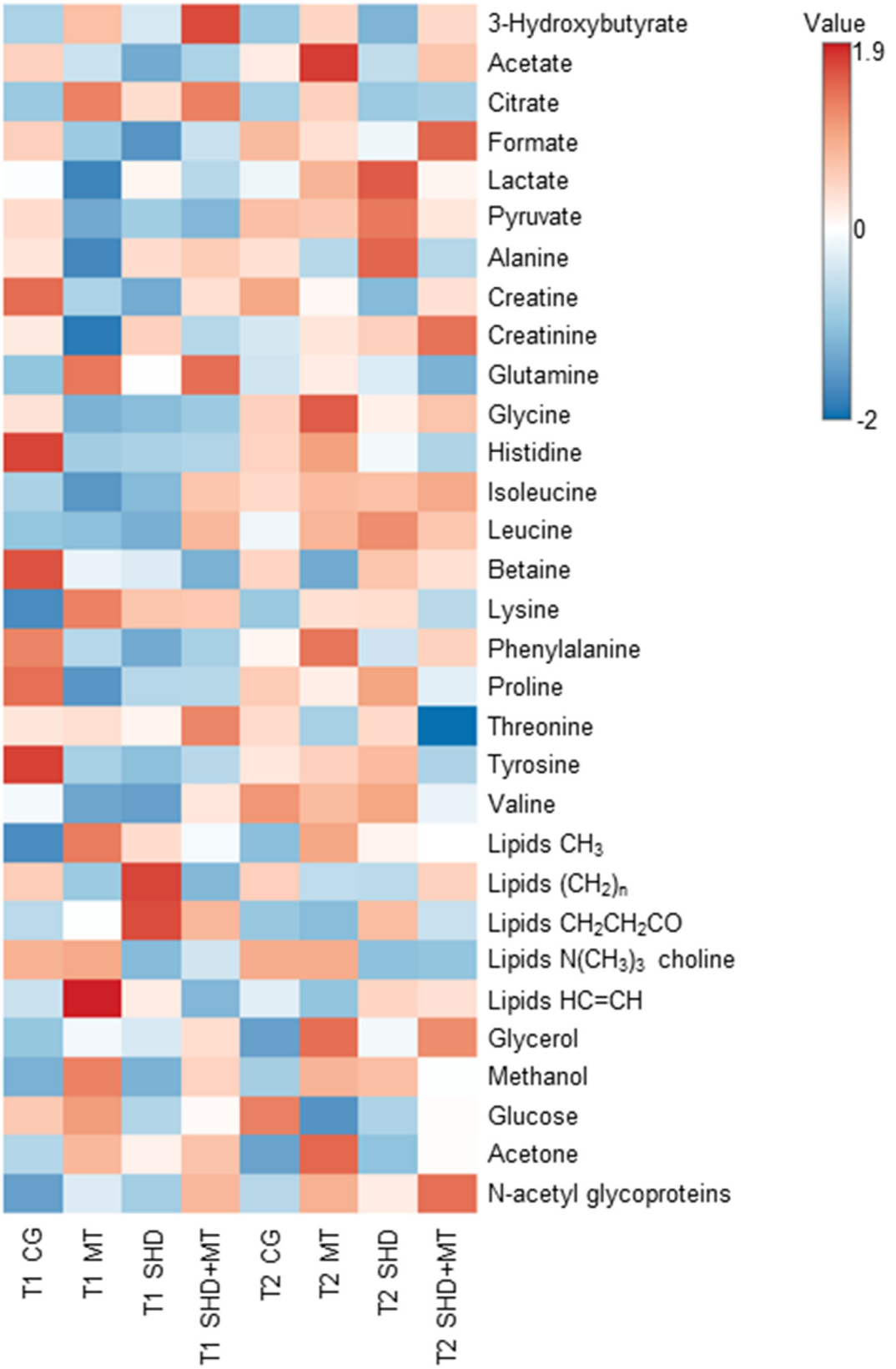

Fig. 1. Heatmap showing the mean levels (normalized peak areas) of metabolites at baseline (T1) and post-intervention (T2) by intervention group. The columns represent each intervention group at T1 and T2, and the rows correspond to the mean normalized area of each metabolite, colored from minimum (dark blue) to maximum (dark red).

Table 3
Intervention impact on plasma metabolite levels, unadjusted and adjusted models.

Plasma metabolites normalized peak area (AU), mean (SD)		CG		MT		SHD		SHD + MT		Adjusted model*		
		Mean difference (T2-T1)	p-value	Mean difference (T2-T1)	p-value	Mean difference (T2-T1)	p-value	Mean difference (T2-T1)	p-value	p (time)	p (group)	p (time*group)
Organic acids	3-Hydroxybutyrate[b]	-1.6×10^{-5} $\pm 2.5 \times 10^{-4}$	0.776	-2.5×10^{-5} $\pm 3.5 \times 10^{-4}$	0.642	-8.6×10^{-5} $\pm 2.5 \times 10^{-4}$	0.132	-1.4×10^{-4} $\pm 5.2 \times 10^{-4}$	0.136	0.476	0.514	0.686
	Acetate[a]	-2.7×10^{-5} $\pm 3.3 \times 10^{-4}$	0.760	2.2×10^{-4} $\pm 4.1 \times 10^{-4}$	**0.043**	7.0×10^{-5} $\pm 3.0 \times 10^{-4}$	0.265	1.4×10^{-4} $\pm 3.5 \times 10^{-4}$	0.131	**0.049**	0.658	0.920
	Citrate[a]	1.8×10^{-5} $\pm 3.7 \times 10^{-4}$	0.855	-1.2×10^{-4} $\pm 3.9 \times 10^{-4}$	0.240	-1.9×10^{-4} $\pm 4.3 \times 10^{-4}$	**0.037**	-3.2×10^{-4} $\pm 4.4 \times 10^{-4}$	**0.009**	0.324	0.967	0.528
	Formate[b]	1.0×10^{-6} $\pm 7.0 \times 10^{-6}$	0.480	7.0×10^{-6} $\pm 2.1 \times 10^{-5}$	0.202	8.0×10^{-6} $\pm 2.0 \times 10^{-5}$	0.075	1.1×10^{-5} $\pm 1.8 \times 10^{-5}$	**0.025**	0.649	0.497	0.238
	Lactate[a]	-1.3×10^{-4} $\pm 1.8 \times 10^{-3}$	0.785	2.5×10^{-3} $\pm 3.5 \times 10^{-3}$	**0.009**	1.5×10^{-3} $\pm 2.4 \times 10^{-3}$	**0.008**	7.6×10^{-4} $\pm 2.7 \times 10^{-3}$	0.252	0.204	0.197	**0.027**
	Pyruvate[a]	4.6×10^{-5} $\pm 2.4 \times 10^{-4}$	0.474	2.8×10^{-4} $\pm 2.2 \times 10^{-4}$	**<0.001**	3.2×10^{-4} $\pm 3.1 \times 10^{-4}$	**<0.001**	2.1×10^{-4} $\pm 1.6 \times 10^{-4}$	**<0.001**	0.799	0.679	**0.002**
Aminoacids and derivatives	Alanine[a]	2.9×10^{-5} $\pm 9.7 \times 10^{-4}$	0.911	5.3×10^{-4} $\pm 1.3 \times 10^{-3}$	0.108	5.8×10^{-4} $\pm 1.3 \times 10^{-3}$	**0.047**	-6.4×10^{-4} $\pm 1.1 \times 10^{-3}$	**0.026**	0.713	0.297	0.062
	Betaine[b]	-3.2×10^{-4} $\pm 1.4 \times 10^{-3}$	0.910	-3.0×10^{-4} $\pm 1.2 \times 10^{-3}$	0.740	2.9×10^{-4} $\pm 1.4 \times 10^{-3}$	0.721	4.3×10^{-4} $\pm 1.0 \times 10^{-3}$	0.118	0.909	**0.017**	0.435
	Creatine[a]	-5.6×10^{-5} $\pm 2.0 \times 10^{-4}$	0.299	9.6×10^{-5} $\pm 3.7 \times 10^{-4}$	0.301	2.0×10^{-5} $\pm 2.9 \times 10^{-4}$	0.745	-1.0×10^{-6} $\pm 3.3 \times 10^{-4}$	0.988	0.135	0.157	0.734
	Creatinine[b]	-2.8×10^{-5} $\pm 1.7 \times 10^{-4}$	0.256	9.6×10^{-5} $\pm 3.3 \times 10^{-4}$	0.227	$0 \pm 3.4 \times 10^{-4}$	0.939	9.4×10^{-5} $\pm 4.0 \times 10^{-4}$	0.602	0.257	0.887	0.685
	Glutamine[a]	2.6×10^{-4} $\pm 8.9 \times 10^{-4}$	0.274	-5.4×10^{-4} $\pm 1.0 \times 10^{-3}$	**0.044**	-1.5×10^{-4} $\pm 1.5 \times 10^{-3}$	0.625	-1.2×10^{-3} $\pm 1.2 \times 10^{-3}$	**<0.001**	0.797	0.769	0.163
	Glycine[b]	4.1×10^{-5} $\pm 5.2 \times 10^{-4}$	0.820	5.9×10^{-4} $\pm 7.4 \times 10^{-4}$	**0.010**	2.6×10^{-4} $\pm 9.8 \times 10^{-4}$	0.161	3.3×10^{-4} $\pm 8.2 \times 10^{-4}$	0.061	0.359	0.735	0.106
	Histidine[a]	-4.6×10^{-5} $\pm 1.2 \times 10^{-4}$	0.174	7.0×10^{-5} $\pm 1.2 \times 10^{-4}$	**0.025**	2.5×10^{-5} $\pm 1.3 \times 10^{-4}$	0.347	-1.0×10^{-6} $\pm 1.4 \times 10^{-4}$	0.987	0.551	0.514	0.056
	Isoleucine[b]	1.0×10^{-4} $\pm 2.9 \times 10^{-4}$	0.164	1.9×10^{-4} $\pm 2.5 \times 10^{-4}$	**0.008**	1.6×10^{-4} $\pm 5.6 \times 10^{-4}$	0.157	2.5×10^{-5} $\pm 2.5 \times 10^{-4}$	0.850	0.963	0.714	0.300
	Leucine[b]	1.4×10^{-4} $\pm 3.7 \times 10^{-4}$	0.177	3.1×10^{-4} $\pm 4.8 \times 10^{-4}$	**0.018**	4.0×10^{-4} $\pm 8.6 \times 10^{-4}$	**0.020**	-2.5×10^{-5} $\pm 4.5 \times 10^{-4}$	0.795	0.550	0.596	0.536
	Lysine[a]	6.4×10^{-4} $\pm 2.2 \times 10^{-3}$	0.275	-8.3×10^{-4} $\pm 3.2 \times 10^{-3}$	0.309	-2.4×10^{-4} $\pm 2.2 \times 10^{-3}$	0.611	-1.1×10^{-3} $\pm 2.0 \times 10^{-3}$	**0.038**	0.474	**0.041**	0.114
	Phenylalanine[a]	-9.0×10^{-5} $\pm 1.9 \times 10^{-4}$	0.080	1.6×10^{-4} $\pm 1.8 \times 10^{-4}$	**0.002**	6.6×10^{-5} $\pm 2.1 \times 10^{-4}$	0.137	1.0×10^{-4} $\pm 1.3 \times 10^{-4}$	**0.004**	0.171	0.102	**0.008**
	Proline[a]	-6.3×10^{-5} $\pm 1.7 \times 10^{-4}$	0.175	1.3×10^{-4} $\pm 2.2 \times 10^{-4}$	**0.030**	1.2×10^{-4} $\pm 2.9 \times 10^{-4}$	0.051	3.0×10^{-5} $\pm 1.9 \times 10^{-4}$	0.522	0.915	0.879	**0.023**
	Threonine[b]	1.1×10^{-5} $\pm 3.6 \times 10^{-4}$	0.865	-1.0×10^{-4} $\pm 4.2 \times 10^{-4}$	0.368	2.4×10^{-5} $\pm 5.0 \times 10^{-4}$	0.808	-2.8×10^{-4} $\pm 5.6 \times 10^{-4}$	0.084	**0.002**	0.850	0.687
	Tyrosine[a]	-8.1×10^{-5} $\pm 1.4 \times 10^{-4}$	**0.044**	7.2×10^{-5} $\pm 2.1 \times 10^{-4}$	0.181	9.8×10^{-5} $\pm 2.1 \times 10^{-4}$	**0.028**	-5.0×10^{-6} $\pm 1.7 \times 10^{-4}$	0.908	0.245	0.690	**0.021**
	Valine[b]	2.2×10^{-4} $\pm 6.5 \times 10^{-4}$	0.118	3.9×10^{-4} $\pm 6.9 \times 10^{-4}$	**0.031**	4.4×10^{-4} $\pm 1.1 \times 10^{-3}$	0.116	-8.5×10^{-5} $\pm 7.3 \times 10^{-4}$	0.653	0.392	0.862	0.627
Lipids	Lipids CH_3[a]	1.4×10^{-3} $\pm 6.0 \times 10^{-3}$	0.379	-9.0×10^{-4} $\pm 1.0 \times 10^{-2}$	0.719	-6.0×10^{-4} $\pm 8.7 \times 10^{-3}$	0.737	2.1×10^{-4} $\pm 5.2 \times 10^{-3}$	0.868	0.780	0.123	0.761
	Lipids $(CH_2)_n$[a]	-5.3×10^{-5} $\pm 2.2 \times 10^{-2}$	0.993	1.2×10^{-3} $\pm 3.3 \times 10^{-2}$	0.885	-9.0×10^{-3} $\pm 3.5 \times 10^{-2}$	0.215	6.3×10^{-3} $\pm 2.7 \times 10^{-2}$	0.358	0.787	0.765	0.863
	Lipids CH_2CH_2CO[a]	-1.5×10^{-4} $\pm 1.0 \times 10^{-3}$	0.579	-5.4×10^{-4} $\pm 1.6 \times 10^{-3}$	0.188	-4.8×10^{-4} $\pm 1.5 \times 10^{-3}$	0.128	-6.6×10^{-4} $\pm 1.6 \times 10^{-3}$	0.111	0.656	0.253	0.454
	Lipids $N(CH_3)_3$ choline[a]	1.9×10^{-4} $\pm 4.7 \times 10^{-3}$	0.881	-9.1×10^{-5} $\pm 5.8 \times 10^{-3}$	0.949	1.9×10^{-4} $\pm 5.9 \times 10^{-3}$	0.877	-1.4×10^{-3} $\pm 6.0 \times 10^{-3}$	0.350	0.085	0.062	0.994
	Lipids HC=CH[b]	3.2×10^{-4} $\pm 3.6 \times 10^{-3}$	0.955	-4.5×10^{-3} $\pm 2.0 \times 10^{-2}$	0.723	4.1×10^{-4} $\pm 5.6 \times 10^{-3}$	0.710	2.3×10^{-3} $\pm 3.8 \times 10^{-3}$	**0.011**	0.783	0.772	0.497
Alcohols	Glycerol[b]	-8.6×10^{-5} $\pm 4.4 \times 10^{-4}$	0.427	3.0×10^{-4} $\pm 4.5 \times 10^{-4}$	**0.023**	4.9×10^{-5} $\pm 6.4 \times 10^{-4}$	0.749	1.6×10^{-4} $\pm 7.4 \times 10^{-4}$	0.478	0.091	0.289	0.163
	Methanol[b]	5.1×10^{-5} $\pm 3.7 \times 10^{-4}$	0.755	-6.0×10^{-5} $\pm 7.5 \times 10^{-4}$	0.943	2.5×10^{-4} $\pm 6.4 \times 10^{-4}$	**0.028**	-6.5×10^{-5} $\pm 3.1 \times 10^{-4}$	0.434	0.152	0.751	0.731
Others	Acetone[b]	-1.6×10^{-4} $\pm 4.6 \times 10^{-4}$	0.280	1.7×10^{-4} $\pm 1.6 \times 10^{-3}$	0.478	-2.8×10^{-4} $\pm 6.3 \times 10^{-4}$	**0.045**	-1.5×10^{-4} $\pm 1.4 \times 10^{-3}$	0.201	0.728	0.548	0.988
	Glucose[b]	2.5×10^{-4} $\pm 1.3 \times 10^{-3}$	0.363	-9.5×10^{-4} $\pm 2.3 \times 10^{-3}$	**0.025**	-1.4×10^{-5} $\pm 2.3 \times 10^{-3}$	0.753	-9.0×10^{-6} $\pm 1.1 \times 10^{-3}$	0981	0.160	0.376	0.164
	N-acetyl glycoproteins[b]	4.6×10^{-4} $\pm 1.6 \times 10^{-3}$	0.069	7.2×10^{-4} $\pm 1.5 \times 10^{-3}$	0.076	6.5×10^{-4} $\pm 1.8 \times 10^{-3}$	0.137	3.9×10^{-4} $\pm 1.9 \times 10^{-3}$	0.356	0.511	0.447	0.265

AU: arbitrary units; T1: first moment of evaluation – baseline/pre-intervention assessment; T2: second moment of evaluation – post-intervention assessment.
[a] Listed p values for Paired Samples T-student Test.
[b] Listed p values for Related-Samples Wilcoxon Signed Rank Test; •Listed p values for within and between-subjects effects assessed by repeated measures ANOVA, adjusted model for baseline age (years), sex, baseline energy intake (kcal/day), total physical activity (MET-min/week), and BMI (kg/m^2); Bolded p-values are significant at the 0.05 level.

results. However, it is noteworthy that exercise groups (MT and SHD + MT) significantly decreased calcium levels which was contrary to expected. In calcium homeostasis during exercise, an increase in serum parathyroid hormone (PTH) is necessary to counteract the decline in serum calcium that is triggered by prolonged sweating [52]. A reduction in post-intervention total PA in these groups can contribute to explain this calcium decrease.

Osmolality had significant differences in the association models and a significant decrease with MT was observed. The greater loss of water (sweating) in consequence of the MT program [53,54], promoted a higher water intake (Table S2), suggestive of a better hydration status in these groups, observed through the biochemical analysis once osmolality increases when dehydrated.

Both Mediterranean Diet [55] and exercise [56] seem to increase serum levels of vitamin B12, which aligns with our results (despite the non-significance). All groups improved vitamin B12 levels, though only MT and SHD + MT showed substantial increased levels. Despite the major sources of vitamin B12 being animal-based products, including meat, fish (whose intake was promoted), eggs, and dairy [57], and the SHD intervention focus on the promotion of a plant-based diet, an increase was still observed in the combined group. However, as no significant differences were observed in the SHD group, it may be suggestive that exercise may have a greater impact on this change.

Fructosamine showed statistical significance only in the SHD, suggesting a potential link to the relevant glucose decrease in this group. Fructosamine is used to evaluate variations in glucose levels over the last weeks when the realization of glycated hemoglobin test it is not possible. High fructosamine values are related to uncontrolled and potentially higher glucose values. Once SHD participants were the ones with higher baseline glucose values and higher intake of added sugars, it seems plausible this greater decrease.

Insulin presented significant differences in the adjusted model for p(time). However, no significant differences were found in insulin nor cortisol, slightly increasing in all intervention groups between assessments. Cortisol increases with age, overweight, obesity [58], and exercise [59] and acts as a counter-regulatory hormone of insulin [58]. The slight increases in cortisol levels in our sample might be possibly related to these factors. Despite the weight reduction in all groups, except CG, participants were still overweight and/or obesity and, although PA levels have globally decreased, our sample still had higher PA levels. Insulin levels might also increase in response either to diet or exercise and given the inter- and intra-variability of dietary intake and PA levels along the days and year, it is plausible that significant differences would be observed with time [60].

The three interventions had different effects on the levels of metabolites involved in amino acid and energy metabolism. The SHD + MT showed changes in several metabolites involved in energy pathways (citrate, pyruvate) and amino acids. Of these, citrate levels showed a consistent significant decrease, whereas pyruvate levels showed a consistent significant increase. These alterations suggest that the SHD + MT interventions collectively improve energy metabolism, possibly by increasing glycolysis and altering tricarboxylic acid (TCA) cycle dynamics. Interestingly, SHD + MT also showed specific alterations in formate and lysine levels, consistent with changes in energy and amino acid metabolism, as well as significant alterations in unsaturated lipid levels, which may indicate an effect on lipoprotein metabolism. The Mediterranean diet, due to its content of MUFA and n-3 PUFA, is associated with higher levels of unsaturated lipids [61,62], including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and linolenic acid [61,63–65]. In our study, an increase in PUFA intake was observed in the SHD + MT group (Table S2). The changes in unsaturated lipids in the SHD + MT group highlight the potential benefits of this strategy for lipid metabolism and cardiovascular health [66]. The observed changes in alanine, glutamine, and lysine suggest significant effects on protein turnover and amino acid utilization, reflecting the body's adaptation to the improved diet and increased exercise [67,68]. Also, these metabolic alterations might be suggestive of a reduction in meat and grain products, the most important food sources of these amino acids [69], partly promoted by the diet intervention. Additionally, as grain products are mainly a carbohydrate source, their decrease is consistent with the reduction in carbohydrate intake in SHD + MT observed in Table S2. SHD + MT was the only intervention group increasing the protein intake (Table S2) and, according to previous literature, higher protein intake levels enhance protein synthesis [70]. Taken together, these results suggest that the combination of diet and exercise interventions may have additive or synergistic effects on human metabolism.

The effect of MT is consistent with previous literature, showing a greater impact on plasma amino acid levels, including branched-chain amino acids (BCAAs) (isoleucine, leucine, valine [71,72]), other essential (histidine, phenylalanine) and non-essential amino acids (glycine, proline), and energy-related metabolites (acetate, lactate, glucose, pyruvate) [19,73]. The increase in BCAAs, other essential, and non-essential amino acids suggest an accumulation of these amino acids in the bloodstream and a less effective protein synthesis [74], in line with previous literature [75], when compared to SHD + MT group. MT participants did not receive any dietary/nutritional advice. Once these amino acids are mainly found in animal food sources, their increase in plasma may also be related to a higher consumption of animal-based foods by these participants [69,76,77]. The significant changes in energy-related metabolites, including increased acetate, lactate, and pyruvate, along with decreased glucose levels, reflect an impact on energy production pathways [67]. These changes suggest that MT improves the aerobic energy system and protein metabolism, contributing to better physical performance and metabolic health. In addition, the reduction in glucose levels suggests improved glycaemic control [78], highlighting the potential of MT in the prevention and management of metabolic diseases.

The Mediterranean Diet-based SHD intervention also showed significant differences in the levels of several energy-related metabolites (citrate, lactate, pyruvate) and, to a lesser extent, in amino acid metabolism (alanine, leucine, tyrosine). The significant changes in energy-related metabolites, including decreased citrate and increased lactate and pyruvate, suggest increased glycolytic activity and energy production efficiency. These changes may reflect a shift towards greater metabolic flexibility, favouring carbohydrate over fat metabolism for energy, as observed in the SHD + MT group [67,68]. Significant alanine, leucine, and tyrosine increases suggest a less efficient protein turnover and muscle protein synthesis: isolated interventions induced significant amino acid increase, while combined intervention induced a reduction, which emphasizes the concomitant effect of diet and exercise in protein synthesis. Also, it was not expected that these amino acids, mainly from animal-based foods, increase in this intervention group [69]. There was a reduction in energy and protein intake in SHD. However, a post-intervention higher adherence to Mediterranean Diet was not observed in this group and, therefore, this might be suggestive of a worse compliance with the intervention and insufficient plant-based foods intake, as well as the predominance of other animal-based food sources. Specific alterations observed in the SHD intervention group included a significant increase in methanol levels and a significant decrease in acetone. The Mediterranean diet, rich in fibre, fruits, and vegetables, can significantly influence the gut microbiota and promote fermentation

processes that could increase methanol production [79,80]. Acetone, a ketone body produced by fatty acid metabolism, can serve as a biomarker for metabolic conditions (e.g., diabetes) and energy use [81]. The observed decrease in acetone levels in the SHD group also suggests a metabolic shift from fat to carbohydrate use for energy production. This shift is consistent with the known benefits of the Mediterranean diet, which is rich in healthy fats and antioxidants [82]. These dietary components are likely to contribute to reduced lipolysis and oxidative stress, thereby reducing free fatty acids' availability for ketogenesis and subsequently reducing acetone levels. Taken together, these changes enhance energy efficiency, support muscle maintenance, and a reduction in the risk of metabolic disorders, highlighting the overall benefits of a Mediterranean dietary pattern.

To the best of our knowledge, this is the first study investigating plasma biomarkers and metabolic alterations induced by a combined Mediterranean Diet-based SHD + MT lifestyle intervention in community-dwelling older adults. A particular strength of our study is the combined approach to dietary patterns and exercise intervention expression parameters, which includes not only two repeated 24-h recall questionnaires and Mediterranean Diet adherence score but different objective measurement techniques - standard biochemical analysis and untargeted NMR-based metabolomics. These approaches allowed for detecting relevant short-term responses to diet and exercise and identifying related metabolite variations. Notably, *a posteriori* control for possible covariates was done in this study.

However, there are limitations to consider. First, it is a quasi-experimental study, thus randomizing was not possible as it was challenging to recruit and to adapt older adults' routines during and after the pandemic situation. Therefore, participants were allocated to the intervention groups according to recruitment and their availability to fulfil the different interventions. This introduces the potential selection biases, as the groups may differ in ways that could affect the outcomes. Yet, in our study intervention groups showed minimal heterogeneity, empowering the findings. The lack of randomization also limits the ability to establish causality between the interventions changes and outcomes generalise results and make causal inferences due to lack of control over extraneous variables, making it difficult to isolate the effects of the independent variables. Likewise, we cannot withdraw the possibility of a higher risk of Type I errors due to our multivariate analysis and comparisons. Reducing the size of the allowable error could control this possible bias, thought great consideration should be taken in multiple testing once the reduction of alpha could, otherwise, inflate the rate of false negatives. Longer and larger interventions across different populations are needed to validate the impact of SHD + MT on plasma biomarkers and metabolome and to identify characteristic metabotypes. Once this is an intervention study, a crossover design would better account for intrasubject variation, as each participant serves as their own control. Dietary intake was assessed through 24-h recall questionnaires that rely on very recent memory. Despite some information could be biased, it aligns with European Food Safety Authority recommendations [36]. Also, data on usual physical activity was not objectively assessed once many participants refused to use an accelerometer, which can impact the results.

5. Conclusions

In conclusion, the combined Mediterranean Diet-based SHD and MT intervention significantly impacted plasma biomarkers and metabolome in community-dwelling older adults. SHD + MT altered the lipid biomarkers and osmolality, with an unexpected decrease in HDL-C and calcium and an improvement in hydration status. SHD + MT significantly altered metabolites associated with energy (common to MT and SHD) and amino acid metabolism (decreased alanine, glutamine, and lysine), improving energy production, and protein turnover and synthesis. MT and SHD mainly increased the levels of essential and non-essential amino acids, supporting previous literature and SHD + MT results on the symbiotic effects of diet and exercise for enhancing protein synthesis. Overall, our study suggests that the combination of SHD and MT provides additive or synergistic benefits by enhancing metabolic health and energy efficiency. Given its broad relevance, it is important to further evaluate how the combination of SHD and MT can contribute to better clinical profiles, such as reducing the risk of cardiovascular and metabolic disorders.

Author contributions

Conceptualisation: J.S., J.C., and R.B.; Data curation: J.S., J.C., and R.B.; Investigation: J.S., A.P., B.O., J.P., A.M., P.P., P.G.d.P., P.M., J.C.; and R.B.; Methodology: J.S., A.P., B.O., J.P., A.M., P.P., P.G.d.P., P.M., J.C., R.B.; Project administration: J.S., J.C., and R.B.; Resources: J.S., J.C., and R.B.; Writing - original draft: J.S.; Writing - review & editing: J.S., A.P., B.O., J.P., A.M., P.P., P.G.d.P., P.M., J.C, and R.B.

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Institutional review board statement

The study protocol was approved by the Ethical Committee of the Faculty of Food and Nutrition Sciences of the University of Porto (ref. 17/2021/CEFCNAUP/2021, 10th of March 2021), the Ethics Committee of Northern Region Health Administration (report CE/2022/71, 7th of July 2022) and by the Data Protection Unit of University of Porto (ref. P-9/2022, 18th of November 2022).

Informed consent statement

Before data collection, the principal researcher obtained informed consent from all interested participants.

Data availability statement

Data sharing does not apply to this article as no datasets were generated or analyzed during the current study.

Conflict of interest

The authors declare that they have no competing interests.

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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.2024.07.025.

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