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ORIGINAL ARTICLE

Clinical Trials and Investigations

Altered proteome profiles related to visceral adiposity may mediate the favorable effect of green Mediterranean diet: the DIRECT-PLUS trial

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Abstract

Objective: The objective of this study was to explore the effects of a green Mediterranean (green-MED) diet, which is high in dietary polyphenols and green plant-based protein and low in red/processed meat, on cardiovascular disease and inflammation-related circulating proteins and their associations with cardiometabolic risk parameters.

Methods: In the 18-month weight loss trial Dietary Intervention Randomized Controlled Trial Polyphenols Unprocessed Study (DIRECT-PLUS), 294 participants with abdominal obesity were randomized to basic healthy dietary guidelines, Mediterranean (MED), or green-MED diets. Both isocaloric MED diet groups consumed walnuts (28 g/day), and the green-MED diet group also consumed green tea (3–4 cups/day) and green shakes (Mankai plant shake, 500 mL/day) and avoided red/processed meat. Proteome panels were measured at three time points using Olink CVDII.

Results: At baseline, a dominant protein cluster was significantly related to higher phenotypic cardiometabolic risk parameters, with the strongest associations attributed to magnetic resonance imaging-assessed visceral adiposity (false discovery

rate of 5%). Overall, after 6 months of intervention, both the MED and green-MED diets induced improvements in cardiovascular disease and proinflammatory risk proteins ($p < 0.05$, vs. healthy dietary guidelines), with the green-MED diet leading to more pronounced beneficial changes, largely driven by dominant proinflammatory proteins (IL-1 receptor antagonist protein, IL-16, IL-18, thrombospondin-2, leptin, prostasin, galectin-9, and fibroblast growth factor 21; adjusted for age, sex, and weight loss; $p < 0.05$). After 18 months, proteomics cluster changes presented the strongest correlations with visceral adiposity reduction.

Conclusions: Proteomics clusters may enhance our understanding of the favorable effect of a green-MED diet that is enriched with polyphenols and low in red/processed meat on visceral adiposity and cardiometabolic risk.

INTRODUCTION

The term "proteomics" refers to the study of large-scale circulating protein expression, derived from genetic transcription and posttranscriptional modifications. This cutting-edge resource can be used for personalized therapies, preventive measures, and disease management [1–7]. Moreover, proteomics, a field that offers a new approach to nutritional science, has the potential to identify an individual's metabolic, physiological, and clinical conditions [1–7], and this could be particularly relevant in the contexts of obesity, inflammation, and cardiovascular disease (CVD) [1–7], as well as in the assessment of dietary intake [2, 8, 9].

A diverse body of literature supports the favorable effect of the Mediterranean (MED) diet, which is rich in polyphenols and based on an increased intake of fruits, vegetables, whole grains, olive oil, nuts, and seeds; this diet is also moderately high in levels of polyunsaturated and monounsaturated fatty acids, which are known to assist in the prevention of CVD risk while decreasing inflammatory responses and visceral adiposity [10–13]. These beneficial effects might be mediated by plant-based polyphenols with antioxidant properties [14], which play a crucial role in alleviating inflammation and reducing mortality risk [15, 16]. Although polyphenols are known to be associated with inhibiting key regulators of proinflammatory cytokines and reducing CVD risk [14, 16, 17], studies on the effect of lifestyle interventions on serum proteome signature patterns are limited.

The present study is a secondary analysis of the Dietary Intervention Randomized Controlled Trial Polyphenols Unprocessed Study (DIRECT-PLUS), an 18-month lifestyle intervention that evaluated the effect of the green Mediterranean (green-MED) diet. Although based on the classic MED diet, the green-MED diet is enhanced with polyphenols and green plant-based proteins and low in red/processed meat. The green-MED diet has been shown to decrease visceral and intrahepatic fat compared with the MED diet [18, 19], reduce age-related brain atrophy, and induce improvements in cardiometabolic risk effects [17–20]. However, the effect of this diet on proteomics profiles has not been thoroughly characterized. Moreover, the identification of proteomics profiles may contribute to our understanding of associations with the favorable effect of the diet. Therefore, the aim of this study was to investigate the effect of the green-MED diet on inflammation and CVD-related circulating proteins and their associations with visceral adipose tissue (VAT) and cardiometabolic risk parameters.

Study Importance

What is already known?

- A green Mediterranean (green-MED) diet, which is high in dietary polyphenols and low in red/processed meat, has been previously shown to promote the loss of visceral and intrahepatic fat, reduce age-related brain atrophy, and induce improvement in cardiometabolic risk effects mediated by the gut microbiome.

What does this study add?

- We identified a dominant cluster of proteins significantly related to increased phenotypic cardiometabolic risk parameters, with the strongest associations attributed to magnetic resonance imaging-assessed visceral adiposity.
- After 6 months of intervention, the two Mediterranean diets (Mediterranean [MED] and green-MED) induced substantial changes in proinflammatory and cardiovascular disease risk marker proteins compared with the control group, with the green-MED diet leading to more pronounced changes.

How might these results change the direction of research or the focus of clinical practice?

- Proteomics, i.e., large-scale studies of protein expression, could enhance our understanding of the effect of lifestyle and dietary modifications while offering a valuable approach to obesity and nutritional science.

METHODS

Study design

The DIRECT-PLUS trial (ClinicalTrials identifier: NCT03020186) was conducted in an isolated workplace (Negev Nuclear Research Center, Dimona, Israel), where a monitored lunch was provided. Of the 378 volunteers, 294 met the following inclusion criteria: 30+ years of age with abdominal obesity (waist circumference [WC]: men, >102 cm; women, >88 cm) or dyslipidemia (triglycerides > 150 mg/dL and high-density lipoprotein [HDL] cholesterol: men, ≤40 mg/dL; women, ≤50 mg/dL). Exclusion criteria included any of the following: an inability to partake in physical activity (PA); serum creatinine levels ≥ 2 mg/dL; disturbed liver function; a major illness that might require hospitalization; pregnancy or lactation; presence of active cancer or chemotherapy treatment within the past 3 years; participation in another trial at that time; current treatment with warfarin (due to the interaction with vitamin K); and implants that would preclude magnetic resonance imaging (MRI). The study protocol for the DIRECT-PLUS trial was approved by the Medical Ethics Board and the Institutional Review Board at the Soroka University Medical Center. All participants signed and submitted an informed consent form, and no financial compensation was offered. In the present study, 290 individuals were included (healthy dietary guidelines [HDG] group, $n = 98$; MED diet group, $n = 97$; and green-MED diet group, $n = 95$).

Randomization and intervention

After undergoing baseline measurements, the participants were randomly assigned to one of the following three dietary intervention groups (1:1:1 ratio), stratified by sex and working sites (to ensure equal workplace-related lifestyle features among groups): basic HDG; isocaloric MED diet; or isocaloric green-MED diet. All groups received PA recommendations, a free gym membership, and educational sessions promoting moderate-intensity PA [11, 18, 19] with ~80% aerobic content (Supplemental Data S1). Periodic nutritional sessions were also held in the workplace. Dietary and PA interventions are fully described in Table S1. Randomization was conducted in a single phase because the interventions were conducted simultaneously, and the participants were aware of their assigned group (open-label protocol). The HDG diet group received basic health-promotion guidelines for maintaining a healthy diet, and the MED diet group was instructed to follow a calorie-restricted, traditional MED diet that was low in simple carbohydrates (similar to the DIRECT and CENTRAL trials) [10, 11]. Both the MED and green-MED diet groups were equally calorie-restricted (men, 1500–1800 kcal/day; women, 1200–1400 kcal/day), and about 40% of their total fat intake was mainly from polyunsaturated and monounsaturated fatty acids and consisted of less than 40 g/day of carbohydrates during the first 2 months, with a gradually increased intake of up to 80 g/day. In addition, both MED diet groups consumed 28 g/day of walnuts (containing ~440 mg of polyphenols per day, i.e., gallic acid equivalents, according to the Phenol-Explorer http://phenol-explorer.eu/food-processing/foods; version 3.6), including mostly ellagitannins and ellagic acid and its derivatives [21–23]. The green-MED diet was richer in polyphenols and green plant-based proteins, and, in addition to being instructed to avoid red/processed meat, participants in this group were also guided to consume 3 to 4 cups/day of green tea, as well as 500 mL of *Wolffia globosa* green shake (Mankai cultivated strain) [18, 19] each evening, which was made from ingredients from their regular diet regimen (fruits, walnuts, or vegetables). The green protein shake was a partial substitute for their evening meal, replacing beef/poultry protein sources. The green tea and Mankai shake provided an additional daily intake of ~800 mg of polyphenols (gallic acid equivalents; Phenol-Explorer and Eurofins laboratory analysis), beyond the polyphenol content in the MED diet. The participants were given green tea, walnuts, and Mankai at their worksite, free of charge.

The participants were instructed to follow their assigned lifestyle intervention for 18 months, including their diet regime and nutritional and PA sessions in the workplace. Conducted with the help of a multidisciplinary team of experts (physicians, clinical dietitians, and fitness instructors), these 90-min sessions were held on a weekly basis during the first month of the intervention and then on a monthly basis for the following 17 months. Participants from all three groups received the same type and intensity of lifestyle educational programs. Text messages with relevant information for each intervention group were sent to the participants at fixed time intervals to enhance motivation. In addition, a website listing all of the information needed regarding the dietary regime and PA was accessible to each intervention group. Adherence was assessed through self-reported dietary intake and submitted at three time points (baseline, 6 months, and 18 months into the intervention). Lifestyle and PA questionnaires were also completed by the participants at these three time points [24]. Finally, the participants' closed workplace enabled convenient monitoring of the freely provided lunches, as well as the presence of an onsite medical clinic. The intervention protocols and additional details regarding the polyphenol content can be accessed elsewhere [18, 19].

In-person data collection visits

Anthropometric parameters (i.e., weight and WC) and blood biomarkers were taken at three time points: baseline and 6 and 18 months into the intervention. Body weight was measured without shoes to the nearest 0.1 kg, and WC was measured halfway between the last rib and the iliac crest to the nearest millimeter through standard procedures using an anthropometric measuring tape. Blood samples were taken after fasting for 12 h. Blood samples were centrifuged, and both blood samples were stored at −80°C. Serum HDL cholesterol and triglycerides (coefficient of variation [CV] of 2.1%) were determined enzymatically with a Cobas 8000 automatic analyzer (Roche). Plasma glucose levels were measured using the Roche GLUC3 (the hexokinase method). Plasma insulin levels were measured using a Roche Elecsis assay. The homeostatic model assessment of insulin resistance was calculated as follows: (insulin [micro-international units per milliliters] × glucose [milligrams per

deciliters])/405. All biochemical analyses were performed at the Institute of Laboratory Medicine of the University of Leipzig, Germany.

Proteomics profiling

Proteomics profiling via the blood samples was conducted through an immunoaffinity proteomics technology using the Olink Biosciences Platform. A targeted Olink CVDII protein panel was analyzed, including 92 proteins, at all three time points (baseline, 6 months, and 18 months). This proximity extension assay (PEA) technique uses an extensive collection of oligonucleotide-labeled, highly specific antibodies in an immuno-polymerase chain reaction method [25]. The final assay readout is presented as arbitrary units, i.e., relative protein quantification as a log2-normalized protein expression, whereby higher values correspond with higher protein expression. Each PEA measurement has a lower limit of detection based on negative controls that were included in each run. A total of 1410 out of 1430 samples (99%) passed quality control, with an intra-assay CV of 6% and an inter-assay CV of 17%. Proteins with ≥20% of samples below the limit of detection were excluded ($n = 2$).

VAT quantification

VAT was assessed at two time points (baseline and 18 months into the intervention) using 3-T MRI scans (Philips Ingenia 3.0 T) [11, 18]. The abdominal fat was quantified using the MATLAB-based semiautomatic software [11, 18] and blinded to the intervention group. The mean VAT was calculated along with two axial sections, L5-S1 and L4-L5. Further information can be found in Supplemental Data S2 and in our previous publication [18].

Statistical analysis

All statistical analyses were performed using R version 3.5.3 and SPSS version 29.0. Continuous variables are presented as mean ± SD. Nominal variables are expressed as numbers and percentages. The Kolmogorov–Smirnov test was used to determine whether variables were normally distributed, and natural log transformations were applied when needed in order to achieve normal distributions. Differences in values over time were examined using a paired-sample *t* test or Wilcoxon test for 18-month changes or, for the three time points, using ANOVA for repeated measures. Differences across groups were tested using ANOVA, the Kruskal-Wallis test, or the χ^2 statistic. Multiple comparisons were adjusted using the Tukey post hoc test (for ANOVA) and Bonferroni correction (for Kruskal-Wallis). General and generalized linear regression models were used for adjustments and interaction models (the specific adjustments are detailed in the *Results* section). Pearson correlations were used to assess associations between proteomics and cardiometabolic parameters (WC, HDL cholesterol, triglycerides, homeostatic model assessment of insulin resistance, and VAT) at two time points, i.e., baseline and 18 months into the intervention (presented as change: time 18 − time 0), and Benjamini-Hochberg correction was used for multiple comparisons, indicating significant associations after adjusting for multiple testing (false discovery rate [FDR] of 5%). Next, to evaluate the effect of dietary intervention on proteomics expression over all three time points and across interventions, multidimensional scaling was applied to extract the first principal coordinate, followed by an estimated linear mixed model, where the fixed effects were group, time, and the interaction between the two groups (group × time), with participant identifier as random. In addition, the data are presented using volcano plots, depicting protein expression at 6 and 18 months into the intervention compared with baseline, with the 10 most significant proteins being colored and corrected for an FDR of 5% ($n = 90$). Next, we focused on the trajectories of the most dominant proteins from baseline (proteins that had ≥5 significant correlations). For each change in protein expression over time and across dietary groups, generalized linear models were used to assess between-group differences, adjusted for age, sex, and weight loss. All *p* values were corrected for multiple comparisons using the Benjamini-Hochberg procedure with an FDR of 5%. Statistical significance was set at a two-sided $\alpha = 0.05$.

RESULTS

Baseline characteristics

Of the 294 participants who began the intervention (as part of the DIRECT-PLUS trial), 290 were eligible for proteomics analysis, and losses were due to invalid blood samples. Most participants were men (88.3%), reflecting the nature of the workplace, with a mean age of 51.1 (SD 10.6) years, and 58.6% had obesity (body mass index [BMI] > 30 kg/m^2). Baseline parameters and proteomics data were similarly distributed across the intervention groups (Table 1).

Baseline correlations of proteomics with cardiometabolic parameters

As part of the baseline analysis (Figure 1), we identified two main circulating protein clusters. Although the first cluster was highly significantly correlated with cardiometabolic parameters, with interleukin (IL)-1 receptor antagonist protein (IL-1RA; FDR of 5%) being the most dominant and significantly correlated protein, the second cluster exhibited inverse associations with cardiometabolic parameters (stem cell factor [SCF], lipoprotein lipase [LPL], and growth hormone [GH]; FDR of 5%). The most dominant proteins with the highest number of correlations with increased cardiometabolic risk parameters (i.e., at ≥5 significant correlations after FDR correction) at baseline were IL-1RA, IL-16, IL-18, thrombospondin-2 (THBS2), leptin, prostasin (PRSS8), galectin-9 (GAL9), fibroblast growth factor 21 (FGF21), angiotensin-converting enzyme 2 (ACE2), hydroxyacid oxidase 1 (HAOX1), and proheparin-binding EGF-like growth factor (HBEGF; Figure 1). VAT

TABLE 1 Baseline characteristics and proteomics levels of the DIRECT-PLUS population across intervention groups ($n = 290$).

	All ($n = 290$)	HDG ($n = 98$)	MED ($n = 97$)	Green-MED ($n = 95$)
Age (y)	51.1 ± 10.6	51.1 ± 10.5	51.5 ± 10.5	50.6 ± 11.0
Men, *n* (%)	256 (88.3)	86 (87.7)	85 (87.6)	85 (89.5)
Weight (kg)	93.7 ± 14.4	92.9 ± 14.7	94.4 ± 13.5	93.7 ± 15.1
BMI	31.3 ± 4.0	31.25 ± 3.8	31.28 ± 4.0	31.35 ± 4.3
Participants with obesity (BMI>30), *n* (%)	170 (58.6)	59 (60.2)	57 (58.8)	54 (56.8)
Systolic blood pressure (mm Hg)	130.3 ± 14.1	130.2 ± 14.3	130.2 ± 12.5	130.5 ± 15.4
Diastolic blood pressure (mm Hg)	81.1 ± 10.3	80.2 ± 11.3	81.8 ± 8.8	81.2 ± 10.5
VAT (cm^2)	131.5 ± 48.8	134.3 ± 49.3	129.6 ± 43.0	130.4 ± 54.2
Blood proteomics levels				
IL-1RA	5.52 ± 0.60	5.53 ± 0.63	5.47 ± 0.55	5.56 ± 0.61
IL-16	6.90 ± 0.40	6.89 ± 0.40	6.84 ± 0.39	6.97 ± 0.39
IL-18	8.78 ± 0.48	8.81 ± 0.49	8.67 ± 0.50	8.87 ± 0.42
THBS2	6.71 ± 0.15	6.72 ± 0.14	6.68 ± 0.15	6.73 ± 0.16
Leptin	8.52 ± 0.85	8.43 ± 0.82	8.55 ± 0.92	8.58 ± 0.81
PRSS8	9.40 ± 0.31	9.39 ± 0.31	9.39 ± 0.31	9.43 ± 0.31
GAL9	9.05 ± 0.29	9.05 ± 0.26	9.02 ± 0.29	9.09 ± 0.31
FGF21	7.66 ± 1.19	7.60 ± 1.19	7.58 ± 1.18	7.81 ± 1.20
ACE2	4.68 ± 0.54	4.70 ± 0.53	4.65 ± 0.55	4.70 ± 0.53
HBEGF	7.63 ± 0.51	7.61 ± 0.46	7.60 ± 0.55	7.67 ± 0.53
HAOX1	6.49 ± 1.30	6.60 ± 1.44	6.35 ± 1.28	6.52 ± 1.18

Note: Values are presented as mean ± SD for continuous variables and as number (percentage) for categorical variables. The baseline characteristics were not significantly different across the randomized intervention groups (evaluated using ANOVA/Kruskal-Wallis test for continuous variables and χ^2 for categorical variables). The presented proteins are the most dominant cardiometabolic risk-related proteins from baseline (with ≥5 significant correlations, corrected for multiple comparisons; Figure 1).

Abbreviations: ACE2, angiotensin-converting enzyme 2; DIRECT-PLUS, Dietary Intervention Randomized Controlled Trial Polyphenols Unprocessed Study; EGF, epidermal growth factor; FGF21, fibroblast growth factor 21; GAL9, galectin-9; HAOX1, hydroxyacid oxidase 1; HBEGF, proheparin-binding EGF-like growth factor; HDG, heathy dietary guidelines; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; IL-1RA, IL-1 receptor antagonist protein; LDL, low-density lipoprotein; MED, Mediterranean; PRSS8, prostasin; THBS2, thrombospondin-2; VAT, visceral adipose tissue.

exhibited the highest number of significant baseline correlations, with 43 protein correlations, compared with WC (31 correlations) and with body weight, which only had 16 significant correlations (Figure 1).

Effect of diets on proteomics following 6 and 18 months

After 6 and 18 months of intervention, participants demonstrated high retention rates, with 98% at 6 months and 89% at 18 months. Overall, at the 6-month assessment, both MED diets had induced substantial changes in proinflammatory and CVD proteins (Figures 2 and 3), with the green-MED diet leading to more prominent changes (Figure 3B). The HDG diet group only showed a significant increase in protein expression (pentraxin-related protein PTX3, α-L-iduronidase [IDUA], lectin-like oxidized low-density lipoprotein receptor 1 [LOX1], proto-oncogene tyrosine-protein kinase SRC, SLAM family member 5 [CD84], carcinoembryonic antigen-related cell adhesion molecule 8 [CEACAM8], serine/threonine-protein kinase 4 [STK4], C-C motif chemokine 3 [CCL3], 2,4-dienoyl-coenzyme A [CoA] reductase 1 [DECR1], and heat shock 27 kDa protein [HSP27]), whereas the MED diet group exhibited both an increase (PTX3, IDUA, STK4, LOX1, and GH) and decrease in protein levels (HAOX1, carbonic anhydrase 5A [CA5A], leptin, PRSS8, and cystatin-like 1 [CSTL1]) and, finally, the green-MED diet group only exhibited a significant decrease in proinflammatory and CVD risk proteins (CSTL1, HAOX1, IL-18, leptin, protein AMBP, PRSS8, spondin-2 [SPON2], IL-1 receptor-like 2 [IL-1RL2], macrophage receptor MARCO, and FGF21). At the 18-month assessment, most proteins had significantly decreased over time (Figure 3C), with similar patterns being seen across groups compared with the 6-month assessment. As seen in Figure 3D, the HDG group exhibited an increase in pro-adrenomedullin (ADM), matrix metallopeptidase 12 (MMP12), AMBP, P-selectin glycoprotein ligand 1 (PSGL1), lymphotactin (XCL1), LPL, decorin (DCN), and bone morphogenetic protein 6 (BMP6) and a decrease in IDUA and HBEGF. However, both MED diets led to a decrease in the proinflammatory proteins (MED diet group increased ADM and decreased IDUA, CEACAM8, poly [ADP-ribose] polymerase 1 [PARP1], CD40 ligand [CD40L], STK4, IL-18, IL-1RA, LOX1, and SRC; green-MED diet group decreased IL-18,

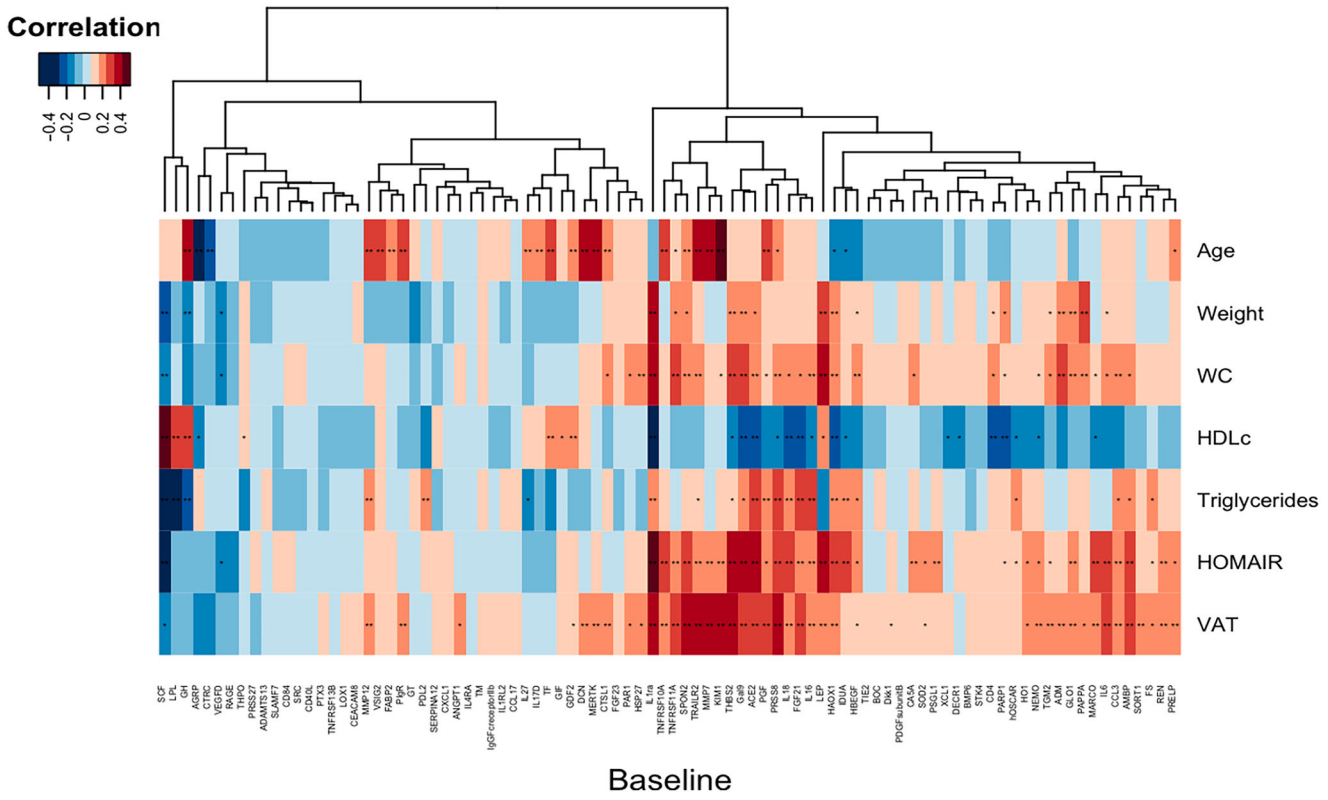

FIGURE 1 Heat map of baseline correlations between plasma proteomics ($n = 90$) and cardiometabolic parameters adjusted for multiple comparisons. Color-coded with direction of correlation (red = positive correlation, blue = negative correlation). Pearson correlation. Benjamini-Hochberg correction was used for multiple comparisons and indicated associations that were significant after adjusting for multiple testing (FDR of 5%). $^{**}p < 0.01$, $^{*}0.01 < p < 0.05$. FDR, false discovery rate; HDLc, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; VAT, visceral adipose tissue; WC, waist circumference.

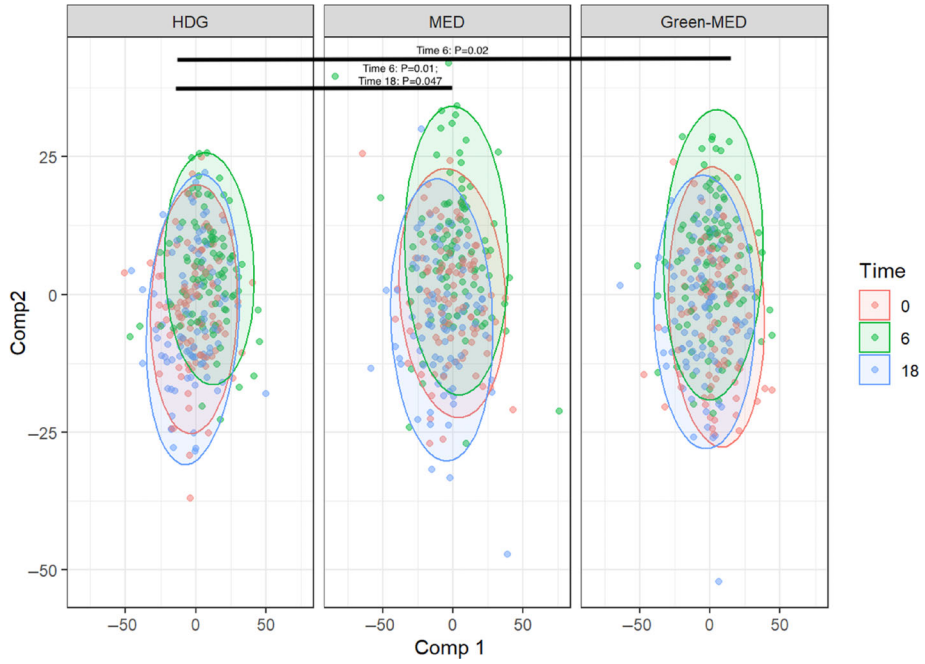

FIGURE 2 Differential protein expression in plasma across intervention groups after 6 and 18 months of follow-up. Multidimensional scaling analysis across intervention groups. Estimate is a linear mixed model where the fixed effects are group, time, and their interaction. In addition, the random effect is the effect of the individual. *P* value adjustment: FDR of 5%, $n = 90$. FDR, false discovery rate; green-MED, green Mediterranean; HDG, healthy dietary guidelines; MED, Mediterranean.

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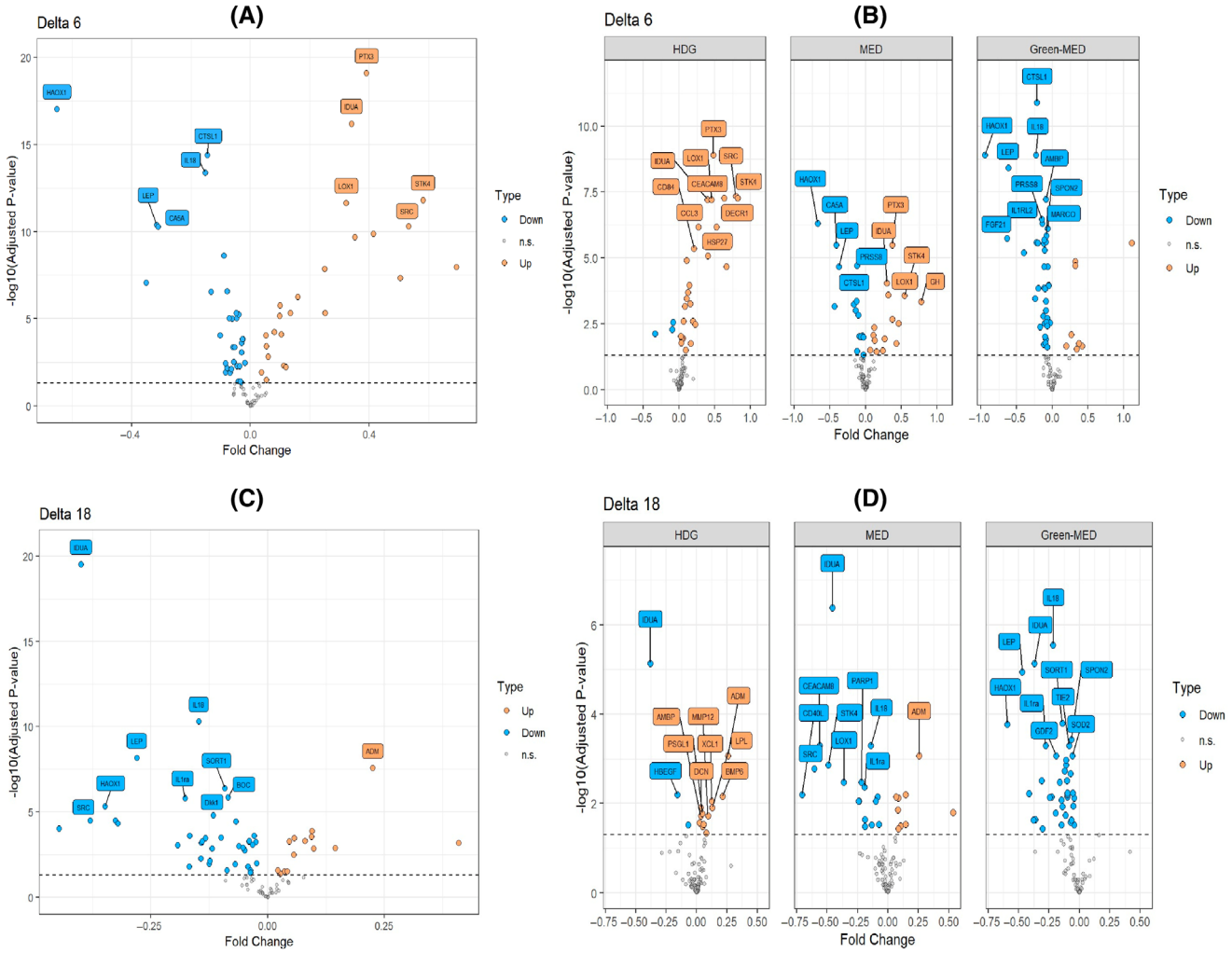

FIGURE 3 Profiling of plasma proteomics after 6 and 18 months of intervention. Volcano plot presenting protein expression after 6 and 18 months compared with baseline. Significant proteins are colored in blue (negative) or orange (positive). Corrected for FDR of 5%, $n = 90$. (A) Protein expression after 6 months. (B) Protein expression after 6 months across intervention groups. (C) Protein expression after 18 months. (D) Protein expression after 18 months across intervention groups. FDR, false discovery rate; green-MED, green Mediterranean; HDG, healthy dietary guidelines; MED, Mediterranean.

IDUA, leptin, sortilin [SORT1], SPON2, TEK tyrosine kinase [TIE2], IL-1RA, HAOX1, superoxide dismutase 2 [SOD2], and growth/differentiation factor 2 [GDF2]; Figure 3D). The significant changes across groups had attenuated after 18 months and FDR correction; however, the MED diet was still significantly different compared with the HDG diet ($p = 0.047$; Figure 2). As seen in Figure 4, when examining the most dominant and proinflammatory proteins from the baseline analysis (proteins that had ≥5 significant correlations), the effect of the green-MED diet led to a significant decrease compared with the HDG and MED diet groups after 6 months of intervention, adjusted for age, sex, and weight loss (IL-1RA, IL-16, leptin, GAL9, PRSS8, and FGF21). THBS2 and IL-18 levels had also significantly decreased compared with the HDG diet group. ACE2, HAOX1, and HBEGF levels after 6 and 18 months had no significant differences across groups, adjusted for age, sex, and weight loss. The full list of all trajectories and mean changes over time and across groups is presented in Table S2.

Proteomics and cardiometabolic parameter changes after 18 months of intervention

As seen in Figure 5, two main clusters of correlations were seen between the 18-month changes in proteomics expression and cardiometabolic parameters. Although leptin change exhibited the highest number of significant correlations with improved cardiometabolic parameters, the SCF change was the most dominant protein to have inverse correlations with cardiometabolic improvement. Although the first proteomics cluster (SCF and GH) had inverse significant correlations with improved cardiometabolic parameters, the second cluster (leptin, PRSS8, HAOX1, IL-1RA, CA5A, FGF21, ACE2, IL-18, and IL-1RL2; proteins with ≥5 significant direct correlations) exhibited direct correlations with reductions in cardiometabolic risk parameters (Figure 5). Finally, the cardiometabolic parameters that had the highest number of significant correlations at baseline and across time included leptin, PRSS8, HAOX1, IL-1RA, FGF21, ACE2, and IL-18.

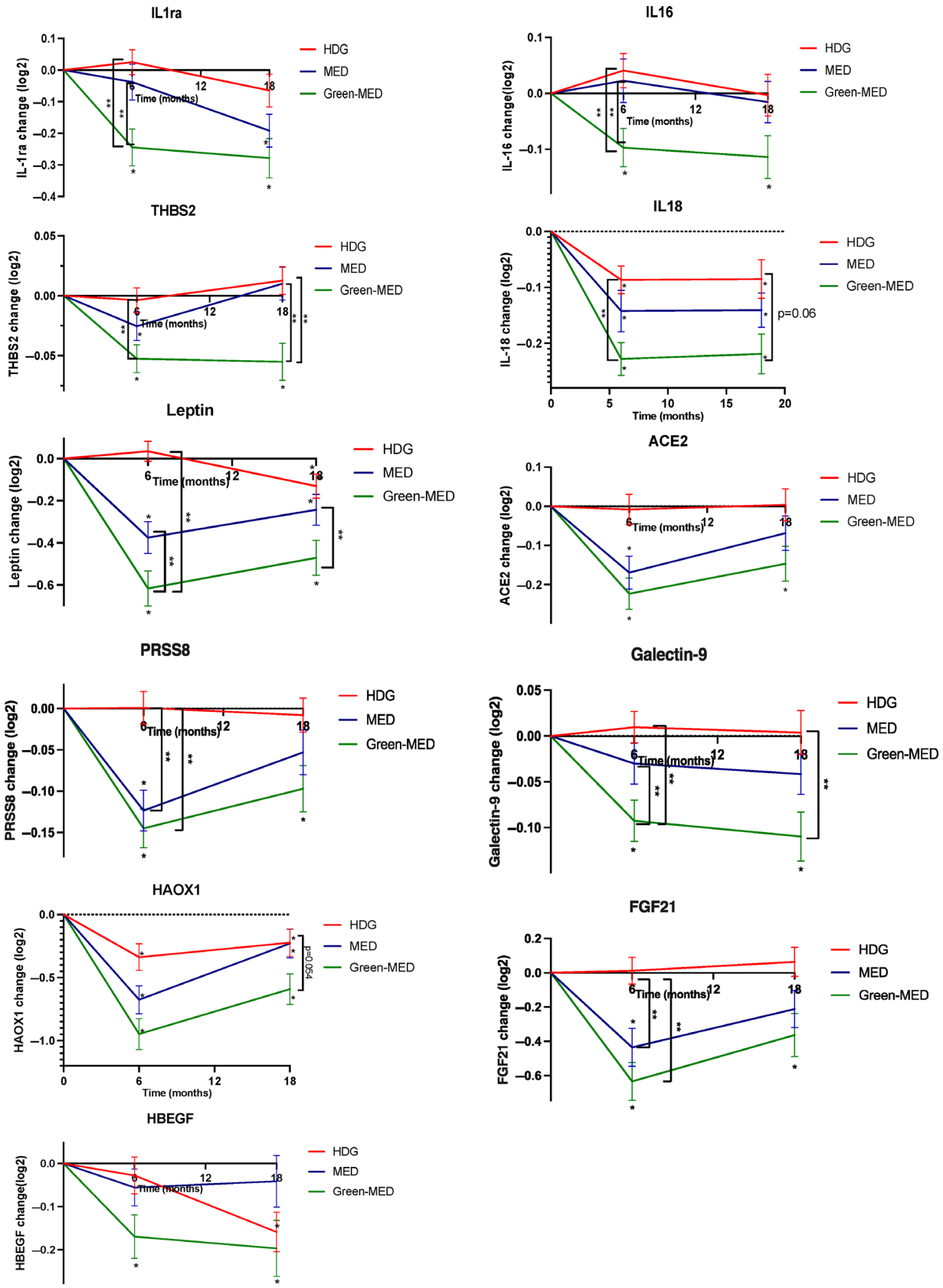

FIGURE 4 The effect of green-MED diet on proteomics profiles, with the highest association with cardiometabolic parameters at baseline adjusted for sex, age, and weight loss. *$p < 0.05$, significant change compared with baseline. **$p < 0.05$, significant across the interventions. Green-MED, green Mediterranean; HDG, healthy dietary guidelines; MED, Mediterranean.

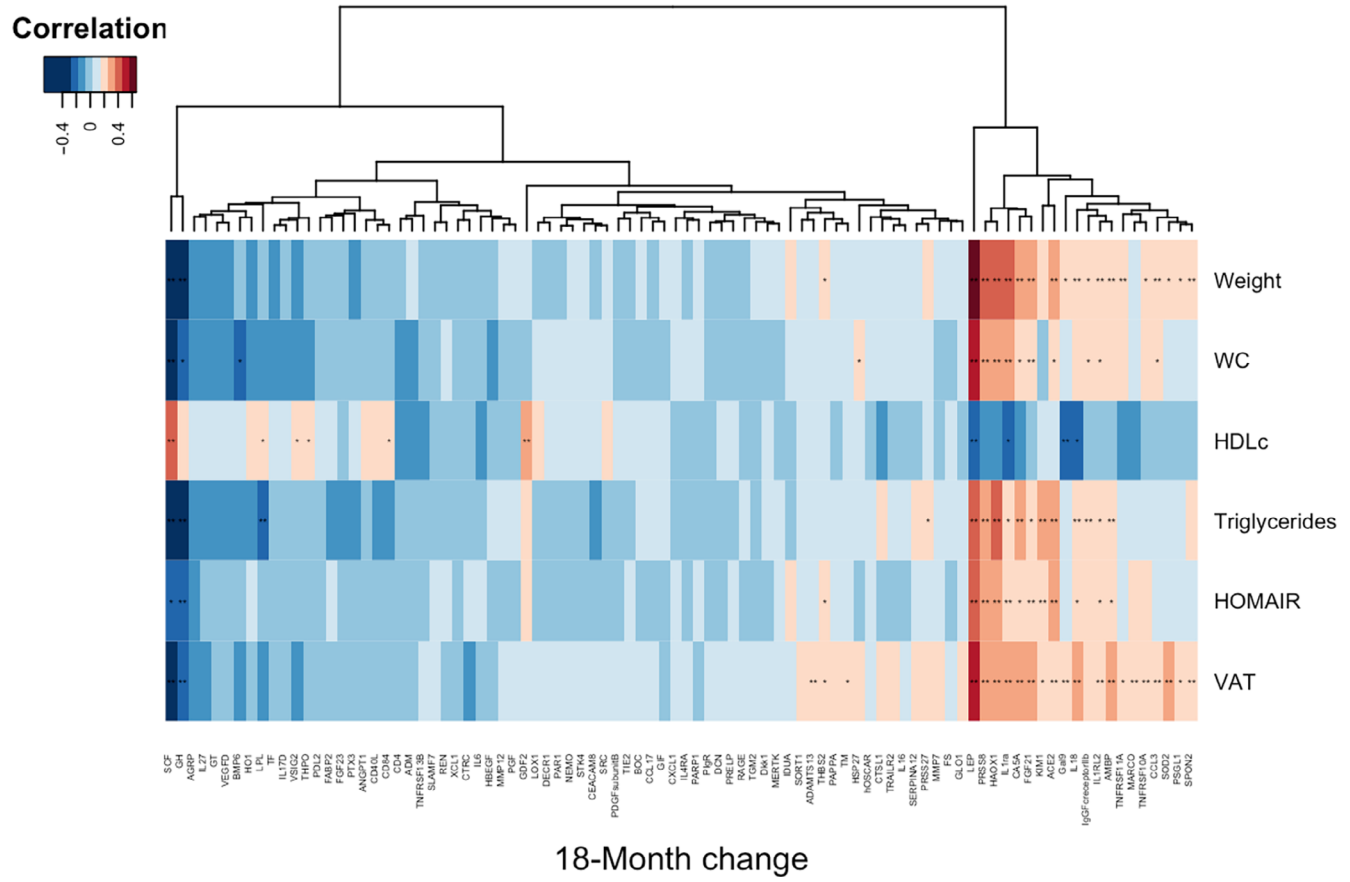

FIGURE 5 Heat map of associations between changes in proteomics and cardiometabolic parameters ($n = 90$) adjusted for multiple comparisons after 18 months of intervention. Color-coded with direction of correlation (red = positive correlation, blue = negative correlation). Pearson correlation. Benjamini-Hochberg correction was used for multiple comparisons, indicated associations significant after adjusting for multiple testing (FDR of 5%). $^{**}p < 0.01$, $^{*}0.01 < p < 0.05$. FDR, false discovery rate; HDLc, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; VAT, visceral adipose tissue; WC, waist circumference.

VAT had the highest correlations with proteomics changes after FDR of 5% and multiple comparisons correction.

DISCUSSION

This 18-month DIRECT-PLUS dietary intervention randomized controlled trial, which included 290 participants, aimed to examine the effects of a green-MED diet, which was rich in dietary polyphenols and green plant-based proteins and low in red/processed meat, on CVD-related proteomics profiles. In addition, we set out to identify circulating proteins that might be associated with the distinct obesity phenotype of cardiometabolic risk factors. We identified an inflammatory and cardiovascular protein cluster that had the most significant direct associations with higher phenotypic cardiometabolic risk parameters at baseline. Overall, following 6 months of intervention, both MED diets induced beneficial changes in the proinflammatory and CVD proteins, with the green-MED diet leading to more prominent changes, largely driven by the proinflammatory cytokines (adjusted for age, sex, and weight loss; $p < 0.05$ for all). After 18 months of intervention (with an 89% retention rate), two major and distinct protein clusters were detected that exhibited opposite correlations with the beneficial cardiometabolic change over time. The first cluster (including SCF and GH changes) showed significant inverse correlations, whereas the second cluster (including leptin, PRSS8, HAOX1, IL-1RA, CA5A, FGF21, ACE2, IL-18, and IL-1RL2) revealed significant direct correlations. VAT area at baseline and reduction over time was found to be the parameter with the highest and strongest correlations with proteomic signatures.

VAT, an intra-abdominal fat depot, can trigger the secretion of specific proinflammatory agents and increase the risk of developing metabolic traits for multiple CVD risk factors [26]. Our randomized controlled trial supports this concept and expands it by identifying VAT as the most dominant parameter at baseline and after 18 months, with a panel of 90 proteins reflecting CVD and inflammation state. The most significant of these proteins included proinflammatory cytokines (including IL-1RA, IL-16, and IL-18) and others (such as leptin, THBS2, PRSS8, GAL9, FGF21, ACE2, HAOX1, and HBEGF). Our findings include some expected VAT associations, especially with the well-established leptin and FGF21 levels [11, 18, 27] and the proinflammatory cytokines IL-16 and IL-18, which have been found to have

higher secretion from VAT than subcutaneous adipose tissue [28, 29]. Another protein that was significantly correlated with VAT was THBS2, a matricellular protein expressed in adipose tissue that has been proposed to contribute to cardiac fibrosis [30] and might be upregulated by nutritionally induced obesity in animal models [31].

Interestingly, IL-1RA, classified as a cytokine with relatively high secretion levels compared with IL-6, tumor necrosis factor (TNF)-α, and IL-1 levels, has anti-inflammatory properties as an antagonist to the proinflammatory cytokine IL-1 [32]. However, in patients with obesity, IL-1RA has been shown to induce leptin resistance, probably by antagonizing leptin at the hypothalamic level [33]. Moreover, when measured in a sample of 117 healthy men without diabetes, IL-1RA was found to be elevated in those with obesity and influenced to a greater extent by visceral rather than subcutaneous adiposity [34]. VAT has also been found to be the best independent predictor of IL-1RA levels [34], whereas plasma IL-1RA levels have been reported to decrease following bariatric surgery [33]. In fact, plasma IL-1RA levels have been found to be elevated in human morbid obesity to a similar extent as with systemic inflammation, which is encountered in inflammatory autoimmune diseases [35]. In the current study, we found that, at baseline, IL-1RA had the strongest correlations with cardiometabolic parameters and VAT accumulation, and its reduction had the strongest correlations with cardiometabolic improvement. However, it is important to mention that we measured relative protein expression and not IL-1RA secretion.

After 18 months of intervention, a protein cluster including an increase in SCF and GH, was found to be correlated with cardiometabolic improvement over time. GH is a peptide hormone released by somatotrophic cells located in the anterior pituitary gland, known to induce insulin-like growth factor 1 (IGF-1) [36]. It facilitates lipolysis in adipose tissue, stimulates protein synthesis in skeletal muscle, and promotes bone growth [37]. It was recently found that GH had a strong inverse association with fat mass index [38]. Increased SCF was found to be associated with better cardiovascular outcomes [39], lower risk of cardiovascular development (myocardial infarction, stroke, and heart failure), and all-cause mortality [40]. It also had a negative association with C-reactive protein (CRP) concentrations [41].

The green-MED diet combines elements that might explain the beneficial effects on inflammation and CVD-related proteomics profiles. For example, the enrichment in polyphenols comes from the additional 800 mg/day derived from the green tea and *Wolffia globosa* Mankai plant shake, with their possible antioxidant and anti-inflammatory properties [42, 43]. Such properties include improvements in endothelial structure through function by transcriptional networks that modulate gene expression while promoting anti-inflammatory mediators and nitric oxide production [44]. Moreover, polyphenols could potentially explain the strong correlations seen in this study between VAT reduction and improvements in inflammation and CVD-related proteomics profiles. Polyphenols have a beneficial effect on adipose tissue by increased fatty acid oxidation, inhibition of adipocytes differentiation, decreased fatty acid synthesis, and increased thermogenesis [45, 46], which could eventually lead to decreased cytokine secretion. An additional component of the MED and green-MED diets that could potentially explain the beneficial reduction seen in the proteomics profiles is red meat consumption, which is associated with increased inflammation [47] and may activate nuclear factor-κB and oxidative stress and produce proinflammatory cytokines [48, 49].

Several limitations should be acknowledged. The low proportion of women reflects the workplace. Additionally, we cannot identify the exact components responsible for the dietary effects because we compared dietary regimens and not specific nutrients. We used a target proteomics analysis related to CVD and inflammation that identified and quantified a limited number of proteins. However, the presented method is well-established, sensitive, reproducible, and quantitative. In addition, the recommended PA was monitored by self-report for all groups and not by direct objective means. The strengths of the study include the relatively large sample size, high retention rate, the novelty of the study, and the use of 3-Tesla MRI measurements for VAT quantification. Furthermore, the closed workplace enabled monitoring of the freely provided lunch and included the presence of an onsite clinic, intense dietary guidance and group meetings with multidisciplinary guidance, and access to polyphenol-rich foods provided at no charge.

In conclusion, the therapeutic benefits of the green-MED diet, which is enriched with polyphenols and low in red/processed meat, that have been demonstrated in previous publications might be explained by the improvement in inflammation and CVD-related proteomics profiles. Proteomics, i.e., large-scale studies of protein expression, could shed new light on the pathophysiologic pathways of obesity on inflammation and CVD profiles and may enhance our understanding of the effect of lifestyle and dietary modifications while offering a valuable approach to nutritional science. Future studies are required to further investigate the mechanisms behind the beneficial effects in the gut-fat-brain axis of the green-MED diet, with additional analysis of targeted and untargeted proteomics. To further explore the potential contribution of circulating proteins related to inflammation and CVD risk, it is necessary to make proteomics a more mainstream technology for clinical research.

AUTHOR CONTRIBUTIONS

Hila Zelicha had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: Iris Shai; conduct of the study: Hila Zelicha, Alon Kaplan, Anat Yaskolka Meir, Ehud Rinott, Gal Tsaban, and Iris Shai; collection, management, analysis, and interpretation of the data: all authors; review and approval of the manuscript: all authors; statistical analysis: Hila Zelicha; and supervision: Iris Shai. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

Matthias Blüher received honoraria for lectures or consultancy from Amgen, AstraZeneca plc, Bayer, Boehringer-Ingelheim, Eli Lilly and Company, Novo Nordisk A/S, Novartis AG, Sanofi S.A., and Pfizer Inc. The other authors declared no conflict of interest.

CLINICAL TRIAL REGISTRATION

ClinicalTrials.gov identifier NCT03020186.

DATA AVAILABILITY STATEMENT

The majority of results corresponding to the current study are included in the article or uploaded as online Supporting Information. No further data are available.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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