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# Effect of green-Mediterranean diet on intrahepatic fat: the DIRECT PLUS randomised controlled trial

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## ABSTRACT

**Objective** To examine the effectiveness of green-Mediterranean (MED) diet, further restricted in red/processed meat, and enriched with green plants and polyphenols on non-alcoholic fatty liver disease (NAFLD), reflected by intrahepatic fat (IHF) loss.

**Design** For the DIRECT-PLUS 18-month randomized clinical trial, we assigned 294 participants with abdominal obesity/dyslipidaemia into healthy dietary guidelines (HDG), MED and green-MED weight-loss diet groups, all accompanied by physical activity. Both isocaloric MED groups consumed 28 g/day walnuts (+440 mg/day polyphenols provided). The green-MED group further consumed green tea (3–4 cups/day) and Mankai (a *Wolffia globosa* aquatic plant strain; 100 g/day frozen cubes) green shake (+1240 mg/day total polyphenols provided). IHF% 18-month changes were quantified continuously by proton magnetic resonance spectroscopy (MRS).

**Results** Participants (age=51 years; 88% men; body mass index=31.3 kg/m<sup>2</sup>; median IHF%=6.6%; mean=10.2%; 62% with NAFLD) had 89.8% 18-month retention-rate, and 78% had eligible follow-up MRS. Overall, NAFLD prevalence declined to: 54.8% (HDG), 47.9% (MED) and 31.5% (green-MED),  $p=0.012$  between groups. Despite similar moderate weight-loss in both MED groups, green-MED group achieved almost double IHF% loss (–38.9% proportionally), as compared with MED (–19.6% proportionally;  $p=0.035$  weight loss adjusted) and HDG (–12.2% proportionally;  $p<0.001$ ). After 18 months, both MED groups had significantly higher total plasma polyphenol levels versus HDG, with higher detection of *Naringenin* and 2-5-dihydroxybenzoic-acid in green-MED. Greater IHF% loss was independently associated with increased Mankai and walnuts intake, decreased red/processed meat consumption, improved serum folate and adipokines/lipids biomarkers, changes in microbiome composition (beta-diversity) and specific bacteria ( $p<0.05$  for all).

**Conclusion** The new suggested strategy of green-Mediterranean diet, amplified with green plant-based proteins/polyphenols as Mankai, green tea, and walnuts, and restricted in red/processed meat can double IHF loss than other healthy nutritional strategies and reduce NAFLD in half.

**Trial registration number** NCT03020186.

## Significance of this study

### What is already known on this subject?

- Non-alcoholic fatty liver disease (NAFLD), a condition affecting 25% of the world population, is reflected by increased intrahepatic fat (IHF)% (>5%) and is associated with elevated liver enzymes, insulin resistance, type 2 diabetes and cardiovascular risk, as well as with decreased gut microbiome diversity and dysbiosis.
- Currently, an evidence-based treatment strategy consists of general weight-loss through lifestyle interventions.

### What are the new findings?

- In this trial, we introduce a new concept of a green Mediterranean diet, further enriched with specific green polyphenols as Mankai, green tea, and walnuts, and restricted in red and processed meat that might lead to significantly double intrahepatic fat loss, as compared with other healthy nutritional strategies.

### How might it impact on clinical practice in the foreseeable future?

- Results from this study may suggest an improved dietary protocol to reduce NAFLD.

## INTRODUCTION

Intrahepatic fat (IHF) accumulation, a result of intracellular triglyceride (TG) deposition in the liver, is promoted by bodily adipose tissue dysfunction and insulin resistance.<sup>1</sup> IHF that exceeds 5%, in the absence of alcohol abuse, defines non-alcoholic fatty liver disease (NAFLD).<sup>2</sup> IHF accumulation is associated with elevated liver enzymes, insulin resistance, type 2 diabetes, cardiovascular risk and extrahepatic malignancies.<sup>2,3</sup> In recent years, the gut microbiome was suggested to have a pivotal role in NAFLD pathogenesis. This association is presumably due to the modulation of hepatic carbohydrate and lipid metabolism, with dysbiosis, that is, aberrant composition of the microbiome community, being a hallmark of the disease.<sup>4,5</sup> NAFLD affects about a quarter of the world population<sup>6</sup> and can progress to the development of steatohepatitis, liver-cirrhosis and hepatocellular carcinoma.<sup>2,7</sup> The



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current evidence-based treatment strategy consists of weight-loss through lifestyle interventions,<sup>8</sup> without specific dietary recommendations, although strong evidence points toward recommending the Mediterranean (MED) diet.<sup>9</sup> MED diet, relatively rich in plant food sources, has been associated with reduced prevalence of NAFLD,<sup>10</sup> improves cardiometabolic and cardiovascular biomarkers, and reduces all-cause mortality.<sup>11–13</sup>

Polyphenols, secondary metabolites of plants with antioxidant properties, are involved in the defence against ultraviolet radiation and pathogenic insults in the plants and have been suggested, in humans, to be protective against several malignancies, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases,<sup>14</sup> as well as reducing hepatic steatosis.<sup>15</sup> The main groups of polyphenols are classified by the number of phenol rings they contain and structural elements and include phenolic acids, flavonoids, stilbenes and lignans.<sup>14</sup> The MED diet has a relatively high content of polyphenols. In the traditional Spanish MED diet, the mean polyphenol intake was estimated to be between ~2500 and 3000 mg/day<sup>16</sup> as compared with ~1000 mg/day in a western-style diet.<sup>17</sup> We, and others, reported a greater decrease in NAFLD with MED diet, as compared with a low-fat diet.<sup>3 18 19</sup> Adherence to vegetarian and plant-based diets was also associated with a lower incidence of NAFLD.<sup>20 21</sup>

In the current 18-month Dietary Intervention Randomized Controlled Trial Polyphenols Unprocessed (DIRECT PLUS), we aimed to examine the effect of MED diet, further enriched with polyphenols and lower in red and processed meat ('green-MED'), on IHF changes, as measured by proton magnetic resonance spectroscopy (H-MRS). Our a priori hypothesis was that a green-MED diet may promote further effectiveness in treating NAFLD, beyond the expected beneficial effects of the MED diet.

## METHODS

### Study design

The 18-month DIRECT-PLUS trial was initiated in May 2017 and was conducted in an isolated workplace (Nuclear Research Center Negev, Dimona, Israel), where a monitored lunch was provided. Most of the clinical and medical measurements and lifestyle-intervention sessions, were performed at the workplace's medical department. Of the 378 volunteers, 294 met the inclusion criteria of age >30 years with abdominal obesity (waist circumference (WC): men >102 cm, women >88 cm) or dyslipidaemia (TG >150 mg/dL and high-density lipoprotein (HDL) cholesterol ≤40 mg/dL for men, ≤50 mg/dL for women). Exclusion criteria are detailed in online supplemental methods 1.

### Randomisation and intervention

All eligible participants who signed consent to participate in the trial and completed the baseline measurements were randomised in a 1:1:1 ratio, stratified by gender and work site (to ensure equal workplace-related lifestyle features between groups), into one of the three intervention groups: healthy dietary guidelines (HDG), MED, green-MED, all combined with physical activity (PA) accommodation. The outline of lifestyle interventions is presented in online supplemental table 1.

The interventions were initiated simultaneously, and participants were aware of their assigned intervention (open-label protocol). All the participants received free gym memberships and educational sessions to engage in moderate-intensity PA,<sup>18</sup> ~80% of which included an aerobic component (online supplemental methods 2).

### HDG group

In addition to PA, participants received standard nutritional counselling to promote a healthy diet and to achieve a similar intervention intensity.

### MED group

In addition to PA, participants were instructed to adopt a calorie-restricted MED diet as described in our previous trials: DIRECT<sup>13</sup> and CENTRAL.<sup>18</sup> The MED diet assigned was rich in vegetables, with poultry and fish replacing beef and lamb. The diet also included 28 g/day of walnuts (containing 440 mg polyphenols/day; gallic acid equivalents (GAE), according to United States Department of Agriculture (USDA) Phenol-Explorer: <http://phenol-explorer.eu/food-processing/foods>, including, mostly, ellagitannins, ellagic acid and its derivatives.<sup>22</sup>

### Green-MED group

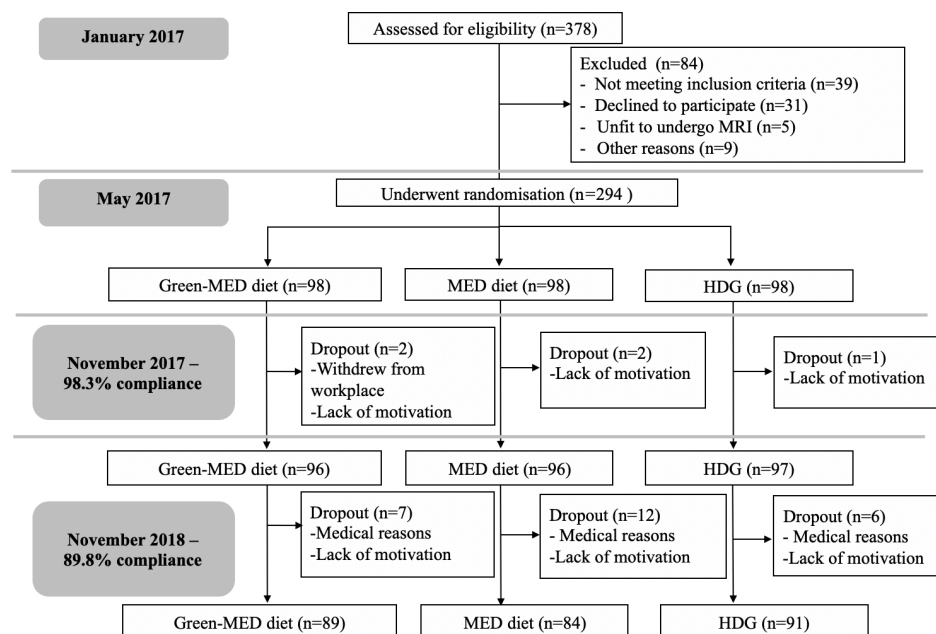
In addition to PA and the provision of 28 g/day walnuts, the green-MED diet was restricted in processed and red meat and was richer in plants and polyphenols. The participants were guided to further consume the following provided items: 3–4 cups/day of green tea and 100 g/day of frozen *Wolffia globosa* (Mankai strain<sup>23 24</sup>) plant frozen cubes, as a green shake replacing dinner. Both green tea and Mankai together provided additional daily intake of 800 mg polyphenols ((GAE), according to Phenol-Explorer and Eurofins lab analysis, including catechins (flavonols)) beyond the polyphenol content in the prescribed MED diet. Both the MED and green-MED diets were equally calorie-restricted (1500–1800 kcal/day for men and 1200–1400 kcal/day for women). A detailed description of the provided polyphenols is available in online supplemental methods 3.

Details regarding the lifestyle interventions and motivation techniques are provided in online supplemental methods 4. All the above polyphenols food sources (Mankai, green tea and walnuts) were provided free of charge and monitored at the on-site clinic.

### Outcome measures

IHF% was assessed at baseline and after 18 months using H-MRS.<sup>25</sup> Localised, single-voxel proton spectra were acquired using a 3.0T magnetic resonance scanner (Philips Ingenia, Best, The Netherlands). The measurements were taken from the frontal part of the right lobe, with a location determined individually for each subject using a surface, receive-only phased-array coil (full protocol is available in online supplemental methods 5). Data were analysed using Mnova software (Mestrelab Research, Santiago de Compostela, Spain) by an experienced physicist blinded to the intervention groups, who also performed visual quality control of fitted spectra. The total hepatic fat fraction in the image was determined as the ratio between the sum of the area under all fat divided by the sum of the area under all fat and water peaks.<sup>26</sup> IHF colour images were produced using PRIDE software (by Philips).

Anthropometric parameters (ie, weight and WC) and blood biomarkers were taken at baseline, after 6 and 18 months of intervention. Assessment of nutritional intake and lifestyle habits was performed using self-reported food frequency questionnaires administered through a computer at baseline, after 6 months, and at the end of the trial.<sup>27 28</sup> Serum folate was measured by the ECLIA competitive approach and was used as a marker for green leaf consumption.<sup>29</sup> We used plasma samples to assess polyphenol levels. All outcomes, including laboratory



**Figure 1** Flow chart of the Dietary Intervention Randomized Controlled Trial Polyphenols Unprocessed study. HDG, healthy dietary guidelines; MED, Mediterranean.

methodology and microbiome analysis, are further detailed in online supplemental methods 6.

### Statistical analysis

The primary outcomes of the DIRECT PLUS study were 18-month changes in IHF%, visceral adipose tissue (VAT), and adiposity (Flow chart of the study is presented in figure 1). Preliminary results indicate that 54% of the participants shared at baseline the top tertile of both—VAT and IHF levels and that after the 18-month intervention, 64% shared the top tertile of greater decline in both. A different report will be dedicated to complete VAT analysis. In the current study, we primarily aimed to assess the effect of the intervention on NAFLD, as evaluated by an 18-month change in IHF% (expressed as a percentage of total liver fat). Second, we evaluated the association of change in liver fat with the change in anthropometric parameters (weight, WC, blood pressure (BP)), blood biomarkers, cardiovascular risk scores, and specific food intake components related to the green-MED diet. Continuous variables are presented as means±SD or as medians and 25th, 75th percentile. Nominal variables are expressed as numbers and/or percentages. The Kolmogorov-Smirnov test was used to determine whether variables were normally distributed. NAFLD cut-off was set to 5% IHF, an acceptable cut-off for NAFLD initial diagnosis with radiological imaging techniques.<sup>2</sup> As a 5.56 cut-off is also appropriate for NAFLD diagnosis,<sup>30</sup> we performed a sensitivity analysis with this cut-off, which yielded similar results. Differences between time points were tested using the Paired sample t-test or Wilcoxon test. Differences between groups were tested using analysis of variance (ANOVA), Kruskal-Wallis test or  $\chi^2$  test. Ln transformations were applied when necessary to achieve normal distribution. Correlations were tested using Spearman or Pearson correlation. Kendall Tau correlation was used to examine p-of-trend. Multiple comparisons were addressed using the Tukey post hoc test (for ANOVA) and Bonferroni correction (for Kruskal-Wallis). For adjustments and interaction models, we used general and generalised linear regression models (with the specific adjustments detailed in the results). Of 294 MRI scans

of the participants, 269 were eligible for IHF% analysis at baseline due to technical reasons. Intention to treat (ITT) analysis was carried according to our previous trials: 18-month analysis for the primary outcome of IHF% included all 269 participants was conducted by imputing the missing observations for 38 individuals with missing data at 18 months by the multiple imputation technique,<sup>31</sup> wherein the following predictors were used in the imputation model: age, sex, baseline weight and WC at 18 months.<sup>18</sup> For missing data of body weight and WC, we used the last observation carried forward for 294 participants.<sup>18</sup> Sample size calculation and microbiome statistical analysis are available in online supplemental methods 7 and 8. Statistical analysis was performed using SPSS (V.25.0) and R (V.3.6.0). Statistical significance was set at a two-sided alpha of 0.05.

## RESULTS

### Baseline characteristics

The mean age of the participants was 51 years. 88% were men, with a mean body mass index (BMI) of 31.3 kg/m<sup>2</sup>. Baseline characteristics are presented in table 1. IHF% (ranged from 0.1% to 44.6%, median=6.6%, mean=10.2%) and NAFLD (IHF>5%) prevalence (62%), did not significantly differ between the three intervention groups ( $p>0.05$  for all). The participants who did not have valid MRI scans at baseline (n=25), did not differ significantly from participants with valid scans (n=269) in terms of gender distribution ( $p=0.99$ ) age ( $p=0.75$ ), baseline weight ( $p=0.65$ ) and WC ( $p=0.44$ ). The participants' median alcohol intake was 0.26 servings/day for men and 0.15 servings/day for women (correspond to 3.64 g/day and 2.1 g/day, respectively<sup>32</sup>). Lifestyle patterns, including daily alcohol and medication usage, were similarly distributed across the groups (online supplemental table 2).

### Adherence to the intervention

The retention rate was 98.3% after 6 months and 89.8% after 18 months. 78% had eligible follow-up MRS scan. Dropout reasons were confined to a lack of motivation and medical reasons

**Table 1** Baseline characteristics of the DIRECT PLUS participants\*

	Entire (n=294)	HDG (n=98)	MED (n=98)	Green-MED (n=98)	P value between groupst
IHF content, %‡	6.6 (3.5, 15.1)	7.0 (3.4, 15.1)	5.9 (3.8, 14.9)	7.7 (3.1, 17.8)	0.62
NAFLD (IHF>5%), %‡	62	63	60	62	0.88
Obese§, %	58.8	60.2	59.2	57.1	0.91
Diabetic¶, %	10.9	10.3	9.3	13.3	0.65
<b>Anthropometric</b>					
Age, years	51.1±10.5	51.10±10.6	51.68±10.4	50.55±10.8	0.76
Men, number (%)	259 (88.1)	86 (87.8)	86 (87.8)	87 (88.8)	0.97
BMI, kg/m <sup>2</sup> †	31.3±4.0	31.2±3.8	31.3±4.0	31.3±4.2	0.99
Weight, kg	93.7±14.3	92.9±14.7	94.5±13.5	93.6±14.9	0.73
Waist circumference, cm	109.7±9.5	109.9±10.3	110.0±9.5	109.3±8.7	0.86
Men	110.6±9.1	110.7±10.1	110.7±9.3	110.4±8.0	0.97
Women	103.3±9.6	103.8±9.7	105.2±9.6	100.8±9.9	0.56
Diastolic-BP, mm Hg	81.1±10.2	80.2±11.3	81.7±8.8	81.3±10.4	0.53
Systolic-BP, mm Hg	130.3±14.0	130.2±14.3	130.1±12.5	130.4±15.2	0.99
<b>Blood biomarkers</b>					
HDL, mg/dL	46.0±11.7	45.4±11.5	47.1±11.1	45.4±12.4	0.51
Men	44.3±10.2	43.4±9.9	46.1±10.1	43.3±10.7	0.13
Women	58.6±13.9	59.6±12.6	54.4±15.7	62.0±13.4	0.42
LDL, mg/dL	125.7±30.1	126.8±32.3	127.0±31.0	123.3±29.2	0.64
TG/HDL ratio**	3.0 (2.0, 4.5)	3.1 (2.0, 4.8)	2.9 (2.0, 4.6)	2.9 (2.0, 4.3)	0.53
Cholesterol/HDL ratio	4.4±1.3	4.4±1.2	4.3±1.3	4.4±1.4	0.82
Fasting glucose, mg/dL**	98.4 (92.3, 106.3)	98.4 (91.9, 105.4)	98.1 (92.4, 106.3)	98.9 (92.4, 107.8)	0.86
Insulin, µU/mL**	13.0 (9.7, 18.9)	13.0 (9.7, 18.9)	13.3 (10.2, 17.8)	12.9 (9.3, 18.1)	0.33
HOMA IR**	3.2 (2.3, 4.6)	3.1 (2.2, 4.9)	3.2 (2.5, 4.4)	3.2 (2.2, 4.5)	0.53
hsCRP, mg/L**	2.5 (1.5, 4.2)	2.3 (1.3, 4.4)	2.6 (1.6, 4.3)	2.4 (1.3, 4.2)	0.58
<b>Liver enzymes and adipokines</b>					
ALT, U/L	34.9±16.8	34.9±20.1	33.1±12.5	35.7±16.8	0.56
AST, U/L	25.6±7.7	25.9±8.6	25.2±6.5	25.8±7.9	0.91
ALT/AST ratio	1.3±0.4	1.4±0.4	1.3±0.4	1.4±0.4	0.60
ALKP, mg/dL	74.2±19.3	73.7±17.3	73.4±18.2	75.4±22.1	0.98
Chemerin, ng/mL	207.9±43.5	205.9±44.1	208.5±43.6	209.4±43.1	0.81
FGF 21, pg/mL	203.0±142.2	196.8±125.3	201.8±162.9	210.4±136.7	0.76
Leptin, ng/mL	14.3±12.0	13.7±12.2	15.0±12.3	14.3±11.5	0.44

\*Values are presented as mean±SD for continuous variables (unless indicated otherwise), and as number and/or % for categorical variables.

†P values according to ANOVA/Kruskal-Wallis test for continuous variables and  $\chi^2$  for categorical variables.

‡Of 269 available H-MRS.

§BMI ≥30 kg/m<sup>2</sup>.

¶Presence of diabetes was defined for participants with baseline fasting plasma glucose levels ≥126 mg/dL or haemoglobin-A1c levels ≥6.5% or if regularly treated with oral antihyperglycaemic medications or exogenous insulin.

\*\*Values presented as median (p25, p75).

ALKP, alkaline phosphatase; ALT, alanine transaminase; ANOVA, analysis of variance; AST, aspartate transaminase; BMI, body mass index; BP, blood pressure; FGF, fibroblast growth factor; HDG, healthy dietary guidelines; HDL, high-density lipoprotein; H-MRS, proton magnetic resonance spectroscopy; hsCRP, high sensitivity C reactive protein; IHF, intrahepatic fat; HOMA IR, homeostatic model assessment of insulin resistance; LDL, Low-density lipoprotein; MED, Mediterranean; NAFLD, non-alcoholic fatty liver disease; TG, triglycerides.

unrelated to the study. Overall, the 18-month dropout rate was not statistically different between the intervention groups ( $p=0.26$ ). Baseline weight, WC and age of those 30 participants who withdrew during the trial did not differ significantly from the 264 completers ( $p=0.4$  for gender distribution,  $p=0.38$  for age,  $p=0.3$  for baseline weight,  $p=0.63$  for baseline WC). No significant difference in PA intensity level (median=28.8 MET/week) was observed between the intervention groups after 18 months of intervention ( $p=0.28$ ). As previously reported<sup>21</sup>, the green-MED diet was distinguished in higher green tea and Mankai green shake intake, along with reduced red meat and poultry intake, as compared with the MED diet ( $p<0.05$  for all comparisons between MED groups). Further information regarding adherence and macronutrient composition is reported

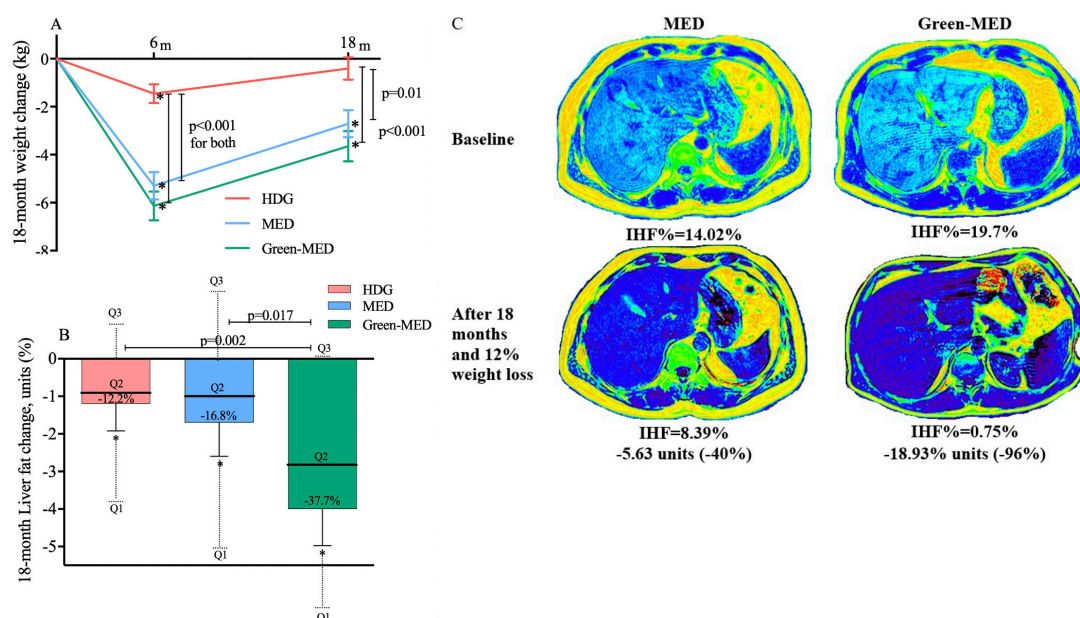
in online supplemental results 1 and online supplemental tables 3 and 4.

### 18-month changes in markers of adherence to intervention: serum folate and plasma polyphenols

Serum folate levels increased across the three intervention groups ( $p$ -of-trend=0.03). The green-MED group participants increased their serum folate level by 1.1 (−0.5, 2.6) ng/dL ( $p<0.001$  vs baseline; median change (25th, 75th percentiles)), an increase that was significantly higher compared with the HDG group (0.4 (−1.0, 1.5) ng/dL,  $p=0.01$  between groups).

Overall, at the end of the intervention, green-MED and MED groups demonstrated significantly higher levels of total





**Figure 2** (A–C) 18-month changes in weight and intrahepatic fat. (A) 18-month absolute change in weight between intervention groups (ITT analysis,  $n=294$ ). (B) 18-month changes in IHF% between intervention groups (ITT analysis, adjusted  $p$  values for age, sex and baseline IHF%;  $n=269$ ). (C) Illustrative MRI: a comparison of two male participants, similar age (46 years) and similar baseline WC (105 cm). Participant A was randomly assigned to the MED groups; participant B was assigned to the green-MED group. Both participants lost about 12% of their initial weight after 18 months and reported consuming at least 5–6 time/week walnuts (reported on 28 g/time). Total plasma polyphenol levels at the end of the intervention were higher in the green-MED participant versus MED participant (0.67 mg/L vs 0.24 mg/L). \*Significant within-group change versus baseline at 0.05 level. Colour liver images were generated using pride software (by Philips). HDG, healthy dietary guidelines; IHF, intrahepatic fat; ITT, intention to treat; MED, Mediterranean; WC, waist circumference.

polyphenols ( $0.47 \pm 0.4$  mg/L for both) as compared with the HDG group ( $0.35 \pm 0.4$  mg/L;  $p < 0.05$  for both MED vs HDG). The following polyphenols were differentially detected between the groups at the end of the intervention: 2-5-dihydroxybenzoic acid (HDG: 11.9%, MED: 37.4%, green-MED: 50.7%;  $p < 0.001$ ) and *Naringenin* (HDG: 4.4%, MED: 30.4%, green-MED: 65.2%;  $p = 0.001$ ).

### 18-month changes in IHF, weight and WC

After 18 months of lifestyle intervention, weight loss (figure 2A) and WC reduction in both green-MED ( $-3.7 \pm 6.3$  kg,  $-6.1 \pm 6.2$  cm;  $p < 0.001$  vs baseline for both) and MED ( $-2.7 \pm 5.6$  kg,  $-5.3 \pm 5.7$  cm;  $p < 0.001$  vs baseline for both) diets were similar, and were higher than the reductions achieved in the HDG group ( $-0.4 \pm 4.7$  kg,  $p = 0.35$  vs baseline;  $-4.0 \pm 5.6$  cm,  $p < 0.001$  vs baseline;  $p < 0.05$  between HDG and MED and green-MED groups for weight loss, with mean differences of  $-2.3$  kg, 95% CI  $-4.2$  to  $-0.4$  and  $-3.2$  kg, 95% CI  $-5.1$  to  $-1.4$ , respectively;  $p = 0.04$  between HDG and green-MED for WC loss with a mean difference of  $-2.1$  cm, 95% CI  $-4.0$  to  $-0.1$ ).

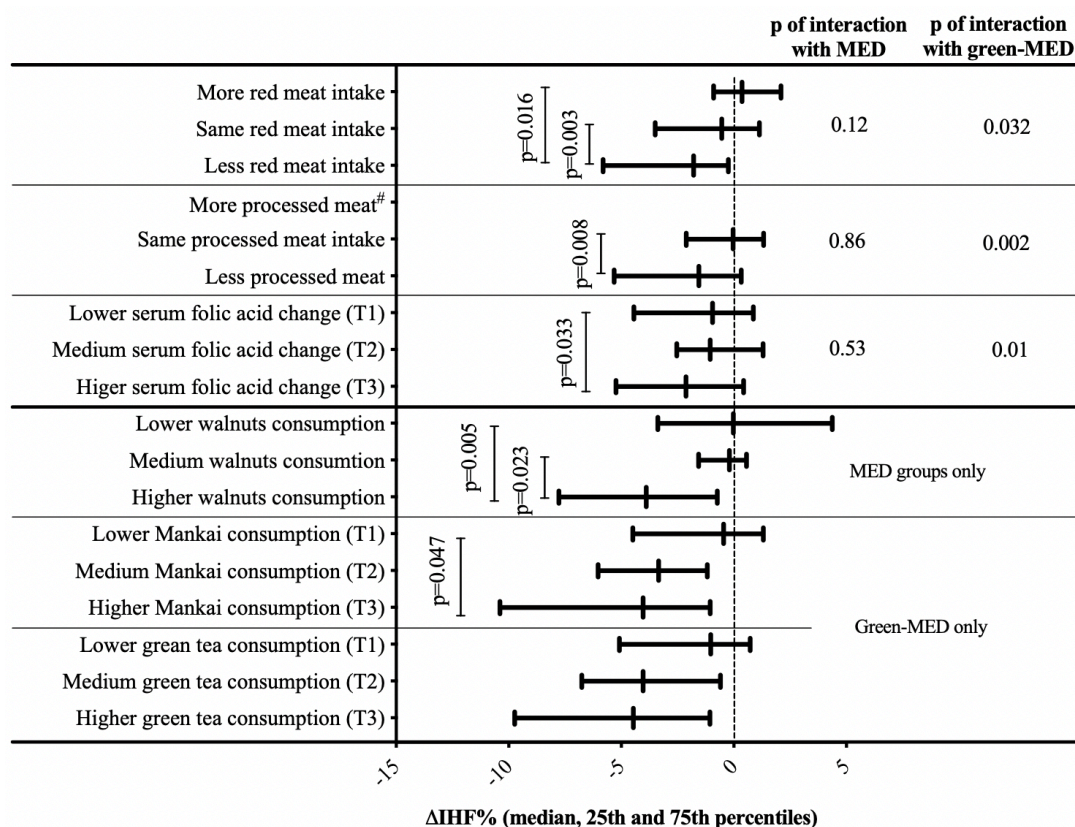
Participants in the green-MED group had a significantly higher reduction in IHF% (median change (25th, 75th change percentiles):  $-2.0\%$  ( $-6.4$ ,  $-0.2$ ) absolute change,  $-38.9\%$  change relative to baseline), as compared with the MED group ( $-1.1\%$  ( $-4.7$ ,  $1.9$ ) absolute change,  $-19.6\%$  relative to baseline,  $p = 0.023$  between groups, adjusted for age, sex and baseline IHF) and HDG ( $-0.7\%$  ( $-2.4$ ,  $1.3$ ) absolute change,  $-12.2\%$ ,  $p < 0.001$  between groups, adjusted). When further adjusted for an 18-month weight loss, the difference remained significant between the two MED groups only ( $p = 0.035$ ). This significant difference between the two MED diets remained after we added PA and energy intake to the statistical model ( $p = 0.047$ ).

Adjustment for VAT change, instead of weight-loss, did not change the results observed (green-MED vs HDG:  $p = 0.006$ ; green-MED vs MED:  $p = 0.029$ ). Further subgroup analysis of IHF% change by the degree of weight loss/VAT reduction is presented in online supplemental figure 1. By the end of the trial, the prevalence of NAFLD reduced from 62% to the following distribution between intervention groups: 54.8% (HDG), 47.9% (MED) and 31.5% (green-MED;  $p = 0.012$  between groups). ITT analysis for the between-group differences (figure 2B) yielded similar results. Further adjustment for weight loss resulted in a significant difference between the two MED group ( $p = 0.024$ ). A comparison between the per-protocol changes and ITT techniques is presented in online supplemental figure 2. Illustrative MRI of the 18-month changes in the two MED diets are presented in figure 2C. Further analysis of 18-month IHF% changes by BMI, age, NAFLD, sex, type 2 diabetes or metabolic syndrome criteria subgroups is presented in online supplemental figure 3.

Since some participants were included in a substudy related and parallel to this trial,<sup>33</sup> we also examined the IHF% change between the substudy intervention groups, with no significant difference observed.

### 'Green component' and IHF loss

To clarify why the green-MED diet was more successful than the MED diet in IHF reduction, we further examined specific food components. IHF% change was inversely correlated with serum folate change ( $r = -0.16$ ,  $p = 0.02$ ). Greater reduction in IHF was observed in participants in the top serum folate change (increase) tertile versus lowest serum folate change, and among participants who reduced red and processed meat ( $p < 0.05$  for all, figure 3). An interaction was observed for red/processed meat



**Figure 3** Changes in IHF across tertiles/categories of dietary components. Mankai shake and green tea tertiles are calculated from the weighted mean of consumption reported after 6 and 18 months of intervention. serum folate tertiles (of 18-month change in serum folate): T1≤−0.41; T2=−0.40 to 1.46; T3≥1.47; Mankai shake tertiles: T1≤1.67/week; T2=1.68 to 3.00/week; T3≥3.01/week; green tea tertiles: T1≤2/day; T2=2.01 to 3.67/day; T3≥3.68/day; walnut consumption categories: low: 0 to 1–3 times/month; medium: 1–2/week to 3–4/week; high: more than 5–6/week. Categories intervention group distribution for walnuts: low consumption: 60% MED, 40% green-MED; medium consumption: 45% MED, 55% green-MED; high consumption: 45% Med, 55% green-MED. Specific between tertiles/consumption group p values are corrected for multiple comparisons. # none of the participants reported on more processed meat. IHF, intrahepatic fat; MED, Mediterranean; T1, lowest tertile; T2, intermediate tertile; T3, highest tertile.

and serum folate with the green-MED diet, that remained significant after further adjustments for age, sex, and either baseline IHF or weight. Further analysis of the reduction in IHF% and diet components is presented in online supplemental figure 4.

Increased intake of both Mankai and walnuts, as reported by the participants, was significantly associated with greater IHF% loss ( $p<0.05$  for all; figure 3). Adjustment for either weight change or baseline IHF level did not materially attenuate the associations ( $p<0.05$  between extreme tertiles). Of note, change in total plasma polyphenols was marginally correlated with IHF% change ( $r=-0.12$ ,  $p=0.09$ ).

### Associations of IHF with cardiometabolic markers

At baseline, IHF% levels were correlated with anthropometric parameters, glycaemic, lipid and liver-related markers ( $p<0.05$  for all; online supplemental figure 5). Further adjustment for age, sex and baseline weight did not affect most of the observed associations.

Eighteen-month weight and WC loss were significantly associated with IHF% loss (table 2). IHF% reduction was associated with a decline in diastolic BP, TG/HDL ratio, cholesterol/HDL ratio, fibroblast growth factor 21 (FGF21) and chemerin ( $p<0.05$  for all biomarkers, consistently in all three statistical models). Eighteenth-month differences in liver-related blood

biomarkers between groups are presented in online supplemental figure 6.

### Intrahepatic fat and the gut microbiome

We next addressed the potential role of the gut microbiome in the observed association between lifestyle intervention and IHF% reduction.

At baseline, IHF% levels were significantly associated with taxonomic composition, as assessed by global structure Permutational analysis of variance (PERMANOVA),  $p=0.008$ , and by the first principal coordinate (PCo) across IHF% tertiles (figure 4A). Concordantly, 18-month change in IHF was found to be associated with the change in the global composition, assessed by the log2 change of all operational taxonomic units (OTUs) (PERMANOVA,  $p=0.037$ ). Aiming to determine whether the microbiome's composition change had a mediatory role in the association between lifestyle intervention and IHF loss, we sought the PCo most highly correlated with IHF change (PC5;  $r=0.25$ ,  $p=0.001$ ; figure 4B, online supplemental figure 7), and evaluated to what extent was it affected by lifestyle intervention. PCo #5, being a surrogate for the compositional shift of the microbiome, differed across the intervention groups ( $p$ -of-trend=0.001), with a significant difference between the HDG group and the MED and green-MED groups ( $p=0.038$  and

**Table 2** Associations between 18-month intrahepatic fat change and 18-month anthropometric parameters and blood biomarkers changes

	Model 1		Model 2		Model 3	
	Beta coefficient	P value	Beta coefficient	P value	Beta coefficient	P value
<b>Anthropometric</b>						
ΔWeight	<b>0.64</b>	<b>5.3e-25</b>	–		–	
ΔWaist circumference	<b>0.56</b>	<b>3.7e-19</b>	–		–	
ΔSystolic BP	<b>0.17</b>	<b>0.01</b>	0.10	0.05	0.07	0.15
ΔDiastolic BP	<b>0.28</b>	<b>1.9e-5</b>	<b>0.16</b>	<b>0.005</b>	<b>0.11</b>	<b>0.04</b>
<b>Glycaemic biomarkers</b>						
ΔGlucose	<b>0.22</b>	<b>0.001</b>	0.11	0.06	0.08	0.15
ΔHOMA IR	<b>0.26</b>	<b>8.8e-5</b>	<b>0.13</b>	<b>0.02</b>	0.04	0.49
ΔInsulin	<b>0.28</b>	<b>2.9e-5</b>	<b>0.14</b>	<b>0.02</b>	0.03	0.54
<b>Lipid biomarkers</b>						
ΔTriglycerides	<b>0.38</b>	<b>5.5e-9</b>	<b>0.22</b>	<b>8.1e-5</b>	0.11	0.056
ΔCholesterol	0.08	0.30	0.08	0.16	0.04	0.41
ΔHDL	<b>–0.38</b>	<b>7.9e-9</b>	<b>–0.20</b>	<b>0.001</b>	<b>–0.11</b>	0.07
ΔLDL	0.04	0.51	0.05	0.40	0.03	0.61
ΔTG/HDL ratio	<b>0.40</b>	<b>3.3E-10</b>	<b>0.25</b>	<b>2.0e-5</b>	<b>0.14</b>	<b>0.02</b>
ΔCholesterol/HDL ratio	<b>0.36</b>	<b>2.6e-8</b>	<b>0.24</b>	<b>1.4e-5</b>	<b>0.15</b>	<b>0.007</b>
<b>Liver enzymes and hepatokines</b>						
ΔALT	<b>0.32</b>	<b>2.0e-6</b>	<b>0.2</b>	<b>0.001</b>	0.1	0.089
ΔAST	<b>0.13</b>	<b>0.049</b>	0.08	0.12	0.07	0.18
ΔALT/AST ratio	<b>0.36</b>	<b>6.2e-8</b>	<b>0.23</b>	<b>9.8e-5</b>	0.10	0.069
ΔALKP	0.03	0.69	–0.001	0.95	–0.008	0.88
ΔFGF21	<b>0.26</b>	<b>9.2e-5</b>	<b>0.15</b>	<b>0.01</b>	<b>0.12</b>	<b>0.03</b>
<b>Adipokines and inflammation</b>						
ΔChemerin	<b>0.18</b>	<b>0.007</b>	<b>0.13</b>	<b>0.02</b>	<b>0.16</b>	<b>0.001</b>
ΔLeptin	<b>0.15</b>	<b>0.03</b>	0.06	0.36	–0.08	0.18
ΔhsCRP	<b>0.17</b>	<b>0.02</b>	<b>0.14</b>	<b>0.01</b>	0.09	0.089

Model 1: adjusted for age, sex, baseline IHF% and intervention group.

Model 2: adjusted for age, sex, baseline IHF% intervention group, and 18-month waist circumference change.

Model 3: adjusted for age, sex, baseline IHF%, intervention group and 18-month weight change.

ALKP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BP, blood pressure; FGF, fibroblast growth factor; HDL, high-density lipoprotein; HOMA IR, homeostatic model assessment of insulin resistance; hsCRP, high sensitivity C reactive protein; LDL, low-density lipoprotein; TG, triglycerides.

$p=0.004$ , respectively) (figure 4B), and no significant difference between the MED groups ( $p=0.268$ ). In a mediation analysis, the compositional shift of the microbiome was estimated to account for 22% of IHF change by the lifestyle interventions (figure 4C).

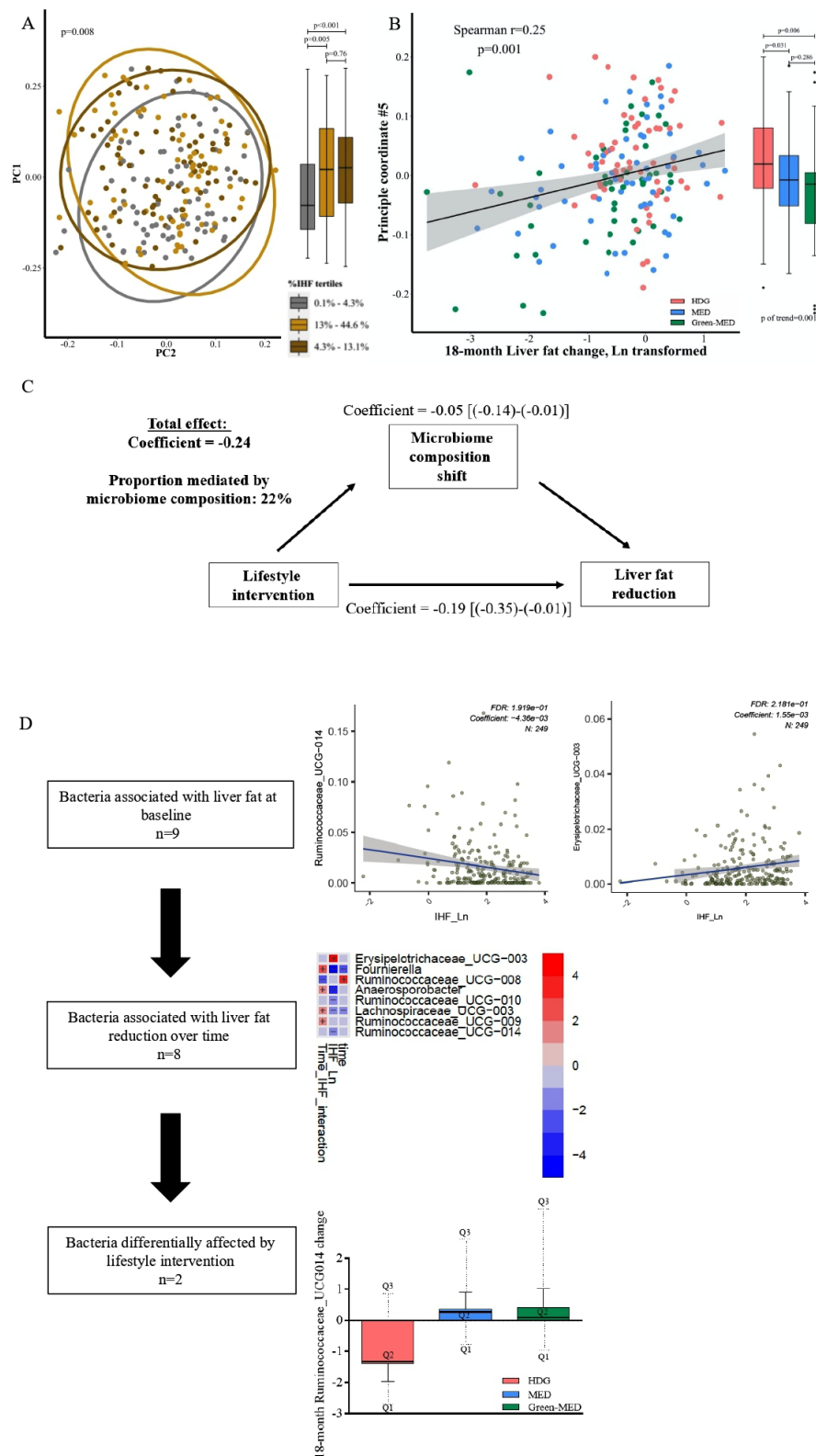
Evaluating the contribution of specific bacteria to this observation, we identified nine genus-level bacteria that were significantly associated with IHF at baseline (5% of genus-level bacteria), including *Fournierella*, *Anaerosporebacter*, *Lachnospiraceae*\_UCG-003 and several genera from the *Ruminococcaceae* family. Among them, eight bacteria were also found to be associated with IHF 18-month change. Next, assessing the effect of lifestyle intervention on these bacteria, the interaction between time and lifestyle intervention group was evaluated. Two specific bacteria, *Fournierella* and *Ruminococcaceae*\_UCG-014 were found to be significantly affected by lifestyle intervention. However, in a mediation analysis, both bacteria were not found as significant mediators between lifestyle intervention and IHF change (figure 4D, online supplemental table 5).

## DISCUSSION

In the current study, we demonstrated that the prevalence of NAFLD was reduced in half by the strategy of exercise and green-MED diet enriched with Mankai and walnuts and restricted in red and processed meat, as reflected by increased plasma polyphenols and serum folate. Also, we found an independent

association between 18-month IHF% reduction and beneficial changes in cardiometabolic, inflammatory parameters, specific gut bacteria and with global microbiota composition, which was also found to have a mediatory role in the association between lifestyle intervention and liver fat reduction. Following our previous trials suggesting that the MED diet is favourable to a low-fat diet in terms of cardiometabolic risk<sup>13 34</sup> and IHF loss,<sup>18</sup> this clinical trial may suggest an effective nutritional tool for the treatment of NAFLD beyond weight loss, a predicament that very little, if any effect, pharmacological treatment exists for.

Several limitations of this study should be considered. First, we had a high proportion of male participants, reflecting the profile of the workplace. This limits our ability to extrapolate our results to women. In addition, NAFLD is almost as prevalent in women as compared with men, and thus gender aspects are not fulfilled by this trial. Also, this study's results may not be extrapolated to a population that is not abdominally obese and/or with dyslipidaemia, or a population with a lower prevalence of NAFLD than seen among our participants. Yet, the high prevalence of liver steatosis is probably a reflection of a sedentary lifestyle and unhealthy eating pattern, as our participants did not report any alcohol abuse. Second, we assessed adherence to the intervention mainly by participants' self-reports. However, serum folate analysis, reflecting green leaf consumption<sup>29</sup> and correlate well with nutritional self-reports,<sup>28</sup> enabled us to objectively estimate



**Figure 4** (A–D) Intrahepatic fat and the gut microbiome. (A) Gut microbiome composition (beta diversity) and IHF% at baseline. Gut microbiome composition and IHF, shown by principal coordinate analysis (PCoA) of UniFrac distances between all baseline samples. Colours denotes 1st (grey) 2nd (yellow) and 3rd (brown) IHF% tertiles. 95% SE ellipses are shown for each tertile. Boxplots on the right describe PCo1 score by IHF% tertile. (B) Gut microbiome composition change and IHF% change. Correlation between principal component 5 (PCo5), the principal coordinate most highly correlated with IHF change (Y axis), and 18-month change in intrahepatic fat. Colours denotes lifestyle intervention group allocation. Boxplots on the right describe PCo5 score by IHF% lifestyle intervention group. (C) Mediation analysis: assessing the proportional mediatory effect of microbiome composition change (measured as PCo5) in the association between lifestyle intervention and IHF% change. (D) Stepwise identification of genus level bacteria associated with: IHF% at baseline (top, two selected bacteria), IHF% 18-month change (middle, heatmap) and with lifestyle intervention (bottom, bar plot, selected bacteria). IHF, intrahepatic fat.



green products' intake. Although we measured plasma polyphenols, these measurements are limited in reflecting polyphenol intake, as only a few phenolic acids, derived from dietary polyphenol metabolism, will be present in overnight, fasted blood samples.<sup>35</sup> The strengths of the study include the closed workplace environment, which enabled monitoring of the provided lunch, the presence of an on-site clinic at the participants' workplace; intense dietary guidance and group meetings with multidisciplinary guidance; access to free-of-charge provided polyphenols; relatively large sample size; high retention rate; and the use of an accurate imaging technique,<sup>30</sup> as compared with other non-invasive methods<sup>36</sup> with high reproducibility between measurements,<sup>37</sup> to quantify IHF%.

According to current guidelines, obese or overweight individuals are advised to undergo a moderate 5%–10% weight reduction by energy restriction.<sup>8,9</sup> NAFLD patients are advised to change their diet (ie, reduce added sugar and reduce saturated fat) and engage in PA, both aerobic and resistance.<sup>8</sup> In our study, participants who were instructed for HDG reduced both WC and IHF%, in accordance with a previous publication,<sup>38</sup> whereas aerobic PA interventions in obese men and women, without weight loss, was found to be useful in the reduction of liver steatosis. The MED intervention in our study had greater efficacy in promoting adiposity (WC and weight) reduction, in addition to IHF% loss, similarly to data previously reported by us,<sup>3,18</sup> where some fat depots, and more specifically IHF%, were effectively reduced by the MED/low carbohydrate diet than the low-fat diet, independently of VAT changes. The green-MED diet achieved the highest IHF loss, within similar weight loss as observed in the MED group, suggesting that diet composition has an effect beyond weight loss. We now add to this knowledge by demonstrating an additional benefit from the green-MED regimen, differed from the MED diet by being rich in green polyphenols and restricted in red and processed meat.

Polyphenols might play a role in reducing liver steatosis by preventing hepatocellular damage through several possible mechanisms, including reducing de novo lipogenesis, increasing fatty acid oxidation and reducing oxidative stress.<sup>15</sup> The MED eating pattern is based mainly on increasing plant-based foods, including olive oil, along with restricted meat consumption. In our trial, we further enriched the diet with provided polyphenols, in addition to the polyphenols naturally found in the MED diet. Participants in the green-MED group were instructed to consume 3–4 cups/day of green tea containing mostly EGCG, associated with reducing liver fat, as well as liver enzymes levels, fibrosis, and inflammation<sup>39</sup> and a daily Mankai green shake containing a mixture of flavonoids, shown to increase fatty acid oxidation in the liver, reduce inflammation by inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells, to increase adiponectin, and to reduce BP.<sup>40</sup> Both MED groups received 28 g/day of walnuts, rich in ellagic acid, shown to improve hepatic status due to antihepatotoxic properties.<sup>41</sup> The participants of the green-MED group had a specific detection of *Naringenin* (demonstrated to have a beneficial effect in liver diseases<sup>42</sup>) and 2-5-dihydroxybenzoic acid (a catabolite of the plant hormone salicylic acid<sup>43</sup>).

In addition to a greater reduction in IHF following a higher intake of polyphenols rich Mankai shake and walnuts, a decrease in red/processed meat and increased folate (probably reflecting Mankai consumption) also led to greater IHF

reduction. Folate is an essential vitamin of the B vitamins family, with several important biological roles (eg, involvement in the DNA synthesis).<sup>44</sup> Low folate levels were previously recognised as an independent risk factor of NAFLD,<sup>45</sup> probably by affecting the expression of genes that might contribute to the accumulation of lipids in the liver.<sup>44</sup> These results suggest that a higher intake of specific polyphenol-serum folate-rich components, in addition to a decrease in red/processed meat, might mediate a reduction of liver fat. We observed known associations of IHF% at baseline with some cardiometabolic-related biomarkers in accordance with our previous report,<sup>3</sup> and an association between IHF change and change in FGF21 (elevated in conditions of obesity and NAFLD<sup>46</sup>). Due to our study's nature, we cannot determine whether the change observed in these markers resulted from the reduction in IHF% and improvement in liver status or is merely a reflection of overall cardiometabolic improvement.

Previous studies have established the role of the gut microbiome in fat storage regulation in general,<sup>47</sup> and NAFLD induction through hepatic fat storage specifically.<sup>5</sup> We described an association between IHF% and microbiome composition at baseline, with a homogenous dysbiotic pattern among the two higher IHF% tertiles (>4.3% IHF) of our cohort. This observation is in accordance with prior evidence, associating NAFLD (>5% IHF) and gut dysbiosis.<sup>4</sup> The family *Ruminococcaceae* has been consistently reported as less abundant in NAFLD,<sup>48,49</sup> a finding we were able to reproduce at baseline in our cohort. Interestingly enough, during our trial IHF reduction was positively correlated with changes in specific *Ruminococcaceae* genera (*Ruminococcaceae\_UCG-014*, *Ruminococcaceae\_UCG-009*) and negatively correlated with change of a specific genus (*Ruminococcaceae\_UCG-008*). This finding warrants further investigation as to the role of *Ruminococcaceae* in NAFLD pathogenesis and resolution. We further report a novel observation, linking IHF% change with a compositional shift in the microbiome over 18 months. This shift, in turn, partially mediated the effect of lifestyle interventions on IHF%. This mediatory effect of gut microbiome composition on IHF reduction constitutes an advancement of the observations made by others, establishing the association between the gut microbiome composition and NAFLD susceptibility.<sup>50</sup>

In conclusion, a green-MED diet, enriched with specific polyphenols and decreased red and processed meat consumption, amplifies the beneficial effect of the MED diet on hepatic fat reduction, beyond weight loss. The results of this study may suggest an improved dietary protocol to treat NAFLD.

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## The effect of green Mediterranean diet on intrahepatic fat; The DIRECT PLUS randomized controlled trial

### **Supplemental Material**

#### **Supplemental Methods:** Exclusion criteria

Exclusion criteria were an inability to partake in physical activity (PA), a serum creatinine level  $\geq 2$  mg/dL, disturbed liver function, a major illness that might require hospitalization, pregnancy or lactation for women, presence of active cancer or undergoing chemotherapy either at present or in the prior three years, participation in another trial, chronic treatment with warfarin (given its interaction with vitamin K), and being implanted with a pacemaker or platinum implant (due to inability to undergo magnetic resonance imaging included in the study design).

#### **Supplemental Methods 2:** Physical activity protocol

The aerobic effort increased gradually, starting with 20 minutes of aerobic training at 65% maximum heart rate, and increased to 45-60 minutes of aerobic training at 80% of maximum heart rate. The full workout program included 45-60 minutes of aerobic training 3-4 times/week; resistance training starting with one set of weights corresponding to 60% of the maximum weight, eventually reached the use of two sets of weights corresponding to 80% of the maximum weight. The resistance training included leg extensions, leg curls, squats, lateral pull-downs, push-ups, shoulder presses, elbow flexions, triceps extensions, and bent leg sit-ups.

#### **Supplemental Methods 3:** Provided polyphenol-rich products

Walnuts [groups Mediterranean (MED), green-MED]: The main polyphenols in walnuts are ellagitannins, ellagic acid, and its derivatives [1]. Walnuts are considered to have a beneficial effect on health maintenance and disease prevention [2]. In addition, Ellagitannin found in nuts was reported to reduce waist circumference (WC), low-density lipoprotein cholesterol (LDL-c), and triglycerides (TG) [3].

Green tea (group green-MED): an unfermented tea produced from the leaves of *Camellia sinensis*, is prepared by drying and steaming the leaves and is a rich source of polyphenols [4]. Most of the polyphenols found in green tea are Catechins (the monomer form of flavanols), mainly epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) [5,6]. Short-term (weeks long) intervention studies and meta-analyses have found an association between administering green tea or its extracts and improvement in cardiometabolic health [7,8], weight reduction [6], and improved cognitive function [9,10].

Wolffia globosa duckweed - Mankai (group green-MED): A specific strain of *Wolffia globosa*, an aquatic plant, which can serve as a plant protein source. In Asian cuisines, *Wolffia globosa* (Mankai cultivated strain) is considered a natural food source or "vegetable meatball" [11]. Nutritionally, Mankai is characterized by high protein content (more than 45% of the dry matter) and the presence of 9 essential and 6 conditional amino acids [12]. In addition, it is a good source of omega-3 fatty acids [13]. The Mankai plant is rich in non-soluble fibers, iron, vitamins, minerals [14], and polyphenols, including catechins, caffeic acid, apigenin, quercetin, naringenin, and kaempferol [15,16]. Mankai provides bioavailable essential amino acids [12], iron [17], vitamin B12 [18], and has beneficial effects on postprandial and fasting glycemic control [19]. We guided the participants to prepare a green Mankai shake with additional ingredients, which also were part of the diet regimen (fruits,



walnuts, or vegetables) each evening. The green protein shake partially substituted for dinner, replacing beef/poultry protein sources.

#### **Supplemental Methods 4: Lifestyle sessions and motivation techniques**

The lifestyle interventions included 90-minute nutritional and PA sessions in the workplace with multidisciplinary guidance (physicians, clinical dietitians, and fitness instructors). These sessions were held every week during the first month, once a month, over the following five months, and every other month until the 18<sup>th</sup> month. All the lifestyle educational programs were provided at the same intensity to all three groups. To keep the participants motivated, text messages with relevant information for each assigned intervention group were sent on fixed time intervals. In addition, a website listing all nutritional and PA information needed by the participants to continue with the intervention was accessible to the participants according to their intervention group.

#### **Supplemental Methods 5: H-MRS by Magnetic Resonance scanner**

In order to quantify and follow intrahepatic (IHF%) changes, we used H-MRS, a reliable tool for liver fat quantification [20]. Localized, single-voxel proton spectra were acquired using a 3.0T magnetic resonance scanner (Philips Ingenia, Best, The Netherlands). The measurements were taken from the right frontal lobe of the liver, with a location determined individually for each subject using a surface, receive-only phased-array coil. Spectra with and without water suppression were acquired using the single-voxel stimulated echo acquisition mode (STEAM) with the following parameters: TR=4000msec, TE=9.0msec, and TM=16.0msec. The receiver bandwidth was 2000Hz, and the number of data points was 1024. Second-order shimming was used. Four averages were taken in a single breath hold for an acquisition time of 16 sec. The voxel size varied somewhat according to anatomy but was approximately 50(AP) × 45(RL) × 54(FH) mm. Water suppression was achieved using the MOIST (Multiple Optimizations Insensitive Suppression Train) sequence consisting of four phase-modulated T1 and B1 insensitive pulses with a 50Hz window. Data analyzed using Mnova software (Mestrelab Research, Santiago de Compostela, Spain) by an experienced physicist blinded to the intervention groups, who also performed visual quality control of fitted spectra. The total hepatic fat fraction in the image was determined as the ratio between the sum of the area under all fat divided by the sum of the area under all fat and water peaks[21]. Inter-class reliability was tested between two different technicians and resulted in an average measure of  $r=0.99$  ( $p<0.001$ ). Intra-class reliability was tested among all baseline scans and resulted in an average measure of  $r=0.96$  ( $p<0.001$ ). Liver fat color images were produced using PRIDE software (by Philips).

#### **Supplemental Methods 6: Further laboratory methodology, anthropometric measurements, lifestyle, plasma polyphenol assessments, and risk scores calculations**

##### Anthropometric parameters and laboratory methodology

Measurements were taken at baseline, after 6 and 18 months of intervention. Height was measured to the nearest millimeter using a standard wall-mounted stadiometer. Bodyweight was measured without shoes to the nearest 0.1kg. WC was measured halfway between the last rib and the iliac crest to the nearest millimetre by standard procedures using an anthropometric measuring tape. Two blood pressure (BP) measurements and resting pulse were recorded after resting, using an automatic BP monitor (Accutorr-4, Datascope) and calculated as the mean of the two measurements taken. Blood samples were obtained at 8:00 AM after a 12-hour fast. The samples were centrifuged and stored at -80°C. Serum total cholesterol (TC; Coefficient-of-variation (CV), 1.3%), High-density lipoprotein cholesterol (HDL-c), LDL-c, and TG (CV, 2.1%) were determined enzymatically with a Cobas-6000

automatic analyzer (Roche). Plasma levels of high-sensitivity C-reactive protein (hsCRP) were measured by ELISA (DiaMed; CV, 1.9%). Plasma glucose levels were measured by Roche GLUC3 (hexokinase method). Plasma insulin levels were measured with an enzyme immunoassay (Immulin automated analyzer, Diagnostic Products; CV, 2.5%). The homeostatic model of insulin resistance (HOMA IR) was calculated as follows:  $\text{insulin}(\mu\text{IU/ml}) \times \text{glucose}(\text{mg/dl}) / 405$  [22]. All biochemical analyses were performed at the University of Leipzig, Germany.

#### Assessment of nutritional intake and lifestyle habits

Self-reported food frequency questionnaires were administered through a computer at baseline, after 6 months, and at the end of the trial [23,24], which included intake assessment of provided items. We followed overall changes in the intake of specific food groups, as described previously [25] and further used lifestyle and validated PA questionnaire [26]. PA intensity levels were measured using metabolic equivalent (MET) units [27].

#### Plasma polyphenols metabolites:

The determination of polyphenol metabolites was performed according to the method of Vrhovsek et al [28] with some modifications. Briefly, a previously developed targeted metabolomic method was performed with an ultra-performance liquid chromatographic system coupled to a tandem mass spectrometry system with electrospray ionization (UHPLC-ESI-MS/MS). Before injection, samples were thawed at 4 °C. Sample preparation was performed using an Ostro™ Pass-through 96-well plate to remove phospholipids and proteins (Waters, Milford, MA, USA). An Ostro™ 96-well plate was fixed on top of a 96-well collection plate. 50 µl of plasma were pipetted into the wells, followed by the addition of 1% formic acid in acetonitrile (3:1 solvent/sample). The mixture was then quickly shaken for 5 minutes to promote protein precipitation. Vacuum (15 in. (~381 mm) Hg) was then applied to the Ostro plate through a vacuum manifold, filtering out the nonphospholipid plasma components. This step was repeated twice to ensure protein precipitation. Then, samples were dried under nitrogen and reconstituted in 100 µl of methanol: water (1:1, v/v), containing hippuric acid D5 (1 µg/ml) as an external standard. Samples were finally transferred to LC vials and injected (2 µL) into the UHPLC-MS/MS system. All solvents were kept at 4 °C prior to their use, and all procedures were carried out in a cold room, assuming that a 4 °C extraction temperature and the relatively short extraction time (10 min) may be favorable for avoiding biological sample degradation and reducing the risk of metabolite precipitation. Quality control (QC) samples were also prepared prior to analysis by pooling a small fraction of all the individual analyzed samples. Data processing was performed using Waters MassLynx 4.1 (Waters, Milford, CT, USA) and TargetLynx software (Waters, Milford, CT, USA). Details of the liquid chromatography and mass spectrometry are described in Vrhovsek et al [28] and Gasperotti et al [29]. The analysis was performed at the Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, Trento, Italy.

#### Fecal samples collection and 16s rRNA sequencing

Fecal samples were collected at baseline and 18 months at the study site, immediately frozen to -20°C for 1-3 days, then transferred to -80°C pending DNA extraction. Following extraction, samples were sequenced on a MiSeq platform following amplification of V3-V4 hypervariable region of the 16S rRNA gene using the primer set 341F/806R, and processed by the DADA2 pipeline. Rare OTUs (< 3% prevalence of all samples) were filtered out. Samples of participants prescribed antibiotic therapy 2 months prior to randomization and samples with less than 103 reads were excluded from the analysis. Analysis was performed at the Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, Trento, Italy.

**Supplemental Methods 7: Sample size calculations**

We based the sample size calculation on the outcomes of a previous trial that resulted in a significant reduction in liver fat [30]:  $6.7 \pm 6.1\%$  reduction in the intervention group (increasing energy expenditure and reducing caloric intake) vs.  $2.1 \pm 6.4\%$  reduction in the control group (encouraged to reduce carbohydrate and fat intake and to engage in physical activity) with a 4.6 difference, pooled variance of 39.085. Calculation for the sample size needed for this trial, with a 5%  $\alpha$  and a 90% power, suggested 39 participants in each intervention group. Considering a 14% expected dropout rate (based on our previous CENTRAL trial [31]), in order to detect differences between intervention groups, we needed a number of 45 participants in each group, and ultimately recruited a number of 98 participants per group ( $\sim 90$  in each group with a valid MRI scan). Sample size calculations were performed using Winpepi software, version 11.6.

**Supplemental Methods 8: Microbiome statistical analysis**

Microbiome composition was assessed based on relative abundance. For composition change, a change matrix was generated by calculating the  $\log_2$  ratio between 18m and baseline, for each taxa and each individual as follows:  $\log_2$  (18m relative abundance/baseline relative abundance). Dissimilarity between samples was measured by the UniFrac distance. Associations between gut microbiome composition and IHF, and IHF% change was assessed by permutational multivariate analysis of variance (PERMANOVA) with the adonis function (R “vegan” package), and by comparing principle coordinate scores across IHF% tertiles. To assess the association between microbiome composition, lifestyle intervention and IHF%, the principle coordinate vector most highly correlated with IHF% was chosen. The mediation analysis was performed by employing the mediation analysis suggested by Imai et al. [32] by the ‘mediate’ package in R (<https://cran.r-project.org/web/packages/mediation/mediation.pdf>). Lifestyle intervention was considered a ranked variable, taking into account the gradual increase of polyphenols, and gradual decrease in red and processed meat across the groups.

For per-taxa analysis, we first aggregated all features to the genus level, and performed quality control filtering for taxonomic and functional features before including them in the subsequent analyses. To be qualified for downstream analyses, a taxonomic feature needed to be detected at a minimum relative abundance of 0.01% in at least 5% of samples. This analysis yielded 180 microbial species that met the criteria. We employed the R package MaAsLin 2 1.0.0 to perform per-feature tests taking into account the compositionality of the data and multiple testing (<https://huttenhower.sph.harvard.edu/maaslin2>). At first, features were selected by their association with  $\ln$  transformed IHF at baseline. In the second phase features were selected by a model taking into account time, IHF and time\*IHF interaction as fixed effects, with each individual as random effect. In the third phase, features were selected by a model taking into account group, time and time\*group interaction as fixed effects, with each individual as random effect. All high-dimensional tests were corrected for multiple hypothesis testing by controlling the false discovery rate (FDR) using the Benjamini-Hochberg method with a target rate of 0.25 for  $q$  values estimated from the per-feature tests.

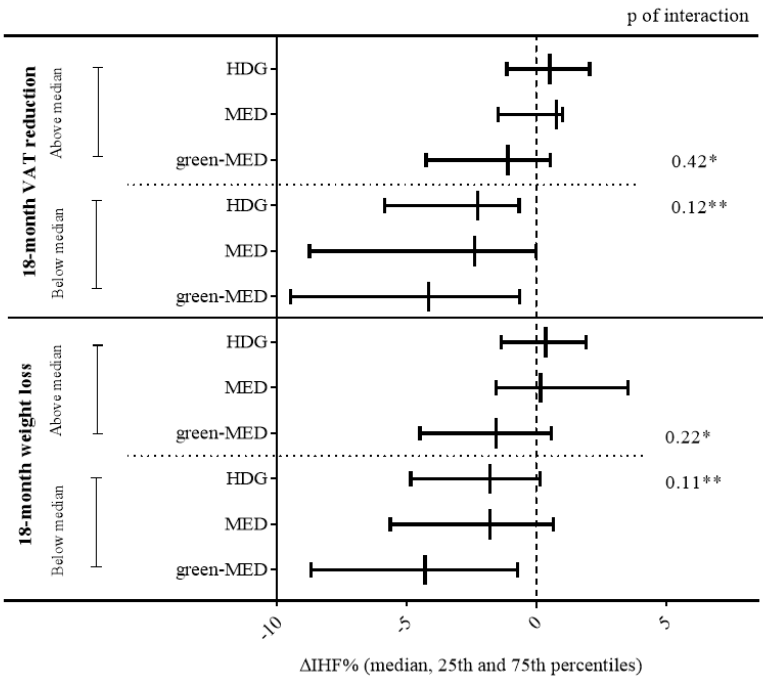
**Supplemental Results 1: Further information regarding the adherence to the intervention**

Among the green-MED group, the 18-month daily green tea consumption weighted average (accounting for the reported consumption after 6 months and the reported consumption after additional 12 months) was  $2.8 \pm 1.6$ /day (median=2.7 cups/day, range 0-8.7) and the weekly consumption weighted average of Mankai was  $2.6 \pm 1.8$ /week (median=2.3 shakes/week, range 0-7). Serum folate change was correlated with higher weekly Mankai intake ( $r=0.41$ ,  $p<0.001$ ), but not with daily green tea intake ( $r=0.08$ ,  $p=0.53$ ). In addition, the green-MED

and MED groups also similarly reduced their carbohydrates consumption ( $-27.9\% \pm 34.0$  and  $-29.8\% \pm 31.3$  respectively, over 18 months;  $p=0.72$ ) as compared to the HDG group ( $p=0.03$  vs. green-MED and  $p=0.01$  vs. MED). Both MED groups also had similar daily walnuts amount ( $p=0.36$ ) and monthly frequency of consumption ( $p=0.52$ ) and were higher as compared with the HDG group ( $p \leq 0.001$  HDG vs. the two MED groups).



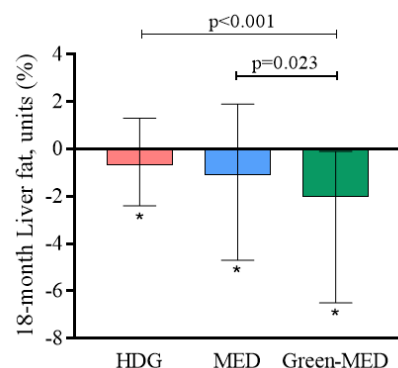
**Supplemental Figure 1:** Subgroup analysis of IHF% change by the degree of weight loss/VAT reduction



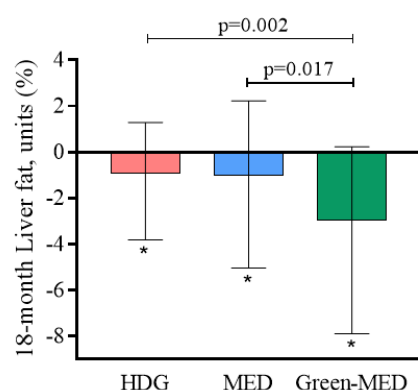
Date presented as medians, 25<sup>th</sup> and 75<sup>th</sup> percentiles of intrahepatic fat. No interactions between the degree of either weight loss or visceral adipose tissue reduction with either MED or green-MED diets were observed. Weight loss and visceral adipose tissue reductions categories are presented as above/below sex-specific median value of 18-month change. Models adjusted to age and baseline IHF. \* p of interaction MED diet with weight/VAT degree of change. \*\* p of interaction green-MED diet with weight/VAT degree of change.

**Supplemental Figure 2:** A comparison between the per-protocol changes and intention to treat techniques for the changes in intrahepatic fat (IHF)

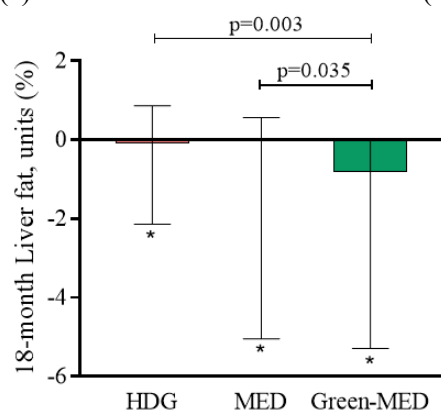
(a) Per-protocol analysis



(b) Multiple imputation (MI)

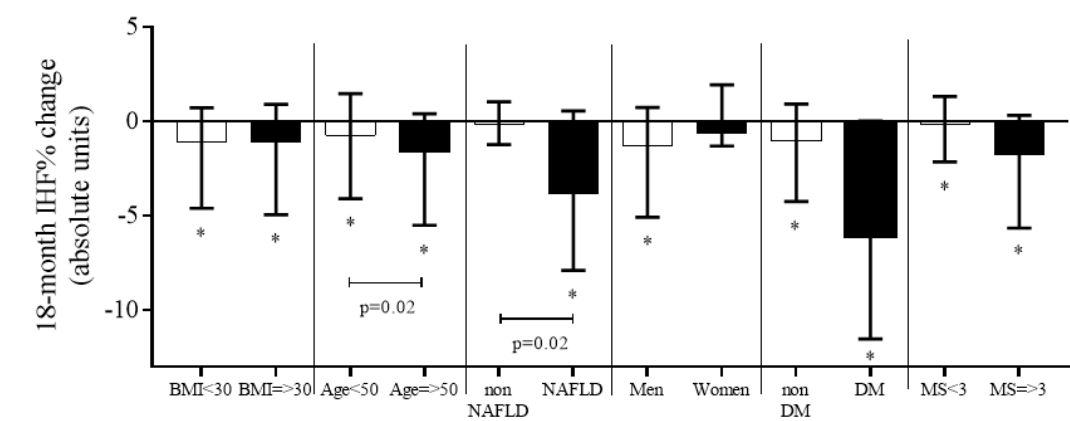


(c) Last observation carried forward (LOCF)



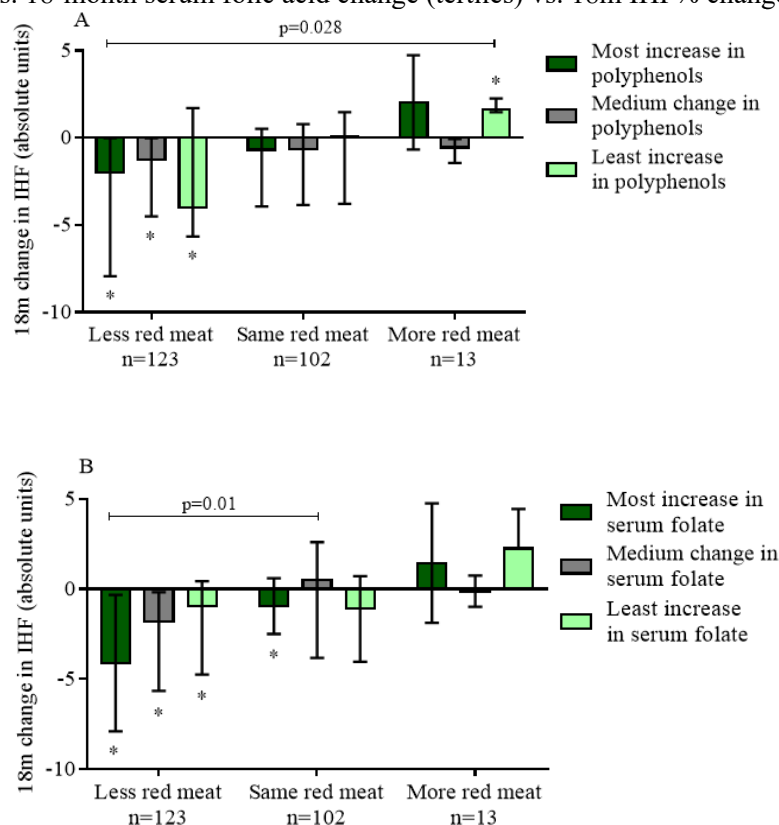
Date presented as medians, 25<sup>th</sup> and 75<sup>th</sup> percentiles of intrahepatic fat. Comparing the per-protocol results to 2 intention to treat techniques (multiple imputation and last observation carried forward) showed that while the significant differences between the intervention groups remained, the effect size (measured as the 18-month change in intrahepatic fat) was reduced, as compared to the per protocol-analysis. This is in accordance with previously published regarding the conservative nature and potential bias of the last observation carried forward technique [33].

**Supplemental Figure 3:** Subgroup analysis of differences in 18-month IHF% change (completers).



Date presented as medians, 25<sup>th</sup> and 75<sup>th</sup> percentiles of 18-month change in IHF. Generalized linear models adjusted for baseline IHF%, 18-month weight change, and intervention group (BMI subgroup differences are adjusted for baseline IHF% and intervention group). The presence of DM was defined for participants with baseline fasting plasma glucose levels≥126mg/dL or hemoglobin-A1c levels≥6.5% or if regularly treated with oral antihyperglycemic medications or exogenous insulin. MS criteria were assessed based on the National Cholesterol Education Program Adult Treatment Panel III criteria. BMI, body mass index; DM, diabetes mellitus; IHF, intrahepatic fat. MS, metabolic syndrome; NAFLD, non-alcoholic fatty liver disease. \* denotes significant difference within-group at p<0.05 level.

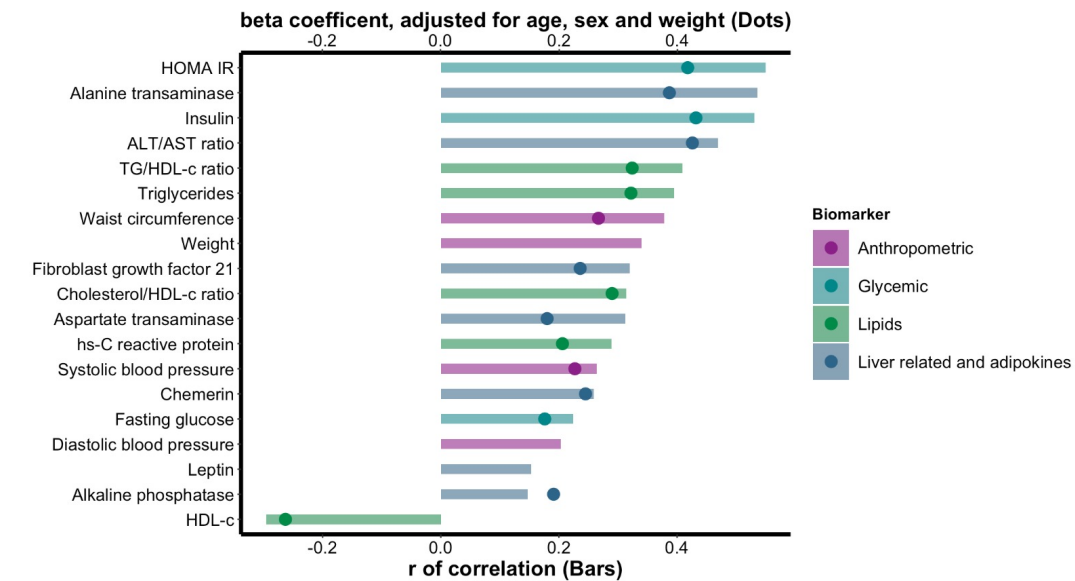
**Supplemental Figure 4: (a)** Red meat consumption change at T18 vs. 18-month total polyphenol change (tertiles) vs. 18m IHF% change. **(b)** Red meat consumption change at T18 vs. 18-month serum folic acid change (tertiles) vs. 18m IHF% change.



Date presented as medians, 25<sup>th</sup> and 75<sup>th</sup> percentiles of 18-month change in IHF. (a) Reductions in IHF% were significantly higher ( $-4.5 \pm 6.9\%$  absolute units) in participants who both reduced red meat consumption and had the greatest increase in plasma polyphenols, as compared with participants who ate more red meat and had the least change in plasma polyphenols ( $2.1 \pm 1.4\%$  absolute units;  $p=0.028$  between groups). No interactions between tertiles of plasma polyphenol change and red meat change were observed. (b) IHF% reduction was greater among participants who reduced red meat and increased serum folate ( $-4.7 \pm 6.6\%$ ), as compared with participants who did not change red meat intake and had at least a moderate change in serum folate level ( $-1.8 \pm 7.5\%$ ). No interactions between tertiles of tertiles of serum folate change and red meat change. \*significant within group, T0 vs. T18. Between-group p values are corrected for multiple comparisons. IHF, intrahepatic fat.

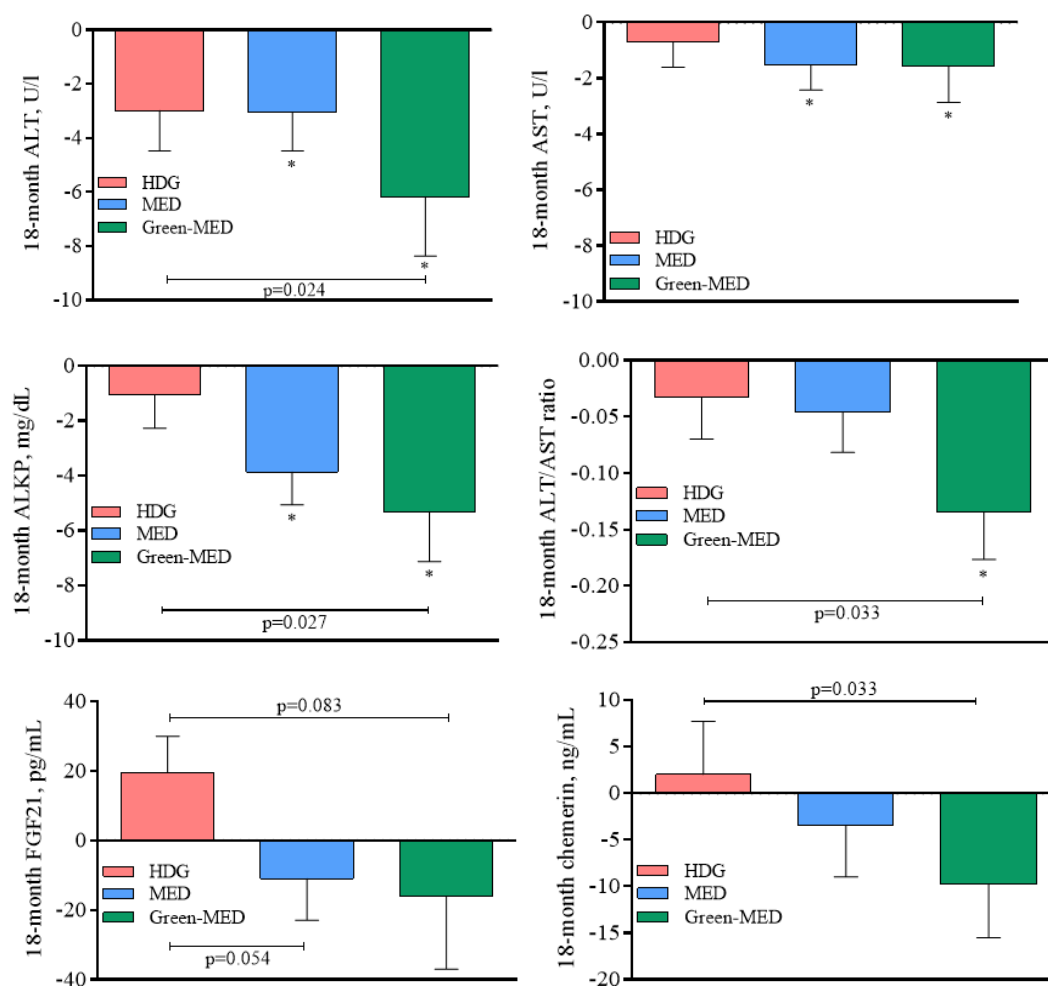


**Supplemental Figure 5:** Associations of intrahepatic fat with cardiometabolic markers



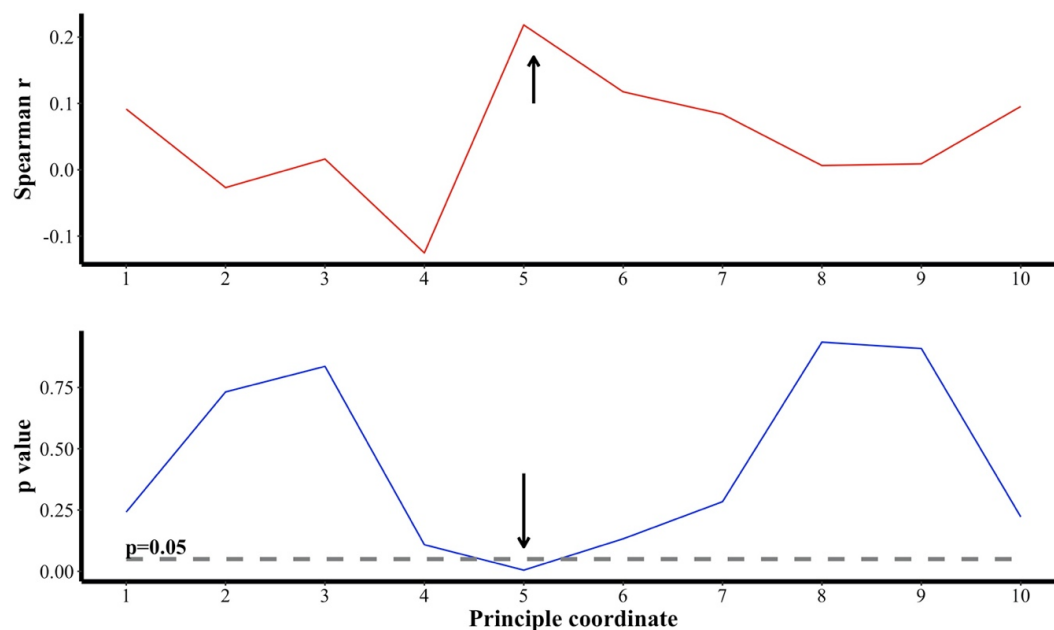
At baseline, IHF% levels were correlated with anthropometric parameters, blood pressure, glycemic, lipid, and liver related markers (presented as bars,  $p<0.05$  for all). When adjusted for baseline body weight, age, and sex (presented as dots on the bars), higher IHF% remained significantly associated with higher WC, systolic BP, fasting blood glucose, insulin, HOMA-IR, TG, TG/HDL-c ratio, TC/HDL-c ratio, hs-CRP, ALKP, ALT, AST, ALT/AST ratio, FGF21 and chemerin ( $p<0.05$  for all). IHF% was found to be inversely associated with HDL-c ( $p<0.001$ ). AST, aspartate transaminase; ALT, alanine transaminase; BP, blood pressure; HDLc, high density lipoprotein cholesterol; HOMA IR, Homeostatic Model Assessment of Insulin Resistance; IHF, intrahepatic fat; TG, triglycerides.

**Supplemental Figure 6:** 18-month change in liver related blood biomarkers, across intervention groups – for participants with valid MRI scans.



Between group p-values are adjusted for age, sex, and baseline level of the biomarker in interest, using General Linear Model. \* significant within group as compared with baseline levels ( $p < 0.05$ ). AST, aspartate transaminase; ALT, alanine transaminase; ALKP, alkaline phosphatase; HDG, healthy dietary guidance; MED, Mediterranean.

**Supplemental Figure 7:** Association between the top 10 principle coordinates (based on UniFrac distance of the log2 change matrix) and IHF change (Ln transformed).



Spearman correlation between IHF change and top 10 principle coordinates (x axis). Top panel shows the correlation coefficient (red line) and bottom panel shows p value (blue line). Arrows point to the most highly correlated principal coordinate (#5;  $r=0.25$ ,  $p=0.001$ ).

**Supplemental Table 1:** Outline of the lifestyle interventions

	<b>HDG</b>	<b>MED</b>	<b>Green-MED</b>
Physical activity	18 months free gym membership 45-60 minutes of aerobic training + resistance training, 3-4 times/week.		
Lifestyle group sessions	18-months group sessions in the workplace, weekly for the first month, and monthly thereafter.		
General dietary guidance	Limit dietary cholesterol, trans-fat, saturated-fat, sugars, and salt and increase intake of vegetables		
Energy, kcal/day	Guidelines for a healthy MED diet with no specific recipes or calorie restriction	1500-1800 kcal/day for men, 1200-1400 kcal/day for women	
Total fat, % of daily consumption		~40% mainly PUFA and MUFA	
Carbohydrates, gr/day		Less than 40 gr/day in the first 2 months with increased gradual intake for up to 80 gr/day	
Specific recommendations		Less/Avoid red and processed meats. Reduced poultry intake	
Polyphenols, mg/day		+440 mg/day [source: provided walnuts (28g/day)]	+1240 mg/day [source: provided walnuts (28 g/day), green tea (3-4 cups/day), Wolffia globosa duckweed (Mankai) shake (100 g/day frozen cubes)]



**Supplemental Table 2:** Lifestyle habits and medications, DIRECT PLUS population, baseline

	<b><u>HDG</u></b> <b><u>(n=98)</u></b>	<b><u>MED</u></b> <b><u>(n=98)</u></b>	<b><u>Green-MED</u></b> <b><u>(n=98)</u></b>	<b><u>All</u></b> <b><u>(n=294)</u></b>	<b><u>p</u></b> <b><u>between</u></b> <b><u>groups<sup>1</sup></u></b>
Smoking, %	19.4	13.3	16.3	16.3	0.51
Shift workers, %	22.4	23.4	23.4	23.1	0.98
PA intensity <sup>2</sup> , %	18.4	29.1	24.4	23.5	0.08
METs/week	(11,41)	(16,50)	(13,38)	(12,42)	
Alcohol <sup>2</sup> , servings/day	0.2 (0.05,0.03)	0.2 (0.05,0.3)	0.2 (0.05,0.3)	0.16 (0.05,0.3)	0.75
<b>Chronic Pharmacotherapy</b>					
Anti-hypertensive, %	14.3	11.2	16.3	13.9	0.59
Cholesterol lowering, %	11.2	8.2	14.3	11.2	0.40
Anti-platelet, %	7.1	3.1	9.2	6.5	0.21
Exogenous insulin, %	1.0	1.0	3.1	1.7	0.27
Oral anti-hyperglycemic, %	6.1	4.1	8.2	6.1	0.49

<sup>1</sup> according to the chi-square test, except for MET and alcohol intake (assessed by Kruskal-Wallis test). <sup>2</sup> Median (25th and 75th percentiles). HDG, healthy dietary guidelines; MED, Mediterranean.

**Supplemental Table 3:** Baseline and 18-month changes in reported dietary intake across intervention groups

	HDG	MED	Green-MED	p between groups
Macronutrients				
Energy				
Energy at baseline (kcal/day)	2193±1180.7	2200.2±1119.3	2065.9±955.8	0.63
Energy change from baseline (kcal/day)	-336±1046	-666±1021	-544±975	0.73
change from baseline, %	-11.6±42.9	-23±27.6	-20±32.0	0.17
Total carbohydrates				
% of energy at baseline	45.3±7.0	44.4±8.5	46.3±7.6	0.24
Change in g/d from baseline, %	-14.0±36.4	-29.8±31.3	-27.9±34.0	0.003
Protein				
% of energy at baseline	20.6±4.0	20.9±4.6	19.9±3.9	0.29
% change out of total energy intake	-5.8±58.3	-12.8±38.4	-14.3±40.2	0.6
Total fat				
% of energy at baseline	34.5±4.5	35.2±4.7	34.5±5.23	0.45
Change in g/d from baseline, %	-7.1±58.5	-15.7±32.0	-10.9±38.4	0.84
Baseline intake of food items, g/day <sup>1</sup>				
Red meat	37.8±31.5	41.8±31.3	40.6±38.7	0.58
Processed meat	13.2±16.6	12.7±12.4	12.3±11.6	0.99
Fish	24.0±22.2	26.6±35.8	19.8±12.7	0.65
Poultry	173.7±138.8	180.4±132.1	152.0±117.7	0.22
Eggs	33.2±28.0	32.0±25.3	33.9±25.9	0.88
Dairy	292.8±250.8	354.7±330.6	286.3±231.0	0.27
Tea (any type)	295.4±380.9	339.5±347.8	327.6±400.4	0.39
Nuts	7.8±9.3	8.6±12.9	8.1±8.6	0.6
Food change frequency <sup>2</sup>				
Red meat				
More	5.9	6.7	3.8	0.012
Same	52.9	46.7	28.2	
Less	41.2	46.7	67.9	
Processed meat				
More	0	0	0	

Same	37.6	34.7	28.2	0.43
Less	62.4	65.3	71.8	
<b>Fish</b>				
More	63.5	69.3	74.4	0.55
Same	35.3	28.0	24.4	
Less	1.2	2.7	1.3	
<b>Poultry</b>				
More	27.1	41.3	26.9	<b>0.002</b>
Same	61.2	52.0	46.2	
Less	11.8	6.7	26.9	
<b>Eggs and dairy</b>				
More	15.3	36.0	35.9	<b>0.02</b>
Same	72.9	56.0	53.8	
Less	11.8	8.0	10.3	
<b>Green Tea</b>				
More	31.8	37.3	82.1	<b>&lt;0.001</b>
Same	58.8	53.3	15.4	
Less	9.4	9.2	2.6	
<b>Nuts</b>				
More	32.9	60.0	56.4	<b>&lt;0.001</b>
Same	61.2	30.7	28.2	
Less	5.9	9.3	15.4	

Data are means  $\pm$  Standard deviations for continuous parameters and percentage for categorical parameters. <sup>1</sup> Data extracted from reported food intake, not part of a recipe. <sup>2</sup> Self-reported as compared with the last food change questionnaire.

**Supplemental Table 4:** Weekly walnut consumption and frequency (reported, time 18):

	HDG	MED	Green-MED	p between groups <sup>1</sup>	p between MED groups <sup>1</sup>
<b>Amount of consumption</b>					
Few	53	16	11	<0.001	0.36
28 g/day	23	46	56		
Bunch	8	11	9		
<b>Frequency of consumption</b>					
less than once a month or never	25	7	6	<0.001	0.52
1-3 times/month	21	14	8		
1-2 times/week	19	12	10		
3-4 times/week	8	8	14		
5-6 times/week	4	13	12		
every day	3	12	19		
2-3 times/day	4	6	7		
4-5 times/day	0	1	0		

<sup>1</sup> Test by Chi-Square test. HDG, healthy dietary guidelines; MED, Mediterranean.

**Supplemental Table 5:** Genus level bacteria associated with IHF, IHF change and lifestyle intervention.

Baseline associations	Genus Level bacteria	Coefficient	Standard error	p value	q value
IHF	Fournierella	-0.0003709	7.65E-05	2.33E-06	0.00041326
IHF	Anaerosporebacter	-0.0002198	6.36E-05	0.00064969	0.05041708
IHF	Lachnospiraceae_U CG-003	-0.0001376	4.07E-05	0.00085453	0.05041708
IHF	Ruminococcaceae_U CG-009	-5.34E-05	1.77E-05	0.00276759	0.12246594
IHF	Ruminococcaceae_U CG-014	-0.0043576	0.00155184	0.00542229	0.19194916
IHF	Erysipelotrichaceae_U CG-003	0.00154705	0.00059427	0.00984842	0.21805458
IHF	Ruminococcaceae_U CG-008	-0.0001195	4.59E-05	0.00985557	0.21805458
IHF	Ruminococcaceae_U CG-010	-0.0008363	0.0003195	0.00945633	0.21805458
IHF	Ruminococcaceae_U CG-002	-0.0040148	0.00158245	0.01185459	0.23314025
<b>18-month change</b>					
IHF	Erysipelotrichaceae_U CG-003	0.04734104	0.01450464	0.00119285	0.01610349
IHF	Fournierella	-0.0058954	0.00173597	0.00075311	0.01610349
IHF	Anaerosporebacter	-0.006328	0.00226281	0.00541248	0.04517776
Time*IHF interaction	Fournierella	0.00466757	0.00185459	0.01254249	0.05707647
Time*IHF interaction	Ruminococcaceae_U CG-008	-0.0090038	0.0038452	0.01982263	0.07645871
IHF	Ruminococcaceae_U CG-010	-0.0079535	0.00435396	0.06847972	0.16808659
IHF	Lachnospiraceae_U CG-003	-0.0033651	0.00179764	0.06193927	0.16808659
Time*IHF interaction	Lachnospiraceae_U CG-003	0.0043736	0.00223972	0.05182528	0.16808659
Time*IHF interaction	Anaerosporebacter	0.00495938	0.00278814	0.07676383	0.17271863
Time*IHF interaction	Ruminococcaceae_U CG-009	0.00576984	0.00336781	0.08760905	0.18195725
IHF	Ruminococcaceae_U CG-014	-0.0216027	0.01302554	0.09799635	0.18899296
<b>Change by Lifestyle intervention</b>					
time*Green-MED interaction	Fournierella	-0.0298969	0.01024317	0.00389043	0.15561715



time*MED interaction	Ruminococcaceae_ UCG-014	0.10581789	0.04643635	0.02362381	0.18899047
time*Green- MED interaction	Ruminococcaceae_ UCG-014	0.09732996	0.04785686	0.04316678	0.21583388

IHF, intrahepatic fat; MED, Mediterranean.

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