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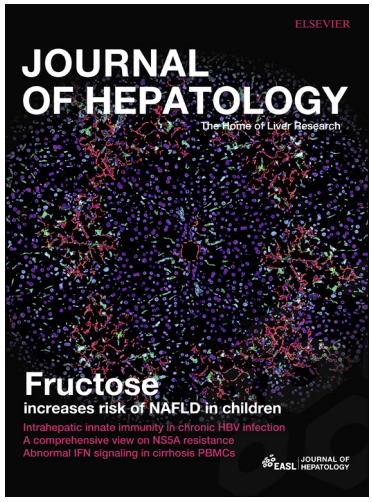

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The Beneficial effects of Mediterranean diet over low-fat diet may be mediated by decreasing hepatic fat content

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ABBREVIATIONS: HFC, hepatic fat content; VAT, visceral adipose tissue; MRI, magnetic resonance imaging; visceral adipose tissue; MED/LC, Mediterranean/low-carbohydrate; LF, low-fat; GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; Chol/HDL, total cholesterol to high density cholesterol ratio; TG/HDL, triglyceride to high density cholesterol ratio; TC, total cholesterol; TG, triglycerides; WC, waist circumference; NAFLD, non-alcoholic fatty liver disease.

CLINICAL TRIAL REGISTRY: ClinicalTrials.gov Identifier: NCT01530724

ABSTRACT

Background and Aim: It is unclear if reduction in hepatic fat content (HFC) is a major mediator of the cardiometabolic benefit of lifestyle intervention, and whether it holds prognostic information beyond visceral adipose tissue (VAT) loss. In the present sub-study, we hypothesized that HFC loss in response to dietary interventions induces specific beneficial effects independent of VAT changes.

Methods: In an 18-month weight-loss trial, 278 participants with abdominal obesity/dyslipidemia were randomized to low-fat (LF) or Mediterranean/low-carbohydrate (MED/LC+28g walnuts/day) diets with/without moderate physical activity (PA). HFC and abdominal fat-depots were measured using magnetic-resonance-imaging at baseline, after 6 (sub-study, n=158) and 18-months.

Results: Of 278 participants [age=48yr;88% men; body-mass-index=30.8kg/m^2; mean HFC =10.2%,(range:0.01%-50.4%)], retention rate was 86.3%. %HFC substantially decreased after 6 [-6.6% absolute-units (-41% relatively)] and 18-months [-4.0% absolute-units (-29% relatively);$p<0.001$ vs. baseline]. Reduction of HFC associated with decreases in VAT beyond weight loss. After controlling for VAT loss, decreased %HFC remained independently associated with reductions in serum gamma-glutamyl-transferase and alanine-aminotransferase, circulating chemerin, and HbA1c ($p<0.05$). While reduction of HFC was similar between PA groups, compared to LF diet, MED/LC induced a greater %HFC decrease ($p=0.036$) and greater improvements in cardiometabolic risk parameters ($p<0.05$), even after controlling for VAT changes. Yet, the greater decreases induced by MED/LC compared to LF diets in triglycerides, TG/HDL ratio and cardiovascular risk score were all markedly attenuated when controlling for HFC changes.

Conclusions: %HFC is substantially reduced by diet-induced moderate weight loss, more effectively by MED/LC diet, beyond VAT changes. HFC loss is associated with specific improved parameters. Beneficial effects of MED/LC diet may be largely mediated by decreases in %HFC rather than VAT loss.

LAY SUMMARY: High hepatic fat content is associated with metabolic syndrome, type two diabetes mellitus, and coronary heart disease. In this CENTRAL 18-months intervention trial, Mediterranean/low-carbohydrate diet induced a greater decrease in hepatic fat content than low-fat diet, and the beneficial health effects were beyond the favorable effects of visceral fat loss.

INTRODUCTION

Beyond total body fat content, fat distribution, both within adipose tissue depots and in ectopic fat deposits, is increasingly shown to determine obesity-related health impact [1, 2]. Visceral adipose tissue (VAT), due to its unique anatomical location, releases free fatty acids (FFA) and adipokines to the liver via the portal vein. Previous studies have demonstrated the inter-relationship between VAT and hepatic fat content (HFC), and indeed, increases in HFC were associated with similar metabolic abnormalities as observed for increases in VAT [3, 4]. In addition, reduction in VAT and HFC are increasingly thought to mediate beneficial cardiometabolic outcomes of weight loss [1, 5]. Though closely associated with HFC, VAT and HFC may uniquely associate with specific effects and independently be linked with cardiometabolic disease risk factors [6]. Interestingly, data from recent studies found that HFC was more strongly associated with obesity's metabolic complications than VAT [7], including the deterioration of glucose tolerance [8], possibly by mediating the link between obesity and metabolic dysfunction [9, 10]. Most recently, the decrease in HFC was associated with diabetes remission [11] .

Diet plays an important role in the accumulation of HFC and VAT [12]. Several short [6, 13] and long-term [14, 15] dietary interventions have suggested that Mediterranean and low-carb diets had favorable effects on their accumulation, but also on glycemic status and lipid biomarkers. Others found no differences between HFC changes induced by diets with different amounts of carbohydrate [16]. The effect of long-term specific lifestyle interventions on HFC and its association with the dynamics of cardiometabolic risk, beyond VAT loss, remains unclear. Notably, recent guidelines for decreasing HFC do not suggest a particular lifestyle strategy, but only endorse weight loss as a general recommendation [17].

In the present sub-study we hypothesized that low-fat (LF) and Mediterranean/low-carbohydrate (MED/LC), similarly-hypocaloric diets, differ in their capacity to induce HFC loss, which

mediates the improvements in cardiometabolic parameters independently of the impact of accompanying decreases in VAT.

MATERIAL AND METHODS

The CENTRAL trial (ClinicalTrials.gov Identifier: NCT01530724) was an 18-month randomized controlled trial [18]. In the first randomization, participants were randomly assigned to one of two calorie-restricted diets for the entire study period: a low-fat (LF) diet[19] (n=139) or a Mediterranean/low-carbohydrate (MED/LC) diet[15] (n=139). In the second randomization, 6 months after initiating the dietary intervention, each diet group was further randomized into added physical activity (PA) groups (LF^{PA+}, MED/LC^{PA+}) or no added PA groups (LF^{PA-}, MED/LC^{PA-}) for another 12 months of intervention (diets were continued throughout, according to the first randomized dietary assignment). This two-stage study design was based on our previous results from the DIRECT study,[15] in which the dietary weight-loss was maximal by 6 months ("rapid weight-loss phase"). Participants were randomized after all had been recruited, in one phase, and after their strata characteristics were defined [18]. The intervention was conducted at a facility with an on-site medical clinic and monitored cafeteria.

Eligibility and Study Design

Inclusion criteria were: abdominal obesity [waist circumference (WC)>40 inches (102 cm) for men and > 35 inches (88 cm) for women], or participants with triglycerides (TG) ≥150mg/dL and high density lipoprotein (HDL-c) <40mg/dL for men and <50mg/dL for women. Candidates were excluded if: they were pregnant or lactating women; had serum creatinine ≥ 2 mg/dl; impaired liver function (≥ 3 fold the upper level of ALAT and ASAT enzymes), active cancer; highly physically active (>3 hours/week) or unable to take part in physical activity, or participation in another trial. The study protocol was approved by the Medical Ethics Board and the Helsinki Committee of the Soroka University Medical Center. All participants provided written informed consent and received no financial compensation or gifts.

Diet Intervention

Both diets aimed for a moderate, long-term, weight loss with restricted intake of *trans*-fats and refined carbohydrates, and an increased intake of vegetables. Lunch was provided exclusively by the workplace cafeteria during the work week, and a dietitian worked closely with the kitchen staff to adjust the diets to the specific diet groups. The 18-month dietary intervention included a 90-minute nutritional session in the workplace with clinical dietitians every week during the first month of the intervention, and every month thereafter. Participants were trained to adhere to their specific diets during the entire week. For the LF diet, the aim was to limit total fat intake to 30% of calories, with up to 10% of saturated fat, and no more than 300 mg/day of cholesterol, and to increase dietary fiber. Participants were counseled to consume whole grains, vegetables, fruits, and legumes and to limit their consumption of additional fats, sweets, and high-fat snacks. The MED/LC diet combined the Mediterranean and low-carbohydrate diets described in our previous weight loss trial (the DIRECT trial)[15]. The diet restricted carbohydrate intake to less than 40 g/day in the first two months (induction phase), and thereafter a gradual increase up to 70 g/day, and increased protein and fat intake, according to the MED diet. The MED/LC diet was rich in vegetables and legumes and low in red meat, with poultry and fish replacing beef and lamb. This group was provided 28 g of walnuts/day [160 Kcal/84% fat, mostly PUFA (omega-3 α-linolenic acid)] starting from the third month, after the induction phase.

Physical Activity Intervention

At the second randomization (after 6 months of dietary intervention), participants assigned to added PA received a free supervised gym membership for the following 12 months. The exercise intervention included monthly educational workshops, and one hour of exercise, three times/week. Participants were guided to engage in 45 minutes of aerobic training at 80% of maximum heart rate and 15 minutes of resistance training at 80% of the one-repetition maximum (1RM) of the weight.

Outcomes

Magnetic resonance imaging (MRI)

MRI was performed using a 3-Tesla magnet (Ingenia 3.0 T, Philips Healthcare, Best, the Netherlands) at baseline, after 6 (only for 157 of participants, randomly selected) and 18 months. The scanner utilized a 3D modified DIXON (mDIXON) imaging technique without gaps (2mm thickness and 2mm of spacing)[18]. The percentage of HFC was assessed in defined area of 2000 mm^2 regions of interest (ROI) method[20] based on measurements of tissue signals [fat/fat + water] using the Fat Ratio Calculation PRIDE software from Philips Medical Systems. We analyzed the liver in two-dimensional 3 cm intervals, referring to each image as a "slice". The number of ROIs in each slice was determined proportionally to the image area. We divided each slice into quarters, and chose ROIs in each of the four quarters in order to represent the entire liver. We determined the mean percentage of fat for each slice and quarter, and then calculated the mean percentage of fat of the liver as a whole. Fat quantification was performed blinded to time-point and treatment group. Reliability of measurements between technicians was measured among 28 images. Inter-observer correlation (2 independent observers) was $r=0.99$, $p<0.001$ and Intra-observer correlations was $r=0.98$, $p<.0001$. Abdominal fat was quantified using MATLAB-based semi-automatic software that was written in-house [21, 22]. Three slices were selected from an intra-vertebral space of L5-S1, L4-L5 and L2-L3 and a continuous line was drawn over the superficialis fascia to differentiate between the deep subcutaneous adipose tissue (SAT) and superficial SAT. Mean VAT, deep SAT and superficial SAT were calculated from the three axial slices.

Clinical, Metabolic and Anthropometric Outcomes

Height (±0.1 cm) was measured using a standard wall-mounted stadiometer. Body weight (±0.1 kg) was measured without shoes. Waist circumference (WC, ±0.1cm) was measured half-way between the last rib and the iliac crest. Fasting blood samples were centrifuged and stored at -80^{o}C. All biomarkers were

assayed in the Leipzig University laboratories, Germany. Fasting plasma glucose (FPG) was measured by Roche GLUC 3 (hexokinase method). Plasma insulin was measured with an enzyme immunometric assay [Immulite automated analyzer, Diagnostic Products, coefficient of variation (CV)=2.5%]. Serum total cholesterol (CV=1.3%), high-density-lipoprotein cholesterol (HDL-c), low-density-lipoprotein (LDL) cholesterol, and triglycerides (CV=2.1%) were determined enzymatically with a Cobas 6000 automatic analyzer (Roche). Homeostasis model assessments of insulin resistance (HOMA-IR) was calculated using HOMA Calculator v2.2.3. Chemerin serum concentrations was determined using a commercially available ELISA kit (Human Chemerin ELISA, Biovendor, Heidelberg, Germany) according to the manufacturer's instructions.

Electronic questionnaires

Adherence to the dietary and PA interventions were evaluated using a validated electronic food-frequency and activity questionnaire (FFQ)[23] as published in our previews publication [18]. The FFQ contains 127 food items, 17 of them with 3 portion-size pictures, and life-style and PA questions, as well as symptoms, adverse-effects, quality of life, medication usage, and safety at baseline and after 6 and 18 months of intervention. The electronic questionnaires were self-administered and helped to ensure completeness of the data by prompting the participant when a question was not answered, and it permitted rapid automated reporting to the group dietitians.

Statistical analysis

The primary outcome of the key CENTRAL study [18], as defined in clinicaltrials.gov, was change in body fat composition. In this sub-study, we aimed to address the influence of HFC reduction by lifestyle interventions, on improvement of cardiometabolic markers, beyond VAT loss. For the 18-month time point, we performed intention-to-treat analyses, including all 278 participants, by imputing the missing observations for all adipose tissues for 38 individuals by the multiple imputation technique [24].

No imputation has been performed for adipose tissues at 6-month time point (only 57%, randomly selected). For missing data of body weight, we used the last observation carried forward. To characterize the entire study population, quantitative variables were expressed as means and standard deviations. All p-values were two-sided and $P<0.05$ considered statistically significant. Analysis of variance with a covariance (ANCOVA) test was used to assess changes in nutrient intakes between the diet strategies. %HFC was ln-transformed at each time point, and the delta was calculated accordingly, allowing us to generate a normal distribution. At baseline, the association (p of trend) of %HFC and cardiometabolic risk parameters across sex-specific quintiles of VAT was tested, in order to estimate baseline relationships between these two main parameters of interest, using univariate linear regression (**Table 1**). Pearson's correlation coefficient was used between continuous variables. We performed multivariate linear regression models to assess changes between diet groups in the dynamics of %HFC after 6 and 18 months, adjusted for age, sex, ln-transformed %HFC at baseline, WC (cm) at baseline, and VAT changes in order to identify independent intervention effects. The association between 18-m changes in %HFC and the dynamics of cardiometabolic risk parameters was tested by linear regression models adjusted for age, sex and the four intervention groups. Next, in separate models, we further adjusted for body weight or VAT changes, each one at the time. We calculate cardiovascular risk using three different scores: The Framingham risk score (FRS) [25], Systematic COronary Risk Evaluation (SCORE) [26], and the American College of Cardiology/American Heart Association (ACC/AHA)- Pooled cohort equations (PCE)[27]. The data was analyzed by SPSS software Version 23.

RESULTS

Baseline characteristics

At Baseline, participants (mean age=48years, 89% men, BMI=30.8±3.8kg/m^2) had 10.2% HFC (median=6.38%), widely ranging between 0.01% and 50.4%. Of the 278 participants, 53% had NAFLD

(HFC above 5%), 40% met the criteria for the Metabolic Syndrome, 75% had abnormal WC and 11% were diabetic. Few participants used medications chronically (anti-platelet=7%, anti-hypertensive=8%, lipid-lowering=12%, oral glycemic-control=3% and insulin treatment=1%), with minor changes during the intervention, that were similar between groups. Characteristics of the CENTRAL study population across intervention groups are shown in **Table 1.** There were no significant differences at baseline between the intervention groups in demographic variables, consumption of energy intake and macronutrients, blood markers, HFC, or abdominal fat sub-depots, but only in VAT area in female.

Dynamics of HFC throughout the intervention

In the entire cohort, HFC substantially decreased after 6 [-6.6% absolute-units (-41% relatively)] and 18 months [-4.0% absolute-units (-29% relatively)] ($p<0.001$ vs. baseline), along with moderate body weight loss (-5.8% and -3.1% after 6 and 18 months, respectively). 18m retention rate was 86.3%. Decreased %HFC directly correlated with loss in all three layers of abdominal sub-depots after 6 and 18 months, when the models were adjusted for age and sex ($p<0.001$ for all). However, when the models were further adjusted for weight loss, decreased %HFC remained associated only with reduction of VAT at 6 ($\beta=0.232$; 95%CI 0.13-0.34) and 18 months ($\beta=0.155$; 95%CI 0.04-0.31), but not with deep-SAT or superficial-SAT changes at 6 and 18 months ($p>0.54$ for all). After controlling for VAT changes, MED/LC diet tended to decrease %HFC more than LF diet after 6 months [MED/LC: -7.3±9.2% vs. LF: -5.8±7.2% (absolute units), $p=0.079$ between diets], a differential effect between the dietary intervention groups that became significant at 18 months of intervention [MED/LC: -4.2±7.1% vs. LF: -3.8±6.7% (absolute units), $p=0.036$ between diets]. Furthermore, the advantageous effect of MED/LC on HFC reduction over LF diet was significant even in non-NAFLD patients (HFC≤5%, $p=0.037$), as in patients with NAFLD (HFC>5%, $p=0.014$). No significant differences were observed between the PA groups ($p=0.32$) for HFC changes after 18 months, with or without adjustment for VAT changes. The changes in HFC over 18-month of intervention across different subgroups of the cohort are shown in **Figure 1**. Higher HFC at

baseline was found, as expected, in males (10.7% vs. 5.8%, p=0.001), in participants with BMI≥30 (12.7% vs. 7.0%, p<0.001) and in those with VAT>30% at baseline (11.5% vs. 8.0%, p=0.007). The relative reductions of %HFC induced by the intervention were higher in males and in patients with BMI≥30 or VAT≥30%, even after controlling for 18-month VAT changes. Interestingly, in a model adjusted for weight loss, the beneficial effect of MED/LC diet over the LF diet was more apparent among males (p=0.016) and in participants with VAT over 30% at baseline (p=0.018), but similarly in both BMI groups. We further performed sensitivity analyses among the participants that completed both 6- and 18-months MRI-body fat measurements (i.e., 6 month sub-study), and a similar pattern was observed (data not shown). Similar results were also found when excluding participants using insulin.

Association between %HFC loss and nutritional intake changes

Overall, during the intervention, participants significantly decreased their energy intake after 6 and 18 months (p<0.001 vs. baseline), but similarly across diet groups [total calorie intake (-26% vs. -22% in the MED/LC diet vs LF diet, respectively, p=0.18)]. Changes in the intake of marco- and micro-nutrients compared to baseline are shown in **Figure 2**. After 18 months of intervention the MED/LC diet greatly decreased intake of carbohydrate and *trans* fat, while the LF diet decreased more the intake of total fat, monounsaturated fat and cholesterol, and tended to decrease more polyunsaturated and saturated fats (p<0.05 for all, **Figure 2a**). In addition, the MED/LC diet increased nuts consumption (p<0.05, **Figure 2b**). Decrease of HFC after 18 months correlated with decreased carbohydrate intake (r=0.175, p=0.009), and with increased fat intake (r=-0.217, p=0.001), as proportions of total calorie intake.

Association between HFC and cardiometabolic risk parameters

In the entire group, significant improvements in cardiometabolic markers were observed after 18 months of intervention, including decreases in total-cholesterol/HDL-c ratio by -0.3 (4.4%), gamma-

glutamyl transferase (GGT) by 12.4U/L (8.2%) and fasting circulating insulin levels by -3.3μU/mL (12.2%), ($p<0.001$ for all vs. baseline). We examined the association between 18-m dynamics of HFC with 18-m changes in hepatic and cardiometabolic parameters using multivariate regression models **(Figure 3)**. In models adjusted for age, sex and intervention group, decreased HFC was associated with decreased GGT (β=0.443; 95%CI 0.32-0.56), alanine aminotransferase (ALT; β=0.253; 95%CI 0.12-0.39), cholesterol/HDL ratio (β=0.226, 95%CI 0.09-0.35), TG/HDL ratio (β=0.209, 95%CI 0.03-0.30) and chemerin (β=0.393, 95%CI 0.26-0.52). To assess the contribution of decreased HFC independent of VAT, the model was further adjusted for VAT changes. Associations between reduction of HFC and lipid parameters were attenuated by adjusting for VAT changes. However, reduction of HFC remained significantly and independently associated with reduced GGT (β=0.330; 95%CI 0.24-0.42), ALT (β=0.189; 95%CI 0.04-0.35) and chemerin (β=0.382; 95%CI 0.23-0.53) even after controlling for VAT changes. When data was stratified to HFC within and above normal range (i.e. <5% and ≥5%, respectively), similar associations between groups were found with GGT, chemerin, and cholesterol/HDL ratio ($p<0.05$ for all). However, while ALT and %HbA1c were associated with HFC loss only for the ≥5% HFC group, insulin levels were associated with HFC in the <5% HFC group (Supp 1).

To compare the impact of losses of HFC, VAT and total weight on improvement in cardiometabolic parameters induced by MED/LC versus LF, we determined how adjustment for those parameters attenuated the differences between the two dietary interventions. Compared to the LF diet, MED/LC diet induced a greater increase in HDL-c (3.3±7.5 vs. 5.6±7.1 mg/dl), and a more pronounced decrease in diastolic BP (1.2±10.1 vs. -1.9±7.5 mmHg), triglycerides (-3.4±43.7 vs. -10.8±28.0 mg/dl), TG/HDL ratio (-0.15±0.4 vs. -0.23±0.4) and cardiovascular risk by the three different scores: Framingham (-0.27±2.2 vs. -0.81±1.9), SCORE (-0.16±1.4 vs. -0.50±1.2), and ACC/AHA score (-0.39±2.7 vs. -1.13±2.5), ($p<0.05$ for all, **Figure 4-** white and black bars). These differences remained significant when body weight and VAT changes were added to the multivariate model (**Figure 4-** colored bars). However, after

adjustment for HFC changes, differences between diets were significantly attenuated, particularly the changes in triglycerides, TG/HDL ratio and in the cardiovascular risk scores. When data was stratified for within and above normal HFC at baseline, the beneficial effect of the MED/LC diet over the LF in reducing cardiovascular risk scores became insignificant, possibly due to the lower power of the analysis. Nevertheless, similar trends were noted in both the normal HFC and abnormal HFC sub-groups (Supp 2a and 2b, respectively). There was no significant effect by the PA intervention on improvement of cardiometabolic parameters or cardiovascular risk.

DISCUSSION

In this long-term lifestyle intervention trial, the MED/LC induced a significantly greater decrease in HFC than the LF diet, even after accounting for the differences in VAT loss. The impact of HFC reduction is highlighted by associated improvements in GGT, ALT, chemerin and HbA1c, which remained significant after adjustment for total weight loss or VAT change. In addition, the MED/LC diet was superior to the LF diet in decreasing cardiometabolic risk, a difference that was attenuated when adjusting for the decrease in HFC, but not following adjustment for weight or VAT.

Our study has several notable limitations. The small number of women (12%) limits our confidence in applying conclusions to females (although expected differences between males and females in various laboratory and fat distribution parameters were detectable). Information regarding adherence to the diets is based on questionnaires and attendance to the diet sessions. However, validated [23] questionnaires were used in order to ensure the highest level of accuracy as possible. In addition, since this study did not include histological tissue analyses, we were unable to trace changes in inflammatory processes in the liver and/or hepatocellular damage (beyond transaminase levels). The accuracy of quantifying liver fat in patients with HFC within the normal range (<5%) had been questioned. However, several recent publications evaluated the accuracy of hepatic proton density fat fraction (PDFF) measurements using MRI, and found that MRI-PDFF is an accurate non-invasive method

for quantifying HFC even within the range below 5% [28, 29].Participants in this trial, although overweight or obese, were relatively healthy (low rate of chronic medicine and only 11% were diabetic), therefore, it may be difficult to extend conclusions to individuals with more advanced liver disease. The strengths of the study include the following: all participants started the study simultaneously (one-phase study design); the use of the 3-T MRI scan; we treated HFC as a continuous variable, enabling us to define the amount of fat even within the normal range (<5%); the relatively long duration of the study and the high rate of adherence.

Despite the moderate weight loss in this study (-3 kg), we observed a highly discernable decrease in HFC that was greater in response to MED/LC diet than to LF diet. Previous studies focused on weight loss as a key factor in reducing HFC and its comorbidities in obese subjects [30, 31]. Therefore, it is not surprising that the guidelines of the American Association for the Study of Liver Diseases [32] suggest weight loss through general nutritional care as first-line intervention and prevention of nonalcoholic fatty liver disease (NAFLD). However, the weight loss phase mostly occurs during the first six months, followed by a weight regain phase, as we previously shown [15]. Adipose tissue expansion during weight gain may result in a decreased insulin response and, thus, increased lipolysis and free fatty acid (FA) production, which support hepatic lipid accumulation [33]. Some recent studies [34, 35] have shown long-term reduction of HFC and improvement in liver markers despite weight gain after dietary weight loss. Bozzetto et al [13, 36] showed that an isocaloric diet enriched in MUFA results in a reduction in HFC (by increasing fat oxidation), independent of weight change. In addition, the beneficial effect of different dietary strategies has demonstrated in previous studies suggesting significant reduction in HFC with minor to moderate weight loss [37] but this was not observed in larger, more recent studies [38]. Nevertheless, data are sparse regarding the effect of long-term specific dietary interventions on HFC, beyond VAT loss. In a randomized study [39], 170 overweight or obese subjects

were randomly-assigned to either reduced fat or reduced carbohydrate, calorie-restricted diets for 6 months, and found similar beneficial effects of the two intervention arms on HFC reduction.

Our results reveal that reductions in liver markers and chemerin are associated with a decrease in HFC, potentially at least partially independent of the VAT-liver axis. Thus, clinically, tracking changes in those biomarkers may reveal changes in HFC that are currently difficult to track directly by imaging or to estimate by other means: Liver biopsy is still considered the current clinical gold standard in this regard [2], but there is an urgent need to rely on non-invasive blood biomarkers. A particularly difficult challenge is to find independent biomarkers that reflect the dynamics of HFC, even beyond VAT. Liver enzyme ALT is the biomarker most commonly used to assess HFC content and liver injury. However, several studies [40, 41] have shown that ALT does not necessarily correlate strongly with HFC or with the severity of liver damage. In a cross-sectional study [40] in which 31% of the subjects had elevated HFC (assessed by MRI), 79% of those had normal levels of ALT. These highlight the urgent need for novel biomarkers indicating HFC. GGT is frequently elevated in patients with NAFLD [41], possibly because increased fat in the liver may induce hepatocellular damage that leads to increased GGT synthesis [42]. Moreover, histological improvement of the liver was associated with reductions in GGT concentrations with weight loss [43]. Yet, GGT has been included only as part of a group of biomarkers used to predict increased HFC [44] and not as an independent biomarker. In the present study, mean GGT and ALT levels at baseline were in the normal range. Therefore, it is possible that considering changes (delta's) in these parameters may be of greater clinical impact than the absolute values in a cross-sectional setting, even within the normal range of these parameters.

Chemerin,[45] is an "adipo-hepatokine" which was found to be associated with obesity and impaired cardio-metabolic state [46]. In a prospective study [47] levels of chemerin were found to be directly correlated with severity of NAFLD in obese subjects. Previously we reported [48] that chemerin dynamics tightly correspond to changes in body weight in the DIRECT trial [15], decreasing during the

weight loss phase and stabilized or increased during the weight maintenance/regain phase. Nonetheless, the link between chemerin and the dynamics of HFC over time has yet to be demonstrated. Notably, the dynamic range of total chemerin levels is low, potentially limiting its use as a biomarker. Possibly, considering different isoforms of this adipo-hepatokine could enhance its sensitivity and specificity as a biomarker for HFC dynamics.

Our analyses suggest that HFC changes, rather than VAT changes, may play a particular role in mediating the greater beneficial effects of MED/LC over the LF dietary intervention. After adjusting for HFC changes, the differences in the association of the diets with improvements in lipid profile and in the cardiovascular risk scores became statistically insignificant. We did not observe such attenuation when controlling for weight or VAT loss. Recent long-term dietary interventions and meta-analyses have shed light on the ability of low-carbohydrate and Mediterranean [14, 15, 49, 50] diets to serve as alternatives to traditional low-fat diets in inducing weight loss and improved cardio-metabolic profile. A previous cross-sectional analysis has also suggested a stronger association of HFC than of VAT with obesity-related cardio-metabolic risk [8]. Moreover, another study that matched subgroups of patients with similar VAT but different HFC, suggested that increased HFC was cross-sectionally associated with insulin resistance [7]. Our current randomized trial results strengthen the notion, and provide evidence, supporting the unique impact of HFC on such risk, showing that these associations occur in response to intervention and are not merely cross-sectional observations. Our finding are also in line with results from mechanistic, fat transplantation studies in mice, in which mesenteric (portally-drained), but not parietal peritoneal (systemically-drained via the vena cava) transplantation induced worse metabolic outcome.[51, 52] However, in humans, conflicting results were obtained on the putative metabolic benefit of omentectomy (surgical VAT reduction) during bariatric surgery.[53] Thus, although HFC partially reflects a downstream consequence of increased VAT, our results strengthen the notion that HFC mechanistically contributes to cariometabolic risk independently of VAT. Moreover, they highlight

the potential value of interventions specifically targeting the hepatic manifestations of obesity, such as LC/MED diet, in diminishing health risks associated with obesity.

The amount of HFC accumulation depends, among other things, on an interaction between hepatic FA uptake, derived from plasma free fatty acid (FFA) released from TG hydrolysis in adipose tissue and circulating TG, and de novo lipogenesis (DNL) [54]. It has been demonstrated that LF, high-carbohydrate diet increased hepatic DNL significantly compared to an isocaloric high-fat, low-carbohydrate diet [55]. Moreover, it is well-established that excessive consumption of sugar, and fructose in particular, leads to dietary carbons channeling directly to the liver, supporting DNL [56]. These mechanisms may also explain the superiority of the MED/LC diet, including a daily intake of walnuts, over the LF diet, regarding the reduction in HFC. Thus, our study highlights the specific potential of MED/LC as a particular dietary strategy to treat NAFLD.

In summary, this sub-study demonstrates how different weight loss strategies may induce favorable dynamics of HFC and consequently improve cardio-metabolic risk. We suggest that improvements in specific easily-tracked blood biomarkers and cardiovascular risk associated with a decrease in HFC, beyond the loss of VAT. Thus, rather than focusing on weight loss only, our findings suggest that LC/MED dietary intervention may be used as a specific approach for the management of NAFLD.

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CONFLICT OF INTEREST STATEMENT

All authors have no conflict of interest to disclose. All authors had full access to all the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis.

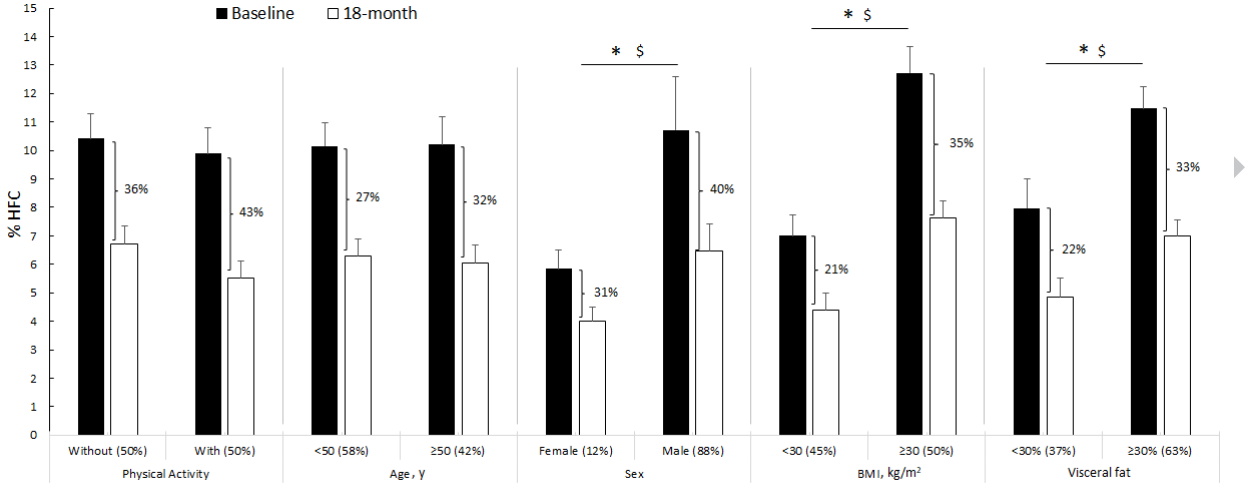
Table 1 - Baseline characteristics of the CENTRAL study population across the four intervention groups, n=278

	Intervention groups				
	Low-fat diet without physical activity (n=76)	Low-fat diet with physical activity (n=63)#	Mediterranean /low-carbohydrate without physical activity (n=73)	Mediterranean/low-carbohydrate with physical activity (n=66)#	All (n=278)
Hepatic fat content (%)	10.8±10.3	9.2±9.0	10.1±10.8	10.5±11.3	10.2±10.4
NAFLD patient (>5%), %	57	56	48	52	53
Age (yr)	49.3±9.3	47.2±9.0	47.0±8.9	47.9±9.8	49.9±9.3
Male, %	84	92	85	95	89
Weight (kg)	90.6±14.1	91.5±12.8	91.6±14.5	92.2±11.9	91.4±13.4
Waist circumference (cm)	105.6±9.4	106.9±8.5	106.4±11.6	108.0±8.5	106.7±9.6
BMI (kg/m^2)	31.1±3.9	30.4±3.5	31.0±4.5	31.0±3.3	30.9±3.8
Systolic Pressure (mmHg)	125±16	122±13	124±18	126±16	124±16
Diastolic Pressure (mmHg)	79±11	78±10	81±12	82±11	80±11
Fasting blood biomarkers					
Glucose (mg/dl)	106.4±17.1	106.7±18.2	107.4±18.3	108.8±18.3	107.3±23.6
HOMA-IR	4.4±2.6	4.7±3.4	4.7±3.8	4.5±3.8	4.6±2.7
Triglycerides (mg/dl)	71.8±41.4	78.7±44.4	73.5±41.9	66.5±41.9	72.6±36.6
LDL-c (mg/dl)	123.2±33.7	124.5±29.9	120.6±34.1	121.1±34.1	122.3±27.1
HDL-c (mg/dl)*					
Male	41.8±11.7	41.2±10.7	40.8±9.0	42.8±8.4	41.6±10.0
Female	54.3±15.2	58.1±24.8	51.3±15.8	63.6±7.3	54.8±16.4
Chol/HDL ratio	5.03±1.90	5.23±1.65	4.95±1.46	4.76±1.46	4.99±1.58
Alk-phosphatase (IU/L)	73.8±17.1	72.5±22.4	70.9±21.6	66.7±21.6	71.0±19.3
ALAT (U/L)	25.3±13.5	29.0±24.7	25.8±12.4	28.8±12.4	27.1±14.3
Gamma-glutamiltransferase (U/L)	25.8±14.3	32.9±24.9	26.9±15.3	28.1±15.3	28.4±18.7
Chemerin (ng/ml)	189.5±22.7	185.7±19.0	185.7±22.4	193.2±22.4	188.5±24.4
Leptin (mg/dl)*					
Male	11.1±7.6	11.8±6.2	11.3±9.0	13.6±7.9	11.9±7.8
Female	41.1±23.0	22.3±6.1	33.7±25.8	30.8±1.3	34.4±21.6
Adiponectin (mg/dl)	9.9±9.4	10.0±9.0	9.6±8.3	13.2±8.3	10.6±12.4
Abdominal fat sub-depots					
Visceral fat (cm^2)*					
Male	180.0±74.2	189.2±62.2	168.0±57.5	189.7±61.4	181.6±64.4
Female	159.7±53.8	92.7±37.9	118.2±67.8	69.7±27.6	125.4±61.7^
Deep-SAT (cm^2)*					
Male	209.5±70.4	219.9±71.8	220.7±87.3	223.0±71.0	218.2±75.2
Female	211.1±52.5	177.2±39.1	213.2±91.7	166.8±33.9	200.1±66.3
Superficial-SAT (cm^2)*					
Male	130.4±58.2	135.6±50.0	133.9±55.6	131.5±47.1	132.8±52.7
Female	224.3±79.1	184.3±44.5	243.0±104.5	179.0±34.2	220.1±82.8

Values in the table are means ± standard deviation. Abbreviations: HDL indicates high-density lipoprotein; HOMA-IR, homeostatic model of insulin resistance; LDL, low-density lipoprotein; Chol, total cholesterol; AKL, alkaline; ALAT, Alanine transaminase; SAT, subcutaneous adipose tissue;. One-way ANOVA test was used to assessed differences between groups ant baseline. * $P < 0.05$ between gender groups. ^ $p < 0.05$ between intervention

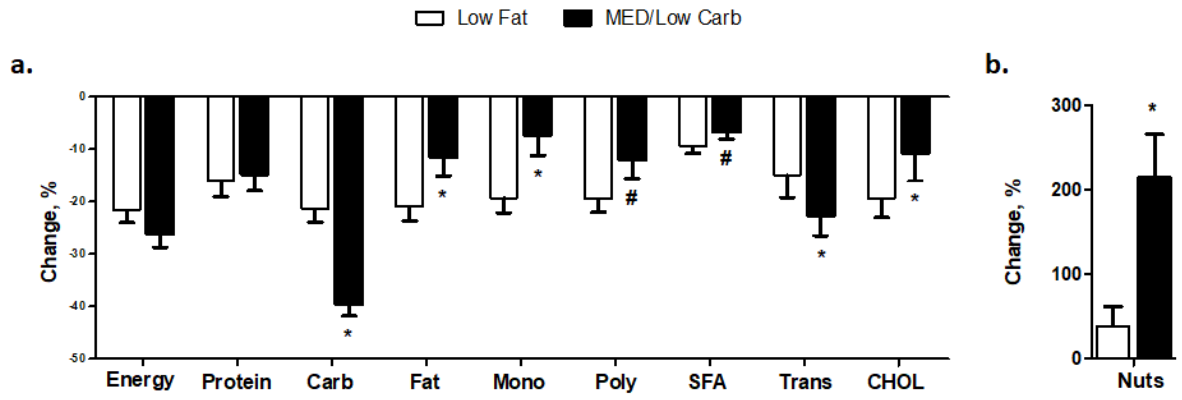
groups. # After 6-months of dietary the intervention (19 dropout), each diet group was further randomized into added physical activity groups or diet only for the last 12-months of intervention.

Figure 1. Hepatic fat content at baseline and after 18 months of intervention by subgroups



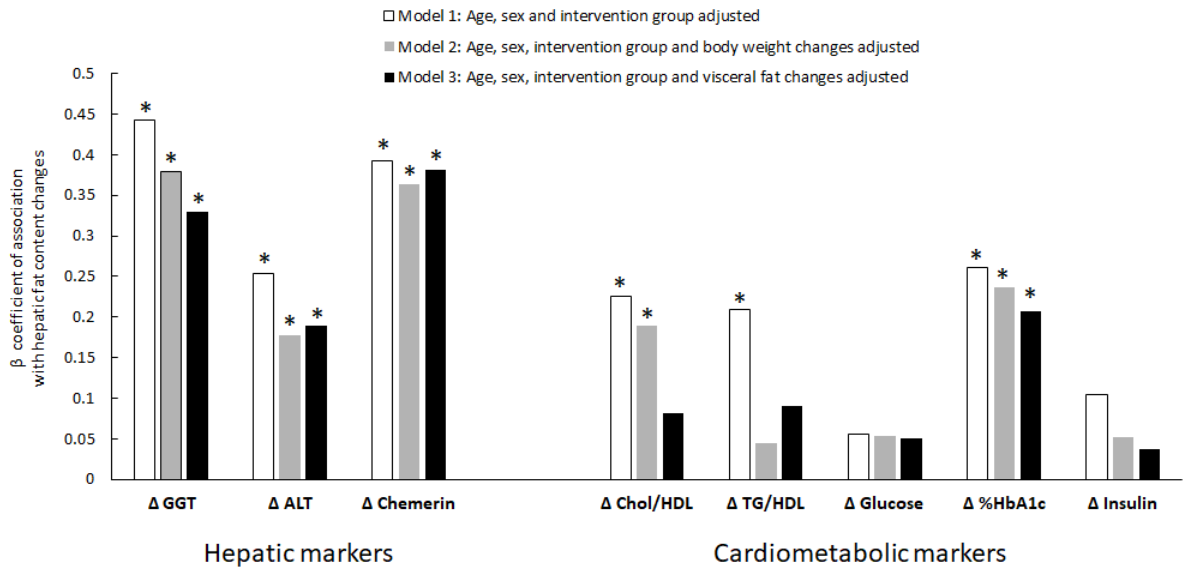
Bar values in the figure are means ± SE at baseline and after 18-month of intervention in hepatic fat content (HFC). Numbers represent relative changes. Data were analyzed using a repeated measures analysis of variance (ANOVA) across all sub groups. * $p<0.05$ for models without adjustment. $ $p<0.05$ for models adjusted for visceral adipose tissue changes. Male and groups with higher body mass index (BMI) and visceral fat at baseline decrease more HFC in both models.

Figure 2. Changes from baseline of the intake of energy and marco- and micro-nutrients between the diet intervention groups



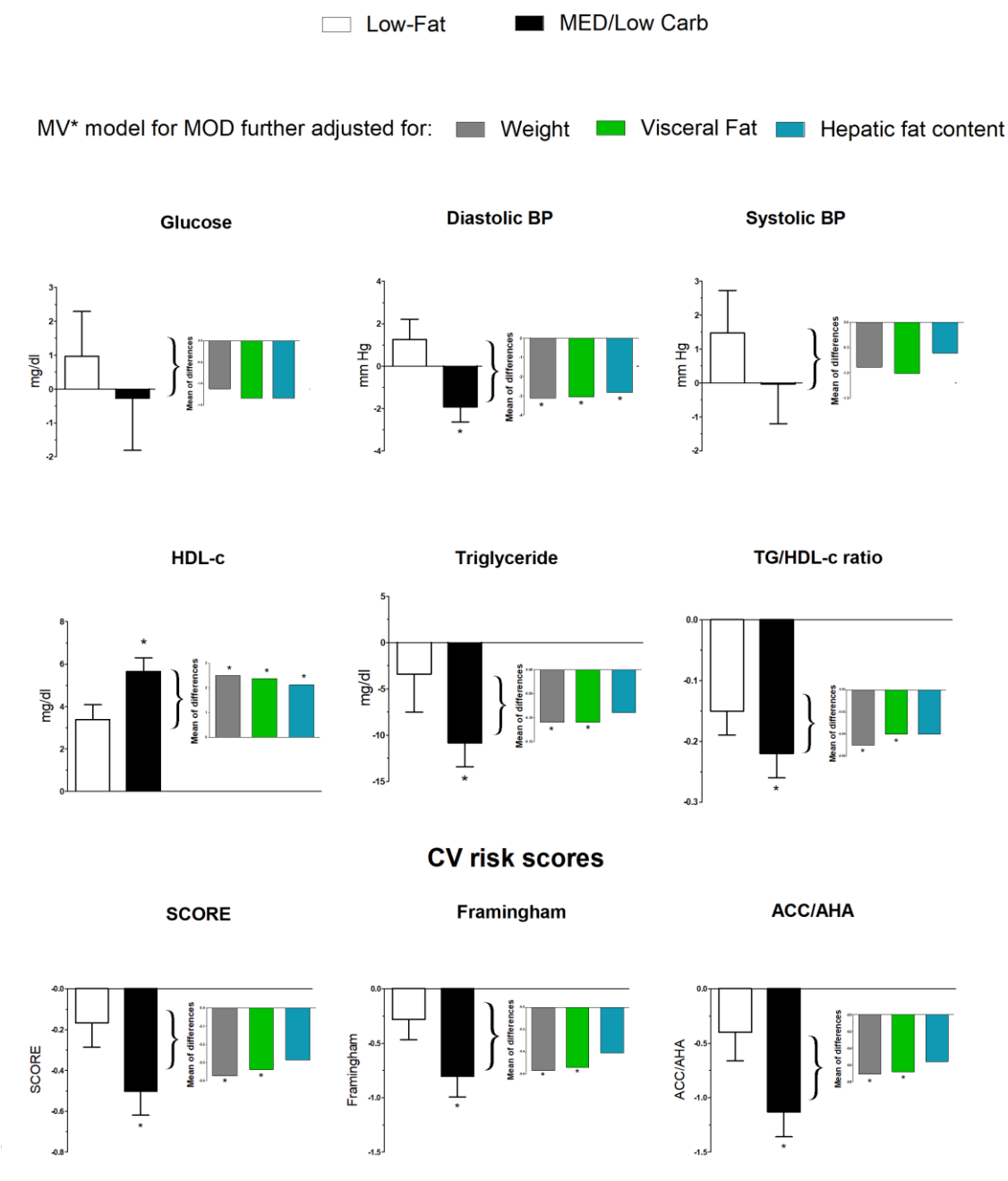
Changes from baseline between the diet intervention groups in marco- and micro-nutrients. The Mediterranean/Low carbohydrate (MED/Low Carb) diet decrease more intake of carb, trans fat and increase (a) more nuts consumption (b). The low fat diet greater decrease intake of total fat, monounsaturated fat (mono) and cholesterol and tended to decrease more polyunsaturated fat (poly) and saturated fat (fat) (a). Analysis of variance with a covariance (ANCOVA) test was used. * $p<0.05$, # $p<0.1$.

Figure 3. Hepatic and cardiometabolic markers associated with 18 months hepatic fat content loss



Values are the β coefficients of the associations between 18-months changes of hepatic fat content and the indicated parameters at the X-axis. Multivariate model adjusted for age, sex and intervention groups (model 1); age, sex, intervention groups and body weight change (model 2); age, sex, intervention groups and visceral fat change (model 3). * p<0.05. GGT- gamma-glutamyl transferase; ALT- alanine aminotransferase; Chol/HDL- total cholesterol to high density cholesterol ratio; TG/HDL- triglyceride to high density cholesterol ratio.

Figure 4. Effect of dietary strategies on cardiometabolic state beyond changes of weight, visceral and hepatic fat content



Values in the figure are means ± SE. The statistical analysis was performed by multivariate general linear regression models adjusted for age, sex, and baseline abdominal obesity. * $p < 0.05$. Mean-of-differences (MOD) model was further adjusted for 18-months weight changes (gray); 18 months visceral fat changes (green); 18 months hepatic fat changes (turquoise). Intention-to-treat analyses, including all 278 participants by multiple imputation (MI) technique. After 18 month of intervention, 38 participants dropped out and had incomplete sets of observations (86.3% adherence). Cardiovascular (CV) risk score includes the Framingham risk score, Systematic Coronary Risk Evaluation (SCORE), and the American College of Cardiology/American Heart Association (ACC/AHA).

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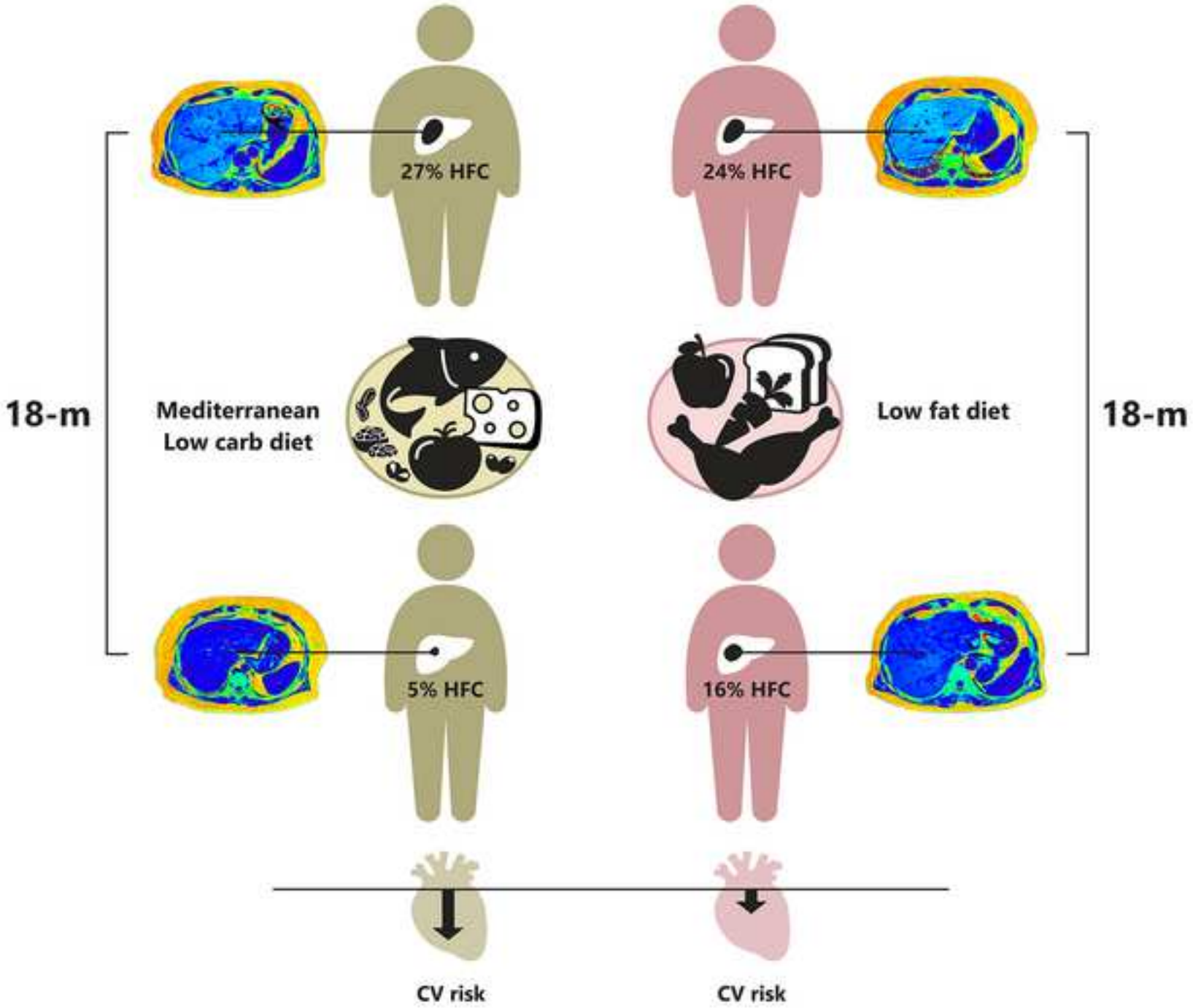
*Graphical Abstract



Highlights

- Mediterranean and low carbohydrate diet greater decrease hepatic fat than the LF diet, beyond visceral fat changes.
- Decrease in hepatic fat is independently associated with specific improved parameters.
- The beneficial effect of Mediterranean diet over the low-fat diet is mainly mediated by decreases in hepatic fat rather than visceral fat loss.