

Effects of lifestyle interventions on epigenetic signatures of liver fat: Central randomized controlled trial

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Abstract

Background and Aims: In the CENTRAL trial context, we found diverse liver fat dynamics in response to different dietary interventions. Epigenetic mechanisms may contribute to the intraindividual variation. Moreover, genetic factors are involved in developing nonalcoholic fatty-liver disease (NAFLD), a disease reflected by an increase in intrahepatic fat (IHF). In this exploratory analysis, we primarily aimed to

Abbreviations: A2MP1, Alpha-2-Macroglobulin Pseudogene 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BMI, body mass index; CDH2, Cadherin 2; CpG, cytosine followed by guanine; CRACR2A, Calcium Release Activated Channel Regulator 2A; DCLK1, Doublecortin Like Kinase 1; EHP1L1, EH Domain Binding Protein 1 Like 1; FARP1, FERM, ARH/RhoGEF And Pleckstrin Domain Protein 1; HDL, High-density lipoprotein cholesterol; HOMA-IR, homeostatic Model Assessment for Insulin Resistance; IHF, Intrahepatic fat; LC, low-carbohydrate; LF, low-fat; MED, Mediterranean; MRI, Magnetic Resonance Imaging; NAFLD, nonalcoholic fatty liver disease; PA, physical activity; ROC, receiver operating characteristic; SNP, Single nucleotide polymorphism; VAT, visceral adipose tissue; WC, waist circumference.

Anat Yaskolka Meir and Maria Keller contributed equally to this study.

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examine the effect of lifestyle interventions on DNA-methylation of NAFLD related genes associated with IHF.

Methods: For 120 participants from the CENTRAL trial, an 18-month regimen of either low-fat (LF) or Mediterranean-low carbohydrate (MED/LC) diets, with or without physical activity (PA+/PA-), was instructed. Magnetic resonance imaging was used to measure IHF%, which was analysed for association with CpG specific DNA-methylation levels of 41 selected candidate genes. Single-nucleotide polymorphisms known to be associated with NAFLD within the studied genes were genotyped by TaqMan assays.

Results: At baseline, participants (92% men; body mass index = 30.2 kg/m²) had mean IHF of 10.7% (59% NAFLD). Baseline-IHF% was inversely correlated with DNA-methylation at individual CpGs within *AC074286.1*, *CRACR2A*, *A2MP1*, *FARP1* ($P < .05$ for all multivariate models). *FARP1* rs9584805 showed association with IHF, with the prevalence of NAFLD and baseline methylation level of the CpG site (cg00071727) associated with IHF%. Following 18-month lifestyle intervention, differential DNA-methylation patterns were observed between diets at cg14335324 annotated to *A2MP1* ($P = .04$, LF vs. MED/LC), and differential DNA-methylation between PA groups within *AC074286.1*, *CRACR2A*, and *FARP1* CpGs ($P < .05$ for all, PA-vs. PA+).

Conclusions: This study suggests epigenetic markers for IHF and potential epigenetic remodeling after long-term lifestyle interventions.

KEYWORDS

Diet, DNA-methylation, genetic variation, nonalcoholic fatty liver disease, physical activity

1 | INTRODUCTION

Increased fat accumulation in the liver in the form of triglycerides is tightly associated with obesity and increased visceral adiposity.¹⁻³ Intrahepatic fat (IHF) accumulation that exceeds 5%, in the absence of alcohol abuse, defines non-alcoholic fatty liver disease (NAFLD).⁴ NAFLD, affecting approximately 25% of the population worldwide, is associated with increased cardiovascular risk, cancer, and mortality^{4,5} and is a major public health concern.⁶

Genome-wide association studies (GWAS) and large candidate gene assessment approaches have improved our understanding of genetic NAFLD predisposition. However, other than a locus on the Patatin-like phospholipase domain-containing 3 gene (*PNPLA3*), the identified candidate loci explain only a small proportion of the fatty liver variance.⁷ Therefore, it is possible that epigenetic patterns, such as the DNA methylation level, which can alter gene expression without changing the DNA sequence,⁸ may further contribute to NAFLD development. Therefore, it is plausible that NAFLD is promoted by epigenetic modifications, potentially mediating between genetic predisposition and environmental factors, as physical activity (PA) and diet.⁹ Indeed, both genetic and epigenetic studies have uncovered numerous genes whose genotype or DNA methylation is related to liver fat accumulation, as well as liver injury and inflammation.¹⁰⁻¹² DNA methylation at specific cytosine followed by guanine

Lay term summary

Previous studies identified novel genes potentially involved in liver fat accumulation and thereby in the development of nonalcoholic fatty liver disease. In this lifestyle intervention trial, we found that different lifestyle interventions (diet or physical activity) may specifically cause DNA methylation changes associated with a heterogeneous response to intervention-induced liver fat reduction. The findings also suggest some relationship between methylation levels of specific CpGs, intrahepatic fat, cardiometabolic risk parameters, and an association with genetic variation of the *FARP1* gene.

(CpG), in addition to other patient characteristics, could predict progression to liver fibrosis.¹³

Epigenetic changes might be induced by several factors such as genetics, environmental, and last but not least, lifestyle factors, as diet and exercise.^{9,14-16} It has been suggested that some nutritional components can lead to epigenetic modifications.¹⁷ These nutritional and dietary factors may affect DNA methylation in two proposed mechanisms, namely, changing the availability of methyl donors or altering

the activity of enzymes involved in DNA methylation (eg, the DNA methyltransferases family of enzymes).⁸ Previous studies suggested that dietary composition may affect epigenetic mechanisms, as adherence to the Mediterranean (MED) diet is associated with methylation of selected genes related to inflammation.¹⁵ In this way, antioxidant and microRNAs are an emerging field of research, especially regarding the epigenetic ability of MED diet.¹⁸ Exercise, both chronic and acute, is an additional lifestyle factor that may also lead to changes in DNA methylation.¹⁶ These changes included both hypo- and hyper-methylation in specific genes related to metabolism and energy usage.

The CENTRAL trial aimed to explore the effect of lifestyle interventions on fat deposit mobilization, offers the unique opportunity to analyse diet-DNA-interactions at the epigenome level. To get more insights regarding the underlying molecular mechanisms of the relationships between lifestyle intervention, intrahepatic fat, and NAFLD, we aimed to examine the effect of lifestyle interventions on epigenetic modifications of liver fat related genes.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

The data for this analysis were taken from the 18-month CENTRAL trial (NCT01530724), which was conducted between October 2012 and April 2014 in a research center workplace in Dimona, Israel, with an onsite clinic and with a monitored provided lunch. Recruitment began in May 2012, and by the start of the trial, 278 sedentary Caucasian individuals were found to be eligible to participate in the study. Inclusion criteria were abdominal obesity or dyslipidemia. Exclusion criteria are described in detail in Information S1. Medical Ethics Board and the Helsinki Committee of the Soroka University Medical Center approved the study protocol. All participants provided written informed consent and received no financial compensation or gifts.

2.2 | Randomization and intervention

The randomization and lifestyle interventions of the CENTRAL study were described in detail elsewhere.¹⁹ Briefly, participants were randomly assigned in two phases: first, into one of two equally hypocaloric diets: a low-fat (LF) diet or a MED/low-carbohydrate (MED/LC) diet with 28 g/day of provided walnuts. Second, after 6 mo of dietary intervention, the two dietary groups were further randomized into diet-only groups (LF, MED/LC) or groups with additional moderate PA intervention, mostly (80%) aerobic.

2.3 | Magnetic resonance imaging (MRI) and clinical measurements

Whole-body magnetic resonance imaging (Ingenia 3.0 T, Philips Healthcare, Best, Netherlands) was used to scan all participants at

baseline (T0) and after 18 mo (T18). Technical description of the scanning procedure, IHF%, and abdominal fat depots acquisitions and clinical measurements are available in Information S2.

2.4 | DNA sampling and extraction

Whole blood samples were taken after an overnight fast at baseline (T0) and 18 mo (T18) after the individuals completed their interventions. Samples were stored at -80°C until DNA was extracted following a standard protocol using proteinase K and 0.2% sodium dodecyl sulfate at Hadassah Hebrew University Medical Center, Jerusalem. Samples were integrity-controlled using gel electrophoresis. Concentrations of double-stranded DNA were measured using Quant-iT PicoGreen dsDNA (Invitrogen, ThermoFisher Scientific, Germany) and Quantus (Promega, Germany) technologies.

2.5 | Sample selection and genome-wide DNA methylation

This is a substudy of the CENTRAL trial (Figure 1), including 120 participants, according to the following criteria: both baseline and 18 mo available blood samples as well as additional consent to genetic analysis. Sample selection was detailed elsewhere²⁰ and in Information S3. Following quality control, bisulfide conversion, amplification, and hybridization on Illumina HumanMethylation850 Bead Chips (Illumina, Inc, San Diego, CA, USA), the Illumina iScan array scanner was used to quantify genome-wide DNA methylation levels at 850K CpG sites per sample on single-nucleotide resolution (GenomeScan, Leiden, Netherlands). Further details regarding genome-wide DNA methylation are presented in Information S3. Cell type composition according to the Houseman approach is presented in Figure S1.

2.6 | Selection of candidate genes related to liver fat

We based the determination of the candidate genes for this study on GWAS catalog hits for NAFLD traits.²¹ According to the representation of such a candidate in Illumina arrays, 2095 different CpGs located among 41 genes were analysed (Table S1).

2.7 | Single nucleotide polymorphisms (SNPs) determination and genotyping

We analysed three SNPs (rs1529093, rs9584805, rs887304) mapping within gene regions of the CpGs, whose methylation associated with IHF% and with blood biomarkers in our baseline analyses [AC074286.1, *FERM, ARH/RhoGEF And Pleckstrin Domain Protein 1 (FARP1)*, *Calcium Release Activated Channel Regulator 2A*

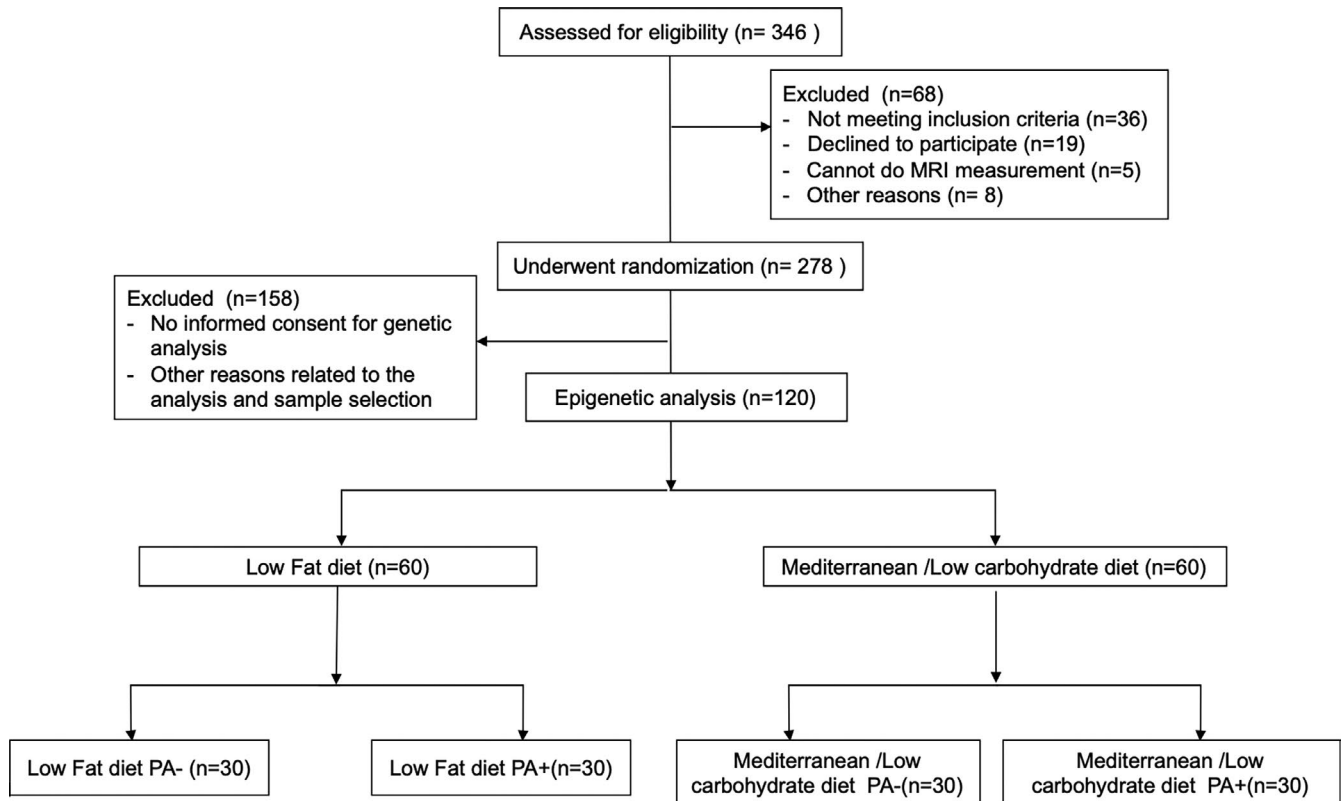


FIGURE 1 A flow diagram of the CENTRAL epigenetic substudy

(CRACR2A)]. These genes were shown to be associated in GWAS with NAFLD,²² and were genotyped in genomic DNA samples extracted from blood using the Allelic Discrimination TaqMan SNP Genotyping System (Applied Biosystems by Life-Technologies Carlsbad, CA, USA) according to the manufacturer's protocol. Water controls were included as nontemplate control (N = 6 per run), and random samples (5%) were genotyped in duplicates on different plates to validate the assay's reproducibility and accuracy. Fluorescence was detected by an ABI 7500 Real-Time PCR system. All variants were in Hardy-Weinberg-equilibrium ($P > .05$).

2.8 | Statistical analysis

This report's primary aim was to examine whether specific lifestyle interventions differentially affect DNA methylation in genes related to hepatosteatosis. We examined diet groups (LF, MED/LC) and PA groups (no PA added (PA-), PA added (PA+)) separately, similarly to previous reported.^{19,23} Secondary aims were to test whether blood DNA methylation levels of genes known to be involved in NAFLD development may reflect IHF storage and whether these relations might be affected by genetic variation. A further exploratory analysis was performed for the association of the candidate CpGs with blood biomarkers. A summary of the analysis is presented in Figure S2. DNA methylation values were used as a continuous variable (normalized beta values). A comparison

between baseline characteristics was performed using *T* test for normally distributed variables, Mann Whitney test otherwise, and chi-square for categorical variables. Pearson and Spearman correlation tests were used to examine the relationships between normal and not normally distributed variables. For correlations control, we used a partial correlation rank test. Where appropriate, non-normally distributed variables were Ln transformed to achieve normal distribution. Benjamini-Hochberg correction²⁴ with a 25% false discovery rate was used to control for multiple comparisons (reported as "q" value) in the exploratory phase, across all the CpG sites within each of the 41 genes, to avoid excessively strict filtering, as further adjustments and multivariate models were introduced in the next stages of the analysis. For NAFLD prediction, the baseline methylation levels of specific CpGs were examined in terms of area under the curve (AUC) to determine which independent variables should be used in the pooled receiver operating characteristic (ROC) analysis. Differences between groups were tested using Generalized linear models. Differences from baseline were expressed as absolute values (T18-T0) or as relative change [(T18-T0)/T0*100]. Within-group changes were tested using paired samples *T* test. All analyses for the SNPs were performed for 3 modes of inheritance (m = minor allele; M = major allele; additive (mm vs. Mm vs. MM), dominant (mm + Mm vs. MM), and recessive (mm vs. Mm + MM)). All statistical analyses were performed using IBM SPSS Statistics Version 27 (IBM, Armonk, NY). Significance was defined as a two-sided *P* value of $P < .05$. The sample size calculation is presented in Information S4.

3 | RESULTS

3.1 | Cross-sectional analyses at baseline

3.1.1 | Characteristics

119 participants had a valid MRI scan. Mean (\pm SD) WC was 107.2 ± 7.1 cm for men and 101.8 ± 15.0 cm for women. Mean high-density lipoprotein cholesterol (HDL) was 41.8 ± 9.1 mg/dl and 53.9 ± 12.4 mg/dl for men and women, respectively. None of the participants reported alcohol abuse, with a mean alcohol intake of

0.25 ± 0.3 servings/day for men and 0.03 ± 0.07 servings/day for women. According to diet groups, the characteristics of participants are presented in Table 1.

3.1.2 | Baseline correlations between methylation levels, intrahepatic fat, and blood biomarkers

At baseline, out of 2095 CpGs examined, IHF% was significantly correlated with 10 CpGs annotating to 6 genes ($q < 0.05$). Of note, no correlation was observed between MRI measured IHF% and *PNPLA3*

TABLE 1 Baseline characteristics of participants

	Entire (n = 120)	Low fat (n = 60)	Mediterranean/low carb (n = 60)	p between groups ^a
Liver fat				
Intrahepatic fat, % ^b	10.7 ± 10.6	10.7 ± 9.7	10.8 ± 11.5	0.96
Fatty liver status %NAFLD	58.8	64.4	53.3	0.22
Male gender, n (%)	110 (92)	54 (90)	56 (93)	0.51
Age, years	48.6 ± 9.3	48.9 ± 9.8	48.2 ± 8.8	0.70
Body mass index, kg/m ²	30.2 ± 3.3	30.2 ± 2.9	30.1 ± 3.7	0.85
Waist circumference, cm	106.7 ± 8.1	106.1 ± 6.8	107.3 ± 9.2	0.42
Smokers (current), %	14	15	13.3	0.75
Type 2 diabetes, %	10	7	13	0.22
Hypertension, % ^c	50	45	55	0.27
Systolic BP, mmHg	125 ± 16	124 ± 15	125 ± 18	0.63
Diastolic BP, mmHg	80 ± 11	78 ± 11	81 ± 11	0.18
Cholesterol, mg/dl	201.1 ± 38.8	204.8 ± 35.6	199.4 ± 41.8	0.63
LDL, mg/dl	122.6 ± 33.2	123.7 ± 31.9	121.5 ± 34.6	0.71
HDL, mg/dl	42.8 ± 10.0	41.9 ± 10.0	43.8 ± 10.0	0.29
Fasting glucose, mg/dl	106.5 ± 16.2	104.3 ± 13.6	108.6 ± 18.3	0.15
Insulin, μ U/ml	18.0 ± 11.3	17.0 ± 10.1	19.0 ± 12.4	0.35
HOMA-IR	4.8 ± 3.5	4.4 ± 2.6	5.3 ± 4.2	0.52
HbA1c, %	5.6 ± 0.5	5.6 ± 0.4	5.6 ± 0.5	0.29
Leptin, ng/ml	14.2 ± 13.5	14.2 ± 11.7	14.2 ± 15.1	0.99
Alanine transaminase, U/l	29.7 ± 13.7	29.3 ± 25.6	30.1 ± 19.3	0.84
Aspartate transaminase, U/l	28.2 ± 16.1	$28.8 \pm 13.$	29.4 ± 10.9	0.65

Abbreviations: BP, blood pressure; HbA1c, Hemoglobin A1c; HOMA IR, Homeostatic Model Assessment for Insulin Resistance; NAFLD, nonalcoholic fatty liver disease.

^aComparison was performed using T test for normally distributed variables, Mann Whitney test otherwise, and chi-square for categorical variables.

^bn = 119 with valid MRI scans.

^cReported on anti-hypertensive medication or have systolic blood pressure > 130 or have diastolic blood pressure > 85.

methylation. Correlations remained significant for 5 CpG sites in 4 genes: *AC074286.1*, *Calcium Release Activated Channel Regulator 2A (CRACR2A)*, *Alpha-2-Macroglobulin Pseudogene 1 (A2MP1)*, *ARH/RhoGEF And Pleckstrin Domain Protein 1 (FARP1)* after adjusting for age, sex, and either baseline body mass index (BMI), WC or visceral adipose tissue (VAT) ($P < .05$ for all, Table 2). For these 5 specific CpGs, we further examined associations with liver enzymes and parameters of glucose and lipid metabolism (Table S2). When adjusted for IHF%, cg 04 614 981 annotated to *CRACR2A* was inversely associated with triglycerides ($\beta = -0.234$) and directly associated with HDL ($\beta = 0.205$; $P < .05$ for both). Also, *FARP1* cg00071727 was found to be associated with triglycerides ($\beta = -0.231$), Alanine transaminase (ALT) ($\beta = -0.21$), and Aspartate aminotransferase (AST) ($\beta = -0.285$; all $P < .05$, adjusted p values).

3.1.3 | NAFLD prediction by specific DNA methylation

As previously reported by us, NAFLD was predicted by WC and specific blood biomarkers.³ Applying this prediction equation in our subcohort resulted in an AUC of 79.5% (95% confidence interval (CI): 70.9, 88.2), an optimal cut-off of 53.7, a sensitivity of 74.2%, and specificity of 79.5%. Constructing a new model using only the 5 specific CpGs significantly correlated with IHF% at baseline resulted in an AUC of 78.3 (95% CI: 69.3, 87.4), with an optimal cut-off of 47.9, a sensitivity of 64.6%, and specificity of 83.1% for the prediction of NAFLD (Figure 2 and Figure S3). Combining these two models (WC, specific blood biomarkers, and all 5 CpGs) resulted in an improved AUC of 87.3% (95% CI: 80.1, 94.5), with an optimal cut-off of 71.0, a sensitivity of 91.9%, and specificity of 79.1%.

3.1.4 | Associations of selected SNPs with intrahepatic fat

Out of all SNPs examined, only rs9584805 located in *FARP1* showed association with baseline IHF levels (dominant mode of inheritance;

$P = .039$, Figure 3A), with the prevalence of NAFLD before and after the intervention (dominant mode of inheritance; T0, $P = .027$ and T18, $P = .044$) (Figure 3B), as well as with baseline methylation level of the CpG site (cg00071727) associated with IHF% (Figure 3C). Other associations of the selected three SNPs with baseline liver fat parameters, anthropometric measures, and blood biomarkers are presented in Table S3a-c.

3.2 | Effects of diet intervention on liver fat and DNA methylation

3.2.1 | Changes in intrahepatic fat following the intervention

After 18 mo, significant reductions in IHF% (-4.7 ± 6.8 absolute units, $P < .001$ vs. baseline), weight (-3.7 ± 5.2 kg, $P < .001$ vs. baseline), and WC (-4.8 ± 5.6 cm; $P < .001$ vs. baseline) were observed, with no differences between the diet groups LF and MED/LC (Information S5). However, between PA groups (PA-/PA+), significant differences in IHF% loss (PA-: -4.4 ± 7.0 absolute units,

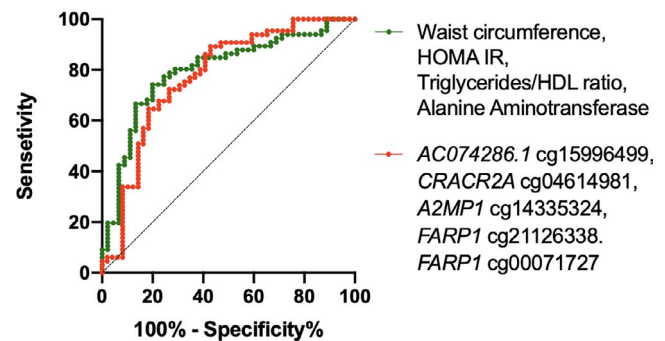


FIGURE 2 Receiver operating characteristic (ROC) curve for the magnetic resonance imaging diagnosis of fatty liver (IHF $\geq 5\%$). Prediction by either waist circumference and blood biomarkers (Triglycerides to HDL ratio, HOMA IR, and Alanine Aminotransferase), or by either 5 CpGs within the following genes: *AC074286.1*, *CRACR2A*, *A2MP1*, and *FARP1*

TABLE 2 MV models for the baseline correlation of IHF% and methylation levels

Location	Gene name	CpG	Model 1		Model 2		Model 3	
			r	p value	r	p value	r	p value
chr2:178257726-178257727	<i>AC074286.1</i>	cg15996499	-0.28	0.003	-0.27	0.005	-0.24	0.01
chr12:3747219-3747220	<i>CRACR2A</i>	cg04614981	-0.26	0.007	-0.25	0.008	-0.24	0.01
chr12:9387271-9387272	<i>A2MP1</i>	cg14335324	-0.23	0.02	-0.26	0.007	-0.26	0.006
chr13:99084698-99084699	<i>FARP1</i>	cg21126338	-0.36	<0.001	-0.32	0.001	-0.26	0.007
chr13:98869894-98869895	<i>FARP1</i>	cg00071727	-0.29	0.002	-0.31	0.001	-0.31	0.001

Note: Model 1: Adjusted for age, sex, and baseline body mass index. Model 2: Adjusted for age, sex, and baseline waist circumference. Model 3: Adjusted for age, sex, and baseline visceral adipose tissue.

Abbreviations: *A2MP1*, Alpha-2-Macroglobulin Pseudogene 1; *CRACR2A*, Calcium Release Activated Channel Regulator 2A; *FARP1*, FERM, ARH/RhoGEF And Pleckstrin Domain Protein 1.

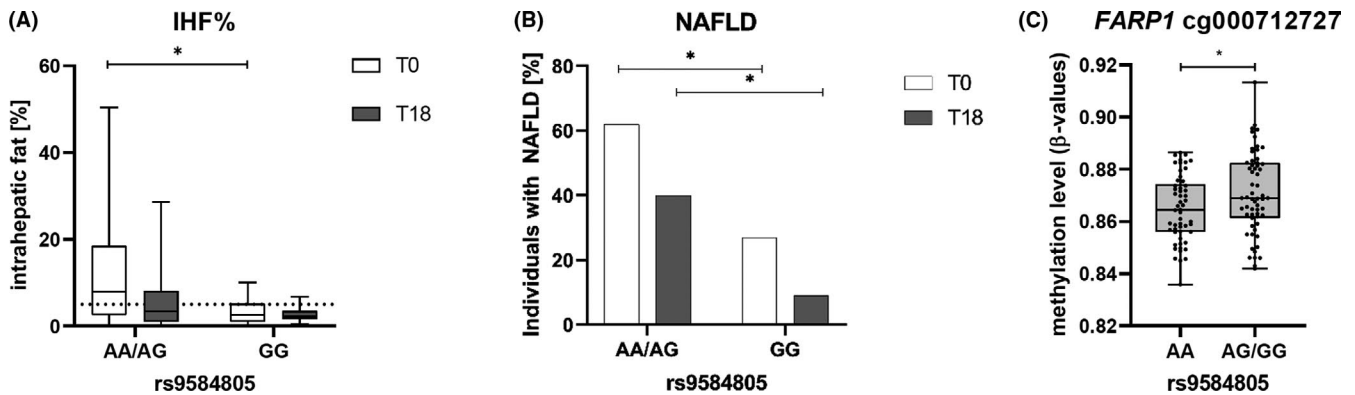


FIGURE 3 (A–C) Differences in *FARP1* SNP for (A) intrahepatic fat, (B) NAFLD, and (C) methylation of cg000712727. (A) The box plot shows intrahepatic fat [%] before (T0) and after the intervention (T18) for the genotypes of the SNP rs9584805 located in intron 2 of *FARP1* assuming the dominant mode of inheritance; The number of individuals: AA/AG (N = 108), GG (N = 11); dashed line marks 5% of intrahepatic fat; *p* values were calculated by Mann Whitney test. (B) The bar chart shows the percentage of NAFLD before (T0) and after the intervention (T18) for each genotype of SNP rs9584805 in the dominant mode of inheritance; The number of individuals: AA/AG (N = 108), GG (N = 11). *P* values were calculated using the Chi-square test. (C) The box plot shows baseline methylation levels (normalized β -values) in the CpG site cg000712727 for the recessive mode of inheritance of SNP rs9584805 located in intron 2 of *FARP1*, the number of individuals: AA (N = 59), AG/GG (N = 60). Whiskers show min and max; the line shows the median, points present individual values. **P* < .05

–5.3%; PA+: -5.0 ± 6.7 absolute units, –31.6%; Figure 4), WC reduction (-3.3 ± 5.2 cm vs. -6.3 ± 5.7 cm; *P* = .02), but not in weight loss (*P* = .11; All models adjusted for baseline levels of the changed parameter) were observed.

3.2.2 | Investigation of DNA methylation changes

For the specific CpGs within *AC074286.1*, *CRACR2A*, *A2MP1*, and *FARP1*, that showed significant baseline correlation with IHF%

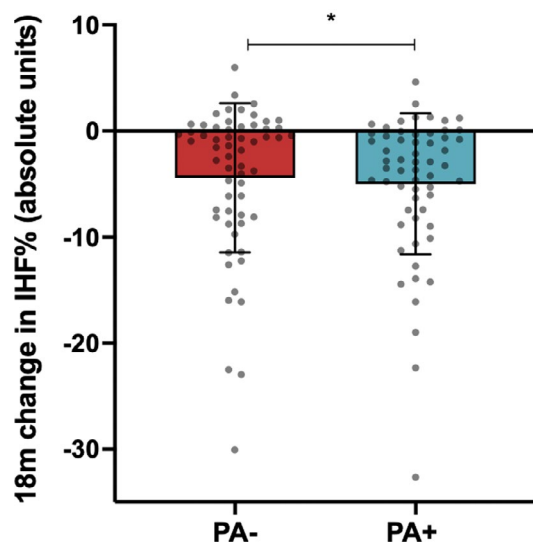


FIGURE 4 18-month difference in IHF reduction between groups of physical activity (no/yes). Differences are calculated for the absolute difference in IHF (T18–T0), adjusted for baseline levels of IHF. Data presented as means \pm SD, points present individual values. IHF, intrahepatic fat; PA, Physical activity. **P* < .05

levels, we further examined 18-months changes of DNA methylation correlations and between lifestyle group differences.

The 18-month change in IHF% was found to be marginally correlated with changes in the following CpGs: (partial correlation, adjusted for baseline IHF and baseline CpG) cg00668495 of *CRACR2A* ($r = 0.174$, *P* = .074), and cg02961200 of *AC074286.1* ($r = -0.177$, *P* = .068). Further adjustment for potential epigenetic drivers (weight loss, PA, and type of diet) completely attenuated these marginal correlations.

Different lifestyle interventions resulted in differential methylation in some CpGs, mostly between the PA groups (Table 3). Adding 18-month weight loss to the multivariate model (accounting for baseline IHF, PA or type of diet, as appropriate, and baseline CpG in-interest) did not affect the differences observed between the PA groups for cg02961200 of *AC074286.1* and cg21126338 of *FARP1* (*P* = .033 and *P* = .02, respectively), and the difference in cg14335324 of *A2MP1* between the diet groups (*P* = .039). Within-group differences (T18 vs. T0) were observed for the following groups and CpGs: a decrease in DNA methylation of cg21126338 at *FARP1* for PA+ (*P* = .014), an increase in methylation of cg00668495 at *CRACR2A* for PA– (*P* = .001), and a marginal difference for cg000712727 methylation-increase at *FARP1* in the PA + group (*P* = .068).

No correlations were observed between the change in the 5 CpGs and liver enzymes, glycemic and lipid biomarkers 18-month changes.

4 | DISCUSSION

The results of this substudy of the CENTRAL trial suggest an association of distinct DNA methylation patterns, genetic variation, and MRI measured intrahepatic fat accumulation. We detected differential methylation between participants adhering to either diet

TABLE 3 Between lifestyle group differences, the 18-month change in specific CpGs

Gene name	CpG	Diet intervention			PA intervention		p value ^a
		LF	MED/LC	p value ^a	PA-	PA+	
AC074286.1	cg15996499	4.0 ± 24.9	1.0 ± 24.4	0.36	6.3 ± 24.4	-1.1 ± 24.5	0.01
CRACR2A	cg04614981	-0.5 ± 3.1	-0.4 ± 2.8	0.78	-1.3 ± 3.0	0.3 ± 2.6	0.03
A2MP1	cg14335324	0.3 ± 1.9	-0.3 ± 1.7	0.04	-0.2 ± 1.7	0.2 ± 2.0	0.096
FARP1	cg21126338	0.2 ± 2.3	0.3 ± 1.9	0.22	-0.3 ± 2.1	0.7 ± 2.0	0.02
FARP1	cg00071727	-0.2 ± 2.1	-0.5 ± 2.2	0.45	-0.1 ± 2.1	-0.5 ± 2.1	0.23

Note: Methylation data presented as the relative 18-month difference (T18-T0/T0*100).

Abbreviations: A2MP1, Alpha-2-Macroglobulin Pseudogene 1; CRACR2A, Calcium Release Activated Channel Regulator 2A; FARP1, FERM, ARH/ RhoGEF And Pleckstrin Domain Protein 1; LF, low fat; MED/LC, Mediterranean/low carbohydrates; PA, physical activity.

^ap value according to Generalized linear models, calculated for the absolute difference in methylation change, adjusted for baseline intrahepatic fat, PA or diet group (as appropriate), and baseline CpG in-interest. A sensitivity analysis using relative change instead of absolute change in CpGs yielded similar results.

or physical activity regimen, which might provide evidence for an epigenetic remodeling following a lifestyle intervention.

At baseline, we searched for specific CpGs correlated with IHF as measured by MRI and further adjusted for other factors tightly associated with IHF. Previous studies showed that DNA methylation signatures might reflect hepatosteatosis and liver fibrosis,^{13,25,26} with differential DNA methylation of certain genes at specific regions. Furthermore, we recently showed that IHF, measured by MRI, is associated with biological age predicted by specific CpGs.²⁷ Next, we examined which biomarkers correlated with these CpGs independent from IHF, thus allowing us to highlight correlations between methylation levels and liver enzymes and cardiometabolic blood indicators. Indeed, previous studies have demonstrated an association between blood DNA methylation of some genes and components related to metabolic syndrome, including blood lipids and markers of glycemia,²⁸⁻³⁰ as well as liver enzymes.³¹ These associations are most conceivably explained because the explored candidate genes are tightly associated with a cardiometabolic state. Despite the strong link between cardiometabolic state and hepatosteatosis, some associations observed were beyond IHF% level, implying that these genes are probably associated with traits other than the fatty liver and might serve as general metabolic epigenetic markers.

Receiver operating characteristic analysis is an appropriate method for determining optimal cut-off values and is commonly used in epidemiological and medical studies.³² Previous studies attempted to generate predictive formulae for NAFLD based on simple biomarkers. Among the entire CENTRAL cohort,³ a ROC analysis demonstrated a joint AUC of 84% for MRI-based fatty liver diagnosis according to WC, ALT, triglycerides/HDL ratio, and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) levels. In this analysis, we used the same markers to predict NAFLD among the 120 participants of our sub-study. This yielded a joint AUC of 79.5% for MRI-based NAFLD diagnosis using simple biomarkers, vs. AUC of 78.3% using blood methylation levels. Thus, we demonstrated that based on DNA methylation in specific CpGs, we could achieve a similar predictive value for NAFLD diagnosis.

Further examining genetic variation in genes that showed an association of baseline methylation levels with IHF and other blood biomarkers revealed associations with, mostly, anthropometric parameters (ie, weight and WC) and lipid profile. Although the *PNPLA3* gene is widely studied in the context of NAFLD, we could not find any significant correlation between methylation at any site of this gene and IHF levels. This might be explained by the limited statistical power to detect significant correlations due to the rather small sample size of our cohort, considering we also adjusted the p-values for multiple comparisons to avoid a type-1 error. Another explanation might be that while *PNPLA3* SNPs were associated with NAFLD and liver fat,³³ with an elevated RNA expression³⁴ and hypermethylation of *PNPLA3*³⁵ among NAFLD patients, the association with liver fat as a continuous parameter is underrepresented. Thus, a possible explanation for not finding an association between IHF and *PNPLA3* methylation is the type of statistical test used, as we used IHF as a continuous parameter and did not compare normal liver fat% and NAFLD groups. *FARP1* locus turned out to be the most promising out of our selected candidates since rs9584805 was the only SNP associated with both liver fat and DNA methylation in its specific CpG sites. Previous human studies on *FARP1* and fatty liver suggested that *FARP1* rs9584805 might be related to NAFLD pathology.³⁶ Another study has demonstrated that other SNPs in *FARP1*, like rs2127779, were associated with obesity in the Korean population.³⁷ Our data suggest strong support regarding the role of *FARP1* in the pathophysiology of NAFLD, which might be mediated by the effects of rs9584805 on liver fat. Although the Causal Inference Test³⁸ did not robustly support the causal role of DNA methylation as a mediator between genotype and outcome (IHF%) in our sample set, methylation-genetic variation cross talk should be considered,³⁹ by, eg, transcription factors binding to gene target promoters gated by promoter methylation.⁴⁰

DNA methylation have been previously described as influenced by weight loss,⁹ race/ethnicity and gender.⁴¹ Moreover, epigenetic dietary effects have been described as influenced

by these factors. A recent study found that gender might have a specific effect on epigenetic rejuvenation when following a MED-style diet.⁴² Among older men, an attenuation of age, reflected by DNA methylation, was observed following weight-loss induced by lifestyle interventions.²⁷ Thus, it is reasonable to suspect that months-long dietary changes and/or IHF reductions due to weight loss might have affected DNA methylation, as revealed with this study's results. Although specific mechanisms explaining the differences observed in methylation of *A2MP1* between diet groups cannot be drawn from such analysis, one can offer plausible explanations. Regarding the fact that differential methylation was achieved regardless of similar IHF and weight reductions among the LF and MED/LC dieters in this sub-study, one possible explanation reflects diet composition. The LF diet was based on American Heart Association guidelines,⁴³ aiming for 30% of calories derived from fat, 10% of calories from saturated fat, and an intake of 300 mg of cholesterol per day. The MED/LC was based on restricted carbohydrates intake (less than 40gr/day in the first 2 mo with increased gradual intake for up to 80gr/ day) and increased protein and fat intake, mostly, but not only, from vegetarian sources, according to the MED diet.^{44,45} Additionally, MED/LC participants received a daily dose of 28g walnut, containing mostly Ellagic acid,⁴⁶ an anti-inflammatory agent that has possibly affected epigenetic modulations.⁴⁷ To examine if these changes are long-lasting and their causality, follow-up measurements and complementary models are required.

Participants who engaged in PA reduced more IHF compared with ones that did not initiate PA intervention. We observed some differential methylation changes between these PA groups. These modest changes in methylation were in different directions (ie, hypo/hyper methylation), suggesting that epigenetic remodeling occurred following initiating PA regimen. Aerobic training may alter the DNA methylation status of blood cells⁴⁸; For example, a small study among 8 trained men showed that acute exercise and dietary supplementation of fatty acids induce DNA methylation measured in whole blood.⁴⁹ Changes in the methylation of *CRACR2A* following initiating PA were described in the context of prostate cancer.⁵⁰ *FARP1* methylation is yet to be described in response to PA intervention. We can now add to the current data our observations regarding DNA methylation changes following initiation aerobic PA.

Several limitations should be considered. Since our study population comprises mostly Caucasian men, the generalizability of this study's findings to other races and women is limited. Similarly, the participants included in this study had abdominal obesity with a high prevalence of NAFLD; therefore, conclusions may not extrapolate to those with normal body weight and liver fat. We used several statistical models for adjustments to overcome this. Additionally, we evaluated blood methylation levels and associated these values with an indirect measure of liver fat. Thus, we cannot draw a real direct link between changes in methylation levels and fat tissue changes or other organs such as the liver. Yet, since obesity is linked with an increase in systemic inflammation, evident in the bloodstream and metabolic organs, methylation in white blood cells might be a

good representative of methylation levels in other organs involved in systemic inflammation. We measured DNA methylation only, without measuring gene expression. Nevertheless, we correlated the normalized beta values with other outcomes to examine a possible link between them. Finally, due to lower frequencies of minor allele carriers for some SNPs, genotype results have to be interpreted with caution since, for example, homozygous GG allele carriers for the *FARP1* SNP are underrepresented in our cohort. The study's strengths include using a relatively large sample size, a one-phase randomized-controlled design, in which all participants started interventions on the same day, an extended period of follow-up, and the use of an accurate, high-quality imaging technique to quantify IHF%.⁵¹

This study results suggest that lifestyle interventions may cause specific DNA methylation changes, which are associated with a heterogeneous response to liver fat reduction. We identified previously unrecognized epigenetic markers for liver fat and liver fat changes and a potential epigenetic remodeling after long-term intense lifestyle interventions. Further analyses targeting these associations' underlying mechanisms are warranted to understand better the role of epigenetic changes in liver disease and liver fat accumulation.

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CONFLICT OF INTEREST

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.

AUTHOR CONTRIBUTIONS

Authors AYM, DS, YG, GT, HZ, IS, ISH designed, and conducted the CENTRAL trial. MK, LM, SB, UC, MB, MS, and PK provided essential materials. AYM, MK, and LM analysed data or performed statistical analysis. AYM, MK, PK, and ISH wrote the paper. AYM, MK, PK, and ISH had primary responsibility for final content. All authors read and approved the final manuscript.

ETHICS APPROVAL STATEMENT

The study protocol was approved by the Medical Ethics Board and the Helsinki Committee of the Soroka University Medical Center.

PATIENT CONSENT STATEMENT

All participants provided written informed consent and received no financial compensation or gifts.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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