Supplementary Information:

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

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Supplementary Information 1: Exclusion criteria for the CENTRAL trial

Exclusion criteria for the CENTRAL trial were as follows: serum creatinine ≥2mg/dL; impaired liver function [≥threefold the upper level of Alanine transaminase (ALT) and Aspartate transaminase (AST)], active cancer, pregnancy or lactation, highly physically active (>3h/week) or unable to take part in physical activity, or participation in another trial.

Supplementary Information 2: MRI, anthropometric measurements and laboratory methods

Technical description of the scanning process:

A 45-minute 3-Tesla Magnetic resonance imaging (MRI, Ingenia 3.0 T, Philips Healthcare, Best, the Netherlands) was used in order to scan all participants at baseline and after 18 months. The scanner utilized a 3D modified DIXON (mDIXON) imaging technique without gaps (2mm thickness and 2mm of spacing), fast-low-angle shot (FLASH) sequence with a multi-echo two-excitation pulse sequence for phase-sensitive encoding of fat and water signals (TR,3.6ms; TE1,1.19ms; TE2,2.3ms; FOV 520×440×80mm; 2×1.4×1mm voxel size). Four images of the phantoms were generated, including in-phase, out-phase, fat and water phase10. A breath-hold technique was used to avoid motion artifacts when the chest and abdomen were scanned. All MRI tests were performed after at least a 2-hour fast to avoid artifacts of fat-disruption in the intestines. In all simultaneous fat depots quantification and comparisons, observers were blinded to time point and group treatment.

IHF% and abdominal fat acquisition:

Quantification of both abdominal and liver fat are described elsewhere [1,2]. Briefly, abdominal fat depots were quantified using a MATLAB-based semi-automatic

program with a continuous line drawn over the fascia superficialis to differentiate deep-subcutaneous adipose tissue (SAT) and superficial-SAT. Mean visceral adipose tissue (VAT), deep-SAT and superficial-SAT areas were calculated from three axial slices: L5-S1, L4-L5 and L2-L3. Intrahepatic fat (IHF) content was calculated using the PRIDE software from Philips Medical Systems. We calculated mean percentages from 2D slices (3cm intervals divided into quarters) by utilizing the region of interest (ROI) approach [3], examining tissue densities (fat/fat+water) based on the fat ratio calculation. We determined the mean fat percentage for each slice and quarter, and for the entire liver.

Anthropometric measurements:

Height was measured at baseline, to the nearest millimeter by using a standard wall-mounted stadiometer. Waist circumference (WC) was measured at baseline and after 18 months, to the nearest millimeter with an anthropometric measuring tape; the measurement was made half-way between the last rib and the iliac crest. Bodyweight was measured monthly without shoes to the nearest 0.1kg.

Clinical and laboratory methods:

Fasting blood samples were taken at baseline and after 18 months at 8:00AM, and were stored at -80°C. 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). Plasma leptin levels were assessed by ELISA

(Mediagnost, CV = 2.4%). All biochemical analyses were performed at the laboratories of the University of Leipzig, Germany.

Supplementary Information 3: Sample selection and genome-wide DNA methylation

Sample selection resulted in 30 subjects per intervention group (60 per diet group) showing the lowest relative weight after 18 months with respect to their initial weight were selected and included in DNA methylation analysis. 500ng of genomic DNA from each sample was bisulfited converted using EZ DNA Methylation Gold Kit (Zymo Research, Netherlands).

DNA Methylation data passed standard quality control for more than 807,5K CpG sites (95%). Since whole blood samples were used for our analysis, we calculated cell type compositions using the Houseman approach [4] adapted to EPIC arrays [5]. However, none of the cell type populations was significantly changed after the intervention (comparing T0 vs T18) (**Supplementary Figure 1**) and therefore additional correction for cell-type composition was not indicated. CpG specific beta values were computed and quantile normalized using Minfi R package [6,7] and further used as a continuous variable.

Supplementary Information 4: Sample size calculations

The calculation for the epigenetic study (with the primary outcome of adiposity changes) was based on a previous intervention study, where significant differences in five different gene methylation were found between high-responders/successors (losing >1.1 BMI standard deviation score) and low-responders/failures (<0.4BMI standard deviation score after treatment) to a 10-week dietary intervention [8]. In that study, the successors lost 9.4kg (a decrease from 92.7±4.1 to 83.3±3.7) as the

failures gained 0.2kg (80.1±4.7 to 80.3±4.7). Based on these results (3 kg of difference after the intervention, pooled variance of 17.88), alpha of 5% and beta of 20%, in order to detect changes in methylation levels, we will need 42 participants in each of our two intervention groups.

Supplementary Information 5: Between diet group differences in intrahepatic fat and anthropometric parameters

In this sub-study, no significant differences were observed in terms of IHF% reductions between the intervention groups LF and MED/LC (-4.9±7.0 absolute-units and -4.5±6.7 absolute units, respectively; p=0.89 between groups), as well as in BMI (-1.6±1.7 kg/m^2 and -1.4.7±1.6 kg/m^2, respectively; p=0.59 between groups) or WC (-4.5±5.7cm vs. -5.2±5.6cm, respectively; p=0.52 between groups).

Supplemental Tables:

Supplementary Table 1: Genes used for this analysis

Identification of candidates according to GWAS in our data - association with

NAFLD:

PNPLA3	*ZNF512*	*FDFT1*	*AC007652.1*	*LINC00322*	*AL645944.1*	*PZP*
SAMM50	*AC074286.1*	*HERPUD2*	*AC138123.1*	*KHDRBS3*	*CDH2*	*DDX60L*
PCSK5	*TEX36*	*SLC4A4*	*NFIC*	*GATAD2A*	*A2MP1*	*CRACR2A*
LPPR4	*SEL1L3*	*GCKR*	*LCP1*	*DCLK1*	*SLC46A3*	*FARP1*
GC	*CACNA2D1*	*NGF*	*SLC38A8*	*MACROD2*	*COL13A1*	
SLC9A9	*TMEM56-RWDD3*	*YIPF1*	*PBX2P1*	*EHBP1L1*	*SVIL*	

Supplementary Table 2: Baseline DNA methylation' significant associations with blood biomarkers

Gene name	CpG	Associated with[1]	IHF adjustment [2]
AC074286.1	cg15996499	↓ HOMA IR (β=-0.219) Insulin (β=-0.206)	-
CRACR2A	cg04614981	↓ HOMA IR (β=-0.201) Insulin (β=-0.191) Glucose (β=-0.186) Triglycerides (β=-0.234) ↑HDL (β=0.24)	↓Triglycerides (β=-0.234) ↑HDL (β=0.205)
FARP1	cg21126338	↓ ALKP (β=-0.282)	-
FARP1	cg00071727	↓ HOMA IR (β=-0.226) Insulin (β=-0.209) Triglycerides (β=-0.232) ALT (β=-0.28) AST (β=-0.334) ↑HDL (β=0.209)	↑Triglycerides (β=-0.231) ALT (β=-0.21) AST (β=-0.285)

Alpha-2-Macroglobulin Pseudogene 1 was not associated with any of the biomarkers in interest. [1] Unadjusted association.[2] Adjusted for baseline IHF. ↓ inverse association, ↑ direct association. ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; *CRACR2A, Calcium Release Activated Channel Regulator 2A; FARP1, FERM, ARH/RhoGEF And Pleckstrin Domain Protein 1*; HOMA IR, Homeostatic Model Assessment for Insulin Resistance; IHF, intrahepatic fat.

Supplementary Table 3(a-c): Associations of the selected three SNP (from the gene regions of the CpGs associated with IFH) with baseline parameters of liver fat, anthropometric measures and blood biomarkers

(a) Associations of SNP rs9584805 located in *FARP1* with baseline parameters of liver fat, anthropometric measures and blood biomarkers

	rs9584805		
	AA (*N*=59)	AG (*N*=49)	GG (*N*=11)
Liver fat			
IHF, %	11.29±10.29	11.49±11.67	3.34±2.84
p-value	**0.039[d]**		
Fatty Liver Status, % NAFLD	64.41	58.33	27.27
p-value[+]	**0.027[d]**		
Anthropometric			
Weight, kg	91.42±9.68	89.53±13.27	86.63±11.23
p-value	n.s.		
Height, cm	174.20±7.88	171.69±8.92	172.36±5.64
p-value	n.s.		
BMI, kg/m²	30.16±2.95	30.33±3.81	29.03±2.36
p-value	n.s.		
Waist circumference, cm	106.49±7.17	107.16±9.06	104.86±8.35
p-value	n.s.		
VAT, cm2	172.89±58.22	183.64±62.85	160.64±58.22
p-value	n.s.		
Deep SAT, cm²	215.29±77.25	206.71±64.22	195.61±46.45
p-value	n.s.		
Superficial SAT, cm²	131.03±45.06	138.01±68.37	131.13±47.96
p-value	n.s.		
Systolic BP, mmHg	126.47±13.54	126.20±17.40	116.45±19.08
p-value	n.s.		

Diastolic BP, mmHg	82.04±8.88	79.53±10.69	76.0±14.49
p-value	n.s.		
Blood Biomarkers			
Cholesterol, mg/dL	202.44±38.26	201.67±41.30	194.35±32.53
p-value	n.s.		
LDL-c, mg/dL	122.50±31.65	124.31±36.84	116.60±26.64
p-value	n.s.		
HDL-c, mg/dL	40.82±8.67	44.02±10.54	49.42±10.40
p-value	**0.012[a]; 0.015[d]**		
Triglycerides mg/dL	78.22±48.40	68.042±33.66	61.04±38.40
p-value	n.s.		
Fasting glucose, mg/dL	105.92±13.69	107.27±18.36	107.67±19.08
p-value	n.s.		
Insulin, µU/mL	19.24±11.21	16.80±10.98	13.52±7.03
p-value	n.s.		
HbA1c, %	5.59±0.43	5.59±0.56	5.58±0.46
p-value	n.s.		
HOMA IR	5.14±3.39	4.63±3.73	3.60±1.76
p-value	n.s.		
Leptin, ng/mL	13.86±10.07	15.74±17.56	9.39±5.26
p-value	n.s.		
ALKP, U/L	74.52±16.04	71.20±20.38	71.12±28.63
p-value	n.s.		
ALT, U/L	30.289±14.75	26.76±11.93	39.52±52.73
p-value	n.s.		
AST, U/L	29.34±11.34	27.49±11.28	32.51±28.12
p-value	n.s.		
GGT, U/L	33.13±14.66	38.31±21.52	32.21±14.26
p-value	n.s.		

Data are presented as mean±SD (standard deviation); *p*-values were calculated using linear regression analyses adjusted for age and sex using the [a]additive, [d]dominant, or [r]recessive mode of inheritance; [+]*p*-value for fatty liver status was calculated using Chi-Square test; ALKP – alkaline phosphatase; ALT - alanine aminotransferase; AST -

aspartate aminotransferase; BMI = body mass index; BP – blood pressure; GGT – gamma glutamyl transferase; HDL-c = high density lipoprotein-cholesterol; IHF – intrahepatic fat; LDL-c = low density lipoprotein-cholesterol; *N* = number; NAFLD – non-alcoholic fatty liver disease; SAT – subcutaneous adipose tissue area; VAT – visceral adipose tissue area

(b) Associations of SNP rs1529093 located in *AC074286.1* with baseline parameters of liver fat, anthropometric measures and blood biomarkers.

	rs1529093		
	TT (*N*=30)	TC (*N*=66)	CC (*N*=24)
Liver fat			
IHF, %	12.42±10.58	9.89±10.42	10.80±11.51
p-value	n.s.		
Fatty Liver Status, % NAFLD	63.33±49.01	53.03±50.29	69.57±47.05
p-value[+]	n.s.		
Anthropometric			
Weight, kg	94.02±12.02	87.87±11.10	92.43±10.40
p-value	n.s.		
Height, cm	174.93±8.24	171.46±7.95	174.77±8.07
p-value	n.s.		
BMI, kg/m²	30.68±3.00	29.86±3.08	30.39±4.20
p-value	n.s.		
Waist circumference, cm	108.69±7.83	104.78±7.48	109.48±8.81
p-value	**0.036**[d]		
VAT, cm2	200.57±60.98	163.61±59.60	180.29±58.60
p-value	n.s.		
Deep SAT, cm²	208.57±76.55	205.17±67.41	229.54±68.27
p-value	n.s.		

Superficial SAT, cm²	119.93±47.33	133.06±52.92	158.59±69.97
p-value	**0.019[d]**		
Systolic BP, mmHg	129.03±16.11	123.32±16.07	126.19±14.69
p-value	n.s.		
Diastolic BP, mmHg	82.92±9.12	79.11±10.60	80.83±10.59
p-value	n.s.		
Blood Biomarkers			
Cholesterol, mg/dL	194.24±25.31	198.11±41.47	217.23±41.01
p-value	**0.072[a]; 0.027[d]**		
LDL-c, mg/dL	115.93±21.76	120.20±36.10	136.87±32.91
p-value	**0.023[a]; 0.018[d]**		
HDL-c, mg/dL	41.77±8.53	44.21±10.62	40.32±9.47
p-value	n.s.		
Triglycerides mg/dL	75.40±40.36	67.42±44.37	82.34±35.19
p-value	n.s.		
Fasting glucose, mg/dL	107.75±12.25	105.16±17.14	108.41±18.10
p-value	n.s.		
Insulin, µU/mL	21.83±13.06	15.94±10.76	19.42±9.65
p-value	n.s.		
HbA1c, %	5.64±0.43	5.59±0.51	5.54±0.47
p-value	n.s.		
HOMA IR	5.91±3.87	4.27±3.45	5.22±2.78
p-value	n.s.		
Leptin, ng/mL	11.63±7.72	14.88±13.32	15.54±18.76
p-value	n.s.		
ALKP, U/L	72.66±15.75	71.29±20.54	77.48±18.80
p-value	n.s.		
ALT, U/L	28.73±14.62	30.72±24.59	27.79±13.04
p-value	n.s.		
AST, U/L	28.59±8.91	29.42±15.36	27.23±13.47
p-value	n.s.		
GGT, U/L	34.39±12.61	35.53±18.76	35.32±20.80
p-value	n.s.		

Data are presented as mean±SD (standard deviation); p-values were calculated using linear regression analyses adjusted for age and sex using the [a]additive, [d]dominant, or [r]recessive mode of inheritance; [+]p-value for fatty liver status was calculated using Chi-Square test; ALKP – alkaline phosphatase; ALT - alanine aminotransferase; AST - aspartate aminotransferase; BMI = body mass index; BP – blood pressure; GGT – gamma glutamyl transferase; HDL-c = high density lipoprotein-cholesterol; IHF – intrahepatic fat; LDL-c = low density lipoprotein-cholesterol; N = number; NAFLD – non-alcoholic fatty liver disease; SAT – subcutaneous adipose tissue area; VAT – visceral adipose tissue area

(c) Associations of SNP rs887304 located in *CRACR2A* with baseline parameters of liver fat, anthropometric measures and blood biomarkers.

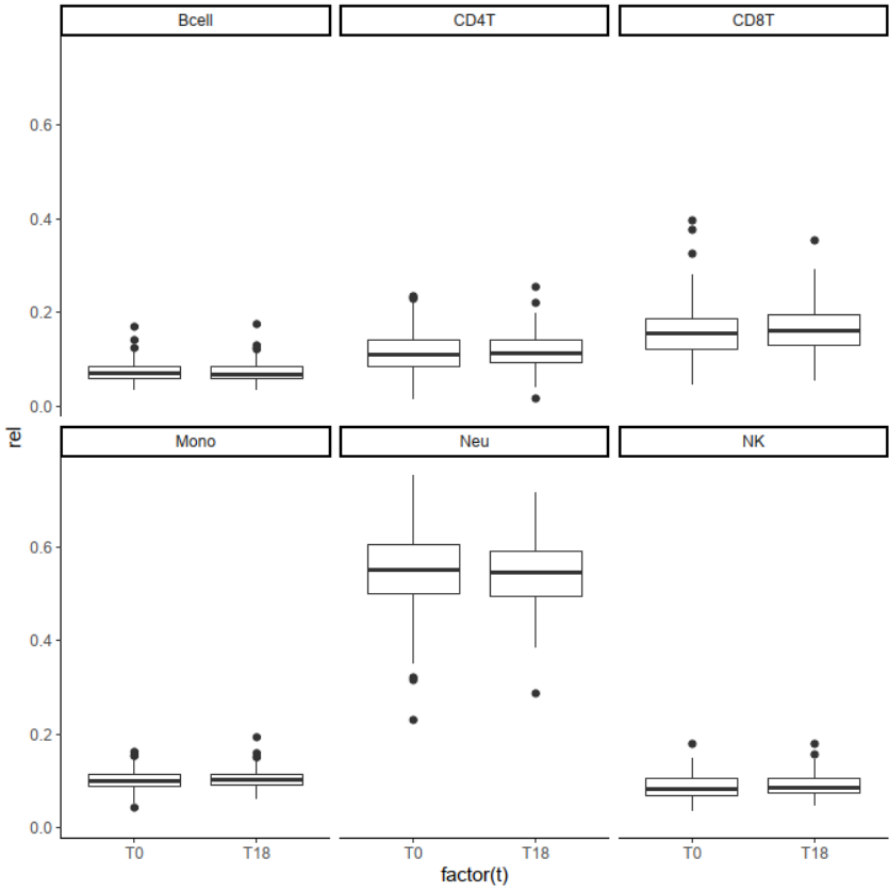
	rs887304		
	CC (*N*=74)	CT (*N*=40)	TT (*N*=6)
Liver fat			
IHF, %	10.97±10.95	10.25±10.53	10.52±8.64
p-value	n.s.		
Fatty Liver Status, % NAFLD	58.90±49.54	57.50±50.06	66.67±51.64
p-value[+]	n.s.		
Anthropometric			
Weight, kg	92.19±11.45	86.92±11.31	89.87±8.02
p-value	**0.034[a]; 0.017[r]**		
Height, cm	173.10±8.15	172.98±8.63	171.75±5.62
p-value	n.s.		
BMI, kg/m²	30.77±3.45	29.02±2.91	30.45±1.98
p-value	**0.045[a]; 0.011[r]**		
Waist circumference, cm	108.05±8.58	104.45±7.06	105.02±3.87

p-value	**0.032[a]; 0.021[r]**		
VAT, cm2	178.88±61.98	174.59±60.77	153.58±60.30
p-value	n.s.		
Deep SAT, cm²	218.21±74.90	199.86±59.49	194.25±70.85
p-value	n.s.		
Superficial SAT, cm²	142.79±60.22	123.64±46.38	112.39±61.07
p-value	**0.018[a]; 0.019[r]**		
Systolic BP, mmHg	124.53±16.86	127.30±14.04	121.92±15.76
p-value	n.s.		
Diastolic BP, mmHg	79.64±10.82	82.43±8.09	76.33±15.44
p-value	n.s.		
Blood Biomarkers			
Cholesterol, mg/dL	199.99±38.58	205.38±40.43	183.01±24.08
p-value	n.s.		
LDL-c, mg/dL	121.92±31.55	125.65±36.92	108.03±24.06
p-value	n.s.		
HDL-c, mg/dL	42.17±10.10	43.74±9.83	45.10±11.01
p-value	n.s.		
Triglycerides mg/dL	72.04±39.91	73.61±45.44	67.03±46.99
p-value	n.s.		
Fasting glucose, mg/dL	106.51±15.49	105.43±17.81	112.55±14.74
p-value	n.s.		
Insulin, µU/mL	18.30±12.46	17.69±9.52	16.76±8.73
p-value	n.s.		
HbA1c, %	5.59±0.48	5.57±0.49	5.79±0.45
p-value	n.s.		
HOMA IR	4.90±3.56	4.78±3.49	4.48±2.06
p-value	n.s.		
Leptin, ng/mL	14.61±14.24	13.63±12.46	12.49±11.87
p-value	n.s.		
ALKP, U/L	71.48±20.51	76.69±16.75	62.35±12.08
p-value	n.s.		
ALT, U/L	30.11±23.99	28.94±14.04	28.97±12.02

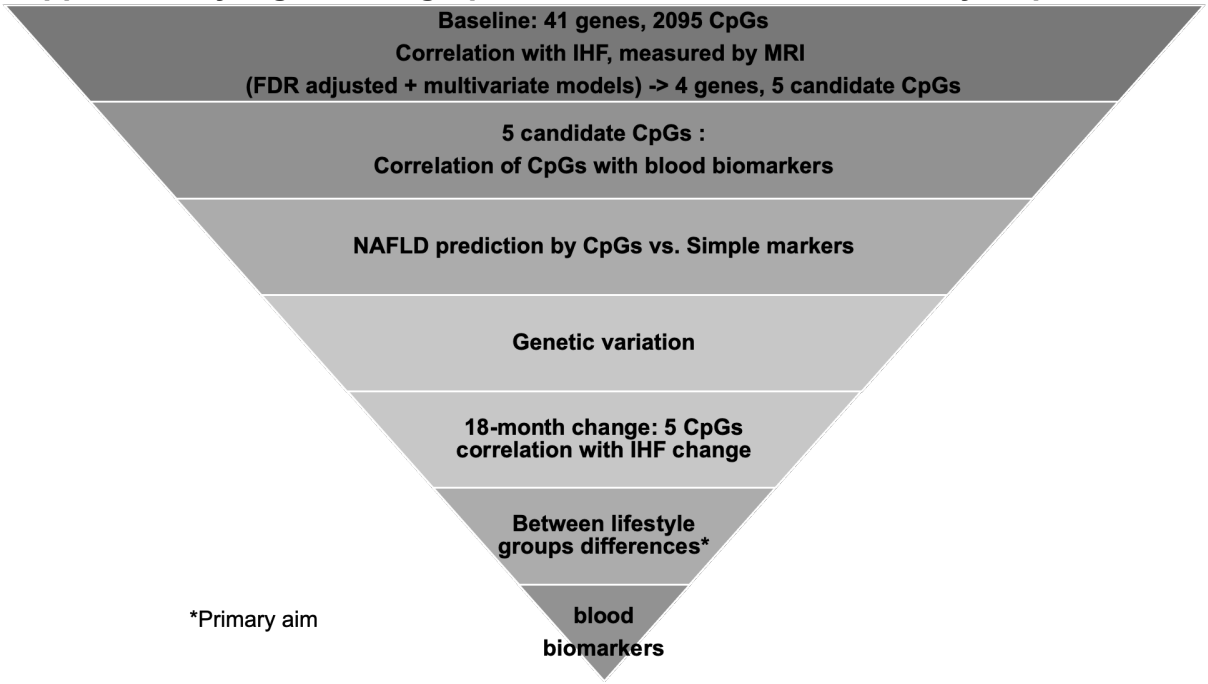
p-value	n.s.		
AST, U/L	28.97±15.48	28.38±10.79	29.12±7.96
p-value	n.s.		
GGT, U/L	35.58±18.15	34.45±17.41	36.11±19.44
p-value	n.s.		

Data are presented as mean±SD (standard deviation); *p*-values were calculated using linear regression analyses adjusted for age and sex using the [a]additive, [d]dominant, or [r]recessive mode of inheritance; [+]*p*-value for fatty liver status was calculated using Chi-Square test; ALKP – alkaline phosphatase; ALT - alanine aminotransferase; AST - aspartate aminotransferase; BMI = body mass index; BP – blood pressure; GGT – gamma glutamyl transferase; HDL-c = high density lipoprotein-cholesterol; IHF – intrahepatic fat; LDL-c = low density lipoprotein-cholesterol; *N* = number; NAFLD – non-alcoholic fatty liver disease; SAT – subcutaneous adipose tissue area; VAT – visceral adipose tissue area.

Supplementary Figure 1: Cell type composition according to the Houseman approach: plotted using ggplot2 and analyzed using Wilcoxon tests in R

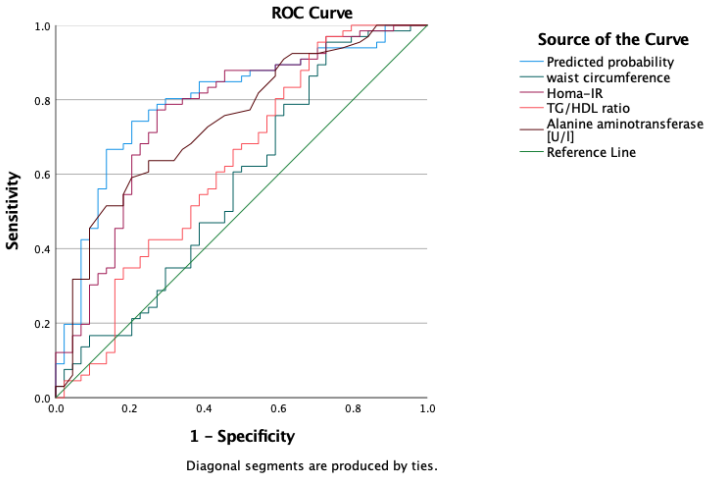


Supplementary Figure 2: A graphical demonstration of the analysis procedure.

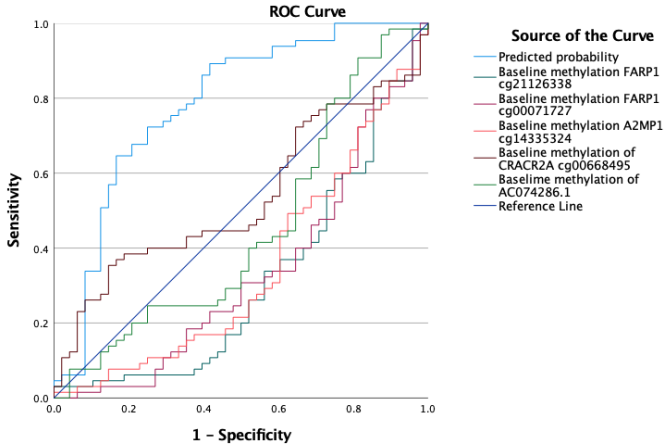


Supplementary Figure 3: Receiver operating characteristic (ROC) curve for the magnetic resonance imaging diagnosis of fatty liver (IHF ≥ 5%).

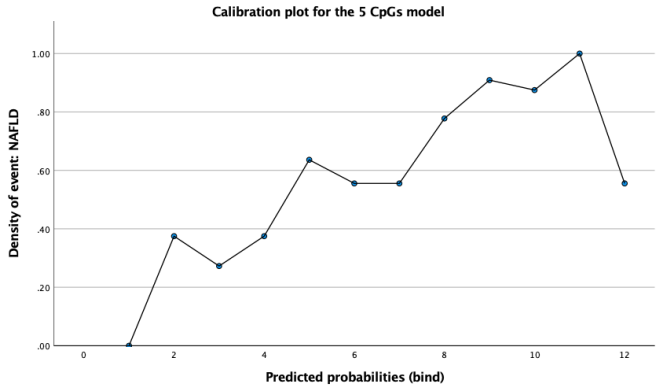
Prediction by waist circumference and simple blood biomarkers:



Prediction by 5 specific CpGs:



Calibration plot for the specific 5 CpGs model:



Values for X axis: low through 0.235186701266456=1; low through 0.293740911910657=2; low through 0.361220293852071=3; low through 0.481828524647340=4; low through 0.529006012393107=5; low through 0.597270291566246=6; low through 0.642988875219034=7; low through 0.704914817550579=8; low through 0.750158141563904=9; low through 0.816052205973293=10; low through 0.886261377029321=11; low through high=12. The calibration plot for the specific 5 CpGs model was manually constructed following the next steps by SPSS version 27.01: Dependent outcome: NAFLD (yes/no), predictor: predicted probabilities based on 5 CpGs; *For the final plot, bind predicted probabilities was used (X axis) and an aggregated mean of NAFLD based on the binned probability variable (Y axis).*

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