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Longitudinal association of peripheral blood DNA methylation with liver fat content: distinguishing between predictors and biomarkers

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Abstract

Background Alterations in DNA methylation (DNAm) have been observed in patients with fatty liver, but whether they are cause or consequence remains unknown. The study aimed to investigate longitudinal association of epigenome-wide DNAm with liver fat content (LFC) in Chinese participants, and explore their temporal relationships.

Methods Data were obtained from 2 waves over a four-year time period of the Shanghai Changfeng Study (discovery, $n=407$ and replication, $n=126$). LFC and peripheral blood DNAm were repeatedly measured using quantitative hepatic ultrasonography and the 850 K Illumina EPIC BeadChip, respectively. Longitudinal and cross-sectional epigenome-wide association studies (EWASs) were conducted with linear mixed model and linear regression model, respectively. Meta-analysis was performed using METAL. Cross-lagged panel analysis (CLPA) was carried out to infer temporal relationships between the significant CpGs and LFC.

Results Longitudinal EWAS identified cg11024682 (*SREBF1*), cg06500161 (*ABCG1*), cg16740586 (*ABCG1*), cg15659943 (*ABCA1*) and cg00163198 (*SNX19*) significantly associated with LFC with $P<1e\text{-}7$. Another 6 of the 22 previously reported CpGs were replicated in the present longitudinal EWAS. CLPA showed longitudinal effects of cg11024682 (*SREBF1*) (β = 0.14 [0.06, 0.23]), cg16740586 (*ABCG1*) (β = 0.17 [0.08, 0.25]), cg06500161 (*ABCG1*) (β = 0.12 [0.03, 0.20]), cg17901584 (*DHCR24*) (β = -0.10 [-0.18, -0.02]), cg00574958 (*CPT1A*) (β = -0.09 [-0.17, -0.01]), cg08309687 (LINC00649) (β = -0.11 [-0.19, -0.03]), and cg27243685 (*ABCG1*) (β = 0.09 [0.01, 0.18]) on subsequent LFC. The effects were attenuated when further adjusting for body mass index. High levels of LFC led to alterations in DNAm of cg15659943

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(*ABCA1*) (β = 0.13 [0.04, 0.21]), cg07162647 (β = -0.11 [-0.19, -0.03]), cg06500161 (*ABCG1*) (β = 0.10 [0.02, 0.18]), and cg27243685 (*ABCG1*) (β = 0.10 [0.02, 0.18]).

Conclusions Blood DNAm at *SREBF1*, *ABCG1*, *DHCR24*, *CPT1A*, and LINC00649 may be predictors of subsequent LFC change. The effects of DNAm at *SREBF1* and *ABCG1* on LFC were partially influenced by obesity. The findings have potential implications in understanding disease pathogenesis and highlight the potential of DNAm for early detection or intervention of fatty liver.

Keywords Liver fat content, DNA methylation, Cross-lagged panel analysis, Longitudinal study

Background

The global prevalence of metabolic dysfunction-associated steatotic liver disease (MASLD) has increased 50.4% over the past 3 decades, and it is estimated to be 38.2% recently [1]. The incidence rate of MASLD was reported to be highest in Mainland China [2]. The presence of MASLD is influenced by geographical region, race, genomic variation and lifestyle factors [3]. For example, genetic variant rs58542926 C>T of *TM6SF2* was reported associated with the full spectrum of MASLD but lower blood lipid levels [4]. Further studies revealed that TM6SF2 mainly localizes to the smooth endoplasmic reticulum, and prevents MASLD by promoting bulk lipidation of apolipoproteins B-containing lipoproteins and very low-density lipoprotein exportation from liver [5, 6].

Epigenetic regulations, especially DNA methylation (DNAm), can also explain part of the variance of MASLD. Using liver biopsies, epigenome-wide association study (EWAS) of MASLD reported that differences in DNAm can distinguish patients with advanced vs. mild MASLD [7, 8], and DNAm alterations associated with MASLD were partially reversible by bariatric surgery [9]. Nevertheless, liver biopsy is invasive, costly, and difficult to standardize and repeat. There have been growing interests in identifying biomarkers from non-invasive imaging techniques and peripheral blood to predict the onset and stratify the severity of MASLD. Using peripheral blood DNA and 450k BeadChip, the largest EWAS of MASLD enrolled 3400 European participants and identified a total of 22 CpGs associated with hepatic fat, such as CpGs annotated to *ABCG1*, *SREBF1*, *SLC7A11* and so on [10]. An EWAS of peripheral leukocytes from 35 Chinese MASLD patients and 30 healthy controls revealed that hypomethylation of *ACSL4* and *CPT1C* was associated with MASLD [11].

However, prior studies mainly focused on cross-sectional associations of DNAm with MASLD or hepatic fat, and were limited by smaller size of detected probes-mostly less than 450,000. Ma et al. investigated potential causal association between the CpGs and MASLD using Mendelian randomization (MR) analyses, and observed a causal relationship between hypomethylated cg08309687 (LINC00649) and increased hepatic fat [10]. The relatively small-scale GWAS used might have led to insufficient statistical power in their MR analyses. Thus, this research was designed to investigate longitudinal associations between DNAm at >700,000 CpGs in peripheral blood and liver fat content (LFC) in a Chinese cohort, and to assess temporal directional relationships between DNAm of the significant CpGs and LFC using cross-lagged panel analysis. It is hypothesized that DNAm alterations of different CpGs might be driver or consequence of LFC change through various pathways.

Methods

Study population and design

The study population and design are presented in Fig. 1. Shanghai Changfeng Study focuses on the risk factors and management of chronic cardiometabolic traits among Chinese residents aged ≥ 45 years in Changfeng community, Shanghai, China [12]. A total of 6595 residents were enrolled at baseline between 2009 and 2012, and 3343 of them were revisited between 2014 and 2017. The discovery cohort included 407 participants with complete DNAm data, among whom 358 and 405 had LFC data at baseline and at follow-up, respectively. Another 126 participants with complete DNAm data were included in the replication cohort, among whom 97 and 125 had LFC data at baseline and at follow-up, respectively. In the longitudinal analyses, the discovery and the replication cohorts included 358 and 96 participants without missing data, respectively.

Measurement of LFC and BMI

Participants received face-to-face interviews and provided basic information including age and sex. Current smokers were defined as smoking ≥ 1 cigarette/day for one or more years, and past smokers were defined as smokers that discontinued smoking for more than 6 months. LFC was measured by trained interviewers using a quantitative hepatic ultrasonography and calculated as LFC (%) = 62.592*standardized ultrasound hepatic/renal ratio + 168.076*standardized ultrasound hepatic attenuation rate-27.863 [13]. Body mass index (BMI) was obtained by dividing body weight by height squared (kg/m^2).

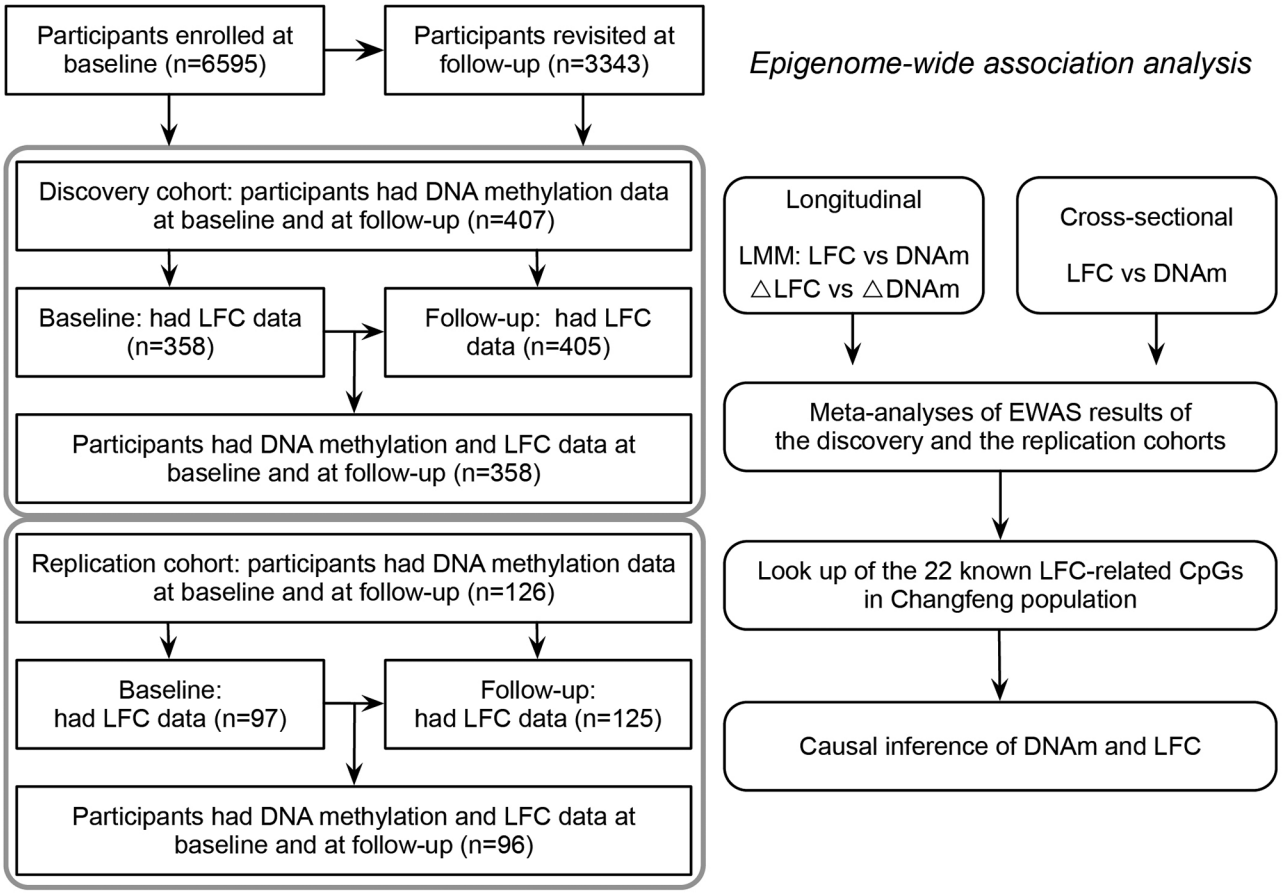

Fig. 1 Study population and design

Blood DNA methylation profiling, quality control, and data preprocessing

Genomic DNA of the study participants was extracted from peripheral blood using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), followed by estimation of purity and concentration using NanoDrop One spectrophotometer and Qubit 3.0 Fluorometer (Thermo Scientific, Waltham, MA, USA). An EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) was used to perform bisulfite conversion of DNA from each sample. Methylation profiles of DNA were measured with Illumina Infinium Methylation EPIC BeadChip (Illumina, San Diego, CA, USA).

Data quality control and preprocessing was conducted with the R package ChAMP separately in the discovery and the replication cohorts [14]. Probes were filtered out with the following criteria: detection $P>0.01$, or >3 beads in ≥5% samples, or non-CpG, or SNP-related, or multi-hit, or non-autosomal. Multidimensional Scaling plot, density plot, and dendrogram plot generated using the function champ.QC were used to further assess data quality. The final clean methylation working dataset contained 711,268 (discovery) and 740,701 (replication) autosomal CpG probes.

Beta value (β, 0–1 scale) was used to represent DNAm level of the CpGs, and normalization of the β value matrix was conducted with the BMIQ method [15]. Batch effects were estimated by using surrogate variable analysis, and were corrected by applying Combat [16] in ChAMP. CpG sites were mapped to human reference GRCh37 and annotated using IlluminaHumanMethylationEPICanno.ilm10b4.hg19. Leukocyte composition of each sample was estimated using the R package EpiDISH [17].

DNA methylation analysis

Longitudinal EWAS of LFC changes

For the 358 and the 96 participants in the two cohorts, DNAm and LFC were repeatedly measured. Longitudinal EWAS was performed using linear mixed models (LMM) in R package limma [18]. Methylation β value and LFC value were entered as the response and the independent variable, adjusting for age, sex, smoking and estimated leukocyte composition at baseline and at follow-up (model 1). Participant ID was entered as a random effect.

In literature reporting longitudinal EWAS of BMI, longitudinally changes in methylation and changes in

BMI were used as independent and response variables [19]. Therefore, the association between longitudinally changes in DNAm and LFC was analyzed using multilinear regression, adjusting for age, sex, follow up time, and smoking status and estimated leukocyte composition at baseline and at follow-up (model 1).

Cross-sectional EWAS of LFC

Cross-sectional EWAS was performed to analyze the associations between DNAm and LFC at baseline and at follow-up, respectively. A multiple linear regression model in limma was used, adjusting age, sex, smoking and estimated leukocyte composition (model 1).

For both longitudinal and cross-sectional analysis, BMI was additionally adjusted in model 2.

Replication study

In the replication cohort, longitudinal and cross-sectional EWAS was conducted, adjusting for the same covariates as those in the discovery cohort in model 1 and model 2.

EWAS meta-analysis

In longitudinal and cross-sectional EWAS, inverse-variance weighted fixed-effects meta-analysis that combined EWAS results from the discovery and the replication cohorts was conducted, using the computationally efficient software METAL [20].

Epigenome-wide significant level was set at $P<1e\text{-}7$ with Bonferroni correction.

Look up of the 22 known CpGs in Changfeng population

Twenty-two CpGs were reported cross-sectionally associated with hepatic fat in previous EWAS in European ancestry [10]. The CpGs were looked up in the present longitudinal EWAS meta-analysis results in model 1 to see whether they were also associated with LFC changes in individuals of Chinese ancestry. Associations between the 22 CpGs and LFC in cross-sectional EWAS meta-analyses at baseline and at follow-up respectively in model 1 are also presented. The significance level was set at 2.3e-3 (0.05/22) using Bonferroni correction.

Methylation quantitative trait loci (meQTL) of CpGs

To explore the potential genetic basis of the identified and replicated CpGs, clumped meQTLs of the markers were looked up in the EPIGEN MeQTL Database [21].

Functional enrichment analysis

Twenty CpGs that were most significantly associated with LFC in the longitudinal meta-analysis in model 1 were collected for the functional enrichment analysis. The CpGs were first annotated to their closest genes with the R package IlluminaHumanMethylationEPICanno.ilm10b4.hg19 [22]. Then, the Gene Ontology (GO) [23], Reactome pathway [24], and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [25] enrichment of the annotated genes were conducted using the R package clusterProfiler v4.8.1 [26] (one-tailed hypergeometric test).

Cross-lagged panel analysis (CLPA)

CLPA is widely used to examine directional influences between intercorrelated and longitudinally changing variables. To test the longitudinal bidirectional relationship between LFC and DNAm, CLPA using longitudinal data of LFC and DNAm was performed, combining the two cohorts. Prior to analysis, LFC values were adjusted for age, sex, and smoking status by linear regression and residuals were Z-transformed; methylation β values were similarly processed but further adjusting for batch effects and estimated leukocyte composition (model 1). BMI was additionally adjusted in model 2. The analysis simultaneously estimated two cross-lagged path coefficients, which represent the effect of baseline LFC (or DNAm) on future DNAm (or LFC) at follow-up. Besides, temporal relationships between DNAm of the CpGs and BMI in model 1 are also presented. R package Lavaan with structural equation model was used to perform CLPA [27]. Model fitting was validated by a goodness-of-fit index > 0.90, a comparative fit index > 0.95, and a standardized root-mean-square residual < 0.08 [28].

Statistical analysis

Numerical variables are presented as mean (standard deviation, SD) for symmetrical distributions, and median [quartile 1, quartile 3] for asymmetric distributions. Differences in normally and nonnormally distributed data were analyzed using Student's t test and the Mann-Whitney U test, respectively. Sex and smoking status are presented as n (%), and the chi-square test was used to compare proportions. All the statistical analyses were performed in the R statistical environment version 4.2.2. All reported p values are two-sided with a significance level of 0.05.

Results

Basic characteristics

The discovery cohort and the replication cohort had mean ages of 61.2 (SD: 7.29 years) and 61.9 (SD: 8.37 years) at baseline, respectively. Approximately 58% and 47% of the participants were female, respectively. The replication cohort had a slightly longer follow-up time than did the discovery cohort (4.39 years vs. 4.10 years). Baseline age, smoking status, BMI, LFC, and longitudinal change of LFC were comparable between the discovery and the replication cohorts (Table 1 and Additional File 1 Supplementary Fig. 1).

Table 1 Basic characteristics of the study participants

	Discovery		Replication		
	Baseline ($n=358$)	Follow-up ($n=405$)	Baseline ($n=97$)	Follow-up ($n=125$)	$P^{\dagger}$
Female, %	208 (58.1)	234 (57.8)	46 (47.4)	59 (47.2)	0.078
Age, years	61.2 (7.29)	65.5 (7.34)	61.9 (8.37)	66.8 (8.60)	0.407
Smoke, %					0.398
Never	292 (81.6)	328 (81.0)	76 (78.4)	93 (74.4)	
Past	9 (2.5)	11 (2.7)	5 (5.2)	11 (8.8)	
Current	57 (15.9)	65 (16.0)	16 (16.5)	21 (16.8)	
BMI, kg/m^2	23.8 (2.96)	24.6 (3.08)	24.0 (2.44)	24.6 (3.05)	0.541
LFC, %	5.72 [2.21, 12.50]	5.75 [2.25, 14.28]	4.73 [1.75, 11.59]	5.87 [2.33, 12.33]	0.193
Follow-up time, years	4.10 (0.63)		4.39 (0.72)		0.003
LFC change, %	1.00 [-2.84, 6.45]		2.79 [-2.31, 7.64]		0.257

$^{\dagger}P$ indicates the significance of differences between discovery and replication cohorts at baseline

Longitudinal EWAS

Longitudinal EWAS of LFC using LMM in the discovery cohort revealed that hypermethylation of cg11024682 (*SREBF1*), cg06500161 (*ABCG1*), and cg16740586 (*ABCG1*) was significantly associated with increased LFC after adjusting for age, sex, and smoking status (Fig. 2A; Table 2), which was successfully replicated in the replication cohort ($P<0.05$ with the same direction of effects). Longitudinal EWAS of LFC using LMM in the replication cohort identified no significant associations (Fig. 2B). The fixed-effects meta-analysis combining EWAS results from the discovery and the replication cohorts revealed 5 significantly associated CpGs ($P<1e\text{-}7$ with the same effect directions in the two cohorts), among which hypermethylation of cg15659943 (*ABCA1*) and cg00163198 (*SNX19*) was also associated with increased LFC (Fig. 2C; Table 2). Further adjusting for BMI attenuated the strength of the associations but the 5 CpGs remained near significantly associated with LFC (Table 2).

Analysis of the association between longitudinal change of LFC and that of DNAm found that hypomethylation of cg09154567 annotated to *SULT4A1* was associated with increased LFC in the replication cohort, but not significant in the discovery cohort or the meta-analysis (Additional File 1 Supplementary Fig. 2 and Table 2).

Functional enrichment

Functional enrichment analysis was conducted for the top 20 CpGs associated with LFC according to the longitudinal meta-analysis results (Table 3). Specifically, the enrichment of genes where the top CpGs located in terms of GO, KEGG, and Reactome pathways was checked. As a result, the following biological processes were significantly enriched: lipid storage and transport (Fig. 3A-B), NR1H2 and NR1H3-mediated signaling (Fig. 3C), ABC transporters and cholesteral metabolism (Fig. 3D), and some other lipid-related functions and pathways.

Cross-sectional EWAS

Cross-sectional EWAS at baseline and at follow-up in the two cohorts was performed, respectively. EWAS in the meta-analysis at follow-up found that hypomethylation of cg07162647 and hypermethylation of cg11024682 (*SREBF1*) and cg06500161 (*ABCG1*) were cross-sectionally associated with higher LFC (Additional File 1 Supplementary Fig. 3 and Table 2).

As shown in Additional File 1 Supplementary Table 1, the significant CpGs identified in the longitudinal analyses were also cross-sectionally associated with LFC with consistent directionality and nominal $P<0.05$ in the meta-analyses at baseline and at follow-up, respectively.

Further adjustment of BMI did not materially change the associations (Table 2 and Additional File 1 Supplementary Table 1). A total of 8 meQTLs were found for the 6 CpGs identified in Changfeng population (Additional File 1 Supplementary Table 2).

Look up of the 22 previously known CpGs

Previous EWAS of hepatic fat in 1,496 participants from the Framingham Heart Study identified 58 significant CpGs with an FDR<0.05, 22 of which were successfully replicated in 1,904 participants from another three cohorts in a sex- and age-adjusted model [10]. Eight of the 22 CpGs remained significant in the longitudinal EWAS using LMM in model 1 after Bonferroni correction, and were also nominally significant in the meta-analyses in cross-sectional EWAS at baseline and at follow-up (Table 4). This suggested that alterations in DNAm detected in peripheral blood could be verified in different ethnic groups, and was associated not only with cross-sectional LFC variation but also with longitudinal LFC changes. A total of 9 meQTLs were found for the 6 CpGs (except for cg11024682 and cg06500161) validated in Changfeng population (Additional File 1 Supplementary Table 3).

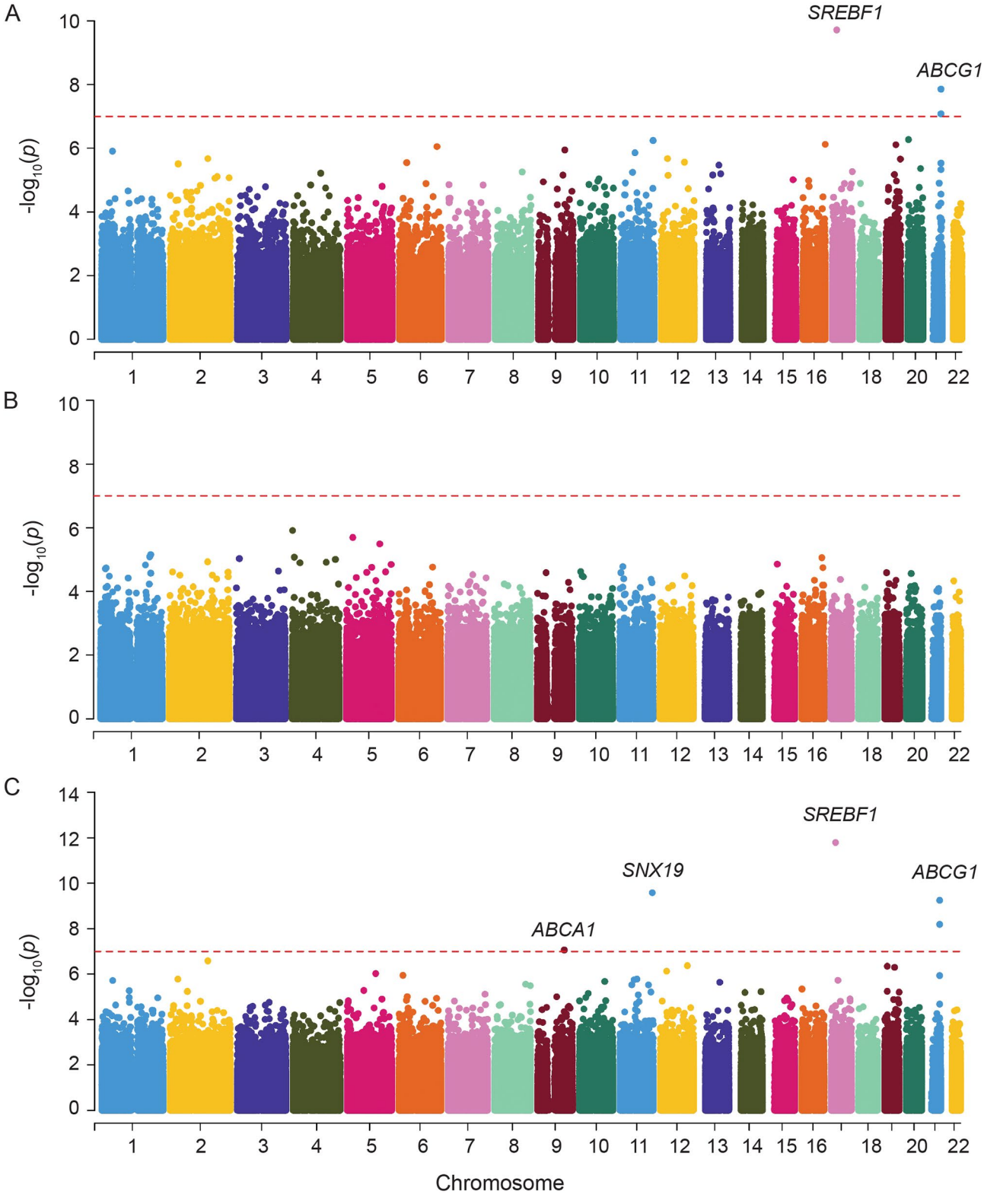

Fig. 2 Manhattan plot of the longitudinal association of peripheral blood-derived DNA methylation with LFC using LMM. Plot was generated using results from the sex-, age-, and smoking-adjusted model in the discovery cohort (**A**), the replication cohort (**B**), and meta-analysis (**C**)

Table 2 Significant CpGs in longitudinal EWAS and cross-sectional EWAS of LFC

CpG	Chr	POS	UCSC_RefGene	Gencode BasicV12_Group	Relation_to_Island	Discovery		Replication		Meta-analysis		*P* adjust BMI†
						Effect	*P*	Effect	*P*	*P*	Effect Direction	
EWAS-LMM: LFC vs. DNAm												
cg15659943	9	107,631,656	*ABCA1*	Body	OpenSea	4.57E-04	1.13E-06	4.78E-04	2.44E-02	**8.70E-08**	++	6.89E-07
cg00163198	11	130,767,760	*SNX19*	3'UTR	OpenSea	5.01E-04	5.73E-07	8.94E-04	5.26E-05	**2.61E-10**	++	**3.04E-09**
cg11024682	17	17,730,094	*SREBF1*	Body	S_Shelf	6.43E-04	**1.93E-10**	6.94E-04	2.09E-03	**1.61E-12**	++	**6.65E-09**
cg16740586	21	43,655,919	*ABCG1*	5'UTR	S_Shore	5.40E-04	**8.32E-08**	5.96E-04	2.21E-02	**6.47E-09**	++	7.39E-06
cg06500161	21	43,656,587	*ABCG1*	5'UTR	S_Shore	4.82E-04	**1.39E-08**	5.17E-04	1.11E-02	**5.64E-10**	++	3.93E-07
Longitudinal EWAS: △LFC vs. △DNAm												
cg09154567	22	44,239,861	*SULT4A1*	Body	OpenSea	-7.25E-05	5.16E-01	-1.34E-03	**7.68E-08**	2.30E-03	--	**2.45E-08**
Cross-sectional EWAS: LFC vs. DNAm												
cg11024682	17	17,730,094	*SREBF1*	Body	S_Shelf	6.35E-04	7.84E-07	9.13E-04	1.59E-03	**4.89E-09**	++	1.71E-06
cg07162647	19	40,466,275		Body	OpenSea	-4.24E-04	3.29E-07	-5.71E-04	1.75E-02	**1.95E-08**	--	1.77E-07
cg06500161	21	43,656,587	*ABCG1*	5'UTR	S_Shore	5.65E-04	1.05E-07	5.05E-04	5.05E-02	**2.17E-08**	++	4.10E-06

P < 1e-7 are marked bold. †*P* in meta-analysis except for cg09154567 (in the replication cohort), further adjust for BMI in model 2, are presented

Cross-lagged panel analysis

CLPA was performed to investigate the longitudinal directional relationships between LFC and DNAm of the 12 identified and replicated CpGs. In model 1 adjusting for age, sex, and smoking status, baseline hypermethylation at cg11024682 (*SREBF1*) (β=0.14 [0.06, 0.23]), cg16740586 (*ABCG1*) (β=0.17 [0.08, 0.25]), cg06500161 (*ABCG1*) (β=0.12 [0.03, 0.20]), cg27243685 (*ABCG1*) (β=0.09 [0.01, 0.18]) and hypomethylation at cg17901584 (*DHCR24*) (β = -0.10 [-0.18, -0.02]), cg00574958 (*CPT1A*) (β = -0.09 [-0.17, -0.01]), cg08309687 (LINC00649) (β = -0.11 [-0.19, -0.03]) predicted increased subsequent LFC; higher LFC led to hypermethylated cg15659943 (*ABCA1*) (β=0.13 [0.04, 0.21]), cg06500161 (*ABCG1*) (β=0.10 [0.02, 0.18]), cg27243685 (*ABCG1*) (β=0.10 [0.02, 0.18]) and hypomethylation at cg07162647 (β = -0.11 [-0.19, -0.03]) (Fig. 4).

Among the 12 identified and replicated CpGs, cg15659943 (*ABCA1*) (β=0.07 [0.02, 0.11]) and cg11024682 (*SREBF1*) (β=0.06 [0.01, 0.10]) showed longitudinal effects on subsequent increased BMI, and BMI was temporally associated with hypomethylation of cg00574958 (*CPT1A*) (β = -0.12 [-0.20, -0.05]) and cg08309687 (LINC00649) (β = -0.09 [-0.15, -0.03]) (Additional File 1 Supplementary Fig. 4).

In model 2 further adjusting for BMI, the temporal effects of cg11024682 (*SREBF1*), cg16740586 (*ABCG1*) and cg06500161 (*ABCG1*) on LFC were almost halved with p values of 0.076, 0.032, and 0.165, which indicated that the effects were partially influenced by increased obesity (Additional File 1 Supplementary Fig. 5). Temporal associations of other CpGs and LFC were not greatly changed (Additional File 1 Supplementary Fig. 5).

Discussion

The present research identified 5 CpGs associated with longitudinal change of LFC, another 1 CpG cross-sectionally associated with LFC, and replicated another 6 previously reported LFC-associated CpGs in longitudinal EWAS in the sex-, age-, and smoking-adjusted model. Among the significant CpGs, 9 showed longitudinal directional associations with LFC, and 8 remained significant after further adjusting for BMI in model 2. The findings are notable in not only identified/replicated DNAm of CpGs longitudinally associated with LFC in Chinese participants, but also distinguished between predictors and biomarkers.

Several cross-sectional EWASs have observed altered DNAm patterns collected from liver biopsies or peripheral blood samples of MASLD patients [29], but longitudinal EWASs of LFC are lacking. In accordance with previous studies, this study likewise showed that genes involved in lipid metabolism and biosynthesis pathways appeared to be the most prominent markers.

Table 3 Top 20 CpGs that most significantly associated with LFC in the longitudinal meta-analysis in model 1

CpG	Chr	POS	UCSC_ RefGene	Gencode BasicV12_Group	Relation to_Island	Zscore	*P*	Effect Direction
cg11024682	17	17,730,094	*SREBF1*	Body	S_Shelf	7.064	1.613E-12	++
cg00163198	11	130,767,760	*SNX19*	Body	OpenSea	6.32	2.608E-10	++
cg06500161	21	43,656,587	*ABCG1*	Body	S_Shore	6.2	5.642E-10	++
cg16740586	21	43,655,919	*ABCG1*	Body	S_Shore	5.804	6.465E-09	++
cg15659943	9	107,631,656	*ABCA1*	Body	OpenSea	5.352	8.702E-08	++
cg03694857	2	152,568,384	*NEB*	Body	OpenSea	5.149	2.614E-07	++
cg13449394	12	109,230,732	*MIR619;SSH1*	Body	OpenSea	5.058	4.248E-07	++
cg10819350	19	10,655,686	*ATG4D*	Body	S_Shore	5.047	4.496E-07	++
cg07162647	19	40,466,275			OpenSea	-5.026	5.014E-07	--
cg27243560	12	25,522,335			OpenSea	4.95	7.413E-07	++
cg21137557	5	115,314,451	*LVRN*	Body	OpenSea	-4.902	9.479E-07	--
cg00857282	6	16,130,727	*MYLIP*	Body	S_Shore	4.867	1.135E-06	++
cg27243685	21	43,642,366	*ABCG1*	Body; 5'UTR	S_Shelf	4.862	1.16E-06	++
cg00574958	11	68,607,622	*CPT1A*	5'UTR	N_Shore	-4.792	1.655E-06	--
cg12748148	2	30,549,065			OpenSea	-4.791	1.658E-06	--
cg13280734	11	57,252,275	*SLC43A1*	3'UTR	S_Shore	4.779	1.763E-06	++
cg12188928	17	27,309,139	*SEZ6*	Body	N_Shelf	4.767	1.87E-06	++
cg10558063	1	44,540,412			OpenSea	4.763	1.906E-06	++
cg07504977	10	102,131,012			N_Shelf	4.743	2.107E-06	++
cg07502358	13	78,168,218	*SCEL*	Body	OpenSea	-4.725	2.297E-06	--

Cg15659943, at which DNAm is longitudinally associated with baseline LFC, is annotated to *ABCA1*. A mild but significant effect of baseline LFC on subsequent hypermethylation at cg06500161 and cg27243685 mapping to *ABCG1* was observed. *ABCA1* and *ABCG1* are members of the ABC transporter superfamily that are essential for the biogenesis of high-density lipoprotein (HDL) and reverse cholesterol transport. ABCA1 functions as an efflux pump of cholesterol from peripheral cells to apolipoprotein A-I and generates the nascent HDL, and ABCG1 facilitates subsequent cholesterol efflux to HDL for further maturation [30, 31]. These implicated that LFC alteration might regulate gene expression involved in reverse cholesterol transport via epigenetic mechanisms.

Cg00163198 is a novel EPIC array marker, and has been described to be related to incident type 2 diabetes [32] and cardiovascular health factors including BMI, blood pressure, glucose, and cholesterol levels [33]. It lies on the intronic region of the Sorting nexin 19 (*SNX19*) gene, which was found to be associated with liver enzymes, lipids, and body fat in a GWAS [34]. *SNX19* enables the binding of lipids and phosphatidylinositol, and plays important roles in insulin secretion [35]. CpG cg00163198 located in an active regulatory region and was significantly correlated with an eQTL of *SNX19* in the adipose tissue (eQTL $P=8.78\times10^{-22}$ by GTEx; meQTL $P=1.93\times10^{-13}$ by Pan-meQTL) [36]. This indicates that the regulation between cg00163198 and *SNX19* is mediated by the eQTL and ultimately affects gene expression.

Evidence on causal relationships between DNAm and LFC or fatty liver is scarce to date. The present study did not conduct MR analysis due to the lack of large GWASs on LFC in Asian populations and insufficient statistical power. Previous studies applying CLPA reported that DNAm is a biomarker rather than a predictor or cause of BMI [37, 38], which was in accordance with the findings of MR [19] and longitudinal enrichment analysis [39]. Using CLPA in a cohort with repeatedly measured data, this study demonstrated that baseline hypermethylation at cg11024682 (*SREBF1*) and the three CpGs (cg16740586, cg06500161, and cg27243685) investigated mapping to *ABCG1* predicted increased subsequent LFC levels. DNAm at cg11024682 (*SREBF1*) was reported associated with hepatic fat [10], BMI [19, 40], type 2 diabetes [41], blood triglyceride levels [42, 43], serum liver enzyme levels [44], and cardiovascular health [33]. Methylation at CpGs mapping to *ABCG1* was reported associated with increased blood lipids, glucose, insulin, and increased risk of diabetes [45, 46]. DNAm of cg27243685 was reported associated with hepatic fat [10, 47]. Animal studies demonstrated that *Abcg1* played a pivotal role in preventing hepatic fat accumulation in mice challenged with high-fat diet [48].

The temporal effects of DNAm of CpGs at *SREBF1* and *ABCG1* on subsequent LFC were reduced after adjusting for BMI. This was consistent with the findings from the cross-sectional EWAS in another study [10] and was not unexpected, as obesity, diabetes, and lipid metabolism are established correlated with each other and are related with MASLD. BMI was positively associated with LFC in

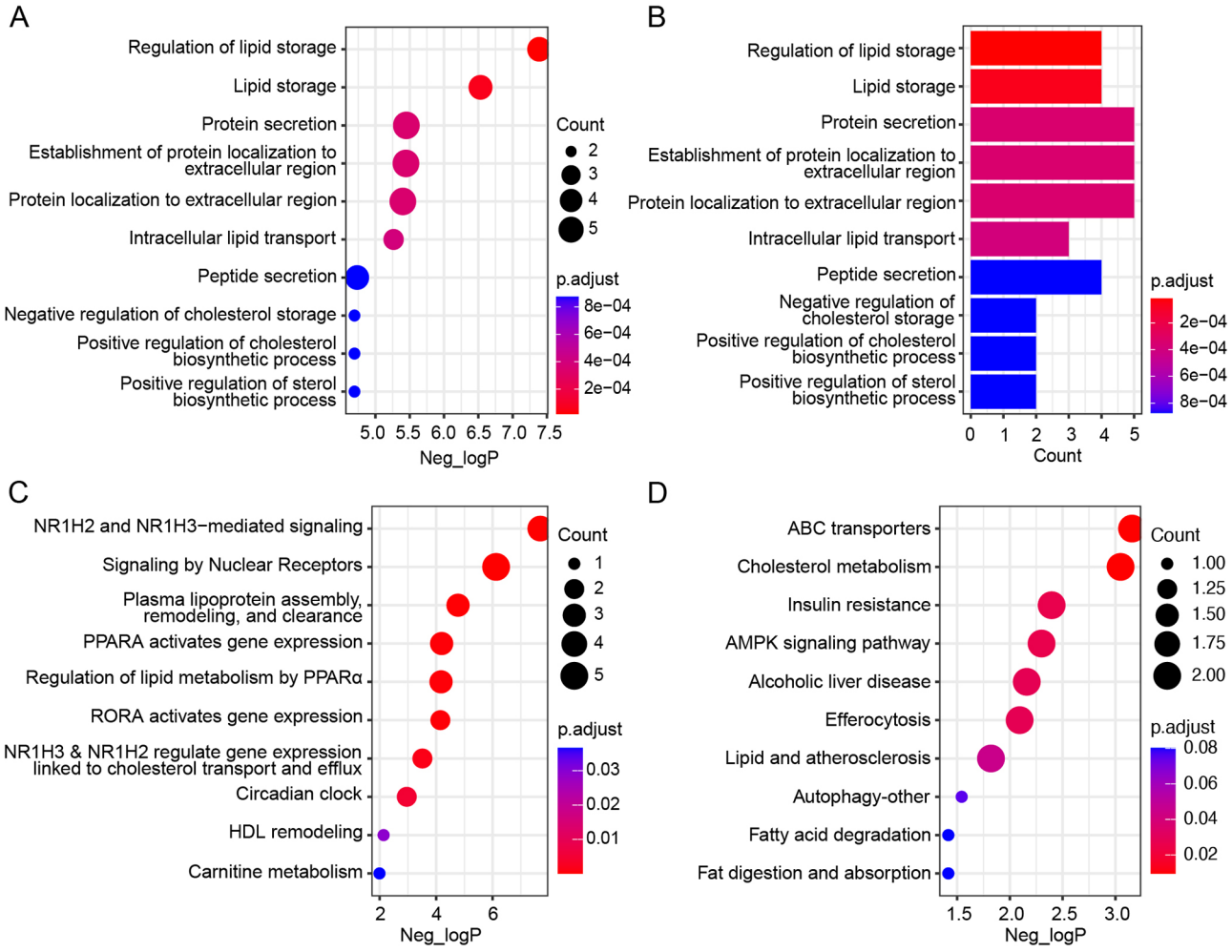

Fig. 3 Functional enrichment of the top 20 CpGs that most significantly associated with LFC. (**A**, **B**) Enrichment of the annotated genes in GO, where the x-axis represents the negative log p value of enrichment significance (**A**) and the number of genes overlapped with the functional term (**B**). (**C**, **D**) Enrichment of the annotated genes in Reactome pathways (**C**) and KEGG pathways (**D**)

Changfeng population with Spearman r=0.32 (P<2.2e-16), and increased obesity might lie on the mechanistic pathway from DNAm of CpGs at *SREBF1* and *ABCG1* to increased LFC. In CLPA, BMI showed weak association with cg11024682 (*SREBF1*) and non-significant associations with the three CpGs (cg16740586, cg06500161, and cg27243685) of *ABCG1*. Together, the results indicated that the effects of DNAm of *SREBF1* and *ABCG1* were mediated by increased BMI as well as other mechanisms, for example, diabetes and lipid metabolism.

Results of CLPA illustrated that hypomethylation at cg08309687 (LINC00649) has a strong prospective effect on increasing LFC, which was in accordance with previous findings from MR analysis [10]. There were four meQTLs at LINC00649 for cg08309687 according to the EPIGEN MeQTL Database. A three-way association by Ma et al. reported that 8% of the association of cg08309687 and hepatic fat was mediated by the expression of *TMEM50B* [10]. In addition, hypermethylation at cg17901584 (*DHCR24*) and cg00574958 (*CPT1A*) were putatively predictors for decreased LFC. *DHCR24* encodes 24-dehydrocholesterol reductase that is involved in the final step of cholesterol synthesis, converting desmosterol into cholesterol [49]. Cg17901584 was also reported associated with metabolic traits such as BMI [19], blood lipids [43], and type 2 diabetes [50].

Strengths and limitations

The strength of this study lies in its prospective nature with repeatedly measured data, and it adds value to EWAS of fatty liver by distinguishing between predictors and biomarkers. This study has potential limitations. First, the modest sample size might result in limited statistical power to detect CpGs with small effect sizes. Second, the study used DNA from peripheral blood as liver tissues were unavailable. However, previous studies demonstrated that quantification of DNAm from peripheral blood or plasma cell-free DNA released by dying

Table 4 Longitudinal and cross-sectional associations of 22 previously known CpGs with LFC in Changfeng population

CpG	Chr	POS	UCSC_RefGene	Gencode BasicV12_Group	Relation_to_Island	Longitudinal		Baseline		Follow-up	
						P†	Effect Direction	*P*	Effect Direction	*P*	Effect Direction
cg09469355	1	2,161,886	*SKI*		S_Shore	5.76E-01	+-	4.45E-01	+-	3.20E-01	--
cg17901584	1	55,353,706	*DHCR24*	TSS1500; 5'UTR	S_Shore	**5.24E-04**	--	7.49E-03	--	7.75E-03	--
cg03725309	1	109,757,585	*SARS*		S_Shore	3.95E-01	+-	9.63E-01	-+	5.65E-01	+-
cg14476101	1	120,255,992	*PHGDH*	5'UTR	S_Shore	5.50E-01	-+	2.02E-01	-+	9.19E-01	-+
cg19693031	1	145,441,552	*TXNIP*	3'UTR; 5'UTR	OpenSea	**1.45E-04**	--	3.16E-02	--	2.44E-03	--
cg06690548	4	139,162,808	*SLC7A11*		OpenSea	4.00E-01	--	1.63E-01	--	9.96E-01	-+
cg05119988	4	166,251,189	*SC4MOL*	5'UTR	S_Shelf	3.57E-01	--	2.77E-01	--	3.61E-01	--
cg03957124	6	37,016,869			S_Shelf	8.56E-02	--	7.81E-01	+-	1.21E-02	--
cg18120259	6	43,894,639			OpenSea	6.05E-02	--	8.78E-02	--	2.77E-01	-+
cg17501210	6	166,970,252	*RPS6KA2*	5'UTR	OpenSea	9.33E-01	-+	2.90E-01	++	2.46E-01	-+
cg21429551	7	30,635,762	*GARS*		S_Shore	5.23E-01	++	8.57E-01	-+	4.18E-01	++
cg11376147	11	57,261,198	*SLC43A1*	3'UTR	OpenSea	2.15E-01	--	3.05E-01	--	2.07E-01	--
cg00574958	11	68,607,622	*CPT1A*	5'UTR	N_Shore	**1.66E-06**	--	3.36E-02	--	9.14E-06	--
cg26894079	11	122,954,435	*ASAM*	1stExon; 3'UTR	OpenSea	9.82E-02	--	3.72E-01	--	8.29E-02	--
cg11024682	17	17,730,094	*SREBF1*		S_Shelf	**1.61E-12**	++	3.81E-05	++	4.89E-09	++
cg14020176	17	72,764,985	*SLC9A3R1*	3'UTR	OpenSea	**9.36E-04**	++	5.29E-02	++	8.95E-03	++
cg19016694	17	80,821,826	*TBCD*	3'UTR	S_Shelf	1.05E-01	--	3.88E-01	--	2.41E-01	--
cg15860624	19	3,811,194	*ZFR2*		Island	1.29E-01	++	5.57E-01	+-	3.24E-01	++
cg08309687	21	35,320,596		TSS1500; 5'UTR	OpenSea	**1.32E-03**	--	1.21E-01	-+	1.29E-03	--
cg27243685	21	43,642,366	*ABCG1*	5'UTR	S_Shelf	**1.16E-06**	++	2.00E-03	+-	4.94E-05	++
cg06500161	21	43,656,587	*ABCG1*	5'UTR	S_Shore	**5.64E-10**	++	5.39E-03	++	2.17E-08	++
cg02711608	19	47,287,964	*SLC1A5*	5'UTR;1stExon	N_Shelf	NA	NA	NA	NA	NA	NA

†*P*<2.3e-3 (0.05/22) are marked bold. Results in meta-analyses of the discovery and the replication cohorts are presented

hepatocytes could reflect the severity of MASLD and could be suggested as an alternative approach with high accuracy to stratify MASLD [11, 51]. Third, experiments are needed to explore the mechanisms involved.

Conclusions

In summary, differences in peripheral blood DNA methylation occur in interindividual variation and intraindividual longitudinal change of LFC. Blood DNAm at *SREBF1*, *ABCG1*, *DHCR24*, *CPT1A*, and LINC00649 may be predictors of subsequent LFC change. The effects of DNAm at *SREBF1* and *ABCG1* were partially influenced by obesity, which implicates the importance of controlling body weight in preventing fatty liver. For clinical perspective, reversible alterations in DNA methylation result from various exposures and lead to changes in gene expression, distinguishing between predictors and biomarkers may be helpful in developing preventive and therapeutic strategies for fatty liver disease.

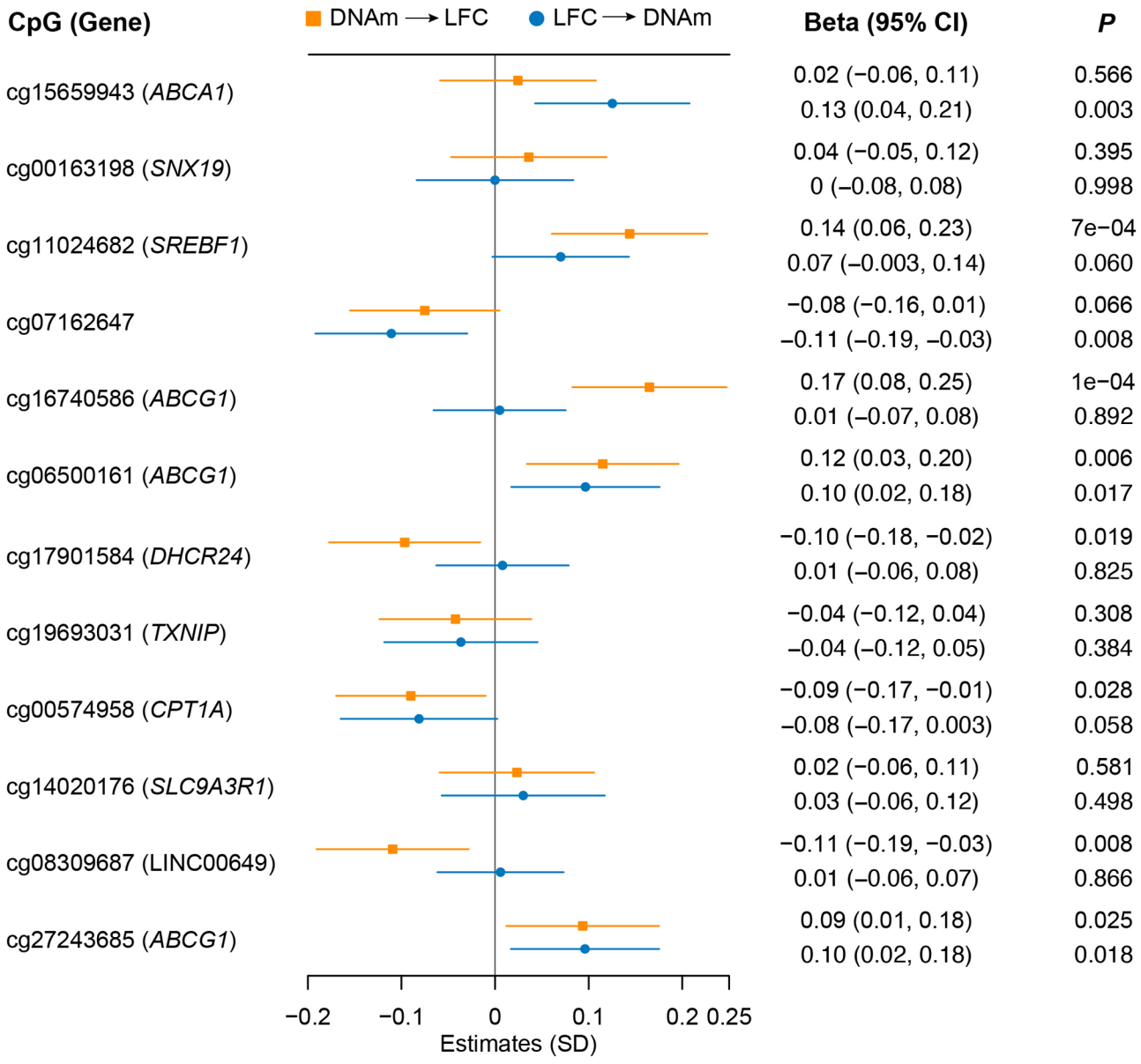

Fig. 4 Cross-lagged path coefficients in longitudinal analysis of LFC and DNA methylation measured at baseline and at follow-up in model 1 adjusted for age, sex, and smoking status

Abbreviations

DNAm	DNA Methylation
LFC	Liver Fat Content
EWAS	Epigenome-Wide Association Study
CLPA	Cross-Lagged Panel Analysis
MASLD	Metabolic Dysfunction-Associated Steatotic Liver Disease
MR	Mendelian Randomization
GWAS	Genome-Wide Association Study
BMI	Body Mass Index
LMM	Linear Mixed Model
MeQTL	Methylation Quantitative Trait Loci
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
SD	Standard Deviation
HDL	High-Density Lipoprotein

Supplementary Information

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Supplementary Material 1

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Author contributions
Study conceptualization, project administration, supervision, writing-review & editing: XG, SW and HL. Data analysis, visualization, interpretation, and writing-original draft: HZ and WL. Data curation: HL, HM, LC, MX, JG and BP. Funding acquisition: XG, SW, WL and HZ.

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Data availability
Summary statistics of the EWAS of LFC in the present study is available from the corresponding authors upon reasonable request.

Declarations

Ethics approval and consent to participate
All participants provided written informed consent, with ethics approval granted by the ethics committee of Zhongshan Hospital affiliated to Fudan University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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