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Gene discovery for high-density lipoprotein cholesterol level change over time in prospective family studies

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Abstract

Backgrounds and aims: Several genes are known to contribute to the levels and metabolism of HDL-C, however, their protective effects in cardiovascular disease (CVD), healthy aging, and longevity are complex and poorly understood. It is also unclear if these genes predict longitudinal HDL-C change. We aimed to identify loci influencing HDL-C change.

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Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atherosclerosis.2020.02.005>.

Methods: We performed a genome-wide association study (GWAS) with harmonized HDL-C and imputed genotype in three family-based studies recruited for exceptional survival (Long Life Family Study), from community-based (Framingham Heart Study) and enriched for CVD (Family Heart Study). In 7738 individuals with at least 2 visits, we employed a growth curve model to estimate the random linear trajectory parameter of age-sex-adjusted HDL-C for each person. GWAS was performed using a linear regression model on HDL-C change accounting for kinship correlations, population structure, and differences among studies.

Results: We identified a novel association for HDL-C with *GRID1* ($p = 5.43 \times 10^{-10}$), which encodes a glutamate receptor channel subunit involved in synaptic plasticity. Seven suggestive novel loci ($p < 1.0 \times 10^{-6}$; *MBOAT2*, *LINC01876-NR4A2*, *NTNG2*, *CYSLTR2*, *SYNE2*, *CTXND1-LINC01314*, and *CYYRI*) and a known lipid gene (*ABCA10*) showed associations with HDL-C change. Two additional sex-specific suggestive loci were identified in women (*DCLK2* and *KCNJ2*). Several of these genetic variants are associated with lipid-related conditions influencing cardiovascular and metabolic health, have predictive regulatory function, and are involved in lipid-related pathways.

Conclusions: Modeling longitudinal HDL-C in prospective studies, with differences in healthy aging, longevity and CVD risk, contributed to gene discovery and provided insights into mechanisms of HDL-C regulation.

Keywords

Longitudinal HDL-C change; GWAS; Longevity; Healthy aging; HDL-C metabolism

1. Introduction

The plasma levels of high-density lipoprotein cholesterol (HDL-C) show a broad distribution at baseline, ranging from less than 0.518 mmol/L (20 mg/dL) to greater than 2.331 mmol/L (90 mg/dL) in general populations [1–3]. Low HDL-C levels (< 1.036 mmol/L (40 mg/dL) in men and < 1.295 mmol/L (50 mg/dL) in women) are indicators of increased risk for cardiovascular events, while elevated HDL-C levels may contribute to atheroprotection [4]. Studies have also shown that the levels of HDL-C are higher among healthy elderly aged 85–89 years [5] or in individuals with exceptional longevity [6,7] compared to younger counterparts. On the contrary, other studies have reported associations between increased all-cause mortality risks and extreme HDL-C levels [1–3,8]. Nevertheless, the thresholds for extreme HDL-C levels varied among these studies that used different ancestries, healthy and behavior conditions, and covariate adjustments for cardiovascular disease (CVD) risk factors.

Genome-wide association studies (GWASs) and candidate gene studies have identified genetic variants associated with HDL-C levels, at baseline [9,10] and with their changes over time [11,12]. Moreover, a study of genetic risk score with 67 SNPs for HDL-C showed association with parental age-at-death in parents surviving to top 1% extreme longevity (mother 98 years, fathers 95 years) [13]. This finding suggests that HDL-C genetic factors contributing to the increase of HDL-C levels also contribute to longevity. Although many of these genetic factors are known to participate in HDL-C metabolism, their protective effects

in healthy aging, longevity, CVD and mortality are quite complex and still poorly understood [3,4]. Furthermore, it is unclear if these or other genetic factors predict HDL-C level change over time.

The aim of the current study is to identify genetic variants for HDL-C level change in three prospective family-based populations using GWAS. These families were recruited for exceptional survival (Long Life Family Study, LLFS), from community-based (Framingham Family Study, FHS) and enriched by CVD (Family Heart Study, FamHS). The power of association tests in these studies may be enhanced by considering harmonization of the phenotype (HDL-C levels) and imputed genotypes across studies, differences among individuals in the HDL-C levels and in protective genetic effects for atherosclerosis and cardiovascular events. Finally, we used bioinformatics to analyze our genetic variants with respect to their functional consequences and regulatory potential in HDL-C metabolism.

2. Materials and methods

2.1. Study descriptions

We examined change in fasting plasma levels of HDL-C in three large family-based studies. In brief, the Long Life Family Study (LLFS, <https://dsgweb.wustl.edu/llfs/>) was designed to determine genetic, behavioral, and environmental factors related to families of exceptionally healthy, elderly individuals. Families were sampled from four clinical centers: Boston University Medical Center in Boston, MA, Columbia College of Physicians and Surgeons in New York City, NY, the University of Pittsburgh in Pittsburgh, PA, USA, and University of Southern Denmark, Denmark. The characteristics, recruitment, eligibility and enrollment were previously described [14,15]. The study started in 2006 and recruited 4953 individuals in 539 two-generational families that demonstrated clustering for exceptional survival in the upper generation. The second clinical exam (2014–2017) recruited 2933 European descent individuals from 528 families. A total of 2002 individuals with DNA and fasting HDL-C levels in two clinical examinations participated in our study.

The Framingham Heart Study (FHS, <https://www.framinghamheartstudy.org/>) is an observational longitudinal population-based cohort study of Framingham (MA) residents predominately of European descent. Our investigation included participants from the Original (Exams 10 and 11) and Offspring (Exams 1 to 8) cohorts, extending from 1971 to 2008. In brief, the Original cohort (N = 5209) was enrolled in 1948. The children and spouses of the Original cohort comprised the Offspring cohort (N = 5124), who were enrolled between 1971 and 1975. The FHS contributed 3841 individuals, 20 years or older at baseline, with DNA and fasting HDL-C levels available in two to up eight clinical examinations (Supplementary Table 1).

The Family Heart Study (FamHS) began in 1992 with the ascertainment of 1200 families, half randomly sampled, and half selected because of an excess of CVD or risk factor abnormalities as compared to age and sex specific population rates. The collection of phenotypes and covariates as well as clinical examination were previously described [16]. In brief, the families, with approximately 6000 individuals, were sampled from four population-based parent studies: the Framingham Heart Study, the Utah Family Tree Study,

and two centers from the Atherosclerosis Risk in Communities study (ARIC: Minneapolis, and Forsyth County, NC). The participants attended the first clinical exam between 1994 and 1996. The second clinical exam (2002–2004) included 2756 European descent individuals from 510 pedigrees. We excluded 189 individuals from FamHS, who had also participated in FHS. The FamHS contributed 1895 individuals with two clinical examinations to our study.

All individuals provided written informed consents to LLFS, FHS or FamHS. Each study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by each study's institution ethics committee on research on humans.

2.2. Phenotype harmonization

HDL-C levels on fasting samples were determined enzymatically after precipitation of low-density lipoprotein cholesterol (LDL-C) and very-LDL-C with heparin-manganese in FHS-Cohort [17], dextran-sulfate in FHS-Offspring [18] and FamHS [19], and directly from serum in LLFS [20]. HDL-C was natural-logarithm transformed. All HDL-C measures (N = 25,146) from FHS were adjusted for age and sex. To avoid the bias of low HDL-C from FamHS enriched CVD individuals or very high HDL-C from LLFS enriched by healthy elderly people, we harmonized HDL-C levels across the three studies. Age and sex regression coefficients from FHS were applied to adjust HDL-C by age and sex in LLFS (N = 4004) and FamHS (N = 3790), and then the values were standardized (0,1) overall. The three harmonized study data summed to 31,940 measures of standardized HDL-C residuals.

2.3. Growth curve estimation

We performed a growth curve analysis using random coefficient linear mixed model [21] to determine the individual trajectory parameters (intercept and slope) of residual-standardized HDL-C for each person to derive the personal linear trajectory HDL-C change. The growth curve model accounted for both fixed and random change effects. The fixed effect was based on the average of HDL-C change for all individuals from our three studies, while the random effects were based on the deviation of HDL-C change for each individual from the overall mean. The linear mixed model simultaneously estimates interindividual differences in intra-individual systematic changes over time. The model estimates: (i) the functional form of the response trait (HDL-C), which partitions and quantifies the variance across individuals and time interval, (ii) the random effects (individual effects of growth parameters), and (iii) the residual error covariance structure. In brief, the linear mixed model estimates the slope, equivalent to the change in HDL-L levels by change in time, which adjusts for different visit intervals. Linear mixed model allows for different numbers of clinical examination per individual and measurements of different individuals taken at different time intervals [21]. To normalize the HDL-C change distribution (slope), we used blom-normal transformation. We defined time interval as the “year of each exam” minus the “year of first exam”. The growth curve estimation was implemented in SAS@9.4 (PROC MIXED).

2.4. Imputation, quality control and genotyping harmonization

We performed imputation based on 1000 Human Genome (1K-HG, phase-3_v5a) in each study separately. For genotyping, LLFS used Illumina Human_Omni-2.5 M (2,225,478 SNVs), FHS used Affymetrix 500K (500,568 SNVs), and FamHS used Illumina

HumMap_550K (547,353 SNPs), Illumina Human_610-Quadv1 (576,888 SNPs) or Illumina Human_1M-Duov3 (1,111,639 SNPs). For LLFS, FHS and FamHS, quality control was performed before imputation and included assessment of Mendelian errors (LOKI_v.2.4.5.; <https://www.stat.washington.edu/thompson/Genepi/Loki.shtml>) and verification of reported pedigree relationships using GRR (<http://csg.sph.umich.edu/abecasis/grr/>). SNPs were used for haplotype phasing and imputation if: call rate > 0.98; minor allele frequency (maf) > 0.01; no deviation from Hardy-Weinberg equilibrium ($p > 1 \times 10^{-6}$), allelic match with 1K-HG; and present in 1K-HG genotyping. After genotype cleaning, we used 1,737,460 SNPs for LLFS, 298,046 SNPs for FHS, and 906,856 SNPs for FamHS. Imputation was implemented using SHAPEIT (v2.837; https://mathgen.stats.ox.ac.uk/genetics_software/shapeit/shapeit.html) and IMPUTE2 (v2.3.2; https://mathgen.stats.ox.ac.uk/impute/impute_v2.html) software. The Cosmopolitan phased haplotypes were used as reference and dosages were derived from 1K-HG (<ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502>).

We completed genotyping harmonization including common SNPs (maf > 0.01) present in the three studies with imputation quality score > 0.8 and with allele frequency differences < 0.2 from 1K-HG, which resulted in 6,523,511 SNVs across LLFS, FHS, and FamHS. The ancestral principal components were generated in the three combined-studies.

2.5. Association and statistical analyses

We performed GWAS implemented by EPACTS (Efficient and Parallelizable Association Container Toolbox, v.3.2.6) which accounts for dependency among family members as a function of their kinship correlations with Emmax (Efficient Mixed-Model Association eXpedited) algorithm. To adjust for genotype batches and changes in kits and secular trends, we corrected the HDL-C change for differences among our studies by including LLFS, FHS, and FamHS as covariates in the model. Principal components (PC1, PC2, PC3 and PC4) were also added in the linear regression model with additive effect of variants on HDL-C change. We carried out GWAS in the combined- and sex-stratified data. We reported a locus to be independent if our sentinel SNP (*i.e.*, most significant) was in low linkage disequilibrium (LD, $r^2 \leq 0.2$), and at least 500 Kb from any variant identified in previous GWASs at a significance of $p < 5 \times 10^{-8}$, and as a suggestive locus with $p < 1.0 \times 10^{-6}$. We compared the significance of SNP-sex interaction to contrast the regression coefficients (β and SE) between men and women for three suggestive SNPs, using a previous sex-stratified GWAS approach, which accounts for Spearman rank men-women-correlations [22]. The significance level for Bonferroni correction (0.05/3 SNPs) was $p < 0.0167$.

Analysis of variance (ANOVA, using SAS®9.4) was employed to compare the mean levels of (unadjusted and residual) HDL-C, among clinical exams and studies. Regional plot was generated with LocusZoom (<http://locuszoom.org/>) for investigation of LD and block structure based on build-138 (hg19), 1K-HG. LDlink (<https://ldlink.nci.nih.gov/>) was accessed to report LD values.

2.6. Function annotation and biological insights

The annotation used for the biological inference work was sourced from NCBI-dbSNP build-138 (hg19) during the analyses and updated to build-151 (hg38). To query whether candidate loci contained variants associated with gene expression (eQTLs) or physical interactions (Hi-C), we used GTEx-Portal (v.7, <https://gtexportal.org/home/>) and GWAS4D (<http://mulinlab.tmu.edu.cn/gwas4d>). For GWAS4D, the ‘combined p ’ is the final regulatory probability that jointly considers both context-free and context-dependent models. Here, we reported the ‘combined p ’ > 0.10 for hepatocellular-carcinoma cell line.

We also investigated the functional annotations of the sentinel SNPs, and whether they resided in regulatory marks, by analyzing information from ENCODE and ROADMAP initiatives as summarized by HaploReg (v.4.1, <http://www.broadinstitute.org/mammals/haploreg/haploreg.php>) and RegulomeDB (v.1.1, <http://regulome.stanford.edu/>).

Some relevant literature for genes residing within ± 500 Kb of our sentinel SNPs and for cardiometabolic risk factors was sourced from NCBI (<https://www.ncbi.nlm.nih.gov/>), UCSC (<http://genome.ucsc.edu/>), GeneCards (<https://www.genecards.org/>), and NHGRI-EBI (<https://www.ebi.ac.uk/gwas/>).

3. Results

3.1. HDL-C level change over time

A total of 7738 individuals contributed to our study with fasting HDL-C levels. LLFS (N = 2002; interval = 5.5–11.2 years) and FamHS (N = 1895; interval = 6.3–9.6 years) participated in 2-time clinical exams, while FHS (interval = 1–36 years) participated in up-to 8-time clinical exams (Supplementary Table 1). Table 1 shows the characteristics of age and HDL-C levels by studies and clinical exams. There are significant differences in the mean HDL-C levels across exams, within each study and across studies.

After phenotype harmonization, the combined-studies produced 32,940 HDL-C residuals. Fig. 1 depicts the distributions and statistics for HDL-C residuals in each study and in the combined-studies. Compared to the community-based FHS, the healthy-aging LLFS had higher mean HDL-C residual levels while the enriched-CVD FamHS had lower levels ($p < 0.05$). Supplementary Fig. 1 shows the means of HDL-C level change (in mmol/L) per 8 years by study and sex.

3.2. Association analysis of HDL-C level change over time

Supplementary Fig. 2 depicts the quantile-quantile plot with deviation between observed and expected p -values for high-signal SNPs. The estimated GWAS inflation factor (λ_{GC}) for HDL-C change was 1.03, indicating absence of genomic inflation, systematic technical bias or population stratification.

We identified one novel locus at genome-wide significance as shown in Table 2 and Manhattan plot (Supplementary Fig. 3). The A-allele of *GRID1*-rs147116709 (10q23.1-q23.2) was associated with increasing mean levels of HDL-C change ($\beta = 0.47 \pm 0.08$, $p = 5.43 \times 10^{-10}$, Supplementary Figs. 4 and 5). Even excluding three individuals homozygous

for the A-allele for rs147116709, the significant association remained ($\beta = 0.44 \pm 0.08$, $p = 1.37 \times 10^{-8}$). To verify whether the association for HDL-C change was mediated by body mass index (BMI), we also included BMI as covariate in the HDL-C adjustment. The BMI-adjusted HDL-C change effect estimate increased slightly ($\beta = 0.49 \pm 0.08$, $p = 3.83 \times 10^{-10}$), despite the smaller sample with BMI measured ($N = 7419$ versus 7738 total sample).

There were seven novel suggestive loci ($p < 1 \times 10^{-6}$) associated with HDL-C change, which included variants in/near *MBOAT2* (2p25.1, $p = 3.49 \times 10^{-7}$), *LINC01876-NR4A2* (2q34.13, $p = 9.06 \times 10^{-7}$), *NTNG2* (9q34.13, $p = 1.11 \times 10^{-7}$), *CYSLTR2* (13q14.2, $p = 7.09 \times 10^{-7}$), *SYNE2* (14q23.2, $p = 2.39 \times 10^{-7}$), *LINC01314-CTXND1* (15q25.1, $p = 7.51 \times 10^{-7}$), and *CYYR1* (21q21.3, $p = 9.35 \times 10^{-7}$). Regional association plots for these chromosome locations are illustrated in Supplementary Figs. 6A–H. In addition, we detected a suggestive (*ABCA10*, $p = 8.75 \times 10^{-7}$, Table 2) and weak associations (e.g., *APOA1-C3-A4-A5*, $p = 7.95 \times 10^{-5}$; *CMIP*, $p = 1.44 \times 10^{-4}$; *LPL*, $p = 2.64 \times 10^{-4}$; *LIPC*, $p = 4.74 \times 10^{-4}$, Supplementary Table 2) for HDL-C change which were previously reported at genome-wide significance for baseline HDL-C, LDL-C, total cholesterol and/or triglycerides levels [9,10].

3.3. Sex-specific association for HDL-C level change over time

Furthermore, we evaluated the sex-specific effect on HDL-C change. We identified two novel suggestive loci in women but not in men in variants of *DCLK2* (4q31.23-q31.3, $p_{\text{women}} = 5.32 \times 10^{-7}$, $p_{\text{men}} = 5.46 \times 10^{-1}$, Table 3 and Supplementary Fig. 7A) and near-*KCNJ2* (17q24.3, $p_{\text{women}} = 5.91 \times 10^{-7}$, $p_{\text{men}} = 8.97 \times 10^{-1}$, Supplementary Figs. 7B) and a genome-wide significance in women and not in men for *GRID1* (rs147116709: $p_{\text{women}} = 3.26 \times 10^{-8}$, $p_{\text{men}} = 1.54 \times 10^{-3}$). The computed p -values testing for difference between men-specific and women-specific associations [22] were significant ($p < 0.0167$) for *DCLK2* and *KCNJ2*; but, there was no significant heterogeneity of association between sexes for *GRID1*.

3.4. Function annotation and biological insights

NCBI-dbSNP, HaploReg, RegulomeDB, GTEx and GWAS4D were accessed to annotate SNPs with respect to their functional consequence and regulatory potential. The findings provided evidence of regulatory features for several of our identified SNPs. On 14q23.2, besides the sentinel intronic-rs11846882 of *SYNE2* ($p = 2.39 \times 10^{-7}$, Supplementary Table 3), there is an association with the missense-rs45453691 ($p = 6.22 \times 10^{-7}$). The rs11846882 and rs45453691 are in low LD ($r^2 = 0.116$), which suggests two independent genetic variants at the same *SYNE2* locus influencing HDL-C change.

Analyses of the sentinel SNPs and the SNPs in high LD with them ($r^2 \geq 0.8$; 76 SNPs within 11 loci, Supplementary Tables 3 and 4) via HaploReg showed 10 SNPs (4 loci) mapped in conserved syntenic regions by GERP and/or SiPhy, 16 SNPs (5 loci) located in promoter histone marks, 52 SNPs (7 loci) in enhancer histone marks, 28 SNPs (6 loci) at DNase hypersensitive sites, 8 SNPs (4 loci) at protein regulatory binding sites, and numerous SNPs (11 loci) located in regulatory protein binding motifs. Some of the predicted functional regulatory elements include *LXR* (liver X-receptor), which functions as regulator of

cholesterol, fatty acid, and glucose homeostasis. *NFAT1* (nuclear factor of activated T-cells) has an essential role in immune function and in development of the cardiac, vascular, and immune systems. *MZF1* (myeloid zinc finger protein-1) is involved in cardiogenesis. SP1 (specificity protein-1) plays an important role in many cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling. NF-kappaB (nuclear factor-kB) has a role in inflammation, immunity and is associated with aging. Foxa1 (forkhead box) is involved in the differentiation and maintenance of liver, pancreas, thyroid, prostate, lung and neural tissues. *GATA2* is a critical regulator of endothelial cell apoptosis and angiogenesis. *HNF4A* (hepatocyte nuclear factor-4 α) plays an essential role in hepatocyte differentiation and liver function, and influences lipid levels. *AFPI* (α -fetoprotein) is involved in cell-specific transcription of the pre-S1 region of the human hepatitis-B. *HDAC2* (histone deacetylase-2) has an important role in transcriptional regulation, cell-cycle progression and developmental events.

There were also 25 SNPs that were suggested for being eQTLs for target genes from GTEx (Supplementary Tables 3 and 4). The best findings were rs28420313 for regulating *DCLK2* in whole blood ($p = 6.78 \times 10^{-14}$), rs990225 for regulating *FAH* in heart ($p = 6.64 \times 10^{-09}$), and rs7855758 for regulating *NTNG2* in muscle ($p = 5.32 \times 10^{-12}$).

Supplementary Table 5 shows the summary of the prioritized functional SNPs from multidimensional analysis of context-specific regulatory variants in hepatocellular-carcinoma cell (HepG2) from GWAS4D. This analysis indicated that SNP rs201265262 (combined $p = 0.248$, $r^2 = 0.81$ with rs990225) on chromosome 15q25.1 had effect in HepG2, significant transcription regulatory binding affinity change, and Hi-C interaction linking to *LINC01314* locus.

In addition, several of our HDL-C change loci (± 500 Kb of the sentinel SNP) have been reported at genome-wide significance with a series of lipid related conditions. A summary of the biological insights including relevant literature and GWAS results is described in Supplementary Material.

4. Discussion

Several loci have been identified for plasma levels of HDL-C using cross-sectional data. However, it is unclear if these loci predict HDL-C level change over time. To investigate whether genetic variants may influence the variation of HDL-C level change over time in middle-aged to elderly individuals, we performed GWAS in three prospective family-based populations recruited for exceptional survival (LLFS), from community-based (FHS), and CVD risk factors (FamHS). The approach of employing growth curve analysis with linear mixed models allowed us to identify one novel locus (*GRID1*), nine novel suggestive loci (*MBOAT2*, *LINC01876-NR4A2*, *NTNG2*, *CYSLTR2*, *SYNE2*, *CTXND1-LINC01314*, *CYYRI*) which includes two SNP-sex interaction (*DCLK2*, and *KCNJ2*), and some loci (e.g., *ABCA10*, *APOA1-C3-A4-A5*, *CMIP*, *LPL*, and *LIPC*) already reported for baseline HDL-C levels [9,10]. Several of these genetic variants were suggested for being eQTLs and/or residing in functional elements, within DNase I hypersensitivity sites, enhancer and promoter histone marks, which predict enrichment in liver, intestine, brain, heart, among

other cell tissues. Some of these genes also participate in lipid-related signaling and metabolic pathways.

It is known that *GRID1* is a receptor gene involved in synaptic transmission by L-glutamate neurotransmitter and has synaptic function, cell–cell adhesion, and glutamate signaling [23]. It has been reported that *GRID1* is also responsible for methylation differences in liver samples between morbidly obese nonalcoholic fatty liver disease patients and controls [24]. Previous GWAS analysis has identified association between *GRID1* variants with BMI [25]. However, the associated variants for HDL-C change (rs147116709) and BMI (rs7899106) [25] were independents (*i.e.*, in low LD: $r^2 = 0.0099$, $D' = 0.13$). Our association results also remained significant after including BMI as covariate in the HDL-C adjustment, indicating that the *GRID1*- HDL-C association was independent of BMI variation. In addition, *GRID1* SNPs (Supplementary Table 3) have shown a potential function as enhancer histone marks, predicting enrichment in intestine, brain, heart, muscle and/or lung. Previous GWASs have also reported suggestive associations ($p < 10^{-6}$) between *GRID1* with cardiac structure and function [26], pulmonary function [27], and anorexia nervosa [28].

It is worth noting the evidence of the missense-rs45453691, which directly implicates *SYNE2*, as regulator of HDL-C change. *SYNE2* encodes a nuclear outer membrane protein that binds cytoplasmic F-actin. The missense-rs45453691 and other high LD SNPs of *SYNE2* (Supplementary Table 3) are located within DNase I hypersensitivity sites, enhancer or promoter histone marks, which predict enrichment in the intestine, fat, brain, heart, muscle, among other cell tissues. The intronic-rs79535137 in *SYNE2* may likely affect binding sites of three genes (*CEBPB*, *STAT3*, and *POL2*). *CEBPB* is a transcription factor regulating the expression of genes in immune and inflammatory responses [29], having a significant role in adipogenesis, gluconeogenic pathway, liver regeneration, and hematopoiesis. *STAT3* is involved in cell growth and apoptosis, while *POL2* encodes RNA Polymerase-II which is responsible for synthesizing messenger RNA in eukaryotes. *SYNE2* has also shown association at genome-wide significance with sphingolipid levels [30], glycerophospholipid levels [31], atrial fibrillation [32–34], blood metabolites [32,35], pulmonary function [36], cognitive function [37], and depression [38]. Nearby of *SYNE2* is *ESR2* (estrogen receptor 2) gene. The estrogen receptor protein binds to 17 β -estradiol or related ligands that interact with specific DNA sequences to activate transcription [39]. *ESR2* has shown association with metabolic syndrome, lipid levels and insulin resistance, and plays a major role in the cardiovascular system [40].

The steroid-thyroid hormone-retinoid nuclear receptor *NR4A2* gene has been implicated in the preservation of the normal smooth muscle cell and endothelial cell characteristics, and inhibition of macrophage activation, resulting in a reduction of susceptibility to progression of atherosclerosis [41]. A study demonstrated that *NR4A* family members (*NR4A1*, *NR4A2*, *NR4A3*) reduce the uptake of oxidized –LDL as well as the inflammatory response in human atherosclerotic macrophages [42]. *NR4A2* haplotypes were also reported to be associated with both HDL-C levels and blood pressure [41].

Chromosome 9q34.13 harbors genes (*NTNG2*, *MED27*, and *TTFI*) implicated in a large variety of biological processes. *NTNG2* (netrin G2) plays a role in synapse formation and

maintenance. *MED27* (mediator complex subunit) is involved in gene expression regulation and required for activity of the enhancer binding protein SP1. *TTF1* (transcription termination factor) enhances cellular statin sensitivity via perturbing cholesterol metabolism [43]. Significant eQTL associations occurred with SNPs of *NTNG2*, *LINC01314* and *DCLK2* with expression in skeletal muscle, heart and/or whole blood. SNPs of *NTNG2* and *LINC01314* may have function at protein regulatory binding sites of genes in hepatocyte nuclear factors (*HNF4A* and *HNF4G*), zinc fingers (*ZNF143*, *ZBTB7A*, *ZNF263*), among others.

The *CYYR1* gene (cysteine and tyrosine rich-1) has shown association with parental extreme longevity in a large UK Biobank data [13]. The same study also suggested association of HDL-C genetic risk scores with extreme longevity. Other important genes at *CYYR1* locus are the *APP* (amyloid beta precursor protein), *ADAMTS1* and *ADAMTS5* (ADAM metalloproteinase with thrombospondin, type 1 and 5 motifs, respectively). *APP* is implicated in the endocytosis of LDL receptor ligands and is required for proper cholesterol homeostasis and amyloid- β clearance in human astrocytes [44]. *ADAMTS1* and *ADAMTS5* are involved in the development of atherosclerosis. They are mediated by *LRPI* (low density lipoprotein-related protein 1) that has been implicated in aortic aneurysm formation in humans and mice [45]. The *ADAMTS-1* expression is induced by lipopolysaccharide and tumor necrosis factor- α , and significantly reduced in presence of HDL subfraction 3. This may suggest a mechanism by which HDL could have a protective effect on endothelial cell and vascular wall function [46]. In addition, some of our candidate genes are known to participate in signaling and metabolic pathways, including *MBOAT2* for glycerolipid metabolism (<http://pathcards.genecards.org/Pathway/4207>), *NR4A2* for gene expression, dopaminergic neurogenesis, and in p53-microRNA-34 network [47], and *FAH* for tyrosine catabolism pathway [48].

Our study identified loci for HDL-C change which have been previously associated at genome-wide significance with several cell types and traits known to be related with HDL-C levels and metabolism (Supplementary Material). Cell types with which our loci have been associated include vascular smooth-muscle cells, leukocytes, platelets, blood metabolites, adipocytes, skeletal muscle myocytes, pancreatic β cells [31,32,35,49]. The identified loci also play a role in cardiovascular risk factors and events, such as adiposity-obesity, type 2 diabetes, blood pressure, PR interval, sudden cardiac arrest, cardiac repolarization, QT interval, atrial fibrillation, inflammation and immunity [9,10,19,25,26,32–34,50]. Furthermore, they have been associated with personality disorders, such as bipolar disease, schizophrenia, neuroticism, cognitive function, disordered eating, and depression [28,37,38,51], and with biomarkers of health-aging and longevity such as higher cognitive function, relative muscular (handgrip) strength and pulmonary function [13,27,36,52]. These findings together may provide biological insights into protective effects of HDL-C in healthy-aging and longevity.

While elevated levels of LDL-C show consistent evidence for causality to the development of atherosclerotic cardiovascular (ASCVD) [53], genetic studies and clinical trials have declined associations between pharmacologically elevate plasma HDL-C levels with anti-atherogenic effects. It is widely known that HDL-C particles play key roles in lipoprotein

metabolism and have effects on numerous cell types influencing cardiovascular and metabolic health [4]. Studies have also suggested that HDL-C particles may be altered in chronic inflammation disorders, including type 2 diabetes and atherosclerosis [4]. In addition, studies have indicated mechanisms of HDL-C mediating longevity via its role in antiatherogenic, anti-inflammatory, and anti-oxidative functions, which may result in systemic improved endothelial function and decreased inflammation [54]. Lower plasma HDL-C were associated with the increased numbers of neurotic plaques and neurofibrillary tangles [55]. Cross sectional study of aging women demonstrated that high HDL-C levels were associated with better cognitive function [56]. The longitudinal study in elderly women over a 12-year period found that lower baseline HDL-C levels were related to a greater risk of cognitive decline at follow-up than those with high HDL-C levels [57]. These findings suggest the potential functionality of HDL-C particles, mediating oxidative and anti-inflammatory proprieties, may attenuate the development of ASCVD and degenerative disorders. However, the impact of genetic and environmental effects on HDL-C metabolism (in conjunction with other lipoproteins) to its cardioprotective potential and metabolic health remain elusive. A further genetic investigation of HDL-C genes with HDL-C particles and other lipoproteins (*e.g.*, LDL-C) may contribute to evaluate whether exist interindividual differences in clinical course over time or responses to interventions in patients with adverse extreme dyslipidemia.

4.1. Strengths/limitations

Our study employed a unique study design by harmonizing phenotype (HDL-C levels) and genotype imputation across our three prospective family-based populations recruited for exceptional survival, community-based and enriched by CVD, which contributed to gene discovery for HDL-C levels. There are however several limitations that are also worth mentioning. Plasma levels of HDL-C are only part of the metabolic and biochemical complexity of the HDL and may not be the relevant cause of anti-atherogenic effects. We cannot discard that some molecular alterations in the HDL-C, or variations in specific HDL components or even in other lipoprotein components (*e.g.*, LDL-C) involved in lipoprotein metabolism might be underestimated or overlooked on HDL-C level change. Individuals with CVD-related risks or other confounding factors that were not handled in our statistical analyses might also influence our HDL-C change findings. We have not corrected (or excluded) secular trends in women for menopausal status and estrogen use. It remains unclear if or how the dynamic changes in estrogen use over the menopausal transition have longitudinal impact on HDL-C level [58]. In addition, no clear differences were observed in baseline mean HDL-C after adjustment for menopausal status in a large population-based cross-sectional study [59]. We have not corrected (or excluded) individuals taking cholesterol medications or other commonly known medications (*e.g.*, cardiovascular medications, antipsychotics, anticonvulsants, hormones and certain immunosuppressive medications; <https://www.ncbi.nlm.nih.gov/books/NBK326739/>) that may impact genetic factors influencing HDL-C levels. However, a GWAS reported that *CETP* gene may be the only detectable locus with common genetic variants that influence HDL-C response to statins, substantially in individuals of European descent [60]. Other lifestyle conditions (*e.g.*, physical activities, smoking, alcohol and food intake) were also not considered here and may impact HDL-C change. There are however several challenges to handle environmental

exposures (e.g., harmonization, frequency, misclassification, and the timing during a person's life trajectory) in GWAS that may produce spurious associations or lack of association due to the reduction in sample size. Our longitudinal study analysis did not consider the potential effects of mortality selection on HDL-C change. This would require more complex joint modeling of longitudinal change in HDL-C and mortality, which is beyond the scope of this study. Potential effects on HDL-C change could also occur because individuals with only one HDL-C measurement or lack of genotype were excluded from our data. External replication in larger and prospective studies is relevant to validate our findings. The generalizability to other populations and/or ancestry groups needs confirmation since the weighting (β -coefficients) of the associated SNPs for HDL-C change in our European ancestry results can differ in other ethnic groups.

In conclusion, we found one novel loci (*GRID1*) and nine novel suggestive loci, including the causal *SYNE2* gene associated with HDL-C level change over time. These loci linked to several lipid related conditions influencing cardiovascular events and metabolic health may shed light on novel insights into the biology of HDL-C metabolism. Our study extends our understanding of protective effects of HDL-C in healthy-aging longevity metabolism and offers an opportunity to enhance the power of gene detection for complex longitudinal traits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Longitudinal HDL-C level change in prospective family-based populations using GWAS contributed to gene discovery and provided insights into mechanisms of HDL-C regulation.
- The gene discovery for HDL-C level change provided biological insights into protective effects of HDL-C in healthy-aging and longevity.
- The HDL-C level change loci have been reported with biomarkers of health-aging and longevity.
- The identified loci for HDL-C level change suggested to play a role in cardiovascular risk factors.
- Some of HDL-C level change genes have participate in lipid-related signaling and metabolic pathways.

Fig. 1. Distributions of HDL-C residuals by study (A) and three combined-studies (B).

Table 1

Descriptive analysis of age and fasting HDL-C levels by cohort studies and clinical exams.

Study	Exam (dates)	Number of individuals	Age (years)	HDL-C (in mmol/L)		
				Mean (S.D.) ^d	Range	Range
LLFS	1 (2006–2009)	2002 ^a	63.9 ± 11.5	1.56436 ± 0.44289 ^e	36.0–102.0	0.4403 ± 3.4706
	2 (2014–2017)	2002	71.7 ± 11.2	0.90650 ± 0.51023	45.0–110.0	0.4662 ± 4.4807
FamHS	1 (1994–1996)	1895 ^a	50.8 ± 12.9	1.28723 ± 0.37814 ^f	25.8–85.7	0.4921 ± 3.1857
	2 (2002–2004)	1895	58.2 ± 12.8	1.25356 ± 0.36778	33.0–93.0	0.4921 ± 2.8490
FHS Original ^{b, c}	1 (1966–1970)	167	58.4 ± 6.9	1.34680 ± 0.39109 ^e	49.0–77.0	0.5698 ± 2.6936
	2 (1968–1971)	167	59.8 ± 6.9	1.31054 ± 0.37555	51.0–78.0	0.5439 ± 3.0303
Offspring ^c	1 (1971–1975)	3418	36.5 ± 9.3	1.32349 ± 0.38073	20.0–70.0	0.4144 ± 3.0562
	2 (1979–1983)	3048	43.8 ± 9.9	1.26133 ± 0.34447	20.0–77.0	0.3626 ± 3.0821
	3 (1983–1987)	3017	48.1 ± 10.0	1.32867 ± 0.34447	21.0–77.0	0.3626 ± 3.2634
	4 (1987–1991)	3181	51.2 ± 10.0	1.28982 ± 0.38332	22.0–81.0	0.4662 ± 3.3929
	5 (1991–1995)	3270	54.6 ± 9.8	1.29500 ± 0.39109	26.0–82.0	0.3626 ± 3.3411
	6 (1995–1998)	3144	58.6 ± 9.7	1.39342 ± 0.44030	29.0–86.0	0.2849 ± 3.3411
	7 (1998–2001)	3083	61.0 ± 9.5	1.39083 ± 0.44030	33.0–89.0	0.4403 ± 3.8073
	8 (2005–2008)	2651	66.4 ± 9.0	1.48666 ± 0.46879	40.0–92.0	0.5439 ± 4.0404

^a Individuals with HDL-C levels in two clinical exams for LLFS and FamHS.

^b Exams 1 and 2 correspond to 10 and 11, respectively, in Original Cohort FHS data.

^c A total of 3841 individuals with HDL-C levels from FHS Original (167) and Offspring (3,674) with two to up eight clinical exams (Supplementary Table 1).

^d Significant mean HDL-C levels among three studies at $p < 0.0001$, and among exams in each study.

^e $p < 0.0001$;

^f $p < 0.005$. S.D.: standard deviation; Range: minimum and maximum values.

Table 2

Association results for HDL-C level change ($p < 1 \times 10^{-6}$).

SNV	Chr	Pos (b138)	Pos (b151)	Gene	Near gene	Role	A1/A2	Freq (A2)	β (S.E.)	p value
rs2048537	2	9154942	9014813	<i>MBOAT2</i>	<i>KIDINS220</i>		C/T	0.55	0.08 (0.02)	3.49×10^{-7}
rs13032427	2	156926963	156070451	<i>LINC01876</i>	<i>NR4A2</i>	intron	C/T	0.22	0.10 (0.02)	9.06×10^{-7}
rs11243647	9	135033545	132158158	<i>NTNG2</i>	<i>NTNG2</i>		A/G	0.21	0.11 (0.02)	1.11×10^{-7}
rs147116709	10	87839709	86079952	<i>GRID1</i>	<i>GRID1</i>	intron	C/A	0.013	0.47 (0.08)	5.43×10^{-10}
rs369681417	13	49383311	48809176	<i>LOC107984559</i>	<i>CYSLTR2</i>		T*/A	0.02	0.31 (0.06)	7.09×10^{-7}
rs11846882	14	64650306	64183588	<i>SYNE2</i>	<i>SYNE2</i>	intron	G/C	0.02	0.32 (0.06)	2.39×10^{-7}
rs990225	15	80539598	80247256	<i>CTXND1-LINC01314</i>	<i>LOC100996492</i>	intron	C/T	0.71	-0.09 (0.02)	7.51×10^{-7}
rs370479509	17	67181057	69184916	<i>ABCA10</i>	<i>ABCA10</i>	intron	T/TAC	0.19	0.11 (0.02)	8.75×10^{-7}
rs71329836	21	27858938	26486620	<i>CYYR1</i>	<i>CYYR1</i>	intron	AT/A	0.15	0.12 (0.02)	9.35×10^{-7}

Sample size of 7738 individuals. SNV: single nucleotide variant; Chr: chromosome; Pos (138): position in dbSNP build-138 (hg19), Pos (151): position in dbSNP build-151 (hg38); gene, near gene, and role in dbSNP build-151 (hg38) annotation; gene reflects near genes at up to +/-500 kb; A1/A2: non-coded (A1) and coded (A2) alleles; Freq (A2): frequency of coded allele; β (S.E.): beta coefficient (standard error) of SNV

T*: insertion (AGAAACTTCCAACCG).

Sex-specific association results for HDL-C level change.

Sex-stratified association model														
Men (N = 3567)				Women (N = 4171)				Combined (N = 7738)		t-Test ^a (Men × Women)				
SNV	Chr/Pos(151)	Gene (Role)	A1/A2	Freq (A2)	β (S.E.)	p value	Freq (A2)	β (S.E.)	p value	Freq (A2)	β (S.E.)	p value	T value	p diff
rs28549386	4/150168879	<i>DCLK2</i> (intron)	T/C	0.30	0.02 (0.03)	5.46 × 10 ⁻¹	0.30	-0.12 (0.02)	5.32 × 10 ⁻⁷	0.30	-0.06 (0.02)	1.52 × 10 ⁻³	3.84	1.24 × 10 ⁻⁴
rs76062157	17/70634590	<i>LOC105371884-KCNJ2</i>	A/G	0.01	-0.01 (0.11)	8.97 × 10 ⁻¹	0.01	-0.53 (0.11)	5.91 × 10 ⁻⁷	0.01	-0.27 (0.08)	4.82 × 10 ⁻⁴	3.30	9.73 × 10 ⁻⁴
rs147116709	10/86079952	<i>GRID1</i> (intron)	C/A	0.01	0.35 (0.11)	1.54 × 10 ⁻³	0.01	0.58 (0.10)	3.26 × 10 ⁻⁸	0.01	0.47 (0.08)	5.43 × 10 ⁻¹⁰	1.53	1.26 × 10 ⁻¹

^aThe computed p -values (p diff) testing for difference between men-specific and women-specific associations [1]. Bonferroni correction p diff < 0.0167 ($=0.05/3$ SNPs); SNV: single nucleotide variant; Chr: position, gene, and role in dbSNP build-151 (hg38) annotation; A1/A2: non-coded (A1) and coded (A2) alleles; Freq (A2): frequency of coded allele; β (S.E.): beta coefficient (standard error) of SNV.