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Genome-wide Linkage Analysis of Carotid Artery Traits in Exceptionally Long-Lived Families

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Abstract

Background and aims: Atherosclerosis develops with age and is partially controlled by genetics. Research to date has identified common variants with small effects on atherosclerosis related traits. We aimed to use family-based genome-wide linkage analysis to identify chromosomal regions potentially harboring rare variants with larger effects for atherosclerosis related traits.

Methods: Participants included 2205 individuals from the Long Life Family Study (LLFS), which recruited families with exceptional longevity from Boston, New York, Pittsburgh, and Denmark. Participants underwent B-mode ultrasonography of the carotid arteries to measure intima-media thickness (IMT), inter-adventitial diameter (IAD), and plaque presence and severity. We conducted residual heritability and genome-wide linkage analyses adjusted for age, age^2, sex, and field center using pedigree-based maximum-likelihood methods in SOLAR.

Results: All carotid traits were significantly heritable with a range of 0.68 for IAD to 0.38 for IMT. We identified three chromosomal regions with linkage to IAD (3q13; max LOD 5.3), plaque

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AUTHOR CONTRIBUTIONS

LLFS Study Design and Implementation: MAP, ABN, JMZ; Phenotype Data QC: EBM, ALK; Genotypic Data QC: RLM, MWK, MAP; Data Analysis: ALK, MKW, LW, MFF; Primary Manuscript Preparation: ALK, JMZ; Critical Manuscript Review and Approval: All authors

CONFLICTS OF INTEREST

None

severity (17q22-q23, max LOD 3.2), and plaque presence (17q24, max LOD 3.1). No common allelic variants within these linkage peaks were associated with the carotid artery traits.

Conclusions: We identified three chromosomal regions with evidence of linkage to carotid artery diameter and atherosclerotic plaque in exceptionally long-lived families. Since common allelic variants within our linkage peaks did not account for our findings, future follow-up resequencing of these regions in LLFS families should help advance our understanding of atherosclerosis, CVD, and healthy vascular aging.

Keywords

carotid ultrasound; atherosclerosis; genome-wide study; linkage analysis; aging

INTRODUCTION

The risk for atherosclerosis increases with age and is the underlying cause of most coronary artery disease and myocardial infarction. While atherosclerosis is accelerated by poor lifestyle, it is also partially determined by genetics, with heritability estimates ranging from 0.1–0.7.[1] Atherosclerosis commonly manifests as clinical cardiovascular disease (CVD) events; however, it is also possible to assess subclinical atherosclerotic burden using carotid ultrasound,[2] which measures the thickness of the arterial wall (intima-media thickness: IMT) and atherosclerotic plaque presence and burden. Additionally, these images can be used to measure arterial diameter, such as inter-adventitial or lumen diameters (IAD and LD, respectively), which reflect early arterial remodeling in response to increased blood pressure and atherosclerosis.[3]

Genome-wide association studies (GWAS) have identified dozens of common genetic variants with small effects on carotid artery ultrasound IMT and plaque.[4–14] However, in order to identify genetic variants with larger effects, which most likely arise from rare variants, family-based linkage analysis is a more powerful study design.[15] Linkage analysis has been very successful in identifying rare variants associated with inherited Mendelian diseases, but has also been used to identify chromosomal regions throughout the genome with linkage to chronic cardiovascular disease.[16] The Long Life Family Study (LLFS)[17] was designed to harness this analytic approach by recruiting families with exceptional longevity in an attempt to identify novel genetic determinants of successful aging, including vascular aging. Indeed, the LLFS participants have a lower prevalence of heart disease and peripheral artery disease compared with other population cohorts.[17] As such, we conducted a genome-wide linkage study of carotid artery ultrasound traits in 2205 men and women from the LLFS in order to identify chromosomal regions harboring genes and variants that are potentially protective against vascular aging.

MATERIALS AND METHODS

Long-Life Family Study

The Long-Life Family Study is a family-based cohort study of exceptional longevity that recruited families at four study centers (Boston, New York, Pittsburgh and Denmark) based on having 2 or more siblings who were exceptionally long-lived (aged 80+ years in the US

and 90+ years in Denmark). The study also aimed to recruit all other siblings of the long-lived individuals, all spouses, and all offspring. Other characteristics of family eligibility, recruitment, and composition have been previously described.[17, 18] In total, the LLFS recruited 4559 men and women from 2006 to 2009. From 2014 to 2017, surviving participants were invited to take part in an in-home follow-up visit that included imaging of the carotid arteries to determine the burden of atherosclerotic plaque and subclinical CVD. In total, 3198 individuals participated in the follow-up visit (86% of survivors) and we have complete data on genetic markers, common carotid artery wall dimensions, and carotid artery plaque severity in 2205 individuals who serve as the basis of the current analysis. The discrepancy in sample sizes corresponds to participants who did not agree to or could not participate in the carotid exams ($N = 598$), had incomplete carotid data ($N = 227$), or had incomplete genetic data ($N = 168$). Written informed consent was obtained from each LLFS participant using forms and procedures approved by each participating institution's Institutional Review Board.

Carotid Artery Ultrasound Measurement

During their in-home LLFS visit, participants underwent B-mode ultrasound imaging of their carotid artery by centrally trained and certified research assistants using a GE LOGIQ 3 BT12 Ultrasound System (GE Healthcare, Wauwatosa, WI) equipped with a high-resolution linear array variable frequency transducer (12L5 6–10 MHz) with a superimposed simultaneous ECG recording for image standardization.

For this study, we analyzed six variables related to carotid artery wall dimensions or plaque taken from the right and left carotid artery ultrasound images. First, we assessed the mean of the far and near wall common carotid artery intima-media thickness (total CCA IMT), as well as, the far wall CCA IMT (FW CCA IMT) alone. We also measured the inter-adventitial diameter (IAD) and the lumen diameter (LD). All carotid dimensions were calculated as the mean of the right and left side mean values. We also calculated mean of the maximum values for each of these carotid measures, but results were similar to those for mean values (data not shown).

Plaque was assessed centrally at the reading center in two ways, first as a dichotomous variable for plaque presence (yes/no) and, second, as a continuous variable for plaque severity (index). Consistent with the Mannheim and ASE consensus statements,[2, 19] plaque presence was defined as a focal area protruding into the vessel lumen that was at least 50% thicker than the adjacent IMT. The plaque index was calculated as a summation of plaque grade (0 (no observable plaque) to 3 (plaque covering 50% or more of the vessel diameter)) in each of 10 sites (right and left side: proximal CCA, distal CCA, carotid bulb, and proximal internal and external carotid arteries). Therefore, this index has a range from 0 (no plaque) to 30 (plaque in every imaged carotid artery site), with greater indices indicating either more numerous lesions, more occlusive lesions, or both. The range for carotid plaque severity index in the LLFS was 0–20.

All ultrasound technicians performed recertification protocols including at least 10 re-scans per technician in order to ensure longitudinal comparability. Intra-class correlation coefficients for the recertifications were excellent (all >0.93). Reproducibility of IMT

measures was good to excellent with an intra-class correlation coefficient between readers of > 0.96. Repeat plaque assessments of 40 study participants yielded a kappa of 0.88. The plaque index was found to be a valid and reproducible measure of carotid atherosclerosis in a number of our study populations, with intra-class correlations ranging from 0.86 to 0.93.[20]

Genotyping, Linkage Markers, and Imputation

The Center for Inherited Disease Research assayed all LLFS subjects who provided DNA samples and consent using the Illumina Human Omni 2.5 v1 chip. Haplotypes were generated from these genotyped data and used to estimate sex-specific multipoint identity-by-descent estimates in Loki[21] for use in linkage analysis. For analysis of variants under the linkage peaks, genotype data were imputed to the haplotype reference consortium (HRC) imputation panel using the Michigan Imputation Server.[22] The HRC was selected due to its appropriateness for the predominantly European ancestry of LLFS and ability to obtain accurate genotype imputation for minor allele frequencies as low as 0.1%.[23] Additional details of the genotyping, quality control, haplotype generation, and imputation in LLFS have been published.[24]

Statistical Analysis

All carotid ultrasound traits were tested for deviations from normality and transformed as necessary. We estimated residual genetic heritability using the maximum likelihood methods as implemented in SOLAR.[25, 26] All heritability analyses were adjusted for age, age^2, sex, and field center. We used histograms to depict differences in mean plaque severity index distribution by generation.

For genome-wide linkage analyses, the significance of a theoretical quantitative trait locus (QTL) was tested with a likelihood ratio test at 1 centi-Morgan (cM) intervals across each autosomal chromosome. All models were adjusted for age, age^2, sex, and field center to control for demographic differences between families, only. No additional covariates were considered as we wanted to elucidate chromosomal regions harboring genetic variants working to influence carotid artery traits through any and all physiologic pathways. Logarithm of the odds (LOD) scores, computed as the $\log_{10}$ of the likelihood ratio, was used to assess the significance of the test. LOD scores greater than 3.0 and 2.5 were considered to represent genome-wide significant and suggestive evidence for QTLs, respectively. We also performed linkage analysis allowing for heterogeneity between families (HLOD) to refine the linkage peaks.[27]

Lastly, we performed regional genetic association analysis under the HLOD-refined, significant linkage peaks using genotyped data that had been imputed to the HRC reference panel as previously described.[24] We tested variants within the HLOD-refined genomic regions that had ≥ 1% minor allele frequency and an imputation info score >0.8 using lmekin in R (for continuous traits) or GEE in SAS (for dichotomous traits) to assess if common variation within the linkage peak regions was associated with the carotid trait of interest. We used a Bonferrroni-corrected alpha for the number of independent tests under each peak as the significance threshold for these analyses. LocusZoom plots[28] were used to visualize the results of the genetic association analyses. We also evaluated the association of

any previously reported candidate SNPs (or SNPs in linkage disequilibrium (r>0.8) with previously reported candidate SNPs) in these regions with the respective carotid artery traits using an individual alpha of 0.05.

RESULTS

Individuals in the LLFS had a mean age of 72 years, with the proband generation (N = 336) being 92 years and the offspring generation (N = 1869) being 63 years (Table 1). Mean carotid IMT was 0.84 mm and 0.86 mm for the total and far wall measurements, respectively, while the mean carotid diameter was 6.10 mm and 7.81 mm for LD and IAD, respectively (Table 2). While 62% of LLFS participants had carotid plaque, the average plaque severity index (range 0–20) was only 2.3, but was greater in the proband generation than the offspring generation (Supplemental Figure). As expected, plaque burden was higher in the much older proband generation (94% prevalence, 5.3 mean severity index) compared to the offspring (56% prevalence, 1.7 mean severity index; data not shown).

All carotid artery traits were significantly heritable and ranged from 0.38 (far wall IMT) to 0.68 (IAD; Table 2). The strongest evidence for linkage was for carotid IAD on chromosome 3 at 120 cM (LOD = 5.3, 3q13.31–13.32). This same genomic region also showed significant evidence of linkage to carotid LD (LOD = 3.9). We also found evidence for linkage to plaque severity index (peak LOD = 3.2 at 82 cM) and any plaque presence (peak LOD = 3.1 at 96 cM) on chromosome 17. In addition, there was one suggestive peak found on chromosome 20 for plaque severity index (peak LOD = 2.5 at 56 cM). There were no genome-wide significant or suggestive linkage peaks for carotid IMT.

To further refine the genomic regions on chromosomes 3 and 17, we used linkage analysis that allowed for heterogeneity between families (HLOD) and selected the top 15% of families contributing to each trait (Table 3; Figure). In these subsets of families, we were able to narrow the linkage regions to 4.5 Mb on average (range: 2.1–5.7 Mb). The HLOD analysis also resolved the signal on chromosome 17 into two separate regions: one for plaque severity index (HLOD = 9.0, q22–23.3) and one for any plaque presence (HLOD = 7.1, 3q24.3). We tested within the HLOD-refined genomic regions for genetic association with all genotyped and imputed common variants (MAF ≥ 0.01) to identify potential genes/variants that might explain the genome-wide linkage signals (Figure 2). After testing all common variants under the peaks in all families, there were no significant associations between common variants in the linkage regions and carotid artery traits. We did not find any nominally significant associations for previously reported candidate SNPs in these regions (all P-value > 0.05).

DISCUSSION

In a cohort of families recruited for exceptional longevity, we demonstrated moderate to strong heritability for several subclinical measures of carotid atherosclerosis, in line with previous reports.[1] Using genome-wide linkage and association analysis, we identified four chromosomal regions with significant evidence of linkage or association with carotid artery traits. In particular, a region on chromosome 3q13 harbors a locus linked to novel measures

of carotid artery diameter. We also found genome-wide evidence of linkage for two independent regions of chromosome 17 (q22 and q23) for carotid plaques: one associated with the presence of any plaque and the other associated with the severity of the plaque. Further analyses narrowed these genomic regions down to regions as small as 2.1 Mb. Additionally, these analyses identified a subset of families with strong evidence for genetic linkage to carotid artery traits and who, therefore, may be valuable targets for further resequencing to identify rare variants in these regions.

Inter-adventitial diameter demonstrated the strongest evidence for linkage on chromosome 3 between q13.31–13.32 (114–118 Mb), which had a max LOD score of 5.3 in the full sample and 7.8 in the top 15% of families contributing to the linkage signal. This region contains 20 annotated genes (Figure 2a), some of which have been previously identified from GWAS as CVD-related loci. For example, a previous GWAS meta-analysis in >7,000 European adults found a SNP in an intron of *CD80* with suggestive genome-wide association (rs112225152; *P*-value = 6×10^{-6}) with carotid plaque burden (current study *P*-value = 0.06).[10] The *CD80* gene has been shown to be upregulated in coronary artery disease (CAD) plaques.[29] Another GWAS on nearly 300,000 participants from the UK Biobank identified an intergenic SNP in this region with suggestive genome-wide association (rs9840892; *P*-value = 2×10^{-6}) with CAD (current study *P*-value = 0.11).[30] The glycogen synthase kinase 3 beta (*GSK3B*) gene, a known negative regulator of glucose homeostasis[31] and member of the Wnt signaling pathway,[32] also lies within the chromosomal region of this linkage peak. While it is unknown what function this gene may have in determining the largely understudied carotid IAD trait, previous GWAS have reported *GSK3B* as a strong locus for high-density lipoprotein cholesterol concentration[33] and there is ongoing research into the underlying link between Wnt signaling and CVD.[34–36] Given the general lack of strong previous genetic association or candidate genes in this chromosomal region for carotid artery IAD, follow-up of this linkage signal may lead to the identification of a novel regulator of arterial size and remodeling.

Initially, it appeared that chromosome 17 harbored a broad region linked to both any plaque presence and plaque severity index. However, HLOD analysis resolved this signal into two independent linkage peaks on chromosome 17: one for plaque severity index (max LOD 3.2 in all; 9.0 in the top 15% of families) at 17q22-q23.2 (57–62 Mb), and the other for any plaque presence (max LOD 3.1 in all; 7.1 in the top 15% of families) at 17q24.3 (70–72 Mb). The refined linkage peak for plaque severity index contains 42 annotated genes and overlaps with a number of previous GWAS, including two previous LLFS GWAS for leukocyte telomere length[24] and high-density lipoprotein cholesterol.[37] There are many potential biological candidates in the region including *ACE*, *BCAS3*, and *VMP1*, among others. The angiotensin I converting enzyme (*ACE*) gene is a well-described contributor to CVD-related pathophysiology in the chromosomal region and genetic variation in *ACE* has been associated with myocardial infarction.[38–40] The *ACE* gene encodes an enzyme that catalyzes angiotensin I into angiotensin II,[41] which is a potent vasopressor that controls BP.[42] However, other genes in the region may be more closely related to carotid plaque severity, such as the microtubule associated cell migration factor (*BCAS3*), which was identified in multiple previous large GWAS for CAD and myocardial infarction.[30, 43, 44] The vacuole membrane protein 1 (*VMP1*) gene encodes a transmembrane protein that regulates

autophagy[45] and has been associated with idiopathic cardiomyopathy[46] and lipoprotein-associated phospholipase A2 activity and mass.[47] There was no significant association between common variation in this chromosomal region with carotid plaque severity, so additional signal refinement and re-sequencing are needed to determine the causal variant(s).

The second region on chromosome 17, which was in linkage with any plaque presence, contains 29 annotated protein coding genes. There were two previously published genome-wide associations for CVD-related traits within this region: coronary artery calcification (CAC)[48] and sudden cardiac arrest.[49] A prominent candidate is SRY-box 9 (*SOX9*), a DNA-binding protein that regulates the transcription of a number of genes, has been linked to cardiac fibrosis,[50] stroke,[51] and aortic valvular calcification.[52] The previous CAC GWAS was a meta-analysis in nearly 6,000 African Americans that found a significant association between a SNP 0.6 Mb upstream from *SOX9* and coronary artery calcification (rs9907236; current study *P*-value = 0.272).[48] However, we did not find significant associations with common variation in this genomic region after adjustment for multiple comparisons. The previous GWAS of sudden cardiac arrest identified a significant association with an intergenic SNP upstream of *CASC17* (rs17718586; current study *P*-value = 0.387),[49] which may suggest there is more than one potential CVD-related variant or locus in this chromosomal region. Thus, further follow-up is needed to determine the causal variant(s) in our families.

A previous linkage analysis[53] reported loci with suggestive evidence of linkage to carotid IMT, including a region on chromosome 17. However, the chromosome 17 regions from the current analysis (80–83cM and 94–97cM) did not overlap with the loci from this previous report (24–31cM). The previous report also highlighted regions on chromosomes 2, 4, 6, 7, 13, and 14 with suggestive evidence of linkage to carotid IMT, but none of those regions overlapped with significant or suggestive loci from the current analysis of any carotid ultrasound trait. As such, we believe the results from the current study may represent genomic regions containing novel variants unique to the LLFS. We are currently sequencing the regions of interest. The family study design of the LLFS will be particularly useful in prioritizing rare variants that segregate within families and with our carotid traits of interest.[16]

The top families identified from this analysis had somewhat healthier carotid arteries than the LLFS sample overall (any plaque: 55% in top families vs. 62% in total sample; plaque severity: mean 1.96 in top families vs. 2.29 in total sample), suggesting that the LLFS may harbor genetic variants responsible for healthy vessel aging. The LLFS was designed to study exceptional longevity and, as such, includes individuals who are somewhat healthier and with less disease variation than their same-aged counterparts, though they still harbor varying degrees of vascular and other disease. In the subset of the healthiest individuals (e.g., those not on lipid-lowering or anti-hypertensive medications), suggestive linkage peaks remained in the same chromosomal locations, even though this analysis excluded 750–980 individuals of medications (data not shown). Nonetheless, we present significant residual genetic heritabilities and genomic loci in linkage with carotid ultrasound traits. Ultimately, we believe this study is strengthened by both the family design – to detect rare variant

effects – and the exceptional longevity design – to research determinants of healthy vessel aging.

In summary, we investigated whether exceptionally long-lived families harbor novel genetic variants for carotid artery traits. In these families, we identified three significant chromosomal regions, all of which contained some genes with previous evidence of association to CVD and atherosclerosis. Follow-up studies of these chromosomal regions and families may advance our understanding of atherosclerosis and CVD etiology, which could lead to future therapeutic options conferring increased longevity with a healthy vasculature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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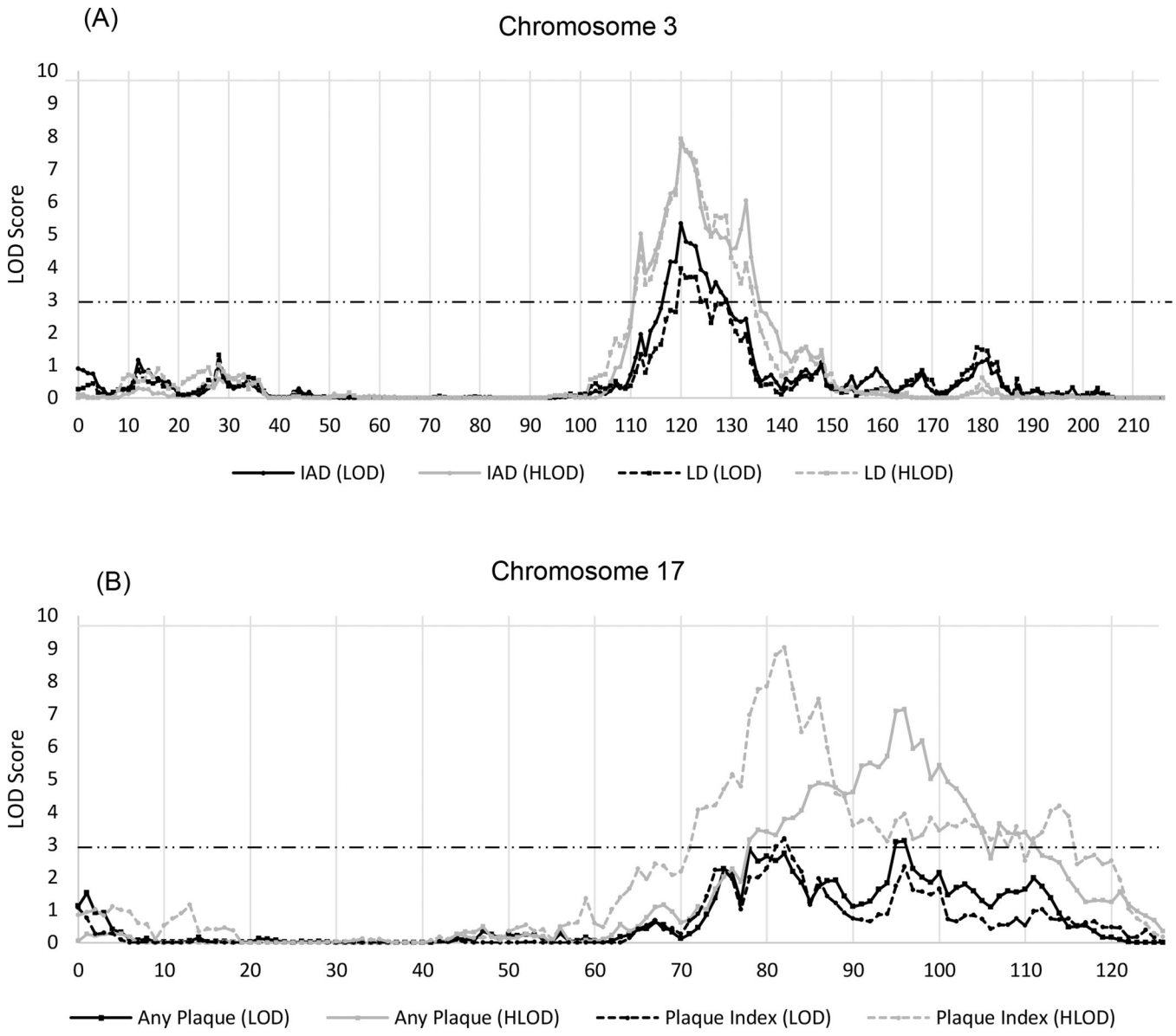

Figure 1. Chromosomes with Significant Evidence of Linkage to Carotid Artery Traits

LOD (black lines) and HLOD (grey lines) scores for carotid artery traits with suggestive or significant evidence of linkage are plotted by centi-Morgan (cM) location for the chromosomes containing the peaks of interest. (A) Chromosome 3 includes plots for IAD (solid lines) and LD (dashed lines). (B) Chromosome 17 includes plots for any plaque presence (solid lines) and plaque severity index (dashed lines). Each plot includes a staggered dashed horizontal line at a LOD of 3.0 representing the threshold for genome-wide significant evidence of linkage.

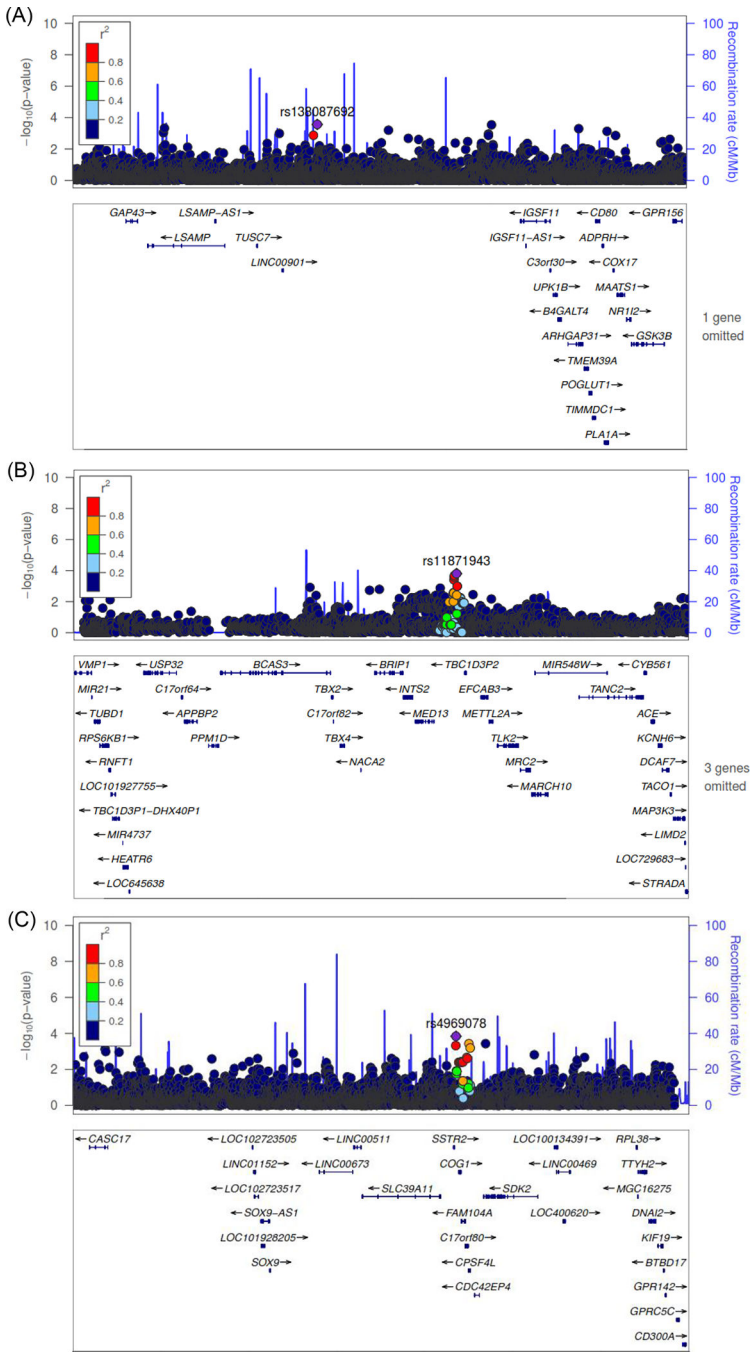

Figure 2. Plots of the Genetic Association Results from Analyses Performed Under the Significant Linkage Peaks for Carotid Artery Traits

Presented are LocusZoom plots for the refined chromosomal regions with significant evidence of linkage to: (A) IAD, (B) plaque severity index, and (C) any plaque presence. All plots depict the respective chromosomal region with genes annotated on the bottom. The genetic association results are plotted as the $-\log_{10}(p\text{-value})$ [left Y-axis] for all tested SNPs in the chromosomal region with the most significant (index) SNP shown as a purple diamond. Also depicted are the linkage disequilibrium values between the index SNP and all

other tested SNPs in the region [see legend] and the recombination rate (cMMb) [right Y-axis].

Table 1.

Characteristics of the LLFS Participants in this Analysis

Characteristic[a]	Overall (*N* = 2205)[b]	Proband Generation (*N* = 336)	Offspring Generation (*N* = 1869)
Age (years)	72.0 ± 11.6 (42–108)	92.4 ± 7.0 (56–108)	63.4 ± 7.8 (42–93)
Female Sex (%)	55.7	59.2	55.0
Study Site (%)			
Boston	24.9	20.2	25.8
New York	18.1	33.3	15.3
Pittsburgh	26.5	35.7	24.9
Denmark	30.5	10.7	25.8

[a]Age is shown as mean ± SD (range), while the other values are shown as *N* (%)

[b]Total number of individuals is 2205, which corresponds to 5184 relative pairs.

Table 2.

Residual genetic heritability and genome-wide linkage results for carotid artery traits

Trait	Mean (SD) or %	Residual Genetic Heritability Analysis[a]			Peak LOD[b]: chr (cM)
		h^2_r	*P*-value	Variance explained by covariates	
Total CCA IMT (mm)	0.86 (0.16)	0.499	1×10^{-17}	0.388	-
FW CCA IMT (mm)	0.84 (0.19)	0.376	6×10^{-11}	0.310	-
IAD (mm)	7.81 (0.89)	0.683	1×10^{-27}	0.350	**5.3: 3 (120)**
LD (mm)	6.10 (0.73)	0.593	2×10^{-21}	0.233	**3.9: 3 (120)**
Any Plaque (%)	61.7	0.402	4×10^{-5}	0.150[c]	**3.1: 17 (96)**; 2.8: 17 (78)
Plaque Index	2.3 (3.0)	0.447	2×10^{-13}	0.299	**3.2: 17 (82)**; 2.5: 20 (56)

[a] All heritability and linkage models were adjusted for age, age^2, sex and field center.

[b] Only peak LODs ≥ 2.5 are shown for each trait and are **BOLD** if ≥ 3.0 representing genome-wide significance.

[c] Covariate R^2 is shown as an estimate of the variance explained by covariates for the dichotomous model of any plaque.

Table 3.

Summary of Genome-wide Linkage Peaks of Interest for Carotid Artery Traits in LLFS

Trait with Max LOD	Full Sample			Trait-Specific Top 15% of Families Only						
	Peak LOD[a]	Chr	cM [range][c]	Peak HLOD[a]	N[b]	Chr	Cytogenetic	cM [range][c]	Basepair Range (Mb)[c]	Genes under Peak (#)[d]
IAD	5.3	3	120 [113–134]	7.8	23; 214	3	q13.31-q13.33	120 [119–123]	114.6–120.3	20
Plaque Index	3.2	17	82 [72–89]	9.0	24; 211	17	q22-q23.3	82 [80–83]	57.7–63.9	71
Any Plaque	3.1	17	96 [90–106]	7.1	26; 263	17	q24.3	96 [94–97]	70.6–72.7	3
Plaque Index	2.5	20	56 [49–71]	6.5	27; 219	20	q12-q13.12	56 [52–57]	40.7–44.7	24

[a] All linkage analyses were adjusted for age, age2, sex, and field center.

[b] Numbers shown for each HLOD analysis as the number of families; number of individuals.

[c] Linkage peak location defined as the position of the peak LOD − 1.0. Mb values are based on sex-averaged cM map and translated into locations on the human genome using assembly version GRCh38.

[d] Known protein coding genes from NCBI RefSeq annotation release GCF_000001405.37_GRCh38.p11.

Chr: chromosome; cM: Centi-morgan; Mb: mega-basepairs