DETAILED MATERIALS AND METHODS

Participants

A case-control study was designed from among 87,980 participants in the Copenhagen General Population Study (CGPS), a general population study initiated in 2003 that is actively recruiting subjects. CGPS examinations included a self-administered questionnaire reviewed by an investigator on the day of attendance, a physical examination, and blood sampling including a sample for DNA analysis. Smokers were active smokers, and diabetes mellitus was diagnosed if self-reported, if insulin or oral hypoglycemic drugs were used, or in the presence of a non-fasting plasma glucose of >11 mmol/L. Body mass index was weight in kilograms divided by height in meters squared.

For the present study, we included all CGPS participants diagnosed with CAVD from 1977 until 2013, and additionally for each CAVD case participant, two matched control participants, dependent on available blood samples (N=2138). LPA KIV-2 genotype was available on 2129 and rs10455872 genotype on 2132 of these participants. CAVD was defined by International Classification of Diseases, 8th edition (ICD-8), codes 424.10, 424.12, 424.18, 424.19, and 10th edition (ICD-10) codes I35.0 and I35.2 and ascertained from the national Danish Patient Registry, and the national Danish Causes of Death Registry; public registers to which all hospitalizations and deaths in Denmark have been reported since 1977 (with outpatients and emergency treatments included from 1995). CAVD cases in the present study were diagnosed from 1982 to 2013, with the large majority diagnosed according to ICD-10 criteria using Doppler echocardiography and according to standard diagnostic criteria following international guidelines (peak transvalvular velocity >2.5 m/s, calculated aortic valve area <2.0 cm2). Controls were free of ischemic cardiovascular disease to exclude undiagnosed early CAVD mistaken for atherosclerotic disease, as symptoms of CAVD including chest pain, palpitations and shortness of breath are also all found in heart disease resulting from ischemic cardiovascular disease and atherosclerosis. Controls were matched to CAVD cases on sex, age (5-year strata), and recruitment-time (1 month strata), and selected randomly by computer from all CGPS participants free of cardiovascular disease at the end of follow-up in 2013.

The CGPS was approved by Herlev Hospital and by a Danish ethical committee, and was conducted according to the Declaration of Helsinki. Participants gave written informed consent.

Laboratory analyses

For the present study, measurements of OxPL-apoB and OxPL-apo(a), as well as lipoprotein(a) measurements were performed on blood samples stored at -80°C Celcius from the date of participant recruitment. Importantly, cases and controls were matched on recruitment date (1 month strata) to ensure comparable storage time of samples.

OxPL-apoB and OxPL-apo(a) levels were measured with a chemiluminescent immunoassay using the murine monoclonal antibody E06 that recognizes the phosphocholine (PC) group on oxidized but not on native phospholipids. E06 similarly recognizes the PC covalently bound to bovine serum albumin (BSA) in PC-BSA. A 1:50 dilution of plasma in 1% BSA in TBS was added to microtiter wells coated with the apoB-100 specific monoclonal antibody MB47, which binds a saturating amount of apoB-100 to each well, and biotinylated E06 was then added to determine the content of OxPL-apoB. These values are reported as nanomolar (nmol/L) PC-OxPL using a standard curve of nM PC equivalents, as recently described. Because each well contains equal numbers of apoB-100 particles, the OxPL-apoB value reflects the absolute content of OxPL per a constant amount of captured apoB lipoprotein. It thus represents an OxPL-apoB value that is independent of plasma levels of apoB-100 or of LDL cholesterol. Furthermore, the assay detects only the subset of OxPL detected by antibody E06 and not all species of OxPL. In prior studies.
this variable was expressed as OxPL/apoB, reflecting the fact that this measure quantitates the number of OxPL moles per unit mass of apolipoprotein B-100 present on microtiter well plates (and not the level in the circulation). The nomenclature is now changed to OxPL-apoB to minimize confusion that this measure represents a ratio of OxPL divided by plasma levels of apoB. Within-person 5-year reproducibility of frozen samples has been shown to be high (r=0.78) and pilot-tests showed that OxPL-apoB levels are stable over 24 hours on ice (intraclass correlation coefficient 0.96) as well as frozen samples stored under long term conditions. OxPL-apo(a) levels were measured in an analogous manner to OxPL-apoB, except that the capture antibody, LPA4, which detects apolipoprotein(a) was used to capture apo(a). The wells were coated with anti-apo(a) antibody, LPA4, plasma was added to saturate the plate with apo(a), and OxPL were then measured on the captured Lp(a) by the use of antibody E06, which only reacts with OxPL and not with the LPA4 antibody. The values are also reported in nmol/L. Please note that the absolute amounts of apoB-100 and apo(a) captured on the wells in the respective assays are different and therefore one can not directly compare the absolute OxPL-apoB and OxPL-apo(a) values.

Lipoprotein(a) total mass was measured on the same frozen samples using the commercial immunoturbidimetric, isoform-insensitive Denka Seiken assay (Denka Seiken, Tokyo, Japan).

At the time of initial CGPS recruitment and blood sampling, enzymatic assays were used on fresh samples to measure plasma levels of total cholesterol and high-density lipoprotein cholesterol. Likewise, high-sensitivity C-reactive protein (hs-CRP) was measured on fresh samples using turbidimetry (Dako) or nephelometry (Dade Behring), and creatinine was measured using the Jaffe method with subsequent estimation of the glomerular filtration rate (eGFR) ad modum CKD-EPI.

The LPA KIV-2 repeat polymorphism was genotyped by real-time PCR analysis on the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) or on the CFX384 Real-time System (Bio-Rad) platform yielding comparable estimates of the sum of repeats on both alleles. Genotyping resulted in an estimate of the total number (sum of repeats on both alleles) of KIV-2 repeats. The single-copy gene albumin was used to normalize for different concentrations of DNA in different samples. Reactions were performed in 10 µL final volume, using 1xTaqMan Universal PCR Master Mix (Applied Biosystems), 900 nmol/L primers, and 200 nmol/L probe. Primer sequences were as follows: KIV-2 for-ward 5’-ATCCAGATGCTTGCGAGCT-3’, KIV-2 reverse 5’-GGACGGAGCAGTTCCCTTCT-3’, albumin forward 5’-ACACGCTTTGGCACAATG-3’, albumin reverse 5’-CCCTGGAATAAGGCCGAGCTAA-3’. The sequence for the FAM labeled KIV-2 probe was 5’-CAACCTGACGCAATGC-3’, while the sequence for the VIC labeled albumin probe was 5’-TGGGTAACCTTTATTTCTCCATC-3’. All samples were run in duplicate for both the KIV-2 assay and the albumin assay. A discrepancy of more than 0.25 in Ct value (threshold cycle of the PCR) for duplicate samples for either the KIV-2 or the albumin assay resulted in a rerun of that sample. The LPA rs10455872 SNP was TaqMan genotyped and in Hardy-Weinberg equilibrium. SNP carriers were minor allele homozygotes and heterozygotes combined; 0.4% and 16%, respectively.

Statistical analyses

We used Stata SE 13.1. A two-sided p<0.05 was considered significant. Kruskal-Wallis and Chi-square tests were used to compare continuous and categorical variables. Spearman’s rank correlation coefficient was used to estimate the linear associations between OxPL-apoB and OxPL-apo(a) and lipoprotein(a) levels. One-way analysis of variance (ANOVA) was used to estimate the contribution of the LPA genotypes to the variation in OxPL-apoB, OxPL-apo(a), and lipoprotein(a) levels (all log transformed due to skewness of the distributions). Cuzick non-parametric test for trend was used to test for differences in OxPL-apoB, OxPL-apo(a), and lipoprotein(a) levels across LPA genotypes.
For further analyses, participants were divided into groups based on tertiles of OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels, and with further top tertile stratification to better examine the risk associated with extreme levels (i.e. 90th-95th and >95th percentiles). Due to the lack of standardization of Lp(a) assays, results are reported according to percentile cutpoints (and mg/dL) to ensure comparability with other studies using different Lp(a) assays and consistent with common practice. For genetic analyses, participants were divided into groups based on KIV-2 repeat percentile groups corresponding to plasma levels percentile groups, or based on rs10455872 carrier status.

We used conditional logistic regression analyses to estimate odds ratios with 95% confidence intervals. Cases and (if possible) two controls were matched perfectly for sex, age (5-year strata), and recruitment time (1 month strata), the latter to ensure similar storage time for samples from cases and controls. Analyses included only appropriately matched cases and controls. In multivariable adjusted analyses, we additionally adjusted for cardiovascular risk factors, i.e. total cholesterol, high-density lipoprotein cholesterol, systolic blood pressure, body mass index, estimated glomerular filtration rate (eGFR), high-sensitivity C-reactive protein (hs-CRP), smoking, and diabetes mellitus. Total cholesterol values were adjusted for the lipoprotein(a) contribution, as done previously. Further, to avoid possible non-linearity in the logit, continuous covariates were separated into eight categories. Information on covariates adjusted for were ~98% complete. For the relatively few participants who lacked information, continuous covariate values were imputed using multiple imputation based on age and sex, and for categorical variables a category for missing was defined. If only individuals with complete data were included, results were similar to those presented. We found no evidence of interaction with sex for Lp(a), OxPL, or LPA measurements on risk of AVS when comparing models with and without 2-factor interactions using maximum likelihood ratio tests; thus, results are presented combined for men and women to maximize statistical power.

Instrumental variable analysis (i.e. a Mendelian randomization analysis integrating the association of LPA genotypes with OxPL-apoB, OxPL-apo(a), or lipoprotein(a), and the association of genotypes with risk of CAVD) based on each of the 2 LPA genotypes separately, or based on the genotypes combined, was used to estimate causal relative risk estimates of CAVD for a doubling in OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels. We used an individual participant data approach, thus including only participants with complete information on genotypes and OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels. First, we estimated the strength of the genotypes as an instrumental variable (i.e. the association of genotype with OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels) by conducting a least-squares regression of levels on genotypes and examining the F-statistics, where F>10 indicates sufficient statistical strength (F-values ranged from 155 to 1086). Second, a causal relative risk was estimated using the multiplicative generalized method of moments estimator implemented in the user-written Stata command "ivpois", which for a binary outcome (i.e. CAVD) estimates the causal relative risk for a unit change in phenotype (i.e. a doubling in OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels). For comparison with genetic estimates, we also estimated the observational multivariable adjusted odds ratio of CAVD for a doubling in OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels.
REFERENCES


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