Haptoglobin Genotype Does Not Confer a Risk of Stroke in Type 1 Diabetes

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Haptoglobin genotype does not confer a risk of stroke in type 1 diabetes

Short title: Haptoglobin genotype in stroke

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Abstract

The exon copy number variant in the haptoglobin gene is associated with cardiovascular and kidney disease. For stroke, previous research is inconclusive. We aimed to study the relationship between haptoglobin Hp1/2 genotype and stroke in individuals with type 1 diabetes from the Finnish Diabetic Nephropathy Study. We included two partially overlapping cohorts: one with haptoglobin genotypes determined using genotyping for 179 stroke cases and 517 matched controls, and the other using haptoglobin genotype imputation for a larger cohort of 500 stroke cases and 3,806 controls. We observed no difference in the Hp1-1, Hp2-1, and Hp2-2 genotype frequencies between the stroke cases and controls, neither in the genotyping nor the imputation cohorts. Haptoglobin genotypes were also not associated with the ischemic or hemorrhagic stroke subtypes. In our imputed haptoglobin cohort, 61% of individuals with stroke died during follow-up. However, the risk of death was not related to the haptoglobin genotype. Diabetic kidney disease and cardiovascular events were common in the cohort, but the haptoglobin genotypes were not associated with stroke when stratified by these complications. To conclude, the Hp1/2 genotypes did not affect the risk of stroke or survival after stroke in our type 1 diabetes cohort.

Type 1 diabetes markedly increases the risk of stroke. This increased risk is observed already at a young age (1) and is mostly driven by the presence and severity of diabetic kidney disease (DKD) (2). Additionally, individuals with type 1 diabetes have a worse prognosis after a stroke (3).

Haptoglobin (Hp) is an abundant plasma protein that binds free hemoglobin released from lysed red blood cells. Thus, haptoglobin both prevents iron loss and protects the cardiovascular system from oxidative heme-bound iron (4). A copy number variant (CNV), rs72294371, in the haptoglobin gene on chromosome 16q22 affects the haptoglobin protein multimerization; haptoglobin circulates as a dimer in individuals homozygous for the minor Hp1 allele, whereas the major Hp2 allele enables the formation of trimers, tetramers, and even six-meres (5). The haptoglobin-hemoglobin complex formed from the Hp2-2 genotype is cleared slowly from the bloodstream, thus, Hp2-2 has weaker antioxidative properties (5). Hp2-2 has also been linked to cholesterol transport defects (6) and HDL dysfunction (7). Most importantly, Hp2-2 is associated with increased risk of cardiovascular disease (8,9), kidney disease (10), and mortality (11) in individuals with diabetes and in the general population (12). However, some studies have showed less convincing or contradictory results (13,14).

Regarding stroke, it remains inconclusive whether the Hp-alleles are associated with stroke, and which allele increases the risk. For example, a study of 124 lacunar stroke cases (*i.e.*, type of cerebral small-vessel disease) and 918 controls found an association between Hp1-1 genotype and stroke in the general population (15), but a study in 378 Finnish individuals with stroke and 1,426 population controls found no excess risk of stroke related to the Hp1-1 (16). On the other hand, stroke survivors with the Hp2-1 or Hp2-2 genotypes had increased cardiovascular mortality (16). In the Finnish 2000 Health survey cohort, Hp2-2 was associated with the pooled cardiovascular disease phenotype, including stroke and transient ischemic attack (TIA) (12). Furthermore, a meta-analysis of studies in individuals with diabetes suggested Hp2-2 as a stroke-risk genotype (17). In contrast, a recent study in 316 individuals with and without diabetes showed no association between Hp-genotypes and

lacunar infarctions (18). The only study conducted in individuals with type 1 diabetes included 607 subjects from the Epidemiology of Diabetes Complications (EDC) Study and suggested Hp1-1 as a risk factor for incident stroke (n=33), but only in individuals with hypertension (19).

Here, we aimed to assess the relationship between the Hp-genotypes and stroke, taking advantage of our well-characterized cohort of 194 incident stroke cases (2). Additionally, we imputed the Hp-alleles to extend the analyses to a larger cohort of 4,345 individuals with type 1 diabetes, including 505 individuals with stroke.

Research Design and Methods

All study participants are part of the prospective nationwide Finnish Diabetic Nephropathy (FinnDiane) Study, initiated in 1997. We analyzed two partially overlapping cohorts: a smaller haptoglobin genotyping cohort and a larger haptoglobin imputation cohort. At baseline, data collection occurred at a regular clinical visit that included for example anthropometric measurements and a thorough review of the individuals' medical history. Additionally, blood and urine samples were collected. For the Hp1/2 imputation cohort, we identified strokes from the Finnish Care Register for Health Care and the Finnish Cause of Death Register (ICD codes in **Supplementary Table 1**)**.** Additional information was gathered from death certificates, FinnDiane visits, and medical files, and all individuals with TIA were excluded. For the Hp1/2 genotyping cohort, the DKD status was assessed based on albumin measurements from two of three timed overnight or 24-hour urine collections. For the Hp1/2 imputation cohort, we retrieved data on kidney failure from the Finnish Care Register for Health Care until the end of 2017, and the data were further complemented with the data from FinnDiane baseline or prospective visit questionnaires or medical files. Data on coronary artery disease (CAD) were gathered from the Finnish Care Register for Health Care and the Finnish Cause of Death Register (**Supplementary Table 1**) or from the FinnDiane study visit questionnaire

(Hp1/2 genotyping cohort). Mortality data by the end of 2017 were obtained from the Finnish Cause of Death Register. The ethics committee of the Helsinki and Uusimaa Hospital District approved the FinnDiane study protocol, which followed the Declaration of Helsinki. All study participants signed an informed consent.

Genotyped Hp1/2 cohort. To select individuals for the Hp1/2 genotyping, we identified 4,173 FinnDiane participants who had type 1 diabetes defined as diabetes diagnosis before the age 40 and insulin treatment initiated within a year from diagnosis, were free of a history of stroke at baseline visit (between years 1997 and 2011), and had their DKD status defined (**Fig. 1**). We identified from the Finnish Care Register for Health Care and death certificates 194 subjects with an incident stroke during follow-up between years 1997 and 2013. All strokes were further verified from medical files and brain imaging by a stroke neurologist (J.P.). Altogether 137 (71%) were ischemic and 57 (29%) were hemorrhagic strokes. These cases had been part of a study described in detail previously (2). We then matched 540 control subjects for age, sex, DKD status, and presence of CAD at baseline (**Fig. 1**). **Supplementary Table 2** presents the characteristics of the matched control subjects compared to those not included in the present study. The group of individuals not included were younger, had lower blood pressure and less diabetic micro- and macrovascular complications, and included fewer males, compared with the matched controls.

Imputed Hp1/2 cohort. We required quality-controlled genome-wide genotyping data, genotyped with the Illumina HumanCoreExome chips and preprocessed at the University of Virginia (20), to be available for the individuals selected for cohort for Hp1/2 imputation. In addition to the stroke cases available from the incident stroke cohort, we identified more cases amongst those who had a stroke either before the FinnDiane baseline visit or until year 2017 according to the Finnish Care Register for Health Care**.** We verified 69.9% of the stroke cases from medical files, 8.4% from the study visit questionnaires, and register data was used for the remaining 21.7%. The familial relationships in the

cohort were calculated with KING v.1.3 software. We included only one individual from the first-degree relatives, prioritizing the relative with a stroke, the parent from the parent-offspring-pair, or randomly one of the siblings. The controls were required to have diabetes duration >10 years, age >35 years, and no history of stroke. Individuals with traumatic brain hemorrhages reported in the FinnDiane visit questionnaires or medical files were excluded from the cases and controls. Altogether, the full phenotypic data were available for 505 stroke cases and 3,840 controls (**Fig. 1**). Additionally, we analyzed four subgroups: individuals with I) no CAD or kidney failure (*n*=3,080, including 157 stroke cases), II) kidney failure (*n*=369, including 86 stroke cases), III) CAD (*n*=436, including 57 stroke cases), or IV) individuals with both CAD and kidney failure (*n*=266, including 45 stroke cases).

Hp genotyping. The Hp CNV significantly affects the haptoglobin gene length. The Hp1 allele contains five exons *vs.* seven exons in the Hp2 allele. This difference is detectable with two polymerase chain reactions (PCRs). Thus, we genotyped the CNV with a PCR method similar to Ijäs and colleagues (12) (**Supplementary Table 3**). The PCR amplicons were analyzed on an agarose gel or with Caliper LabChip GX instrument (PerkinElmer, MA, USA) at the Finnish Institute of Molecular Medicine to read the Hp1-1, Hp2-1, or Hp2-2 genotypes from the gel figures or Caliper results.

Hp imputation. Boettger and colleagues (21) developed an imputation protocol for the Hp CNV. This imputation method can also separate the "S" and "F" alleles (Hp1S, Hp1F, Hp2FF, Hp2FS, and Hp2SS) that differ in a few amino acid residues and cause haptoglobin protein to run slower (S) or faster (F) in the gel electrophoresis (22). The imputation reference panel comprised 274 unrelated individuals of European origin from the 1000 Genomes Utah residents (CEPH) with Northern and Western European ancestry (CEU), Iberian populations in Spain IBS, and Tuscans from Italy (TSI) populations. The panel included 1,277 variants (chr16:71,088,193–73,097,663; GRCh37/hg19) genotyped with Illumina OMNI 2.5 SNP array, and the variants in the Hp CNV region

(chr16:72,090,310–72,093,744) had been replaced with Hp1/2 alleles determined with droplet-digital PCR. We renamed some variants with the corresponding rs-number (*e.g.*, SNP16-69,664,602 to rs144319423). Thereafter, we used PLINK v.1.9 to extract genotyped variants located at chr16:72,090,310–72,093,744, with Hardy–Weinberg equilibrium (HWE) $P<0.001$ and >0.90 genotyping success rate for variants and individuals from our GWAS data. We converted the 146 variants overlapping with the imputation reference panel to Beagle-format (.bgl) with PLINK and imputed the Hp1/2 alleles with Beagle 3.2 software similar to Boettger *et al.* (nsamples=15, niterations=15, maxwindow=2000). The median imputation info-score was >0.99 for all alleles Hp1S, Hp1F, Hp2FS, and Hp2SS, whereas the rare HP2FF allele was absent. The Hp1/2 genotypes (Hp1-1 = Hp1S-Hp1S, Hp1S-Hp1F, or Hp1F-Hp1F; Hp2-2 = Hp2SS-Hp2SS, Hp2SS-Hp2FS, or Hp2FS-Hp2FS; Hp2-1 = Hp1S-Hp2SS, Hp1S-Hp2FS, Hp1F-Hp2SS, or Hp1F-Hp2FS) were coded for 500/505 stroke cases and 3,806/3,840 controls (total 99.1% success rate), with both Hp1/2 alleles imputed with ≥0.70 probability. With an expected Hp1 allele frequency of 38% in the Finnish population (16) and $P=0.05$, we can detect an OR of 1.54 between the cases and controls or HR=1.20 in a prospective analysis, with 80% power.

Statistical analyses. We aimed to conduct both time-to-event analyses and case-control analyses in our Hp genotyping and imputation cohorts, as presented in **Fig. 2.** We analyzed normally distributed continuous variables with Student's *t*-test or ANOVA, and the non-normally distributed continuous variables with the non-parametric Mann–Whitney *U*-test or Kruskal–Wallis test. The difference in categorical variables between groups was tested with the χ^2-test. The association between haptoglobin genotype and stroke was analyzed with χ^2-test, logistic regression, and with the Cox proportional-hazards model. In the genotyped Hp cohort, the follow-up until stroke started at the individual's baseline study visit, and at the time of diabetes diagnosis for the imputation cohort. Analyses were performed with R versions 3.6–4.0, and a *P*-value below 0.05 was considered statistically significant.

Data and Resource Availability. The datasets analyzed in the current study are not publicly available because the FinnDiane study protocol does not allow the sharing of individual-level data. The scripts used in the data analysis are available from the corresponding author upon reasonable request.

Results

Genotyped Hp1/2 cohort

The stroke cases and controls were matched for age, DKD stage, and CAD at baseline, but cases had higher systolic blood pressure (SBP), lower body mass index (BMI), poorer glycemic control, and a higher proportion of current smokers, when compared to the controls (**Supplementary Table 4**). The genotyping success rate of the haptoglobin polymorphism was 0.95, thus, 179 (92%) stroke cases and 517 (96%) controls remained in further analyses. The haptoglobin Hp1-1 genotype was observed in 14.9% (n=104), Hp2-1 in 45.5% (n=317), and Hp2-2 in 39.5% (n=275) of the individuals, and the frequencies followed the HWE (P>0.05). The Hp genotype frequencies did not differ between the incident stroke cases (Hp1-1: 13.4%, Hp2-1: 48.6%, and Hp2-2: 38.0%) and controls (Hp1-1: 15.5%, Hp2-1: 44.5%, Hp2-2: 40.0%; χ^2-test P=0.602), and neither did the allele frequencies (Hp1: 37.7% in both groups). The baseline clinical characteristics of individuals with different Hp genotypes were similar (**Table 1)**.

Time-to-event analysis. The median follow-up time in the genotyped Hp cohort was 10.7 (interquartile range (IQR) 5.5–13.4) years. Time-to-event analysis showed no difference between the Hp genotypes (number of Hp2-alleles, additive model) and the risk of stroke in an unadjusted analysis (HR 1.01 [95%CI 0.82, 1.25], P=0.905, **Fig. 3A**) or in a model with baseline diabetes duration, SBP, BMI, HbA_{1c}, and retinal photocoagulation as covariates (HR 0.98 [0.79, 1.22], P=0.872). Furthermore, the Hp-genotype was not a risk factor in separate time-to-event analyses for ischemic

or hemorrhagic stroke, adjusted for the same covariates (ischemic stroke: HR 0.92 [0.72, 1.18], *P*=0.517; all hemorrhagic strokes: HR 1.25 [0.82, 1.90], *P*=0.298) or when analyzing lacunar infarcts or intracranial and subarachnoid hemorrhage separately (Supplementary Table 5). A fully-adjusted model with baseline diabetes duration, SBP, BMI, HbA_{1c}, retinal photocoagulation in addition to LDL-cholesterol, triglycerides, eGFR, high-sensitivity CRP concentration, and current smoking as covariates gave similar non-significant findings for the Hp1/2 genotype (data not shown).

Survival after stroke. During follow-up**,** 209 (40%) controls and 120 (67%) stroke cases died. In cases, the Hp-genotype frequencies were similar in those who died (Hp1-1: 12.5%; Hp2-1: 45.8%, Hp2-2: 41.7%) and those who stayed alive (Hp1-1: 15.3%; Hp2-1: 54.2%, Hp2-2: 30.5%, *P*=0.351). The median follow-up time after an incident stroke was 5.4 (IQR 1.2–9.1) years. In the 35 individuals who died after a hemorrhagic stroke, the time-to-death was significantly shorter (median 0.05 [IQR 0.00–3.06] years) compared to the 85 individuals who died after an ischemic stroke (3.7 [1.2–7.1] years, *P*<0.001). Altogether 66% (*n*=23) of the deaths after a hemorrhagic stroke occurred within 30 days from the stroke, whereas the same proportion for the ischemic strokes was 21% (*n*=18).

Survival analyses did not show Hp1/2 genotype (coded as a number of Hp2 alleles) as a risk factor for death after a hemorrhagic stroke (HR 1.30 [95%CI 0.79, 2.17], *P*=0.303) or an ischemic stroke (HR 1.16 [0.84, 1.61], *P*=0.368), or in a combined analysis of all strokes together (HR 1.24 [0.95, 1.63], *P*=0.118, **Fig. 3B**). Similarly, haptoglobin genotype was not a risk factor for short-term (<30 days) or long-term (≥30 days) mortality after an ischemic or hemorrhagic stroke (data not shown).

Imputed Hp1/2 cohort

The stroke cases had a lower age at diagnosis of type 1 diabetes, more kidney failure and CAD at the time of the stroke and included fewer women, when compared to individuals without stroke (all $P<0.0001$, **Supplementary Table 6**). On the other hand, the cases were younger and had a shorter duration of diabetes at the time of stroke when compared to the age and duration of the controls at the end of the follow-up (death or December 31, 2017; $P<0.003$). We conducted the following analyses in 500 stroke cases and 3,806 controls with successfully imputed Hp1/2 genotypes.

In the imputation cohort, the frequency of the minor Hp1 allele was 36.1%, and the genotype frequencies followed the HWE ($P>0.05$) both in stroke cases (Hp1-1: 13.8%, Hp2-1: 47.8%, and Hp2-2: 38.4%) and controls (Hp1-1: 13.4%, Hp2-1: 45.0%, and Hp2-2: 41.6%).

The concordance of imputed and genotyped Hp1/2. Altogether 609 individuals had successfully determined Hp1/2 genotypes with both PCR genotyping and imputation (**Supplementary Fig. 1**). In this group, the concordance between the imputed genotypes and the genotyped was high: 92.6% for Hp1-1, 96.7% for Hp2-1, and 97.5% for Hp2-2 (**Supplementary Table 7**).

Hp and stroke. The Hp1/2 genotype frequencies were similar in stroke cases and controls ($P=0.237$, sex and age-adjusted; **Table 2**). Additionally, the Hp1/2 genotype frequencies were similar in 263 individuals with an ischemic stroke, 83 people with hemorrhagic stroke, and in 149 individuals with unclassified stroke, when compared together or separately with the control group without stroke (all $P>0.05$, sex- and age-adjusted logistic regression, **Table 2**). The Hp2-2 genotype carriers had similar age-at-stroke in individuals with an ischemic stroke (Hp1-1: 52.3 years, Hp2-1: 51.5 years, and Hp2-2: 50.1 years, ANOVA $P=0.225$), a hemorrhagic stroke (Hp1-1: 50.6 years, Hp2-1: 49.9 years, and Hp2-2: 48.0 years, $P=0.342$), or among all cases including the 149 non-classified strokes as well ($n=500$, Hp1-1: 51.4 years, Hp2-1 years: 51.0, and Hp2-2: 49.3 years, $P=0.084$, **Table 3**). Additionally, the Hp2 allele was not a significant risk factor for stroke in the Cox proportional-hazards models, when analyzing all strokes, or ischemic or hemorrhagic strokes with follow-up

starting at diabetes diagnosis and sex and age at diabetes diagnosis as covariates (*e.g.,* risk of any stroke, increasing number of Hp2 alleles HR 0.93 [0.82, 1.06], *P*=0.260)

Analyses stratified by CAD and kidney failure. Other diabetic complications were common among the stroke cases; 48.2% had kidney failure (33.2% prior to stroke) and 44.0% had CAD (22.0% prior to stroke) (**Table 3)**. The Hp2-2 genotype frequencies were similar in individuals with kidney failure (Hp2-2: 43.1% *vs*. 40.9%), when compared to individuals without kidney failure and in individuals with CAD (Hp2-2: 42.8% *vs*. 40.9%) when compared to individuals without CAD (*P*>0.05 in χ^2-test comparing the Hp genotype frequencies, or in logistic regression models adjusted for sex and age at the time of the event [cases] or at the end of the follow-up [controls]). When analyzing the sub-groups of individuals stratified by the presence of CAD and kidney failure, there was a trend of lower age at stroke in individuals with the Hp2-2 genotype (**Table 3**). This difference was significant within 121 individuals with kidney failure prior to stroke (Hp1-1: 51.8 years, Hp2-1: 49.3 years, Hp2-2: 46.6 years, *P*=0.012). However, time-to-event analysis with follow-up starting at diabetes diagnosis did not support Hp2 as a stroke risk allele (data not shown). Furthermore, we did not observe any significant differences in Hp genotype frequencies between the stroke cases and controls in these four comorbidities subgroups (**Table 3**). For example, the 45 individuals who had both CAD and kidney failure prior to stroke had similar Hp1/2 frequencies when compared to the 221 individuals who had CAD and kidney failure but no stroke (unadjusted *P*=0.638; sex- and age-adjusted *P*=0.551).

Hp1/2 F and S alleles. In the imputation cohort, the frequencies of different "S" and "F" Hp-alleles were Hp1S: 19.5%, Hp1F: 16.6%, Hp2FS: 60.7%, and Hp2SS: 1.3%. These frequencies were similar to those reported for the 119 individuals from the European CEU population (21): Hp1S: 22.7%, Hp1F: 13.9%, Hp2FS: 60.5%, and Hp2SS: 2.9% (χ^2-test *P*=0.083). With respect to stroke, the exact Hp-genotype frequencies in stroke cases and controls were similar within each Hp1/2 genotype group (*P*>0.05 both unadjusted and sex- and age-adjusted; **Supplementary Table 8**).

Survival after stroke. In the imputation cohort, the median follow-up time after a suffered stroke was 6.0 (IQR 1.6–12.8) years during which altogether 305 individuals (61%) died. Those who died had more often kidney failure (73.9% vs. 49.0%, $P<0.0001$) and CAD (70.0% *vs.* 53.9%, $P<0.0001$) compared to individuals who stayed alive. Of note, 68.9% of the kidney failures but only 48.2% of the CAD events had occurred prior to stroke. Dying from cardiovascular causes accounted for 76.1% of all deaths in individuals with stroke.

The Hp1/2 genotype frequencies were similar in individuals who died after stroke compared to individuals who stayed alive, and the number of Hp2-alleles was not a risk factor for death in a Cox proportional hazards model adjusted for sex and age at stroke (HR 1.03 [0.87, 1.22], $P=0.722$). Similarly, haptoglobin genotype did not affect the survival after an ischemic stroke or a hemorrhagic stroke, when analyzed separate (data not shown). Since prior comorbidities, especially kidney failure, affects the risk of death, we added the presence of kidney failure (prior to stroke) as a predictor variable to the Cox model. In this analysis, kidney failure almost tripled the risk of dying after stroke (HR 2.99 [2.35, 3.80], $P<0.0001$), while the Hp1/2 genotype did not affect the risk of death (HR 0.97 [0.82, 1.15], $P=0.755$). Additionally, prior CAD was a risk factor for dying after stroke (HR 1.46 [1.10, 1.94], $P=0.008$), in a model with sex, age at stroke, and the Hp1/2 genotype, which was non-significant. Cardiovascular mortality was similar after any stroke, ischemic stroke, or hemorrhagic stroke in the different Hp1/2 genotype carriers.

Discussion

Our study in Finnish individuals with type 1 diabetes found no association between haptoglobin gene CNV and stroke. Both case-control - and time-to-event analyses showed a similar risk of stroke and stroke subtypes in the carriers of Hp1-1, Hp2-1, and Hp2-2 genotypes. Additionally, survival after a stroke was not affected by the Hp1/2 genotype.

In type 1 diabetes, our study analyzing 179 stroke cases with genotyped Hp alleles and 500 stroke cases with imputed Hp genotypes is thus far the largest conducted. While some earlier studies in the general population and diabetes cohorts have shown increased stroke risk associated either with Hp2-2 or Hp1-1 genotypes, our results follow those studies showing no association between haptoglobin CNV and stroke (16,18). Similar to our results, Costacou and colleagues (19) found no association between Hp1/2 genotypes and the risk of stroke in the EDC cohort. They detected, however, higher incidence of stroke in individuals with the Hp1-1 genotype when compared to the Hp2 allele carriers (Hp2-1 and Hp2-2) in a subgroup of individuals with more recent disease onset (diagnosis year ≥1965) and hypertension (<10 individuals with stroke) (19). We failed to replicate this finding in our cohort (258 individuals, of which 75 had a stroke, fulfilling these criteria in our cohort; data not shown).

We sought to stratify for comorbidities since the Hp2-2 genotype has been associated with CAD and kidney failure while Hp1-1 is the previously suggested risk genotype for stroke incidence in type 1 diabetes (19). Nevertheless, the Hp genotype was not associated with stroke in individuals without comorbidities, nor in individuals with both CAD and kidney failure. We noticed, however, a significant trend towards younger age of stroke in the Hp2-2 genotype carriers in individuals with prior kidney failure. Time-to-event analyses in individuals with kidney failure showed, however, no excess risk of stroke in Hp2-2 genotype carriers.

Haptoglobin CNV might have several biological functions in stroke. An *in vitro* study suggested that Hp1-1 might be associated with poorer endothelial repair potential in individuals that suffered a lacunar stroke, the most common type of ischemic stroke (23). After a hemorrhagic stroke, the rapid clearance of free hemoglobin by haptoglobin is essential for recovery (24). Therefore, possibly due to poorer free hemoglobin clearance capacity or some other vascular related function, the Hp2-2 genotype seems to increase cardiovascular mortality after a stroke in the general population (16).

However, in the type 1 diabetes context, we found no excess risk of death after a hemorrhagic stroke (or any stroke) related to the Hp2-2 genotype. As a limitation, however, we had only limited number of hemorrhagic stroke cases. Further, cardiovascular mortality after stroke was similar regardless of the Hp1/2 genotype.

In addition to having poorer antioxidant properties, the Hp2 allele has been associated with higher cholesterol concentrations. In our study, both total cholesterol and LDL-cholesterol were the highest in the individuals with the Hp2-2 genotype. This non-significant trend was seen in both stroke cases and controls and individuals with or without lipid-lowering medication. Similarly, in the Diabetes Control and Complication Trial (DCCT) type 1 diabetes cohort, Hp2-2 carriers showed a non-significant trend for higher LDL and total cholesterol concentrations (14). As not being the main phenotype of interest of our study, we analyzed the lipid variables only in our smaller cohort with genotyped Hp alleles (n=696) and comprehensive baseline phenotypic data available. Therefore, further studies in a larger cohort are needed to confirm the effect of Hp CNV on lipid concentrations in type 1 diabetes.

More interestingly, the same study (14) in the DCCT cohort found an association between the Hp2-2 genotype and CAD only in individuals belonging to the secondary cohort (diabetes duration 1-15 years at recruitment and early diabetic retinopathy) and intensive glucose control treatment arm, while their whole DCCT cohort analysis showed no excess CAD risk for Hp2-2 genotype carriers. Similarly, Hp2-2 was not a risk factor for combined CAD and stroke end points in individuals with elevated HbA_{1c} (13). Our study found no association between Hp1/2 genotype and CAD, but other studies have shown Hp2-2 as a CAD risk genotype in type 1diabetes (8,9) or in individuals with elevated HbA_{1c} (26).

Similarly, individuals with kidney failure had similar Hp1/2 frequencies in our cohort. An earlier study in type 1 diabetes showed Hp2-2 to be a risk factor for kidney failure and kidney function

decline (10). We could also see such a trend in our genotyped Hp1/2-alleles cohort, where the Hp2-2 genotype carriers tended to have a lower estimated glomerular filtration rate (P=0.097) – a measure of kidney function.

Since no single SNP correlates well with the Hp exon CNV (highest r^2=0.44), the European SNP reference panel for the Hp1/2 genotype imputation (21) is a great resource that enables Hp imputation in large cohorts with genome-wide SNP data. In our study, the imputation of Hp alleles performed well. The imputation method provides additional subdivision to "S" and "F" Hp1 and Hp2 alleles, which in our study did not associate with the risk of stroke, as the genotype frequencies were similar in cases and controls. Our study is among the first studies (21,25) to analyze the imputed S and F alleles.

Hp1/2 CNV primarily affects the haptoglobin protein multimerization and function but is also modestly associated with haptoglobin concentration in the blood – Hp2-allele associated with lower haptoglobin levels. Our main hypothesis was, however, that the structural effects of the Hp1/2 CNV are stronger in stroke susceptibility than the effect on the haptoglobin concentration, which is more strongly regulated by other genetic variants (27−29).

An asymptomatic cerebral small vessel disease characterized with *e.g.,* cerebral microbleeds are common in individuals with type 1 diabetes (30). As a limitation, we did not study these silent brain manifestations as part of this study. Therefore, some of our non-stroke controls likely had asymptomatic brain manifestations. To conclude, previous studies on Hp1/2 genotype and stroke were contradictory and inconclusive. Our study suggests that Hp CNV is not associated with the risk of stroke in type 1 diabetes, nor with stroke subtypes or survival after stroke.

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Author Contributions CF, NS, LT, and PHG conceptualized the project. AS conducted the laboratory investigation. AS and EHD performed the imputation. AS, SHH, CF, VH, JP, and LT curated the data. AS analyzed the data. AS and LT wrote the original draft of the manuscript and all authors critically revised and edited the manuscript. PHG is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Soedamah-Muthu SS, Fuller JH, Mulnier HE, Raleigh VS, Lawrenson RA, Colhoun HM. High risk of cardiovascular disease in patients with type 1 diabetes in the U.K.: a cohort study using the general practice research database. *Diabetes Care* 2006;29:798–804

2. Hägg S, Thorn LM, Putaala J, Liebkind R, Harjutsalo V, Forsblom CM, et al. Incidence of stroke according to presence of diabetic nephropathy and severe diabetic retinopathy in patients with type 1 diabetes. Diabetes Care 2013;36:4140–6.

3. Putaala J, Curtze S, Hiltunen S, Tolppanen H, Kaste M, Tatlisumak T. Causes of death and predictors of 5-year mortality in young adults after first-ever ischemic stroke: the Helsinki Young Stroke Registry. Stroke 2009;40:2698–703

4. Smith A, McCulloh R. Hemopexin and haptoglobin: allies against heme toxicity from hemoglobin not contenders. *Front Physiol* 2015;6:187. Available from: https://www.frontiersin.org/article/10.3389/fphys.2015.00187

5. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem* 1996;42:1589–600

6. Asleh R, Miller-Lotan R, Aviram M, Hayek T, Yulish M, Levy JE, et al. Haptoglobin genotype is a regulator of reverse cholesterol transport in diabetes in vitro and in vivo. *Circ Res* 2006;99:1419–25

7. Asleh R, Blum S, Kalet-Litman S, Alshiek J, Miller-Lotan R, Asaf R, et al. Correction of HDL dysfunction in individuals with diabetes and the haptoglobin 2-2 genotype. *Diabetes* 2008;57:2794–800

8. Levy AP, Hochberg I, Jablonski K, Resnick HE, Lee ET, Best L, et al. Haptoglobin phenotype is an independent risk factor for cardiovascular disease in individuals with diabetes: The Strong Heart Study. *J Am Coll Cardiol* 2002;40:1984–90

9. Costacou T, Ferrell RE, Orchard TJ. Haptoglobin genotype: a determinant of cardiovascular complication risk in type 1 diabetes. *Diabetes* 2008 ;57:1702–6

10. Costacou T, Ferrell RE, Ellis D, Orchard TJ. Haptoglobin genotype and renal function decline in type 1 diabetes. *Diabetes* 2009;58(12):2904–9

11. Costacou T, Evans R, Orchard T. Glycaemic control modifies the haptoglobin 2 allele-conferred susceptibility to coronary artery disease in Type 1 diabetes. *Diabet Med* 2016;33:1524–7

12. Ijäs P, Saksi J, Soinne L, Tuimala J, Jauhiainen M, Jula A, et al. Haptoglobin 2 allele associates with unstable carotid plaque and major cardiovascular events. *Atherosclerosis* 2013;230:228–34

13. Pechlaner R, Kiechl S, Willeit P, Demetz E, Haun M, Weger S, et al. Haptoglobin 2-2 genotype is not associated with cardiovascular risk in subjects with elevated glycohemoglobin-results from the Bruneck Study. *J Am Heart Assoc* 2014;3:e000732

14. Orchard TJ, Backlund J-YC, Costacou T, Cleary P, Lopes-Virella M, Levy AP, et al. Haptoglobin 2-2 genotype and the risk of coronary artery disease in the Diabetes Control and

Complications Trial/Epidemiology of Diabetes Interventions and Complications study (DCCT/EDIC). *J Diabetes Complications* 2016;30:1577–84

15. Staals J, Pieters B, Knottnerus IL, Rouhl RP, Van Oostenbrugge RJ, Delanghe JR, et al. Haptoglobin polymorphism and lacunar stroke. *Curr Neurovasc Res* 2008;5:153–8

16. Ijäs P, Melkas S, Saksi J, Jula A, Jauhiainen M, Oksala N, et al. Haptoglobin Hp2 Variant Promotes Premature Cardiovascular Death in Stroke Survivors. *Stroke* 2017;48:1463–9.

17. Vardi M, Blum S, Levy AP. Haptoglobin genotype and cardiovascular outcomes in diabetes mellitus - natural history of the disease and the effect of vitamin E treatment. Meta-analysis of the medical literature. *Eur J Intern Med* 2012 Oct;23(7):628–32.

18. Lempiäinen J, Ijäs P, Niiranen TJ, Kaste M, Karhunen PJ, Lindsberg PJ, et al. Haptoglobin Hp1 Variant Does Not Associate with Small Vessel Disease. *Brain Sci* 2019;10:18.

19. Costacou T, Secrest AM, Ferrell RE, Orchard TJ. Haptoglobin genotype and cerebrovascular disease incidence in type 1 diabetes. *Diab Vasc Dis Res* 2014;11:335–42

20. Syreeni A, Sandholm N, Sidore C, Cucca F, Haukka J, Harjutsalo V, et al. Genome-wide search for genes affecting the age at diagnosis of type 1 diabetes. *J Intern Med* 2021;289:662–74

21. Boettger LM, Salem RM, Handsaker RE, Peloso GM, Kathiresan S, Hirschhorn JN, et al. Recurring exon deletions in the HP (haptoglobin) gene contribute to lower blood cholesterol levels. *Nat Genet* 2016;48:359–66

22. Carter K, Worwood M. Haptoglobin: a review of the major allele frequencies worldwide and their association with diseases. *Int J Lab Hematol* 2007;29:92–110

23. Rouhl RPW, van Oostenbrugge RJ, Damoiseaux JGMC, Debrus-Palmans LL, Theunissen ROMFIH, Knottnerus ILH, et al. Haptoglobin phenotype may alter endothelial progenitor cell cluster formation in cerebral small vessel disease. *Curr Neurovasc Res* 2009;6:32–41

24. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: secondary brain injury. *Stroke* 2011;42:1781–6

25. Zheng NS, Bastarache LA, Bastarache JA, Lu Y, Ware LB, Shu X-O, et al. A common deletion in the haptoglobin gene associated with blood cholesterol levels among Chinese women. *J Hum Genet* 2017;62:911–4

26. Cahill LE, Levy AP, Chiuve SE, Jensen MK, Wang H, Shara NM, et al. Haptoglobin Genotype Is a Consistent Marker of Coronary Heart Disease Risk Among Individuals With Elevated Glycosylated Hemoglobin. *J Am Coll Cardiol.* 2013;61:728–37

27. Bjornsson E, Helgason H, Halldorsson G, Helgadottir A, Gylfason A, Kehr B, et al. A rare splice donor mutation in the haptoglobin gene associates with blood lipid levels and coronary artery disease. *Hum Mol Genet*. 2017;15:2364–76

28. Froguel P, Ndiaye NC, Bonnefond A, Boutia-Naji N, Dechaume A, Siest G et al. A genome-wide association study identifies rs2000999 as a strong genetic determinant of circulating haptoglobin levels. *PLoS One* 2012;7:e32327

29. Pietzer M, Wheeler E, Carrasco-Zanini J, Raffler J, Kerrison ND, Oerton E et al. Genetic architecture of host proteins involved in SARS CoV-2 infection. *Nat Commun* 2020;16:6397

30. Thorn LM, Shams S, Gordin D, Liebkind R, Forsblom C, Summanen P et al. Clinical and MRI Features of Cerebral Small-Vessel Disease in Type 1 Diabetes. *Diabetes Care* 2019;42:329–30

Table 1: Clinical characteristics in the haptoglobin Hp1/2 genotype groups.

	Hp1-1	**Hp2-1**	**Hp2-2**	***P****
N	104	317	275	
Baseline				
Women, %	38	42	36	0.433
Age, years	45.4 ± 9.7	45.8 ± 9.1	45.1 ± 9.8	0.534
Diabetes duration, years	28.4 ± 9.9	31.3 ± 9.4	29.7 ± 9.5	0.845
Age at diagnosis of diabetes, years	14.3 (10.0–21.6)	13.2 (8.0–19.1)	13.1 (9.0–21.4)	0.068
Body mass index, kg/m^2	25.8 ± 3.9	24.9 ± 3.6	25.7 ± 3.7	0.502
HbA_{1c}, %	8.8 ± 1.6	8.6 ± 1.4	8.6 ± 1.5	0.217
HbA_{1c}, mmol/mol	73 ± 17	70 ± 15	70 ± 16	0.217
Systolic blood pressure, mmHg	146 ± 18	146 ± 22	147 ± 21	0.636
Diastolic blood pressure, mmHg	84 ± 11	81 ± 11	83 ± 11	0.913
Antihypertensive therapy, %	79	75	78	0.641
RAAS blockers, %	43	54	57	0.057
Aspirin, %	35	28	34	0.233
Warfarin, %	1.0	1.0	1.9	0.732
Lipid-lowering therapy, %	25	23	29	0.241
Total cholesterol, mmol/L	5.15 ± 0.94	5.14 ± 1.03	5.28 ± 1.13	0.149
Triglycerides, mmol/L	1.24 (0.90–1.80)	1.22 (0.86–1.74)	1.25 (0.9–1.96)	0.583
HDL cholesterol, mmol/L	1.26 ± 0.39	1.29 ± 0.41	1.24 ± 0.41	0.408
LDL cholesterol, mmol/L	3.25 ± 0.88	3.22 ± 0.90	3.36 ± 1.01	0.160
with lipid-lowering therapy	2.97 ± 1.03	2.98 ± 0.85	3.09 ± 0.99	0.508
without lipid-lowering therapy	3.34 ± 0.81	3.28 ± 0.90	3.46 ± 0.98	0.164
Serum high-sensitivity CRP, mg/L[†]	2.94 (1.51–5.32)	2.40 (1.42, 4.85)	2.03 (1.28, 3.80)	0.220
Serum creatinine, µmol/L	91 (71–143)	93 (73–130)	99 (76–176)	0.064
eGFR, mL/min/1.73m^2	76 (46–99)	76 (49–97)	70 (38–96)	0.097
Albumin excretion rate[‡], mg/24h	81 (9–365)	42 (11–239)	74 (15–386)	0.375
Microalbuminuria[§], %	12.5	21.8	16.4	0.061
Macroalbuminuria[§], %	34.6	30.0	34.9	0.395
Kidney failure, %	30.8	27.8	28.0	0.831
Retinal photocoagulation, %	68	69	67	0.924
Amputation or peripheral artery bypass, %	15	12	12	0.601
Coronary artery disease, %	11	11	13	0.729
History of myocardial infarction, %	7.7	5.7	8.0	0.507
Current smoking, %	21	23	22	0.877
History of smoking, %	70	65	63	0.477
Follow-up				
Incident stroke, %	23	27	25	0.602
Ischemic stroke, %	19	21	18	0.664
Lacunar stroke, %	6.7	8.9	8.1	0.786
Hemorrhagic stroke, %	5.9	9.8	9.8	0.533
Intracranial hemorrhage, %	2.9	6.0	6.0	0.412

	Subarachnoid hemorrhage, %	1.9	1.9	1.5	0.857
	Multiple any stroke, %	13	23	16	0.385
Died, %		44	45	51	0.368

Data are mean ± standard deviation or median (IQR).

[*]*P* value from a χ^2-test, ANOVA, or Kruskal–Wallis test.

[†]Serum high-sensitivity C-reactive protein measurement was available for 71% of the cohort

[‡] One time-point urinary albumin excretion data were available for 52% of the total cohort

[§]The definition of microalbuminuria (*i.e.*, moderately increased albumin excretion) was urinary albumin excretion rate (AER) between 30 and 300 mg/24h or 20 and 200 μg/min. Macroalbuminuria (*i.e.*, severely increased albumin excretion) was AER >300 mg/24h or >200 μg/min.

Table 2: Haptoglobin genotype frequencies in the imputed Hp cohort stroke cases and controls

	n	Haptoglobin genotype			Un-adjusted *P**	Adjusted†	
		Hp1-1 (%)	Hp2-1 (%)	Hp2-2 (%)		OR [95% CI]	*P*
All stroke cases	500	13.8	47.8	38.4	0.374	0.92 [0.80,1.06]	0.237
Ischemic stroke	263	16.4	45.6	38.0	0.304	0.87 [0.73,1.04]	0.130
Hemorrhagic stroke	83	8.4	50.6	41.0	0.352	1.09 [0.79,1.52]	0.600
Unclassified stroke	149	12.8	49.7	37.6	0.517	0.93 [0.73,1.18]	0.551
Stroke controls	3,806	13.4	45.0	41.6	NA	NA	NA

**P* value from a χ^2-test comparing the genotype frequencies in the cases to the control group

† Odds ratio, its 95% confidence intervals, and *P* value from a logistic regression analysis. The model included stroke as a dependent variable and Hp genotypes (0, 1, or 2 Hp2 alleles, an additive model), sex, and age (at stroke for cases and at the end of the follow-up for the controls) as covariates.

Table 3: Haptoglobin genotype frequencies in stroke cases and controls in the haptoglobin imputation cohort

Prior complications*	Stroke	*n*	Haptoglobin genotype (%)			*P*†	*P*‡	Age (years)§			*P*‖
			Hp1-1	Hp2-1	Hp2-2			Hp1-1	Hp2-1	Hp2-2	
Whole cohort											
No co-morbidities, kidney failure, CAD, or both	Stroke	500	13.8	47.8	38.4	0.374	0.237	51.4 ± 11.3	51.0 ± 10.6	49.3 ± 11.0	0.084
	No Stroke	3,806	13.4	45.0	41.6			53.9 ± 11.4	53.2 ± 10.3	53.6 ± 10.7	0.955
Grouping according to prior comorbidities											
No comorbidities	Stroke	269	14.9	50.2	34.9	0.152	0.086	48.4 ± 12.0	48.5± 10.3	48.5± 12.7	0.987
	No Stroke	2,923	13.4	45.6	41.0			52.6 ± 11.1	52.2 ± 10.1	52.4 ± 10.5	0.976
Kidney failure	Stroke	121	11.6	42.2	46.3	0.621	0.507	51.8± 8.6	49.3± 8.1	46.6 ± 7.4	0.012
	No Stroke	283	14.8	42.8	42.4			52.0 ± 8.3	51.7 ± 8.4	51.8 ± 9.5	0.951
CAD	Stroke	65	9.2	55.4	35.4	0.223	0.337	61.0 ± 8.2	59.9 ± 10.0	55.7 ± 11.3	0.128
	No Stroke	379	11.6	43.8	44.6			65.8 ± 11.2	61.0 ± 9.7	61.9 ± 9.3	0.167
Kidney failure and CAD	Stroke	45	20.0	37.8	42.2	0.638	0.551	57.5 ± 7.7	56.9 ± 9.3	53.2 ± 6.2	0.134
	No Stroke	221	14.5	41.6	43.9			55.5 ± 9.0	56.9 ± 8.4	56.6 ± 9.3	0.705

*Grouping is based on the events (kidney failure with replacement therapy or coronary artery disease that occurred prior to stroke. Comorbidities after stroke (in cases) were ignored.

†*P* value from a χ^2-test

‡*P* value from a logistic regression analysis. The model included stroke as a dependent variable and Hp genotypes (0, 1, or 2 Hp2 alleles, an additive model), sex, and age (at stroke for cases and at the end of the follow-up for the controls) as covariates.

§Mean (± SD) age at stroke in cases and age at the end of the follow-up in controls

‖*P* value from comparison of age in Hp genotype groups with ANOVA

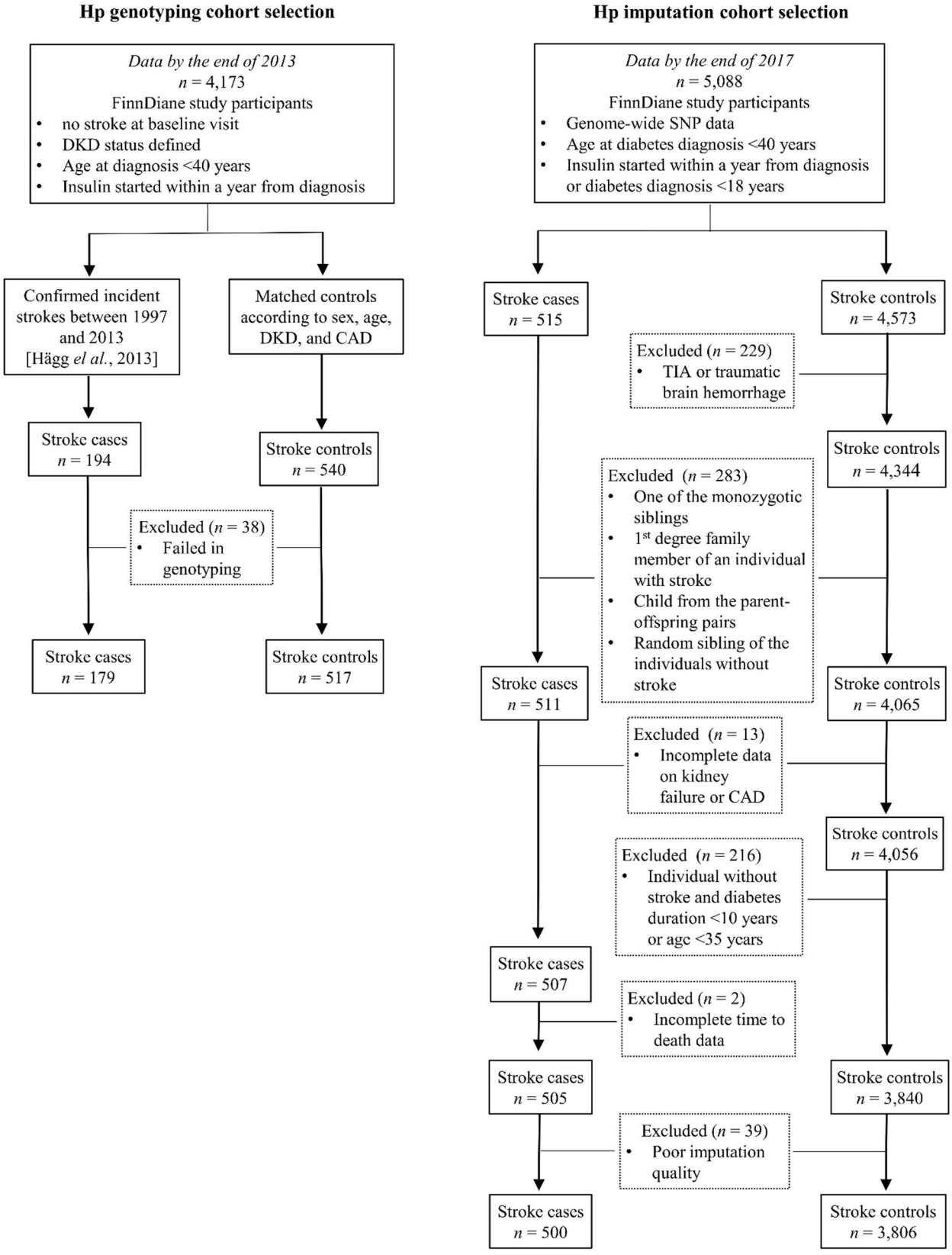

Figure 1. Selection of the cohorts for the haptoglobin gene copy number variant genotyping and imputation. Abbreviations: SNP=single nucleotide polymorphism, TIA=transient ishemic attack.

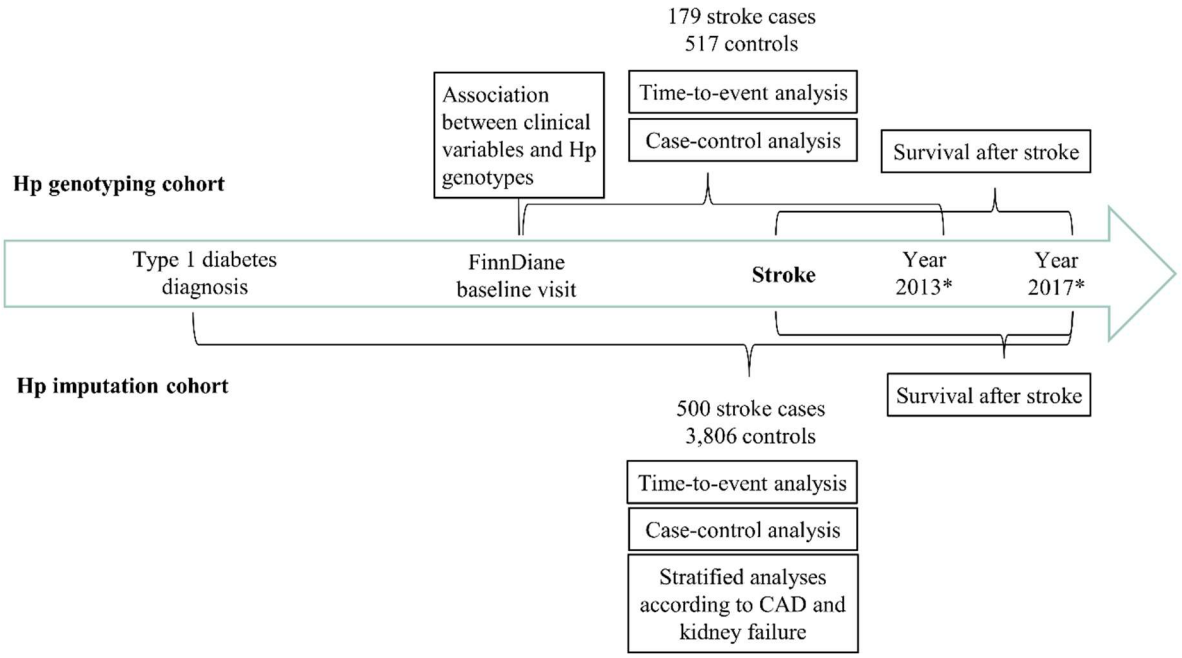

Figure 2. Analyses conducted in the Hp genotyping and Hp imputation cohorts. *For the Hp genotyping cohort, cases were strokes in the register data and verified from medical papers between FinnDiane baseline visit and 31 December, 2013. In the Hp imputation cohort, all register data was collected until 31 December, 2017. We verified 69.9% of the strokes from medical records, 8.4% from the FinnDiane study visit questionnaires, and register data was used for the remaining 21.7%.

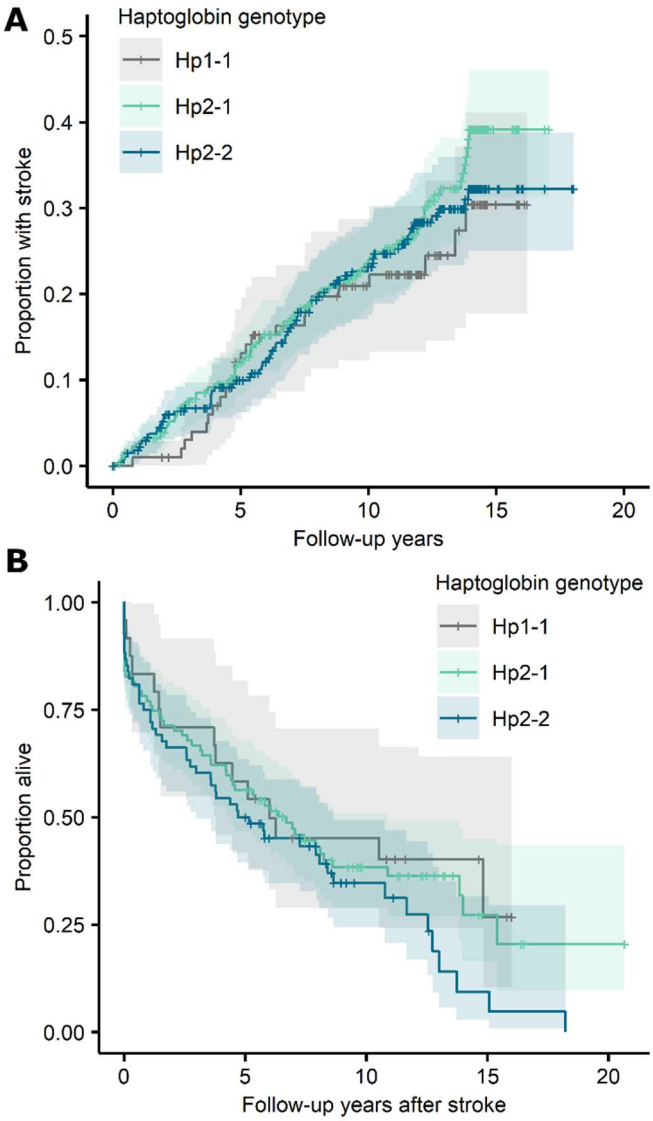

Figure 3. Kaplan–Meier curves for Hp1/2 genotypes and (A) the risk of stroke after the baseline and (B) survival after a stroke. No differences between the haptoglobin genotypes were detected in these analyses (P>0.05). The shaded colors define the 95% confidence intervals for the curves.