Haptoglobin Hp2 Variant Promotes Premature Cardiovascular Death in Stroke Survivors

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Background and Purpose—Haptoglobin (Hp) is an acute phase plasma protein protecting tissues from oxidative damage. It exists in 2 variant alleles (*hp1/hp2*) giving rise to 3 protein isoforms with different biochemical properties and efficiency to limit oxidative stress. We previously found that *hp2* variant is associated with stroke risk in the patients with carotid stenosis and the risk of ischemic cardiovascular events in a general population cohort. This study examined the hypothesis that Hp genotype is associated with general cardiovascular risk in patients with stroke.

Methods—Hp was genotyped in SAM study (Helsinki Stroke Aging Memory, n=378). A total of 1426 individuals ascertained from a nationally representative cross-sectional health survey served as population controls.

Results—Hp genotype frequencies were 15.6% (*hp1-1*), 44.2% (*hp1-2*), and 40.2% (*hp2-2*) in patients with stroke. During a mean of 7.5-year follow-up after first-ever stroke, *hp2* carriers had a substantially higher rate of cardiac deaths (24.5% versus 8.5%; *P*=0.006) and a trend toward more fatal strokes (23.5% versus 13.6%; *P*=0.122). The combined risk of ischemic cardiovascular deaths was 2.4-fold higher among *hp2* carriers (95% confidence interval, 1.28–4.43) after adjustment for major cardiovascular risk factors.

Conclusions—*Hp2* allele is associated with premature ischemic cardiovascular deaths after first-ever ischemic stroke. (***Stroke*. 2017;48:1463-1469. DOI: 10.1161/STROKEAHA.116.015683.**)

Key Words: cardiovascular diseases ■ carotid artery diseases ■ haptoglobins ■ prognosis ■ stroke

The principal defense against the deleterious effects of free hemoglobin (Hb) released during hemorrhage is provided by haptoglobin (Hp), an acute phase plasma protein and important physiological antioxidant.[1,2] Hp regulates vascular health by several means. It binds to Hb forming a soluble complex which is cleared through endocytosis by the macrophage scavenger receptor CD163 initiating anti-inflammatory signaling cascades.[1] Heme is degraded by heme oxygenase 1 to carbon monoxide (CO), iron (Fe^{2+}), and biliverdin to be further metabolized to bilirubin, a potent tissue antioxidant. CO functions as a slow vasodilator and can replace NO in damaged atherosclerotic vessel wall. Fe^{2+} has widespread effects on gene expression through the network of iron-response elements and proteins. To this end, Hp–CD163–heme oxygenase 1 pathway has been shown to influence important vasoregulatory targets, such as vascular tone, balance between apoptosis and proliferation of vessel wall cells, and protection against oxidative damage and inflammation related to local Hb accumulation.

The Hp locus in the chromosome, 16q22, is polymorphic. Hp gene carries a common 1.7 kb copy number variant resulting in 2 variant alleles in humans, *hp1* and *hp2*. They give rise to 3 protein isoforms, Hp1-1, Hp1-2, and Hp2-2, which differ remarkably in their biochemical properties and also in their efficiency to counteract oxidative stress. Hp1-1 occurs as a small circular molecule that binds Hb in 3:1 proportion and efficiently clear free Hb. In contrast, Hp1-2 and Hp2-2 form sheet structures or complex linear structures, respectively, where both binding to Hb and CD163 receptor are compromised. Hp2 allele is common in some subtropical regions and may yield selective advantage given to its greater protection against malaria falciparum infection.[3] However, it is becoming obvious that other properties of hp2 allele present cardiometabolic risks to the aging population of the modern world. In

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addition to attenuated heme-recycling and antioxidant properties, Hp2-2 binds with high affinity to human apolipoprotein A-I[4–6] and apolipoprotein E[7] in high-density lipoprotein and thereby seems to interfere with cholesterol levels and reverse cholesterol transport (RCT). In line, hp2 allele has been associated with vascular complication in diabetic subjects.[2,8–10]

Our earlier observations demonstrated that hp2 allele (Hp1-2 and Hp2-2 protein phenotypes) associate with the risk of ischemic stroke in patients with carotid stenosis (ie, symptomatic carotid stenosis) and also with the risk of acute myocardial infarction and ischemic stroke in a general population cohort.[11] Staals et al[12] have associated Hp1-1 phenotype with lacunar stroke in young patients devoid of silent lacunar infarcts or extensive white matter lesions. Hp1-1 genotype was also linked with increased stroke risk in type I diabetics, in a prospective study by Costacou et al.[13] This study was therefore designed to examine whether hp2 allele genotypes associate with specific stroke subtypes and are there variants that accelerate myocardial infarction, stroke, and cardiovascular death.

Patients and Methods

More details on study cohorts and genotyping can be found in the online-only Data Supplement.

Helsinki Stroke Aging Memory Study

Stroke subjects belong to SAM study (Helsinki Stroke Aging Memory), which is a prospective, cross-sectional study examining the cognitive, functional, and emotional consequences of ischemic stroke.[14,15] The cohort includes 486 consecutive patients aged 55 to 85 years who were admitted to Helsinki University Hospital between 1993 and 1995 because of suspected stroke and fulfilling the preset inclusion criteria. Patients underwent comprehensive clinical and cognitive evaluation. DNA was available from 378 patients and brain magnetic resonance imaging from 396 patients. The patients were followed for a mean of 7.5±4.0 years until September 2006 using extensive national registers.[15] Long-term survival data in September 2006 were obtained from Statistics Finland. Of the original 486 patients, 347 (71.4%) had died. The causes of death based on *International Classification of Diseases, Ninth Revision* and *Tenth Revision* classifications were obtained and divided further into cardiac, brain-related (ischemic stroke, bleeding, dementia), cancer, infection, trauma, and other categories. In 9 cases, the cause of death could not be determined, and these were excluded when analyzing the data. The demographics of SAM patients are presented in Table 1 and the frequencies of ischemic cardiovascular deaths in Table 2.

Health 2000 Survey

As a population control, we used participants derived from the nationally representative Health 2000 survey, which is an epidemiological health examination survey performed in Finland from fall 2000 to spring 2001 by the National Public Health Institute (www.terveys2000.fi) as detailed also in our previous study.[11] The overall study cohort was a 2-stage stratified cluster sample (8028 patients) representing the entire Finnish population aged ≥30 years. Here, we included 1516 patients aged 45 to 74 from the Cardiovascular diseases, substudy of the Health 2000 survey. These subjects were invited to undergo a thorough cardiovascular examinations including carotid ultrasound. The original sample size was 1867, and the participation rate was 82%. DNA was available from 1426 individuals.

Genotyping

DNA was extracted from peripheral blood leukocytes. Hp genotypes were determined by a modification of a method described by Koch et al.[16] Details are given in the online-only Data Supplement.

Statistical Methods

Statistical analyses were performed by IBM SPSS Statistics 22 software. Statistical analysis included (1) bivariate analysis of association between Hp alleles and genotypes and all stroke and stroke subtypes (using Health 2000 samples as controls) and clinical variables using independent samples *t* test for interval variables and either χ^2 or Fisher exact test for nominal variables. (2) Cox proportional hazards model of the effect of hp2 allele on the risk of fatal ischemic stroke or cardiac death during follow-up. Time was expressed in years to either event (death due to ischemic stroke or cardiac cause) or the end of follow-up (September 21, 2006 or the time of death because of other causes). Major classical risk factors for cardiovascular disease (age, sex, smoking, hypertension, diabetes mellitus, atrial fibrillation, and low-density lipoprotein cholesterol) were entered into the model (Table II in the online-only Data Supplement). A receiver operating characteristic curves were created by plotting the probability distributions for 4 logistic regression models described in Table III in the online-only Data Supplement. Significance of the difference between the areas under receiver operating characteristic curves was calculated by the method described by Hanley and McNeil.[17]

Ethical Issues

The SAM study has been approved by the Ethics committee of the Department of Clinical Neurosciences, Helsinki University Hospital and Health 2000 survey by the Ethics committees of Hospital District of Helsinki and Uusimaa and the National Public Health Institute.

Results

The genotype frequencies were 15.6% for *hp1-1*, 44.2% for *hp1-2*, and 40.2% for *hp2-2* in the SAM cohort and 14.3%, 48.6%, and 37.1%, respectively, in the Health 2000 cohort (Table 3). Both distributions were within Hardy–Weinberg equilibrium (P=0.247 in SAM and P=0.361 in Health 2000). The allele frequencies of *hp1* were 0.38 and 0.39 and *hp2* were 0.62 and 0.61 in SAM and Health 2000, respectively. There were no significant differences in genotype or allele frequencies between the patients with stroke and population controls (Table 3).

There were no differences in the frequency of risk factors between stroke patients with different Hp genotypes with the exception that male *hp2-2* carriers smoked less often than male *hp1-1* and *hp1-2* carriers (P=0.017; Table 1).

Male patients with stroke displayed higher frequency of *hp2-2* genotype than female patients with stroke (46.0% versus 34.4%; P=0.042) who on the other hand had more *hp1-2* genotype (50.3% versus 38.1%). In women, a history of acute myocardial infarction was associated with *hp2* allele (*hp1-2* and *hp2-2*; P=0.016). There were no differences in Hp genotypes between different stroke subtypes classified according to TOAST (Trial of ORG 10172 in Acute Stroke Treatment; Table 1).

Carriers of *hp2* allele had significantly higher rate of cardiac deaths during the follow-up period than *hp1-1* carriers (odds ratio [OR], 3.43; 95% confidence interval [CI], 1.32–8.90). In males, the risk was equally high in *hp1-2* (OR, 2.99; 95% CI, 0.81–11.05) and *hp2-2* (OR, 2.85; 95% CI, 0.78–10.36) carriers, but in females, the risk was higher among heterozygote *hp1-2* (OR, 5.83; 95% CI, 1.29–26.46) than among hp2-2 (OR, 1.44; 95% CI, 0.28–7.48; Table 2) carriers. There was a trend toward higher frequency of death because of ischemic stroke in *hp2* carriers (OR, 1.80; 95% CI, 0.80–4.03),

Table 1. Demographics of the SAM Study (Helsinki Stroke Aging Memory) Patients

	Hp Genotype			*P* Value	Hp Allele	*P* Value
Variable*	*hp1-1*	*hp1-2*	*hp2-2*	(Genotypes)†	*hp2*	(*hp1-1* vs *hp2*)‡
Genotype/allele frequency	15.6	44.2	40.2		84.4	
Sex (% males)	50.8	43.1	57.2	0.042	49.8	1.000
Age	70.4±6.6	71.9±8.3	70.5±7.6	0.211	71.3±8.0	0.356
Smoking (current or former)	60.3	50.9	48.3	0.304	49.7	0.154
Males	76.7	80.6	60.5	0.017	69.6	0.516
Low education (<6 y)	30.9	30.7	29.5	0.964	30.2	1.000
Hypertension	44.1	49.1	48.7	0.809	48.9	0.571
Diabetes mellitus	18.6	25.1	21.7	0.572	23.5	0.500
Probable metabolic syndrome§	18.6	25.7	21.1	0.463	23.5	0.500
Total cholesterol, mmol/L	5.5±1.1	5.6±1.2	5.5±1.2	0.930	5.6±1.2	0.795
LDL-C	3.7±1.1	3.7±1.0	3.7±1.0	0.929	3.7±1.0	0.918
HDL-C	1.1±0.4	1.2±0.3	1.1±0.3	0.845	1.1±0.3	0.914
Total cholesterol/HDL-cholesterol	5.40±2.57	5.17±1.67	5.20±1.74	0.751	5.20±1.71	0.526
LDL/HDL-cholesterol	3.71±2.21	3.47±1.40	3.53±1.42	0.614	3.49±1.41	0.347
Log(triglyceride/HDL cholesterol)	0.11±0.26	0.12±0.25	0.09±0.28	0.898	0.12±0.28	0.677
Myocardial infarction	15.3	19.8	19.7	0.771	19.7	0.475
Females	0.0	16.8	13.8	0.036	15.6	0.016*
Atrial fibrillation	16.9	19.9	17.8	0.861	18.9	0.856
Cardiac failure	20.3	22.3	21.7	0.985	22.0	0.865
Peripheral arterial disease	10.2	12.6	14.5	0.717	13.0	0.673
TOAST stroke subtype						
Large vessel stroke	22.0	16.8	16.4	0.593	16.6	0.350
Cardioembolic	8.5	7.8	4.6	0.379	6.3	0.567
Small vessel disease	10.2	15.0	14.5	0.689	14.7	0.420
Nondetermined	59.3	60.5	64.5	0.681	62.4	0.664

HDL indicates high-density lipoprotein; hp, haptoglobin; LDL, low-density lipoprotein; and TOAST, Trial of ORG 10172 in Acute Stroke Treatment.
*Mean (±SD) is shown for continuous variables and percentage for nominal variables.
†Fisher exact test between Hp genotypes.
‡χ^2 test between *hp1-1* and *hp2* genotypes.
§Patient was defined as having probable metabolic syndrome if he/she had ≥3 of the following: (1) triglycerides ≥1.7 mmol/L, (2) HDL cholesterol <1.0 mmol/L in men and <1.3 mmol/L in women, (3) diagnosis of hypertension or blood pressure medication, (4) fasting glucose ≥5.6 mmol/L or diagnosis of diabetes mellitus or diabetes mellitus medication.

especially in women (OR, 2.40; 95% CI, 0.67–8.63). The combined risk of death from ischemic stroke or cardiac cause was significantly increased in *hp2* carriers (OR, 3.24; 95% CI, 1.65–6.34). Again, the risk was equally high in male *hp1-2* (OR, 2.50; 95% CI, 0.97–6.44) and *hp2-2* (OR, 2.46; 95% CI, 0.97–6.21) carriers, but in females, the risk was highest among *hp1-2* heterozygote subjects (OR, 5.79; 95% CI, 1.93–17.39). There was a trend toward higher all-cause mortality in *hp1-2* and *hp2-2* carriers (*P*=0.061, data not shown).

There were only 82 diabetic stroke patients, and in their group, *hp2* allele was associated with increased risk of ischemic cardiovascular death. The risk was highest in the carriers of *hp2-2* genotype (OR, 8.51; 95% CI, 1.55–46.86) but was also clearly elevated in *hp1-2* (OR, 5.57; 95% CI, 1.06–29.36) compared with *hp1-1* subjects.

When adjusted for major cardiovascular risk factors (age, sex, hypertension, diabetes mellitus, atrial fibrillation, and low-density lipoprotein cholesterol), the combined risk of ischemic cardiovascular death was 2.4-fold elevated in *hp2* carriers (95% CI, 1.28–4.43). The size of the effect of *hp1-2* and *hp2-2* genotypes was comparable to classical cardiovascular risk factors (Table II in the online-only Data Supplement). The addition of *hp2* allele to either of the multivariate models increased the discrimination power of the model (defined as area under curve [receiver operating characteristic curve analysis]; Figure and Table III in the online-only Data Supplement). Adding *hp2* allele to classical risk factors (model 3 in Table III in the online-only Data Supplement) increased the area under curve from 0.699 to 0.727, but this did not reach statistical significance.

Table 2. Premature Ischemic Cardiovascular Deaths at Follow-Up in the SAM Study (Helsinki Stroke Aging Memory) Patients

Outcome at Follow-Up*	*hp1-1*	*hp1-2*	*hp2-2*	*hp2*	All	*P* Value (Genotypes)†	*P* Value (*hp1-1* vs *hp2*)‡	OR (*hp1-2*) §	OR (*hp2-2*)‖
Ischemic cardiovascular death	22.0	53.3	42.1	48.0	43.9 (166)	0.000	0.000	3.93 (1.94–7.99)	2.62 (1.28–5.36)
Females	17.2	58.9	38.5	50.6	45.5 (86)	0.000	0.001	5.79 (1.93–17.39)	2.55 (0.82–7.96)
Males	26.7	45.8	44.8	45.3	42.3 (80)	0.171	0.071	2.50 (0.97–6.44)	2.46 (0.97–6.21)
No diabetes mellitus	22.9	52.8	35.3	40.8	40.8 (119)	0.000	0.006	3.48 (1.57–7.70)	1.93 (0.86–4.32)
Diabetes mellitus	18.2	54.8	66.7	60.0	54.7 (47)	0.019	0.020	5.57 (1.06–29.36)	8.51 (1.55–46.86)
Cardiac death	8.5	29.9	18.4	24.5	22.0 (83)	0.001	0.006	4.55 (1.71–12.10)	2.40 (0.88–6.57)
Females	6.9	33.7	10.8	24.4	21.7 (41)	0.000	0.048	5.83 (1.29–26.46)	1.44 (0.28–7.48)
Males	10.0	25.0	24.1	24.5	22.2 (42)	0.214	0.095	2.99 (0.81–11.05)	2.85 (0.78–10.36)
Brain infarct death	13.6	23.4	23.7	23.5	22.0 (83)	0.241	0.122	1.67 (0.71–3.91)	1.94 (0.83–4.57)
Females	10.3	25.3	27.7	26.3	23.8 (45)	0.173	0.095	2.13 (0.57–8.00)	2.82 (0.73–10.84)
Males	16.7	20.8	20.7	20.8	20.1 (38)	0.940	0.805	1.40 (0.45–4.39)	1.44 (0.47–4.41)

hp indicates haptoglobin; and OR odds ratio.
*Frequency (%) of outcome for different Hp genotypes. The number of all cases is given in parenthesis.
†Fisher exact test between Hp genotypes.
‡χ^2 test between hp1-1 and hp2 genotypes.
§Odds ratios with 95% confidence intervals of outcome for genotype hp1-2 (hp1-1 as a reference genotype) adjusted for age and sex if not included in the model.
‖Odds ratios with 95% confidence intervals of outcome for genotype hp2-2 (hp1-1 as a reference genotype) adjusted for age and sex if not included in the model.

Discussion

In this study, we demonstrate evidence for the association of *hp2* allele of the haptoglobin gene with ischemic cardiovascular death after first-ever ischemic stroke. Overall, the association was strongest in female and diabetic stroke patients. We note a consistent trend toward better discrimination power when *hp2* allele is added to other cardiovascular risk factors (Figure). This raises the possibility that Hp genotype or phenotype could be used in risk stratification to identify stroke survivors who need enhanced monitoring of cardiovascular risk factors and more intensive management strategies.

The results from this stroke cohort replicate our earlier finding in a population-based cross-sectional health survey, the Health 2000 study, where *hp2* allele was associated with a 2.2-fold elevated risk of ischemic cardiovascular events during the 10 years follow-up period of middle-aged volunteers.[11] This study confirms that the association between *hp2* allele and ischemic cardiovascular events also applies to high-risk stroke population. Our earlier finding of an association of *hp2* allele with symptomatic carotid stenosis and the tight association between myocardial infarction seen both in our earlier study and in this stroke cohort supports the hypothesis that *hp2* allele increases the risk of sudden atherothrombotic events both in cerebral and cardiac circulation. However, we could not confirm an association of Hp genotypes with pathogenic stroke subtypes classified according to TOAST, not between *hp1-1* and lacunar stroke reported by Staals et al[12] neither *hp2* and large vessel disease in general.

Initially, haptoglobin Hp2-2 phenotype was associated with a larger size of myocardial infarct.[18] After this, Hp genotypes have been tested against different cardiovascular manifestations, but later reports[3] conflicted in reporting this association to Hp1-2[19] or Hp1-1 phenotype,[20] or reporting no association.[21] There are several possible explanations for these inconsistent findings. Except for the Framingham Heart Offspring Study (n=3273)[19] and Bruneck Study (n=806),[21] the sample sizes in all older studies were relatively small, up to 200 patients. The definition of outcome varied across the studies. In the Bruneck Study, the combined cardiovascular disease outcome was uncommon (n=123) and included intracerebral hemorrhage, the pathogenesis of which clearly differs from that of myocardial infarction and is driven by hypertension rather than by atherosclerosis. In Framingham Heart Offspring Study, angina pectoris patients without a history of myocardial infarction were also included. In our study, we did not detect association between Hp genotypes and intracerebral hemorrhage, including subarachnoid hemorrhage (data

Table 3. Hp Genotype and Allele Frequencies (%) in the SAM Study (Helsinki Stroke Aging Memory) and Health 2000 Cohorts

Cohort	Hp Genotypes			*hp2* Allele	*P* Value (Genotypes)*	*P* Value (*hp1-1* vs *hp2*)†
	hp1-1	*hp1-2*	*hp2-2*			
SAM	15.6	44.2	40.2	84.4	0.312	0.513
Health 2000	14.3	48.6	37.1	85.7		

hp indicates haptoglobin.
*Fisher exact test between Hp genotypes.
†χ^2 test between hp1-1 and hp2 genotypes.

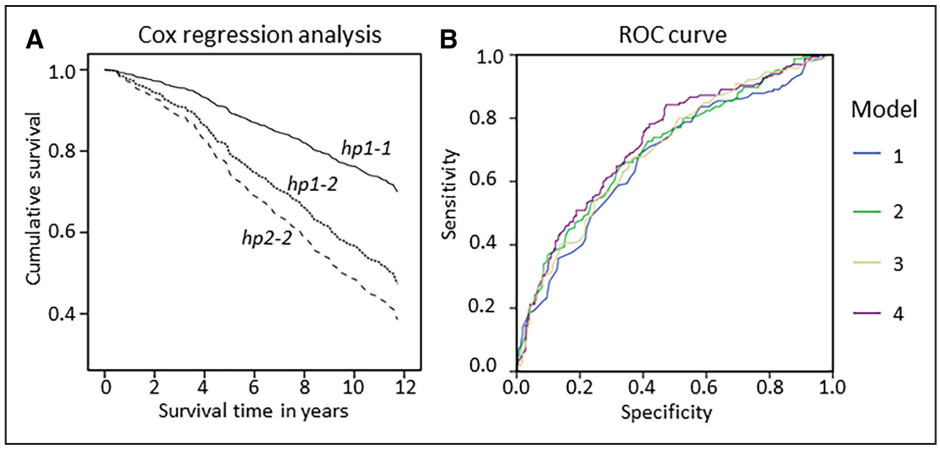

Figure. A, Cox proportional hazards model of the effect of haptoglobin (Hp) genotypes on the risk of ischemic cardiovascular death during the follow-up period. Time is expressed in years to either event or the end of follow-up (time of censoring or death because of other cause). Major classical risk factors for cardiovascular disease (age, sex, smoking, hypertension, diabetes mellitus, atrial fibrillation, and low-density lipoprotein cholesterol) were entered into the model (Table II in the online-only Data Supplement). **B**, Receiver operating characteristic (ROC) curves were created by plotting the probability distributions for 4 logistic regression models in Table III in the online-only Data Supplement.

not shown) and based on our previous study,[11] the association is strongest between acute atherothrombotic cardiovascular events, such as myocardial infarction and large artery atherosclerotic stroke, rather than chronic atherosclerotic manifestations. This substantiates the importance of careful definition of phenotype in the genetic studies of stroke, the pathogenesis of which is heterogeneous compared with myocardial infarction.

During the last 2 decades, the focus has shifted to the effect of Hp polymorphism in diabetic individuals because *hp2* genotype seems to aggravate the deleterious vascular effects during hyperglycemia.[2,22,23] The association was first reported in a cohort of type I diabetics where *hp2* allele was associated with diabetic nephropathy and the prevalence of restenosis after coronary angioplasty.[24] In Strong Heart study, a longitudinal population-based study of American Indians, the *hp2-2* genotype was associated with an OR of 5.0 to cardiovascular disease compared with *hp1-1* in diabetic subjects, whereas *hp1-2* showed intermediate risk.[8] In a recent meta-analysis of 5 studies involving 1829 patients with diabetes mellitus, the pooled OR for cardiovascular disease was 2.03 (95% CI, 1.46–2.81) among *hp2-2* genotype subjects when compared with subjects with other genotypes.[22] Furthermore, the risk increases among individuals with elevated HbA(1c).[23] In the present stroke cohort, the frequency of ischemic cardiovascular deaths was significantly higher in both diabetic and nondiabetic *hp2* carriers compared with *hp1-1* carriers, but *hp2-2* seemed to be more strongly associated in diabetics whereas *hp1-2* in nondiabetics. Similar trend was noted in the Framingham Heart Offspring Study.[19] This could be explained by different biochemical properties of Hp1-2 and Hp2-2 protein isoforms but remains to be confirmed in future studies. On the contrary, we did not find association between *hp1* allele and small vessel disease (by TOAST classification) in diabetics which was reported earlier by Costacou et al[13] in type I diabetic patients. However, the number of diabetics in our study cohort was low (n=86) and we could not differentiate between type I and II diabetes mellitus, and thus our study is underpowered to detect specific associations in type I diabetics.

Haptoglobin *hp2* genotype may increase cardiovascular risk by impairing cellular capacity to alleviate heme-induced oxidative stress and interfering with RCT, thereby promoting accumulation of oxidatively modified low-density lipoprotein into the vessel wall. Hp2-2 has been shown to lead to an impairment of Hb clearance in atherosclerotic plaques after intraplaque hemorrhage and increased oxidative, inflammatory, and angiogenic responses,[25,26] as well as oxidation of low-density lipoprotein.[27] Hb–Hp2-2 complexes bind tightly to apolipoprotein A-1 of high-density lipoprotein while high-density lipoprotein becomes proinflammatory and loses its capacity for RCT.[5,6] Furthermore, the Hp gene cluster has emerged as an important locus influencing cholesterol levels.[28–30] Recently, it was suggested that Hp locus drives cholesterol levels by regulating Hp serum levels and oxidation of apolipoprotein E, thus representing another route of effect on RCT.[31]

Could the detrimental effect of *hp2* be alleviated in high-risk individuals? Interesting recent data suggest that the impaired RCT process in *hp2-2* individuals could be enhanced by antioxidant α-tocopherol (ie, vitamin E treatment). In a meta-analysis of 2110 diabetic patients in 3 trials, the OR for nonfatal myocardial infarction, stroke, or cardiovascular death was 0.66 (95% CI, 0.48–0.9) in favor of the vitamin E treatment in patients with h*p2-2*.[22] This effect was genotype specific; α-tocopherol improved high-density lipoprotein function in *hp2-2* carriers but appeared to affect lipid peroxides and lipoprotein subfractions among *hp1* allele carriers adversely.[32] α-Tocopherol has not yet been tested in nondiabetics, and its effect on heterozygote *hp1-2* individuals is unclear. In principle, this kind of personalized genotype-directed treatment of individuals at high risk of cardiovascular events, such as in the secondary prevention of acute myocardial infarction and ischemic stroke, could be tested for clinical efficacy.

Limitations

There are certain limitations in this study. The large proportion of stroke cases with undetermined pathogenesis may be the reason why we cannot conclude on the association of hp2

allele with specific TOAST stroke subtypes in SAM cohort. At the time of the recruitment of the SAM cohort (in 1990s), carotid duplex or ambulatory ECG were not included in the diagnostic workup of patients with stroke in our hospital, for which we do not have true knowledge on the prevalence of carotid stenosis or atrial fibrillation. Another limitation is the cohort size that limits the power for subgroup analysis. On the other hand, the strengths of our study are the extent of brain magnetic resonance imaging and long follow-up time which all minimize the number of stroke mimics in our cohort. Finally, there is a possibility of selection/survival bias because the SAM cohort was formed 3 months after the index stroke when patients with most severe strokes have died.[15] This may have led to a decreased number of patients with stroke, especially females with severe atherothrombotic and cardioembolic strokes, and may even underlie the relative paucity of hp2-2 females in the SAM cohort compared with men (Table 2). If such bias exists, it would have led to underestimation of the effect of hp2 allele rather than type I error.

Summary

We show that *hp2* allele of the haptoglobin gene increases the risk of ischemic cardiovascular death after first-ever ischemic stroke by 2.4-fold even after adjustment with major cardiovascular risk factors. The risk is high for both *hp1-2* and *hp2-2* genotypes but especially elevated in *hp1-2* females and in *hp2-2* diabetics. We found no significant difference in Hp genotypes between patients with stroke and population controls or between stroke subtypes classified according to TOAST.

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Disclosures

None.

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Haptoglobin Hp2 Variant Promotes Premature Cardiovascular Death in Stroke-Survivors

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ONLINE SUPPLEMENTS

Helsinki Stroke Aging Memory Study (SAM)

The SAM study patients were recruited from ischemic stroke patients admitted to Helsinki University Hospital between 1.12.1993 - 31.3.1995 (n=1149). Because the goal of the study was to examine post-stroke cognitive decline and dementia in long-term follow-up, young (under 55 years of age, n=285) and elderly (over 85 years of age, n=88) patients were excluded. In addition, patients not living in Helsinki (n=158) and non-Finnish speakers (n=3) were excluded since participation in the study required several visits to the hospital and neuropsychological examination in Finnish. In addition, 71 patients died soon after admission before they were recruited to the study. From this final cohort (n=571), 82 patients (12.8%) refused to participate giving a final cohort size of 486. More women (67.1% vs 49.2%) and those that we were still in the hospital (60% vs 16.8%) refused to participate. Blood sample for DNA extraction was obtained from 378 patients and DNA was gained from all blood samples.

Health 2000 Survey

In Health 2000 study, 1867 randomly selected inhabitants in the vicinity of five Finnish University hospitals were invited to participate in the study, of whom 1526 accepted to participate. Participants were examined during years 2000-2001. Blood samples could be obtained from 1523 and DNA from 1426 individuals. The main reason for not obtaining DNA sample that the participant refused to participate in the DNA study. Approximately 0.5% of DNA samples were lost because the blood sample was not preserved or extraction was not successful.

There were no significant differences between individuals that gave DNA sample compared to those that did not either in the SAM cohort or in Health 2000 survey. Data for the SAM cohort is shown below in Table I.

Table I. Demographics of patients that gave DNA sample compared to those that did not in the SAM cohort.

Variable	No DNA sample	DNA sample available	*P*
n	108	378	
sex (%males)	50.0	52.8	0.663
age	71.5 ± 7.1	71.1 ± 7.8	0.679
Total cholesterol	5.47 ±1.08	5.56 ± 1.17	0.602
LDL cholesterol	3.07 ± 0.98	3.69 ±1.03	0.936
Hypertension	44.4	48.1	0.514
Diabetes	31.5	22.8	0.076
History of myocardial	19.4	19.0	0.891
Cardiac failure	23.1	21.8	0.793
Atrial fibrillation	25.9	18.6	0.103
ASO	8.3	13.0	0.239
Large vessel disease	17.5	20.4	0.481
Small vessel disease	9.3	14.0	0.255
Cardiac embolism	4.6	6.6	0.650
Follow-up			
Cardiac death	15.7	22.0	0.178
Brain infarct death	25.0	22.0	0.516
Brain death	26.9	23.8	0.527
Combined outcome	40.7	43.9	0.583

DNA extraction

DNA was extracted from peripheral blood leucocytes. Blood samples were drawn and transferred to -20°C for storage until DNA extraction. In Health 2000 Survey the samples were collected during 2000-2001 and DNA was extracted during years 2000-2005 (maximum 5 years from sampling). In SAM study samples were collected during 1994-1995 and DNA was extracted in one batch during spring 2000 (5-6 years after sampling).

In both cohorts DNA was extracted by salt precipitation with minor differences. DNA quality was good with A260/280 (DNA purity) generally around 1.8. DNA stocks were stored in TE-buffer in -20°C. Fresh working dilutions of DNA were made for Hp genotyping.

Genotyping of Hp common polymorphism

Hp alleles were amplified in two different PCR reactions and separated by agarose gel electrophoresis. Hp1 was detected using primers A 5’-GAGGGGAGCTTGCCTTTCCATTG-3' and B 5'-GAGATTTTTGAGCCCTGGCTGGT-3' (HpAB-PCR) and hp2 using primers C 5'-CCTGCCTCGTATTAACTGCACCAT-3' and D 5'- CCGAGTGCTCCACATAGCCATGT-3' (HpCD-PCR). The amplicon lengths were 1757 bp for hp1 and 3481 bp for hp2 in HpAB-PCR and 349 bp for hp2 in

HpCD-PCR. HpAB-PCR was performed in a 30 µl total PCR volume containing 100 ng of DNA, 0,5 µM primers, 200 µM dNTP's, 0.8 M betaine, 1x Phusion® buffer and 1 U of Phusion® polymerase (New England BioLabs™). The cycling conditions for HpAB-PCR were: initial denaturation at 98°C for 3 minutes, 35 cycles of amplification at 98°C for 15 seconds, 69°C for 30 seconds, 72°C for 1 minute for hp2, and final extension at 72°C for 10 minutes. HpCD-PCR was performed in a 30 µl total PCR volume containing 50 ng of DNA, 0.5 µM primers, 200 µM dNTP's, 0.8 M betaine, 1x Dynazyme® buffer and 1 U of Dynazyme® polymerase. The cycling conditions for HpCD-PCR were: initial denaturation at 95°C for 2 minutes, 35 cycles of amplification at 95°C for 30 seconds, 63°C for 20 seconds, 72°C for 1 minute, and final extension at 72°C for 5 minutes. The HpAB and HpCD amplicons were loaded in adjacent wells and size-separated in 0.7% agarose gel with DNA size marker. Each sample was analysed twice.

Genotypes were determined by two independent readers blinded to clinical data. Discrepant samples were reanalysed. Genotyping was successful in all samples of SAM and 97.7% of Health 2000 participants.

Table II. Cox proportional hazards model of ischemic cardiovascular deaths in the Helsinki Stroke Aging Memory Study (SAM) patients

Covariate	B	SE	P	Exp(B)	95% CI
Age	0.102	0.014	0.000	1.108	1.078-1.138
Male sex	0.401	0.201	0.046	1.494	1.007-2.217
Hypertension	0.155	0.168	0.358	1.167	0.839-1.624
Diabetes	0.493	0.188	0.009	1.638	1.132-2.369
Atrial fibrillation	0.408	0.208	0.050	1.505	0.001-2.260
Not smoking	-0.192	0.194	0.324	0.825	0.568-1.208
LDL-C	-0.119	0.090	0.189	0.888	0.744-1.060
hp1-2 genotype	0.981	0.326	0.003	2.668	1.407-5.058
hp2-2 genotype	0.737	0.333	0.027	2.090	1.088-4.014

Table III. ROC curve models

Model	Variables	AUC*	SE	P	95% CI
1	age, sex	0.678	0.028	0.000	0.623-0.732
2	model 1 + Hp genotype	0.705	0.027	0.000	0.652-0.758
3	age, sex, diabetes, atrial fibrillation	0.699	0.027	0.000	0.646-0.752
4	model 3 + Hp genotype	0.727	0.026	0.000	0.675-0.778

*Area under the curve – value

†Variables with significant effect on Cox proportional hazards model (Table II) were included in the models 3 and 4.