Haptoglobin Phenotype Is an Independent Risk Factor for Cardiovascular Disease in Individuals With Diabetes: The Strong Heart Study

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OBJECTIVES	The goal of this study was to determine if the haptoglobin phenotype was predictive of cardiovascular disease (CVD) in diabetic mellitus (DM).
BACKGROUND	Cardiovascular disease is the most frequent, severe, and costly complication of type 2 DM. There are clear geographic and ethnic differences in the risk of CVD among diabetic patients that cannot be fully explained by differences in conventional CVD risk factors. We have demonstrated that a functional allelic polymorphism in the haptoglobin gene acts as a major determinant of susceptibility for the development of diabetic microvascular complications.
METHODS	We sought to determine if this paradigm concerning the haptoglobin gene could be extended to CVD in DM. We tested this hypothesis in a case-control sample from the Strong Heart study, a population-based longitudinal study of CVD in American Indians. Haptoglobin phenotype was determined by polyacrylamide gel electrophoresis in 206 CVD cases and 206 matched controls age 45 to 74 years. Median follow-up was six years.
RESULTS	In multivariate analyses controlling for conventional CVD risk factors, haptoglobin phenotype was a highly statistically significant, independent predictor of CVD in DM. The odds ratio of having CVD in DM with the haptoglobin 2-2 phenotype was 5.0 times greater than in DM with the haptoglobin 1-1 phenotype ($p = 0.002$). An intermediate risk of CVD was associated with the haptoglobin 2-1 phenotype.
CONCLUSIONS	This study suggests that determination of haptoglobin phenotype may contribute to the algorithm used in CVD risk stratification, and in evaluation of new therapies to prevent CVD in the diabetic patient. (J Am Coll Cardiol 2002;40:1984–90) © 2002 by the American College of Cardiology Foundation

Cardiovascular disease (CVD) is the most frequent, severe, and costly complication of type 2 diabetes mellitus (DM) (1). It is the leading cause of death among patients with type 2 DM regardless of DM duration (2). Population-based studies have shown that the relative risk of CVD in DM is several fold higher compared with those without DM (3). This increased risk appears to be even more striking in women (3). Risk factors such as hypertension, hyperlipidemia, and cigarette smoking independently increase the risk to the DM patient of developing CVD, but the effect of DM appears to be independent of conventional risk factors (3).

Although the incidence of CVD is higher in DM compared with non-DM in all populations studied, there are geographic and ethnic differences in the risk of CVD among DM patients that cannot be fully explained by differences in conventional CVD risk factors between these

groups (4). These studies suggest that genetic factors could contribute to differences in susceptibility to CVD among individuals with DM. One such factor is a functional allelic polymorphism in the haptoglobin gene. In man, there are two general classes of alleles for the haptoglobin gene, designated 1 and 2 (5,6). The haptoglobin 2 allele appears to have arisen from the 1 allele early in human evolution and to have spread in the world population as a result of selective pressures related to resistance to infectious agents (7,8). Haptoglobin alleles differ dramatically in their relative frequency among different ethnic groups (9). The protein products of the two haptoglobin alleles differ both in biophysical and biochemical properties. A key difference between the alleles is that the protein product of the 1 allele is a more potent antioxidant compared with that produced by the 2 allele (10).

We have demonstrated that haptoglobin phenotype is predictive of development of microvascular complications in DM (11–13). Specifically, we have shown that patients who are homozygous for the haptoglobin 1 allele are at decreased risk for developing retinopathy and nephropathy. This effect, at least for nephropathy, has been observed in both type 1 and type 2 DM and the relevance strengthened by the finding of a gradient effect with respect to the number of haptoglobin 2 alleles and the development of nephropathy

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Abbreviations and Acronyms

BP = blood pressure
BMI = body mass index
CI = confidence intervals
CVD = cardiovascular disease
DM = diabetes mellitus
HDL = high-density lipoprotein
Hp 1-1 = individuals who are homozygous for the 1 allele
Hp 2-2 = individuals who are homozygous for the 2 allele
Hp 2-1 = individuals who are heterozygous at the haptoglobin locus
LCAT = lecithin-cholesterol acyl transferase
LDL = low-density lipoprotein
SHS = Strong Heart Study

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(13). Furthermore, the haptoglobin phenotype may be predictive of development of macrovascular complications in DM. We have shown that development of restenosis after percutaneous coronary angioplasty is significantly decreased in DM patients with the 1-1 haptoglobin phenotype (11–14). Previous retrospective and cross-sectional studies examining haptoglobin phenotype and coronary artery disease in the general population have yielded conflicting results (15–17). The role of haptoglobin phenotype in the development of atherosclerotic coronary artery disease in DM has not been studied.

American Indians, previously thought to be resistant to developing coronary artery disease, are presently experiencing CVD in epidemic proportions (18). The increased incidence of CVD has been attributed to the sharp increase in type 2 DM in this population (1,2). The Strong Heart Study (SHS) has examined the incidence, prevalence, and risk factors of CVD in American Indian populations in three geographic areas since 1988 with continued surveillance to the present time (18). The relative genetic homogeneity of this population, along with the high prevalence of DM, permits a unique opportunity for identification of genetic factors that may contribute to CVD in DM. Accordingly, we aimed to determine the relative risk of CVD in DM according to haptoglobin phenotype in a case-control sample from the SHS.

METHODS

Detailed descriptions of the SHS study design, survey methods, and laboratory techniques and the participating Indian communities have been published (18–20). All SHS participants gave consent for utilization of their blood samples in genetic studies. In addition, institutional review boards of individual tribes and of the tribal communities approved use of stored blood samples for the determination of haptoglobin phenotype.

The SHS study cohort consists of 4,549 individuals ages 45 to 74 years who were seen at the first examination conducted between July, 1989 and January, 1992. Partici-

pation rates of all eligible tribe members averaged 64%. Nonparticipants were similar to participants in age and self-reported frequency of DM. Reexamination rates for those alive at the second examination (July 1993 to December 1995) averaged 89% and at the third examination (July 1997 to December 1999) averaged 88%. However, surveillance for cardiovascular events by review of hospital records and tribal records was also performed on participants who did not attend the second and third examinations such that greater than 99% of all cardiovascular events occurring in the SHS cohort were detected (20).

Blood samples were collected in the presence of EDTA, and the plasma was stored at −80°C. Standardized blood pressure (BP) measurements were obtained, and electrocardiograms were recorded and coded as previously described (19,20). Participants were classified as diabetic according to WHO criteria (21).

Deaths in the SHS cohort between 1988 and the present were identified through tribal and hospital records and by direct contact by study personnel with participants and their families. Copies of death certificates were obtained from state health departments and ICD-9 coded centrally by a nosologist. Possible CVD deaths were initially identified from death certificates as described previously (22). Cause of death was investigated through autopsy reports, medical records abstractions, and informant interviews as described previously (22).

Medical records were reviewed at each examination to identify nonfatal cardiovascular events, definite myocardial infarction, and definite CVD (18,23) that had occurred since the previous examination. Records of those who did not participate in the second or third examination were also reviewed to maximize event ascertainment. For all potential CVD events or interventions, medical records were reviewed by trained medical record abstractors. Blinded review of abstracted records by other physician members of the Morbidity Review Committee showed >90% concordance in diagnosis.

Definition of case-control sample. This case-control sample was designed to examine the relationship between CVD and haptoglobin phenotype. A total of 206 CVD cases and 206 controls (matched for age, gender, and geographic area) were included in the analysis. Incident cases of fatal and nonfatal CVD were ascertained during ongoing surveillance between 1989 and 1998 (18). Cases in this report consisted of one-half of all CVD cases identified during this surveillance period. These cases were selected at random. Controls were selected at random from all age, gender, and center-matched individuals without CVD at the time of case ascertainment.

Haptoglobin phenotyping. The haptoglobin gene on chromosome 16 exists as two allelic variants (5,6). The haptoglobin phenotype refers to the distinct set of polymeric haptoglobin molecules produced from the two classes of haptoglobin alleles. The polymeric nature of the haptoglobin molecule (dimer, linear, or cyclic polymer) is dependent

on the haptoglobin alleles because the protein product of the 1 allele is monovalent, combining with only one other haptoglobin monomer, while the protein product of the 2 allele is bivalent, combining with two other haptoglobin monomers (6). A signature pattern of polymeric species is, therefore, obtained from individuals who are homozygous for the 1 allele (Hp 1-1), homozygous for the 2 allele (Hp 2-2), or are heterozygous at the haptoglobin locus (Hp 2-1). This unique pattern of polymeric species first demonstrated over 45 years ago by Smithies (24) by gel electrophoresis remains the most common method used today for phenotyping haptoglobin. Accordingly, we phenotyped haptoglobin from 10 μl of plasma by polyacrylamide gel electrophoresis according to established methods (25). Haptoglobin phenotyping was performed without knowledge concerning participant case/control status. Because diabetes is associated with posttranslational modification of serum proteins, there was concern whether the haptoglobin phenotype of diabetic patients would accurately reflect the correct haptoglobin allele. We observed the same three unambiguous banding patterns in nondiabetic and diabetic patients. Furthermore, proof of coincidence in diabetic patients of the haptoglobin phenotype and the haptoglobin genotype was demonstrated by 100% concordance in over 300 diabetic and nondiabetic patients in which we have typed haptoglobin both from the plasma and from DNA by polymerase chain reaction.

Statistical analysis. Cardiovascular disease risk factors including age, gender, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, systolic BP, body mass index (BMI), diabetes, smoking status, family history of CVD, and geographic area were compared between cases and controls as well as between the three haptoglobin phenotypes. In addition, DM-associated characteristics including fasting glucose and insulin levels, HbA1c, DM duration, and family history of DM were compared between cases and controls as well as between the three haptoglobin phenotypes. Univariate and multinomial logistic regression modeling was performed to determine if these CVD risk factors and DM characteristics were related to phenotype. The likelihood ratio test was used to evaluate individual model parameters.

Consistent with the case-control design, conditional logistic regression was used to model the probability of having a CVD event for diabetic and nondiabetic patients by the three haptoglobin phenotypes. A diabetes-haptoglobin phenotype interaction term was coded using two indicator variables, one for patients with diabetes and another for patients without diabetes. Nested models were constructed, first with only the DM-haptoglobin variables, then adjusted for DM characteristics (fasting glucose and insulin levels, HbA1c, and family history of DM), and then for DM characteristics and CVD risk factors (LDL cholesterol, HDL cholesterol, triglycerides, systolic BP, BMI, smoking status, and family history of CVD). Adjustment was designed to account for known and postulated biological relationships among specific factors in the disease pathway. Adjustment for known CVD risk factors in the presence of haptoglobin permits testing of the hypothesis that haptoglobin is related to CVD independently of known CVD risk factors, and whether this relationship is similar in diabetic and nondiabetic participants. Model fit was assessed by an analysis of residuals. We report odds ratios and 95% confidence intervals (CI) for the probability of a CVD event for each haptoglobin phenotype in diabetic and nondiabetic individuals.

RESULTS

Table 1 shows the clinical characteristics of the case-control sample according to CVD risk factors and DM characteristics. Cases and controls were matched for age, gender, and geographic area. These data are consistent with our previous finding in this population that DM, LDL cholesterol, triglycerides, and hypertension are all independent predictors of CVD (18).

The distribution of the haptoglobin phenotypes according to case-control and DM status is shown in Table 2. The distribution of the haptoglobin phenotypes in the case-control sample was consistent with the Hardy-Weinberg expectation (p = 0.98). We found no significant difference between the haptoglobin phenotypes for any of the CVD risk factors or DM characteristics as determined both by univariate analysis and by multinomial logistic regression analysis. There was no significant difference in the duration of DM between the haptoglobin phenotypes.

Table 3 shows conditional logistic regression analysis of the odds of CVD for each of the haptoglobin phenotypes in DM and non-DM individuals before and after adjustment for DM characteristics and CVD risk factors. After adjustment for DM characteristics and CVD risk factors, participants with DM and an Hp 2-2 phenotype were 4.96 (95% CI, 1.85 to 13.33) times more likely to have had a CVD event than those with DM and an Hp 1-1 phenotype (p = 0.002) and 3.04 (95% CI, 1.30 to 7.09) times more likely to have had a CVD event than those with DM and an Hp 2-1 phenotype (p = 0.010). Moreover, participants with DM and an Hp 2-1 phenotype were 1.63 (95% CI, 0.74 to 3.63) times more likely to have had a CVD event than those with DM and a Hp 1-1 phenotype although this was not statistically significant. This analysis was also performed including only cases and controls with diabetes, and the results were the same although the CIs were wider. Taken together, these data suggest a graded risk associated with the number of haptoglobin 2 alleles on development of CVD in DM that is independent of conventional CVD risk factors and DM characteristics. Finally, in patients without DM, we observed a trend of borderline statistical significance showing that the non-DM patients with a haptoglobin phenotype of 2-2 were 2.73 (95% CI, 0.81 to 9.26) times more likely to have had a CVD event than non-DM with a 1-1 haptoglobin phenotype (p = 0.107).

Table 1. CVD Risk Factors by Case-Control Status

CVD Risk Factors	Controls			Cases		
	Mean	STD		Mean	STD	
Age	59.16	8.01		60.09	8.08	
LDL cholesterol	112.1	31.44		123.0	40.47	
(Percentile)	**25th**	**50th**	**75th**	**25th**	**50th**	**75th**
Systolic BP (mm Hg)	115	124	137	120	131	148
BMI (kg/m²)	26	30	34	27	30	33
HbA1c (% tot Hb)	4.0	4.0	8.3	4.0	7.2	10.0
Fasting glucose (mg/dl)	100	119	178	110	149	230
Insulin (uU/ml)	9.0	16.0	25.0	11.8	18.7	28.9
HDL (ng/dl)	37	42	52	34	41	49
Triglycerides (ng/dl)	82	119	159	92	134	203
	n	**%**		**n**	**%**	
Female gender	102	49.51		102	49.51	
Diabetes	93	45.15		146	70.87	
Current smoker	136	66.02		143	69.42	
Family hx DM	131	63.59		145	70.39	
Family hx CVD	119	57.77		148	71.84	
Center						
OK	74	35.92		74	35.92	
SD	73	35.44		73	35.44	
AZ	59	28.64		59	28.64	

The 25th, 50th, and 75th percentiles denote the 1st, 2nd, and 3rd quartile values for that variable (i.e., LDL) present in that patient group (control or case).

AZ = Arizona; BMI = body mass index; BP = blood pressure; CVD = cardiovascular disease; DM = diabetes mellitus; HDL = high-density lipoprotein; hx = history of; LDL = low-density lipoprotein; OK = Oklahoma; SD = South Dakota; STD = standard deviation.

DISCUSSION

This case-control study demonstrates that haptoglobin phenotype is a significant predictor of CVD in individuals with DM. Individuals with the Hp 2-2 phenotype had significantly higher odds of CVD compared with individuals with the Hp 2-1 or Hp 1-1 phenotypes. This relationship persisted after adjustment for known CVD risk factors and DM characteristics. This study, therefore, suggests that determination of the haptoglobin phenotype may contribute to the algorithm used in CVD risk stratification and in the evaluation of new therapies to prevent CVD in the diabetic patient. A theranostic application of the knowledge that a diabetic patient has the Hp 2-2 phenotype would be to more aggressively manage concomitant cardiovascular risk factors in such a patient.

Our findings are inconsistent with a recently reported prospective study from Belgium demonstrating increased cardiac mortality in patients with the Hp 1-1 phenotype [17]. Three differences between the two studies may explain the apparently inconsistent results. First, only 7% of the Belgian cohort had DM as opposed to nearly 60% of the SHS cohort. The DM subgroup in the Belgian cohort was too small to be able to determine relative risk by haptoglobin phenotype in DM. The interaction of DM and haptoglobin phenotype on risk of CVD is a key finding in our study. Second, the Belgian study examined a single cardiovascular end point, CVD mortality; our report includes both fatal and nonfatal cardiovascular events and may, therefore, provide greater insight into the relationship between haptoglobin and CVD [20]. Finally, only 35% of eligible patients were enrolled in the Belgian study [17], potentially leading to selection bias in that study. In contrast, the SHS enrollment was approximately twice that of the Belgian study, suggesting a greater degree of generalizability of the findings [20]. Because our study cohort includes a significant percentage of individuals of advanced age, we cannot rule out the effect of selection bias in this study due to survivorship. Selective mortality may have introduced a bias if DM carriers of the haptoglobin 1 allele were more likely to die before they entered the study and could be phenotyped. Thus, DM patients with Hp 1-1 who were most susceptible to CVD may have self-selected out of the study due to early mortality. The

Table 2. Distribution of Hp Types by Case-Control and Diabetes Status

Hp Type	Cases	Controls
	n (%)	n (%)
DM		
1-1	32 (49)	33 (51)
2-1	64 (59)	44 (41)
2-2	50 (76)	16 (24)
Nondiabetic		
1-1	9 (26)	25 (74)
2-1	24 (33)	49 (67)
2-2	27 (41)	39 (59)

Note that this was a matched case-control study. Statistical analysis presented in the manuscript were carried out on matched pairs, and this table ignores matched pairs.

DM = diabetes mellitus; Hp = haptoglobin.

Table 3. Conditional Logistic Regression Predicting the Probability of a CVD Event

Unadjusted Variables	OR	95% CI	p Value
DM and Hp 2-1 (vs. DM and Hp 1-1)	2.32	(1.27–4.23)	0.006
DM and Hp 2-2 (vs. DM and Hp 1-1)	5.08	(2.37–10.89)	<0.001
DM and Hp 2-2 (vs. DM and Hp 2-1)	3.26	(1.67–6.37)	<0.001
No DM, Hp 2-1 (vs. no DM, Hp 1-1)	0.63	(0.33–1.20)	0.159
No DM, Hp 2-2 (vs. no DM, Hp 1-1)	1.10	(0.53–2.30)	0.795
No DM, Hp 2-2 (vs. no DM, Hp 2-1)	0.75	(0.40–1.38)	0.350

Adjusted for DM Characteristics Only			
Variables	OR	95% CI	p Value
DM and Hp 2-1 (vs. DM and Hp 1-1)	1.86	(0.93–3.69)	0.078
DM and Hp 2-2 (vs. DM and Hp 1-1)	3.90	(1.68–9.09)	0.002
DM and Hp 2-2 (vs. DM and Hp 2-1)	2.10	(1.00–4.40)	0.049
No DM, Hp 2-1 (vs. no DM, Hp 1-1)	1.40	(0.48–4.09)	0.542
No DM, Hp 2-2 (vs. no DM, Hp 1-1)	2.31	(0.76–7.05)	0.141
No DM, Hp 2-2 (vs. no DM, Hp 2-1)	1.65	(0.73–3.75)	0.228

Adjusted for DM Characteristics and CVD Risk Factors			
Variables	OR	95% CI	p Value
DM and Hp 2-1 (vs. DM and Hp 1-1)	1.63	(0.74–3.63)	0.228
DM and Hp 2-2 (vs. DM and Hp 1-1)	4.96	(1.85–3.33)	0.002
DM and Hp 2-2 (vs. DM and Hp 2-1)	3.04	(1.30–7.09)	0.010
No DM, Hp 2-1 (vs. no DM, Hp 1-1)	1.46	(0.44–4.87)	0.542
No DM, Hp 2-2 (vs. no DM, Hp 1-1)	2.73	(0.81–9.26)	0.107
No DM, Hp 2-2 (vs. no DM, Hp 2-1)	1.88	(0.73–4.80)	0.190

Cases and controls were matched for age, gender, and geographical center. Statistical modeling adjusted for the following DM characteristics and CVD risk factors. DM characteristics: HbA1c, fasting glucose and insulin, and family history of diabetes. CVD risk factors: low-density lipoprotein and high-density lipoprotein cholesterol, triglycerides, cigarette use, systolic blood pressure, body mass index, and family history of CVD.

CI = confidence interval; CVD = cardiovascular disease; DM = diabetes mellitus; Hp = haptoglobin; OR = odds ratio.

Belgian study cited above appears to support this idea, but this is not the case (17). Although the number of DM patients in the Belgian study was too small to permit meaningful statistical analysis, the cardiovascular mortality rate in DM patients with Hp 2-2 or Hp 2-1 was double that in Hp 1-1. Furthermore, population-based analysis of haptoglobin type stratified by age has failed to show an increased mortality rate among carriers of the 1 allele (26).

We cannot rule out the possibility that our findings are not due to a polymorphism in haptoglobin but rather could be explained by linkage disequilibrium with another gene that is in close proximity to the haptoglobin gene. One gene of interest is the LCAT (lecithin-cholesterol acyl transferase) gene, which is found in close proximity to the haptoglobin gene on chromosome 16 (27). Deficiency of the LCAT gene product, a rare recessive condition, has been associated with premature atherosclerosis (27). It is important to note that our results suggest an association between functionally distinct phenotypes of the haptoglobin gene product and CVD. This is distinct from linkage disequilibrium, which is the result not only of physical proximity of loci, but also of many other factors related to the evolutionary history of nucleotide diversity in the region. In order for the association we have reported to reflect an association based on linkage disequilibrium with LCAT or any other gene, it would be necessary to define the relevant polymor-

phisms in the LCAT gene and/or establish the pattern of linkage disequilibrium across the whole region based on the generation of haplotypes.

Several functions have been assigned to the haptoglobin protein that may impact on development of atherosclerotic CVD. It has been appreciated for over 60 years that a major function of serum haptoglobin is to bind free hemoglobin (5). This interaction is thought to help scavenge iron, and prevent its loss in the urine, and to serve as an antioxidant, thereby protecting tissues against hemoglobin-mediated tissue oxidation (6). The antioxidant capacity of the different haptoglobin phenotypes has been shown to differ, with the Hp 1-1 protein appearing to confer superior antioxidant protection as compared with the other forms of the protein (6,10). Such an antioxidant hypothesis is particularly intriguing given the apparent important role of oxidative stress in the development of diabetic vascular complications (28). Potentially further amplifying differences in the oxidative protection afforded by the different types of haptoglobin are gross differences in the size of the haptoglobin protein present in individuals with the different phenotypes. Haptoglobin 1-1 is markedly smaller than Hp 2-2 and may, thus, be better able to sieve into the extravascular compartment and prevent hemoglobin-mediated tissue damage at sites of vascular injury (6). Haptoglobin has also been demonstrated to play a role as an immunomodulator that

may not be unrelated to its role in hemoglobin metabolism (6).

The SHS population is a distinct ethnic group with unique metabolic characteristics associated with a high degree of obesity and other components of the metabolic syndrome, including diabetes. However, DM has not been shown to have unique physiology in American Indians. Indeed, current American Diabetes Association and WHO definitions of DM are largely based on data collected in American Indian communities. The fact that fundamental clinical practice guidelines are based on data from American Indians indicates that data from these communities are generalizable to other populations. In support of the generalizability of findings in this report are previous studies in which we demonstrated that the haptoglobin phenotype is predictive of the development of diabetic microvascular complications in a Semitic population of diabetics (11–13). Furthermore, we have recently found in a one-year follow-up study after coronary artery stent placement of over 900 diabetic patients in Germany that patients with the 1-1 Hp phenotype have significantly less myocardial infarction and less need for target vessel revascularization as compared with patients with the Hp 2-1 or Hp 2-2 phenotypes (Roguin A, Koch W, Kastrati A, Schomig A, Levy AP, unpublished data). Thus, although the anthropometric and metabolic profiles of American Indians are unique, the role of DM in CVD and the potentially modifying role of haptoglobin in this pathway are similar across ethnicity.

Haptoglobin phenotype appears to be less predictive of CVD in the non-DM population as compared with the DM population. This may be due to differences in the relative importance of antioxidant protection between DM and non-DM patients. While oxidative modification of proteins has been implicated in the pathogenesis of atherosclerotic disease in the non-DM as well as in the DM patient, the individual with DM is under considerably more oxidative stress than the individual without DM (28). Genetic differences in the endogenous antioxidant status, as determined by the haptoglobin phenotype, may be, therefore, of considerable importance in DM.

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