The Risk of Coronary Heart Disease Associated With Glycosylated Hemoglobin of 6.5% or Greater Is Pronounced in the Haptoglobin 2-2 Genotype

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ABSTRACT

BACKGROUND Research targeting glycosylated hemoglobin A1c (HbA1c) to <6.5% to prevent coronary heart disease (CHD) events has conflicting results. We previously observed the haptoglobin (Hp) Hp2-2 genotype is associated with a ~10-fold increased CHD risk among individuals with HbA1c ≥6.5%, and thus might be useful in identifying those at high risk of CHD who would benefit from maintaining HbA1c <6.5%.

OBJECTIVES This study sought to model whether HbA1c ≥ 6.5% in the Hp2-2 genotype is associated with CHD in a prospective case-control study nested within the Health Professionals Follow-Up Study (HPFS).

METHODS HbA1c concentration and Hp genotype were determined for 695 incident cases of CHD from 1994 to 2010 and matched control participants. Logistic regression models calculated relative risk (RR) and 95% CI, for the first and second halves of follow-up, adjusting for confounding variables. A dataset from the Nurses’ Health Study served as a replication cohort.

RESULTS The prevalence of the Hp2-2 genotype in HPFS was 39%. Compared with HbA1c <6.5%, the RR of CHD for HbA1c ≥6.5% for the Hp2-2 genotype over full follow-up was 3.07 (95% CI: 1.37 to 6.86) to 3.88 (95% CI: 1.31 to 11.52) during the first half of follow-up and 2.16 (95% CI: 0.61 to 7.61) in the second half. The corresponding RRs for the Hp1-1 + Hp2-1 genotypes were: full follow-up, 2.19 (95% CI: 1.14 to 4.24); first half, 1.60 (95% CI: 0.73 to 3.53); and second half, 4.72 (95% CI: 1.26 to 17.65).

CONCLUSIONS In 2 independent cohorts, the risk of CHD associated with HbA1c ≥6.5% is pronounced in the Hp2-2 genotype, particularly in early cases. The Hp2-2 genotype may identify individuals at greatest CHD risk from hyperglycemia. (J Am Coll Cardiol 2015;66:1791–9) © 2015 by the American College of Cardiology Foundation.
large, randomized, controlled trials that have targeted glycosylated hemo-
globin A1c (HbA1c) to a concentration of <6.5% among participants with diabetes
mellitus have found significant reduction in cardiovascular outcomes in some (1,2) but
not all (3,4) studies. This inconsistency may be due in part to different unknown charac-
teristics among patient subgroups, which have not yet been explored (5). The common
haptoglobin (Hp) copy number variant (CNV) (rs72294371) has 3 genotypes (Hp1-1, Hp2-1,
and Hp2-2) with frequencies that differ among populations (6) and may explain
potentially the differences in the efficacy of strict glycemic control between study populations. The
primary function of Hp is to protect against oxidative damage from extracorpuscular hemoglobin (7). The 3
Hp genotypes produce structurally different Hp proteins, of which the Hp2-2 is the least functional—an impairment that is further accentuated when hemoglobin is glycosylated (8). Currently available
genome-wide association study (GWAS) technologies have not been found to capture the Hp CNV polymor-
phism, explaining why the association of this polymorphism with cardiovascular disease in the setting of elevated blood glucose has not been investigated in cohorts screened only by GWAS (9–11).

In 2 independent populations, we have observed that participants with both the Hp2-2 genotype (ho-
mozygous for presence of CNV) and HbA1c ≥6.5% had a 10-fold higher risk of coronary heart disease (CHD)
compared with those with at least 1 Hp1 allele and HbA1c <6.5% (12). However, a recent analysis of car-
diovascular disease among adults in the Bruneck study did not observe a similar association (13). The
discrepancy may be due to several study design fac-
tors, especially the combined endpoint of CHD and stroke (the majority of cases were stroke), because stroke
has been associated with the Hp1-1 genotype rather than the Hp2-2 genotype (14,15). However, the
Bruneck study is of interest because it contained repeated measures of HbA1c and provides suggestive
evidence for a potential time-dependent bias against the Hp2-2 genotype as participants aged that supports
previous findings of increased longevity among individuals with the Hp1-1 genotype (16,17). Thus, if
Hp2-2 individuals are at increased risk of early disease and death, it strengthens the growing evidence
behind the hypothesis that targeted screening and treatment for the Hp2-2 genotype in cases of HbA1c
≥6.5% could prevent coronary events.

In the present study, we used a large prospective case-control study nested within a cohort of healthy
men at baseline to further examine the interaction between the Hp polymorphism and HbA1c concen-
tration on risk of CHD, testing the hypothesis that targeted screening for the Hp2-2 genotype in the
setting of HbA1c ≥6.5% may be prudent. Because previous long-term studies have reported that the
association between HbA1c and the risk of CHD is strongest in the earliest years of follow-up (18),
we examined the first and second halves of follow-up separately as well as together. We then sought replication
of our full analysis in a complementary, but independent, cohort of women.

METHODS

COHORT: THE HEALTH PROFESSIONALS FOLLOW-UP
STUDY. The Health Professionals Follow-up Study (HPFS) is a prospective cohort of 51,529 male health
professionals who were 40 to 75 years of age at baseline in 1986. Information on anthropometric and lifestyle factors is obtained through self-administered ques-
tionnaires every 2 years and diet every 4 years. Blood samples were collected from participants free of cancer and without prior cardiovascular events in 1993 to
1995. Men who had an incident myocardial infarction (MI) or fatal CHD between the date of blood draw and
January 2010 were identified and matched 1:1 with control participants with the same age, smoking sta-
tus, and month of blood draw. Participants were excluded from the final sample in the present analysis if they were missing data on the exposures or the
outcome. Using 2-tailed tests, the type I error probability of 0.05, a prevalence in control participants of
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METHODS
obtained through self-administered questionnaires every 2 years and diet every 4 years. From 1989 to 1990, a blood sample was provided by 32,826 women. Women who had an incident MI or fatal CHD between the date of blood draw and June 2004 were identified and matched 1:1 with control participants for age, smoking status, fasting status, and month of blood draw, as described elsewhere (20). Details of the genotype frequencies and baseline characteristics of the specific sample used in this present study have been previously reported (12), but the hypothesis tested is new in the present study. Using 2-tailed tests, the type I error probability of 0.05, a prevalence in control participants of 0.39 for the Hp2-2 genotype, and our final NHS sample size of 400 cases matched 1:1 to control participants, we have >80% power to detect an odds ratio of 1.50.

**Hp Typing.** Although recently given the identifier rs72294371 (21), the Hp polymorphism is not a single nucleotide polymorphism; it is a CNV defined by the absence (Hp1 allele) or presence (Hp2 allele) of a 1.7 kb partial in-frame intragenic duplication of exons 3 and 4. Hp type was determined in both cohorts by protein gel electrophoresis of hemoglobin-enriched serum (22). This procedure produces a fingerprint banding pattern for each Hp type and has been shown to correspond completely with the Hp genotype (23). Currently available GWAS platforms do not include the Hp CNV polymorphism (9–11). Genotype frequencies in the HPFS were in Hardy-Weinberg equilibrium in the whole sample (p = 0.08) and also within cases (p = 0.28) and control participants (p = 0.17) separately. Genotype frequencies in the NHS were also in Hardy-Weinberg equilibrium, as reported previously (12).

**CHD Case Assessment.** As previously detailed in the HPFS (24) and the NHS (25), CHD is similarly defined in the 2 datasets as nonfatal MI or fatal CHD, but does not include the development of coronary atherosclerosis which has not resulted in an acute coronary syndrome. MI was diagnosed on the basis of the criteria of the World Health Organization (symptoms plus either diagnostic electrocardiographic changes or altered levels of cardiac enzymes) (26). After a participant reported a nonfatal event on a questionnaire, the case was confirmed through the review of medical records by physicians blinded to the self-report. Deaths were identified from state vital records and the National Death Index or reported by the participant’s next of kin or the postal system. Fatal CHD was confirmed by an examination of hospital or autopsy records.

**Statistical Analysis.** Participant characteristics were compared between cases and control participants using Student t tests for continuous variables and chi-square tests for categorical variables. For skewed variables (physical activity, alcohol, high sensitivity C-reactive protein, high-density lipoprotein [HDL] cholesterol, HbA1c, and triglycerides), p values from log-transformed analyses and median and interquartile ranges were determined. The primary hypothesis we tested was that HbA1c ≥6.5% in the risk genotype (Hp2-2) is associated with CHD in a prospective nested case-control study. Because of the nested case-control study design, relative risks (RRs) of CHD were estimated by incidence rate ratios from logistic regression with adjustment for the matching factors. In addition to matching factors, analyses were adjusted for body mass index, alcohol, physical activity, parental MI before the age of 60, diet quality score (the 2010 Alternate Healthy Eating Index) (27), history of high cholesterol, history of high blood pressure, and medications for high blood pressure. We used an a priori cutpoint of 6.5% for HbA1c, because this is the level for complications from hyperglycemia as established by the International Expert Committee composed of members of the American Diabetes Association, the European Association for the Study of Diabetes, and the International Diabetes Federation (28). Because of the low frequency of the Hp1-1 genotype (~15%) and the structure (29) and function (30) of the different Hp proteins, we used a common approach of combining the Hp1-1 and Hp2-1 genotype for most analyses (13). Because a single measure of HbA1c at baseline has been shown to be differently associated with short-term CHD risk than long-term CHD risk (18), we conducted analyses within halves of follow-up (first 8 years and second 8 years), in addition to examining the full follow-up period. To pool the risk estimates from multiple study cohorts, we used the weighted average of regression estimates in a random-effects meta-analysis, and tested for between-study heterogeneity (31). All analyses were conducted using SAS software version 9.3 (SAS Institute, Cary, North Carolina), at a 2-tailed alpha level of 0.05.

**Results**

The distribution of Hp genotype frequencies in the HPFS cohort was 16% (Hp1-1), 45% (Hp2-1), and 39% (Hp2-2). The Hp genotype frequency did not differ between cases and control participants. Baseline characteristics by case-control status are described in Table 1. As expected, cases drank less alcohol and had a lower diet quality than control participants, and had higher body mass index, family history of CHD prevalence of hypertension, diabetes, hypercholesterolemia, and greater medication use at baseline.
For the Hp2-2 genotype and the Hp1 allele carriers separately, Table 2 presents the multivariate-adjusted RR and 95% CI of CHD when HbA1c was ≥6.5% compared with when HbA1c <6.5%. HPFS participants with the Hp2-2 genotype had a multivariate RR of 3.07 (95% CI: 1.14 to 4.24) among participants with both HbA1c ≥6.5% and the Hp2-2 genotype (12). When we reanalyzed the NHS with identical methods to our present HPFS analysis and pooled results, our findings were strong and consistent. The risk of CHD associated with HbA1c ≥6.5% for the Hp2-2 genotype was substantial (RR: 10.59; 95% CI: 2.34 to 47.91), but even potentially greater when we restricted the analysis to the first 8 years after blood draw (RR: 28.62; 95% CI: 3.27 to 250.72), and was not significant after the first 8 years of follow-up (RR: 4.98; 95% CI: 0.54 to 46.16) (Table 2). Among NHS participants, the RR of CHD for HbA1c ≥6.5% was not significant for the Hp1-1-Hp2-1 genotypes (RR: 2.12; 95% CI: 0.98 to 4.60). Pooling the results from the HPFS and NHS substantially increased the power and highlighted the differences in the RR of CHD for HbA1c ≥6.5% among the Hp 2-2 genotypes (RR: 8.38; 95% CI: 1.25 to 56.32) and Hp1 carriers (RR: 2.12; 95% CI: 1.02 to 4.39) during the first 8 years of follow-up. The multivariate-adjusted p values for the tests of interaction were not significant. Results were not appreciably altered by further adjustment for cholesterol-lowering medications, such as statins, including those taken during the follow-up period.

In keeping with the format of previous analyses (12), pooled risk of CHD was calculated for HPFS and NHS participants together, with participants grouped by the combination of their Hp genotype and HbA1c status (Online Figure 1). Compared with participants who carried an Hp1 allele and had HbA1c <6.5%, those with the Hp2-2 genotype and HbA1c ≥6.5% had a 4.25-fold increased risk of CHD (95% CI: 1.05 to 17.22), whereas those with the Hp2-2 genotype and HbA1c <6.5% did not have a significant risk of CHD (RR: 0.92; 95% CI: 0.69 to 1.23).

**DISCUSSION**

In a prospective, nested, case-control study design with 16 years of follow-up, HbA1c ≥6.5% was a strong and significant predictor of CHD among men with the Hp2-2 genotype, especially in the cases most proximal to blood draw. Our results are strengthened...
by the replication of these findings in women, using data from a second cohort.

In 2 independent cohorts with a broad range of HbA1c concentrations (the Israel Cardiovascular Events Reduction With Vitamin E Study, and the same NHS dataset used as a replication cohort in the present study), we have reported previously that individuals with the Hp2-2 genotype and HbA1c ≥6.5% had a >10-fold increased risk of CHD compared with those with an Hp1 allele and HbA1c <6.5% (12). Previously reported results from the Strong Heart Study data support additional replication (12,32). However, a recent longitudinal analysis of cardiovascular disease among adults in the Bruneck study did not report that HbA1c ≥6.5% in the Hp2-2 genotype was predictive of cardiovascular disease (13). The authors did not present directly the RR of CHD for HbA1c ≥6.5% among the Hp2-2 subgroup, but did present a nonsignificant risk (multivariate-adjusted RR: 0.35; 95% CI: 0.08 to 1.52) for the Hp2-2 versus Hp2-1+Hp1-1 genotypes in the strata of participants with HbA1c ≥6.5%. The discrepancy in findings may be due to several distinguishing factors. For example, the Bruneck study included participants with prevalent disease at baseline and had a small number of cases during the 15 years of follow-up (123 cases, among which only 48 cases were CHD). The endpoint of CHD was combined with stroke, an endpoint that has been associated with the Hp1-1 genotype rather than Hp2-2 (14,15). It has been suggested that the protection conferred by the Hp1-1 genotype

### TABLE 2

<table>
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<th>Haptoglobin Genotype</th>
<th>HbA1c &lt;6.5%</th>
<th>HbA1c ≥6.5%</th>
<th>HbA1c &lt;6.5%</th>
<th>HbA1c ≥6.5%</th>
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<td>3.07 (1.37-6.86)</td>
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<tr>
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<td>21/11</td>
<td>135/150</td>
<td>19/5</td>
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<tr>
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<td>1.60 (0.73-3.53)</td>
<td>1.00 (Ref.)</td>
<td>3.88 (1.31-11.52)</td>
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<td></td>
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<td>102/116</td>
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<td>1.00 (Ref.)</td>
<td>2.16 (0.61-7.61)</td>
<td>0.42</td>
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<tr>
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<td>All participants</td>
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<td></td>
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</tr>
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<td>N, cases/control participants</td>
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<td>372/418</td>
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<td>46/16</td>
<td>182/231</td>
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<td>RR (95% CI)</td>
<td>1.00 (Ref.)</td>
<td>2.12 (1.02-4.39)</td>
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<td>8.38 (1.25-56.32)</td>
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<td>190/187</td>
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<td>RR (95% CI)</td>
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<td>2.03 (0.41-10.13)</td>
<td>1.00 (Ref.)</td>
<td>2.64 (0.88-7.92)</td>
<td>0.78</td>
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The multivariate model is adjusted for matching factors (age and smoking) and also: body mass index, alcohol, physical activity, parental coronary heart disease before the age of 60 years, diet quality score, history of high cholesterol, history of high blood pressure, and medications for high blood pressure.

HPFS = Health Professionals Follow-Up Study; NHS = Nurses Health Study; Ref. = reference group; RR = relative risk; other abbreviations as in Table 1.
against CHD development is connected to function of Hp as the scavenger of free hemoglobin, whereas the role of Hp in angiogenesis may confer the protection against stroke associated with the Hp2-2 phenotype (33,34).

A single biomarker at baseline may be a better proxy for exposure over the first several years, and possibly become less correlated with exposure over a longer period of time. In a recent 14-year study of HbA1c and risk of CHD, when we stratified according to the midpoint of follow-up, the association was strongest among cases and control participants in the first half (first 7 years) of follow-up, even when limited to only participants with HbA1c $< 6.5\%$ (18). In the present analysis, when we analyzed the HPFS data in halves of follow-up (two 8-year-long studies instead of one 16-year-long study), we observed an increased risk of CHD in the Hp2-2 participants with HbA1c $\geq 6.5\%$ in the first half, but not the second half of follow-up, and an increased risk of CHD in the Hp1-1/Hp2-1 participants with HbA1c $\geq 6.5\%$ in the second half of follow-up but not the first half. We observed the same pattern in the NHS data as well. It is possible that our single measurement of HbA1c may not be a strong predictor of risk beyond 8 years. In our pilot studies, HbA1c did have a strong intraclass correlation of 0.73 over a 3-year period ($r$ between draws = 0.88; $n = 83$), but when HbA1c was measured in blood samples collected 10 years apart, the intraclass correlation was 0.45 ($r$ between draws = 0.68; $n = 244$), which suggests good within-person reproducibility of HbA1c over periods of time as long as a decade, but that this reproducibility still decays over time. The fact that the correlations between draws are higher than the intraclass correlations indicates that the HbA1c is changing in the same direction for participants. Indeed, HbA1c levels are associated positively with age, even among populations without impaired fasting glucose (35,36), suggesting that HbA1c $\geq 6.5\%$ may be a weaker marker of risk among older populations. However, the increase in HbA1c with age is considered to be modest (37) and age-specific diagnostic and treatment criteria do not exist.

If individuals with the Hp2-2 genotype are more susceptible to CHD, they may have cardiovascular events at an earlier age and have overall reduced longevity than the other genotypes, creating a survivor bias or depletion of susceptibles that may explain in part why we observe a stronger association in the earlier follow-up years and even a reverse pattern in the later years. Analyses in Bruneck participants stratified by age displayed an interesting pattern: in participants age 75 years or older only, the risk of cardiovascular disease for the Hp2-2 type compared with the Hp1-1 type was 0.11 (95% CI: 0.01 to 0.95) among those with HbA1c $\geq 6.5\%$, suggesting a potential survivor bias against the Hp2-2 genotype with increased age. This is consistent with previous studies that suggest increased longevity among individuals with the Hp1-1 genotype (16,17).

**BIOLOGICAL MECHANISMS.** The large difference in size of the Hp CNV’s 2 alleles, Hp1 and Hp2, results in structurally and functionally different proteins being formed by each of the 3 genotypes (38). Hb released intravascularly from erythrocytes is rapidly bound by Hp protein to form an Hp–Hb complex that is cleared by the monocyte/macrophage scavenger receptor CD163. However, this clearance by CD163 is impaired in Hp2-2 individuals as well as under hyperglycemic conditions, resulting in increased amounts of circulating Hp2-2:Hb complex in Hp2-2 individuals with hyperglycemia (8,39) (Central Illustration). Further, the glycosylation of hemoglobin impairs the ability of the Hp2-2 protein to act as an antioxidant, thus resulting in increased oxidative activity of the glycosylated Hp2:Hb complex (8). This pro-oxidant Hp2-2:Hb complex can bind to HDL and produce reactive oxygen species that oxidize HDL and its related components, such as apolipoprotein A, glutathione peroxidase, and lecithin-cholesterol acytransferase, thereby decreasing the function of HDL as both an antioxidant and in its role in reverse cholesterol transport, and paradoxically turning the HDL into a proatherogenic prothrombotic molecule (8,39-43). Our results were stronger in our analysis of women than of men, potentially due to higher HDL among women and thus more influence from the dysfunctional HDL associated with the Hp2-2 genotype in the setting of hyperglycemia. However, we were unable to assess this potential sex difference directly in the present study.

**IMPLICATIONS.** Replication is required to confirm findings from the present study, and should be attempted in a randomized trial targeting HbA1c to concentrations <6.5%, such as the ACCORD (Action to Control Cardiovascular Risk in Diabetes) study (4), the ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation) trial (1), and the VADT (Veterans Affairs Diabetes Trial) (3). These studies have reported conflicting results regarding the efficacy of achievement of HbA1c concentrations of <6.5% to prevent cardiovascular disease, an inconsistency that may be due in part to different unknown characteristics among patient subgroups, which have not yet been explored, as recently suggested in a meta-analysis (5). Unfortunately, currently available versions of single nucleotide polymorphism chips and
tagging single nucleotide polymorphisms cannot be
used to query the common Hp polymorphism (9), so
replication or confirmation of our findings by GWAS is
not possible. However, several genotyping and phe-
notyping methods exist that easily allow for direct
assessment of the Hp CNV polymorphism and could
be used to determine Hp genotype in ACCORD,
ADVANCE, or VADT. Such an investigation, with a
follow-up length of 3 to 5 years, could further
decrease the risk of survival bias for the Hp2-2 ge-
notype subgroup, and could also reflect a more con-
temporary sample than our own, due to medication
use and biomarker concentrations that better reflect
current clinical settings. If our findings are replicated,
Hp genotyping could potentially assist in identifying
the genetically susceptible individuals who would
most benefit from targeted clinical management of HbA1c.

**STUDY STRENGTHS AND LIMITATIONS.** Strengths of the present analysis include comprehensive data gathered prospectively with a long duration of follow-up, replication in a second cohort, and a validated Hp genotyping method. The main limitation of the present study is that we only had a single measurement of HbA1c, taken at baseline, which became less proximal during each year of follow-up. Thus, random error caused by normal fluctuations and increases in HbA1c with age (35,36) could cause underestimation of true RRs, or the biomarker may lose effectiveness as a measure of glycemia over time. Although we had a large number of cases and our results were significant, we lost power due to multiple stratifications, and the confidence intervals that we observed are wide, thus the exact risk estimate is difficult to estimate with much precision, and our main hypothesis needs confirmation within an intervention study design. Tests of interaction were underpowered, especially when adjusted for covariates. Bias from residual confounding is a potential limitation; however, our cases and control participants were matched on age and founding is a potential limitation; however, our cases and control participants were matched on age and smoking (the 2 strongest CHD risk factors), and our multivariate model showed little attenuation by known CHD risk factors. Another important limitation is that the participants we studied are predominantly Caucasian, so it is unknown whether the associations we observed would be similar in non-Caucasian populations.

**CONCLUSIONS**

In 2 independent cohorts, the risk of CHD associated with HbA1c ≥6.5% was intensified in individuals with the Hp2-2 genotype, an association that was pronounced in the earlier half of cases in this prospective nested case-control study. If replicated, the results of the present study suggest that targeted screening for the Hp polymorphism among individuals with HbA1c ≥6.5% could help to identify the patients who would most benefit from interventions to lower glycemia to HbA1c concentrations of <6.5%. The present study also suggests that aging and time-dependent bias limit observational research of the Hp polymorphism, and thus results would best be confirmed within a randomized clinical trial study design.

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**REFERENCES**


KEY WORDS acute myocardial infarction, coronary disease, epidemiology, genetic association, glycoproteins

APPENDIX For a supplemental figure, please see the online version of this article.