Abdominal aortic aneurysm (AAA) is a documented lethal disorder in older adults, the risk of rupture with which increases with aneurysm size. In normal aortas, the lamellar structure of media is the thickest layer and is an orderly array of collagen, elastin, and smooth muscle cells. Degeneration of this matrix leads to destruction of the lamellar structure of media and adventia, which in turn results in gradual aortic dilatation. Inflammation is associated with disruption of the orderly structure of the aortic media and appears to play a fundamental role in the progression and development of AAA.

Haptoglobin (Hp) is the major hemoglobin (Hb)-binding protein in humans and other mammals, and is one of the few acute phase proteins that exhibits increased synthesis during inflammation and is conserved in all vertebrate species studied. Hp is an extremely potent antiox-ident that directly inhibits low-density lipoprotein oxidation. Structurally, Hp is tetrameric with (β-α-α-β) joined by disulfide linkages between the two α and β chains. Three Hp phenotypes, 1-1, 2-1, and 2-2 share the same two β chains. Functional differences between the Hp phenotypes have been demonstrated; these appear to have important biological and clinical consequences.

Controversy exists with regard to the relationship between Hp phenotypes and the prevalence of cardiovascular disease. Findings from the Framingham offspring cohort suggest that there is decreased prevalence of coronary heart disease (CHD) in diabetic individuals with allele 2 (Hp 2-1 and Hp 2-2). In contrast, an increased prevalence of CHD was found in nondiabetic subjects with the Hp 2-1 phenotype compared with those with the Hp 1-1 phenotype. In another study, it was reported that the risk of cardiovascular disease in patients with sleep apnea aged <55 years with the Hp 2-2 phenotype was 2.32-fold higher than that in corresponding patients with the 2-1 phenotype. Further to this, it has been found that myocardial infarction patients with Hp 2-2 have more frequent left ventricular failure than patients with Hp 1-1 and Hp 2-1. Several other studies have reported relationships between the Hp 2-1 phenotype and the occurrence of AAA.

Although Hp phenotypes have been frequently reported to be associated with cardiovascular diseases, human plasma Hp concentrations, which may be associated with diseases and of diagnostic significance, have rarely been reported. This is almost certainly a reflection of the difficult purification procedures necessary for Hp immunoasay, although we have previously assayed this protein...
in humans and reported that there is significant between-
phytotype variability in plasma Hp levels.17

It would be interesting to know more about the rela-
tionships between plasma Hp levels, Hp phenotype, and
AAA. Hence, the aims of this study were to determine: a) the
association (if any) between Hp phenotype and AAA; and b) plasma Hp levels in AAA and non-AAA individuals
with different Hp phenotypes.

METHODS

Patients. The study participants were consecutively
enrolled between January 2009 and December 2009 in the
Divisions of Cardiovascular Surgery and Cardiology of
Taipei Veterans General Hospital. The cohort consisted of
45 patients with AAA and 49 non-AAA subjects.

Elective open repair operation or endograft stent im-
plantation was performed whenever informed consent was
obtained after consensus indications of surgery were ex-
plained to the patients with AAA. During the same period,
patients with positive thallium-201 myocardial perfusion
scans admitted with suspected coronary artery disease but
showing normal or insignificant coronary artery disease
(<40% luminal stenosis), normal ventricular diameter (as
determined by both coronary arteriography and left ven-
triculography), and normal abdominal aorta diameter (as
determined by aortographic examination) were catego-
rized as non-AAA. Patients with acute or chronic infectious
diseases and malignancy were excluded from the study.

Analysis of lipid profiles. Blood samples were ob-
tained (before surgical intervention) from all patients fol-
lowing overnight fasting (12 hours) and mixed with 0.1%
ethylenediamine tetraacetic acid. Total cholesterol, high
density lipoprotein, low density lipoprotein, and triglyc-
eride levels were analyzed enzymatically using commercial
reagents (CHOP-PAP Method; Merck Scientific Corpora-
tion, Germany). ApoAI concentration was determined by
high sensitivity enzyme-linked immunosorbent assay
(AlerCHEK, Inc, Portland, Me).

Measurement of human C-reactive protein (CRP)
plasma levels. The IMMAGE Immunochemistry Systems
CRPH reagent (Beckman Coulter, Inc, Fullerton, Calif),
based on the highly sensitive Near Infrared Particle Immu-
noassay rate methodology, was used to measure CRP. In
this assay, an anti-CRP coated particle binds to CRP in the
patient sample, resulting in the formation of insoluble
aggregates causing turbidity. The rate of aggregate forma-
tion is directly proportional to the concentration of CRP in
the sample.18

Hp phenotyping. Hb was isolated from lyzed red
blood cells. Hp phenotyping was conducted by native
polyacrylamide gel electrophoresis with Hb-supplemented
plasma. This assay has been described in detail in our
previous publication.17

Hp purification. Hp purification was performed us-
ing Ab-affinity chromatography. The affinity column (with
a 10 mL bed volume at room temperature) was washed
with 50 mL of phosphate buffered saline. The bound
materials were further washed with 50 mL of 0.02 M
phosphate buffer containing 0.2 M NaCl (pH 7.4), and
then eluted with 50 mL of a freshly prepared 0.15 M NaCl
solution (pH 11). Five milliliters of each fraction was
collected in a tube containing 0.25 mL of 1M Tris-HCl
buffer (pH 6.8) to facilitate neutralization of solution.

Pooled fractions containing Hp were then concen-
trated to a final volume of 1 mL using Centricon tubes
(Millipore, Cork, Ireland) and filtered through a 0.45-μm
membrane. Finally, the protein was run on a gel-filtration
HPLC Superose-12 column (1 × 30 cm; Pharmacia, Upp-
sala, Sweden). The homogeneity of each isolated Hp type
was >95% as determined by sodium dodecyl sulfate poly-
acrylamide gel electrophoresis.

Lyophilized Hp was stored at −80°C until analysis.

Preparation of anti-Hp monoclonal antibodies. Six
monoclonal antibodies (8B1-3A, W1-11G, 2-3H, G2D-
7G, 12B-1, and 4A2-4H) against human Hp were pro-
duced and characterized as previously described.17 Mono-
clonal antibody 8B1-3A, which possesses the highest
binding affinity for Hp, was selected for preparation of the
affinity column. Briefly, 120 mL of cultured medium from
the 8B1-3A hybridoma was precipitated in 50% saturated
ammonium sulfate. The precipitate was dissolved in 12 mL
of phosphate buffered saline (PBS) containing 0.02 M
phosphate and 0.15M NaCl (pH 7.4). The solution was
then dialyzed exhaustively in PBS to remove the remaining
ammonium sulfate. This was followed by dialysis in cou-
ing buffer containing 0.1M NaHCO3 and 0.6M NaCl,
(pH 8.3). All monoclonal antibody binding epitopes were
directed against the β-chain.

Measurement of human-free Hp plasma levels.
Human-free Hp plasma levels were measured using a
phenotype-matched standard sandwich enzyme-linked
immunosorbent assay, which has been comprehensively
described in a previous publication.17

Statistical analysis. Normal distributed continuous
variables are presented as the mean ± standard deviation.
Abnormally distributed continuous variables are presented
as the median (interquartile range). Categorical variables
are presented as the number in each category and the
percentage. Data were compared between groups by inde-
pendent samples t test (normally distributed continuous
variables), Mann-Whitney U test (abnormally distributed
continuous variables) and X2 or Fisher’s exact test (categori-
ical variables). Two-way analysis of variance was performed
to examine the relationship between age and Hp concen-
tration with regard to AAA grouping (AAA or non-AAA).
Subsequent analysis of covarance was performed due to the
lack of a significant interaction effect. All analyses were
performed using SAS statistical software (version 9.1.3;
SAS Inc, Cary, NC). All tests were two-sided, with differ-
ces being considered significant when P < .05.

RESULTS

A total of 94 subjects were recruited during the study
period. Table 1 summarizes the characteristics of the 94
subjects with respect to AAA grouping (ie, AAA or non-
AAA). There were no between-group differences in terms
Table I. Summary of demographic characteristics, laboratory data, and Hp phenotypes for the 94 subjects included in this study

<table>
<thead>
<tr>
<th></th>
<th>Non-AAA (n = 49)</th>
<th>AAA (n = 45)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>74 (72, 79)</td>
<td>76 (73, 80)</td>
<td>.404</td>
</tr>
<tr>
<td>Male gender</td>
<td>41 (84)</td>
<td>39 (87)</td>
<td>.684</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.8 ± 3.4</td>
<td>23.7 ± 2.9</td>
<td>.106</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>180.8 ± 32.9</td>
<td>178 ± 45</td>
<td>.771</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>103 (72, 163)</td>
<td>114 (90, 145)</td>
<td>.389</td>
</tr>
<tr>
<td>High density lipoprotein cholesterol (mg/dL)</td>
<td>37 (30, 50)</td>
<td>40 (34, 48)</td>
<td>.585</td>
</tr>
<tr>
<td>Low density lipoprotein cholesterol (mg/dL)</td>
<td>111 ± 34</td>
<td>110 ± 38</td>
<td>.942</td>
</tr>
<tr>
<td>Hp concentration (ng/mL)</td>
<td>186 ± 108</td>
<td>254 ± 158</td>
<td>.017</td>
</tr>
<tr>
<td>C-reactive protein concentration (mg/dL)</td>
<td>0.476 ± 1.086</td>
<td>1.107 ± 2.073</td>
<td>.0496</td>
</tr>
<tr>
<td>Smoking</td>
<td>24 (50)</td>
<td>31 (69)</td>
<td>.064</td>
</tr>
<tr>
<td>Drinking</td>
<td>16 (34)</td>
<td>15 (33)</td>
<td>.943</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2 (4)</td>
<td>7 (16)</td>
<td>.084</td>
</tr>
<tr>
<td>Hypertension</td>
<td>14 (29)</td>
<td>32 (71)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hp phenotype</td>
<td></td>
<td></td>
<td>.141</td>
</tr>
<tr>
<td>1-1</td>
<td>4 (8)</td>
<td>2 (4)</td>
<td></td>
</tr>
<tr>
<td>2-1</td>
<td>22 (45)</td>
<td>13 (29)</td>
<td></td>
</tr>
<tr>
<td>2-2</td>
<td>23 (47)</td>
<td>30 (67)</td>
<td></td>
</tr>
</tbody>
</table>

AAA, Abdominal aortic aneurysm; Hp, haptoglobin.
Continuous variables are presented as median (interquartile range) or mean ± standard deviation; categorical are presented as number (%).

Table II. Plasma Hp concentrations stratified with respect to Hp phenotype and whether the patient did or did not have AAA

<table>
<thead>
<tr>
<th>Hp phenotype</th>
<th>Non-AAA (n = 49)</th>
<th>AAA (n = 45)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>105 ± 60</td>
<td>276 ± 143</td>
<td>.089</td>
</tr>
<tr>
<td>2-1</td>
<td>226 ± 123</td>
<td>288 ± 193</td>
<td>.256</td>
</tr>
<tr>
<td>2-2</td>
<td>163 ± 86</td>
<td>238 ± 144</td>
<td>.024b</td>
</tr>
</tbody>
</table>

AAA, Abdominal aortic aneurysm; Hp, haptoglobin.
Data are presented as mean ± standard deviation and were compared by independent samples t tests.
Indicates a statistically significant between groups difference (P < .05).

Table III. Plasma Hp concentrations stratified with respect to hypertension and whether the patient did or did not have abdominal aortic aneurysm

<table>
<thead>
<tr>
<th>Hypertension</th>
<th>Non-AAA (n = 49)</th>
<th>AAA (n = 45)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>182 ± 117</td>
<td>333 ± 195</td>
<td>.019b</td>
</tr>
<tr>
<td>Yes</td>
<td>196 ± 85</td>
<td>220 ± 128</td>
<td>.517</td>
</tr>
</tbody>
</table>

AAA, Abdominal aortic aneurysm; Hp, haptoglobin.
Data are presented as mean ± standard deviation and were compared by independent samples t tests.
Indicates a statistically significant between groups difference (P < .05).

of age, gender distribution, body mass index, smoking or drinking habits, diabetes history, Hp phenotype distribution, or any biochemical measure. Patients with AAA had significantly higher concentrations of Hp than their non-AAA counterparts (P = .017), and a significantly higher percentage of these patients also suffered from hypertension (P < .001). The concentration of CRP was marginally higher (P = .049) in AAA patients.

Table II summarizes plasma Hp concentrations with respect to phenotype and AAA status. Plasma Hp concentrations for the 2-2 phenotype were significantly higher in patients with AAA compared with those who did not have AAA (P = .024). Plasma concentrations of Hp in phenotype 1-1 and 2-1 patients were somewhat higher in those with AAA, but this trend did not reach statistical significance.

The effect of hypertension on the association between AAA and plasma Hp concentration is shown in Table III. In patients without hypertension, plasma Hp concentration was significantly higher in the AAA group (P = .019), but in patients with hypertension, the plasma Hp concentration was similar in both AAA and non-AAA groups. Hypertension was not associated with Hp levels in the absence of AAA (P = .069).

As age has been reported to be related to AAA,19 the effect of age on plasma Hp levels in the 53 patients with the 2-2 phenotype was examined. There was no significant interaction between age and the occurrence of AAA (ie, the correlation between these variables was similar for both the AAA and non-AAA groups [data not shown]). Subsequent analysis of covariance revealed that AAA status was the only significant factor affecting Hp concentration. For the 2-2 phenotype, plasma Hp concentrations were significantly higher in patients with AAA compared with subjects who did not have AAA (P = .031). Age was not significantly associated with plasma Hp level (P = .380; Fig). In the study cohort, aspirin was prescribed in 13 AAA patients and eight non-AAA patients. In the total patient population, no statistically significant difference in Hp con-
concentration was found between patients taking aspirin and those not taking aspirin ($P = 0.195$). When patients were subgrouped into AAA and control (non-AAA) patients, no difference in Hp concentration was found between control patients taking aspirin and those not taking this drug ($P = 0.960$). However, AAA patients taking aspirin had lower Hp concentrations than those not taking aspirin ($P = 0.006$).

**DISCUSSION**

In this study, we examined the relationships between plasma Hp levels and Hp phenotype in patients who did and did not have AAA. While there were some trends toward differences in phenotypic distribution between the AAA and non-AAA individuals, these did not reach statistical significance. Of note, however, plasma Hp levels were significantly higher in 2-2 phenotype patients with AAA compared with their non-AAA counterparts with the same Hp phenotype. This effect was independent of age. Also, in AAA patients, Hp levels were significantly higher in patients not taking aspirin than in those taking this anti-inflammatory drug. This result indirectly shows that the increase in Hp concentration is closely related to the inflammatory response that leads to AAA pathology.

Hypertension has been documented as a risk factor like other clinical risk factors, including smoking, advanced age, male gender, chronic obstructive pulmonary disease, and family history that predisposes individuals to degenerative diseases in the arterial wall. In our study, a significant interaction between hypertension and AAA was found, but hypertension gave no additional effect to Hp concentration for AAA diagnosis. Therefore, it seems that Hp is an independent risk factor for AAA.

In humans there exist two classes of alleles for Hp, designated one and two. The Hp polymorphism is a common polymorphism. A geographic ethnic difference in Hp phenotypes has been reported between Western and Asian populations. In Western populations, it has been reported that 16% of individuals have the 1-1 phenotype, 36% the 2-2 and 48% the 2-1. In contrast, the frequency of the 1 to 1 phenotype has been found to be lower in Asian populations, while the frequency of 2 to 2 is higher. The findings from the current study regarding phenotype distribution are in agreement with those previously reported.

In the present study, there was an increased (but non-significant) tendency for individuals with AAA to have the 2-2 phenotype. This finding contrasts with that reported by Norrgard and colleagues, who found that there was an increased frequency of the 2-1 phenotype in Northern Swedish individuals with AAA. The reason for this inconsistency is unclear, but may be related to ethnicity. It also must be acknowledged that a larger number of patients should be studied to confirm our findings.

Other studies have reported increased incidences of disease in individuals with the Hp 2-2 phenotype. Ashle et al reported that diabetic individuals homozygous for the Hp 2 allele (Hp 2-2) had a five-fold greater risk of cardiovascular disease compared with diabetic individuals homozygous for the Hp 1 allele (Hp 1-1). These researchers also found that hemoglobin within plaque derived from microvascular hemorrhage was cleared more slowly from plaques associated with the Hp 2-2 phenotype as compared with Hp 1-1 plaques. This suggests that the Hp 2-2 phenotype is associated with an increased risk of atherosclerotic cardiovascular disease. In a recently published study of diabetic transgenic mice, it was found that the Hp 2-2 phenotype was associated with increased mortality and more severe cardiac remodeling 30 days after myocardial infarction. In addition, Hp 2-2 has been associated with refractory hypertension, a predictor of diabetic nephropathy, is associated with a higher risk of mortality in tuberculosis, and a lower hepatitis B level than other phenotypes.

The Hp 2-2 phenotype differs structurally from the other two phenotypes in that these phenotypes are either dimers (Hp 1-1) or form linear polymers (Hp 2-1) while the 2-2 phenotype polymerizes into rosettes of varying complexity. Scavenging of Hb by endocytosis of Hb-Hp complexes has been found to occur via the CD163 receptor. Hp 1-Hb complexes stimulate anti-inflammatory macrophages, while Hp 2-Hb complexes do not. Strauss and Levy reported that casein kinase II activity was increased following the binding of Hp 1-1 Hb to CD163. Reduced clearance of the macrophage-Hp-Hb complex, favoring iron deposition, oxidative stress, and active macrophage accumulation was found in individuals with the Hp 2-2 phenotype. Impaired Hb clearance capacity has
also been found in Hp 2-2 diabetic individuals with plaque rupture propensity.34,35

Increased matrix metalloproteinases (MMP)-9 and MMP-2 activity is associated with destruction of the elastic laminae of arteries and aneurysm formation in humans.34 Complexes of MMP-9 and Hp have been detected in sera of cows with acute inflammation, but not in clinically normal cows or cows with chronic disease.36,37 The interaction between Hp and MMP in aneurysm development has not been fully investigated. A functional study performed by de Kleijn et al revealed that Hp inhibits the activity of MMP-2 by 50% and MMP-9 by 30% in cultured arterial smooth muscle culture cells, but increases the production of gelatin in arterial smooth muscle cells in Hp knockout mice.36,37 These findings suggest that Hp may play a role in the regulation of the arterial restructuring.

All of the above observations suggest that individuals with specific Hp phenotypes may respond differently to biological insults such as inflammation and vessel wall degeneration. The synthesis of Hp may reflect a regulatory response to these pathologic processes. In the present study, the finding that plasma Hp concentrations were significantly higher in patients with AAA of the 2-2 phenotype compared to corresponding non-AAA individuals is of interest and agrees with our previous findings demonstrating that significant between-phenotype variability in plasma Hp levels occurs.17 However, in this case-control observational study, the study cohort was recruited from a hospital-based population, was relatively small in size (with a narrow age range), and although the two study arms had similar demographics, some unknown bias might have occurred because the patients were not randomly selected. If the results of this study are confirmed and extended by a further larger scale, more stringently designed study, Hp phenotype and concentration may be potential biomarkers of abdominal aortic aneurysm development that can be used clinically in association with other biomarkers.

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AUTHOR CONTRIBUTIONS
Conception and design: J-PP, C-CS, SM, S-TL
Analysis and interpretation: J-PP, T-MC
Data collection: J-PP, S-CCho, S-TL
Writing the article: J-PP
Critical revision of the article: J-PP, SM
Final approval of the article: J-PP
Statistical analysis: J-PP, S-CChi
Obtained funding: J-PP, C-CS, S-TL
Overall responsibility: J-PP, T-MC, C-CS, S-CChi, S-CCho, SM, S-TL

REFERENCES