Haptoglobin Phenotypes in Epilepsy, Sayed M.H. Sadrzadeh, Yasi Saffari, and Jafar Bozorgmehr (Department of Laboratory Medicine, University of Washington, Harborview Medical Center, Seattle, WA 98104; * author for correspondence: fax 206-731-3930, e-mail sadrzade@u.washington.edu)

Seizures occur in ~5% of people, and recur in >20% of that 5% (1, 2). The etiologies of most seizures are unknown, and head trauma is implicated in only 5–10% of cases (3). Blood or blood components, specifically iron, may be etiologically important; intracranial injection of hemoglobin (4), lysed erythrocytes (5), iron-containing proteins (5), or iron salts (6) produced chronic focal spike activity in rodents and cats. Because microhemorrhagic events occur in the central nervous system of all people, inadequate clearance of iron-rich (7) hemoglobin might underlie development of some seizure disorders.

Haptoglobin binds free hemoglobin and removes it from the circulation (8), thus preventing iron loss and kidney damage during hemolysis (9). Haptoglobin contains B- (heavy; 40 kDa) and α- (light; α1 = 8.9 kDa and α2 = 16 kDa) chains. Humans are polymorphic for haptoglobin, with three major phenotypes: Hp 1-1, Hp 2-2, and the heterozygous Hp 2-1 (10). The β-chains are identical in all, with variations dependent on different α-chains. Hp 1-1 expresses only the α1-chain and is the smallest form (86 kDa). Hp 2-1 and Hp 2-2 express α2-chains, which can form polymers of 86–300 kDa (Hp 2-1) and up to 900 kDa (Hp 2-2) (10). Hp 1-1 is biologically the most effective in binding free hemoglobin and suppressing inflammatory responses associated with extracellular (free) hemoglobin (9). In contrast, Hp 2-2 is the least effective (11). The plasma concentrations of haptoglobin are highest in individuals with Hp 1-1 and lowest in those with Hp 2-2, with intermediate concentrations in Hp 2-1 individuals (9).

Haptoglobin also has antioxidant (12), angiogenic (13), and anti-inflammatory effects (11, 14). Furthermore, haptoglobin has a role in regulation of immune responses (15) by suppressing release of cytotoxins from T-helper type 2 cells and regulating T-helper type 1/T-helper type 2 balance (15).

If hemoglobin (or its iron) is involved in the etiology of seizures, then inadequate removal of hemoglobin (by haptoglobin) may be important. We postulated that functional differences between the haptoglobin phenotypes might be related to the severity and frequency of seizure attacks in patients with epilepsy. In this study, we investigated the serum concentrations of haptoglobin and the distribution of haptoglobin phenotypes in people with and without epilepsy and examined the relationship of haptoglobin phenotypes with C-reactive protein (CRP), which, like haptoglobin, is an acute-phase protein.

We studied 92 patients (59 men and 33 women), with a mean age of 43 (range, 21–87) years, who had one or more idiopathic seizures per month and who were treated at our medical center. Controls were 100 volunteers (62 men and 38 women), with a mean age of 44 years. No participants had intravascular hemolysis, liver disease, or trauma. The diagnosis of recurrent idiopathic epilepsy was based on clinical status and electroencephalography results. The study was approved by the local Institutional Review Board.

Phenotyping of haptoglobin was performed by gel electrophoresis followed by peroxidase staining (16). Mobilities of haptoglobin in the samples were compared with authentic samples of Hp 1-1 and Hp 2-2 for phenotype identification. Concentrations of haptoglobin and CRP (17) were measured by fixed-time immunonephelometry with reagents and instrumentation from Dade Behring.

Data are presented as the mean (SE). We used the t-test with Welch’s correction to assess significance of differences of means. Analysis of differences in haptoglobin phenotype distributions in patients and controls was done by χ² test.

Haptoglobin phenotype 2-2 was significantly associated with recurrent seizures (P < 0.001), being present in 67% of the patients and in only 35% of controls. Hp 2-1 and 1-1 were present in 18% and 13% of patients, respectively, and in 50% and 15% of controls. Haptoglobin was undetectable in two patients (2%). The distributions of haptoglobin types were in Hardy–Weinberg equilibrium. The association of Hp 2-2 with seizure attacks persisted when patients were compared with ethnically matched controls (Tables 1 and 2 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue6; P < 0.05).

Haptoglobin concentrations were significantly higher (P < 0.0001) in patients [1.41 (0.08) g/L] than in controls [1.04 (0.04) g/L].

Serum haptoglobin concentrations in patients differed significantly from concentrations in controls when analyzed in relation to their phenotypes (Table 1).

Because haptoglobin is an acute-phase protein, we measured serum CRP in all participants. CRP was significantly higher in patients than in controls [10.1 (1.5) and 1.4 (0.3) mg/L, respectively; P < 0.0001]. Not only was pooled serum CRP significantly different in patients vs
controls, but also in individual phenotypes (Table 1). In addition, we found statistically significant correlations between serum CRP and haptoglobin concentrations within each individual phenotype, that is, in the Hp 2-2, Hp 2-1, and Hp 1-1 groups individually ($r = 0.99$).

The role of iron and its oxidative capabilities in tissue damage is well documented (18), and iron-containing proteins such as hemoglobin can initiate or enhance oxidative processes (19). Increased accumulation of iron in the brain and defective antioxidant defenses have been linked to both Parkinson and Alzheimer diseases (19). We previously showed that hypohaptoglobinemia was associated with high incidence and frequency of seizures in patients with idiopathic familial epilepsy (20). Defective haptoglobin-mediated clearance of free hemoglobin from the central nervous system could lead to hemoglobin-dependent central nervous system damage.

The major hazard posed by iron-containing compounds is in facilitating the formation of reactive oxygen species (21, 22). Hemoglobin, in the presence of a source of superoxide anions and hydrogen peroxide, can catalyze the formation of one or more reactive species resembling hydroxyl radicals (22). Free hemoglobin also enhances the peroxidation of purified arachidonic acid and other polyunsaturated fatty acids within normal cell membranes (23). Furthermore, purified hemoglobin or crude erythrocyte lysates, in the absence of superoxide anions or hydrogen peroxide, cause rapid peroxidation of crude murine brain homogenate (24).

CRP is a marker for inflammatory processes, tissue damage, and infection (25). The increase in CRP in our patients suggests that they suffered from inflammation in addition to their seizure disorders. Although we found a correlation between different haptoglobin phenotypes and serum CRP, we do not know whether there is a direct association between inflammation and the pathogenesis of seizure disorders. Haptoglobin is a marker for inflammation (9), and it also was increased. We believe that identification of haptoglobin phenotypes in addition to analysis of serum haptoglobin concentrations may better identify the presence of inflammation: a Hp 2-2 phenotype may indicate the presence of an inflammatory process because Hp 2-2 is more associated with inflammation than Hp 2-1 or Hp 1-1. Indeed, Hp 2-2 type has been associated with several clinical conditions, such as atherosclerosis, cancer, infection, and neurologic disorders (11, 26). In addition, Hp 2-2 complexed with free hemoglobin has a high affinity for CD 163 receptors on macrophages (27). Furthermore, it has been suggested that the uptake and internalization of haptoglobin–hemoglobin complex by macrophages leads to increased oxidative stress in macrophages and, more importantly, delocalization of iron (28), which can further enhance oxidative stress via generation of reactive oxygen species. Although one report denies the enhanced oxidative stress in macrophages after internalization of haptoglobin–hemoglobin complex (29), most of the data in this field support the association of Hp 2-2 and inflammatory processes, and most pathologic conditions (cancer, atherosclerosis, and neurologic disorders) associated with Hp 2-2 are associated with inflammation.

In aggregate, our data clearly show an association of Hp 2-2 with the presence of seizures in patients with epilepsy. At present we do not know the mechanism for this phenomenon. We hypothesize that a defective clearing system (such as Hp 2-2), which cannot effectively remove free hemoglobin after microhemorrhagic events in the central nervous system, leads to iron accumulation and increased oxidative stress in tissue. Therefore, enhanced oxidative stress with minimum antioxidant defense can lead to tissue injury and inflammation, which may have a role in the etiology of seizure attacks in this patient population. More work is needed for better understanding of the role of haptoglobin in the pathophysiology of epilepsy.

### Table 1. Serum haptoglobin and CRP concentrations according to haptoglobin phenotype in patients and controls.

<table>
<thead>
<tr>
<th>Haptoglobin phenotype</th>
<th>Mean (SE) haptoglobin, g/L</th>
<th>Mean (SE) CRP, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subjects</td>
<td>Controls</td>
</tr>
<tr>
<td>Hp 2-2</td>
<td>1.31 (0.08)a</td>
<td>1.01 (0.09)</td>
</tr>
<tr>
<td></td>
<td>(n = 62)</td>
<td>(n = 35)</td>
</tr>
<tr>
<td>Hp 2-1</td>
<td>1.62 (0.27)c</td>
<td>1.03 (0.05)</td>
</tr>
<tr>
<td></td>
<td>(n = 11)</td>
<td>(n = 50)</td>
</tr>
<tr>
<td>Hp 1-1</td>
<td>1.71 (0.24)e</td>
<td>1.16 (0.1)</td>
</tr>
<tr>
<td></td>
<td>(n = 12)</td>
<td>(n = 15)</td>
</tr>
</tbody>
</table>

*Comparisons with controls: a $P = 0.01$; b $P = 0.0001$; c $P = 0.02$; d $P = 0.0018$; e $P = 0.03$; f $P = 0.022$.

### References

Evaluation of a clinically inapparent adrenal mass led to tests for pheochromocytoma (1) with findings of increased urinary metanephrine and normetanephrine but normal plasma metanephrines and catecholamines.

A 45-year-old woman presented with pulmonary embolism and thrombosis of the left arm. Her recent medical history included removal of the right breast because of cancer with postoperative locoregional radiotherapy and chemotherapy. She had a known spina bifida and an history included removal of the right breast because of cancer with postoperative locoregional radiotherapy and chemotherapy.

After treatment with fractionated heparin and acenocumarol the patient was further evaluated for the presence of tumor recurrence or distant metastases as an explanation for her thromboembolism. Mammography showed no signs of malignancy. The computed tomography (CT) scan of the thorax showed a pulmonary embolism but no evidence of metastases. The abdominal CT scan revealed a 2.5-cm hypodense mass in the right adrenal gland, most likely an adenoma. Because the differential diagnosis of adrenal masses with low attenuation on CT includes functional tumors, especially pheochromocytoma (2), the functionality of the adrenal mass was determined.

Cortisol was 0.09 and 0.07 μmol/L after a repeated 1-mg dexamethasone suppression test. The 24-h urinary excretion of cortisol was 37.9 nmol, which made the presence of glucocorticoid excess unlikely. Serum potassium was within reference values, and renin and aldosterone were not increased, which together with the normal blood pressure excluded primary aldosteronism. Urinary excretion of metanephrines, measured as described previously (3), was increased on repeated occasions [normetanephrine (NMN), 36.450 nmol/24 h (reference values <2900 nmol/24 h); metanephrine (MN), 10.256 nmol/24 h (reference values <1000 nmol/24 h)], suggesting the presence of a pheochromocytoma. However, both fractionated plasma-free metanephrines, which may be preferred for the diagnosis of pheochromocytoma in high-risk patients (4), and plasma catecholamines were not clearly increased [MN, 312 pmol/L (reference values <600 pmol/L); MN, 264 pmol/L (reference values <300 pmol/L); adrenaline, 0.04 nmol/L (reference values <0.3 nmol/L); noradrenaline, 2.84 nmol/L (reference values <3.0 nmol/L)]. Because of the patient’s impaired mobility and anxiety, no metadionobenzylguanidine scan was performed.

Close inspection of the urinary chromatograms of this patient revealed, in addition to an extra peak at an unusual location, a striking decrease (10- to 15-fold) in the internal standard (4-O-methyltyramine) peak compared with urinary chromatograms of other patients. In contrast, the heights of the MN and NMN peaks were in the usual range. Because calculation of the concentrations of MN and NMN was based on the internal standard principle, in which a constant ratio between recoveries of the analytes to be measured and the internal standard is assumed for processed samples and calibrators (3), very high estimates of MN and NMN resulted. Thus, either the recovery of MN and NMN followed that of the internal standard, meaning that the high result was correct, or the internal standard principle did not hold in this urine sample and underestimation of the procedural recovery, rather than increased MN and NMN concentrations, was responsible for the strongly increased MN and NMN values calculated from the chromatography data.