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An enzyme linked immunosorbent assay (ELISA) for the determination of the human haptoglobin phenotype

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Conflict of interest statement

Authors' conflict of interest disclosure: APL and the Rappaport Institute are the owners of a patent for haptoglobin phenotype testing for the determination of vascular complications in diabetic individuals. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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Abstract

Background—Haptoglobin (Hp) is an abundant serum protein which binds extracorpuscular hemoglobin (Hb). Two alleles exist in humans for the Hp gene, denoted 1 and 2. Diabetic individuals with the *Hp 2-2* genotype are at increased risk of developing vascular complications including heart attack, stroke, and kidney disease. Recent evidence shows that treatment with vitamin E can reduce the risk of diabetic vascular complications by as much as 50% in *Hp 2-2* individuals. We sought to develop a rapid and accurate test for Hp phenotype (which is 100% concordant with the three major Hp genotypes) to facilitate widespread diagnostic testing as well as prospective clinical trials.

Methods—A monoclonal antibody raised against human Hp was shown to distinguish between the three Hp phenotypes in an enzyme linked immunosorbent assay (ELISA). Hp phenotypes obtained in over 8000 patient samples using this ELISA method were compared with those obtained by polyacrylamide gel electrophoresis or the TaqMan PCR method.

Results—Our analysis showed that the sensitivity and specificity of the ELISA test for Hp 2-2 phenotype is 99.0% and 98.1%, respectively. The positive predictive value and the negative predictive value for Hp 2-2 phenotype is 97.5% and 99.3%, respectively. Similar results were obtained for Hp 2-1 and Hp 1-1 phenotypes. In addition, the ELISA was determined to be more sensitive and specific than the TaqMan method.

Conclusions—The Hp ELISA represents a user-friendly, rapid and highly accurate diagnostic tool for determining Hp phenotypes. This test will greatly facilitate the typing of thousands of samples in ongoing clinical studies.

Keywords

diabetes; ELISA; haptoglobin phenotype; pharmacogenomics; vitamin E

Introduction

Haptoglobin (Hp) is an abundant serum glycoprotein which binds free hemoglobin (Hb) with very high affinity ($K_d = 10^{-14}$ M). A major function of Hp is to neutralize the pro-oxidant iron present in the heme portion of Hb and prevent free radical formation that occurs in the presence of hydrogen peroxide. In humans there exist two alleles for Hp (*Hp 1* and *Hp 2*; rs72294371) which give rise to three genotypes (*Hp 1-1*, *Hp 2-1,* and *Hp 2-2)*. Each genotype gives rise to a unique phenotype which is characterized by a distinctive banding pattern seen following polyacrylamide gel electrophoresis. It has now been established that the Hp phenotype is a marker for the development of vascular complications in individuals suffering from diabetes [1]. Specifically, it has been shown that diabetic individuals of the Hp 2-2 phenotype are at significantly increased risk of developing vascular complications including myocardial infarction, stroke, and kidney disease. Furthermore, recent work has shown that Hp 2-2 diabetic individuals experience a 30%–40% decrease in cardiovascular complications following treatment with vitamin E [2]. We therefore sought to develop a rapid and accurate enzyme linked immunosorbent assay (ELISA) for the determination of Hp phenotype.

An earlier attempt at developing this assay resulted in the isolation of a single chain antibody which was able to accurately distinguish between the three Hp phenotypes in a sandwich ELISA assay [3]. This reagent proved to be unsatisfactory for large-scale analyses due to low yields obtained from bacterial cultures and poor stability of the single chain antibody. In this paper, we report the isolation of a monoclonal antibody (mAb) with greatly

improved yield and stability that can distinguish between the different Hp phenotypes with even greater accuracy. This improved assay will facilitate ongoing clinical and biochemical studies of Hp and may also be used for selecting appropriate treatment regimens for diabetic patients.

Development of an antibody based assay for distinguishing between the three Hp phenotypes was not straightforward due to the nature of the Hp polymorphism. As shown in Figure 1, the *Hp 1* allele contains 5 exons. A 1.7-kb intragenic duplication event of exons 3 and 4 of the *Hp 1* allele gave rise to an *Hp 2* allele containing seven exons (see Figure 1). The nature of the inframe duplication event is such that no unique sequences are present in the *Hp 2* allele compared to the *Hp 1* allele, with the exception of the junction between exons 4 and 5 of the *Hp 2* allele. Attempts at preparing antibodies to this unique sequence have been unsuccessful in our laboratory. Similarly, there are no unique amino acid sequences in *Hp 1* that do not appear in *Hp 2*. Therefore, the simplest approach of preparing antibodies to unique epitopes in each allelic protein product was not possible in this case. However, Hp proteins present in individuals of different genotypes differ significantly in quaternary structure, as will be described below. The ELISA described in this report probably differentiates between the Hp phenotypes based on these higher order structural variations.

Hp is synthesized as a single polypeptide chain which becomes cleaved into an α and β chain. The α and β chains become disulfide linked, thus forming the Hp monomer. Owing to gene duplication, there exist two types of α chains in humans, α 1 and α 2. Intermolecular disulfide bridges can form between α chains of each monomer, creating Hp polymers. The α 1 chain forms one intermolecular linkage. Therefore, in *Hp 1-1* individuals one observes Hp 1-1 homodimers exclusively (see Figure 2). By contrast, the α 2 chain forms two disulfide linkages resulting in linear heteropolymers in the case of *Hp 2-1* individuals and cyclic homopolymers in the case of *Hp 2-2* individuals. Non-denaturing polyacrylamide gel electrophoresis studies show that Hp 2-1 polymers on average are smaller than Hp 2-2 polymers. Electron microscope scanning studies of Hp indicate that polymers from *Hp 2-1* individuals have an average of 3.4 monomer units, whereas polymers from *Hp 2-2* individuals have an average of 4.5 monomer units [4].

Differences in the quaternary structure of the different Hp polymers were exploited for the purpose of distinguishing between the three Hp phenotypes (Hp 1-1, 2-1, and 2-2).

In this assay, a microtiter plate is coated with a mAb to Hp. Serum containing Hp is allowed to bind to the mAb, after which the same mAb conjugated to horseradish peroxidase (HRP) is allowed to bind to the captured Hp. The amount of Hp is then quantified by spectrophotometry following addition of a colorimetric substrate for HRP. According to this strategy Hp 1-1 dimers would be expected to bind at most one HRP-conjugated mAb because the other epitope present in the dimer would already be bound by the mAb on the surface of the plate. By contrast, Hp 2-1 and Hp 2-2 polymers would be expected to bind approximately one to seven HRP-conjugated mAb molecules due to the additional subunits present in these polymers. Owing to the larger size of the Hp 2-2 polymers, the level of intensity of the HRP signal in the ELISA for Hp 2-2 should be greater than that for Hp 2-1, which in turn should be greater than that for Hp 1-1. We predicted that these differences in secondary antibody binding would make it possible to distinguish between the different Hp phenotypes reliably and accurately.

Materials and methods

mAbs

Affinity purified human Hp 1-1 was used to immunize Balb/c mice and hybridomas were established according to published procedures. See Supplemental Data 1, which accompanies the article at http://www.degruyter.com/view/j/cclm.2013.51.issue-8/issue-files/cclm.2013.51.issue-8.xml, for screening and characterization of hybridoma supernatants. One of these mAbs, designated as 4G12, was selected for use in the Hp ELISA.

Preparation of antibody coated plates and conjugate

See Supplemental Data 2

Hp ELISA protocol

See Supplemental Data 3

Assay validity

The internal validity of the ELISA assay was determined by testing one sample of each genotype as well as a blank sample eight times using a single lot of reagents each day over a period of 10 days.

Serum samples

In total, 8059 serum samples from seven separate clinical studies were obtained and stored at −20 °C [5–11]. See Supplemental Data 4 for additional information.

Gel electrophoresis/peroxidase assay

Hp typing was determined from 10 μL of hemoglobin enriched serum by polyacrylamide gel electrophoresis [12].

PCR TaqMan assay for Hp genotype

The TaqMan method [13] was used to analyze DNA samples obtained from the RASS (Renal and retinal effects of enalapril and losartan in type 1 diabetes) study [7] and the DCCT (Diabetes Control and Complications Trial) study [11]. See Supplemental Data 5 for more information.

Operators

The ELISA was performed by two individuals (N.S.L. and O.L.). Gel electrophoresis was performed by one individual (Y.A.). The TaqMan method was performed by one individual (B.B.). The readers of the results for each method were blinded to the results of the other two methods.

Statistics

The sensitivity, specificity, and positive and negative predictive values of the ELISA test were calculated for the three different Hp types (1-1, 2-1, and 2-2). The McNemar's χ^2-test was used to compare the sensitivities and specificities of the ELISA test with alternative tests.

Results

Antibody screening

Screening of hybridoma supernatants made from spleen cells of Hp 1-1 immunized mice revealed the existence of two classes of mAbs. Class I antibodies detected serum Hp 1-1 and Hp 2-2 at equal intensity and class II antibodies reacted with a much lower intensity to Hp 1-1 than Hp 2-2. This class distinction became evident only when the hybridoma culture medium containing the mAbs was tested at a dilution of 1:2000 or greater (see Supplemental Data 1). Several class II antibodies were tested as capture antibodies and HRP-conjugated detection antibodies. The combination which resulted in the greatest distinction between Hp phenotypes was antibody 4G12 when used for both capture and detection.

The analytical sensitivity or background level of the kit is 0.050, determined by taking the mean of ten buffer blank determinations plus two standard deviations (0.044 + 2 ×0.003 = 0.05).

The absorbance cut-off range for the Hp 2-2 phenotype was determined by testing 411 samples of known Hp genotype and analyzing the data by using receiver operating characteristic (ROC) plots. It was established empirically that the Hp 2-2 cut-off value for each assay is determined by multiplying the absorbance of the Hp 2-2 control by an adjustment factor of 0.6. The adjustment factor accounts for run-to-run as well as day-to-day variation. The absorbance cut-off value is determined for each assay (ELISA plate) by running an Hp 2-2 positive control sample on that plate. For example, if the absorbance of the Hp 2-2 positive control was 3.0, the cut-off value would be 1.8, meaning that any sample with absorbance above 1.8 would be designated Hp 2-2. The absorbance cut-off value for Hp 1-1 was established empirically to be 0.1, meaning that any sample with an absorbance <0.1 was designated Hp 1-1. Samples with absorbances in the range of 0.1 to 1.8 were designated Hp 2-1.

Assay validity

We calculated the percent coefficient of variation for eight replicate samples for each Hp genotype on 10 different days. These ranged from 4.62 to 53.4 (median 11.1) for Hp 1-1, 0.61 to 1.88 (median 0.895) for Hp 2-1, and 0.38 to 1.74 (median 1.01) for Hp 2-2.

Limit of detection

Hp 2-2 samples were diluted from 1:10 to 1:4000 and run in both the electrophoresis assay and the ELISA. The samples were correctly identified as Hp 2-2 down to a dilution of 1:50 by gel electrophoresis and down to a dilution of 1:1000 by ELISA. Hp 1-1 and Hp 2-1 samples were also diluted from 1:10 to 1:4000 and were correctly identified as non-Hp 2-2 by the ELISA at all dilutions tested.

Interfering substances

Endogenous substances found in blood and exogenous substances (common and prescription drugs) were evaluated for interference in the Hp typing ELISA. Six serum samples, two of each Hp phenotype (Hp 1-1, Hp 2-1, and Hp 2-2), with Hp ELISA absorbance ranging from 0.045 to 2.521, were spiked with potential interfering substances. Table 1 lists the substances and the levels tested at which no appreciable interference was observed.

Comparison with gel electrophoresis

Serum samples were analyzed to compare the performance of the ELISA with polyacrylamide gel electrophoresis, the established reference method in use for over 60

years. Some serum samples (13/8059) analyzed by gel electrophoresis could not be assigned an Hp type and therefore could not be compared with the ELISA method. Out of a total of 8046 samples which gave a result by gel electrophoresis, there were 201 mismatches between the ELISA method and the gel electrophoresis method, which corresponds to a 97.50% overall correlation. Four samples were Hp 2-1M (modified) by gel electrophoresis. This phenotype is the result of a mutation in the *Hp 2* promoter, resulting in very low expression. All of these samples scored as Hp 2-1 in the ELISA. Although these samples scored on the low end of the Hp 2-1 cut-off value, there were not enough samples to designate a unique range for the Hp 2-1M phenotype.

Statistical analysis of the comparative results

To calculate the sensitivity and specificity of the ELISA it was necessary to determine the number of true positive, true negative, false positive, and false negative results, as will be explained below. It should be noted that a positive result in the ELISA for Hp 1-1 is a simultaneous negative result for Hp 2-1 and Hp 2-2. Similarly, a positive result for Hp 2-1 is a negative result for Hp 1-1 and Hp 2-2, and a positive result for Hp 2-2 is also a negative result for Hp 1-1 and Hp 2-1. The data were further categorized as shown in Table 2. A false positive is a positive result in the ELISA which did not match a positive result in the gel assay. A false negative is a negative result obtained in the ELISA which did not match a negative result in the gel assay. A true positive is a positive result obtained in the ELISA which matched a positive result in the gel assay, and a true negative is a negative result in the ELISA which matched a negative result in the gel assay.

Using the data from Table 2 the following parameters were calculated. Sensitivity: the proportion of true positives in a group of affected individuals. Specificity: the proportion of true negatives in a group of unaffected individuals. Positive predictive value (PPV): the probability that a person has the condition when a positive test result is observed. Negative predictive value (NPV): the probability that a person does not have the condition when a negative test result is observed. These results are summarized in Table 3. See also Supplemental Data 6 for the ROC curve comparing the ELISA and gel electrophoresis assays.

Comparison with TaqMan

A second method, known as TaqMan [13], has also been used to assign Hp genotypes. This method is very attractive in that many samples can be tested rapidly and simultaneously in an automated manner. Samples (n = 1610) were analyzed using the TaqMan method, the ELISA method, and the gel electrophoresis method. The sensitivities of detection of Hp 2-2 for the ELISA and TaqMan in this cohort of patients were 98.70% and 82.20%, respectively, and the specificities were 99.29% and 85.08%, respectively. For sensitivity and for specificity, the ELISA method was significantly superior to the TaqMan method ($p < 0.0001$). See Supplemental Data 6 for the ROC curve comparing TaqMan and ELISA methods.

Discussion

This paper describes the development of a rapid and accurate ELISA which distinguishes between the three Hp phenotypes based on differences in polymeric protein structure. The use of a mAb in this assay has several advantages compared with the single chain antibody used in our previous assay [3], which include greatly improved yields from cell culture and increased stability of the antibody. In addition, the mAb has increased affinity for Hp which allows for coating of the microtiter plates at a concentration of 1 μg/mL mAb compared with 100 μg/mL single chain antibody and use of the secondary, conjugated antibody at a

concentration of 0.04 μg/mL instead of 0.8 mg/mL. We found that the current Hp ELISA can predict the correct Hp phenotype in >97.5% of cases when compared with the well-established gold standard method of gel electrophoresis (see PPV, Table 3). Similarly, the current Hp ELISA is accurate in predicting when an individual is not of a particular Hp phenotype in >97% of cases (see NPV, Table 3). For example, an ELISA result of Hp 2-1 or Hp 1-1 also indicates a negative result for Hp 2-2. This is particularly important for individuals who are not Hp 2-2 and should not receive high dose vitamin E supplements, as this treatment may be detrimental in non-Hp 2-2 individuals [2, 14].

Hp phenotypes determined using the ELISA in samples from the ICARE (the Israel Cardiovascular Vitamin E) study were further analyzed by preparing primary composite plots and Kaplan-Meier plots and comparing them to similar plots obtained using phenotypes determined by the gel electrophoresis method [1]. These plots demonstrated that the ELISA is comparable with the reference method in assessing cardiovascular risk in diabetics. This result indicates that the Hp ELISA may be used to reliably determine the effect of Hp phenotype on diabetic vascular disease.

The Hp polymorphism is unique in that no distinctive sequences distinguish between the genotypes. Rather, a genetic duplication results in larger polymers in individuals carrying the *Hp 2* allele. We found that the signal ratio in our ELISA for the different Hp phenotype s was 22:10:1 for Hp 2-2/Hp 2-1/Hp 1-1, respectively. This result is surprising, given the fact that the average number of monomer units in each of the aforementioned Hp polymers is 4.5, 3.4, and 2 [4]. Several features may be contributing to the increase in the observed signal ratio. First, the same mAb is used to capture Hp onto the bottom of a microtiter plate as well as to detect the bound Hp. Those epitopes bound to the plate will not be available for binding to the secondary conjugated antibody. This strategy allows for a proportionately higher signal for the larger Hp 2-2 containing polymers. Second, the epitope which is recognized by the mAb may be in the duplicated region of the molecule, making this bias even more exaggerated for Hp 2 containing polymers. Third, the cyclic nature of the Hp 2-2 polymers compared with the linear structure of the Hp 2-1 and Hp 1-1 polymers may play an important role in this assay, possibly by inhibiting the binding of more than one epitope per polymer to the antibody coated plate, and thus leaving more epitopes available for secondary antibody binding.

The Hp ELISA described in this paper has a number of advantages over the gel electrophoresis method. First, it is approximately fourfold more efficient time wise and less labor intensive. It takes a single operator approximately 3 h to assay 184 samples for the ELISA compared with 6 h to assay 96 samples for the gel assay. Second, the ELISA is more sensitive. Serum samples may be diluted down to 1:1000 for the ELISA compared with 1:50 for the gel assay. Third, the ELISA does not require working with hazardous materials such as polyacrylamide and benzidine.

Another attractive method for Hp typing is the TaqMan PCR assay. Although this method has the advantage of being very efficient, it has a number of disadvantages. First, it is less sensitive and less specific than the ELISA for analyzing Hp genotype, as noted above. Second, to avoid DNA contamination, sample preparation should be carried out in a laminar flow hood, further making this method more labor intensive and requiring special equipment. Third, it requires the use of a real-time PCR machine which is approximately five times more costly than an ELISA reader.

The Hp ELISA has a number of limitations including the inability to differentiate between individuals with no Hp (anhaptoglobinemia, which occurs in approximately 0.1% of Caucasian populations but can be as high as 30% in some African populations) and

individuals with Hp 1-1 phenotype. Similarly, the ELISA may give erroneous results in individuals with acutely elevated levels of Hp such as during acute inflammation or sepsis. Finally, the ELISA has a relatively high coefficient of variation for Hp 1-1 (median 11%), which is reflected in the lower sensitivity of 91% for Hp 1-1 compared with 97.2% for Hp 2-1 and 99.1% for Hp 2-2. Note, however, that incorrect assignment of Hp 1-1 individuals as Hp 2-1 would not affect the clinical value of the ELISA test because only Hp 2-2 individuals are selected for potential treatment with vitamin E.

The importance of this assay for continuing clinical research in diabetics cannot be underestimated. It has now been well established in numerous studies that diabetics carrying the Hp 2-2 phenotype are at increased risk of developing vascular complications [2]. The reason for this elevated risk is thought to involve a decrease in the antioxidant capability of the Hp 2-2 protein, either by rendering the heme iron in the Hp-Hb complex more accessible to oxidative reactions or by inefficiently clearing redox active Hb from the circulation promptly [15, 16]. The end result is that Hp 2-2 diabetics experience increased oxidative modification of proteins such as high-density lipoprotein to which the Hp 2-2-Hb complex is bound [17]. The use of antioxidant therapy to alleviate the toxic effects of oxidative modification in Hp 2-2 diabetics was therefore a logical avenue to pursue.

Numerous studies investigating the potential benefit of vitamin E have reported a positive therapeutic effect, whereas many others report no beneficial effect. A large meta-analysis reported in 2005 which analyzed 19 studies including over 135,000 patients concluded that whereas vitamin E treatment (< 400 IU/day) has no significant effect on mortality, high doses (>400 IU/day) of vitamin E may increase mortality [18]. We propose that vitamin E has not been effective in preventing vascular disease due to the lack of appropriate patient selection. We therefore tested the theory that vitamin E may be beneficial in a subset of patients, namely diabetics of the Hp 2-2 phenotype. Several retrospective studies as well as one prospective study indicate that vitamin E can reduce vascular disease in Hp 2-2 diabetics by 30%–50% [2, 14]. Larger clinical studies are essential to strengthen this encouraging finding.

It could be argued that all diabetics should take vitamin E without the need for Hp phenotype testing. Recent studies in our laboratory, however, as well as the meta-analysis mentioned above [2, 14] suggest that there may be a detrimental effect of vitamin E treatment in non-Hp 2-2 diabetic individuals. Hp phenotyping may therefore be critical in determining whether a diabetic patient should be a candidate for vitamin E therapy. In summary, the Hp ELISA kit described here should be instrumental in determining the risk of vascular disease in individuals with diabetes and in evaluating potential pharmacogenetically targeted preventive treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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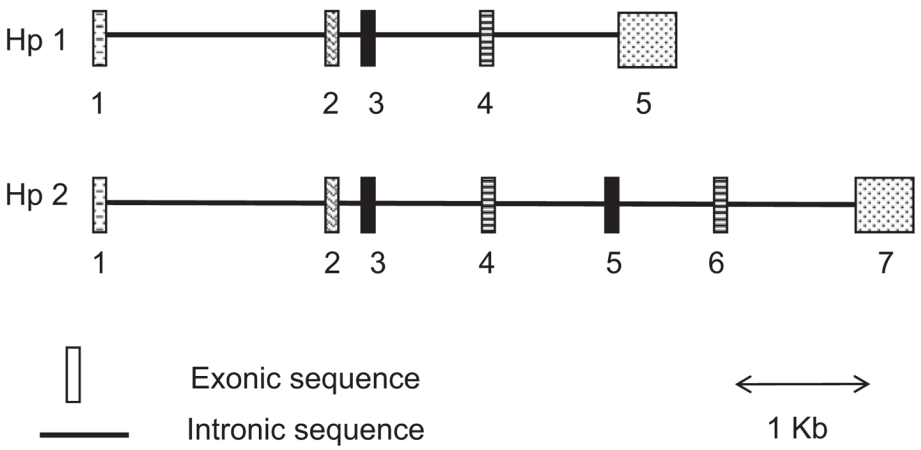

Figure 1. Schematic diagram of the genomic structure of Hp alleles 1 and 2

Exonic sequences are denoted by shaded boxes and are numbered below. Intronic sequences are denoted by a dark line. Exons 3 and 4 of the *Hp 1* allele have been duplicated in the *Hp 2* allele, giving rise to exons 3–6.

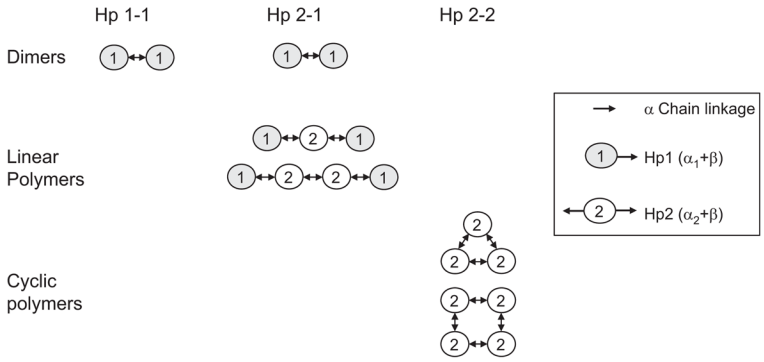

Figure 2. Schematic diagram of Hp polymer structure

Hp 1 α chains form one disulfide bond linkage, whereas Hp 2 α chains form two disulfide bond linkages. *Hp 1-1* individuals carry homodimers, *Hp 2-1* individuals carry homodimers of the Hp 1 type as well as linear heteropolymers of varying sizes. *Hp 2-2* individuals carry cyclic homopolymers of varying sizes.

Table 1

Acceptable levels of endogenous interfering substances.

Endogenous interfering substance	Acceptable level
Bilirubin	342 μM
Cholesterol	12.8 mM
Triglyceride	22.5 mM
Hemoglobin	12.8 g/L
Ascorbic acid (vitamin C)	342 μM
Atorvastatin (Lipitor)	20 μM
Niacin	6500 μM
Pravastatin	10 μM
Warfarin	32.5 μM
Acetaminophen	1324 μM
Tolbutamide	2.37 mM
Aspirin	3.62 mM
Fenofibrate	125 μM
Diphenhydramine	19.6 μM
Lisinopril	0.74 μM
Motormen	310 μM

Table 2

Categorization of the results obtained in the ELISA.

Category[a]	Hp 1-1	Hp 2-1	Hp 2-2
False positives	12	103	86
False negatives	73	95	33
True positives	935	3516	3394
True negatives	6924	4329	4456

[a]For the purposes of this analysis a positive result for haptoglobin (Hp) 1-1 is also a negative result for Hp 2-1 and Hp 2-2. By analogy, a positive result for Hp 2-1 is also a negative result for Hp 1-1 and Hp 2-2, and a positive result for Hp 2-2 is a negative result for Hp 1-1 and Hp 2-1. In this table, a false positive is a positive result in the ELISA which did not match a positive result in the gel assay. A false negative is a negative result obtained in the ELISA which did not match a negative result in the gel assay. A true positive is a positive result obtained in the ELISA which matched a positive result in the gel assay, and a true negative is a negative result in the ELISA which matched a negative result in the gel assay.

Table 3

Characteristics of the ELISA kit (n = 8046 evaluable samples).[a]

	Hp[b] 2-2	Hp 2-1	Hp 1-1
Sensitivity	99.0 (98.6–99.3)	97.4 (96.8–97.9)	92.8 (91.0–94.2)
Specificity	98.1 (97.7–98.5)	97.7 (97.2–98.1)	99.8 (99.7–99.9)
PPV	97.5 (96.9–98.0)	97.2 (96.5–97.7)	98.8 (97.7–99.3)
NPV	99.3 (99.0–99.5)	97.9 (97.4–98.3)	99.0 (98.7–99.2)

[a] Numbers reported are percentages and 95% confidence intervals are indicated in parentheses.

[b] Hp, haptoglobin; NPV, negative predictive value; PPV, positive predictive value.