Supplemental Data

Supplemental Data 1

Screening of monoclonal anti Hp antibodies

Hybridoma supernatants were screened in a direct ELISA at a dilution of 1:2 against Hp 1-1 using HRP-conjugated anti-IgG antibodies. The strongest reacting supernatants (140 out of 480) were rescreened against all three Hp types in a direct ELISA. In total, 21 hybridomas were selected for strongest reactivity and IgG isotype. These antibodies were rescreened in a sandwich ELISA using rabbit anti-human Hp (1 μg/well) as the capture antibody, followed by purified human Hp 1-1, 2-1, or 2-2 protein, followed by hybridoma supernatant (1:2), followed by HRP-conjugated anti-IgG antibodies. At a dilution of 1:2 all but 2 of the 21 antibodies gave strong signals with all 3 Hp types (data not shown). At a dilution of 1:2000, two classes of antibodies were found. Class I antibodies reacted equally well with both Hp 1-1 and Hp 2-2, whereas class II antibodies reacted more strongly with Hp 2-2 than with Hp 1-1. These results are summarized in Table S1. A subset of hybridomas was selected for further analysis in a sandwich ELISA at greater supernatant dilution (1:4000 and 1:5000) against all three Hp types. Those results are summarized in Table S2. Note that monoclonal 4G12 shows the greatest differential reactivity between the different Hp types.

Supplemental Data 2

Preparation of antibody coated plates and conjugate

Purified monoclonal 4G12 was coupled to microtiter plates at 1 μg/mL in 0.2 M sodium bicarbonate buffer pH 9, 0.1 mL/well. Plates were incubated overnight at room temperature and blocked with 1% BSA in phosphate buffer overnight at room temperature. The plates were stabilized with 2.5% sucrose solution for 4 h and dried in a vacuum dryer overnight. Dried plates were stored in plastic bags with desiccant. HRP-conjugated 4G12 was prepared by treating 1 mg of purified 4G12 monoclonal antibody with sodium periodate followed by treatment with sodium borohydride. The conjugate was dialyzed and purified on an S-300 column and diluted to 5 – 10 μg/mL in conjugate dilution buffer containing 50% StabilZyme® HRP Conjugate Stabilizer in H2O with 0.1 mg/mL mouse IgG. StabilZyme® was purchased from SurModics Pharmaceuticals, Birmingham, AL, USA. The conjugate was used at a dilution of 250.

Supplemental Data 3

Hp ELISA protocol

All steps are carried out at room temperature. Serum samples are diluted 1:10 in sample diluent buffer (PBS

Table S1 Characterization of hybridoma supernatants at a dilution of 1:2000 in a sandwich ELISA against Hp 1-1 and Hp 2-2.a

<table>
<thead>
<tr>
<th>mAb</th>
<th>Class</th>
<th>Buffer</th>
<th>1-1</th>
<th>2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A12</td>
<td>I</td>
<td>0.177</td>
<td>3.321</td>
<td>3.811</td>
</tr>
<tr>
<td>1D1</td>
<td>I</td>
<td>0.160</td>
<td>3.132</td>
<td>3.332</td>
</tr>
<tr>
<td>1E9</td>
<td>I</td>
<td>0.147</td>
<td>3.899</td>
<td>4.000</td>
</tr>
<tr>
<td>2A9</td>
<td>I</td>
<td>0.160</td>
<td>3.839</td>
<td>3.934</td>
</tr>
<tr>
<td>2E4</td>
<td>I</td>
<td>0.151</td>
<td>3.692</td>
<td>3.759</td>
</tr>
<tr>
<td>3A1</td>
<td>I</td>
<td>0.144</td>
<td>2.144</td>
<td>2.228</td>
</tr>
<tr>
<td>3B5</td>
<td>I</td>
<td>0.154</td>
<td>3.172</td>
<td>3.024</td>
</tr>
<tr>
<td>3E6</td>
<td>I</td>
<td>0.140</td>
<td>2.281</td>
<td>2.235</td>
</tr>
<tr>
<td>3E12</td>
<td>I</td>
<td>0.148</td>
<td>3.123</td>
<td>3.569</td>
</tr>
<tr>
<td>4D12</td>
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<td>0.160</td>
<td>3.826</td>
<td>3.960</td>
</tr>
<tr>
<td>5D3</td>
<td>I</td>
<td>0.139</td>
<td>2.858</td>
<td>2.742</td>
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<tr>
<td>1E10</td>
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<td>1.755</td>
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<tr>
<td>1G1</td>
<td>II</td>
<td>0.143</td>
<td>1.964</td>
<td>2.403</td>
</tr>
<tr>
<td>2B3</td>
<td>II</td>
<td>0.143</td>
<td>1.603</td>
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<td>3H3</td>
<td>II</td>
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<td>1.573</td>
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<tr>
<td>4F9</td>
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<td>0.645</td>
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<td>0.722</td>
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<tr>
<td>4D3</td>
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<tr>
<td>4D6</td>
<td>II</td>
<td>0.128</td>
<td>2.847</td>
<td>3.402</td>
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</table>

aValues in the buffer, 1-1, and 2-2 columns are for OD at 450 nm.

Table S2 Characterization of hybridoma supernatants in a sandwich ELISA at dilutions of 1:4000 and 1:5000 against all three Hp types.a

<table>
<thead>
<tr>
<th>mAb</th>
<th>Class</th>
<th>Buffer 1-1</th>
<th>2-1</th>
<th>2-2</th>
<th>Buffer 1-1</th>
<th>2-1</th>
<th>2-2</th>
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<tr>
<td>1E9</td>
<td>I</td>
<td>0.122</td>
<td>2.506</td>
<td>2.605</td>
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<td>2E4</td>
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<td>0.120</td>
<td>2.464</td>
<td>2.360</td>
<td>2.486</td>
<td>0.119</td>
<td>2.900</td>
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<tr>
<td>3E6</td>
<td>I</td>
<td>0.128</td>
<td>1.384</td>
<td>1.249</td>
<td>1.245</td>
<td>0.115</td>
<td>1.684</td>
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<td>5D3</td>
<td>I</td>
<td>0.122</td>
<td>1.417</td>
<td>1.265</td>
<td>1.302</td>
<td>0.145</td>
<td>1.764</td>
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<td>4G12</td>
<td>II</td>
<td>0.117</td>
<td>0.415</td>
<td>1.128</td>
<td>1.452</td>
<td>0.117</td>
<td>0.495</td>
</tr>
</tbody>
</table>

aValues in the buffer, 1-1, and 2-2 columns are for OD at 450 nm.
with 1% BSA and 0.1% Tween-20) and 100 μL added to a microtiter dish coated with 4G12 mAb directed against Hp. The plate is incubated for 30 min on a plate shaker set at 750 rpm. After washing the wells five times with 300 μL wash buffer (PBS with 0.5% Tween-20) and tapping the plate upside down on absorbent paper after each wash, 100 μL 4G12 mAb conjugated to HRP is added at a dilution of 1:250 in conjugate dilution buffer and placed on a shaker at 750 rpm for 1 h. After extensive washing as described above, 3′,3′,5,5′-tetramethylbenzidine (TMB) substrate is added for 15 min with shaking at 750 rpm. Stop solution (100 μL 1 N HCl) is added and the absorbance read at 450 nm within 15 min.

Supplemental Data 4

Description of serum samples

Serum samples (n=8059) from seven separate clinical studies were obtained and stored at –20°C. These studies were: the Women’s Health Study (WHS) (n=818) [5], the Coronary Artery Calcification in Type I Diabetes (CACTI) Study (n=1379) [6], the Renal and retinal effects of enalapril and losartan in type 1 diabetes (RASS) study [7], the Israel Cardiovascular Vitamin E (ICARE) study [8], the Nurse’s Health Study (NHS) (n=607) [9], the Diabetes-Dementia study (n=391) [10], and the Diabetes Control and Complications Trial (DCCT) (n=1455) [11]. All these studies were done in compliance with the Helsinki Declaration of 1975, as revised in 1996. All analyses were carried out from August 2009 to March 2011. The interval between analysis of a given sample by gel electrophoresis and ELISA was several days to several months.

Supplemental Data 5

TaqMan PCR method

This method [13] relies on accumulation of a PCR product which spans the unique Hp 2-2 exon 4–5 junction. The Hp 2-2 specific product is normalized to a second PCR product from the promoter region of the Hp gene which is present in all individuals regardless of Hp genotype. The results are expressed as a ratio of Hp 2-2 specific PCR product divided by the ubiquitous PCR product. Hp 2-2 individuals should score a ratio which is twice that of Hp 2-1 individuals, whereas Hp 1-1 individuals should score a ratio of zero.

Supplemental Data 6

Receiver operator characteristic (ROC) curves

The ROC curves comparing the ELISA and TaqMan to the gel electrophoresis method are as follows.

- ROC curve for ELISA predicting that a given sample is Hp 2-2 or not Hp 2-2. Samples are from the DCCT study. Reference method is gel electrophoresis method. AUC (area under the curve) is 0.999 (95% CI=0.996–1.000).

- ROC curve for TaqMan predicting that a given sample is Hp 2-2 or not Hp 2-2. Samples are from the DCCT study. Reference method is gel electrophoresis method. AUC is 0.873 (95% CI=0.853–0.893).