Assessment of highly sensitive C-Reactive Protein levels as diagnostic discriminator of Maturity Onset Diabetes of the Young due to HNF1A mutations

Running title: hsCRP in subtypes of diabetes

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Objective: Despite the clinical importance of an accurate diagnosis in individuals with monogenic forms of diabetes, restricted access to genetic testing leaves many patients undiagnosed. Recently, common variation near the HNF1 homeobox A (HNF1A) gene was shown to influence C-reactive protein levels in healthy adults. We hypothesised that serum levels of highly-sensitive CRP (hsCRP) could represent a clinically-useful biomarker for the identification of HNF1A mutations causing Maturity Onset Diabetes of the Young (MODY).

Research Design and Methods: Serum hsCRP was measured in subjects with HNF1A-MODY (n=31), autoimmune diabetes (n=316), type 2 diabetes (n=240), Glucokinase (GCK-) MODY (n=24) and non-diabetic individuals (n=198). The discriminative accuracy of hsCRP was evaluated through receiver operating characteristic (ROC) curve analysis, and performance compared to standard diagnostic criteria. Our primary analyses excluded ~11% of subjects in whom the single available hsCRP measurement exceeded 10mg/l.

Results: Geometric mean (standard deviation range) hsCRP levels were significantly lower (p≤0.009) for HNF1A-MODY individuals [0.20 (0.03-1.14) mg/l] than any other group: autoimmune diabetes 0.58 (0.10-2.75), type 2 diabetes 1.33 (0.28-6.14), GCK-MODY 1.01 (0.19-5.33), non-diabetic 0.48 (0.10-2.42) mg/l. The ROC-derived C-statistic for discriminating HNF1A-MODY and type 2 diabetes was 0.8. Performance of hsCRP, either alone or in combination with current diagnostic criteria, was superior to current diagnostic criteria alone. Sensitivity and specificity for the combined criteria approached 80%.

Conclusions: Serum hsCRP levels are markedly lower in HNF1A-MODY than other forms of diabetes. HsCRP has potential as a widely-available, cost-effective screening test to support more precise targeting of MODY diagnostic testing.

Mutations in Hepatocyte Nuclear Factor 1-alpha (HNF1 homeobox A; HNF-1alpha; HNF1A) gene represent the most common cause of Maturity-onset diabetes of the young (MODY), and are estimated to account for ~2% of all diabetes (1). Demonstrating that an HNF1A mutation is responsible for diabetes in a given individual has important clinical implications for both the patient and their relatives: for example, in contrast to typical type 2 diabetes, low-dose sulphonylureas rather than metformin should be first-line treatment (2; 3). Despite the clinical value of an accurate molecular diagnosis, many individuals with HNF1A-MODY are never tested, and are consequently misclassified as having type 1 or type 2 diabetes.

The principal barriers to the implementation of systematic diagnostics for monogenic forms of diabetes include the high cost and restricted availability of genetic testing. Currently, patients are typically selected for molecular testing on the basis of non-specific clinical features such as age of onset, parental history of diabetes (4) and/or a clinical presentation which is otherwise atypical for the assumed aetiology (such as an apparent absence of insulin resistance in an
individual presumed to have type 2 diabetes (5)). However, the performance of these criteria is such that it is difficult to combine acceptable levels of specificity and sensitivity. Consequently there would be considerable value in identifying additional screening tools, which, in conjunction with existing clinical and biochemical markers, would assist selection of cases that merit further investigation, including HNF1A sequencing. Since a relative beta-cell defect is common to all forms of diabetes, the best prospects for identifying such a marker may rely on the extra-pancreatic manifestations of an HNF1A mutation. HNF1A encodes the transcription factor HNF-1alpha, initially identified in the liver, where it is involved in regulation of a large number of genes (6), and also expressed in the pancreas, gut and kidney. There have been several previous attempts to identify HNF1A-MODY biomarkers, most based on candidates initially highlighted by studies on Hnf1a knockout mice (6; 7). However the candidates examined, including apolipoprotein M (8-10), aminoaciduria (11; 12), complement components (13) and glycosuria (14-16), have either not demonstrated sufficient sensitivity and/or specificity to warrant further evaluation or have thus far translated into a clinically-useful biomarker. Recently, genome-wide association studies have revealed that common variants mapping near the HNF1A gene on chromosome 12q24 are associated with small alterations in serum C-reactive protein (CRP) levels in healthy adults (17; 18). The presence of HNF-1alpha binding sites in the CRP promoter (19) suggests the effect on CRP is mediated through altered regulation of HNF1A expression. Moreover, a loss of HNF1A binding has been shown to result in a loss of CRP expression (19). If so, it follows that the rare, but large-effect loss of function mutations in HNF1A responsible for MODY, might be expected to lead to more substantial reductions in serum CRP levels. This would be analogous to observations involving the glucokinase (GCK) gene: whilst a common GCK-promoter variant is associated with a modest (~0.06 mmol/L) effect on fasting plasma glucose levels in healthy adults (20), rare pathogenic mutations in GCK are responsible for a far larger (~2 mmol/L) increase (21). We aimed to test whether individuals with HNF1A-MODY have reduced serum CRP levels compared to those with other forms of diabetes, and to establish whether this could provide a useful diagnostic marker.

RESEARCH DESIGN AND METHODS
The subjects included in this study are more fully-described in the online-appendix available at http://care.diabetesjournals.org. Briefly, we included 31 cases of HNF1A-MODY, 24 of GCK-MODY, 275 with classic type 1 diabetes, 41 with latent autoimmune diabetes of adulthood (LADA), 240 with young-onset type 2 diabetes (diagnosed up to 45 years of age) and 198 non-diabetic individuals. The study was approved by the Oxfordshire Local Research Ethics Committee and all subjects gave informed consent. Serum high-sensitivity CRP (hsCRP) levels were measured using a wide range latex-enhanced immunoturbidimetric assay on an ADVIA 2400 analyser (Siemens Healthcare Diagnostics Ltd, Frimley, UK), with a quoted method linearity of 0.03 to 160 mg/L. Imprecision, expressed as CV%, at concentrations >0.05 mg/L was <10% and at 23.5 mg/L was <1%. Glutamic acid decarboxylase antibodies (GADA) were measured by a
radioimmunoassay using $^{35}$S-labelled full-length GAD65, with results expressed in WHO units/ml derived from a standard curve calibrated from international reference material (National Institute for Biological Standards and Control code 97/550). Samples were considered positive if they had levels above 14 WHO units/ml (97.5th percentile of healthy school children) (22).

**Analysis.** HsCRP values for all subjects were inspected. Means and standard deviations for the two autoimmune groups (type 1 diabetes and LADA) were very similar, so these were combined for further analyses. Supplementary Figure 1 documents the distribution of individual hsCRP levels in all subjects. Median (IQR) values for hsCRP are also reported in Table 1, along with other clinical characteristics of the groups. In line with previous studies (23-24), we considered that hsCRP values $>$10mg/l were likely in many instances to represent an acute inflammatory response, and, prior to any analysis, had decided to exclude such values from our primary analyses. From the 809 subjects with hsCRP values, 90 (11%) with hsCRP $>$10mg/l were removed: 3 (10%) with HNF1A-MODY, 22 (7%) with autoimmune diabetes, 53 (22%) with type 2 diabetes and 12 (6%) of the non-diabetic subjects. For subsequent analyses, all variables were log-transformed. Differences between the hsCRP value in HNF1A-MODY and other groups were evaluated using the T-test, both before and after adjustment for BMI (since BMI was correlated with hsCRP). As certain drugs, particularly statins and aspirin, can lower CRP levels (25), we performed a further analysis after removing subjects treated with either. Other variables (gender, age at sampling, HbA1c, duration of diabetes, ethnic origin) were not associated with hsCRP levels in our data, and were therefore not included as covariates. We also examined whether hsCRP level was affected by the type of mutation (nonsense vs. missense) or the isoform of HNF1A affected by missense mutations. Finally, since some of the MODY subjects were related to each other, we sought to account for non-independence in two ways. First, we reanalysed using only a single individual from each family (either the proband, or if they were not available the youngest person in the family) and second, by including family-membership as a covariate into the analysis.

The performance of hsCRP as a diagnostic test for HNF1A-MODY was examined by ROC curve analysis and compared to standard clinical criteria. Sensitivities and specificities were calculated from our data and then used to model the effect of using hsCRP ± clinical criteria as a screening test for HNF1A mutations in a patient group of apparent type 2 diabetes diagnosed up to 45 years (Supplementary Table 2). For this purpose we assumed that 4% of the group were misclassified HNF1A-MODY subjects, based on observations we have made in a large undifferentiated group of young-onset type 2 subjects (Owen, McCarthy unpublished data).

All statistical analysis was performed in SPSS v16 and a p value of $<$0.05 was considered significant.

**RESULTS**

The results confirm our hypothesis that hsCRP levels are significantly lower in HNF1A-MODY cases compared to all other groups, including non-diabetic subjects (Table 1, Figure 1a). Geometric mean (standard deviation range) unadjusted hsCRP values for HNF1A-MODY subjects (after excluding those
with hsCRP>10mg/l – see METHODS) were 0.20 (0.03-1.14) mg/l, compared to 1.33 (0.28-6.14) mg/l for type 2 diabetes, 0.58 (0.10-2.75) mg/l for autoimmune diabetes, 1.01 (0.19-5.33) mg/l for GCK-MODY and 0.48 (0.10-2.42) mg/l for non-diabetic subjects (p≤0.009 for all pairwise comparisons with HNF1A-MODY cases). The geometric mean (standard deviation range) value for the other diabetic groups combined was 0.81 (0.15-4.50) mg/l, significantly greater than for the HNF1A-MODY group (p=0.00003).

Use of statin and/or aspirin therapy had negligible effects on the comparisons with the HNF1A-MODY group (Table 1). In the type 2 diabetes group hsCRP level was the same in the statin/aspirin users compared to the non-users (p=0.27), while in the type 1 diabetes group those taking statins and/or aspirin had a higher hsCRP than non-users (p=0.004). Thus it seems unlikely that use of these drugs would lower hsCRP towards the range seen in the HNF1A-MODY group. In contrast, adjustment for BMI had a large impact on the estimated means (Table 1) and, given the correlation between BMI and CRP ($r^2=0.28$, p<10^{-6} for controls), abolished much of the difference observed between the type 2 diabetes and other groups. However, the differences in hsCRP levels between HNF1A-MODY and the other groups were preserved (p≤0.01). Estimated mean adjusted hsCRP (95% CI) levels for HNF1A-MODY compared to the other diabetic groups combined were 0.28 (0.16-0.49) vs. 0.82 (0.71-0.94) mg/l, p=0.003. Reanalysis accounting for shared family membership (see METHODS) did not alter the magnitude or significance of the most important between group differences (e.g. using the probands only, HNF1A-MODY vs. type 2 diabetes p=7x10^{-6}, HNF1A-MODY vs. autoimmune diabetes p=0.009). There was no difference between the hsCRP levels seen in families segregating missense mutations (11 families) as compared to those featuring a premature stop codon(8 families, p=0.6). As all but 2 of the missense mutations were in exons represented in all three HNF1A isoforms, we could reach no conclusions concerning the relationship between CRP levels and HNF1A isoform.

The differences in mean values between HNF1A-MODY and the other groups are striking, but this does not necessarily translate into clinical utility. We therefore evaluated the performance of hsCRP levels as a potential diagnostic test for distinguishing HNF1A-MODY from other kinds of diabetes (Supplementary Tables 1 and 2). Figure 1b displays the (cumulative) distribution of hsCRP values for individuals in the different subject groups. In our data, a diagnostic threshold value of 0.4mg/l (the vertical line on Figure 1b) equates to a sensitivity of 71% and specificity of 77% for distinguishing HNF1A-MODY from type 2 diabetes. These figures are revised to 65% and 82% respectively if subjects with hsCRP≥10mg/l are not excluded. Inclusion of autoimmune and GCK-MODY cases reduces the specificity of the hsCRP threshold of 0.4mg/l to 63%. Using a hsCRP threshold below 0.4mg/l improves specificity (and would therefore reduce the number of individuals with type 2 diabetes who might undergo “unnecessary” HNF1A sequencing), but this comes at the expense of sensitivity (i.e. more HNF1A-MODY cases are missed). The consequences of different hsCRP criteria on these measures of sensitivity and specificity, in our data, are illustrated in Supplementary Table 1. The ROC-derived C-statistic (a measure of discriminative accuracy) for unadjusted
hsCRP levels was 0.80 for distinguishing HNF1A-MODY from type 2 diabetes (Figure 1c) and 0.75 for distinguishing HNF1A-MODY from all other diabetes subtypes combined. We compared the performance of hsCRP (again excluding those with hsCRP>10mg/l) to current diagnostic criteria including age of diabetes onset ≤25 years and a first degree family history of diabetes. In our data set, these existing criteria had lower sensitivity (58%) than hsCRP testing but they were highly specific for the discrimination of HNF1A-MODY and type 2 diabetes (only 6% of the latter met these criteria). The combination of existing diagnostic criteria or an hsCRP level of ≤0.2mg/l produced, in our data set, a sensitivity of 79% and specificity of 83% for distinguishing HNF1A-MODY from type 2 diabetes, a considerable improvement in sensitivity (p=0.035) on traditional criteria alone with little loss of specificity (Supplementary Table 1).

Using these criteria as a screening test for selection of cases for further investigation would lead to sequencing of 20% of type 2 diabetic subjects diagnosed up to 45 years with a detection rate of HNF1A mutations of 16% of those sequenced (Supplementary Table 2).

DISCUSSION
We have shown for the first time that subjects with HNF1A-MODY maintain substantially lower levels of serum hsCRP levels than individuals with other forms of diabetes, or non-diabetic controls. By demonstrating these effects of rare coding mutations in HNF1A, our findings therefore extend recent observations that common variants near HNF1A are associated with CRP levels. In so doing, our study confirms that the common variant associations are almost certainly mediated through alterations in HNF1A transcription. However, the observation that some HNF1A-MODY individuals had hsCRP levels over 10 mg/l (including one of 52 mg/l) demonstrates that HNF1A haploinsufficiency is not sufficient to prevent substantial elevation in CRP levels, presumably as part of an acute inflammatory response.

We have shown that as a stand-alone diagnostic test to select cases for HNF1A-MODY sequencing, hsCRP performs reasonably well in our data set, with a C-statistic of 0.80 for differentiating HNF1A-MODY from young-onset type 2 diabetes. In clinical use, it seems likely that hsCRP levels would be combined with other clinical and biochemical data within a broader diagnostic algorithm. We have shown that such a combination of clinical criteria and hsCRP can achieve sensitivities and specificities around 80% (Supplementary Table 1). We are aware of the dangers of over-fitting, and clearly these performance metrics need to be evaluated in independent datasets. We also note that to take full advantage of the sensitivity afforded by this combination, would require molecular diagnostic testing of around 20% of those diagnosed with apparent type 2 diabetes below 45 years (Supplementary Table 2). It is worth emphasising that in this study we focused on individuals with a relatively early diagnosis of type 2 diabetes on the basis that these constitute the group in which the diagnostic differentiation from HNF1A-MODY is most pertinent.

One point for discussion is how values outside the normal range for the assay should be handled. By removing all individuals with hsCRP above 10mg/l, we sought to avoid the loss of discriminatory accuracy associated with what we assume to be acutely elevated hsCRP levels. Our current recommendation
would be for individuals with elevated CRP levels to have a repeat measure some weeks later, allowing any acute inflammatory response to subside. As our study did not include serial measurements of hsCRP, future studies will be needed to explore this particular issue. It is entirely plausible that the low-grade chronic elevation of CRP which is characteristic of type 2 diabetes would enhance the discriminatory performance of repeated over single hsCRP measures. Whilst we included reasonably large collections of subjects with HNF1A- and GCK-MODY, the very low prevalence of HNF4A-MODY means that subjects with this form of MODY were not available for comparison. HNF4A-MODY presents with a very similar beta-cell phenotype to HNF1A-MODY, but many of the extrapancreatic manifestations are distinct (4). As the CRP promoter lacks any HNF-4 alpha binding sites, it seems probable that hsCRP levels in HNF4A-MODY subjects will not be reduced: if future studies confirm this, hsCRP levels may provide useful diagnostic differentiation between HNF1A and HNF4A-MODY. In conclusion, we have shown that subjects with pathogenic mutations in HNF1A have significantly lower serum levels of hsCRP than those with other types of diabetes. Although these findings require validation in independent datasets, and extension to other rarer subtypes of MODY, hsCRP emerges from this study as the most promising diagnostic biomarker for HNF1A-MODY identified to date. Since hsCRP estimation is already widely-available at low cost in many routine pathology services, it should be relatively easy to incorporate this biomarker into diagnostic pathways. We expect this to lead to better targeting of molecular diagnostic testing for monogenic forms of diabetes, and for consequent improvements in detection rates to result in more effective treatment of this currently under-diagnosed condition.

**Author Contributions:** KRO: contributed to the conception and design of study, acquisition, analysis and interpretation of data, wrote and revised the manuscript and approved the final version.
GT: contributed to the acquisition, analysis and interpretation of data, revised the manuscript and approved the final version.
TJJ, FK and AJF: contributed to the acquisition of data, revised the manuscript and approved the final version.
MIMC: contributed to the conception and design of study, analysis and interpretation of data, revised the manuscript and approved the final version.
ALG: contributed to the conception and design of study, acquisition, analysis and interpretation of data, wrote and revised the manuscript and approved the final version.

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The authors declare no conflicts of interest relevant to this article.

REFERENCES


Figure Legends

Figure 1a-c
The following analyses include 28 subjects with HNF1A-MODY, 294 with Autoimmune diabetes, 187 with Type 2 diabetes, 24 with GCK-MODY and 198 non-diabetic controls. Values of hsCRP >10mg/l are excluded.

Figure 1a
Geometric mean hsCRP levels for the different groups; error bars show 95% confidence intervals.

Figure 1b
Cumulative percentage plot for hsCRP levels in the different groups. The dotted reference line corresponds to a hsCRP value of 0.4mg/l. HsCRP levels are plotted on a log_{10} scale.

Figure 1c
Receiver operated characteristic curve illustrating the capacity of hsCRP to distinguish between HNF1A-MODY and type 2 diabetes. The C-statistic (area under the curve) for this comparison is 0.8.
Figure 1b

Cumulative percentage

hsCRP mg/l

HNF1A-MODY
Non diabetic
Autoimmune
GCK-MODY
Type 2 diabetes

Figure 1c

Sensitivity

1 - Specificity

hs CRP, A = 0.80
### Table 1 Characteristics of subjects

<table>
<thead>
<tr>
<th></th>
<th>HNF1A-MODY</th>
<th>Autoimmune Diabetes</th>
<th>Type 2 Diabetes</th>
<th>GCK-MODY</th>
<th>Non-diabetic</th>
<th>p</th>
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<tr>
<td></td>
<td>n=31</td>
<td>n=316</td>
<td>n=240</td>
<td>n=24</td>
<td>n=198</td>
<td></td>
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<tr>
<td>% Male</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>*Age of diagnosis yr</td>
<td>35.5</td>
<td>56.3</td>
<td>55.0</td>
<td>41.6</td>
<td>46.5</td>
<td></td>
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<tr>
<td>*Duration of diabetes yr</td>
<td>13.8 (4.0-48.1)</td>
<td>11.7 (5.8-23.7)</td>
<td>8.5 (2.4-30.8)</td>
<td>13.1 (6.8-25.2)</td>
<td>-</td>
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<tr>
<td>*HbA1c %</td>
<td>7.1 (5.9-8.5)</td>
<td>N/A</td>
<td>8.0 (6.5-9.9)</td>
<td>6.8 (5.6-8.3)</td>
<td>N/A</td>
<td>*5x10^-5</td>
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<tr>
<td>% treated with aspirin</td>
<td>16.1</td>
<td>7.5</td>
<td>28.1</td>
<td>12.5</td>
<td>0</td>
<td>*0.002</td>
</tr>
<tr>
<td>% treated with statin</td>
<td>22.6</td>
<td>29.7</td>
<td>63.8</td>
<td>8.3</td>
<td>1.0</td>
<td>*3x10^-6</td>
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<tr>
<td><strong>Median hsCRP mg/l</strong></td>
<td>0.1 (0.03-1.14)</td>
<td>0.58 (0.10-2.75)</td>
<td>1.33 (0.28-6.14)</td>
<td>1.01 (0.19-5.33)</td>
<td>0.48 (0.10-2.42)</td>
<td>*&lt;1x10^-6</td>
</tr>
<tr>
<td>*hsCRP mg/l (hsCRP&lt;10 mg/l, unadjusted)</td>
<td>n=20</td>
<td>n=294</td>
<td>n=187</td>
<td>n=24</td>
<td>n=186</td>
<td>*&lt;1x10^-6</td>
</tr>
<tr>
<td>p value vs. HNF1A-MODY</td>
<td>0.11 (0.02-0.50)</td>
<td>0.45 (0.07-2.75)</td>
<td>1.61 (0.30-8.59)</td>
<td>0.95 (0.16-5.42)</td>
<td>0.48 (0.09-2.45)</td>
<td>*&lt;1x10^-6</td>
</tr>
<tr>
<td>**hsCRP mg/l (hsCRP&lt;10 mg/l, adjusted for BMI)</td>
<td>n=28</td>
<td>n=294</td>
<td>n=187</td>
<td>n=24</td>
<td>n=186</td>
<td>*&lt;1x10^-6</td>
</tr>
<tr>
<td>p value vs. HNF1A-MODY</td>
<td>0.27 (0.15-0.46)</td>
<td>0.76 (0.64-0.91)</td>
<td>0.76 (0.60-0.96)</td>
<td>0.95 (0.47-1.89)</td>
<td>0.57 (0.46-0.71)</td>
<td>*&lt;1x10^-6</td>
</tr>
</tbody>
</table>

Values shown are *Geometric mean (SD range), ¶ median (IQR), ** Estimated marginal means (95%CI). *Age of sampling for control subjects.
P value compares all groups and was calculated by ‡ ANOVA, § Chi square test (diabetic groups only), # Kruskal Wallis test.