Author's response to reviews

Title: Serum levels of Pancreatic Stone Protein (PSP)/reg1A as an indicator of beta-cell apoptosis suggest an increased apoptosis rate in hepatocyte nuclear factor 1 alpha (HNF1A-MODY) carriers from the third decade of life onward

Authors:

Siobhan Bacon (siobhanbacon@gmail.com)
Peyh M Kyithar (pkyithar@hotmail.com)
Jasmin Schmid (jschmid@rcsi.ie)
Syed R Rizvi (syedrizvi@hotmail.com)
Caroline Bonner (cbonner@rcsi.ie)
Rolf Graf (grafr@nz.ie)
Jochen H.M Prehn (jprehn@rcsi.ie)
Maria M Byrne (mbyrne@mater.ie)

Version: 2 Date: 29 March 2012

Author's response to reviews: see over
Response to the Reviewers’ Comments.

We thank both reviewers for their constructive comments and suggestions.

Reviewer 1:

Major compulsory revisions

1. The family controls are the optimal control group for this study. However we agree with the reviewer’s concerns regarding the inclusion of subjects with IGT in the family control group. In fact only one subject had IGT (all others had normal glucose tolerance) and this subject has now been excluded from the control group. This subject belonged to a pedigree with a novel mutation S352fsdelG. This mutation co-segregates with diabetes (Kyithar et al., Diabetes Metabolism, 2011, 37:512-519).

As suggested by the reviewer, we now use the larger, normally distributed historical control group for main comparisons of PSP/reg1A serum levels. As expected, there is also a significant difference when compared to the historical control group (p=0.0008). This information has been included in the abstract (lines 20-21) and in the results section (page 6, paragraph 1, line 7). As reviewer two was concerned about the study size we added 4 additional subjects to the HNF1-A MODY group and 6 additional subjects to the type one diabetic group (page 5, paragraph 1, line 9).

2. When directly compared there was no difference between the GCK vs. HNF1A-MODY group (p=0.55) or T1DM group (p=0.34). This information has now been added to the manuscript (page 8, paragraph 3, lines 27-28) and to the amended Figure 4. While the medians of PSP were significantly different between the HNF1A-MODY and controls (p=0.0008) and subjects with T1DM and the controls (p=0.0007), there was no significant difference between the GCK group and the controls (p=0.1641). However, we do appreciate the limitations of these data, and now discuss the finding in relation to the GCK group more carefully in the
3. We did not find a significant correlation between PSP/reg1A and the duration of diabetes in the HNF1A-MODY group. However, we consider age to be the more important parameter in our analysis as HNF1A-MODY may remain undiagnosed for years. In contrast, the duration of type 1 diabetes obviously can be accurately determined. Interestingly, we did not find a significant correlation between PSP/reg1A and duration of diabetes in the T1DM group, suggesting ongoing beta-cell destruction during disease as discussed in the manuscript.

Fasting C-peptide did not correlate with PSP/reg1A levels, nor did AUC for C-peptide during the OGTT in any of the groups studied. However, the correlation analysis may be hampered by the quality of the C-peptide assay. We therefore chose not to show these data in this study.

4. We agree with the reviewer. We used only non-parametric statistics as PSP was not normally distributed in the groups tested. All results are now reported in medians and IQR as requested. This has been changed in the abstract, results, and figure legends.

5. As suggested by the reviewer, we have included a paragraph to the discussion section regarding the use of PSP/reg1A as a biomarker for beta-cell apoptosis (page 9, paragraph 2, lines 13-22).

Minor Essential Revisions

1. The abstract now states ‘HNF-1A MODY carries have reduced pancreatic beta-cell mass, partially due to […]’ to be conform with the main part of the manuscript.

2. Abstract results now state the full p value as opposed to p<0.05.
3. This sentence in the Background section (paragraph 2, lines 23-24) has been re-phrased to state ‘Mutations in HNF1A are usually detected later in life when they are incidentally discovered through a screening programme or if subjects become symptomatic.’

4. The use of the word ‘cohort’ has been replaced with ‘group’ throughout the manuscript.

5. The number of index cases/families from which subjects were drawn from is now included in the subjects section (page 5, paragraph 1, lines 3-6). The HNF1A-MODY group consisted of 13 different families and the GCK-MODY group represented 6 different families. HNF1A mutations included L17H, G207D, P291fsinsC, S352fsdelG, F426X, P379T, and IVS7-6G>A, and R200Q/N. GCK mutations included D160N, Y61X, A378V, L146fs, Ile293Arg, and p.Asp311fs.

6. The OGTT was not performed in the type 1 diabetes mellitus group as it was not appropriate to discontinue insulin in this group. This is now clarified in the methods section [page 5, line 23; ‘A 75 g OGTT was performed on subjects (excluding subjects with type 1 diabetes mellitus) after a 12-h overnight fast with measurement of glucose’].

7. The results paragraphs have been amended to remove background and interpretation.

Results section, page 7, paragraph 1, lines 3-5 as stated below have been removed from results section and added to the discussion page 9, paragraph 1, lines 3-5.
The Irish HNF1A-MODY cohort has been recently characterized [6]. This study reported on a mutation identification rate of 30.5% among Irish adults clinically selected for HNF1A-MODY from attendees at the diabetes clinic.

Therefore, as insulin secretion deteriorates, PSP/reg1A levels are stimulated.

We found a positive correlation between age and PSP/reg1A (r=0.40, p=0.02) suggesting that beta-cell apoptosis may be increased with ageing.

Defining initial onset of diabetes in individuals is subject to great variability particularly in those subjects with HNF1A-MODY /HNF4A-MODY and GCK mutations as they may be asymptomatic for some time preceding diagnosis.

Heterozygous loss of function GCK mutations in subjects result in a stable beta cell defect which is present in-utero. There is a very small deterioration in fasting plasma glucose from birth resulting in a less severe form of diabetes than that caused either by HNF1A-MODY or type 1 diabetes mellitus.

suggesting that beta-cell apoptosis may not be a prominent contributor to GCK-MODY.
8. The correct units for C-peptide as performed in our laboratory are mcg/L. We cannot detect levels of C-peptide <0.5mcg/L. The fasting C-peptide levels were <0.5mcg/L in all type one subjects apart from 3 subjects. However PSP levels were not different between the two groups. We have removed fasting C-peptide in the type 1 diabetic group in Table 1 as it only represents 3 subjects.

9. We agree that the GCK group will be assumed to be hyperglycaemic from birth. We have therefore altered Table 1 accordingly.

10. The figures and table legends have been edited to remove interpretation. The data is now being presented in median and IQR, and the comparisons for the non-significant results are now indicated.

**Discretionary Revisions**

The abstract is now 279 words following amendment, and the heading for the Methods section has been added.
Reviewer 2:

Major compulsory revisions

1. Monogenic diabetes is a rare disorder accounting for approximately 2% of all diabetes as previously reported. Previous international single centres have published with comparable numbers on similar topics to this current research article. We have added an additional 4 subjects with HNF1A-MODY which increases the size of the monogenic diabetes group to n=50 which is a large number for a relatively small population size in Ireland. Power calculations were not performed retrospectively since it is a rare genetic disorder and therefore, we wish to report any findings on the sample size available to our research group. We have also added an additional 6 subjects with type 1 diabetes mellitus to the study group as stated in the results section (page 5, paragraph 1, line 9).

2. We have previously provided biological evidence in insulinoma cell lines and transgenic mice models of HNF1A-MODY that beta cells undergoing apoptosis induce the expression of the PSP/reg gene in a caspase-dependent manner in neighbouring beta-cells (Bonner et al., Diabetes, 2010, 59:36199-206). This induction was inhibited in cells treated with capase inhibitors. Paraffin embedded pancreatic sections from 5 month old diabetic mice expressing HNF1A-MODY in beta cells also demonstrated elevated expression of PSP/reg throughout the islets compared with wild-type mice. Islet cells with elevated PSP/reg immunoreactivity were positioned in the vicinity of cells displaying apoptotic nuclear morphology, suggesting that PSP/reg1A gene expression was induced during apoptosis in animal models. One of the core findings of the present study is that PSP/reg1A serum levels may be used as a non-invasive marker to evaluate the extent of beta cell apoptosis during disease progression and an indirect marker of beta cell mass in humans. This information has been added to the discussion (page 9, paragraph 2, lines 13-22).

3. In this manuscript, we did not use a cut-off value to distinguish dichotomous data. It was simply a statistical analysis correlating a state of disease with a value (PSP). We would have provided a ROC-curve to determine an
optimal cut-off value and subsequently would also estimate sensitivity and specificity. However the purpose of the manuscript was to demonstrate that the disease activity/mutation correlates with PSP as a serum marker for beta cell apoptosis. It is not intended that PSP/reg1A would act as a biomarker to distinguish between diabetes types.

The technical specificity of the PSP/reg1A assay has been determined: addition of 0.5 ng/ml to diluted serum from different individuals gave a recovery of 101 +/- 20% (n=19). The intra-plate and inter-plate variability is less than 5% and 10% respectively. This has been added to the methods section (page 6, paragraph 1, lines 13-15).

**Minor essential revisions**

1. We did not perform correlation analysis between the groups, however we did demonstrate the difference between median PSP/reg1A levels as illustrated in amended figure 4 and as outlined in our response to Reviewer 1 (Major compulsory revision, question 2).

2. The AUC for insulin was correlated with PSP/reg1A for all groups, however, the only significant correlation was with HNF1A-MODY.

   GCK (rho=0.21, p=0.56)

   Normoglycaemic controls (rho=-0.04, p=0.90)

   HNF1A-MODY (rho=-0.40, p=0.02)

   An OGTT was not completed in the subjects with type 1 diabetes mellitus as it is not possible to discontinue insulin in this population. This information has been added to the manuscript (page 5, line 23).
3. We agree that total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides are not good markers of lipotoxicity, and therefore the statement on lipotoxicity as being represented by a cholesterol profile has been deleted from page 7, paragraph 3, line 21.

4. In clinical practice white cell count, and neutrophil count are the most widely used test for establishing if an acute infection is present.

5. Fasting C-peptide did not correlate with PSP/reg1A levels, nor did AUC for C-peptide during the OGTT in any of the groups studied. However, the correlation analysis may be hampered by the quality of the C-peptide assay. We therefore chose not to show these data in this study.

6. The sub-analysis of PSP/reg1A in the HNF1A-MODY group into those above and below the age of 25 years was used as previously published studies have clinical guidelines that characterize HNF1A-MODY as in general being diagnosed before the age of 25 years.

There is no significant correlation between PSP/reg1A and duration of diabetes.

7. The glucose levels during the OGTT has been added to Table 2.