Author's response to reviews

Title: Association of obesity risk SNPs in PCSK1 with insulin sensitivity and proinsulin conversion

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Author's response to reviews: see over
We thank the reviewers for their critical and helpful comments. We revised the manuscript accordingly and answered all issues point by point.

Reviewer 1

This paper therefore makes three major claims:
1. Two missense SNPs at PCSK1 (one of which has been shown to impair enzymatic activity) influence proinsulin to insulin conversion.
2. These SNPs are not associated with obesity measures in this cohort.
3. The proinsulin-raising allele may also increase insulin sensitivity through unknown mechanisms.

With respect to #1, the finding is relevant but expected. With regard to #2, as the authors acknowledge their cohort is likely underpowered to replicate the previously reported result; as recently presented in international meetings (e.g. ASHG October 2009, oral abstract by Speliotes et al.) the GIANT Consortium has demonstrated an association of PCSK1 SNPs with body mass index at P ~ 10e-5 – though not genome-wide significant, more convincing than the negative result shown here.

Ad #1) We agree with this point.
Ad #2) To our opinion, the issue whether PCSK1 SNPs contribute to alterations in BMI is not ultimately clarified. The unpublished study reported by the reviewer points to an association that does not reach the required genome wide significance level, and further independent replications are unfortunately still lacking. However, we agree with the reviewer, that our study was clearly underpowered to replicate this finding. Moreover, it was not the primary aim of this study to replicate associations of PCSK1 SNPs with BMI.

Finally, a major problem concerns the unexpected result described in #3. If a genetic variant truly impairs prohormone convertase activity, as shown in a prior publication, then carriers of that variant will display relatively higher levels of proinsulin and lower levels of endogenous insulin (as shown here). But the primary reason is a conversion defect, not heightened insulin sensitivity. Because insulin is used in the numerator for HOMA-IR and in the denominator for the Matsuda insulin sensitivity index, it is natural that the proinsulin-raising and insulin-lowering allele will also seem to be associated with lower HOMA-IR and higher insulin sensitivity; but in this scenario, these indices do not accurately reflect the insulin resistant state of the organism, because there is a primary insulin synthetic defect. In order to eliminate such confounding, statistical adjustment is not enough: one ought to study measures of insulin sensitivity that are derived from the exogenous administration of insulin, as in the hyperinsulinemic euglycemic clamp.

Thank you for this important suggestion. We now additionally report euglycemic hyperinsulinemic clamp derived insulin sensitivity in the tables and in the text. These data confirm our previous associations with insulin sensitivity.
Therefore, this paper seems to be reduced to one interesting but expected result, an ultimately false negative statement on obesity, and a novel claim on insulin sensitivity that is based on a physiological measure that seems inappropriate in this context, and should therefore be removed as currently framed.

We agree with the reviewer, that the association of \textit{PCSK1} SNPs rs6232 and rs6235 with proinsulin conversion was expected but it was not yet shown by others. Moreover, after inclusion of the hyperinsulinemic euglycemic clamp data, a major novelty of this study may be the association of \textit{PCSK1} SNP rs6232 with insulin sensitivity.

1. Adjust the obesity analyses and their interpretation to the likely fact that \textit{PCSK1} confers a modest effect on obesity when analyzed in large enough samples, as recently reported by the GIANT Consortium

   Thank you for the comment. We adjusted the obesity interpretation in the introduction and discussion sections and removed “obesity-independent” from the title.

2. Remove the analyses for insulin sensitivity as being inadequate in this context

   As suggested, we now additionally report insulin sensitivity derived from hyperinsulinemic-euglycemic clamps which confirm our OGTT-derived insulin sensitivity data.

3. Report linkage disequilibrium between the two SNPs

   We now report linkage data in the Methods section.

4. The decision to analyze rs6232 under a dominant model seems arbitrary, as a low minor allele frequency does not influence the mode of risk transmission. An additive model should be provided, and the use of a dominant model justified on biological/genetic rather than statistical grounds

   We now report additive and dominant models for both SNPs.

5. Discussion, 2nd paragraph: please change the first sentence to reflect that alleles (and not SNPs) are associated with higher stimulated proinsulin levels, as you correctly do in the rest of the manuscript

   Thank you for this hint, we changed the sentence accordingly.

Discretionary revisions:
The authors might consider including a figure plotting insulin secretion vs proinsulin levels by genotype group, to indicate whether the presence of the risk allele impairs the ability of beta cells to secrete mature insulin (see Ingelsson et al. Diabetes 2010 online, Fig. 1 as an illustration).

   Thank you for this suggestion. We prepared according figures and additionally plotted AUC Insulin against AUC Proinsulin. These figures are attached to this revision. Since no SNP effects are visible in this analysis, probably due to the lack
of these SNP’s association with insulin secretion, we would prefer not to include these figures in the manuscript.

**Reviewer 2**

Major revisions
- It is not stated whether one or two-sided tests were used for the linear regression, only that one-sided tests were used for the power calculations. Two-sided tests should be used for all calculations. Power should be calculated for the statistical tests that were used or at least a close approximation. The effect sizes calculated seem to be Cohen’s d, which should be stated, and should be expressed in standard deviations.
  - We now performed power analyses using two tailed t-tests and changed the manuscript accordingly.
  - Even though we compared two genotype groups, all other analyses were performed using multiple linear regression analysis which does not allow discrimination between one- and two-sided tests.
  - The JMP software package uses raw effect sizes rather than standardized effect sizes, as required for Cohen’s d.

Also, please clarify which phenotypes were ln-transformed, in the methods part is says only the ones that were not normally distributed; in the tables it says all.
- Since none of the phenotype measurements were normally distributed, we ln-transformed all of them prior to statistical analysis. We changed this part of the methods section.

It says in the methods that the subjects were selected from a larger study. How was this selection done?
- Selection was done based on the availability of all phenotype data used for the analyses. Especially proinsulin levels for all time points during the OGTT were not available for all subjects in the Tübingen Family Study cohort. We added this information in the first paragraph of the methods section.

Minor revisions

The first sentence of the abstract gives the impression that the role of PCSK1 in neuropeptide processing is the main focus of the manuscript.
- Thank you for this hint, we changed “neuropeptide processing” to “peptide processing” in the first sentence of the abstract.

- “First-phase insulin was determined from the OGTT as described earlier [19] by calculating $1283 + 1.829 \times \text{Ins30} - 138.7 \times \text{Glc30} + 3.772 \times \text{Ins0}$.” Please cite the original article instead.
  - We now cite the original article (Stumvoll et al.).
“Insulin sensitivity during the OGTT was estimated as proposed by Matsuda and DeFronzo [20].” Please, write the full formula.

We now report the full formula in the methods section.

-“The similar findings with two different measurements of insulin sensitivity as well as the inverse influence on 120-min blood glucose argue against a mere statistical type 1 error.” These are related phenotypes and not independent evidence.

Since we now find significant associations with two independent measures for insulin sensitivity, i.e., insulin sensitivity derived from the OGTT and insulin sensitivity derived from hyperinsulinemic euglycemic clamp, we are pretty sure that these findings are not mere type 1 errors, however we agree that the association with 120-min blood glucose may be a logical consequence of the SNP’s effect on insulin sensitivity and thus might not be an independent phenotype.

**Reviewer 3**

The manuscript addresses an interesting question and extends previous work by investigating measures of proinsulin secretion/ conversion – obvious outcomes of interest when studying genetic variation in PCSK1. My main comments relate to the design of the study and choice of parameters used to assess and compare associations with the different outcomes.

Study design/ power: As the authors acknowledge themselves, this study is underpowered. Of all common genetic loci identified to be associated with obesity levels to date, FTO is by far the strongest – according to the authors’ calculations the current study is not even adequately powered to detect effects of FTO, let alone any variants with weaker effects, as would be expected for the two SNPs in question. Effect sizes for BMI and waist do actually appear rather large (e.g. table 2), despite the lack of statistical significance, this should be acknowledged. The absence of significant associations does therefore not allow any inference about a potential lack of effects of these PCSK1 variants, an important limitation.

We agree with the reviewer, that with our sample size, the absence of significant associations with obesity does not allow any inference about a potential lack of
effects of these 

In general, due to the on average small effects sizes of individual genetic variants influencing complex traits and lack of power of single studies, the past few years have seen growing efforts of different research groups to join forces in order to increase power and minimise false negative reports. I would encourage the authors to consider this option given the significant lack of power of the present study. Likewise, I do not see why the current study needs to end with the conclusion that further replication is needed. This should be actively pursued in the current study.

It was not the aim of this study to confirm previously reported associations of the SNPs with obesity. To replicate the obesity effects, we agree with the reviewer that joining larger consortia would have been very helpful. Since this was not our primary aim, we now weakened the negative finding on associations with obesity. Instead, our hypothesis was an implication of the SNP’s in beta-cell functions due to the biological role of this gene’s product in insulin maturation. In this regard, our study appears sufficiently powered to show effects of the SNPs on insulin maturation. Of course, replication of this finding is required, but we are not aware of other cohorts with proinsulin measurements every 30 minutes during the OGTT.

Effect sizes versus significance: Throughout the report, the authors focus on statistical significance instead of effect sizes and directions, a shortcoming particularly when considering the limited power of this study. It would be preferable to compare the effect sizes observed in the present study to those from earlier reports and to consistently describe and discuss effects on the different traits for the obesity “risk” alleles.

Since the study was underpowered to detect associations with obesity, we would prefer not to discuss these data in detail in the manuscript. We now address the low power in the limitations section. Furthermore, we now report the effect sizes of our significant results.

Minor comments:
Could the authors please state why one tailed t-tests were used?
We now performed power analyses using two tailed t-tests and changed this part in the methods section of the manuscript accordingly. All other analyses were performed using multiple linear regression analysis which does not allow discrimination between one- and two-sided tests.
Associate Editor

The reviewers addressed important concerns which, if addressed, will strengthen the paper. Also, authors should be cautious about possible overinterpretation of association findings of PCSK1 variants with increased insulin sensitivity. In this respect, authors are strongly encouraged to provide the data on insulin sensitivity assessed during the euglycemic hyperinsulinemic clamp.

We now additionally report euglycemic-hyperinsulinemic clamp derived insulin sensitivity. These data confirmed our data from the OGTT.