Author's response to reviews

Title: Genetic analyses of bone morphogenetic protein 2, 4 and 7 in congenital combined pituitary hormone deficiency

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Author's response to reviews: see over
Dear Dr Koltowska-Haggstrom,

Thank you very much for the helpful comments. We carefully considered the concerns of the reviewers and revised our manuscript accordingly. We hope we satisfactorily responded to all critical comments and the manuscript will be suitable for publication in BMC Endocrine Disorders.

Please find attached a copy of our revised manuscript “Genetic analyses of bone morphogenetic protein 2, 4 and 7 in congenital combined pituitary hormone deficiency”. We have highlighted all changes and new material in our paper.

The material is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration.

Thank you for considering this manuscript.

Sincerely,

Anke Tönjes, M.D.
Reviewer: Daniel Kelberman

1. The major concern relates to the nomenclature used to describe the variants which is of paramount importance to avoid any confusion as to the specific variant bases and amino acid changes. The authors have stated Ensembl gene accession numbers for each of the three genes, however BMP7 in particular has multiple transcripts and it is unclear as to which the stated variants c.611+3366C>T and c.959-2T>C are referring to. The numbering used should be clearly defined for each variant relative to the accession number for a given transcript so there is no ambiguity. The latter change in particular should be checked carefully as it appears to this reviewer that base c.959 is the first base of exon 5, therefore the variant c.959-2 should refer to the invariant AG splice acceptor site preceding this exon. Seeing as any variation at this position is likely to affect splicing these details need to be checked and clarified. I would recommend checking the naming of all variants with the Mutalyzer program. On a similar note, all amino acid variation should be prefixed "p." in the manuscript.

We completely agree with the reviewer. Since each of the investigated genes includes several splice variants we indicated the transcript the variants refer to. To clearly notify this for the reader we now added the transcript IDs and accession numbers of the protein in Table 1. We also prefix all amino acid variation with “p.” in the revised manuscript.

With regard to the variant c.959-2T>C, the reviewer is absolutely right, the second base preceding exon 5 of the transcript ENST00000395863 (coding for longest protein biotype) is located in a position likely to affect splicing. Unfortunately, none of the used programs (Mutpred, SNPs&GO, mutation taster, fathmm) allowed to predict the functional consequences of this variant (please see new table 3).

In course of the revision of the manuscript all results of sequencing were re-assessed by an independent person. Three results required further validation by re-sequencing. The potential splice site mutation c.959-2T>C was not proved and is deleted in the revised manuscript.

2. The authors acknowledge the limitations of bioinformatic prediction tools, however there are a wealth of alternative programs that assess other aspects of genetic variants beyond sequence conservation. I would recommend, in the absence of any experimental data, using a variety of different programs available to try to assess pathogenicity (eg. Mutpred, SNPs&GO, mutation taster, fathmm) to obtain a consensus from multiple different models. It would also be good to provide parental genotype information, but I appreciate this is not always possible.

Unfortunately, we do not have any parental information available. Given the retrospective character of the study we are not able to access further data for most of the patients. We state this issue as limitation of the study in the manuscript (Discussion; page 8).

However, as suggested by the reviewer, we assessed potential functional consequences of all variants predicting amino acid exchange by employing several programs publicly available programs such as Mutpred, SNPs&GO, mutation taster and fathmm. These results are now included in the results and discussion section of the revised manuscript and are summarized in an additional table (Table 3).

1. The PCR primers used for screening should be made available in the manuscript or supplementary information rather than being available "on request."
We thank the reviewer for this suggestion and now provide the PCR primers and conditions in a supplementary table (additional information 1).

2. It would be interesting if the authors included information on the genetic variation identified in the patients in the screening of other genes listed in the Additional information. The authors state this analysis did not reveal any aberrant results but it would be good to include in the table the variation that was observed.

Sequencing was performed by a third party. Results are available there and the complete list of variants within all genes screened will be made available on request for each patient.

3. Page 5, the term "infinite omega" with regard to PAML analyses should be defined.

Omega is defined as $\omega = \frac{dN}{dS}$ (nonsynonymous changes divided by synonymous changes). The absence of synonymous changes in the data leads to the infinite omega, as there is a positive number divided by zero. A likelihood ratio test (LRT) against the model with the average omega reveals that $P<0.005$, underlining that we have a strongly conserved gene.

**Reviewer 2: Frederic Castinetti**

- to perform additional TF gene sequencing in the patients with BMP's SNP (to be sure that they are not carrying a mutation of a known TF gene)

We agree with the reviewer that we cannot exclude that variation in other transcription factors contributes to the phenotype. Therefore, we screened the patients for mutations in several well established players in the control of pituitary function such as PIT1, PROP1, HESX1, LHX3 (and partially LHX4, Gli2, OTX2, SOX2). However, even though well aware that the list of these genes might be much longer, we need to acknowledge that additional gene sequencing would be beyond the scope of this manuscript.

- to give additional data about family (and ideally perform sequencing)

We completely agree but unfortunately, we do not have any parental information available. Given the retrospective character of the study we are not able to access further data for most of the patients. We state this issue as limitation of the study in the manuscript (Discussion; page 8).

- to give more insights on the way BMP4 mutations would lead to a pituitary phenotype without severe organ defects taking into account their early roles in brain/body development (unless there might be a way, maybe by collaboration with another group, to perform functional studies.) To summarize, if the idea is original, the research part in this paper might be too preliminary to lead to a publication currently. Moreover, to my knowledge, BMPs are not pituitary specific, and this makes me skeptical about the possibility of having isolated pituitary phenotype with such mutations (if they are real mutations)

There is a substantial variability in the clinical presentation of patients with combined pituitary hormone deficiency even if the same gene is affected and even in subjects with identical mutations. Intrafamilial penetrance can range from high to incomplete and it is not possible to draw direct conclusions form the clinical manifestation to the potential genotype. This indicates the remarkable
influence of the genetic background, incomplete penetrance, highly variable expressivity, environmental factors and possibly stochastic events. Also co-occurring mutations in interacting genes have to be taken into account. (e.g. Pfaffle R & Klammt J. Pituitary transcription factors in the aetiology of combined pituitary hormone deficiency. Best Pract Res Clin Endocrinol Metab. 2011;25(1):43-60).

This issue is now discussed on page 8 in the revised manuscript.

A few points to consider more in detail as major revisions

1. It is hard to imagine that BMP2 mutations leading to heart defects and very early death in mice could lead to an isolated phenotype of CPHD in Humans. Are there any known mutations of BMP2 lading to isolated organs dysfunction in Humans?

There is a recent report of involvement of BMP2 in syndromic forms of cleft palate (Williams et al. Am J Med Genet A. 2012;158A:2616-20. Cleft palate in a multigenerational family with a microdeletion of 20p12.3 involving BMP2). However, to our knowledge there has not been performed a systematic screening for variation in BMP2 in subjects with pituitary malformation.

2. How about phenotyping PROP1 and POU1F1 in the 19th patient?

We completed genetic screening also for the 19th subjects. In PIT1, PROP1, HESX1 and LHX3 we did not detect any relevant mutation. We added this information in Table 1.

3. Can the authors predict what would be the consequence of BMP4 mutation? It is a highly conserved sequence, but is it located in a functional domain, a DNA interaction zone…

As indicated in the manuscript, PAML analyses showed an overall strong conservation of the gene. Positional analyses further indicated that most positions are conserved or strongly conserved. Position number 300 is highly conserved (page 5, results for BMP4 analysis). According to www.uniprot.org the variant is located within the protein chain, but more detailed information is not available yet. As a matter of fact, we are currently conducting functional studies to investigate the consequences of this variant in more detail.

4. Ok to consider that bmp4 mutation could induce the skeletal phenotype. For this reviewer, this is clearly not an evidence for the role of BMP4 mutation in pituitary phenotype.

We thank the reviewer for raising this point. However, there is a substantial variability in the clinical presentation of patients with combined pituitary hormone deficiency even if the same gene is affected. This indicates the influence of the genetic background, incomplete penetrance, environmental factors and possibly stochastic events.

5. Patient 5 (who seems to be the more interesting) could take benefit from sequencing of other genes coding for TF, taking into account the non specific pituitary phenotype (before considering that BMP4 mutation could lead to the pituitary phenotype)

The reviewer is right and we completely agree that other genes could be involved in the phenotype as well. We therefore performed a mutation screening in genes frequently associated
with abnormalities of pituitary function (PIT1, PROP1, HESX-1). We are aware that by including only a few genes the data remain inconclusive. However, we believe that even by extending the list of studied genes by further candidates there would be no guarantee that further players will be identified. Thus, a systematic approach including whole genome/exome sequencing strategies would be desirable here, which however, would definitely go beyond the current scope of our manuscript.

This issue is now discussed on page 8 of the revised manuscript.

6. I might have missed it in the paper... Was the BMP4 SNP hetero or homozygous?

The BMP4 variant c.899G>C was heterozygous in one of the investigated subjects (patient 5). This point is now clearly stated in the results section of the revised manuscript (page 5).

Minor revision
Individual data are needed in the manuscript, and not as an additional table. Another possibility could be to include a summarized table with only the patients with BMP SNPs.

We now provide the table including individual data in the manuscript (Table 1).