nature portfolio

Peer Review File

QSOX2 Deficiency-induced short stature, gastrointestinal dysmotility and immune dysfunction

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is a very interesting article about a potential new growth disorder due to recessive variants in QSOX2. The mechanistic data is beautiful, and the authors have put together a nice biological story about the effects of QSOX2 deficiency. This is totally novel information. The human genetic data is much weaker. It is definitely possible that these patients are on a clinical spectrum due to QSOX2 deficiency, but it is hard to be 100% certain about this.

The two families presented clearly have overlapping features but a somewhat distinct clinical and biochemical presentation. It is interesting to note that 2 additional probands have been identified with recessive variants in QSOX2 (lines 186-188). It would be great if those clinical data could be included in this manuscript as it could give us a better picture of the clinical spectrum. However, if this data is not going to be included in the manuscript, I think you should delete these lines as we have no basis to evaluate that evidence. If those patients clinical and biochemical data matched the first family, it would greatly strengthen the argument.

The lack of biochemical data on P3 and P4 is a shame. It would be very helpful to at least get an IGF-1 level on these two individuals.

The human genetic evidence supporting the pathogenicity of the T352M variant is not strong. There was no effect of this variant in the UK Biobank in an additive model and the 31 homozygotes did not show a clear phenotype. This means that this variant must have a minimal effect on function. Therefore, it is hard to justify how this variant is causing such a severe phenotype in Family 1, even in trans with a frameshift variant. You try to address this with splicing hypothesis, but do you have any evidence to show that this correlates with height in the actual human carriers?

I think the response to growth hormone is relatively good. This along with the normalization of the IGF-1 makes it more difficult to understand this as a growth hormone insensitivity situation as one would expect based on the proposed mechanism.

Reviewer #2 (Remarks to the Author):

Maharaj et al describe 4 cases from 2 families in whom biallelic variants in QSOX2 are associated with short stature, gastrointestinal dysmotility and immune dysfunction. P1 and P2 from family 1 are identical twins, and display a consistent phenotype. P3 from family 2 is homozygous for a single amino acid deletion variant, which her father (P4) is heterozygous for, but also carries a de novo QSOX2 missense variant which may account for his phenotype. The authors go on to show that

QSOX2 variants impair nuclear translocation of STAT5B, a transcription factor responsible for transcription of growth associated genes such as IGF1. Of note, STAT5B mutations are associated with a similar growth disorder described herein. The authors show patient-derived cells treated with recombinant IGF-1 may overcome the observed STAT5B defects. The authors also show defects in mitochondrial morphology and electron transport chain function.

The manuscript is generally well written and describes a novel cause of post-natal growth failure with sufficient evidence to show causality of most of the variants identified, with mechanistic insight. However, I feel there are aspects of the manuscript that would benefit from further detail, clarification or description.

N.B. It would be useful if the authors could include page and line numbers to assist with the review process.

Results

1. Index family 1, paragraph 2 - "the disparate peak GH response in P1 may be explained by significant technical difficulties". The GH-provocation testing data isn't provided so it's difficult to make any sense of this. It would also be useful to know what the technical difficulties were and why the authors feel these contributed to the results observed.

2. Index family 1, para. 3 - "Intriguingly, the top candidate variants were...". How did the authors prioritise variants? What made these variants top of that list. Were any other biallelic variants in the list that could be considered?

3. Index family 2, para.3 - the authors identified an additional de novo variant in P4 (father of P3), in addition to him being heterozygous for the p.F474del variant that is homozygous in P3. Very little additional information is provided for P4 other than his short stature. Does P4 have the gastrointestinal dysmotility and immune dysfunction aspects of the phenotype too? These are very briefly in Table 1, but without much detail. Do the authors know whether the variants are in cis or trans? Do the authors have any additional functional data to implicate the de novo p.D574Y variant as pathogenic? This variant is not included in any of the functional studies, and the authors only state that SIFT and PolyPhen2 predicts it to be pathogenic - does this imply other software does not predict it to be pathogenic?

4. Index family 2, para.4 - "Of note, 2 additional probands with recessively inherited variants in QSOX2...". Do the authors intend to add these to this manuscript following peer review? If not, then I

don't feel this sentence adds anything to the manuscript and should be removed - unless there is further evidence added to support pathogenicity of said variants.

5. The UK biobank analysis is intriguing - particular that it reveals the p.T352M found in P1/P2 is found in 31 individuals as a homozygous variant, all of whom had adult heights within the normal range. A subsequent analysis of the Finnish population identified the variant in 15 individuals as homozygous, some of whom showed a slight reduction in height (range -0.1 to -2.5 st. dev.). The authors suggest this may be due to altered splicing caused by the variant and show an additional splicing product by in vitro splicing assays, which would under NMD. However, all functional assays appear to show the T352M variant has a similar effect on QSOX2 function as the V325Wfs*26 variant. Either way, it's not clear to me why individuals homozygous for the variant don't have the same phenotype as P1-P3. If P4 only shows a short-stature phenotype does this imply missense variants give a milder phenotype? I think this aspect of the manuscript needs clarifying and discussing further.

6. Have the authors ruled out a larger deletion/structural variant/ CNV in P1/P2 to add further evidence that the p.T352M variant is the only other potentially pathogenic variant?

Discussion

7. para.2 - the authors provide coordinates of the polymorphisms investigated, but they don't provide which genome build these map to. Please could the genome build be provided.

Methods

8. Protein Structure Modelling - please provide the version number for PyMOL (currently listed as X.X)

9. There are no methods for the exome/genome sequencing - was this done by the authors for P1/P2 or was this undertaken elsewhere. Either way, additional information should be provided on how this was generated and analysed in order to reach the conclusion that the QSOX2 variants are the most likely cause of disease.

Figures

10. Fig 2 & Fig 4 - the scale bars in the IF panels are very difficult to see and there's no indication in the legend what they represent. Please could the scale be made clearer in these images.

Reviewer #3 (Remarks to the Author):

In a highly interesting manuscript, the authors describe in two pedigrees and 4 patients a novel human disease, autosomal recessive QSOX2 deficiency, which leads to GHI, low IGF-1 and prominent immune/ gastrointestinal dysregulation. Thus, this will highly likely be coined an inborn error of immunity as well. While the focus of the manuscript is on the effect of described variants to growth/IGF-1 metabolism and to the hitherto unrecognized interaction between QSOX2 and STAT5B as well as to QSOX2's novel role as a gatekeeper for regulation of import into nucleus of pSTAT5B, as a regulator of mitochondrial integrity and energy metabolism, and in causing a GH-induced mitochondriopathy, it rather superfluously discusses its effects on immunity. While the main findings seem sound (and its findings in FINRISK cohort and UKBB further add to the weight of evidence), I have improvements to suggest that would make the discussion and etiopathogenesis of the described immune dysregulation more balanced and sounder. Report is however generally well written.

In abstract you state "a definitive role of QSOX2 in modulating human growth likely due to impairment of STAT5B downstream activity and mitochondrial dynamics leading to growth failure, immune dysregulation and gut dysfunction". However, I find no direct proof that mitochondrial dysfunction would be involved in the etiopathogenesis of patients' immune dysregulation? While thus this may occur, due to the high energy consumption by immune responses, you should revise the sentence?

Autosomal recessive and autosomal dominant/dominant negative STAT5B deficiencies cause increased IgE levels. However, a true hyper-IgE phenotype is missing in patients, see https://doi.org/10.1007/s10875-022-01289-3, please use clearly increased IgE levels instead to avoid confusion. Above-mentioned inborn errors recapitulate not only the GH insensitive growth failure, but also atopic eczema, and AR STAT5B deficiency also the immune defects with autoimmunity (e.g. lymphocytic interstitial pneumonitis, other organ-specific autoimmunity). These disease should be mentioned in the introduction more clearly and comprehensively. Intriguingly, also somatic STAT5B GOF cause HES with eosinophilia and atopic dermatitis. Thus, clearly many of the described immune features have to do with STAT5B downstream effects, this should be clarified and deepened in discussion (and further strengthens the proposed mechanisms in QSOX2 deficiency).

Also, in Table 1, you give IgE levels in kU/L, while more conventionally values are given either in IU/mL or kU/mL, please revise to avoid confusion.

Of course, since this is clearly an inborn error of immunity, the article would be further strengthened by data explaining more deeply the etiopathogenesis of QSOX2's one main feature, immune dysregulation as well. The somewhat curious combination of dysmotility and gastrointestinal inflammatory problems (usually leading to diarrhea) described in QSOX2 deficient patients clinically resembles those described in APECED patients (see for example, doi: 10.3389/fimmu.2023.1172369), with dysregulated IFN responses. As is known, anti-IFN antibodies are common in APECED (against type I +/- type III), and there is data emerging, mostly still only available as meeting abstracts, that describe the key role of excessive IFN-gamma production (type II) in its etiopathogenesis and consequent treatment responses). Similar GI phenotype plus further lichen planus (please clarify if it affected skin and/or mucosal membranes) in P3 suggests that maybe IFN-gamma production could be increased in these patients? Slow transit constipation is associated with increased IFN-gamma signature, in various disease states.

Finding the etiopathogenesis of patients' immune dysregulation could open avenues to treat these patients (e.g., JAKinibs). IFN signature one could study rather easily by using Nanostring assay in blood and/or patients´ fibroblasts (doi: 10.1089/jir.2017.0127.), which clearly are available. Clearly, one internationally likely finds many similar patients in the future.

Rebuttal: NCOMMS-23-41144-T for Nature Communications

The authors are very grateful for the reviewers' feedback and have addressed each of the reviewer's comments in detail below. The changes to the manuscript are highlighted in the revised version.

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It would be great if those clinical data could be included in this manuscript as it could give us a better picture of the clinical spectrum. However, if this data is not going to be included in the manuscript, I think you should delete these lines as we have no basis to evaluate that evidence. If those patients clinical and biochemical data matched the first family, it would greatly strengthen the argument.

We'd like to thank Reviewer 1 for their comments and suggesting further evaluation of additional probands with new genomic variants. Of the two additional probands, we were only able to successfully consent and analyse one kindred. However, this expands our cohort to 5 affected individuals from 3 families. Phenotypic characterisation of Proband 5 is discussed in **lines 162-179 and Table 1**:

> "Proband P5, a British Caucasian male (**Figure 1A**) was enrolled in the U.K. 100,000 Genomes Project at 4.8 years with postnatal growth restriction, failure to thrive and motor developmental delay. He was born appropriate for gestational age and demonstrated early postnatal growth retardation associated with poor feeding and frequent infections in the neonatal period. The patient presented at the age of 2.5 years with delayed fine and gross motor development, dystonic posturing, eczema, hyper-pigmented skin macules, short stature and gastro-oesophageal reflux (**Table 1**). Biochemically, the proband demonstrated features of primary IGF-1 deficiency (growth hormone insensitivity) with an IGF-1 SDS of -2.0 associated with an adequate GH peak of 11 µg/L on provocation testing (**Table 1**). Interferon (IFN) gene profiling revealed no evidence of interferonopathy but IFI27 levels were elevated. (**Table 1**).
> Interrogation of the 100,000 Genomes Project rare disease cohort revealed that P5 harboured bi-allelic compound heterozygous variants in *QSOX2*; A paternally inherited missense variant, (*c.2048G>A*, p.R683Q) with a MAF of 0.000003989 (gnomAD; no homozygotes) and predicted deleterious by several computational platforms (SIFT, PolyPhen-2 and CADD) and; a maternally inherited single amino acid substitution, (*c.881A>G*, p.K294R) with a MAF of 0.00001971 (gnomAD; no homozygotes), predicted damaging by CADD and Mutation taster but tolerated by SIFT and PolyPhen-2".

In vitro characterisation of these variants revealed findings that were concordant with the original variants (**Supplementary Figure 3 and lines 272-277**).

> "compound heterozygous variants in proband 5 showed reduced expression upon immunoblotting and immunofluorescence, when compared to wild-type (WT) QSOX2 (**Supplementary Figure 3 A, B**). Similar to other characterised variants, STAT5 phosphorylation was unaltered in response to GH but nuclear localisation was attenuated and mutant interaction with WT-STAT5B was markedly reduced (**Supplementary Figure 3C-F**)".

2. The lack of biochemical data on P3 and P4 is a shame. It would be very helpful to at least get an IGF-1 level on these two individuals.

We concur with Reviewer 1's statement. However, both probands from Kindred 2 declined biochemical testing, consenting only for DNA extraction and analysis. However, Proband 5, the newest patient included in the cohort, demonstrated biochemical features of primary IGF-1 deficiency (growth hormone insensitivity) with an IGF-1 SDS of -2.0 associated with an adequate GH peak of 11 µg/L on provocation testing (**lines 168-170**, **Table 1**).

3. The human genetic evidence supporting the pathogenicity of the T352M variant is not strong. There was no effect of this variant in the UK Biobank in an additive model and the 31 homozygotes did not show a clear phenotype. This means that this variant must have a minimal effect on function. Therefore, it is hard to justify how this variant is causing such a severe phenotype in Family 1, even in trans with a frameshift variant. You try to address this with splicing hypothesis, but do you have any evidence to show that this correlates with height in the actual human carriers?

We acknowledge the validity of Reviewer 1's concerns and have approached this via a multi-pronged strategy.

We demonstrated *in vitro,* an impact of this variant on splicing:

Results (lines 207-218): "*In vitro* splicing assays (**Supplementary Figure 1C**) revealed the presence of two transcripts (**Supplementary Figure 1D**) for the homozygous p.T352M variant, one consistent with unaltered splicing (489bp) and a smaller transcript demonstrating exon 8 skipping (359bp) (**Supplementary Figure 1E**). This aberrantly spliced transcript, which likely occurs due to naturally weak canonical splice sites, is predicted to result in a frameshift p.N319Kfs*51, possibly undergoing degradation by nonsense mediated mRNA decay. Notably, in patient (P2) fibroblasts which harbour this variant in heterozygosity, RT-PCR using coding primers spanning exons 7-9 of *QSOX2* revealed the presence of two transcripts, one consistent with wild type product (320bp) and a minor, smaller transcript, (190bp) consistent with the skipping of exon 8 (130bp) (**Supplementary Figure 1E**). These observations support low occurrence of abnormal splicing events due to SNP rs61744120 (*QSOX2 c.1055C>T* variant)".

The security of an *in vitro* splicing assay engenders reproducibility of a mis-splicing event, which in this case was complete exon skipping and generation of an alternate transcript. However, *in vivo*, background genetic heterogeneity and population level variability may render this impact highly unpredictable. Indeed, mistranslation of the p.T352M protein in humans may produce unintended

consequences, where identical genotypes may produce phenotypic variance even at tissue level due to stochastic gene expression (**see Discussion lines 344-350**).

Given the pleiotropic nature of this disorder, phenotypic variability was inevitably anticipated. The degree of short stature appeared most pronounced in compound heterozygotes (4/5 probands) when compared to the lone simple recessive homozygote (P3) within our cohort. Since the impact of p.T352M homozygosity on height is highly variable, it seemed possible that phenotypic discordance may be due to interallellic complementation[31,32]. The QSOX2 protein may have altered functionality or negative interaction between two distinct mutants *in trans* as opposed to identical mutant subunits, which may have the unintended consequence of partial phenotypic rescue or positive complementation; i.e. the heteromultimer is less functionally active than the homomultimer. **This has been further discussed in the manuscript (Lines 351-359)**.

Protein modelling:

QSOX2 was modelled using the IntFOLD7 and MultiFOLD servers and protein-ligand interactions were modelled using the IntFOLD7 server. The PINOT server was used to verify interaction partners of QSOX2 and the DISOPRED3 server was used to predict disordered and protein-binding regions. The p.T352M variant occurs within the active FAD/sulfhydryl oxidase domain and STAT5B was verified to be an interacting partner of QSOX2 via this active domain. This variant is liable to produce local folding changes inside the sulfhydryl oxidase interacting domain, which may indirectly attenuate its interactivity with STAT5B (**Lines 280-299** and **Supplementary Figure 4**).

4. I think the response to growth hormone is relatively good. This along with the normalization of the IGF-1 makes it more difficult to understand this as a growth hormone insensitivity situation as one would expect based on the proposed mechanism.

The spectrum of growth hormone insensitivity (GHI) is very wide, ranging from "classical" (this appears to be the focus of Reviewer 1's comments) to atypical (such as our cases). Indeed, clinically, we see more "atypical" GHI than classical cases. Interestingly, the response to rhGH therapy is also unpredictable and even in cases of severe GHI, falls on a continuum.

Partial GH insensitivity and responsiveness to GH therapy is well-established. Furthermore, biochemical indices (IGF-I, GH and IGFBP3) can be very variable. The senior authors have published several reviews discussing this very topic (including Storr *et. al*., 2019 - Endocrine Reviews).

We now report probands with milder phenotypes akin to dominant negative STAT5B heterozygotes but associated with a novel regulatory interactor of STAT5B which, when absent, blunts STAT5B-mediated regulation of IGF-1 expression by impairing STAT5B nuclear translocation. Although, there is clear evidence of improved growth trajectory in probands 1 and 2 following commencement of early hGH therapy (Figure 1B), the catch-up growth is modest compared to children treated with hGH for GH deficiency (Bang *et al*., 2011), suggesting a degree of GH resistance consistent witht the biochemical phenotype. The target height centile for the twins is the 50th (0.02 SDS) and despite >2 years of therapy they are still growing below the 2nd centile. **This growth pattern is typical of children with a disorder with the GH resistance spectrum.**

Reviewer #2 (Remarks to the Author):

Maharaj et al describe 4 cases from 2 families in whom biallelic variants in QSOX2 are associated with short stature, gastrointestinal dysmotility and immune dysfunction. P1 and P2 from family 1 are identical twins, and display a consistent phenotype. P3 from family 2 is homozygous for a single amino acid deletion variant, which her father (P4) is heterozygous for, but also carries a de novo QSOX2 missense variant which may account for his phenotype. The authors go on to show that QSOX2 variants impair nuclear translocation of STAT5B, a transcription factor responsible for transcription of growth associated genes such as IGF1. Of note, STAT5B mutations are associated with a similar growth disorder described herein. The authors show patient-derived cells treated with recombinant IGF-1 may overcome the observed STAT5B defects. The authors also show defects in mitochondrial morphology and electron transport chain function.

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N.B. It would be useful if the authors could include page and line numbers to assist with the review process.

We would like to thank Reviewer 2 for this comment – we have now inserted line numbers throughout the manuscript.

Results

1. Index family 1, paragraph 2 - "the disparate peak GH response in P1 may be explained by significant technical difficulties". The GH-provocation testing data isn't provided so it's difficult to make any sense of this. It would also be useful to know what the technical difficulties were and why the authors feel these contributed to the results observed.

Thank you for highlighting this. There were several issues with GH-provocation testing (using glucagon stimulation). Mostly due to multiple attempts at cannulation prior to stimulation. Proband 1 (P1) became hypoglycaemic early on, with a nadir blood glucose of <3.3mmol/L necessitating abandoning testing before a 'potential peak GH level' was attained. Typical for young children with severe GHD or GH insensitivity, there was a history of recurrent spontaneous hypoglycaemia in both probands 1 and 2. This may have led to a degree of desensitisation and an inability to mount a prodigious GH response to hypoglycaemia (Kelly *et al.,* 2008). Furthermore, the test was only performed once (given the age and distress incurred to the patient) and multiple sources of evidence show poor sensitivity/specificity of single provocation studies and variable responses depending on the stimulus used (Kelly *et al*., 2008, Chesover *et al*., 2016). Further details have been added to **Table 1's legend** (and referred to in the manuscript – **line 99**).

2. Index family 1, para. 3 - "Intriguingly, the top candidate variants were...". How did the authors prioritise variants? What made these variants top of that list. Were any other biallelic variants in the list that could be considered?

Whole exome sequencing of Probands 1 and 2 was conducted by the Otogenetics Corporation using an Illumina HiSeq 2500 platform. Downstream analysis was conducted using Ingenuity variant analysis (https://variants.ingenuity.com/qci/). Variants with a call quality ≥ 20 were retained whilst common

variants with an allele frequency ≥ 0.5% in databases such as gnomAD, ExAC, NHLBI ESP and 1000 genomes project were excluded unless designated as known disease-causing variants. Deep intronic variants (>20bp into the intron), and those predicted to be pathogenic, likely pathogenic or variants of uncertain significance as computed by ACMG guidelines were kept. Variants associated with gain of function as well as loss of function frameshift, indel, missense, nullizygous, splice site (up to 20bases into intron), copy number loss and deleterious to a microRNA were retained. Variants that were homozygous, heterozygous, heterozygous_ambiguous and homozygous in both probands were prioritised (This pipeline has been included in the **Materials and methods section, lines 476-489**).

Using a recessive disease model the top candidates were the compound heterozygous variants found in QSOX2. Additional variants of interest included (details summarised in **Supplementary Table 4**):

1. Heterozygous *PNPO* (*c.98A>T*, p.D33V) missense variant, MAF 0.0185% gnomAD (European) with a CADD score of 32 and predicted damaging by SIFT and Polyphen-2. Variants in *PNPO* are associated with pyridoxamine 5-prime-phophate oxidase deficiency, an autosomal recessive disease-causing epileptic encephalopathy. Both probands were heterozygous for this variant **(heterozygosity does not confer a disease phenotype**) and their phenotype was not consistent with this disorder.

2. Heterozygous *NPC1* (*c.3019C>G*, p.P1007A) missense variant, MAF 0.0194% gnomAD (European) with a CADD score of 26.3 and predicted damaging by SIFT and Polyphen-2. Recessively inherited variants in *NPC1* are associated with Niemann-Pick Type C disease. Similar to the *PNPO* variant detected, zygosity and phenotype of the probands were incongruous with this disorder **(heterozygosity does not confer a disease phenotype**).

3. Index family 2, para.3 - the authors identified an additional *de novo* variant in P4 (father of P3), in addition to him being heterozygous for the p.F474del variant that is homozygous in P3. Very little additional information is provided for P4 other than his short stature. Does P4 have the gastrointestinal dysmotility and immune dysfunction aspects of the phenotype too? These are very briefly in Table 1, but without much detail. Do the authors know whether the variants are in cis or trans? Do the authors have any additional functional data to implicate the de novo p.D574Y variant as pathogenic? This variant is not included in any of the functional studies, and the authors only state that SIFT and PolyPhen2 predicts it to be pathogenic - does this imply other software does not predict it to be pathogenic?

Thank you for highlighting this. The variants p.F474del and p.D574Y likely occur *in trans* in Proband 4, given the absence of the p.D574Y variant in Proband 3 (daughter of Proband 4, homozygous for p.F474del variant) and the consanguineous spouse of Proband 4 (heterozygous for p.F474del variant). We have added more detail to the manuscript and conducted functional characterisation of the p.D574Y variant *in vitro* (**Results section; Lines 272-277 and Supplementary Figure 3**):

"The missense variant (p.D574Y) occurring in trans with p.P474del, and the compound heterozygous variants in proband 5 showed reduced expression upon immunoblotting and immunofluorescence, when compared to wild-type (WT) QSOX2 (**Supplementary Figure 3 A and B**). Similar to other characterised variants, STAT5 phosphorylation was unaltered in response to GH but nuclear localisation was attenuated and mutant interaction with WT-STAT5B was markedly reduced (**Supplementary Figure 3C-F**)".

Additionally, *in silico* protein modelling was undertaken. QSOX2 was modelled using the IntFOLD7 and MultiFOLD servers and protein-ligand interactions were modelled using the IntFOLD7 server. The PINOT server was used to verify interaction partners of QSOX2 and the DISOPRED3 server was used to predict disordered and protein-binding regions. The p.D574Y variant occurs within the active FAD/sulfhydryl oxidase domain and STAT5B was verified to be an interacting partner of QSOX2 via this active domain. The p.D574Y variant may affect local domain structure and/or binding of QSOX2 to STAT5B. This has been added to the manuscript (**Results section, lines 280-299**). This was demonstrated by the markedly reduced interaction of this variant with WT-STAT5B on nanoluciferase complementation assays (**Supplementary Figure 3F**).

4. Index family 2, para.4 - "Of note, 2 additional probands with recessively inherited variants in QSOX2...". Do the authors intend to add these to this manuscript following peer review? If not, then I don't feel this sentence adds anything to the manuscript and should be removed - unless there is further evidence added to support pathogenicity of said variants.

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"Proband P5, a British Caucasian male (**Figure 1A**) was enrolled in the U.K. 100,000 Genomes Project at 4.8 years with postnatal growth restriction, failure to thrive and motor developmental delay. He was born appropriate for gestational age and demonstrated early postnatal growth retardation associated with poor feeding and frequent infections in the neonatal period. The patient presented at the age of 2.5 years with delayed fine and gross motor development, dystonic posturing, eczema, hyper-pigmented skin macules, short stature and gastro-oesophageal reflux (**Table 1**). Biochemically, the proband demonstrated features of primary IGF-1 deficiency (growth hormone insensitivity) with an IGF-1 SDS of -2.0 associated with an adequate GH peak of 11 µg/L on provocation testing (**Table 1**). Interferon (IFN) gene profiling revealed no evidence of interferonopathy but IFI27 levels were elevated. (**Table 1**).

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In vitro characterisation of these variants revealed findings not dissimilar to previous variants as demonstrated in **Supplementary Figure 3** and **lines 272-277** of the manuscript.

"compound heterozygous variants in proband 5 showed reduced expression upon immunoblotting and immunofluorescence, when compared to wild-type (WT) QSOX2 (**Supplementary Figure 3 A, B**). Similar to other characterised variants, STAT5 phosphorylation was unaltered in response to GH but nuclear localisation was attenuated and mutant interaction with WT-STAT5B was markedly reduced (**Supplementary Figure 3C-F**)".

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6. Have the authors ruled out a larger deletion/structural variant/ CNV in P1/P2 to add further evidence that the p.T352M variant is the only other potentially pathogenic variant?

Thank you for this comment. The authors considered this as a possibility. Hence, microarray-based comparative genomic hybridisation was undertaken in both P1 and P2, which revealed no significant copy number variations. We concluded that a larger deletion/structural variant/CNV was not contributing to the phenotype of the probands/twins (**line 116- 118**).

Discussion

7. para.2 - the authors provide coordinates of the polymorphisms investigated, but they don't provide which genome build these map to. Please could the genome build be provided.

The polymorphisms mentioned map to the GRCh38 or hg38 genome build (included **in Discussion, line 337**).

Methods

8. Protein Structure Modelling - please provide the version number for PyMOL (currently listed as X.X)

This has been amended to PyMOL v2.3.3 (https://pymol.org/2/) in **Materials and methods, line 501**.

9. There are no methods for the exome/genome sequencing - was this done by the authors for P1/P2 or was this undertaken elsewhere. Either way, additional information should be provided on how this was generated and analysed in order to reach the conclusion that the QSOX2 variants are the most likely cause of disease.

This has now been addressed. Please see Reviewer 2's second comment.

Figures

10. Fig 2 & Fig 4 - the scale bars in the IF panels are very difficult to see and there's no indication in the legend what they represent. Please could the scale be made clearer in these images.

Images were obtained using the 63x oil objective of the confocal Laser scanning microscope 710. Larger versions of these images, **with clearer embedded scales** will now be made available as supplementary data.

Reviewer #3 (Remarks to the Author):

In a highly interesting manuscript, the authors describe in two pedigrees and 4 patients a novel human disease, autosomal recessive QSOX2 deficiency, which leads to GHI, low IGF-1 and prominent immune/ gastrointestinal dysregulation. Thus, this will highly likely be coined an inborn error of immunity as well. While the focus of the manuscript is on the effect of described variants to growth/IGF-1 metabolism and to the hitherto unrecognized interaction between QSOX2 and STAT5B as well as to QSOX2's novel role as a gatekeeper for regulation of import into nucleus of pSTAT5B, as a regulator of mitochondrial integrity and energy metabolism, and in causing a GH-induced mitochondriopathy, it rather superfluously discusses its effects on immunity. While the main findings seem sound (and its findings in FINRISK cohort and UKBB further add to the weight of evidence), I have improvements to suggest that would make the discussion and etiopathogenesis of the described immune dysregulation more balanced and sounder. Report is however generally well written.

Firstly, we would like to thank Reviewer 3 for the comments and would like to acknowledge that we agree with their assessment that the immune dysregulation should be discussed in further detail and this has been addressed in the revised manuscript – see below for further details.

1. In abstract you state "a definitive role of QSOX2 in modulating human growth likely due to impairment of STAT5B downstream activity and mitochondrial dynamics leading to growth failure, immune dysregulation and gut dysfunction". However, I find no direct proof that mitochondrial dysfunction would be involved in the etiopathogenesis of patients' immune dysregulation? While thus this may occur, due to the high energy consumption by immune responses, you should revise the sentence?

The authors agree and this has been revised: "Altogether, QSOX2 deficiency modulates human growth by impairing GH-STAT5B downstream activities and mitochondrial dynamics, which contribute to multi-system dysfunction."

2. Autosomal recessive and autosomal dominant/dominant negative STAT5B deficiencies cause increased IgE levels. However, a true hyper-IgE phenotype is missing in patients, see https://doi.org/10.1007/s10875-022-01289-3, please use clearly increased IgE levels instead to avoid confusion.

Agreed: We have amended to "elevated IgE levels" (**line 141**).

3. Above-mentioned inborn errors recapitulate not only the GH insensitive growth failure, but also atopic eczema, and AR STAT5B deficiency also the immune defects with autoimmunity (e.g. lymphocytic interstitial pneumonitis, other organ-specific autoimmunity). These disease should be mentioned in the introduction more clearly and comprehensively. Intriguingly, also somatic STAT5B GOF cause HES with eosinophilia and atopic dermatitis. Thus, clearly many of the described immune features have to do with STAT5B downstream effects, this should be clarified and deepened in discussion (and further strengthens the proposed mechanisms in QSOX2 deficiency).

The spectrum of immune disease secondary to STAT5B genetic variation has now been included in the introduction and discussion in lines 41-44 and 368-400 respectively.

Introduction -

"STAT5B loss of function of variants lead to immune dysregulation which exists on a continuum with features ranging from eczema, opportunistic infections, progressive immunodeficiency, autoimmunity and pulmonary compromise[4–9]".

Discussion -

"Altered STAT5B activity is ubiquitously associated with maladaptive immune signalling. Congenital dominant negative and loss of function *STAT5B* variants give rise to a wide phenotypic spectrum ranging from mild eczema to autoimmune disease that can potentially lead to fatal pulmonary fibrosis and respiratory failure[7]. Unlike endocrine profiles which show an absolute association between loss-of-function *STAT5B* variants and IGF-I deficiency, abnormalities in immunological profiles are often variable, even between siblings carrying the same pathological homozygous *STAT5B* variant[34,35]. Indeed, since many cytokines activate STAT5B, impacts on immunity are expected. For example, a syndrome of surfactant accumulation due to dysregulated GM-CSF signalling in alveolar macrophages, with features of lymphocytosis, bronchiectasis and fibrosis, was associated with a homozygous frameshift *STAT5B c.1680delG* variant[8]. Other reports of STAT5B-associated immune deficiencies due to cytokine dysregulation include pronounced T-cell lymphopenia, altered NK cell maturation, and impaired humoral immune dysregulation[17,35–38]. Intriguingly, a previously reported autosomal recessive *STAT5B c.1102insC* truncating variant[5,39] and a recently reported truncating variant, *c.1453delG*[40], were both associated with relatively normal immune profiles lacking the severe immune deficiency typically associated with loss of function *STAT5B* defects. It is of note that somatic gain of function *STAT5B* variants are associated with allergic inflammation and large granular cell leukaemia[9]. Collectively, the link between STAT5B and immune function appears inextricable, but still mechanistically not well understood.

In our cohort, growth failure is universal, and while the patients have variable presentations of altered immunity, eczema appears to be a highly penetrant feature, similar in patients with STAT5B deficiency[4]. Although the downstream impact of attenuated STAT5B nuclear localisation contributed to the phenotypes, the association of immunodeficiency and gastrointestinal dysfunction with loss of *QSOX2* prompted investigation into interferon signalling given overlap with excessive interferon states such as APECED[41]. However, an interferon (IFN) signature gene assay conducted on probands 1, 2 and 5 revealed no evidence of interferonopathy in peripheral blood samples. Interestingly, a concordant downregulation of SIGLEC1 (sialic acid binding Ig like lectin 1) was of note in P1 and P2. SIGLEC1 is a key regulator of phagocytic function and its deficiency is implicated in the pathogenesis of obstructive pulmonary disease[42]. IFI27, elevated in P5, is a pro-apoptotic protein present at the mitochondrial membrane and implicated in IFN-dependent modulation of mitochondrial permeability[43]. Further work is necessary to characterise these findings in the context of QSOX2 insufficiency".

4. Also, in Table 1, you give IgE levels in kU/L, while more conventionally values are given either in IU/mL or kU/mL, please revise to avoid confusion.

This has been amended to IU/mL (**Table 1**).

5. Of course, since this is clearly an inborn error of immunity, the article would be further strengthened by data explaining more deeply the etiopathogenesis of QSOX2's one main feature, immune dysregulation as well. The somewhat curious combination of dysmotility and gastrointestinal inflammatory problems (usually leading to diarrhea) described in QSOX2 deficient patients clinically resembles those described in APECED patients (see for example, doi:

10.3389/fimmu.2023.1172369), with dysregulated IFN responses. As is known, anti-IFN antibodies are common in APECED (against type I +/- type III), and there is data emerging, mostly still only available as meeting abstracts, that describe the key role of excessive IFN-gamma production (type II) in its etiopathogenesis and consequent treatment responses). Similar GI phenotype plus further lichen planus (please clarify if it affected skin and/or mucosal membranes) in P3 suggests that maybe IFN-gamma production could be increased in these patients? Slow transit constipation is associated with increased IFN-gamma signature, in various disease states. Finding the etiopathogenesis of patients' immune dysregulation could open avenues to treat these patients (e.g., JAKinibs). IFN signature one could study rather easily by using Nanostring assay in blood and/or patients´ fibroblasts (doi: 10.1089/jir.2017.0127.), which clearly are available. Clearly, one internationally likely finds many similar patients in the future.

Thank you for the pertinent and helpful suggestions. Our analysis of the Type 1 Interferon-inducible gene expression in peripheral blood samples from probands 1, 2 and 5 are detailed in the results section (**lines 111-114**) and the discussion (as seen above under comment 3, **lines 368-400**).

Results –

Regarding probands 1 and 2: "An interferon (IFN) signature gene assay revealed equivocal Type I IFN-inducible gene expression but both twins demonstrated significant downregulation of SIGLEC1 (sialic acid binding Ig like lectin 1)**".**

Regarding Proband 5: "Interferon (IFN) gene profiling revealed no evidence of interferonopathy but IFI27 levels were elevated".

Discussion -

"Although the downstream impact of attenuated STAT5B nuclear localisation contributed to the phenotypes, the association of immunodeficiency and gastrointestinal dysfunction with loss of *QSOX2* prompted investigation into interferon signalling given overlap with excessive interferon states such as APECED[41]. However, an interferon (IFN) signature gene assay conducted on probands 1, 2 and 5 revealed no evidence of interferonopathy in peripheral blood samples. Interestingly, a concordant downregulation of SIGLEC1 (sialic acid binding Ig like lectin 1) was of note in P1 and P2. SIGLEC1 is a key regulator of phagocytic function and its deficiency is implicated in the pathogenesis of obstructive pulmonary disease[42]. IFI27, elevated in P5, is a pro-apoptotic protein present at the mitochondrial membrane and implicated in IFN-dependent modulation of mitochondrial permeability[43]. Further work is necessary to characterise these findings in the context of QSOX2 insufficiency".

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded very thoughtfully to my initial comments. The additional information about patient #5 is extremely helpful and adds to the level of evidence in the study. The explanation about the variable splicing is well thought out. I have no further questions. Overall, this is a highly novel and insightful manuscript.

Reviewer #2 (Remarks to the Author):

I thank the authors for updating their manuscript based on the comments from peer review and commend them for providing such detailed responses.

My queries have been addressed by the authors in both the updated manuscript and the response to reviewers rebuttal. I have no further comments or recommendations.

Reviewer #3 (Remarks to the Author):

The revised version of the manuscript has greatly improved immunologically. The authors now convincingly show that this indeed is a novel IEI, and that it largely recapitulates STAT5B AR and DN phenotypes and their phenotypic heterogeneity, as it should.

On top of the other fitting changes (a.o. intro, results) I found the new parts of discussion on lines 405-420 balanced and generally very well written.

I however have one suggestion left:

on line 417, could you for clarity switch from "negative interaction" into the more exact and unambiguous expression "negative interallelic complementation" and on line 419 use the opposite, "postive interallelic complementation"?

In my opinion, the authors have otherwise revised the manuscript highly satisfactorily, the added data nicely also adds evidence to both endocrinologic and immunologic phenotypes, and in a balanced way addresses the intricacies of transcript factor biology and the tricks in that trade, like stochastic gene expression.

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Response: We would like to thank Reviewer 1 for this generous feedback.

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Response: We would like to thank Reviewer 2 for this generous feedback.

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Response: We would like to thank Reviewer 3 for their detailed comments. We have edited the discussion to accommodate Reviewer 3's feedback as follows:

"Given the pleiotropic nature of this disorder, phenotypic variability was inevitably anticipated. The degree of short stature appeared most pronounced in compound heterozygotes (4/5 probands) when compared to the lone simple recessive homozygote (P3) within our cohort. Since the impact of p.T352M homozygosity on height is highly variable, it seemed possible that phenotypic discordance may be due to interallellic complementation[32,33]. The QSOX2 protein may have altered functionality or **negative interallelic complementation** between two distinct mutants *in trans* as opposed to identical mutant subunits, which may have the unintended consequence of partial phenotypic rescue or **positive interallelic complementation** i.e. the heteromultimer is less functionally active than the homomultimer".