Thiffault and colleagues describe the identification of a new patient with mutations in the PIGN gene. This gene encodes glycosylphosphatidylinositol (GPI) ethanolamine phosphate transferase 1 (PIGN), a protein involved in GPI-anchor biosynthesis. PIGN is expressed in various tissues, and pathogenic variants in PIGN have been reported to be associated with a broad range of clinical phenotypes, including multiple congenital anomalies, dysmorphic features, developmental delay, hypotonia and epilepsy.

Here is reported the identification of a 2 years old male with hypotonia, global development delay, and focal epilepsy. Trio-whole exome sequencing revealed heterozygous variants in PIGN, as well as a heterozygous variant in the SCNA1 gene. This variant was already identified when the patient was around 5 months of age, after that targeted NGS analysis for an epilepsy gene panel was performed. In addition, the authors performed flow-cytometry analysis on blood granulocytes, after staining for both FLAER and CD59, to investigate the expression of GPI anchors and GPI-anchored proteins, respectively. Based on all of their observations, Thiffault and colleagues interpreted the reported case as a PIGN-related disease, although dysmorphic features were not present. Nevertheless, as they report it themselves, this observation is in line with recent reports describing a genotype-phenotype correlation for PIGN-deficiency, even suggesting that major congenital abnormalities might not be a core feature of PIGN disorders.

Nevertheless, after careful reading and evaluation of this manuscript, I have a few major comments which unfortunately prevent me of recommending to have this manuscript approved for publication in its current form.

General comment: the line numbers don't fit with the actual lines of the manuscript, making the reading and mostly the reviewing of the manuscript very confusing. Please correct.

Abstract (page 02):

1. Line 7: please indicate within brackets that GPI stands for glycosylphosphatidylinositol.

2. Line 27: please reformulate to indicate that staining for FLAER and CD59 was performed by flow-cytometry. Also mention that FLAER stands for fluorescent aerolysin and that they respectively were used to investigate the expression of GPI anchors and GPI-anchored proteins.
3. Line 32: use the GPI abbreviation for glycosylphosphatidylinositol. You should be consistent in using either the abbreviation or the full name throughout the manuscript.

Background (page 03):

4. Line 36: please add a reference for the publications linking germline pathogenic variants for genes involved in the biosynthesis and/or remodeling of GPI anchors with human disorders.

5. Line 44: Multiple Congenital Anomalies-Hypotonia-Seizures syndrome 1 (MCAHS1) is an autosomal recessive disorder, and so is Fryns syndrome. Both disorders have actually been reported to be associated to PIGN-deficiency. Please specify that MCAHS1 has been linked to PIGN deficiency, since MCAHS2 is linked to PIGA defects, and MCAHS3 to variants in PIGT. Please mention something as well about Fryns syndrome, with adequate references.

Case presentation:

6. Where the alkaline phosphatase levels checked? AP levels are usually elevated in the case of GPI-deficiency, resulting in hyperphosphatasia. Even as such elevated AP levels aren't a systematic hallmark of GPI-deficiency, it still might provide additional evidence for the pathogenicity of the identified variants for PIGN.

7. Page 06: it might be useful to add a table, describing the retained variants in the patients, including the mutations at both the transcript and protein levels, their inheritance pattern, and the scoring of their pathogenicity according to in silico software, e.g. Polyphen2, Alamut, SIFT, Mutation Taster. In addition, the authors could add the sequencing results of both the proband and his parents for both SCNA1 and PIGN genes.

8. Page 06 line 39: "(…) it is of the type expected to be pathogenic, resulting in a truncating or degraded product". I agree that this mutation will result in the generation of a premature stop codon, and consequently in the transcription of a severely truncated protein, most likely rapidly degraded. Nevertheless, the sentence is very approximate and poorly written, please reformulate.

9. Page 06 line 48: "(…) predicted to be deleterious by in silico programs". Which programs were used, what are their respective scores? Please see comment #7 and add this in information in the manuscript.

10. Page 06 line 56: "(…) the missense variant was interpreted as a variant of unknown significance". I agree that just on the basis of the given scores of in silico programs and the fact this variant has not been reported before you first considered classify it as VUS. Nevertheless, you decided to proceed to functional studies with flow cytometry to gain more insight into its pathogenicity as written on the following page, line 07 "(…) the pathogenicity of the missense variant called for functional studies to prove causality before interpreting this genotype as pathogenic". I would reformulate this paragraph, since I find it confusing. The reader indeed really gets the idea that your decision is to exclude this variant from your analysis.
11. Page 07 lines 9-12: "in order to examine the effect of the p.Arg95Gln variant on the function of PIGN (...)". I totally disagree with this statement. This flow cytometry experiment allows you to look at the global function/dysfunction of PIGN in the patient's cells, e.g. the impact of BOTH identified mutations. Please reformulate. If you want to look at the pathogenicity of the p.Arg95Gln variant alone, you also should have analyze blood cells of the father too, since you report it is paternally-inherited. Even though a single heterozygous variant for PIGN can't lead to disease of course. Please reformulate.

12. Page 07 lines 14-16: I'm not sure I understand your experiment. You mention that "granulocytes were gated after staining with mouse anti-human CD59 and FLAER". Can you explain your experimental protocol please? Normally you should first gate your different cell populations to isolate them and consequently analyze the fluorescence of your staining of interest, e.g. in your case CD59 and FLAER. I'm skeptical about your results…. Which markers were used for population isolation? Which gating parameters?

13. Page 07 line 16: see comment #2. Please correct

14. Page 07 line 16: if you use the abbreviation FC for flow cytometry, please mention on the precedent line the abbreviation within brackets.

15. Page 07 line 19: what do you mean by "performed on a research basis"? Furthermore, you mention that the experiment was performed on blood granulocytes but in figure 02 are presented the results for both erythrocytes and granulocytes… Please update those results/comments.

16. Page 07 line 21: "demonstrated a shift in this patient's granulocytes, indicating (...)". Which staining are you talking about? FLAER, CD59? As I already mentioned it before, the two stainings are used to visualize two different things, either the abundance of GPI-anchor at the cell surface, or the abundance of a specific kind of GPI-anchored protein. Please be more accurate in the description of your results. In addition, did you consider staining with another marker, specific for granulocytes, such as CD24? If not, why? CD59 is actually a marker present at the cell surface of both erythrocytes and granulocytes.

Figure 02:

17. The figure is very confusing and of poor resolution. My initial comment is for the legend which is absolutely not clear. Why to not call the different plots control, patient, mother, negative control? In addition, the color codes in the right panel do not refer to the same samples than in the two first panels. Why?

18. Moreover, you report that you observe the fluorescence levels of CD59 in both the left (erythrocytes) and the right panel (granulocytes). However, while you look at anti-CD59 for red cells, you look at APC-A for granulocytes. Can you explain? APC is a fluorescent molecule, but coupled to which protein? What are you looking at in this panel?
19. The whole point of the flow cytometry experiment is to demonstrate functional impairment of the identified PIGN mutations on both GPI and GPI-anchored protein expression in patient's blood cells. But according to your results, while you observe a small shift (less than a half log) for CD59 levels in granulocytes (assuming you're looking at CD59 in this panel, which isn't clear) there are absolutely no differences in erythrocytes. Why don't you comment on that?

20. Still in the right panel, while you observe a shift towards the left in patient's cells compared to control cells, you observe a positive shift of the same amplitude for mother's cells. Once again, why no comments about that? This is assuming that the central plot in dark green in the one of the control cells (and hence should be blue), since the colors are not very clear on this panel.

Conclusions:

21. Page 07 lines 40-45: please reformulate. This section is poorly written.

22. Page 07 lines 55-59: "A recent genotype-phenotype correlation has been suggested, (…) our patient further supports this". This is maybe my major comment of this manuscript, why don't you actually discuss that? If your report is in line with the work of Fleming and coworkers, I don't understand that in a similar way than what they described, you don't compare: (i) the clinical phenotype of your patient to the one of all PIGN-deficient patients described so far, similarly to what they did and (ii) the types of mutations. The two mutations you identified are both localized in the phosphodiesterase domain of PIGN. To my knowledge, only the patient reported by Ohba and coworkers (2014) reported such a case, with the combination of both a missense and a stop mutation in this domain. Please discuss that.

23. Page 08 lines 04-51: This part is quite lengthy and doesn't bring much information to the manuscript. I think that by now the added value of WES in diagnostic is not to be proven anymore, especially for the identification of inborn errors of metabolism. Please shorten this part and focus your discussion more on the genotype-phenotype correlation.

24. Page 08 lines 53-58 and page 09: see comment #22. Please discuss the added value of the reported case and how the identified mutations can lead to such a mild phenotype. In addition, how is Fryns syndrome providing further evidence for the genetic heterogeneity of PIGN deficiency? Please develop.

General conclusion:

Thiffault and colleagues report the interesting case of a 2-year-old male patient presenting compound heterozygous mutations for PIGN. In addition, the patient doesn't present congenital anomalies nor obvious dysmorphic features, suggesting - in correlation with recent reports - that those might not be core features of PIGN deficiency.

* The general opinion I have about this manuscript is that the authors wrote it in a hurry and didn't take time to carefully analyze their data and the added value of their case in
comparison to what have been published so far. Once again a pity since their findings seem to be in line with the recent publications suggesting genotype-phenotype correlation for PIGN deficiency.

* Although the pathogenicity of the identified stop mutation is undeniable, the data presented in support of a GPI-anchor deficiency (consecutively to the compound heterozygosity, hence indirectly proving pathogenicity of the missense variation) are not very robust and should be strengthened.

* A major point bothering me is that while the authors identified a variant in SCN1A, already being associated to autosomal dominant epilepsy, and predicted to be possibly pathogenic (score of 0.917 in Polyphen-2) they don't discuss anything about it. Is this variant be responsible for the epilepsy in the patient? Or is the epilepsy part of the phenotype due to the PIGN deficiency? This should be discussed in the manuscript. Actually, epileptic seizures in GPI-deficient patients have already been described to be drug-resistant (Kuki et al., 2013) while the seizures reported in this patient stopped after administration of levetiracetam and topiramate (page 05, lines 16-19). So are the authors sure that the epileptic seizures are part of the phenotype due to the PIGN mutations?

In conclusion, the data presented in this report is interesting and deserve to be considered by the scientific community, but at this stage I think that the manuscript isn't ready for publication yet. The main comments listed in this review should be addressed for the manuscript to be further considered for publication.

**Are the methods appropriate and well described?**
If not, please specify what is required in your comments to the authors.

Yes

**Does the work include the necessary controls?**
If not, please specify which controls are required in your comments to the authors.

Yes

**Are the conclusions drawn adequately supported by the data shown?**
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