Author’s response to reviews

Title: Mutation analysis of PALB2 in BRCA1 and BRCA2-negative breast/ovarian cancer families from Eastern Ontario, Canada

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Author’s response to reviews: see over
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Re: Hereditary Cancer in Clinical Practice, MS: 5369106512096138

Title: Mutation analysis of PALB2 in BRCA1 and BRCA2-negative breast and/or ovarian cancer families from Eastern Ontario, Canada

Dear Editor,

We thank the reviewers for their thoughtful critique of our manuscript. Please find enclosed a revised version we have amended in light of the reviewers’ comments. Our responses to the reviewers’ comments are outlined below. All changes to the manuscript have been tracked and all major changes have been highlighted.

The References Section has been edited to include 6 new references which have been highlighted (10, 18,19,20,26,27). In addition, 5 references (the old 22,23,26,27 and 32) have been deleted. For the sake of reading clarity the deletions of these 5 references is not tracked in the appended revised version.

We hope that these changes will address the reviewers’ comments and that the paper will be found suitable for publication in Hereditary Cancer in Clinical Practice. We look forward to hearing from you.

Reviewer: Melissa Southey

1) The use of the word “moderate” risk gene is unfortunate. Certainly much of the literature and lay reports refer to mutations in this gene as “moderate risk” but this is unfortunate, given the lack of evidence for such a designation. Could the authors be more balanced in this assessment of risk in the text - could PALB2 be simply a “breast cancer susceptibility gene” (eg first line of abstract, second para of the introduction)?

We agree that at this point the penetrance and risks associated with mutations in PALB2 remain ill-defined. We have edited the manuscript (p.3 and p.5) accordingly.

2) In the era of gene panel testing the second sentence of the abstract appears to be inaccurate. Testing of PALB2 could be done along side the testing for BRCA1 and BRCA2 and be very efficient? Also it is not the prevalence (frequency) of PALB2 mutations that are most challenging in genetic counseling-rather it is the uncertainly around the penetrance and treatment options.

We agree that the advent of multiplex panels has recently increased the efficiency of testing PALB2 in the hereditary cancer clinic. We have adjusted the abstract (p.3) to reflect that genetic counseling rather than the prevalence is the greatest challenge to PALB2 clinical testing.

3) Second para of the introduction: “2.3 to as high as 6.0 depending of the methods used”…do the authors mean statistical methods or molecular methods or study design of something else by “methods used”?

The study design, specifically the mutation and population under study, was the greatest influence in these relative risks. We have adjusted the manuscript for clarity (p.5).
4) The last para of the introduction would benefit from reference to the onset of panel testing in diagnostic services and what impact this is having on this subject.

We agree with the reviewer’s observation that the recent implementation of panel testing in hereditary cancer clinics has made testing PALB2 more efficient. We have added a sentence to the introduction to reflect this (p.5).

5) Material and Methods: I think a subheading for the
   a. N1096S testing description and
   b. The LOH methods description
would be helpful for clarity.

We agree; sub-headings have been included in the Material and Methods section (p.8,9).

6) Results and discussion para 5: The cumulative risk of breast cancer is not supported/reported in all references cited (5,7,11,12,13,14,19) this text should be adjusted for accuracy.

We have reviewed the references and adjusted the text for accuracy (p.12).

7) Results and discussion para 7: The work has been conducted on research participants rather than “patients”, in my opinion.

We agree; the text has been edited on p.14.

8) Results and discussion para 7: It is interesting that the literature has a number of reports of ER-PR-/basal like breast cancers in PALB2 mutation carriers – but how many of these are related to the one PALB2 mutation (ie the Finnish founder PALB2 c.1592delT)? Could this tumour phenotype be a feature of this specific mutation?

We have reviewed the available literature and found reports suggesting that the triple negative phenotype is over-represented in series not limited to individuals carrying the Finnish founder mutation. The paragraph pertaining to the immunohistochemical features of the tumours in our series has been rewritten p.14).

Reviewer: Katherine Nathanson

In this manuscript Hartley et al used HRM/Sanger and MLPA del/dup analysis to identify variants in the PALB2 gene in families with breast and/or ovarian cancer. They identified two pathogenic mutations and three missense variants which they have classified as probably pathogenic. As noted in the Introduction, defining the patient populations for whom to offer PALB2 genetic testing is of utmost importance in the new era of clinical testing for moderate risk breast cancer genes. However, while Supplementary Table 1 proves some information, the definition of the patient population studied is quite confusing throughout the manuscript. The title states “...breast/ovarian cancer families...” ; the Abstract-Methods “family histories of breast and/or ovarian”; the Abstract-Conclusions “breast/ovarian”; and the Introduction last paragraph “breast and ovarian cancer pedigrees”. In the discussion, the statement is made that “It should be noted that the majority (139/175) of the families we screened did not contain any reported cases of ovarian cancer”. As this manuscript is purporting to help genetic testing decisions, the patient population should be
consistently and accurately defined throughout. The authors have used multiple ‘sample sets of convenience’.

We agree with this comment. The population being studied is indeed families with cases of breast and/or ovarian cancer who meet clinical criteria in Ontario for BRCA1 and BRCA2 sequencing and MLPA and have had negative results. We have made numerous changes to the manuscript in order to ensure the patient population is described accurately throughout.

Additionally, it is not at all clear at all that the missense mutations are pathogenic, as the calls are based on very limited analysis, which is not even consistent. Finally, one of the truncating mutations is in the last coding exon and due to its location, it is a variant of uncertain significance, not a deleterious mutation, without additional evaluation. Thus, the conclusions of the paper about PALB2 mutations are vastly overstated.

1) SIFT and Polyphen2 are insufficient to classify the PALB2 missense variants as “predicted pathogenic” and to make the statement “While the variants are possibly pathogenic functionally...”. Predicted pathogenic reflects a posterior probability of likelihood of disease, which is not demonstrated by the authors. The conclusions are particularly problematic given that c.1846G>C (p.D616H) does not segregate with disease or show LOH in the tumor and c.3418T>G (p.W1140G) does not show LOH in the tumor. Their analysis need to be redone using additional pathogenicity softwares, including ones that have been validated as likely more predictive than SIFT and Polyphen2, such as MutationTaster and Condel. Their conclusions regarding the missense mutations are overstated.

We agree with the reviewer that further evidence for the pathogenicity of the missense variants would be helpful. We have redone the analysis of the three missense mutations using both MutationTaster and Condel. Both prediction algorithms classified all three missense mutations to be likely pathogenic.

<table>
<thead>
<tr>
<th>PALB2 variant</th>
<th>MutationTaster</th>
<th>Condel</th>
<th>SIFT</th>
<th>PPH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.1846G&gt;C (p.Asp616His)</td>
<td>0.981 (disease causing)</td>
<td>0.990 (deleterious)</td>
<td>0 (deleterious)</td>
<td>0.999 (deleterious)</td>
</tr>
<tr>
<td>c.3287A&gt;G (p.Asn1096Ser)</td>
<td>0.997 (disease causing)</td>
<td>0.857 (deleterious)</td>
<td>0.05 (deleterious)</td>
<td>0.901 (deleterious)</td>
</tr>
<tr>
<td>c.3418T&gt;G (p.Trp1140Gly)</td>
<td>1 (disease causing)</td>
<td>0.990 (deleterious)</td>
<td>0 (deleterious)</td>
<td>0.999 (deleterious)</td>
</tr>
</tbody>
</table>

As for the c.3507_3508delTC (p.H1170Ffs*19) mutation, we thank Dr. Nathanson for the astute observation that because this mutation is located in the final exon (exon 13) of PALB2, it is unlikely to be subject to NMD. Indeed, sequencing of RNA from cultured LCLS showed expression of the mutant allele (which we now show in Supplementary Figure S3), which indicates that mRNA derived from the mutated allele is not subject to nonsense-mediated decay. The frameshift introduces 19 new amino acids that are not seen in the native protein (shown now in Figure S1B), creating a mutant protein that, if expressed, will have 1189 amino acids, compared to 1186 amino acids in wild-type protein. Interestingly, this protein is now longer than the native protein. The predicted structure of PALB2 includes an amino-terminal coiled-coil structure and a carboxy-terminal WD40-repeats motif that has the characteristics of a seven-bladed beta-propeller, a domain commonly involved in protein-protein interactions. The WD40-repeats domain on amino acids 836-1186 provides a binding site for the N-terminus of BRCA2. So mutations that alter the sequence of this region, even if they do not result in nonsense-mediated decay of the resulting protein, are quite likely to lead to alteration of the structure of WD40-repeats (as we show in Figure S1), and many are known to be pathogenic. Overall, there is reasonably convincing
evidence that the novel mutation c.3507_3508delTC (p.H1170Ffs*19) we describe here is deleterious, probably as a result of disruption of the BRCA1-PALB2-BRCA2 interaction, resulting in defective homologous repair of double-stranded breaks.

2) What is the reasoning for testing only the c.3287A>G (p.N1096S) variant in the additional case population? The rationale should be clarified.

The c.3287A>G variant was identified in a patient of Portuguese ancestry. We therefore wondered if it was a founder mutation in the Portuguese population. A BRCA1 and BRCA2-negative cohort was therefore screened. No additional patients were identified with the variant. The manuscript has been edited (p.11) to reflect our rationale.

3) The description of the different sample sets is very difficult to follow. The authors appear to have included every convenient sample set that they get their hands on. Have they excluded patients with BRCA1/2 mutations? – it is not stated in the manuscript.

We appreciate the reviewers comment. We have made efforts to clarify the sample sets throughout the paper as per this reviewer’s first comment. With regards to the question of whether patients with BRCA1 and BRCA2 mutations were excluded, we feel that it is clearly stated in the methods that the cohort was “selected participants affected with breast and/or ovarian cancer who had been previously screened for BRCA1 and BRCA2 mutations” and we “excluded individuals with pathogenic mutations.”

4) The discussion of tumor morphology is confusing – the authors point out six references that did not find associations of PALB2 mutations with HR negative breast tumors but then say that TNBC is overrepresented in PALB2 cancers as per reference 4. The last sentence of this paragraph that it is interesting that the probands have 24% TNBC is also unclear.

This section of the manuscript has been edited for clarity (p.14).

5) Discussion page 13: The last sentence of the last full paragraph is too much of a stretch given the small data set.

We agree that this sentence may be too hypothetical. It has been deleted from the manuscript (p.15).

6) The evidence that the second truncating mutation is truly deleterious is weak, as I am sure the authors are aware. The mutation would not lead to nonsense mediated decay, but add an extra 19 amino acids. Although it may be pathogenic, and the authors provide evidence suggesting that it could be, there are examples of such changes (albeit in other genes) that are no pathogenic. The authors are stretching it, classifying it as a class 4 variant (VUS, likely pathogenic) is likely more accurate.

We have performed additional analyses of the c.3507_3508delTC (p.H1170Ffs*19) variant which confirm the absence of NMD (data shown in new Supplementary Figure 3). We believe however that the position of the variant just 5’ to the well established c.3549C>G (p.Tyr1183*) mutation provides further evidence of pathogenicity. Text related to these analyses and our interpretation as well as additional references have been added to the Molecular Methods, and Results and Discussion sections (pages 9-10, 13).
Minor Comments

1) Abstract: “...but not in other tumors from that family or in tumors from carriers of other mutations” – this statement is misleading as in the text tumors from two of the variant carriers were not available. We agree with this comment and have edited the abstract to reflect that only “available” tumors were tested for LOH (p.3).

2) Abstract: “In our cohort, all clearly pathogenic...” Even with the n defined at the end of the sentence, “all” is misleading as it is only two mutations, would replace “all” with “both”. In addition, the final statement is too strong of a recommendation given the small data set. Same for Conclusion page 16 “Each of the families” would be better clarified as “Both”.

The abstract has been edited to read “both clearly pathogenic mutations” (p.3).

3) The HGVS full name is given for the variants in some locations and only the cDNA change is given in other locations; when referring to the missense mutations, it is more clear to give the full name at all times.

The manuscript has been edited to so that the HGVS full name is included for all missense mutations throughout the document.

4) Discussion page 14: There is no proof that the 11yo daughter with melanoma carries the PALB2 mutation.

It is true that the 11yo daughter has not been tested for the PALB2 variant given her young age. We have edited the manuscript so that this is explicitly mentioned (p.15).