Genetics in Medicine

Risks of breast and ovarian cancer for women harboring pathogenic missense variants in \( \textit{BRCA1} \) and \( \textit{BRCA2} \) compared with those harboring protein truncating variants.

--Manuscript Draft--

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Corresponding Author: David Goldgar
UNITED STATES
First Author: Hongyan Li
Order of Authors:
Hongyan Li
Christoph Engel
Miguel de la Hoya
Paolo Peterlongo
Drakoulis Yannoukakos
Luca Livraghi
Paolo Radice
Mads Thomassen
Thomas V.O. Hansen
Anne-Marie Gerdes
Henriette Nielsen
Sandrine Caputo
Alberto Zambelli
Ake Borg
Angela Solano
Abigail Thomas
Michael Parsons
Antonis Antoniou
Goska Leslie
Xin Yang
Georgia Chenevix-Trench
Trinidad Caldes
Ava Kwong
Inge Pedersen
Charlotte Lautrup
Esther John
Mary Beth Terry
John Hopper
Abstract: Germline genetic testing for BRCA1 and BRCA2 variants has been part of clinical practice for over two decades. However, no studies have compared the cancer risks associated with missense pathogenic variants (PVs) relative to those for protein-truncating (PTC) variants.
Methods: We collected 582 informative pedigrees segregating one of 28 missense PVs in BRCA1 and 153 pedigrees segregating one of 12 missense PVs in BRCA2. We analyzed 324 pedigrees with PTC variants in BRCA1 and 214 with PTC variants in BRCA2. Cancer risks were estimated using modified segregation analysis.

Results: Estimated BC risks were markedly lower for women over age 50 carrying BRCA1 missense PVs compared to BRCA1 PTCs (HR 3.9 [2.4 – 6.2] vs. 12.8 [5.7 – 28.7] for PTC variants; p = 0.01), particularly for missense PVs in the BRCT domain (HR = 2.8 [1.4, 5.6]; p = 0.005). The HR for women over age 50 was 3.9 [2.0 – 7.2] for women heterozygous for missense PVs compared to 7.0 [3.3 – 14.7] for those harboring PTC variants.

Conclusion: These results have important implications for counseling of at-risk women who harbor missense PVs in the BRCA1/2 genes.
July 17, 2021

Robert D. Steiner, M.D.
Editor in Chief, Genetics in Medicine

Dear Dr. Steiner,

We are re-submitting our manuscript entitled “Risks of breast and ovarian cancer for carriers of pathogenic missense variants in BRCA1 and BRCA2 compared with carriers of protein truncating variants” to Genetics in Medicine as an Article. We have modified the manuscript in accordance with the reviewers’ and editor’s comments and feel that as a result it is much improved. We also have added the information on the Ethics Approval to the manuscript.

You may note that there were some Reviewer suggestions that we judged were impossible or not desirable as we mention in our response. As before, we very much look forward to publishing this work in GIM.

Sincerely Yours,

David E. Goldgar, Ph.D.
Research Professor Emeritus, Department of Dermatology
Investigator, Huntsman Cancer Institute
University of Utah School of Medicine
RE: GIM-D-21-00505, entitled "Risks of breast and ovarian cancer for women harboring pathogenic missense variants in BRCA1 and BRCA2 compared with those harboring protein truncating variants."

Editorial Office Requirements:
GIM has preferred usage for certain terms (detailed here https://www.nature.com/gim/authors-and-referees/preparation-of-submissions#terminology). We ask you to check your article to ensure those terms are used correctly.

- heterozygote (rather than carrier) when referring to a person's genotype/genetic test result. This is intended to deal with the issue that "heterozygote" describes the presence of a variant, regardless of classification, but "carrier" typically implies that the variant is disease-causing. We want descriptions of variants to use the terms heterozygous, homozygous, hemizygous, etc in order to have no ambiguity. If "heterozygous" is only implied by saying "carrier" then that is not precise at the level of a genetic variant.

Table 1 and 4 still contain "carrier" and "non-carrier" as terms - please provide more accurate terminology.

Response: We have modified this as follows: Instead of Carrier we now use Heterozygous and instead of Non-Carrier we use Wild-Type in the headings of Table 1. We hope this is acceptable.

Response to Reviewer Comments:

Before addressing the Specific Comments of the two Reviewers I would like to outline some changes made to the analyses. C64R, G1738R and BRCA2:D2723H are now analyzed separately as presented in the Tables. The other variants that were previously grouped with them at the same residues are now listed in the three relevant 'other categories. We chose to do this to focus on the single variants where there were substantial numbers of families and to avoid any contamination from other variants with potential splicing defects. This also allowed us to include only Italian families for the C64R and (almost) all Greek families for the G1738R founder variants. We have also eliminated Table 5 and moved this information to a Supplementary Table 2.

Reviewer #1: The authors have undertaken a large analysis of missense variants that have been classified as LP/P excluding those with evidence of splicing. The analysis shows a potential continuum of risk that is generally lower than for PTC variants. The number of PTC families is rather low and oddly there were 2/24 OCs that were phenocopies. The authors use UK rates and have adjusted for this in sensitivity analysis but I think a separate analysis should be performed on the predominantly Eastern European BRCA1 c.181T>G,p.[Cys61Gly. This if adjusted looks to have similar BC rates to PTCs and may be higher for OC. This is an important analysis and demonstrates that missense variants should not be treated as the same as PTCs as in ATM and CHEK2 but also that each missense should not be treated as the same as each other. It would be a useful addition to comment on the rates of phenocopies with fits with their low risks of BRCA1 Ring domain residue 64 and their potential downgrading of the BRCA2 variant.

Response: It is difficult to really get a handle on this without considering cancers occurring in those untyped individuals who may have high probabilities of being non-carriers based on pedigree position and genotypes of relatives. We have added data related to this to the discussion about the C64R and W2626C variants.
Specific comments

1. Abstract: 'In BRCA2 the HR for women over age 50 was 3.9 for women heterozygous for missense PVs compared to 7.0 for those harboring PTC variants.' Why are there no 95% CIs for the HRs when these are provided for BRCA1

Response: We have now added the CIs for the BRCA2 HRs.

2. 'Cumulative risks of breast cancer to age 80 were 72% for BRCA1 heterozygotes and 69% for BRCA2 heterozygotes; corresponding risks of ovarian cancer were 44% and 17%.' Please provide 95% CIs

Response: We have now changed the cited risks from age 80 to age 70 (to be more comparable to our results in Table 3 and added the CIs here as well.

3. 'Through the work of the ENIGMA (Evidence-Based Network Investigating Germline Mutant Alleles) consortium7 and others, ~60 missense variants have now been classified as pathogenic by multifactorial methods8 using such lines of evidence as co-segregation, family history of index cases in clinical testing series10-11, and tumor histopathology12 to (classify) variants of uncertain significance (VUS).'- I presume you mean 'reclassify'?

Response: I guess this all depends on whether VUS is considered a classification. In the early days, VUS were termed 'unclassified' so what ENIGMA etc. did was to 'classify' them. But I take your point and have changed this to 'classified/reclassified'.

4. Also Are all these pathogenic or some 'likely pathogenic'?

Response: As shown in the Supplemental Table 1., these have different reclassifications by different groups. Some testing companies have considered them Pathogenic while others have been more conservative and classified them as 'Likely Pathogenic'. The interested reader can compare the classifications in Supp. Table 1, or look them up on ClinVar/BRCA_Exchange. The vast majority of these variants are quite convincingly classified as Pathogenic by all/most sources.

5. 'Indeed, the BRCA1 variant c.5096G>A;p.[Arg1699Gln] was shown to be associated with lower risks of breast cancer (~20% by age 70)14,15 and had reduced function in some assays14' -Need to clarify reduced function compared to absent function. Otherwise in context could be reduced compared to truncating.

Response: We now specify the activity in terms of % wild-type transcriptional activation in the 293T cell line which was 78% of wild-type compared to 45% and 10% for two variants in the present study (R1699W and A1708E, respectively).

6. Methods: 'ClinVar17 (at least two submitters denoting it Pathogenic/Likely Pathogenic with none calling it Benign or Likely Benign).' -This is problematic. A number of ClinVar variants would fit this criteria with the majority of submitters still submitting as class 3. If these are included they should be treated as a separate category in analysis if a greater proportion of submitters classify as class 3.

Response: This is of course complicated by the fact that all ClinVar submitters' classifications are not considered equal. Our approach has been to focus on submitters with at least one 'star' as determined by ClinVar and we rely most heavily on the ENIGMA expert-panel classifications (3-star). In addition to the data in Supp Table 1., we have re-examined each variant and verified that none of the variants have a majority of these classifications as VUS.

7. '324 pedigrees with (loss of function variants) in BRCA1 and 214 with PTC variants in
BRCA2' Please be consistent 'loss of function variants' could be 'missense' if there is reduced function. Should be PTC for both BRCA1 and BRCA2
Response: Thanks for pointing out this error – we have corrected this sentence.

8. 'Because only a very small subset of the contributed families provided data on risk reducing salpingo-oophorectomy (RRSO) we chose not to censor women at this age; this may lead to an underestimation of ovarian cancer risk but this effect would be expected to be the same for the PTC variant pedigrees as well as the missense PV set.' -Not necessarily as PV carriers for PTCs will have been told about high OC risk whereas missense may not. So, uptake of RRSO may well have been higher in PTC.
Response: The reviewer makes a good point – we have added a sentence to the discussion about this, although we note that many of the BRCA1 pathogenic missense variants were C61G which has been identified as pathogenic since the late 1990s

9. 'For both breast and ovarian cancer analyses the baseline population incidence rates were assumed to be those for the UK organized in eight 10-year birth cohorts (Cancer Incidence in Five Continents Reports32)' -This will likely underestimate HRs for missense variants in countries with lower BC incidence with high submission rates such as the variants from Italy and Greece
Response: We have reanalyzed the Italian and Greek variants (C64R and G1738R, respectively) using rates specific to those countries (although these countries do not have nationwide cancer registry data) and we did not observe any major differences. We prefer to use the same rates for all analyses to make comparisons between variants easier. In addition some variants have a wide geographic distribution, further complicating the issue.

10. 'assuming UK rates in the women born between 1970 and 1979;' -This will again underestimate risks for those born <1970 with missense variants
Response: We acknowledge this point but feel that the HR estimate used in the Cumulative risk calculations will adjust for this.

11. 'However, there was some evidence of heterogeneity associated with the BRCA2 variants in the DBD with HRs for women over age 50 varying from 1.7 (0.4, 6.9) for 7878G>C;p.[Trp2626Cys]' -this is very low. Please stipulate evidence for pathogenicity especially considering the only tested OC was negative (table 1).
Response: We commented on the evidence for pathogenicity and results of functional assays for this variant on page 19. We have elaborated on the reasons for these lower estimates somewhat in the revised text.

12. Please discuss phenocopy rates as a surrogate. The rates for c.190T>C;c.190T>G;c.191G>A; p.[Cys64Arg/Gly/Tyr] are extremely high at 10/25 ie 40%. This suggests that these residues are relatively low risk and will dilute the effect of other ring domain missense such as c.181T>G,p.[Cys61Gly] with only 14/128. The overall rate for phenocopies outside these 3 residues is 27/227 compared to 10/25 (p<0.0001)
Response: We are not sure what you mean exactly by surrogate. While the data you cite does bear on this question, it is difficult to interpret this in isolation since the HR analysis takes into account the ages at diagnosis of these cases and more importantly the weighted genotype probabilities of the large number of untyped breast cancer cases in these families. Certainly if there is true risk heterogeneity between variants within a class, it would make the combined data of limited interpretability. However if the observed differences are random variation then the combined result does have value. For this reason we prefer to keep it in. We note that in studies of largely PTC variants, combined results are combined even though there is substantial evidence of risk heterogeneity based on position within the gene as cited
13. "The risks associated with the BRCA2 p.[Trp2626Cys] missense PV for breast and ovarian cancer is significantly reduced compared to both BRCA2 PTC variants or other BRCA2 missense PVs analyzed in this study (Table 2)." - The evidence from this analysis that this variant has any effect is very limited. There is no significant increased risk for either BC or OC and the OC risk is based entirely on untested OC cases. If the OC cases were assumed to have the variant entirely based on a priori risk at birth rather than inference based on OC would there have been any increase?

**Response:** We are not sure of the point the reviewer is making here. The reviewer is correct in that there is no evidence in this data set that W2626C is associated with an increased risk of breast or ovarian cancer. However, the information contained in the 34 pedigrees with this variant may have been insufficient to demonstrate any such effects. We discussed this variant in some detail on page 18 of the submitted manuscript. and conclude that it is possibly associated with only a two or three-fold risk of BC. We recommended downgrading the classification of this variant to VUS at the present time pending additional analyses.

14. "Based on these analyses, differences in rates from our assumed UK rates are an unlikely explanation for the findings." - Although this may be true to some extent and there was only one Eastern European submitter (Lithuania) most of the c.181T>G,p.[Cys61Gly] are likely of Eastern European origin and are likely to have been recent migrants. Please assess this variant separately for the much lower Eastern European rates. Already this variant is not significantly lower for BC incidence than PTCs.

**Response:** Among the 316 C61G informative pedigrees, in addition to the 277 families from the German Consortium, there were 39 submitted from a variety of countries/regions including 20 families from 7 other European countries, South America (3), USA (11), and Australia (5). Thus it is difficult to see exactly which rates to use, as some of the countries/regions do not have complete cancer registries. Although the reviewer is correct about the probable origin of this variant, one would normally use the rates corresponding to the place of birth or residence during key developmental periods, at least for breast cancer. In response to your comment however, I have analyzed the effect of lower rates for the Italy and Greece founder variants, C64R and G1738R, assuming 20% lower rates than the UK rates assumed in the main analysis. The HRs changed very little (e.g. for G1738R in the Greek population the HRs were 22.9, 3.6, and 14.7 using the UK rates (table 2) and 28.8, 3.7, and 15.9 under rates assumed to be uniformly 20% lower than the UK. For the Italian C64R founder the estimated HRs were 3.7, 4.1, 99 vs. 3.7, 4.0, 100 and with 20% lower incidence rates they were 3.7, 4.0 and 125. In our view, and based on data from Cancer Incidence data over time, the major source of variation in rates is change over time and we have adjusted for this by using birth cohort specific incidence rates.

15. "The results shown in Tables 2 and 3 clearly indicate that missense PVs in both functionally important domains (RING and BRCT) in BRCA1 are associated with lower risks of breast cancer than PTC variants" - yes as a group but not for c.181T>G,p.[Cys61Gly] especially given above argument

**Response:** Although the difference between C61G overall and the GC-HBOC PTC variants fell short of statistical significance, we note that when restricted to the 277 families with this variant that were ascertained from the same GC-HBOC centers as the PTC families (matched for Center within Germany, in fact), the difference was significant as shown in table 2.

16. "Although data are more limited, it appears that ovarian cancer risks are comparable (though on average perhaps slightly lower) for BRCA1 to those found in published risk estimates for PTCs1-3" Again NOT for c.181T>G,p.[Cys61Gly]
Response: The HRs for C61G and PTC were not statistically significantly different, and when restricted to the GC-HBOC C61G the point estimates were nearly identical.

17. ‘Further, these risks will depend on family history, their polygenic risk score (PRS), and lifestyle factors.’ References please
Response: We have added two references that are relevant to this point.

18. ‘This variant is denoted in ClinVar as pathogenic by four clinical laboratories, and as likely pathogenic by another.’ -There are currently 19 entries and well over 5 by clinical laboratories. Please update.
Response: we now include all relevant classifications from ClinVar for this variant, in the text. As noted in response to your point 6 above, we only consider ClinVar classifications with at least one-star reliability. For the c.7878 variant, there are 9 Pathogenic, 3 Likely Path, and 1 VUS for the 13 submitters who meet this criterion.

19. Please comment on potential for dominant negative missense as in ATM and TP53 which give higher risks than for PTCs. Is this possible in BRCA1 or BRCA2? This is also an argument why all missense should not necessarily be grouped together
Response: The reviewer makes an interesting point. We do not have any experimental evidence for such a dominant negative effect but we note that in our study of BRCA missense, we find an opposite effect than in TP53 and ATM, with missense having generally lower risks than PTC variants. We very much agree with the last statement about grouping of variants with potentially very different site-specific cancer risks.

Reviewer #2: The goal of this project was to assess the impact on risks of breast and ovarian cancer of "pathogenic" and "likely pathogenic" missense mutations in BRCA1 and BRCA2, taking advantage of large numbers of families available through ENIGMA, CIMBA, and other consortia. Specifically, Goldgar and colleagues selected 40 missense mutations in BRCA1 or BRCA2, defined as pathogenic or likely pathogenic by their own or ClinVar criteria, in the RING domain of BRCA1, the BRCT domain of BRCA1, and the DNA binding domain of BRCA2, then calculated odds ratios for breast cancer dx before age 50, for breast cancer dx age 50 or older, and for ovarian cancer, compared to incidence in the UK. Their results suggest that risks of breast cancer after age 50 associated with these missenses, while highly significant and certainly clinically meaningful, are lower than risks associated with BRCA1 and BRCA2 truncating mutations. In contrast, risks of breast cancer before age 50 and of ovarian cancer are about the same for these missenses and for loss-of-function mutations.

Comments. Since the first days after the cloning of BRCA1, it has been clear that a few BRCA1 missense mutations had devastating clinical effects. Cys61Gly and Met1775Arg were among the first BRCA1 mutations identified, and the informative families that harbored them led to very strong suspicion that each was causal for the cancer phenotype. However, given the rarity of individual missense mutations, and the fact that few large informative families were enrolled in subsequent years, these effects have been difficult to measure accurately for other missense mutations. It will be a service to the field to have more robust estimates of cancer risks due to clearly damaging missense mutations in BRCA1 and BRCA2.

The need for these results also highlights a limitation of ClinVar that is obvious to its users, but not to the outside world; namely, that "pathogenic" and "likely pathogenic" are definitions based on gene and protein function, not on clinical consequence, and that ClinVar and similar sites do not provide a mechanism for assessing variable severity of mutations that share the label "pathogenic" or "likely pathogenic." This project implicitly addresses that
problem and potentially will be very useful to clinical practice.

However, in order to be useful to either clinical practice or to additional biological understanding, some revisions are necessary.

**Response:** We thank the reviewer for their comments above and their appreciation of our work. Below we respond to the specific comments that were raised.

C1. Table 1 column 2, and the associated sections of the text, cite numbers of "informative families." This is misleading. Based on the data of this table 1, most of the "families" are in fact probands with an affected relative but no additional genetic information. For example, the table indicates 405 "informative families" with RING domain mutations, but only 199 genotyped affected relatives in all 405 families combined. Inference from relatives who could not be genotyped is appropriate, but column 2 should be re-named "probands" and the next six columns "relatives with breast cancer" and "relatives with ovarian cancer."

Then, so that the reader can review the central data of the project, create a Supplementary Table listing every family individually, with the cancer, age at dx, and mutation of the proband (who is correctly excluded from analysis), and each genotyped relative, with her relationship to the proband, her cancer and age at diagnosis, and her genotype. This is not all the relevant information but is the core of it.

**Response:** We agree and have changed the column headings as suggested. However, it should be noted that in a few cases the index case/proband is an unaffected individual who was sequenced and in whom the variant was first identified. More importantly, we note that much of the information comes from the ages of genotyped relatives who are, at time of testing are unaffected with cancer. All families in this table are informative in the statistical sense that they have at least one individual in the family genotyped for the variant in addition to the index case.

With respect to the proposed Supplementary table listing all genotyped individuals in the data set (presumably by Variant), we think this is a bit too much information and may very well run afoul of privacy laws. Such a table would contain ~5500 entries so it is difficult to see how this would be of use to anyone. As mentioned in the text, we are happy to share the complete (de-identified) dataset with anyone interested to do their own analyses (where privacy rules permit).

C2. In the Methods section, indicate how probabilities of carrier/non-carrier status were estimated for relatives who could not be genotyped. Since not-genotyped relatives are the great majority of subjects, the method of inference is obviously critical. Simply referring to the 2020 JCO paper is not sufficient, and only marginally relevant. Please describe in detail the approach used by Dr Goldgar for this project.

**Response:** We are confused by this comment and are not sure what is requested. The 2020 JCO paper is quite relevant since essentially the same method/computer program for estimation of HRs that was used in that study was used in ours, and is based on a method that has been around since the early 1970s for analyzing pedigree data.

In essence, we calculate the likelihood of the observed pedigree data (genotypes and phenotypes) as a function of the parameters (in our case the HRs for breast<50, breast>50, and ovarian cancer based on the Cox-survival analysis model as described in the text) and then use numerical searching techniques to find the values of these parameters that maximize this likelihood. This analysis incorporates each individual in the pedigree by calculating the probabilities of each variant genotype based on the genotypes of other relatives in the pedigree. In addition to the JCO paper, we referenced the computer algorithm that implement these calculations. Further information about the basic underlying...

C3. Do not pool the mutations, at least not at first. For Table 2, calculate the odds ratio for each mutation individually, as has been done for a few. Based on the supplementary table, analyses included 11 missenses in the BRCA1 RING domain, 17 missenses in the BRCA1 BRCT domain, and 12 missenses in the BRCA2 DNA binding domain. Please give the individual odds ratios and confidence intervals for each of these 40 mutations for each outcome of Table 2: breast cancer dx<50, breast cancer dx>50, and ovarian cancer. Certainly for the most rare mutations the confidence limits will be very wide, but the reader should see all the point estimates and their confidence limits, so as to judge for him/herself when pooling is appropriate. The goal here is not to reach an arbitrary significance level but to see the pattern across missenses and for which is evidence is individually strong. Comment in the text on which missenses appear to be similar to each other in clinical effect. 

Response: We thank the reviewer for this suggestion. Unfortunately with the exceptions of the more frequent variants in our data set, the methods we are using to estimate the breast and ovarian Hazard Rates will not converge with more limited data (or if they do they will have such large standard errors as to be useless). However based on your comment we have decided to not pool the C64R with the much rarer C64G and C64Y variants, especially given that C64R seems to be largely an Italian variant. Similarly we now restrict our analysis to G1738R in which all families are either submitted from Greece or from areas with large Greek populations (e.g. Melbourne Australia). The C64G and C64Y variants are now included in the Other RING Domain variant group and the G1738E pedigree is placed in the Other BRCT Domain group. Similarly in BRCA2, we now have analyzed D2723H alone and added the D2723G pedigrees to the Other DNA Binding domain. We feel that this is a more pure analysis.

C4. Drop the estimates of lifetime risks (Table 3). Let the odds ratios for the three outcomes speak for themselves. The lifetime risk estimates suffer from very small sample sizes at older ages. 

Response: It should be noted that these ‘lifetime risks’ as shown in table 3 are derived from the estimated HRs and population incidence rates; they are not estimated directly from the data (although the HRs in fact are estimated from the retrospective analysis of cumulative incidence in the pedigrees). They are included just to show what risks might be hypothetically used in counseling based on the <50 and ≥50 estimated HRs. True cumulative risks estimates can only come from large prospective trials of carriers of pathogenic missense variants for which insufficient data exists at present. We will clarify that these risks are hypothetical and based on the HR estimates and somewhat arbitrary’ background incidence rates, but prefer to keep Table 3 in the manuscript.

C5. It is interesting BRCA1 missense mutations and BRCA1 loss-of-function mutations are equally associated with ER-negative tumors among breast cancer patients (Table 4). 

Response: Yes we agree but think that this issue could benefit from even larger data sets such as the population-based series in Dorling et al. and Hu et al. in NEJM. Certainly the biological processes that determine the histopathological type of cancer could differ from the mechanisms that influence overall cancer risk.

C6. It is misleading to suggest that mutations of BRCA1 Cys64 are "low risk for breast cancer." These mutations yielded extremely high risks for ovarian cancer (OR > 100) It is likely that competing risks led to fewer women with these mutations being dx with breast
cancer. The analysis attempted to adjust for competing risks, but there are limits to statistical adjustment for an odds ratio > 100. Omit this sentence from the abstract, and in the text, comment on the difference in breast and ovarian cancer risks for this mutation, not on a "low risk" of breast cancer (among women who developed ovarian cancer.)

Response: Given that typically ovarian cancer occurs later than breast cancer, we do not believe this could be explained by competing risks. Moreover, the most striking thing about C64R is the relatively low HR for women under 50 compared to not only the PTC variants but to the other missense as well. This is unlikely to be affected by a high risk of ovarian cancer. We also note that However, we agree that saying ‘low risk of breast cancer’ somehow implies it has a protective effect and have modified the text to address this oversight.

C7. Move Table 5 to the supplement. Text Table 2 will be much larger with all mutations included individually, and repeating the results for one of many possible variations in analytic approach need not be included in the main text.

Response: We agree and will move Table 5 to the Supplemental Material.

C8. Over-use of acronyms makes the text almost impossible to read, even for a specialist. For online publication, ink is not a limiting expense. Write out PTC, DBD, PV, LOF, LRT, BC, OC, and BPM each time they appear. (BRCA1, BRCA2, RING, and BRCT are accepted as acronyms for names and are fine.)

Response: While indeed, ink is not a limiting expense, we were (and perhaps with other suggested changes are now over) right up against the word limit in the submitted version and to do as you suggest would add a not inconsiderable number of words. I would rather not remove useful descriptive text if I can help it. If the Journal will allow a few more words, I will do as you suggest. I agree that too many acronyms are distracting – I often find myself going back to where they are first defined to remind myself of the definition of some term or another.
Editor: Please respond to the Reviewers' comments. Regarding the comments of Reviewer #2, please elaborate on the statistical methods (this can be added to the Supplement) and reduce the use of acronyms throughout. You may increase the word count by ~ 10% in order to achieve this.

Response: First, thank you for allowing a few extra words; this will be helpful in replacing some acronyms with appropriate text. I have prepared a Supplementary Methods document but I am somewhat unclear if this is what the reviewer had in mind, as he/she was not very specific about this point.

Reviewer Comments:

Reviewer #1: The authors have overall responded well to reviewer comments

There are still two areas that need addressing

1. The authors have not commented on the low number of PTCS used as controls. Why so few. what was the selection criteria? 'As a comparison group, we also received a matching set of informative PTC variant pedigrees from the large German Consortium for Hereditary Breast and Ovarian Cancer (GC-HBOC).'-What does a matching set mean? You have also used German controls but use UK rates, please comment on how you tally this? Please put something in discussion regarding this

Response: As we state in Methods, all PTC variant pedigrees were contributed by the German Consortium (GC-HBOC). In the original call for pedigrees from the ENIGMA centers, we did not ask for pedigrees with PTCs as our main focus was on estimating risks for pathogenic missense variants. It was only later, when we saw some hint that the cancer risks were somewhat lower for these variants that we thought it would be a good idea to examine some PTC variants. Rather than go back to the 40+ different centers that contributed originally, it was convenient to get these pedigrees from the GC-HBOC consortium because of the high-quality data and more importantly because they had contributed by far the highest number of missense PV pedigrees. Note that we compare separately the 277 GC-HBOC C61G pedigrees to the 324 GC-HBOC BRCA1 PTC pedigrees in the various tables. Here, 'matching set' means that for every GC-HBOC missense pedigree we attempted to gather a PTC pedigree from the same center; basically they were matched for clinical center, of which there are 17 such centers in the German Consortium. We have clarified this in the text on page 9 of the re-revised manuscript. Because of the wide geographic variation in the origin of the pedigrees in the study as a whole, we chose not to use country specific rates, as we pointed out in our response 9 to reviewer 2 in the previous response, specifically: We have reanalyzed the Italian and Greek variants (C64R and G1738R, respectively) using rates specific to those countries (although these countries do not have nationwide cancer registry data) and we did not observe any major differences. We prefer to use the same rates for all analyses to make comparisons between variants easier. In addition some variants have a wide geographic distribution, further complicating the issue.

As we described in the Results section, we performed sensitivity analyses regarding the assumed population incidence rates and showed that the results did not differ substantially as a function of underlying rates (20% higher and 20% lower than assumed UK rates). Specifically to your question, we looked at German incidence rates in Cancer Incidence in Five Continents and the only data available with complete cancer incidence
data appeared to be from the Hamburg region, and thus may not be representative of other areas in Germany. However, in general, they did not differ very much from the UK rates.

2. The authors responded that they only considered ClinVar entries from starred labs. This needs to go in the methods

**Response:** We have added this to the Methods on Page 9 of the re-revised MS.

Reviewer #2: It remains the opinion of this reviewer that this important paper would be more widely read - and certainly more widely understood - if the statistical methods were explained (rather than citing references that, despite the authors' protestations, are inadequate for the purpose) and if the text were written in English rather than in acronyms. There are multiple places in the text where superfluous wording could be removed to make room for an explanation of the approach and for words rather than acronyms. But these are issues of judgement, so I leave the call to these experienced authors.

**Response:** We have eliminated many (but not all) of the Acronyms. As to the more detailed methods, we are at somewhat of a loss as to what could/should be done here. We have tried our best in a supplemental methods section that provides some of the mathematical underpinnings of the statistical approach, but we are not totally sure it is what the reviewer is looking for.
Risks of breast and ovarian cancer for women harboring pathogenic missense variants in BRCA1 and BRCA2 compared with those harboring protein truncating variants.

Hongyan Li,¹,⁶⁴ Christoph Engel,²,⁶⁴ Miguel de la Hoya,³ Paolo Peterlongo,⁴ Drakoulis Yannoukakos,⁵ Luca Livraghi,⁵,⁷ Paolo Radice,⁸ Mads Thomassen,⁹ Thomas V.O. Hansen,¹⁰ Anne-Marie Gerdes,¹⁰ Henriette R. Nielsen,¹¹ Sandrine M. Caputo,¹²,¹³ Alberto Zambelli,⁶ Ake Borg,¹⁴ Angela Solano,¹⁵ Abigail Thomas,¹⁶ Michael T. Parsons,¹⁷ Antonis C. Antoniou,¹⁸ Goska Leslie,¹⁸ Xin Yang,¹⁸ Georgia Chenevix-Trench,¹⁷ Trinidad Caldes,³ Ava Kwong,¹⁹-²⁰ Inge Søkilde Pedersen,²¹-²³ Charlotte K. Lautrup,²²,²³ Esther M. John,²⁴ Mary Beth Terry,²⁵,²⁶ John L. Hopper,²⁷ Melissa C. Southey,²⁸-³⁰ Irene L. Andrulis,³¹,³² Marc Tischkowitz,³³ Ramunas Janavicius,³⁴,³⁵ Susanne E. Boonen,⁹ Lone Kroedrup,⁹ Liliana Varesco,³⁶ Ute Hamann,³⁷ Ana Vega,³⁸ Edenir I. Palmero,³⁹,⁴⁰ Judy Garber,⁴¹ Marco Montagna,⁴² Christi J. Van Asperen,⁴³ Lenka Foretova,⁴⁴ Mark H. Greene,⁴⁵ Tina Selkirk,⁴⁶ Pal Moller,⁴⁷,⁴⁸ Amanda E. Toland,⁴⁹ Susan M. Domchek,⁵⁰ Paul James,⁵¹,⁵² Heather Thorne,⁵² Diana M. Eccles,⁵³, Sarah M. Nielsen,⁵⁴ Siranoush Manoukian,⁵⁵ Barbara Pasini,⁵⁶ Maria A. Caligo,⁵⁷ Conxi Lazo,⁵⁸ Judy Kirk,⁵⁹ Barbara Wappenschmidt,⁶⁰,⁶¹ ENIGMA Consortium, CIMBA Consortium, Amanda B. Spurdle,¹⁷ Fergus J. Couch,⁶²,⁶⁵ Rita Schmutzler,⁶⁰,⁶¹,⁶⁵ David E. Goldgar¹,⁶³,⁶⁵,*

¹ Cancer Control and Population Science, Huntsman Cancer Institute, University of Utah, Salt Lake City, USA.
² Institute for Medical Informatics, Statistics and Epidemiology, University of Leipzig, Leipzig, Germany.
³ Molecular Oncology Laboratory, CIBERONC, Hospital Clínico San Carlos, IdISSC (Instituto de Investigación Sanitaria del Hospital Clínico San Carlos), Madrid, Spain.
4 IFOM - the FIRC Institute of Molecular Oncology, Genome Diagnostics Program, Milan, Italy.
5 National Centre for Scientific Research 'Demokritos', Molecular Diagnostics Laboratory, INRARESTES, Athens, Greece.
6 Medical Oncology Unit, ASST Papa Giovanni XXIII, 24127, Bergamo, Italy.
7 University of Siena, Siena, Italy
8 Unit of Molecular Bases of Genetic Risk and Genetic Testing, Department of Research, Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milan, Italy.
9 Department of Clinical Genetics, Odense University Hospital, Odense, Denmark.
10 Department of Clinical Genetics, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark.
11 Department of Clinical Genetics, Sygehus Lillebaelt, Vejle Hospital, Denmark
12 Service de Génétique, Institut Curie, Paris, France.
13 Paris Sciences Lettres Research University, Paris, France.
14 Division of Oncology and Pathology, Department of Clinical Sciences Lund, Lund University, Lund, Sweden.
15 INBIOMED, Faculty of Medicine, University of Buenos Aires, CONICET and Genotyping Laboratory, Department of Clinical Chemistry, CEMIC, Buenos Aires, Argentina.
16 Department of Health Sciences Research, Mayo Clinic, Rochester, MN, USA.
17 Department of Genetics and Computational Biology, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia.
18 Centre for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK
19 Cancer Genetics Centre, Hong Kong Hereditary Breast Cancer Family Registry, Happy Valley, Hong Kong.
20 The University of Hong Kong, Department of Surgery, Pok Fu Lam, Hong Kong.
21 Molecular Diagnostics, Aalborg University Hospital, Aalborg, Denmark.

23 Clinical Cancer Research Center, Aalborg University Hospital, Aalborg, Denmark.

23 Departments of Clinical Medicine and Clinical Genetics, Aalborg University, Aalborg, Denmark.

24 Department of Epidemiology & Population Health and Stanford Cancer Institute, Stanford University School of Medicine, Stanford, CA, USA.

25 Department of Epidemiology, Columbia University, New York, New York, USA.

26 Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, New York.

27 Centre for Epidemiology and Biostatistics, School of Population and Global Health, The University of Melbourne, Victoria, Australia.

28 Monash University, Precision Medicine, School of Clinical Sciences at Monash Health, Clayton, Victoria, Australia.

28 The University of Melbourne, Department of Clinical Pathology, Melbourne, Victoria, Australia.

30 Cancer Council Victoria, Cancer Epidemiology Division, Melbourne, Victoria, Australia.

31 Lunenfeld-Tanenbaum Research Institute of Mount Sinai Hospital, Fred A. Litwin Center for Cancer Genetics, Toronto, ON, Canada.

32 University of Toronto, Department of Molecular Genetics, Toronto, ON, Canada.

33 Department of Medical Genetics, National Institute for Health Research Cambridge Biomedical Research Centre, University of Cambridge, Cambridge, UK.

34 Faculty of Medicine, Institute of Biomedical Sciences, Dept. Of Human and Medical Genetics, Vilnius University, Vilnius, Lithuania

35 State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania.

36 Unit of Hereditary Cancer, IRCCS Ospedale Policlinico San Martino, Genoa, Italy.

37 German Cancer Research Center (DKFZ), Molecular Genetics of Breast Cancer, Heidelberg,
Germany.

38 Fundación Pública galega Medicina Xenómica-SERGAS, Grupo de Medicina Xenómica-USC, CIBERER, IDIS, Santiago de Compostela, Spain.

39 Molecular Oncology Research Center, Barretos Cancer Hospital, São Paulo, Brazil.

40 Faculdades Pequeno Príncipe, Instituto de Pesquisa Pelé Pequeno Príncipe, Curitiba, Brazil.

41 Dana-Farber Cancer Institute, Cancer Risk and Prevention Clinic, Boston, MA, USA.

42 Immunology and Molecular Oncology Unit, Veneto Institute of Oncology IOV - IRCCS, Padua, Italy.

43 Department of Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands.

44 Masaryk Memorial Cancer Institute, Department of Cancer Epidemiology and Genetics, Brno, Czech Republic.

45 Clinical Genetics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD, USA.

46 NorthShore University HealthSystem, University of Chicago, Evanston, IL, USA.

47 Department of Tumor Biology, Institute of Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo 0424, Norway.

48 Center for Hereditary Tumors, HELIOS-Klinikum Wuppertal, University of Witten-Herdecke, Wuppertal 42283, Germany.

49 Department of Cancer Biology and Genetics, The Ohio State University, Columbus, OH, USA.

50 Basser Center for BRCA, Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA, USA.

51 Familial Cancer Centre, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia.

52 Sir Peter MacCallum Department of Oncology, The University of Melbourne, Victoria, Australia.

53 Faculty of Medicine, University of Southampton, Southampton, UK.
Center for Clinical Cancer Genetics, The University of Chicago, Chicago, IL

Department of Medical Sciences, Medical Genetics Unit, University of Torino, 10126 Torino, Italy.

Unit of Medical Genetics, Department of Medical Oncology and Hematology, Fondazione IRCCS Istituto Nazionale dei Tumori, 20133 Milano, Italy

SOD Genetica Molecolare, University Hospital, Pisa, Italy.

ONCOBELL-IDIBELL-IDIBGI-IGTP, Catalan Institute of Oncology, CIBERONC, Hereditary Cancer Program, Barcelona, Spain.

Familial Cancer Service, Crown Princess Mary Cancer Centre, Westmead Hospital, Sydney Medical School, University of Sydney, Centre for Cancer Research, The Westmead Institute for Medical Research, Westmead, NSW, Australia

Center for Hereditary Breast and Ovarian Cancer, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany.

Center for Integrated Oncology (CIO), Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany.

Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA.

Department of Dermatology, Huntsman Cancer Institute, University of Utah School of Medicine, Salt Lake City, UT, USA.

These authors contributed equally to this work.

*Correspondence: david.goldgar@hsc.utah.edu*

**KEYWORDS:** BRCA1, BRCA2, Missense Variants, Cancer Risks

**SUMMARY**
Purpose: Germline genetic testing for BRCA1 and BRCA2 variants has been part of clinical practice for over two decades. However, no studies have compared the cancer risks associated with missense pathogenic variants (PVs) relative to those for protein-truncating (PTC) variants.

Methods: We collected 582 informative pedigrees segregating one of 28 missense PVs in BRCA1 and 153 pedigrees segregating one of 12 missense PVs in BRCA2. We analyzed 324 pedigrees with PTC variants in BRCA1 and 214 with PTC variants in BRCA2. Cancer risks were estimated using modified segregation analysis.

Results: Estimated BC risks were markedly lower for women over age 50 carrying BRCA1 missense PVs compared to BRCA1 PTCs (HR 3.9 [2.4 – 6.2] vs. 12.8 [5.7 – 28.7] for PTC variants; p=0.01), particularly for missense PVs in the BRCT domain (HR=2.8 [1.4, 5.6]; p=0.005). In BRCA2 the HR for women over age 50 was 3.9 [2.0 – 7.2] for women heterozygous for missense PVs compared to 7.0 [3.3 – 14.7] for those harboring PTC variants. BRCA1 p.[Cys64Arg] and BRCA2 p.[Trp2626Cys], were associated with particularly low risks of BC compared to other PVs.

Conclusion: These results have important implications for counseling of at-risk women who harbor missense PVs in the BRCA1/2 genes.

INTRODUCTION

Germline genetic testing for BRCA1 (OMIM 113705) and BRCA2 (OMIM 600185) variants has long been part of clinical practice. Initially restricted to families that met strict criteria for such testing, the advent of panel tests by a number of clinical diagnostic testing services that has led to the use of these tests in a much broader group of individuals. Once a pathogenic germ-line variant (PV) has been identified women are counseled about their risks of breast, ovarian, and
other cancers and appropriate screening or surgical prevention strategies are discussed. In most situations these cancer risk estimates are based on studies of large collections of families or relatives of breast cancer cases largely unselected for family history; typically these analyses have pooled all pathogenic variants, irrespective of variant type, under the assumption that all such variants are associated with the same risks. The vast majority of the pathogenic BRCA variants included in these studies were variants predicted to result in a transcript encoding a protein termination codon that is subject to nonsense-mediated decay or encoding truncated inactive protein (for simplicity, these will be referred to hereafter as protein truncating variants). The most recent estimates for BRCA1/2 risks associated with pathogenic variants come from the large prospective study of BRCA pathogenic variant heterozygotes conducted by the IBCCS/PROF-SC/kConFab group. Cumulative risks of breast cancer to age 70 were 66% (95% CI 61 – 72) for BRCA1 heterozygotes and 69% (55 – 68) for BRCA2 heterozygotes; corresponding risks of ovarian cancer were 41% (33 – 50) and 15% (10 – 23). There is also accumulating evidence from genotype/phenotype studies that even protein truncating variants may not all be associated with the same risks, depending on their position within the gene.

Analysis of missense variants poses a particular problem because most such variants are expected, a priori, to be of little clinical significance. Through the work of the ENIGMA (Evidence-Based Network Investigating Germline Mutant Alleles) consortium and others, ~60 missense variants have now been classified/reclassified as pathogenic by multifactorial methods using such lines of evidence as co-segregation, family history of index cases in clinical testing series, and tumor histopathology to classify variants of uncertain significance.

To date, all predicted missense substitution variants that have been classified on the basis of genetic data as pathogenic (excluding those that act by disruption of normal splicing) reside in one of three domains of these proteins: the RING domain of BRCA1 (nucleotides 4 – 294); the
BRCA1-C-Terminal (BRCT) repeats in *BRCA1* (nt 4987-5577) and the DNA Binding domain (DBD) of *BRCA2* (nt 7669-9558). However it is not clear if pathogenic missense changes in these important domains are associated with the same levels of risk of breast and ovarian cancer as the protein-truncating/null variants that have been the subject of most studies designed to estimate these risks. Indeed, the *BRCA1* variant c.5096G>A;p.[Arg1699Gln] was shown to be associated with lower risks of breast cancer (~20% by age 70)\(^{14,15}\) and had reduced function in a transcriptional activation assay in the 293T (78% of wild-type activity compared to 45% for the p.[Arg1699Trp] variant included in this study)\(^{14}\). Similarly, functional and case-control analyses have identified the *BRCA2* p.[Tyr3035Ser] variant as a hypomorphic allele associated with only a 2.5-fold increased risk of breast cancer (95% CI 1.05 – 6.05)\(^{16}\).

In order to comprehensively examine the risks associated with established (e.g. those classified as such in ClinVar\(^{17}\), BRCA Challenge\(^{18}\), ENIGMA\(^7\)) missense pathogenic variants in *BRCA1* and *BRCA2*, a large series of pedigrees segregating missense pathogenic variants were collected through the ENIGMA and CIMBA (Consortium of Investigators of Modifiers of BRCA1/2)\(^{19}\) consortia and compared through detailed statistical and genetic analyses with a set of pedigrees segregating protein truncating variants. In addition the histopathological profiles of breast tumors of patients carrying *BRCA1* pathogenic variants and protein truncating variants were compared.

Here we demonstrate that pathogenic missense variants, especially in *BRCA1*, are associated with a reduced risk of breast cancer compared to that for protein truncating variants, particularly at older ages.

**MATERIAL & METHODS**
Through the ENIGMA\textsuperscript{7} Consortium we put out a broad call for pedigree and tumor pathology information from families with a \textit{BRCA1} or \textit{BRCA2} missense variant that, at the time of initiation of the study, had evidence in favor of pathogenicity; this initial list comprised 58 such variants. We also obtained qualifying families from the CIMBA\textsuperscript{8} consortium where complete pedigree information was available. Criteria for submission of families for this study were:

1) Index individual (proband) with a pathogenic or likely pathogenic missense variant in \textit{BRCA1} or \textit{BRCA2}, as determined by ENIGMA through multifactorial analysis\textsuperscript{13}, or ClinVar\textsuperscript{17} (at least two certified (at least * ) submitters denoting it Pathogenic/Likely Pathogenic with none calling it Benign or Likely Benign).

2) Informative for segregation analysis, i.e., at least one non-proband individual tested for the variant;

As a comparison group, we also received a set of 538 pedigrees segregating protein truncating variants from the large German Consortium for Hereditary Breast and Ovarian Cancer (GC-HBOC). These pedigrees were matched by specific clinical center and date of ascertainment/testing to the GC-HBOC missense variant pedigrees.

\textit{Variant Selection}

Pedigrees were contributed in which the index case had one of 58 selected missense variants. Of these, 18 variants were excluded from the analysis as they were determined at the time of analysis to not meet the specified criteria, either because they were found to be acting primarily through disruption of normal splicing and/or other evidence that cast doubt on their classification as pathogenic missense variants. The remaining 40 variants consisted of 11 variants in the
BRCA1 RING domain, 17 in the BRCA1 BRCT domain and 12 in the BRCA2 DBD. Supplementary Table 1 provides detailed genetic and functional information about the 40 included variants. One BRCA1 missense variant (p.[Cys64Gly]) and two BRCA2 missense variants (p.[Glu2663Val] and p.[Asp2723Gly]) show experimental and bioinformatics evidence of pathogenicity through both protein effects and potential splicing effects20,21. Because these variants showed strong deleterious effects in functional assays22-24 and only partial splicing defects25,26 they were included in our analyses.

Family Data

A total of 1146 missense pathogenic variant pedigrees and 543 protein truncating variant pedigrees from 45 contributors representing 17 countries from around the world were submitted for this study. The requested information included pedigree data, age at testing/death/current age, age at diagnosis of breast cancer, age at diagnosis of ovarian cancer, as well as any available information on breast tumor grade, ER, PR, and HER2 status, and prophylactic surgeries. Loss of function variant pedigrees (326 BRCA1 and 217 BRCA2) and 527 of the missense variant pedigrees (including 279 c.181T>G;p.[Cys61Gly]) were contributed by the GC-HBOC network. In addition, we also had relatively large contributions of two founder variants (BRCA1:c.190T>C;p.[Cys64Arg])27 with 51 informative families submitted from Italy and (BRCA1:c.5212G>A;p.[Gly1738Arg])28 with 21 families submitted from Greece. Of the 1689 total submitted families, 391 were excluded from all analyses because they proved to not be informative (308 families) for the planned analyses, did not have one of the 40 final selected variants (93), or had unclear pedigree structures or multiple variants (14).

After the exclusions described above, the data used for the analyses here included 582 informative pedigrees with pathogenic missense variants in BRCA1, 153 with pathogenic
missense variants in BRCA2, 324 pedigrees with protein truncating variants in BRCA1 and 214 with protein truncating variants in BRCA2. Summary information of the characteristics of the set of families included in the study for each of the included variants is shown in Table 1.

**Imputation of Missing Ages and Censoring**

To avoid any biases due to differential information by submitting center (and hence BRCA variant), we imputed missing ages at last follow-up/death and years of birth using the program PedPro\textsuperscript{29} that uses the ages/years of birth of close relatives to assign an age or year of birth to individuals missing such information. Those with imputed ages ≥90 years were imputed to be age 65 since these were likely in older generations without reliable data, and those imputed to be 80-89 were censored at age 80. To be conservative, we assumed that 324 cases with missing ages at diagnosis of breast or ovarian cancer were diagnosed at age at their last follow-up/age at death (including imputed ages). For women with breast cancer and ovarian cancer we considered them as having both cancers if the breast cancer occurred first; women with breast cancer following ovarian cancer were treated as only having ovarian cancer and censored at the age of ovarian cancer diagnosis. Age at bilateral prophylactic mastectomy (BPM) was available in the GC-HBOC data set, in several smaller centers, and in the 36 families contributed from the CIMBA consortium, but not available in the remainder of the dataset. Women without breast or ovarian cancer were censored at their age at last observation or, if deceased, at age at death. When such information was provided, women known to have had a prophylactic mastectomy were censored at age of surgery. Because only a very small subset of the contributed families provided data on risk reducing salpingo-oophorectomy (RRSO) we chose not to censor women at this age; this may lead to an underestimation of ovarian cancer risk but this effect would be expected to be the same for the protein truncating variant pedigrees as well as the missense pathogenic variant set, particularly for the p.[Cys61Gly] variant which has been known to be pathogenic for over 20 years.
Estimation of Risk

Breast and ovarian cancer risks were estimated by maximum likelihood analysis using modified segregation analysis with the MENDEL package of programs\(^{30}\) as implemented for breast, ovarian and other cancers in the recent analyses of families with pathogenic variants in \textit{PALB2}.\(^{31}\) In the present analyses we estimated risks for breast and ovarian cancer jointly, censoring each affected individual at their age at first breast or ovarian cancer. For each dataset, we estimated the hazard ratio (HR) associated with development of breast cancer in the following two age groups: <50 and \(\geq 50\) years. Due to the more limited data for ovarian cancer in this dataset, we assumed a constant HR across age. Thus, in each analysis three parameters were estimated simultaneously. For both breast and ovarian cancer analyses the baseline population incidence rates were assumed to be those for the UK organized in eight 10-year birth cohorts (Cancer Incidence in Five Continents Reports\(^{32}\)) to account for the increasing temporal trends in these cancers over time. In order to adjust for the ascertainment process of the families, the likelihood of observing the pedigree phenotypes and variant genotypes was calculated conditional on the pedigree phenotypes and the genotype of the index case; all information for risk estimation thus comes from the distribution of genotypes (i.e., genotype status) among the phenotypes of the family members of the proband. From the resulting parameter estimates of BC and OC, relative risk, age-specific cumulative risk estimates were calculated from the cumulative incidence \(\Lambda(t) = 1 - \exp(-\Lambda(t))\), assuming UK rates in the women born between 1970 and 1979; the corresponding confidence intervals were calculated using a parametric bootstrap. Heterogeneity of breast or ovarian cancer risk between variants in the same domain was tested by a likelihood ratio test (LRT) of model likelihoods. Comparisons of parameter estimates between missense variants (or groups of variants) and the
corresponding estimate for the protein truncating variant pedigrees was done by constructing a Z-test based on the parameter estimates and their respective standard errors.

RESULTS

**BRCA1 and BRCA2 Risk estimates**

Table 2 presents the results of the modified segregation analyses in which we estimate the risks of breast and ovarian cancer relative to UK population incidence rates. Based on the estimated Hazard Ratios (HR), the biggest difference in risk between pathogenic missense and protein truncating variants was in the risk of breast cancer among women over age 50. For example, for BRCA1 RING domain variants the HR for women 50 and older was roughly half the corresponding estimate for the BRCA1 protein truncating variants. Of note also, the estimated HR for c.181T>G, [p.Cys61Gly] in women 50+ for the subset of 217 informative pedigrees with this variant from the GC-HBOC clinical network was significantly different from the corresponding HR for BRCA1 protein truncating variant pedigrees ascertained from the same GC-HBOC clinical centers (p=0.049). The three groups of BRCA1 RING domain variants as shown in Table 2 did not reveal statistically significant heterogeneity in the estimated HRs ($X^2 = 10.0, 6 \text{ df}; p=0.12$) by likelihood ratio test (LRT)). For BRCA1 BRCT domain missense variants the HR for women 50 and older was significantly reduced compared to protein truncating variant heterozygotes (2.0 vs. 12.8; $p=0.005$). There was no evidence of significant heterogeneity between variants within the BRCT domain and there was no significant difference between the overall estimated models for the RING and BRCT domains ($X^2 = 5.48; 3 \text{ df}; p=0.14$).
No significant difference was observed in the HR of all BRCA2 missense pathogenic variants in the DBD compared to the estimated cancer risks for protein truncating variants\textsuperscript{2,3}. However, there was some evidence of heterogeneity associated with the BRCA2 variants in the DBD with HRs for women over age 50 varying from 1.7 (0.4, 6.9) for c.7878G>C:p.[Trp2626Cys], to 5.9 (2.2, 16.3) for the well-established missense pathogenic variant p.[Asp2723His], and 7.0 (3.3, 14.7) for the BRCA2 protein truncating variants.

When we examined the risks to women over age 60 (data not shown), we observed even lower risks for breast cancer compared to protein truncating, although reduction in sample size increased the standard errors, resulting in wider confidence Intervals. For example, for the RING and BRCT domain pathogenic variant heterozygotes the HRs were 2.8 (1.1, 7.3) and 2.0 (0.6, 6.6), respectively, versus 16.5 (4.3, 63.2) in the BRCA1 protein truncating pedigrees in our study. For BRCA2 pathogenic DBD missense variants, the HR for women over age 60 was 2.0 (0.6, 6.3) vs. 5.3 (1.6, 17.9) in the BRCA2 protein truncating pedigrees.

In general the estimated HRs for ovarian cancer were quite similar in the various pathogenic missense variant analyses although risks were somewhat reduced for the BRCA1 BRCT variants but this was not statistically significant. Estimated cumulative risks of BC to age 50 and age 70 and of OC to age 70 for the primary sets of variants are shown in Table 3. Globally, risks to age 50 did not vary substantially between missense pathogenic variants and their protein truncating variant counterparts However the estimated risks to age 70 were more variable, due to the HRs for ages greater than 50 being in general lower for the missense variants.

*Estrogen Status of Breast Tumors with BRCA1 Variants*

ER status was available for 361 breast tumors from women harboring a BRCA1 pathogenic missense variant. These included 250 from missense variants in the RING domain (220 of
which were from p.[Cys61Gly] heterozygotes) and 111 in the BRCT domain, to compare to 210 tumors from BRCA1 protein truncating heterozygotes (Table 4). There was no significant difference in the frequency of ER negative tumors among breast cancers from BRCA1 missense pathogenic variant women compared to the tumors from women heterozygous for a protein truncating variant. Individually, only p.[Ala1708Glu] had a nominally significantly lower frequency of ER negative tumors (52% vs. 72%; p=0.027) compared to tumors from women with protein truncating variants, although this was not significant when corrected for the number of comparisons made.

**BC risk associated with specific variants**

We found that the breast (but not ovarian) cancer risks associated with two specific missense pathogenic variants were lower than that for other BRCA1 missense pathogenic variants. The HR for women under age 50 associated with the p.[Cys64Arg] missense pathogenic variants was 3.7 (95% CI 1.4,9.5) and that for women ≥50 was 4.1 (0.9, 18.3). In particular, the HR for younger women was substantially lower than that for other RING and BRCT variants. We tested this variant against the estimated risk for the p.[Cys61Gly] variant assuming a constant HR and found it to be significantly different (Z-test; p=0.015). These lower estimated risks are consistent with the observation of 10 BC cases in these families who tested negative for the variant, mean age at diagnosis of 48.6) compared with 14 non-proband cases that tested positive (mean age at diagnosis of 43.9). Moreover, among 13 women unaffected with breast or ovarian cancer over the age of 60 tested for the variant, almost half (6/13) were found to be positive, also indicating a lower penetrance. Thus the evidence suggests that this BRCA1 missense pathogenic variant has substantially reduced breast cancer risk (but not necessarily ovarian cancer) compared to other pathogenic variants while still higher than the population incidence rates. The risks associated with the BRCA2 p.[Trp2626Cys] missense variant for breast and ovarian cancer is significantly reduced compared to both BRCA2 protein truncating variants or
other \textit{BRCA2} missense pathogenic variants analyzed in this study (Table 2). Among 31 female relatives who tested positive for the p.[Trp2626Cys] variant in the 34 pedigrees there were only three cases of breast cancer and one ovarian cancer. Moreover, among 22 unaffected women over age 60 who tested for the variant, 13 were found to be positive for the p.[Trp.2626Cys] variant, also indicating that this variant has reduced penetrance.

\textit{Sensitivity Analyses for Imputed Age}

Reanalysis of the data assuming all individuals with missing year of birth were born in the period 1960-1969 (thus eliminating cohort differences in incidence rates for these individuals) resulted in only a minimal effect on the estimates since the vast majority of the missing ages were in individuals who were not tested or closely related to a tested individual, and thus did not contribute heavily to the analysis. Individuals with missing current age/age at death were excluded in these analyses as well. Results of these analyses are shown in Supplementary Table 2. For example, for the \textit{BRCA1} protein truncating set of pedigrees, the HRs for BC<50 years, BC≥50 years and OC were 16.4, 12.1, and 26.3, respectively without the missing age imputation and 17.0, 12.8 and 27.1 (Table 2) using imputed age data.

\textit{Sensitivity Analyses population incidence rates}

In these analyses we assumed age- and cohort-specific cancer incidence rates for the UK. In order to see how our results and conclusions could be affected by possible differences in rates we re-analyzed the larger subsets of pedigrees assuming both rates that were 20\% higher and 20\% lower than these (across all ages and birth cohorts). Given that the vast majority of the pedigrees submitted came from Western European clinical centers, this should account for incidence rate differences among the various populations included here. The parameter
estimates for the \textit{BRCA1} BRCT domain variants, RING domain variants and protein truncating variants from the GC-HBOC resource were on average 5% higher when incidence rates were assumed to be 20% lower and estimates were 4% lower when rates were set to be 20% higher. The largest differences were seen for the ln(HR) estimates for ovarian cancer which were 11% higher for the set of \textit{BRCA1} RING domain variants when incidence rates were assumed to be 20% lower. Based on these analyses, differences in rates from our assumed UK rates are an unlikely explanation for the findings.

\section*{DISCUSSION}

Here we report a large international collaborative study examining the risks of breast and ovarian cancer for women harboring a pathogenic missense variants in the \textit{BRCA1} and \textit{BRCA2} genes. We benefited from a large national study of hereditary breast and ovarian cancer in Germany (GC-HBOC) that contributed approximately half of the missense pathogenic variant families and a series of families that segregated protein truncating variants in \textit{BRCA1} and \textit{BRCA2} in order to form comparison groups for the missense variants in each gene. The results shown in Tables 2 and 3 clearly indicate that missense pathogenic variants in both functionally important domains (RING and BRCT) in \textit{BRCA1} are associated with lower risks of breast cancer than protein truncating variants. Although data are more limited, it appears that ovarian cancer risks are comparable (though on average perhaps slightly lower) for \textit{BRCA1} to those found in published risk estimates for protein truncating variants\textsuperscript{1-3}, and also comparable to those estimated from the protein truncating variant pedigrees from GC-HBOC. Although associated with lower breast cancer risks, missense pathogenic variants in \textit{BRCA1} had similar hormone receptor status profiles as their protein truncating counterpart; importantly the families studied here were largely tested before TN status was a stand-alone criterion for \textit{BRCA} testing, suggesting a lack of ascertainment bias based on pathology. Most \textit{BRCA2} pathogenic missense
variants appeared to be associated with lower risks compared to the \textit{BRCA2} protein truncating families in the over 50 age group (Table 2); however these differences were not statistically significant and were of smaller magnitude than the parallel comparisons for \textit{BRCA1} variants.

\textit{Clinical considerations}

Our results provide evidence of the convergence of moderate- to high-risk classes that no longer seem to be distinct but rather describe a risk continuum. Indeed we have previously shown that the hypomorphic \textit{BRCA1} variant p[Arg1699Gln] is associated with reduced levels of breast cancer risk compared to the average \textit{BRCA1} truncating variant\textsuperscript{15,16}. That raises the question of what clinical measures to offer for different levels of risk. The estimated cumulative risks to age 70 of 70\% for \textit{BRCA1} protein truncating variants compared to 39\% for the combined group of missense pathogenic variants in the BRCT repeat domains (primarily p.[Ala1708Glu] and p.[Arg1699Trp] are significantly different and may very well affect a woman's choice of prevention/screening options, in particular bilateral prophylactic mastectomy. Further, these risks will depend on family history, their polygenic risk score (PRS)\textsuperscript{33}, and lifestyle factors\textsuperscript{34}. We believe that these new risk estimates for \textit{BRCA1} missense variants should be incorporated into comprehensive risk prediction tools such as BOADICEA\textsuperscript{35}. Although, on average slightly lower, the ovarian cancer risks associated with both \textit{BRCA1} and \textit{BRCA2} missense pathogenic variants did not seem to differ markedly from that for the protein truncating variants (with the possible exception of pathogenic variants in the BRCT domain of \textit{BRCA1}). For cancer-free women over age 50, many of the \textit{BRCA1} pathogenic missense variants are associated with relative breast cancer risks closer to those estimated for pathogenic variants in genes such as \textit{ATM}, \textit{CHEK2}, and other moderate risk genes, indicating that surveillance might be an optimal approach. However, given the high ovarian cancer risks of 36\% by age 70 for RING domain missense pathogenic variants, we recommend that women heterozygous for such
these variants should be counseled the same with respect to ovarian cancer as women with
BRCA1 protein truncating variants in terms of recommendations for RRSO. Clinically, these
data strengthen the importance of communicating not only the cumulative lifetime risks but also
risks within a manageable timespan, e.g., 10-year risks.

For the BRCA1 p.[Cys64Arg] variants we propose counseling heterozygotes similarly to those
proposed for the BRCA1 p.[Arg1699Gln] hypomorphic variant. The p.[Cys64Arg] variant has
been classified as pathogenic by the majority of clinical laboratories so it will be important to
collect more genetic data in order to clarify the status of this variant and its associated cancer
risks.

We also noted particularly low breast cancer risk estimates for BRCA2 p.[Trp2626Cys]. The
functional effects of this variant have been examined and although it was classified as
functionally deleterious, it was near the boundary between deleterious and intermediate
function. This variant is denoted in ClinVar as pathogenic by nine clinical laboratories, and as
likely pathogenic by three others, with a single lab reporting it in ClinVar as a Variant of
Uncertain Significance. This variant has also been evaluated in a number of other recent
studies. Based on family histories of 12 individuals heterozygous for p.[Trp2626Cys] in a large
clinical testing set, we estimated odds of 16:1 against pathogenicity and in the large
OncoArray breast cancer case-control series this variant was identified in 16/75350 cases
and 5/52793 controls (OR=2.2 95% CI 0.8 – 7.8; p=0.1) which is consistent with the estimate
reported here (K. Michailidou, personal communication). Taken together the available evidence
indicates that caution should be exercised in counseling individuals harboring this variant as a
pathogenic BRCA2 variant until further genetic and functional studies can be performed.

Limitations and Caveats
The primary limitation inherent in our study is that families were submitted from a wide variety of countries and clinical centers that could vary widely with respect to ascertainment criteria, cascade testing, and prospective follow-up. Although in our analysis, we adjust for the phenotypes of all pedigree members and relied only on the genotype status of non-proband family members, it is possible that different practices in different centers could affect the risk estimates. However, the consistency of our results together with the direct comparison of p.[Cys61Gly] with protein truncating variant pedigrees from the GC-HBOC resource make it highly unlikely that systematic biases could explain our findings.

A second limitation of this study is the lack of complete data on prophylactic surgery, and potentially differing rates of uptake across countries. In order to ensure that the observed differences in risk were not due to the censoring of women at BPM in some of the data sets and not others, we repeated analyses ignoring the BPM information. In particular we were concerned about the effect in the BRCA1 and BRCA2 reference sets as they were used as comparison to the missense pathogenic variants. The mean difference between age at BPM and current age/age at death was only 2.9 years (95% CI 1.9 – 3.9) indicating that differential information with regard to BPM would not alter our conclusions.

Conclusions

Our analyses of ~1250 informative pedigrees have demonstrated that BRCA1 missense pathogenic variants are associated with smaller increased risks of breast cancer in women over age 50 when compared to variants predicted to result in complete loss of function. The risk reduction is less pronounced for BRCA2 in such women. We have also shown that specific variants are associated with particularly low risks compared to other BRCA pathogenic variants: BRCA1 p.[Cys64Arg] and BRCA2 p.[Trp2626Cys] for breast cancer and possibly BRCA1
p.[Ala1708Glu] for ovarian cancer. Interestingly, the histopathology of breast cancers from patients carrying pathogenic missense variants in \textit{BRCA1} showed similar rates of ER negative/triple negative status as the cancers in protein truncating patients. Future studies should focus on the functional basis for the reduction in relative risks for older women who harbor a pathogenic missense variant in \textit{BRCA1}, and to a lesser extent, \textit{BRCA2}.

\textbf{Supplemental Data}

There are two supplementary tables are associated with this article. Supplementary Table 1 (Excel) provides detailed information on the 40 variants analyzed in this study. Supplementary Table 2 displays the HR estimates excluding individuals with missing age data and assumes individuals with missing year of birth information were born between 1960-1969.

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Data and Code Availability

Data requests for files used in the analysis should be made to D. Goldgar although European GDPR regulations may not permit pedigree data to be transferred outside the EU.

Declaration of Interests
F.J.C. has received consulting fees from Astrazeneca.

A.S. has received consulting fees from Pfizer and Astra Zeneca.

The remaining authors declare no competing interests.

**Ethics**

This study was covered under Amendment to Project P1051 (PI Prof. Amanda Spurdle) approved by the Human Research Ethics Committee at QIMR Berghofer, Brisbane Australia, Oct 7 2020. All patients gave consent to have their data used for research purposes and all studies received local Ethics Committee approvals. Data from all centers were de-identified prior to analysis at the coordinating center.

**Web Resources**

Brca Exchange: [https://brcaexchange.org/](https://brcaexchange.org/) Accessed 2/15/2021


Author Contributions

Conceptualization: D.G., A.S., F.C., R.S., B.W.


Formal Analysis: H.L., D.G.

Funding Acquisition F.C., A.S.

Methodology: D.G., A.C., X.Y., M.P., H.L.

Writing – original draft: D.G.

Writing – review & editing: All Authors
References


histopathological predictors of BRCA1 and BRCA2 mutation status: a large-scale analysis of breast cancer characteristics from the BCAC, CIMBA, and ENIGMA consortia. Breast Cancer Res. 16(6):3419 PubMed PMID: 25857409; PubMed Central PMCID: PMC4352262.


Table 1. Description of variants and families included in the risk estimation analyses. Index cases are excluded from tabulations.

<table>
<thead>
<tr>
<th>Variant/Group</th>
<th>Informative Families</th>
<th>Breast Cancer</th>
<th>Ovarian Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heterozygous</td>
<td>Wild-Type</td>
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<tr>
<td><strong>BRCA1</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GC-HBOC BRCA1 PTC</td>
<td>324</td>
<td>85</td>
<td>7</td>
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<tr>
<td><strong>RING Domain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.181T&gt;G, p.[Cys61Gly]</td>
<td>316</td>
<td>114</td>
<td>14</td>
</tr>
<tr>
<td>c.190T&gt;C; p.[Cys64Arg]</td>
<td>55</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Other RING Domain¹</td>
<td>34</td>
<td>13</td>
<td>3</td>
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<tr>
<td><strong>All RING Domain</strong></td>
<td>405</td>
<td>141</td>
<td>27</td>
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<tr>
<td>GC-HBOC c.181T&gt;G, p.[Cys61Gly]</td>
<td>277</td>
<td>81</td>
<td>9</td>
</tr>
<tr>
<td><strong>BRCT Domain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.5095C&gt;T, p.[Arg1699Trp]</td>
<td>43</td>
<td>11</td>
<td>2</td>
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<tr>
<td>Variant</td>
<td>c.5123C&gt;A, p.[Ala1708Glu]</td>
<td>c.5212G&gt;A, p.[Gly1738Arg]</td>
<td>Other BRCT Domain</td>
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<td></td>
<td>56</td>
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<td>0</td>
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<td>36</td>
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<tr>
<td></td>
<td>177</td>
<td>74</td>
<td>10</td>
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<tr>
<td></td>
<td>582</td>
<td>215</td>
<td>37</td>
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</tr>
<tr>
<td>All DBD Missense</td>
<td>153</td>
<td>49</td>
<td>11</td>
</tr>
</tbody>
</table>

**BRCA1 Missense**

- **Other BRCT Domain**: 42 changes
- **All BRCT Domain**: 177 changes
- **All BRCA1 Missense**: 582 changes

**BRCA2**

- **GCHBOC BRCA2 PTC**: 214 changes
- **DNA Binding Domain**
  - c.7878G>C, p.[Trp2626Cys]: 34 changes
  - c.8167G>C, p.[Asp2723His]: 33 changes
  - c.9154C>T, p.[Arg3052Trp]: 20 changes
- **Other B2 DBD**: 66 changes
- **All DBD Missense**: 153 changes
1 c.53T>C, p.[Met18Thr]; c.65T>C, p.[Leu22Ser]; c.110C>A, p.[Thr37Lys]; c.115T>C, p.[Cys39Arg]; c.116G>A, p.[Cys39Tyr]; c.130T>A, p.[Cys44Ser]; c.131G>T, p.[Cys44Phe]; c.190T>G, p.[Cys64Gly]; c.191G>A, p.[Cys64Tyr]

c.5053A>G, p.[Thr1685Ala]; c.5054C>T, p.[Thr1685Ile]; c.5089T>C, p.[Cys1697Arg]; c.5117G>A, p.[Gly1706Glu]; c.5143A>C, p.[Ser1715Arg]; c.5213G>A, p.[Gly1738Glu]; c.5216A>T, p.[Asp1739Val]; c.5291T>C, p.[Leu1764Pro]; c.5297T>G, p.[Ile1766Ser]; c.5309G>T, p.[Gly1770Val]; c.5324T>A, p.[Met1775Lys]; c.5324T>G, p.[Met1775Arg]; c.5363G>T, p.[Gly1788Val]; c.5513T>A, p.[Val1838Glu]

2 c.5053A>G, p.[Thr1685Ala]; c.5054C>T, p.[Thr1685Ile]; c.5089T>C, p.[Cys1697Arg]; c.5117G>A, p.[Gly1706Glu]; c.5143A>C, p.[Ser1715Arg]; c.5213G>A, p.[Gly1738Glu]; c.5216A>T, p.[Asp1739Val]; c.5291T>C, p.[Leu1764Pro]; c.5297T>G, p.[Ile1766Ser]; c.5309G>T, p.[Gly1770Val]; c.5324T>A, p.[Met1775Lys]; c.5324T>G, p.[Met1775Arg]; c.5363G>T, p.[Gly1788Val]; c.5513T>A, p.[Val1838Glu]

3 c.7879A>T, p.[Ile2627Phe]; c.7940T>C, p.[Leu2647Pro]; c.7958T>C, p.[Leu2653Pro]; c.7988A>T p.[Glu2663Val]; c.8165C>G, p.[Thr2722Arg]; c.8168G>A, p.[Asp2723Gly]; c.8243G>A, p.[Gly2748Asp]; c.9004G>A, p.[Glu3002Lys]; c.9227G>A, p.[Gly3076Glu]
Table 2. Hazard Ratio (HR) estimates by variant/group of variants.

<table>
<thead>
<tr>
<th>Variant/Variant Group</th>
<th>Breast Cancer Dx&lt;50</th>
<th>Breast Cancer Dx≥50</th>
<th>Ovarian Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td><strong>BRCA1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-HBOC BRCA1 PTC</td>
<td>17.0 (9.4, 30.5)</td>
<td>12.8 (5.7, 28.7)</td>
<td>27.1 (8.6, 85.2)</td>
</tr>
<tr>
<td>RING Domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.[Cys61Gly]</td>
<td>14.6 (8.8, 24.3)</td>
<td>7.6 (3.6, 16.4)</td>
<td>41.2 (19.0, 89.3)</td>
</tr>
<tr>
<td>p.[Cys64Arg]</td>
<td>3.7 (1.4, 9.5)*</td>
<td>4.1 (0.9, 18.3)</td>
<td>99.9 (37.8, 264)</td>
</tr>
<tr>
<td>Other RING Domain</td>
<td>12.0 (3.2, 37.4)</td>
<td>2.9 (0.5, 17.0)</td>
<td>23.9 (4.6, 119)</td>
</tr>
<tr>
<td>All RING Domain</td>
<td>11.5 (7.2, 18.3)</td>
<td>5.8 (3.0, 11.3)</td>
<td>41.0 (20.9, 80.4)</td>
</tr>
<tr>
<td>GC-HBOC p.[Cys61Gly]</td>
<td>15.3 (8.3, 28.2)</td>
<td>3.7 (1.5, 9.4)*</td>
<td>27.1 (7.2, 102.8)</td>
</tr>
<tr>
<td><strong>BRCA2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-HBOC BRCA2 PTC</td>
<td>10.4 (5.9, 19.8)</td>
<td>7.0 (3.3, 14.7)</td>
<td>13.1 (3.7, 45.6)</td>
</tr>
<tr>
<td><strong>DNA Binding Domain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.[Trp2626Cys]</td>
<td>5.0 (0.3, 92.0)</td>
<td>1.7 (0.4, 6.9)</td>
<td>2.1 (0.1, 32.8)</td>
</tr>
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<td>------------------</td>
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<td>--------</td>
</tr>
<tr>
<td>p.[Asp2723His]</td>
<td>8.5 (2.5, 28.7)</td>
<td>5.2 (1.5, 18.6)</td>
<td>15.0 (2.1, 109)</td>
</tr>
<tr>
<td>p.[Arg3052Trp]</td>
<td>8.1 (0.9, 73.3)</td>
<td>3.5 (0.4, 30.0)</td>
<td>4.1 (0.3, 115)</td>
</tr>
<tr>
<td>Other DBD</td>
<td>9.5 (3.8, 23.7)</td>
<td>5.3 (2.1, 13.2)</td>
<td>5.6 (1.4, 21.8)</td>
</tr>
<tr>
<td>All DBD</td>
<td>8.3 (2.2, 30.8)</td>
<td>3.9 (2.0, 7.2)</td>
<td>5.5 (2.0, 14.8)</td>
</tr>
</tbody>
</table>

*p<0.05; **p<0.01 for test of parameter vs. corresponding parameter for LOF variants.*
Table 3. Theoretical Cumulative Risks for selected groups of variants based on HR estimates and UK incidence rates (1960-1969).

<table>
<thead>
<tr>
<th>Variant Group</th>
<th>Breast Cancer</th>
<th>Ovarian Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Risk to Age 50 (95% CI)</td>
<td>Risk to Age 70 (95% CI)</td>
</tr>
<tr>
<td>GC-HBOC BRCA1 PTC</td>
<td>0.33 (0.20, 0.51)</td>
<td>0.70 (0.51, 0.89)</td>
</tr>
<tr>
<td>All RING Domain</td>
<td>0.21 (0.14, 0.31)</td>
<td>0.46 (0.33, 0.61)</td>
</tr>
<tr>
<td>All BRCT Domain</td>
<td>0.30 (0.18, 0.44)</td>
<td>0.39 (0.27, 0.54)</td>
</tr>
<tr>
<td>All BRCA1 Missense</td>
<td>0.24 (0.17, 0.32)</td>
<td>0.40 (0.32, 0.49)</td>
</tr>
<tr>
<td>GC-HBOC BRCA2 PTC</td>
<td>0.22 (0.12, 0.37)</td>
<td>0.51 (0.34, 0.69)</td>
</tr>
<tr>
<td>All DBD</td>
<td>0.18 (0.09, 0.31)</td>
<td>0.36 (0.24, 0.51)</td>
</tr>
<tr>
<td>Variant/Group</td>
<td>Number of Tumors</td>
<td>Number ER Positive (%)</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>GCHBOC BRCA1 PTC</td>
<td>210</td>
<td>59 (28)</td>
</tr>
<tr>
<td>p.[Cys61Gly]</td>
<td>216</td>
<td>55 (25)</td>
</tr>
<tr>
<td>p.[Cys64Arg]</td>
<td>21</td>
<td>4 (19)</td>
</tr>
<tr>
<td>Other RING Domain&lt;sup&gt;1&lt;/sup&gt;</td>
<td>11</td>
<td>2 (18)</td>
</tr>
<tr>
<td>p.[Arg1699Trp]</td>
<td>26</td>
<td>8 (31)</td>
</tr>
<tr>
<td>p.[Ala1708Glu]</td>
<td>29</td>
<td>14 (48)</td>
</tr>
<tr>
<td>p.[Gly1738Arg]</td>
<td>33</td>
<td>9 (27)</td>
</tr>
<tr>
<td>Other BRCT Domain</td>
<td>23</td>
<td>6 (26)</td>
</tr>
<tr>
<td>All BRCA1 Missense</td>
<td>359</td>
<td>98 (27)</td>
</tr>
</tbody>
</table>
Risks of breast and ovarian cancer for women harboring pathogenic missense variants in \textit{BRCA1} and \textit{BRCA2} compared with those harboring protein truncating variants.

Hongyan Li,\textsuperscript{1,\textasciitilde64} Christoph Engel,\textsuperscript{2,\textasciitilde64} Miguel de la Hoya,\textsuperscript{3} Paolo Peterlongo,\textsuperscript{4} Drakoulis Yannoukakos,\textsuperscript{5} Luca Livraghi,\textsuperscript{6,7} Paolo Radice,\textsuperscript{8} Mads Thomassen,\textsuperscript{9} Thomas V.O. Hansen,\textsuperscript{10} Anne-Marie Gerdes,\textsuperscript{10} Henriette R. Nielsen,\textsuperscript{11} Sandrine M. Caputo,\textsuperscript{12,13} Alberto Zambelli,\textsuperscript{6} Ake Borg,\textsuperscript{14} Angela Solano,\textsuperscript{15} Abigail Thomas,\textsuperscript{16} Michael T. Parsons,\textsuperscript{17} Antonis C. Antoniou,\textsuperscript{18} Goska Leslie,\textsuperscript{18} Xin Yang,\textsuperscript{18} Georgia Chenevix-Trench,\textsuperscript{17} Trinidad Caldes,\textsuperscript{3} Ava Kwong,\textsuperscript{19-20} Inge Søkilde Pedersen,\textsuperscript{21-23} Charlotte K. Lautrup,\textsuperscript{22,23} Esther M. John,\textsuperscript{24} Mary Beth Terry,\textsuperscript{25,26} John L. Hopper,\textsuperscript{27} Melissa C. Southey,\textsuperscript{28-30} Irene L. Andrulis,\textsuperscript{31,32} Marc Tischkowitz,\textsuperscript{33} Ramunas Janavicius,\textsuperscript{34,35} Susanne E. Boonen,\textsuperscript{9} Lone Kroeldrup,\textsuperscript{9} Liliana Varesco,\textsuperscript{36} Ute Hamann,\textsuperscript{37} Ana Vega,\textsuperscript{38} Edenir I. Palmero,\textsuperscript{39,40} Judy Garber,\textsuperscript{41} Marco Montagna,\textsuperscript{42} Christi J. Van Asperen,\textsuperscript{43} Lenka Foretova,\textsuperscript{44} Mark H. Greene,\textsuperscript{45} Tina Selkirk,\textsuperscript{46} Pal Moller,\textsuperscript{47,48} Amanda E. Toland,\textsuperscript{49} Susan M. Domchek,\textsuperscript{50} Paul James,\textsuperscript{51,52} Heather Thorne,\textsuperscript{52} Diana M. Eccles,\textsuperscript{53} Sarah M. Nielsen,\textsuperscript{54} Siranoush Manoukian,\textsuperscript{55} Barbara Pasini,\textsuperscript{56} Maria A. Caligo,\textsuperscript{57} Conxi Lazaro,\textsuperscript{58} Judy Kirk,\textsuperscript{59} Barbara Wappenschmidt,\textsuperscript{60,61} ENIGMA Consortium, CIMBA Consortium, Amanda B. Spurdle,\textsuperscript{17} Fergus J. Couch,\textsuperscript{62,65} Rita Schmutzler,\textsuperscript{60,61,65} David E. Goldgar\textsuperscript{1,63,65}.

1 Cancer Control and Population Science, Huntsman Cancer Institute, University of Utah, Salt Lake City, USA.

2 Institute for Medical Informatics, Statistics and Epidemiology, University of Leipzig, Leipzig, Germany.

3 Molecular Oncology Laboratory, CIBERONC, Hospital Clínico San Carlos, IdISSC (Instituto de Investigación Sanitaria del Hospital Clínico San Carlos), Madrid, Spain.
4 IFOM - the FIRC Institute of Molecular Oncology, Genome Diagnostics Program, Milan, Italy.
5 National Centre for Scientific Research 'Demokritos', Molecular Diagnostics Laboratory, INRAGES, Athens, Greece.
6 Medical Oncology Unit, ASST Papa Giovanni XXIII, 24127, Bergamo, Italy.
7 University of Siena, Siena, Italy
8 Unit of Molecular Bases of Genetic Risk and Genetic Testing, Department of Research, Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milan, Italy.
9 Department of Clinical Genetics, Odense University Hospital, Odense, Denmark.
10 Department of Clinical Genetics, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark.
11 Department of Clinical Genetics, Sygehus Lillebaelt, Vejle Hospital, Denmark
12 Service de Génétique, Institut Curie, Paris, France.
13 Paris Sciences Lettres Research University, Paris, France.
14 Division of Oncology and Pathology, Department of Clinical Sciences Lund, Lund University, Lund, Sweden.
15 INBIOMED, Faculty of Medicine, University of Buenos Aires, CONICET and Genotyping Laboratory, Department of Clinical Chemistry, CEMIC, Buenos Aires, Argentina.
16 Department of Health Sciences Research, Mayo Clinic, Rochester, MN, USA.
17 Department of Genetics and Computational Biology, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia.
18 Centre for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK
19 Cancer Genetics Centre, Hong Kong Hereditary Breast Cancer Family Registry, Happy Valley, Hong Kong.
20 The University of Hong Kong, Department of Surgery, Pok Fu Lam, Hong Kong.
Molecular Diagnostics, Aalborg University Hospital, Aalborg, Denmark.

Clinical Cancer Research Center, Aalborg University Hospital, Aalborg, Denmark.

Departments of Clinical Medicine and Clinical Genetics, Aalborg University, Aalborg, Denmark.

Department of Epidemiology & Population Health and Stanford Cancer Institute, Stanford University School of Medicine, Stanford, CA, USA.

Department of Epidemiology, Columbia University, New York, New York, USA.

Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, New York.

Centre for Epidemiology and Biostatistics, School of Population and Global Health, The University of Melbourne, Victoria, Australia.

Monash University, Precision Medicine, School of Clinical Sciences at Monash Health, Clayton, Victoria, Australia.

The University of Melbourne, Department of Clinical Pathology, Melbourne, Victoria, Australia.

Cancer Council Victoria, Cancer Epidemiology Division, Melbourne, Victoria, Australia.

Lunenfeld-Tanenbaum Research Institute of Mount Sinai Hospital, Fred A. Litwin Center for Cancer Genetics, Toronto, ON, Canada.

University of Toronto, Department of Molecular Genetics, Toronto, ON, Canada.

Department of Medical Genetics, National Institute for Health Research Cambridge Biomedical Research Centre, University of Cambridge, Cambridge, UK.

Faculty of Medicine, Institute of Biomedical Sciences, Dept. Of Human and Medical Genetics, Vilnius University, Vilnius, Lithuania

State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania.

Unit of Hereditary Cancer, IRCCS Ospedale Policlinico San Martino, Genoa, Italy.

German Cancer Research Center (DKFZ), Molecular Genetics of Breast Cancer, Heidelberg,
38 Fundación Pública galega Medicina Xenómica-SERGAS, Grupo de Medicina Xenómica-USC, CIBERER, IDIS, Santiago de Compostela, Spain.

39 Molecular Oncology Research Center, Barretos Cancer Hospital, São Paulo, Brazil.

40 Faculdades Pequeno Príncipe, Instituto de Pesquisa Pelé Pequeno Príncipe, Curitiba, Brazil.

41 Dana-Farber Cancer Institute, Cancer Risk and Prevention Clinic, Boston, MA, USA.

42 Immunology and Molecular Oncology Unit, Veneto Institute of Oncology IOV - IRCCS, Padua, Italy.

43 Department of Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands.

44 Masaryk Memorial Cancer Institute, Department of Cancer Epidemiology and Genetics, Brno, Czech Republic.

45 Clinical Genetics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD, USA.

46 NorthShore University HealthSystem, University of Chicago, Evanston, IL, USA.

47 Department of Tumor Biology, Institute of Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo 0424, Norway.

48 Center for Hereditary Tumors, HELIOS-Klinikum Wuppertal, University of Witten-Herdecke, Wuppertal 42283, Germany.

49 Department of Cancer Biology and Genetics, The Ohio State University, Columbus, OH, USA.

50 Basser Center for BRCA, Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA, USA.

51 Familial Cancer Centre, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia.

52 Sir Peter MacCallum Department of Oncology, The University of Melbourne, Victoria, Australia.

53 Faculty of Medicine, University of Southampton, Southampton, UK.
Center for Clinical Cancer Genetics, The University of Chicago, Chicago, IL

Department of Medical Sciences, Medical Genetics Unit, University of Torino, 10126 Torino, Italy.

Unit of Medical Genetics, Department of Medical Oncology and Hematology, Fondazione IRCCS Istituto Nazionale dei Tumori, 20133 Milano, Italy

SOD Genetica Molecolare, University Hospital, Pisa, Italy.

ONCOBELL-IDIBELL-IDIBGI-IGTP, Catalan Institute of Oncology, CIBERONC, Hereditary Cancer Program, Barcelona, Spain.

Familial Cancer Service, Crown Princess Mary Cancer Centre, Westmead Hospital, Sydney Medical School, University of Sydney, Centre for Cancer Research, The Westmead Institute for Medical Research, Westmead, NSW, Australia

Center for Hereditary Breast and Ovarian Cancer, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany.

Center for Integrated Oncology (CIO), Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany.

Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA.

Department of Dermatology, Huntsman Cancer Institute, University of Utah School of Medicine, Salt Lake City, UT, USA.

These authors contributed equally to this work.

These authors contributed equally to this work.

Correspondence: david.goldgar@hsc.utah.edu

KEYWORDS: BRCA1, BRCA2, Missense Variants, Cancer Risks

SUMMARY
Purpose: Germline genetic testing for BRCA1 and BRCA2 variants has been part of clinical practice for over two decades. However, no studies have compared the cancer risks associated with missense pathogenic variants (PVs) relative to those for protein-truncating (PTC) variants.

Methods: We collected 582 informative pedigrees segregating one of 28 missense PVs in BRCA1 and 153 pedigrees segregating one of 12 missense PVs in BRCA2. We analyzed 324 pedigrees with PTC variants in BRCA1 and 214 with PTC variants in BRCA2. Cancer risks were estimated using modified segregation analysis.

Results: Estimated BC risks were markedly lower for women over age 50 carrying BRCA1 missense PVs compared to BRCA1 PTCs (HR 3.9 [2.4 – 6.2] vs. 12.8 [5.7 – 28.7] for PTC variants; p=0.01), particularly for missense PVs in the BRCT domain (HR=2.8 [1.4, 5.6]; p=0.005). In BRCA2 the HR for women over age 50 was 3.9 [2.0 – 7.2] for women heterozygous for missense PVs compared to 7.0 [3.3 – 14.7] for those harboring PTC variants. BRCA1 p.[Cys64Arg] and BRCA2 p.[Trp2626Cys], were associated with particularly low risks of BC compared to other PVs.

Conclusion: These results have important implications for counseling of at-risk women who harbor missense PVs in the BRCA1/2 genes.

INTRODUCTION

Germline genetic testing for BRCA1 (OMIM 113705) and BRCA2 (OMIM 600185) variants has long been part of clinical practice. Initially restricted to families that met strict criteria for such testing, the advent of panel tests by a number of clinical diagnostic testing services that has led to the use of these tests in a much broader group of individuals. Once a pathogenic germ-line
variant (PV) has been identified women are counseled about their risks of breast, ovarian, and other cancers and appropriate screening or surgical prevention strategies are discussed. In most situations these cancer risk estimates are based on studies of large collections of families\(^1\) or relatives of breast cancer cases largely unselected for family history\(^2\); typically these analyses have pooled all pathogenic variants, irrespective of variant type, under the assumption that all such variants are associated with the same risks. The vast majority of the pathogenic BRCA variants included in these studies were variants predicted to result in a transcript encoding a protein termination codon that is subject to nonsense-mediated decay or encoding truncated inactive protein (for simplicity, these will be referred to hereafter as protein truncating variants\(^{PTC}\)). The most recent estimates for BRCA1/2 risks associated with \(\text{PV}^{\text{pathogenic}}\) variants come from the large prospective study of BRCA \(\text{PV}^{\text{pathogenic}}\) variant heterozygotes conducted by the IBCCS/PROF-SC/kConFab group\(^3\). Cumulative risks of breast cancer to age 70 were 66\% (95\% CI 61 – 72) for BRCA1 heterozygotes and 69\% (55 – 68) for BRCA2 heterozygotes; corresponding risks of ovarian cancer were 41\% (33 – 50) and 15\% (10 – 23). There is also accumulating evidence from genotype/phenotype studies that even \(\text{PTC}^{\text{protein truncating}}\) variants may not all be associated with the same risks, depending on their position within the gene\(^3\)\(^6\).

Analysis of missense variants poses a particular problem because most such variants are expected, \(\text{a priori}\), to be of little clinical significance. Through the work of the ENIGMA (Evidence-Based Network Investigating Germline Mutant Alleles) consortium\(^7\) and others, ~60 missense variants have now been classified/reclassified as pathogenic by multifactorial methods\(^8\) using such lines of evidence as co-segregation\(^9\), family history of index cases in clinical testing series\(^10\)(\(^11\), and tumor histopathology\(^12\) to classify variants of uncertain significance (\(\text{VUS}\))\(^13\).
To date, all predicted missense substitution variants that have been classified on the basis of genetic data as pathogenic (excluding those that act by disruption of normal splicing) reside in one of three domains of these proteins: the RING domain of BRCA1 (nucleotides 4 – 294); the BRCA1-C-Terminal (BRCT) repeats in BRCA1 (nt 4987- 5577) and the DNA Binding domain (DBD) of BRCA2 (nt 7669 – 9558). However it is not clear if pathogenic missense changes in these important domains are associated with the same levels of risk of breast and ovarian cancer as the protein-truncating/null variants that have been the subject of most studies designed to estimate these risks. Indeed, the BRCA1 variant c.5096G>A;p.[Arg1699Gln] was shown to be associated with lower risks of breast cancer (~20% by age 70)\textsuperscript{14,15} and had reduced function in a transcriptional activation assay in the 293T (78% of wild-type activity compared to 45% for the p.[Arg1699Trp] variant included in this study)\textsuperscript{14}. Similarly, functional and case-control analyses have identified the BRCA2 p.[Tyr3035Ser] variant as a hypomorphic allele associated with only a 2.5-fold increased risk of breast cancer (95% CI 1.05 – 6.05)\textsuperscript{16}.

In order to comprehensively examine the risks associated with established (e.g. those classified as such in ClinVar\textsuperscript{17}, BRCA Challenge\textsuperscript{18}, ENIGMA\textsuperscript{7}) missense PV\textsubscript{pathogenic variants} in BRCA1 and BRCA2, a large series of pedigrees segregating missense PV\textsubscript{pathogenic variants} were collected through the ENIGMA and CIMBA (Consortium of Investigators of Modifiers of BRCA1/2)\textsuperscript{19} consortia and compared through detailed statistical and genetic analyses with a set of pedigrees segregating PTP\textsubscript{protein truncating} variants. In addition the histopathological profiles of breast tumors of patients carrying BRCA1 PV\textsubscript{pathogenic variants} and PTP\textsubscript{protein truncating} variants were compared.

Here we demonstrate that pathogenic missense variants, especially in BRCA1, are associated with a reduced risk of breast cancer compared to that for PTP\textsubscript{protein truncating} variants, particularly at older ages.
MATERIAL & METHODS

Through the ENIGMA\textsuperscript{7} Consortium we put out a broad call for pedigree and tumor pathology information from families with a BRCA\textit{1} or BRCA\textit{2} missense variant that, at the time of initiation of the study, had evidence in favor of pathogenicity; this initial list comprised 58 such variants. We also obtained qualifying families from the CIMBA\textsuperscript{8} consortium where complete pedigree information was available. Criteria for submission of families for this study were:

1) Index individual (proband) with a pathogenic or likely pathogenic missense variant in BRCA\textit{1} or BRCA\textit{2}, as determined by ENIGMA through multifactorial analysis\textsuperscript{13}, or ClinVar\textsuperscript{17} (at least two certified (at least *) submitters denoting it Pathogenic/Likely Pathogenic with none calling it Benign or Likely Benign).

2) Informative for segregation analysis, i.e., at least one non-proband individual tested for the variant;

As a comparison group, we also received a matching set of 538 pedigrees segregating informative PTCprotein truncating variants pedigrees from the large German Consortium for Hereditary Breast and Ovarian Cancer (GC-HBOC). These pedigrees were matched by specific clinical center and date of ascertainment/testing to the GC-HBOC missense variant pedigrees.

\textit{Variant Selection}

Pedigrees were contributed in which the index case had one of 58 selected missense variants. Of these, 18 variants were excluded from the analysis as they were determined at the time of
analysis to not meet the specified criteria, either because they were found to be acting primarily through disruption of normal splicing and/or other evidence that cast doubt on their classification as pathogenic missense variants. The remaining 40 variants consisted of 11 variants in the BRCA1 RING domain, 17 in the BRCA1 BRCT domain and 12 in the BRCA2 DBD. Supplementary Table 1 provides detailed genetic and functional information about the 40 included variants. One BRCA1 missense variant (p.[Cys64Gly]) and two BRCA2 missense variants (p.[Glu2663Val] and p.[Asp2723Gly]) show experimental and bioinformatics evidence of pathogenicity through both protein effects and potential splicing effects\textsuperscript{20,21}. Because these variants showed strong deleterious effects in functional assays\textsuperscript{22-24} and only partial splicing defects\textsuperscript{25,26} they were included in our analyses.

Family Data

A total of 1146 missense \textit{PV}\textit{pathogenic variant} pedigrees and 543 \textit{PTC}\textit{protein truncating} variant pedigrees from 45 contributors representing 17 countries from around the world were submitted for this study. The requested information included pedigree data, age at testing/death/current age, age at diagnosis of breast cancer, age at diagnosis of ovarian cancer, as well as any available information on breast tumor grade, ER, PR, and HER2 status, and prophylactic surgeries. Loss of function variant pedigrees (326 BRCA1 and 217 BRCA2) and 527 of the missense variant pedigrees (including 279 c.181T>G;p.[Cys61Gly]) were contributed by the GC-HBOC network. In addition, we also had relatively large contributions of two founder variants (\textit{BRCA1:c.190T>C};p.[Cys64Arg])\textsuperscript{27} with 51 informative families submitted from Italy and (\textit{BRCA1:c.5212G>A};p.[Gly1738Arg])\textsuperscript{28} with 21 families submitted from Greece. Of the 1689 total submitted families, 391 were excluded from all analyses because they proved to not be informative (308 families) for the planned analyses, did not have one of the 40 final selected variants (93), or had unclear pedigree structures or multiple variants (14).
After the exclusions described above, the data used for the analyses here included 582 informative pedigrees with pathogenic missense variants in *BRCA1*, 153 with pathogenic missense variants in *BRCA2*, 324 pedigrees with **PTCprotein truncating** variants in *BRCA1* and 214 with **PTCprotein truncating** variants in *BRCA2*. Summary information of the characteristics of the set of families included in the study for each of the included variants is shown in Table 1.

**Imputation of Missing Ages and Censoring**

To avoid any biases due to differential information by submitting center (and hence *BRCA* variant), we imputed missing ages at last follow-up/death and years of birth using the program PedPro\textsuperscript{29} that uses the ages/years of birth of close relatives to assign an age or year of birth to individuals missing such information. Those with imputed ages $\geq 90$ years were imputed to be age 65 since these were likely in older generations without reliable data, and those imputed to be 80-89 were censored at age 80. To be conservative, we assumed that 324 cases with missing ages at diagnosis of breast or ovarian cancer were assumed to be diagnosed at age at their last follow-up/age at death (including imputed ages). For women with breast cancer and ovarian cancer we considered them as having both cancers if the breast cancer occurred first; women with breast cancer following ovarian cancer were treated as only having ovarian cancer and censored at the age of ovarian cancer diagnosis. Age at bilateral prophylactic mastectomy (BPM) was available in the GC-HBOC data set, in several smaller centers, and in the 36 families contributed from the CIMBA consortium, but not available in the remainder of the dataset. Women without breast or ovarian cancer were censored at their age at last observation or, if deceased, at age at death. When such information was provided, women known to have had a prophylactic mastectomy were censored at age of surgery. Because only a very small subset of the contributed families provided data on risk reducing salpingo-oophorectomy (RRSO) we chose not to censor women at this age; this may lead to an underestimation of
ovarian cancer risk but this effect would be expected to be the same for the PTCprotein truncating variant pedigrees as well as the missense PVpathogenic variant set, particularly for the p.[Cys61Gly] variant which has been known to be pathogenic for over 20 years.

Estimation of Risk

Breast and ovarian cancer risks were estimated by maximum likelihood analysis using modified segregation analysis with the MENDEL package of programs30 as implemented for breast, ovarian and other cancers in the recent analyses of families with PVpathogenic variants in PALB2.31 In the present analyses we estimated risks for breast and ovarian cancer jointly, censoring each affected individual at their age at first breast or ovarian cancer. For each dataset, we estimated the hazard ratio (HR) associated with development of breast cancer in the following two age groups: <50 and ≥50 years. Due to the more limited data for ovarian cancer in this dataset, we assumed a constant HR across age. Thus, in each analysis three parameters were estimated simultaneously. For both breast and ovarian cancer analyses the baseline population incidence rates were assumed to be those for the UK organized in eight 10-year birth cohorts (Cancer Incidence in Five Continents Reports32) to account for the increasing temporal trends in these cancers over time. In order to adjust for the ascertainment process of the families, the likelihood of observing the pedigree phenotypes and variant genotypes was calculated conditional on the pedigree phenotypes and the genotype of the index case; all information for risk estimation thus comes from the distribution of genotypes (i.e., genotype status) among the phenotypes of the family members of the proband. From the resulting parameter estimates of BC and OC, relative risk, age-specific cumulative risk estimates were calculated from the cumulative incidence Λ(t): F(t) = 1 - exp(-Λ(t)), assuming UK rates in the women born between 1970 and 1979; the corresponding confidence intervals were calculated using a parametric bootstrap. Heterogeneity of breast or ovarian cancer risk between variants in
the same domain was tested by a likelihood ratio test (LRT) of model likelihoods. Comparisons of parameter estimates between missense variants (or groups of variants) and the corresponding estimate for the PTC protein truncating variant pedigrees was done by constructing a Z-test based on the parameter estimates and their respective standard errors.

RESULTS

BRCA1 and BRCA2 Risk estimates

Table 2 presents the results of the modified segregation analyses in which we estimate the risks of breast and ovarian cancer relative to UK population incidence rates. Based on the estimated Hazard Ratios (HR), the biggest difference in risk between pathogenic missense and PTC protein truncating variants was in the risk of breast cancer among women over age 50. For example, for BRCA1 RING domain variants the HR for women 50 and older was roughly half the corresponding estimate for the BRCA1 PTC protein truncating variants. Of note also, the estimated HR for c.181T>G, [p.Cys61Gly] in women 50+ for the subset of 217 informative pedigrees with this variant from the GC-HBOC clinical network was significantly different from the corresponding HR for BRCA1 PTC protein truncating variant pedigrees ascertained from the same GC-HBOC clinical centers (p=0.049). The three groups of BRCA1 RING domain variants as shown in Table 2 did not reveal statistically significant heterogeneity in the estimated HRs (X²=10.0, 6 df; p=0.12) by likelihood ratio test (LRT)). For BRCA1 BRCT domain missense variants the HR for women 50 and older was significantly reduced compared to PTC protein truncating variant heterozygotes (2.0 vs. 12.8; p=0.005). There was no evidence of significant heterogeneity between variants within the BRCT domain and there was no significant difference
between the overall estimated models for the RING and BRCT domains ($X^2 = 5.48; 3 \text{ df}; p=0.14$).

No significant difference was observed in the HR of all BRCA2 missense PTVpathogenic variants in the DBD compared to the estimated cancer risks for PTVprotein truncating variants$^{2,3}$. However, there was some evidence of heterogeneity associated with the BRCA2 variants in the DBD with HRs for women over age 50 varying from 1.7 (0.4, 6.9) for c.7878G>C;p.[Trp2626Cys], to 5.9 (2.2, 16.3) for the well-established missense PTVpathogenic variant p.[Asp2723His], and 7.0 (3.3, 14.7) for the BRCA2 PTVprotein truncating variants.

When we examined the risks to women over age 60 (data not shown), we observed even lower risks for breast cancer compared to PTVprotein truncating, although reduction in sample size increased the standard errors, resulting in wider confidence Intervals. For example, for the RING and BRCT domain PTVpathogenic variant heterozygotes the HRs were 2.8 (1.1, 7.3) and 2.0 (0.6, 6.6), respectively, versus 16.5 (4.3, 63.2) in the BRCA1 PTVprotein truncating pedigrees in our study. For BRCA2 pathogenic DBD missense variants, the HR for women over age 60 was 2.0 (0.6, 6.3) vs. 5.3 (1.6, 17.9) in the BRCA2 PTVprotein truncating pedigrees.

In general the estimated HRs for ovarian cancer were quite similar in the various pathogenic missense variant analyses although risks were somewhat reduced for the BRCA1 BRCT variants but this was not statistically significant. Estimated cumulative risks of BC to age 50 and age 70 and of OC to age 70 for the primary sets of variants are shown in Table 3. Globally, risks to age 50 did not vary substantially between missense PTVpathogenic variants and their PTVprotein truncating variant counterparts However the estimated risks to age 70 were more variable, due to the HRs for ages greater than 50 being in general lower for the missense variants.

*Estrogen Status of Breast Tumors with BRCA1 Variants*
ER status was available for 361 breast tumors from women harboring a \textit{BRCA1} pathogenic missense variant. These included 250 from missense variants in the RING domain (220 of which were from p.[Cys61Gly] heterozygotes) and 111 in the BRCT domain, to compare to 210 tumors from \textit{BRCA1} \textsc{P}T\textsc{C}protein truncating heterozygotes (Table 4). There was no significant difference in the frequency of ER negative tumors among breast cancers from \textit{BRCA1} missense \textsc{P}V\textsc{V}pathogenic variant women compared to the tumors from women heterozygous for a \textsc{P}T\textsc{C}protein truncating variant. Individually, only p.[Ala1708Glu] had a nominally significantly lower frequency of ER negative tumors (52\% vs. 72\%; \textit{p}=0.027) compared to tumors from women with \textsc{P}T\textsc{C}protein truncating variants, although this was not significant when corrected for the number of comparisons made.

\textit{BC risk associated with specific variants}

We found that the breast (but not ovarian) cancer risks associated with two specific missense \textsc{P}V\textsc{V}pathogenic variants were lower than that for other \textit{BRCA1} missense \textsc{P}V\textsc{V}pathogenic variants. The HR for women under age 50 associated with the p.[Cys64Arg] missense \textsc{P}V\textsc{V}pathogenic variant was 3.7 (95\% CI 1.4,9.5) and that for women \(\geq 50\) was 4.1 (0.9, 18.3). In particular, the HR for younger women was substantially lower than that for other RING and BRCT variants. We tested this variant against the estimated risk for the p.[Cys61Gly] variant assuming a constant HR and found it to be significantly different (Z-test; \(p=0.015\)). These lower estimated risks are consistent with the observation of 10 BC cases in these families who tested negative for the variant, mean age at diagnosis of 48.6) compared with 14 non-proband cases that tested positive (mean age at diagnosis of 43.9). Moreover, among 13 women unaffected with breast or ovarian cancer over the age of 60 tested for the variant, almost half (6/13) were found to be positive, also indicating a lower penetrance. Thus the evidence suggests that this \textit{BRCA1} missense \textsc{P}V\textsc{V}pathogenic variant has substantially reduced breast cancer risk (but not
necessarily ovarian cancer) compared to other \textit{PV}pathogenic \textit{variants} while still higher than the population incidence rates. The risks associated with the \textit{BRCA2} p.[Trp2626Cys] missense-\textit{PV} \textit{variant} for breast and ovarian cancer is significantly reduced compared to both \textit{BRCA2 PTCprotein truncating} variants or other \textit{BRCA2} missense \textit{PV}pathogenic \textit{variants} analyzed in this study (Table 2). Among 31 female relatives who tested positive for the p.[Trp2626Cys] variant in the 34 pedigrees there were only three cases of breast cancer and one ovarian cancer. Moreover, among 22 unaffected women over age 60 who tested for the variant, 13 were found to be positive for the p.[Trp.2626Cys] variant, also indicating that this variant has reduced penetrance.

\textit{Sensitivity Analyses for Imputed Age}

Reanalysis of the data assuming all individuals with missing year of birth were born in the period 1960-1969 (thus eliminating cohort differences in incidence rates for these individuals) resulted in only a minimal effect on the estimates since the vast majority of the missing ages were in individuals who were not tested or closely related to a tested individual, and thus did not contribute heavily to the analysis. Individuals with missing current age/age at death were excluded in these analyses as well. Results of these analyses are shown in Supplementary Table 2. For example, for the \textit{BRCA1 PTCprotein truncating} set of pedigrees, the HRs for BC<50 years, BC≥50 years and OC were 16.4, 12.1, and 26.3, respectively without the missing age imputation and 17.0, 12.8 and 27.1 (Table 2) using imputed age data.

\textit{Sensitivity Analyses population incidence rates}

In these analyses we assumed age- and cohort-specific cancer incidence rates for the UK. In order to see how our results and conclusions could be affected by possible differences in rates
we re-analyzed the larger subsets of pedigrees assuming both rates that were 20% higher and 20% lower than these (across all ages and birth cohorts). Given that the vast majority of the pedigrees submitted came from Western European clinical centers, this should account for incidence rate differences among the various populations included here. The parameter estimates for the \textit{BRCA1} BRCT domain variants, RING domain variants and \texttt{PTCprotein} \texttt{truncating} variants from the GC-HBOC resource were on average 5% higher when incidence rates were assumed to be 20% lower and estimates were 4% lower when rates were set to be 20% higher. The largest differences were seen for the \texttt{ln(HR)} estimates for ovarian cancer which were 11% higher for the set of \textit{BRCA1} RING domain variants when incidence rates were assumed to be 20% lower. Based on these analyses, differences in rates from our assumed UK rates are an unlikely explanation for the findings.

\textbf{DISCUSSION}

Here we report a large international collaborative study examining the risks of breast and ovarian cancer for women harboring a pathogenic missense variants in the \textit{BRCA1} and \textit{BRCA2} genes. We benefited from a large national study of hereditary breast and ovarian cancer in Germany (GC-HBOC) that contributed approximately half of the missense \texttt{PVpathogenic variant} families and a series of families that segregated \texttt{PTCprotein truncating} variants in \textit{BRCA1} and \textit{BRCA2} in order to form comparison groups for the missense variants in each gene. The results shown in Tables 2 and 3 clearly indicate that missense \texttt{PVpathogenic variants} in both functionally important domains (RING and BRCT) in \textit{BRCA1} are associated with lower risks of breast cancer than \texttt{PTCprotein truncating} variants. Although data are more limited, it appears that ovarian cancer risks are comparable (though on average perhaps slightly lower) for \textit{BRCA1} to those found in published risk estimates for \texttt{PTCprotein truncating variants}\textsuperscript{1-3}, and also comparable to those estimated from the \texttt{PTCprotein truncating set-variant e} of pedigrees from
GC-HBOC. Although associated with lower breast cancer risks, missense **PVpathogenic variants** in *BRCA1* had similar hormone receptor status profiles as their **PTCprotein truncating** counterpart; importantly the families studied here were largely tested before TN status was a stand-alone criterion for *BRCA* testing, suggesting a lack of ascertainment bias based on pathology. Most *BRCA2* pathogenic missense variants appeared to be associated with lower risks compared to the *BRCA2**PTCprotein truncating** families in the over 50 age group (Table 2); however these differences were not statistically significant and were of smaller magnitude than the parallel comparisons for *BRCA1* variants.

**Clinical considerations**

Our results provide evidence of the convergence of moderate- to high-risk classes that no longer seem to be distinct but rather describe a risk continuum. Indeed we have previously shown that the hypomorphic *BRCA1* variant p[Arg1699Gln] is associated with reduced levels of breast cancer risk compared to the average *BRCA1* truncating variant\(^\text{15,16}\). That raises the question of what clinical measures to offer for different levels of risk. The estimated cumulative risks to age 70 of 70% for *BRCA1**PTCprotein truncating** variants compared to 39% for the combined group of missense **PVpathogenic variants** in the BRCT repeat domains (primarily p.[Ala1708Glu] and p.[Arg1699Trp] are significantly different and may very well affect a woman’s choice of prevention/screening options, in particular bilateral prophylactic mastectomy. Further, these risks will depend on family history, their polygenic risk score (PRS)\(^\text{33}\), and lifestyle factors\(^\text{34}\). We believe that these new risk estimates for *BRCA1* missense variants should be incorporated into comprehensive risk prediction tools such as BOADICEA\(^\text{35}\). Although, on average slightly lower, the ovarian cancer risks associated with both *BRCA1* and *BRCA2* missense **PVpathogenic variants** did not seem to differ markedly from that for the **PTCprotein truncating** variants (with the possible exception of **PVpathogenic variants** in the BRCT domain of
BRCA1. For cancer-free women over age 50, many of the BRCA1 pathogenic missense variants are associated with relative breast cancer risks closer to those estimated for pathogenic variants in genes such as ATM, CHEK2, and other moderate risk genes, indicating that surveillance might be an optimal approach. However, given the high ovarian cancer risks of 36% by age 70 for RING domain missense PTPathogenic variants, we recommend that women heterozygous for such these variants should be counseled the same with respect to ovarian cancer as women with BRCA1 PTCprotein truncating variants in terms of recommendations for RRSO. Clinically, these data strengthen the importance of communicating not only the cumulative lifetime risks but also risks within a manageable timespan, e.g., 10-year risks.

For the BRCA1 p.[Cys64Arg] variants we propose counseling heterozygotes similarly to those proposed for the BRCA1 p.[Arg1699Gln] hypomorphic variant\textsuperscript{16}. The p.[Cys64Arg] variant has been classified as pathogenic by the majority of clinical laboratories so it will be important to collect more genetic data in order to clarify the status of this variant and its associated cancer risks.

We also noted particularly low breast cancer risk estimates for BRCA2 p.[Trp2626Cys]. The functional effects of this variant have been examined and although it was classified as functionally deleterious, it was near the boundary between deleterious and intermediate function\textsuperscript{36}. This variant is denoted in ClinVar as pathogenic by nine clinical laboratories, and as likely pathogenic by three others, with a single lab reporting it in ClinVar as a–\textsuperscript{Variant of Uncertain Significance\textsuperscript{VUS}}. This variant has also been evaluated in a number of other recent studies. Based on family histories of 12 individuals heterozygous for p.[Trp2626Cys] in a large clinical testing set, we estimated odds of 16:1 against pathogenicity\textsuperscript{11} and in the large OncoArray\textsuperscript{37} breast cancer case-control series this variant was identified in 16/75350 cases and 5/52793 controls (OR=2.2 95% CI 0.8 – 7.8; p=0.1) which is consistent with the estimate reported here (K. Michailidou, personal communication). Taken together the available evidence
indicates that caution should be exercised in counseling individuals harboring this variant as a pathogenic BRCA2 variant until further genetic and functional studies can be performed.

**Limitations and Caveats**

The primary limitation inherent in our study is that families were submitted from a wide variety of countries and clinical centers that could vary widely with respect to ascertainment criteria, cascade testing, and prospective follow-up. Although in our analysis, we adjust for the phenotypes of all pedigree members and relied only on the genotype status of non-proband family members, it is possible that different practices in different centers could affect the risk estimates. However, the consistency of our results together with the direct comparison of p.[Cys61Gly] with PTC protein truncating variant pedigrees from the GC-HBOC resource make it highly unlikely that systematic biases could explain our findings.

A second limitation of this study is the lack of complete data on prophylactic surgery, and potentially differing rates of uptake across countries. In order to ensure that the observed differences in risk were not due to the censoring of women at BPM in some of the data sets and not others, we repeated analyses ignoring the BPM information. In particular we were concerned about the effect in the BRCA1 and BRCA2 reference sets as they were used as comparison to the missense PV pathogenic variants. The mean difference between age at BPM and current age/age at death was only 2.9 years (95% CI 1.9 – 3.9) indicating that differential information with regard to BPM would not alter our conclusions.

**Conclusions**

Our analyses of ~1250 informative pedigrees have demonstrated that BRCA1 missense PV pathogenic variants are associated with smaller increased risks of breast cancer in women
over age 50 when compared to variants predicted to result in complete loss of function. The risk reduction is less pronounced for BRCA2 in such women. We have also shown that specific variants are associated with particularly low risks compared to other BRCA pathogenic variants: BRCA1 p.[Cys64Arg] and BRCA2 p.[Trp2626Cys] for breast cancer and possibly BRCA1 p.[Ala1708Glu] for ovarian cancer. Interestingly, the histopathology of breast cancers from patients carrying pathogenic missense variants in BRCA1 showed similar rates of ER negative/triple negative status as the cancers in PTC protein truncating patients. Future studies should focus on the functional basis for the reduction in relative risks for older women who harbor a pathogenic missense variant in BRCA1, and to a lesser extent, BRCA2.

Supplemental Data

There are two supplementary tables are associated with this article. Supplementary Table 1 (Excel) provides detailed information on the 40 variants analyzed in this study. Supplementary Table 2 displays the HR estimates excluding individuals with missing age data and assumes individuals with missing year of birth information were born between 1960-1969.

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Data and Code Availability
Data requests for files used in the analysis should be made to D. Goldgar although European GDPR regulations may not permit pedigree data to be transferred outside the EU.

**Declaration of Interests**

F.J.C. has received consulting fees from Astrazeneca.

A.S. has received consulting fees from Pfizer and Astra Zeneca.

The remaining authors declare no competing interests.

**Ethics**

This study was covered under Amendment to Project P1051 (PI Prof. Amanda Spurdle) approved by the Human Research Ethics Committee at QIMR Berghofer, Brisbane Australia, Oct 7 2020. All patients gave consent to have their data used for research purposes and all studies received local Ethics Committee approvals. Data from all centers were de-identified prior to analysis at the coordinating center.

**Web Resources**

Brca Exchange: [https://brcaexchange.org/](https://brcaexchange.org/) Accessed 2/15/2021


HCI Priors database: http://priors.hci.utah.edu/PRIORS/BRCA/viewer.php?gene=BRCA1
accessed 2/15/2021
Author Contributions

Conceptualization: D.G., A.S., F.C., R.S., B.W.


Formal Analysis: H.L., D.G.

Funding Acquisition F.C., A.S.,

Methodology: D.G., A.C., X.Y., M.P., H.L.

Writing – original draft: D.G.

Writing – review & editing: All Authors
References


histopathological predictors of BRCA1 and BRCA2 mutation status: a large-scale analysis of breast cancer characteristics from the BCAC, CIMBA, and ENIGMA consortia. Breast Cancer Res. 16(6):3419 PubMed PMID: 25857409; PubMed Central PMCID: PMC4352262.


Table 1. Description of variants and families included in the risk estimation analyses. Index cases are excluded from tabulations.

<table>
<thead>
<tr>
<th>Variant/Group</th>
<th>Informative Families</th>
<th>Breast Cancer</th>
<th>Ovarian Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heterozygous</td>
<td>Wild-Type</td>
</tr>
<tr>
<td>BRCA1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-HBOC BRCA1 PTC</td>
<td>324</td>
<td>85</td>
<td>7</td>
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<tr>
<td>RING Domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.181T&gt;G, p.[Cys61Gly]</td>
<td>316</td>
<td>114</td>
<td>14</td>
</tr>
<tr>
<td>c.190T&gt;C; p.[Cys64Arg]</td>
<td>55</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Other RING Domain¹</td>
<td>34</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>All RING Domain</td>
<td>405</td>
<td>141</td>
<td>27</td>
</tr>
<tr>
<td>GC-HBOC c.181T&gt;G, p.[Cys61Gly]</td>
<td>277</td>
<td>81</td>
<td>9</td>
</tr>
<tr>
<td>BRCT Domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.5095C&gt;T, p.[Arg1699Trp]</td>
<td>43</td>
<td>11</td>
<td>2</td>
</tr>
</tbody>
</table>

¹ Including single families reported as BRCT.
<table>
<thead>
<tr>
<th>BRCT Domain</th>
<th>c.5123C&gt;A, p.[Ala1708Glu]</th>
<th>56</th>
<th>17</th>
<th>0</th>
<th>84</th>
<th>3</th>
<th>1</th>
<th>19</th>
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<tr>
<td></td>
<td>c.5212G&gt;A, p.[Gly1738Arg]</td>
<td>36</td>
<td>25</td>
<td>2</td>
<td>39</td>
<td>12</td>
<td>0</td>
<td>9</td>
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<tr>
<td>Other BRCT Domain^2</td>
<td>42</td>
<td>21</td>
<td>6</td>
<td>59</td>
<td>10</td>
<td>0</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>All BRCT Domain</td>
<td>177</td>
<td>74</td>
<td>10</td>
<td>227</td>
<td>38</td>
<td>1</td>
<td>78</td>
<td></td>
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<tr>
<td>All BRCA1 Missense</td>
<td>582</td>
<td>215</td>
<td>37</td>
<td>889</td>
<td>69</td>
<td>1</td>
<td>221</td>
<td></td>
</tr>
<tr>
<td>BRCA2</td>
<td>GCHBOC BRCA2 PTC</td>
<td>214</td>
<td>48</td>
<td>9</td>
<td>376</td>
<td>5</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>DNA Binding Domain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.7878G&gt;C, p.[Trp2626Cys]</td>
<td>34</td>
<td>3</td>
<td>0</td>
<td>44</td>
<td>0</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>c.8167G&gt;C, p.[Asp2723His]</td>
<td>33</td>
<td>18</td>
<td>3</td>
<td>66</td>
<td>2</td>
<td>0</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>c.9154C&gt;T, p.[Arg3052Trp]</td>
<td>20</td>
<td>9</td>
<td>2</td>
<td>39</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Other B2 DBD^3</td>
<td>66</td>
<td>19</td>
<td>6</td>
<td>121</td>
<td>6</td>
<td>1</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>All DBD Missense</td>
<td>153</td>
<td>49</td>
<td>11</td>
<td>270</td>
<td>8</td>
<td>2</td>
<td>47</td>
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</tr>
</tbody>
</table>
1. c.53T>C, p.[Met18Thr]; c.65T>C, p.[Leu22Ser]; c.110C>A, p.[Thr37Lys]; c.115T>C, p.[Cys39Arg]; c.116G>A, p.[Cys39Tyr]; c.130T>A, p.[Cys44Ser]; c.131G>T, p.[Cys44Phe]; c.190T>G, p.[Cys64Gly]; c.191G>A, p.[Cys64Tyr]

2. c.5053A>G, p.[Thr1685Ala]; c.5054C>T, p.[Thr1685Ile]; c.5089T>C, p.[Cys1697Arg]; c.5117G>A, p.[Gly1706Glu]; c.5143A>C, p.[Ser1715Arg]; c.5213G>A, p.[Gly1738Glu]; c.5216A>T, p.[Asp1739Val]; c.5291T>C, p.[Leu1764Pro]; c.5297T>G, p.[Ile1766Ser]; c.5309G>T, p.[Gly1770Val]; c.5324T>A, p.[Met1775Lys]; c.5324T>G, (p.[Met1775Arg]); c.5363G>T, p.[Gly1788Val]; c.5513T>A, p.[Val1838Glu]

3. c.7879A>T, p.[Ile2627Phe]; c.7940T>C, p.[Leu2647Pro]; c.7958T>C, p.[Leu2653Pro]; c.7988A>T p.[Glu2663Val]; c.8165C>G, p.[Thr2722Arg]; c.8168G>A, p.[Asp2723Gly]; c.8243G>A, p.[Gly2748Asp]; c.9004G>A, p.[Glu3002Lys]; c.9227G>A, p.[Gly3076Glu]
Table 2. Hazard Ratio (HR) estimates by variant/group of variants.

<table>
<thead>
<tr>
<th>Variant/Variant Group</th>
<th>Breast Cancer Dx&lt;50</th>
<th>Breast Cancer Dx≥50</th>
<th>Ovarian Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td><strong>BRCA1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-HBOC BRCA1 PTC</td>
<td>17.0 (9.4, 30.5)</td>
<td>12.8 (5.7, 28.7)</td>
<td>27.1 (8.6, 85.2)</td>
</tr>
<tr>
<td>RING Domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.[Cys61Gly]</td>
<td>14.6 (8.8, 24.3)</td>
<td>7.6 (3.6, 16.4)</td>
<td>41.2 (19.0, 89.3)</td>
</tr>
<tr>
<td>p.[Cys64Arg]</td>
<td>3.7 (1.4, 9.5)*</td>
<td>4.1 (0.9, 18.3)</td>
<td>99.9 (37.8, 264)</td>
</tr>
<tr>
<td>Other RING Domain</td>
<td>12.0 (3.2, 37.4)</td>
<td>2.9 (0.5, 17.0)</td>
<td>23.9 (4.6, 119)</td>
</tr>
<tr>
<td>All RING Domain</td>
<td>11.5 (7.2, 18.3)</td>
<td>5.8 (3.0, 11.3)</td>
<td>41.0 (20.9, 80.4)</td>
</tr>
<tr>
<td>GC-HBOC p.[Cys61Gly]</td>
<td>15.3 (8.3, 28.2)</td>
<td>3.7 (1.5, 9.4)*</td>
<td>27.1 (7.2, 102.8)</td>
</tr>
<tr>
<td><strong>BRCT Domain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.[Arg1699Trp]</td>
<td>10.4 (2.7, 39.4)</td>
<td>2.0 (0.2, 21.8)</td>
<td>31.5 (5.1, 195)</td>
</tr>
<tr>
<td>p.[Ala1708Glu]</td>
<td>12.1 (4.3, 34.0)</td>
<td>4.9 (1.3, 18.3)</td>
<td>5.2 (0.8, 33.8)</td>
</tr>
<tr>
<td>p.[Gly1738Arg]</td>
<td>22.8 (9.2, 56.9)</td>
<td>3.6 (1.4, 8.9)</td>
<td>14.7 (3.8, 57.8)</td>
</tr>
<tr>
<td>Other BRCT Domain</td>
<td>15.0 (6.0, 37.5)</td>
<td>2.1 (0.7, 6.2)**</td>
<td>18.8 (6.3, 56.2)</td>
</tr>
<tr>
<td>All BRCT Domain</td>
<td>14.8 (8.7, 25.1)</td>
<td>2.8 (1.4, 5.6)**</td>
<td>15.2 (7.6, 30.4)</td>
</tr>
<tr>
<td><strong>All BRCA1 Missense</strong></td>
<td>13.1 (9.2, 18.9)</td>
<td>3.9 (2.4, 6.2)**</td>
<td>21.7 (12.4, 38.0)</td>
</tr>
<tr>
<td><strong>BRCA2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-HBOC BRCA2 PTC</td>
<td>10.4 (5.9, 19.8)</td>
<td>7.0 (3.3, 14.7)</td>
<td>13.1 (3.7, 45.6)</td>
</tr>
<tr>
<td>DNA Binding Domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.[Trp2626Cys]</td>
<td>5.0 (0.3, 92.0)</td>
<td>1.7 (0.4, 6.9)</td>
<td>2.1 (0.1, 32.8)</td>
</tr>
<tr>
<td>Protein</td>
<td>Value 1 (Min, Max)</td>
<td>Value 2 (Min, Max)</td>
<td>Value 3 (Min, Max)</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>p.[Asp2723His]</td>
<td>8.5 (2.5, 28.7)</td>
<td>5.2 (1.5, 18.6)</td>
<td>15.0 (2.1, 109)</td>
</tr>
<tr>
<td>p.[Arg3052Trp]</td>
<td>8.1 (0.9, 73.3)</td>
<td>3.5 (0.4, 30.0)</td>
<td>4.1 (0.3, 115)</td>
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<tr>
<td>Other DBD</td>
<td>9.5 (3.8, 23.7)</td>
<td>5.3 (2.1, 13.2)</td>
<td>5.6 (1.4, 21.8)</td>
</tr>
<tr>
<td>All DBD</td>
<td>8.3 (2.2, 30.8)</td>
<td>3.9 (2.0, 7.2)</td>
<td>5.5 (2.0, 14.8)</td>
</tr>
</tbody>
</table>

* p<0.05; ** p<0.01 for test of parameter vs. corresponding parameter for LOF variants.
Table 3. Theoretical Cumulative Risks for selected groups of variants based on HR estimates and UK incidence rates (1960-1969).

<table>
<thead>
<tr>
<th>Variant Group</th>
<th>Breast Cancer</th>
<th>Ovarian Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Risk to Age 50 (95% CI)</td>
<td>Risk to Age 70 (95% CI)</td>
</tr>
<tr>
<td>GC-HBOC BRCA1 PTC</td>
<td>0.33 (0.20,0.51)</td>
<td>0.70 (0.51,0.89)</td>
</tr>
<tr>
<td>All RING Domain</td>
<td>0.21 (0.14,0.31)</td>
<td>0.46 (0.33,0.61)</td>
</tr>
<tr>
<td>All BRCT Domain</td>
<td>0.30 (0.18, 0.44)</td>
<td>0.39 (0.27,0.54)</td>
</tr>
<tr>
<td>All BRCA1 Missense</td>
<td>0.24 (0.17, 0.32)</td>
<td>0.40 (0.32, 0.49)</td>
</tr>
<tr>
<td>GC-HBOC BRCA2 PTC</td>
<td>0.22 (0.12,0.37)</td>
<td>0.51 (0.34,0.69)</td>
</tr>
<tr>
<td>All DBD</td>
<td>0.18 (0.09,0.31)</td>
<td>0.36 (0.24,0.51)</td>
</tr>
</tbody>
</table>
Table 4. ER status in tumors of *BRCA1* PV pathogenic variant heterozygotes.

<table>
<thead>
<tr>
<th>Variant/Group</th>
<th>Number of Tumors</th>
<th>Number ER Positive (%)</th>
<th>Number ER Negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCHBOC BRCA1 PTC</td>
<td>210</td>
<td>59 (28)</td>
<td>151 (72)</td>
</tr>
<tr>
<td>p.[Cys61Gly]</td>
<td>216</td>
<td>55 (25)</td>
<td>161 (75)</td>
</tr>
<tr>
<td>p.[Cys64Arg]</td>
<td>21</td>
<td>4 (19)</td>
<td>17 (81)</td>
</tr>
<tr>
<td>Other RING Domain(^1)</td>
<td>11</td>
<td>2 (18)</td>
<td>9 (82)</td>
</tr>
<tr>
<td>p.[Arg1699Trp]</td>
<td>26</td>
<td>8 (31)</td>
<td>18 (69)</td>
</tr>
<tr>
<td>p.[Ala1708Glu]</td>
<td>29</td>
<td>14 (48)</td>
<td>15 (52)</td>
</tr>
<tr>
<td>p.[Gly1738Arg]</td>
<td>33</td>
<td>9 (27)</td>
<td>24 (73)</td>
</tr>
<tr>
<td>Other BRCT Domain</td>
<td>23</td>
<td>6 (26)</td>
<td>17 (74)</td>
</tr>
<tr>
<td>All BRCA1 Missense</td>
<td>359</td>
<td>98 (27)</td>
<td>261 (73)</td>
</tr>
</tbody>
</table>
Conflict of Interest Notification Page.

Fergus J. Couch has received consulting fees from Astrazeneca.

Angela Solano has received consulting fees from Pfizer and Astra Zeneca.

The remaining authors declare no competing interests.
**Supplementary Table 2.** Hazard Ratio (HR) estimates by variant/group of variants assuming birth cohort rates from 1960-1969 for individuals missing year of birth.

<table>
<thead>
<tr>
<th>Variant/Variant Group</th>
<th>Breast Cancer Dx&lt;50 HR (95% CI)</th>
<th>Breast Cancer Dx≥50 HR (95% CI)</th>
<th>Ovarian Cancer HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRCA1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-HBOC BRCA1 PTC</td>
<td>16.4 (9.2, 29.4)</td>
<td>12.1 (5.3, 27.4)</td>
<td>26.3 (9.1, 75.8)</td>
</tr>
<tr>
<td><strong>RING Domain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-HBOC p.[Cys61Gly]</td>
<td>15.3 (8.6, 27.3)</td>
<td>3.6 (1.4, 9.2)</td>
<td>27.1 (7.7, 96.0)</td>
</tr>
<tr>
<td>All p.[Cys61Gly]</td>
<td>15.2 (9.2, 25.1)</td>
<td>8.1 (3.8, 17.3)</td>
<td>42.5 (18.9, 95.7)</td>
</tr>
<tr>
<td>p.[Cys64Arg]</td>
<td>3.2 (1.3, 7.9) *</td>
<td>2.1 (0.5, 8.6)</td>
<td>11.9 (1.3, 111)</td>
</tr>
<tr>
<td>Other RING Domain</td>
<td>7.9 (2.6, 24.0)</td>
<td>4.8 (1.1, 22.1)</td>
<td>23.7 (5.9, 95.8)</td>
</tr>
<tr>
<td><strong>All RING Domain</strong></td>
<td>10.9 (6.8, 17.5)</td>
<td>5.4 (2.7, 10.8)</td>
<td>36.2 (16.5, 79.8)</td>
</tr>
<tr>
<td>GC-HBOC p.[Cys61Gly]</td>
<td>15.3 (8.6, 27.3)</td>
<td>3.6 (1.4, 9.2)</td>
<td>27.1 (7.7, 96.0)</td>
</tr>
<tr>
<td><strong>BRCT Domain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.[Arg1699Trp]</td>
<td>10.7 (2.6, 43.5)</td>
<td>2.3 (0.2, 28.9)</td>
<td>29.5 (5.9, 147)</td>
</tr>
<tr>
<td>p.[Ala1708Glu]</td>
<td>14.9 (5.0, 44.7)</td>
<td>6.5 (1.8, 23.7)</td>
<td>4.5 (0.8, 25.6)</td>
</tr>
<tr>
<td>p.[Gly1738Arg]</td>
<td>26.5 (11.9, 59.1)</td>
<td>4.1 (1.2, 13.6)</td>
<td>16.0 (3.6, 71.1)</td>
</tr>
<tr>
<td>Other BRCT Domain</td>
<td>17.1 (6.4, 45.5)</td>
<td>2.2 (0.7, 6.7) *</td>
<td>17.0 (5.0, 57.8)</td>
</tr>
<tr>
<td><strong>All BRCT Domain</strong></td>
<td>17.3 (10.1, 29.6)</td>
<td>3.2 (1.6, 6.5) *</td>
<td>16.3 (7.7, 34.6)</td>
</tr>
<tr>
<td><strong>BRCA2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-HBOC BRCA2 PTC</td>
<td>9.9 (5.2, 18.8)</td>
<td>7.4 (3.5, 15.6)</td>
<td>13.5 (3.9, 46.1)</td>
</tr>
<tr>
<td><strong>DNA Binding Domain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.[Trp2626Cys]</td>
<td>3.4 (0.1, 89.4)</td>
<td>1.8 (0.4, 8.0)</td>
<td>2.3 (0.2, 35.7)</td>
</tr>
<tr>
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<tr>
<td>------------------</td>
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<td>--------</td>
</tr>
<tr>
<td>p.[Asp2723His]</td>
<td>8.8 (2.4, 32.4)</td>
<td>3.2 (0.7, 14.6)</td>
<td>17.1 (2.0, 150)</td>
</tr>
<tr>
<td>p.[Arg3052Trp]</td>
<td>7.3 (0.8, 68.3)</td>
<td>3.8 (0.4, 37.5)</td>
<td>3.2 (0.2, 62.0)</td>
</tr>
<tr>
<td>Other DBD</td>
<td>17.2 (5.8, 51.1)</td>
<td>8.6 (2.9, 25.5)</td>
<td>9.6 (2.0, 46.5)</td>
</tr>
<tr>
<td>All Binding Domain</td>
<td>9.9 (4.9, 20.4)</td>
<td>3.9 (2.3, 19.9)</td>
<td>6.7 (2.0, 46.5)</td>
</tr>
</tbody>
</table>
**Supplementary Statistical Methods**

**Statistical Methods**

*Ascertainment adjustment*

We adjusted for ascertainment for each family separately by employing an assumption-free approach\(^1-3\). We divided the data for each family into two parts depending on whether the data could be relevant to the ascertainment (F1) or not (F2). The conditional likelihood \(L=\text{Pr}(F1, F2)/\text{Pr}(F1)\) was then maximized, where \(\text{Pr}(F1, F2)\) is the probability of the observed data in the entire pedigree and \(\text{Pr}(F1)\) is the probability of the observed data in the component relevant to the ascertainment. F1 and F2 may include both phenotypes and genotypes. For families ascertained through multiple affected family members, F1 included the genotype of the proband and phenotypes of all the family members that led to the ascertainment, which in our case were all pedigree members. F2 consist of all pedigree phenotype and genotype data. Non-informative families, in which no additional information beyond the data relevant to the ascertainment was available (i.e. those with no additional individuals tested for the variant other than the proband), were excluded from the analysis.

*Genetic models fitted*

The primary objective was to estimate the cancer-specific hazard ratios risks (HR) for *BRCA1* and *BRCA2* pathogenic missense variant heterozygotes relative to the population incidences (for breast and ovarian), and compare these to the parallel estimates obtained for protein-truncating variant heterozygotes. Pedigree likelihoods were constructed and maximized using the pedigree analysis software Mendel version
In these analyses the models were parameterized in terms of the HR defined as:

\[
HR(t, k) = \frac{i_{BRCA+}(t, k)}{i_{\text{pop}}(t, k)}
\]

where \(i_{\text{pop}}(t, k)\) denotes the population incidence at age \(t\) for cohort \(k\) and \(i_{BRCA+}(t, k)\) denotes the cancer incidence for the \(BRCA\) pathogenic variant heterozygotes at age \(t\) born in cohort \(k\). The cancer incidence for individual \(i\) at age \(t\) was assumed to be birth cohort specific, and to depend on the underlying genetic effects though a model of the form

\[
\lambda_i(t, k) = \lambda_0(t, k) \exp(\beta(t, k)G_i),
\]

where \(\lambda_0(t, k)\) is the baseline incidence at age \(t\) for cohort \(k\), \(\beta(t, k)\) represents the log-HR associated with the \(BRCA\) pathogenic variant at age \(t\) for cohort \(k\), \(G_i\) is an indicator variable taking values 1 for \(BRCA\) pathogenic variant heterozygotes and 0 for individuals wild-type for the variant. In all models the \(BRCA\) pathogenic variant frequency was assumed to be 0.05% (0.0005). However, since all the analyses involved conditioning on the genotype of the proband in each family in order to adjust for ascertainment, the exact value of the assumed variant frequency has little impact on the estimated HR. Each female was assumed to be at risk of developing breast or ovarian cancer. The probability of developing each individual cancer was assumed to be independent of the probability of developing any of the other cancers, conditional on the underlying genotype. Follow-up after the first cancer diagnosis was not considered within each of these groups the HR was assumed to be constant within the interval. For breast cancer in the main analysis, we estimated the HR in two age groups: under age 50 and ages 50-80. For ovarian cancer due to limited computational/statistical power we assumed a constant relative risk to age 80. We fitted the above models using calendar- and cohort-specific population cancer for the UK. The reported incidences are reported in five-year age intervals. To avoid large variations in the incidences between adjacent age intervals, we smoothed the incidences using the locally weighted regression LOWESS approach and derived eight cohort-specific incidences by assuming each individual was born at the midpoint of
each assumed cohort period (1915 for the first cohort and 1985 for the last cohort). The overall cancer incidences over all assumed genetic effects in the models were constrained to agree with the population age-specific incidences.

Nested models were compared using the likelihood ratio test (LRT), and individual parameters were compared using a simple Z-test based on the estimates and their standard errors. All statistical tests were two sided.

References


Corresponding Author Name: David Goldgar
Manuscript Number: GIM-D-21-00505

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  - definition of ‘center values’ as median or mean;
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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Human Research Ethics Committee at QIMR Berghofer, page 24</td>
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</table>

12. Include a statement confirming that informed consent was obtained from all subjects.

<table>
<thead>
<tr>
<th>Text AND page number from manuscript</th>
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</thead>
<tbody>
<tr>
<td>All subjects gave informed consent, page 24</td>
</tr>
</tbody>
</table>

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14. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent).

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<td>NA</td>
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</table>

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   c. Crystallographic data for small molecules
   d. Microarray data

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</tbody>
</table>

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