Title: Co-observation of germline pathogenic variants in different breast cancer predisposition genes: results from analysis of the BRIDGES sequencing dataset

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Summary

Co-observation of a gene variant with a pathogenic variant in another gene that explains the disease presentation has been designated as evidence against pathogenicity for commonly used variant classification guidelines. Multiple variant curation expert panels have specified, from consensus opinion, that this evidence type is not applicable for the classification of breast cancer predisposition gene variants. Statistical analysis of sequence data

for 55,815 individuals diagnosed with breast cancer from the BRIDGES sequencing project was undertaken to formally assess the utility of co-observation data for germline variant classification. Our analysis included expected loss-of-function variants in 11 breast cancer predisposition genes, and pathogenic missense variants in *BRCA1*, *BRCA2,* and *TP53*. We assessed whether co-observation of pathogenic variants in two different genes occurred more or less often than expected under the assumption of independence. Co-observation of pathogenic variants in each of *BRCA1, BRCA2,* and *PALB2* with the remaining genes was less frequent than expected. This evidence for depletion remained after adjustment for age at diagnosis, study design (familial versus population-based), and country. Co-observation of a variant of uncertain significance in *BRCA1*, *BRCA2,* or *PALB2* with a pathogenic variant in another breast cancer gene equated to supporting evidence against pathogenicity following criterion strength assignment based on the likelihood ratio, and showed utility in reclassification of missense *BRCA1* and *BRCA2* variants identified in BRIDGES. Our approach has applicability for assessing the value of co-observation as a predictor of variant pathogenicity in other clinical contexts, including for gene-specific guidelines developed by ClinGen Variant Curation Expert Panels.

Main text

The American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) classification guidelines consist of a series of evidence-based criteria (or codes) that can be applied as support for or against the pathogenicity of a variant[1]. The original description for the supporting benign criterion BP5 is “variant found in a case with an alternate molecular basis for disease”. That is, when a variant of unknown pathogenicity is observed in an individual with a specific phenotype that is explained by a pathogenic variant (PV) in another gene, this co-observation is considered supporting evidence against pathogenicity for the variant under clinical assessment[1].

After the introduction of the ACMG/AMP guidelines, the Clinical Genome Resource (ClinGen) Variant Curation Expert Panels (VCEPs) developed tailored variant curation specifications for some hereditary disease genes[2]. These criteria are available through the ClinGen Criteria Specification (CSpec) Registry (see web resources). Review of the publicly available CSpec specifications for hereditary cancer genes identified variability for the BP5 criterion (summarized in **Table S1**), such as: usage only when co-observed with certain genes (*CDH1*, MIM [192090])[3, 4]; usage in certain personal, familial and/or disease-specific context (*APC*, MIM [611731] and *PTEN*, MIM [601728])[5, 6]; recommendation against usage (*ATM* MIM [607585], *DICER1* MIM [606241], *PALB2* MIM [610355], *RUNX1* MIM [151385] and *TP53*, MIM [191170])[7-9]; or use to capture other clinical evidence data (*BRCA1*, MIM [113705] and *BRCA2*, MIM [600185])[10].

To date, individuals with a PV in each of two different breast cancer (MIM: 114480) predisposition genes, have typically been reported with no clearly distinctive clinical features with potential for aiding in variant classification, although there are limited reports suggesting somewhat younger average age at onset for these individuals. As such, the justification used by the VCEP for not considering the BP5 co-observation criterion is that individuals with PVs in both *BRCA1* and *BRCA2* (or in conjunction with pathogenic variants in other breast-ovarian cancer susceptibility genes) do occur. Furthermore, carrying dual *BRCA1* and *BRCA2* PVs is not associated with an unusual clinical presentation; rather, these individuals present with clinical features more typical of a sole *BRCA1* PV carrier[11]. Here, and throughout, we refer to a PV carrier as an individual with a PV affecting a single allele (heterozygous), or both alleles (homozygous or compound heterozygous). Similar arguments have been used against the usage of BP5 for the curation of *PALB2* variants. It is nevertheless theoretically possible to estimate, from the PV carrier frequency in large datasets, if co-observation of a variant with a PV in a different gene occurs more or less often than expected by chance, information that may provide statistically-derived evidence for or

against pathogenicity. Therefore, we undertook a study to formally assess the utility of the benign supporting ACMG/AMP criterion BP5 for the interpretation of germline variants in breast cancer predisposition genes.

This research was approved by the QIMR Berghofer Human Research Ethics Committee (P1051). Analyses were based on 55,815 female individuals diagnosed with breast cancer from 43 studies in the Breast Cancer Association Consortium (BCAC) and included in the BRIDGES germline targeted sequencing dataset[12]. Details of the study design, sequencing methodology and variant calling have been described previously[12], and information about the studies included in our analysis are summarized in **Table S2**. This study did not generate new datasets, but the original source data is available from the Breast Cancer Association Consortium (BCAC) via application to the Data Access and Coordination Committee (https://bcac.ccge.medschl.cam.ac.uk/).

The BRIDGES sequencing panel covered 35 genes, but only the 11 genes with an established breast cancer risk association were considered in these analyses. Pathogenic variants: in *BRCA1* (GenBank: NM_007294.4), *BRCA2* (GenBank: NM_000059.4) and *PALB2* (GenBank: NM_024675.4) are high-risk for breast cancer (odds ratio (OR) > 4.0); those in *ATM* (GenBank: NM_000051.4) and *CHEK2* (GenBank: NM_007194.4, MIM [604373]) are associated with moderate breast cancer risk (OR > 2.0); those in *BARD1* (GenBank: NM_000465.4, MIM [601593]), *RAD51C* (GenBank: NM_058216.3, MIM [602774]) and *RAD51D* (GenBank: NM_002878.4, MIM [602954]) are associated with triple-negative breast cancer (OR > 5)[12]; and those in *CDH1* (NM_004360.5), *PTEN* (NM_000314.8) and *TP53* (GenBank: NM_000546.6) are associated with cancer predisposition syndromes that include increased breast cancer risk. *BRIP1* MIM [605882], a gene included in the remit for curation by the ClinGen Hereditary Breast Ovarian Pancreatic VCEP, based on a clear association with ovarian cancer (MIM: 167000) risk, was specifically excluded from our analysis based on lack of evidence for association with breast cancer risk (overall or by subtype) in the BRIDGES and CARRIERS studies[12, 13]. Variant gene and molecular consequence annotation was performed using the Ensembl Variant Effect Predictor (VEP) GRCh37 online portal (June 2022)[14]. Statistical analyses and figure generation were performed using R version 4.3.1 with the *tidyverse* (v2.0.0)[15], *logistf* (v1.26.0), and *cowplot* (v1.1.1) packages.

PVs were defined as single-nucleotide variants or insertions/deletions that are expected loss-of-function (LoF) as per the original ACMG/AMP designation for the PVS1 criterion (initiation codon loss, frameshift, stop gain, or splice site ±1,2 dinucleotide variants), evaluated in conjunction with the ClinGen recommendations for the PVS1 criterion[1, 16], the gene-specific CSpec recommendations as at May 2023 (*ATM*, *BRCA1*, *BRCA2*, *CDH1*, *PALB2*, *PTEN* and *TP53*) and/or ClinVar classification where available[17]. Specifically, 61 variants identified in 1,177 individuals were excluded as PVs for analyses as follows: expected LoF located in last exon or last 50 bp of the penultimate exon; expected LoF variant listed within gene relevant CSpec recommendations as “PVS1_N/A” or “PVS1_Supporting”; ±1,2 dinucleotide variants located in the last splice site motif for mature mRNA or affecting the penultimate exon (*BRCA1* and *CHEK2* only); ±1,2 dinucleotide variants in *BRCA1* that were excluded from the original BRIDGES analysis[12, 18]; expected LoF variants with a ClinVar[17] classification (last reviewed May 2023) of (likely) benign or uncertain significance with a review status of “criteria provided, multiple submitters, no conflicts” or “reviewed by expert panel”. Since individual variant-level data were available for *BRCA1*, *BRCA2* and *TP53*, pathogenic missense variants in these genes with convincing evidence for pathogenicity (see **Table S3** for rationale) were also included in co-observation analysis[12, 17, 19].

Manual review of the BRIDGES sequencing data was undertaken for breast cancer affected individuals originally called as harboring homozygous PVs or compound heterozygous PVs in the same gene, excluding *CHEK2*. After this review and removal of likely sequencing artifacts, two individuals were identified to each harbour two *ATM* PVs (zygosity unknown), and a single individual was found to harbour two *in cis* stop gain *RAD51D* variants. Although not manually reviewed, 15 individuals harbored either homozygous or compound heterozygous *CHEK2* variants, with the majority involving the *CHEK2*:c.1100del variant. Otherwise all other variants included in our analysis were considered as heterozygous in accordance with their original call.

We analyzed the co-observation of PVs among 11 breast cancer risk genes in the BRIDGES breast cancer affected dataset. The probability of observing the actual number of observed co-observations was first calculated based upon the observed frequency of PVs in each comparator under the assumption of independence (see supplementary information). We then estimated the expected range of co-observations by calculating the lower and upper 95% confidence interval (CI) limits for co-observations based on sampling error, assuming no interaction. The actual number of co-observations was designated as depleted if it was less than the expected lower 95% confidence co-observation value, or enriched if it was greater than the expected upper 95% confidence value. Statistical evidence for departure from a multiplicative model for co-observation for each gene-gene pairing was determined by computing Fisher's exact test p-values.

In order to estimate the likelihood ratio (LR) toward pathogenicity for each gene-gene pairing, we compared the occurrence of each gene-gene pairing versus single variant occurrence, with the 95% CIs calculated as previously described (see supplementary information)[20]. An LR of ≤ 0.48 was considered to meet at least supporting benign evidence, based on thresholds recommended from Bayesian modeling of the ACMG/AMP[21].

To mimic the practical use of clinical variant data arising from gene panel sequencing, we then re-assessed genes shown to have evidence for depletion of co-observation with at least one other breast cancer gene (namely, *BRCA1*, *BRCA2*, *PALB2*, *ATM* or *CHEK2*) for co-observation of a PV in that single gene against the remaining genes assessed. As a further analysis, we then repeated the comparisons for *BRCA1*, *BRCA2, PALB2, ATM,* and *CHEK2* against all other genes after excluding PVs in high-risk genes (*BRCA1*, *BRCA2* and *PALB2)*.

Logistic regression analysis was conducted to investigate possible confounding of the observed interactions by participant selection criteria based on overall study design of familial versus population-ascertainment (as designated for the original BRIDGES study), age at breast cancer diagnosis, and study country[12]. These analyses excluded 412 individuals for whom age at diagnosis was missing. The response variable was PV status in the first comparator, and explanatory variables were PV status in the second comparator gene/s, age at diagnosis, study design, and country.

Our findings were then applied to rare *BRCA1* and *BRCA2* missense variants observed within the BRIDGES breast cancer affected dataset, excluding those missense variants already classified as pathogenic for our co-observation analysis (**Table S3**). To minimize overlap with other ACMG/AMP criteria, only variants with a frequency lower than that required to meet at least the BS1_Supporting criterion were considered (global 95% CI filter allele frequency < 0.00002 with sufficient coverage in any of exome (version 2.1.1), genome (version 2.1.1) or genome (version 3.1.2) gnomAD datasets[22, 23] modeled against the *BRCA1* and *BRCA2* CSpec recommendations (V1.1.0). Any of these rare missense variants that were found in co-observation with a PV in another gene were classified following the CSpec *BRCA1* and *BRCA2* specifications using publicly available information[24-41], and ACMG/AMP criterion weights aligned to points as per published recommendations[42]. The impact on change in classification with addition of co-observation evidence was recorded.

Across the 11 breast cancer genes there were 1,261 unique PVs observed in 3,832 individuals (**Figures 1A and 1B**). Approximately 6.9% of individuals carried at least one PV, with PVs in *BRCA2* (2.0% of individuals) being the most frequent (**Figure 1B**). The *CHEK2*:c.1100del (p.Thr367MetfsTer15) variant was the most common individual PV, observed in 1.4% of all individuals and 19.8% of PV carriers. Co-observation of PVs was seen in 50 individuals, involving all genes except *CDH1* and *PTEN* (**Figure 1C**).

No individual was found to carry PVs in three or more genes. All co-observation instances represented unique variant pairs, except the co-observation of *TP53*:c.1010G>A (p.Arg337His) with *CHEK2*:c.1100del (p.Thr367MetfsTer15) in two individuals. Another three PVs, in addition to the former *CHEK2* and *TP53* variants,

were involved in multiple co-observation events: *BRCA1*:c.5266dup (p.Gln1756ProfsTer74); *CHEK2*:c.444+1G>A and *RAD51D*:c.451C>T (p.Gln151Ter). Furthermore, *CHEK2*:c.1100del was the most common variant involved in co-observation instances. All PVs occurring in co-observation events were in the heterozygous state. The list of PV co-observation events, including variant details, is provided in **Table S4**.

Most (80.2%) of the 55,815 individuals included in our analysis were from studies that ascertained participants independently of family history of cancer (population-based studies), with only 19.8% from familial studies (**Table S2**). The reported age at breast cancer diagnosis ranged from 17 to 98 years (average 54.8 years) for the 55,403 individuals with age information available. Although the age of breast cancer diagnosis did differ significantly with regards to overall PV carrier status (carriers mean 49.9 years vs non-carriers 55.1 years, $p<2.2e-16$, two-sided Student's t-test), there was no significant difference in age of breast cancer diagnosis between single and dual PV carriers (single carriers mean 49.9 years vs dual carriers mean 49.0 years, $p=0.584$). The proportion of individuals with familial ascertainment was 28.6% in carriers versus 19.7% in non-carriers.

The actual count of PV co-observation fell within the expected 95% CI range for most pairwise gene comparisons, and there was no evidence for departure from a multiplicative model based on the Fisher's exact test (summarized in **Table S5**). There was evidence for the depletion of PV co-observation for five gene combinations: *BRCA1* and *BRCA2*; *BRCA1* and *PALB2*; *BRCA2* and *ATM*; *BRCA2* and *CHEK2*; and *BRCA2* and *PALB2* (**Table 1**). This evidence for depletion of co-observation remained when comparing each of *BRCA1*, *BRCA2*, *PALB2*, *ATM* and *CHEK2* against all other breast cancer genes. Statistical evidence ($p<0.05$) for departure from a multiplicative model was seen for co-observation of *BRCA1* and *BRCA2* in the gene pair analysis, and for each of *BRCA1*, *BRCA2*, *PALB2, ATM* and *CHEK2* against all other breast cancer genes.

Co-observation of PVs in *BRCA1*, *BRCA2*, *PALB2*, *ATM,* and *CHEK2* with PVs in other genes was then assessed after excluding the designated high-risk variant genes (*BRCA1*, *BRCA2,* and *PALB2*) from the comparison gene group. For these analyses evidence of depletion remained for *BRCA1*, *BRCA,2* and *PALB2* based on comparison of observed counts to the expected range (95% CI), reaching statistical significance based on the Fisher's exact test for *BRCA1* ($p=0.04$) and *BRCA2* ($p=0.02$) but not *PALB2* ($p=0.06$). These findings indicate that high-risk PVs in *BRCA1*, *BRCA2,* and *PALB2* drove the depletion findings observed for *ATM* and *CHEK2*.

Logistic regression analysis showed that evidence for depletion of PV co-observation was not confounded by age at diagnosis, study design or country of origin. That is, significant evidence for depletion from the crude analysis (either gene-gene or single gene-other genes) remained after adjustments for these variables (**Table S6**).

The estimated LR toward variant pathogenicity was ≤ 0.48 for co-observation of a PV in *BRCA1*, *BRCA2* or *PALB2* with a PV in any other gene (**Table 1**). This was also true for *ATM* co-observation with a PV in any other gene, but the interaction was driven by the high-risk PV genes. This information has applicability for the classification of variants of uncertain significance (VUS) identified within the BRIDGES breast cancer affected dataset; that is co-observation of a VUS in any one of *BRCA1*, *BRCA2* or *PALB2* (but not *ATM*) with a PV in any of the other breast cancer panel gene considered in this analysis would provide at least benign supporting evidence in ACMG/AMP classification of the co-observed VUS.

There were 30 rare *BRCA1* (out of 618 total) and 61 rare *BRCA2* (out of 1,454 total) missense variants found to be co-observed with a PV in another gene in the BRIDGES breast cancer affected cohort (**Table S7**). Slightly less than half of these co-observations (40.7%) involved a *BRCA1* missense variant with a *BRCA2* PV, or vice versa. After application of the CSpec *BRCA1* and *BRCA2* specifications, these missense variants were classified as: benign (n = 24), likely benign (n = 53), VUSs (n = 5), or likely pathogenic (n = 1). Likely benign classification was mostly based upon application of the BP1_Strong criterion (variant located outside of a functional domain with no predicted impact on splicing). However, upon addition of evidence from our co-observation analysis (equivalent to benign supporting evidence, or -1 point in the Bayesian classification framework) the initial classification was changed for five

missense variants (6.0%, 5/83); four increased in classification certainty from likely benign to benign, and one moved from likely pathogenic to VUS.

Pathogenic variant co-observations have been described previously for most gene combinations examined in our analysis, including between the high-risk variant genes *BRCA1*, *BRCA2* or *PALB2* and moderate-risk variant genes such as *ATM* or *CHEK2*[43-53]. Moreover, rare instances of concomitant observations of PVs in three different cancer predisposition genes have also been reported, including a report of three PVs in breast cancer risk genes (*BRCA1* c.5266dupC, *PALB2* c.3771C>T and *TP53* c.1010G>A) in sisters with early-onset breast cancer [47, 54, 55]. Consistent with these previous observations, our data provided no significant indication (p=0.584) that dual carriers of PVs in different genes have a markedly different phenotypic presentation, including obviously different distribution of age at diagnosis, compared to carriers of a single PV. However, the findings do provide statistical evidence for depletion of co-observation of PVs in *BRCA1*, *BRCA2* and *PALB2* with PVs in other breast cancer genes, where the complete list of breast cancer genes included *BRCA1*, *BRCA2*, *ATM*, *CHEK2*, *CDH1*, *PALB2*, *BARD1, PTEN, RAD51C, RAD51D* and *TP53*. This depletion, relative to the frequency expected if co-occurrence was independent, persisted after adjustment for age at diagnosis, study design, and country of origin. Despite no striking differences in clinical presentation for dual carriers, our findings provide statistical justification for application of the BP5 code for co-observation of a VUS with a PV in the context of at least some gene combinations. In this dataset, co-observation of a VUS in *BRCA1* (LR 0.36), *BRCA2* (LR 0.39) or *PALB2* (LR 0.18) with a PV in another breast cancer gene equates to at least supporting evidence against pathogenicity following criterion strength assignment based on an LR of 0.23-0.48:1[21].

Similar depletion findings have been reported previously, from the analysis of a smaller cohort of familial breast cancer affected individuals (5,280 for *CHEK2*:c.del1100C analysis; 1,411 for *ATM* analysis). Turnbull *et al* observed lower than expected frequency of co-observation of *BRCA1* and *BRCA2* PVs with PVs in selected exons of *ATM*, and with the *CHEK2*:c.del1100C variant[56]. These authors speculated that for individuals with functional abrogation of *BRCA1* or *BRCA2*, limited additional risk for breast cancer is conferred by LoF variants in genes such as *ATM* or *CHEK2*, which lie upstream in the homologous recombination pathway. An alternative explanation may be that nearly all tumors in *BRCA1, BRCA2* and *PALB2* carriers occur in cells that have undergone inactivation of the wild-type allele, whereas this is a much less consistent mechanism in tumors arising in *ATM* and *CHEK2* PV carriers[57-61]. These biological hypotheses could be investigated in future work by examining patterns of somatic loss of the co-occurring genes in tumors. Nevertheless, while a biological explanation for the observation of depletion can be speculated, statistically the results indicate that when a PV is present in a high-risk variant breast cancer gene the effect of an additional PV is not multiplicative. Instead the risk associated with PVs in the high-risk variant breast cancer genes in combination with each other or a moderate-risk gene was attenuated by 40-80% compared to the expectation based on the OR estimated in the BRIDGES study[12].

These analyses were based on a large cohort of individuals diagnosed with breast cancer the majority from studies that did not select participants based on family history. It is important to note that the degree of depletion, and hence the appropriate likelihood ratio, may differ in other contexts: for example, if the study participants were selected for triple-negative breast cancer, or ovarian cancer. We found no evidence for depletion of co-observed PVs in moderate-risk variant genes with approximately 2-fold cancer risk, including those involving *ATM* and *CHEK2* where the numbers of PV carriers in the dataset are similar to those seen for high-risk variant genes. However, since the expected depletion is likely to be smaller in absolute terms for moderate-risk genes, larger better-powered studies may be required to rule out presence of depletion.

Our statistical analysis identified depletion of co-observation of PVs in the *BRCA1, BRCA2,* and *PALB2* high-risk variant breast cancer genes compared to other genes commonly included in clinical breast cancer gene panels. These findings indicate that the *BRCA1*, *BRCA2,* and *PALB2* gene-specific classification criteria could allow for co-observation to be applied as benign supporting evidence for rare variants that do not already meet benign frequency

criteria, if justified by cohort-specific calibration. For this study specifically, identification of a VUS in *BRCA1*, *BRCA2* or *PALB2*, in an individual with a PV in another breast cancer gene (here listed as *BRCA1*, *BRCA2*, *PALB2, ATM*, *CHEK2*, *BARD1*, *RAD51C, RAD51D, CDH1*, *PTEN,* and *TP53*), could be used to provide supporting evidence against pathogenicity for that VUS. Our review of missense VUSs in the BRIDGES breast cancer affected individuals indicated that the BP5 criterion could be applied for 4.4% (91/2,072) of rare *BRCA1* or *BRCA2* missense variant observations in this cohort. Inclusion of this evidence type strengthened classification of approximately 5% of these variant observations. Moreover, it provided the only clinical evidence against pathogenicity for many of the variants found in co-observation with another PV in our dataset. The application of this data type may have even more value for classification of *PALB2* variants, since this gene is relatively understudied compared to *BRCA1* and *BRCA2*.

Calibration of this data type as a predictor against variant pathogenicity for other disease gene panels may highlight its value in variant classification for other clinical contexts. To promote dataset-specific calibration of any group of hereditary disease genes, we provide an accessible example Excel calculator to determine relevance and strength level for co-observation evidence for a given dataset. This calculator is provided as **Table S8** to assist the reader in determining the relevance and strength level for co-observation evidence in other similar sequencing datasets. These findings have the potential to justify use – or non-applicability – of co-observation data for existing gene-specific criteria developed for other breast cancer genes included in our analysis.

Data and code availability

Information to replicate the findings of this study are shown in the supplementary material.

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Supplemental Tables

Table S1. Overview of BP5 ACMG/AMP specifications for different hereditary cancer genes.
Table S2. Summary of BRIDGES studies included in the analysis.
Table S3. Rationale for inclusion of pathogenic missense variants in *BRCA1*, *BRCA2* and *TP53*.
Table S4. List of pathogenic variant co-observation events.
Table S5. Evidence for depletion of co-observation of pathogenic variants, and estimated likelihood ratio towards variant pathogenicity for co-observation.
Table S6. Results from logistic regression analysis adjusting for age, study design and study country as potential confounder of co-observation depletion for *BRCA1*, *BRCA2, PALB2, ATM* and *CHEK2.*
Table S7. Rare *BRCA1* and *BRCA2* missense variants that were co-observed with a pathogenic variant in another breast cancer gene.
Table S8. Template for analysis of depletion/enrichment of co-observed pathogenic events, and to estimate likelihood ratio towards pathogenicity for co-observation.

Author contributions

Conceptualization and methodology: ALD, KM, PAJ, ABS. Resources and data acquisition: all authors. Data curation, formal analysis and visualization: ALD, KM, MTP, CF, MN, PAJ, ABS. Writing original draft: ALD, KM, PD, DE, PAJ, ABS. Writing review and editing: all authors. Final approval of manuscript: all authors.

Declaration of interests

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Web resources

ClinGen Criteria Specification Registry, https://cspec.genome.network/cspec/ui/svi/
Breast Cancer Association Consortium (BCAC), https://bcac.ccge.medschl.cam.ac.uk/
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
gnomAD, https://gnomad.broadinstitute.org/
Ensembl Variant Effect Predictor (VEP) online portal, https://grch37.ensembl.org/Homo_sapiens/Tools/VEP
logistf R package, https://CRAN.R-project.org/package=logistf
cowplot R package, https://CRAN.R-project.org/package=cowplot

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Figure Legends

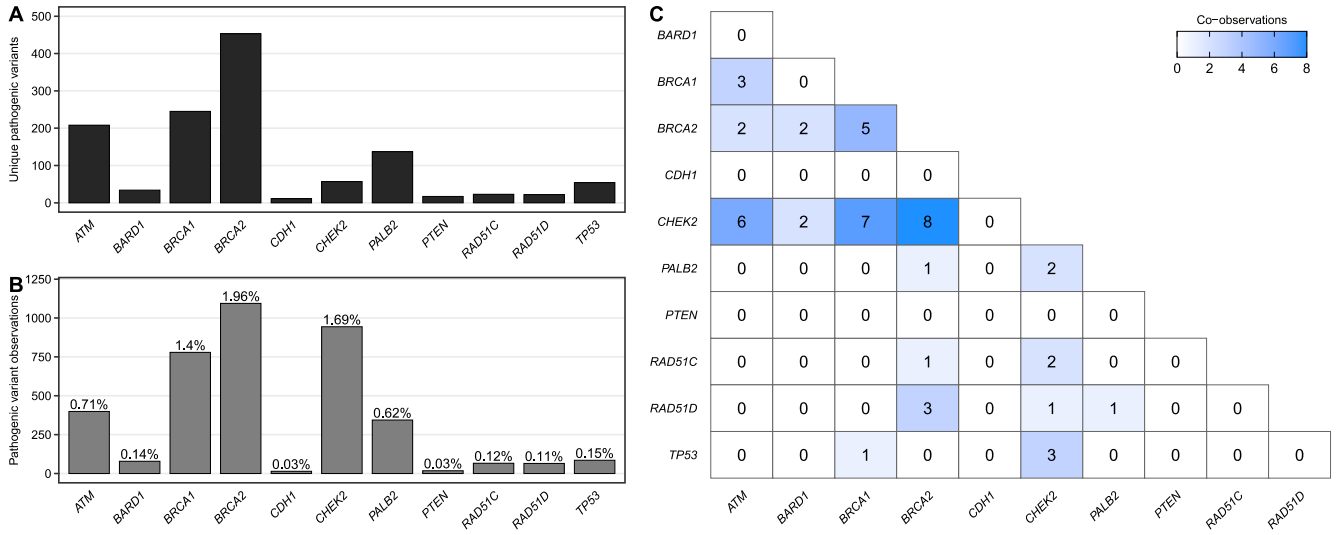

Figure 1. Observation of pathogenic variants (PVs) in hereditary breast cancer genes within the BRIDGES breast cancer affected cohort.
The BRIDGES breast cancer affected cohort consists of 55,815 individuals. (A) Number of unique PVs observed in the cohort. (B) Number of PVs observed within the cohort with the proportion of individuals with at least one PV in the designated gene given above bars. (C) Number of PV co-observations, shown for each gene pair. PVs in *CDH1* or *PTEN* were not co-observed with a PV in another gene. Final figure formatting performed using Inkscape (version 0.92.3).

Table 1. Genes with evidence for depletion of co-observation of pathogenic variants (PVs) in BRIDGES breast cancer affected cohort.

Comparison Gene A vs Gene B		Co-observed PVs	PV in Gene A only	PV in Gene(s) B only	Expected count individuals with co-observed PVs (binomial 95% CI)	Fisher's Exact p-value	Likelihood ratio (LR)	LR meets at least supporting benign evidence (≤ 0.48)[a]
Pairwise comparisons								
BRCA1	*BRCA2*	5	763	1072	8 - 23	0.027	0.32 (0.13 - 0.77)	Yes
	PALB2	0	763	340	1 - 10	0.062	0.10 (0.01 - 1.62)	Yes[b]
BRCA2	*PALB2*	1	1072	340	2 - 12	0.070	0.15 (0.02 - 1.03)	Yes
	ATM	2	1072	386	3 - 14	0.109	0.26 (0.06 - 1.02)	Yes
	CHEK2	8	1072	911	10 - 27	0.052	0.43 (0.22 - 0.86)	Yes
Single gene versus combined all other genes (*ATM*, *BRCA1*, *BRCA2, CDH1*, *CHEK2*, *BARD1*, *PALB2*, *PTEN*, *RAD51C*, *RAD51D*, *TP53*)[c]								
BRCA1	All other genes	16	763	3053	31 - 56	0.001	0.36 (0.22 - 0.59)	Yes
BRCA2		22	1072	2738	40 - 69	<0.001	0.39 (0.26 - 0.60)	Yes
PALB2		4	340	3488	13 - 31	0.001	0.18 (0.07 - 0.47)	Yes
ATM		11	386	3435	15 - 35	0.029	0.43 (0.24 - 0.79)	Yes
CHEK2		31	911	2890	36 - 64	0.045	0.62 (0.43 - 0.88)	No
Single gene versus combined all other genes excluding the high-risk variant genes (*ATM*, *CDH1*, *CHEK2*, *BARD1*, *PTEN*, *RAD51C*, *RAD51D*, TP53)[d]								
BRCA1	All other genes (exclude high-risk)	11	763	1621	14 - 33	0.041	0.47 (0.26 - 0.84)	Yes
BRCA2		16	1072	1621	22 - 44	0.021	0.48 (0.30 - 0.79)	Yes
PALB2		3	340	1621	5 - 17	0.057	0.28 (0.09 - 0.88)	Yes
ATM		6	386	1229	4 - 15	0.454	0.66 (0.29 - 1.47)	No
CHEK2		14	911	696	6 - 20	1.000	1.14 (0.68 - 1.93)	No

[a] LR ≤ 0.48 was considered as meeting at least supporting benign evidence based upon a Bayesian modelling of the ACMG/AMP criteria proposed by Tavtigian *et al*[21].

[b] Haldane correction was applied for likelihood calculation.

[c] Gene A versus all other genes listed excluding Gene A.

[d] Gene A versus all other genes listed excluding Gene A; other genes list excludes high risk variant genes *BRCA1*, *BRCA2* and *PALB2*.

Abbreviations: CI, confidence interval; LR, Likelihood ratio; PV, pathogenic variant