Analysis of rare disruptive germline mutations in 2135 enriched BRCA-negative breast cancers excludes additional high-impact susceptibility genes


Background: Breast cancer has a significant heritable basis, of which ~60% remains unexplained. Testing for BRCA1/BRCA2 offers useful discrimination of breast cancer risk within families, and identification of additional breast cancer susceptibility genes could offer clinical utility.

Patients and methods: We included 2135 invasive breast cancer cases recruited via the Breast and Ovarian Cancer Susceptibility Collaboration, a retrospective UK study of familial breast cancer. Eligibility criteria: female, white European ethnicity, and one of: (i) breast cancer family history, (ii) bilateral disease, (iii) young age of onset (<30 years), and (iv) comitant ovarian cancer.

We undertook exome sequencing of cases and carried out gene-level burden testing of rare damaging variants against those from 51 377 ethnicity-matched population controls from gnomAD.

Results: 159/2135 (7.4%) cases had a qualifying variant in an established breast cancer susceptibility gene, with minimal evidence of signal in other cancer susceptibility genes. Known breast cancer susceptibility genes PALB2, CHEK2, and ATM were the only genes to retain statistical significance after correcting for multiple testing. Due to the enrichment of hereditary cases in the series, we had good power (>80%) to detect a gene of BRCA1-like risk [odds ratio (OR) = 10.6] down to a population minor allele frequency of $4.6 \times 10^{-5}$ (1 in 10 799, less than one-tenth that of BRCA1) and of PALB2-like risk (OR = 5.0) down to a population minor allele frequency of $2.8 \times 10^{-5}$ (1 in 1779, less than half that of PALB2). Power was lower for identification of novel moderate penetrance genes (OR = 2-3) like CHEK2 and ATM.

Conclusions: This is the largest case-control whole-exome analysis of enriched breast cancer published to date. Whilst additional breast cancer susceptibility genes likely exist, those of high penetrance are likely to be of very low mutational frequency. Contention exists regarding the clinical utility of such genes.
INTRODUCTION
Female breast cancer is now the most common cancer with 2.3 million cases diagnosed annually worldwide and a lifetime risk of ~15%. The heritable basis of the disease is well established and evidenced by the increased risk in relatives of cases. It is now well recognized that the genetic architecture of breast cancer susceptibility encompasses a broad spectrum, from common polymorphisms individually conferring small risks through to breast cancer susceptibility genes (BCSGs; Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2022.09.152) characterized by multiple disparate mutations. BCSGs are typically categorized by their disease penetrance, for example, as very high penetrance [odds ratio (OR) ≥ 10], high (OR ≥ 5), or moderate/intermediate (OR ≥ 2).

It is estimated that ~41% of the heritable risk of breast cancer has been deciphered. Of the 23% attributable to rare variants, the majority is accounted for by BRCA1 and BRCA2 (17%), with other established BCSGs (PALB2, ATM, CHEK2, TP53, STK11, PTEN, NF1, CDH1, BARD1, RAD51C, RAD51D) collectively accounting for about 6%. It is estimated that 18% of genetic risk of breast cancer resides in common variants identified by genome-wide association studies (GWAS), with statistical modelling suggesting a further 23% is potentially tractable by larger GWAS. Of the 59% of unexplained heritable risk, over half (36%) is thus potentially ascribable to hitherto unidentified rare variants. Whilst many of these variants may be non-coding and/or of low penetrance, there is considerable interest in the component that may be enshrined in further susceptibility genes of moderate to high penetrance which are both mechanistically important as well as potentially relevant to the clinical management of patients and their families. Numerous studies have used next-generation sequencing (NGS) technologies to search this space for novel BCSGs (Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2022.09.152), though few if any convincing candidates to date have been identified.

To look for novel BCSGs, we carried out germline whole-exome sequencing (WES) of 2135 BRCA1/BRCA2-negative genetically enriched breast cancer cases. We used a three-phase analytical strategy (Figure 1). Firstly, we determined the contribution of pathogenic variants in established autosomal dominant cancer susceptibility genes (CSGs; n = 86 genes). Secondly, we assessed the contribution of candidate genes implicated on the basis of involvement in DNA repair (n = 276 genes), and/or a known oncogenic role in cancer (n = 686 genes) by carrying out burden analyses collapsing at the gene level. Finally, we carried out exome-wide rare-variant burden testing, undertaking correction for cumulative performance of multiple tests.

PATIENTS AND METHODS
Subjects and data
We included BRCA1/BRCA2-negative women with a diagnosis of pathologically confirmed invasive breast cancer (n = 2430), recruited to the Institute of Cancer Research Breast and Ovarian Cancer Susceptibility (BOCS) study. Eligibility criteria included one of: (i) a significant family history of breast cancer (breast cancer in at least one first-degree relative or two second-degree relatives); (ii) bilateral breast cancer; (iii) early-onset breast cancer (<30 years); and (iv) concomitant ovarian cancer. Cases were assigned a ‘genetic enrichment score’ based on allele sharing to reflect the degree of personal and family history of breast cancer as follows: bilateral disease in the proband received a score of 1, with additional points assigned for disease in first-degree relatives (0.5 each), second-degree relatives (0.25 each), and third-degree relatives (0.125 each). We used a score of ≥1.5 to define a set of ‘high-risk’ cases (n = 855).

The proportion of cases of self-reported Ashkenazi Jewish ancestry was <1%.

Written informed consent was obtained from all participants and the research was approved by the London Multicentre Research Ethics Committee (MREC/01/2/18).

For controls, we used WES data from 51377 non-Finnish European non-cancer individuals from the Genome Aggregation Database (gnomAD) (date of accession 21 May 2020).

Whole-exome sequencing and quality control
Germline DNA from cases underwent WES using Illumina technology. Data processing, variant calling, and annotation were carried out according to Genome Analysis Toolkit (GATK) best practices. We excluded cases based on quality, cryptic familial duplicates, presence of occult BRCA1/BRCA2 mutations, and ancestry analysis using principal components (Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2022.09.152).

Case-control variant set alignment
Variant-level exclusions are described in Supplementary Table S4, available at https://doi.org/10.1016/j.annonc.2022.09.152 and Supplementary methods, available at https://doi.org/10.1016/j.annonc.2022.09.152. Analyses were restricted to rare variants with a minor allele frequency (MAF) < 0.5%.

We controlled against spurious inflation of the burden test statistic by filtering the bottom fifth centile of variants based on the GATK variant quality metric QualByDepth (QD) and restricting analyses to base positions with comparable levels of
Statistical analyses

We carried out collapsed gene-level burden analyses in: (i) autosomal dominantly acting CSGs \((n = 86)\)^11,12; (ii) DNA repair genes \((n = 276)\)^13; (iii) somatic cancer driver genes \((n = 686)\)\(^{14}\); and (iv) the exome \((n = 19,651)\) (Supplementary Table S6, available at https://doi.org/10.1016/j.annonc.2022.09.152). A single class of test was carried out for the CSGs, comprising high-confidence protein truncating variants (HC-PTVs, as defined by Loss-of-Function Transcript Effect Estimator\(^{15}\) plus variants (regardless of variant consequence or LOFTEE annotation) classified as pathogenic/likely pathogenic (P/LP) on ClinVar with one or more stars using The American College of Medical Genetics and Genomics criteria.\(^{16}\) A single class of test was also carried out for missense/in-frame variants at recurrently somatically mutated residues in cancer driver genes (as defined in oncoKB; Supplementary Table S7, available at https://doi.org/10.1016/j.annonc.2022.09.152). For the DNA repair/exome-wide analyses, two classes of test were carried out: class I (HC-PTVs only) and class II (HC-PTVs + missense...
The proportion of REVEL-qualifying missense variants that were also pathogenic in ClinVar in known CSGs was 9/172 (5.2%).

Association was measured using Fisher’s exact test (two-sided) comparing case-control proportions with a qualifying variant. For declaring statistical significance, we imposed a Bonferroni correction of \( P < 0.05 \) for the number tests carried out, accounting for cumulative testing in candidate-based followed by exome-wide analyses. For replication, we used WES data from UK Biobank comprising 15 017 female breast cancer cases and 199 479 healthy women with no previous history of cancer, assigning \( P < 0.05 \) as nominal evidence of replication. Analyses were carried out in R (v4.0.3).

**Calculation of study power**

Disease allele frequency in controls was taken as the baseline, and the frequency in cases was determined by a weighted average of the predicted enrichment in cases with bilateral disease and/or an affected first-degree relative. Fisher’s exact test (two-sided) was carried out for each sampling of cases and controls, carried out 10 000 times for each frequency/relative risk combination.

Additional methodological information is available in the Supplementary methods, available at https://doi.org/10.1016/j.annonc.2022.09.152.

**RESULTS**

The final case dataset comprised 2135 BRCA-negative breast cancer cases, of which 838 (39%) had bilateral disease, 1628 (76%) had a significant family history of breast cancer (at least one first-degree relative or two second-degree relatives), 183 (9%) had breast cancer diagnosed \( \leq 30 \) years, and 261 (12%) had concomitant ovarian cancer (Figure 2). The cases were enriched for bilaterality, family history, and early-onset disease compared to unselected cases from the literature (Supplementary Figure S4, available at https://doi.org/10.1016/j.annonc.2022.09.152). Mean age at first diagnosis was 48 years (compared to the UK average of \( >60 \) years).  

**Frequency of pathogenic germline variants in cancer susceptibility genes**

A total of 159/2135 (7.4%) of cases had a qualifying variant (HC-PTV and/or ClinVar P/LP) in one of the 13 established BCSGs, including 44 (2.1%) in PALB2, 41 (1.9%) in ATM, and 53 (2.5%) in CHEK2 (Figure 3, Supplementary Table S8, available at https://doi.org/10.1016/j.annonc.2022.09.152). Eleven cases (0.5%) had a variant in genes associated with estrogen receptor (ER)-negative disease: BARD1 (5, 0.2%), RAD51C (4, 0.2%), RAD51D (2, 0.1%). Eleven cases (0.5%) had a variant in a rare pleiomorphic cancer susceptibility gene, comprising TP53 (7, 0.3%), CDH1 (1, 0.05%), NF1 (2, 0.1%), and PTEN (1, 0.05%). There was no significant difference in the age of breast cancer diagnosis, burden of family history, or frequency of bilateral disease between cases with and without a qualifying variant (\( P > 0.05 \) in relevant statistical test: see Supplementary methods, available at https://doi.org/10.1016/j.annonc.2022.09.152).

There were no significant associations with breast cancer amongst the other CSGs, though there was an appreciable case frequency of qualifying variants in some, notably MSH6 (10 cases) and BRI1 (5 cases), genes previously proposed as BCSGs (Figure 3, Supplementary Table S8, available at https://doi.org/10.1016/j.annonc.2022.09.152). Excluding the 13 established BCSGs, the frequency of qualifying variants in CSGs was not significantly elevated in cases (53/2135 or 2.5%) compared to controls (1365/51 377 or 2.7%) (\( P = 0.68 \)).

**Candidate gene burden testing**

Cognizant of the impact of correction for multiple testing, we firstly considered targeted candidacy-based approaches. On the functional basis of genes implicated to date in breast

![Figure 2. Distribution of case phenotypic features. Distribution of phenotypes across 2135 probands with invasive breast cancer where 'Family history' denotes at least one first-degree or two second-degree relatives affected with breast cancer, 'Ovarian' denotes the proband also being diagnosed with invasive ovarian cancer, 'Young onset' denotes the proband's first cancer being diagnosed at age <30, and 'Bilateral' denotes a diagnosis of bilateral breast cancer.](https://doi.org/10.1016/j.annonc.2022.09.152)
cancer susceptibility, we first considered 276 genes involved in DNA repair and carried out burden analysis for rare variants (MAF < 0.5%) of class I (HC-PTVs only) and class II (HC-PTVs and deleterious non-synonymous variants). PALB2, CHEK2, and ATM emerged from this analysis as strongly associated (Figure 3, Supplementary Table S8, available at https://doi.org/10.1016/j.annonc.2022.09.152). WTN was the highest ranked (non-BCSG) DNA repair gene by P value, driven particularly by the HC-PTV frequency, but the association did not retain significance following Bonferroni correction (class I test: 10/2135, 0.47% cases versus 73/51,377, 0.14% controls, \( P = 0.002 \)). There was only nominal statistical support on querying of the UK Biobank data for association of this gene with breast cancer, (\( P = 0.0087 \)), which again did not withstand correction for multiple testing (Supplementary Table S9, available at https://doi.org/10.1016/j.annonc.2022.09.152). Excluding the known BCSGs, collapsing across the remaining 263 genes involved in DNA repair the signal of association was not significant on analysis of variants of class I \( (10/2135 \times 73/51,377 \times 0.5\%) \) of class I (HC-PTVs only) and class II (HC-PTVs and deleterious non-synonymous variants) overlapping with known oncogenic residues \( (81/2135 \times 73/51,377 \times 0.5\%) \) of class II (HC-PTVs only) and class II (HC-PTVs and deleterious non-synonymous variants). For all three genes, the ORs for breast cancer risk with class I variants were substantially inflated compared to published estimates from unselected breast cancers,\(^{26-28}\) commensurate with the inflation predicted on the basis of the genetic enrichment in our cases (Supplementary Table S8, available at https://doi.org/10.1016/j.annonc.2022.09.152). Amongst the top 20 associations ranked by the smallest \( P \) value were \( C20orf141 \) (class I and class II) and \( EXPH5 \) (class I and class II), but the associations were not significant following Bonferroni correction. Furthermore, neither these genes nor any others in the top 20 associations showed evidence of replication in the UK Biobank \( (P > 0.1); \) Supplementary Table S9, available at https://doi.org/10.1016/j.annonc.2022.09.152).

Next, on the basis of exemplar genes associated with both autosomal dominant cancer susceptibility and somatic oncogenic mechanisms [e.g. \( RET \) mutations predisposing to thyroid cancer and \( KIT \) mutations predisposing to gastrointestinal stromal tumor (GIST)], we considered 686 cancer driver genes for which recurrent somatic hotspot mutations have been reported. On burden analysis for rare missense/in-frame variants (MAF < 0.5%) overlapping with known oncogenic residues (Supplementary Figure S5, available at https://doi.org/10.1016/j.annonc.2022.09.152, Supplementary Table S8, available at https://doi.org/10.1016/j.annonc.2022.09.152), there were no significant associations after correcting for multiple testing.

**Exome-wide rare-variant burden analysis**

We carried out exome-wide burden testing, again collapsing at the gene level for association of rare variants (MAF < 0.5%) of each of class I and class II. Again, only for \( PALB2 \) (classes I and II), \( CHEK2 \) (classes I and II), and \( ATM \) (class II only) were the associations significant following Bonferroni correction (Figure 3, Supplementary Table S8, available at https://doi.org/10.1016/j.annonc.2022.09.152). For all three genes, the ORs for breast cancer risk with class I variants were substantially inflated compared to published estimates from unselected breast cancers,\(^{26-28}\) commensurate with the inflation predicted on the basis of the genetic enrichment in our cases (Supplementary Table S8, available at https://doi.org/10.1016/j.annonc.2022.09.152). Amongst the top 20 associations ranked by the smallest \( P \) value were \( C20orf141 \) (class I and class II) and \( EXPH5 \) (class I and class II), but the associations were not significant following Bonferroni correction. Furthermore, neither these genes nor any others in the top 20 associations showed evidence of replication in the UK Biobank \( (P > 0.1); \) Supplementary Table S9, available at https://doi.org/10.1016/j.annonc.2022.09.152).

Notable amongst the top 20 ranked gene associations in this analysis was \( PPM1D \). Eroneously reported originally as a BCSG, \( PPM1D \) is characterized by somatic mosaic mutations arising in hematopoietic cells in response to chemotherapy.\(^{26-28}\) Consistent with somatic mosaicism in blood
cells, the PPM1D VAFs detected in this study were skewed downwards for PTVs compared to synonymous variants (Kolmogorov–Smirnov P value = 0.11, Mood’s median P value = 0.17; Supplementary Figure S6, available at https://doi.org/10.1016/j.annonc.2022.09.152).

There was likewise no evidence of association for any genes on subsequent restriction to a smaller set of 855 ‘high-risk’ cases (see Methods, Supplementary Table S8, available at https://doi.org/10.1016/j.annonc.2022.09.152).

On gene-set enrichment analysis of gene ontology terms and Kyoto Encyclopedia of Genes and Genomes pathways amongst genes with a class I/class II test P value ≤ 0.01,29,30 multiple gene groups showed significant associations, primarily related to DNA replication and repair, but once corrected for the signal of association for constituent known BCSGs no gene groups showed significant residual association (Supplementary Table S10, available at https://doi.org/10.1016/j.annonc.2022.09.152).

Power analyses
Recent large-scale genetic epidemiologic analyses have confirmed BRCA1 as being of almost twice the penetrance but much lower mutational frequency than BRCA2 (BRCA1: OR = 10.6, population MAFcombined = 0.10% or 1 in 494; BRCA2: OR = 5.9, population MAFcombined = 0.16% or 1 in 306). However, PALB2 (OR = 5.0, population MAFcombined = 0.07% or 1 in 667) has substantially lower ‘impact profile’ (penetrance × mutational frequency) with risk in the rough range of BRCA2 but a mutational frequency similar to BRCA1. Taking into account the enrichment as well as the size of our case and control series, we had very good power (90%) to identify a new high-impact BCSG conferring a BRCA2-like breast cancer risk (OR = 10.6) with population-combined mutation frequency (MAFcombined) down to (i) 0.0043% (or 1 in 11 628) in the targeted DNA repair gene analysis or (ii) MAFcombined of 0.0056% (or 1 in 8945) in the WES analysis. For a new gene of PALB2-like (OR = 5) or ATM-like (OR = 2.1) breast cancer risk, we had 90% power to identify a gene of MAFcombined down to (i) 0.026% (1 in 1946) or 0.37% (1 in 135), respectively, in the DNA repair analysis and (ii) 0.034% (1 in 1475) and 0.5% (1 in 100) in the WES analysis (Figure 4, Tables 1 and 2).

These estimates are likely to be inherently conservative, for although we have accounted for bilateral disease and the first affected first degree relative in our series where present, we have not accounted for the additional family members with breast cancer, young age of cases, and concomitant occurrence of ovarian cancer.20

Power calculations representing variability in population MAFCombined, the largest breast WES study published to date, and expansion of the current study with increasing sample size, are presented in Supplementary Tables S11, S12, and S13, available at https://doi.org/10.1016/j.annonc.2022.09.152, respectively.

DISCUSSION
In our series of 2135 enriched BRCA-negative female breast cancer cases, only 159 (7.4%) have a P/LP variant in an established BCSG. The vast majority (138/159, 87%) of these P/LP variants were in three genes: PALB2, CHEK2, and ATM. As would be anticipated from our ascertainment framework, the contribution of P/LP variants in genes associated with pleiomorphic tumor syndromes was very modest (0.5% of cases). Furthermore, given that P/LP variants in BARD1, RAD51C, and RAD51D are of relatively low frequency even in the triple-negative breast cancers with which they are associated, their contribution was predictably low in this series ascertained agnostic to histology (0.5% of cases). The frequency in cases of P/LP variants in other autosomal dominant CSGs (2.5%) was not elevated compared to controls (2.7%), suggesting firstly that these variants are largely incidental to etiology of these breast cancers and secondly that amongst other established CSGs there is minimal unexplained residual pleiotropy for breast cancer.

In our subsequent exploration of residual breast cancer genetic susceptibility attributable to rare variants, aside from ‘rediscovery’ of established BCSGs PALB2, CHEK2, and ATM, no gene attained statistically significant signal of association at the respective thresholds for either the candidate-based approaches or the exome-wide approach. Furthermore, there was no evidence for association found in a replication series (UK Biobank) for top-ranking genes with nonsignificant signals of association.

To our knowledge, our study is the largest case-control analysis reported to date in enriched breast cancer cases, and better powered for discovery of a new rare high-penetrance susceptibility gene than the largest reported case-control analysis to date of unselected breast cancers (7859 cases/117 456 controls)19 (Supplementary Tables S2 and S12, available at https://doi.org/10.1016/j.annonc.2022.09.152).

Contextualization of our findings with previous studies
In the 1990s, linkage analysis in modest numbers of multi-case pedigrees enabled identification of BRCA1 followed by BRCA2.31-34 Subsequent linkage analyses in larger series of BRCA1- and BRCA2-negative breast cancer families yielded no reproducible signals.35 Thereafter followed case-control mutational screening for genes functionally related to BRCA1 and BRCA2, necessarily limited to modest numbers of samples on account of the low-throughput technologies available. Through these experiments, PALB2, CHEK2, and ATM were identified as BCSGs, with the less reproducible associations for BARD1, RAD51C, and RAD51D subsequently confirmed as exclusive to ER-negative tumors.25,36-38

It was widely anticipated that the advent of NGS would allow identification of the elusive additional BCSGs. Initial applications of WES typically involved case-only segregation-type analyses of multiple members of breast cancer families (Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2022.09.152). With wider public availability of control data, NGS breast cancer gene discovery studies progressed on to case-control analyses, with the vast majority focused on targeted sets of DNA repair genes selected on the basis of biological candidacy.39-41 Few case-control WES
analyses for breast cancer have been reported. In 2021, Wang et al. reported the largest WES case-control analysis in breast cancer to date comprising 7859 unselected breast cancer cases compared to 117,456 controls in UK Biobank.19 Overall, despite cancer to date comprising 7859 unselected breast cancer cases et al. reported the largest WES case-control analysis in breast analyses for breast cancer have been reported. In 2021, Wang et al. reported the largest WES case-control analysis in breast cancer to date comprising 7859 unselected breast cancer cases compared to 117,456 controls in UK Biobank.19 Overall, despite almost a decade of application of NGS via family-only, candidate, and exome-wide case-control approaches, including the study presented here, none has yielded robust novel associations of genes with breast cancer susceptibility.

Power analyses from this study demonstrate that whilst entirely plausible that additional high-penetrance BCSGs exist, (i) for genes of BRCA1-like risks, the MAF\textsubscript{combined} will be vanishingly low, and (ii) for any gene in the ~5-fold range of risk, the population mutational frequency is likely to be less than half that of PALB2 (i.e. <1 in 1500 in the population). These sobering power analyses contextualize the failure of those modest-sized early linkage studies to identify PALB2 and of the WES familial segregation studies to yield reproducible findings.

Conversely, our study was substantially less well powered for genes of more modest effect sizes, for which the use of enriched cases also offers less benefit (Tables 1 and 2). For truncating variants in CHEK2, large genetic epidemiologic studies have demonstrated OR of 2.5 and population analyses for breast cancer have been reported. In 2021, Wang et al. reported the largest WES case-control analysis in breast cancer to date comprising 7859 unselected breast cancer cases compared to 117,456 controls in UK Biobank.19 Overall, despite almost a decade of application of NGS via family-only, candidate, and exome-wide case-control approaches, including the study presented here, none has yielded robust novel associations of genes with breast cancer susceptibility.

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MAF\textsubscript{combined} of 0.31% (1 in 163).25 Our power to detect a gene of this risk/MAF\textsubscript{combined} profile was 95%, consistent with CHEK2 coming out in our analysis as statistically significant for class I. However, for ATM we only had 12% power for detection of class I variants as the penetrance is lower (OR = 2.1) and the population mutational frequency is half that of CHEK2 [MAF\textsubscript{combined} = 0.14% (1 in 350)]. Thus, it is entirely plausible that numerous additional moderate penetrance BCSGs exist of impact profiles (penetrance × mutational frequency) akin to that of ATM and up to that of CHEK2, for which discovery studies to date (including ours) have been underpowered and/or not included in previously studied gene sets.

High-penetrance genes are widely agreed to offer high clinical utility. Even recognizing variation in background polygenic contribution, the breast cancer risks are deemed sufficiently high to dichotomize management within families, with aggressive interventions such as risk-reducing mastectomies justified in family members carrying the mutation and restoration to near-population risk for those without. Contention persists regarding the utility for risk stratification offered by genes of more moderate penetrance, especially when conferring only sub-type-specific risks and/or lacking recurrent founder mutations.

**Limitations of the study**

Data regarding receptor status and/or histopathology were only available for a subset of patients, thus limiting power for histologically driven subtype analyses. The gnomAD series is only available as summary-level count data; as such, equivalently stringent sample- and variant-level quality control for the case data were required to address the possibility of inflation due to systematic genotyping error and potential differences in depth of coverage. This also meant we were unable to carry out robust analyses for recessive and co-dominant susceptibility.

Another limitation was definitions by which missense variants were included as pathogenic/deleterious. For the CSG analysis, we included only missense variants classified on ClinVar as pathogenic/likely pathogenic with at least one star. Thus, any variants assigned on ClinVar as having ‘conflicting interpretations’ were excluded. Likewise, for the DNA repair/exome-wide analyses, application of a threshold of REVEL > 0.7 was inevitably an imperfect filter, likely resulting both in inclusion of neutral variants and exclusion of deleterious variants.

Additionally, we did not include exon-level (or greater) deletions and duplications in our analyses. Such variants (which are inherently more difficult to detect from short-read NGS data) are thought to comprise a non-trivial fraction of disease-associated germline alterations in cancer susceptibility genes. In the case of BRCA1 and BRCA2, they are thought to account for somewhere between 10% and 20% of all alterations in those genes. Thus, any putative novel breast cancer gene where structural variation is the predominant mutation type would be missed by our analyses. Our power analyses, therefore, do not account for any
gain in power that would be afforded by the inclusion of such variants.

**Future studies**

Future analysis of 15,000 similarly enriched breast cancer cases against 400,000 ethnicity-matched controls would be fully powered (>99%) to identify all genes that might exist down to an ATM-like profile of risk and mutational frequency, as well as all genes of a PALB2-like risk (OR = 5), down to a population MAFcombined of 0.0075% (1 in 6702) (Supplementary Table S13, available at https://doi.org/10.1016/j.annonc.2022.09.152).

**Conclusions**

This is the largest study to date of enriched BRCA-negative breast cancer probands, in which 7.4% were found to carry a BCSCG pathogenic mutation. With appropriate correction for multiple tests, aside from ‘rediscovery’ of known BCSCGs, neither the targeted DNA repair gene analysis nor the WES approach yielded robust additional BCSCGs. Outstanding BCSCGs of high penetrance (OR > 5) must be extremely rare. However, many genes up to a CHEK2/ATM-like risk may exist. Whilst a WES study involving 5- to 10-fold more enriched breast cancer samples is feasible and would allow comprehensive survey of all such genes, there is no unanimous agreement regarding utility of clinical testing for genes that are so rare or of such modest penetrance.

**ACKNOWLEDGEMENTS**

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**Table 1. Power to discover genes of effect size and population frequency equivalent to known breast cancer susceptibility genes**

<table>
<thead>
<tr>
<th>Gene effect size</th>
<th>Published population frequency of gene (PTVs)</th>
<th>Power to detect (%) in study of 2135 cases versus 51 377 controls</th>
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<tbody>
<tr>
<td></td>
<td>Population MAF</td>
<td>Population frequency</td>
</tr>
<tr>
<td>BRCA1-like (OR = 10.6)</td>
<td>1.01E-03</td>
<td>1 in 494</td>
</tr>
<tr>
<td>BRCA2-like (OR = 5.9)</td>
<td>1.63E-03</td>
<td>1 in 306</td>
</tr>
<tr>
<td>PALB2-like (OR = 5)</td>
<td>7.49E-04</td>
<td>1 in 667</td>
</tr>
<tr>
<td>CHEK2-like (OR = 2.5)</td>
<td>3.07E-03</td>
<td>1 in 163</td>
</tr>
<tr>
<td>ATM-like (OR = 2.1)</td>
<td>1.43E-03</td>
<td>1 in 350</td>
</tr>
</tbody>
</table>

Power afforded by study of 2135 cases and 51 377 controls to discover genes of the OR/MAF profile of BRCA1, BRCA2, PALB2, CHEK2, and ATM with Bonferroni correction for multiple tests, aside from a BCSG pathogenic mutation. With appropriate correction for undertaking targeted (N = 286 DNA repair genes) or exome-wide analysis (N = 19 651 genes), comparing use of enriched versus unenriched cases.

**Table 2. Population minor allele frequency at which genes with effect size equivalent to known breast cancer susceptibility genes are discoverable**

<table>
<thead>
<tr>
<th>Gene effect size</th>
<th>DNA repair gene analysis</th>
<th>WES analysis</th>
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<tbody>
<tr>
<td></td>
<td>Population MAF</td>
<td>Population frequency</td>
</tr>
<tr>
<td>BRCA1-like (OR = 10.6)</td>
<td>4.30E-05</td>
<td>1 in 11 628</td>
</tr>
<tr>
<td>BRCA2-like (OR = 5.9)</td>
<td>1.70E-04</td>
<td>1 in 2924</td>
</tr>
<tr>
<td>PALB2-like (OR = 5)</td>
<td>2.60E-04</td>
<td>1 in 1946</td>
</tr>
<tr>
<td>CHEK2-like (OR = 2.5)</td>
<td>1.90E-03</td>
<td>1 in 260</td>
</tr>
<tr>
<td>ATM-like (OR = 2.1)</td>
<td>3.70E-03</td>
<td>1 in 135</td>
</tr>
</tbody>
</table>

Genes population frequency (MAFcombined) for genes of equivalent effect size to BRCA1, BRCA2, PALB2, CHEK2, and ATM for which this study of 2135 enriched cases versus 51 377 controls had 90% and 80% power to detect an association. Bonferroni correction applied for undertaking targeted (N = 286 DNA repair genes) or exome-wide analysis (N = 19 651 genes). OR for BRCA1, BRCA2, PALB2, CHEK2, and ATM are derived from Dorling et al. [25].
Cancer Research and Royal Marsden ras part of a joint entity referred to as the National Institute of Health Research Specialist Biomedical Research Centre for Cancer.

DISCLOSURE
The authors have declared no conflicts of interest.

REFERENCES

APPENDIX

Breast and Ovarian Cancer Susceptibility Collaboration

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