The Prevalence And Clinical Significance Of Mutations *BRIP1, BARD1, PALB2* And *NBN* Genes In Women With Ovarian Cancer

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Abstract:

Several genes functioning in DNA repair pathways confer susceptibility to epithelial ovarian cancer (EOC). We evaluated the prevalence of coding sequence mutations in 4 DNA repair genes - *BRIP1*, *BARD1*, *NBN1*, and *PALB2* - in 3,236 invasive EOC cases and 3,431 controls of European ancestry, and in 2,133 *BRCA1/BRCA2* negative, unaffected women from the UK Familial Ovarian Cancer Screening Study (UKFOCSS), a clinical screening trial for ovarian cancer. We found significant evidence of an increased frequency of deleterious mutations in *BRIP1* in cases (0.9 percent) compared to controls (0.09 percent) (P=0.0001), but no differences in case/control mutation frequency for *BARD1* (P=0.39), *NBN1* (P=0.61), or *PALB2* (P=0.078). We identified a non-random distribution of mutations in the *BRIP1* gene (P=0.0085) indicating there is a correlation between mutation location and occurrence of ovarian cancer. *BRIP1* mutation carriers tended to be diagnosed at a later age (P=0.072), with high-grade serous disease (P=0.049), and at a later stage (P=0.093) compared to non-carriers. Using data from non-family based case-control studies we estimated the relative risks associated with *BRIP1* mutations to be 11 for invasive EOC (95% confidence interval (CI) 3.2 – 34; $P = 1 \times 10^{-4}$) and 14 for high-grade serous ovarian cancer (95% CI 4.0 – 45; $P = 2 \times 10^{-5}$). We also performed segregation analysis using family data from case-control studies and from UKFOCSS, estimating the average relative risks in *BRIP1* mutation carriers to be 3.4 (95% CI: 2.1 - 5.5, $p=7\times10^{-7}$) compared to the UK general population. These data have implications for genetic risk prediction and clinical intervention strategies for the prevention of ovarian cancer.

INTRODUCTION

Several susceptibility genes for epithelial ovarian cancer (EOC) with varying frequencies and penetrance in the population have been identified. The strongest known genetic risk factors are deleterious alleles in the *BRCA1* and *BRCA2* genes which cause the hereditary breast-ovarian cancer syndrome[1]. The average cumulative risks of EOC by age 70 years are estimated to be 39 per cent in *BRCA1* carriers and 11 per cent in *BRCA2* carriers [2]. Other susceptibility genes associated with increased ovarian risk have also been identified, including the mismatch repair genes *MSH6*, *MSH2* and *MLH1*, which are associated with familial hereditary non-polyposis colorectal cancer (HNPCC). More recently, rare deleterious alleles associated with an increased risk of ovarian cancer have been reported for *RAD51C*[3] and *RAD51D*[4]. The estimates of the magnitude of risk associated with these alleles were similar to the risk associated with *BRCA2* mutations. A protein truncating variant of *BRIP1* with a frequency of 0.4 per cent in the Icelandic population, based on the analysis an analysis of 318 Icelandic ovarian cancer cases has also been shown to be associated with ovarian cancer risk [5]; but *BRIP1* has not been demonstrated to be an important cause of ovarian cancer in other populations. In addition, multiple common low-penetrance susceptibility alleles conferring relative risks of less than 1.5-fold have been found using genome wide association studies (GWAS)[6-12].

It is estimated that the known genetic risk factors account for less than half the excess familial risk of ovarian cancer[13] suggesting that other genetic risk factors await discovery. The unexplained familial risk is unlikely to be due to other high penetrance genes because *BRCA1* and *BRCA2* cause most multi-case ovarian cancer families. The remainder is probably due to a combination of as yet undiscovered common genetic variants conferring weak effects and/or uncommon alleles conferring weak to moderate relative risks (less than 10-fold).

Some studies have already identified plausible susceptibility genes using high throughput sequencing of case series. For example, Walsh and colleagues sequenced 21 “tumor suppressor genes” in 360 ovarian cancer cases and identified truncating mutations in several genes including known susceptibility genes (e.g. *BRCA1*, *BRCA2* and *RAD51C*) and other candidate genes (including *BARD1*, *BRIP1*, *NBN* and *PALB2*)[14]. The main weaknesses of this study were the modest sample size and the absence of appropriate controls. Thus, the population prevalence and ovarian cancer risk (if any) associated with rare mutations in these genes are not known. Nevertheless, these genes are

currently included on commercially available gene testing panels for ovarian cancer, including the OvaNEXT™ panel marketed by Ambry Genetics™ and the Breast/Ovarian Cancer Panel marketed by GeneDX™.

As *BARD1*, *BRIP1*, *NBN* and *PALB2* have been reported as putative ovarian cancer susceptibility genes and their translated proteins involved in the BRCA1/BRCA2 DNA repair pathways, they are strong candidates for ovarian cancer susceptibility. BARD1 (BRCA1-associated RING domain protein 1) interacts with BRCA1 *in vivo* and *in vitro* and is essential for BRCA1 stability. BRIP1 is a Fanconi anemia group protein (FANCJ), which is important in normal DSB repair function in breast cancer. NBN is a member of the NBS1/MRE11/RAD50 (MRN) DSB repair complex, which recognizes DNA damage and rapidly relocates to DSB sites and forms nuclear foci. Finally PALB2 (partner and localizer of BRCA2) is another Fanconi anemia group protein (FANCN), which binds to and co-localizes with BRCA2 in nuclear foci permitting the stable intra-nuclear localization and accumulation of BRCA2. Germline mutations in *PALB2* are associated with an increased risk of developing breast cancer [15] and PALB2-deficient cells are sensitive to PARP inhibitors [16]. The aim of this study was to establish whether or not rare protein truncating variants in *BARD1*, *BRIP1*, *NBN* and *PALB2* are associated with an increased risk of ovarian cancer in populations of European origin.

MATERIAL AND METHODS

Study subjects

We included 3,374 cases and 3,487 controls in this study. These were from eight ovarian cancer case-control studies (AOC, HJO, HMO, LAX, MAY, SEA, STA, UKO), one familial ovarian cancer registry from the USA (GRFOCR) and one case series (RMH). These studies have been described previously (e.g Pharoah and colleagues, 2013 [10]). Cases with high-grade serous cancer from each study were preferentially selected for sequencing because high-penetrance alleles of *BRCA1* and *BRCA2* predispose to this subtype. We also selected a small number cases with other tumour histologies and a family history of ovarian cancer (**Table 1**). Also studied were 2,000 unaffected women that are part of the UK Familial Ovarian Cancer Screening Study (UKFOCSS) [17]. The eligibility criteria for UKFOCSS recruitment is women older than 35 years with an estimated minimum lifetime risk of ovarian cancer of 10 per cent based on a family history of ovarian and/or breast cancer and/or the presence of known predisposing germline gene mutation (*BRCA1*, *BRCA2*, and mismatch repair genes) in the family. Subjects were recruited between June, 2002, and September, 2010 from 44 UK regional centers with screening and outcome data were collected prospectively. Seven of the nine studies had been previously screened for *BRCA1* and *BRCA2* mutations and known mutation carriers were excluded. All studies had ethics committee approval, and all participants provided informed consent.

Sequencing

Target sequence enrichment was performed using 48.48 Fluidigm access arrays and 4-primer chemistry for addition of barcode and adapter sequences during the PCR amplification. All target specific regions were less than 200bp for complete sequencing using 100 bp pair-end sequencing on the Illumina HiSeq2000 according to the manufacturer's protocol (Illumina Inc, San Diego, CA). A total of 138 amplicons were designed and the average fragment size was 184 base pairs (range 102-200) (**Supplementary Table 1**). The numbers of amplicons for *BARD1, BRIP1, NBN,* and *PALB2* were 26, 45, 29, and 38 respectively.

Sequenced reads were de-multiplexed using standard Illumina software. We used the Burrows-Wheeler Aligner (BWA) [18] for sequencing read alignment against the human genome reference sequence (hg19). The Genome Analysis Toolkit (GATK) [19] was used for base quality-score

recalibration, local indel realignment, and substitution, insertion/deletion (INDEL) discovery. Variants were only considered for further analysis if they satisfied the set of recommended GATK filters, as described in the GATK best practices guide. ANNOVAR [20] was used to annotate the sequence variation detected. The transcript identifiers used for mutation annotation of *BARD1, BRIP1, NBN,* and *PALB2* were NM_000465.3, NM_032043.2, NM_002485.4, and NM_024675.3 respectively. Variant alternate allele frequency was defined as the fraction of alternative allele bases compared to the total number of bases at the variant locus. We applied the following thresholds for variants calling: the minimum coverage is 15, a variant will be called if (1) coverage ≥500 and alternate allele frequency ≥10%, or (2) 250 ≤coverage <500 and alternate allele frequency ≥15%, or (3) 30 ≤coverage <250 and alternate allele frequency ≥20%, or (4) 15 ≤coverage <30 and alternate allele frequency ≥30%. The thresholds for coverage and alternate allele frequency used in variant calling were defined previously based on results from sequencing of positive controls with known variants [21].

One hundred and thirty eight cases, 56 controls and 167 UKFOCSS participants were excluded because <80 per cent of the target bases from these samples had read depth ≥15. The average percent of coding region and splice sites screened at 15X coverage for the remaining 8,667 samples for *BARD1, BRIP1, NBN,* and *PALB2* genes were 92.2, 96.1, 96.9 and 99.2 per cent respectively.

Deleterious mutation identification and validation

Deleterious variants were defined as those predicted to result in protein truncation (frameshift, splice site and nonsense mutations). We used the programme MaxEntScan to identify splice site variants most likely to affect gene splicing [22]. Splice site variants with a MaxEntScan score that differed from the score for the consensus sequence by more than 40% were assumed to affect splicing. Sequencing alignments were visually inspected using the Integrative Genomic viewer (IGV) [23] to confirm presence of deleterious variants. We performed Sanger sequencing using standard methods for validation in independent PCR products of all potentially deleterious truncating variants.

Statistical methods

We tested for association between deleterious mutations and ovarian cancer risk using unconditional logistic regression adjusted for geographical region of origin (Australia, continental Europe, the United Kingdom and the USA). Odds ratios and associated 95 per cent confidence intervals (95% CI)

were also calculated. The association between mutation position and case-control status was tested using a Wilcoxon rank-sum test. The inheritance patterns of disease and genotypes in families were used to estimate the ovarian cancer risk conferred by *BRIP1* loss-of-function mutations, with the use of modified complex-segregation- analysis methods [24]. Pedigree likelihoods were constructed with the use of the pedigree-analysis software Mendel, version 3.3 [25], and a maximum- likelihood approach was used to obtain parameter estimates.

We used a model in which genetic susceptibility to ovarian cancer was due to *BRIP1* loss-of-function mutations with a residual component representing other familial effects (polygenic effect). Under each model, the incidence of ovarian cancer for person *i* was dependent on the underlying *BRIP1* genotype and polygenotype (i.e., the genotype under the polygenic model) through a model of the form

$$\lambda_i(t) = \lambda_0(t) \exp [\beta g_i + P_i],$$

where $g_i = 0$ for persons without a *BRIP1* mutation; $g_i = 1$ for carriers of a deleterious *BRIP1* variant; P_i is the residual familial component, assumed to be normally distributed with mean 0 and variance $\sigma_R 2$; and β is the log relative risk for carriers of a deleterious *BRIP1* variant relative to the base- line ovarian-cancer incidence, $\lambda_0(t)$. Note that this is not equivalent to the relative risk of ovarian cancer for a *BRIP1* mutation carrier relative to an average non-carrier in the population. We constrained the sum of the variance in ovarian-cancer risk due to *BRIP1* mutations and the residual variance, $\sigma_R 2$, to agree with external estimates of the total familial ovarian-cancer variance, $\sigma_P 2$, following the procedure described in detail elsewhere [26]. The total familial variance, $\sigma_P 2$, was assumed to be equal to 1.43 a value estimated in previous ovarian-cancer segregation analyses [Jervis: submitted].

Our analysis included both cases and controls that had a *BRIP1* mutation screening result and where available all the relevant family data. For the UKFOCSS families, we used only families for which the pedigree data were available (N=1,693). Failure to adjust for the methods of ascertainment could lead to a biased estimation of cancer risk, because families were ascertained in multiple ways. We used an ascertainment assumption–free approach in which we evaluated each family separately [27]. This involved dividing the data for each family into two components, one containing all the data potentially relevant to the ascertainment (F_1), and the other containing all the data not relevant to the ascertainment (F_2). For each family, we modeled the conditional likelihood, L, as being equal to $P(F_1, F_2)/P(F_1)$, where $P(F_1, F_2)$ is the probability of observing all data in the pedigree, and $P(F_1)$ is the

probability of observing only data relevant to ascertainment in the pedigree. Specifically, for families identified through screening for *BRIP1* mutations in population-based series of ovarian-cancer cases or controls, we modeled the conditional likelihood of observing the phenotype and genotype of the index and where available all family phenotypes, given the disease status, and age at diagnosis/last follow only of the index case or control. For the UKFOCSS families identified through multiple affected members, we maximized the likelihood of observing the phenotypes and genotypes in the family, given all the disease phenotypes in the family. The models were parameterized in terms of the natural logarithm of the ratio of cancer incidence in *BRIP1* mutation carriers relative to baseline cancer incidence and the *BRIP1* deleterious mutation frequency. Parameters were estimated with the use of the maximum-likelihood method, and variances were obtained from the observed information matrix.

For the main analysis, the phenotype of each female family member was defined by her age at ovarian-cancer diagnosis or, if she was unaffected, her age at last follow-up. Women were followed from 20 years of age until the age at diagnosis of ovarian, bilateral prophylactic oophorectomy, age at death, age at last follow-up, or 80 years of age, whichever occurred first. Only women diagnosed with ovarian cancer were assumed to be affected. Separate models were investigated where we modelled susceptibility to both ovarian and breast cancer which allowed for a breast cancer log-relative risk parameter. In these, women were followed from 20 years of age until the age at diagnosis of ovarian cancer, breast cancer, bilateral prophylactic oophorectomy, age at death, age at last follow-up, or 80 years of age, whichever occurred first.

We identified multiple missense variants that have an unknown functional effect on the protein. We exclude all missense variants with a minor allele frequency of > 1 per cent from further analyses as large-scale genome-wide association studies have shown that the relative risks conferred by common susceptibility allele are small (< 1.3) and thus not detectable by the smaller sample size of this targeted sequencing study. The statistical power to detect single rare alleles by association, even if they confer larger risk (RR > 2) is still modest. We therefore used the rare admixture likelihood (RAML) burden test [28] to test for association on a gene-by-gene basis. The RAML combines the information across multiple rare variants to increase statistical power and allows for alleles associated with either an increased or a decreased risk. We classified variants with frequency ≤ 1 per cent into three groups: (1) deleterious variants as defined previously (these were excluded from the

RAML analyses); (2) variants predicted to have a damaging effect on protein function by 2 out of 3 prediction tools - SIFT (score <0.05), polyphen-2 (classified as possibly damaging/probably damaging) and Provean (score<-2.5); (3) variants with probable benign effects. Only subjects with a call rate greater than 80 per cent for missense variants and variants with a call rate greater than 80 per cent with genotype frequencies consistent with Hardy-Weinberg equilibrium ($P>10^{-5}$) were included in these analyses.

RESULTS

BARD1, BRIP1, NBN and *PALB2* deleterious mutation prevalence in ovarian cancer cases and controls

Sequence information for the coding region and splice site boundaries of the *BARD1*, *BRIP1*, *NBN* and *PALB2* genes was available for 3,236 EOC cases (of which 91 per cent were serous and 78 per cent were high-grade serous) and 3,431 controls after quality control analysis. The characteristics of these individuals by study are summarized in **Table 1**. We identified predicted, deleterious mutations in 52 EOC cases (1.6 percent) and 16 controls (0.47 per cent) in the four genes combined (**Table 2**). There was a significantly higher frequency of mutations in cases compared to controls for *BRIP1* (30/3,227 cases, 0.9 percent; 3/3,444, controls, 0.09 percent; P=0.0001). Based on the modified segregation analysis the evidence for association between *BRIP1* mutations and ovarian cancer risk was even stronger ($P=7\times10^{-7}$). There were no significant differences in mutation frequency in cases compared to controls for mutations in *BARD1* (4 cases, 0.12 per cent; 2 controls, 0.06 per cent, P=0.39), *NBN* (9 cases, 0.28 per cent; 8 controls, 0.23 per cent, P=0.61) or *PALB2* (9 cases, 0.28 per cent; 3 controls, 0.09 per cent, P=0.078) (**Table 2; Supplementary Table 2**). The distribution of mutations throughout each gene is illustrated in **Figure 1**.

We also evaluated the prevalence of mutations in these genes in individuals from the UK Familial ovarian cancer screening study (UKFOCSS). After quality control analysis and exclusion of women that screened positive for *BRCA1* and *BRCA2* mutations, sequence data were available for 2,000 subjects. We identified 25 predicted deleterious mutations in these four genes (1.25 per cent) (**Table 4**, **Supplementary Table 4**). Mutations were significantly more prevalent in UKFOCSS subjects than in the controls for the other studies controls for *BRIP1* (12 mutations, 0.60 percent) and *PALB2* (7 mutations, 0.35 percent) genes (P=0.0008 and 0.045 respectively**).** There were no differences in mutation frequency in UKFOCSS subjects and controls for *BARD1* (3 mutations, 0.15 percent, P=0.15) or *NBN* (3 mutations, 0.15 percent, P=0.76) (**Table 2**).

Genotype-phenotype associations in *BRIP1*

We identified a non-random distribution of mutations in the *BRIP1* gene (P=0.0085) indicating there is a correlation between mutation location and occurrence of ovarian cancer (**Figure 1A**). Of the 30 predicted truncating *BRIP1* mutations identified in ovarian cancer cases, 29 are located in the first

two thirds of the gene (between nucleotides 68 and 2508) and are predicted to truncate the protein before the BRCA1 binding domain. The mutations outside this region occur at nucleotides 3607, which would be predicted to retain the binding BRCA1 domain. The three truncating *BRIP1* mutations identified in controls were all located 3' of nucleotide 2508 (**Figure 1a; Supplementary table 3**).

***BARD1, BRIP1, NBN* and *PALB2* missense variants and ovarian cancer risk**

In addition to truncating deleterious mutations, we also identified 372 non-synonymous coding variants in these 4 genes with an overall frequency of less than 1 per cent. We used the *in silico* software programs SIFT, Polyphen-2 and Provean (Adzhubei et al. 2010, Sim et al. 2012 and Choi et al. 2012) to predict if any of these non-synonymous coding variants are likely to have a deleterious impact on the predicted protein of each gene. One hundred and fifty-three missense variants were classified as potentially deleterious using 2 out of 3 of these *in silico* tools; 32 variants in *BARD1*, 49 variants in *BRIP1*, 40 variants in *NBN* and 32 variants in *PALB2* (**Supplementary Table 5**). Based on these variants, we compared the relative burden in cases and controls for each gene using the rare admixture maximum likelihood test (RAML)[28]. We found some evidence for association with increased risk of ovarian cancer for missense variants in *BRIP1* (P=0.02) but no associations with risk for missense variants in *BARD1*, *NBN* or *PALB2*.

Clinical-pathological characteristics associated with *BRIP1* carrier status

We evaluated associations between *BRIP1* mutation carrier status and age at diagnosis, histological subtype and family history of ovarian and or breast cancer (**Table 3**). *BRIP1* mutation carriers tended to be diagnosed at a later age of onset compared to all other case subjects in the study; the average age at diagnosis was 63.8 years in carriers and 58.0 years in non-carriers (P=0.072). All of the 30 *BRIP1* mutation carriers we identified were in cases of the serous subtype of which 25 were high-grade serous. The difference in *BRIP1*-carrier frequency between high-grade serous and other subtypes was nominally significant (P=0.049). *BRIP1* mutation carriers were also more likely to be diagnosed with late stage disease - 92 percent of carriers were stage 3 or 4 compared 81 percent in non-carriers (P=0.093). Fifty per cent (6/12) of the *BRIP1* carriers from UKFOCSS had at least two family members with ovarian cancer compared to 28 per cent (553/1,919) of the 1,919 non-carriers**.** This difference was not significant (P=0.11). The proportion of *BARD1*, *NBN* and *PALB2* carriers with a strong family history was similar to the proportion in non-carriers (**Table 4)**

Ovarian cancer risks associated with *BRIP1* mutations

We estimated the relative risk of ovarian cancer associated with deleterious mutations in the *BRIP1* gene as odds ratios, using data from the case-control studies that were not family based. The relative risk of ovarian cancer was 11 (95% confidence interval (CI) 3.2 – 34; P = 1 x 10^{-4}), whereas the relative risk of high-grade serous ovarian cancer was 14 (95% CI 4.0 – 45; P = 2 x 10^{-5}). Using modified segregation analysis that included the case-control study family data where available and the UKFOCSS family data, we estimated the average ovarian cancer relative risk compared to the UK general population to be 3.4 (95% CI: 2.1 - 5.5, p=7×10^{-7}). The estimated *BRIP1* truncating variant frequency was 0.0012 (95%CI: 0.0007 - 0.002) corresponding to a mutation carrier frequency of 1 in 416. Based on this model, the estimated cumulative ovarian cancer risk by age 80 was 5.8 per cent (95% CI: 3.6 - 9.1%). Models that allowed for breast cancer risk to be associated with *BRIP1* variants yielded similar results for the ovarian cancer risk (RR compared to general population ~3.9, p=1×10^{-8}), but there was no significant evidence of association with breast cancer risk (breast cancer RR=1.5, 95%CI: 0.86-2.6, p=0.15). The effects of the predicted splice site variants were not confirmed by sequencing cDNA. However, for three of them simple exon skipping is predicted to cause in-frame deletion of 58, 74 and 97 amino acids (**Supplementary Table 2**). All three variants were identified in cases. Even if these are reclassified as non-pathogenic the case-control association with *BRIP1* carrier status is only slightly attenuated (OR = 9.5, 95% CI 2.9 – 31; P = 0.0002).

DISCUSSION

This study has catalogued the occurrence of coding sequence variants in the *BARD1*, *BRIP1*, *NBN* and *PALB2* genes in ovarian cancer cases and controls, and provides estimates of the prevalence of germline variants in these genes in individuals of European ancestry. We found strong evidence that protein truncating mutations in *BRIP1* are associated with an increased risk of epithelial ovarian cancer (P=0.0001 for case-control analysis; $P=7x10^{-7}$ for the segregation analysis for the segregation analysis with additional evidence for an increased carrier frequency in women at high risk of ovarian cancer because of a family history (P=0.0008)). The increased ovarian cancer risk for *BRIP1* mutation carriers may be restricted to the high-grade serous epithelial subtype. This is highly plausible given that *BRIP1* protein is known to interact with *BRCA1/BRCA2* in homologous recombination and double strand DNA break repair pathways, and that *BRCA1* and *BRCA2* mutation carriers are also strongly associated with the high-grade serous subtype [21, 29].

The estimated relative risk for *BRIP1* from the case-control studies was substantially higher than that from the segregation analysis (11 compared to 3.4). There are several possible reasons for this difference. First, sampling error may be an explanation as the confidence intervals for the first estimate (3.2 – 34) includes the point estimate from the segregation analysis. In addition, the estimate from the case-control studies is not a true estimate of the average relative risk for all EOC because there had been preferential inclusion of serous cases from the case-control studies and the relative risk is higher for serous OC than other subtypes. In contrast the relative risk estimate from the segregation analysis is an estimate of the relative risk for all subtypes as it is based on the occurrence of ovarian cancer as reported in the relatives of study participants. We have no information of disease sub-types for these cases. It is also possible that there is some under-reporting of ovarian cancer family history in families that would result in an underestimate of risk in the segregation analysis.

Mutation location has been shown to influence disease risks for several genes, including *BRCA1* [30, 31]} and *BRCA2* [32 , 33]. We found evidence of a non-random distribution of truncating mutations in *BRIP1*. All but one of the mutations we identified in cases are predicted to truncate the protein prior to the BRCA1 binding domain suggesting a functional rationale underlying this mutation distribution. BRIP1 normally interacts with BRCA1 through the BRCT repeats at the c-terminal end of BRCA1. The BRCT domain, which appears to act as a phospho-protein binding domain [34], is found predominantly

in proteins involved in cell cycle checkpoint functions responsive to DNA damage [35]. Our data imply that loss of this interaction is critical in mediating ovarian cancer development, perhaps by impairing response to DNA damage in ovarian/fallopian tube epithelial cells; but truncating mutations to not appear to confer similarly high risks of breast cancer[36] and so loss of the BRIP1-BRCA1 interaction may only be critical in ovarian cancer precursor cells. Truncated forms of BRIP1 that either retain the BRCA1 binding region or occur after amino acid 836 might be stable and so retain the ability to bind to BRCA1 and maintain DNA damage response. By this rationale, the one deleterious *BRIP1* mutation in an ovarian cancer case occurring 3' of the BRCA1 binding domain (G3607X) may be functionally null or of much lower penetrance, similar to the K3326X variant at the 3' end of BRCA2 [37].

BARD1, *NBN* and *PALB2* have all been suggested as susceptibility genes for epithelial ovarian cancer (EOC), because of their critical role in biological pathways involved in ovarian tumorigenesis, and because some studies have shown evidence of truncating mutations in these genes in EOC cases from small studies (n<1000 cases) without population matched, unaffected controls. We have shown that the prevalence of protein truncating mutation in *BARD1*, *NBN* and *PALB2* was very low in populations of European origin suggesting that these genes are unlikely to contribute substantially to ovarian cancer risk. However, because of the low mutation frequency in these genes we cannot rule out that they confer modest risks of ovarian cancer. For example, if the observed difference in carrier frequencies for *BARD1* (0.0012 in cases and 0.0006 in controls, which is equivalent to an odds ratio of 2) were real, over 40,000 cases and 40,000 controls would be needed to detect this difference with 80 per cent power at a Type 1 error rate of 5 per cent. Similarly 9,000 cases and 9,000 controls would be needed to detect the observed carrier frequency difference in *PALB2* (0.0028 and 0.0009). Our data emphasize the need for caution in interpreting the findings from case only studies. For example, *NBN* was suggested to be an ovarian cancer susceptibility gene based on the analysis of 360 ovarian cancer cases without an appropriate comparator control group [14]. We found that deleterious *NBN* mutations were moderately prevalent in cases (0.28 per cent) but they were similarly prevalent in controls (0.23 percent), suggesting that testing for *NBN* mutations is of little clinical value for ovarian cancer risk prediction.

It is likely that we have underestimated the true prevalence of deleterious variants in these genes because our sequencing approach was not 100 per cent sensitive. While our sequencing method enables rapid and high throughout analysis of candidate genes in thousands of samples, it did not

provide complete sequence coverage of each gene in all samples (mean coverage 92 to 99 per cent). In addition, large genomic deletions and rearrangement mutations occur relatively frequently in some genes. Currently available software is not able to detect these type of mutations using NGS data derived from PCR based library amplification. Finally, we did not include missense variants in our risk estimates because we cannot be certain of their pathogenicity in the absence of definitive functional assays.

In an era of cost-effective panel testing of multiple genes using next generation sequencing, *BRIP1* mutation analysis could be rapidly implemented as part of a program of clinical genetic testing followed by prophylactic surgery (salpingo-oophorectomy). Whether or not there is clinical utility in testing unaffected women for truncating mutations in *BRIP1* is not clear, given the uncertainties in our risk estimates. Assuming the "best" estimate to be the point estimate from the segregation analysis, the lifetime risk of epithelial ovarian cancer in *BRIP1* carriers would be 5.8 per cent. Whether or not this level of risk would warrant preventive surgery is open to question. However the true risk may be lower than this. It has been suggested that 80 per cent confidence limits are more appropriate than 95 per confidence limits for the purposes of clinical decision making. The lower 80 per cent confidence limit for lifetime risk estimate is 4.3 per cent, which might not be sufficiently high to warrant intervention. On the other hand, these risk estimates are the risk for the average *BRIP1* mutation carrier and are likely to be modified by the presence of other lifestyle and genetic risk factors. The log-additive model on a relative risk scale for interaction between risk factors has been shown to fit well for interactions between risk alleles and lifestyle risk factors. Eighteen common risk alleles for ovarian cancer have now been identified [6-12]. Women at the 80th centile of the polygenic risk distribution based on these 18 alleles would have an expected lifetime risk of 7.2 per cent (80% CI 5.3 – 9.7), assuming the log-additive model also applies to *BRIP1* carriers. If we incorporate other risk factors for ovarian cancer into the polygenic model – specifically oral contraceptive pill use, tubal ligation, parity, a history of endometriosis and family history after removing the component due to the known genes – the lifetime risk at the 80th centile of the risk distribution is even greater (8.2 per cent; 80% CI 6.0 - 11). *BRIP1* mutation testing in women with high-grade serous ovarian cancer might also have clinical utility through targeted treatment. Poly (ADP-ribose) polymerase (PARP) inhibitors, which cause synthetic lethality in cells with defective DNA double-strand break repair by homologous recombination, are currently being evaluated in women

with *BRCA1* and *BRCA2* associated ovarian cancer. PARP inhibitors might also prove useful in ovarian cancer cases with mutations in other DNA repair genes, but further work will be needed to establish this. *BARD1*, *BRIP1*, *NBN* and *PALB2* are now components of commercially available gene panel tests that are advertised for use in breast/ovarian cancer families. The lack of evidence for association of *BARD1*, *NBN* and *PALB2* and the uncertainty around the *BRIP1* risk estimates would suggest that implementation of mutations testing for these genes in a clinical setting is premature.

In summary, we have found strong evidence that deleterious germline mutations in *BRIP1* are associated with a moderate increase in the risk of epithelial ovarian cancer. Because of the very low frequency of mutations in *BARD1* and *PALB2*, we cannot rule out that these genes also confer susceptibility to ovarian cancer, but *NBN* mutations do not appear to predispose to ovarian cancer. These data may have clinical implications for risk prediction and prevention approaches for ovarian cancer in the future if confirmed by other studies; but this study highlights the critical need for accurate risk estimation of candidate susceptibility genes based on very large sample sizes before genes of moderate penetrance have clinical utility in cancer prevention.

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Table and Figure Legends

Table 1: Characteristics of ovarian cancer case-control populations analyzed in this study

Table 2: Truncating mutations in *BRIP1*, *BARD1*, *NBN* and *PALB2* identified in ovarian cancer cases and controls and in the UKFOCSS subjects

Table 3: Mutation frequency by clinical characteristics in ovarian cancer cases

Table 4: Mutation frequency by family history in UCKFOCSS subjects

Figure 1. Distribution of predicted 'deleterious' mutations in the *BRIP1* (**A**), *BARD1* (**B**), *NBN* (**C**) and *PALB2* (**D**) genes identified in ovarian cancer cases and controls. The location of each mutation is shown with respect to domains of functional significance in the translated protein and the exon structure of the coding sequence. Mutations in cases are illustrated in black, mutations in controls are in green. Also shown is where a mutation was identified in more than individual (e.g. x2, x3 etc)

Supplementary Table 1: Target regions for *BARD1, BRIP1, NBN* and *PALB2* sequencing.

Supplementary Table 2: Predicted deleterious mutations identified in ovarian cancer cases and controls

Supplementary 3: Deleterious mutations identified in *BARD1, BRIP1, NBN, PALB2* in UKFOCSS subjects

Supplementary table 4: Non-synonymous variants identified in *BARD1*, *BRIP1*, *NBN* and *PALB2*. Variants are those in which at least two of three measures of pathogenicity (Polyphen, Sift and PROVEAN) indicate they are possibly deleterious.