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The contribution of rare germline variants to the immune landscape of breast cancer

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ABSTRACT

Background

Many breast cancer predisposition genes are involved in DNA damage repair, leading to genome instability that can impact immunosurveillance, neoantigen formation, and the composition of the tumor immune microenvironment.

Methods

Here, we explored associations between germline protein truncating variants (PTVs) in 34 (putative) breast cancer predisposition genes, of which 26 involved in DNA damage repair, with the abundance of four immune cell markers, i.e., CD8+, FOXP3+, CD20+ and CD163+, across 7,969 invasive breast tumors of women of European ancestry.

Results

The most apparent associations were those of CD163, a marker of M2-like tumor-associated macrophages, with genes involved in double- and single-strand break DNA repair, and with the 12 known breast cancer predisposition genes combined. Specifically, DNA damage repair genes, *BRCA1*, *BRCA2*, *PALB2*, *RAD51D*, and *MSH6* were associated with a 1.3 to 2-fold abundance of CD163-positive cells. Estrogen receptor status was found to mediate associations to a limited extent.

Conclusions

Our findings support a role of rare pathogenic germline variants involved in DNA damage repair, and particularly those predisposing to breast cancer, in the immune landscape of breast tumors. These insights may help guide the development of immunomodulatory strategies for breast cancer prevention and treatment.

KEY WORDS

Rare germline variants; tumor immune microenvironment; homologous recombination repair, macrophages.

BACKGROUND

In breast cancer, the composition of the tumor immune microenvironment, namely the type and abundance of lymphocytes, monocytes/macrophages and neutrophils, influences tumor progression (1). A high number of tumor-infiltrating lymphocytes has been associated with improved responsiveness to neoadjuvant chemotherapy alone (2) or in combination with immunotherapy (3). However, the unique activities of individual lymphocytic and monocytic subtypes suggest nuanced associations with clinical outcomes. For instance, elevated levels of cytotoxic T-cells (CD8-positive cells) are associated with improved patient survival (4, 5). Increased levels of pro-tumorigenic immune cell types such as regulatory T-cells (Treg-cells; FOXP3-/CD25-positive cells) and M2 tumor-associated macrophages (M2-TAMs; CD163-/CD206-positive cells) are associated with worse clinical outcomes (6, 7). Contradictory evidence exists on the association between abundance of B-cells (CD20-/CD19-positive cells), and breast cancer prognosis (reviewed by (8)). However, a recent meta-analysis suggests that increased B-lymphocyte abundance correlates with improved overall and breast cancer-specific survival (9).

The abundance of immune cells is associated with intrinsic tumor characteristics, particularly the estrogen receptor (ER)-status of the tumor. ER-positive tumors tend to have a lower number of tumor-infiltrating lymphocytes compared to ER-negative tumors (10). Among the ER-negative subtype, triple-negative tumors with increased levels of tumor-infiltrating lymphocytes associate with a more favorable survival

benefit even in the absence of chemotherapy (11-13). Importantly, germline genetic factors may also influence the abundance of anti- and pro-tumorigenic immune cells (14). Tumors (specifically triple-negative ones) in carriers of germline pathogenic variants in *BRCA1*, exhibit a heightened mutational burden resulting in an increased lymphocytic infiltration (15). Similarly, an association study has linked *BRCA1* germline variants with increased dendritic cells, interferon-gamma, MHC-I and STAT1 immune features (14). Additionally, a pre-clinical study has suggested that breast tissue of non-affected *BRCA1/2* pathogenic variant carriers has a higher immune infiltration compared to non-carriers (16). This evidence agrees with the hypothesis that genomic instability due to DNA damage repair deficiency, which might be caused by germline variants, leads to altered tissue immune microenvironment (16, 17). There is also, seemingly contrasting, evidence that patients with pathogenic variants in *BRCA1/2* do not seem to benefit more from immunotherapy compared to non-carriers patients (18). Another, preclinical, study showed an association with response to immunotherapy for truncating mutations in *BRCA2*, but found no association for *BRCA1* (19).

Understanding the role of germline pathogenic variants that predispose to breast cancer, specifically those in the DNA damage repair domain, in shaping the immune microenvironment of tumor development and progression, will contribute to preventative and treatment strategies for high-risk groups of women. In this analysis, we used data from the BRIDGES study (20), which characterized and quantified risk association of rare protein truncating variants (PTVs) and missense variants (MSVs) in 34 (putative) breast cancer predisposition genes, 26 of which are involved in DNA damage repair. Approximately 8,000 cases from BRIDGES had data on a series of immune markers obtained from standardized scoring of tumor tissue microarrays. The main

objective of this study was to investigate the association between rare germline variants in these DNA damage repair genes and the abundance of CD8-, CD20-, FOXP3- and CD163-positive cells. The extent to which these associations might be mediated by subtype, based on ER-status, was also investigated. In addition, we assessed associations in the subgroup of triple-negative breast cancers. A secondary objective was to assess whether any of the remaining eight genes, unrelated to DNA damage repair but including some involved in PI3K signaling, cell adhesion and proliferation, were associated with the composition of the tumor immune microenvironment.

METHODS

Patients

Women diagnosed with breast cancer from 11 studies participating in the BRIDGES study within the Breast Cancer Association Consortium (BCAC) were included (20). Analyses were restricted to women of self-reported European ancestry, diagnosed with primary invasive breast cancer and at least 18 years of age at diagnosis (Additional file 1: Fig. S1). Demographic data and tumor characteristics were obtained from the central BCAC database: each individual study collected data that was later centralized, after quality control and harmonization (BCAC database version 15.01). All women involved provided written informed consent, and each study met local ethical committee and/or institutional review board approvals.

Patient and tumor characteristics

Tumor data for a total of 7,969 women were included, of which 7,232 had scores for CD8, 7,326 for CD20, 7,239 for FOXP3, and 7,213 for CD163 immunohistochemical staining. A flow diagram describing inclusion and exclusion criteria is shown in Additional file 1: Fig. S1. The number of cases included for each of the 11 studies are given

in Additional file 2: Table S1, while the baseline characteristics of all participants, and across immune cell types, are shown in Additional file 1: Table S3. The median age at diagnosis was 55 years of age [(Min–Max): 18 – 93]; 22% of the tumors were ER-negative, 71% ER-positive tumors and 7% with unknown ER-status (Additional file 1: Table S3). Subgroup analysis was performed on 765 (approximately 11%) tumors that were triple negative. The distributions of relevant tumor characteristics were similar across the slightly different subsets of patients with available data for each immune cell type.

Quantification of immune cell abundance

Each study collected formalin-fixed, paraffin-embedded tissue blocks containing primary breast tumor tissue. Whole tumor slides were obtained from the tumor blocks and representative tumor areas were manually identified after staining with hematoxylin and eosin. Tissue microarrays were constructed from the tumors by collecting tissue cylinders with a diameter ranging from 0.6 to 2 millimeter at each study site (Additional file 2: Table S1). Tumor tissues were from surgical resections, prior to commencement of adjuvant therapy; except potentially from 3.2% of the cases that were noted in the database to have received neo-adjuvant chemotherapy. Each study sent unstained tissue microarray slides for central immunohistochemical staining of CD8, FOXP3, CD20 and CD163 at the Cancer Research UK Cambridge Institute (Additional file 2: Table S2; Additional file 1: Table S3). Tissue microarray sections that were successfully stained using cytoplasm monoclonal antibodies for CD8, CD20 or CD163; and nuclear monoclonal antibody for FOXP3 were scanned using Leica Aperio AT2 at the Cancer Research UK Cambridge Institute (Additional file 1: Fig. S2). Digitized slides were quality controlled and linked to data in the central BCAC database.

Quantification of positively stained areas of core images from de-arrayed tissue microarrays was performed using a custom HALO algorithm (21). The abundance of immune cells was quantified across the entire core, by combining stromal and intra-tumoral regions. The abundance of immune cells in each core was assessed as the percentage of the total tissue area occupied by positively stained cells. This score was defined as the ratio of the area covered by positive staining out of the total area composing the tumor core. Tumor area was composed by positively stained regions, non-stained areas and artifact regions that were attributed to overstaining, non-specific staining, widespread debris, or tissue folding. Any image that contained more than 40% of the total region detected as artifact was excluded. For each patient, a single score on each immune marker was obtained by averaging across all cores for that patient. Only a fraction of tumors ($n = 755$ of 7,969 tumors) had a single image available, and for these tumors scores from the single score was used. The distributions of percentages of positively stained area for the four immune cell types are shown in Additional file 1: Fig. S3.

Genotype and quality control

Details of sequencing, variant calling and quality control have been described previously (20). Briefly, genotype data for PTVs and rare MSVs were obtained for 34 (putative and established) breast cancer predisposition genes, using a targeted panel. Of these, 12 were considered definitively associated with breast cancer risk in the BRIDGES study and/or analyses based on the ClinGen clinical validity framework: *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *CDH1*, *CHEK2*, *PALB2*, *PTEN*, *RAD51C*, *RAD51D*, *STK11*, *TP53* (22). The *CHEK2* c.1100delC PTV was also analyzed separately because of its abundance and earlier reports of strong associations with breast cancer risk (23).

MSVs that were described as deleterious by having a Phred-like Combined Annotation-Dependent Depletion (phred-CADD; version 1.4) score higher than 20 were included (24). This score predicts how much more deleterious a MSV is compared to the remaining human genome. A score higher than 20 suggests that a MSV is likely to be among the top 1% of deleterious substitutions (25). In a previous study involving BRIDGES genotype data (26), the best method to classify missense variants differed per gene, but Phred-CADD showed good overall performance. In line with the previous BRIDGES studies published, for *BRCA1*, *BRCA2*, and *TP53* MSV classification we used the more reliable ClinGen/ENIGMA guidelines (20), and for *ATM* and *CHEK2* MSV classification we combined multiple in-silico algorithms (26).

Classification criteria for DNA damage repair genes

Among the 34 genes included in the BRIDGES gene panel, 26 are associated with DNA damage repair either directly or indirectly. Among these genes, 16 genes are involved in double-strand break repair as part of the homologous recombination pathway, seven genes are involved in single-strand break repair (i.e., Fanconi anemia/DNA crosslinking, mismatch repair or base excision repair) and three genes are indirectly involved in DNA damage repair, either as DNA damage sensors (*ATM* and *CHEK2*) or as downstream targets (*TP53*). The remaining eight genes are involved in proliferation (*NF1* and *STK11*), PI3K signaling (*AKT1*, *PIK3CA* and *PTEN*), epigenetic regulation (*MEN1*) or cell adhesion (*CDH1* and *EPCAM*). A detailed annotation of the function of each of the 34 genes included in the analyses and a grouping in terms of four DNA repair types are available in Additional file 1: Table S4.

Statistical analyses

In the primary analyses we evaluated the association between each immune marker and PTVs of each gene. We also included analyses of MSVs. As individual variants are rare, we performed burden analyses in which the genotype of each gene was defined as 1 or 0 according to whether the individual carried a germline variant in the gene. Non-carriers were defined as women without pathogenic germline variants in the gene of interest, irrespective of germline variants in other genes. For illustration purposes, raw immune cell abundances grouped by germline genotype for the 12 breast cancer predisposition genes are shown in Additional file 1: Fig. S4. A sensitivity analysis was conducted in which the non-carriers were only women with no other pathogenic germline mutation, either PTV or MSV, in any other gene to investigate whether individual gene effects were attenuated if the non-carrier group included individuals with PTVs or MSVs in other genes. We also performed analyses based on the burden of variants across a set of genes, grouped by the mechanisms of DNA damage repair in which they are involved. This approach aimed to identify associations that might be missed due to a low number of cases for each individual gene. Similarly, we also grouped the 12 known breast cancer predisposition genes together to identify their association with immune abundance.

The percentage of immune cells was transformed to a proportion by scaling it to a non-inclusive unit interval score (0,1). For samples where the score was either 0 or 1, 10^{-5} was added or subtracted, accordingly. Associations between the percentage of immune cells and germline genotypes were assessed using beta regression with a logit link (27). Beta regression was chosen as it accurately models outcomes bounded by the

unit interval. The regression coefficients can be interpreted as the change in the log(odds) of the proportion of stained cells, per unit increase in the covariate (28, 29).

The primary analyses included study and age at breast cancer diagnosis as covariates (reported here as unadjusted models). To assess the extent to which the association between germline variants and immune cell abundance is mediated by the ER-status of the tumor, an adjusted model including ER-status of the tumor as an additional covariate was included. This model will highlight which associations are influenced by ER subtype. If an association from the unadjusted model remains largely unchanged and statistically significant in the adjustment model, it indicates that the observed effect is irrespective of the ER-status. Subgroup analysis was performed for women with triple-negative breast cancer (defined as ER-negative, progesterone receptor (PR)-negative, and HER2-negative). We did not perform subgroup analysis on ER-positive samples because of the low abundance of immune cells in these tumors, often referred to as “cold tumors” (30), coupled with the low variability of the immune abundance in our data (Additional file 1: Fig. S3). Given the low prevalence of the PTVs, we report statistical significance at a nominal level of 0.05; though to account for the many comparisons and small numbers in some analyses we focused our discussion on those with $p < 0.01$ and with more than 10 cases. All analyses were performed in R v.3.6 (31) using the *betareg* v.3.1.4 R package (32).

RESULTS

The number of cases, OR and 95% CI for all associations between germline genotype and abundance of immune cell types are provided in Additional file 1: Tables S5-S12.

Double-strand break repair genes

Compared to non-carriers, carriers of germline PTVs in four individual double-strand break repair genes, *BRCA1*, *BRCA2*, *PALB2*, and *RAD51D*, were found to be associated with breast cancer immune cell abundance (Fig. 1; Additional file 1: Fig. S3 and Fig. S5; Additional file 1: Tables S5-S12). PTVs in *BRCA1* were associated with higher FOXP3-positive and CD163-positive cells [OR (95%CI): 1.25 (1.04–1.5) $p=0.017$ and 1.66 (1.41–1.95) $p<0.001$, respectively]. PTVs in *BRCA2* were associated with CD8-positive and CD163-positive cells [OR (95% CI): 1.27 (1.09-1.49) $p=0.003$ and 1.29 (1.11-1.5) $p=0.001$, respectively]. PTVs in *PALB2* and *RAD51D* were associated with CD163-positive cells [OR (95% CI): 1.36 (1.08-1.73) $p=0.01$ and 1.71 (1.05-2.79) $p=0.031$, respectively]. MSVs in *MRE11* were also associated with CD163-positive cells. There was no evidence of consistent or strong associations between PTVs or MSVs in other genes with any of the immune cell types when considering all tumors together. In analyses adjusting for ER-status of the tumor (Additional file 1: Fig. S5; Tables S5-S12), albeit slightly attenuated, most of the observed associations between rare germline variants and breast cancer immune cell abundance remained statistically significant.

When pooling all genes involved in double-strand break repair, carriers of PTVs had a 1.2- and 1.3-fold mean increase in the abundance of CD8-positive and CD163-positive cells [OR (95% CI): 1.16 (1.06-1.26) $p=0.001$ and 1.29 (1.19-1.4) $p<0.001$, respectively; Fig. 2, Additional file 1: Tables S13-S16]. Sensitivity analyses comparing carriers to non-carriers of any PTVs or MSVs in these genes, did not change associations (Additional file 1: Tables S17-S20).

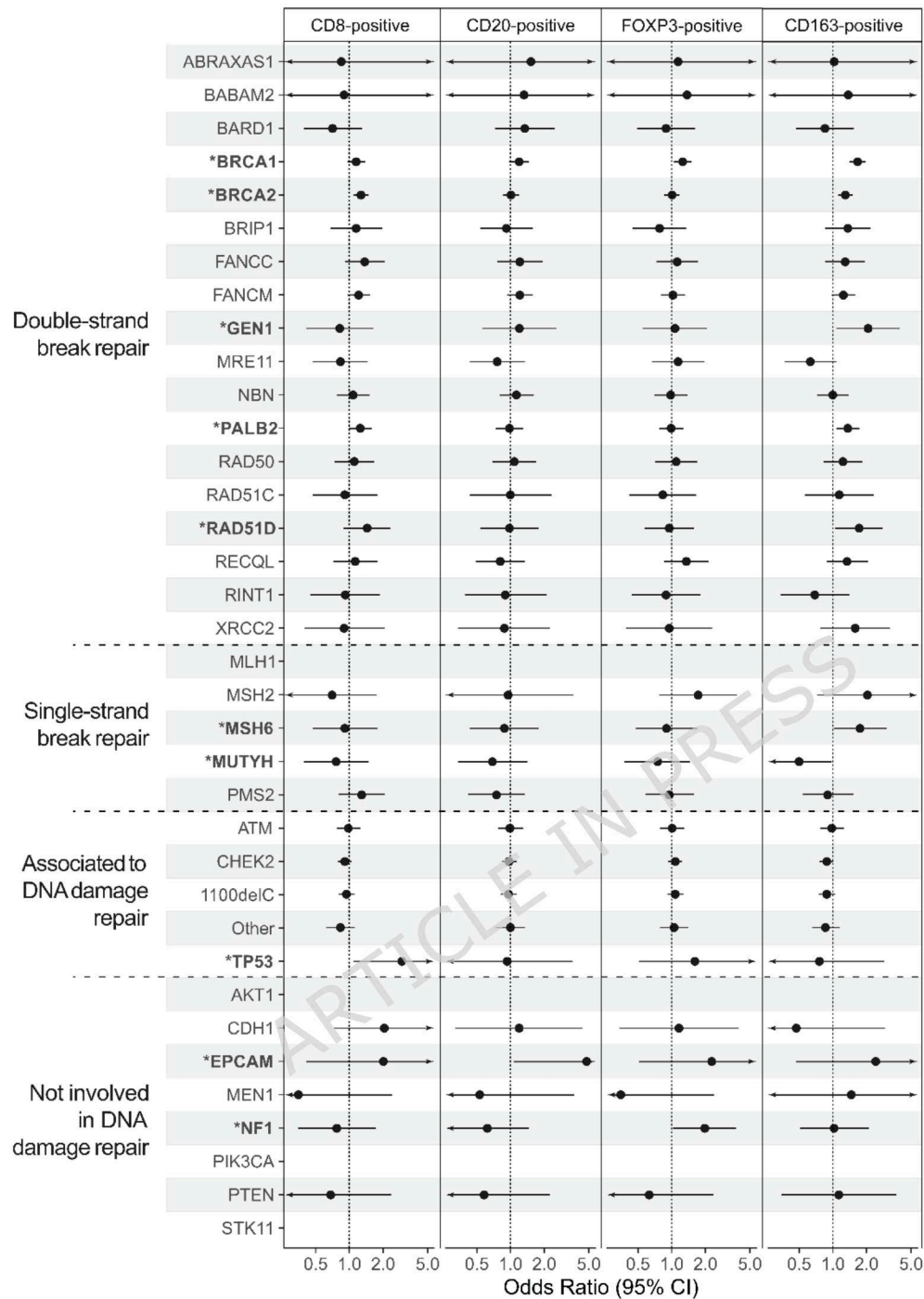


Figure 1. Associations of protein truncating variants with the abundance of four immune cell types in breast cancer grouped by DNA damage repair mechanism.

Odds ratios and 95% confidence intervals (95% CIs) were derived from beta-regression models adjusted for study and age at breast cancer diagnosis. The four panels represent four immune cell types: CD8-, CD20-, FOXP3- and CD163-positive. The dotted lines

separate the 34 genes according to the corresponding mechanisms of DNA damage repair. The dots and lines represent the odds ratio and 95% CIs. Estimates for *MLH1*, *AKT1*, *PIK3CA*, and *STK11* are missing because no PTVs were observed for those genes. The arrows at the extremes of the lines represent a confidence interval that goes outside the plot boundaries (0.3 - 5). Statistically significant genes (p-value < 0.05) for at least one of the immune cell types have an asterisk (*) and are highlighted in bold. For each gene the exact values and number of women included in the analyses are available in Additional file 1: Tables S5-S12. Abbreviations: CI, confidence intervals.

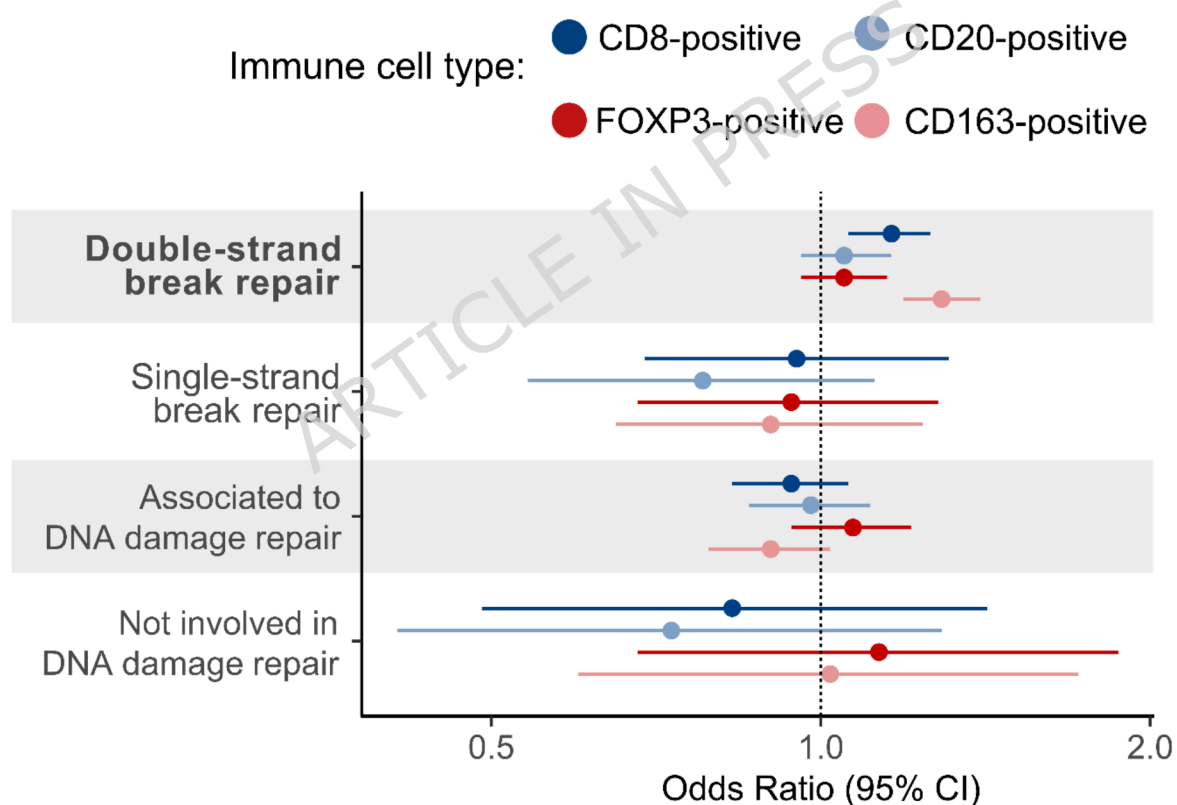


Figure 2. Associations of protein truncating variants with the abundance of four immune cell types in breast cancer. Forest plot pooled together across the four mechanisms of DNA damage repair. Odds ratios and 95% confidence intervals (95% CIs) were derived from beta-regression models adjusted for study and age at breast

cancer diagnosis. Anti-tumorigenic immune cell types: CD8-positive and CD20-positive cells are presented in blue and light blue, respectively. Pro-tumorigenic immune cell types: FOXP3-positive and CD163-positive cells are presented in red and pink, respectively. The dots and lines represent the odds ratio and 95% confidence intervals. Statistically significant mechanisms ($p\text{-value} < 0.05$) for at least one of the immune cell types are highlighted in bold. For each mechanism the exact values and number of women included in the analysis are available in Additional file 1: Tables S13 - S16. Abbreviations: CI, confidence intervals.

The triple-negative subgroup analysis showed that carriers of germline PTVs and/or MSVs in three double-strand break repair genes compared to non-carriers were associated with breast cancer immune cell abundance only in triple-negative breast cancer (Additional file 1: Fig. S6, Tables S21-S24), but numbers of carriers were small in those analyses. With the exception of *RAD51D*, the significant associations found in the overall analysis were not significant in the triple-negative subgroup.

Other DNA damage repair genes

Carriers of germline PTVs and/or MSVs in mismatch repair or single-strand break repair genes compared to non-carriers were to a more limited extent found to be associated with breast cancer immune cell abundance with some evidence for *MLH1* and *MSH6*, but not for *MSH2* and *PMS2* (Fig. 1; Additional file 1: Tables S5-S24).

Established breast cancer predisposition genes in aggregate

When aggregating germline PTVs in any of the 12 known breast cancer predisposition genes, i.e., *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *CDH1*, *CHEK2*, *PALB2*, *PTEN*,

RAD51C, *RAD51D*, *NF1* and *TP53*, carriers were associated with breast cancer immune cell abundance compared to non-carriers (Fig. 3). PTV carriers had a 1.15-fold mean increase in the abundance of CD163-positive immune cells (OR (95%CI) 1.15 (1.07-1.25)). There was no evidence of association between aggregated PTVs (or MSVs) with the abundance of CD8-, CD20- or FOXP3-positive immune cell abundance (Additional file 1: Table S25).

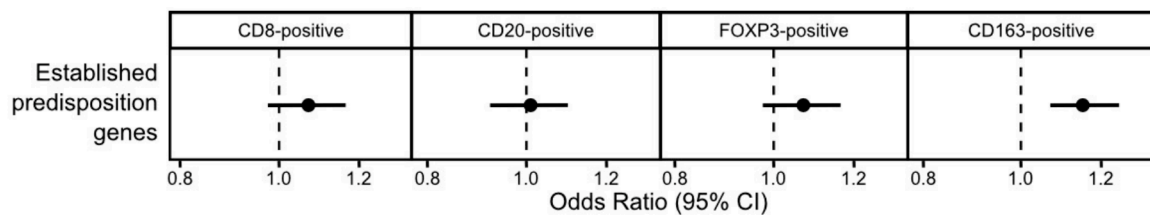


Figure 3. Forest plot showing the association of protein truncating variants grouping 12 genes that are associated with breast cancer risk together with the abundance of four immune cell types in breast cancer. Odds ratios and 95% confidence intervals were derived from beta regression models adjusted for study and age at breast cancer diagnosis. The four panels represent four immune cell types: CD8-, CD20-, FOXP3- and CD163-positive. The dots and lines represent the odds ratio and 95% confidence intervals. Analyses included protein truncating variants in *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *CDH1*, *CHEK2*, *PALB2*, *PTEN*, *RAD51C*, *RAD51D*, *NF1* and *TP53*.

DISCUSSION

The risk of developing breast cancer is strongly associated with deleterious variants in predisposition genes, many of which play a role in DNA damage repair and related pathways (20, 26). Beyond impacting risk, germline PVs have also been shown to influence tumor biological behavior, predisposing more strongly to risks of aggressive (triple-negative and/or high-grade) breast cancers (33). The immune composition of the

tumor microenvironment has emerged as an important component of breast tumor biological behavior, but the impact of germline PVs on tumor immune composition is less-well understood. In this study, we explored the association between rare germline variants and the abundance of four immune cell types that have been shown to associate with breast cancer prognosis (4-7, 9). We found most convincing evidence for associations between germline variants in DNA double-strand break repair in aggregate, and in particular *BRCA1*, *BRCA2*, *PALB2*, *RAD51D*, and CD163-positive cell abundance in the tumor. When PTVs across all genes involved in homologous recombination were grouped, we observed an association with CD8-positive and CD163-positive cells. In alignment with recent evidence (14, 34), these associations suggest that CD8-positive and CD163-positive cell abundance in breast tumors might be linked to a heritable genetic component beyond *BRCA1* and *BRCA2*.

In our study, PTVs in *BRCA1* were associated with an increased abundance of FOXP3-positive and CD163-positive cells, which tend to promote an immunosuppressive tumor microenvironment. Contrastingly, results from a previous pan-cancer analysis by Sayaman and colleagues that investigated germline genetic contribution to the immune landscape of cancer, including breast cancer, showed that *BRCA1* germline variants were associated with elevated interferon and major histocompatibility complex class-I traits, which play a role in immune activation (14). These apparent contradictions demonstrate the complex biology of immune mechanisms in mammary carcinogenesis. Conceivably, our observation of increased abundance of CD163 and FOXP3-positive cells in relation to PTVs in *BRCA1* might reflect tumor promoting chronic inflammatory processes, while the observations by Sayaman and colleagues might reflect ongoing cancer immunoediting, processes that might coexist during tumor development and progression (35). The

association of PTVs in *BRCA1* with CD163-positive cells slightly attenuated after adjusting for the ER-status of the tumor, suggesting that the ER-status might be partially mediating the association. We also found PTVs in *BRCA2* to be associated with increased levels of CD8-positive and CD163-positive cells. This is consistent with recent evidence that *BRCA2*-deficiency results in an increase of interferon responses through cGAS/STING micronuclei accumulation (36), which ultimately can regulate macrophage polarization and T-cell differentiation (37). Similarly, long-term exposure to *BRCA2* abrogation in cancer-derived human cells was associated with an upregulation of interferon-stimulated genes (38). Moreover, pre-clinical studies demonstrated that healthy breast lobules of *BRCA1/2* germline carriers have 2.3 times more CD8-positive T-cells compared to non-carriers (16). The associations of PTVs in *BRCA2* with CD8-positive and CD163-positive cells remained largely unchanged after adjusting for ER-status of the tumor, suggesting that ER-status might not be a mediator. These associations were not statistically significant in the triple-negative subgroup analysis, which was expected considering the low abundance of *BRCA2* carriers in that subgroup. Moreover, it is important to note that other genetic, epigenetic, and non-genetic mechanisms might contribute to the immunogenicity of triple-negative breast cancer and further studies will be required to disentangle these. Recent evidence showed that ER-positive tumors display reduced number of immune cells, low HLA class I expression and favor the recruitment of lymphoid or myeloid cells other than T-cells (30). The few immune-rich ER-positive tumors show an increased relative fraction of macrophages compared to immune-low ER-positive tumors (39).

While the direct effect of some of the genes we studied on breast cancer immune cell abundance remain largely unknown, evidence in other cancer types might suggest

potential mechanisms of action. For example, *BRCA1-PALB2* deficiency has been associated with T-lymphocyte infiltration and the activation of cGAS-STING in macrophages in hepatocellular carcinoma (40). In ovarian cancer, patients with germline homologous recombination deficiency were enriched for CD68+ tumor associated macrophages and CD3+ lymphocytes, with the latter associated with longer median overall survival (41). These findings concur with reports showing that impairment of homologous recombination increases the rate of DNA defects, potentially leading to an enhanced immunogenic response against tumors (42) and higher leukocyte fractions (14). In sum, these results suggest that partial impairment of innate DNA damage repair can lead to increased immunological signaling and recruitment of CD163-positive cells.

Variants in breast cancer risk genes, especially those involved in homologous recombination, are more strongly associated with risk for ER-negative and in particular triple-negative breast cancer (33). These subtypes show both increased immune infiltration (43) and a higher frequency of homologous recombination deficiency (44). In our analyses, most associations between germline variants and immune cell abundance remained significant after adjustment for ER-status. In the triple-negative subgroup analysis, the association of PTVs in *RAD51D* with CD163-positive cells remained significant, but associations for other genes failed to reach significance. Reduced power due to smaller sample sizes may explain this for some genes, but this will need to be confirmed in larger datasets of triple-negative breast cancers.

While our study is the largest association analysis of rare MSVs and PTVs in breast cancer predisposition genes and immune cell abundance, it remains open to further improvement. For example, to our surprise we did not observe a statistically significant

association between PTVs in *BRCA1* and CD8-positive cells, even though the association [OR (95% CI): 1.15 (0.96-1.39)] was in the same direction as previously reported (15). While we observe an association between CD163-positive cells and grouping germline genetic variants on breast cancer risk genes, the effect is mainly driven by *BRCA1*, *BRCA2*, *PALB2* and *RAD51D*. This may be due to limited power for several genes, although our cohort was large. Another possible explanation for the absence of some expected associations might be the leftward-skewed distribution of the percentages, where limited variability in the outcome makes it challenging to detect the effect of rare germline variants. This might result from utilizing tissue microarrays instead of whole-slide images to estimate immune cell abundance, as tissue microarrays have been shown to markedly underestimate immune cell levels compared to the original whole-slide from which they are derived (45). Other potential limitations of using TMAs include intratumoral heterogeneity and lack of spatial information regarding where the cores were sampled in the tissues. Notably, however, emerging data, including two studies from the current analysis, suggest that evaluation of immune cell subsets on TMAs may closely approximate tumor biology assessed on whole sections (46). However, further studies might be required to clarify the etiologic relevance of spatial immune localization in breast tissues. Due to the original data source and study design (aimed to detect novel breast cancer genes) our analysis focused exclusively on (putative) predisposition genes, potentially excluding genes with a strong effect on immune response, such as *IL7* (47, 48).

Beyond impacting immune composition, it is important to be aware that perturbations in *BRCA1* may also impact the function of immune effector cells. For example, in-vivo evidence suggests that cytotoxic T-cell antitumor immune activity is

impaired in *BRCA1*-knockout mice (49). Similarly, macrophages with deficiency in nucleotide excision repair show functional and morphological abnormalities, displaying heightened glucose intake and pro-inflammatory stimuli (50). Collectively, these prior results suggest that DNA damage repair deficiencies can compromise immune cell function and exhibit a bi-directional effect, influencing the activity of immune cell as well as their interaction with tumor cells, which is something we could not address in our analyses.

CONCLUSIONS

Our findings underpin a potential driver role of rare pathogenic germline variants involved in DNA damage repair, and particularly those predisposing to breast cancer, in the immune landscape of breast tumors, most notably with CD163-positive cells. With further elucidation, these findings have the potential to improve the selection of patients that may benefit from immunomodulatory therapies, especially considering recent developments pointing towards tumor associated macrophages as potential targets for PD-1/PD-L1 immunotherapy (51). Our results lend support to the notion that germline impairment of DNA damage repair machinery interferes with the immunological component of breast cancer.

ABBREVIATIONS

- MSV – (likely) Deleterious missense variants
- PTV – Protein truncating variants
- ER – Estrogen receptor
- PR - Progesterone receptor
- OR – Odds ratios

- CI – Confidence intervals

DECLARATIONS

Ethics approval and consent to participate

All women involved provided written informed consent. Each study met local approval by the ethical committee and/or institutional review board listed in Additional file 2: Table S1. All studies conform to the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the current study are not publicly available due to patient privacy and legal constraints but are available from the authors on reasonable request. The tissue-microarray data are a subset of data previously deposited in the European Genome-Phenome Archive, <https://ega-archive.org/datasets/EGAD50000002125>. Requests for individual-level data (genetic, tissue-microarray immunohistochemistry or immune cell density) must be submitted for consideration to the BCAC Data Access Co-ordinating Committee (bcac@medschl.cam.ac.uk). All code supporting the results of this study are publicly available on GitHub at: https://github.com/SchmidtGroupNKI/RareGermline_ImmuneBC (52).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization and design: MKS, AMA, PPP, MGC, SC. Data support: AB, RK. Statistical analysis: FRR. Data interpretation and writing of first draft: FRR, SC, MKS. Data contribution: all other authors. All authors read and approved the final manuscript.

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SUPPLEMENTAL INFORMATION

All supplemental information was provided by the authors.

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Additional file 1: **Fig. S1.** Flow chart detailing the inclusion criteria and number of women that were included in the study. **Fig. S2.** Examples of core images from tissue microarrays that were used for immune marker quantification. **Fig. S3.** Distribution of the number of tumors according to the percentage of positive immunohistochemistry-stained area in the tumor for the four immune cell types. **Fig. S4.** Logit-transformed marker percentages stratified by PTV carrier status of twelve breast cancer predisposition genes. **Fig. S5.** Association of protein truncating variants with the abundance of four immune cell types in breast cancer with and without adjustment for estrogen receptor status of the tumor. **Fig. S6.** Associations of protein truncating variants, grouped by DNA damage repair mechanism, with the abundance of four immune cell types for women diagnosed with triple-negative breast cancer. **Table S3.** Patient characteristics for each of the immune cell type datasets. **Table S4.** Functional annotation of the 34 genes included in the BRIDGES panel. **Table S5.** Associations of protein truncating variants in 34 (putative) predisposition genes with the abundance of CD8-positive cells in all breast cancers. **Table S6.** Associations of (likely) deleterious missense variants in 34 (putative) predisposition genes with the abundance of CD8-positive cells in all breast cancers. **Table S7.**

Associations of protein truncating variants in 34 (putative) predisposition genes with the abundance of CD20-positive cells in all breast cancers. **Table S8.** Associations of (likely) deleterious missense variants in 34 (putative) predisposition genes with the abundance of CD20-positive cells in all breast cancers. **Table S9.** Associations of protein truncating variants in 34 (putative) predisposition genes with the abundance of FOXP3-positive cells in all breast cancers. **Table S10.** Associations of (likely) deleterious missense variants in 34 (putative) predisposition genes with the abundance of FOXP3-positive cells in all breast cancers. **Table S11.** Associations of protein truncating variants in 34 (putative) predisposition genes with the abundance of CD163-positive cells in all breast cancers. **Table S12.** Associations of (likely) deleterious missense variants in 34 (putative) predisposition genes with the abundance of CD163-positive cells in all breast cancers. **Table S13.** Associations of protein truncating variants and (likely) deleterious missense variants grouped across mechanisms of DNA damage repair with the abundance of CD8-positive cells in all breast cancers. **Table S14.** Associations of protein truncating variants and (likely) deleterious missense variants grouped across mechanisms of DNA damage repair with the abundance of CD20-positive cells in all breast cancers. **Table S15.** Associations of protein truncating variants and (likely) deleterious missense variants grouped across mechanisms of DNA damage repair with the abundance of FOXP3-positive cells in all breast cancers. **Table S16.** Associations of protein truncating variants and (likely) deleterious missense variants grouped across mechanisms of DNA damage repair with the abundance of CD163-positive cells in all breast cancers. **Table S17.** Associations of protein truncating variants and (likely) deleterious missense variants with the abundance of CD8-positive cells; sensitivity analyses only including non-carriers without any other germline mutation in any other gene. **Table S18.** Associations of protein truncating variants and (likely) deleterious missense variants with the

abundance of CD20-positive cells; sensitivity analyses only including non-carriers without any other germline mutation in any other gene. **Table S19.** Associations of protein truncating variants and (likely) deleterious missense variants with the abundance of FOXP3-positive cells; sensitivity analyses only including non-carriers without any other germline mutation in any other gene. **Table S20.** Associations of protein truncating variants and (likely) deleterious missense variants with the abundance of CD163-positive cells; sensitivity analyses only including non-carriers without any other germline mutation in any other gene. **Table S21.** Associations of protein truncating variants and (likely) deleterious missense variants with the abundance of CD8-positive cells in triple-negative breast cancers. **Table S22.** Associations of protein truncating variants and (likely) deleterious missense variants with the abundance of CD20-positive cells in triple-negative breast cancers. **Table S23.** Associations of protein truncating variants and (likely) deleterious missense variants with the abundance of FOXP3-positive cells in triple-negative breast cancers. **Table S24.** Associations of protein truncating variants and (likely) deleterious missense variants with the abundance of CD163-positive cells in triple-negative breast cancers. **Table S25.** Associations of protein truncating variants and (likely) deleterious missense variants grouped for the 12 known breast cancer predisposition genes with the abundance of the CD8-, CD20, FOXP3- and CD163-positive cells in all breast cancers.

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Additional file 2: **Table S1.** B-CAST study design information. **Table S2.** Details of staining for the four immune markers.

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