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**Title:** Pathogenic Variants in *CHEK2* are Associated with an Adverse Prognosis in Symptomatic Early Onset Breast Cancer

**Short Title:** Germline *CHEK2* Mutations and Prognosis

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Abstract

PURPOSE: Checkpoint Kinase 2 (*CHEK2*) is frequently included in multi-gene panels, we describe the associated outcomes amongst carriers of *CHEK2* pathogenic variants in young symptomatic breast cancer patients.

PATIENTS AND METHODS: 2344 participants in the Prospective Outcomes in Sporadic Versus Hereditary Breast Cancer (POSH) Study were included. Participants had a diagnosis of primary invasive breast cancer at the age of 40 years or younger. Summary statistics were used to compare tumour characteristics amongst *CHEK2*+ carriers with *CHEK2*-. Kaplan Meier curves were used to demonstrate Overall Survival (OS) and Distant Disease Free Survival (DDFS).

RESULTS: Overall, 53/2344 (2.3%) of cases had a pathogenic *CHEK2* variant. *CHEK2*+ associated tumours were significantly more likely to be grade 2, Oestrogen Receptor (ER) and Progesterone Receptor (PR) positive compared to *CHEK2*- (grade 2, 28/52 (53.8%) versus 803/2229 (36.0%) (p=0.029)). *CHEK2* associated tumours were significantly more likely to present with nodal involvement (N1, 37/53 (69.8%) versus 1169/2253 (51.9%) (p=0.0098) and demonstrated a trend towards multifocality. A higher proportion of *CHEK2*+ with invasive breast cancer were obese compared to *CHEK2*- (28.3% versus 18.8%, p=0.039). Univariate and multivariable analysis revealed that Overall Survival (OS) and Distant Disease Free Survival (DDFS) was significantly worse in *CHEK2*+ versus *CHEK2*- (OS HR, 1.58 (95%CI, 1.01-2.48 (p=0.043))).

CONCLUSIONS: This work highlights the adverse prognosis associated with breast cancer in *CHEK2* pathogenic variant carriers. It also identifies a potential association between obesity, family history and breast cancer risk in young *CHEK2* gene carriers.

Pathogenic Variants in *CHEK2* are Associated with an Adverse Prognosis in Symptomatic Early Onset Breast Cancer

Introduction

Advances in Next Generation Sequencing Technology (NGS) and a reduction in cost has widened access to cancer genomic technology including gene panel testing in routine clinical practice.1 A Consensus Guideline published by the American Society of Breast Surgeons in 2019 advocates the routine testing of *BRCA*1, *BRCA*2 and *PALB2* along with other high and moderate risk breast cancer susceptibility genes to facilitate practice standardisation in the context of hereditary cancer susceptibility.2 Despite this consensus opinion, there is residual concern regarding the true clinical validity and utility of moderate risk genes in the context of patient care. One moderate risk gene which remains a focus for such evaluation is Checkpoint Kinase 2 (*CHEK2*).

*CHEK2* is a serine/threonine kinase which functions as a tumour suppressor gene necessary for cell cycle checkpoint regulation, the inhibition of cellular proliferation and activation of DNA repair pathways or apoptosis.3-5 Pathogenic variants in *CHEK2* abolish the protein kinase activity and confer a moderate increase in breast cancer risk which is 2-3 fold above the baseline population level.5-11 *CHEK2* pathogenic variants are most prevalent amongst individuals of European descent and several founder mutations exist.5,12,13 Of these, *CHEK2* c.1100delC is the most frequently identified amongst Northern Europeans with an observed population frequency of 0.5-1%.4,6,7

At present, there is limited data regarding the effects of a germline *CHEK2* mutation on breast tumour biology. In general, *CHEK2* associated tumours are considered more likely to be Grade 2, Oestrogen Receptor (ER) and Progesterone Receptor (PR) positive compared to *CHEK2-*. 4,6,14-16 A trend towards bilateral disease at presentation with higher levels of nodal involvement has also been suggested indicating a potentially more aggressive underlying tumour biology which may influence patient outcome.16,17

The primary aim of this study is to describe the histopathological tumour phenotype of *CHEK2* associated breast cancers in a young onset, symptomatic cohort. The secondary aim is to determine whether a germline *CHEK2* pathogenic variant differentially influences breast cancer survival compared to sporadic controls in early onset breast cancer.

Methods: Sample Population

The sample population has been obtained from the Prospective Outcomes in Sporadic Versus Hereditary Breast Cancer (POSH) Study (UKCRN ID: 1137).18,19  POSH was a large prospective multi-centre prospective cohort study which recruited 3095 women from 127 UK hospitals between 1st Jan 2000 and 31st January 2008.18,19 The study was designed to evaluate which factors influence prognosis and treatment response, in women diagnosed with early onset breast cancer. 18,19  Ethical approval was from the South and West Multi-Centre Research Ethics Committee (MREC 00/6/69).

Inclusion required a primary diagnosis of invasive breast cancer at the age of 40 years or younger.18,19 In total, 3021 of the 3095 participants recruited into the POSH study were eligible for further analysis.

Diagnostic pathology data were collected for all patients including tumour size, stage, grade, multifocality and ER, PR and HER2 receptor status. Genomic DNA was extracted from whole blood and a customised gene panel using a multiplex amplicon based library preparation system (Fluidigm Access Array™).20 Extracted DNA was available for 2907 (96%) of recruits. NGS was performed using an Illumina platform.21 Sequencing targeted exonic regions and the exon/intron boundaries for the panel of genes which included *CHEK2, BRCA1*, *BRCA2, PALB2, ATM and TP53*.21

In total 3021 of the 3095 participants recruited into the POSH study were eligible for further analysis. Of these, 2344 (78%) were included in the analysis population for this study and 677 (22%) were excluded. Participants were excluded due to missing genotyping data (n=159), a germline pathogenic variant in *BRCA*1, *BRCA*2, *PALB2*, *ATM* or *TP53* (n=400), M1 stage disease (n=74), age 41-50 years (n=42) and missing primary tumour data (n=2). Samples failed NGS either due to inferior quality or low concentration DNA (Supplement 1).

Methods: Bioinformatics Analysis

The POSH study utilised two validated bioinformatics pipelines for the identification of pathogenic variants in *CHEK2*. The Burrows Wheeler Aligner, BWA-MEM, was used to align sequence data to the reference human genome (build hg19) and create a Binary Alignment/Map (BAM) file.22,23 GATK was used for base quality recalibration and indel realignment. To ensure the accuracy of variant identification, reads with low mapping quality scores (less than phred 20), unmapped reads, failed primary alignments and reads failing platform or vendor quality checks were removed. Duplicate reads were kept because the amplicon based sequencing method generates legitimate duplicates. SAMtools and GATK Unified Genotyper were used to identify probable variants. 24-27 The resulting variant call files (VCF) were annotated using ANNOVAR.28

Variants were classified using the American College of Medical Genetics and Genomics (ACMG) guidelines.29 This assigns pathogenicity based upon several factors including population frequency, segregation, presence in databases of functional significance and *in-silico* predictions. Filters were applied to identify those variants which were clearly or highly likely to be pathogenic (ACMG Class 4 and 5) or protein truncating (frameshift, stop-gain or stop-loss) with a minor allele frequency (MAF) of less than 1% in the genome aggregation database (gnomAD).30,31 All filtered variants were manually reviewed and compared with the assigned pathogenicity in ClinVar.32 Variants of Uncertain Clinical Significance and hypomorphic alleles were classified as mutation negative. Confirmatory testing was completed by Sanger Sequencing. For the purpose of this analysis, *CHEK2* pathogenic variant carriers were categorised as *CHEK2*+ and non-carriers as *CHEK2-.*

Methods - Statistical Analysis

Statistical Analysis was performed according to a pre-specified statistical analysis plan (SAP). Summary statistics were used to describe the cohort. The Mann-Whitney test was used for continuous variables and the Pearson χ2 test for categorical variables to identify any specific differences between *CHEK2*+ mutation carriers and *CHEK2-*. Kaplan Meier curves were used to demonstrate Overall Survival (OS) and Distant Disease Free Survival (DDFS). Differential survival between *CHEK2*+ and *CHEK2*- groups was compared using a univariate Cox regression model. Multivariable analyses (MVA) was also performed using Cox regression.

Results

The complete analysed cohort comprised 2344 participants diagnosed with a primary invasive breast cancer under the age of 40 years (supplement 2). A confirmed *CHEK2* pathogenic variant was found in 53/2344 (2.3%) of this study cohort (1.9% (53/2744) of the whole POSH cohort).

The Northern European founder *CHEK2* c.1100delC was the most frequently identified pathogenic variant accounting for 36/53 (67.9%) of all *CHEK2* pathogenic variants. In total, 3 participants also possessed a germline pathogenic variant in either *BRCA*2 (N=2) or *PALB2* (N=1). We identified 28 individuals with Variants of Uncertain Significance in *CHEK2*. This included 27 missense variants (including 6 instances of the low penetrance variant c.470T>C, p.Ile157Thr) and 1 in-frame deletion.4 In routine clinical practice, individuals with low penetrance risk alleles or variants of uncertain significance would be managed as mutation negative so these cases were included in the control group (supplement 3).

Overall, *CHEK2+* associated tumours were significantly more likely to be grade 2 at presentation compared to *CHEK2-* (grade 2 28/52 (53.8%) versus 803/2229 (36.0%) (p=0.029), Table 1). There was no difference in baseline tumour size between *CHEK2*+ and *CHEK2*- (p=0.73).

*CHEK2+* associated tumours also displayed significantly higher levels of nodal involvement compared to *CHEK2-*. In total, 37/53 (69.8%) of *CHEK2+* presented with N1 stage disease versus 1169/2253 (51.9%) of *CHEK2-* (p=0.0098). In addition, *CHEK2+* associated tumours demonstrated a trend towards multifocality at presentation compared to *CHEK2*- (22/52 (42.3%) versus 624/2085 (29.9%) (p=0.055)).

We compared baseline tumour grade, size and focality between *CHEK2* c.1100delC carriers and all other truncating variant carriers and found no significant difference (table 1 and figure 1).

*CHEK2* related tumours were significantly more likely to be ER positive and PR positive compared to *CHEK2-* (table 1). In total, 47/53 (88.7%) of *CHEK2* associated tumours were ER positive compared to 1557/2279 (68.3%) of *CHEK2-* (p=0.0016) and 33/42 (78.6%) of *CHEK2+* associated tumours were PR positive compared to 1084/1848 (58.7%) of *CHEK2*- (p=0.0094). *CHEK2+* were also significantly less likely to have a triple negative breast cancer (TNBC) compared to *CHEK2-* (p=0.0022). In total, 1/53 (1.9%) of *CHEK2+* associated tumours were TNBC compared to 417/2291 (18.2%) of *CHEK2-* tumours (table 1 and figure 2). There was no significant association with HER2 receptor status and *CHEK2* genotype.

The median age of cancer onset was 37 years (IQR 34-39 years) for *CHEK2*+ mutation carriers, and 37 years (IQR 34-39 years) for *CHEK2*-. The majority of *CHEK2*+ carriers were Caucasian 50/53 (94.3%). There was no association between family history of breast cancer and median BOADICEA score comparing *CHEK2+* and *CHEK2*- (supplement 2). In total, 36/51 (70.6%) of all *CHEK2+* had no family history of breast cancer. The median BOADICEA score was 0.03 for both *CHEK2+* and *CHEK2*- ((*CHEK2+*, IQR 0.02-0.07) versus (*CHEK2-*, IQR 0.02-0.05) (p=0.86)). However we noted that a higher proportion of *CHEK2* + with invasive breast cancer were obese compared to *CHEK2-* (28.3% versus 18.8%, p=0.039).

The majority of patients received adjuvant chemotherapy. The most frequent regimen included anthracyclines with or without an additional of taxane. There were no significant differences in the treatment received between *CHEK2+* and *CHEK2*- (table 2). However, a non-significant trend towards mastectomy was identified amongst *CHEK2+*. In total 36/53 (67.9%) amongst *CHEK2+* underwent mastectomy as the primary surgical intervention versus 1122/2291 (49.0%) amongst *CHEK2*- (p=0.054).

The median duration of follow up was 8.2 years. Contralateral breast cancers were more frequently observed in *CHEK2*+ compared to *CHEK2-* (S4). A contralateral breast cancer was observed in 5/53 (9.4%) of *CHEK2+* at 10 years compared to 85/2291 (3.7%) of *CHEK2-*. Of the 5 *CHEK2+* individuals with contralateral breast cancer, 2 had bilateral disease at presentation and a further participant was found to have a contralateral breast cancer in the same year as their primary breast cancer diagnosis.

Subgroup analysis revealed that the observed increase in contralateral breast cancer risk observed amongst *CHEK2+* carriers occurred in the context of familial breast cancer. In total, 3/15 (20.0%) of *CHEK2*+ with a positive family history of breast cancer developed a contralateral breast cancer compared to 1/36 (2.8%) *CHEK2+* without family history. Family history data was missing for one individual with a contralateral breast cancer. This difference was apparent in the first five years following breast cancer diagnosis. The contralateral breast cancer rates observed amongst *CHEK2+* without a family history of breast cancer where similar to the *CHEK2*- with or without a family history (table 3). Furthermore, 3/5 *CHEK2*+ individuals with contralateral disease had a family history of breast cancer and were obese.

Univariable Analysis (UVA) identified significantly worse Overall Survival (OS) in *CHEK2+* versus *CHEK2-* (HR, 1.58 (95%CI, 1.01-2.48 (p=0.043)) (figure 3). At 5 years, OS was 75.1% (95% CI, 60.9-84.7) amongst *CHEK2+* versus 85.1% (95% CI, 83.5-86.5) in *CHEK2-*. At 10 years, OS was 60.7% (95% CI, 42.5-74.8) amongst *CHEK2+* versus 70.2% (95% CI, 67.8-72.5) in *CHEK2-*. The observed difference in OS between *CHEK2+* and *CHEK2-* was maintained after adjustment for known prognostic factors including age at diagnosis, BMI, grade, maximum invasive size (cm), hormone receptor status, nodal involvement, ethnicity and taxanes in a multivariable analysis (HR 1.65 (95%CI, 1.05-2.59 (p=0.03)).

Distant Disease Free Survival (DDFS) was also significantly worse in *CHEK2+* versus *CHEK2-* UVA, HR, 1.62 (95%CI, 1.06-2.48 (p=0.025)) (Supplement 4). At 5 years, DDFS was 61.8% (95% CI, 47.2-73.4) amongst *CHEK2+* versus 77.7% (95% CI, 75.9-79.4) in *CHEK2-*. At 10 years, DDFS was 56.8% (95% CI, 41.8-69.3) amongst *CHEK2+* versus 69.0% (95% CI, 66.7-71.2) in *CHEK2-* (Supplement 4). The observed difference in DDFS between *CHEK2+* and *CHEK2-* was maintained after adjustment for known prognostic factors in a multivariable analysis (HR 1.60 (95%CI, 1.04-2.46 (p=0.033)).

## Discussion

## Analyses of 2344 participants within the POSH cohort, has identified that pathogenic truncating variants in *CHEK2* are present in 1.9% of unselected early onset breast cancers within a UK population. *CHEK2* c.1100delC is the most frequently identifiable variant, accounting for 67.9% of all truncating mutations. These figures are consistent with Decker et al. who identified a truncating *CHEK2* variant in 1.6% of unselected breast cancer cases within a large UK population based study.6 *CHEK2* c.1100delC was the most frequent variant accounting for 81% of truncating mutations.6

We have determined that patients with a germline *CHEK2* pathogenic variant who develop breast cancer have an adverse outcome with reduced OS and DDFS compared to those without, a relationship which persists after adjustment for known prognostic factors. We also noted that 4/74 (5.7%) CHEK+ individuals removed from the analysis because they presented with M1 disease, carried a pathogenic CHEK2 variant compared with 74/3095 (2.4%) of the total patients in the POSH study Whilst the numbers are small, this observation is supportive of future work to better define the relationship between CHEK2 mutational status and outcome in early onset breast cancer.

Our results are consistent with Schmidt et al. who found that *CHEK2*, c.1100delC mutation carriers had a worse recurrence free and breast cancer specific survival than *CHEK2-* (HR 1.7 95% CI, 1.2-2.4 (p=0.006)) and (HR 1.4 95% CI, 1.0-2.1 (p=0.072)) respectively but after multi-variable analysis the difference was no longer statistically significant.8 Wesicher et al. also found that ER positive *CHEK2* c.1100delC carriers within the BCAC consortium had a significantly increased risk of breast cancer specific death which persisted after multi-variable analysis (HR 1.63 (95% CI, 1.24 to 2.15) p<0.001).16 They also identified a 2.8 fold risk of a second breast cancer (HR 2.8 (95% CI, 2.00 - 3.83 p<0.001).16

In our study, *CHEK2* associated breast cancers are significantly more likely to be Grade 2, ER and PR positive compared to age matched controls with no difference in HER2 expression. They are not associated with a TNBC phenotype. These results are consistent with other reports of histopathological tumour phenotype associated with germline mutations in *CHEK2*. We were not able to classify into intrinsic subtypes as gene expression data were not available for the POSH study.

Decker et al found that *CHEK2* associated tumours were significantly more likely to be ER-positive OR=3.42 (95% CI, 2.33 - 5.21 (p=1.5x10-11)).6 Cybulski et al. also found that *CHEK2*-associated cancers were significantly more likely to be oestrogen and progesterone receptor positive (69.7% versus 63.1% (p=0 .002)) and (77% versus 68.7% (p<0.001)) respectively.15 Weischer et al. also found a significantly higher frequency of oestrogen and progesterone receptor positive breast cancers in c.1100delC carriers than *CHEK2-* (63% versus 57% (p<0.001)) and (46% versus 43% (p=0.01)) respectively.16 Couch et al found no *CHEK2* pathogenic variants amongst 1,824 patients presenting with triple negative breast cancer.33 The strong association of a germline *CHEK2* pathogenic variant with ER positive disease may support the use of oestrogen receptor blockade such as Tamoxifen as chemoprophylaxis in patients with a *CHEK2* related breast cancer risk.15

Within the POSH cohort, family history did not predict the presence of a germline *CHEK2* pathogenic variant. This is consistent with a low-moderate overall increase in risk compared to population average.

A higher proportion of *CHEK2+* with invasive breast cancer were obese than CHEK2-. It is well recognised that obesity is associated with an increased risk of post-menopausal breast cancer but it has not been associated with an increased risk in pre-menopausal breast cancer.34 In 2017, The Premenopausal Breast Cancer Collaborative Group assessed the BMI associated breast cancer risk in 758,592 premenopausal women and found an inverse correlation between age and the associated risk. The results of this study, suggest a potential association in breast cancer patients between *CHEK2* related genetic risk and obesity.35,36 We had previously shown that BMI was associated with an adverse prognosis after breast cancer diagnosis, independent of other known risk factors, we therefore included BMI in the multivariable analysis.37

Invasive breast cancers occurring in the context of a pathogenic *CHEK2* variant demonstrated a trend towards multifocality with significantly higher levels of nodal involvement at presentation. Our study confirmed the findings of Cybulski et al. who found that *CHEK2* associated cancers demonstrated higher levels of nodal involvement. 14,15 Multifocal tumour pathology is likely to be one of the key drivers for more frequent mastectomy rather than breast conserving treatment amongst *CHEK2* carriers.

The contralateral breast cancer rate amongst *CHEK2* pathogenic variant carriers was almost twice that of *CHEK2-* at both 5 and 10 years. Although the absolute numbers of cases was small, we noted that *CHEK2* carriers with a family history, had a contralateral breast cancer rate that was higher than non-carriers. Within the POSH cohort, family history was not an independent predictor of outcome. 38

In 2004, De Bock et al. reported that at 5 years, a contralateral breast cancer had developed in 21% of *CHEK2* c.1100delC carriers compared to 4% of *CHEK2-* representing an almost 6-fold increase in risk.39 Decker et al. found that *CHEK2* associated tumours were significantly more likely to be bilateral at presentation (OR=3.27 (95% CI 1.66 - 5.83) p=0.0014).6 This was further supported by Kilpivaara et al. who also noted a strong association with bilateral disease at presentation.14,15 Our study notes the presentation with bilateral disease particularly in the context of other risk factors (obesity and family history)

There are several strengths of this study. Participants were ascertained shortly after diagnosis through oncology clinics and presented with symptomatic rather than screen detected breast cancers. The clinical data available is comprehensive and allows multivariable data analysis. There was no systematic bias in selecting patients for the study or for genotyping and the cohort is representative of the general UK breast cancer population.18 Furthermore, carriers of additional high-risk breast cancer susceptibility genes could be excluded from the analysis allowing a clean comparison between *CHEK2* pathogenic variant carriers and non-carriers. However the study sampled a very specific population of early onset (under the age of 40 years) breast cancer patients and therefore may potentially not be representative of all age groups.

*CHEK2* is commonly included in multigene panel testing, and most frequently identified in the context of a patient presenting with breast cancer. Although the numbers are small, we observed a higher rate of a contralateral breast cancer associated with a pathogenic *CHEK2* variant. The increased incidence appears to be confined to carriers with a family history of breast cancer. For carriers with no family history, the incidence of contralateral breast cancer is no greater than the incidence in a non-carrier population.

*CHEK2* pathogenic variant carriers also presented with tumours more likely to metastasise, manifest as higher nodal involvement and poorer overall survival. This is in contrast to analysis in the same cohort which showed that prognosis was not altered in a multivariable analysis of *BRCA1* or *BRCA2* carriers compared with *CHEK2-*.40

Including *CHEK2* genotyping as part of population risk stratified approaches to inform targeted screening and improve early diagnosis is aspirational. The current approach for managing moderate breast cancer risk within the UK is annual mammograms from the age of 40 years.41 The use of chemoprophylaxis may be effective given the high proportion of hormone receptor positive breast cancers.41However, neither measure has yet been tested in this particular group of patients.

Our study highlights the importance of including effective measures to address lifestyle risk factors, particularly around maintaining a healthy body weight, for premenopausal women at increased breast cancer risk.

Conclusion

In summary, this work describes the characteristics and clinical outcomes for patients who present with invasive early onset breast cancer and carry a *CHEK2* pathogenic variant. Since a pathogenic *CHEK2* variant is likely to be identified in approximately 2% of Caucasian breast cancers patients, including those aged 40 years or younger, clinicians should be aware of the adverse prognosis and potential effect of family history on contralateral cancer risk in planning cancer treatment. It also highlights the importance of preventing and managing obesity in the context of breast cancer susceptibility.

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Figures and Tables

**Figure 1:** **Baseline Histopathological Characteristics of the *CHEK2* Cohort:** Comparison of the tumour focality, grade and lymph node involvement between *CHEK2* truncating variant carriers and non-*CHEK2* carriers.

**Figure 2: Hormone Receptor Status of the *CHEK2* Cohort:** Comparison of ER, PR and HER2 receptor status between *CHEK2* truncating variant carriers and non-*CHEK2* carriers. Values represented as percentage of the cohort. Samples derived from the POSH Cohort.

**Figure 3:** **Kaplan Meier Plot of Overall Survival** Kaplan-Meier Plot demonstrating Overall Survival (OS) for *CHEK2* truncating variant carriers versus *CHEK2-* following univariable analysis.

**Table 1:** **Baseline Histopathological Characteristics of the *CHEK2* Cohort** Comparison of the tumour focality, grade, lymph node involvement, ER, PR and HER2 receptor status between *CHEK2* truncating variant carriers and non-*CHEK2* carriers. †Assessment of statistical significance were performed using the Mann-Whitney test for continuous variables and a Pearson χ2 test for categorical variables. Samples derived from the POSH Cohort.

**Table 2: Treatment Characteristics of the CHEK2 Cohort:** Comparison of the treatment protocol in relation to genotype. †Assessment of statistical significance were performed using the Mann-Whitney test for continuous variables and a Pearson 2 test for categorical variables. Samples derived from the POSH Cohort.

**Table 3 Contralateral Breast Cancer Risk in Association with *CHEK2*:** Contralateral breast cancer risk at both 5 and 10 years for *CHEK2* truncating variant carriers versus *CHEK2-*. The presence of a family history (FH+) was associated with an increased contralateral breast cancer risk.