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One in three highly selected Greek patients with breast cancer carries a loss-of-function variant in a cancer susceptibility gene

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ABSTRACT

Background Gene panel testing has become the norm for assessing breast cancer (BC) susceptibility, but actual cancer risks conferred by genes included in panels are not established. Contrarily, deciphering the missing hereditability on BC, through identification of novel candidates, remains a challenge. We aimed to investigate the mutation prevalence and spectra in a highly selected cohort of Greek patients with BC, questioning an extensive number of genes, implicated in cancer predisposition and DNA repair, while calculating gene-specific BC risks that can ultimately lead to important associations.

Methods To further discern BC susceptibility, a comprehensive 94-cancer gene panel was implemented in a cohort of 1382 Greek patients with BC, highly selected for strong family history and/or very young age (<35 years) at diagnosis, followed by BC risk calculation, based on a case–control analysis.

Results Herein, 31.5% of patients tested carried pathogenic variants (PVs) in 28 known, suspected or candidate BC predisposition genes. In total, 24.8% of the patients carried *BRCA1/2* loss-of-function variants. An additional 6.7% carried PVs in additional genes, the vast majority of which can be offered meaningful clinical changes. Significant association to BC predisposition was observed for *ATM, PALB2, TP53, RAD51C* and *CHEK2* PVs. Primarily, compared with controls, *RAD51C* PVs and *CHEK2* damaging missense variants were associated with high (ORs 6.19 (Exome Aggregation Consortium (ExAC)) and 12.6 (Fabulous Ladies Over Seventy (FLOSSIES)), p<0.01) and moderate BC risk (ORs 3.79 (ExAC) and 5.9 (FLOSSIES), p<0.01), respectively.

Conclusion Studying a large and unique cohort of highly selected patients with BC, deriving from a population with founder effects, provides important insight on distinct associations, pivotal for patient management.

INTRODUCTION

Loss-of-function (LoF) variants in the highly penetrant genes, *BRCA1* and *BRCA2*, are the major players for breast and ovarian cancer susceptibility; individuals carrying such pathogenic variants (PVs) face an increased lifetime risk for cancer diagnoses.[1] The implementation of *BRCA1* and *BRCA2* genetic testing into clinical practice has enabled the identification of individuals at high risk and the application of tailored management guidelines, significantly improving both cancer prevention and survival.[2]

Mutations in *BRCA1/2* genes account for 5%–10% of breast cancer (BC) cases. This can be slightly higher in populations with strong founder effects, such as the Ashkenazi Jewish, or to a lesser extent, Greek.[3–6] Testing for *BRCA1/2*, for more than two decades, revealed an important limitation; a substantial number of individuals with early diagnosis and/or strong family history receive a negative test result. Further investigation on high-risk families, along with advances in sequencing technologies, have led to the characterisation of additional BC predisposition alleles in genes, most of which encode for proteins involved in DNA repair through homologous recombination.[7]

Nowadays, a number of high penetrant genes, that is, *TP53* and *PALB2*, genes of intermediate penetrance, that is, *CHEK2* and *ATM*, as well as syndromic genes, that is, *CDH1*, *PTEN* and *STK11*, are included in gene panels. Alongside, multigene panel testing has replaced, to a large extent, traditional single gene testing, where additional genes, PVs of which predispose for other types of cancers or are low penetrant, are analysed. In this context, the probability of receiving an unclear or inconclusive result increases significantly, while the referring clinician's major concern remains the clinical actionability of the genetic testing results, mainly due to lack of defined management guidelines.[8]

We therefore sought to investigate the mutation prevalence and spectra in a highly selected cohort of Greek patients with BC diagnosed at a very young age (<35 years) and/or with strong family history, implementing a comprehensive 94 gene panel, while calculating gene-specific BC risks. Both the extensive number of genes questioned, most of which are implicated in cancer predisposition and DNA repair and the stringent patient selection criteria set, provide important benefits for identification of

novel BC candidates and revelation of important associations to BC susceptibility.

PATIENTS AND METHODS

Patient selection

The study cohort included 1382 Greek patients with BC having strong family history (at least three cancer diagnoses of breast, ovarian or pancreatic cancer from the same side of the family) and/or diagnosed <35 years. All patients were index, were not related and have been retrospectively selected from referrals to Molecular Diagnostics Laboratory of National Centre for Scientific Research (NCSR) 'Demokritos' between the years 1999 and 2017. Written informed consent was obtained from all individuals prior to genetic testing. The study was in agreement with the 1975 Helsinki statement.

Testing for Greek *BRCA1* founder alleles

Initial screening involved testing for the five Greek founder and one recurrent *BRCA1* mutations, as previously described.[3 9] This includes (1) three *BRCA1* large genomic rearrangements (LGRs), all of which disrupt the BRCA1 C-terminus domain and are confined to the Greek population[4 5] and (2) three single nucleotide variants, namely, c.5212G>A, c.5266dupC and c.5251C>T0.[3]

Genomic capture and massively parallel sequencing using Trusight cancer panel

Germline DNA is enzymatically fragmented, adaptor tagged, indexed and captured to target the 1736 genomic regions of 94 cancer predisposing genes using TruSight Cancer Panel, following manufacturer's instructions (Illumina, San Diego, USA). The complete gene list is shown in the online supplementary table 1.

Amplified libraries were evaluated qualitatively and quantitatively using Fragment Analyzer (Advanced Analytical Technologies, Heidelberg, Germany). Indexed libraries were sequenced on MiSeq using the Standard V2 kit performing 150 base paired-end reads, while FASTQ, BAM and VCF files were generated through Illumina MiSeq Reporter; annotation was performed against the human reference genome GRCh38 using VariantStudio V.3 (Illumina). The minimum base and amplicon coverage were 50×, and 100×, respectively, while the mean read depth was 182×. All PVs were confirmed by Sanger sequencing.

Multiplex ligation-dependent probe amplification (MLPA)

Detection of large deletions and duplications among the genes tested, excluding the Greek founder *BRCA1* LGRs, was not sufficiently reliable through NGS-based testing. Therefore, SALSA MLPA kit P002, P045, P190 and P056 were used to assess LGRs involving *BRCA1*, *BRCA2, CHEK2* and *TP53* genes, respectively, following manufacturer's instructions (MRC-Holland, Amsterdam, the Netherlands).

Evaluation of missense variants

Missense variants identified herein have been evaluated and classified based on data deriving from: (1) their characterisation based on in vivo and/or in vitro functional assay(s), (2) classification based on ClinVar[10] and (3) prediction models (SIFT, PolyPhen, AlignGVGD, PastCons, Phylop and Mutation Taster), providing (1) strong and (2, 3) supporting evidence, respectively, based on the American College of Medical Genetics and Genomics (ACMG) classification guidelines.[11] Detailed description of the rare missense variants classified as pathogenic is illustrated in the online supplementary table 2.

Statistical analysis and case–control study

Quantitative variables are presented as mean±1 SD, while categorical variables as frequency. Non-parametric Wilcoxon rank-sum test was used to compare age association between mutations per gene. Comparisons of categorical variables between groups were performed using Pearson's χ^2 test. ORs were calculated using the Fisher's exact test. P values were corrected for multiple comparison using false discovery rate method and were considered statistically significant when <0.05.

Cumulative LoF variants per gene were directly compared with the non-The Cancer Genome Atlas (TCGA) exomes, non-Finnish European (NFE) Exome Aggregation Consortium (ExAC) data set,[12] and, when applicable, to the Fabulous Ladies Over Seventy (FLOSSIES) European-American data set (https://whi.color.com/). ExAC–NFE variants with allele frequency >0.2% and low-quality variants were filtered out. For both data sets, only pathogenic/likely PVs by ClinVar were included. Individuals carrying LGRs, double LoF variants and the low-risk *CHEK2* variants: p.(Ile157Thr) and p.(Ser428Phe) were excluded from analyses.

Two separate analyses were performed for *TP53* mutations, one including all carriers and one for those with BC diagnosis <35 years.

RESULTS

Prevalence of LoF variants

The detection rate of LoF variants among the 1382 patients with BC with strong family history and/or young age at BC diagnosis (<35 years) tested was 31.5% (436/1382). In total, 446 LoF variants have been identified in 436 individuals in 28 genes and more specifically, *BRCA1*, *BRCA2*, *ATM*, *BLM*, *BRIP1*, *CDKN2A*, *CHEK2*, *DICER1*, *ERCC3*, *FANCC*, *FANCD2*, *FANCI*, *FANCL*, *FANCM*, *MLH1*, *MSH6*, *NBN*, *NF1*, *PALB2*, *PMS2*, *PTEN*, *RAD51C*, *RAD51D*, *RECQL4*, *SDHB*, *SDHC*, *SLX4* and *TP53*. *BRCA1/2* LoF variants dominated the variant spectra, identified in 24.8% (343/1382) of the patients tested. *BRCA1* Greek founder variants accounted for approximately half (11.4%; 158/1382) of the *BRCA1/2* variants identified. A detailed list of the variants, along with patients' age at BC diagnosis, personal and family history are illustrated in the online supplementary table 3.

A considerably high number of the tested patients (6.7%; 93/1382) harbour PVs in known, suspected or candidate BC genes, other than *BRCA1/2*. Detected mutations scatter among 26 genes with frequencies recorded as follows: 22 in *CHEK2* (1.6%), 18 in *ATM* (1.3%), 11 in *PALB2* (0.8%), eight in each of *TP53* and *RAD51C* (0.6%), four in *RAD51D* (0.3%), three in each of *PTEN*, *MSH6* and *NBN* (0.2% each), two in each of *BRIP1*, *ERCC3, FANCL*, *FANCM*, *PMS2* and *SLX4* (0.15% each) and one in each of *BLM*, *CDKN2A*, *FANCC*, *FANCD2*, *FANCI*, *RECQL4*, *DICER1*, *MLH1*, *NF1*, *SDHB* and *SDHC* (0.07%). Interestingly, the detection rate of PVs in established cancer genes alone, that is, *BRCA1*, *BRCA2*, *TP53, PTEN*, *PALB2*, *ATM* and *CHEK2* is 29.3%. Noteworthy, 16 missense variants in five genes, namely, *BRCA1*, *BRCA2*, *CHEK2, TP53* and *CDKN2A*, were classified as pathogenic, based on the detailed evaluation for variant classification, following ACMG guidelines (online supplementary table 2). A graphical illustration of the distribution of damaging variants among the 28 genes identified in this study is shown in figure 1.

Excluding *BRCA1/2* and based on current knowledge, LoF variants in genes conferring high BC risk, that is, in *PTEN*, *PALB2* and

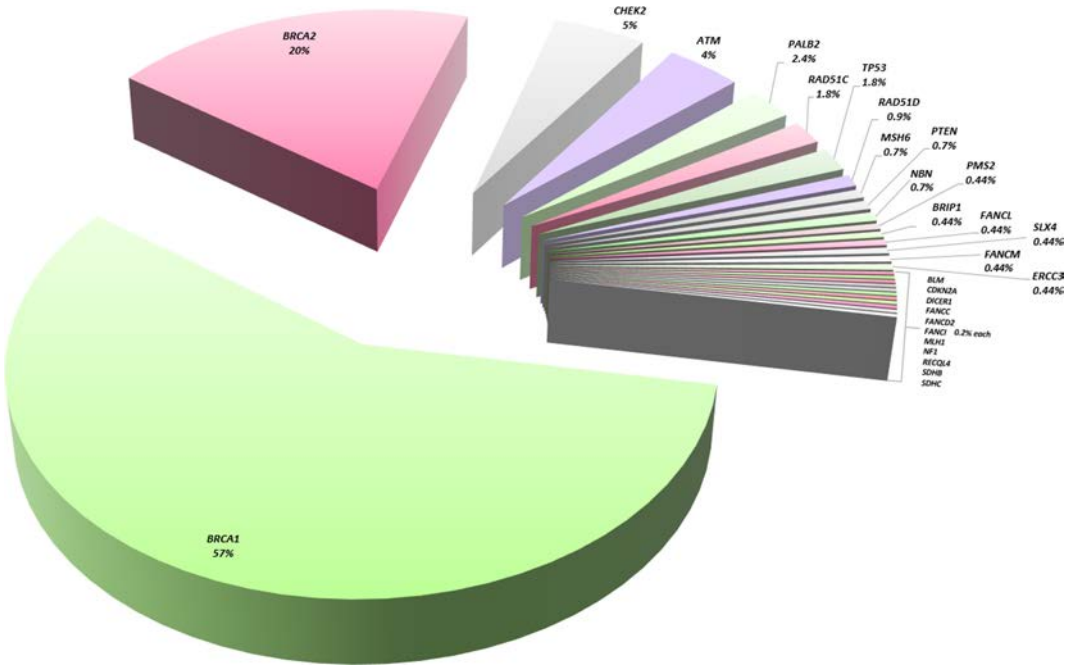

Figure 1 Distribution of loss-of-function variants, in known, candidate and suspected breast cancer predisposing genes.

TP53, were identified in 1.6% of the patients tested. Damaging variants in genes associated with moderate (*ATM* and *CHEK2*) and suspected BC risk (*SLX4*, *NBN*, *BLM*, *ERCC3*, *FANCM*, *RAD51C*, *RAD51D*, *MSH6*, *PMS2*, *MLH1* and *NF1*) were identified in 2.9% and 2.2% of the patients tested, respectively. Variants in candidate BC genes, proteins of which are either involved in the Fanconi anaemia pathway (*FANCC*, *FANCD2*, *FANCI*, *FANCL* and *BRIP1*) or in genes associated with known syndromes (*SDHB*, *SDHC*, *CDKN2A, RECQL4* and *DICER1*), were detected in 0.85% of the tested individuals (figure 2).

LGRs were evaluated through non-NGS experimentation, in four genes namely, *BRCA1*, *BRCA2*, *TP53* and *CHEK2*. In total, 5.7% (79/1382) of the patients in our cohort carried LGRs, with only 0.7% (10/1382) of them being different from the Greek

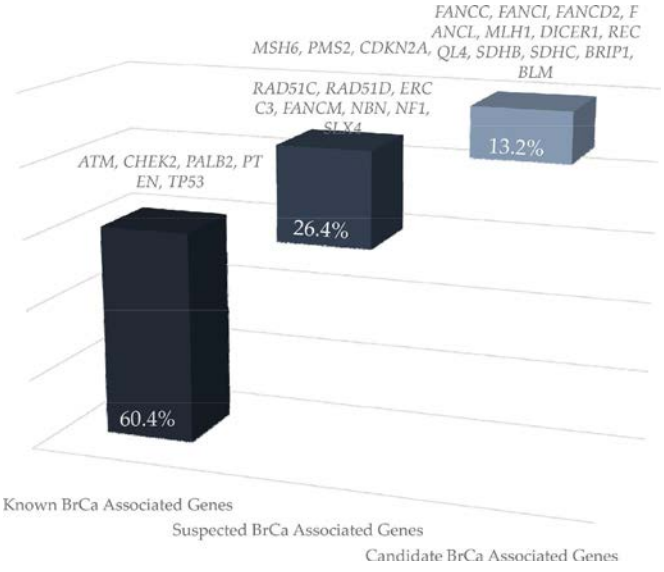

Figure 2 Loss-of-function variant distribution in known, suspected or candidate genes associated with breast cancer, other than *BRCA1* and *BRCA2*.

BRCA1 founders. These were one *BRCA1* deletion affecting the non-coding exon 1 and coding exon 2 (based on LRG_292), two *BRCA2* deletions (affecting exons 12 and 13 and exon 3 (based on LRG_293)), three novel *CHEK2* deletions (affecting promoter and exon 1, exon 6 and exons 2 and 3 of the gene (based on LRG_302)) and a *TP53* deletion of the promoter and the non-coding, exon 1 of the gene (based on LRG_321).

In addition to the LoF variants, the low-risk BC variant, *CHEK2* c.470T>C and the hypomorphic *BRCA2* missense variant, c.9104A>C, were detected in 12 and two individuals, respectively, representing a frequency of 0.9% and 0.15%, respectively.

Case–control analysis for breast cancer risk estimations

Associations between detected LoF variants per gene were compared with controls extracted from the ExAC (non-Finnish/non-TCGA) and FLOSSIES (European-American) data sets.

TP53 deleterious variants were associated with high BC risk (OR 4.79; 95% CI 1.77 to 11.2, p<0.01 and OR 11.2; 95% CI 2.87 to 75.64, p<0.01) when compared with ExAC and FLOSSIES controls, respectively. *PALB2* LoF variants were also significantly associated with high BC risk (OR 8.03; 95% CI 2.81 to 20.42, p<0.01 and OR 6.3; 95% CI 1.81 to 22.75, p<0.01) when compared with ExAC and FLOSSIES controls, respectively. Interestingly, pathogenic *RAD51C* variants showed a significant association to high BC risk (OR 6.19; 95% CI 2.23 to 15.03, p<0.01 and OR 12.6; 95% CI 2.87 to 75.64, p<0.01) when compared with ExAC and FLOSSIES controls, respectively, while *ATM* deleterious variants were significantly associated with moderate BC risk (OR 3.41; 95% CI 1.87 to 5.86, p<0.01 and OR 4.12; 95% CI 2.0 to 8.3, p<0.01) when compared with ExAC and FLOSSIES controls, respectively.

Intriguingly, the association of LoF *CHEK2* variants neither was associated with clinically relevant BC risk nor was statistically significant when compared with ExAC (OR 1.66; 95% CI 0.98 to 2.67, p=0.11), while was associated with moderate BC risk, when compared with FLOSSIES (OR 2.67; 95% CI 1.44 to 4.68, p<0.01). When performing a subanalysis that included only missense LoF *CHEK2* variants, not only reached statistical

Table 1 Prevalence and ORs for breast cancer risk of LoF variants in genes between selected Greek patients with breast cancer and reference controls from ExAC and FLOSSIES.

	Demokritos cases					Controls (ExAC/FLOSSIES)					Cancer risk (ExAC/FLOSSIES)		
Gene	Truncating	Missense	Total	Cases (n)	Freq%	Truncating	Missense	Total	Controls (n)	Freq%	OR	95% CI	P value
ATM	13	3	16	1355	1.18	78/14	15/7	93/21	26862/7325	0.35/0.29	3.41/4.12	1.87 to 5.86/2 to 8.3	6.12×10^{-5}/2.45×10^{-5}
CHEK2	7	12	19	1355	1.40	155/28	63/11	218/39	25819/7325	0.84/0.53	1.66/2.63	0.98 to 2.67/1.44 to 4.68	0.11/**0.003**
CHEK2 miss	0	12	12	1355	0.89	0/0	63/11	63/11	26974/7325	0.23/0.15	3.79/5.9	1.86 to 7.12/2.38 to 14.78	**1.2×10^{-3}/2.45×10^{-4}**
PALB2	7	0	7	1355	0.52	17/6	0/0	17/6	26435/7325	0.06/0.08	8.03/6.3	2.81 to 20.42/1.81 to 22.75	**7.16×10^{-3}/0.003**
RAD51C	5	2	7	1355	0.52	19/2	3/1	22/3	26361/7325	0.08/0.04	6.19/12.6	2.23 to 15.03/2.87 to 75.64	**0.002/4.73×10^{-4}**
TP53	2	6	7	1355	0.52	1/0	28/3	29/3	26889/7325	0.11/0.04	4.79/12.6	1.77 to 11.2/2.87 to 75.64	**0.001/4.73×10^{-4}**

CNV carriers, double LoF variant carriers and the low-risk *CHEK2* variants: p.(Ile157Thr) and p.(Ser428Phe) were excluded from the analysis. Genes that had at least seven LoF variants were included. ExAC data extracted were non-TCGA/non-Finnish. FLOSSIES data extracted were on European-Americans.
Bold numbers indicate statistically significant findings.
ExAC, Exome Aggregation Consortium;FLOSSIES, Fabulous Ladies Over Seventy; Freq, variant observed frequency;LoF, loss-of-function; NA, not applicable; TCGA, The Cancer Genome Atlas; miss, missense.

significance but also provided clinically relevant associations to moderate BC susceptibility (OR 3.79; 95% CI 1.86 to 7.12, p<0.01) when compared with ExAC and moderate to high BC susceptibility (OR 5.9; 95% CI 2.38 to 14.78, p<0.01) when compared with FLOSSIES.

The combined prevalence of LoF variants per gene (<7 variants), along with ORs for BC risk between selected Greek patients with BC patients and controls from ExAC and FLOSSIES are summarised in table 1, while a detailed table of ORs derived from the case–control study for all genes is listed in the online supplementary table 4. Therefore, ORs derived from the case–control analysis for only *CHEK2*, *ATM*, *PALB2*, *TP53* and *RAD51C* are presented on a Forest plot diagram in figure 3.

Families with damaging variants in established hereditary cancer syndromes

Highly penetrant LoF variants were identified in *TP53* (0.6%) and *PTEN* (0.2%), associated with Li-Fraumeni syndrome (LFS) and Cowden syndromes, respectively. No mutations were detected in *STK11* and *CDH1* genes known to cause Peutz-Jeghers and Hereditary Diffuse Gastric Cancer syndromes, respectively.

All individuals carrying *TP53* mutations (variant allele frequencies ranged from 46% to 51%) had very young age at diagnosis (range 20–36 years); however, ascertainment bias on age selection should be noted. Among them, 11 BC emerged, seven of which were human epidermal growth factor receptor 2 positive. Surprisingly, only 2/8 initially fulfilled the revised Chompret criteria for *TP53* genetic testing,[13] followed by two more cases, only after a family relative was diagnosed with an LFS-related cancer; therefore, half of the carriers fulfilled the criteria after later follow-up.

Among three *PTEN* carriers, only one (F975) fulfilled the testing criteria for Cowden syndrome, due to her clinical features (gastrointestinal hamartomas from young age, thyroid and endometrial cancer diagnosis). Notably, proband F1097, who is a *PTEN* carrier, also carries a *PALB2* deleterious mutation. Available family pedigrees for *TP53* and *PTEN* carriers are summarised in the online supplementary figure 1.

Moreover, six individuals (6/1382; 0.4%) harboured damaging variants in Lynch-related genes and specifically, three *MSH6*, two *PMS2* and one *MLH1* LoF variants were detected. Interestingly, two patients (1178 *MLH1* carrier and 1272 *MSH6* carrier) had family histories of both breast and gastrointestinal cancers, while patient 2514 (*PMS2* carrier) had breast and ovarian cancer and no Lynch-related cancers diagnoses among family relatives.

Individuals carrying two LoF variants

In total, 0.7% (10/1382) of the individuals tested were found to carry two LoF variants in two genes, of which three had combinations of variants both associated with high BC risk, that is, *BRCA1* and *BRCA2*, *BRCA1* and *PALB2* and *PTEN* and *PALB2* (F543, F330 and F1097, respectively). Four cases combined damaging variants conferring high BC risk with moderate breast or ovarian cancer risks, namely, *PALB2* and *ATM*, *BRCA2* and *ATM*, *BRCA2* and *RAD51C* and *PALB2* and *SLX4* (F776, F882, F2540 and F2364). Another case (F2070) had a *BRCA2* along with a *RECQL4* LoF variant, for which little evidence on BC association exists. Single cases involved combinations of *ERCC3*/*FANCC* and *BRCA2*/*SDHC* LoF variants (F2516 and F1281, respectively).

Interestingly, two patients were also diagnosed with epithelial ovarian cancer (F2070 and F2364). Since most of these gene combinations are either rare or unique, it is rather difficult to investigate whether these individuals present a more severe phenotype, when compared with single mutation carriers, while information on gene mutations can be rather useful for targeted testing and appropriate surveillance on family relatives. All the aforementioned PVs are depicted in blue colour in the online supplementary table 3, while available pedigrees are displayed in the online supplementary figure 2.

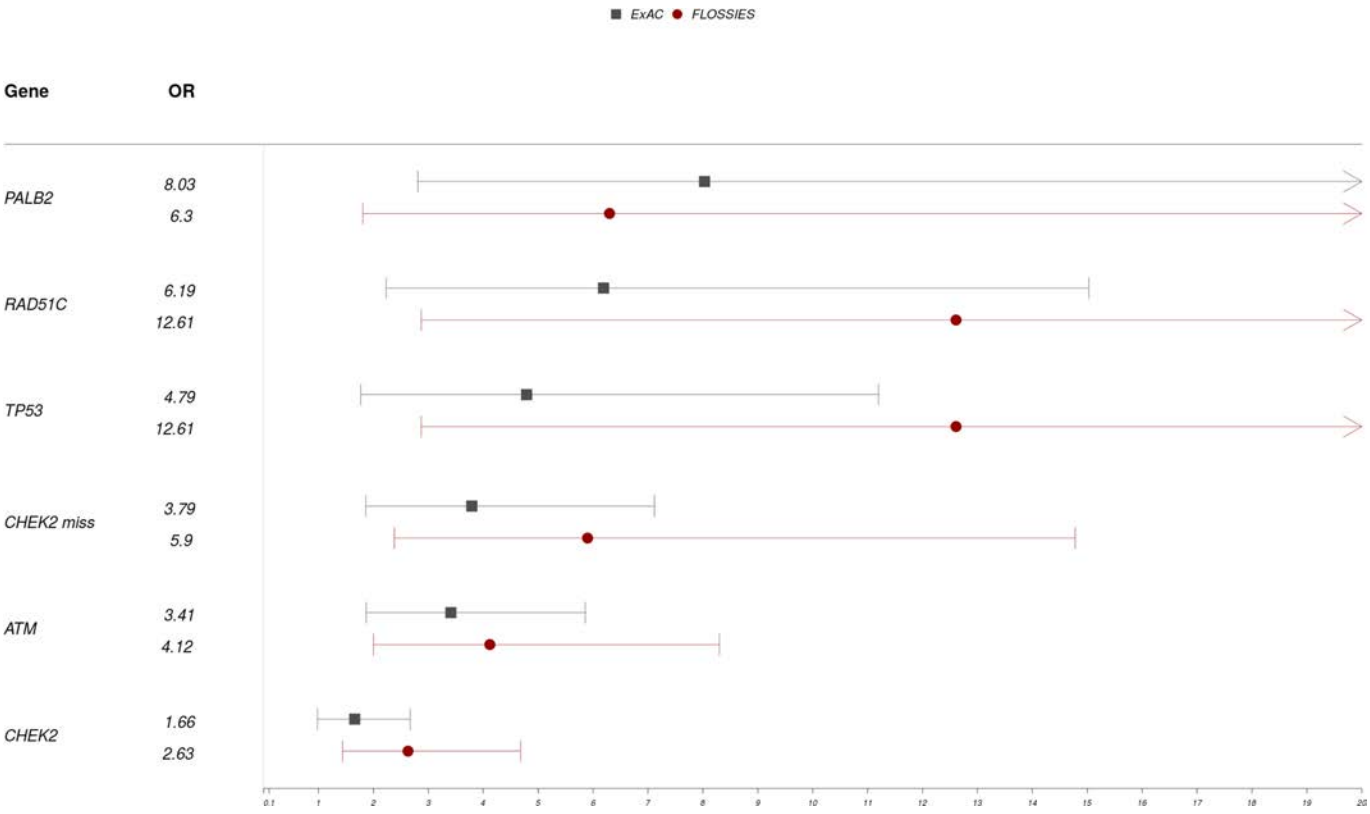

Figure 3 Forest plot illustrating OR for breast cancer between selected Greek patients with breast cancer and controls from ExAC and FLOSSIES. Notes: CNV carriers, double LoF variant carriers and the low risk CHEK2 variants: p.(Ile157Thr) and p.(Ser428Phe) were excluded from the analysis. Genes that had at least seven LoF variants were included. ExAC data extracted were non-TCGA/non-Finnish Europeans.FLOSSIES data extracted were European-Americans. ExAC, Exome Aggregation Consortium; FLOSSIES, Fabulous Ladies Over Seventy; LoF, loss of function; miss, missense; TCGA, The Cancer Genome Atlas.

Alleles in genes, other than *BRCA1* and *BRCA2*, with possible founder effect in Greek patients

During this large-scale analysis, a number of PVs were seen recurrently. This is not surprising, since the Greek population is characterised by founder effects, as already reported.[3] Four detected variants specifically, *PALB2* (c.2257C>T), *ATM* (c.1215delT), *RAD51C* (c.706-2A>C) and *CHEK2* (c.549G>C) have been encountered in four, apparently non-relative families, each, while *CHEK2* c.499G>A has been detected in three, apparently non-relative families. Although these observations suggest possible founder effect(s), additional studies are required to elucidate this.

Association of gene LoF variants with age at primary BC diagnosis, secondary cancer diagnosis and histopathology characteristics

Possible association of age at first and second primary BC diagnosis, ovarian or other cancer and triple-negative breast cancer (TNBC) diagnosis with PVs in *BRCA1*, *BRCA2*, *PALB2*, *ATM*, *CHEK2*, *TP53* and *RAD51C* were investigated. The small number of mutation carriers in other genes deterred us from pursuing further analysis.

The analysis results of the are summarised in table 2.

BRCA1 and *TP53* PVs were associated with younger age at first BC diagnosis (mean age 38.74±9.2; p<0.01 and 30.3±6.2; p=0.024, respectively). As expected, *BRCA1* and *BRCA2* carriers were more likely to develop a second primary BC (p<0.01 and p=0.036, respectively) and to be diagnosed with ovarian cancer (p<0.01 and p=0.013). LoF *BRCA1* variants were clearly associated with TNBC diagnosis (p<0.01).

TP53 mutation carriers had a statistically significant higher incidence of second primary BC tumours than non-carriers (p=0.035), while *CHEK2* mutation carriers had a significantly higher incidence of other cancer diagnosis, beyond breast or ovarian (p=0.046).

RAD51C deleterious variants seem to be associated with a second BC diagnosis, illustrated by the trend towards significance (p=0.057). Incidence of second primary tumours, other than breast or ovarian, is on the cusp of statistical significance (p=0.063) among *TP53* mutation carriers. These results indicate the need of further investigation in larger datasets.

Clinical actionability of LoF variants in other than *BRCA1* and *BRCA2*

Based on the January 2019 National Comprehensive Cancer Network (NCCN) consensus guidelines,[14] clinical management and surveillance will be modified for the majority (85/93) of the non-*BRCA1/2* mutation carriers (in alphabetical order, *ATM*, *BRIP1*, *CHEK2*, *MLH1*, *MSH6*, *NBN*, *PALB2*, *PMS2*, *PTEN*, *RAD51C*, *RAD51D*, *TP53*, *SDHB* and *SDHC*). Herein, breast and ovarian cancer surveillance could be altered in 4.5% (carriers of *PALB2*, *ATM*, *CHEK2*, *TP53*, *PTEN* and *NBN* PVs) and 1.3% (carriers of *BRIP1*, *RAD51C*, *RAD51D*, *MLH1* and *MSH6* PVs), respectively. Cancer surveillance for other sites (colon, endometrium, abdomen, thyroid) could be modified in 2.96% (carriers of *CHEK2*, *TP53*, *PTEN*, *PMS2*, *MLH1*, *MSH6*, *SDHB* and *SDHC* PVs) of the initial cohort. A fundamental aspect of this study was the possibility for targeted testing of the familial mutation in 114 family relatives of the aforementioned

Table 2 Association of age at primary breast cancer diagnosis, incidence of secondary cancer diagnosis and histopathology with *BRCA1*, *BRCA2*, *PALB2*, *ATM*, *CHEK2*, *TP53* and *RAD51C* LoF variants

		Non-carriers (n=931)	*BRCA1* (n=254)	*BRCA2* (n=81)	*PALB2* (n=11)	*ATM* (n=18)	*CHEK2* (n=25)	*TP53* (n=12)	*RAD51C* (n=7)
Mean age at first BrCa Dx	Mean age±SD	41.49±10.9	38.74±9.2	40.95±9.1	41.36±10.1	42.67±8.5	42.36±8.9	30.3±6.2	43.86±7.9
	P value	–	**0.00084**	0.9253	0.8932	0.3004	0.4179	**0.02391**	0.3432
Second BrCa Dx	No	134	60	19	2	5	6	6	3
	Frequency, %	14.39	23.62	23.46	18.18	27.78	24.00	50.00	42.86
	P value	–	**0.001499**	**0.03598**	1	0.17	0.242	**0.03498**	0.05747
OvCa Dx	No	26	45	7	0	0	0	1	1
	Frequency, %	2.79	17.72	8.64	0.00	0.00	0.00	8.33	14.28
	P value	–	**0.0004998**	**0.01349**	1	1	0.6402	0.2634	0.4253
Other Cancer Dx	No	28	11	2	0	2	3	2	0
	Frequency, %	3.00	4.33	2.47	0.00	11.11	12.00	16.66	0.00
	P value	–	0.3273	1	1	0.11	**0.04648**	0.063	1
		Non-carriers (n=931)	*BRCA1* (n=254)	*BRCA2* (n=81)	*PALB2* (n=8)	*ATM* (n=16)	*CHEK2* (n=22)	*TP53* (n=10)	*RAD51C* (n=7)
TNBC	No	151	169	9	2	1	1	2	3
	Frequency, %	16.22	66.54	11.11	25	6.25	4.55	20.00	42.86
	P value	–	**0.0004998**	0.2509	0.6062	0.3408	0.1719	1	0.09495

All double mutation carriers have been excluded from the analysis; for PALB2, ATM, CHEK2, TP53 and RAD51C, available data from family relatives carrying LoF alleles have been included in the analysis.
Bold numbers indicate statistically significant findings.
Dx, diagnosis;LoF, loss-of-function; TNBC, triple-negative breast cancer.

mutations carriers, in genes beyond *BRCA1/2;* of which, 42.9% (49/114) tested positive for the familial PV.

Taken together, 6.1% of the individuals included in this study, along with 49 of their family relatives, would be given the opportunity to adjust their clinical management. This is in addition to *BRCA1/2* carriers, who already receive tailored management strategies. Detailed numbers of tested individuals, along with relevant surveillance guidelines per gene are summarised in table 3.

DISCUSSION

This is an original study questioning an extensive number of genes, known to be implicated in cancer predisposition and DNA repair, and their possible association to BC predisposition, in a highly selected cohort of Greek patients with BC. Through this approach, integrated knowledge on BC susceptibility is acquired; from providing a spectrum of variants in known genes, to proposing novel BC candidates, while providing estimates on BC risks.

Among 1382 patients with very young BC diagnosis (<35 years) and/or strong family history, 31.5% harboured LoF variants in at least one gene. Other than *BRCA1/2*, 4.5% of patients carried variants in genes conferring high and moderate BC risk, that is, *CHEK2*, *ATM*, *TP53* and *PALB2*, with detected frequencies being in line with previous studies.[15–17]

For the first time herein, *RAD51C* LoF variants show a clear association to BC predisposition, irrespectively of breast histology subtype, while a trend towards secondary BC diagnosis was observed. Damaging *RAD51C* and *RAD51D* variants are known to confer elevated ovarian cancer risks, but their association to BC susceptibility is often disputed. *RAD51D* LoF variants have been associated with moderate BC susceptibility in several

Table 3 Clinical actionability by site of loss-of-function variants in genes other than *BRCA1 and BRCA2*

Gene	Total no. of carriers	Relatives tested (n)(carriers)	Breast cancer surveillance	Ovarian cancer surveillance	Other cancer surveillance
CHEK2	**43**	**21** (**9**)	Yes	No increased risk	Yes; colon
PALB2	**23**	**29** (**12**)	Yes	Insufficient evidence	Insufficient evidence
ATM	**25**	**21** (**7**)	Yes	No increased risk	Insufficient evidence for PanCa/PrCa
TP53	**20**	**12** (**6**)	Yes	No increased risk	Yes; multiple sites
RAD51C	**10**	**2** (**2**)	Insufficient evidence	Yes	Insufficient evidence
NBN	**6**	**6** (**3**)	Yes	Insufficient evidence	Insufficient evidence
PTEN	**4**	**4** (**1**)	Yes	No increased risk	Yes; endometrium, thyroid, colon
RAD51D	**4**	**2** (**0**)	Insufficient evidence	Yes	Insufficient evidence
PMS2	**4**	**4** (**2**)	Insufficient evidence	Low/no increased risk	Yes; colon, endometrium,
MSH6	**3**	**2** (**0**)	Insufficient evidence	Yes	Yes; endometrium, gastrointestinal tract
BRIP1	**3**	**6** (**1**)	No increased risk	Yes	Insufficient evidence
MLH1	**1**	**1** (**0**)	Insufficient evidence	Yes	Yes; endometrium, gastrointestinal tract
SDHB/C	**2**	**1** (**0**)	Insufficient evidence	No increased risk	Yes; paragangliomas
Total	**148**	**111**	**121**	**21**	**77**
			Out of which 65 index	Out of which 18 index	Out of which 41 index

Bold numbers indicate statistically significant findings.

studies, even though the number of carriers was limited, while *RAD51C* damaging variants did not meet statistical significance for association.[16 18–20] Quite recently though, an association of both *RAD51C* and *RAD51D* PVs to TNBC diagnosis specifically, was reported.[21] The association observed in our study can be possibly attributed to the strict patient selection, but also to the relatively large number of *RAD51C* damaging variants identified, possibly due to a Greek founder effect. If this is further validated, it will be of clinical importance for the appropriate follow-up and management of these individuals.

Primarily, a statistically significant association of *CHEK2* missense LoF variants and moderate BC susceptibility, arose. This observation might be due to our population-specific elevated frequency of *CHEK2* damaging missense variants, thus allowing statistical associations, while we have been able to evaluate the effect of these missense variants through the implementation of a functional assay.[22] On the contrary, when comparing altogether *CHEK2* LoF variants, with controls, the association was marginally moderate, and was in compliance with previous reports.[16 18 19]

We have been able to replicate the established associations of *BRCA1* and *TP53* LoF variants to early age and second primary BC diagnosis.[18 23] Interestingly, second primary tumour diagnosis, beyond breast or ovarian cancer, was found to be associated with the presence of *CHEK2* LoF variants. *CHEK2* variants have been identified among young individuals diagnosed with colorectal cancer[24] and have been previously associated with increased colorectal cancer risk.[25] Moreover, prostate[26] and gastric cancer risk[27] seems to be elevated among *CHEK2* carriers. Studies like the current assist in defining these important associations, since although colon surveillance is proposed by NCCN guidelines for *CHEK2* carriers, evidence for other cancer-specific risks is limited.[14]

Mutations in the 'syndromic' cancer genes, *TP53*, *PTEN*, *STK11* and *CDH1* were observed at considerably variable frequencies, corresponding to the extreme low end of the prevalence reported in most studies.[17 28] Herein, no *CDH1* or *STK11* damaging alleles were identified, while the prevalence of *PTEN* and *TP53* mutations was 0.2% and 0.6%, respectively, mainly attributed to the prominent syndromic features that are frequently associated with mutations in these genes, allowing direct single gene testing.[13]

A very important observation that emerged though, involved the weakness of the criteria for LFS genetic testing. In our series, only half of *TP53* carriers fulfilled the revised Chompret criteria at diagnosis. As already indicated, testing for LFS based solely on the Chompret criteria will miss a significant number of *TP53* mutation carriers,[29 30] leading to severe implications for proper patient management, that is, avoidance of radiation due to increased risk for secondary radiation-induced malignancies. It is very likely that a rare, differential or divergent or attenuated LFS subgroup, characterised by early-onset BC, exists and does not fit to the typical LFS phenotype of cancer diagnoses at a very young age, while as recently suggested,[30] the LFS phenotypic spectrum will be fully uncovered in the near future. Based on these, LFS testing criteria should be amended to recommend *TP53* genetic testing in all female patients with BC diagnosed <35 years.

An important aspect of this study lies within the clinical translation of the produced results, an issue that is on the spotlight since the incorporation of panel testing in clinical setting. Beyond *BRCA1/2*, the vast majority (91.3%) of the identified carriers, will be offered cancer screening and/or preventive measures, based on current gene-based NCCN guidelines.[14] More specifically, a third of the mutation carriers can receive tailored recommendations for multiorgan cancer screening following established guidelines, that is, *TP53*, *PALB2*, *PTEN*, *MLH1*, *MSH6, PMS2, SDHB* and *SDHC,* even in the absence of gene-specific phenotypic characteristics. The benefit of these findings is augmented through the additional testing of family relatives, of which herein, 49 carried the familial mutation and would be therefore given the opportunity to receive more appropriate clinical management.

Noteworthy, 2% of patients in our cohort carried deleterious variants in genes that have been previously suspected to be associated with BC predisposition, while LoF variants in candidate BC genes, either involved in DNA damage repair through the Fanconi anaemia pathway or associated with other known cancer syndromes, were identified in 0.8% of patients in our cohort. Of these, LoF variants in *ERRC3*[31] and *FANCM*[32] stand out, since they have been recently associated with BC predisposition. These data, due to the rarity of these variants, should be handled with caution though and their possible association to BC should be interrogated through large case–control studies.

This study is limited to patients with BC of Greek descent, with the cancer risk estimations deriving from comparisons with selected controls from the ExAC and the FLOSSIES data set, which can provide legitimate estimates for the purposes of this study, but do not derive from a balanced case–control study. Another limitation of our study involves the low number of variants in multiple genes, a fact that does not provide the adequate statistical power to draw safe conclusions on whether a LoF variant of a gene can be associated with BC. Moreover, although the gene panel implemented herein is quite extensive, *BARD1*, PVs of which have been associated with both breast and ovarian cancer predisposition has not been evaluated.

Overall, this study reports a high rate of cancer predisposing variants, followed by calculated risks in known and suspected BC genes, while proposing a number of novel candidates, in a strictly selected cohort of Greek patients with BC. The genetic diversity of the Greek population, simultaneously harbouring ancient founder alleles and characterised by genetic heterogeneity, can assist to BC risk estimation conferred by damaging variants in specific genes, while paving the way for novel BC associations, through the identification of population-specific alleles in additional genes.

Delineating the actual cancer risk associated with specific gene variants will ultimately lead to the development of appropriate clinical management guidelines. As such, the clinical value for both patients with cancer and healthy mutation carriers will be maximised, while moving away the scepticism regarding the clinical validity of multigene panel testing.

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