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EPIDEMIOLOGY

Germline deleterious mutations in genes other than *BRCA2* are infrequent in male breast cancer

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Abstract

Purpose Male breast cancer (MBC) is a rare cancer entity, with mutations in *BRCA1* and *BRCA2 genes* accounting for ~ 10% of patients. Multiple-gene sequencing has already entered clinical practice for female breast cancer, whereas the performance of panel testing in MBC has not been studied extensively. Therefore, the aim of this study was to evaluate the clinical utility of panel testing for MBC, by the largest gene panel used so far, through investigation of patients deriving from a population with known founder effects.

Methods Genomic DNA from one hundred and two Greek MBC patients, unselected for age and family history, was used to prepare libraries which capture the entire coding regions of 94 cancer genes.

Results Loss-of-function (LoF) mutations were found in 12.7% of the cases, distributed in six genes: *BRCA2, ATM, BRCA1, CHEK2, PMS2,* and *FANCL. BRCA2* mutations were the most frequent, followed by *ATM* mutations, accounting for 6.9 and 2%, respectively, while mutations in other genes were detected in single cases. Age at diagnosis or family history was not predictive of mutation status. Beyond mutations in established breast cancer predisposing genes, LoF mutations in *PMS2* and *FANCL* among MBC patients are reported here for the first time.

Conclusions Our findings, using the largest gene panel for MBC patients so far, indicate that *BRCA* testing should be the primary concern for MBC patients. Until sufficient evidence arises from larger studies, multiple-gene panels may be of limited benefit for MBC and their families, at least for MBC patients of specific descent.

Keywords Male breast cancer · BRCA1 · BRCA2 · NGS · Hereditary cancer

Introduction

Male breast cancer (MBC) is a rare cancer entity, representing just 1% of all breast cancer cases [1, 2] and with the tendency to be diagnosed at an advanced stage, in part due to lack of awareness. Dedicated research studies on male breast cancer are limited, mainly due to the rarity of the disease, and therefore, MBC is currently being treated based on the knowledge extrapolated from female breast cancer (FMB). Quite recently, a large set of MBC cases was retrospectively centrally analyzed, showing that breast cancers arising in men are usually ER-, PR-, and AR-positive, Luminal B-like/HER2-negative [3].

MBC risk increases with hormonal abnormalities and advanced age, as well as with the number of first-degree relatives diagnosed with breast cancer [4]. More importantly, cancer-predisposing mutations, specifically in *BRCA1* and

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BRCA2 genes, have been long-standing the key genetic risk factor for MBC diagnosis. Loss-of-function (LoF) mutations in *BRCA1* and *BRCA2* genes confer cumulative lifetime MBC risks of 1–5 and 5–0%, respectively, with *BRCA2* mutations occurring more frequently [5, 6].

The prevalence of mutations in MBC in the post-BRCA era has been mainly assessed by individual efforts, with LoF mutations in *PALB2* and *CHEK2* mainly been reported. Specifically, through population-specific studies, the *CHEK2* c.1100delC mutation was linked to tenfold increased risk for MBC [7, 8], while *PALB2* mutations are reported to increase MBC risk by eight times [9]. In addition to that, males carrying LoF mutations in the syndromic genes *NF1* and *PTEN* have been reported to be at increased risk for MBC diagnosis, but these studies are small and have ascertainment bias [10, 11].

The evolution of sequencing technologies, along with the implementation of panel testing in clinical practice, has enabled the identification of multiple breast cancer-predisposing mutations in additional genes, the majority of which involved female breast cancer (FBC) cases. Depending on the stringency of the selection criteria, mutation in genes other than *BRCA1* and *BRCA2* can account for 4–11% of FBC cases [12–15].

On the contrary, data deriving from comprehensive testing among MBC cases are limited, with the recent report by Pritzlaff et al., being the largest published, so far [16]. Through utilization of various gene panels, pathogenic or likely pathogenic variants were identified in sixteen genes in 18.1% of MBC patients, of various ethnicities, tested. The great majority of variants (12.3%) lied within *BRCA1* and *BRCA2*, while *CHEK2* was the second more frequent gene, detected in 4.1% of the cases.

To better understand the contribution and association of mutations in cancer genes to MBC, we interrogated a cohort of 102 Greek MBC patients, unselected for family history or age at diagnosis, by the largest tested so far, comprehensive gene panel that includes 94 cancer genes. The aim of this study was to evaluate the clinical utility of implementation of panel testing, at least for some genes, among MBC cases through investigation of patients deriving from a specific population with known founder effects.

Patients and methods

Patient cohort

One hundred and two male individuals diagnosed with breast cancer and treated mainly at Papageorgiou Hospital of Thessaloniki and the University Hospital of Heraklion, both in Greece, were invited to participate and to donate their biological material for future research purposes. Patients were unselected for family history and age at diagnosis. The study was approved by the Bioethics committee of NCSR "Demokritos," as well as both hospitals' ethic committees, in agreement with the 1975 Helsinki statement, revised in 1983. Genetic counseling was mandatory prior to genetic testing, and written informed consent was obtained from all individuals before performing genetic analysis.

Analysis of 94 cancer genes through next-generation sequencing

Genomic DNA, extracted from whole blood using the salt-extraction procedure [17], was used to prepare indexed libraries to target the sequence of 94 cancer predisposing genes using the Illumina Trusight Cancer Panel and was sequenced on a MiSeq analyzer (Illumina, San Diego, USA). FASTQ, BAM, and VCF files were produced through Basespace (Illumina, San Diego, USA). The minimum base and amplicon coverage was 50× and 100× respectively. All called variants of interest were confirmed by Sanger sequencing.

Variant annotation and classification

Called variants were annotated by VariantStudio version 3 (Illumina, San Diego, USA) against the human reference genome GRCh38. Variants were filtered and classified based on the recommendations published by American College of Medical Genetics and Genomics (ACMG) [18]. More specifically, all variants considered of unknown significance (VUS), with minor allele frequency (MAF) lower than 1%, have been assessed for their pathogenicity with the use of five in silico software (Align-GVGD, SIFT, PolyPhen, Mutation Taster and PhastCons). Therefore, the evolutionary amino acid conservation, biochemical and transactivation consequence of the amino acid change, as well as the effect on canonical splicing have been interrogated, while testing by functional assays has been also considered. Based on the data collected from all the aforementioned methods, each VUS has been categorized with a scale 1–5, with 1 and 5 can be considered polymorphic and pathogenic, respectively.

Multiplex ligation-dependent probe amplification (MLPA)

All samples were subsequently tested by MLPA for the detection of large genomic rearrangements. Specifically, SALSA MLPA KIT P002 and SALSA MLPA KIT P090 (MRC-Holland, Netherlands) were used for the *BRCA1 and BRCA2* genes, respectively, according to the manufacturer's instructions.

Results

Patient characteristics

This study cohort included 102 MBC cases (mean age at diagnosis: 62.8 ± 12 years). Among them, a small proportion (16/102; 15.6%) was diagnosed at a young age (< 50 years). Histology reports were available for eighty-two individuals; information on histology type, hormone receptors, and HER2 status was extrapolated. The majority of tumors (73/82; 89%) were estrogen receptor-positive; three cases were triple negative (3/82; 3.65%), while 14.6% (12/83) showed HER2 amplification. The most common histology type was ductal (80/82; 97.5%, of which two in situ diagnoses), with other types being rare (papillary and lobular, seen once and twice, respectively).

In total, 14.7% (15/102) of the patients were diagnosed with a second primary cancer, of which gastrointestinal malignancies (colorectal and duodenal cancer), represented one-third of them. Other cancer types involved are prostate, thyroid, pancreatic, bladder, laryngeal, and non-Hodgkin's lymphoma. Interestingly, two of these patients were diagnosed with a metachronous breast cancer.

Mutation prevalence

Definitely pathogenic mutations were identified in 12.7% (13/102) of the patients tested, distributed in six cancer susceptibility genes. *BRCA2* was the most commonly mutated gene, accounting for more than half of the mutations identified (7/102; 6.9%), followed by *ATM* (2/102; 1.96%). LoF mutations in all other genes (*BRCA1*, *CHEK2, PMS2,* and *FANCL*) were single events. The mutation distribution is illustrated in Fig. 1.

Two individuals (2/102; 1.96%) were found to carry a *MUTYH* monoallelic variant (p.Arg245His) and an *APC* allele p.Ile1307Lys, respectively, which are both associated with slight increase in lifetime colorectal cancer risk. Pathogenic variants in *PALB2, NF1,* and *PTEN* genes, which have been previously associated with MBC, have not been detected during this study. All definitely pathogenic mutations identified during this study, along with age at diagnosis, available clinicopathological data, and family history, are summarized in Table 1.

Tumor pathology and characteristics of mutation carriers

Among the thirteen MBC carriers, the histology subtypes were ductal (10/12 available; 83.3%), lobular and papillary (1/12 available; 8.3%, each). The clinical subtypes included ten (90.9%) luminal and one (9.1%) HER2-positive breast cancers.

Additional characteristics of mutation carriers

The median age of breast cancer diagnosis in mutation carriers was 61.3 ± 11.3 years, which was not statistical significantly different to that of non-carriers (63 ± 12.1 years; $p = 0.64$). Second primary cancer diagnosis was reported in two cases, pancreatic cancer and multiple myeloma, both in *BRCA2* carriers. Strong family history was reported in three cases, again all of which carried *BRCA2* mutations, while four cases reported at least one family relative to breast cancer diagnosis. Selected pedigrees are illustrated in Fig. 2.

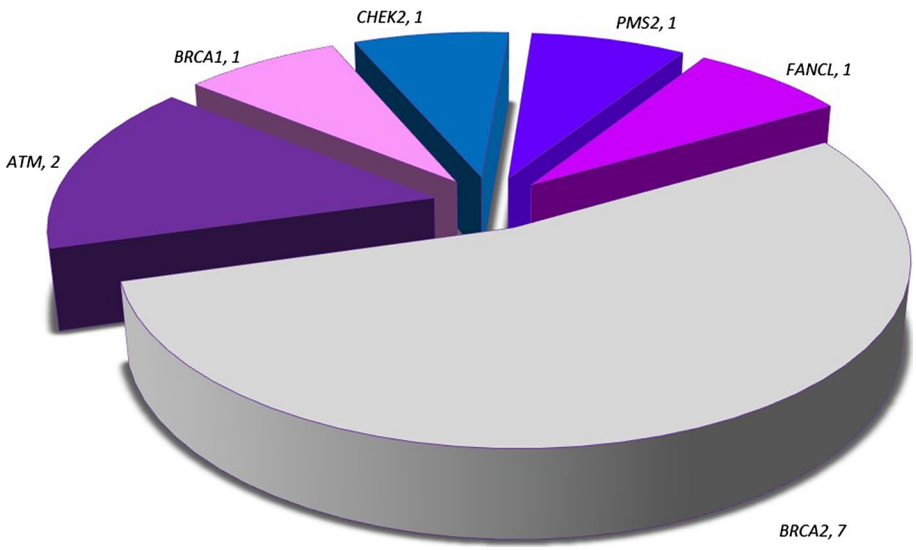

Fig. 1 Proportions of male breast cancer patients with germline loss-of-function mutations in *BRCA2, ATM, BRCA1, CHEK2, PMS2,* and *FANCL* genes

Table 1 Deleterious mutations identified in male breast cancer cases, along with tumor characteristics and details on family history

Patient number	Gene	Variant (DNA level)	Variant (protein level)	Age of onset	Histology type	Grade	ER	PR	HER2	Lymph node positivity	Other primary	Family history
1040	*BRCA2*	c.2339 C > G	p.Ser780Ter	71	Ductal	2	+	+	–	No	PanCa	1× BrCa, 2× PanCa
1346	*BRCA2*	c.8992_9025del34	p.Ser2998 fs*19	78	Ductal	3	+	+	–	No	No	no FH
630	*BRCA2*	c.9097dupA	p.Thr3033 fs*11	61	Ductal	3	+	+	–	No	No	1× BrCa
683	*BRCA2*	c.2808_2811delACAA	p.Lys936 fs*23	44	Lobular	3	+	–	–	Yes	Multiple myeloma	4× BrCa
144	*BRCA2*	c.1796_1800delCTTAT	p.Thr598 fs*1	66	Ductal	2	+	–	NA	No	No	3× BrCa
2284	*BRCA2*	c.6275_6276delTT	p.Leu2092 fs*7	67	Ductal	NA	+	+	–	No	No	No FH
2271	*BRCA2*	c.6941delC	p.Thr2314 fs*2	67	Ductal	3	+	+	–	Yes	No	No FH
821	*BRCA1*	c.5266dupC	p.Gln1756 fs*74	55	Ductal	3	+	+	+	Yes	No	No FH
2273	*ATM*	c.1215delT	p.Asn405 fs*15	76	Ductal	3	+	+	–	No	No	No FH
1355	*ATM*	c.6679C > T	p.Arg2227Cys	53	Ductal	3	+	+	–	No	No	1× BrCa
548	*PMS2*	c.2523 G > A	p.Trp841Ter	69	Ductal	3	+	+	NA	NA	No	No FH
2648	*FANCL*	c.2 T > C	p.Met1?	51	Ductal	2	+	+	–	No	No	1× BrCa
2276	*CHEK2*	c.499G > A	p.Gly167Arg	41	Papillary	3	+	+	–	Yes	No	1× BrCa

RefSeq: *ATM*: NM_000051.3; *BRCA1*: NM_007294.3; *BRCA2*: NM_000059.3; *CHEK2*: NM_007194.3; *FANCL*: NM_001114636.1; *PMS2*: NM_000535.5

Change of clinical care based on panel test result

In total, an additional 2% of the patients tested will be offered amended surveillance protocols based on their test result. More specifically, *PMS2* and *CHEK2* carriers are potential candidates for gastrointestinal and colorectal cancer screening, respectively. This proportion can be doubled if specific guidelines are proposed for male *ATM* mutation carriers, specifically for prostate and pancreas surveillance for which at the moment there is insufficient evidence.

Variants of unknown clinical significance—variant classification

Assessment and classification have been performed for all detected variants located in gene coding regions, having MAF below 1%. Nine variants were identified in eight cancer susceptibility genes, have been considered suspicious, and/or have a potential to be associated with pathogenicity. Of these, *BRCA1* p.Gly1727Ala has been classified as likely pathogenic, due to its rarity and the aggravating predictions of all tools used. The remaining missense changes were considered as variants of unknown significance, due to the conflicting evidence that is currently available, and were seen as follows: *ATM* (2), *BRIP1* (1), *CHEK2* (1), *MLH1* (1), *MSH2* (1), *NBN* (1), and *PALB2* (1). All the above information is summarized in Table 2.

Discussion

In this study of unselected males with breast cancer, of Greek descent, 12.7% carried germline LoF alleles in six genes, previously associated with cancer predisposition. Traditionally, early age at cancer diagnosis, multiple primary cancers, and breast cancer family history are red flags for genetic testing referral. Interestingly, this does not seem to be the case for MBC. In line with previously published studies [16, 19], age at MBC diagnosis cannot be used as indicator of an underlying genetic defect. The study was designed to collect unselected MBC cases and, therefore, patient follow-up for secondary diagnosis, as well as detailed family history were not collected prior to analysis. Therefore, a limitation of our study is that statistical associations cannot be accurately performed and appropriately addressed for these cofactors.

BRCA2 mutations dominate the mutation spectrum, with a prevalence of 6.9%. Previous studies have reported *BRCA2* mutations in 4–20% of MBC patients [13, 20, 21], with the upper end most likely involving ascertainment bias due to selection based on family history and population-specific alleles. The prevalence reported as 11 and 12%, in a recent large series of MBC [16] and in an Italian multicenter study

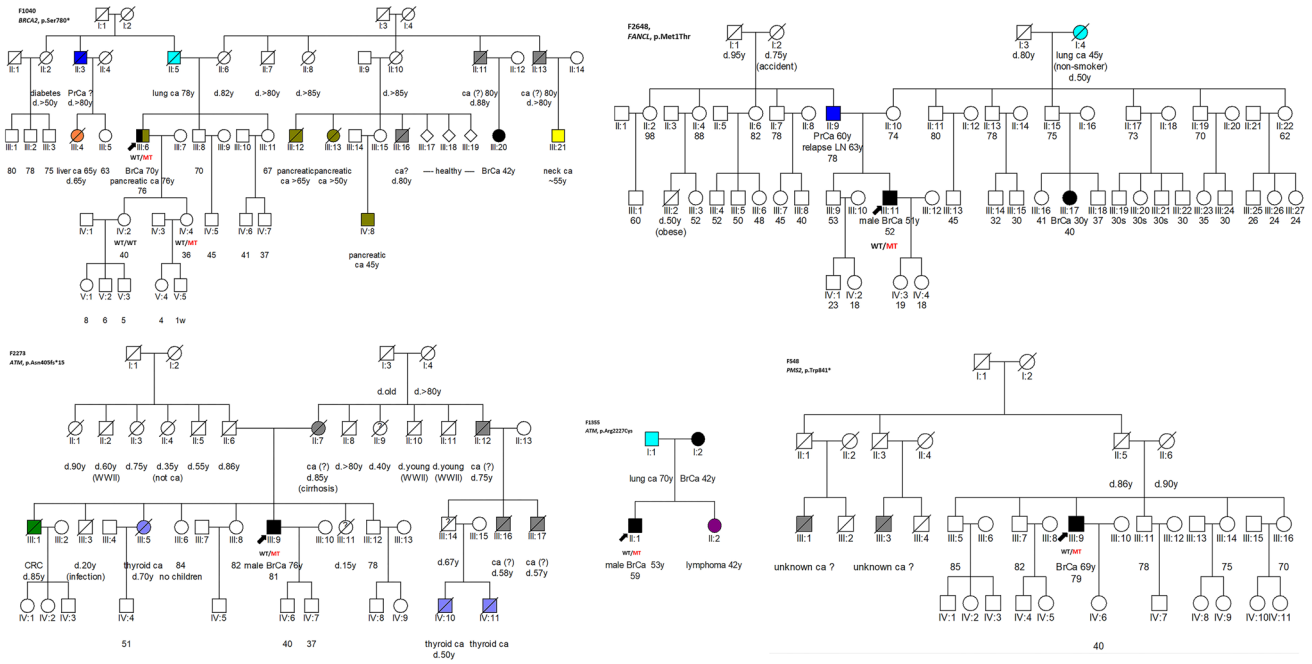

Fig. 2 Selected pedigrees of male breast cancer families, carrying LoF mutations. Probands are represented by the arrow, while breast cancer patients are colored in black. *BrCa* breast cancer, *Ca* cancer, *CRC* colorectal cancer, *PrCa* prostate cancer, *WT/MT* allele carriers of mutations, *WT/WT* wild type for the familial mutation

[22], respectively, can be considered representative for mixed populations due to a large number of patients included and is higher than ours. This can be attributed to the genetic peculiarity of Greeks, characterized by *BRCA1* strong founder effects, as shown through the extensive studies performed on female breast and ovarian cancer patients [23, 24]. Similarly, the *BRCA2* mutation rate in Finnish MBC patients, another population with strong founder effects, was reported as 7.8% [25], which is comparable to ours.

The most frequent mutations in genes other than *BRCA1* and *BRCA2* were seen in *ATM* and *CHEK2*, which have been previously associated with both MBC and FBC. In our study, *ATM* mutations were the second most prevalent, accounting for 2% of the cases. Heterozygous *ATM* mutations have been associated with two- to fourfold increased risk for FBC [26, 27], while they also seem to increase pancreatic [28] and, possibly, ovarian cancer risk [29]. While there is no evidence for elevated risk for other cancers in *ATM* heterozygotes, this is the third published report of *ATM* mutation in MBC patients. Through multigene panel testing, *ATM* mutations were detected in six MBC patients (1.5%), of which one was an ataxia telangiectasia patient and two carried a pathogenic *BRCA2* mutation [16]. Therefore, it seems that *ATM* mutations can be rare predisposing events for MBC, indicating that increased breast cancer surveillance can be proposed for male carriers, as for female carriers, based on National Comprehensive Cancer Network (NCCN) guidelines (https://www.nccn.org/).

Whether *CHEK2* mutations confer increased risk for breast cancer is in the spotlight for many years. Clear associations and risks mostly derive from data on c.1100delC mutation, which is associated with a fourfold increased breast cancer risk in women and can be found in significant proportion among high-risk breast cancer patients in populations with founder effects. More specifically, among Finnish FBC and MBC patients, the prevalence is approximately 5 [30] and 5.9%, respectively [31]. In our series, a single *CHEK2* LoF, but not truncating, allele (p.Gly167Arg) was identified. The low prevalence of *CHEK2* mutations can be due to the rarity of c.1100delC allele in Mediterranean populations and especially, in individuals of Greek descent [32]. Quite recently, protein-truncating *CHEK2* variants were associated with a 3.8-fold increased MBC risk and identified in ~ 2% of the MBC patients studied, indicating a clear association among *CHEK2* truncating variants and MBC [16]. On the contrary, association with other LoF *CHEK2* alleles is possible but questionable, mainly due to small number of carriers, and requires further investigation.

Interestingly, LoF mutations in two genes, which encode for proteins that are involved in DNA repair, and more specifically FANCL (Fanconi anemia pathway) and PMS2 (mismatch repair (MMR) pathway), that are not established predisposing breast cancer genes, have been identified. To the best of our knowledge, this is the first report of inactivating mutations among MBC patients. Specifically, the change in the initiator codon of *FANCL*

Table 2 Variants of unclassified significance along with the results of the in silico tools used and the available data on functional assays

Patient number	Gene	Variant (DNA level)	Variant (protein level)	GVGD	Sift	Polyphen	Mutation taster	Conservation (phastCons)	Clinical significance ClinVar	class
1583	*ATM*	c.6503C > T	p.Ser2168Leu	C25	Damaging	Probably damaging	Disease causing	1	Uncertain significance	3
241	*ATM*	c.8187A > C	p.Gln2729His	C15	Damaging	Probably damaging	Disease causing	1	Uncertain significance	3
2275	*BRCA1*	c.5180G > C	p.Gly1727Ala	C55	Damaging	Probably damaging	Disease causing	1	–	4
1740	*BRIP1*	c.550G > T	p.Asp184Tyr	–	Damaging	Probably damaging	Disease causing	0.948	Uncertain significance	3
2276	*CHEK2*	c.1441G > T	p.Asp438Tyr	C25	Damaging	Probably damaging	Disease causing	1	Uncertain significance	3
2270	*MLH1*	c.28C > T	p.Arg10Trp	C35	Damaging	Probably damaging	Disease causing	1	–	3
2278	*MSH2*	c.681A > C	p.Arg227Ser	C65	Damaging	Possibly damaging	Disease causing	0.994	–	3
2222	*NBN*	c.511A > G	p.Ile171Val	C25	Damaging	Probably damaging	Disease causing	1	Possible low penetrance pathogenic variant	3
2283	*PALB2*	c.2720A > G	p.Glu907Gly	C0	Damaging	Probably damaging	Disease causing	0.961	Uncertain significance	3

has been detected in a MBC patient diagnosed at a relatively young age, who also had family history of breast cancer. A borderline association with increased FBC has been reported among Czech high-risk breast cancer patients carrying a four-base pair duplication in *FANCL* (c.1096_1099dupATTA) [33], located in the last exon of *FANCL*. This association was further doubted in a subsequent study on German breast cancer patients, where it was reported as a possible low-risk allele [34], mostly due to the likely hypomorphic nature of the specific allele. On the contrary, the mutation reported herein is pathogenic based on ACMG guidelines [35] and is predicted to alter normal protein FANCL production, therefore can be associated with both FBC and MBC susceptibilities.

Moreover, breast cancer predisposition conferred by mutations in MMR genes, including *PMS2,* has been questioned for long time. Quite recently, through a modified segregation analysis in a significant number of *PMS2* carriers, the standardized incidence ratio of 3.8 for FBC risk showed to be significant. Due to the low number of carriers, this cannot be a definite association, but can be taken into account, especially in families that show breast cancer clustering [36]. Consistently, through a retrospective evaluation of patients who undergone panel testing, 11.9% carried MMR mutations alone and had breast cancer diagnosis only, with *MSH6* and *PMS2* mutations being statistically significant more frequent than *MLH1* and *MSH2* mutations, suggesting a possible breast cancer predisposition [37].

Germline LoF mutations among *MLH1*, *MSH2*, *MSH6,* and *PMS2* genes are now known to confer variable lifetime cancer risks, with *PMS2* mutations proposed to be associated with an attenuated Lynch syndrome phenotype, characterized by lower penetrance, later age at diagnosis, and lower risks for extracolonic Lynch syndrome-associated cancers [37–39]. Therefore, the cancer phenotypic spectrum associated with *PMS2* mutations may be different, involving predisposition to breast cancer, as well. In the absence of a clear association of breast cancer to *PMS2* mutations, the surveillance of this *PMS2* carrier and his family relatives who will be tested positive for this mutation possibly need to be altered. Following the NCCN guidelines, gastrointestinal and gynecological surveillance is proposed for carriers, while chemoprevention by aspirin intake can be also proposed (https://www.nccn.org/). As a whole, clinical actionability will be proposed for ~ 2% of the MBC patients included in this study, based on the available evidence and current guidelines.

Interestingly, pathogenic variants in the genes *PALB2, NF1,* and *PTEN,* those have been previously reported among MBC cases [10, 11, 40], have not been identified through our study. This can be attributed to the relatively small size of our cohort, but can be indicative of the rarity or non-association of these genes with MBC pathogenesis.

In conclusion, this is an extensive evaluation of a comprehensive multicancer gene panel in a cohort of Greek MBC cases, unselected for family history or age at diagnosis. Our findings indicate that genetic testing for *BRCA2* and *BRCA1* can be sufficient for these patients, if taking into account prompt for change of care, which will be offered to only 2% of patients in our cohort beyond BRCA carriers. Although mutations in new genes that can be possibly associated with breast cancer predisposition emerged from this study, genetic events in genes, other than *BRCA2* and *BRCA1,* involve rare cases. Until the production of sufficient evidence and clinical guidelines, from larger studies, it seems that gene panel testing may be of limited benefit for MBC patients of specific descent.

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Compliance with ethical standards

Conflict of interest The authors have no disclosures/conflict of interest to declare.

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