Insight into genetic susceptibility to male breast cancer by multigene panel testing: Results from a multicenter study in Italy

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Breast cancer (BC) in men is rare and genetic predisposition is likely to play a relevant role in its etiology. Inherited mutations in *BRCA1/2* account for about 13% of all cases and additional genes that may contribute to the missing heritability need to be investigated. In our study, a well-characterized series of 523 male BC (MBC) patients from the Italian multicenter study on MBC, enriched for non-*BRCA1/2* MBC cases, was screened by a multigene custom panel of 50 cancer-associated genes. The main clinical-pathologic characteristics of MBC in pathogenic variant carriers and non-carriers were also compared. *BRCA1/2* pathogenic variants were detected in twenty patients, thus, a total of 503 non-*BRCA1/2* MBC patients were examined in our study. Twenty-seven of the non-*BRCA1/2* MBC patients were carriers of germline pathogenic variants in other genes, including two *APC* p.Ile1307Lys variant carriers and one *MUTYH* biallelic variant carrier. *PALB2* was the most frequently altered gene (1.2%) and *PALB2* pathogenic variants were significantly associated with high risk of MBC. Non-*BRCA1/2* pathogenic variant

carriers were more likely to have personal ($p = 0.0005$) and family ($p = 0.007$) history of cancer. Results of our study support a central role of *PALB2* in MBC susceptibility and show a low impact of *CHEK2* on MBC predisposition in the Italian population. Overall, our data indicate that a multigene testing approach may benefit from appropriately selected patients with implications for clinical management and counseling of MBC patients and their family members.

What's new?

While multigene panel testing for breast cancer predisposition has been performed extensively in females, its use in male breast cancer (MBC) patients has been much more limited, despite a likely role for genetic predisposition in MBC. In this multicenter study in Italy, panel testing involving 50 cancer-associated genes identified germline pathogenic variants in about 5 percent of *BRCA1/2*-negative MBC patients. In non-*BRCA1/2* MBC, the most frequently mutated genes were *PALB2* and *ATM*, with *PALB2* mutations having a major impact on MBC risk. By comparison, mutations in *CHEK2* had little impact on MBC predisposition in the Italian population.

Introduction

Breast cancer (BC) in men is a rare disease if compared to BC in women. It represents less than 1% of all BCs and less than 1% of all cancers in men.[1] The annual incidence of male BC (MBC) is estimated at less than 1 per 100,000 men.[2] In Italy about 500 men were estimated to be diagnosed with BC in 2017.[3] About 20% of MBC patients have family history of BC and more than 20% develop a second non-breast tumor,[4] thus pointing to a relevant role of the genetic component in MBC susceptibility. Inherited mutations in *BRCA1* and, more commonly, in *BRCA2* predispose to MBC and account for about 13% of all cases.[5] There is also some evidence indicating that *CHEK2* and *PALB2* germline mutations may increase MBC risk but, to date, they seem to account for a small proportion of MBC cases.[6–8] Thus, many questions still remain regarding MBC genetic susceptibility and additional genes that may contribute to the missing heritability need to be investigated.

Over the last two decades clinical genetic testing has become widespread as several genes have been associated with increased risk of BC.[9] Next-generation sequencing (NGS) technology has enabled massive parallel sequencing of multiple cancer susceptibility genes simultaneously in a large number of patients, at relatively low cost. A broad range of next-generation panels that evaluate BC- or multiple cancer-associated genes, is now available from genetic testing laboratories.[10–18] Genes frequently included in testing panels comprise high-penetrance BC genes, such as *BRCA1* and *BRCA2*, moderate/low-penetrance BC genes, such as *PALB2*, *CHEK2* and *ATM*, mismatch repair genes, such as *hMLH1* and *hMSH2*, and genes associated with hereditary cancer syndromes, such as *CDH1, PTEN, STK11* and *TP53*.

Results from multigene panel testing indicate that gene variants associated with BC risk are individually rare and this has introduced new clinical challenges, as evidence-based risk estimates for some genes, included in multigene panels, may not be available and can be significantly modified by the specific family history of BC.[10,19,20]

Thus far, multigene panel testing for BC predisposition has been extensively performed in female BC patients but rarely in MBC patients.[7,8,11] In the present study, we aimed to further examine genetic susceptibility to BC in men, analyzing a large series of Italian MBC patients, enriched for *BRCA1/2* mutation negative patients, using a custom multigene panel. Specific aims of the study were to: -expand the spectrum of MBC susceptibility genes, -assess the yield of germline pathogenic variants in *BRCA1/2* mutation negative MBC patients through multigene panel testing, -examine predictors of pathogenic variants in non-*BRCA1/2* genes.

Materials and Methods

Study population

The present study benefits from a well-characterized series of MBC cases from 13 Italian Investigator Centers, enrolled in the frame of the ongoing Italian multicenter study on MBC.[21] A total of 523 MBC cases, unselected for age at diagnosis and family history of cancer, for which enough quantity and good quality of genomic DNA were available to perform a multigene panel testing, were included in our study. Overall, the sample set included 443 MBC cases previously tested negative for *BRCA1/2* germline mutations by automated Sanger sequencing, otherwise by a combination of screening methods such as protein truncation test (PTT), single-strand conformation polymorphism (SSCP) analysis and denaturing high-performance liquid chromatography (DHPLC)[21] and 80 newly recruited MBC cases with no prior *BRCA1/2* mutation testing. All MBC cases have been characterized for the main clinical-pathologic characteristics, including: age at diagnosis, first-degree family history (FH) and personal history (PH) of cancer, tumor histological type, stage (TNM classification), grade, nodal status, estrogen and progesterone receptor (ER/PR), HER2 and Ki67/MIB1 expression, as previously described.[22] For each patient, samples of blood or DNA from peripheral blood leukocytes were collected. DNA from blood samples was extracted using ReliaPrep Blood gDNA Miniprep System (Promega,

Madison, Wisconsin, USA), according to the manufacturer's instructions.

The study was approved by Local Ethical Committee (Sapienza University of Rome, Prot. 669/17) and informed consent for using information and biological samples was obtained from all participants to the study.

Gene selection

A custom multigene panel, sequencing all exons and flanking intronic sequences of 50 cancer-associated genes (Supporting Information Table S1), was specifically designed. Genes were selected to include: 1) known high- and moderate-breast and ovarian cancer (BC/OC) susceptibility genes; 2) proposed BC/OC susceptibility genes; 3) genes associated with BC risk identified by Genome Wide Association Studies (GWASs); 4) genes associated with cancers frequently observed in families with MBC (i.e. prostate, colon, pancreatic, gastrointestinal cancers and melanoma) and with hereditary cancer syndromes.

NGS analysis

Briefly, genomic regions were prepared in paired-end libraries using the Nextera Rapid Capture Custom Enrichment kit (Illumina, San Diego, California, USA), pooled and loaded into the MiniSeq system (Illumina) for automated cluster generation, sequencing and data analysis, including variant calling. In particular, read alignment was performed using Burrows-Wheeler Aligner (BWA) software, while variant calling was performed using the Genome Analysis Toolkit (GATK). In our study, paired-end reads of 300 (150x2) base pair per sample were obtained and a minimum of 95% of the on-target regions was covered to a depth of at least 200x. Results were annotated and filtered using Illumina Variant Studio software version 2.2 against the human reference genome GRCh37.

Variant classification

Variants were classified as pathogenic/likely pathogenic (collectively termed pathogenic), or benign/likely benign, based on the published American College of Medical Genetics and Genomics (ACMG) recommendations.[23] All variants with minor allele frequency (MAF) lower than 1% and not meeting the criteria for benign and pathogenic or the criteria were contradictory, were classified as Variant of Uncertain Significance (VUS). All pathogenic variants detected by NGS were validated by double-stranded Sanger sequencing (primer sequences are available upon request). Variants were named according to Human Genome Variation Society nomenclature (HGVS, hpp://www.hgvs.org).

Statistical analysis

Case–control study, for estimation of MBC risk associated with pathogenic variants, was performed by univariate logistic regression analysis and MBC risk was assessed by the Odds Ratio (OR) and its corresponding 95% confidence interval (CI). Two independent publicly accessible datasets, the European-

American population in Exome Variant Server (EVS) dataset (evs.gs.washingron.edu/) and the Non-Finnish European (NFE) population in the Exome Aggregation Consortium (ExAC) dataset (exac.broadinstitute.org/), excluding samples from The Cancer Genome Atlas (TCGA), were used as controls for case–control association studies.

All ExAC and EVS non-PASS variants were excluded. All remaining loss-of-function (nonsense, frameshift, +/−1,2 splice) variants and any missense variant defined as pathogenic in ClinVar, were selected for analysis.

For selected genes for which significant association with high risk of MBC emerged by case–control studies using ExAc and EVS, the non-cancer, NFE male population in the Genome Aggregation Database (gnomAD) dataset (gnomad.broadinstitute.org/), was used as control for a specific case–control association study, considering only loss-of-function variants. An additional dataset including whole exome sequencing data of 300 Italian healthy male individuals,[21] was specifically interrogated for all the pathogenic variants identified in MBC cases.

Clinical history and pathologic characteristics were compared between pathogenic variant carriers and non-carriers. Fisher exact test and t-test were used where appropriate. A p value <0.05 was considered statistically significant. All statistical analyses were performed with the R software (www.r-project.org).

Results

Clinical-pathologic characteristics of MBC patients included in the study

A total of 523 MBC patients from the ongoing Italian multicenter study on MBC were included in our study. Clinical-pathologic characteristics of MBC patients are provided in Table 1. Overall, mean age at first BC diagnosis was 62 years (range 22–91 years), 87 cases (16.7%) had first-degree FH of BC/OC and 230 (44.1%) of any cancer. PH of other cancers, mostly prostate, colorectal and bladder cancer, was observed in 99 cases (18.9%). The majority of male breast tumors were invasive ductal carcinomas (83.9%), ER and PR positive (93.6% and 88.1%), HER2 negative (80.5%) and Ki67/MIB1 low (56.5%).

Multigene panel testing in MBC patients

Multigene panel testing was performed in 523 MBCs, including 80 cases with no prior BRCA1/2 testing and 443 cases previously tested negative for BRCA1/2 germline mutations. Overall, 47 MBC patients were pathogenic variant carriers (Fig. 1).

A total of 42 pathogenic variants distributed in 16 of 50 genes, including BRCA1, BRCA2, PALB2, ATM, CHEK2, RAD51D, RAD51C, NF1, BARD1, BLM, CASP8, FANCM, RAD50, APC, EPCAM and MUTYH (Supporting Information Table S2) were identified. Two patients were found to carry two pathogenic variants, including one biallelic MUTYH carrier (compound heterozygous) and one RAD51C/MUTYH carrier (double heterozygous).

Table 1. Clinical-pathologic characteristics of the 523 MBCs analyzed in our study

Characteristic[1]	No.	%
Testing history		
BRCA1/2 negative	443	84.7
No prior *BRCA1/2* testing	80	15.3
Mean age at diagnosis ± SD (range)	62.0 ± 11.9 (22–91)	
First-degree family history of BC/OC[2]		
Negative	435	83.3
Positive	87	16.7
First-degree family history of cancer		
Negative	292	55.9
Positive	230	44.1
Personal history of cancer in addition to BC		
Negative	424	81.1
Positive	99	18.9
Tumor histotype		
Invasive ductal carcinoma	375	83.9
In situ ductal carcinoma	38	8.5
Invasive lobular carcinoma	6	1.3
Medullary carcinoma	1	0.2
Other	27	6.1
TNM stage		
0–1	198	54.4
2	108	29.7
3–4	58	15.9
Histologic grade		
1	46	12.9
2	211	58.9
3	101	28.2
Lymph node status		
Negative	229	62.7
Positive	136	37.3
ER status		
Negative	26	6.4
Positive	381	93.6
PR status		
Negative	48	11.9
Positive	355	88.1
HER2 status		
Negative	260	80.5
Positive	63	19.5
Ki67/MIB1 status		
Low	179	56.5
High	138	43.5

[1]Some data for each pathologic characteristic are not available.
[2]BC: Breast Cancer, OC: Ovarian Cancer.

BRCA1/2 pathogenic variants were detected in 13 of the MBC cases with no prior *BRCA1/2* testing and in seven of MBC cases previously tested negative, for a total of 20 *BRCA1/2* pathogenic

variant carriers (Fig. 1). Overall, 503 MBC patients were negative for *BRCA1/2* pathogenic variants (from now on, called non-*BRCA1/2* MBCs).

Pathogenic variants in non-*BRCA1/2* genes were detected in four of the MBC cases with no prior *BRCA1/2* testing and in 23 of the MBC cases previously tested negative for *BRCA1/2* mutation, for a total of 27 non-*BRCA1/2* MBC patients (Fig. 1). Overall, pathogenic variants in non-*BRCA1/2* genes were detected in 5.4% (27/503) non-*BRCA1/2* MBC patients.

Among the non-*BRCA1/2* genes, *PALB2* and *ATM* were the most frequently mutated genes. In particular, of the 27 non-*BRCA1/2* MBC patients, six were *PALB2* carriers and three *ATM* carriers (Fig. 2). Overall, among the 503 non-*BRCA1/2* MBC patients, the frequency of *PALB2* pathogenic variants was 1.2% and of *ATM* pathogenic variants was 0.6%.

Among the other non-*BRCA1/2* BC/OC susceptibility genes examined, pathogenic variants of *BARD1, BLM, CHEK2, FANCM* and *RAD51D* were each detected in two (0.4%) non-*BRCA1/2* MBC patients, and pathogenic variants of *CASP8, NF1, RAD50* and *RAD51C* were each detected in one (0.2%) non-*BRCA1/2* MBC case (Fig. 2). One of the two *CHEK2* carriers had the *CHEK2* c.1100delC variant and both the two unrelated *RAD51D* carriers had the c.293delA variant (Supporting Information Table S2).

Pathogenic variants in genes not closely related to BC predisposition, including *APC, EPCAM* and *MUTYH* were also identified in non-*BRCA1/2* MBC patients (Fig. 2). In particular, two unrelated MBC cases had the *APC* c.3920T>A variant and one case had biallelic *MUTYH* c.536A>G and c.721C>T variants (Supporting Information Table S2). The MBC patient with biallelic *MUTYH* pathogenic variants had phenotypic manifestations of *MUTYH*-associated adenomatous polyposis (MAP), whereas none of the two MBC patients with *APC* c.3920T>A variant had phenotypic features associated with familial adenomatous polyposis (FAP) or had first-degree FH of FAP (Table 2). Monoallelic *MUTYH* pathogenic variants were also detected and reported in another study.[24]

No pathogenic variants were found in the other genes examined, including genes associated with hereditary cancer syndromes, such as *TP53, CDH1, PTEN* and *STK11*.

Overall, excluding the *MUTYH* biallelic variant carrier with MAP phenotype and the two *APC* c.3920T>A variant carriers, due to lower associated cancer risk,[11,25] pathogenic variants in non-*BRCA1/2* genes were detected in 4.8% (24/503) non-*BRCA1/2* MBC patients.

The majority of MBC cases who were carriers of pathogenic variants in non-*BRCA1/2* genes had a first-degree FH of a combination of cancers including BC, and PH of other cancers in addition to BC (Table 2). As expected for MBC, the vast majority of non-*BRCA1/2* MBC cases were ER+/PR+/HER2- and only one case, specifically a *FANCM* MBC case, was a triple negative (ER-/PR-/HER2-) BC (Table 2).

A total of 120 different VUS distributed in 34 of the 50 genes analyzed (Supporting Information Table S3), were
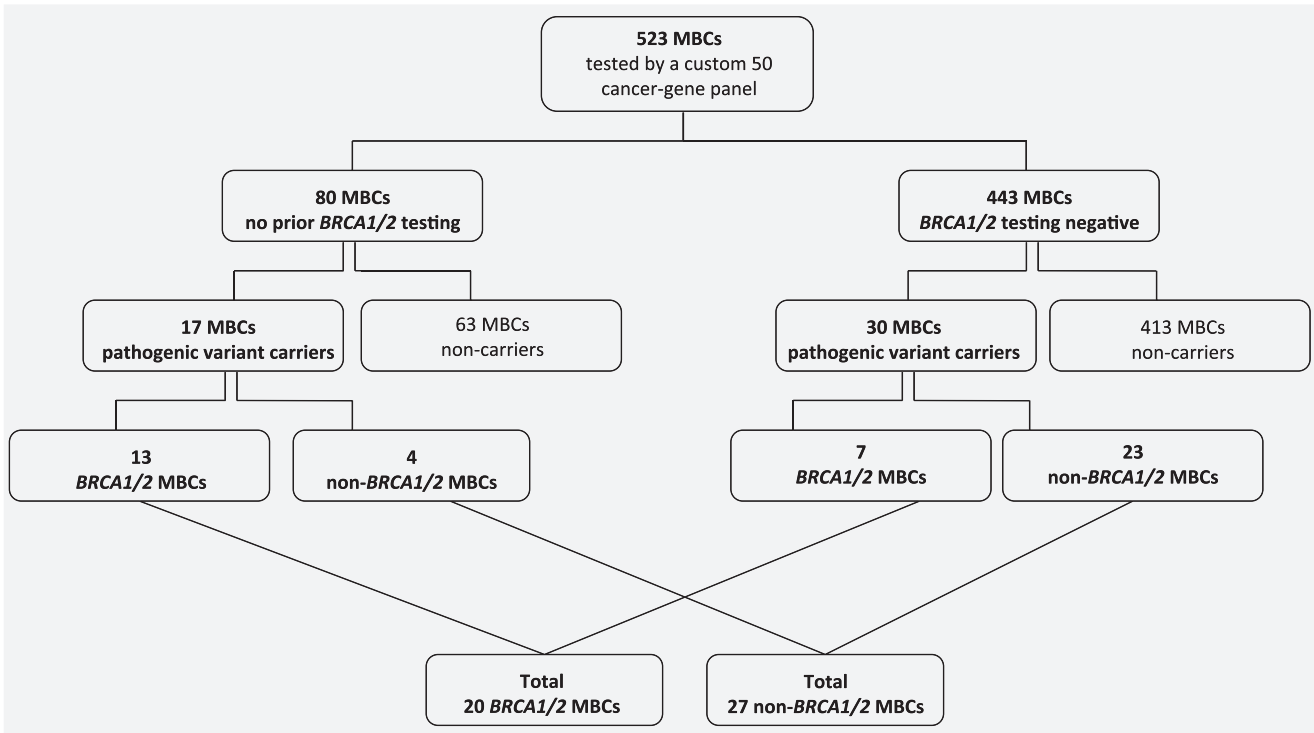
Figure 1. Diagram showing the number of MBC patients analyzed and the number of MBC patients with pathogenic variants identified in *BRCA1/2* and non-*BRCA1/2* genes.

identified in 110 of the 523 MBC patients (21%). Overall, 10 of the 110 cases with VUS harbor also pathogenic variants. The presence of two or more VUS was detected in 22/110 (20%) cases, including two pathogenic variant carriers (Supporting Information Table S4).

The majority of VUS were identified in *ATM*, *BRCA2* and *SLX4* genes and were respectively observed in 23 (4.4%), 14 (2.7%) and 12 (2.3%) of the 523 MBCs. A significant number of VUS were also found in *CHEK2* and *BLM* genes and were observed in 10 (1.9%) and 8 (1.5%) of the 523 MBCs, respectively (Supporting Information Fig. S1).
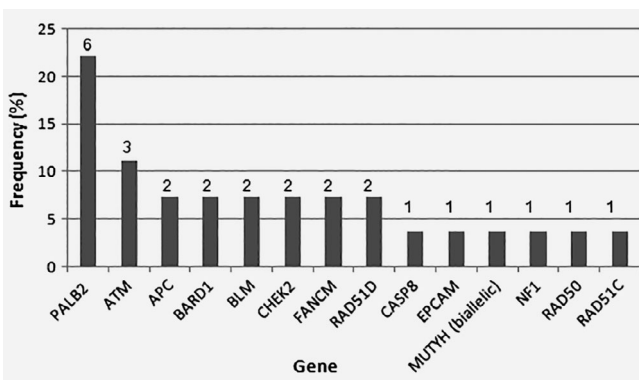

Figure 2. Distribution and frequency of pathogenic variants identified in the 27 non-*BRCA1/2* pathogenic variant carriers. The number of carriers is reported for each gene.

Gene-specific risk of MBC

Association between the pathogenic variants identified in non-*BRCA1/2* genes and MBC risk was assessed by case–control studies based on sequencing results from 503 non-*BRCA1/2* MBC patients and EVS and ExAc controls (Table 3). The cases with *MUTYH* biallelic variants and *APC* c.3920T>A variant were not included in the analyses. Variants in *PALB2* were significantly associated with high risk of MBC (EVS: OR 17.30; 95% CI: 4.31–69.36; $p < 0.0001$; ExAc: OR 11.20, 95% CI: 4.63–27.11, $p < 0.0001$). Significant association also emerged for the *RAD51D* variants and high MBC risk (EVS: OR 8.58; 95% CI: 1.21–61.4; $p = 0.01$; ExAc: OR 10.18; 95% CI: 2.22–46.58; $p = 0.0002$). The non-cancer NFE male population in the gnomAD dataset was also interrogated for the genes for which significant associations emerged. Specifically, 29,543 and 27,259 gnomAD non-cancer NFE male controls were interrogated for *PALB2* and *RAD51D*, respectively. Both the associations between variants in *PALB2* and *RAD51D* and high risk of MBC were confirmed (*PALB2*: OR 9.63, 95% CI: 4.04–22.91, $p < 0.0001$; *RAD51D*: OR 6.04, 95% CI: 1.4–26.11; $p = 0.006$). No significant associations were observed between pathogenic variants in the other genes analyzed and MBC risk. An additional dataset including whole exome sequencing data of 300 Italian healthy male individuals, was specifically interrogated for all the pathogenic variants identified in MBC cases. None of pathogenic variants identified in MBC cases was found in Italian healthy male individuals.

Cancer Genetics and Epigenetics

Table 2. Non-*BRCA1/2* pathogenic variants detected in 27 MBC cases and clinical-pathologic characteristics of carriers

Case ID	Gene	Nucleotide change	Age of onset	First-degree family history of cancer (age)	Personal history of other cancer (age)	Tumor histotype	ER	PR	HER2	Ki67/MIB1
#164	*APC*	c.3920T›A	56	Non-Hodgkin lymphoma (55)		Invasive ductal	+	+	+	−
#26	*APC*	c.3920T›A	54	Breast (40)		Invasive ductal	+	+	−	−
#318	*ATM*	c.1402_1403delAA	74		Prostate (73)	Invasive ductal	+	+	−	+
#11	*ATM*	c.1523delT	60	Breast (49, 72); Prostate (62)	Colorectal (62); Lung	Invasive ductal	+	+	−	−
#401	*ATM*	c.2151_2152insT	38	Colorectal (31)		Invasive ductal	+	+	−	+
#116	*BARD1*	c.158+1G›T	68	Breast (60); Liver (40); Chondroma (43)		Invasive ductal	+	na	na	na
#285	*BARD1*	c.1765dupG	79	Colorectal (75)	Kidney (63)	Medullary	+	+	−	+
#388	*BLM*	c.98+1G›C	63			Invasive ductal	+	+	−	+
#476	*BLM*	c.1828_1829insT	60	Prostate (71,78)		Invasive ductal	+	+	−	−
#354	*CASP8*	c.280C›T	57	Lung (75)		Invasive ductal	na	na	na	na
#199	*CHEK2*	c.1100delC	36	Prostate (70)		Invasive ductal	+	+	+	+
#363	*CHEK2*	c.1427C›T	72	Liposarcoma (49)	Melanoma (66)	Invasive ductal	+	+	−	−
#132	*EPCAM*	c.13C›T	52		Prostate (67)	Invasive ductal	+	+	−	−
#56	*FANCM*	c.1432C›T	41	Melanoma (81)		*In situ* ductal	−	−	−	na
#163	*FANCM*	c.1972C›T	55	Breast (46); Colorectal (23)	Skin (58)	Other (intracystic papillary)	+	+	na	−
#227[1]	*MUTYH*	c.536A›G; c.721C›T	51	Melanoma (26)	Colorectal (41)	Invasive ductal	+	+	−	−
#49[2]	*NF1*	c.574C›T	54	Breast (60); Condrosarcoma (58); Non-Hodgkin lymphoma (55)		Invasive ductal	−	+	na	−
#141	*PALB2*	c.419delA	76	Male breast (66); Glioma (48); Gastric (74)	Melanoma (65)	Invasive ductal	+	+	−	+
#20	*PALB2*	c.1140_1143delTCTT	38	Lung (69); Paget's Disease (30)		Invasive ductal	+	+	−	+
#523	*PALB2*	c.1984A›T	60	Breast (80)	Lung (66); Prostate (67)	Invasive ductal	−	+	na	−
#405	*PALB2*	c.2167_2168delAT	85	Breast (61); Gastric (60); Melanoma (73)		Invasive ductal	+	+	−	+
#232	*PALB2*	c.2257C›T	44	Breast (79); Colorectal (71)		Invasive ductal	+	+	+	−
#47	*PALB2*	c.3332delC	70	Breast (34, 45, 63)		Invasive ductal	na	na	na	na
#409	*RAD50*	c.1238_1241delAACT	46			Invasive ductal	+	+	−	−
#478[3]	*RAD51C*	c.905-2_905-1delAG	82	Colorectal (50)		Invasive ductal	+	+	−	−
#195	*RAD51D*	c.293delA	62		Tongue (56)	Invasive ductal	+	+	−	+
#432	*RAD51D*	c.293delA	77	Breast (52); Laringeal (50)	Sarcoma (77)	Invasive ductal	+	+	na	+

[1]Compound heterozygote.
[2]NF1 (Neurofibromatosis type 1) patient.
[3]Double heterozygote: *RAD51C*/*MUTYH* c.536A›G (p.Tyr179Cys).
na: not available; +: positive; −: negative.

Clinical-pathologic characteristics of MBC patients with and without germline pathogenic variants in non-*BRCA1/2* genes

Clinical-pathologic characteristics of carriers and non-carriers of pathogenic variants in non-*BRCA1/2* genes were compared excluding the cases with *MUTYH* biallelic variants and *APC* c.3920T>A variant. As shown in Table 4, the mean age at diagnosis was similar between pathogenic variant carriers (60.4 years, range 36–85 years) and non-carriers (62 years, range 22–91 years). Pathogenic variant carriers were more likely to have FH of cancer ($p = 0.007$). On the other hand, FH of BC/OC was noticeably lacking in the majority of pathogenic variant carriers (22/24, 91.7%). A significant association also emerged between carriers and PH of cancer besides BC ($p = 0.0005$). No statistically significant differences emerged between carriers and non-carriers with regards to tumor characteristics.

Discussion

We investigated genetic susceptibility to BC in men, analyzing a large series of Italian MBC cases through a custom multigene panel designed to include genes known and suggested to be associated with increased BC/OC risk and genes associated with cancers frequently observed in families with MBC. Despite increasing utilization of multigene panel in diagnostic testing for BC, to date, there is a limited number of studies investigating the impact of mutations in genes other than *BRCA1/2* in MBC susceptibility. One study retrospectively assessed the diagnostic yield of multigene panel testing using data from 512 MBC patients tested for 16 genes.[7] Other studies, performing multigene panel testing in MBC patients, examined a limited number of patients, ranging from 22 to 102.[8,11] In our study, we performed an extensive evaluation of

Table 3. Analysis of MBC risk associated with pathogenic variants in panel genes

| Gene | MBC cases, No. 503 | | EVS controls, No. 4,300 | | ExAC controls | | | MBC Risk[1] | |
	Mutated No.	Mutation frequency %	Mutated No.	Mutation frequency %	Mutated No.	Total No.	Mutation frequency %	MBC *vs.* EVS OR (95% CI), p[2]	MBC *vs.* ExAC OR (95% CI), p[2]
ATM	3	0.6	12	0.28	92	26,868	0.34	2.14 (0.6–7.6), 0.2	1.75 (0.55–5.5), 0.3
BARD1	1	0.2	-	-	21	26,504	0.08	-	2.50 (0.34–18.7), 0.4
BLM	2	0.4	12	0.28	47	26,470	0.18	1.43 (0.32–6.39), 0.6	2.20 (0.54–9.27), 0.3
CASP8	1	0.2	3	0.07	7	27,081	0.025	2.85 (0.3–27.48), 0.3	**7.70 (0.95–62.7), 0.02**
CHEK2	2	0.4	21	0.49	164	25,406	0.64	0.81 (0.19–3.48), 0.8	0.60 (0.15–2.48), 0.5
EPCAM	1	0.2	2	0.05	14	25,154	0.055	4.28 (0.39–47.3), 0.2	3.60 (0.5–27.26), 0.2
FANCM	2	0.4	18	0.42	174	26,479	0.66	1.07 (0.25–4.6), 0.9	0.60 (0.15–2.46), 0.5
NF1	1	0.2	4	0.09	25	26,501	0.09	2.14 (0.24–19.2), 0.5	2.11 (0.29–15.6), 0.5
PALB2	6	1.2	3	0.07	29	26,941	0.10	**17.30 (4.31–69.36), <0.0001**	**11.20 (4.63–27.11), <0.0001**
RAD50	1	0.2	12	0.28	52	26,830	0.19	0.70 (0.09–5.49), 0.7	1.03 (0.14–7.44), 0.1
RAD51C	1	0.2	-	-	31	26,774	0.11	-	1.72 (0.23–12.6), 0.6
RAD51D	2	0.4	2	0.04	10	25,309	0.04	**8.58 (1.21–61.4), 0.01**	**10.18 (2.22–46.58), 0.0002**

[1]Cancer risk was assessed by the Odds Ratio (OR) and its corresponding 95% confidence interval (CI), calculated by univariate logistic regression analysis.
[2]*p* Value <0.05 in bold text.

a large multigene panel, including 50 cancer-associated genes, in a well-characterized series of 523 MBC cases from a single Country, making this the largest collection reported to date of MBC patients all undergoing a comprehensive multigene panel testing. Furthermore, compared to previous studies, our study benefits from a large series of MBC cases with an accurate and extensive characterization for clinical and pathological data collected by a geneticist and validated by relevant sources, mainly local cancer and mortality registries.

As expected, *BRCA1/2* pathogenic variants were the most frequent mutations found in MBC patients with no prior *BRCA1/2* testing (16.3%). In particular, *BRCA2* pathogenic variants were identified in 12.5% of the cases, thus confirming the role of *BRCA2* as the key gene associated with increased risk of developing BC in men. *BRCA1/2* pathogenic variants were also detected in 1.6% of MBC cases previously tested negative for *BRCA1/2*. Specifically, all the cases detected with *BRCA1/2* pathogenic variants by NGS and previously tested and labeled negative, had been analyzed by PTT, SSCP and DHPLC. These results show that these screening methods may lead to false negatives and that NGS is more sensitive in detecting *BRCA1/2* sequence variants. Thus, our results highlight the need to re-assess patients using new NGS technologies.[26]

Among non-*BRCA1/2* genes a significant role of *PALB2* in MBC susceptibility emerged. We have previously shown that *PALB2* plays a relevant role in high-risk, non-*BRCA1/2* MBC cases.[27] In the present study, more than 1% of non-*BRCA1/2* MBC cases, unselected for FH of BC/OC had a germline *PALB2* pathogenic variant. *PALB2* pathogenic variants were frequently found in MBC patients with first-degree FH of cancers in addition to BC, suggesting that hereditary MBC does not

necessarily appear in BC/OC families only and that MBC may be instrumental in the identification of *PALB2*-like families. In our study, we also showed that pathogenic variants in *PALB2* were associated with a high risk of MBC, ranging from 9.63 to 17.30-fold increased, according to the datasets used as controls. Thus, the estimated MBC risk of *PALB2* pathogenic variants in our study population was higher than those previously reported, ranging from 6.60 to 8-fold increased risk.[7,28] Overall, these results reinforce previous evidence and extend the role of *PALB2* in MBC susceptibility, drawing attention to its relevance in MBC genetic testing.

ATM was the second most frequently altered gene in our MBC series with pathogenic variants identified in 0.6% of non-*BRCA1/2* MBC cases. These results are in line with recent studies reporting heterozygous *ATM* variants in MBC with a frequency ranging from 0.5% to 1.96%.[7,8,11] In our study, no significant association between *ATM* pathogenic variants and increased MBC risk emerged, in agreement with previous data.[7] Larger collaborative studies are needed to further estimate BC risk in men with *ATM* variants.

CHEK2 pathogenic variants were found in 0.4% of our MBC cases. Germline mutations in *CHEK2*, particularly, the *CHEK2* c.1100delC variant, increase the risk of developing MBC.[29] In our study, we found a lower frequency of *CHEK2* pathogenic variants compared to those reported in other MBC series, ranging from 1% to 9%.[7,8,11,29–31] In particular, the *CHEK2* c.1100delC variant was detected in only one case of our MBC series (0.2%). No significant association between *CHEK2* pathogenic variants and increased risk of MBC was observed. Overall, these results support our previous data indicating that *CHEK2*, and in particular the *CHEK2* c.1100delC variant, does not play a relevant role in

Cancer Genetics and Epigenetics

Table 4. Clinical-pathologic characteristics of non-*BRCA1/2* MBCs: comparison between non-*BRCA1/2* pathogenic variant carriers and non-carriers

Characteristic [1]	Non-*BRCA1/2* pathogenic variant carriers (No. 24)[2]		non-carriers (No. 476)		p-value[4]
	No.	%	No.	%	
Mean age at diagnosis ± SD (range)	60.4 ± 14.7 (36–85)		62.0 ± 11.8 (22–91)		0.6
First-degree family history of BC/OC[3]					
Negative	22	91.7	400	84.2	
Positive	2	8.3	75	15.8	0.3
First-degree family history of cancer					
Negative	7	29.2	272	57.3	
Positive	17	70.8	203	42.7	**0.007**
Personal history of cancer in addition to BC					
Negative	13	54.2	393	82.6	
Positive	11	45.8	83	17.4	**0.0005**
Tumor histotype					
Invasive ductal carcinoma	21	87.4	334	83.0	
In situ ductal carcinoma	1	4.2	36	9.0	
Invasive lobular carcinoma	0	-	6	1.5	
Medullary carcinoma	1	4.2	0	-	
Other	1	4.2	26	6.5	0.1
TNM stage					
0–1	7	50.0	184	55.3	
2	5	35.7	95	28.5	
3–4	2	14.3	54	16.2	0.9
Histologic grade					
1	2	11.8	43	13.2	
2	10	58.8	196	60.3	
3	5	29.4	86	26.5	0.9
Lymph node status					
Negative	12	70.6	209	63.0	
Positive	5	29.4	123	37.0	0.6
ER status					
Negative	3	13.6	21	5.8	
Positive	19	86.4	344	94.2	0.1
PR status					
Negative	1	4.8	43	11.9	
Positive	20	95.2	319	88.1	0.5
HER2 status					
Negative	15	88.2	233	80.3	
Positive	2	11.8	57	19.7	0.5
Ki67/MIB1 status					
Low	10	50.0	163	58.0	
High	10	50.0	118	42.0	0.5

[1]Some data for each pathologic characteristic are not available.
[2]*APC* variant carriers (No. 2) and *MUTYH* biallelic variant carrier (No. 1) were excluded from the analysis.
[3]BC: Breast Cancer, OC: Ovarian Cancer.
[4]*p* Value ‹0.05 in bold text.

BC genetic predisposition in the Italian population[32] and, in particular, in MBC.[33]

Pathogenic variants in *RAD51C* and *RAD51D* were also detected in our study. To the best of our knowledge, this is the first study reporting germline pathogenic variants of these two genes in MBC cases. To date, the role of *RAD51C* and *RAD51D* as moderate OC susceptibility genes is well-established, whereas their contribution to BC risk is less clear.[9,18] Interestingly,

Cancer Genetics and Epigenetics

we found the same variant of RAD51D (c.293delA) in two unrelated cases and indication of an association with MBC risk emerged with an estimated risk increased from 6.04 to 10.80-fold, according to the datasets used as controls. Overall, our findings may add evidence on a possible role of RAD51D as BC susceptibility gene.[14]

A pathogenic variant in NF1 was found in one MBC patient. The relationship between neurofibromatosis type 1 (NF1) and BC in women is known,[34] by contrast, the concurrent presentation of NF1 and BC in men is a very rare phenomenon. To the best of our knowledge, only five other cases of NF1 and MBC have been reported.[35–38] Thus, our results emphasize the need to perform further studies to elucidate the link between these two rare diseases, as it could improve the clinical management of patients affected by NF1. Moreover, there is evidence suggesting that pathogenic variants in NF1 may confer resistance to antiestrogen treatment in BC.[39] This can be particularly relevant in clinical management of men with BC as the vast majority of male breast tumors are hormone receptor-positive,[22,40] therefore MBC patients often receive antiestrogen therapy.

Pathogenic variants in genes proposed as BC susceptibility genes, including BARD1, BLM, CASP8, FANCM and RAD50,[9,41,42] were found in our MBC series with a frequency ranging from 0.2% to 0.4%. To date, the penetrance and the clinical spectrum associated with these genes are not well-characterized[11] and, with the exception of FANCM,[21] the impact of these genes in MBC predisposition remains largely unknown. Our findings may suggest a possible role of these genes in MBC susceptibility, however, further studies are needed to add evidence on their role in BC.

The majority of pathogenic variants identified in our study were in genes belonging to the homologous recombination (HR) mechanism functionally linked to BRCA1/2.[9] There is evidence that germline mutations in genes involved in HR mechanism, such as PALB2, ATM and RAD51C, are associated with sensitivity to PARP inhibitors (PARPi).[43,44] Overall, our results highlight the central role of HR pathway in MBC susceptibility, with a possible impact on therapeutic management of MBC patients.

Pathogenic variants in genes considered not closely related to BC predisposition, including APC, EPCAM and MUTYH, were also detected in our MBC cases. We identified the well-known colorectal cancer-associated APC c.3920T>A (p. Ile1307Lys) variant[45] in two unrelated MBC cases with no personal and family history of FAP syndrome. This variant has been reported as a candidate low penetrance BC risk gene or genetic modifier in BRCA1/2 cases.[13,25,46] Further studies are needed to elucidate if the APC p.Ile1307Lys variant can play a role as low penetrance allele in MBC susceptibility. We also identified biallelic pathogenic variants of MUTYH in a MBC patient with phenotypic manifestation of MAP. To our knowledge, this is the second MBC case reported associated to

MAP syndrome.[47] These findings suggest that MBC may be part of the tumor spectrum associated with MAP syndrome, with implication in clinical management of the patients and their relatives.

In agreement with other reports on multigene panel testing in MBC,[7,8] in our study, no pathogenic variants were found in genes associated with hereditary cancer syndromes, including TP53. TP53 pathogenic variants have been reported among women with BC, who have had panel testing, with a frequency ranging from 0.3% to 1.9%.[13,17,48] These findings, while indicating that TP53 may not play a significant role in MBC, suggest that men with clinical history suggestive of Li-Fraumeni syndrome would have had TP53 testing first[49] instead of multigene panel testing for BC, as BC often appears at older age in men than in women.

Overall, we found pathogenic variants only in a fraction of the genes analyzed, some of which not previously associated with BC risk. These results indicate that the identification of the more appropriate genes for the genomic screening of MBC patients is essential in order to develop a comprehensive and specific BC susceptibility panel.

In order to examine predictors of identifying pathogenic variants in non-BRCA1/2 genes, we compared clinical-pathologic characteristics between pathogenic variant carriers and non-carriers and showed that carriers were more likely to have PH of other cancers in addition to BC and FH of cancer, compared to non-carriers. These findings suggest that multigene testing approach may benefit from appropriately selected patients, especially those with a personal or family history of cancer, allowing for testing at-risk families. The association between the presence of non-BRCA1/2 pathogenic variants and PH and FH of cancer observed in our study needs to be further investigated in larger studies, as more intensive surveillance might be justified in carriers with important implications for clinical management of MBC patients and their family members.

The identification of non-BRCA1/2 pathogenic variants in MBC patients could guide cancer surveillance and prevention recommendations both for the affected men and their relatives. To date, National Comprehensive Cancer Network (NCCN) guidelines are only available for the clinical management of men with BRCA1/2 germline pathogenic variants.[50] On the other hand, NCCN guidelines are also available for women with germline pathogenic variants in non-BRCA1/2 genes, such as PALB2, ATM, CHEK2 and NF1.[50] Our results indicate the need to perform further collaborative studies in non-BRCA1/2 MBCs in order to provide data that may be instrumental in establishing guidelines for the clinical management of men carriers of pathogenic variants in these genes.

Although a large series of MBC cases was analyzed, the power of our study may be insufficient in order to identify smaller risk effects. Moreover, information on tumor characteristics was not available for all cases. Thus, some associations may be underestimated. Larger-scale collaborative multicenter

studies are needed to investigate any possible association with rarer variants and to provide a more precise MBC risk estimate.

In conclusion, results from our study support a central role of *PALB2* in MBC susceptibility and confirm a low impact of *CHEK2* on MBC predisposition in the Italian population. Our findings also highlight the importance of NGS panels to identify genes involved in MBC susceptibility and to better define the fraction of MBC cases due to genetic predisposition.

Author contributions
PIR and VZ: drafted the study, performed NGS and statistical analyses and interpreted the results; VS: performed statistical analyses, and interpreted the results; VV: performed Sanger sequencing validation and interpreted the results; IZ, SB, GM, AMS, MGT, AR, LV, GG, CC, DC, LC, AV, BB, JA, SM, MM, PP, PAR and DP: recruited samples and collected clinical-pathologic data; LO: conceived, designed and coordinated the study and drafted the study. All authors reviewed, edited and approved the study for publication.

Cancer Genetics and Epigenetics

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Cancer Genetics and Epigenetics