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# Familial breast cancer and DNA repair genes: Insights into known and novel susceptibility genes from the GENESIS study, and implications for multigene panel testing

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**Key words:** breast cancer, exome sequencing, multigene panel testing, variant, case-control study

**Abbreviations:** 95%CI: 95% confidence interval; BC: breast cancer; ER: estrogen receptor; HBOC: hereditary breast and ovarian cancer; IGV: integrative genomics viewer; LoF: loss-of-function variant; MAF: minor allele frequency; MV: missense variant; OR: odds ratio; P: probability value; PR: progesterone receptor; SNV: single nucleotide variant; VUS: variant of uncertain clinical significance; WES: whole-exome sequencing.

Additional Supporting Information may be found in the online version of this article.

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Pathogenic variants in *BRCA1* and *BRCA2* only explain the underlying genetic cause of about 10% of hereditary breast and ovarian cancer families. Because of cost-effectiveness, multigene panel testing is often performed even if the clinical utility of testing most of the genes remains questionable. The purpose of our study was to assess the contribution of rare, deleterious-predicted variants in DNA repair genes in familial breast cancer (BC) in a well-characterized and homogeneous population. We analyzed 113 DNA repair genes selected from either an exome sequencing or a candidate gene approach in the GENESIS study, which includes familial BC cases with no *BRCA1* or *BRCA2* mutation and having a sister with BC ( $N = 1,207$ ), and general population controls ( $N = 1,199$ ). Sequencing data were filtered for rare loss-of-function variants (LoF) and likely deleterious missense variants (MV). We confirmed associations between LoF and MV in *PALB2*, *ATM* and *CHEK2* and BC occurrence. We also identified for the first time associations between *FANCI*, *MAST1*, *POLH* and *RTEL1* and BC susceptibility. Unlike other associated genes, carriers of an *ATM* LoF had a significantly higher risk of developing BC than carriers of an *ATM* MV ( $OR_{LoF} = 17.4$  vs.  $OR_{MV} = 1.6$ ;  $p_{Het} = 0.002$ ). Hence, our approach allowed us to specify BC relative risks associated with deleterious-predicted variants in *PALB2*, *ATM* and *CHEK2* and to add *MAST1*, *POLH*, *RTEL1* and *FANCI* to the list of DNA repair genes possibly involved in BC susceptibility. We also highlight that different types of variants within the same gene can lead to different risk estimates.

#### What's new?

Pathogenic variants in *BRCA1* and *BRCA2* only explain the genetic cause of about 10% of hereditary breast and ovarian cancer families, and the clinical usefulness of testing other genes following the recent introduction of cost-effective multigene panel sequencing in diagnostics laboratories remains questionable. This large case-control study describes genetic variation in 113 DNA repair genes and specifies breast cancer relative risks associated with rare deleterious-predicted variants in *PALB2*, *ATM*, and *CHEK2*. Importantly, different types of variants within the same gene can lead to different risk estimates. The results may help improve risk prediction models and define gene-specific consensus management guidelines.

## Introduction

Women from families with multiple cases of breast or ovarian cancer have a higher risk of developing breast cancer (BC) during their lifetime than women in the general population.<sup>1</sup> Since the identification of *BRCA1* and *BRCA2* in the late 1990s, genetic testing has become a routine clinical assessment for individuals with clinical features suggestive of a hereditary breast and ovarian cancer (HBOC) predisposition. However, a pathogenic variant in one of these two genes is found in only 10% of patients attending the cancer genetics clinics.<sup>2</sup> HBOC families with no pathogenic variants in the known predisposition genes present challenges for molecular genetic diagnostics. In such families, predictive testing for healthy relatives, genetic counseling and preventive medical management are hampered.<sup>3</sup>

Paradoxically in recent years, the introduction of cost-effective multigene panel sequencing in diagnostics laboratories has led to an explosion of genetic data in cancer-prone families. Very often, 20–25 genes or more are being screened in parallel to *BRCA1* and *BRCA2* in HBOC families,<sup>4</sup> but data attributing a causative role for potentially deleterious variants identified in many of these genes are usually lacking, and precise risk estimates per variant category (e.g. loss-of-function variants (LoF) or likely deleterious missense variants (MV)) have not been determined yet.

To date, the screened genes include known cancer susceptibility genes associated with a moderate (2- to 5-fold) to high relative risk of BC (>5-fold) because of their direct or indirect functional link with *BRCA1* and *BRCA2*, such as *PALB2*,<sup>5–8</sup> *ATM*,<sup>9,10</sup> and *CHEK2*.<sup>11</sup> Other DNA repair genes, such as *RAD51* paralogs,<sup>12–14</sup> *RINT1*<sup>15</sup> and genes of the *MRE11A-RAD50-NBN* complex<sup>16</sup> are also screened although replication studies conducted in independent at-risk populations are lacking or failed to replicate initial findings.<sup>17,18</sup> The interpretation of the genetic data is also complicated. Most of the known pathogenic variants associated with BC are LoF (i.e. frameshift, stop-gain and canonical splice variants predicted to result in a truncated protein)<sup>19</sup> but a significant fraction of BC risk may be attributable to rare MV in some genes such as *ATM*, *CHEK2* and *TP53*,<sup>9,11,20</sup> and this fraction may be even bigger than that attributable to LoF.<sup>21</sup>

In the light of these issues, whether identifying a LoF or a MV in genes usually screened in parallel to *BRCA1* and *BRCA2* is clinically useful remains questionable. We therefore conducted a study to estimate BC relative risks associated with rare potentially pathogenic variants in DNA repair genes in a large well-characterized and homogeneous population.

## Participants, Material and Methods

### Study participants

The study population consisted of women participating in GENESIS (GENE SISTers), a French resource for familial BC research.<sup>22</sup> In summary, 1,721 women affected with infiltrating

**Table 1.** Distribution of cases and controls by age, by population ancestry/ethnicity and by family history of cancer

	Controls (N = 1,199)	All cases (N = 1,207)	Pseudo-incident <sup>1</sup> cases (N = 663)
Age <sup>2</sup> (years)			
30	9	0	0
31–40	61	20	13
41–50	265	269	171
51–60	429	465	250
61	435	453	229
Mean age (SE)	56.1 (0.3)	57.5 (0.3)	56.6 (0.4)
Range	25–83	31–90	31–85
Population ancestry/ Ethnicity			
Caucasian	1,176	1,152	631
Ashkenazi	0	4	4
African/Afro-Caribbean	1	3	1
Arab/Berber/Asian/Arab +Berber	4	13	6
Mixed origin	9	18	7
Unknown	9	17	14
Number of cancers in family			
Breast cancer (C50 + D05.1) <sup>3</sup>			
0	851	0	0
1	261	0	0
2	68	294	167
3	14	467	252
4	3	280	161
5	1	104	46
6	0	46	28
7	1	11	6
8	0	5	3
Ovarian cancer (C56)			
0	1,164	1,139	623
1	33	61	34
2	1	6	6
3	0	1	0
5	1	0	0
Cancers from Lynch syndrome spectrum <sup>4</sup>			
0	781	678	396
1	306	335	158
2	81	130	74
3	20	44	23
4	5	14	8
5	5	5	4
6	1	0	0
7	0	1	0

<sup>1</sup>Cases diagnosed with BC less than 5 years before enrollment in GENESIS.

<sup>2</sup>Age at diagnosis for cases and age at inclusion for controls.

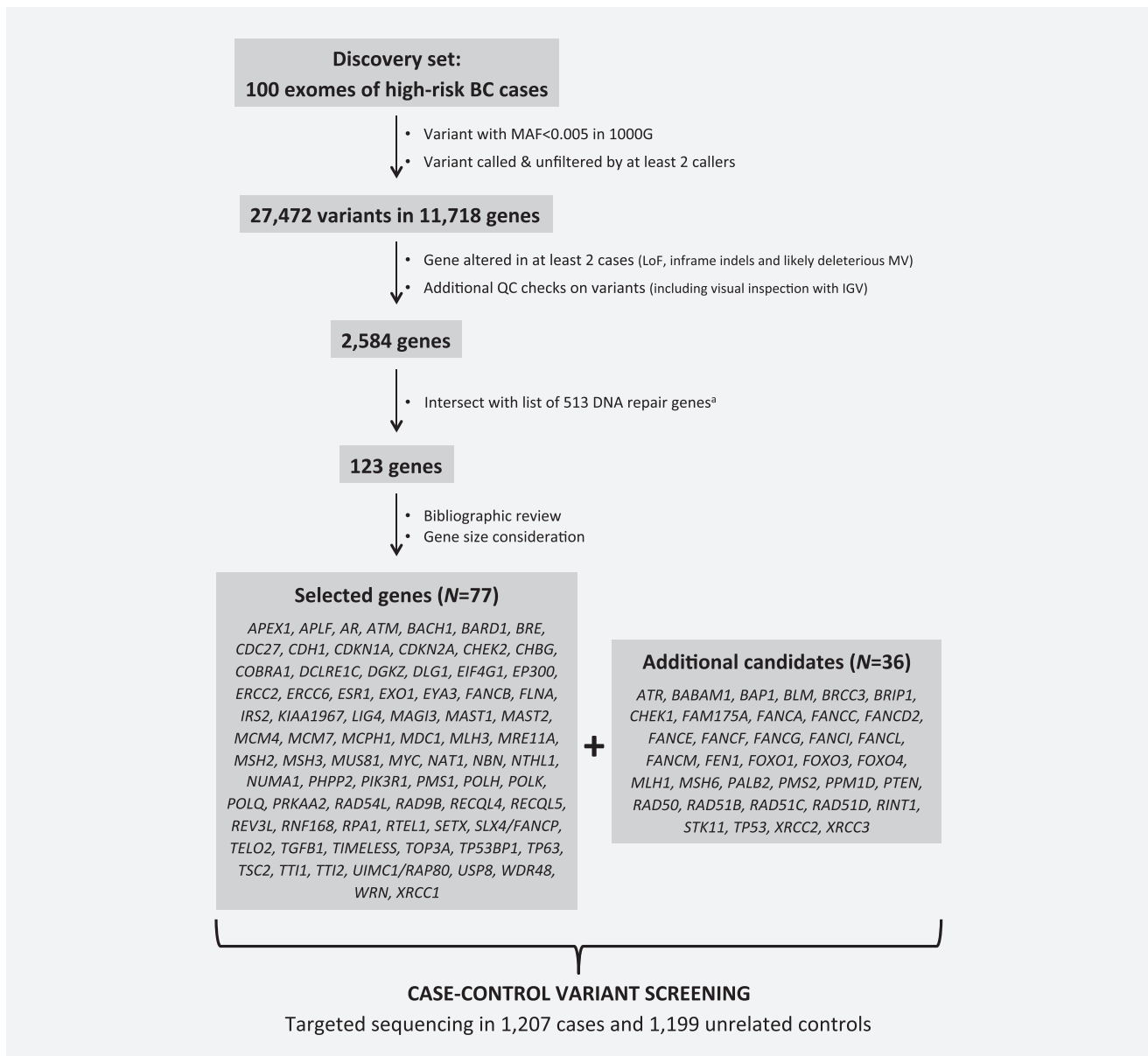
<sup>3</sup>Number after excluding GENESIS index case and one affected sister.

<sup>4</sup>Colon (C18), small bowel (C17), rectum (C19, C20), endometrium (C53, C54, C55), ovary (C56), stomach (C16), bile duct cancers (C24).

mammary or ductal adenocarcinoma, not carrying a pathogenic variant in *BRCA1* and *BRCA2*, and having a sister with BC were enrolled in GENESIS between 2007 and 2013 through the national network of cancer genetics clinics (<http://www.unicancer.fr/en/unicancer-group>). Affected sisters ( $N = 826$ ) and 1,419 unrelated cancer-free friends or colleagues of index cases (controls) were also included. These latter were aged-matched ( $\pm 3$  years) to cases at interview. Blood samples, clinical and epidemiological data were collected for each participant. Written informed consent for our study was obtained from all participants. GENESIS study protocol and genetic subsequent studies were approved by the appropriate ethics committee (CCP Ile-de-France III) and by the French data protection authority (CNIL).

**Selection of cases for the gene discovery stage.** Women included in the WES stage consisted of 100 unrelated cases presenting with a strong personal or family history of BC. Among those, 53 cases belonged to a family with at least one case having developed bilateral BC, 43 cases had at least three sisters affected with BC, and 11 cases had a male first-degree relative affected with BC. Seven of the selected cases fulfilled two of these criteria. The mean age at diagnosis of first BC was 48.4 (range: 28–71 years). Ninety-eight of the cases were of European origin.

**Selection of subjects for BC risk assessment.** After quality control procedures, we analyzed DNA from 1,207 unrelated



**Figure 1.** Strategy for genes prioritization. <sup>a</sup>The DNA repair genes list was compiled from ACSN maps (<https://acsncurie.fr/>), KEGG (<http://www.genome.jp/kegg/pathway.html>) and after review of literature.

cases (none of whom had been included or were related to an individual who was included in the discovery stage), and 1,199 unrelated controls. The screened sample represented 77% of the GENESIS population. Distribution of cases and controls by age, ethnicity and number of cancers in the family is shown in Table 1.

### DNA preparation

Peripheral blood samples (10 mL) were collected in the presence of anticoagulant (sodium citrate), and genomic DNA (gDNA) extracted from peripheral blood leukocytes with a standard inorganic method<sup>23</sup> using the DNA extractor Autopure-LS

(Qiagen). DNA handling (normalization and aliquoting) was done using a TECAN EVO instrument.

### Sequencing, data processing and variant discovery

Library preparation, whole exome sequencing (WES) and gene panel sequencing were performed at the Centre National de Recherche en Génomique Humaine (Evry, France). Exomes were captured using the SureSelectXT Human All Exon V5 kit, and coding exons of candidate genes were captured using a custom SureSelect target Enrichment system (Agilent Technologies, Santa Clara, CA, USA). Final libraries were sequenced on an Illumina HiSeq 2500 instrument with 100-bp

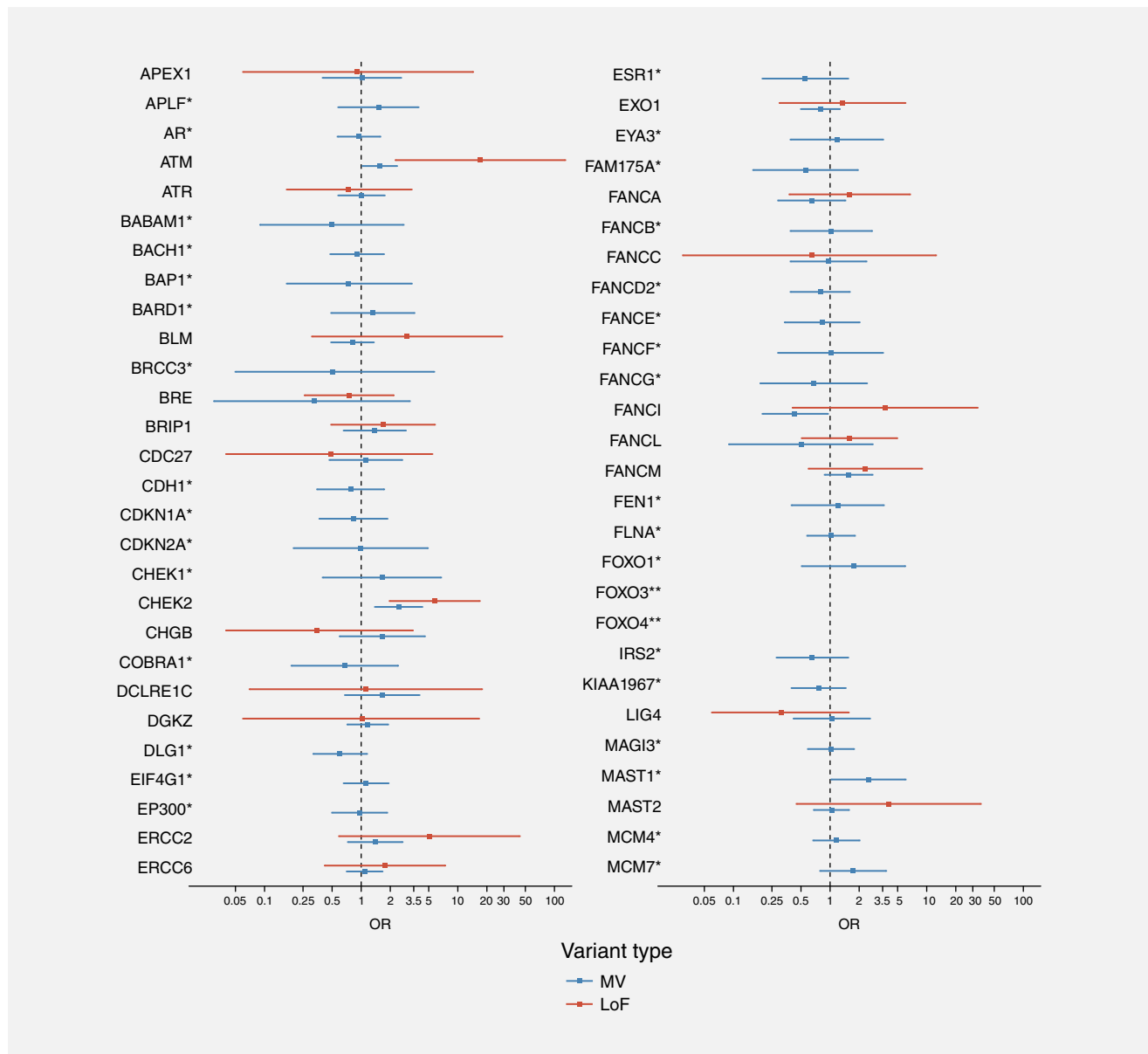


Figure 2. Result of the association tests per variant type for the 113 genes. Legend: \*, no LoF identified in cases and/or in controls; \*\*, no LoF and no likely deleterious MV identified in cases and/or in controls.

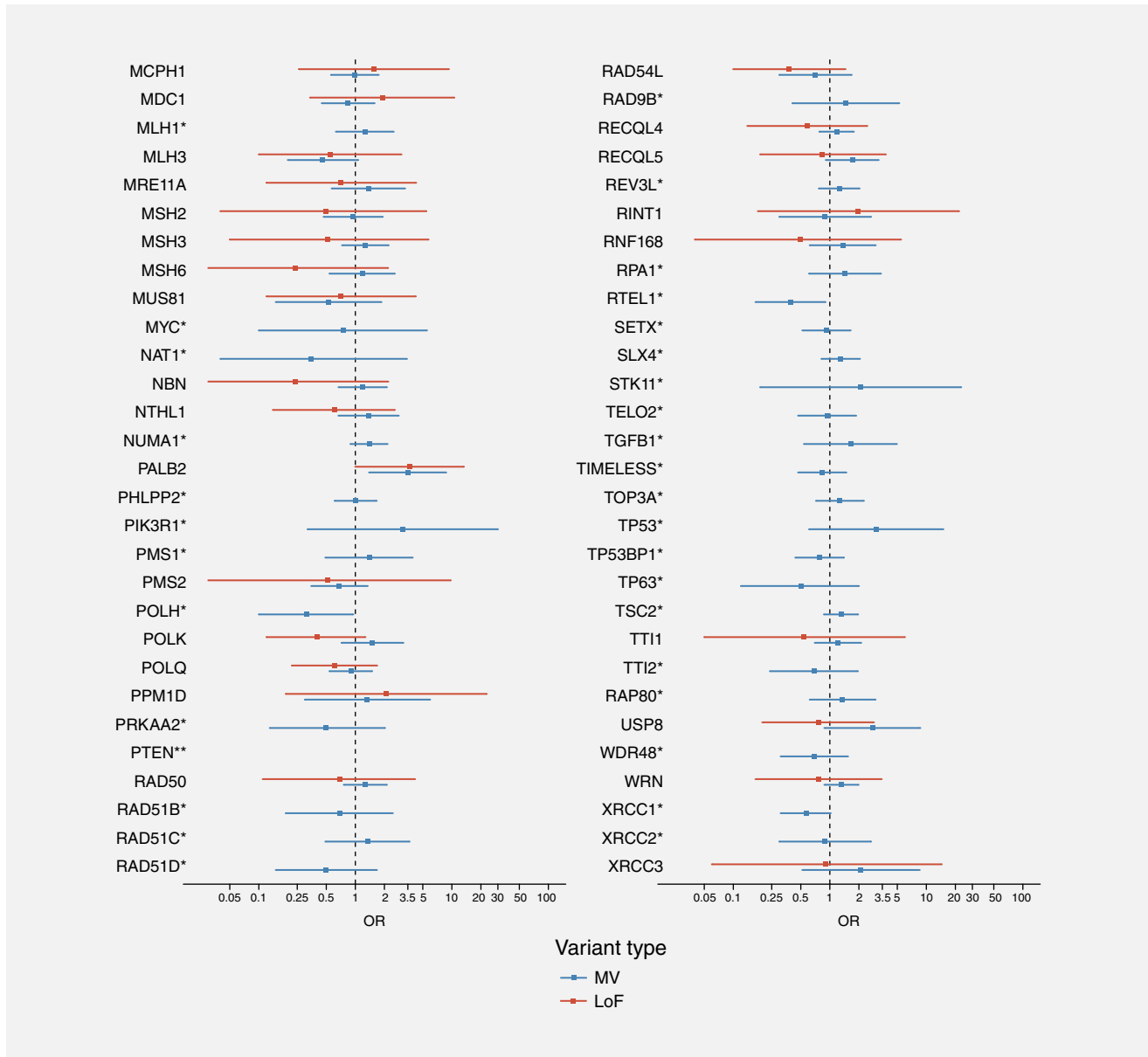


Figure 2. (Continued)

paired end reads. Reads were mapped to the reference genome hg19 (1,000 Genomes GRCh37 build) using BWA aln v0.6.2<sup>24</sup> with the after parameters: two mismatches allowed in the 32 bp-seed alignment and four mismatches allowed in the whole read, one alignment reported per read. For the exome-sequenced samples, Picard Tools v.1.103 (<http://picard.sourceforge.net>) was used to remove duplicate reads in order to mitigate biases introduced by data generation steps such as PCR amplification. SNV and insertions/deletions (indels) were then called using three strategies: *i.* Freebayes (v0.9.9.2),<sup>25</sup> *ii.* GATK' Unified Genotyper (v2.2)<sup>26</sup> on original alignment files and *iii.* on preprocessed alignment files using GATK modules for local realignment around indels and base quality score

recalibration using databases of known polymorphisms, after best practice recommendations. All variant calls were applied using a minimum base quality of 17 and a minimum mapping quality of 10. A dedicated filtering process was applied per sample to prioritize variants of interest: variants covered by less than 5× and with phred quality of less than 30 were discarded, while only heterozygous variants (defined by an allelic ratio between 20 and 80%) and variants detected by at least two calling strategies were considered. For some complex events (indels or SNVs falling in repeated sequences) we also performed visual inspection of aligned reads using the Integrative Genomics Viewer (IGV)<sup>27,28</sup> to reduce the risk of false positives and help characterizing them.



Table 2. Distribution of rare variants and results of the association tests for the 7 genes showing association with BC ( $p \leq 0.05$ )

Gene	Any variant				LoF				Likely deleterious MV						
	Control carriers	Case carriers	OR <sup>1</sup> (95%CI)	p-Value	OR <sup>2</sup> (95%CI)	p-Value	Control carriers	Case carriers	OR <sup>1</sup> (95%CI)	p-Value	Control carriers	Case carriers	OR <sup>1</sup> (95%CI)	p-Value	P <sub>het</sub> <sup>3</sup>
	ATM	40	77	1.9 (1.3, 2.9)	0.001	1.8 (1.2, 2.7)	0.003	1	16	17.4 (2.3, 132)	0.006	39	61	1.6 (1.0, 2.3)	0.04
CHEK2	22	62	3.0 (1.9, 5.0)	0.00001	2.9 (1.7, 4.7)	0.0004	4	21	5.8 (2.0, 16.9)	0.001	18	41	2.4 (1.4, 4.3)	0.002	0.14
FANCI	22	13	0.6 (0.3, 1.2)	0.13	0.5 (0.3, 1.1)	0.08	1	4	3.7 (0.4, 33.6)	0.24	21	9	0.4 (0.2, 1.0)	0.04	0.04
MAST1	8	17	2.2 (0.9, 5.1)	0.07	2.0 (0.9, 4.7)	0.11	1	0	-	-	7	17	2.5 (1.0, 6.0)	0.04	-
PALB2	9	30	3.5 (1.7, 7.5)	0.001	3.2 (1.5, 6.9)	0.002	3	10	3.6 (1.0, 13.3)	0.05	6	20	3.5 (1.4, 8.7)	0.008	0.95
POLH	13	5	0.4 (0.1, 1.1)	0.07	0.3 (0.1, 1.0)	0.04	0	1	-	-	13	4	0.3 (0.1, 1.0)	0.04	-
RTEL1	20	8	0.4 (0.2, 0.9)	0.03	0.4 (0.2, 0.8)	0.02	0	0	-	-	20	8	0.4 (0.2, 0.9)	0.03	-

<sup>1</sup>Reference group: noncarriers of a variant in the tested gene; adjusting for ethnicity and age at inclusion.  
<sup>2</sup>Reference group: noncarriers of a variant in the tested gene; adjusting for ethnicity, age at inclusion and number of altered genes (continuous).  
<sup>3</sup>p-Value of the likelihood ratio test to test for heterogeneity between the effect of LoF and MV.

The joint variant calling file was finally annotated with refGene regions, SNV effects, as well as 1,000 Genomes (1000G) minor allele frequency (MAF; august 2015 version) using Annotator (October 2015 version, <http://annovar.openbioinformatics.org>). Only exonic or splicing variants presenting a MAF below 0.5% in all represented ethnic groups of the 1000G database were kept in further analyses. In the gene discovery stage, variants carried by more than five cases and absent from 1000G were excluded as they were likely to represent a sequencing artifact or an unreported common polymorphism in our population. We then only considered LoF, inframe indels and conserved MV (PhyloP score > 0)<sup>29</sup> predicted as deleterious by SIFT<sup>30</sup> and/or PolyPhen2<sup>31</sup> in the analyses. In the case-control study, CADD<sup>32</sup> (for all genes) and Align-GVGD (for ATM, CHEK2 and PALB2)<sup>33</sup> were also used to annotate and filter MV. In this latter stage, we applied a supplementary filter on MAF in order to exclude any variant with MAF > 0.5% in controls, as we observed that the MAF of some variants was underestimated in the public databases.

**Statistical analyses**

All LoF and MV with a phred CADD score equal or above 20 were considered in the analyses. Their frequencies among cases and controls were compared by unconditional logistic regressions, adjusting for ethnicity and age at inclusion in GENESIS. To address issues of multiple testing, by examining 113 genes and applying a Bonferroni correction, statistical significance was defined as  $p < 0.0004$ .

We used Likelihood ratio tests to test for heterogeneity according to variant type (LoF vs. MV), and polytomous logistic regressions to test for heterogeneity according to hormone status and according to time between inclusion and diagnosis.

All statistical tests were two-sided and data was analyzed using STATA, version 11.2 (StataCorp, College Station, TX).

**Results**

**Gene discovery stage**

WES was performed on 100 unrelated high-risk women to identify candidate genes. The average read depth achieved for target regions was 110X (range: 55X-207X). At least 80% (average: 96.45%; range: 79.77%-99.35%) of the capture target regions were covered by 20 or more sequenced reads for all samples (Supporting Information Fig. S1). Whole exome sequencing performance and variants count for each sequenced DNA sample is shown in Supporting Information Table S1. After initial single nucleotide variants (SNV) filtering steps, an average of 18.2 (11-29) LoF, 6.8 (1-14) inframe indels and 133.7 (105-170) MV were identified per individual. In total, 2,584 genes were altered by such variants in at least two unrelated cases. Among those, 123 genes were known to be involved in DNA repair pathways. After having reviewed the potential involvement of each of them in cancer etiology or progression, we selected 77 genes for further investigation in the case-control study. This gene panel was completed with



Table 3. Analyses by hormone receptor status and restricted to pseudo-incident cases, for the 7 genes associated with BC ( $P \leq 0.05$ )

Gene	Analysis <sup>1</sup>	Any variant			LoF			Likely deleterious MV		
		Case carriers	OR <sup>2</sup> (95%CI)	p-Value	Case carriers	OR <sup>2</sup> (95%CI)	p-Value	Case carriers	OR <sup>2</sup> (95%CI)	p-Value
<b>ATM</b>	ER positive	47	2.1 (1.4, 3.3)	0.001	9	17.7 (2.2, 140)	0.006	38	1.7 (1.1, 2.8)	0.02
	ER negative	8	2.1 (0.9, 4.5)	0.07	2	20.8 (1.9, 232)	0.01	6	1.6 (0.7, 3.8)	0.31
	PR positive	38	2.1 (1.3, 3.4)	0.001	9	22.0 (2.8, 174)	0.003	29	1.6 (1.0, 2.7)	0.06
	PR negative	12	1.7 (0.9, 3.4)	0.10	2	10.9 (1.0, 120)	0.05	10	1.5 (0.7, 3.0)	0.27
	HER2 positive	3	1.3 (0.4, 4.2)	0.70	0	-	-	3	1.3 (0.4, 4.3)	0.68
	HER2 negative	24	1.8 (1.1, 3.1)	0.02	6	19.2 (2.3, 160)	0.006	18	1.4 (0.8, 2.5)	0.27
	Pseudo-incident cases <sup>3</sup>	41	1.9 (1.2, 2.9)	0.006	8	15.6 (1.9, 125)	0.01	33	1.5 (1.0, 2.5)	0.08
<b>CHEK2</b>	ER positive	39	3.5 (2.1, 6.0)	0.000004	16	7.9 (2.6, 23.9)	0.0003	23	2.5 (1.3, 4.7)	0.004
	ER negative	3	1.3 (0.4, 4.5)	0.64	1	2.5 (0.3, 22.2)	0.42	2	1.1 (0.2, 4.8)	0.91
	PR positive	35	3.9 (2.2, 6.7)	0.000001	17	10.5 (3.5, 31.3)	0.00003	18	2.4 (1.2, 4.7)	0.01
	PR negative	6	1.6 (0.7, 4.1)	0.29	0	-	-	6	2.0 (0.8, 5.2)	0.14
	HER2 positive	5	3.8 (1.4, 10.4)	0.009	1	4.3 (0.5, 39.1)	0.2	4	3.7 (1.2, 11.3)	0.02
	HER2 negative	27	4.1 (2.3, 7.3)	0.000002	11	9.0 (2.8, 28.4)	0.0002	16	3.0 (1.5, 6.0)	0.002
	Pseudo-incident cases <sup>3</sup>	37	3.3 (1.9, 5.6)	0.00001	12	5.9 (1.9, 18.4)	0.002	25	2.7 (1.5, 5.0)	0.001
<b>FANCI</b>	ER positive	9	0.7 (0.3, 1.6)	0.44	3	4.7 (0.5, 46.3)	0.19	6	0.5 (0.2, 1.3)	0.17
	ER negative	0	-	-	0	-	-	0	-	-
	PR positive	8	0.9 (0.4, 1.9)	0.70	2	4.7 (0.4, 52.0)	0.21	6	0.7 (0.3, 1.7)	0.39
	PR negative	1	0.2 (0.0, 1.8)	0.17	1	4.3 (0.2, 79.5)	0.33	0	-	-
	HER2 positive	1	0.7 (0.1, 5.4)	0.75	1	15.1 (0.7, 319)	0.09	0	-	-
	HER2 negative	3	0.4 (0.1, 1.4)	0.20	0	-	-	3	0.4 (0.1, 1.5)	0.19
	Pseudo-incident cases <sup>3</sup>	6	0.5 (0.2, 1.2)	0.11	2	2.8 (0.2, 32.8)	0.42	4	0.4 (0.1, 1.0)	0.06
<b>MAST1</b>	ER positive	8	1.8 (0.7, 5.0)	0.23	0	-	-	8	2.1 (0.8, 5.9)	0.15
	ER negative	3	3.8 (1.0, 14.4)	0.05	0	-	-	3	4.3 (1.1, 16.8)	0.04
	PR positive	5	1.2 (0.4, 3.9)	0.79	0	-	-	5	1.7 (0.5, 5.3)	0.38
	PR negative	4	3.0 (0.9, 10.0)	0.08	0	-	-	4	3.4 (1.0, 11.9)	0.05
	HER2 positive	2	4.2 (0.9, 20.0)	0.08	0	-	-	2	4.7 (1.0, 23.1)	0.06
	HER2 negative	2	0.8 (0.2, 3.7)	0.75	0	-	-	2	0.9 (0.2, 4.3)	0.89
	Pseudo-incident cases <sup>3</sup>	8	1.9 (0.7, 5.0)	0.20	0	-	-	8	2.1 (0.8, 6.0)	0.14
<b>PALB2</b>	ER positive	21	4.6 (2.1, 10.1)	0.0002	8	5.4 (1.4, 20.4)	0.01	13	4.2 (1.6, 11.1)	0.004
	ER negative	1	1.1 (0.1, 8.8)	0.93	0	-	-	1	1.6 (0.2, 13.7)	0.65
	PR positive	14	3.7 (1.6, 8.7)	0.002	3	2.4 (0.5, 11.9)	0.29	11	4.4 (1.6, 11.9)	0.004
	PR negative	7	4.9 (1.8, 13.4)	0.002	5	11.1 (2.6, 47.4)	0.001	2	2.0 (0.4, 10.0)	0.40
	HER2 positive	0	-	-	0	-	-	0	-	-
	HER2 negative	13	4.7 (2.0, 11.1)	0.0004	3	3.4 (0.7, 16.7)	0.14	10	5.3 (1.9, 14.8)	0.001
	Pseudo-incident cases <sup>3</sup>	15	3.2 (1.4, 7.3)	0.007	5	3.2 (0.8, 13.6)	0.11	10	3.1 (1.1, 8.7)	0.03
<b>POLH</b>	ER positive	4	0.6 (0.2, 1.8)	0.30	1	-	-	3	0.4 (0.1, 1.5)	0.19
	ER negative	0	-	-	0	-	-	0	-	-
	PR positive	4	0.7 (0.2, 2.2)	0.60	1	-	-	3	0.5 (0.2, 1.9)	0.34
	PR negative	0	-	-	0	-	-	0	-	-
	HER2 positive	0	-	-	0	-	-	0	-	-

(Continues)

Table 3. Continued

Gene	Analysis <sup>1</sup>	Any variant			LoF			Likely deleterious MV		
		Case carriers	OR <sup>2</sup> (95%CI)	p-Value	Case carriers	OR <sup>2</sup> (95%CI)	p-Value	Case carriers	OR <sup>2</sup> (95%CI)	p-Value
	HER2 negative	1	0.2 (0.0, 1.8)	0.20	1	-	-	0	-	-
	Pseudo-incident cases <sup>3</sup>	1	0.1 (0.0, 1.1)	0.06	0	-	-	1	0.1 (0.0, 1.1)	0.06
<b>RTEL1</b>	ER positive	5	0.5 (0.2, 1.2)	0.12	0	-	-	5	0.5 (0.2, 1.2)	0.12
	ER negative	0	-	-	0	-	-	0	-	-
	PR positive	5	0.6 (0.2, 1.5)	0.30	0	-	-	5	0.6 (0.2, 1.5)	0.27
	PR negative	0	-	-	0	-	-	0	-	-
	HER2 positive	2	1.4 (0.3, 6.6)	0.61	0	-	-	2	1.5 (0.3, 6.6)	0.61
	HER2 negative	2	0.3 (0.1, 1.3)	0.10	0	-	-	2	0.3 (0.1, 1.3)	0.10
	Pseudo-incident cases <sup>3</sup>	7	0.6 (0.3, 1.5)	0.30	0	-	-	7	0.6 (0.3, 1.5)	0.31

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, HER2 gene amplification.

<sup>1</sup>ER, PR and HER2 status were available for 788 (67%), 742 (63%), and 475 (40%) of sequenced GENESIS cases.

<sup>2</sup>Reference group: noncarriers of a variant in the tested gene; adjusting for ethnicity and age at inclusion.

<sup>3</sup>Cases diagnosed with BC less than 5 years before enrollment in GENESIS (N = 663).

36 genes currently included in commercial BC multigene panels. The workflow for gene prioritization and the final list of analyzed genes are shown on Figure 1.

### Breast cancer risk assessment

Targeted sequencing of the 113 genes identified 3,930 distinct variants with MAF lower than 0.5% in GENESIS controls. These included 264 LoF variants (155 in cases and 132 in controls) and 1,994 MV bioinformatically predicted to alter protein function or stability (1,213 in cases and 1,103 in controls). The distribution of the number of altered genes per subject, according to their case/control status is shown on Supporting Information Figure S2. There was no overall difference in the distribution of number of altered genes between cases and controls ( $\chi^2$  (6df) = 10.0,  $p = 0.12$ ) and only a modest increased risk of BC for subjects with at least two altered genes as compared to subjects with 0 or 1 altered gene (OR = 1.2; 95%CI, 1.0–1.4;  $p = 0.02$ ). Nevertheless, we performed sensitivity analyses adjusted on the number of altered genes in the subsequent analyses. For each gene, we aggregated all rare LoF and MV, and also examined separately the two types of variants. The distribution of variants in cases and controls, per gene, and associated risk estimates are provided in Supporting Information Table S2, and results for all genes are summarized in Figure 2. Among those, *PALB2*, *ATM*, *CHEK2*, *FANCI*, *MAST1*, *POLH* and *RTEL1* showed significant association with BC although only *CHEK2* remained associated with BC after correcting for multiple testing.

Both LoF and MV in *PALB2*, *ATM* and *CHEK2* were associated with an increased BC risk, but carriers of an *ATM* LoF had a significantly higher risk of developing BC than carriers of an *ATM* MV (OR<sub>LoF</sub> = 17.4 vs. OR<sub>MV</sub> = 1.6;  $p_{\text{Het}} = 0.002$ ) (Table 2). In contrast for *CHEK2* and *PALB2* there was no significant heterogeneity

in risk according to variant type (*CHEK2*: OR<sub>LoF + MV</sub> = 3.0; 95% CI 1.9–5.0; *PALB2*: OR<sub>LoF + MV</sub> = 3.5; 95%CI 1.7–7.5).

The increased risk associated with variants in *MAST1* was essentially driven by carrying a MV (OR<sub>MV</sub> = 2.5; 95%CI 1.0–6.0). Likewise, the decreased risk associated with variants in *FANCI*, *POLH* and *RTEL1* was driven by carrying a MV (*FANCI*: OR<sub>MV</sub> = 0.4; 95%CI, 0.2–1.0; *POLH*: OR<sub>MV</sub> = 0.3; 95%CI 0.1–1.0; *RTEL1*: OR<sub>MV</sub> = 0.4; 95%CI 0.2–0.9). Intriguingly, four cases and one control carried a *FANCI* LoF, leading to a nonsignificant increase in BC risk (OR<sub>LoF</sub> = 3.7 vs. OR<sub>MV</sub> = 0.4;  $p_{\text{Het}} = 0.04$ ) (Table 2).

Furthermore, we found that carriers of a LoF in *CHEK2* were diagnosed with BC at a younger age than noncarriers of a *CHEK2* variant (46.7 years vs. 52.1 years,  $p_{\text{T test}} = 0.007$ ). Such a difference was not observed for carriers of a MV (50.1 years vs. 52.1 years,  $p_{\text{T test}} = 0.16$ ). For all other genes, no difference in age at diagnosis was observed (data not shown).

### Breast cancer risk by hormone receptor status

Estrogen receptor (ER), progesterone receptor (PR) and *HER2* gene amplification status of the breast tumor were available for 67%, 63% and 40% of the sequenced cases, respectively. For *FANCI*, *POLH* and *RTEL1*, there were too few observations in each stratum to draw any conclusion on whether carrying a likely deleterious variant was associated with a particular status. Information on HER2 status among carriers of a variant in any of the genes was too scarce to conclude. In the analyses comparing BC risk according to ER or PR status, none of the heterogeneity tests was significant. However, *CHEK2* variants were associated with a higher BC risk point estimate for ER-positive (OR = 3.5) compared to ER-negative BC (OR = 1.3); a similar pattern was observed for PR status (Table 3). *PALB2* variants were also associated with a higher

**Table 4.** Distribution of MV and results of the association tests for *ATM*, *CHEK2* and *PALB2* using Align-GVGD classifiers (Tavtigian et al.<sup>33</sup>)

Gene	Variant class	Controls	Cases	OR <sup>1</sup> (95%CI)	p-Value
<i>ATM</i>	Noncarriers	1,129	1,102	Ref.	
	C0	43	47	1.1 (0.7, 1.6)	0.75
	C15-C65	26	42	1.7 (1.0, 2.7)	0.05
<i>CHEK2</i>	Noncarriers	1,174	1,137	Ref.	
	C0	7	23	3.4 (1.5, 8.0)	0.005
	C15-C65	14	26	2.0 (1.1, 3.9)	0.03
<i>PALB2</i>	Noncarriers	1,181	1,168	Ref.	
	C0	10	14	1.3 (0.6, 3.0)	0.51
	C15-C65	5	15	3.3 (1.2, 9.0)	0.02

<sup>1</sup>Reference group: noncarriers of a variant in the tested gene; adjusting for ethnicity and age at inclusion.

BC risk point estimate for ER-positive (OR = 4.6) compared to ER-negative BC (OR = 1.1). *MAST1* MV were associated with a higher point estimate for ER-negative (OR = 4.3) compared to ER-positive BC (OR = 2.1).

### Sensitivity analyses

Because GENESIS cases are prevalent cases, i.e. diagnosed with BC sometimes several years before being invited to participate in the study, cases carrying a pathogenic variant in a gene associated with a poor prognosis would have had less chance to be included and would be therefore under-represented. To examine a survival bias, we excluded cases with a delay between diagnosis of BC and inclusion in GENESIS of greater than 5 years and performed analyses on pseudo-incident cases ( $N = 663$ ). There was no significant heterogeneity in BC risk according to time between diagnosis and inclusion for *ATM*, *CHEK2*, *PALB2*, *FANCI*, *MAST1* and *POLH* (Table 3). The observed decreased BC risk associated with carrying a MV in *RTEL1* seemed different, although not statistically significant, between cases diagnosed more than 5 years before inclusion and cases diagnosed less than 5 years before inclusion ( $OR_{>5\text{years}} = 0.1$  vs.  $OR_{\leq 5\text{years}} = 0.6$ ;  $p_{\text{Het}} = 0.09$ ). Therefore, we cannot conclude here that *RTEL1* variant carriers have a poorer survival, but more studies are warranted to clarify this.

There was no change in BC risk estimates when adjusting for the number of self-reported cancer cases in the family (BC, ovarian cancer or cancers from the Lynch syndrome spectrum; data not shown) or for the number of altered genes (Table 2). Finally, results by hormone receptor status were unchanged when restricting the analyses to pseudo-incident cases (data not shown).

### Discussion

By analyzing 113 DNA repair genes selected from either a WES or a candidate gene approach in familial BC cases and a well-identified control group, we confirmed associations

between variants predicted to impair protein function in *PALB2*, *ATM* and *CHEK2* and occurrence of BC. We found that the risk associated with *ATM* variants can greatly vary according to variant type ( $OR_{\text{ATM-LoF}} \sim 10 \times OR_{\text{ATM-MV}}$ ). The risk conferred by LoF would still significantly differ from the risk conferred by MV in this gene even if the number of *ATM* LoF carriers was four times higher in controls. There were no *ATM* LoF that were observed more than once in cases, and thus none could contribute disproportionately to the risk estimate (Supporting Information Table S3).

We also identified four genes (*FANCI*, *MAST1*, *POLH* and *RTEL1*) that had not been previously associated with BC susceptibility. Should the association with BC be confirmed in larger studies, the decreased BC risk associated with MV in *FANCI*, *POLH* and *RTEL1* is puzzling. Because of a difference in BC risk between cases included more than 5 years after BC diagnosis and those included less than 5 years after BC diagnosis, we concluded that *RTEL1* alterations might be associated with a poor prognosis. The same hypothesis was tested for carriers of a variant in *FANCI* or *POLH* and results were unchanged. Another possible explanation could be that *FANCI* and *POLH* are associated with another syndrome than the HBOC syndrome. Therefore due to recruitment criteria in GENESIS, carriers of a variant in one of these genes would be under-represented among the cases of our study.

Apart from *FANCI*, which we included in our panel because it belongs to the same Fanconi Anemia gene family as *BRCA1/FANCS*, *BRCA2/FANCD1* and *PALB2/FANCN*, we investigated *MAST1*, *POLH* and *RTEL1* because they had been highlighted in the WES exploratory stage, as were the known BC genes *ATM* and *CHEK2*. This demonstrates the efficiency of our approach to prioritize new candidate genes. Another strength of our study is that the full coding sequence of the genes was screened in all subjects using the same sequencing techniques and platforms, thus avoiding biases that may arise when only the variants identified in cases are tested in controls. Moreover, unlike many other studies, controls were specifically recruited for the GENESIS study. They were cancer-free friends or colleagues belonging to the same birth cohort as the cases, and were therefore naturally matched on many unmeasured potential confounders.

The literature on *MAST1*, *POLH* and *RTEL1* has indicated relationships between these genes and carcinogenesis. Putative germline pathogenic variants in *MAST1*, encoding a microtubule-associated serine–threonine kinase, were described in familial lung cancer cases.<sup>34</sup> Transcriptome analysis of BC cell lines and tissue identified somatic recurrent rearrangements involving *MAST1* and over-expression of the resulting gene fusion.<sup>35</sup> Inactivating variants of *POLH*, which encodes the translesion DNA polymerase  $\eta$  are responsible for Xeroderma Pigmentosum, a rare recessive syndrome associated with hypersensitivity to sunlight and a high frequency of skin cancers at an early age.<sup>36,37</sup> Of note, a germline *POLH* MV (c.2074A>G) was identified by WES in a Lebanese BC-prone family<sup>38</sup> but this variant, predicted to be

benign by various tools, was not found in our population. *RTEL1* encodes the regulator of telomere elongation helicase 1, which is essential for telomere maintenance and regulation of homologous recombination. Germline biallelic *RTEL1* variants are responsible for dyskeratosis congenita and its severe variant Hoyeraal-Hreidarsson syndrome,<sup>39</sup> while heterozygous carriers with pulmonary fibrosis,<sup>40–42</sup> myelodysplasia and liver disease have been described.<sup>43</sup> Thus, our findings on *FANCI*, *MAST1*, *POLH* and *RTEL1* would warrant further investigation in other large-scale studies on familial BC and the setup of functional studies to assess the impact of the variants.

There was some discrepancy between our findings on *ATM* in GENESIS and previous findings from a pooled analysis of seven case-control studies that included the Breast Cancer Family Registry (BCFR) study<sup>9</sup> in terms of risk estimates. In these latter analyses, we had found that carriers of a likely deleterious MV may be at higher risk of BC than LoF carriers.<sup>9</sup> One explanation could be the difference in the tool predicting the impact of the MV. We therefore reanalyzed GENESIS data for MV in *ATM*, *CHEK2* and *PALB2* using Align-GVGD<sup>33</sup> which was used in the BCFR<sup>9,44</sup> and other studies.<sup>45,46</sup> Results obtained with Align-GVGD were consistent with those obtained with CADD (Table 4). Another explanation could be the difference in the ages at diagnosis between the studies. In GENESIS, carriers of an *ATM* LoF or MV were diagnosed on average at age 50.0 (SD = 2.6 years) and 50.9 (SD = 1.2 years), respectively, while most of the cases in the pooled analysis were diagnosed before age 46. Even if in GENESIS carrying an *ATM* MV was associated with a higher BC risk point estimate in early-onset cases ( $OR_{\leq 45\text{years}} = 2.2$  vs.  $OR_{>45\text{years}} = 1.2$ ,  $p_{\text{Het}} = 0.06$ ), the risk estimate associated with MV remained much lower than the risk estimate associated with LoF. No difference in BC risk was observed for LoF when stratifying by age ( $OR_{\leq 45\text{years}} = 18.6$  vs.  $OR_{>45\text{years}} = 17.9$ ,  $p_{\text{Het}} = 0.95$ ). Of note, the MV c.7271T>G (p.Val2424Gly), which had been associated with a substantial BC risk in several studies,<sup>47–49</sup> and in particular in the Australian population, was not found in GENESIS cases and was found only once in controls. Moreover, there was no evidence that the observed association was driven by a specific variant (either a LoF or a MV) in our study set (Supporting Information Table S3). Conversely, the *CHEK2* LoF c.1100delC was quite prevalent in the French population (Supporting Information Table S3). However, when excluding carriers of this variant in the analysis we found that other *CHEK2* variants (both LoF and MV) contribute significantly to BC risk ( $OR = 2.5$ ; 95%CI, 1.5–4.4;  $p = 0.0004$ ).

There was also a noteworthy difference in *PALB2* BC relative risk estimates when compared to those estimated by Antoniou *et al.*<sup>8</sup> for carriers of a LoF. This may be explained by the difference in populations under study. Indeed, Antoniou *et al.* estimated BC risk from highly selected *PALB2* families with uncertainty in modes of ascertainment. Although analyses were performed using modeling to reduce bias, their *PALB2* families may have aggregated many other unmeasured genetic and

nongenetic BC risk factors. Another notable finding in our study is the relatively high OR associated with MV in *PALB2*, which have not been systematically assessed in previous reports. Interestingly, the *PALB2* MV c.2816T>G (p. Leu939Trp) was quite frequent in our study population: half of the *PALB2* MV carriers carried this variant (12 out of 20 carriers in cases, and 3 out of 6 in controls;  $OR_{c.2816T>G} = 4.3$ ; 95% CI, 1.2–15.4;  $p = 0.02$ ). To understand the origin of its high prevalence, which could explain our ability to detect its effect, we performed a haplotype analysis of the 16p12.2 locus containing *PALB2* on GENESIS carriers of the c.2816T>G variant using MERLIN.<sup>50</sup> In a companion study, genotypes of 38 SNPs localized +/-500 kb around the variant were obtained with the iCOGS array in the GENESIS population (authors' unpublished data). This latter analysis suggests that the c.2816T>G variant could be a founder allele in the French BC cases (data not shown). A first study showed that this variant falls in the *PALB2* WD40 domain and results in altered *PALB2*-BRCA2 binding.<sup>51</sup> However, it was then demonstrated that it does not disrupt the HR-mediated DNA repair activity of *PALB2*.<sup>52</sup> Two case-control studies conducted in the German population<sup>53</sup> and in the British population<sup>6</sup> on over 800 and 923 familial BC cases, respectively, showed that the c.2816T>G variant was not associated with BC. More recently, a multicenter case-control study conducted by the Breast Cancer Association Consortium (BCAC) on 42,671 invasive BC and 42,164 controls did not show any association with this variant ( $OR_{c.2816T>G} = 1.05$ ; 95%CI 0.83–1.32),<sup>49</sup> leading to the conclusion that the *PALB2* c.2816T>G variant should be classified as a likely neutral variant. However, even if we cannot rule out a false-positive result in our study, one asset of the GENESIS population is to be homogeneous, in contrast with the countries heterogeneity in BCAC. Thus, further investigation on this variant may be worthwhile. Nonetheless, the risk estimates associated with *PALB2* MVs was not driven by this peculiar variant (after excluding carriers of c.2816T>G,  $OR_{MV} = 3.1$ ; 95%CI, 1.2–7.9;  $p = 0.02$ ).

With respect with the confirmed BC genes, deleterious variants in *ATM*, *CHEK2* and *PALB2* are present in 14% of GENESIS BC cases, of whom 1% carry a LoF in the actionable gene *PALB2* and another 1% carry a *PALB2* MV that would, for the time being, probably be considered as a variant of uncertain clinical significance in a cancer clinics setting. To further evaluate the potential clinical significance of variants considered in our case-control analysis, we determined how many of the 264 LoF and 1,994 likely deleterious MV identified in the 113 genes were present in ClinVar.<sup>54,55</sup> Only 78 (3.5%) were reported as "pathogenic" or "likely pathogenic" variants (Supporting Information Fig. S3). They were located in 30 genes including *PALB2*, *TP53*, *CDH1*, *RAD51C*, *RAD51D*, *MLH1*, *MSH2* and *PMS2* for which specific management guidelines have been elaborated in France in some specific cancer predisposition contexts (<http://www.unicancer.fr/recherche/les-groupes-recherche/groupe-genetique-et-cancer-ggc>). This illustrates that although ClinVar is a useful



tool for variant interpretations, the majority of variants that will be identified by multigene panel testing will be unreported, and efforts to facilitate sharing of data, in particular observation in unaffected populations, together with methods for variant assessment and expertise in specific disease areas are needed.

We chose to investigate each gene independently in order to be able to compare our relative risk estimates with those of other published data. However, we also performed the analyses adjusting for the number of altered genes, and no significant change in relative risk estimates was observed (Table 2). Nevertheless, one cannot exclude that there is a residual quantitative effect shift of number of altered genes in cases as compared to controls (Supporting Information Fig. S2). A potential polygenic effect might be further investigated in larger studies where a larger number of genes can be tested.

In conclusion, this case-control study conducted in a well-characterized population allowed us to describe genetic variation in 113 DNA repair genes and to specify BC relative risks associated with rare deleterious-predicted variants in *PALB2*, *ATM* and *CHEK2*. More generally, this work highlights the importance of considering all types of variants in the analyses, as their contribution might differ from one gene to another. Further family-based studies and gene-specific prospective cohorts are needed to assess absolute risk for carriers of a deleterious variant by taking into account other factors such as family history of cancer, polygenic models or even environmental/lifestyle factors. They will be useful to improve risk prediction models and will help defining gene-specific consensus management guidelines.

### Authors' contribution

Conceived and designed the experiments: RO, FD, AB, JFD, FL. Performed the experiments: LB, NM, ALR. Analyzed the data: EG, ALR, NA, FL. Contributed reagents/materials/analysis tools: MGD, DG, VM, CL, JC, NS. Coordinated the GENESIS study: SM, DSL, NA. Included GENESIS participants, collected data: SEM, DLG, JB, NM, MM, EC. Invited GENESIS index cases: OCH, DL, CP, SFF, CC, TF, FE, AC, CA, AF, LG, JT, AF, JC, SG, IM, FS, SAB, JML, CL, SLD, HD, YJB, ML, PP, LVB, VB, PB, EL, CM, CN, CD, JPF, PG, LF, AL, BB,

OC, MG, V, IC, DSL. Wrote the paper: EG, NA, FL. All authors read and approved the final version of the study.

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