Identification of a novel pathogenic variant in PALB2 and BARD1 genes by a multigene sequencing panel in triple negative breast cancer in Morocco

Abdelilah Laraqui1,2, Mathias Cavaillé3,4, Nancy Uhrhammer3,4, Oubaida ElBiad1,5, Yannick Bidet3,4, Hicham El Rhaftouli2, Hicham El Anaz1, Driss Moussaoui Rahali6, Jaouad Kouach6, Khaled Guelzim6, Bouabid Badaoui5, Abderrahman AlBouzidi7, Mohammed Ouakabi7, Rachid Tanz8, Yasser Sbitti8, Mohammed Ichou8, Khaled Ennibi1, Yassine Sekhsokh2, Yves-Jean Bignon3,4

1. Unité de séquençage, Centre de virologie, des maladies infectieuses et tropicales, Hôpital militaire d’Instruction Mohammed V, Faculté de Médecine et de Pharmacie, Université Mohammed V, Rabat, Maroc.
2. Laboratoire de Recherche et de Biosécurité P3, Hôpital Militaire d’Instruction Mohammed V, Faculté de Médecine et de Pharmacie, Rabat, Maroc.
3. Laboratoire Diagnostique Génétique et Moléculaire, Centre Jean Perrin, 58 rue Montalembert, Clermont-Ferrand, France.
4. INSERM, U1240 Imagerie Moléculaire et Stratégies Théranostiques, Université Clermont Auvergne, Clermont-Ferrand, France.
5. Laboratoire de Biodiversité, Écologie et Génome, Faculté des Sciences, Université Mohammed V, Rabat, Maroc.
6. Service de Gynécologie Obstétrique, Hôpital Militaire d’Instruction Mohammed V, Faculté de Médecine et de Pharmacie, Rabat, Maroc.
7. Laboratoire d’Anatomopathologie, Hôpital Militaire d’Instruction Mohammed V, Faculté de Médecine et de Pharmacie, Rabat 10000, Maroc.
8. Service d’Oncologie Médicale, Hôpital Militaire d’Instruction Mohammed V, Faculté de Médecine et de Pharmacie, Rabat, Maroc.

Corresponding author: Abdelilah LARAQUI, Laboratoire de Recherche et de Biosécurité P3, Hôpital Militaire d’Instruction Mohammed V, Rabat, Maroc. Tel: (212) 678 682 530; E-mail: loranjad@yahoo.fr.

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Abstract

Pathogenic variants (PVs) in BRCA genes have been mainly associated with an increasing risk of triple negative breast cancer (TNBC). The contribution of PVs in non-BRCA genes to TNBC seems likely since the processing of homologous recombination repair of double-strand DNA breaks involves several genes. Here, we investigate the susceptibility of genetic variation of the BRCA and non-BRCA genes in 30 early-onset Moroccan women with TNBC.

Methods: Targeted capture-based next generation sequencing (NGS) method was performed with a multigene panel testing (MGPT) for variant screening. Panel sequencing was performed with genes involved in hereditary predisposition to cancer and candidate genes whose involvement remains unclear using Illumina MiSeq platform. Interpretation was conducted by following the American College of Medical Genetics and Genomics-Association for Molecular Pathology (ACMG-AMP) criteria.

Results: PVs were identified in 20% (6/30) of patients with TNBC. Of these, 16.7% (5/30) carried a BRCA PV [10% (3/30) in BRCA1, 6.7% (2/30) in BRCA2] and 6.6% (2/30) carried a non-BRCA PV. The identified PVs in BRCA genes (BRCA1 c.798_799delTT, BRCA1 c.3279delC, BRCA2 c.1310_1313del, and BRCA2 c.1658T>G) have been reported before and were classified as pathogenic. The identified founder PVs BRCA1 c.798_799del and BRCA2 c.1310_1313delAAGA represented 10% (3/30). Our MGPT allowed identification of several sequence variations in most investigated genes, among which we found novel truncating variations in PALB2 and BARD1 genes. The PALB2 c.3290dup and BARD1 c.1333G>T variants are classified as pathogenic. We also identified 42 variants of unknown/uncertain significance (VUS) in 70% (21/30) of patients with TNBC, including 50% (21/42) missense variants. The highest VUS rate was observed in ATM (13%, 4/30). Additionally, 35.7% (15/42) variants initially well-known as benign, likely benign or conflicting interpretations of pathogenicity have been reclassified as VUS according to ACMG-AMP.

Conclusions: PALB2 and BARD1 along with BRCA genetic screening could be helpful for a larger proportion of early-onset TNBC in Morocco.

Key words: Triple negative breast cancer, Next Generation Sequencing, Multigene panel testing, ACMG-AMP guidelines, Moroccan Population
Introduction

Breast cancer (BC) is the most commonly diagnosed malignancy and the leading cause of cancer death among women worldwide. An estimated 19.3 million new cases and 10 million cancer deaths occurred in 2020 [1]. According to the GLOBOCAN Cancer Tomorrow prediction tool, incident cases are expected to increase by more than 46% by 2040 [2]. The increasing global BC burden is mainly observed in low and medium human development index (HDI) countries [3], particularly women under the age of 50. Marked changes in lifestyle, socio-cultural contexts, and built environments are having a major impact on the prevalence of risk factors for BC burden in low and medium HDI countries [4]. In North Africa (i.e., in Morocco, Algeria, Tunisia, Libya and Mauritania), BC has rapidly overtaken cervical cancer as the most commonly diagnosed cancer [5]. The incidence among women aged 15-49 is lower than in Western countries, but the very low incidence among women over 50, combined to the young age pyramid of North Africa, makes the relative proportions of young patients substantially higher (50-60% versus 20% in France) [5]. The size and grade of breast tumors are increased, while the median age of onset (48) is more than ten years younger than the European/North American median of 61 [6]. Moreover, the relative frequency of triple negative and inflammatory breast cancer is also higher in North Africa [5].

BC is a heterogeneous and polygenic disease that can be divided into different molecular sub-types based on histological and genomic features. Increasing steroid hormone receptors expression (estrogen and/or progesterone receptors) defines different BC subtypes using immunohistochemistry (IHC) markers together with clinic-pathologic indexes. Approximately 70% of BCs are estrogen receptors (ER) positive (ER+) and/or progesterone receptor (PR) positive (PR+) tumors [7]. The human epidermal growth factor receptor type (HER) 2 amplification defines a second type, with an incidence of about 20% of BCs [8]. The remaining belongs to the triple negative breast cancer (TNBC) subtype, i.e. those that do not over-express ER, PR or HER2. TNBC is associated with advanced-stage disease and higher-grade tumors at diagnosis and is associated with an increased recurrence risk and poor five-year survival rates relative to other BCs [9].

Current evidence suggests the association between specific molecular subtypes and BRCA mutational status. BRCA1 PV carriers mainly increase TNBC, whereas BRCA2 carriers are more likely to increase ER+ and/or PR+ tumors [10]. Besides BRCA genes, an increasing number of studies have investigated genetic predisposition to TNBC using gene panel analysis [11,12]. PVs in non-BRCA genes have been observed in women with TNBC, and subsequent studies showed that PVs in BARD1, BRIP1, PALB2, RAD51C, and RAD51D are more common in TNBC compared to other BC subtypes [13]. PVs in established BC genes as well as other cancer susceptibility genes were identified in 14.4% (8.4% BRCA genes and 6.0% non-BRCA) of TNBC patients. PVs in BARD1, BRCA1, BRCA2, PALB2, and RAD51D were associated with high risk (Odds Ratio (OR)> 5.0) of TNBC. PVs in BRIP1, RAD51C, and TP53 were associated with moderate risk (OR > 2) of TNBC [11]. Moreover, PVs in BARD1, RAD51C, and RAD51D placed the patient at moderate risk for ER-BC and TNBC. Conversely, PVs in ATM, CDH1, and CHEK2 were associated with ER+ BC. Additionally, there was a higher prevalence of PVs in BRCA1, BRCA2/ and PALB2 observed with TNBC compared with ER+ BC, 8.13% versus 1.84%, respectively [14].

In North Africa, BRCA mutations frequency varied widely from ≈1% (Morocco) in sporadic BC [15] to 37.5% (Tunisia) in hereditary breast or/and ovarian cancer patients (HBOC) [16]. The pooled prevalence of BRCA-PVs among HBOC was 16% [17]. However, the contribution of BRCA and non-BRCA genes to TNBC has not yet been determined. In a recent study from Morocco, it has been reported that 22% of TNBC patients harbor PVs in the BRCA genes using Ion AmpliSeq BRCA1 and BRCA2 Panel [18]. Thus, there is a great need to investigate the frequency and importance of PVs in BRCA and non-BRCA genes among TNBC patients. Herein, we present the first Next Generation Sequencing (NGS)-based Multigene Panel Test (MGPT) study of 30 early onset (≤ 41 years of age) Moroccan women with TNBC. Panel sequencing was performed with genes involved in hereditary predisposition to cancer and candidate genes whose involvement remains unclear. This provides an advantage to map susceptibility genes for TNBC in North Africa.

Methods

Study subjects

Newly diagnosed women with TNBC were identified at the department of Obstetrics and Gynecology of Mohammed V Military Teaching Hospital in Rabat. TNBC patients were chosen according to the following criteria: age at diagnosis ≤ 41 years without family history or belonging to a family history. This family history of cancer was defined as diagnosed BC or OC in first- and second-degree relatives. Clinico-pathological data of
TNBC, including tumor site, histological type and grade and TNM classification were collected at BC diagnosis. A total of 30 early onset TNBC patients were enrolled between January 2020 and June 2020, including 18 (60%) without a family history and 12 (40%) with a family history. Informed consent was obtained from all participants at the time of peripheral blood draw. Patients consenting to participate in the study completed epidemiology and family history questionnaires and donated 10 ml of blood for genetic analyses.

**Immunohistochemical analysis**

The status of ER, PR, and HER2 receptors was determined using IHC analysis. Briefly, IHC analysis to determine ER and PR status was performed using standard procedures on 4 μm sections of paraffin embedded tissue specimens stained with the monoclonal antibodies 6F11 and 1A6 for ER and PR, respectively. Nuclear staining 1% was considered a positive result. The Hercept test was carried out in the institute since 2007 and was determined for all patients during the course of this study. Assays are scored with a 4-tiered system (0 − 3+). HER2 positivity was defined as strong complete membrane staining in at least 10% of tumor cells. Patients were considered HER2 if they had IHC 3+ by DAKO Hercept test. Tumors exhibiting equivocal HER2 expression, denoted as 2+ membranous staining of tumor cells, are confirmed by fluorescence in situ hybridization (FISH) at an outside laboratory. A signal ratios (HER2: CEP17) of ≥ 2.2 were classified as amplified. In the absence of positive FISH data, tumors scored 2+ by IHC were considered negative for HER2.

**DNA isolation**

Genomic DNA was isolated from 200 μl peripheral blood anti-coagulated with Ethylene diamine tetra acetic acid on Blood DNA Maxi kit (Qiagen, Hilden, Germany) following the manufacturer’s manual. DNA concentrations were assessed with the dsDNA HS assay kit by the Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA).

**Gene panel testing**

We performed targeted capture sequencing with a gene panel that is associated with high, intermediate and low cancer risk and candidate genes whose involvement remains unclear. Gene panel was designed by “Département d’Oncogénétique, Centre Jean Perrin, Clermont-Ferrand, France” according to the literature. The mode of inheritance is dominant in AIP, APC, ATM, BAP1, BARD1,BMPR1A, BRCA1, BRCA2, BRIP1, CASR, CDC73, CDH1, CDK4, CDKN2A, CHEK2, EPCAM, FANCM, FH, FLCN, MAX, MCM10, MEN1, MET, MITF, MLH1, MSH2, MSH6, MUTYH, NBN, NF1, NF2, PALB2, PMS2, POLD1, POLE, PTEN, RAD50, RAD51C, RAD51D, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, SMAD4, SMARCA4, STK11, TMEM127, TP53, and VHL. Additional genes such as BRK1, FAM175, GREM1, MLH3, MRE11, MSH2, NTH11, PMS1, RAD51, RAD51B, RINT1, RNF3, RNF43 and WRN were also included in the testing panel as candidate genes. The genes with a known association with BC are ATM, BARD1, BRCA1, BRCA2, CHEK2, MSH6, NF1, PALB2, PTEN, RAD51C, RAD51D, and TP53. A list of analyzed genes is provided in Table 1.

**Panel sequencing**

Sonic fragmentation of DNA was performed on a Bioruptor instrument (Diagenode). Kapa HTP library preparation and SeqCap EZ Choice probes and reagents (Roche) were used for library preparation and capture. Briefly, 20ng of genomic DNA was fragmented and processed by end-repairing, A-tailing and adapter ligation of paired-end indexed adapters, and a 7-cycle pre-capture PCR amplification. Further, the libraries were enriched through hybrid capture based method using specific probes. This was followed by PCR based enrichment, cleanup, and quantification of double stranded DNA using high sensitivity Qubit (Invitrogen, USA) measurement. Agencourt AMPure XP beads (Beckman Coulter, USA) were widely used for PCR amplicon purification and DNA size selection. The quality of fragmentation, library, and capture were evaluated using the Agilent 2100 Bioanalyzer system. A final library concentration ranging from 8 to 10 pM was used to carry out cluster generation and was sequenced on a Standard Flow Cell using V2 sequencing reagent kit (300 cycles) on MiSeq Instrument (Ilumina, San Diego, CA). A typical sequencing run consists of 12 samples. All steps were performed following the providers’ guidelines. No analysis of exons 11 to 15 of PMS2 and exons 1, 13, and 14 of SDHA, and no quantitative analysis of WRN exon 10 were performed, due to high identity with paralogs genes.

**Bioinformatics Analysis**

De-multiplexing was performed using bcl2fastq2 Conversion Software (Illumina). Sequencing reads were aligned to the University of California Santa Cruz hg19 reference genome assembly by Burrows-Wheeler Aligner [19]. Recalibration of base quality scores (BaseRecalibrator) and realignment (Realigner-TargetCreator, IndelRealigner) were carried out using Genome Analysis Toolkit (GATK) and PICARD tools.
as recommended by Eurogentest guidelines [20]. A variant calling was made using GATK Haplotype-Caller and annotated using Ensemble Variant Effect Predictor score ≥ 30, reads.

Variants were filtered for quality depth ≥ 30x, and presence in ≥ 20% of

Table 1. Triple negative breast cancer susceptibility genes

| Gene   | Genomic location | Coding transcript | Tumor types                                                                 | Cancer syndrome                                      |
|--------|------------------|-------------------|------------------------------------------------------------------------------|-------------------------------------------------------|
| AIP    | 1q13.3            | NM_003977.2       | Pituitary adenoma                                                             | pituitary syndrome                                    |
| APC    | 5q22.2            | NM_000038.5       | colorectal, pancreatic, desmoid, hepatoablastoma, glioma, other CNS          | adenomatous polyposis coli; Turcot syndrome           |
| ATM    | 11q22.3           | NM_000051.3       | leukaemia, lymphoma, medulloblastoma, glioma                                 | ataxia-telangiectasia                                 |
| BAP1   | 3p21.1            | NM_004656.2       | mesothelioma, uveal melanoma                                                 | tumor predisposition syndrome                         |
| BARD1  | 2q35              | NM_004655.2       | ovarian cancer, breast cancer, endometrioid cancer                            | ovarian cancer, breast cancer, endometrioid cancer    |
| BMPRIA | 10q23.2           | NM_004329.2       | gastrointestinal polyps                                                       | juvenile polyposis                                     |
| BRCA1  | 17q21.31          | NM_000294.3       | breast, ovarian                                                               | hereditary breast/ovarian cancer                      |
| BRCA2  | 13q13.1           | NM_000593.9       | breast, ovarian, pancreatic, leukaemia                                        | hereditary breast/ovarian cancer                      |
| BRK1   | 3p25.3            | NM_018462         | renal                                                                        | Von Hippel-Lindau (VHL) syndrome                      |
| BRIP1  | 17q25.2           | NM_032403.2       | AML, leukaemia, breast                                                       | Fanconi anaemia J, breast cancer susceptibility       |
| CASR   | 3q21.1            | NM_004883.3       | parathyroid, colorectal                                                       | hyperparathyroidism-jaw                               |
| CDC73  | 1q3.1             | NM_024529.4       | Parathyroid adenoma, multiple ossifying jaw fibroma                           | hyperparathyroidism-jaw tumour syndrome               |
| CDH1   | 16q22.1           | NM_004360.3       | gastric                                                                      | familial gastric carcinoma                            |
| CDK4   | 12q14.1           | NM_000075.3       | melanoma                                                                     | familial malignant melanoma                           |
| CDKN2A | 9p21.3            | NM_000077.4       | melanoma, pancreatic                                                           | familial malignant melanoma                           |
| CHEK2  | 22q12.1           | NM_007194.3       | breast                                                                        | Familial breast cancer                                |
| EPCAM  | 2p21              | NM_002354.2       | colorectal                                                                   | colorectal cancer, hereditary non polyposis, type 8   |
| FANCM  | 14q21.3           | NM_020937.2       | breast, ovarian                                                               | hereditary breast/ovarian cancer                      |
| FAM17S | 4q21.23           | NM_004329.2       | breast, ovarian                                                               | hereditary breast/ovarian cancer                      |
| FHI    | 1q43              | NM_000143.3       | leiomyosarcoma, renal                                                         | leiomyosarcoma and renal cell cancer                  |
| FLCN   | 17p11.2           | NM_149097.5       | renal, fibrofolliculomas, trichodiscomas                                      | Birt-Hogg-Dube syndrome                               |
| GREM1  | 15q13.3           | NM_013372         | colorectal                                                                    | hereditary mixed polyposis syndrome                   |
| MAX    | 14q23.3           | NM_002382.4       | pheochromocytoma                                                              | pheochromocytoma, susceptibility to                   |
| MCI1   | 16q24.3           | NM_002386.3       | skin                                                                         | familial malignant melanoma                           |
| MEN1   | 11q13.1           | NM_000244.3       | Thyroid adenoma, pituitary adenoma, pancreatic islet cell, carcinoid          | multiple endocrine neoplasia type 1                   |
| MET    | 7q31              | NM_00127500.1     | lung, gastric, colon                                                           | lung, gastric, and colon cancer                       |
| MIF    | 3p13              | NM_000248.3       | melanoma                                                                      | melanoma, cutaneous malignant, susceptibility to     |
| MLH1   | 3p22.2            | NM_000249.3       | colorectal, endometrial, ovarian, central nervous system                      | hereditary non-polyposis colorectal cancer, Turcot syndrome |
| MLH3   | 14q24.3           | NM_001040108.1    | colorectal, endometrial                                                       | colorectal cancer, hereditary nonpolyposis, type 7,  |
| MLHJ   | 11q22.3           | NM_000591.4       | colorectal                                                                    | Endometrial Cancer                                    |
| MRE11  | 11q21             | NM_000591.4       | colorectal                                                                    | colorectal cancer                                     |
| MSIN2  | 17p11.2           | NM_000251.2       | colorectal, endometrial, ovarian                                              | hereditary non-polyposis colorectal cancer            |
| MSIN6  | 2p11              | NM_000251.2       | colorectal, endometrial, ovarian                                              | hereditary non-polyposis colorectal cancer            |
| MUTYH  | 1p34.1            | NM_00128425.1     | colorectal                                                                    | adenomatous polyposis coli                            |
| NTHL1  | 14q24.3           | NM_00140108.1     | colorectal, breast                                                             | colorectal cancer, and colorectal polyposis.         |
| NBN    | 8q21.3            | NM_002485.4       | non-Hodgkin lymphoma, glioma, medulloblastoma, rhabdomyosarcoma               | nijmegen breakage syndrome                            |
| NF1    | 17q12.2           | NM_000267.3       | neurofibroma, glioma                                                           | neurofibromatosis type 1                               |
| NF2    | 22q12.8           | NM_000268.3       | neurofibroma, acoustic neuroma                                                | neurofibromin 2 (merlin)                              |
| PALB2  | 16p12.2           | NM_024675.3       | Wilms tumour, medulloblastoma, AML, breast                                    | Fanconi anaemia N, breast cancer susceptibility       |
| PMS1   | 2q31-q33          | NM_000534.4       | Colorectal, endometrial, ovarian, medulloblastoma, glioma                     | hereditary nonpolyposis colorectal cancer type 3       |
| PMS2   | 7q22.1            | NM_000535.5       | Colorectal, endometrial, ovarian, medulloblastoma, glioma                     | hereditary non-polyposis colorectal cancer, Turcot syndrome |
| POLD1  | 19q13.33          | NM_001256849.1    | colorectal                                                                    | Lynch syndrome                                        |
| POLE   | 12q24.3           | NM_006231.2       | colorectal                                                                    | colorectal cancer, adenomatous colorectal polyps, family |
| PTEN   | 10q23.31          | NM_000314.4       | Harmatoma, glioma, prostate, endometrial                                       | Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome   |
| RAD50  | 5q31.1            | NM_005723.2       | breast cancer                                                                  | Nijmegen breakage syndrome-like disorder              |
| RAD51  | 15q15.1           | NM_002875.5       | lung, colon, breast cancer                                                    | lung adenocarcinoma, glioblastoma, colon adenocarcinoma, breast invasive ductal |
| RAD51B | 14q23-q24.2       | NM_002877.5       | lung, skin, bladder, endometrial, prostate                                    | lung adenocarcinoma, cutaneous melanoma, bladder       |
| RAD51C | 17q22             | NM_058216.1       | breast, ovarian                                                                | adenocarcinoma, prostate adenocarcinoma               |
| RAD51D | 17q12             | NM_002878.3       | breast, ovarian                                                                | breast-ovarian cancer, familial, susceptibility to     |
| RE1    | 10q11.21          | NM_020975.4       | medullary thyroid, papillary thyroid                                            | multiple endocrine neoplasia 2A/2B                    |

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**Interpretation of variants**

An Interpretation of variants was conducted by following the classification system recommended by the American College of Medical Genetics and Genomics-Association for Molecular Pathology (ACMG-AMP) Standards and Guideline for the Interpretation of Sequence Variants [22]. The process can result in 1 of 5 classifications: benign, likely benign, unknown/uncertain significance (VUS), likely pathogenic, and pathogenic. Likely benign and benign variants were not clinically reported. All classifications were ultimately evaluated by AL, MC and referred to NU. The clinical significance of each sequence variant was also based on a set of criteria such as allele frequency as well as the information from clinical genome databases including ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

**In silico prediction**

A potential clinical effect of VUS was evaluated by the analysis of the severity of the amino acid changes and their conservation across species. These analyses were performed using the Mutation Taster through Alamut® Visual v.2.11.0 including Alignment-Grantham variation Grantham deviation (Align GVGD; http://agvgd.iarc.fr/agvgd_input.php), Polymorphism Phenotyping-2 (Poly-Phen-2; http://genetics.bwh.harvard.edu/pph2/), and Sorting Intolerant from Tolerant (SIFT; http://blocks.fhcrc.org/sift/SIFT.html) scores. The Alamut interactive software provides results and/or links to the following databases used for variant annotation: Exome Aggregation Consortium (ExAC), Genome Aggregation Database (gnomAD: https://gnomad.broadinstitute.org/), Database of Short Genetic Variation (dbSNP) and ClinVar.

**Complementary analysis**

Identified PVs were confirmed on a second patient sample. PVs in BRCA1 and BRCA2 genes were examined by NGS with Ion AmpliSeq BRCA1 and BRCA2 Panel (Life Technologies). The Sanger sequencing was performed for the novel PV in PALB2 and BARD1 genes using a 3500xl instrument and Big Dye terminator kit 3.1 (Applied Biosystems).

**Statistical analysis**

The statistical analysis used the chi-squared test, with p< 0.05 taken as the threshold for a significant difference.

**Results**

The mean age at diagnosis of TNBC was 38 (± 2.8) years. Most TNBC patients were diagnosed with infiltrating ductal carcinoma (IDC). The Scarff-Bloom-Richardson grades II and III were predominant (33.3% and 36.7% of cases, respectively). Axillary lymph nodes contained metastasis (N+) in 5 (16.7%) cases. Unilateral BC was diagnosed in all patients, and one (3.3%) had both BC and OC. The characteristics of the early onset TNBC patients are summarized in Table 2.

| Gene   | Genomic location | Coding transcript | Tumor types                                   | Cancer syndrome                          |
|--------|------------------|-------------------|----------------------------------------------|------------------------------------------|
| RNF43  | 17q23.2          | NM_017763.4       | breast cancer, colon                         | breast cancer, Lynch syndrome            |
| SDHA   | 5p15             | NM_004168.2       | colon, breast, pancreatic, endometrial, lung | colon adenocarcinoma, breast cancer, pancreatic adenocarcinoma, endometrial, lung adenocarcinoma |
| SDHAF2 | 11q12.2          | NM_017841.2       | lung, colon, breast, pancreatic, bladder    | lung adenocarcinoma, colon adenocarcinoma, breast cancer, pancreatic adenocarcinoma, bladder urethelial carcinoma |
| SDHB   | 1p36.13          | NM_003000.2       | paraganglioma                                | familial paraganglioma                   |
| SDHC   | 1q23.3           | NM_003000.2       | paraganglioma, pheochromocytoma             | familial paraganglioma                   |
| SDHD   | 11q23.1          | NM_003002.2       | paraganglioma, pheochromocytoma             | familial paraganglioma                   |
| SMAD4  | 18q21.2          | NM_005359.5       | gastrointestinal polyp                      | juvenile polyposis                       |
| SMARCA4| 19p13.3          | NM_00112844.1     | lung, colon, endometrial, bladder, breast   | lung adenocarcinoma, colon adenocarcinoma, endometrial endometrioid adenocarcinoma, bladder urethelial carcinoma, breast invasive ductal carcinoma |
| STK11  | 19p13.3          | NM_000455.4       | jejunal hamartoma, ovarian, testicular,    | Peutz-Jeghers syndrome                   |
| TMEM127| 2q11.2           | NM_017849.3       | pheochromocytoma, renal cell carcinoma      | pheochromocytoma, susceptibility to       |
| TP53   | 17p13.1          | NM_000546.5       | breast, sarcoma, adrenocortical carcinoma,  | Li-Fraumeni syndrome                     |
| VHL    | 3p25.3           | NM_000551.3       | renal, haemangiomma, pheochromocytoma       | Von Hippel-Lindau syndrome                |
| WRN    | 8p12             | NM_000553.4       | colon, gastric                               | peritoneal cancer, colon cancer, and stomach cancer |

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identified PVs. The BRCA1 c.798_799del (p.Ser267LysfsX19) variant was found in two unrelated TNBC patients. Both carriers were diagnosed less than 38 years of age and showed a strong family history of BC. The BRCA1 c.798_799delTT variant, located in exon 11, is a frame-shift variant including two small deletions, two bases (TT) deletion. The deletion causes a frame-shift which changes a Serine to a Lysine at codon 267, and creates a premature stop codon at position 19 of the new reading frame. The BRCA1 c.798_799delTT variant, previously reported as BRCA1 917_918delTT using alternate nomenclature, has been reported in association with familial and early-onset BC and OC and has been described as a North African identified founder variant [15, 23-26].

| Characteristics          | Patients |
|--------------------------|----------|
| Age                      | 38 (± 2.8) |
| Family history           |          |
| Positive                 | 18 (60%) |
| Negative                 | 12 (40%) |
| Histologic               |          |
| IDC                      | 19 (63.3%) |
| Lobular                  | 7 (23.3%) |
| Medullar                 | 2 (6.7%) |
| Others                   | 2 (6.7%) |
| Tumour size              |          |
| T1                       | 5 (16.7%) |
| T2                       | 10 (33.3%) |
| T3                       | 11 (36.7%) |
| T4                       | 4 (13.3%) |
| SBR grading              |          |
| I                        | 7 (23.3%) |
| II                       | 10 (33.3%) |
| III                      | 11 (36.7%) |
| IV                       | 2 (6.7%) |
| Lymph node status        |          |
| Positive                 | 5 (16.7%) |
| negative                 | 7 (23.3%) |

IDC: invasive ductal carcinoma; SBR: Scarff-Bloom-Richardson.

Table 2. Demographic and clinical-pathologic characteristics of 30 TNBC samples

The BRCA1 c.3279delC (p.Tyr1094IlefsX15) variant was detected in a young woman diagnosed with TNBC at the age of 37. The BRCA1 c.3279delC variant, located in exon 11, is a frame-shift variant including one small deletion, one base (C) deletion. The deletion causes a frame-shift which changes a Tyrosine to an Isoleucine at codon 1094, and creates a premature stop codon at position 15 of the new reading frame. Using alternate nomenclature, The BRCA1 c.3279delC variant would be defined as BRCA1c.3390delC.

The BRCA2 c.1310_1313delAAGA (p.Lys437Ilefs) variant, a specific founder variant from the North-East of Morocco [27], was detected in early onset women diagnosed with both TNBC (at the age of 35) and OC (at the age of 38). The BRCA2 c.1310_1313delAAGA variant, located in exon 10, causes a frame-shift which changes a Lysine to an Isoleucine at codon 437 and creates a premature stop codon at position 22 of the new reading frame. It has previously reported as BRCA2 1310del4, BRCA21537del4, and BRCA21538del4 using alternate nomenclature.

The BRCA2 c.1658T>G (p.Leu553Ter) variant, located in exon 11, causes a non-sense substitution. It was observed in one patient without family history, diagnosed with TNBC at the age of 36. In databases, the BRCA2c.1658T>G variant was associated with breast-ovarian cancer, hereditary cancer-predisposing syndrome, or hereditary breast or/and ovarian cancer syndrome.

Our panel sequencing allowed identification of several sequence variations in most investigated genes, among which we found novel variation in PALB2 and BARD1 genes. The PALB2 c.3290dup (p.Lys1098) variant has not been reported before in the BIC database. The PALB2 c.3290dup variant was detected in a young patient diagnosed with TNBC at an age ≤ 36 years. Family history was negative in the PV carrier. Thus, we concluded that this PV is not linked to family history of BC and/or OC. The PALB2 c.3290dup variant is a frame-shift variation due to the insertion of C nucleotide at acid 3290 of codon 1097 in exon 12, which is predicted to lead to a premature stop codon 1098 and a truncated protein. The BARD1 c.1333G>T (p.Glu445), that has not been reported previously, is classified as pathogenic (Class 5). The BARD1 c.1333G>T variant has not been reported before in the BIC database. The BARD1 c.1333G>T variant occurred with BRCA2 c.1658T>G (p.Leu553Ter) in early onset TNBC women diagnosed with pancreatic cancer at the age of 32. The early disease onset and having two cancers in this case may be the result of harboring two PVs in BRCA2 and BARD1 genes. Details of PVs detected by NGS-based MGPT in our study are reported in Table 3.

We also identified 42 VUS in 70% (21/30) of TNBC patients, including 50% (21/42) missense variants. The other VUSs were characterized as intronic or synonymous variants. A high rate was expected given the number of genes included in our panel gene. The BC susceptibility gene carrying the highest number of VUS was ATM (13%,4/30). Additionally, 35.7% (15/42) variants initially well-known as benign, likely benign or conflicting interpretations of pathogenicity have been reclassified as VUS according to the ACMG-AMP classification. Details of the identified VUS are shown in Table 4.

http://www.jgenomics.com
Table 3. Pathogenic variants and likely pathogenic variant detected by NGS based-MGPT in Moroccan TNBC patients

| Age at diagnosis | Affected gene | Nucleotide change | Amino acid change | Type of variant | Rs number | ClinVar Database | ACMG Classification |
|------------------|---------------|-------------------|-------------------|----------------|-----------|------------------|---------------------|
| 1907L0132        | c.798_799del  | p.Ser267fs        | Frameshift         | Pathogenic     | rs80337724 | 5                |                     |
| 1907L0133        | c.3279del     | p.Tyr1094Ilefs    | Frameshift         | Pathogenic     | rs397590050 | 5                |                     |
| 1907L0141        | c.1310_1313del| p.Lys431Ilefs     | Frameshift         | Pathogenic     | rs80339277 | 5                |                     |
| 1907L0145        | c.1658G>T     | p.Leu553Ter       | Nonsens            | Pathogenic     | rs876659627 | 5                |                     |
| 1907L0146        | c.3290dup     | p.Lys1098         | Frameshift         | Not reported   | Not reported| Not reported     |                     |

ACMG: American College of Medical Genetics and Genomics.

Table 4. Variant of unknown/uncertain significance detected by NGS based-MGPT in Moroccan TNBC patients

| Gene     | Sequence variant | Amino acid change | Type of variant | Rs number | ClinVar Database | ACMG Classification |
|----------|------------------|-------------------|-----------------|-----------|------------------|---------------------|
| APC      | c.-151G>C        | Non coding        | 5 Prime UTR     | rs1029997545 | Likely benign    | 3                   |
| APC      | c.325C>T         | p.Arg109Trp       | Missense        | Not reported| Not reported     | 3                   |
| APC      | c.295C>T         | p.Arg99Trp        | Missense        | rs139196838 | Conflicting interpretations of pathogenicity | 3 |
| APC      | c.835-41A>G      | Non coding        | Intron Variant  | Not reported| Not reported     | 3                   |
| APC      | c.781-41A>G      | Non coding        | Intron Variant  | Not reported| Not reported     | 3                   |
| ATM      | c.1959C>G        | p.Cys532Trp       | Missense        | rs5963548   | Conflicting interpretations of pathogenicity | 3 |
| ATM      | c.1810C>T        | p.Pro606Ser       | Missense        | rs2227922   | Conflicting interpretations of pathogenicity | 3 |
| ATM      | c.9002G>A        | p.Ser300Asn       | Missense        | rs587781431 | VUS               | 3                   |
| ATM      | c.8560C>T        | p.Arg2854Cys      | Missense        | rs201958469 | VUS               | 3                   |
| BARD1    | c.1028C>T        | p.Thr341Ile      | Missense        | rs201032007 | Conflicting interpretations of pathogenicity | 3 |
| BRC4A    | c.3587C>T        | p.Thr196Ile      | Missense        | rs80336944  | VUS               | 3                   |
| BRIP1    | c.415T>G         | p.Ser139Ala       | Missense        | rs202072866 | VUS               | 3                   |
| BMP1A    | c.431-30A>G      | Non coding        | Intron Variant  | Not reported| VUS               | 3                   |
| CDKN2A   | c.13T>A          | p.Phe51Ile        | Intron Variant  | rs776987532 | VUS               | 3                   |
| CDKN2A   | c.36T>A          | p.His123Gln       | Missense        | rs6413463   | Conflicting interpretations of pathogenicity | 3 |
| FANCM    | c.1667A>G        | p.Asp556Gly       | Missense        | rs148810507 | VUS               | 3                   |
| FANCM    | c.1576C>G        | p.Leu526Val       | Missense        | rs144215747 | VUS               | 3                   |
| MET      | c.841T>G         | p.Phe281Val       | Missense        | Not reported| Not reported     | 3                   |
| MIF      | c.-28C>T         | Non coding        | 5 Prime UTR     | Not reported| Not reported     | 3                   |
| MLH3     | c.3746C>T        | p.Ser1245Phe      | Missense        | rs139265757 | Benign            | 3                   |
| MSH2     | c.965G>T         | p.Gly322Val       | Missense        | rs987188    | VUS               | 3                   |
| MSH4     | c.2540A>T        | p.Glu847Val       | Missense        | Not reported| Not reported     | 3                   |
| NBN      | c.425A>G         | p.Asn142Ser       | Missense        | rs769414    | Conflicting interpretations of pathogenicity | 3 |
| NF1      | c.8161-45A>C     | Non coding        | Intron Variant  | rs17879551  | Likely benign    | 3                   |
| NF1      | c.8098-45A>C     | Non coding        | Intron Variant  | rs17879551  | Likely benign    | 3                   |
| NTHL1    | c.86G>T          | p.Pro291Leu       | Missense        | Not reported| Not reported     | 3                   |
| PMS1     | c.-99G>T         | Non coding        | 5 prime UTR     | rs577363454 | VUS               | 3                   |
| PMS2     | c.250A>C         | p.Thr84Pro        | Missense        | rs1554304938| VUS               | 3                   |
| POLD1    | c.883G>A         | p.Val295Met       | Missense        | rs19954019  | Conflicting interpretations of pathogenicity | 3 |
| POLD1    | c.2388+5G>A      | Non coding        | Intron Variant  | rs750858275 | VUS               | 3                   |
| POLD1    | c.1014C>G        | p.Cys338Trp       | Missense        | Not reported| Not reported     | 3                   |
| RAD50    | c.2354C>T        | p.Ala765Val       | Missense        | Not reported| Not reported     | 3                   |
| RAD51    | c.1080C>G        | p.Cys330Trp       | Missense        | Not reported| Not reported     | 3                   |
| RET      | c.1756T>C        | Leu586Phe         | Missense        | rs777604634 | VUS               | 3                   |
| RNF43    | c.172A>G         | Thr58Ala          | Missense        | Not reported| VUS               | 3                   |
| RNF43    | c.2054A>C        | Thr685Asn         | Missense        | Not reported| Not reported     | 3                   |
| RNF43    | c.611C>T         | Non coding        | Missense        | rs2636625   | Not reported     | 3                   |
| SDHA     | c.1367C>T        | Ser456Leu         | Missense        | rs76869145  | Benign            | 3                   |
| SMARCA4  | c.722_733del     | Gly241_Pro244del  | Inframe Deletion | rs568390760 | Conflicting interpretations of pathogenicity | 3 |
| SMARCA4  | c.722_733del     | Gly241_Pro244del  | Inframe Deletion | rs568390760 | Conflicting interpretations of pathogenicity | 3 |
| WRN      | c.1530_1532del   | Glu510del         | Initiator Codon | rs781777438 | VUS               | 3                   |
| WRN      | c.3785C>G        | Thr1262Arg        | Missense        | rs78488552  | Conflicting interpretations of pathogenicity | 3 |

ACMG: American College of Medical Genetics and Genomics; VUS: variant of uncertain significance.

Discussion
The absence of specific molecular markers for TNBC has made the targeted treatments extremely challenging and the death rates very high compared to the other BC subtypes. The NGS offers several clinical applications in cancer and precision oncology that are significant for risk predictors, early detection of disease, diagnosis by sequencing and medical imaging, accurate prognosis, biomarker identification and identification of therapeutic targets for novel drug discovery [28]. To identify TNBC patients who
might benefit from treatment strategies, BRCA and non-BRCA genes testing through NGS could lead to a more accurate prediction of the responsiveness to platinum and poly (ADP-ribose) polymerase (PARP) inhibitors. Thus, this strategy should be considered in management and precision medicine [29]. Recent data have hypothesized that patients with advanced-stage TNBC associated with PVs in BRCA genes might be specifically sensitive to PARP inhibition; both Olaparib and Talazoparib are currently approved for such situation [30,31]. Moreover, there is some evidence that adding platinum-agents in the neoadjuvant setting improves the pathologic complete response [32,33]. The role of PARP inhibitors in the setting of non-BRCA associated cancers has been limited. Recently, Lapatinib plus Veliparib therapy have a manageable safety profile and promising antitumor activity in advanced TNBC cancers has been limited. Robertson et al. showed that diagnosis of TNBC below 50 years would be a suitable age threshold for BRCA testing and may be a cost effectiveness strategy [42]. Lu et al. recommended genetic testing for TNBC patients diagnosed before the age of 50, a population with BRCA PV frequency of 17.5%. The authors also highlighted data showing that the estimated BRCA PV frequency of individuals with any type of BC diagnosed before the age of 40 is 11% [43]. Overall, testing in the UK has an unusually high threshold in comparison with the other European countries, where in all cases <41 years would be eligible for screening [44].

When stratified by family history, our study showed that 13% (4/30) early onset TNBC patients with a family history were identified with PVs in BRCA genes, compared to 3.3% (1/30) without a family history. Couch et al. revealed that 12.2% (66/539) TNBC patients with a family history carry BRCA PVs, compared to 8.6% (83/969) patients without a family history [12]. Hartman et al. identified 21 BRCA PVs (13 in BRCA1 and 8 in BRCA2) in a cohort of 199 unselected women with TNBC and providing an overall prevalence rate of 10.6%. Additionally, 5.2% (8/153) PVs were found in 153/199 patients without significant family history [45]. In an unselected cohort study in 77 TNBC patients, it was found that 19.5% (15/77) had BRCA PVs including 15.6% (12/77) in BRCA1 and 3.9% (3/77) in BRCA2 [46]. In a Canadian TNBC cohort (n=54) with no familial BC aggregation, 9% (5/54) with PVs in BRCA1 and 2% (1/54) of patients with PVs in BRCA2 were detected [47]. Lu et al. stated the importance of BRCA testing regardless of family history as few female family members or small families can mask genetics relative to BC and OC [43].

Muendlein et al. conclude that the prevalence of BRCA PVs is high in TNBC patients and are not restricted to young women or patients with a positive family history [48]. Although family history is commonly used to identify individuals with a possible predisposition to BC, Zang et al. showed that family history could not predict an underlying predisposition cancer syndrome in most patients. Furthermore, some individuals with cancer have de novo mutations, whereas others inherit them with incomplete penetrance; where, the family history is
likely to be negative [49].

**TNBC and non BRCA-genes**

In recent years it has become clear that truncating PALB2 variants have been shown to be associated with a high risk for breast, ovarian and pancreatic cancers [50-52]. In our investigation, the truncating variant PALB2 c.3290dup was found in 3.3% (1/30) of TNBC cases. According to the ACMG-AMP guidelines, the PALB2 c.3290dup variant was characterized as pathogenic (class 5) and considered predisposing for TNBC. Family history was negative in a PALB2 c.3290dup carrier. Genetic testing for PALB2 would provide another key genetic marker to identify women at elevated risk of TNBC regardless of their family history of BC. Our results suggest that the PALB2 gene contributes to the risk of TNBC in North Africans and highlights the need to identify PVs in the PALB2 gene which has a significant impact on an individual’s risk of TNBC in younger women.

Similar observations are made when comparing the frequency of PVs reported here to that reported in the study of Zanati et al. in which 4.3% of TNBC patients carried PVs in PALB2 [52]. The observed frequency of PALB2 PVs in our study (3.3%) and the last one appears to be higher compared to other populations (1.3-1.4%) [11,13]. Low prevalence of PALB2 germline mutation (~1%) was observed in 347 Australian TNBC women, similar to the prevalence of PALB2 germline mutation of 1% in familial non-BRCA genes BC cohorts [11]. PALB2 PVs might be overrepresented in patients with TNBC in earlier studies performed in European cohorts [54,55]. It is estimated that 30% [56] and 34% [57] of BC patients with a germline PALB2 PV have a TNBC subtype. Shimelis et al. found that PVs in PALB2 were found to be associated with a high-risk of TNBC with an OR of 14.41 (95%CI; 9.27-22.60) and were enriched in patients with TNBC compared to non-TNBC tumors with an OR of 2.12 (95%CI; 1.63-2.74) [11]. The relatively poor survival from BC in patients with a PALB2 PV was not attributable to the high prevalence of triple-negative phenotype [57]. Due to the lower PV carrier frequency in the PALB2 gene in the population, broad-based studies are needed to refine the genetic testing criteria and the management of the patients and their family members.

**BARD1 targeted sequencing studies** showed that PVs in BARD1 gene are enriched in TNBCs, which are associated with higher rates of recurrence, progression, and mortality [12,13]. BARD1 PVs were increased by more than threefold in TNBC cases (0.67%) compared to non-TNBC cases (0.18%), suggesting that BARD1 is a predominantly TNBC predisposition gene [11]. In our study, the BARD1c.1333G>T variant was identified in 3.3% (1/30) of TNBC women. According to the ACMG-AMP guidelines, BARD1 c.1333G>T was characterized as pathogenic (class 5).

Similar observations are made when comparing the frequency of BRAD1PVs reported here to that reported in previous studies. De Brakeleer et al. suggest that TNBC patients are enriched for PVs in BRAD1 as compared to control samples and high BC risk families when they identified four harbored variants in BARD1, of which two protein-truncating variants (c.1347A>G and c.1972C>T) have been confirmed as pathogenic [58]. In a study of 105 women with TNBC from a trial exploring the anti-tumor activity of neoadjuvant carboplatin/docetaxel chemotherapy, BARD1 PVs were detected in two TNBC patients (1.9%) [59]. The Analysis of 1824 TNBC patients unselected for age or family history of BC led to the identification of 0.5% (9/1824) cases with a BARD1-truncating variant [12]. In Buys’s study, the prevalence of BARD1 PVs was higher among women with TNBC (3.3%) than among women with non-TNBC (1.7%) [13]. Shimelis et al. identified 25 individuals harboring BARD1 PVs (0.61%) and reported an OR of 5.92 for TNBC cases of African American and Caucasian populations [11]. Rofes et al. identified ten BARD1 PV carriers from 680 TNBC patients (carrier frequency = 0.9%), resulting in an OR = 5.40 [60]. Similar observations were reported in other studies [61,62], indicating that BARD1 is a risk gene for TNBC. Although the BARD1 gene offers a new hope for improving the TNBC therapy, the low number of BARD1 PV carriers, the non-identification of a recurrent hotspot variant, a study with an sufficient sample size, a lack of geographically matched population controls have complicated the interpretation of the results and could hamper the strength of the association of BARD1 PVs with TNBC risks [56]. Further studies in larger cohorts will be necessary to more precisely assess the BARD1-associated risk with this tumor phenotype.

**TNBC and VUS**

VUS represent a significant proportion of variants identified in clinical genetic testing, which account for about 40% of the total variants [63]. In our study, 42 VUS were identified in 70% (21/30) of TNBC patients including 50% (21/42) distinct misense variants. The gene with the highest VUS frequency was the ATM (13%, 4/30). The ATM is a very large gene and is one of the genes with more identified VUS (40%) including missense, in-frame, or synonymous mutations [63]. In our MGPT, the genes that contain more VUS are among the top ten genes cited in the literature with the highest number of
variants submitted. A previous study has shown that the rate of VUSs was proportional to the number of genes analyzed in the MGPT in TNBC [64]. Identification of the VUS has become a daily fact of life when tailoring genetic counseling, but little guidance is available for how best to approach them, and limited data are available on how they are affecting the medical practice and the well-being of cancer patients [65]. In the lack of reliable clinical information or functional evidence the VUS remains non-informative in risk management and decision making.

Management recommendations for diagnostic and treatment decision-making for the carriers of PVs in BRCA and non-BRCA genes have been established. Recommendations are focused on a combination of annual magnetic resonance imaging (MRI) and mammography for women with familial risk or BRCA PV and a history of BC [66-68]. Guidelines also made recommendations for the treatment of individuals with a BRCA carrier or those with a strong familial risk of developing BC. More recent European and US NCCN guidelines have updated recommendations regarding BRCA-targeted PARP inhibitor therapy in BC [69]. Current evidence suggests that the BC risk for PALB2 mutation carriers may overlap with that for BRCA2 mutation carriers, particularly in the context of a significant family history [55]. Accordingly, recently introduced NCCN practice guidelines suggest that a PALB2 carrier should undergo a MRI or a mammography screening [70,71]. Several studies have shown that the BARD1 can potentially become a new target for BC treatment. Li et al. have reported that the BARD1 BRCT domain interacts with PAR, which results in a subsequent recruitment of the BARD1-BRCA1 complex to the damaged DNA [72]. A higher BARD1 and BRCA1 expression is associated with a worse prognosis of early BC patients, especially the ones that received a radiotherapy, indicating the potential use of PI3K inhibitors to reverse chemoresistance and radioresistance in ER+ BC patients [73]. Mammography and MRI remain the fundamental imaging modalities for the high and very high-risk BC patients. An interesting approach might be radiogenomics, which brings together the clinical assessment, imaging results, and the genetic background [74]. This approach would be of interest in relation to the immunohistochemical staining of the BARD1 gene, which in turn can be imaged in magnetic resonance scans [75].

Limitations

Our study has some limitations that should be acknowledged. The sample size was relatively small. Thus, our data underscore the need for larger series to better understand the frequency and the contribution of PVs in BRCA, PALB2 and BARD1 genes in patients with early onset TNBC. Despite the reduced sample size in our cohort, our findings support those deleterious PVs in PALB2 and BARD1 were enriched in TNBC patients. Our analysis does not include copy number variants. Although having many advantages, large sequencing panels still have limitations compared to the traditional Sanger sequencing test or smaller NGS panels in cancer precision. In our MGPT, Some genes or exons are not well captured and therefore are not covered, resulting in some variants within these regions going undetected and hence being refractory to analysis. Despite these limitations, our findings may help in implementing effective strategies for BRCA, PALB2 and BARD1 PVs testing in TNBC patients.

Conclusion

Defects in homologous recombination DNA repair genes that may be targeted with PARP inhibitors occur in 60 to 69% of TNBC. PARP repairs the damaged DNA and renders the tumor highly sensitive to platinum-based chemotherapy. With the rise of NGS, it is possible to target multiple genes rapidly and simultaneously in a cost-effective manner. Defining groups of TNBC patients with BRCA, PALB2 and BARD1 PVs is important for the clinical management of patients because several new treatment strategies are being evaluated for related tumors. Overall, an improvement in the detection rate by using an extensive panel analysis determines the hereditary cancer to which the patients and families are exposed based on their history and genetics as part of a targeted therapy [76].

Supplementary Material

Supplementary figure s1. http://www.jgenomics.com/v09p0043s1.pdf
Supplementary table S2. http://www.jgenomics.com/v09p0043s2.xlsx
Supplementary table S3. http://www.jgenomics.com/v09p0043s3.xlsx

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Author contributions
AL, NU, OE, YS and Y-JB designed the study. AL, NU, MC, and OE wrote this paper. JK, DMR, KG, MI, RT, YS, MO, and AA provided samples. AL, MC, NU, YB, and RH analyzed the data. RH and HA reviewed the manuscript. All authors read and approved the manuscript.

Competing Interests
The authors have declared that no competing interest exists.

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