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# Identification of germline pathogenic variants in DNA damage repair genes by a next-generation sequencing multigene panel in BRCAX patients.

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**ABSTRACT**

Background

Approximately 5-10% of breast carcinomas have been related to hereditary conditions and are attributable to pathogenic variants in the *BRCA1* and *BRCA2* genes, which is referred to as hereditary breast and ovarian cancer (HBOC) syndrome. The inclusion of additional genes that can be related to HBOC syndrome is under intense evaluation due to the high proportion of patients with HBOC criteria who do not present pathogenic mutations in *BRCA* genes, named BRCAX, despite having high clinical suspicion of hereditary cancer. The main aim is to identify new potentially pathogenic gene variants that may contribute to HBOC to improve the efficiency of routine diagnostic tests in this hereditary condition.

Methods

A retrospective cohort of 77 HBOC BRCAX patients was analyzed by next-generation sequencing using a targeted multigene panel composed of 25 genes related to hereditary cancer and deficiencies in DNA repair pathways.

Results

We found 9 variants in 7 different genes, which were confirmed by automated sequencing. Six variants were classified as pathogenic or likely pathogenic. Three of them were located in the *PALB2* gene, one in the *BRIP1* gene, one in the *BARD1* gene and 1 in the *RAD50* gene. In addition, three variants of uncertain significance (VUS) were detected in the *TP53, CHEK2,* and *CDH1* genes.

Conclusions

We identified that 8% of BRCAX patients were carriers of pathogenic variants in genes other than *BRCA1* and *BRCA2*. Therefore, wide gene panels, including clinically actionable genes, should be routinely used in the screening of HBOC in our population. We observed differences from other studies in the prevalence of mutated genes, most likely due to differences in the selection criteria of the probands and in the population analyzed. The high incidence of deleterious variant detection in *PALB2* supports its significant role in breast cancer susceptibility and reinforces its inclusion in the HBOC genetic diagnostic process.

**KEYWORDS:**

Hereditary breast and ovarian cancer; *BRCA1*; *BRCA2*; next-generation sequencing; DNA repair; genetic testing

## 1. INTRODUCTION

Breast cancer is a common disease, affecting over one million women worldwide every year (Bray et al. 2018). Though the vast majority of breast cancers are sporadic in nature, approximately 5–10% of them are due to autosomal dominant inheritance of a specific genetic mutation (Apostolou and Fostira 2013). Hereditary breast and ovarian cancer syndrome (HBOC) is a well-known syndrome caused by germline *BRCA1* and *BRCA2* pathogenic mutations. These two genes are the most well-studied and best-known genes implicated in hereditary breast cancer susceptibility. However, mutations in *BRCA1* and *BRCA2* account for no more than 20% of all inherited breast cancers (Easton 1999; Easton and Peto 1990; Ford et al. 1998; Shih et al. 2002). The remaining 80% are so-called BRCAX patients because they are high-risk breast and/or ovarian cancer patients, negative for *BRCA1* and *BRCA2* mutations. Other pathogenic variants have also been described in high or moderate breast cancer (BC) susceptibility genes since they are mainly associated with other cancer syndromes: *ATM* (ataxia telangiectasia), *TP53* (Li-Fraumeni syndrome), *PTEN* (Cowden syndrome), *CDH1* (hereditary diffuse gastric cancer), *STK11* (Peutz-Jeghers syndrome), *PALB2* or *CHEK2* (both related to breast cancer) (Frey and Pothuri 2017; González and Honrubia 2018).

Although the "gold-standard" technique used to detect mutations is automatic sequencing based on Sanger technology, next-generation sequencing (NGS) has been widely introduced not only because of its flexibility and speed but also because it is cost- and time-effective. With the emergence of multigene panel analyses, more genetic counseling units have included this type of genetic study in some specific diseases, such as hereditary cancer or neonatal pathologies (Brunelli et al. 2017). Specifically, in hereditary breast cancer, the most extensive panels used include between 20 to 30 genes linked to this disease. Many of these genes code for proteins involved in the homologous recombination repair (HRR) process and other protein co-factors necessary for the successful repair of DNA (Frey and Pothuri 2017). Most of them were selected because both germline and somatic pathogenic variants in *BRCA* genes and in HRR-related genes (*ATM, BARD1, BRIP1, CHEK1, CHEK2, FAM175A, MRE11A, NBN, PALB2, RAD51C, RAD51D*) can deregulate HRR pathways. Indeed, some of these DNA repair pathways are currently being targeted by emerging therapies (Coyne, Chen, and Kummar 2011; Pujade-Lauraine et al. 2017).

The discovery of new genes involved in the susceptibility to hereditary cancers is constantly under investigation, and a great number of new germline mutations have been identified, which allows for a deeper knowledge of the mechanisms that predispose patients to cancer. Therefore, the detection of germline and somatic pathogenic variants that lead to protein malfunctioning is useful not only for primary and secondary prevention in hereditary cancer but also for the selection of cancer treatment. Thus, the need to identify the contribution of other genes in hereditary breast cancer is crucial. In fact, in cancer care units, genetic studies are well-established diagnostic tools to determine the origin of a cancer aggregation in a family to achieve early detection and to apply the most appropriate preventive measures (Bermejo-

Pérez, Márquez-Calderón, and Llanos-Méndez 2007). Additionally, the importance of identifying alterations in these genes lies in the capacity of the detection of high-risk asymptomatic individuals than can benefit from cancer prevention programs (Godet and Gilkes 2017). Likewise, patients carrying tumors with *BRCA1* and *BRCA2* mutations may also benefit from certain targeted antineoplastic therapies (Godet and Gilkes 2017).

This study analyzed 25 genes involved in other highly prevalent hereditary cancer syndromes in a cohort of breast cancer patients without *BRCA1* and *BRCA2* pathogenic variants. Our aim was to identify new potentially predisposing gene variants in cancer susceptibility genes to improve the efficiency of routine diagnostic tests.

## 2. MATERIAL AND METHODS

### 2.1. Patients and samples

The selected participants were 77 women who were patients in the Genetic Counseling Unit of the Oncology Institute of South Catalonia (IOCS). These patients fulfill the HBOC criteria to undergo genetic testing of the *BRCA1* and *BRCA2* genes according to the Spanish Society of Medical Oncology guidelines (Llort et al. 2015). A 10-mL blood sample was collected from each patient in an EDTA blood collection tube, and DNA was extracted using the commercial Gentra® PureGene DNA Isolation Kit according the manufacturer's instructions (Qiagen, Barcelona, Spain). The *BRCA1* and *BRCA2* genes were analyzed by sequencing and multiple-ligation-dependent probe amplification (MLPA), and none of the detected variants were classified as pathogenic variants according to the ENIGMA Consortium classification criteria (version 2.5.1 29 June 2017) and the 5-tiered classification system proposed by Plon et al. (Plon et al. 2008). The median age of cancer onset was 47 years (range 29-72 years). Personal and family clinical data of the patients were collected, as well as the histology, hormonal receptor status and Her-2 expression in the tumor. The study was approved by the Clinical Research Ethics Committee of Sant Joan University Hospital (Reus, Spain), and written informed consent was obtained from all participants.

### 2.2. Targeted NGS

To perform targeted NGS, we modified the Ion AmpliSeq™BRCA Reflex, Hereditary Cancer Research panel (ThermoFisher Scientific) described by Hirotsu and colleagues (Hirotsu et al. 2015) that includes 25 genes related to hereditary cancer, most of which are considered moderate and low penetrance breast cancer genes (Shannon and Chittenden 2012). Using Ion

AmpliSeq designer software (ThermoFisher Scientific), we expanded the target amplicon length to 375 base pairs to increase the number of samples analyzed in one chip. The panel now includes 479 primer pairs distributed into two pools, with an amplicon range between 125 and 375 base pairs, with a minimum exon padding of 5 base pairs that enables the detection of splicing variants and with 99.54% coverage (322 bases missed, most of them in the MUTYH gene). *BRCA1* and *BRCA2* were not included since they have been previously analyzed. The 25 genes included in the panel are AP*C, ATM, BARD1, BMPR1A, BRIP1, CDH1, CDK4, CDKN2A, CHEK2, EPCAM, MLH1, MRE11A, MSH2, MSH6, MUTYH, NBN, PALB2, PMS2, PTEN, RAD50, RAD51C, RAD51D, SMAD4, STK11,* and *TP53,* which are relevant for a considerable number of hereditary cancer syndromes.

Libraries were generated by multiplex PCR amplification of 20 ng of DNA from each sample as described previously (Hirotsu et al. 2015; Simbolo et al. 2015) with the Ion One Touch 2 System (Ion Torrent™) and the Ion Personal Genome Machine (PGM™) system (Ion Torrent™).

### 2.3. Data analysis, variant prioritization and *in silico* analysis

The obtained sequences were aligned against the UCSC hg19 human reference genome sequence, and variant calling was performed using Torrent Suite Software v3.6 (Ion Torrent™). Genetic variant annotation of indels, single nucleotide variants and splice-site variants was performed by Ion Reporter Software v5.2 (Ion Torrent™). Regions with at least 20-fold depth of coverage were used to call variants. To prioritize variants detected by NGS-based analysis, we used wANNOVAR (Chang and Wang 2012; Wang, Li, and Hakonarson 2010) to obtain those variants that were not present in all the samples. Alignments were verified with Integrative Genomics Viewer (IGV) to visually inspect the detected variants (Robinson et al. 2011), and variants were identified with Alamut Visual version 2.9.0 (Interactive Biosoftware, Rouen, France, www.interactive-biosoftware.com).

### 2.4. Sanger sequencing

All variants detected by NGS that were suspected to be pathogenic or probably pathogenic were confirmed by bidirectional Sanger sequencing using genomic DNA and primer pairs flanking the variant position (primer sequences and PCR conditions available upon request). Sequencing was performed by capillary electrophoresis on an ABI3500 Genetic Analyzer (Applied Biosystems).

We also analyzed the missing regions or regions with insufficient read depth coverage (under 20X) for reliable heterozygous variant detection by capillary electrophoresis.

### 2.5. Variant classification

To classify the detected variants, we used the American College of Medical Genetics and Genomics (ACMG) classification standards and guidelines (Richards et al. 2015). The five categories included the following: pathogenic, likely pathogenic, uncertain significance, likely benign and benign, and the 5-tier classification system previously described (Plon et al. 2008). Variants were classified as variants of uncertain significance (VUS) when no functional data were available or the risk was not clearly established according to the literature or gene databases. The following public databases were used for the interpretation of the variants: ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Ensembl (https://www.ensembl.org/index.html), Varsome (https://varsome.com/), InSIGHT (https://www.insight-group.org/variants/databases/), LOVD (https://databases.lovd.nl/shared/genes) and the Human Genome Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php). Last access: December 2018.

## 3. RESULTS

Our study included the analysis of 25 genes associated with hereditary cancer syndromes with breast cancer as a clinical manifestation. These 25 genes were analyzed in 77 patients fulfilling criteria of hereditary breast cancer and a breast cancer diagnosis: 43 patients were noncarriers of pathogenic variants or VUS in *BRCA1 or BRCA2* genes, and 32 patients were carriers of VUS. We obtained a total coverage of 99.54% of the selected regions, a mean depth of coverage of 615X and an average coverage uniformity of 96%. Two samples were excluded due to low coverage (less than 20-fold).

We identified 11 putative pathogenic variants, all were resequenced by Sanger sequencing.

Six variants were classified as pathogenic or likely pathogenic variants, and three were considered VUS (Table 1). The pathogenic or likely pathogenic variants were located in the *PALB2* gene (3 variants), *BRIP1* gene (1 variant), *BARD1* gene (1 variant) and *RAD50* gene (1 variant). *PALB2* was the most commonly mutated gene in our participants, with three variants detected (c.2257T>C, c.2834+1G>C, and c.1965dup). Two unique frameshift variants were detected in *BARD1* (c.1509del) and *BRIP1* (c.1702_1703del) and a nonsense variant was identified in *RAD50* (c.2116C>T). The three VUS were detected in *CDH1* (c.44_46dup), *TP53* (c.847C>T) and *CHEK2* (c.190G>A) genes.

In addition, we detected two false positive variants, the *PMS2* c.2337_2338insT variant located in a homopolymer region, and the *PMS2* c.2492dup variant detected due to interference with the homologous pseudogene *PMS2CL.*

No pathogenic variants or VUS were identified in the rest of the genes analyzed.

## 4. DISCUSSION

In this study, we sought to determine the feasibility of analyzing hereditary cancer-related genes other than *BRCA1* and *BRCA2* in our breast cancer population. Here, we present results from a multigene panel test for pathogenic variants in 25 genes of hereditary cancer among 77 patients who had negative test results for the *BRCA1* and *BRCA2* genes. Pathogenic or likely pathogenic variants were identified in 8% of the patients. These findings were similar to those obtained in other studies of hereditary breast cancer (Moran et al. 2017). The pathogenic variants identified were found in high- and moderate-risk genes conferring breast cancer and were also related to cancers that were present in personal and family histories of the patients selected for our study. Genes that harbor pathogenic variants are included in the clinical guidelines with management recommendations, such as the National Comprehensive Cancer Network (NCCN) guidelines (Saam et al. 2015).

The identification of a pathogenic variant in a HBOC patient can result in multiple benefits. Traditionally, only *BRCA* genes were considered when a genetic study was indicated in a patient suggestive of HBOC; however, a considerable percentage of patients who fulfill the criteria of HBOC do not harbor pathogenic variants in the *BRCA1* and *BRCA2* genes, as it occurs with the 77 patients analyzed in the present study. Due to the linkage of different cancer syndromes with common clinical outcomes, a wide spectrum of candidate genes has been established. For that reason, targeted sequencing of gene panels by NGS has been demonstrated to be widely useful not only for patient's clinical management but also for secondary prevention in healthy relatives (Susswein et al. 2016).

The analysis of 25 genes associated with several cancer syndromes has retrieved a diagnostic yield of 8%. We found a high frequency (4%) of pathogenic mutations in the *PALB2* gene which is in accordance with other studies previously reported. For instance, founder pathogenic variants in *PALB2,* in women with breast cancer and with a family history of breast cancer, account for approximately 4% (Janatova et al. 2015). The *PALB2* c.2257C>T pathogenic variant was found in a patient with breast cancer diagnosed at the age of 56 (Figure 1A). We were not able to obtain DNA samples from other family members affected with cancer, so we could not perform carrier analyses in relatives. The *PALB2* c.2834+1G>C is a splice variant classified in the databases as likely pathogenic due to its position in the canonical donor site and its absence in the general population (Landrum et al. 2016). The patient was a woman diagnosed with triple-negative breast cancer at age 31. We were only able to obtain a sample from her mother, who was also diagnosed with breast cancer and carrier of this variant. We observed that an uncle of the proband's mother was diagnosed with pancreatic cancer (Supplementary Figure 1), but we did not have a DNA sample to perform the carrier analysis. The *PALB2* c.1965dup variant detected in a breast cancer patient has not been previously described. We could not analyze its presence in any affected relative because DNA samples were not available for analysis (Supplementary Figure 2). Interestingly, this patient, in addition to the *PALB2* variant, also carried a controversial variant in the *BRCA2* gene, classified as a variant of

uncertain significance or likely pathogenic (class 4) by different authors. However, although this *BRCA2* variant has been classified as likely pathogenic in the databases by its position in a canonical donor site, assuming a negative effect in the *splicing*, in a previous study, we could not conclude its pathogenic effect due to the lack of alteration effect in the splicing process (Rodríguez-Balada et al. 2016). This finding reinforces the fact that the *PALB2* gene is strongly related not only to breast cancer but also to pancreatic cancer, as previously reported (Hofstatter et al. 2011). The *PALB2* gene product acts as a tumor suppressor and interacts closely with both *BRCA1* and *BRCA2* genes during the double-strand DNA repair process (Hofstatter et al. 2011). In addition, Antoniou et al. suggested that the breast cancer risk for *PALB2* mutation carriers may overlap with that for *BRCA2* mutation carriers (Antoniou et al. 2014). The *PALB2* gene, partner and localizer of *BRCA2*, also known as *FANCN*, is also related to the Fanconi anemia pathway, as well as *BRIP1* and *RAD51C*, all of which are considered to increase the risk of breast cancer. Some studies estimate that pathogenic variants in *PALB2* are associated with a two to four times higher risk of breast cancer (Antoniou et al., 2014). The *PALB2* gene is one of the most mutated genes after *BRCA1* and *BRCA2* genes in HBOC (Buys et al., 2017; Tedaldi et al., 2017).

Our results support the incorporation of *PALB2* in the gene panel for the clinical genetic testing of HBOC.

The *RAD50* c.2116C>T is a nonsense variant (p.Arg706Ter) found in a proband with bilateral breast carcinoma without known familial history of cancer (Figure 1B). This variant has been previously described as pathogenic in public variant databases as ClinVar, Ensembl, HGMD and Varsome. Germline mutations in the *RAD50* gene, although seen at low frequencies and known to be population specific, can be qualified as a novel candidate gene for conferring breast cancer susceptibility in a subset of non-*BRCA1* and *BRCA1/2* families (Apostolou and Fostira 2013). However, their clinical impact has yet to be determined.

*BARD1* c.1509del is a frameshift variant identified in one patient affected by bilateral breast cancer (Supplementary Figure 3). As far as we know, this variant is not described in the databases, but *in silico* predictions show that it creates a premature translational stop signal at codon 520 of the protein, so it is expected to result in an absent or disrupted protein product; consequently, we can considered it as likely pathogenic. BRCA1 and BRCA2 possess an amino-terminal RING-finger motif that facilitates BARD1/BRCA1 heterodimer formation. This stabilizes both proteins and is essential for the expression of the tumor suppressor functions of BRCA1 (Wu et al. 1996). In fact, loss-of-function variants in this gene are known to be pathogenic (Cimmino, Formicola, and Capasso 2017); however, although *BARD1* mutations can confer cancer susceptibility, larger studies are essential to confirm this.

The *BRIP1* c.1702_1703del variant identified in the studied population (Supplementary Figure 4) is a frameshift variant previously classified as likely pathogenic by *in silico* prediction (Esteban-Jurado et al. 2016). Rafnar et al. genotyped this deletion in cancer patients and control subjects from Spain. They showed that this *BRIP1* deletion is very rare (allelic frequency 0.03% in control subjects, N = 1,780), but it is associated with a greatly increased risk of ovarian

cancer (OR = 25, P = 0.016) and a significant risk of breast cancer (OR = 12, P = 0.0079) (Rafnar et al. 2011). *BRIP1* encodes a protein that was identified as a binding partner of BRCA1 and was investigated as a breast cancer predisposing gene (Apostolou and Fostira 2013). Although germline mutations in the *BRIP1* gene have been described as conferring a moderate risk for ovarian cancer, other authors suggested that the role of *BRIP1* in breast cancer pathogenesis remains controversial (Weber-Lassalle et al. 2018), and its potential effects cannot be excluded yet.

Three unique VUS were detected in the *CDH1*, *TP53* and *CHEK2* genes. These VUS are missense or duplication variants. *CDH1* c.44_46dup is an in-frame variant found in a breast cancer patient that produces a gain of one amino acid (p.Leudup) (Supplementary Figure 5). It shows a frequency of 0.00083% in population databases (ExAC, rs141568342), so insufficient evidence exists to classify it. The *TP53* c.847C>T (Supplementary Figure 6) missense variant is mainly classified in the databases as VUS because of the lack of evidence to consider it pathogenic. We also identified a *CHEK2* c.190G>A missense variant (p.Glu64Lys) in a patient with a personal and familial history of breast cancer (Supplementary Figure 7), which is not described in databases, and we did not find evidence to consider it as likely pathogenic. Although the amino acid change can be critical by the physicochemical differences in these variants, their presence in population databases and the lack of the possibility to perform segregation analysis make their classification impossible.

In this study, we described that 8% of BRCAX patients are carriers of pathogenic variants in other genes, particularly *PALB2, BARD1, BRIP1* and *RAD50,* all of which are functionally related to *BRCA1* and *BRCA2* (Clark et al. 2012; Buys et al. 2017; Tedaldi et al. 2017,Nunziato M et al. 2019). Some of these genes were known to be related to ovarian cancer but not breast cancer until now, which reinforces the idea of not segregating gene panels according to specific phenotypes, which takes the possibility of overlapping phenotypes into account. We found VUS and pathogenic variants in genes related to other cancer syndromes, highlighting the need to create multigenic panels, taking into account the high- and moderate-risk genes of the main hereditary cancer syndromes, as well as genes that are important in the DNA repair pathways. In this setting, massive genetic studies have a short track, so results in databases are still not completely available and there is a considerable number of VUS. This panel approach provided some VUS that would require the use of a prioritization system to select those with the highest probability of being associated with risk. This drawback will be solved in the near future with the current implementation of these panels in laboratories and with the collection and collation of VUS in databases, leading to more information to determine the putative pathogenicity of these variants.

A limitation of this approach is that it was not possible to analyze Copy Number variants (CNVs) and to determine the presence of large rearrangements because with this approximation, we did not achieve the necessary coverage uniformity of the amplicons to perform it. Another possibility

would be the use of different techniques, such as MLPA, to detect these rearrangements, but currently, this is not a cost-effective alternative for panels that include a large number of genes.

## 5. CONCLUSIONS

In conclusion, our study obtained an increment of the diagnostic yield in patients with HBOC syndrome reinforcing the utility of multigene panels in patients who previously underwent noninformative genetic screening of *BRCA* genes. Further studies are warranted to better gauge whether the non-BRCA1/2 familial clusters might contain important single gene subsets associated with higher cancer risk and the role of the candidate genes in the susceptibility to HBOC.

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FIGURES CAPTION:

FIGURE 1.

Pedigrees of the families carrying pathogenic variants and Alamut® Visual BAM viewer screenshot and electropherogram of the variant identified. A: *PALB2* c.2257C>T variant. B: *RAD50* c.2116C>T variant. Cancer and age diagnoses are indicated in affected patients. The arrow indicates the index case analysed.

SUPPLEMENTARY FIGURE 1

Pedigree of the family carrying the *PALB2* c.2834+1G>C likely pathogenic variant, Alamut® Visual BAM viewer screenshot and the electropherogram of the variant identified. Cancer and age diagnoses are indicated in affected patients. The arrow indicates the index case analysed.

SUPPLEMENTARY FIGURE 2

Pedigree of the family carrying the *PALB2* c.1965dup likely pathogenic variant, Alamut® Visual BAM viewer screenshot and electropherogram of the variant identified. Cancer and age diagnoses are indicated in affected patients. The arrow indicates the index case analysed.

SUPPLEMENTARY FIGURE 3

Pedigree of the family carrying the *BARD1* c.1509del likely pathogenic variant ,Alamut® Visual BAM viewer screenshot and electropherogram of the variant identified. Cancer and age diagnoses are indicated in affected patients. The arrow indicates the index case analysed.

SUPPLEMENTARY FIGURE 4

Pedigree of the family carrying the *BRIP1* c.1702_1703del likely pathogenic variant, Alamut® Visual BAM viewer screenshot and electropherogram of the variant identified. Cancer and age diagnoses are indicated in affected patients. The arrow indicates the index case analysed.

SUPPLEMENTARY FIGURE 5

Pedigree of the family carrying the *CDH1* c.44_46dup variant of uncertain significance, Alamut® Visual BAM viewer screenshot and electropherogram of the variant identified. Cancer and age diagnoses are indicated in affected patients. The arrow indicates the index case analysed.

SUPPLEMENTARY FIGURE 6

Pedigree of the family carrying the *TP53* c.847C>T variant of uncertain significance, Alamut® Visual BAM viewer screenshot and electropherogram of the variant identified. Cancer and age diagnoses are indicated in affected patients. The arrow indicates the index case analysed.

SUPPLEMENTARY FIGURE 7

Pedigree of the family carrying the *CHEK2* c.190G>A variant of uncertain significance, Alamut® Visual BAM viewer screenshot and electropherogram of the variant identified. Cancer and age diagnoses are indicated in affected patients. The arrow indicates the index case analysed.

| CANCER TYPE | AGE AT DIAGNOSIS (years) | FAMILY HISTORY OF CANCER (Age at diagnosis) | GENE (Transcript) | NUCLEOTIDE CHANGE | PROTEIN CHANGE | PATHOGENICITY* | BRCA1 / BRCA VARIANTS IDE |
|---|---|---|---|---|---|---|---|
| BREAST | 56 | BREAST (73);<br>BREAST (78);<br>HEAD AND NECK (80);<br>BREAST(78);<br>BREAST (42);<br>GASTRIC (60~) | ***PALB2***<br>(NM_024675.3) | c..2257C>T | p.Arg753Ter | **PATHOGENIC** | ***BRCA2***: c..886 |
| BILATERAL BREAST | 45; 55 | - | ***RAD50***<br>(NM_005732.3) | c..2116C>T | p.Arg706Ter | **PATHOGENIC** | **None** |
| BREAST | 31 | BREAST (44);<br>GASTRIC (40);<br>PANCREATIC (63) | ***PALB2***<br>(NM_024675.3) | c..2834+1G>C | | **LIKELY PATHOGENIC** | **None** |
| BREAST | 47 | BREAST (54);<br>PANCREATIC (65);<br>LUNG (44);<br>BREAST (40~) | ***PALB2***<br>(NM_024675.3) | c..1965dup | p.Pro656SerfsTer7 | **LIKELY PATHOGENIC** + | **None** |
| BILATERAL BREAST | 50; 52 | BREAST (63);<br>LIVER (64);<br>HEAD AND NECK (63) | ***BARD1***<br>(NM_001282543.1) | c..1509del | p.Val504LeufsTer17 | **LIKELY PATHOGENIC**+ | **None** |
| BREAST | 44 | BREAST (46);<br>BREAST (41) | ***BRIP1***<br>(NM_032043.2) | c..1702_1703del | p.Asn568TrpfsTer9 | **LIKELY PATHOGENIC** | ***BRCA2:*** c..572 |
| BREAST | 47 | BILATERAL BREAST (66; 70);<br>BREAST (70);<br>PROSTATE (70~) | ***TP53***<br>(NM_000546.4) | c..847C>T | p.Arg283Cys | **VUS** | ***BRCA1***: c..-18 |
| BREAST | 50 | BREAST (43);<br>LUNG (78);<br>LEUKEMIA (26);<br>ESOPHAGUS (80);<br>GASTRIC (50) | ***CHEK2***<br>(NM_001005735.1) | c..190G>A | p.Glu64Lys | **VUS** | **None** |
| BREAST | 57 | OVARIAN (79);<br>LUNG (53);<br>GASTRIC (75);<br>BREAST (¿); | ***CDH1***<br>**(**NM_001317184.1) | c..44_46dup | p.Leu15dup | **VUS** | ***BRCA2***: c..778 |

**Table1**. Pathogenic variants, likely pathogenic variants and VUS identified among 77 individuals, who previously tested negative for pathogenic BRCA1/BRCA2 variants.

* Pathogenicity as listed in ClinVar, InSIGHT, LOVD or HGMD databases and categorized according to the ACMG.

+ Pathogenicity according to the ACMG guidelines for variants classification.

HIGHLIGHTS:

- The diagnostic yield of the analysis of 25 genes related to hereditary cancer and DNA repair pathways in BRCAX patients is 8% in the studied population.
- Multigene panels including other genes besides *BRCA1* and *BRCA2* should be included as a diagnostic tool in genetic testing approaches
- *PALB2* has to be included in gene panels for the clinical genetic testing of Hereditary Breast and ovarian cancer