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Study on the inheritance of genetic predispositions in women with breast and ovarian cancer in the Bulgarian population and the importance of the identified genetic variants for the development of a genetic counselling approach for these diseases

A B S T R A C T

from a dissertation for granting of the educational and scientific degree DOCTOR

PhD Program: Medical Genetics

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Scientific jury:

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The present work is dedicated to my mother and my sister.

The dissertation is presented in 172 pages and contains 20 figures and 28 tables. The bibliography comprises 465 references, of which 5 in Cyrillic and 460 in Latin.

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The dissertation has been approved and referred for public defence by the Department of Medical Genetics, Medical University - Pleven, held on 03.12.2024.

The materials related to the defense are available in the Scientific Department of the Faculty of Pharmacy, MU-Pleven and on the university website - www.mu-pleven.bg.

Composition of the scientific jury:

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The public defense will take place on 25.02.2025 at 13.00 h. in "Ambroise Pare" Hall of Telecommunication Endoscopy Center, Medical University - Pleven.

CONTENT

ABBREVIATIONS USED

AK - amino acid
AML - Acute Myelogenous Leukemia
APC - Adenomatosis polyposis coli
AT - Ataxia teleangectasia
BC - breast cancer
BIC - Breast Cancer Information Core
BS - Bloom syndrome
ClinVar - Database of clinically significant genetic variants
CRC - colorectal cancer
DNA - deoxyribonucleic acid
EC - endometrial carcinoma
EOC - endometrioid ovarian carcinoma
ER - estrogen receptor
ESMO - European Society for Medical Oncology
FA - Fanconi anaemia
FBC - familial breast cancer
FIGO - International Gynecology and Obstetrics Organization
FOC - familial ovarian cancer
GnomAD - The Genome Aggregation Database
GWAS - Genome Wide Association Study
HBC - hereditary breast cancer
HBOC - hereditary breast and ovarian cancer
HER2 - Human epidermal factor receptor 2
HGSOC - high-grade serous ovarian carcinoma
HNPCC - hereditary nonpolyposis colorectal cancer
HOC - hereditary ovarian cancer
HRD - Homologous recombination deficiency
HRT - hormone replacement therapy
IDC - invasive ductal carcinoma
IHC - immunohistochemistry
ISH - in-situ hybridization
LFL - Li Fraumeni Syndrome - like
LFS - Li Fraumeni Syndrome
LGSOC - Highly differentiated serous ovarian carcinoma
LLS - Lynch-like syndrome
LOF - loss of function
LOH - loss of heterozygosity
LS - Lynch syndrome
MAPK - mitogen-activated protein kinase
MINAS - Multilocus Inherited Neoplasia Allele Syndrome
MMR - mismatch repair of DNA
MOC - mucinous ovarian carcinoma
MRI - Magnetic Resonance Imaging
MS - microsatellites
MSI - microsatellite instability
NCCN - National Comprehensive Cancer Network
NER - nucleotide excision repair
NGS - Next Generation Sequencing
NSAIDs - non-steroidal anti-inflammatory drugs
NSGC - National Society of Genetic Counselor
NST - non-special type
OC - ovarian carcinoma
OR - odds ratio
P/LP - pathogenic/likely pathogenic
PC- prostate cancer
PCR - Polymerase chain reaction
PGT - Preimplantation genetic test
PnC - pancreatic cancer
PR - progesterone receptor
PRS - Polygenic Risk Assessment
PV - pathogenic variant
RRM - risk-reducing mastectomy
RRSO - risk-reducing salpingo-oophorectomy
SBC - sporadic breast cancer
SEOC - synchronous endometrial and ovarian carcinoma
SNP - Single nucleotide polymorphism
COC - clear cell ovarian carcinoma
SOC - sporadic ovarian cancer
SOC- serous ovarian cancer
TC - thyroid cancer
TNBC - triple-negative breast cancer
XP - Xeroderma pigmentosum
XR- homologous recombination

INTRODUCTION

Hereditary forms of breast and ovarian cancer account for only a small proportion, around 15%, of all cases of these diseases, but the individual and social impact far exceeds this figure. The discovery of the genetic causes of cancer in individual patients enables the personalization of treatment with the increasing prevalence of target therapies and the identification of risks at other sites with a view to early prevention. This reduces the side effects of the patient's treatment and thus the financial burden on society by saving unnecessary, costly drugs in the treatment of the primary site and also by treating possible other sites at an early stage. The discovery of an inherited pathogenic variant in a patient associated with an increased risk of breast and ovarian cancer personalises the risks for her relatives and geometrically increases the positive effect of its discovery. Those relatives in whom the familial variant is confirmed can carry out active and effective prophylaxis, and those relatives in whom such inheritance is not confirmed are relieved of the fear of a possible disease.

Revealing the genetic nature of hereditary breast and ovarian cancer was until recently limited to a few high-risk genes. Today, with the advent of new genomic technologies - massive parallel sequencing - the spectrum of genes involved in hereditary predisposition is constantly growing, posing new challenges to both pharmaceutical companies looking for new molecules for target therapy and genetic counsellors personalising risks according to the diagnosis, family history and characteristics of the individual patient.

I. OBJECTIVE

To investigate the frequency and profile of pathogenic variants in susceptibility genes in women with breast or ovarian cancer from the Bulgarian population and to develop an approach for genetic counselling for these diseases.

II. TASKS

1. To investigate, in women with breast cancer (BC), the following main characteristics:
    - 1.1. Age of diagnosis.
    - 1.2. Reproductive and clinical features
    - 1.3. Presence of familial cancer.
    - 1.4. Histological and molecular characteristics of tumors
2. To investigate the frequency and profile (type and molecular characteristics) of pathogenic variants in cancer predisposition genes in women with BC:
    - 2.1. In the total group of women with BC.
    - 2.2. In different groups of women, distributed according to the age of diagnosis.
    - 2.3. In the group of women with familial BC.
    - 2.4. In the group of women with TNBC.
3. To investigate, in women with ovarian cancer (OC), the following main characteristics:
    - 3.1. Age of diagnosis.
    - 3.2. Reproductive and clinical characteristics.
    - 3.3. Family history.
    - 3.4. Histological features of tumors.
4. To investigate the frequency and profile (type and molecular characteristics) of pathogenic variants in cancer predisposition genes in women with OC.
5. To develop an approach to genetic counseling for women with BC/OC depending on the carrier status of pathogenic variants in cancer predisposition genes.

III. MATERIAL AND METHODS

1. General characteristics and main patient groups

Women diagnosed with breast cancer (BC) or ovarian cancer (OC) were enrolled in the present study and recruited retrospectively and prospectively from 2011 to 2022. To accomplish the different objectives and achieve the set goal, we studied different groups of patients.

For tasks 1 and 2, a total of 203 patients with BC (from different families, no established relationship) were studied. Of these, 144 women were prospectively recruited in the period from January 2019 to December 2022), these were women who visited the Medical-Genetic Counseling Centre at the "University Hospital - Dr. Georgi Stransky", Pleven. The remaining 59 women (29.1%) were recruited retrospectively, from the registry of the Oncology Centre at the University Hospital - Dr. Georgi Strensky - Pleven (registered women for the period from January 2009 to December 2013 and from January 2019 to December 2020). To all live patients diagnosed with BC, recruited retrospectively, were sent letters-invitations to participate in the study. All women who responded to this invitation and accepted to participate were included.

Patients with BC were divided into groups according to age at diagnosis, family history and IHC status of the tumour cells. According to age at diagnosis, women were divided into six age groups - 20-29 years, 30-39 years, 40-49 years, 50-59 years, 60-69 years and a group of women diagnosed after the age of 70. The established criteria (according to ESMO and NCCN) for familiality were used to define patients with familial BC.

Based on the IHC status of the tumour cells, we defined four groups corresponding to the surrogate classification of the molecular subtypes of BC, namely luminal A-like, luminal B-like, HER2-enriched and TNBC.

For tasks 3 and 4, a total of 67 women with OC (from different families, no established relationship) were studied. Of these, 18 women (27%) were retrospectively recruited from the registry of the Oncology Centre of the University Hospital Dr. GeoBCi Stransky in Pleven. They also accepted the invitation (invitation letter) to voluntarily participate in the study. The remaining 49 women were prospectively recruited in the period from January 2019 to December 2022, and all of these women were diagnosed, operated and treated at the Oncology Clinic of Dr. Georgi Stransky University Hospital in Pleven.

All women who agreed to participate in the study signed a consent form approved by the MU-Pleven Ethics Committee.

2. Main methods used in this study

2.1. Questionnaire method - Questionnaires were prepared with information on: passport details, sex, age, health status, occupational conditions, reproductive history, pregnancy history, menopause and hormone replacement therapy, lifestyle, personal medical history, personal breast cancer treatment, family history of breast cancer or other associated cancers. A questionnaire was administered to each woman who accepted to participate in the study using the interview method.

2.2 Genealogical method - A family tree containing information related to the presence of malignancy was constructed for each patient who met the inclusion criteria for the study and included the relatives of at least three generations of the proband. After construction of the family tree and genealogical analysis based on it, familial cases of BC or OC were determined. Based on the genealogical analysis, groups of women with BC were defined according to family history.

2.3. Molecular genetic method (DNA analysis) - All patients selected for genetic testing had biological material taken - venous blood, about 5ml taken in a vacuum flask with EDTA. Genomic testing was performed using Next Generation Sequencing (NGS) methods.

Main stages of genetic analysis

2.3.1. DNA isolation - Isolation of genomic DNA from venous blood was performed automated, using a ready-to-use MagCore Genomic DNA Whole Blood kit (Ref: MGB400 02, RBC Bioscience), according to the protocol for use. The accuOCcy of the DNA isolation method is guaOCnteed by a Certificate provided by the DNA isolation kit supplier. After isolation, the DNA is stored at -80º in the DNA bank of the Centre of Competence - Leonardo da Vinci, MU-Pleven.

2.3.2. Massively parallel sequencing (Next Generation Sequencing - NGS) - Genetic analysis of genomic DNA from all patients was performed using the method of massively parallel sequencing (NGS). This method allows simultaneous sequencing (reading) of many genes from many patients. A targeted panel of Trusight Cancer-associated genes (Illumina©) was used for library preparation. Library preparation from all study patients was performed according to a protocol provided by the manufacturer. The pan-hereditary cancer target panel contains oligosamples for 94 genes associated with increased cancer susceptibility. The constructed libraries were sequenced on the Illumina NextSeq 550 platform with a 2x150 bp configuration. Genetic analysis performed with this method detected small genetic rearrangements - single nucleotide substitutions and small insertions and deletions (indels) and was not informative for large genomic rearrangements.

2.3.3. Analysis of the sequencing data - The sequenced fragments obtained from the patients were compared with the human reference genome hg19. The output data files (gVCF)

were imported into BaseSpace Variant Interpreter (Illumina©). Custom filters were created to improve variant annotation and interpretation, including a minimum read depth of 20x per variant and at variant exclusion of no clinical relevance. To classify the variants found, the American College of Medical Genetics and Genomics (ACMG) five-level classification was used: pathogenic (P), likely pathogenic (LP), variant of uncertain clinical significance (VUS), likely benign (LB), and benign (B). For the purpose of classifying the variants found, established pathogenicity criteria were used - detailed in Table 1.

Table 1. Pathogenicity criteria for genetic variants.

Criteria for pathogenicity	Category
Very strong	**PVS1** - variant leading to loss of protein function (nonsense, frameshift, canonical±1 or 2 splice sites, initiation codon, exon/exon deletion)
Strong	**PS1** - amino acid substitution, at a site where a pathogenic variant has been previously reported **PS2** - de novo variant, proven by segregation analysis **PS3** - presence of experimental evidence of pathogenicity of the variant **PS4** - variant incidence in affected individuals is higher than incidence in healthy controls
Moderate-strength	**PM1** - localized hotspot of pathogenic variants **PM2** - absent in healthy controls, or extremely low frequency **PM3** - detected in *trans* with another pathogenic variant (for recessive conditions) **PM4** - leading to protein length change (for in-frame mutations) or protein elongating variants **PM5** - a novel amino acid substitution at a site where a pathogenic variant has been reported **PM6** - assumed de novo variant, no segregation analysis conducted
Supporting	**PP1** - co-segregation with disease, with many affected relatives in one family **PP2** - missense variant in a gene with few benign missense variants, in a gene for which missense variants are known to be a pathogenic mechanism **PP3** - lots of computer-processed evidence of variant pathogenicity **PP4** - Patient's clinical picture is highly specific for a pathogenic variant in this gene **PP5** - Lots of evidence in the literature for pathogenicity of the variant, but the lab can't prove it independently

Variants detected automatically were verified in different databases: ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), dbSNP (https://www.ncbi.nlm.nih.gov/snp/) and Ensembl (https://www.ensembl.oBC/index.html).

2.4. Genetic counselling - Genetic counselling was done to all the women included in the study. For those of them in whom we found a pathogenic (P)/likely pathogenic (LP) variant, testing of relatives for the founded specific variant was also recommended in order to personalize prophylaxis recommendations for them.

2.5. Statistical data processing -

The statistical analysis of the obtained data was performed using the following methods:

- Frequency analysis of changes including absolute frequencies in percentages
- Graphical analysis
- A statistically significant association between two variables was determined using the $\chi 2$ test of association (Chi-square test of association).
- The strength of the relationship between two variables was assessed by *Pearson*'s *Phi (for 2x2 cross-tabulations)* and *Cramer*'s *V (for cross-tabulations larger than 3x3). For 2x3 or 3x2 cross-tabulations, either Pearson*'s *Phi or Cramer*'s *V coefficients were used (giving equal magnitude of the strength of the relationship).*

The confidence interval (95% CI) was calculated in the statistical processing of the incidence data. A *p-value* for statistical significance of the data was determined, with $p<0.05$ values considered significant. Statistical processing was performed using SPSS for Windows, v.29.0.2.0

IV. RESULTS AND DISCUSSION

1. Age, reproductive, familial and clinical characteristics of the women with BC and histological and molecular characteristics of tumours

1.1. Age characteristics

The study group included a total of 203 women with a histologically defined diagnosis of BC. The mean age at diagnosis of BC was 46.5 years (range, 23 to 79 years). The women were divided into six age groups: diagnosed between 20-29yrs inclusive; between 30-39yrs; between 40-49yrs; between 50-59yrs; between 60-69yrs and over 70yrs. The distribution of the women studied is shown in Figure 1 and is as follows: diagnosed between 20-29 years, 3.9% (n=8); between 30-39 years, 20.7% (n=42); between 40-49 years, 42.9% (n=87); between 50-59 years, 19.2% (n=39); between 60-69 years, 10.8% (n=22); and over 70 years, 2.5% (n=5).

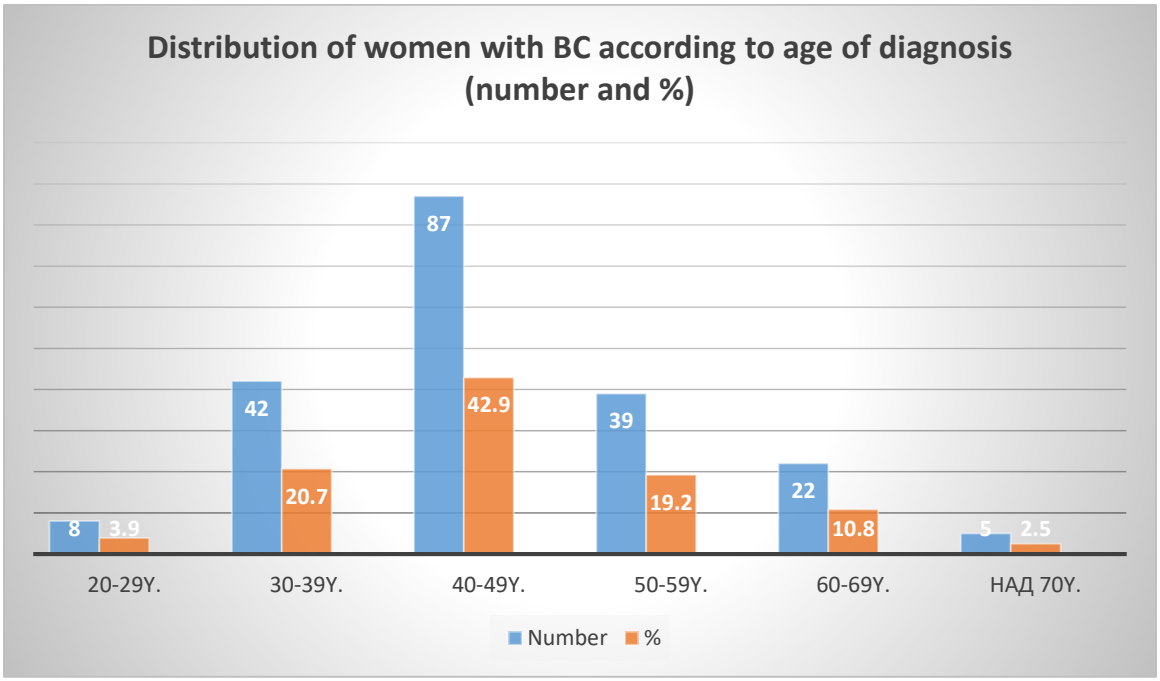


Figure 1. Distribution of study women by age at diagnosis of BC (by number, %).

The present study confirms that women diagnosed with BC between the ages of 40 and 49 make up a large proportion of patients with this disease and that they are overlooked due to the current legislation in Bulgaria and the age restriction. On the other hand, the survival rate for BC correlates strongly with the age at which the disease is diagnosed, with a lower survival rate in patients under the age of 50. In the group of patients diagnosed over 70 years of age, the survival rate was the lowest, but this was related to advanced age and the presence of comorbidities.

The high number of affected women in the 40-49 age group, as indicated by both the Bulgarian National Cancer Registry and the results of the present study, and the demonstrably lower survival rate in this age group make it necessary to reconsider the age of entry for BC screening in our population and to start screening at the age of 40 rather than at 50, as is currently the case.

1.2. Reproductive history and BMI

Menarche, reproduction and menopause

It has been shown that early menarche (before the age of 12) and late menopause (after the age of 55) increase the risk of developing BC. In our study, the mean age of menarche was 13.3 years, with 1% (n=2) of the women studied reporting early menarche. The mean age of first birth in the women studied was 23.1 years, with 16 women (7.9%) being nulliparous, 11 of these women were of reproductive age and only 5 were not. The statistical analysis revealed no correlation between the age of first pregnancy or the absence of pregnancy and the degree of differentiation of the tumor, an indicator of a more aggressive disease course *($\chi^{(2)}(16,203)=8.22$, $p=0.631$)*, or between the age at diagnosis and the degree of differentiation *($\chi^{(2)}(10,203)=13.57$, $p=0.607$).*

BMI

In the present study, 28.6 % (56 women) were classified as overweight (25≤BMI <30) and 14.8 % (30 women) as obese (BMI ≥ 30). A high BMI has been shown to correlate with an increased risk of BC, regardless of whether the diagnosis is made before or during menopause. This is confirmed by the present study, in which there was almost no difference in the proportion of obese women (BMI ≥ 30) between the age groups (up to 60 years). In the age groups after 60 years, the proportion of obese women tends to almost double (27.3% (n=6) in women aged 60-69 years, 25% (n=1) in those over 70 years). A correlation was sought between the two factors BMI and age at diagnosis, and a statistically significant, strongly positive association was found *($\chi^2(10,203)=22.54$, $p=0.013$, $N=203$, Cramer's $V=0.24$)*, confirming the literature evidence that women diagnosed after the age of 50 are characterized by a higher BMI, i.e., that BMI is a factor in the diagnosis of cancer. i.e. that BMI is a factor that plays a role in the etiology of BC in patients diagnosed over 50.

Tumor cell receptor status (in terms of ER and PR) was also influenced by BMI in the group of women diagnosed at menopause. The proportion of postmenopausal women with hormone receptor-positive tumor cell status and obesity (BMI≥ 25) was twice as high (64.7%, n=22) compared to the proportion (31.3%, n=5) of the same tumor type in postmenopausal women without obesity (BMI < 25). No such difference was found in the group of

premenopausal women. In this respect, our study confirms the results of other studies for the European population, according to which there is a positive association between obesity and hormone receptor-positive breast tumors in women diagnosed during menopause.

1.3. Family history

According to the literature, a family history is found on average in about 15-20% of BC cases, demonstrating the multifactorial etiology of the disease resulting from the interaction of genetic and non-genetic factors, with the risk in women increasing with the number of affected relatives (in both parental lines). In the present study, a genealogical analysis was carried out on all 203 women, with relatives of at least three generations included in the analysis. The prevalence of familiality (first-degree relatives with BC) was **15.8%** (n=32 women), which is consistent with the literature data. Six women (3%) had first- and second-degree relatives affected at the same time, and only one woman (0.5%) had first-, second- and third-degree relatives with BC at the same time. The highest proportion (73%, n=16) of familial BC cases (presence of a first-, second- or third-degree relative with BC) was found in the group of women diagnosed between 60and 69 years of age (Table 2) (Figure 2).

The likely explanation for the highest proportion of familial cases in the 60-69 age group is that for a large proportion of patients diagnosed after the age of 60, the reason for accepting the invitation to participate in our study was precisely the presence of a family history of BC and the desire of these patients to determine both their own risk and the risk of their healthy relatives.

In the genealogical analysis, 5.4% (n=11) of the women were found to have relatives with so-called associated cancers. Associated cancers are those in which a common genetic cause was found, such as OC, prostate and pancreatic cancer. Adding this type of familiality results in a cumulative **21.2%** of familial cases (first-degree relatives with BC or other associated cancers).

Table 2. Familiality and histo-molecular characteristics of tumors in the studied women with BC in different age groups.

Age of diagnosis of BC	Number /%	Fertility for BC (n/% of number of women in the respective age group)					Familiality for other cancers(n/% of the number of women in the respective age group)			Surrogate molecular subtype (n/% of the number of women in the respective age group)				Degree of differentiation (n/% of the number of women in the respective age group)		
		I degree	II degree	III degree	Total - I/II/III -	I+II	I degree	II degree	III degree	Luminal A like	Luminal B like	HER2+	TNBC	G1	G2	G3
< 29 years	8 (3.9%)	0	0	0	0	0	0	0	0	(25%)	5 (62,5%)	0	1	1(12,5%)	3(37,5%)	(50%)
30-39 years	42 (20.7%)	2 (4,8%)	9 (21,4%)	2 (4,8%)	13 (31%)	1 (2,4%)	4 (9,5%)	4 (9,5%)	0	(52,4%)	6 (14,3%)	2 (4,8%)	12 (28,6%)	3(7,1%)	21(50%)	18 (42,9%)
40-49 years	87 (42.9%)	15 (17,2%)	14 (16%)	4 (4,6%)	33 (37,8%)	4 (4,6%)	6 (6,9%)	13 (15%)	0	50 (57,5%)	21 (24,1%)	3 (3,4%)	13 (14,9%)	13(15%)	45(52%)	29 (33%)
50-59 years	39 (19.2%)	8 (20,5%)	4 (10,3%)	2 (5.1%)	13 (35,9%)	0	0	2 (5,1%)	1 (2,6%)	22 (56,4%)	7 (17,9%)	4 (10,3%)	6 (15,4%)	3(7,7%)	26(66,6%)	10 (25,7%)
60-69 years	22 (10.8%)	6 (27,3%)	6 (27,3%)	4 (18,2%)	16 (72,8%)	1 (4,5%)	1 (4,5%)	1 (4,5%)	0	13 (59,1%)	5 (22,7%)	1 (4,5%)	3 (13,6%)	2(9,1%)	15(68,2%)	5 (22,7%)
> 70 years	5 (2.5%)	1 (20%)	0	0	1(20%)	0	0	1 (20%)	0	(20%)	2 (40%)	0	2 (40%)	0	4(80%)	1 (20%)
Total	203 (100%)	32 (15,8%)	33 (16,3%)	12 (5,9%)	76 (37,5%)	6 (3,0%)	11 (5,4%)	21 (10,3%)	1(0,5%)	110 (54,2%)	46 (22,7%)	10 (4,9%)	37 (18,2%)	22 (10,9%)	114 (56,2%)	67 (33%)

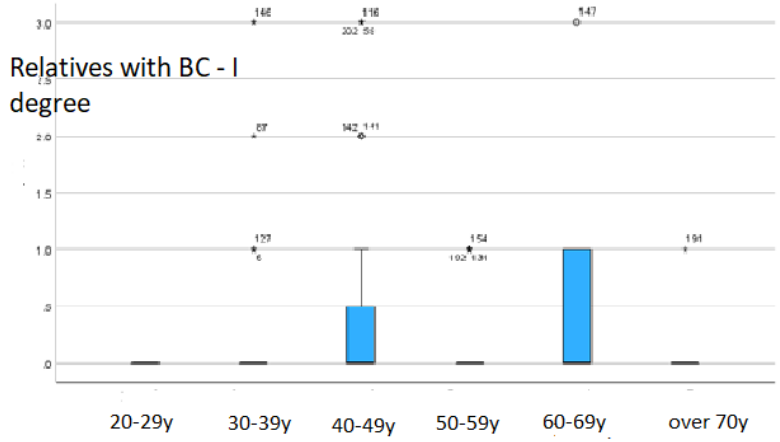


Figure 2. Distribution of familial cases of BC (as relative proportion), in different age groups

1.4. Clinical characteristics

Our study confirmed the data from the literature and revealed a higher incidence of left breast involvement, accounting for 53.7% (n=109) of the women studied, with a mean age at diagnosis of 46 years (Table 3). Right breast involvement was found in 42.9% (n=87) of women, with a younger mean age at diagnosis (40.4 years). Bilateral involvement was found in 7 women (3.4%).

Table 3. Distribution of women, according to the location of BC

Localization	Number	Share (%)
Left breast	109	53.7
Right breast	87	42.9
BilateOCl involvement	7	3.4
Total	203	100.0

1.5. Histological and molecular features of tumours

In our study cohort of women, confirming the Literature data, we found the highest proportion of the so-called histologic type "no special type" (NST), namely 81.3% (n=165) of women (Table 4). This type is diagnosed by default if the histology of the tumor does not fall into any of the special histological type groups. About 25% of invasive breast carcinomas are known to have special histologic features and growth forms that are diagnosed as special types (invasive lobular, tubular, mucinous, neuroendocrine, etc.). In the cohort of women we studied, tumors of specific histological types were found in about 20: lobular (8.9%, n=18); ducto-lobular (5.9%, n=8); medullary (2.4%, n=5); gelatinous (1.5%, n=3); metaplastic, sebaceous, micropapillary and lymphoepithelioma-like, 0.5% each (n=1) (Table 4).

Table 4. Distribution of women with BC, depending on the histological type of tumour

Histological type	Number of women	Share (%)
Ductal (NST)	165	81.3
Lobular	18	8.9
Ducto-Lobular	8	3.9
Dr. type	12	5.9
Total	203	100.0

The molecular classification of invasive BC is independent of the histological classification and is based on RNA expression in different tumors. The molecular

classification used today distinguishes between four subtypes - Luminal A-like, Luminal B-like, HER2-enriched and basal-like.

The surrogate classification of the molecular subtypes of BC based on IHC markers is used in clinical practice. The IHC surrogate classification (based on ER, PR, HER2 and KI67 receptor status) is used to determine the molecular subtypes in the study patients. In the present study, we found (Table 5): luminal A-like in 54.2% (n=110); luminal B-like in 22.7% (n=46); HER2-enriched subtype in a low proportion, compared to literature data, in about 5% (n=10); basal-like (TNBC, triple negative) in 18.2% (n=37)

Our finding of a low proportion of the HER2-enriched subtype is most likely due to the fact that, according to Literature, around 30 % of HER2-enriched BC is incorrectly classified as HER2-negative on the basis of IHC and/or FISH. Secondly, hereditary forms are not discussed in this subtype, i.e. patients with this subtype of BC would be less likely to see a genetic counselor. Therefore, the group investigated in our study was selected (women in whom a hereditary form of BC was suspected). In the HER2-enriched subtype group, a high proportion of G3 tumors were found, 60% (n=6), while G1 was found in only one patient (10%). However, due to the small number of women in this group, no definitive conclusions can be drawn.

Of the other three subtypes, the highest proportion of low-differentiated tumors (G3), in almost half of the cases, was found in TNBC, while the highest proportion of highly differentiated tumors (G1) was found in the group of women with Luminal A subtype, which partly explains the poor prognosis in TNBC and the good prognosis in Luminal A subtype.

Table 5: Distribution of women with BC according to surrogate molecular subtype

Surrogate molecular subtype	**Number of women**	**Share (%)**	**Proportion (%) of G1 tumours of the defined subtype**	**Proportion (%) of G3 tumours of the defined subtype**
Luminal A-like	110	54.2	12,6	25,5
Luminal B-like	46	22.7	11,0	34,8
HER2 enriched	10	4.9	10,0	60,0
Triple Negative	37	18.2	3,0	48,6
Total	203	100.0	XXX	XXX

The statistical analysis revealed no correlation between the molecular surrogate subtype and the degree of differentiation *($\chi^{(2)}$(6,203)=10.86, p=0.093)*, which in turn is most likely due to the fact that the degree of differentiation is part of the overall biological characteristics of the tumors

From the analysis of the results obtained, it can be summarized that the most common subtype was Luminal A, except in the age group under 30 years, where the most common

histological type was Luminal B (in about 63%) (Table 2). The most common tumors were moderate or poorly differentiated, with the proportion of poorly differentiated tumors being highest in women diagnosed before the age of 30 years and decreasing steadily in the other age groups and in women diagnosed after the age of 70 years. This is consistent with the statement that tumors in young patients are poorly differentiated and have a more aggressive disease course.

2. Frequency and profile (type and molecular characteristics) of pathogenic/likely pathogenic (P/LP) variants in cancer predisposition genes among the study group of women with BC

2.1. In the total group of women with BC

Genetic testing for cancer predisposition, especially BC predisposition, has become an integral part of medical practice. Until recently, genetic testing was mainly performed in patients with a strong family history of BC, in young patients or in patients with TNBC. In addition, patients had to meet certain criteria to be tested and genetic testing was limited to the two highly penetrant genes *BRCA1* and *BRCA2*. The limitations of this selection approach meant that only a limited number of patients were tested for a limited number of genes, which was unefficient for patients. The National Comprehensive Cancer Network (NCCN) has established one of the globally recognized standards for genetic testing for breast cancer predisposition. Recent updates to the standard include pancreatic cancer as part of hereditary cancer with *BRCA1/2* etiology, which previously included BC and OC. Even with such an expansion, any type of criteria would be a limitation rather than a tool, as a large proportion of carriers would be missed if we were to strictly follow these criteria. On this basis, the American Society of Breast Surgeons (ASBrS) recommends that all women with BC should be tested for a genetic predisposition to BC. In our study, all women with BC, regardless of age, family history, personal medical history or ICH phenotype, were screened with a gene panel comprising 94 predisposition genes. A summary of the clinical data and the results of the genomic analysis of all 203 women with BC is shown in Table 6

Table 6: Prevalence of P/LP variants in cancer predisposition genes among women with BC, according to different clinical, histological and familial characteristics.

Characteristics of the studied women with BC	**All** patients No(%)	Patients with P/LP variants in ***BRCA1/2*** No (%)	Patients with P/LP variants in **other high-penetrant genes** No (%)	Patients with P/LP variants in genes with **moderate penetrance** No (%)	Patients **without** P/LP variants in susceptibility genes No (%)
No patients	203 (100)	20 (9.9)	2 (1.0)	22 (10.8)	159 (78.3)
Age at diagnosis (D)					
Average age of D (min-max)	46.5 (23-79г.)	38.2 (26-61г.)	40.5 (32-49г.)	49.3 (32-79г.)	47.2 (23-79г.)
Histological type					
Invasive ductal	165 (81.3)	15 (75.0)	1 (50.0)	19 (86.4)	130(81.8)
Invasive lobular	18(8.9)	2 (10.0)	1 (50.0)	2 (9.1)	13(8.2)
Ducto-Lobular	8 (3.9)	2 (10.0)	0	1 (4.5)	5(3.1)
Other special invasive types	12 (5.9)	1 (5.0)	0	0	11(6.9)
Degree of tumor differentiation					
Highly differentiated (G1)	20 (9.9)	0	0	2 (9.1)	18 (11.3)
Moderately differentiated (G2)	114 (56.2)	11 (55.0)	1 (50.0)	12 (54.5)	90 (56.6)
Low differentiated (G3)	69 (33.9)	9 (45.0)	1 (50.0)	8 (36.4)	51 (32.1)
Surrogate molecular subtype (St Gallen 2013					
Luminal A-like	110 (54.2)	5 (25.0)	1 (50.0)	13 (59.1)	91 (57.2)
Luminal B-like	46 (22.7)	2 (10.0)	1 (50.0)	4 (18.2)	39 (24.5)
HER2 - positive (non-luminal)	10 (4.9)	1 (5.0)	0	1 (4.5)	8 (5.0)
TNBC	37 (18.2)	12 (60.0)	0	4 (18.2)	21 (13.3)
Another BC					
Yes	3 (1.54)	0	0	0	3 (1.9)
No	200 (98.5)	20 (100)	2 (100)	22 (100)	155 (97.5)
Average age of D (min-max)	41.7 (40-44г.)	-	-	-	41.7 (40-44г.)
BC in a first degree relative					
Yes	32 (15.8)	2 (10.0)	1 (50.0)	4 (18.2)	25 (3.0)
No	171 (84.2)	18 (90.0)	1 (50.0)	18 (81.8)	134 (84.2)
OC in relatives of the first degree					
Yes	4 (2.0)	2 (10.0)	0	0	2 (1.3)
No	199 (98.0)	18 (90.0)	2 (100)	22 (100)	157 (98.7)
Other associated carcinomas in I degree relatives					
Yes	6 (3.0)	1 (5.0)	0	4 (21.7)	1 (0.6)

No	197 (97.0)	19 (95.0)	2 (100.0)	18 (78.3)	158 (99.4)
BC in a II degree relatives					
Yes	37 (18.2)	7 (5.0)	0	6 (26.1)	24 (15.1)
No	166 (81.8)	13 (95.0)	2 (100)	16 (73.9)	135 (84.9)

P/LP* variants in the genes predisposing to BC were found in 44 (**21.6%**) of all 203 women examined. PVs were found in high penetrance genes in 22 (**10.8%**) of the women, which represents 50% of all women with hereditary forms of BC. In 20 of the women (9.9%), the PVs were found in *BRCA1/2* genes, of which 13 (6.4%) were found in *BRCA1* and 7 (3.4%) in *BRCA2* (Table 7 and Figure 3). In one of the women carrying a *BRCA2* mutation, PVs were combined in two other susceptibility genes - moderately penetrant (Multi-locus Inherited Neoplasia Allele Syndrome (MINAS) - *BRCA2*, *CHEK2*, *ATM*). Two of the women (1%) carried pathogenic variants in the *PALB2* gene.

Table 7: Prevalence of P/LP variants in predisposing genes, according to penetrance, among the total group of women with BC.

Carrier state of PV in predisposing genes	**Frequency**	**Share (%)**
None	159	78.3
BRCA1/2	20	9.9
Other high-penetrant genes	2	1.0
Moderate-penetrant genes	22	10.8
Total	203	100.0

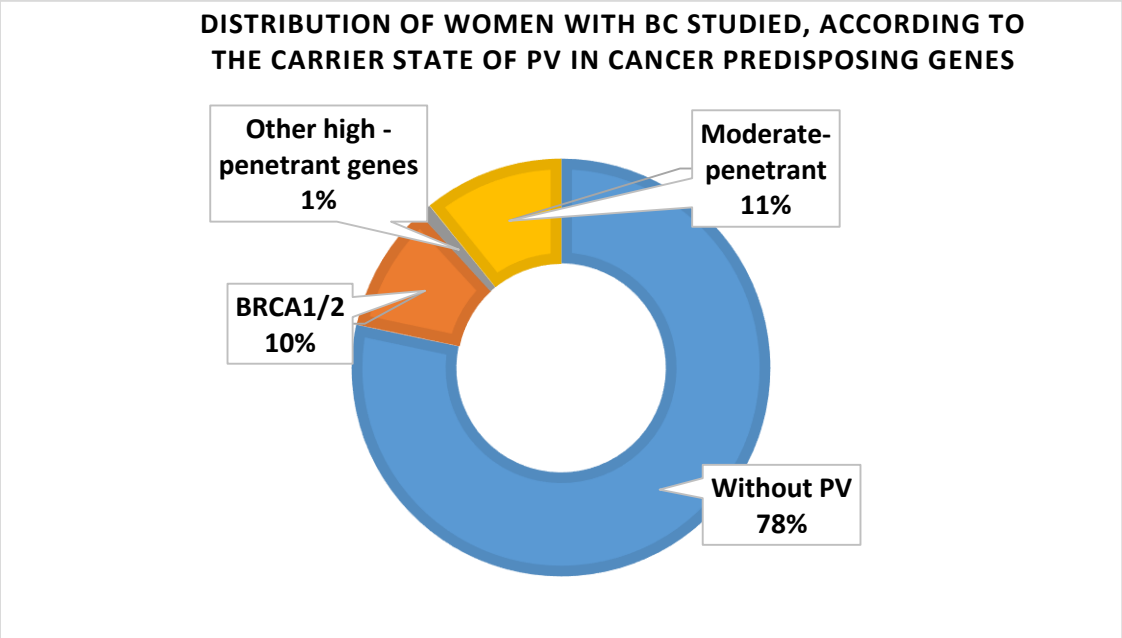


Figure 3. Distribution of studied women with BC, according to the PV carrier state in cancer predisposing genes.

**P/LP* variants - for ease of description in this study we will use only the abbreviation PV, and will consider both classes of variants - pathogenic (P) and likely pathogenic (LP)

In the remaining 22 women (**10.8%** of all women studied and 50% of women with NBC), we found P/HR variants in susceptibility genes with moderate penetrance (*ATM, BLM,CHEK2, BRIP1, CDKN2A, ERCC5, RAD51C, FANCM, FANCG, FANCI, FANCL*), and in one of these women, we found combined PV carrier state in two moderate-penetrance genes (MINAS - *CHEK2* and *ERCC5*). Detailed information on the detected PVs in cancer predisposing genes is presented in Table 8 and Figure 4.

Table 8: Frequency of PV carrier status in specific predisposing genes in the total group of women with BC

Gene	Number of women	Share (%)
None	160	78,8
BRCA1	13	6,4
BRCA2	5	2,5
PALB2	2	1,0
FANCM	1	0,5
FANCL	1	0,5
RAD51C	1	0,5
CHEK2	10	4,9
FANCI	1	0,5
FANCG	1	0,5
ATM	2	1,0
BRIP1	1	0,5
CDKN2A	1	0,5
BLM	2	1,0
MINAS	2	1,0
Total	203	100,0

We looked for correlations between various clinical and histological features in the women with BC and PV in the predisposition genes.

There was a statistically significant strong positive association between age at menarche and PV carrier status *($\chi^{(2)}(24,203)=35.07$, $p=0.013$, $N=203$, Cramer's $V=0.34$)*, which in fact confirms that age at menarche is genetically determined, and plays a role in the pathogenesis of the development of BC, due to the involvement of hormonal factors, in the etiology of this type of cancer.

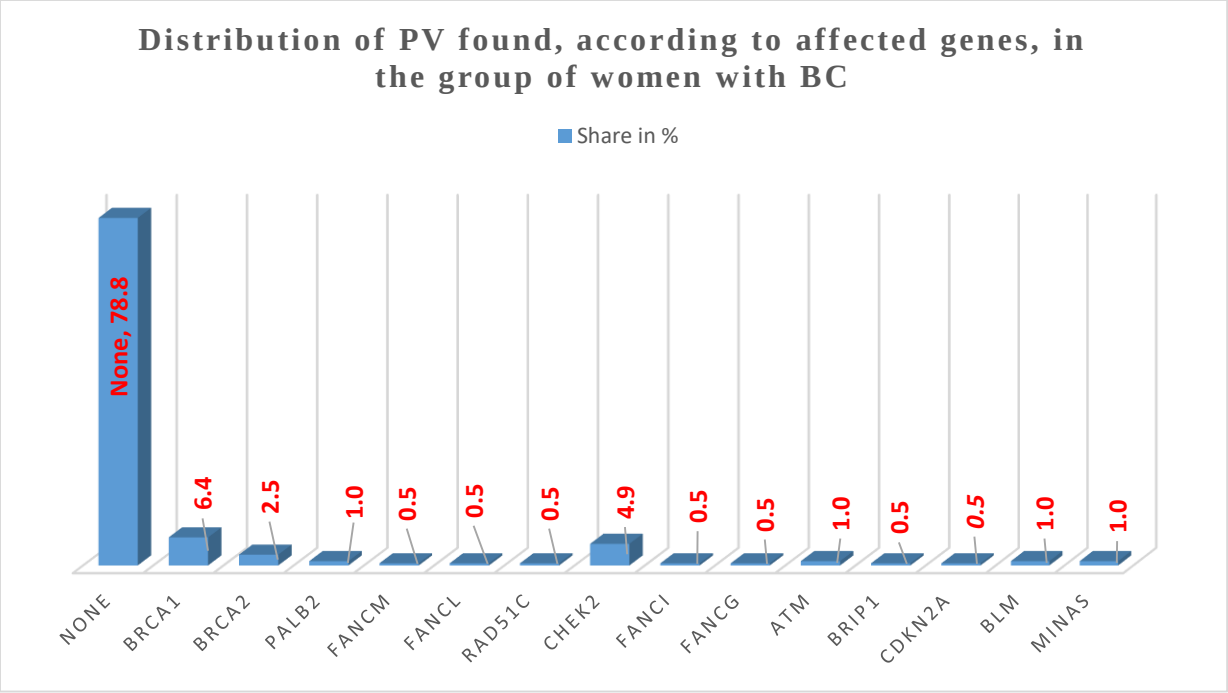


Figure 4. Distribution of genes with found PVs in the total group of women studied with BC

A correlation between histological type and PV carrier was sought but not found *($\chi^{(2)}(9,203)=8.28$, $p=0.506$)*, nor was a correlation found between histological variant, degree of differentiation and carrier status of PV in predisposing genes *($\chi^{(2)}(6,203)=3.64$, $p=0.730$)*. The explanation is most likely that other genetic and non-genetic factors as well as different levels of regulation of gene activity are also important for tumour development with corresponding histological features and degrees of differentiation. For this reason, the presence of a specific genetic variant is not decisive for the histological type of tumour. However, a very strong positive association was found *($\chi^{(2)}(3,203)=21.56$, $p<0.001$, $N=203$, Cramer's $V=0.326$)* between the hormone receptor status of the tumor and the PV carrier status, with ER-positive tumors being associated with PV in moderately penetrant genes, whereas in women with ER-negative tumors we have found more frequent germline mutations in highly penetrant genes and genetic screening limited to these genes is justified. In ER-positive tumors, a genetic screening approach using NGS methods is more effective, as PV transmission is more frequently detected in genes with intermediate penetrance. A method such as NGS, which enables the simultaneous testing of a large number of genes for predisposition, allows for more effective genetic diagnosis, prognosis and personalization of treatment, especially in the more common cases of ER-positive tumors.

In contrast to the lack of correlation between histological type and PV in predisposing genes, a moderate positive association was found between surrogate molecular tumor type and carrier status *($\chi^{(2)}(9,203)=27.85$, $p=0.001$, $N=203$, Cramer's $V=0.214$)*. Table 9 and

Figure 5 show the distribution of PV carrier state, according to their penetrance, among women with different molecular subtypes of tumor.

Table 9. Distribution of PV in moderate-penetrant genes in different surrogate molecular subtypes of BC

		Number of women with a genetic defect in moderate-penetrant genes (%)	Total number of women examined with the corresponding histological type
Surrogate molecular subtype	Luminal A-Like	13 (11,8%)	110
	Luminal B-Like	4 (8,7%)	46
	HER2 enriched	1 (10%)	10
	Triple Negative	4 (10,8%)	37
Total		22 (10,8%)	203

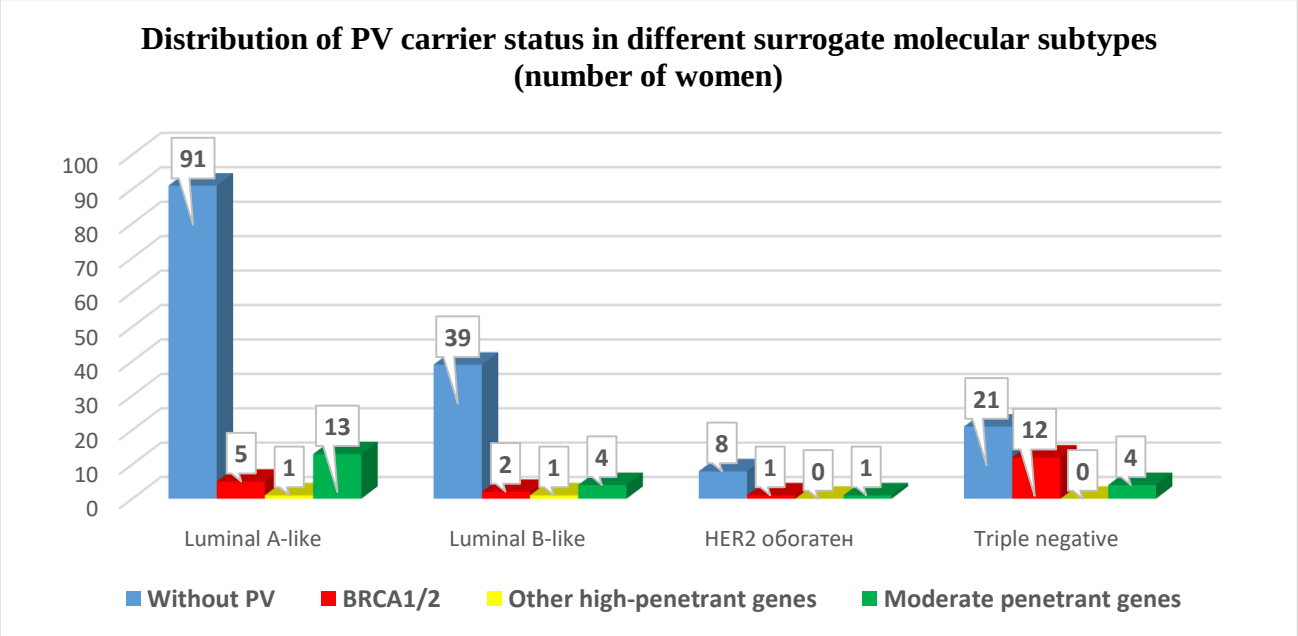


Figure 5 Distribution of PV carrier status in predisposition genes, according to penetrance, among women with different surrogate molecular tumor subtypes. Values shown in the figure reflect actual number of women.

Our finding of a statistically significant strong association between molecular subtype and PV carrier in predisposing genes confirms that it is no coincidence that nowadays the molecular subtype of the tumor and not the histological type is the guiding factor for the diagnostic and therapeutic plan in patients with BC. Carrier status of PVs in predisposition genes also define specific molecular processes in the breast cells that can lead them out of the normal growth and proliferation processes and lead to malignant transformation. Therefore, it is of utmost importance to investigate the molecular mechanisms of cell transformation, in particular the predisposition of somatic cells to such transformation (the presence of germline mutations in predisposition genes), in order to better understand the processes of tumorigenesis and to be able to modify (treat) them more effectively.

2.1.1. High-penetrant genes

In the present study, the frequency of *BRCA* mutations found (9.9 %) was twice lower than that found in an earlier study conducted for the Bulgarian population (19.5 %). This discrepancy is probably due to the fact that the earlier study with Bulgarian patients was conducted on a selected group of women with BC, in accordance with the recommendations of the Breast Cancer Linkage Consortium (BCLC) and the NCCN for genetic testing for cancer predisposition. Despite this apparent contradiction, the frequency of PV variants found in our study corresponds to that published for an unselected group of BC patients in the Serbian population. In that study, the frequency of PV in *BRCA1* was 7.6%, compared with 6.4% in our study. The frequency of detected pathogenic variants in *BRCA2* in our study was 3.4%, while it was estimated at 4.8% for the Serbian population. In another study in Israel, an almost identical overall frequency was found for both *BRCA* genes (9.3 %). The PVs found in *BRCA1* have different molecular characteristics - nonsense (nonsense, generating a stop signal), missense (missense, with change in meaning), frameshift (frameshift, with change in reading frame), inframe (inframe, without change in reading frame) and splice mutations (splice cite, affecting splice sites). The most common variant was **c.5266dup** (Gln1756ProfsTer74), which was found in 6 women (3% of all women tested). The next most common variant was **c.5062_5064del** (Val1688del), which was found in 3 women. The following variants were detected in one woman (0.5%): **c.181T>G** (Cys61Gly); **c.2019del** (Glu673AspfsTer28); **c.5333-1G>A**. The most common *BRCA1* mutation we found, c.5266dupC in exon 20, is the second most common mutation in the Breast Cancer Information Core (BIC) database and the most common in Central and Eastern Europe. In the group of women with BC, PVs were also found in another high-penetrant gene, *PALB2*. *The PALB2* variants we found (**c.172_175delTTGT** and **c.509_510del**) are known PVs with the

characteristic of a frameshift mutation, both of which result in the creation of a premature stop signal in protein chain synthesis

Detailed information on all women with PVs found in high-penetrant BC predisposition genes with clinical, familial, histological and molecular characteristics are detailed in Appendix 1

2.1.2. Moderate-penetrant genes

In the present study, 22 of 44 women with BC (all carriers of PV in predisposing genes) were found to have 25 PVs in 11 genes with intermediate penetrance. This suggests that 50% of the hereditary BC forms in the present study are due to a genetic variant in the intermediate penetrance cancer predisposition genes. A detailed description of the women with BC and PV found in the moderate penetrant genes and their clinical, familial, histologic and molecular characteristics can be found in Appendix 2.

2.2. In women diagnosed with BC in different age groups

The analysis of our data on the frequency of carriers and the type of PV in the genes predisposing to BC in women diagnosed at different ages shows that mutations in *BRCA1/2* were most frequently found in those diagnosed before the age of 40, in about two-thirds of cases (in 13 out of 19 women in this age group with PV). In the age group between 40 and 50 years, almost equal numbers of mutations were found in genes with high penetrance (57%, n=8 of 14 women with PV) and in genes with moderate penetrance (43%, n=6). In women diagnosed after the age of 50, PV was found more frequently in moderate-penetrant genes (80 % of the PV found, n=9 of 11 women with PV). The detailed distribution of PVs found in susceptibility genes in the desktop study by age group is shown in Table 10.

We searched for an association between the age of diagnosis in the studied patients and the penetrance of the affected gene and found that there was a statistically significant strong association between the two parameters ($_{\chi}(2)(15,203)=32.79$, p=0.005, N=203, Phi=0.402).

Table 10. Distribution of patients, from different age groups, according to the carrier status of PV in genes for predisposition to BC

Age of diagnosis of BC	**Number (n)/%**	**Patients with P/LP variants in BRCA1/2 №(%)**	**Patients with P/LP variants in other high-penetrant genes №(%)**	**Patients with P/LP variants in genes with moderate penetrance №(%)**	**Patients without P/LP variants in susceptibility genes №(%)**
< 29 years old	8 (3.9%)	4 (50%)	0	0	4 (50%)
30-39 years	42 (20.7%)	9 (21.4%)	1 (2.4%)	5 (12%)	27 (64.2%)
40-49 years	87 (42.4%)	5 (5.7%)	1 (1.1%)	8 (9.2%)	73 (83.9%)
50-59 years	39 (19.3%)	1(2.6%)	0	3 (7.7%)	35 (89.7%)
60-69 years	22 (10.9%)	1 (4.5%)	0	5 (22.7%)	16 (72.7%)
> 70 years	5 (2.6%)	0	0	1 (20%)	4 (80%)
Total	203 (100%)	20 (9.9%)	2 (1%)	22 (10.8%)	159 (78.3%)

2.3. In women with familial BC

In our study, FBC was found in **15.8%** (n=32) (affected first-degree relative with BC, regardless of age of diagnosis), with an established carrier status of PV in BC predisposing genes **of 21.9%** (n=7). The distribution of PVs in the highly and moderately penetrant genes was almost equal, with 9.4% (n=3) carrying the mutation in the highly penetrant genes: *BRCA1* - c.5266dup (n=1), *BRCA2* (n=1)- c.3975_3978dup, *PALB2* (n=1) - c.172_175del, and 12.5% (n=4) were found to have PVs in the moderate-penetrant genes: *FANCG*(n=1) - c.1760+2T>A; *CHEK2* (n=2) - c.444+1G>A and c.470T>C; *BLM* (n=1)- c.1642C>T.

A prevalence of FBC was calculated, taking into account not only first-degree relatives with FBC but also those with OC, and a proportion of **17.7%** (n=36) was found. In these cases a higher prevalence of carriers of PV were found **- 25%** (n=9), compared to the prevalence found when considering familiality for BC only (21.9%). The PVs found were again evenly distributed between high and moderate penetrance genes, 14.3% (n=5) and 12.9% (n=4), respectively.

The mutation spectrum found in the two groups of women, one with family history only for BC, the other with family history for both BC and OC, differed in the frequency of PV carrier state in the *BRCA1/2* genes. A higher proportion of PV in the *BRCA* genes was found when the family history analysis also took into account the relatives with OC (44.4%,

n=4 of all cases of FBC and PV in the predisposing genes), compared with the proportion of carriers among women with family history for BC only (28.6%, n=2).

Familial cases that were found to have total relatives with BC (n=32) and relatives with OC and other associated carcinomas (PC, PnC) (n=11) accounted for a total of **21.2%** (n=43) (Table 2). The estimated proportion of hereditary form pf BC in this group was **32.6%** (n=14), and again PVs were evenly distributed between the highly and moderate penetrant genes, at 14.3% (n=6) and 19.0% (n=8), respectively.

From the analysis performed in the three groups with familiality (1st degree relative): 1) for BC only; 2) for BC and OC; 3) for BC, OC and other associated carcinomas, it appeared that the highest detection of hereditary forms of BC occurred when the genealogical analysis considered relatives not only with BC and OC but also with other associated carcinomas. Thus, we found that the proportion of PV carriers in the third group of studied women (32.6%, n=14) was about one-third higher than that in the group of women with a relative with only BC (21.9%, n=7).

After our statistical processing of the results and looking for a correlation between family history and PV carrier status in the predisposition genes for BC, we found a strong positive correlation between I degree relatives (BC/OC or other associated carcinomas) and PV carrier state in the predisposition genes *($\chi^{(2)}(9,203)=31.06$, $p< 0.001$, $N=203$, $Phi=0.391$).*

We also looked for such an association in the presence of an affected relatives of II or III degree, but found none *($\chi^{(2)}(9,203)=14.01$, $p=0.12$ and $\chi^{(2)}(9,203)=10.02$, $p=0.35$, respectively).*

2.4. In women with TNBC

We found an overall prevalence of carriers of PV, in predisposition genes, among the total group of women with TNBC of **43.2%** (n=16/37), with PV in *BRCA1/2* found in about one/third of women with TNBC (32.4%, n=12/37), and the proportion of PV in moderate-penetrant genes was 10.8% (n=4/37). The proportion of PV we found indicates that almost half of TNBC cases are hereditary forms, with two-thirds of these forms due to PV in the *BRCA1/2* gene (n=12/37, 75% of all PV detected in TNBC).

Summary of our own survey results for women with BC

A summary statistical analysis of all clinical, familial, histologic, and genetic results was performed for all women with BC in our study cohort.

Two clusters emerged: the first consisted of 156 women (76.8% of all women studied) and the second of 47 women (23.2%) (Figure 6). A total of 17 criteria (including all clinical, histologic, familial, and genetic characteristics discussed in this chapter) were used to define the two clusters.

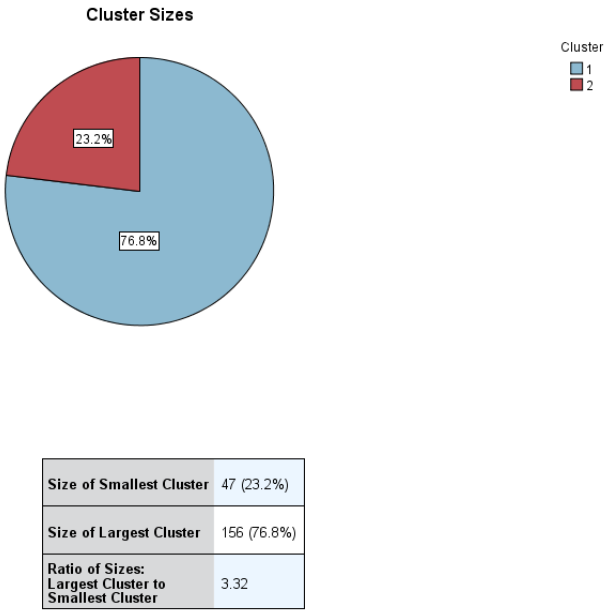


Figure 6. Clusters among the study group of women with

To summarize, we can state that our study group of women can be formally divided into two groups. The first group is three times more larger and has the following characteristics: Tumors are predominantly ER (+) (in 99.4 % of cases), moderately differentiated (59.6 %), mostly surrogate molecular subtype Luminal A-like (in 70.5 %), with predominantly germline PV carrier in moderate penetrance (most frequently *CHEK2*). The second group of women was characterized by predominantly ER(-) tumors (100 %), which were low differentiated (in 51.1 %), most frequently had a triple negative phenotype (in 78.7 %), and the most frequently found PV were in highly penetrant predisposing genes (most frequently *BRCA1*).

3. Age, reproductive, familial, histological and clinical characteristics of the studied women with OC

Age characteristics

We divided all the women with OC into six age groups according to the age of diagnosis (similar to women with BC) as follows: between 20-29 years; between 30-39 years; 40-49 years; 50-59 years; 60-69 years and over 70 years. The detailed distribution of women in these age groups is shown in Table 11 and Figure 7.

Table 11. Distribution of women with OC, according to age of diagnosis

Age group	Number of women	Share /in %/
20-29г.	2	3.0
30-39г.	1	1.5
40-49г.	12	17.9
50-59г.	21	31.3
60-69г.	21	31.3
over 70.	10	14.9
Total	**67**	100.0

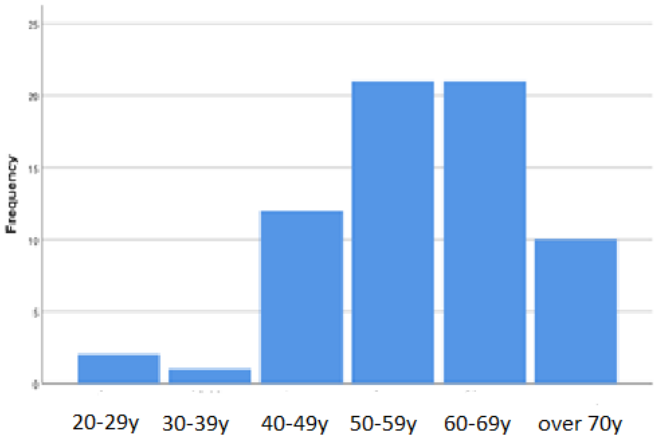


Figure 7: Percentage distribution of women with OC by age group

It was found that most women were diagnosed in the 50-59 and 60-69 age groups, with the two groups together accounting for almost two-thirds (62.6%, n=42) of our study cohort. Women diagnosed under the age of 40 years were only 3 (4.5%)(Table 11).

BMI and age of menarche

About two-thirds (66.7%, n=44) of women with OC were found to have a BMI ≥ 25, supporting the role of overweight as a risk factor for developing this disease. To confirm this conclusion, statistical processing of the data was performed and a correlation between age of diagnosis of OC and BMI was sought. A very strong, statistically significant, positive relationship was found between the two parameters *($\chi^{(2)}$(561,67)=654.09, p=0.004, N=67, Cramer's V=0.758).*

We found the mean age of menarche in the study group of women to be 13.7 years (OCnge 11 to 16 years), and looked for an association between age of menarche and age of diagnosis of OC. Statistical significance was not reached *(p=0.52),* probably due to the small number of women in the study cohort with OC.

Family history

In the study group of 67 women with BC, only 11.9% (n=8) of these cases were found to have a family history of BC/OC. According to other population-based studies, the incidence of familial OC is higher, reaching up to 20%. The reason for the lower incidence of familial cases that we found is probably related to the fact that OC is usually detected late in the disease course when other foci are already present and it is sometimes difficult to identify the primary site. Therefore, some familial cases remain unrecognised or are mistakenly attributed to a different location.

Histological characteristics of tumors

The results of this study confirmed the literature data that SCOC was the most frequently diagnosed histological type of OC, being found in 67.2% (n=45) of the women studied. The mean age of diagnosis of OC in these women was found to be 62.6yrs. The data on the distribution of different histological types of OC, among the study cohort, is presented in Table 12.

Table 12. Distribution of histological types of OC among the group of women with this disease

Histological type	**Number of women**	**Share in %**
HGSOC	45	67.2
LGSOC	5	7.5
Endometrioid	9	13.4
Serous+endometrioid	2	3.0
Mucinous	4	6.0
Clear Cell	2	3.0
Total	67	100.0

The 7.5% (n=5) prevalence of LGSOC we found (Table 12) is slightly higher than the prevalence reported in the literature of about 5%. This discrepancy is most likely due to the fact that our study cohort with OC is relatively small, resulting in an unrealistic overestimation of the proportion of patients with LGSOC. Endometrioid tumors were represented in our study cohort with a proportion of 13.4% (n=9) (Table 12), is consistent with literature data (10-20%). Two of the women studied (3.0%) were found to have both endometrioid and serous components in the tumor. Mucinous ovarian carcinoma (MOC) was detected with a proportion of 6% (n=4) (Table 12), which is comparable to reported data in the literature. Clear cell ovarian carcinoma (COC) is represented in approximately 5% of all patients with OC in the United States. Women of Asian descent are most commonly affected (~11% of all patients with OC), and it is less common in African American

women (~3%) or women of Caucasian descent (~5%). The found prevalence of COC among the women studied by us was only 3% (n=2), (Table 12) which is consistent with the European population.

4. Frequency and profile (type and molecular characteristics) of P/LP variants in cancer predisposition genes among the studied group of women with OC

We found, in the studied cohort, germline PVs in the genes predisposing to OC in 18 women (**26.9%**, out of 67 women with OC) (Figure 8), which is consistent with the literature data on the proportion of hereditary OC (HOC)

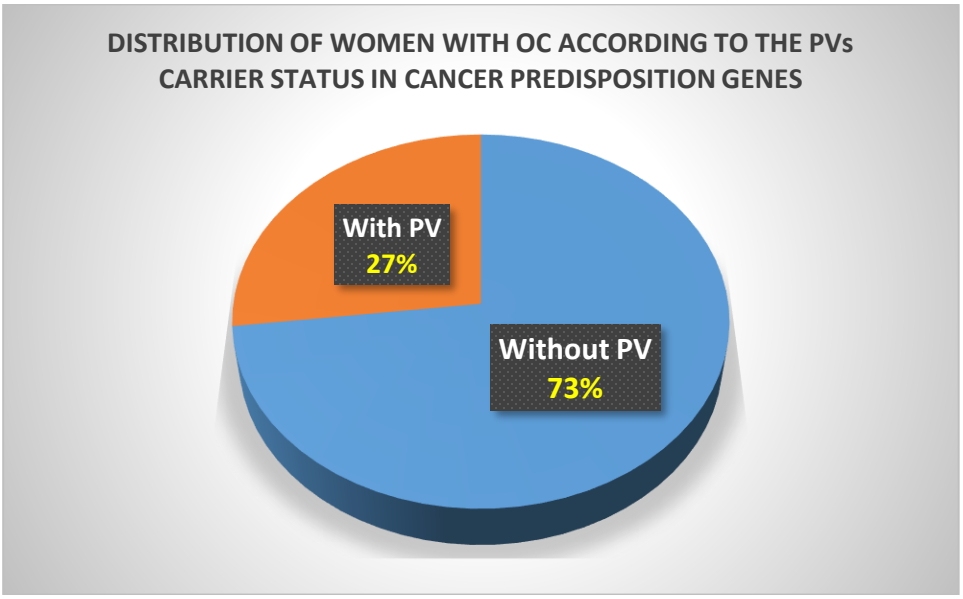


Figure 8: Distribution of women with OC according to PV carrier status in cancer predisposition genes.

The impact of germline mutations on cancer risk is variable and depends on the penetrance of the gene. Depending on the magnitude of the risk conferred by PV carrier status in OC predisposing genes, we divide predisposition genes into high-penetrant genes (>5-20-fold risk for OC), genes with moderate penetrance (1.5-5-fold risk), and low-penetrant gene loci that do not independently confer a clinically significant increase in risk.

The genetic factors conferring the highest risk of developing OC, *BRCA1/2*, are the cause of the autosomal dominant cancer syndrome, Hereditary Breast and Ovarian Cancer (HBOC). Pathogenic germline variants in *BRCA1* and *BRCA2* confer a cumulative risk of 39-63% and 17-27%, respectively, for the development of OC, as well as an increased risk for PC and PnC. The incidence of this hereditary cancer syndrome we found in the study group of women with OC was 9% (n=6) of all cases with OC and one/third (33.3%) of cases with

HOC, which represents a slightly lower proportion than reported in the literature, as the PVs were found only in the *BRCA1* gene. In 5 of the women this was the only PV found, while in the sixth woman we found combined carrier status with PV in another predisposing gene (MINAS). The median age of diagnosis found in our study in *BRCA1* mutation carriers was 51.7 years (OCnge 41-66 years), which is almost 10 years less than the median age of diagnosis in patients without pathogenic variants in the predisposing genes (59.5 years). This, confirms the literature data of early onset of OC in *BRCA1* positive women. Familial cases among *BRCA* mutation carriers were found in 66.7% (n=4) of cases. The most frequent histological type of OC in *BRCA1* PV carriers is HGSOC due to the involvement of these genes in the DNA repair mechanism called homologous recombination (HR), which is a major pathogenetic factor in carcinogenesis in this histological variant. Our study confirmed the literature data by finding HGSOC in 83.3% of all *BRCA1* mutation carriers (n=5). The sixth patient was also found to have this histological type, but with foci of endometrioid carcinoma, and this woman was found to carry an additional genetic factor (PV in the *CHEK2* gene). A reverse analysis of these results showed that of all women with proven HGSOC (n=45), approximately 11.1% (n=5) were found to carry the *BRCA1* mutation, which is consistent with results from other previous studies among unselected groups of women with OC

The BRIP1, *RAD51C* and *RAD51D* genes are also involved in HR processes and are considered to be moderate penetrant with respect to risk for OC. The cumulative risk they carry is about 5.2-9% for *RAD51C* and 10-12% for *RAD51D* mutations, respectively. For *BRIP1* mutation carriers, the literature-estimated cumulative risk for OC is 5.8%. Other genes involved in HR, such as *PALB2*, *ATM*, *NBN*, and *CHEK2*, may also be involved in ovarian carcinogenesis, a moderate risk of developing OC and are classified as genes with moderate penetrance, and are therefore increasingly included in multigene cancer panels.

There are other inherited cancer syndromes that are also associated with a risk of developing OC. Lynch syndrome (LS) is one such type. Carcinogenesis in LS causes the following features of tumours - microsatellite instability (MSI) (tested by PCR), loss of MMR proteins (by IHC) and a large number of somatic mutations, all of the above collectively combined under the term MMR deficiency. The widespread introduction of 'universal screening' for LS (all cases with colorectal cancer -CRC and all cases of endometrial cancer - EC diagnosed before age 60 to be tested for MMR deficiency) has led to an increasing number of suspected cases of LS - MMR-deficient tumours without a germline mutation in the MMR genes. These cases are attributed to the so-called Lynch-like syndrome (LLS). The etiology of LLS is not yet clear, but three possible mechanisms are suspected: a) germline mutation in other genes involved in MMR that may also cause MMR deficiency in tumour tissue, b) germline mutation in MMR genes that cannot be identified due to limitations of the

DNA test performed, c) molecular process in tumour cells causing the same deficiency. LLS patients are therefore a heterogeneous group that includes sporadic cases with biallelic MMR deficiency and hereditary cases associated with pathogenic germline variants in other genes involved in DNA repair. Carriers of hereditary MMR deficiency and their carrier relatives are at high risk for a second primary cancer and should be referred for prophylaxis. Conversely, individuals with somatic mutations in MMR genes and their relatives are not at increased risk. The genetic basis of hereditary LLS is not yet fully understood. With the advent of next-generation sequencing (NGS) and the ability to test many cancer predisposing genes simultaneously, many genes have now been shown to be associated with hereditary cases of LLS - *MUTYH*, genes involved in regulating cellular activity (*EXO1*, *POLD1*, *RCF1* and *RPA1*), *BUB1* and *BUB3*, *SETD2*, *WOC*, *BARD1*, and other genes that disrupt genomic integrity

Our study found an equal proportion of patients with OC and LS, 3% (n=2, one case with PV in *MSH2* and one case with PV in *PMS2* gene) and OC and LLS, also 3% (n=2, both cases with PV found in *WRN* gene). The results of the present study, show that LS and LLS, occupy a large proportion, in total about 22% of the hereditary forms of OC.

In summary, about half of the cases of HOC (43.3% - 33.3% for HBOC and 11% for LS and LLS combined, respectively) are due to autosomal dominant cancer syndromes - Hereditary Breast and Ovarian Cancer and Lynch Syndrome. In addition, about 11% of HOC3 is due to LLS still under investigation.

The remaining cases of HOC (45.7%) were the result of PV in other genes predisposing to HOC: high-penetrant - *RAD51D*, moderate-penetrant - *TP53*, *FANCE*, *FANCM*, *FANCL*, *FANCG*, *ERCC3*, *ATM*, *NBN*, *WOC*, *CHEK2*. Detailed distribution of women with OC and PV carriers according to penetrance and specific predisposing gene is presented, respectively, in Table 13 and Table 14.

Table 13. Distribution of women with OC and PV according to penetrance of the predisposing gene.

Genes, depending on penetrance	**Number of women**	**Share (%)**
None	49	73.1
BRCA1/2	5	7.5
Other high-penetrant genes	2	3.0
Moderate-penetrant	8	11.9
MINAS	3	4.5
Total	67	100.0

Table 14. Distribution of women with OC and PV carriers according to the specific predisposing gene.

Gene	Number of women carrying a genetic defect	Share (%)
None	49	73.1
ATM	1	1.5
BRCA1	5	7.5
CHEK2	1	1.5
FANCL	1	1.5
FANCM	1	1.5
MINAS	3	4.5
MSH2	1	1.5
NBN	1	1.5
PMS2	1	1.5
RAD51D	1	1.5
WRN	2	3.0
Total	67	100.0

We found that the mean age at diagnosis of women carrying PV in the predisposing genes was 53.5 years, 6 years younger than the mean age at diagnosis of women without PV (59.5 years). We found a strong positive correlation, with statistical significance, between age at diagnosis and PV carrier status in the predisposition genes for OC *($\chi^{(2)}$(132,67)=161.097, p=0.043, N=67, Cramer's V=0.775)*, confirming the literature evidence that carrier status of genetic defects in the susceptibility genes for OC is associated with younger age at diagnosis of this disease.

Regarding the distribution of PVs according to their penetrance, the results of the present study showed that the incidence of HOC due to germline PVs only in high-penetrant genes was almost equally distributed 38.9% (n=7, PVs in *BRCA1, RAD51D, MSH2*) and PVs only in moderate-penetrant genes 44.4% (n=8, PVs in *ATM, CHEK2, FANCL, FANCM, NBN, PMS2, WRC*). The proportion of MINAS in HOC was found to be 16.7% (n=3). The distribution of cases with OC according to the penetrance of the PVs found is presented in Figure 9

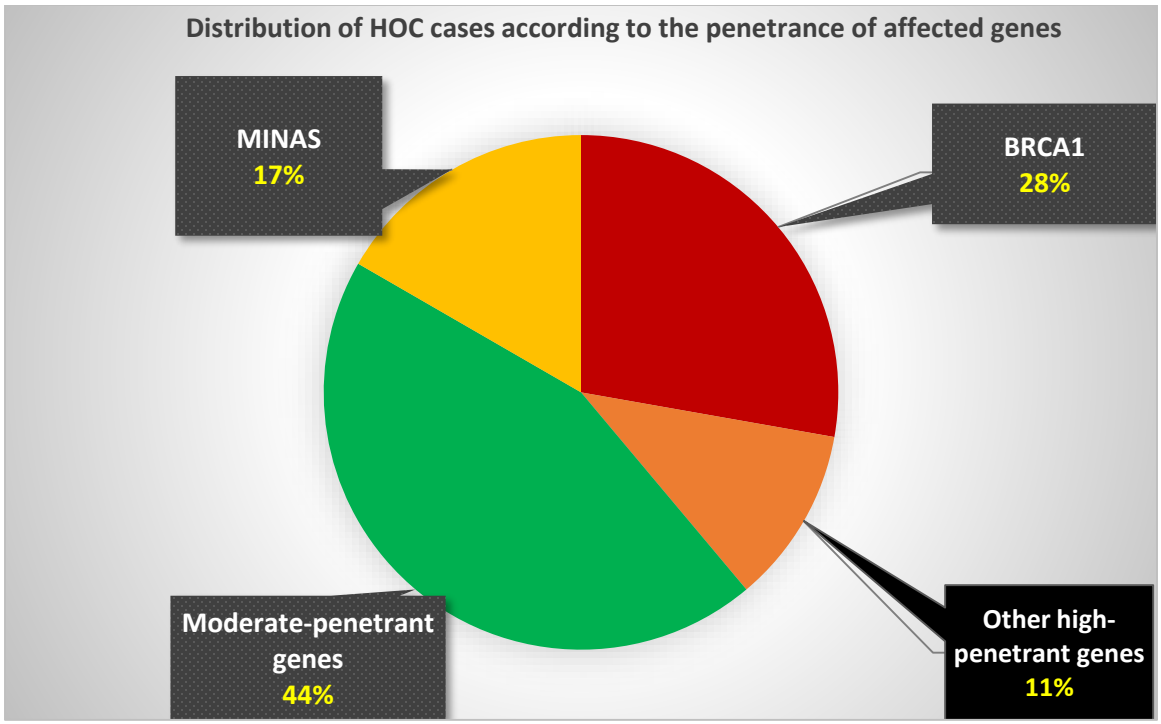


Figure 9. Distribution of HOC cases according to the penetrance of the affected genes.

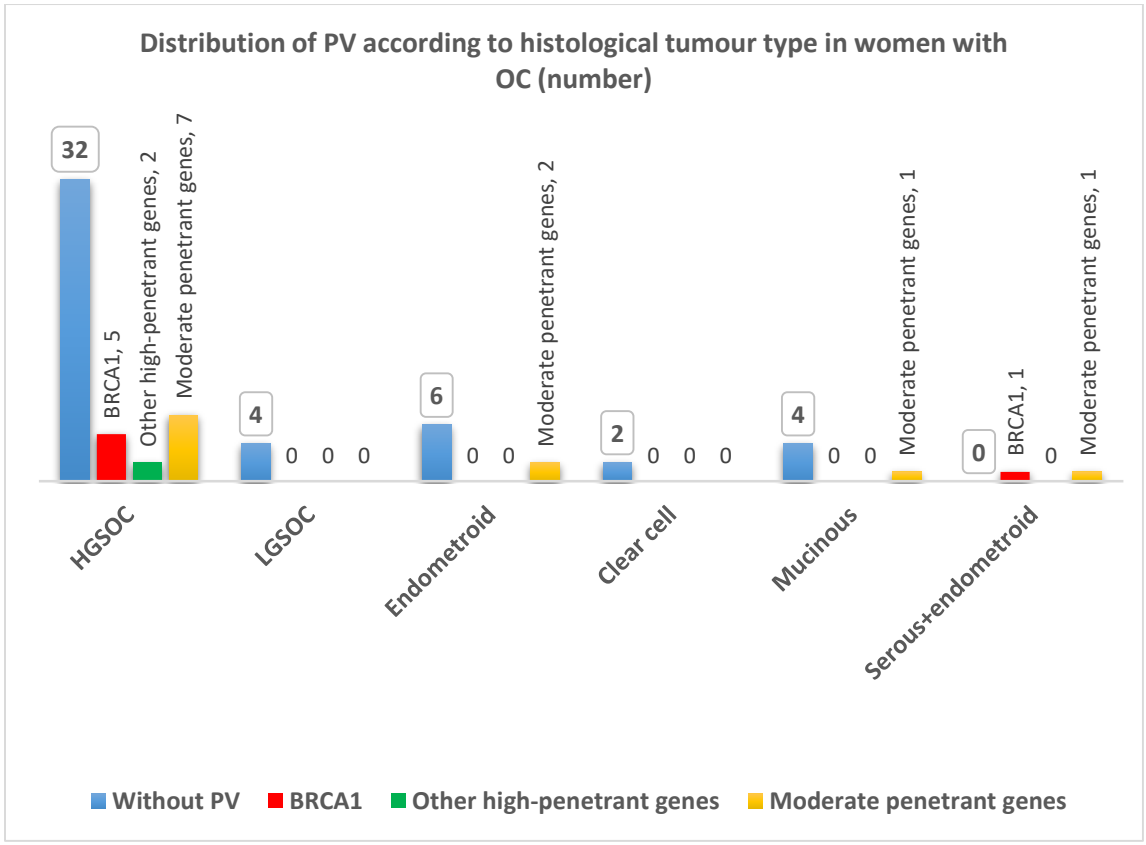


Figure 10. Distribution of carriers of genetic defects with different penetrance in different histological types of OC (number of women)

Regarding the relationship between the PVs found and the histological type of the tumor, it is remarkable, in our study (see Figure 10), that among the cases of HGSOC, *BRCA* genes and moderate-penetrant genes play almost an equal role, whereas in the endometrioid histological type, mainly moderate-penetrant genes and high-penetrant genes other than *BRCA* play a role.

We looked for a correlation between the specific gene in which we found PV and tumor histology, but found none *(p=0.084)*. In contrast, we found a statistically significant strong positive association between gene penetrance and tumor histology *($\chi^{(2)}$(20,67)=52.12, p<0.001, N=67, Phi=0.882*), with PVs in high-penetrant genes correlating more strongly with serous histological type.

Interestingly, in all cases of mixed serous and endometrioid tumors, combined carrier status of PV was found in more than one cancer predisposing gene (MINAS), which is most likely explained by the presence of more than one pathogenetic pathway leading to the development of OC in these patients. MINAS cases will be discussed in detail in Appendix 5 of this paper.

This study also found several cases of synchronous endometrial and ovarian cancer (SEOC) (both primary cancers diagnosed within 6 months). SEOC accounts for about 5% of endometrial cancers and 10-20% of ovarian cancers. SEOC accounts for 50-70% of all synchronous gynecologic cancers in women. The characteristic histology of SEOC is endometrioid adenocarcinoma of both the endometrium and ovary, and this histologic variant has been described in about 70% of cases of synchronous tumors with these two locations. SEOC has been found to be a more common finding in patients with LS and LLS, compared to women without these syndromes. In our study, a total of four women with LS and LLS were found, and in three of them (75%) the diagnosis was SEOC, confirming the literature evidence that the presence of SEOC is one of the clinical features of hereditary gynecologic cancer syndromes (LS, LLS) and is important for genetic counseling in such patients as well as in their at-risk relatives

4.1. High-penetrant

In the present study, we found a prevalence of germline mutation in high-penetrant genes among women with OC of **12%** (n=8 out of 18 cases of OC). This proportion is relatively lower than the reported incidence of 15-20% for other populations, and the lower proportion, in our study, appeared to be mainly on account of the lower incidence of *BRCA1/2* mutations (some of the women carrying BRCA mutations were pre-screened by pharmaceutical companies in connection with the target therapy with PARB-inhibitors and had no motive to undergo genetic testing with us). The median age at diagnosis that we found for women carrying PV in the high-penetrant genes, 52.4 years, was about 8 years younger than women who did not carry PV in the predisposition genes. PVs were found in three high-penetrant genes, *BRCA1*, *RAD51D*, and *MSH2*.

A detailed description of the clinical, histological and familial characteristics of women with OC and PV carrier status in high-penetrant genes is presented in Appendix 3

4.2. Moderate-penetrant genes

In the present study, 13 PVs in 11 moderate-penetrant genes were found in 11 women with OC, out of the 18 women who were found to have a hereditary form of OC (carriers of PVs in predisposing genes). This showed that almost two-thirds (62%, n=11) of the hereditary forms of OC in the present study were due to a genetic variant in the moderate-penetrant cancer predisposition genes. We found a prevalence of germline mutation carrier in moderate-penetrant genes among women with OC of 14.9% (n=10 cases), and this accounted for more than half (55.6%) of women carrying PV in predisposing genes (18 cases in total). One of the moderate-penetrant PV carriers (n=1/11) was found to have combined with PV in the *BRCA1* gene. The median age at diagnosis of female carriers of PV in the moderate-penetrant genes was found to be 54.4 years (OCnge 34-72 years), which was about 5 years younger than that of non-carriers (59.5 years). The PVs found were in the *TP53*, *FANCE, FANCM, FANCL, FANCG, ERCC3, ATM, WOC, NBN, PMS2*, and *CHEK2* genes

A detailed description of the clinical, histological and familial characteristics of women with OC and PV in high-penetrant cancer predisposing genes is presented in Appendix 4.

Summary of the results of our own survey of women with OC

The statistical analysis was carried out for the entire group of women with OC and all the characteristics examined. Two clusters emerged, although they are not very clear. They differ in seven main characteristics. The first group of women was the larger and consisted of 35 women (58.2% of all 67 women with OC in the study) and the second of 28 women (41.8%). The larger group included women most commonly diagnosed in the 50-59 age group, with the average age of this group at diagnosis being 61.23 years. None of the women in this group were found to have a familial predisposition or PV in the genes that predispose to OC. The second group comprised women most commonly diagnosed in the 40-49 age group, with an average age at diagnosis of 52.39 years, 10 years younger than the women in the first group. The main characteristics that distinguish this group from the first are the presence of family history (all women with family history for BC/OC belong to this group) and the presence of hereditary predisposition (all women with germline PV in the predisposition genes belong to this group). The definition of these two groups in the women with OC studied showed that OC is more frequently diagnosed in women in adulthood (after the age of 60), but that a familial and/or hereditary predisposition is more likely to be present in women diagnosed before the age of 50.

Summary and discussion of the results from the general study group of women with BC/OC

In the discussion so far, we have considered the spectrum of genes and the molecular characterization of the genetic defects found separately for the group of women with BC and the group of women with OC. The purpose of this separation was to personalize the genetic counseling of patients with these diseases, taking into account the individual clinical and family history as well as the histological and immunohistochemical characterization of the tumors. However, the genetic etiology of the hereditary forms of BC and OC is common. Recently, it has been accepted to group the hereditary forms of both diseases under the name Hereditary Breast and Ovarian Cancer (HBOC). With the advent of new genomic technologies, the spectrum of genes for predisposition to BC and OC is constantly increasing. The results of the present study on the genetic etiology of the hereditary forms of BC and OC confirmed that the same genes are involved in both diseases. The two groups of women with BC and OC were pooled and the frequency and molecular characterization of genetic variants in susceptibility genes were investigated in the entire study cohort. The frequency of molecular characteristics of each PV found in the pooled group of women with BC/OC is shown in Table 15.

Table 15. Frequency and molecular characteristics of PVs found in susceptibility genes among all women with BC/OC studied.

Gene	Genetic syndrome with which it is associated	Type of inheritance	Phenotype in patients of the present study	Transcript	HGVS variant nomenclature	Place of the variant	Protein - variant nomenclature	Type of variant	Clinical relevance (ACMG)	Number of carriers	Proportion in % of the total
BRCA1	HBOC/FA	AD/AR	**BC/OC**	NM_007294.4	c.5266dup	Exon: 19/23	Gln1756ProfsTer74	Frameshift Indels	Pathogenic	8	3.0
BRCA1	HBOC/FA	AD/AR	**BC/OC**	NM_007294.4	c.181T>G	Exon: 4/23	Cys61Gly	Missense	Pathogenic	2	0.7
BRCA1	HBOC/FA	AD/AR	**BC**	NM_007294.4	c.5062_5064del	Exon: 16/23	Val1688del	InfOCme deletion	Pathogenic	3	1.1
BRCA1	HBOC/FA	AD/AR	**BC/OC**	NM_007294.4	c.5333-1G>A	Exon:		Splice acceptor	Pathogenic	2	0.7
BRCA1	HBOC/FA	AD/AR	**BC**	NM_007294.4	c.2019del	Exon: 10/23	Glu673AspfsTer28	Frameshift Indels	Pathogenic	2	0.7
BRCA1	HBOC/FA	AD/AR	**OC**	NM_007294.4	c.3700_3704del	Exon: 10/23	p.(Val1234GlnfsTer8)	Frameshift Indels	Pathogenic	1	0.4
BRCA1	HBOC/FA	AD/AR	**OC (MINAS)**	NM_007294.4	c.5497G>A	Exon: 23/23	Mr. (Val1833Met)	Missense	Pathogenic	1	0.4
BRCA2	HBOC/FA	AD/AR	**BC**	NM_000059.4	c.9097dup	Exon: 23/27	Thr3033AsnfsTer11	Frameshift Indels	Pathogenic	1	0.4
BRCA2	HBOC/FA	AD/AR	**BC (MINAS)**	NM_000059.4	c.5851_5854del	Exon: 11/27	Ser1951TrpfsTer11	Frameshift Indels	Pathogenic	1	0.4
BRCA2	HBOC/FA	AD/AR	**BC**	NM_000059.4	c.9682del	Exon: 27/27	Ser3228ValfsTer21	Frameshift Indels	Pathogenic	1	0.4
BRCA2	HBOC/FA	AD/AR	**BC**	NM_000059.4	c.6955A>T	Exon: 13/27	ABC2319Ter	Stop gained	Pathogenic	1	0.4
BRCA2	HBOC/FA	AD/AR	**BC**	NM_000059.4	c.3975_3978dup	Exon: 11/27	Ala1327CysfsTer4	Frameshift Indels	Pathogenic	1	0.4
BRCA2	HBOC/FA	AD/AR	**BC**	NM_000059.4	c.7975A>G	Exon: 17/27	ABC2659Gly	Missense	Pathogenic	1	0.4
BRCA2	HBOC/FA	AD/AR	**BC**	NM_000059.4	c.658_659del	Exon: 8/27	Val220IlefsTer4	Frameshift Indels	Pathogenic	1	0.4
PALB2	HBOC/FA	AD/AR	**BC**	NM_024675.4	c.509_510del	Exon: 4/13	ABC170IlefsTer14	Frameshift Indels	Pathogenic	1	0.4
PALB2	HBOC/FA	AD/AR	**BC**	NM_024675.4	c.172_175del	Exon: 3/13	p.(Gln60ABCfsTer7)	Frameshift Indels	Pathogenic	1	0.4
FANCM	FA	AR	**BC**	NM_020937.4	c.1139_1140del	Exon: 6/23	p.(ABC380IlefsTer14)	Frameshift Indels	Likely pathogenic	1	0.4

									(**Unreported en**)		
FANCM	FA	AR	**OC**	NM_020937.4	c.1972C>T	Exon: 11/23	p.(ABC658Ter)	Stop gained	Likely pathogenic	1	0.4
FANCL	FA	AR	**BC/OC**	NM_001114636.1	c.1111_1114dup	Exon: 14/14	p.(Thr372AsnfsTer13)	Frameshift Indels	Likely pathogenic	2	0.7
FANCG	FA	AR	**BC**	NM_004629.2	c.1760+2T>A	Exon:		Splice donor	Likely pathogenic (**Unreported**)	1	0.4
FANCG	FA	AR	**OC (MINAS)**	NM_004629.2	c.1538G>A	Exon: 12/14	p.(ABC513Gln)	Missense	Likely pathogenic	1	0.4
FANCI	FA	AR	**BC**	NM_001113378.2	c.3645C>G	Exon: 34/38	Mr. (Tyr1215Ter)	Stop gained	Likely pathogenic	1	0.4
FANCE	FA	AR	**OC (MINAS)**	NM_021922.3	c.1239dup	Exon: 7/10	p.(Pro414SerfsTer54)	Frameshift Indels	Likely pathogenic	1	0.4
BRIP1 (FANCJ)	HBOC/FA	AD/AR	**BC**	NM_032043.3	c.3201C>A	Exon: 20/20	p.(Cys1067Ter)	Stop gained	Likely pathogenic (**Unreported**)	1	0.4
RAD51D			**OC**	NM_002878.4	c.803G>A	Exon: 9/10	p.(Trp268Ter)	Stop gained	Pathogenic	1	0.4
RAD51C	FA	AR	**BC**	NM_058216.3	c.931del	Exon: 7/9	p.(Ile311TyrfsTer3)	Frameshift Indels	Likely pathogenic (**Unreported**)	1	0.4
TP53	LFS	AD	**OC (MINAS)**	NM_000546.6	c.1148_1149del	Exon: 11/11	Mr. (Leu383HisfsTer8)	Frameshift Indels	Likely pathogenic (**Unreported**)	1	0.4
MSH2	LS	AD	**OC**	NM_000251.3	c.1386+1G>A	Exon:		Splice donor	Pathogenic	1	0.4
WOC	LLS/Werner syndrome	?/AR	**OC**	NM_000553.6	c.4109del	Exon: 34/35	p.(Asn1370ThrfsTer23)	Frameshift Indels	Likely pathogenic	1	0.4
WOC	LLS/Werner syndrome	?/AR	**OC**	NM_000553.6	c.1105C>T	Exon: 9/35	p.(ABC369Ter)	Stop gained	Likely pathogenic	1	0.4
PMS2	LS	AD	**OC**	NM_000535.7	c.163+1G>T	Exon:		Splice donor	Pathogenic	1	0.4
ERCC5	XP	AR	**BC (MINAS)**	NM_000123.4	c.495del	Exon: 5/15	p.(Trp165CysfsTer5)	Frameshift Indels	Likely pathogenic (**Unreported**)	1	0.4
ERCC3	XP	AR	**OC (MINAS)**	NM_000122.2	c.325C>T	Exon: 3/15	p.(ABC109Ter)	Stop gained	Pathogenic	1	0.4
ATM	AT	AR	**BC**	NM_000051.4	c.1564_1565del	Exon: 10/63	p.(Glu522IlefsTer43)	Frameshift Indels	Pathogenic	1	0.4
ATM	AT	AR	**BC**	NM_000051.4	c.7475T>G	Exon: 50/63	Mr. (Leu2492ABC)	Missense	Likely pathogenic	1	0.4
ATM	AT	AR	**BC (MINAS)**	NM_000051.4	c.2131_2132dup	Exon: 14/63	p.(Asn711LysfsTer25)	Frameshift Indels	Likely pathogenic	1	0.4
ATM	AT	AR	**OC**	NM_000051.4	c.8147T>C	Exon: 55/63	Mr. (Val2716Ala)	Missense	Pathogenic	1	0.4
NBN	NBS	AR	**OC**	NM_002485.5	c.2140C>T	Exon: 14/16	p.(ABC714Ter)	Stop gained	Pathogenic	1	0.4
BLM	BS	AR	**BC**	NM_000057.4	c.1642C>T	Exon: 7/22	Mr. (Gln548Ter)	Stop gained	Pathogenic	2	0.7
CDKN2A	Cancer syndrome with melanoma	AD	**BC**	NM_000077.5	c.71G>C	Exon: 1/3	p.(ABC24Pro)	Missense	Pathogenic	1	0.4
CHEK2		**?**	**BC/BC (MINAS)**	NM_007194.4	c.470T>C	Exon: 4/15	p. (Ile157Thr)	Missense	Pathogenic	8	3.0
CHEK2		**?**	**BC/OC (MINAS)**	NM_007194.4	c.444+1G>A	Exon:		Splice donor	Pathogenic	2	0.7
CHEK2		**?**	**BC (MINAS)**	NM_007194.4	c.917G>C	Exon: 9/15	Mr. (Gly306Ala)	Missense	Likely pathogenic	1	0.4
CHEK2		**?**	**BC**	NM_007194.4	c.433C>T	Exon: 3/15	p.(ABC145Trp)	Missense	Likely pathogenic	1	0.4
CHEK2		**?**	**OC**	NM_007194.4	c.1427C>T	Exon: 13/15	p.(Thr476Met)	Missense	Likely pathogenic	1	0.4

In the total group of 270 women (203 with BC and 67 with OC), we found PV in predisposition genes in 23% (n=62) of women, which is consistent with the proportion of inherited forms found in the groups of women with BC (21.6%, n=44) and OC (26.9%, n=18) separately. A total of 45 PVs were found in 22 susceptibility genes (Table 15) (Figure 11). Six variants, previously unreported in global genomic databases, were found in the following ***FANCM, FANCG, BRIP1 (FANCJ), RAD51C, TP53*** and ***ERCC5,*** resulting in a **13.3%** proportion of novel variants, twice the proportion of novel variants reported in similar analyses performed for other populations. The high proportion (13.3%) of novel variants detected in our study is evidence of the genetic heterogeneity of the Bulgarian population with respect to the etiology of HBOC.

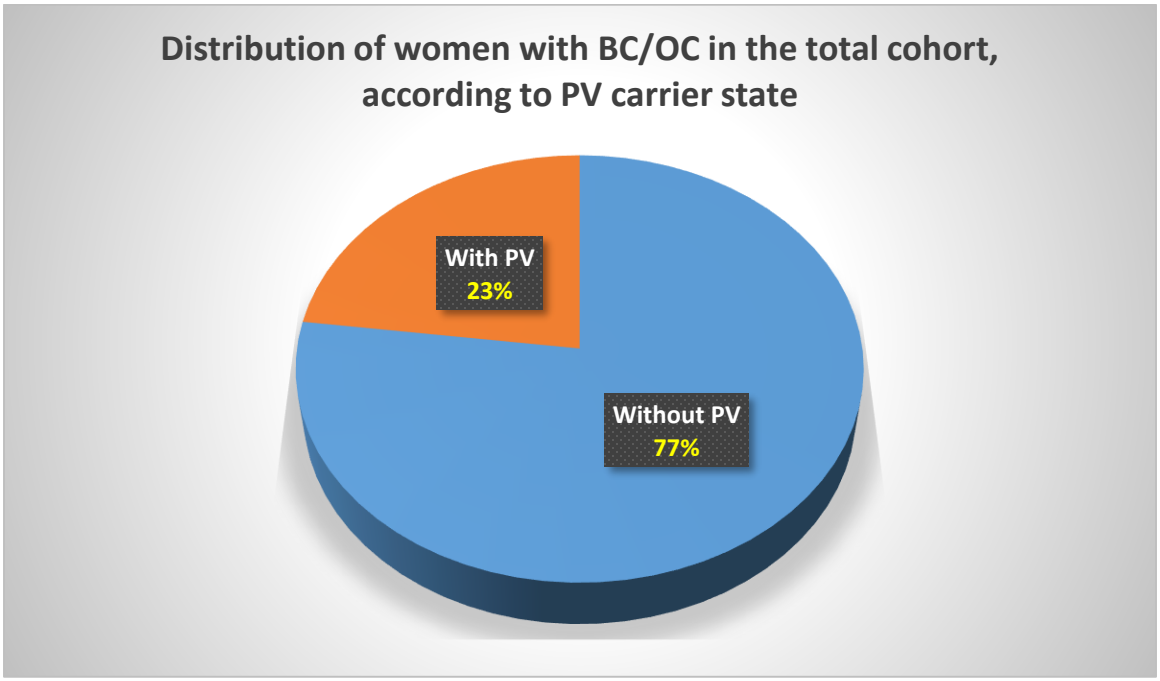


Figure 11. Distribution of women with BC/OC in the overall study cohort, according to PV carrier status in cancer predisposing genes.

In our study, we calculated the frequency of PV in each HBOC susceptibility genes in both the total study group of women with BC/OC (n=270) and in the group of women found to carry PV (with HBOC) (n=62). The frequencies found in the two groups of women were compared with literature data to attempt to define a spectrum of affected genes in women with BC/OC for the Bulgarian population (Table 16).

Table 16. Distribution of PVs found, by gene, in the total study group of women with BC/OC

Gene	Genetic syndrome with which it is associated	Type of inheritance	Number of PVs in the total study group of women with BC/OC	Number of carriers in the total study group of women with	Percentage of all women surveyed (270 women in total)	Proportion in % of unselected women with BC/OC according to literature data	Proportion in % of all women with HBOK (62 women in total)	Percentage of women with HBOC according to literature data
BRCA1 (FANCS)	HBOC/FA	AD/AR	7	19	**7.0**	5-15%	**30.6**	4.
BRCA2 (FANCD1)	HBOC/FA	AD/AR	7	7	**2.6**		**11.3**	
PALB2 (FANCN)	HBOC/FA	AD/AR	2	2	**0.7**	1-3%	**3.2**	
FANCM	FA	AR	2	2	**0.7**		**3.2**	2.2-3.
FANCL	FA	AR	1	2	**0.7**		**3.2**	
FANCG	FA	AR	2	2	**0.7**		**3.2**	
FANCI	FA	AR	1	1	**0.4**		**1.6**	
FANCE	FA	AR	1	1	**0.4**		**1.6**	
BRIP1 (FANCJ)	FA	AR	1	1	**0.4**		**1.6**	
RAD51D			1	1	**0.4**		**1.6**	
RAD51C	FA	AR	1	1	**0.4**		**1.6**	
TP53	LFS	AD	1	1	**0.4**		**1.6**	
MSH2	LS	AD	1	1	**0.4**		**1.6**	
WOC	LLS/Werner syndrome	?/AP	2	2	**0.7**		**3.2**	
PMS2	LS	AD	1	1	**0.4**		**1.6**	
ERCC5	XP	AR	1	1	**0.4**		**1.6**	
ERCC3	XP	AR	1	1	**0.4**		**1.6**	
ATM	AT	AR	4	4	**1.5**		**6.5**	
NBN	NBS	AR	1	1	**0.4**		**1.6**	
BLM	BS	AR	1	2	**0.7**	<1%	**3.2**	
CDKN2A	Cancer syndrome with melanoma	?	1	1	**0.4**		**1.6**	
CHEK2			5	13	**4.8**	1-5%	**21.0**	

We divided the genes with PVs found in this study into several groups, depending on the function of the proteins they encode.

Genes involved in DNA repair of double-strand breaks

BRCA1/2

BRCA1/2 genes are the most common cause of HBOC, affecting all races and ethnic groups. We found a prevalence of PV in *BRCA1* and *BRCA2* in the overall group with BC/OC of 9.6% (n=26 of 270 women), with germline mutations in *BRCA1* accounting for 7% (n=19/270) of BC/OC cases and PV in *BRCA2* accounting for 2.6% (n=7/270) of cases. In our study, in the total cohort of women studied, about 10% of cases were due to *BRCA* PV, and the remaining 13% were for PV in other predisposing genes. The found frequency of PV in *BRCA* genes is in complete agreement with known literature data, as well as with data from a recently published large study of nearly 4600 patients with BC/OC.

The PVs we found in the *BRCA* genes accounted for a large proportion of all PVs found in the present study (38.8%, n=26/67 for both genes and 28.4% for *BRCA1* and 10.4% for *BRCA2* separately, respectively). This high frequency found, showed that the majority of HBOC in Bulgarian women is also due to PV in *BRCA1/2* genes.

The most common *BRCA1* mutation found was **c.5266dupC** in exon 20, the second most common mutation in the Breast Cancer Information Core (BIC) database, and the most common in Central and Eastern Europe. It was found among high-risk families with BC/OC in Poland with an incidence of 34%, in Russia - 14%, Hungary - 14%, Slovenia - 13%, in the Jewish Ashkenazi population -10%, Greece - 8%, Germany - 4%, Italy - 3%. In practice, it is absent in Spain and Portugal and occurs with low frequency in the Netherlands, Belgium and Scandinavian countries We found the frequency of this mutation to be 3% in the total group of women studied and 13% in the group of women with HBOC. In Russia, Belarus, Poland, Latvia, Czech Republic, Greece and Lithuania this mutation represents 94%, 73%, 60%, 55%, 37-52%, 46% , 34% of all *BRCA1* mutations found, respectively.

The results of our study show that for the Bulgarian population this mutation accounts for 42.1% of all found PV in *BRCA1*. Five of the PVs in the *BRCA1* gene were found in more than one carrier (c.5266dup; **c.181T>G; c.5333-1G>A; c.5062_5064del; c.2019del**), the first three were found in women with BC and women with OC, while the other two (**c.3700_3704del; c.5497G>A**) were found only once each, and only in patients with OC (Table 15). The highest frequencies in *BRCA1* were PV c.5266dup (3% of the total cohort of women with BC/OC, 13% of the group of women with HBOC) and c.5062_5064del (0.7% of the total cohort of women with BC/OC, 4.8% of women with HBOC).

In *BRCA2*, a total of 7 PVs were found in 7 carriers, with no presence of recurrent PVs. This is partly related to the lower incidence *of BRCA2* compared to *BRCA1* due to the lower penetrance of *BRCA2*. Germline PVs in *BRCA1* are associated with a 57-65% and 39-

44% lifetime risk, respectively, for BC and OC. In addition, PVs in *BRCA1* are also associated with an increased risk for PC and PnC. The risks conferred by germline PVs in *BRCA2* are lower (compared to *BRCA1*), 45-55% and 11-18% lifetime risk for BC and OC, respectively.

Other Fanconi anemia genes

The BRCA1 and *BRCA2* genes (formally *FANCS* and *FANCD1*, respectively) also belong to the FA gene group, although their involvement in this disease is a matter of debate, as the biallelic state of *BRCA* mutations has a lethal effect during the embryonic period. All proteins encoded by FA genes play a tumor-suppressor role, creating a complex that is activated upon double-strand DNA damage and is involved in repair by the mechanism of HR. There are numerous reports in the literature that FA monoallelic PV in many FA genes associates with HBOC, but those for which there is multiple evidence for this are *PALB2* and *BRIP1*. In the present study, PVs were found in the following FA genes (excluding *BRCA1/2*) - *PALB2*, *FANCM*, *FANCL*, *FANCG*, *FANCI*, *FANCE*, *BRIP1 (FANCJ)*, *RAD51C*. Our detection of PV carriers in FA genes (without *BRCA1/2*) in the total group of women with BC/OC was 4.4% (n=12 out of 270 women), accounting for 17.9% (n=12/62) of all BC/OC cases.

We found a prevalence of PV *PALB2* carriers in the total cohort of women with BC/OC was 0.7% (n=2/270), and accounted for 3.2% (n=2/62) of all cases of HBOC, and was significantly lower when compared with literature data (approximately 10% of HBOC cases).

Monoallelic PVs in *PALB2* result in an increased risk of BC for both sexes, which was recently estimated at 53% lifetime risk for women and 1% for men. Carriers of germline PVs in *PALB2* have also been found to have a slightly increased risk of 5% for OC and 2-3% for PC. In the present study, both female carriers of PV in *PALB2* had BC.

The frequency of another FA gene, *BRIP1*, that we found was consistent with the literature data in both the total group of women with BC/OC studied and the group of women with HBOC (Table 16). Of the remaining FA genes, which according to literature data are rare genetic defects, we found a relatively higher frequency (.7%, each) in *FANCM*, *FANCL*, and *FANCG*.

ATM

The prevalence of PV in the *ATM* gene was found to be 1.5% (n=4) of all women studied and 6.5% of women with HBOC, which is consistent with data in the literature (Table 16). All four detected variants are different, which once again proves the genetic heterogeneity of the Bulgarian population. While the involvement of the PV in *ATM* gene in tumorigenesis in BC and PnC has been proven, its role in relation to OC is still under investigation. Three of the four female carriers of PV in *ATM* were diagnosed with BC, which is consistent with literature data.

RAD51 paralogs - ***RAD51C, RAD51D***

Recent studies have shown that germline mutations in *RAD51C* carries a higher mean cumulative risk for OC of 6.94 (between 20%-40%), compared to a risk for BC of 1.93. Some studies have found that the incidence of PV in *RAD51C* is between 0.4-2.9% in cases of hereditary BC/OC, while other studies have found no PV in *RAD51C* in BC/OC. This discrepancy is likely due to differences in the genetic analysis methodology used in different studies, the majority of which do not use next-generation sequencing methods. We found the frequency of PVs in RAD51C in the total group of women with BC/OC to be 0.4% (n=1), and in the group of women with HBOC the proportion of these PVs was estimated to be 1.6%. The PV found in RAD51C was in a woman with the less common localization for such PV, namely, BC, which on the one hand shows the advantages of the DNA analysis methodology we used and on the other hand confirms the genetic heterogeneity of the Bulgarian population. However, the found , frequency is low, which, with the genetic analysis technology used, indicates that PVs in RAD51C are rare in our population.

NBN

In the present study, one woman was found to be a carrier of PV in the *NBN*, making the frequency of carriership for the entire study group of women only 0.4% (n=1), confirming the data that genetic defects in this gene are rare in hereditary forms of BC and OC.

CHEK2

In the present study, PVs in the *CHEK2* gene were the second most frequent among women in the total group with a prevalence of 21% (n=13/62). Five variants with different molecular characteristics were found, and one of them, p. (Ile157Thr), was found in 8 women (with a frequency in the total group of studied women of 3% and a proportion of 12.9% of HBOC cases). This variant is found in relatively high frequency in different European populations and carries a moderate to low risk for developing HBOC. In our study, this variant was found only in cases of women with BC, and one of the patients had combined carrier status of PV in other predisposing genes (MINAS).

Genes involved in DNA repair of single-stOCnded nucleotide repeats (NER) - *ERCC3, ERCC5*

We found a low prevalence of PV in genes involved in repair mechanisms by excision of mispaired nucleotides, 0.4% (n=1) each for *ERCC3 and ERCC5*, in the entire study group of women with BC/OC and 1.6% in the group of women with HBOC. Which can be partly

explained by their lower penetrance, i.e. they are associated with lower risks of developing HBOC.

Genes involved in DNA repair of single nucleotide substitutions (MMR) - *MSH2, PMS2*

Lynch syndrome (LS) is an autosomal dominant cancer syndrome with a high risk of developing endometrial and CRC. Patients with PV in the LS genes (*MLH1, MSH2, MSH6, PMS2*) are at risk for developing other tumors such as OC, with a lifetime risk estimated at 4-12%. While the histology of tumors in OC resulting from germline *BRCA* mutations is serous type, that of LS is endometrioid. Whether LS associates with BC has not yet been clarified. In the present study, PVs in genes involved in the repair of single nucleotide errors were found in 0.8% (n=2), only in patients with OC, and in both patients histology was endometrioid carcinoma. The role of genes encoding proteins involved in single-nucleotide repair in the etiology of HBOC is predominantly with respect to the development of OC and less for the development of BC, and the results of our study confirm this.

Other genes

BLM, WRN, TP53

The observed prevalence of PV in the ***BLM*** gene was 0.7% (n=2), and both women were diagnosed with BC. The variant in both women (from different families) was the same. This variant is known to have a founder effect in the European population, which is most likely true for the Bulgarian population in particular. PV in ***WRN*** were also found in two female patients, with a carrier rate of 0.7% (n=2) in the total study group of women. The two women had OC, an endometrial histological type that is characteristic of the so-called Lynch-like syndrome (described in detail in the previous chapter).

We found that the prevalence of PV in ***TP53*** was only 1.6% in the group of women with HBOC, with an expected proportion of about 20% according to literature data. This discrepancy can be explained by the fact that in Bulgarian women with HBOC, *TP53* is very rarely affected

In summary, the genes involved in the repair processes of double-strand breaks, and HR in particular, are the major contributors (in 83.6% of cases) to the etiology of H. BRCA1/2 has the highest frequency of PVs, and they are an etiologic factor in about 39% of HBOC cases. We found a lower frequency of germline mutations in PALB2 and TP53, suggesting that these two genes play a minor role in the spectrum of HBOC in Bulgarian patients in contrast to other populations. For the other affected genes (except BRCA1/2), a relatively higher incidence in women with HBOC was found in the present study, although reliable literature data are not available for some of them due to their extreme rarity. This

relatively higher incidence may be explained, on the one hand, by the DNA analysis technique we used, which allows a more complete and comprehensive investigation of the genetic etiology of HBOC, and, on the other hand, by the genetic heterogeneity of our population. In addition, six new PVs were found in our study that were not previously recorded in global databases of genetic variants, which, together with the large number of PVs found with different molecular characteristics, supports the conclusion that the Bulgarian population is genetically heterogeneous.

5. Development of an approach for genetic counselling depending on the carrier status of the P/LP variant in the susceptibility genes in patients with BC/OC

5.1. Genetic counselling in cancer

The need for genetic counseling in cancer patients has increased dramatically in recent years with the introduction of new sequencing technologies, as the amount of information that needs to be evaluated, analyzed and implemented/applied in the diagnosis and treatment of these patients, as well as in the prevention for them and their relatives, is increasing.

The traditional approach to identifying patients with a genetic predisposition involves risk assessment and genetic testing for predisposition with mandatory genetic counseling before and after the genetic test result.

Initial genetic counselling

During the initial genetic consultation (prior to genetic testing), the genetic counsellor obtains and analyses information about the patient's personal medical history, reproductive history, lifestyle and family history.

Determining the risk

Risk assessment models

There are risk assessment models for BCs and WNs that use empirical data and software processing to determine the probable risk of a particular individual (Gail's, Claus's, etc.).

Genetic testing

Primary genetic counselling includes selecting the appropriate genetic test that will provide the most accurate picture of the genetic aetiology of BC/OC in the individual patient.

Secondary genetic counselling

Secondary genetic counselling (performed after the genetic test) includes interpreting the results of the genetic test, discussing treatment and prevention options as well as the patient's risk of recurrence or involvement of another site and prevention for the patient and their at-risk relatives.

5.2. Approach to genetic counselling for PV in genes with high penetrance

Genetic counseling regarding the management of patients in whom germline P/VP variants are found in highly penetrant predisposing genes is based on generally accepted recommendations (NCCN, ESMO).

5.3. Approach to genetic counselling for PV in genes with moderate penetrance

Multigene panels contain genes with moderate penetrance. For many of these genes, there is limited data on the extent of cancer risk, and there are currently no consistent and clear guidelines for the medical management of these patients and their relatives. The approach to genetic counselling in these cases is determined by a variety of factors.

5.3.1 Depending on the discovered gene

In the entire cohort of women with BC and OC (n=270), we found that **23.7%** (n=64) had hereditary cancer. About half, 48.4% (n=31), of the PVs involved genes causing autosomal dominant cancer syndromes. About one third of all detected pathogenic variants (29.7%, n=19 of 64 female PV carriers) were in genes that cause autosomal recessive monogenic diseases (Fanconi anaemia, Xeroderma pigmentosum, Ataxia teleangectasia, Nijmegen syndrome, Bloom syndrome, etc.) in the homozygous state (biallelic mutation), while in the heterozygous state they lead to a predisposition of the carrier to develop a certain type of cancer. The detection of PV in recessive disease genes leads to the need to discuss reproductive options as part of genetic counselling to prevent the birth of a child with an autosomal recessive syndrome. Genetic counselling recommendations are limited to genetic testing of the partner and, if PV is found in the same gene, prenatal diagnosis during a subsequent pregnancy.

5.3.2. Depending on *the PV detected* in the

Some P/LP variants in a gene may confer a higher or lower risk than other P/LP variants in the same gene.

Variants in the *CHEK2* gene were found to be the second most frequent in the total cohort of women studied, after those in the *BRCA1* gene.

In general, LOF variants in *CHEK2* are characterized by a pathogenic effect. In contrast, missense variants have different effects (from pathogenic to benign) and their effect depends on whether a critical protein domain is affected. Several missense variants, p.I157T, p.S428F and p.T476M, are associated with a lower risk of BC compared to loss-of-function variants. Studies provide evidence for a role of *CHEK2* in thyroid cancer development. It has been suggested that in terms of family history, there is an association between thyroid cancer and BC; previous history of thyroid cancer is a risk factor for breast cancer and vice versa (relative risks in the range 1.7-12.4), and for the variant p.I157T, the estimated relative risk is about 2.8-fold.

In our study of women with BC, the most frequently detected PV in *CHEK2* was the missense variant c.470T>C (p.I157T). However, primary genetic counseling revealed thyroid carcinoma in two of the women. One of the patients was found to have a familial first-degree relative (mother) diagnosed at age 59 with thyroid cancer, and the second patient was diagnosed with papillary thyroid cancer herself, 2 years before she was diagnosed with BC. In both women, genetic analysis found PV carrying a relatively lower risk for both BC and thyroid, but taking into account personal medical history in one case and family history in the other, the genetic counseling assumed that the risks in these two families for both cancers were empirically higher and that the relatives of these women should take more active prophylactic measures for both BC and thyroid cancer.

5.4. Approach to genetic counselling in case of a negative (indeterminate) genetic test result

A negative (indeterminate) genetic test result does not always mean the absence of PV in the predisposition genes. Genetic test results are considered indeterminate when it is not possible to accurately interpret them in terms of their clinical relevance to risk. There are two types of indeterminate results: They are potentially false-negative results or the variants found are of uncertain clinical significance (VUS). A potentially false-negative result is a genetic test result that is classified as negative either due to limitations of the genetic testing method used (e.g. the presence of large genomic alterations that cannot be detected by sequencing methods) or due to an insufficient database at the time of analysis - i.e. it is likely that a gene is affected that is not yet known in the genetic community. A further uncertainty in the genetic test result arises if a genetic variant of uncertain clinical significance (VUS) is detected. In these cases, the laboratory cannot interpret the genetic variant found as pathogenic or non-pathogenic (benign) and therefore cannot specify its clinical significance regarding cancer risk. In some cases, it takes some time before additional information on a particular variant is collected in databases, on the basis of which the variant can then be classified as benign or pathogenic. Until then, however, the genetic test result is presented to the patient as negative.

If the genetic test result is negative, the genetic counsellor can use software-supported models to determine empirical risks based on personal medical, reproductive and family history.

5.5. Approach to genetic counselling for carriers of PV variants in more than one gene for predisposition to BC/OC - Multilocus Inherited Neoplasia Allele Syndrome (MINAS)

Advances in genomic technology have made it possible to test patients for PV in many predisposition genes simultaneously. In 2016, Whitworth first proposed the term Multilocus Inherited Neoplasia Allele Syndrome (MINAS) for patients carrying PV in more than one predisposition gene. In our study, MINAS was found in 1.9% (5/270) of all screened women with BC and OC, and 40% (2/5) of these women were found to have combined transmission with PV in BRCA genes. The second most commonly affected gene was the CHEK2 gene, which we found in a further 40% (2/5) of MINAS cases, which is entirely consistent with the data from McGuigan's rebuttal study. A detailed description of the MINAS cases we found can be found in Appendix 5.

Summary

Genetic counseling of patients with hereditary forms of breast or ovarian cancer is of critical importance, both in terms of selecting a treatment strategy for the patient and in terms of the effectiveness of prevention of other cancers. The approach chosen by the genetic counselor depends on the penetrance of the affected gene, the molecular characterization of the pathogenic variant and the possible coexistence with other PVs. It is crucial for the patient that the genetic counselor collects all clinical, histological, familial and genetic information in order to personalize the risks in relation to other organs and to establish the most appropriate diagnostic and prophylactic plan. The genetic counseling approach is illustrated schematically in Figure 12.

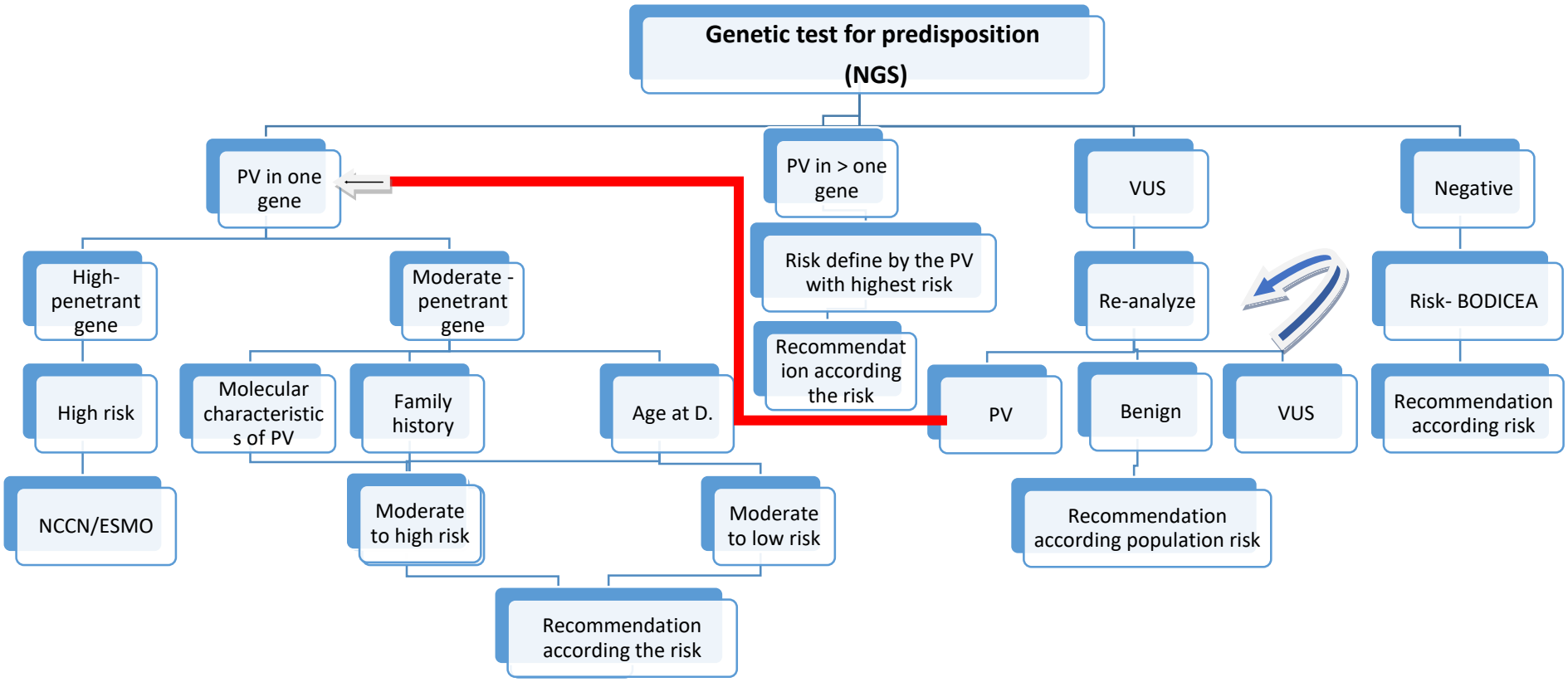


Figure 12. Approach to genetic counselling in patients with BC/OC

SUMMARY OF THIS STUDY

Breast cancer is the most common type of cancer in women, while ovarian cancer is one of the most malignant - with an aggressive course and a low survival rate, which determines the medico-social significance of these two malignancies. Their aetiology is complex and involves the participation of genetic and environmental factors that interact with each other in a complex way. The inherited forms of these two diseases are based on germline mutations in the cancer predisposition genes.

We found that the incidence of hereditary breast and ovarian cancer is 22% and 27%, respectively, almost twice as high as the data published in the literature from other studies. This is most likely due to the novel genomic technology used in the present study, which enables the simultaneous sequencing of a large number of genes associated with hereditary cancer.

A broad genetic spectrum was found in the hereditary forms of BC or OC, both in terms of genes affected and PVs found in them (47 PVs in 14 genes and 21 PVs in 14 genes, respectively). New PVs were found that were not previously recorded in global genome databases: 5 (11% of all detected) in BC and 1 (5% of all detected) in OC. The present study has shown that genetic screening for predisposition to BC/OC, which has been introduced for some populations and covers the most common genetic defects in a limited number of genes (mainly BRCA1/2), is not effective in a heterogeneous (from a genetic point of view) population such as the Bulgarian one.

In the hereditary forms of BC/OC, the study revealed an almost equal involvement of high and medium penetrance genes in the development of cancer. In young women (diagnosed <40 years of age), genes with high penetrance play a greater role in hereditary susceptibility to BC/OC, while in women diagnosed after 40 years of age, genes with medium penetrance play a greater role. It is confirmed that the BRCA1/2 and CHEK2 genes are most frequently affected.

Based on the results of the present study, a comprehensive approach to genetic counseling for hereditary forms of BC/OC has been developed. The role of the genetic counselor is crucial in HBOC with PV in genes of intermediate penetrance. In these cases, the analysis of all aggregated information (family history, affected gene, PV found) allows the personalization of the risk for the patient or his/her relative and the development of the most appropriate strategy for effective treatment of the underlying disease and prevention.

The results of the present study point to the need for a multidisciplinary approach in clinical practice for patients with hereditary breast or ovarian cancer and their relatives, integrating the knowledge and practical skills of physicians from different specialties to improve the diagnosis, treatment and prevention of these malignancies.

V. CONCLUSIONS

1. Regarding the main characteristics in women with BC:
   1.1. The age group most commonly affected (43% of cases) was those aged 40-49.
   1.2. The more common localization (54% of cases) is the left breast.
   1.3. Family history was found in 16% of all BC cases.
   1.4. It was confirmed that the most common histological type was NST (81% of cases) and the most common surrogate subtype (54%) was Luminal A-like. The proportion of TNBC was higher (18%) than that reported in the literature.
2. Regarding the frequency and profile of PV in cancer predisposition genes, in women with BC:
   2.1. The prevalence of PV in genes predisposing to BC in the total group of women was 22%. The found higher frequency than that reported in the literature is due to the new NGS technology used, which proves its advantages over traditional genetic analysis methods previously used in BC. Highly and moderate penetrant genes were equally affected, 11% (6% in *BRCA1*, 4% in *BRCA2* and 1% in *PALB2*) and 11% (*CHEK2* was the most frequently affected gene, accounting for 5% of cases), respectively.
   2.2. The highest prevalence of PV (38%) was found among patients diagnosed by age 39 years. We confirmed the literature data that in the same age group, *BRCA1/2* genes were most frequently affected (in 68.4% of cases with PV), whereas in the group of women after 40, moderate-penetrant genes were most frequently found to be involved in (72% of all PV found in this group).
   2.3. In the group of women with familial BC - 22% of them were carriers of PV, with a similar proportion of high- and moderate-penetrant genes, respectively - 43% and 57%. The incidence of overt PV in BRCA genes increases to 44% if relatives with OC are also considered in the family history analysis. This confirms the similar genetic aetiology (involvement of BRCA1/2 genes) of both diseases (BC and OC), and makes genealogical analysis of these diseases mandatory to consider family history for both conditions.
   2.4. Among women with TNBC, PVs were 43.2%, with about two-thirds in the BRCA1 gene.
3. Regarding the main characteristics in women with OC:
   3.1. It was confirmed that the most affected age group was between 50-69 years and we found the mean age of diagnosis to be 57.54 years.
   3.2. Overweight was confirmed to be involved in the etiology of OC, with 2/3 of women studied (67%) having a BMI ≥ 25.
   3.3. Familial OC accounts for about 12% of all cases.

3.4. It is confirmed that the most common histological type is HGSOC (67% of cases).

4. The prevalence of PV in susceptibility genes in the overall group of women with OC was 27%, with 9% of PVs in BRCA1/2, 3% in other high-penetrant genes, and 15% in moderate-penetrant genes.
5. In the total cohort of women studied with BC and OC, 45 PVs (in 22 genes) were found, 6 of them (13.3%, in the ***FANCM, FANCG, BRIP1 (FANCJ), RAD51C, TP53*** and ***ERCC5*** genes) previously unreported in global genomic databases. This demonstrates the high heterogeneity of genetic defects for these diseases in the Bulgarian population.
6. Hereditary multilocus neoplasia syndrome (MINAS) was found in 2% of all women with HBOC, and in 40% of these cases one of the variants was in the BRCA genes.
7. The peculiarities of the genetic profile of the Bulgarian population confirm the need for more comprehensive genetic screening, using the new genomic NGS technologies, which would increase the detection of individuals with a predisposition/risk for developing these cancers, with a view to more effective prevention, early diagnosis and treatment.
8. In contrast to women with HBOC due to PV in high-penetrant genes, in whom genetic counseling mainly adheres to generally accepted recommendations, in women with PV in moderate-penetrant genes, the role of the genetic counselor is critically important in order to personalize risk and build the most appropriate treatment strategy for the underlying disease and prevention of other possible localizations.
9. Genetic counseling for BC and OC is a major approach to detect inherited forms of these diseases. Summarizing and analyzing information from available clinical and familial data, along with the result of genetic testing, allows the genetic counselor to more accurately assess the risk in the individual patient and his or her relatives, providing guidance for more effective early diagnosis, treatment, and prevention.

VI. CONTRIBUTIONS

Contributions of a scientific and original nature

1. For the first time, a comprehensive study of genetic defects in a broad spectrum of 94 susceptibility genes in women with breast or ovarian cancer from the Bulgarian population has been conducted in our country using next-generation sequencing methods.
2. The data for the Bulgarian population on the frequency and the profile of pathogenic variants in the genes for predisposition to BC and OC are supplemented.
3. New pathogenic variants have been discovered that have not been described before for the Bulgarian population, as well as those that have never been described in world databases.
4. New scientific data are added regarding the role of PV in moderate penetrantgenes in the etiology of cancers with other sites found in relatives of women with FBC and FOC.
5. New scientific evidence is being added on the role of genetic factors (mutations in susceptibility genes) in BC and OC, confirming their similar genetic etiology.

Contributions of an applied nature

1. A comprehensive approach to genetic counseling of patients with hereditary BC/OC and their at-risk relatives has been developed for early diagnosis, more effective treatment and prevention of these diseases.
2. It was found that in a heterogeneous population such as the Bulgarian one, next-generation sequencing (with a single-month complete analysis of a specific set of genes) would be the most appropriate and effective method for application in the framework of genetic screening for HBOC.
3. The study substantiates the need for a thorough genealogical analysis in cases of HBOC, taking into account the familial basis for both conditions.
4. The responsibilities and the role of the genetic counsellor as an integral part of the multidisciplinary team involved with patients with BC or OC in the following stages are confirmed: interpretation of family data and selection of the appropriate genetic test for testing; analysis of the detected variants and, based on the carrier status of the PV, determination of treatment guidelines; detection of relatives at risk.

VII. PUBLICATIONS AND PARTICIPATION IN SCIENTIFIC EVENTS RELATED TO DISSERTATION

Publications

- **Zornica B. Kamburova**, Katia S. Kovacheva, Savelina L. Popovska, Maria N. Simeonova, Genealogy of families with breast cancer - a tool to identify women at risk - J Biomed Clin Res, **2012**: Vol 5, 2; 139-147 p.;
- Kovacheva K., **Z. Kamburova**, S. Popovska, I. Ivanov, M. Simeonova, P. Angelova. Study of the carrier state for five BRCA1/BRCA2 deleterious mutations in Bulgarian women with breast cancer. J Biomed Clin Res, **2013,** vol 6, 2:
- Kovacheva K, **Kamburova Z**, Popovska S, Dimitrov D, Ivanov I, Simeonova M, Deliyski T. Prevalence of five BRCA1/2 mutations in Bulgarian breast cancer patients. Journal of Biomedical and Clinical Research. **2018**;11(2):123-127. /ISSN 1313-9053/
- **Kamburova, Z.B.**, Dimitrova, P.D., Dimitrova, D.S., Kovacheva, K.S., Popovska, S.L., Nikolova, S.E. Lynch-like syndrome with germline WRN mutation in Bulgarian patient with synchronous endometrial and ovarian cancer. Hered Cancer Clin POCct 21, 13 (**2023**). https://doi.oBC/10.1186/s13053-023-00257-1

Participation in scientific forums abroad

- **Z. Kamburova**, K. Kovacheva, S. Popovska, I. Ivanov, M. Simeonova, R. Dodova, D. Dacheva, P. Angelova, V. Mitev, A. Mitkova, R. Preliminary study of BRCA1/BRCA2 mutations in Bulgarian women with breast cancer. The European Human Genetics Conference 2014, J12.022, published in European jouOCal of human genetics, **2014,** Vol. 22, Suppl-1,
- Dodova R., D. Dacheva, M. Taushanova, S. Valev, **Z. Kamburova**, K. Kovacheva, C. Timcheva, S. Christova, A. Mitkova, R. Kaneva. BRCA1/2 mutation screening in Bulgarian patients with triple negative breast - P019, The European Breast cancer Conference 2014, Publisher of abstOCcts - European journal of cancer, Vol 50, Suppl 2, **2014**

Participation in scientific forums in Bulgaria

- Kovacheva K., **Kamburova Z.,** Popovska S., Ivanov I., Simeonova M. Susceptibility gene mutations in Brest Cancer. 10th International Medical Scientific conference for Student and Yong Doctors, 17-20 October **2012**, Pleven, Bulgaria.
- Kovacheva K., **Kamburova Z.,** Petrova I., Dimitrov D., Popovska S., Ivanov I., Simeonova M., Deliiski T., Dimitrova N., Gatsev O., Petrov C., Hadyieva E., Sabev I., Iordanov S. Clinical Cases of Familial Brest Cancer in Bulgarien Patients.10th International Medical Scientific conference for Student and Yong Doctors, 17-20 October **2012**, Pleven,
- Kovacheva K., **Z. Kamburova**, M. Simeonova, S. Popovska, I. Ivanov, D. Dimitrov, I. Petrova, , T. Genetic screening for BRCA1/2 point mutations and large genomic rearrangements in Bulgarian women with breast cancer. Jubilee scientific conference - 40 years of Medical University Pleven, 30 Oct-1 Nov, **2014**. Pleven

APPENDIX 1 - Clinical, familial, histologic and molecular features of all BC cases in which PVs were found in highly penetrant cancer predisposition genes[1](* - new genetic variant not reported in the world databases)

[1] m./f. designations indicate in which parental line the affected relatives are

ID	BMI	Age of menarche	Age of 1st burden.	I - degree relatives	II - degree relatives	III - degree relatives	Age of D. of BC	Localization	Histological result	Degree of differentiation	Molecular surrogate subtype	Gene	Transcript	Variant	Type of variant	Classification of the variant
1-94	22.5	14	19				44	Right breast	NST	G2	TNBC	***BRCA1***	NM_007294.4	**c.2019del** p.(Glu673AspfsTer28) Exon: 10/23	Frameshift indels	Pathogenic (PVS1, PS3)
2-9	25	14	29	CRC f	BC-60 f		30	Left breast	NST	G3	TNBC	***BRCA1***	NM_007294.4	**c.2019del** p.(Glu673AspfsTer28) Exon: 10/23	Frameshift indels	Pathogenic (PVS1, PS3)
1-86	23.2	14	32		BC-38		35	Left breast	ducto-lobular	G2	TNBC	***BRCA1***	NM_007294.4	**c.5062_5064del** p.(Val1688del) Exon: 16/23	Inframe deletion	Pathogenic (PVS1, PS3)
1-95	20.8	13	20				47	Left breast	ducto-lobular	G2	TNBC	***BRCA1***	NM_007294.4	**c.5062_5064del** p.(Val1688del) Exon: 16/23	Inframe deletion	Pathogenic (PVS1, PS3)
6-13	20.4	14	39	SC-78 f	BC-67 f	OC-54 m	52	Right breast	NST	G2	Luminal A	***BRCA1***	NM_007294.4	**c.5062_5064del** p.(Val1688del) Exon: 16/23	Inframe deletion	Pathogenic (PVS1, PS3)
D5	20.1	12	23		SC-49 f		31	Left breast	NST	G3	TNBC	***BRCA1***	NM_007294.4	**c.5266dup** p.(Gln1756ProfsTer74) Exon: 19/23	Frameshift indels	Pathogenic (PVS1, PS3)
1-11	18.7	13	28	BC-64+OC-52; BC-33	BC-38		45	Left breast	NST	G3	TNBC	***BRCA1***	NM_007294.4	**c.5266dup** p.(Gln1756ProfsTer74) Exon: 19/23	Frameshift indels	Pathogenic (PVS1, PS3)
1-58	22.5	14	19	CRC-52			39	Left breast	NST	G2	TNBC	***BRCA1***	NM_007294.4	**c.5266dup** p.(Gln1756ProfsTer74) Exon: 19/23	Frameshift indels	Pathogenic (PVS1, PS3)

ID	BMI	Age of menarche	Age of 1st burden.	I - degree relatives	II - degree relatives	III - degree relatives	Age of D. of BC	Localization	Histological result	Degree of differentiation	Molecular surrogate subtype	Gene	Transcript	Variant	Type of variant	Classification of the variant
6-90	25.9	15	22	OC-56 m			43	Left breast	Medullary carcinoma with marked lympho plasma. cellular infiltration	G2	HER2 - enriched	***BRCA1***	NM_007294.4	**c.5266dup** p.(Gln1756ProfsTer74) Exon: 19/23	Frameshift indels	Pathogenic (PVS1, PS3)
2-2	20.4	12	24	PnC-59 f			41	Right breast	NST	G3	TNBC	***BRCA1***	NM_007294.4	**c.5266dup** p.(Gln1756ProfsTer74) Exon: 19/23	Frameshift indels	Pathogenic (PVS1, PS3)
1-87	22.7	14	27		EC-65		34	Left breast	lobular carcinoma	G3	TNBC	***BRCA1***	NM_007294.4	**c.5266dup** p.(Gln1756ProfsTer74) Exon: 19/23	Frameshift indels	Pathogenic (PVS1, PS3)
5-10	18.7	15	22				38	Left breast	NST	G3	TNBC	***BRCA1***	NM_007294.4	**c.5333-1G>AExon:**	Splice acceptor	Pathogenic (PVS1, PS3)
1-21	19.8	13	26		BC in male -60		29	Right breast	NST	G3	TNBC	***BRCA1***	NM_007294.4	**c.181T>G** p.(Cys61Gly) Exon: 4/23	Missense	Pathogenic (PS3, PS4, PM2)
6-48	29	13	19	BC-38 m		BC-40 m	61	Right breast	NST	G2	TNBC	***BRCA2***	NM_000059.4	**c.3975_3978dup** p.(Ala1327CysfsTer4) Exon: 11/27	Frameshift Indels	Pathogenic (PVS1, PS3)
7-16	27.3	14	27	SC-66 f			38	Right breast	NST	G3	Luminal A	***BRCA2***	NM_000059.4	**c.658_659del** p.(Val220IlefsTer4) Exon: 8/27	Frameshift Indels	Pathogenic (PVS1, PS3)

ID	BMI	Age of menarche	Age of 1st burden.	I - degree relatives	II - degree relatives	III - degree relat.	Age of D. of BC	Localization	Histological result	Degree of differentiation	Molecular surrogate subtype	Gene	Transcript	Variant	Type of variant	Classification of the variant
3-13	20.8	14	26	OC-51 m	BC-50 m; BC-47 m		37	Left breast	NST	G2	Luminal A	***BRCA2***	NM_000059.4	**c.6955A>T** p.(ABC2319Ter) Exon: 13/27	Stop gained	Pathogenic (PVS1, PS3)
7-13	30.1	12	28		CRC-65 m		38	Right breast	NST	G2	Luminal A	***BRCA2***	NM_000059.4	**c.7975A>G** p.(ABC2659Gly) Exon: 17/27	Missense	Pathogenic (PS1, PS3, PS4)
1-18	27.9	12	25		BC-35		29	Right breast	lobular	G2	Luminal A	***BRCA2***	NM_000059.4	**c.9097dup** p.(Thr3033AsnfsTer11) Exon: 23/27	Frameshift indels	Pathogenic (PVS1, PS3)
3-7	20.6	12	Non para				27	Right breast	NST	G2	Luminal B	***BRCA2***	NM_000059.4	**c.9682del** p.(Ser3228ValfsTer21) Exon: 27/27	Frameshift Indels	Pathogenic (PVS1, PS3)
2-18	19.5	14	Non para				26	Right breast	NST	G3	Luminal B	***BRCA2/ CHEK2/ ATM***	NM_000059.4/ NM_007194.4/ NM_000051.4	**c.5851_5854del** p.(Ser1951TPCfsTer11) Exon: 11/27// **c.470T>C** p.(Ile157Thr) Exon: 4/15//c.**2131_2132dup** p.(Asn711LysfsTer25) Exon: 14/63	Frameshift Indels/ Missense/ Frameshift Indels	Pathogenic (PVS1, PS3)/Likely pathogenic (PS1, PP1, PP3)/Likely pathogenic (PVS1, PM2)
6-54	28	14	18	BC-70 m	PCn-72d. f		49	Left breast	lobular	G3	Luminal A	***PALB2***	NM_024675.4	**c.172_175del** p.(Gln60ArgfsTer7) Exon: 3/13	Frameshift Indels	Pathogenic (PVS1, PS3)
2-13	19.8	10	26				32	Left breast	NST	G2	Luminal B	***PALB2***	NM_024675.4	**c.509_510del** p.(Arg170IlefsTer14) Exon: 4/13	Frameshift Indels	Pathogenic (PVS1, PS3)

APPENDIX 2 - Clinical, familial, histologic and molecular features of all BC cases in which PVs were found in moderate-penetrant predisposition genes [2] (***** - new genetic variant not reported in the world databases).

[2] m./f. designations indicate in which parental line the affected relatives are

ID	BMI	Age of menarche	Age of 1st burden.	I - degree relatives	II - degree relatives	III - degree relat.	Age of D. of BC	Localization	Histological result	Degree of differentiation	Molecular surrogate subtype	Gene	Transcript	Variant	Type of variant	Classification of the variant
6-80	27	12	20	PnC-49 f	PC -78 f; SC-m		36	Right breast	NST	G2	Luminal A	***ATM***	NM_000051.4	**c.1564_1565del** p.(Glu522IlefsTer43) Exon: 10/63	Frameshift Indels	Pathogenic (PVS1, PS4, PM2, PM3)
6-23	22.3	12	Non para	PC-63 f	sarcoma of Ewing-55f;LC-50f		42	Left breast	NST, papillary	G2	Luminal A	***ATM***	NM_000051.4	**c.7475T>G** p.(Leu2492ABC) Exon: 50/63	Missense	Likely pathogenic (PS3,PM1,PM2,PP3,PP4)
6-89	28.4	14	26	BC-80 m; melanoma-50 f	BC-70 m; BC-72 f; CRC-70 f; SC-50 f	BC-70 f	69	Right breast	NST	G3	Luminal B	***BLM***	NM_000057.4	**c.1642C>T** p.(Gln548Ter) Exon: 7/22	Stop gained	Pathogenic (PVS1, PM2, PM3)
7-23	21.3	14	18				47	Right breast	NST	G3	TNBC	***BLM***	NM_000057.4	**c.1642C>T** p.(Gln548Ter) Exon: 7/22	Stop gained	Pathogenic (PVS1, PM2, PM3)
6-81	44.1	11	Non para	PC+CRC f; EC+CRC m	BC-60 m		62	Left breast	NST	G3	Luminal A	***BRIP1****	NM_032043.3	**c.3201C>A** p.(Cys1067Ter) Exon: 20/20	Stop gained	Likely pathogenic (PVS1, PM2)*
6-85	33.1	12	19	Ca intestine-68 f	CRC-72 f		38	Left breast	NST	G1	TNBC	***CDKN2A***	NM_000077.5	**c.71G>C** p.(Arg24Pro) Exon: 1/3	Missense	Pathogenic (PS1, PS3)

ID	BMI	Age of menarche	Age of 1st burden.	I - degree relatives	II - degree relatives	III - degree relatives	Age of D. of BC	Localization	Histological result	Degree of differentiation	Molecular surrogate subtype	Gene	Transcript	Variant	Type of variant	Classification of the variant
2-16	27.5	12	28				62	Left breast	NST	G2	Luminal B	***CHEK2***	NM_007194.4	**c.433C>T** p.(Arg145Trp) Exon: 3/15	Missense	Likely pathogenic (PS4, PS3)
3-16	26	12	20	CRC-69 f			53	Left breast	NST	G2	Luminal A	***CHEK2***	NM_007194.4	**c.444+1G>A**	Splice donor	Pathogenic (PVS1, PS3)
6-86	32.1	15	18	BC-37		SC 70 f	65	Left breast	NST	G2	Luminal A	***CHEK2***	NM_007194.4	**c.444+1G>A**	Splice donor	Pathogenic (PVS1, PS3)
1-46	20.5	11	18		BC-70		44	Right breast	lobular	G2	Luminal A	***CHEK2***	NM_007194.4	**c.470T>C** p.(Ile157Thr) Exon: 4/15	Missense	Likely pathogenic (PS1, PP1, PP3)
6-88	28.9	14	22	BC-35			51	Right breast	NST	G3	Luminal A	***CHEK2***	NM_007194.4	**c.470T>C** p.(Ile157Thr) Exon: 4/15	Missense	Likely pathogenic (PS1, PP1, PP3)
7-14	22.9	14	23				32	Right breast	NST	G2	Luminal A	***CHEK2***	NM_007194.4	**c.470T>C** p.(Ile157Thr) Exon: 4/15	Missense	Likely pathogenic (PS1, PP1, PP3)
8-14	31.6	16	21		BC-60 m	BC-75 m	62	Right breast	NST	G1	HER2 - enriched	***CHEK2***	NM_007194.4	**c.470T>C** p.(Ile157Thr) Exon: 4/15	Missense	Likely pathogenic (PS1, PP1, PP3)
D3	21.9	14	30	TC-59 m	BC-40 m		37	Left breast	NST	G3	Luminal A	***CHEK2***	NM_007194.4	**c.470T>C** p.(Ile157Thr) Exon: 4/15	Missense	Likely pathogenic (PS1, PP1, PP3)
D4	23.8	11	29	PC+SC f	BC-70 f; BC-63 f		49	Right breast	lobular	G2	Luminal A	***CHEK2***	NM_007194.4	**c.470T>C** p.(Ile157Thr) Exon: 4/15	Missense	Likely pathogenic (PS1, PP1, PP3)

ID	BMI	Age of menarche	Age of 1st burden.	1sn Family	Family 2sn	Family 3sn	Age of D. of BC	Localization	Histological result	Degree of differentiation	Molecular surrogate subtype	Gene	Transcript	Option	Type of variant	Classification of the variant
1-60	23.1	14	20	CRC-72			79	Left breast	NST	G2	Luminal B	***CHEK2***	NM_007194.4	**c.470T>C** p.(Ile157Thr) Exon: 4/15	Missense	Likely pathogenic (PS1, PS3)
6-42	25.1	14	27	LC-62 f			44	Right breast	NST	G1	Luminal A	***ERCC5*/ CHEK2***	NM_000123.4/ NM_007194.4	**c.495del** p.(TPC165CysfsTer5) Exon: 5/15/ **c.917G>C** p.(Gly306Ala) Exon: 9/15	Frameshift Indels/Missense	Likely pathogenic (PVS1, PM2)*/Likely pathogenic (PS4,PS3,PM2)
6-2	22.8	14	23	bilateral BC-37 m			47	Left breast	ducto-lobular	G2	Luminal A	***FANCG****	NM_004629.2	**c.1760+2T>A**	Splice donor	Likely pathogenic (PVS1, PM2, PM5)*
6-1	27.9	15	17				46	Left breast	NST	G3	Luminal B	***FANCI***	NM_001113378.2	**c.3645C>G** p.(Tyr1215Ter) Exon: 34/38	Stop gained	Likely pathogenic (PVS1, PM2)
1-20	20.3	14	21		PC-80 m; PC-55 f		45	Left breast	NST	G3	TNBC	***FANCL***	NM_001114636.1	**c.1111_1114dup** p.(Thr372Asnfs Ter13) Exon: 14/14	Frameshift Indels	Likely pathogenic (PVS1, PM2, PM3)
1-2	17.1	12	24			CRC-82	38	Right breast	NST	G2	TNBC	***FANCM****	NM_020937.3	**c.1139_1140del** p.(Arg380IlefsTer14) Exon: 6/23	Frameshift indels	Likely pathogenic (PVS1, PM2)*
1-34	29.1	12	23				55	Left breast	NST	G2	Luminal A	***RAD51C****	NM_058216.3	**c.931del** p.(Ile311TyrfsTer3) Exon: 7/9	Frameshift Indels	Likely pathogenic (PVS1, PM2)*

APPENDIX 3 - Clinical, familial, histologic and molecular features of all OC cases in which PVs were found in highly penetrant cancer predisposition genes[3](* - new genetic variant not reported in the world databases)

[3] m./f. designations indicate in which parental line the affected relatives are

ID	BMI	Age menarche	Age 1 st pregn.	Diagnosis	Age of D.	Histological result	Degree of differentiation	Family history	Gene	Transcript	Variant	Type of variant	Classification of the variant
1-63	30	15	20	Ca ovarii	59	serous	G3		***BRCA1***	NM_007294.4	**c.181T>G** Mr. (Cys61Gly) Exon: 4/23	Missense	Pathogenic (PS3, PS4, PM2)
1-73	34	14	19	Ca ovarii	47	serous	G3	OC	***BRCA1***	NM_007294.4	**c.3700_3704del** p.(Val1234GlnfsTer8) Exon: 10/23	Frameshift Indels	Pathogenic (PVS1, PS3, PM2)
1-7	20	16	21	Ca ovarii	46	serous	G3	BC, OC	***BRCA1***	NM_007294.4	**c.5266dup** p.(Gln1756ProfsTer74) Exon: 19/23	Frameshift Indels	Pathogenic (PVS1, PS3, PM2)
1-55	24	14	28	Ca ovarii	51	serous	G3	OC	***BRCA1***	NM_007294.4	**c.5266dup** p.(Gln1756ProfsTer74) Exon: 19/23	Frameshift Indels	Pathogenic (PVS1, PS3, PM2)
3-23	30	16	25	Ca ovarii	41	serous	G3	BC	***BRCA1***	NM_007294.4	**c.5533dup** p.(Tyr1845LeufsTer35) Exon: 23/23	Frameshift Indels	Pathogenic (PVS1, PS3, PM2)
1-67	29	15	20	Ca ovarii	66	serous+endometroid	G3		***BRCA1/ CHEK2***	NM_007294.4 /NM_007194.4	**c.5497G>A** Mr. (Val1833Met) Exon:**23/23//c.444+1G>A** Exon:	Missense/Splice donor	Pathogenic (PS3, PS4,PM2)/Pathogenic (PVS1, PS3)
1-5	28	11	20	Ca ovarii/Ca endometrii (CEOC)	49	endometroid of ovary and uterus	G2		***MSH2***	NM_000251.3	**c.1386+1G>A** Exon:	Splice donor	Pathogenic (PVS1, PS3, PM2)
5-14	24	14	25	Ca tubae	60	serous	G3		***RAD51D***	NM_002878.4	**c.803G>A** p.(TPC268Ter) Exon: 9/10	Stop gained	Pathogenic (PVS1, PS3, PM2)

APPENDIX 4 - Clinical, familial, histologic and molecular features of all OC cases in which PVs were found in highly penetrant cancer predisposition genes[4](* - new genetic variant not reported in the world databases)

[4] m./f. designations indicate in which parental line the affected relatives are

ID	BMI	Age menarche	Age 1va brem.	Diagnosis	Age of D.	Histological result	Degree of differentiation	Family	Gene	Transcript	Option	Type of variant	Classification of the variant
1-78	22	14	19	Ca ovarii	61	serous	G2	PC, LC	***ATM***	NM_000051.4	**c.8147T>C** p.(Val2716Ala) Exon: 55/63	Missense	Pathogenic (PS3, PS4, PM2)
5-11	23	13	24	Ca ovarii	60	serous	G3		***CHEK2***	NM_007194.4	**c.1427C>T** p.(Thr476Met) Exon: 13/15	Missense	Likely pathogenic (PS3, PM2)
1-71	28	11	34	Ca ovarii	34	serous+ endometroid	G3	lary nx canc er, PC	***FANCG/ ERCC3***	NM_004629.1/ NM_000122.2	**c.1538G>A** p.(Arg513Gln)Exon: 12/14 //**c.325C>T** p.(Arg109Ter)Exon: 3/15	Missense/ Stop gain	Likely pathogenic (PS4, PM3)/ Pathogenic (PVS1, PM2)
5-3	24	15	20	Ca tubae	45	serous	G3		***FANCL***	NM_001114636.1	**c.1048_1051del** p.Gln350fs Exon: 14/14	Stop gained	Likely pathogenic (PVS1, PM2)
5-13	28	13	23	Ca ovarii	60	serous	G2		***FANCM***	NM_020937.7	**c.1972C>T** p.(Arg658Ter) Exon: 11/23	Stop gained	Likely pathogenic (PVS1, PM2)
5-4	28	16	22	Ca ovarii	45	biateral serous carcinoma	G3		***NBN***	NM_002485.5	**c.2140C>T** p.(Arg714Ter) Exon: 14/16	Stop gained	Pathogenic (PVS1, PS3, PM2)
6-67	26	14	28	Ca ovarii/Ca endometrii (SEOC)	52	serous	G3		***PMS2***	NM_000535.7	**c.163+1G**>T Exon:	Splice donor	Pathogenic (PVS1, PM2, PM3)
1-47	26	12	22	Ca ovarii	72	serous	G3	BC	***TP53*/ FANCE***	NM_000546.6/ NM_021922.3	**c.1148_1149del*** p.(Leu383HisfsTer8) Exon 11/11/c**.1239dup** p.(Pro414SerfsTer54) Exon: 7/10	Frameshift Indels /Frameshift Indels	Likely pathogenic (PS4, PM3)/Pathogenic (PVS1, PM2)
5-20	26	12	21	Ca ovarii	52	endometroid	G2		***WRN***	NM_000553.6	**c.1105C>T** p.(Arg369Ter)Exon: 9/35	Stop gained	Pathogenic (PVS1, PM2, PM3)

3-22	30	15	Non para	Ca ovarii/Ca endometrii (SEOC)	52	endometroid	G2		***WRN***	NM_000553.6	**c.4109del** p.(Asn1370ThrfsTer23) Exon: 34/35	Frameshift Indels	Pathogenic (PVS1, PS3, PM2)

APPENDIX 5 - CLINICAL CASES WITH MINAS

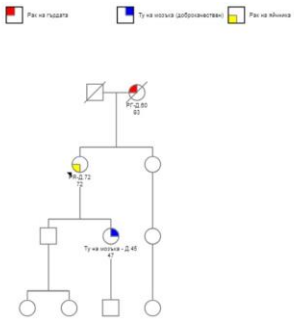
Clinical case 1-47

Anamnestic data:

This is a woman diagnosed at age 72 with serous ovarian carcinoma invasive ductal carcinoma. Reproductive history: menarche at age 12, five pregnancies (first at 22, last at 38), two live births, breastfed for 1 month. The reason for hospitalization was the reduction of body weight by about 20 kg in 2 months. Imaging studies (ultrasound, MRI) showed tumor involvement of both ovaries, involvement of the paraaortic and parahilar lymph nodes and involvement of the peritoneum. Examination of the ascitic fluid shows serous ovarian carcinoma, G3. The patient refused treatment.

Genealogical analysis:

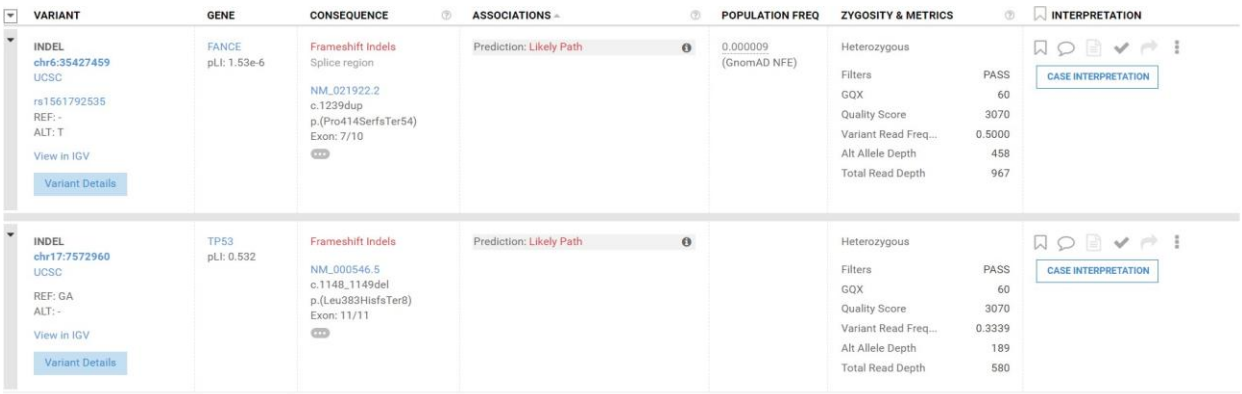
10 blood relatives of 4 generations in both parental lines were covered. A first-degree relative (mother) diagnosed with BC and another first-degree relative (daughter) diagnosed with brain tumor (benign) were identified.

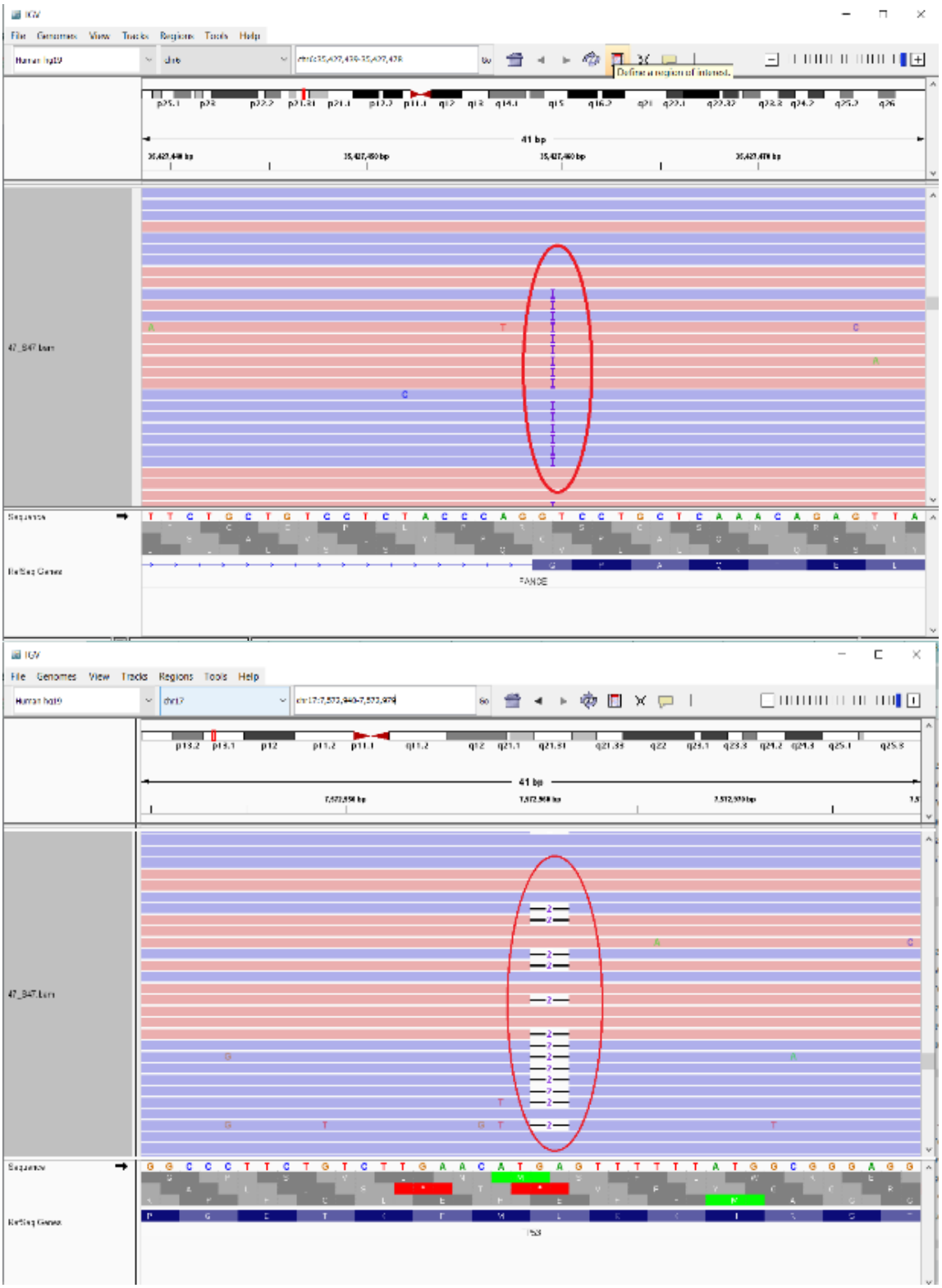


Genetic analysis:

Conducted with next-generation sequencing. Pathogenic variants were detected in:

1. ***TP53*** **- c.1148_1149del p.(Leu383HisfsTer8**
2. ***FANCE*** **- c.1239dup p.(Pro414SerfsTer54)**

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Interpretation of detected variants:

In ***TP53*** **- The** variant c.1148_1149del p. (Leu383HisfsTer8) we found is novel and not previously described in the literature. It represents a deletion of 2 nucleotides - GA and results in a frameshift read (frameshift variant). The variant is absent in population databases of healthy individuals and has not been previously described in the literature in patients with BC/OC. It is classified as Likely pathogenic.
In ***FANCE*** **-** Our discovered variant c.1239dup p. (Pro414SerfsTer54) creates a premature translational stop signal (p.Pro414Serfs*54) in the *FANCE* gene. It is expected to result in a missing or disrupted protein product. Loss-of-function variants in *FANCE* are known to be pathogenic (PMID: 11001585, 17924555). This variant is present in population databases (gnomAD 0.0009%). The variant has been described in the literature in patients with BC/OC (PMID: 27913932). It is classified as pathogenic.

Genetic counseling recommendations:

The recommendations of the genetic counseling, due to the presence of PVs in two predisposing genes, was tailored primarily to the PV carrying the higher risk (in this case it was *TP53*). Taking into account the molecular characteristics of the variant found in *TP53* (located in the last exon) and the family and personal medical history (OC and BC were diagnosed at a more mature age), the recommendations are as follows

- ✓ ***About the patient***

❖ The following prophylactic measures are recommended (based on NCCN and the patient's family history):

- ❖ Clinical examinations of the mammary glands every 6-12 months
- ❖ Annual mammograms or breast MRIs

✓ ***For the relatives*** of the patient (daughter and sister) - in order to refine the risk in them, it is necessary to test for carrier state, the variant found in the patient and subsequent genetic consultation.

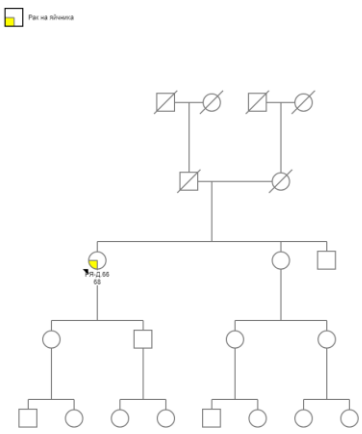
Clinical case 1-67

Anamnestic data:

This is a woman diagnosed at the age of 66 with ovarian cancer. Reproductive history: menarche at 15, two pregnancies (at 20 and 24), two live-born children, breastfed for about 1 year each. Tumor formation detected in the pelvis on routine gynecological examination. Total hysterectomy and omentectomy performed. From histology - low-differentiated adenocarcinoma, partly serous papillary, partly endometrioid

Genealogical analysis:

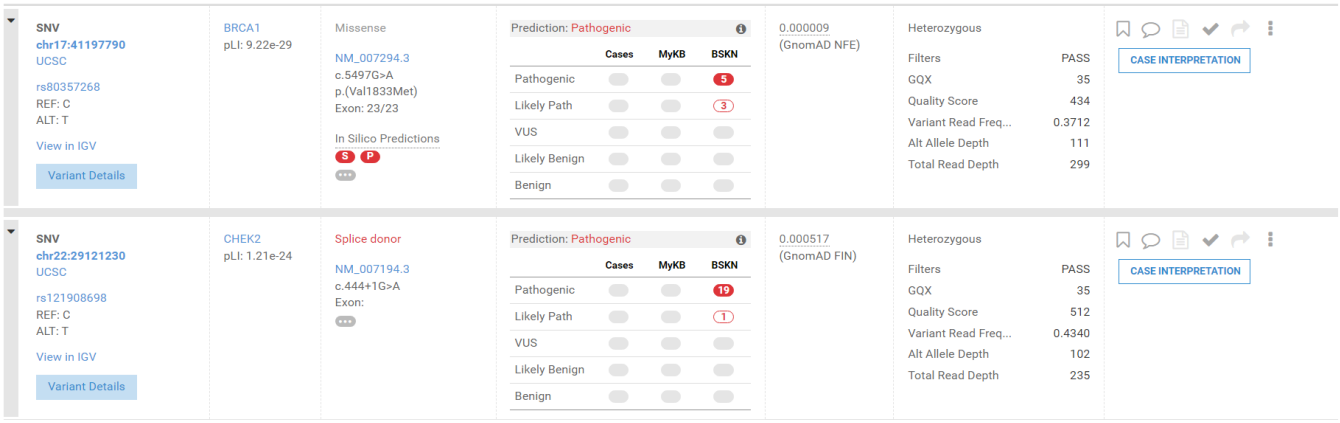
Twenty-one blood relatives of 5 generations in both parental lines were covered. No familial cancer was found.

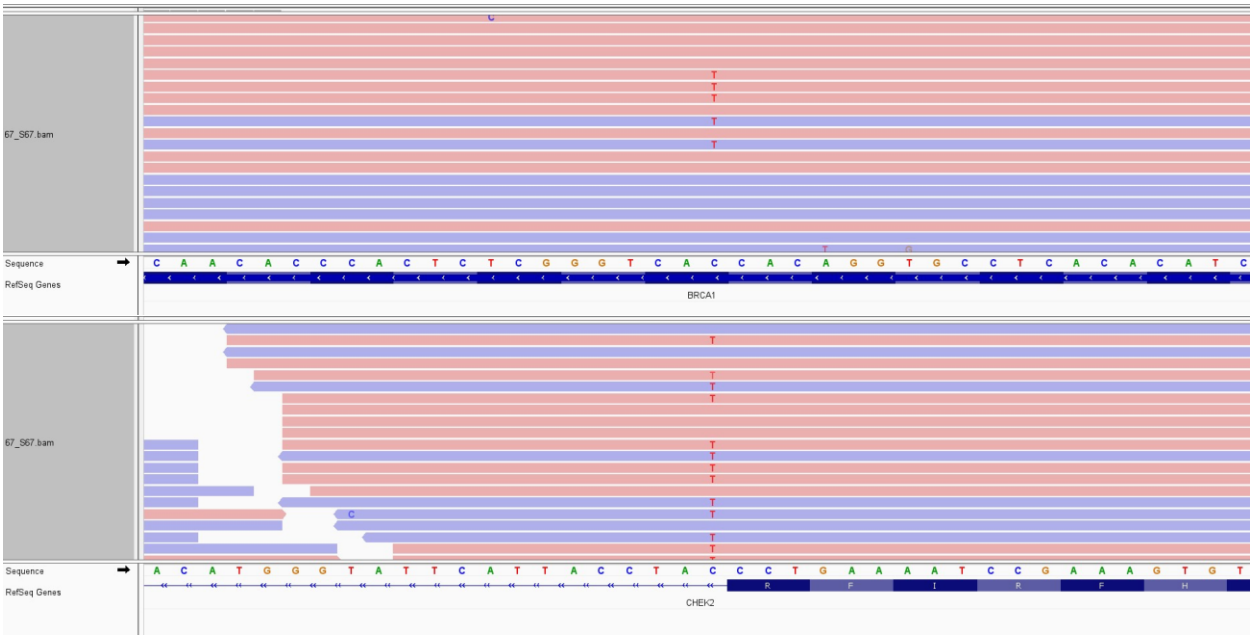


Genetic analysis:

Conducted with next-generation sequencing.

1. ***BRCA1*** - c.5497G>A p.(Val1833Met)
2. ***CHEK2*** - c.444+1G>A





Interpretation of detected variants

1.***BRCA1*** - The variant p.V1833M (also known as c.5497G>A), located in coding exon 22 of the *BRCA1* gene, results from a G to A substitution at nucleotide position 5497. The valine in codon 1833 is replaced by methionine, an amino acid with very similar properties. This variant has been detected at a relatively higher frequency in cohorts of Greek patients with BC and/or OC (Stavropoulou AV, PLoS ONE 2013;8(3):e58182; Apessos A et al. Cancer Genet 2018 01;220:1-12; Papamentzelopoulou M et al. Cancer Genet. 2019 Sep;237:90-96). The work of Rowling et al. both experimentally and functionally demonstrated the destabilization of the BRCT2 domain as a result of this variant, resulting in loss of function (Rowling PJ, J. Biol. Chem. 2010 Jun;285(26):20080 -7). Functional assays show reduced activity compared to wild type (Carvalho M, Mutat. Res. 2009 Jan;660(1-2):1-11, Woods et al, 22).

2.***CHEK2*** **-** This variant disrupts a donor splicing site in intron 3 of the *CHEK2* gene. RNA analysis indicates that disruption of this site causes altered splicing and may result in a missing or disrupted protein product. This variant is present in population databases (SC121908698, gnomAD 0.05%) and has a higher frequency than expected for a pathogenic variant. Disruption of this splice site has been described in individual(s) at increased risk (OR=2.3-3.5) for familial breast cancer or increased risk (OR=2.5) for prostate cancer (PMID: 15492928, 19030985, 24713400; 21876083 12533788). Studies have shown that disruption of this splice site leads to activation of a cryptic splice site, creating a premature termination codon (PMID: 12533788; Invitae). The resulting iRNA is expected to undeBCo decay. For these reasons, this variant is classified as pathogenic

The recommendations of the genetic counseling, due to the presence of PV in two predisposing genes, is primarily tailored to the PV carrying the higher risk (in this case it is *BRCA1*). Considering the additional PV carrier status in *CHEK2* and the lack of family history, the recommendations are as follows

✓ ***About the patient***

The following prophylactic measures are recommended (according to NCCN guidelines):

- Clinical examinations of the mammary glands every 6-12 months
- Annual mammograms or breast MRIs (for women aged 30-75)
- Risk-reducing mastectomy (removal) of the breasts
- Ultrasound examinations of abdominal organs once a year

✓ ***For the relatives*** of the tested patient - in order to refine the risk in them, it is necessary to test for carrier status, the variant found in the patient and subsequent genetic counseling.

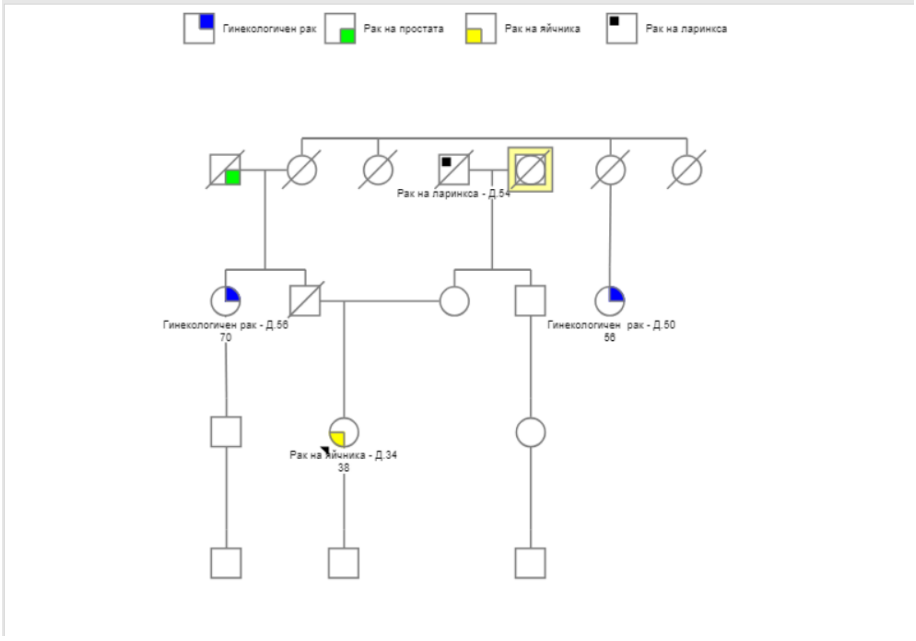
Clinical case 1-71

Anamnestic data:

This is a woman diagnosed at the age of 34 with ovarian cancer. Reproductive history: menarche at age 11, one pregnancy (at age 34), one live born child, breastfed for 2 years and 2 months. At age 34, she underwent surgery for an endometrioid cyst which histology revealed to be a well-differentiated endometrioid and locally serous ovarian carcinoma. Due to rupture of the cyst at the time of surgery, the patient was treated with 4 courses of Carboplatin monotherapy

Genealogical analysis:

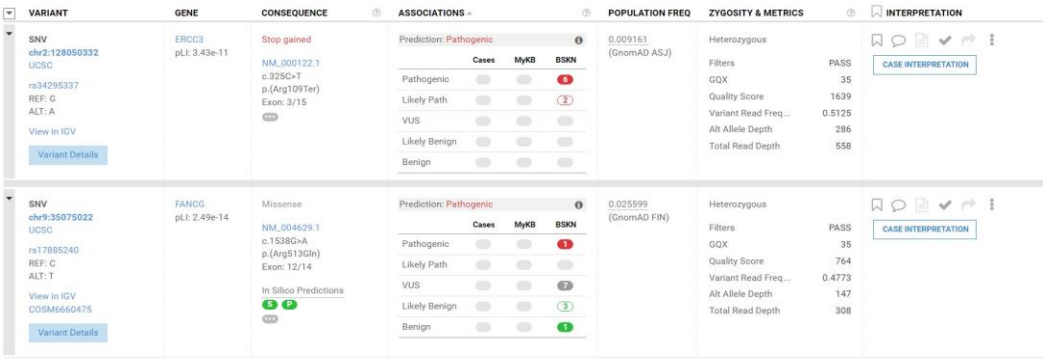
Eighteen blood relatives of 4 generations in both parental lines were covered. There was a Grade II relative (paternal sister) diagnosed with gynecologic cancer, without knowing the exact localization, at the age of 56 years, another Grade II relative (paternal grandfather) diagnosed with prostate cancer.

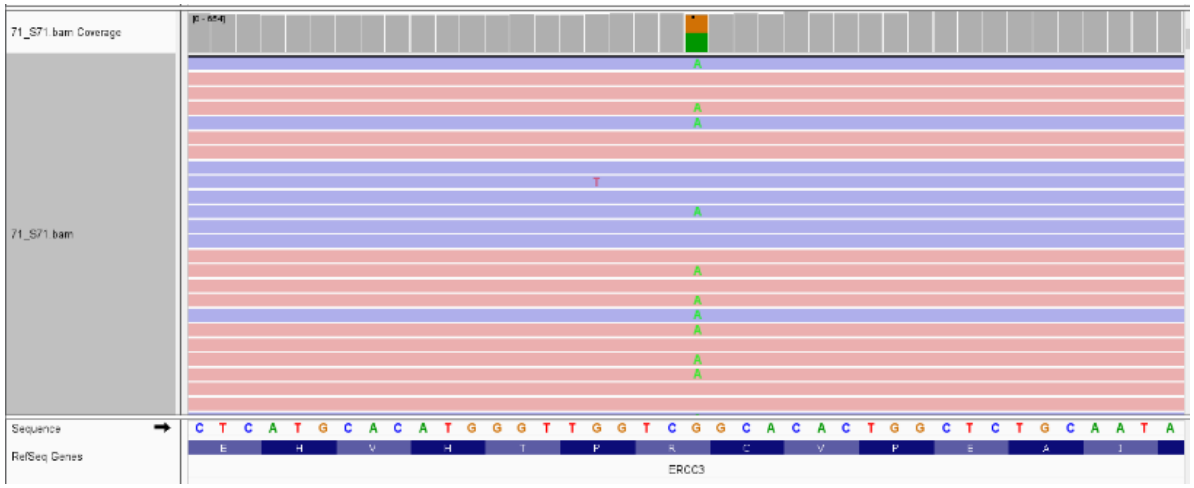


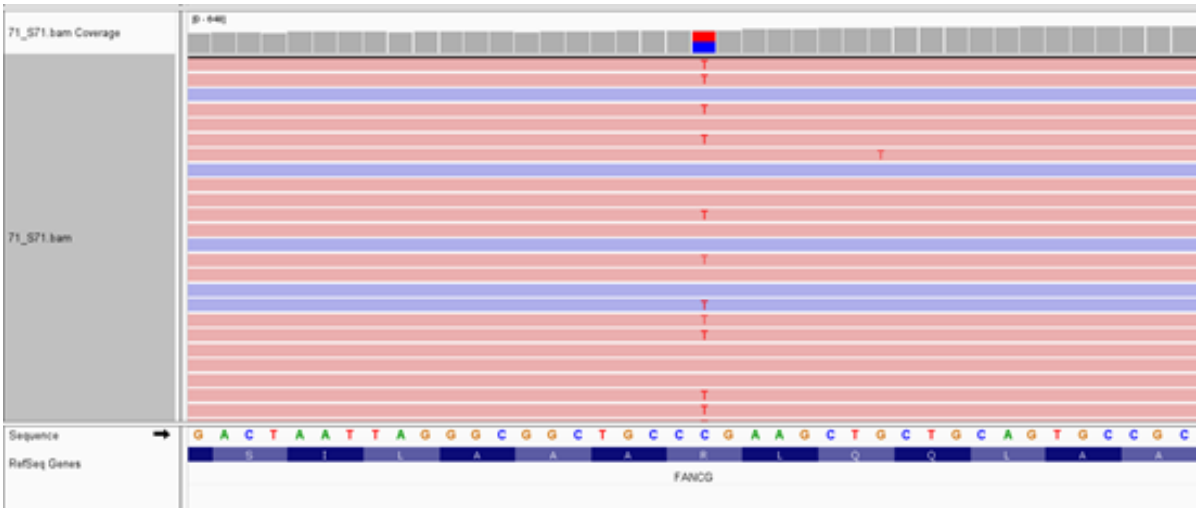
Genetic analysis:

Conducted with next-generation sequencing.

1. ***ERCC3*** - c.325C>T p.(Arg109Ter)
2. ***FANCG*** - c.1538G>Ap.(Arg513Gln)







Interpretation of detected variants

1. ***ERCC3*** - The variant detected creates a premature translational stop signal (p.Arg109*) in the *ERCC3* gene. Expected to result in a missing or disrupted protein product. Loss-of-function variants in *ERCC3* are known to be pathogenic (PMID: 16947863). This variant is present in population databases (rs34295337, gnomAD 0,0002%). This premature translational stop signal has been described in patients with breast cancer, skin cancer and/or UV-sensitive syndrome (PMID: 26023681, 27004399, 27655433). For these reasons, this variant is classified as pathogenic (less). A biallelic mutation in this gene leads to the development of the autosomal recessive syndrome, Xeroderma pigmentosum.
2. ***FANCG*** - The *FANCG* gene is one of the genes causing Fanconi anemia and is involved in homologous recombination processes. According to the ClinVar database(1) , this variant has a conflicting interpretation, but in a study done among children with Acute Myeloid Leukemia (AML)(2) , this variant was classified as pathogenic and has been shown functionally and experimentally to be pathogenic with respect to AML. With respect to other cancers, the studies performed do not reach statistical significance because this variant is rare (reported population frequency in ClinVar - 0.00300 (T). The variant detected represents an amino acid substitution in a moderately conserved region of the protein encoded by *FANCG*.

In our patient, this variant was co-carried with a pathogenic variant (p.Arg109*) in the *ERCC3* gene. The results of a study done on the carrier state of PV in *ERCC3*, among women with BC and/or OC shows the median age of diagnosis in women with OC only is 65 years . In the patient we studied, the diagnosis was made at a very young age of 34 years, and in addition, genealogical analysis revealed affected relatives with gynecologic carcinoma, which suggests that other genetic factors played a role in the polygenic complex predisposing to OC in this particular patient. For these reasons, we assumed that the variant in the *FANCG* gene was a low penetrance allele involved in the polygenic complex in ovarian cancer in this particular patient and classified it as likely pathogenic.

Genetic counseling recommendations:

The recommendations of the genetic counseling, due to the presence of PV in two predisposing genes, was tailored primarily to the PV carrying the higher risk (in this case it was *ERCC3*):

✓ ***About the patient***

❖ The following prophylactic measures are recommended (based on NCCN and the patient's family history):

- Clinical examinations of the mammary glands every 6-12 months
- Annual mammograms or breast MRIs
- Annual full body dermatological clinical examinations

❖ The following prophylactic measures are recommended with regard to the carrier state of a pathogenic variant for autosomal recessive disease (in the case of the patient there are two):

As a carrier of the autosomal recessive disease mutation Fanconi anemia and Xeroderma pigmentosum, the patient is at risk of passing the genetic variants found in these two genes to her children. In order to refine the risk to the children, the patient's partner should also be tested for carrier state of pathogenic variants in the same genes.

✓ ***<u>For the relatives</u>*** of the patient (mother and son) - in order to refine the risk in them, it is necessary to test for the carrier status of the variants found in the patient and subsequent genetic counselling.

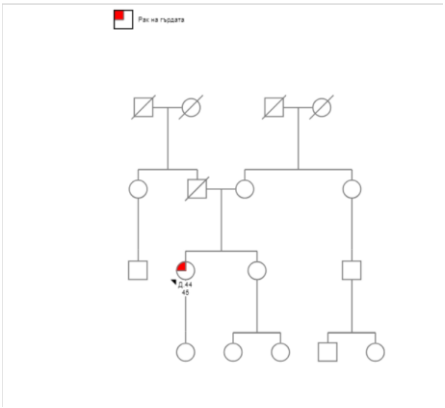
Clinical Case 6-42

Anamnestic data:

This is a woman diagnosed at age 44 with invasive ductal carcinoma. Reproductive history: menarche at 14 yeaSC, four pregnancies, one live birth, not breastfed. At the age of 44 yeaSC she felt a lump in her right mammary gland. Radical mastectomy of right breast with lymph node dissection performed. From the histological result- invasive ductal carcinoma and 12 lymph nodes were examined - 2 of them showed diffuse metastasis. Immunohistochemistry (IHC): ER(+), PR(+), HER2(-). She underwent neoadjuvant and adjuvant chemotherapy and postoperative radiotherapy as decided by the oncology committee.

Genealogical analysis:

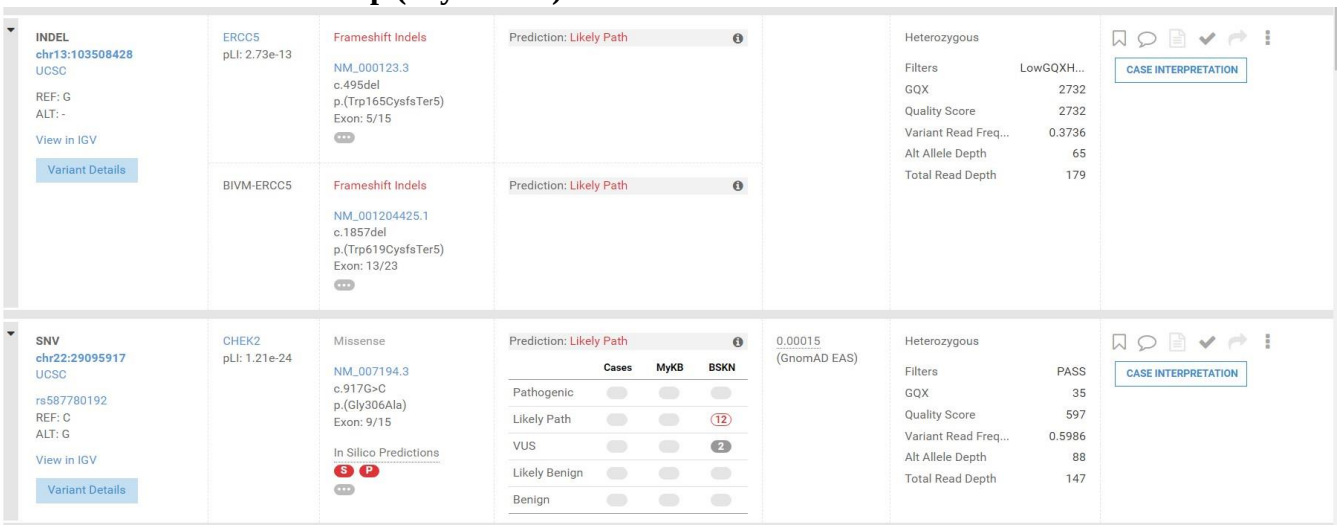
Seventeen blood relatives of 4 generations in both parental lines were covered. No relatives with cancer were found.

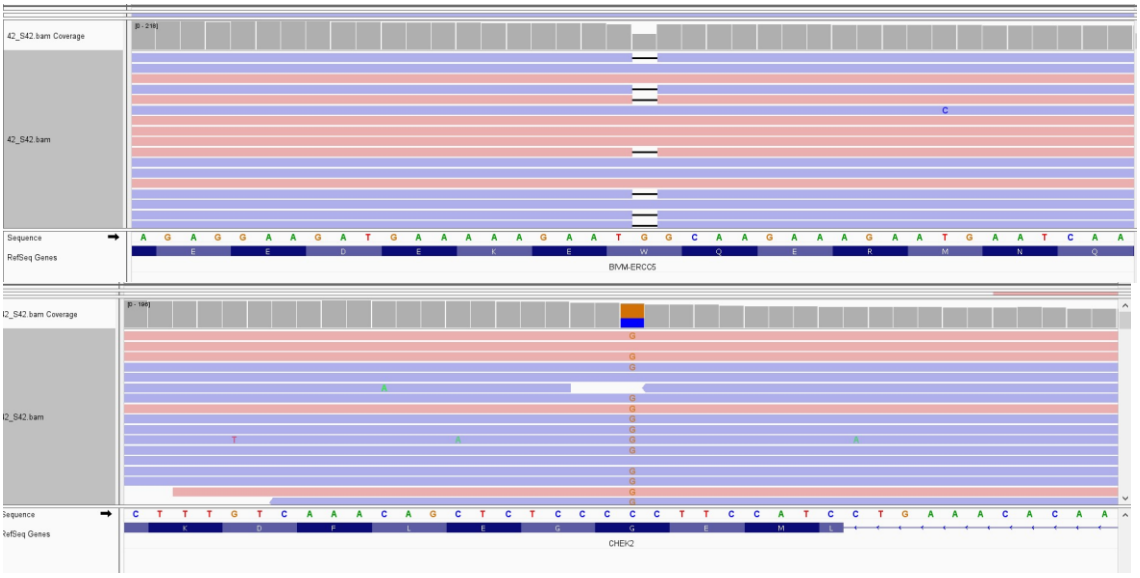


Genetic analysis:

Conducted with next-generation sequencing. **Pathogenic variants** were detected **in**

1. **ERCC5 - c.495del p.(TPC165CysfsTer5)**
2. **CHEK2 - c.917G>C p.(Gly306Ala)**





Interpretation of detected variants

ERCC5 - The detected variant c.495del p.(Trp165CysfsTer5) is novel and not previously reported in the database (ClinVar). It represents a deletion of a single nucleotide in exon 5, resulting in a frameshift readout and creating a premature stop signal. The creation of a premature stop in protein synthesis is a well-known pathogenic mechanism for Xeroderma pigmentosum, complementation group G. This variant has not been detected and described so far, both in patients and healthy individuals. For these reasons, the variant is classified as Likely pathogenic.

CHEK2 - The variant c.917G>C p. (Gly306Ala) exists in population databases at an extremely low frequency (GnomAD - 0.00015), evidence of its pathogenicity. In the literature, the variant has been reported in patients with BC (PMIDs: 26681312 (2015), 27751358 (2016), 28486781 (2017), 28580595 (2018), 30128536 (2018), 30303537 (2019), 32068069 (2020), 32658311 (2021)), RY (PMID: 30322717 (2018)), melanoma (PMID: 33050356 (2020)), and colorectal cancer (PMID: 31118792 (2019)). Functional studies confirm the pathogenicity of the variant (PMID: 31050813 (2019))

Genetic counseling recommendations:

The recommendations of the genetic counseling, due to the presence of PV in two predisposing genes, was tailored primarily to the PV carrying the higher risk (in this case it was *ERCC5*):

- ✓ ***<u>About the patient</u>***
- ❖ The following prophylactic measures are recommended:
  - ➢ Clinical examinations of the mammary glands every 6-12 months
  - ➢ Annual mammogram or MRI of the left mammary gland
- ❖ The following prophylactic measures are recommended <u>with regard to the carrier status of a pathogenic variant for an autosomal recessive disease (in the case of the patient Xeroderma pigmentosum):</u>

As a carrier of the autosomal recessive disease mutation, the patient is at risk of passing on the genetic variant found to her children. In order to refine the risk to the children, the patient's partner should also be tested for carrier status of pathogenic variants in the same genes.

- ✓ ***<u>For the relatives</u>*** of the patient (mother, sister and daughter) - in order to refine the risk in them, it is necessary to test for carrier status of the variants found in the patient and subsequent genetic counselling.

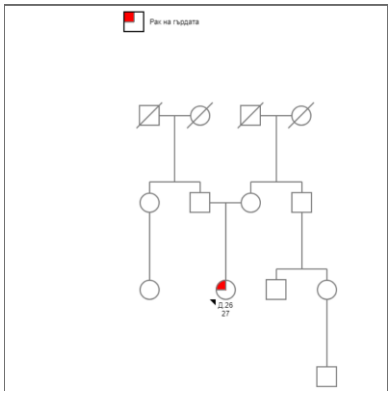
Clinical Case 2-18

Anamnestic data:

The patient was diagnosed with invasive ductal carcinoma of the mammary gland at the age of 27 years. Immunohistochemical examination of tumor tissue indeterminate HER2 status, repeat study recommended after surgical treatment.

Genealogical analysis:

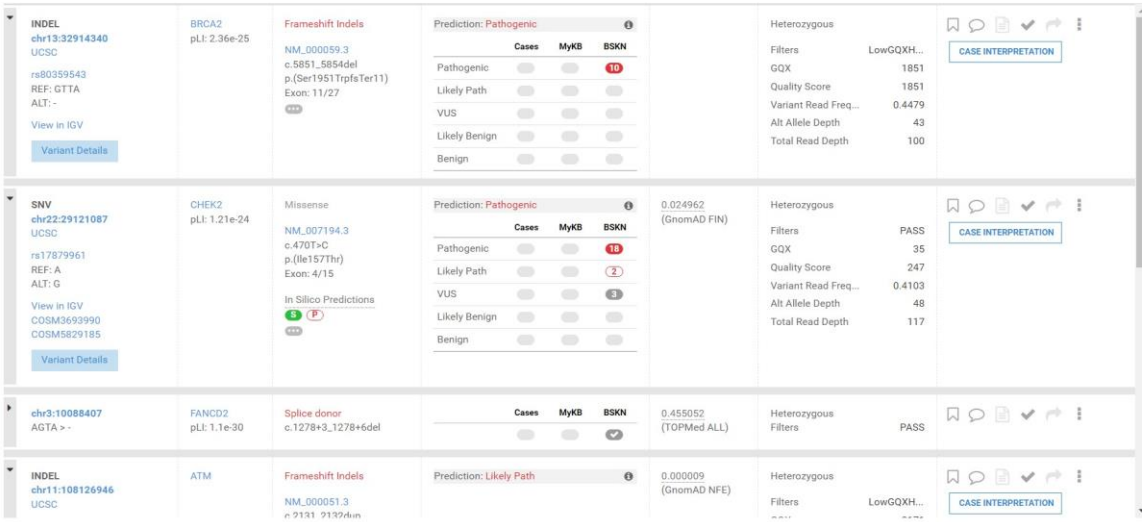
Thirteen blood relatives of 4 generations in both parental lines were covered. No familiality for cancer was found

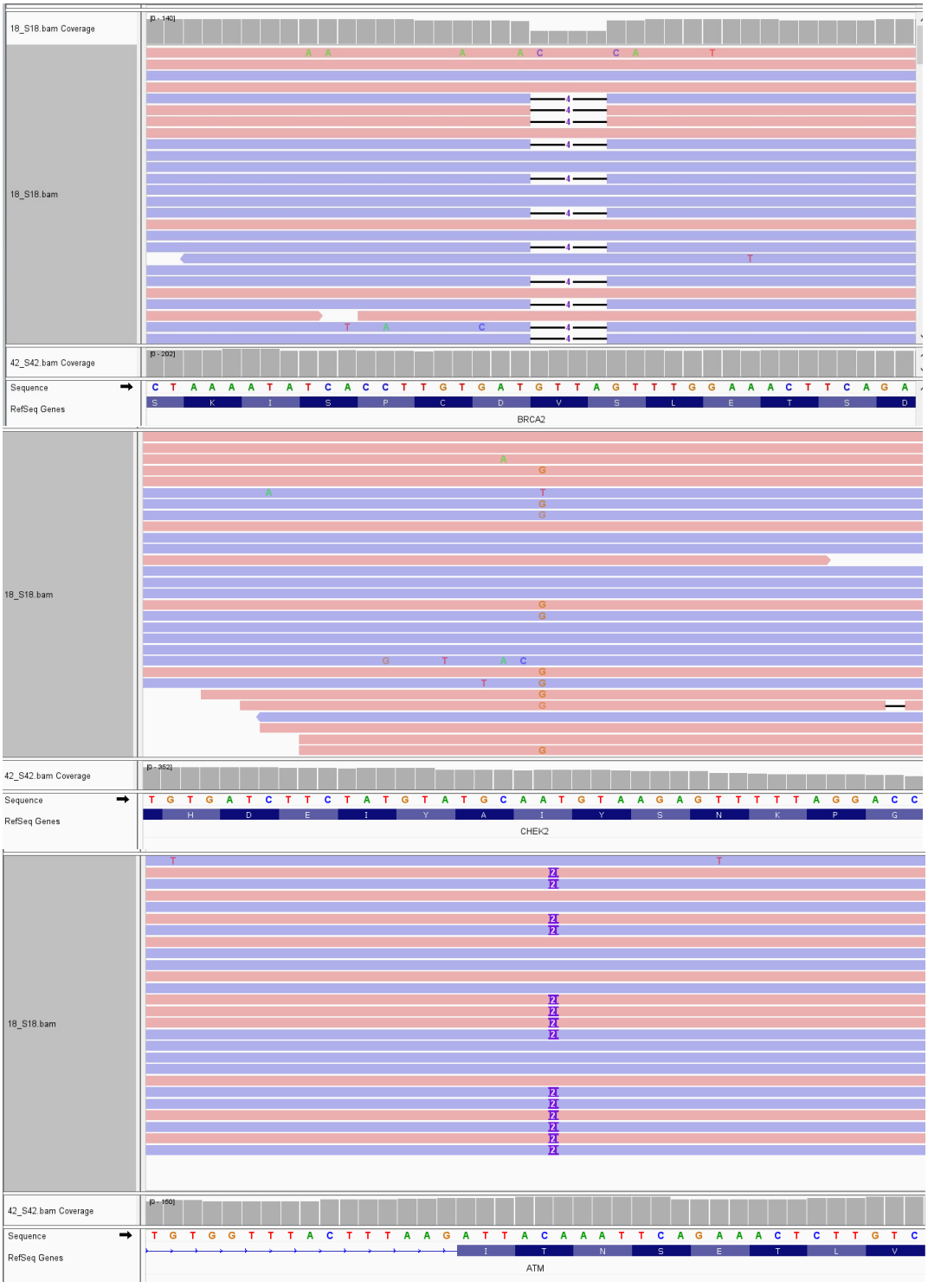


Genetic analysis:

Conducted with next-generation sequencing. **The pathogenic variants** were detected:

1. ***BRCA2* - c.5851_5854delAGTT**
2. ***CHEK2* - c.**
3. ***ATM* - c.2131_2132dupAA**




18_S18.bam Coverage
18_S18.bam
42_S42.bam Coverage
Sequence
RefSeq Genes
BRCA2
18_S18.bam
42_S42.bam Coverage
Sequence
RefSeq Genes
CHEK2
18_S18.bam
42_S42.bam Coverage
Sequence
RefSeq Genes
ATM

Interpretation of detected variants

BRCA2 The variant c.5851_5854delAGTT detected in the patient is pathogenic, localized in the coding exon 10 of the *BRCA2* gene, it is a deletion of 4 nucleotides from position 5851 to 5854. The variant results in a pro-reading frame change upon translation and leads to the creation of a premature stop codon (p.S1951Wfs*11). It has been described in the literature in many families with breast and/or ovarian cancer in different populations (Vaidyanathan K et al. J. Biosci. 2009 Sep;34:415-22; Papi L et al. Breast Cancer Res. Treat. 2009 Oct;117:497-504; Kwong A et al. Breast Cancer Res. Treat. 2009 Oct;117:683-6; Juwle A et al. Med. Oncol. 2012 Dec;29:3272-81; Dodova RI et al. BMC Cancer. 2015 Jul;15:523).

CHEK2 - The variant detected in the patient represents an isoleucine to threonine substitution at position 157 in the more lipopeptide chain of *CHEK2* (p.Ile157Thr). Isoleucine is located in a moderately conserved stretch and has relatively little physicochemical difference with threonine. It was detected in population databases at a frequency of 2.6%. In a large study involving several thousand cases and controls, it was shown to have a slightly increased (up to 1.5-fold) risk of developing breast cancer (low penetrance) (PMID: 22799331, 23713947). Experimental studies have shown that this missense variant alters the binding of *CHEK2* protein to Cdc25A, BRCA1 and p53 in vitro and may exhibit a dominant-negative effect at the cellular level despite not altering CHEK2 protein kinase activity (PMID: 11298456, 11571648, 15239132, 12049740, 22419737).

ATM - The variant c.2131_2132dupAA p. (Asn711LysfsTer25) detected in the patient is Likely pathogenic, localized in the coding exon 14 of the *ATM* gene and leads to the creation of a premature stop signal, thus Likely interfering with the synthesis of a normal protein product. The variant is rare and has a frequency found only in the European population of 0.000009. It does not exist in the ClinVar database, and has not been previously described in the literature.

Genetic counseling recommendations:

It has not been established how the carrier status of three pathogenic variants in three different breast cancer risk genes affects the ultimate risk of developing breast cancer due to the small number of such cases described in the literature. For this reason, the genetic counseling committee assumes that in the case of patient, recommendations should be tailored to the pathogenic variant carrying the highest risk for breast cancer development- **c.5851_5854delAGTT** in ***BRCA2*** [Sukumar J, Kassem M, Agnese D, PilaSCki R, Ramaswamy B, Sweet K, Sardesai S. Concurrent germline *BRCA1*, *BRCA2*, and *CHEK2* pathogenic variants in hereditary breast cancer: a case series. Breast Cancer Res Treat. 2021 Apr;186(2):569-575. doi: 10.1007/s10549-021-06095-w. Epub 2021 Jan 28. PMID: 33507482; PMCID: PMC7990865].

Genetic counseling recommendations

✓ ***About the patient***

The following prophylactic measures are recommended (according to NCCN guidelines):

- Clinical examinations of the other mammary gland every 6-12 months
- Annual mammogram or MRI of the other mammary gland (for women between 30-75 years)
- Risk-reducing radical mastectomy (removal) of the affected breast

- Risk-reducing mastectomy (removal) of the other breast
- Risk-reducing oophorectomy (removal of the ovaries) between the ages of 35-40, after the completion of reproduction in the patient.
- For patients refusing risk-reducing oophorectomy, transvaginal ultrasonography in combination with CA-125 testing is recommended
- In relation to carrying a pathogenic variant in the *ATM* gene, the patient has a 25% risk of having children with the autosomal recessive disorder ataxia-telangiectasia (in case her partner also carries a pathogenic variant in the *ATM* gene). It is recommended that the patient's partner be tested for carrier status of pathogenic variants in the *ATM* gene before reproduction.

✓ ***For the relatives*** of the tested patient - in order to refine the risk in them, it is necessary to test for carrier status, the patient's intentions, variant and subsequent genetic counseling.