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HEREDITARY BREAST AND OVARIAN CANCER GENETICS: GENETIC TESTING IN SERBIA[1]

НАСЛЕДНИ КАРЦИНОМ ДОЈКЕ И ЈАЈНИКА: ГЕНЕТИЧКО ТЕСТИРАЊЕ У СРБИЈИ

***Abstract**: Breast cancer makes up 25% of all new cancers in women globally. Even though it usually occurs by chance (sporadic breast cancer), 5-10% of all breast cancer cases belong to hereditary breast cancer. It is usually characterized by many cancer cases (breast and/or ovarian), earlier age of onset, multiple primary and bilateral or multifocal cancers. Up to 30% of hereditary breast and ovarian cancers harbor a mutation in high risk breast cancer susceptibility genes BRCA1 or BRCA2. Besides BRCA genes, there are also other genes with the smaller effect on the risk.*

Since May 2016, 161 patients have been tested for the presence of mutations in the 19-gene panel at the Institute for Oncology and Radiology of Serbia. The majority of the mutations were in BRCA1 (15/161), BRCA2 (7/161) and PALB2 (7/161). Pathogenic mutations were also detected in ATM (2/161), CHEK2 (4/161) and TP53 (1/161). Variants of unclassified significance (VUS) were detected in BRCA2 (6/161), ATM (5/161), PMS2 (6/161), NBN (5/161) and PALB2 (5/161). 25 family members were tested for particular family mu-

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tation using targeted Sanger sequencing. Additional 6 mutations were detected in BRCA1, 1 in BRCA2, 3 in PALB2, 1 in CHEK2 and 1 in ATM gene. New sequencing technology lowered the cost of genetic testing and enabled higher access to genetic testing and genetic counseling for middle and lower-income countries. Our results of multi-gene germline testing showed the importance of widening the spectrum of the genes that should be offered for detecting hereditary predisposition in Serbia.

***Сажетак**: Карцином дојке чини 25% случајева свих карцинома код жена. Иако се најчешће јавља по принципу случајности – спорадично, 5-10% свих карцинома дојке спада у групу која се назива наследни карцином дојке. Наследни карцином дојке се најчешће карактерише великим бројем случајева карцинома (дојке и/или јајника) у породици, настанком болести раније у животу, мултиплим примарним карциномима, као и билатералним и мултифокалним карциномима. 20-30% случајева наследног карцинома дојке повезано је са постојањем штетних мутација у високо ризичним генима BRCA1 и BRCA2. Поред ових гена, постоје и други гени који са нижим ризиком доприносе настанку наследне болести.*

Од маја 2016. године, на Институту за онкологију и радиологију Србије, тестиран је 161 пацијент на присуство мутација у панелу од 19 гена. Највећи број мутација пронађен је у BRCA1 (15/161), BRCA2 (7/161) и PALB2 (7/161) генима. Такође, детектоване су штетне мутације и у ATM (2/161), CHEK2 (4/161) и TP53 (1/161) генима. Висок проценат генетичких варијанти непознатог клиничког значаја (VUS) пронашли смо у BRCA2 (6/161), ATM (5/161), PMS2 (6/161), NBN (5/161) и PALB2 (5/161) генима. 25 чланова породица тестирали смо на присуство породичних мутација употребом Сангеровог секвенцирања. Додатно смо пронашли 6 мутација у BRCA1, 1 у BRCA2, 3 у PALB2, 1 у CHEK2 и 1 у ATM гену.

Нова технологија секвенцирања смањила је цену генетичког тестирања и омогућила већи приступ генетичком тестирању и генетичком саветовању. Наши резултати указују на значај тестирања ширег панела гена у одређивању наследне предиспозиције за карцином дојке и јајника. Такође, наши резултати указују на то да би испитаницима пореклом из Србије свакако требало понудити шири панел гена у циљу прецизнијег дефинисања наследне предиспозиције.

***Keywords**: genetic testing, genetic counseling, hereditary breast and ovarian cancer, NGS, panel*
***Кључне речи**: генетичко тестирање, генетичко саветовање, наследни карцином дојке и јајника, NGS, панел*

INTRODUCTION

According to the American Cancer Society, breast cancer makes up 25% of all new cancers in women globally. The majority of new breast cancer diagnoses and deaths occur in developing countries with the rates that have been steadily increasing in recent decades. In developed countries breast cancer is second to lung cancer for cancer-related deaths in women. Survival rates greatly vary worldwide, ranging from 80% in North America, Sweden and Japan, to around 60% in the middle income countries and below 40% in low-income countries (Coleman et al., 2008). The lack of the early detection programs, adequate diagnosis and treatment might explain the low survival rates in less developed countries.

Most of the breast cancers (70-75%) occur by chance alone. These cases are called sporadic cancers and people with this type of disease typically do not have relatives with the same type of cancer. Sporadic breast cancer might be caused by age, reproductive and hormonal, factors lifestyle choices, environmental conditions or other non-inherited factors. It usually happens later in life and family members of the affected person have the same cancer risk as general population (Figure 1).

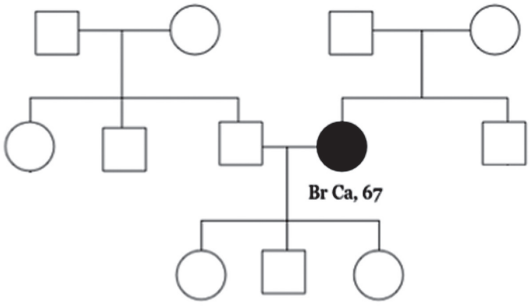

Figure 1 Sporadic breast cancer. BrCa-Breast Cancer

In some families (15-20% of cases) we see more cancer cases than it can be expected if it happened by chance alone. So-called familial cancer is likely caused by a combination of multiple minor genetic and environmental risk factors. People with familial cancer may have one or more relatives with the same type of cancer; however, there is no clear pattern of inheritance in these families. The cancer usually appears later in life, and even though the family members of the affected person have

somewhat increased risk for developing breast cancer, genetic testing is unlikely to be helpful for these families (Figure 2).

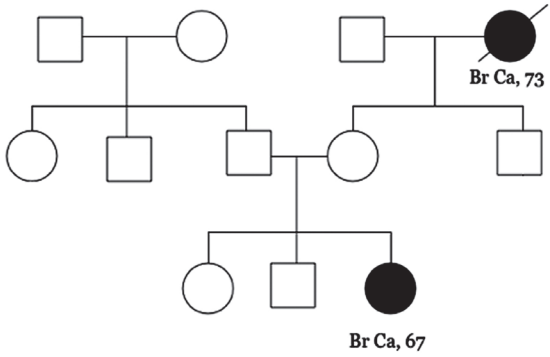

Figure 2 Familial breast cancer. BrCa-Breast Cancer

Only 5-10% of all breast cancer cases belong to the group that is called hereditary breast cancer. These families are characterized by many cancer cases (same type or related type of cancer), earlier age of disease onset, multiple primary cancers and bilateral or multifocal breast cancers (Figure 3). Up to 20-30% of hereditary breast cancer cases harbor a mutation in breast cancer susceptibility genes *BRCA1* or *BRCA2* (Couch, Nathanson, & Offit, 2014). Cancer usually occurs when genetic mutation (altered gene) is passed down in the family from parent to child. Cancer risks in hereditary cancer families are much higher than in the general population. Also, the risk for early onset cancers as well as cancers on multiple sites significantly increases for the mutation carriers.

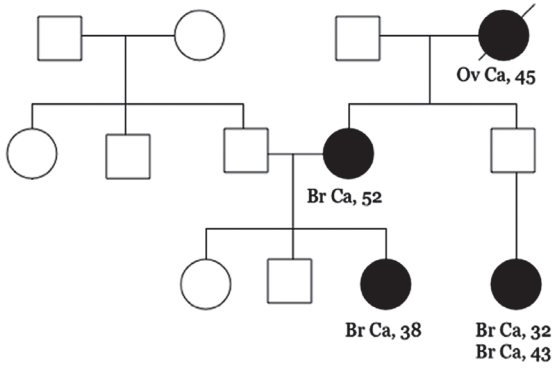

Figure 3 Hereditary breast cancer. BrCa-Breast Cancer, OvCa-Ovarian Cancer

Hereditary breast and ovarian cancer (HBOC) can be classified into two following groups: 1) clustering of both breast and ovarian cancers and 2) site specific breast or ovarian cancer in families. The distinction between these two groups might be useful on the clinical level, but is not supported on the genetic level since breast cancer susceptibility genes predispose to both types of disease. Most of the hereditary cancer cases come from the families with the clustering of both breast and ovarian cancers. The diagnosis of HBOC relies upon the following characteristics:

– Increasing number of affected family members (usually breast and ovarian cancers)- two or more relatives under the age of 50; three or more relatives with breast cancer at any age
– Early cancer onset (younger than 35)
– An excess of bilateral disease
– Ovarian cancer at any age
– Two primary breast cancers
– Male breast cancer
– Triple negative breast cancer diagnosed under the age of 60
– Pancreatic cancer with breast or ovarian cancer
– Ashkenazi Jewish ancestry
– A previously identified BRCA mutation in the family

BREAST CANCER SUSCEPTIBILITY GENES (BRCA1 AND BRCA2)

At the end of 20th century, many efforts have been made in order to discover the underlying cause of the breast cancer clustering in families. In 1991, complex segregation analysis have shown that highly penetrant susceptibility allele could explain this specific pattern of disease clustering (Claus, Risch, & Thompson, 1991). The same year, *BRCA1* gene was discovered in the 17q21 region of the chromosome and was linked to hereditary breast and ovarian cancers. *BRCA1* was cloned in 1994 and germline mutations in this gene were detected in many HBOC cases (Miki et al., 1994). The second breast cancer gene was isolated in 1995, it was called *BRCA2* and was shown to be involved in male breast cancer and other family cases not attributed to *BRCA1* (Tavtigian et al., 1996). Both *BRCA1* and *BRCA2* belong to the group of tumor

suppressor genes expressed in the wide range of tissues with the main role in DNA repair of double-strand breaks by homologous recombination (Krejci, Altmannova, Spirek, & Zhao, 2012). *BRCA1* has also been shown to play a role in a cell cycle checkpoint control, ubiquitination and chromatin remodeling (Venkitaraman, 2002). More than a thousand of different mutations are described in both *BRCA* genes with no mutational hotspots- they are spread throughout the whole coding region. Most known mutations are listed in The Breast Cancer Information Core (BIC) database and are available on the internet. The most frequent pathogenic mutations are small insertions and deletions which cause premature truncation of the protein (Narod & Foulkes, 2004). Large genomic rearrangements (deletions or duplications of whole exons) contribute with a small portion to *BRCA* mutational landscape. Most of the discovered mutations are unique for a given family, however, a number of founder mutations common in defined populations have been identified. For example, Ashkenazi Jewish harbor mutations (185delAG and 5382insC in *BRCA1* and 6174 delT in *BRCA2*) that can be passed through even 46 generations or more (Neuhausen et al., 1996). *BRCA1* founder mutations have been reported in other populations as well, such as Netherlands (Peelen et al., 1997), Sweden (Johannsson et al., 1996) and Norway (Dorum, Hovig, Tropé, Inganas, & Moller, 1999).

Pathogenic mutations in *BRCA1* and *BRCA2* genes confer high risk of breast, ovarian and contralateral breast cancers although the precise magnitude of these risks is uncertain. Risks for breast cancer for *BRCA1* mutation carriers are estimated in the range of 40% to 87% and for *BRCA2* mutation carriers for 18% to 88%. Risks for ovarian cancer for *BRCA1* mutation carriers are estimated in the range of 22% to 65% and 10% to 35% for *BRCA2* mutation carriers (Antoniou et al., 2003; Evans et al., 2008; Risch et al., 2001; Wacholder, Struewing, Hartge, Greene, & Tucker, 2004). The observed variations in terms of the magnitude of risks may be explained by different study methods and different populations. Also, these risks may vary by the age at diagnosis, family history, type and site of the mutation, lifestyle factors as well as the existence of genetic modifiers or other familial factors that influence cancer risk. According to the EMBRACE study which is one of the largest prospective studies reporting cancer risks in *BRCA1* and *BRCA2* mutation

carriers, the average cumulative risks by age 70 for *BRCA1* carriers are estimated to be 60% for breast cancer, 59% for ovarian cancer and 83% for contralateral breast cancer (Mavaddat et al., 2013). For *BRCA2* carriers, 55% is breast cancer risk, 16.5% ovarian cancer risk and 62% contralateral breast cancer risk (Mavaddat et al., 2013).

BRCA1 related breast cancers are usually invasive ductal carcinomas (with higher frequency of medullary carcinomas), often of higher grade than sporadic cancers and usually hormone receptor negative (Larsen, Thomassen, Gerdes, & Kruse, 2014). 11% of medullary carcinomas carry *BRCA1* germline mutations (Eisinger et al., 1998). By contrast, excess of invasive lobular and tubular carcinomas has been reported for *BRCA2* mutation carriers. Similarly to *BRCA1*, most *BRCA2* tumors are grade 2/3 with high mitotic rates (Mavaddat et al., 2012). Unlike *BRCA1* tumors, most *BRCA2* tumors seem to be more similar to sporadic tumors with relation to expression of IHC markers. Most of *BRCA2* tumors show luminal phenotype by ER and PR over expression and cytokeratins CK8 and CK18 (Larsen et al., 2014). Regarding ovarian cancers, the vast majority of those associated with germline *BRCA* mutations are high-grade and advanced stage serous carcinomas (Boyd et al., 2000). Low-grade serous carcinoma and noninvasive micro-papillary serous carcinoma do not seem to be related to *BRCA* germline mutations (Girolimetti et al., 2014).

Recent evidence suggests that the presence of germline *BRCA* mutation defines a subgroup of patients whose immediate clinical management could be influenced by this information. One of the characteristics of *BRCA1* and *BRCA2* mutated cells is hypersensitivity to DNA crosslinking agents such as cisplatin and carboplatin (Bhattacharyya, Ear, Koller, Weichselbaum, & Bishop, 2000). In the context of the cancers where this type of systemic therapy is routinely used (for example ovarian cancer), the presence of germline *BRCA* mutation is a good predictive factor. On the other hand, data suggest that taxanes, used in both adjuvant and metastatic setting in women with breast cancer, might not be as effective in *BRCA* mutated cancers (Smith & Isaacs, 2011). Tumor cells lacking functional *BRCA1* or *BRCA2* are unable to effectively repair double strand breaks due to disruption of their HR pathway, and thus are more sensitive to PARP inhibitors *in vitro* than wild type cells (Farmer et al., 2005). This phenomenon is used for de-

velopment of low toxicity drugs that selectively affects only those cells that have lost *BRCA* associated repair function, namely tumor cells, whereas normal tissue is unaffected. Olaparib (Lynparza, AstraZeneca Pharmaceuticals LP), a poly (ADP-ribose) polymerase (PARP) inhibitor, was firstly approved by the US Food and Drug Administration (FDA) for the treatment of advanced ovarian cancer in patients with germline *BRCA*-mutations in 2014. In January, 2018, FDA granted approval to olaparib tablets for the treatment of patients with pathogenic or suspected pathogenic germline *BRCA*-mutated, HER2-negative metastatic breast cancer who have been treated with chemotherapy either in the neoadjuvant, adjuvant, or metastatic setting.

THE ERA OF NEW SUSCEPTIBILITY GENES

Even though mutations in high risk *BRCA* genes are often related to hereditary disease, it has been shown that even 67% families with 4 or more site specific cases of breast cancers are not linked to *BRCA1* or *BRCA2*. It turned out that mutations in these high penetrance genes can explain only as much as 5% of cases with strong inherited component (Apostolou & Fostira, 2013). Many explanations for this phenomenon called missing heritability have been suggested, including the existence of larger number of genetic variants with the smaller effect on risk. According to the polygenic model of HBOC all cancer predisposing genes can be categorized in three groups according to their relative risk for cancer: high penetrance genes (cancer relative risk higher than 5), intermediate penetrance genes (cancer relative risk 1.5-5) and low penetrance alleles (cancer relative risk lower than 1.5) (Apostolou & Fostira, 2013). *BRCA1* and *BRCA2* together with *TP53, PTEN, STK11* and *CDH1* genes belong to the high penetrance genes which mutations confer to the risk up to 20 times higher than the one for general population. Mutations in these genes, although clinically very important, are very rare with the frequencies lower than 1%. (Mavaddat, Antoniou, Easton, & Garcia-Closas, 2010). In 2014, important research has been published in the *New England Journal of Medicine* on the *PALB2* gene (*Partner And Localizer of BRCA2)* which encodes a protein that works with BRCA2 to repair damaged DNA and stop tumor growth. It was previously thought that mutations in *PALB2* contributes to a slightly increased risk for breast cancer, however, a study showed that women under 40 years and with an

abnormal *PALB2* gene had a risk of breast cancer that was 9.47 times higher than average (Leeneer et al., 2014). In women with *PALB2* mutation, breast cancer risk was 8 to 9 times higher than average in women ages 20 to 39, 6 to 8 times higher than average in women ages 40 to 60, 5 times higher than average in women older than 60. By age 70, women with pathogenic mutation in *PALB2* gene had a 33% risk of developing breast cancer even when they have no family history of the disease. The risk is higher for those who have two or more first-degree relatives with breast cancer and goes up to 58%. This research concluded that the breast cancer risk for *PALB2* mutation carriers, even in the absence of family history should be classified as high and similar to *BRCA2* breast cancer risk. The group of intermediate penetrant genes is consisted of rare alleles (~1%) which mutations confer relative risk between 1.5 and 5 (*ATM, CHEK2, RAD51, RAD51C, XRCC1, BRIP1*...). The third group consists of a number of common breast cancer susceptibility loci that have been associated with a slightly increased risk of breast cancer (relative risk lower than 1.5). The most common genetic changes in low penetrant genes are single nucleotide polymorphisms (SNPs) with high frequencies (~40%), but with the small individual effect on breast cancer risk. Most of these low-susceptibility loci have been found through genome wide association studies (GWAS) (*MAP3K1, FGFR2, LSP1, TNRC19,* and *H19*). Although the actual contribution of these common susceptibility loci in HBOC is debatable, the identification of such alleles can explain a subset of breast cancer cases. In addition, similarly to *BRCA1/2*, genes that are related to the DNA repair mechanisms might be used as potential targets for PARP inhibition.

BREAST CANCER IN SERBIA

In Serbia, 4600 new breast cancer cases have been diagnosed annually. More than one third of all of these cases has been diagnosed at the Institute for Oncology and Radiology of Serbia (IORS). Around 200 patients have been diagnosed with the advanced disease. According to data from Cancer Registry of Central Serbia from 2013, the cancer incidence rate in males was 274.7 per 100.000 population, and in females 234.8 per 100.000 population. After the District of Sumadija with the highest cancer rates (346.4/100.000 males and 322.7/100.000 females), district of Pirot showed second highest cancer rates for both

males (340.1/100.000) and females (284.0/100.000). According to the Cancer Register data men were mostly diagnosed with and died of cancer of bronchus and lung, colon and rectum cancer and prostate cancer. In women, the most frequent sites of malignant tumors were breast, cervix and bronchus and lung (Institut za javno zdravlje Srbije, 2016).

Cancer register for District of Pirot collects the incidences and mortality data and publish them once in two years. The analysis of data in the period from 1996 to 2005 shows averagely 473 new cancer patients annually: 233 (53%) men and 204 (47%) women with around 240 deaths caused by cancer: 139 (57.7%) men and 102 (42.3%) women. Leading localizations for cancer in men are lung (19% new cases, 24.7% deaths) and colorectal (16.5% new cases, 17% deaths) cancers. Among women, leading localization is by far breast cancer (24.4% new cases, 22.3% deaths) followed by colorectal cancer (10.6% new cases, 7% deaths) and lung cancer (6.9% new cases, 14% deaths). Thanks to the strategies for prevention and early detection, cervical cancer is in the fourth place with 4.8% newly diagnosed cases annually.

GENETIC TESTING AND GENETIC COUNSELING

Individuals with family and personal history suggestive for a hereditary disease should be referred to a genetic counselor at the Institute for Oncology and Radiology of Serbia (IORS) for a comprehensive evaluation. Based on the clinical situation and detailed pedigree analysis the decision on the genetic testing is individually based and requires a high index of suspicion for a particular gene/group of genes. In general, when a family history is suggestive, it is best to test the individual with a cancer diagnosis, as this increases the probability of a positive test result (Shiovitz & Korde, 2015). In case of a HBOC syndrome in the family, we recommend testing of a wider gene panel. During pre-test genetic counseling and after a detailed analysis of personal and family medical history, patients are being informed about the course of testing and about possible outcomes. All risks and benefits of genetic testing are explained to the patients who decide whether he/she wants to be tested and sign specifically designed informed consent for genetic testing that is conducted at IORS.

Standard clinical *BRCA1* and *BRCA2* genetic testing in the previous years has been carried out using PCR amplification and Sanger

sequencing. However, new Next Generation Sequencing (NGS) technology enabled us implementation of a wider gene panel testing as well as simultaneous analysis of many DNA samples. NGS also allows millions of fragments of DNA to be sequenced in a single run versus Sanger sequencing which only produces one forward and one reverse read. NGS has become routine technology in the Laboratory for Molecular Genetics which enabled higher throughput of the samples as well as lower cost per sample. The comparison between two technologies is shown in Table 1.

Table 1 The comparison between Sanger sequencing and NGS

	Sanger Sequencing	Next Generation Sequencing (NGS)
Sequencing strategy	Many separate reactions for sequencing exons/parts of the exons of a single gene for a single patients	One single reaction for simultaneous sequencing of many genes for many patients
Sample preparation	Many independent steps: PCR, clean-up, Cycle Sequencing, precipitation, capillary sequencing	DNA library preparation for many samples at the same time using complex protocols
Results	One read per sample (low depth of coverage)	Thousands and millions reads per sample (high depth of coverage)
Benefits	High precision. Useful for targeted sequencing of specific regions of the gene.	Highly cost-effective, fast and efficient by simultaneous analysis of many samples. Able to sequence large gene panels for many samples simultaneously. Able to sequence whole genomes in one reaction.
Challenges	Expensive and time consuming due to many different reactions.	Challenging interpretation of the abundance of data. Because of the higher error rate high coverage is needed for the accuracy and precision. The need for the bioinformatics support. Expensive instrumentation. The need for highly trained professionals.

In case when the result of genetic testing shows a pathogenic mutation it usually means the existence of the risk for developing breast/ovarian cancers that is higher than in general population. The actual risk is estimated based on the gene where mutation is found, the type of the mutation and its position, as well as on the clinical information for a particular patient. In case a patient has already been diagnosed with breast/ovarian cancer, pathogenic mutation can indicate higher probability for secondary malignancies. These pathogenic mutations are inherited in autosomal dominant way which means that there is a probability of 50% for the offspring to inherit pathogenic mutation.

Besides clearly pathogenic mutations, genetic testing can reveal genetic variations with unknown clinical impact. Variants of uncertain clinical significance (VUS) represent a particular challenge since their clinical significance cannot be inferred from sequence information alone (Eccles et al., 2015). Disclosure of a VUS requires special attention and highly experienced genetic counseling teams. There are many *in silico* models aiming to postulate the functional significance of VUS (Thompson et al., 2013). Even so, current recommendations are to still treat these variants as those of unknown significance until they are officially classified as either pathogenic or benign (Eccles et al., 2015).

In case pathogenic mutation in one of the predisposing genes has previously been detected in the family, the family members should be tested for the presence of that particular mutation. For those family members that have already developed a disease, positive results of this type of testing usually indicates higher risk for developing secondary malignancies. In case the family member is healthy but carries the family mutation, risk for developing breast/ovarian cancer for this person is higher than in general population. The level of risk depends on the gene where mutation is found, the type of the mutation and its position, as well as on the family history for the particular person. If a family member tests negative for family mutation, this doesn't mean that there is no risk for developing breast/ovarian cancer. The risk for developing disease is the same as the risk for every other person in the general population. Also, there is a small chance that mutation exists in other genes that are not tested but contributes to the higher risk for breast/ovarian cancers.

RESULTS OF GENETIC TESTING FOR HBOC IN SERBIA

Since May 2016, when we introduced NGS technology at the Laboratory for Molecular Genetics at IORS, we have tested 161 patients for the presence of mutations in 19 genes (*BRCA1*, *BRCA2, ATM, BRIP1, CDH1, CHEK2, MSH2, MLH1, MSH6, PMS2, EPCAM, NBN, NF1, PALB2, PTEN, RAD51C, RAD51D, STK11, TP53)* that are recommended by the *National Comprehensive Cancer Network* (NCCN) guidelines for HBOC. All tested patients had either strong family history of breast and ovarian cancers or early breast or ovarian cancer (before the age of 35). According to the BRCAPRO software, all patients had BRCA mutation carrier probability equal or higher than 10%. All patients went through pre-test genetic counseling and signed informed consent for testing.

Nextera DNA Library Preparation Kit in combination with TruSight® Cancer Panel (Illumina, San Diego, USA) was used for the enrichment of the coding sequence and exon/intron boundaries. NGS was performed on MiSeq Sequencing System (Illumina) according to manufacturer's protocol. Secondary data analysis and base calling was performed by MiSeq Reporter Software 2.5.1. VCF v4.1 files generated during secondary analysis of sequencing data were imported into Illumina Variant Studio software for variant annotation and filtering. Classification of detected variations was done through Illumina Variant Interpreter Software (Illumina) and Geneticist Assistant (Soft Genetics). Detected variations were annotated according to the databases (ClinVar, BIC, HGMD, BRCAShare, LOVD) and available published literature.

Table 2 shows number of detected pathogenic mutations in the 19 genes in the cohort of our patients that were tested by NGS during the two years of using this technology. The most of the mutations were detected in *BRCA1* (15/161), *BRCA2* (7/161) and *PALB2* (7/161) genes. Besides these genes, pathogenic mutations were also detected in *ATM* (2/161), *CHEK2* (4/161) and *TP53* (1/161) genes. Along with pathogenic mutations, we also detected the number of VUS in these genes (Table 3). High percentages of VUS were detected in *BRCA2* (6/161), *ATM* (5/161), *PMS2* (6/161), *NBN* (5/161) and *PALB2* (5/161) genes. All of the patients who carry VUS in one of the 19 genes are encouraged to visit our lab once a year for the eventual reclassification of the detected variant.

Table 2 Pathogenic mutations and VUS in 19 genes recommended by the NCCN guidelines detected in 161 patients by NGS

Gene	Case patients (n=161)	
	Carrier No. (%)	Non carrier No. (%)
BRCA1		
Mutation	16 (10)	145 (90)
VUS	2 (1.2)	159 (98.8)
BRCA2		
Mutation	7 (4.3)	154 (95.7)
VUS	6 (3.7)	155 (96.3)
ATM		
Mutation	2 (1.2)	159 (98.8)
VUS	5 (3.1)	156 (96.9)
BRIP1		
Mutation	0 (0)	161 (100)
VUS	0 (0)	161 (100)
CDH1		
Mutation	0 (0)	161 (100)
VUS	2 (1.2)	159 (98.8)
CHEK2		
Mutation	4 (2.5)	157 (97.5)
VUS	3 (1.9)	158 (98.1)
MSH2		
Mutation	0 (0)	161 (100)
VUS	3 (1.9)	158 (98.1)
MLH1		
Mutation	0 (0)	161 (100)
VUS	1 (0.6)	160 (99.4)
MSH6		
Mutation	0 (0)	161 (100)
VUS	0 (0)	161 (100)
PMS2		
Mutation	0 (0)	161 (100)
VUS	6 (3.7)	155 (96.3)

EPCAM		
Mutation	0 (0)	161 (100)
VUS	1 (0.6)	160 (99.4)
NBN		
Mutation	0 (0)	161 (100)
VUS	5 (3.1)	156 (96.9)
NF1		
Mutation	0 (0)	161 (100)
VUS	2 (1.2)	159 (98.8)
PALB2		
Mutation	7 (4.3)	154 (95.7)
VUS	5 (3.1)	156 (96.9)
PTEN		
Mutation	0 (0)	161 (100)
VUS	0 (0)	161 (100)
RAD51C		
Mutation	0 (0)	161 (100)
VUS	1 (0.6)	160 (99.4)
RAD51D		
Mutation	0 (0)	161 (100)
VUS	3 (1.9)	158 (98.1)
STK11		
Mutation	0 (0)	161 (100)
VUS	0 (0)	161 (100)
TP53		
Mutation	1 (0.6)	160 (99.4)
VUS	2 (1.2)	159 (98.8)

Besides 161 patients that were tested by NGS, we used Sanger sequencing for targeted sequencing of previously reported family mutations. 25 family members of the patients with confirmed pathogenic mutations were tested for that particular family mutation using targeted Sanger sequencing. Additional 6 mutations were detected in *BRCA1*, 1 in *BRCA2*, 3 in *PALB2*, 1 in *CHEK2* and 1 in *ATM* gene.

The overall mutational frequency in 186 patients tested by both NGS and Sanger sequencing at IORS in period from June 2016. until May 2018. is shown in Figure 4.

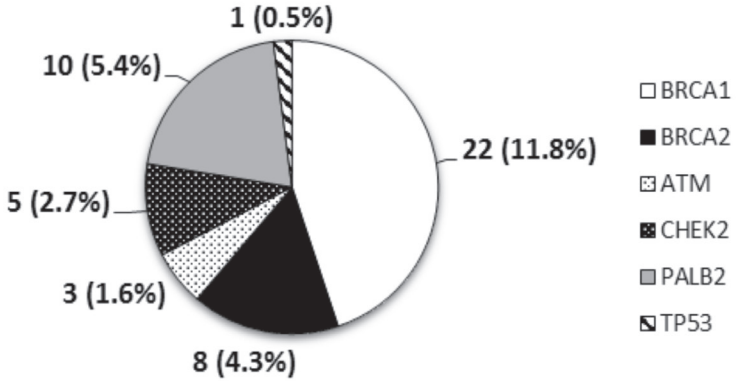

Figure 4 The overall frequency of pathogenic mutations detected in 186 patients tested by both NGS and Sanger sequencing

The most frequent pathogenic mutations were frameshift (57% of all detected pathogenic mutations) caused by addition or deletion of a base par/s. The number and the type of detected pathogenic mutations are presented in the Table 3.

Table 3 The type of the detected mutations

Gene	Type of the mutation		
	Missense	*Nonsense*	*Frameshift*
BRCA1	6/22 (27.3%)	3/22 (13.6%)	13/22 (59.1%)
BRCA2	1/8 (12.5%)	4/8 (50%)	3/8 (37.5%)
ATM	0	0	3/3 (100%)
CHEK2	2/5 (40%)	0	3/5 (60%)
PALB2	0	4/10 (40%)	6/10 (60%)
TP53	1/1 (100%)	0	0

THE IMPORTANCE OF GENETIC TESTING AND FUTURE DIRECTIONS

Genetic testing is a powerful tool that allows for the detection of *BRCA* and non-*BRCA* germline mutations in individuals at high risk of breast and ovarian cancer and those who have already developed the disease. On one hand, genetic testing is very important for determining the level of risk in high-risk patients and on the other for the individualization of treatment for those who already developed the disease (Cavic, Krivokuca, Jankovic, & Radulovic, 2015). For mutation carriers, several options are available for early detection of the disease and for managing cancer risk such as enhanced screening, prophylactic (risk-reducing) surgery, and chemoprevention. Enhanced screening may increase the chance of detecting breast cancer at an early stage when it may have a better chance of being treated successfully. Surgical procedures might help in reducing the cancer risk for those who carry pathogenic mutations in high penetrance genes. Because of this, surgical procedures are often described as "risk-reducing" rather than "preventive". Research demonstrates that women who underwent bilateral prophylactic salpingo-oophorectomy had a nearly 80% reduction in risk of dying from ovarian cancer, a 56% reduction in risk of dying from breast cancer (Domchek et al., 2010).

Regardless of whether a person receives positive or negative test result, there are many benefits to genetic testing. Benefits of receiving negative test result include the knowledge that there is no enhanced genetic risk for that particular cancer and a sense of relief knowing that one's children are not at risk of inheriting family's cancer susceptibility. A positive test result allow people to make informed decisions about their future health care, including regular medical follow up and taking steps to reduce cancer risk.

Before NGS technology, sequencing was time consuming and expensive, it was limited to targeted sequencing and as such was mostly the privilege of the developed countries. However, since NGS was introduced, the cost of genetic testing and timeframe for reporting results has dramatically reduced. Consequently, carrier probability threshold for genetic testing for *BRCA1/2* mutations was moved from 20% to 10% since more genetic centers were able to provide this service. Even though lowering the threshold for genetic testing directly impact on the

number of people that can be identified with pathogenic mutations, we have to keep in mind that genetic testing for HBOC is still not recommended for general population. Mutations in breast cancer genes are rare in general population, and probably only account for only about 2% of breast cancer cases overall. Because of the low frequency of pathogenic mutations and high costs of sequencing and data analysis, as well as a small proportion of women with a family history of breast and ovarian cancers, it is currently impractical to test all women with breast cancer. The stronger the woman's family history of cancer, the higher the chance she will harbor a pathogenic mutation in one of the cancer related genes. At the IORS, the criteria from National Guidelines for diagnostics and treatment of breast cancer are used for carrier status information. The threshold is set based on the national and international guidelines so it can pick up a significant proportion of mutation carriers while at the same time keep specificity of testing as high as possible. Genetic testing is offered at IORS if the patient's carrier probability exceeds the established threshold which is 10%. In circumstances where no living affected family member is available to offer a direct diagnostic test, we also offer genetic testing to unaffected patients but only for those who have a substantial risk of being mutation carriers.

Our results of multi-gene germline testing showed the importance of widening the spectrum of the genes that should be tested for detecting hereditary predisposition in Serbia. *BRCA1* and *BRCA2* genes are still those whose mutations are the most prevalent among high risk patients (11.8% and 4.3% respectively). However, high frequency of *PALB2* pathogenic mutations (5.4%) in Serbian population shows the importance of implementing this gene into routine clinical testing. The fact that *PALB2* has recently been announced as high penetrance gene shows that adequate clinical guidelines for the carriers of *PALB2* pathogenic mutations should be implemented in Serbia as soon as possible.

We have no precise data on the geographical origin of the tested patients. The main reason for this is that they usually have been referred to IORS from the clinics where they receive therapy or do the medical follow up rather than from the local hospitals and health centers where they were born. The most of our patients have been referred from the institutions from Belgrade or Vojvodina. There have been no referrals from the institutions from the District of Pirot since we started genet-

ic testing using new technology. There is couple of reasons why we should encourage referrals from this part of Serbia. Firstly, district of Pirot showed second highest cancer rates for both males and females, with the breast cancer as the most frequent type of disease among women. Genetic testing could help in defining the subgroup of patients that could benefit the most from the adequate clinical measures which will in the long run, help reduce deaths from hereditary breast and ovarian cancer in this part of Serbia. Secondly, defining the spectrum and frequency of genetic variations in hereditary breast and ovarian cancer genes in district of Pirot would be interesting from the scientific point of view. The distribution of mutations, particularly *BRCA1* and *BRCA2* is usually population specific. In some countries as well as ethnic communities *BRCA1/2* mutation spectrum is limited to a few founder mutations. There are also mutations that are specific for certain families. These mutations appear in a large number of cases but only in one family and we call them family specific mutations. The knowledge of the genetic structure of a particular population is important for developing effective screening protocols and more efficient approach for the individualization of genetic testing. Knowledge on the geographic distribution of genetic variations in Serbia, including District of Pirot can have impact on the management of hereditary cancer families on local and national healthcare system level. It might also help in developing targeted genetic tests that would be more affordable and cost-effective.

Multi-gene germline genetic testing for HBOC is increasingly relevant but it is not without challenges and limitations. According to the Thompson et al., because of the low frequency of non-BRCA mutations multiple-gene germline panels may provide clinical misinformation and harm at the individual patient level especially with mutation carriers lacking the classic phenotype associated with a cancer syndrome, as their cancer risk may be lower than that of previously estimated (Thompson et al., 2016). Another major challenge is adequate variant classification and interpretation of variants of unclassified significance (VUS). These variants are rare in *BRCA* genes nowadays due to the increase in *BRCA* testing in diverse populations. Also, thanks to the lower costs of genetic testing, the large efforts have been made in variants classification which results in lower frequency of VUS. However, we are now dealing with VUS in genes other than *BRCA1* and *BRCA2* and

with huge accumulation of sequences with no proven clinical significance. Further complications arise when two genetic laboratories provide conflicting interpretations of the same genetic variant which raises major concerns about the appropriate clinical management strategy for a patient with such conflicting results (Balmaña et al., 2016). One way in trying to resolve this important issue is making data available in publically available mutational databases and registries.

Previous decade has yielded major technology advancement with the great potential benefit for patients and families with high risk of hereditary breast cancer. Studies from diverse populations and genetic variability within the populations are essential to understand the true prevalence of breast cancer susceptibility genes in both affected and unaffected families so proper clinical guidelines can be introduced. Another important approach is focus on clinical trials for the treatment and prevention of hereditary breast cancer. Given the magnitude of this disease it is of great benefit for all parties to understand the role of genetic testing in hereditary breast and ovarian cancer especially the indications and interpretations associated with such testing.

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РЕЗИМЕ

Иако се највећи број карцинома дојке јавља спорадично у породицама, 5-10% случајева припада групи коју називамо наследни карцином дојке. Наследни карцином дојке се најчешће карактерише великим бројем случајева карцинома (дојке и/или јајника) у породици, настанком болести раније у животу, мултиплим примарним карциномима, као и билатералним и мултифокалним карциномима. 20-30% случајева наследног карцинома дојке и јајника повезано је са постојањем штетних мутација у високо ризичним генима BRCA1 и BRCA2. Ове штетне мутације се преносе са родитеља на децу и свако дете има вероватноћу од 50% да их наследи. Штетне мутације у BRCA1/2 генима доприносе повећаном ризику за оболевање од карцинома дојке, контралатералног карцинома дојке као и карцинома јајника. Ризик за карцином дојке износи 40%-87% а за карцином јајника 22%-65% код носиоца штетних мутација у BRCA1 гену. Носиоци штетних мутација у BRCA2 гену имају ризик за карцином дојке од 18%-88% а за карцином јајника 10%-35%. Варијације у опсегу ризика код носилаца BRCA штетних мутација зависе од испитиване популације, животног доба у коме је пацијент оболео, постојање породичне историје болести, врсте мутације као и њене тачне позиције у гену. На ризик за оболевање могу утицати други генетички фактори а та-

кође и начин живота и утицаји спољашње средине. Поред утврђивања ризика за оболевање код здравих особа, информација о присуству BRCA мутација код већ оболелих помаже клиничком збрињавању пацијената у контексту одабира адекватне терапије.

Поред BRCA гена, постоје и други гени који са мањим ризиком доприносе настанку наследног карцинома дојке и јајника. На основу полигеног модела, сви гени који доприносе наследном карциному дојке и јајника се могу сврстати у 3 групе: високоризични (BRCA1, BRCA2, TP53, PTEN, STK11 и CDH1), гени са средњим ризиком (ATM, CHEK2, RAD51, RAD51C, XRCC1, BRIP1...) и генетичке варијанте које носе низак ризик за оболевање (MAP3K1, FGFR2, LSP1, TNRC19….). Најновије студије указују да мутације у PALB2 гену доприносе високом ризику за оболевање од карцинома дојке што овај ген сврстава у групу високо ризичних. Жене носиоци PALB2 мутација имају ризик од 33% за оболевање од карцинома дојке чак иако немају породичну историју болести. Овај ризик расте до 58% код особа које имају два или више сродника оболелих од карцинома дојке.

Од маја 2016. године, на Институту за онкологију и радиологију Србије, тестирали смо 161 пацијента на присуство мутација у панелу од 19 гена. Највећи број мутација пронашли смо у BRCA1 (15/161), BRCA2 (7/161) и PALB2 (7/161) генима. Такође, детектовали смо штетне мутације и у ATM (2/161), CHEK2 (4/161) и TP53 (1/161) генима. Висок проценат генетичких варијанти непознатог клиничког значаја (ВУС) пронашли смо у BRCA2 (6/161), ATM (5/161), PMS2 (6/161), NBN (5/161) и PALB2 (5/161) генима. Додатно смо тестирали 25 чланова породица на присуство породичних мутација употребом Сангеровог секвенцирања. Пронашли смо додатних 6 мутација у BRCA1, 1 у BRCA2, 3 у PALB2, 1 у CHEK2 и 1 у ATM гену.

Већина тестираних пацијената воде порекло са територије северне Србије. Још увек није било пацијената који су упућени са територије Пиротског округа. Један од разлога зашто би требало подстаћи упућивање пацијената из овог дела Србије на тестирање је тај што је Пиротски округ, након Шумадијског, водећи по броју оболелих од карцинома и код мушкараца и код жена. Управо је карцином дојке водећа локализација карцинома код жена у Пиротском округу. Генетичко тестирање помогло би у дефинисању адекватних клиничких мера што би могло да допринесе смањењу броја оболелих и броја умрлих у овом делу Србије. Такође, дефинисање спектра и учесталости мутација унутар наше популације, укључујући и Пиротски округ, било би занимљиво и са

научне стране јер би могло да нам открије генетичку структуру популације као и евентуално постојање оснивачких мутација. Информација о географској дистрибуцији генетичких варијанти у Србији могла би да помогне и у развијању нових, циљаних тестова који били доступнији широј популацији.

Генетичко тестирање олакшава идентификацију особа под ризиком за оболевање од наследног карцинома дојке и јајника као и адекватно клиничко збрињавање особа које су већ оболеле. Редовније клиничко праћење, профилактичка хирургија и хемопревенција су неке од мера које омогућавају рану детекцију болести као и смањење ризика за настанак болести код носилаца штетних мутација. Обзиром на распрострањеност карцинома дојке у Србији, веома је важно разумети индикације за тестирање, улогу генетичког тестирања у смислу наследне предиспозиције за оболевање као и интерпретацију резултата и генетичко саветовање.

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