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RĪGAS STRADIŅA UNIVERSITĀTE

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THE ROLE OF GERMLINE *BRCA1* FOUNDER MUTATIONS AND SOMATIC *TP53* MUTATIONS IN THE TRIPLE-NEGATIVE BREAST CANCER SUBTYPE

Summary of the Doctoral Thesis
Speciality – Surgery

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Doctoral Thesis was carried out in Pauls Stradins Clinical University hospital, Rīga Stradiņš University Department of Surgery and Oncology Institute, Riga, Latvia

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LIST OF ABBREVIATIONS

AC – Doxorubicin, cyclophosphamide

BRCA1 – Breast cancer susceptibility gene 1

BRCA2 – Breast cancer susceptibility gene 2

BCT – Breast-conserving therapy

CEF – Cyclophosphamide, epirubicin, 5-fluorouracil

CEP 17 – Centrometric Probe for chromosome 17

CK5/6 – Cytokeratin 5/6

CMF Cyclophosphamide, methotrexate, 5-fluouracil

DNA – Deoxyribonucleic acid

DRFS – Distant recurrence-free survival

EGFR – Epidermal growth factor receptor

ER – Oestrogen Receptor

FAC – 5-fluouracil, doxorubicin, cyclophosphamide

FFPE – Formaline-fixed paraffin-embedded

FISH – Fluorescence in situ hybridization

HER2/neu – Human Epidermal Growth Factor Receptor 2

IHC – Immunohistochemistry

LRR – Locoregional recurrence

LRFS – Locoregional recurrence-free survival

NCCN – The National Comprehensive Cancer Network

PARP – Poly (adenosine diphosphate) ribose polymerases

pCR – Pathologic complete response

PR – Progesterone Receptor

SNP – The Single Nucleotide Polimorphism

TAC – Docetaxel, doxorubicin, cyclophosphamide

TP53 – Tumor protein 53

1. INTRODUCTION

Triple-negative breast cancer is a heterogeneous clinicopathological entity defined as an oestrogen (ER), progesterone (PR) and HER2/ neu negative breast cancer [*Bauer et al*., 2007]. Triple-negative breast cancer is estimated as an immunohistochemical surrogate of basal-like breast cancer subtype, but it should be mentioned that there is no complete overlap between the two groups [*Rakha et al*., 2009]. Triple-negative breast cancer accounts for approximately 10–20% of all breast cancer subtypes [*Bauer et al*., 2007]. As triple-negative breast cancer is hormone receptor and HER2/neu negative there is no targeted treatment available for this cancer subtype and a standard chemotherapy remains a basic systemic treatment option with no optimal cytotoxic regimen recommended. Inspite of relative chemosensitivity of this cancer subtype it is characterized by agressive clinical behavior with high recurrence and deaths rate, especially in the first five years after diagnosis [*Carey et al*., 2007]. Therefore, a further subclassification of triple-negative breast cancer is needed to develop a new targeted treatment to improve prognosis in these unfavorable cancer subtype.

In previous studies a strong relationship between *BRCA1* mutation-associated tumors and triple-negative breast cancer has been manifested, approximately 57–88% of all *BRCA1*-related tumours are triple-negative or/and basal-like [*Foulkes et al*., 2003; *Reis-Filho et al*., 2008]. The prevalence/incidence of germline *BRCA1/2* mutations in the triple-negative breast cancer subtype is relatively high, accounting for 10.6–19.5% in unselected patients' group [*Gonzalez-Angulo et al*., 2010; *Evans et al*., 2011]. *BRCA1*-mutated tumours carrier a dysfunctional DNA double-strand break repair mechanism and therefore is thought to be sensitive to platinum-based chemotherapy regimens and to inhibitors of the poly(ADP-rybosil)-polymerase [*Kennedy et al*., 2004; *Farmer et al*., 2005]. Theoretically, this agents could be

a new treatment options also for triple-negative breast cancer subtype and at the moment several clinical trials are now underway to figure out a therapeutic benefit of DNA-damaging agents and PARP inhibitors in this breast cancer subtype [*Silver et al*., 2010]. The role of carrying a *BRCA1* mutation could be crucial to guide a treatment strategy and to design further clinical trials.

However, previous studies showed contradicting and limited results with similar or worse outcomes for affected *BRCA* mutation carriers [*Stoppa-Lyonnet et al*., 2000; *Robson et al*., 2004; *Brekelmans et al*., 2006; *Rennert et al*., 2007; *Lee et a1*., 2011; *Bayraktar et al*., 2011; *Gonzalez-Angulo et al*., 2011]. Other potential agent for targeted treatment could be p53 or components of the p53 signaling pathway [*Turner et al*., 2013]. Approximately 60–88% of triple-negative / basal-like or *BRCA1*-related breast cancers have *TP53* mutations [*Shah et al*., 2012; *Dumay et al*., 2013]. Experimental models of breast cancer in mice revealed that tumors carrying *TP53* mutations show more aggressive clinical behavior [*Lang et al*., 2004]. The clinical studies showed contraversial results about the predictive and prognostic value of p53 protein overexpression/ *TP53* somatic mutations [*Pharoah et al*., 1999; *Overgaard et al*., 2000; *Goffin et al*., 2003; *Olivier et al*., 2006]. The majority of studies used immunohistochemistry(IHC) of p53 protein to detect alternations in the *TP53* gene, but this method failed to provide sufficiently accurate results and demonstrated lower prognostic value, if compared with a complementary DNA(cDNA)-based sequencing [*Sjorgen et al*., 1996, *Norberg et al*., 1998]. According to the last update of recommenadations for use of tumor markers of the American Society of Clinical Oncology p53 measurements are not currently recommended for routine clinical practice [*Harris et al*., 2007]. Therefore, further investigation of the breast cancer subclass-specific prognostic and predicative potential of different types of *BRCA1* and *TP53* mutations is required.

1.1. The aim of the research

To investigate the prognostic significance of carrying a two germline *BRCA1* founder mutations (4153delA and 5382insC) and somatic *TP53* mutations in patients with triple-negative breast cancer.

1.2. Research objectives

1. To evaluate the clinicopathological characteristics of the triple-negative *BRCA1* founder mutations negative breast cancers.
2. To evaluate the locoregional recurrence (LRR) rate and the impact of the type of surgery on distant recurrence-free and breast cancer-specific survival in the triple-negative *BRCA1* founder mutations negative group.
3. To evaluate the prognostic implication of carrying the *BRCA1* germline founder mutations among triple-negative breast cancer patients.
4. To identify prognostic factors for distant recurrence-free and breast cancer-specific survival in the triple-negative breast cancer group.
5. To evaluate the spectrum of somatic *TP53* mutations and its impact on prognosis in the triple-negative breast cancer group.

1.3. Scientific assumptions or working hypothesis

Positive germline *BRCA1* founder mutation status and presence of somatic *TP53* mutations may allow to identify the specific subsets of triple-negative breast cancer with different biological, prognostic features and response to treatment.

1.4. Scientific and practical novelty

In our study we showed that positive *BRCA1* founder mutation status in the triple-negative breast cancer significantly improve prognosis and could be used as independent favorauble prognostic factor. Sporadic *TP53* mutations could be used as prognostic factor for worse survival outcomes in the triple-negative breast cancer group.

1.5. Personal contribution

The author was involved in all stages of the study, including the study design, breast cancer diagnostic, surgery, postoperative patients management, multidisciplinary meetings. Clinical data collection from medical and pathological records, data annual update, data entering into electronic database, literature review, all stages of somatic *TP53* mutations verification, scientific measurements, data statistical analysis were performed by the author.

1.6. Ethics statement

All patients gave their written informed consent for genetic testing. The study protocol was approved by the Ethical Commettee of Rīga Stradiņš University.

2. MATERIAL AND METHODS

2.1. The study design

2943 patients (~50% of all breast cancer cases registered in Latvia between 2005–2011) with invasive breast cancer between 2005–2011 underwent genetic testing for *BRCA1/2* mutations at the Rīga Stradiņš University's Oncology Institute. In the study only patients who met all inclusion and exclusion criteria were included.

Inclusion criteria were:

1) invasive breast cancer in stage I–IV;

2) ER and PR defined as ER/PR – 0%, HER2 – 0;1+; luminal A breast cancer, defined as ER/PR positive, HER2 – 0;1+, Ki-67 < 14; luminal B HER2 negative, defined as ER/PR positive, HER2 – 0;1+, Ki-67 ≥ 14 [*Hammond et al*., 2010; *Goldhirsch et al*., 2011];

3) underwent definitive surgery between 2005–2011;

4) tested for *BRCA1/2* mutations;

5) in the case of positive *BRCA1* germline mutation, only patients with two founder mutations (5382insC and 4153 delA) (Table 2.1.1.);

2.1.1. Table

Spectrum of *BRCA1* founder mutations included in the study

BRCA1 founder mutations	N = 39	(%)
5382ins C	29	74.4
4153delA	10	25.6

6) signed informed consent forms to participate in the study;

7) available clinical data.

Exclusion criteria were:

1) inflammatory breast cancers;

2) with a history of ovarian or other advanced cancers;

3) *BRCA2* mutation carriers.

Consecutive 258 patients were deemed eligible for study.

The prospective phase of the study.

All patients were classified into four groups according to *BRCA1* mutation status and immunohistochemical subtypes of breast cancer defined at the 2011 St. Gallen Consensus [*Goldhirsch et al*., 2011] :

- 78 *BRCA1* mutation negative triple-negative breast cancers operated in Riga Eastern Clinical University Hospital between 2005–2007 and in Pauls Stradins Clinical University hospital between 2005–2011;

- 86 *BRCA1* mutation negative luminal A breast cancers opereted in Pauls Stradins Clinical University hospital between 2005–2011;

- 56 *BRCA1* mutation negative luminal B HER2 negative *BRCA1* mutation negative breast cancers (Table 2.1.2.) opereted in Pauls Stradins Clinical University hospital between 2005–2011;

- 38 *BRCA1* mutation positive triple-negative breast cancers opereted in Pauls Stradins Clinical University hospital, Riga Eastern Clinical University Hospital and Daugavpils Regional Hospital between 2005–2011.

2.1.2. Table

Expression of ER, PR, HER2 and Ki-67 in tumors of 78 *BRCA1* mutation negative triple-negative breast cancer, *BRCA1* mutation negative 86 luminal A, *BRCA1* mutation negative 56 luminal B HER2 negative and 38 *BRCA1* mutation positive triple-negative breast cancer patients

Characteristics	*BRCA1* negative TNBC*	*BRCA1* negative Luminal A	*BRCA1* negative Luminal B HER2* negative	*BRCA1* positive TNBC
ER*				
Average	0%	85.3%	83.1%	0%
PR*				
Average	0%	63.5%	53.9%	0%
HER2/neu*				
0;1+	78 (100%)	86 (100%)	56 (100%)	39 (100%)
2+	0 (0%)	0(0%)	0 (0%)	0 (0%)
3+	0 (0%)	0(0%)	0 (0%)	0 (%)
Ki-67 status				
Average	52.2%	6.9%	28.9%	58.4%

TNBC – Triple-negative breast cancer, ER – Oestrogen, PR – Progesterone, HER2/neu – Human epidermal growth factor receptor

The retrospective phase of the study: 66 triple-negative *BRCA1* germline positive or negative breast cancer patients opereted in Pauls Stradins Clinical University hospital and Riga Eastern Clinical University Hospital between 2005–2011 with available paraffin-embedded blocks were included.

2.2. The pathological examination

The prospective phase of the study:

258 breast cancer specimens from women undergoing surgery for primary invasive breast cancer between 2005–2011 in Pauls Stradins Clinical University Hospital, Daugavpils Regional Hospital and between 2005–2007 Riga Eastern Clinical University Hospital were collected. Tissue samples were fixed in 10% neutral buffered formalin. Tissue sample were processed and embedded in paraffin blocks. Histological parameters of all cases were reviewed by breast pathologists. Histological type and grade of ductal breast cancers was determined for each case according to the Bloom-Richardson histological system.

The retrospective phase of the study:

paraffin-embedded blocks were retrospectively obtained from Pauls Stradins Clinical University hospital and Riga Eastern Clinical University Hospital.

2.3. Immunohistochemistry

Estrogen (ER) and progesterone (PR) status and Ki-67 index were determined using standard immunohistochemistry (IHC). The evaluation of ER alpha and PR assays were performed according to the American Society of Clinical Oncology/ College of American Pathologists (ASCO/CAP) guideline recommendations for immunohistochemical testing of ER/PR. ER alpha and PR status were considered negative if immunoperoxidase staining of tumor cell nuclei was 0% [*Hammond et al*., 2010].

The expression of ER, PR and proliferation marker Ki-67 was evaluated in the tumor cell nuclei. Ki-67 index below 14% was considered as low and Ki-67 index equal or over 14% was considered as high [*Goldhirsch et al*., 2011].

HER2/neu was assessed through IHC (Monoclonal Mouse Anti-Human HER2-pY-1248, Clone PN2A, Code Nr. M 7269). The assessment of HER-2/neu expression was carried out using the *HercepTest* kit according to the manufacturer's instructions. IHC is scored on a qualitative scale from 0 to 3+, based on interpretation of staining intensity, with 0 and 1+ classified as negative (0 – was considered, if no staining or staining of the tumor cells membrane were less than 10%, and 1+, if more than 10% of the tumor cells membrane stained partly) and 3+ classified as positive (3+ – was defined, as uniform intense membrane staining of > 30% of invasive tumor cells).

Specimens with equivocal HER2/neu IHC (2+) test results (a moderate complete membrane staining observed in more than 10% of the tumor cells), were confirmed by fluorescence in situ hybridization (FISH).

All IHC and FISH tests were performed in the Department of Pathology at Pauls Stradins Clinical University Hospital or/and Riga Eastern Clinical University Hospital.

2.4. Molecular diagnostics

2.4.1. *BRCA1/2* germline founder mutations

BRCA1/2 testing results were obtained from prospectively registered database of the Riga Stradins University's Oncology Institute.

230 (89.1%) patients were tested for germline *BRCA1* founder mutations at the time of the surgery, 23 (8.9%) patients were tested before surgery and 5 (2%) patients were tested within 1 year after surgery.

2.4.2. Detection of sporadic *TP53* gene mutations

Purification of genomic DNA from FFPE tissue was performed using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution. Somatic *TP53* mutations were analysed in exons 5–8 using a RT-PCR assay with subsequent high resolution melt analysis (HRM). The reaction was run on Rotor Gene 6000™ real-time system (Qiagen, Germany). In the study method described by *Krypuy et al.,* was used. HRM curve analysis was performed with Rotor-Gene Q Series Software 1.7. Mutations detected by RT-PCR/HRM were confirmed by DNA sequencing using Genetic Analyzer 3130 (Applied Biosystems) according to the standard protocol.

Data analysis was performed using Applied Biosystems software for DNA sequencing, SeqScape and NCBI BLAST. For interpretation of the results several databases were used: SNP-NCBI (National Center for Biotechnology Information), IARC *TP53* (International Agency of Research on Cancer) and COSMIC (Catalogue of Somatic Mutations In Cancer).

2.5. Data collection

Clinical data were obtained from the patients' medical records and entered into electronic database. The data were completed at diagnosis and updated annually. Survival data were supplemented with Latvian cancer registry data-prospective database of Centre for Disease Control and Prevention.

2.6. Follow-up

The median follow-up from the original diagnosis until analysis was 36 (range, 8–85) months in the triple-negative *BRCA1* mutation non-carriers, 41

(range, 8–86) months in the triple-negative *BRCA1* mutation carriers, 45 (range, 24–96) months in the *BRCA1* negative luminal A group and 43 (range, 29–73) months in the *BRCA1* negative luminal B HER2 negative group.

2.7. Outcomes

The outcomes were analysed in all 258 patients. The complete pathologic response (pCR) was defined as no evidence of residual invasive breast cancer and ductal carcinoma in situ both in the breast and lymph nodes. Locoregional recurrence (LRR) was defined as clinical and histological documented recurrence in the ipsilateral breast, chest wall or regional lymphnodes (axillary, supraclavicular, internal mammary). Locoregional recurrence-free survival (LRFS) was defined as the time from diagnosis to clinical and histological documented evidence of local recurrence. Distant recurrence was defined as clinical and radiographical evidence of distant relapse. Distant recurrence-free survival (DRFS) was defined as the time from diagnosis to first evidence of distant recurrence. The DRFS was censored at the data of the last follow-up if no distant recurrence were observed. The breast cancer-specific survival was calculated from data of diagnosis until death due to breast cancer.

2.8. Statistical methods

Statistical analysis was performed using the statistical software SPSS version 16.0. In the present study a chi-square, Fisher's exact test, independent samples t-test, one-way analysis of variance (ANOVA), univariate and multivariate Cox proportional hazards models were used. The breast cancer-specific survival was estimated using the Kaplan-Meier method and compared

by a long-rank test. $P \leq 0.05$ was considered to indicate a statistically significant difference.

3. RESULTS

3.1. The clinicopathological characteristics and estimates of survival outcomes in the triple-negative luminal A, luminal B HER2 negative breast cancers

3.1.1. The clinicopathological characteritics of sporadic triple-negative, luminal A, luminal B HER2 negative breast cancers

The median age at diagnosis in the triple-negative breast cancer group was 54.3 years, in the luminal A breast cancer group the mean age at diagnosis was 60.1 years and in the luminal B HER2 negative breast cancer group the mean patients' age was 57.2. Patients in the triple-negative breast cancer group was statistically significantly younger than in the luminal A group ($P < 0.004$).

The majority of triple-negative, luminal A and luminal B HER2 negative breast cancers were classified as ductal carcinomas. Triple-negative subgroup was more likely to have medullary breast cancer. Triple-negative breast cancer group was more likely to have grade III tumors than luminal A and B HER2 negative breast cancers. In the triple-negative breast cancer group there was a statistically significantly higher Ki-67 expression (52.2%) compared to luminal A (6.9%) and luminal B HER2 negative (28.9%) breast cancer groups ($P < 0.0001$).

In the triple-negative breast cancer group the mean tumor size was a statistically significantly larger than in the luminal A breast cancer group (32.9 mm versus 23.8 mm, respectively; $P < 0.002$). A statistically significantly higher proportion of patients in the luminal A breast cancer had T1 and T2 stage than in the triple-negative and luminal B HER2 negative breast cancers. The rate of lymph node negativity was statistically significantly higher in the luminal A subtype than in the triple-negative and luminal B HER2 negative subtypes. Luminal A breast cancers were more likely to be diagnosed in stage I

than triple-negative and Luminal B HER2 negative breast cancers. A higher proportion of patients with triple-negative and luminal B HER2 negative breast cancer were diagnosed in stage III compared to luminal A breast cancer. There was a significantly positive correlation between tumor size and a positive lymph node status in the luminal A and B HER2 negative breast cancers. In contrast, in the triple-negative breast cancer group there was no correlation between tumor size and positive lymph node status ($P = 0.17$) among patients with tumors of < 5 cm, compare to luminal A and B HER2 negative ($P < 0.002$ and $P < 0.026$, respectively).

There was no statistically significant difference in performed type of surgery between breast cancer subtypes ($P = 0.15$). A statistically significantly higher proportion of patients in the luminal A breast cancer group underwent sentinel node biopsy, compare to patients in the luminal B HER2 negative and triple-negative breast cancer groups ($P < 0.02$). A statistically significantly higher proportion of patients in the triple-negative breast cancer group received chemotherapy compare to luminal A and luminal B HER2 negative breast cancers. The chemotherapy regimens most commonly used in all breast cancer subtypes were anthracycline-based, anthracycline+taxane-based and CMF.

A significantly higher proportion of patients in the triple-negative group received neoadjuvant chemotherapy compare to luminal A and luminal B HER2 negative groups.

63 (80.8%) patients in the triple-negative breast cancer group, 51 (59.3%) patients in the luminal A group and 39 (69.6%) patients in the luminal B HER2 negative group received adjuvant radiation therapy ($P < 0.03$).

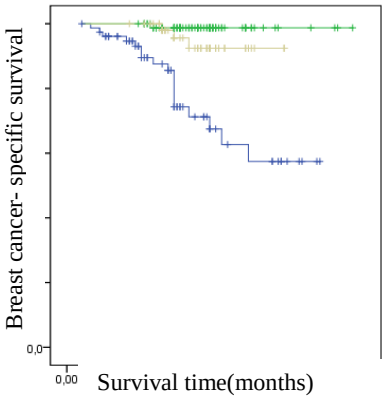
3.1.2. Estimates of survival outcomes in the sporadic triple-negative, luminal A and luminal B HER2 negative breast cancer groups

There was no statistically significant difference in the LRR rate between triple-negative, luminal A and luminal B HER2 negative groups (3 (3.9%) versus 2 (2.3%) versus 0 (0%), respectively; P = 0.34). The LRFS was 5.7 months (range, 4–8 months) in the triple-negative breast cancer group and 27.5 months (29 and 26 months) in the luminal A group.

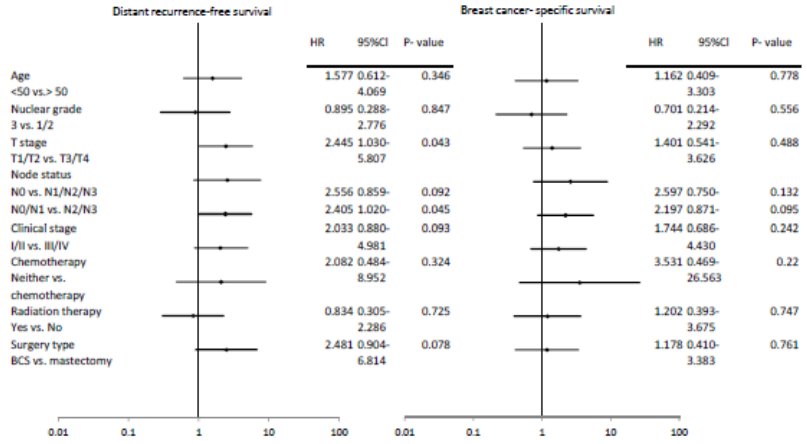
A higher proportion of triple-negative breast cancer patients experienced distant recurrence compared with luminal A and luminal B HER2 negative breast cancer patients (P < 0.0001). The DRFS was 32.2 months (range, 685 months) in the triple-negative breast cancer group, 45 months (range, 11–96 months) in the luminal A group and 42 months (range, 7–73 months) in the luminal B HER2 negative group. There was no statistically significant difference between groups in incidence of sites of distant recurrence.

Triple-negative breast cancer patients were more likely to die from breast cancer than Luminal A and luminal B HER2 negative breast cancer patients (18 (23.1%) versus 1 (1.2%) and 3 (5.4%) respectively; P < 0.02). Luminal A and luminal B HER2 negative breast cancer patients had a statistically significant higher breast cancer-specific survival than non-carriers (98.8% in the luminal A group, 94.6% in the luminal B HER2 negative group and 76.9% in the triple-negative group, P < 0.0001) (Figure 3.1.2.2.).

In the univariate analyses, clinical T stage 3 and 4 (HR = 2.445;95% Cl:1.030–5.807; P < 0.043) and positive lymph node status (HR = 2.405; 95% Cl:1.020–5.670; P < 0.045) was associated with a higher risk of distant recurrence, no statistically significant effect of evaluated risk factors on breast cancer-specific survival was found (Figure 3.1.2.3.).



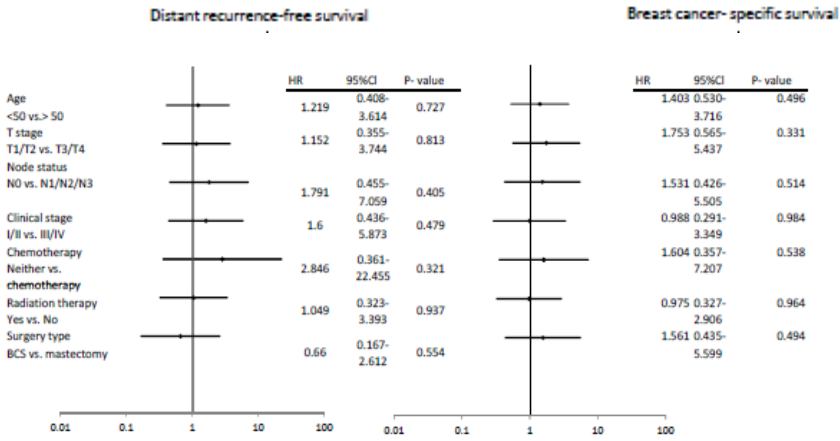
3.1.2.2. Figure. Survival curves of *BRCA1* negative triple-negative breast cancers (blue line), luminal A breast cancers (green line) and luminal B HER2 negative breast cancers (yellow line). P < 0.0001



3.1.2.3. Figure. Univariate Cox proportional hazards model for distant recurrence-free survival and breast cancer-specific survival

HR – hazard ratio, Cl – confidence interval, BCT – breast-conserving surgery

In the multivariate analysis Cox proportional hazards model no statistically significant effect of evaluated risk factors on distant recurrence-free survival and breast cancer-specific survival was found (Figure 3.1.2.4.).



3.1.2.4. Figure. Multivariate Cox proportional hazards model for distant recurrence-free survival and breast cancer-specific survival
HR – hazard ratio, Cl – confidence interval, BCS – breast-conserving surgery

3.2. The clinicopathological characteristics and estimates of survival outcomes in the triple- negative breast cancer *BRCA1* mutation carriers and non-carriers

3.2.1.The clinicopathological characteristics of triple-negative breast cancer *BRCA1* mutation carriers and non-carriers

The median age at diagnosis in the triple-negative breast cancer *BRCA1* mutation positive group was 48.8 years compared to 54.3 years in the triple-negative *BRCA1* mutation negative group ($P < 0.034$).

Invasive ductal carcinoma was the most common histological type in both groups, but *BRCA1* mutation non-carriers were more likely to have

invasive lobular carcinomas. The majority of triple-negative *BRCA1* mutation carriers and non-carriers were grade III tumors. There was no statistically significant difference in Ki-67 expression between triple-negative *BRCA1* mutation positive and negative breast cancer groups (59.8% versus 52.2%, respectively; P = 0.27).

The tumor size was 36.2 mm in the triple-negative *BRCA1* mutation positive group and 32.9 mm in the *BRCA1* mutation negative group (P = 0.47). There was no statistically significant difference in relation to T stage and stage of the disease between two groups. There were a higher proportion of lymph node negative patients in the triple-negative *BRCA1* mutation-carriers group compared to non-carriers group (P < 0.004).

There was no statistically significant correlation between tumor size and positive lymph node status among patients with tumors of < 5 cm both in the triple-negative *BRCA1* positive (P = 0.079) and *BRCA1* negative groups (P = 0.17).

A higher proportion of triple-negative *BRCA1* mutation carriers compared to *BRCA1* mutation non-carriers underwent mastectomy (32 (84.2%) versus 42 (53.9%), respectively; P < 0.001). There were no difference in performed lymphadenectomy (P = 0.80) and sentinel node biopsy (P = 0.94) between triple-negative *BRCA1* mutation carriers and non-carriers.

There was no statistically significant difference between two groups in the proportion of patients, who received chemotherapy and the type of received chemotherapy regimens. The chemotherapy regimens used in the triple-negative *BRCA1* mutation carrriers and non-carriers were anthracycline-based, anthracycline+taxane-based, CMF, platine-based. 9 (23.7%) of patients in the triple-negative *BRCA1* mutation carriers received neoadjuvant chemotherapy compared to 22 (28.2%) in the triple-negative *BRCA1* mutation non-carriers (P = 0.62). Triple-negative *BRCA1* mutation non-carriers more likely received radiation therapy compared to *BRCA1* mutation carriers (61 (78.2%) versus 22

(57.9%), respectively; P < 0.027). 3 (3.9%) patients in the triple-negative *BRCA1* carriers group and 2 (5.3%) patients in the *BRCA1* non-carriers group underwent bilateral salpingo-oophorectomy under the age of 50 years. Prophylactic mastectomy was performed in 3 (7.7%) *BRCA1* mutation carriers. Patients with positive *BRCA1* mutation experienced more bilateral breast cancers than non-carriers (6 (15.8%) versus 2 (2.6%), respectively; P < 0.016).

3.2.2. Estimates of survival outcomes in the triple-negative *BRCA1* carriers and non-carriers

There was no statistically significant difference in the LRR rate between *BRCA1* mutation non-carriers and carriers (3 (3.9%) versus 1 (2.6%), respectively; P = 0.80). The LRFS was 5.7 months (range, 4–8 months) in the *BRCA1* mutation non-carriers group and 20 months in the *BRCA1* mutation carriers group.

A higher proportion of *BRCA1* mutation non-carriers experienced distant recurrence compared with mutation carriers (22 (28.2%) versus 4 (10.5%), respectively; P < 0.03). There was no statistically significant difference between the two groups in incidence of sites of distant recurrence. *BRCA1* mutation non-carriers were more likely to die from breast cancer than *BRCA1* mutation carriers (18 (23.1%) versus 2 (5.3%), respectively; P < 0.014). *BRCA1* mutation carriers had a statistically significant higher breast cancer-specific survival than non-carriers (94.9% in the *BRCA1* mutation carriers and 76.9% in the *BRCA1* mutation non-carriers, P < 0.02) (Figure 3.2.2.1.). The development of bilateral breast cancer didn't significantly impact the survival outcomes (HR = 0.040; 95% Cl:0.001–4.804; P = 0.59).

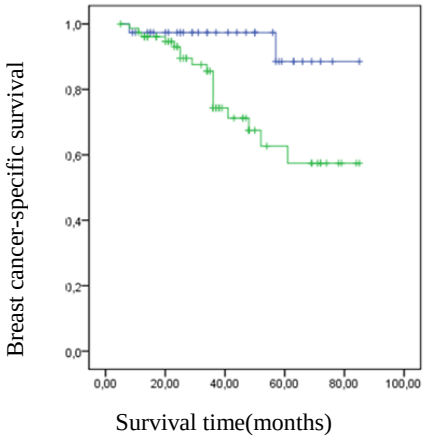
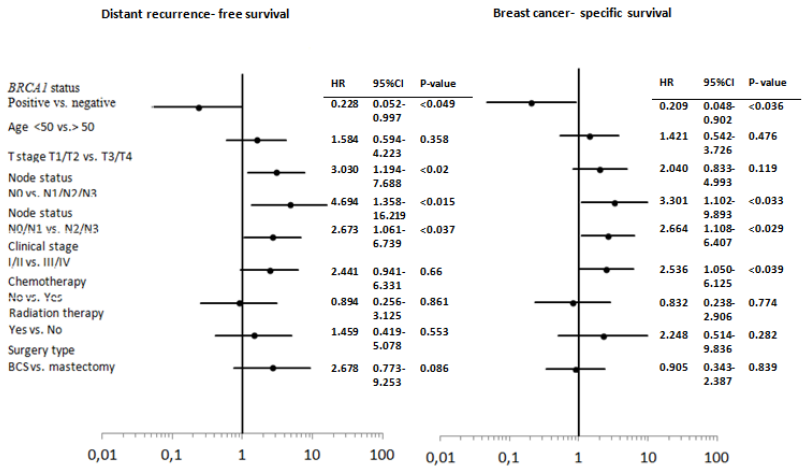


Figure 3.2.2.1. Survival curves of triple-negative *BRCA1* mutation carriers (blue line) and triple-negative *BRCA1* mutation non-carriers (green line). P < 0.02

In the univariate analyses, clinical T stage 3 and 4 (HR = 3.030; 95% Cl:1.194–7.688; P < 0.02) and positive lymph node status (HR = 4.694; 95% Cl:1.358–16.219; P < 0.015) were associated with a higher risk of distant recurrence, but *BRCA1* positive status (HR = 0.228; 95% Cl:0.052–0.997; P < 0.049) was associated with deacreased risk of distant recurrence (Figure 3.2.2.2.).

In multivariate analysis Cox proportional hazards model *BRCA1* positive status was independent favorable prognostic factor for distant recurrence-free survival (HR = 3.301; 95% Cl:1.102–9.893; P < 0.033) (Figure 3.2.2.3.).

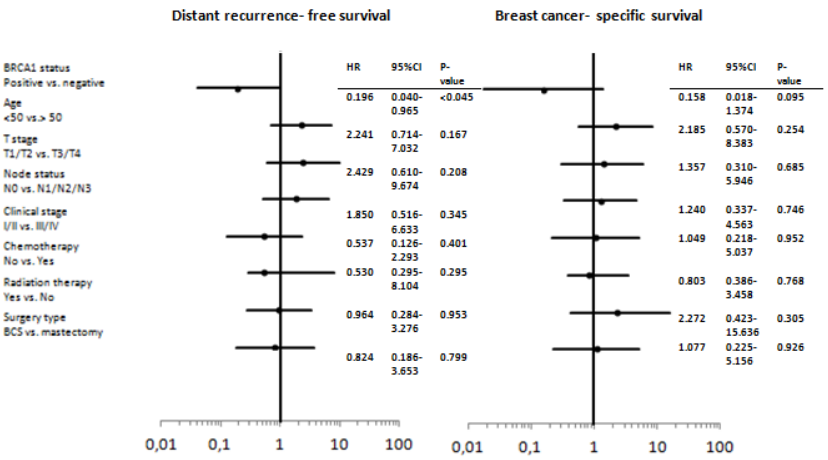


3.2.2.2. Univariate Cox proportional hazards model for distant recurrence-free survival and breast cancer-specific survival

HR – hazard ratio, Cl – confidence interval, BCT – breast-conserving surgery

In the univariate analyses, clinical stage III and IV (HR = 2.536; 95% Cl:1.050–6.125; P < 0.039) and positive lymph node status (HR = 3.301; 95% Cl:1.102–9.893; P < 0.033) were associated with increased risk of breast cancer-specific death, but positive status (HR = 0.209; 95% Cl:0.048–0.902; P < 0.036) was associated with decreased risk of breast cancer-specific death (Figure 3.2.2.2.).

In the multivariate analysis Cox proportional hazards model no statistically significant effect of evaluated risk factors on breast cancer-specific survival was found (Figure 3.2.2.3.).



3.2.2.3. Multivariate Cox proportional hazards model for distant recurrence-free survival and breast cancer-specific survival

HR – hazard ratio, Cl – confidence interval, BCS – breast-conserving surgery

3.3. Sporadic *TP53* mutations in the triple-negative breast cancer

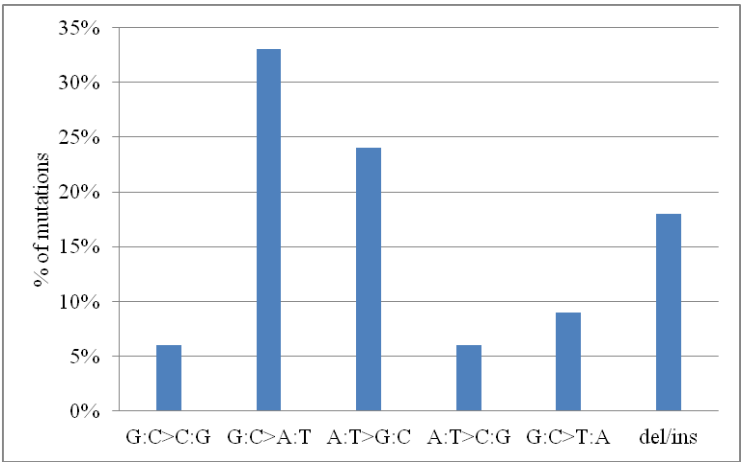
3.3.1. Spectrum of *TP53* sporadic mutations in the triple-negative breast cancer *BRCA1* germline mutations non-carriers and carriers

A total of 66 primary triple-negative breast tumors were screened for mutations in *TP53* exons 5 to 8 using real-time PCR with subsequent HRM and direct bi-directionally DNA sequencing performed on RT-PCR-positive specimens. *TP53* sporadic mutations were found in 2 6 (39.4%) tumors. There was no statistically significant difference in the *TP53* mutations rate between triple-negative *BRCA1* mutation non-carriers and carriers (22 (40%) versus 4 (36.4%), respectively; P = 0.84).

In a total of 26 tumors with at least one *TP53* sporadic mutation, 33 *TP53* mutations (27 (81.8%) point mutations, 5 (15.2%) deletions, 1 (3%) insertion) were detected. Triple-negative breast cancers exhibited a high rates

of G:C > A:T(33.3%) mutations and A:T > C:G (24.2%) mutations. The distribution of the types of *TP53* mutations are shown in Figure 3.3.1.1. There was no statistically significant difference in the types of *TP53* mutations between triple-negative *BRCA1* carriers and non-carriers (P = 0.29). There were 4 (66.7%) transitions in the triple-negative *BRCA1* carriers group compared to 15(55.6%) in the *BRCA1* non-carriers group (P = 0.66). The triple-negative *BRCA1* carriers group harboured 1 (16.7%) transversion mutation compared to 6 (22.2%) in the *BRCA1* non-carriers group (P = 0.83). There was no insertions/deletions identified in the *BRCA1* carriers group compared to 6 (22.2%) identified in the *BRCA1* non-carriers group (P = 0.27).

In one triple-negative *BRCA1* germline negative patient 3 different *TP53* sporadic mutations (1 deletion, 1 transition, 1 transversion) in exons 5, 6 and 7 were detected. There was 5 (83.3%) *TP53* missense deleterious mutations in the triple-negative *BRCA1* carriers compared to 11 (68.8%) *TP53* missense deleterious mutations in the *BRCA1* non-carriers group (P = 0.08). A significantly higher proportion of *TP53* mutations were detected in 8 exon compared to 7, 6 and 5 exons (15 (45%) in exon 8 compared to 7 (21.2%) in exon 7, 5 (15%) in exon exon and 6 (18.2%) in exon 5; P < 0.0017). In the triple-negative *BRCA1* carriers all 6 (100%) *TP53* mutations were identified in 7 and/or 8 exons compared to 16 (48.5%) *TP53* mutations in the non-carriers, but this difference didn't reach statisticall significance(P = 0.067). We identified three novel sporadic *TP53* mutations (c.510 ins TAG in exon5, c.446del C in exon 5 and c.864 delT in exon 8 which are not described in the COSMIC and IARC TP53 databases.



3.3.1.1.Figure. The types of the *TP53* sporadic mutations in the triple-negative *BRCA1*carriers/non-carriers group
Del / ins – deletions / insertions

3.3.2. The association between *TP53* sporadic mutations and clinicopathological characteristics in the triple-negative breast cancer group

The median age at diagnosis in the triple-negative *TP53* positive group was 53.3 years (range, 28–80 years) compared to 52.8 years (range, 31–79 years) in the triple-negative *TP53* negative group (P = 0.88). There was no statistically significant difference in the size of the tumor between triple-negative *TP53* positive and negative groups (30.9 mm versus 33.6 mm, respectively; P = 0.28). No statistically significant difference was found between triple-negative *TP53* positive and negative group on percentage of cases of ductal (18 (6%) versus 32 (80%), respectively; P = 0.08) and lobular carcinoma (3 (11.5%) versus 6 (5%), respectively; P = 0.72). A higher proportion of patients in the triple-negative *TP53* positive group had a medullary carcinoma compared to *TP53* negative group, but this difference

didn't reach statisticall significance (3 (11.5%) versus 1 (2.5%), respectively; P = 0.19). 5 (12.5%) patients in the triple-negative *TP53* mutations negative group had a grade II and 27 (67.5%) patients had a grade III tumors compared to 2(7.7%) patients with grade II tumors and 17 (65.4%) patients with grade III tumors in the triple-negative *TP53* positive group (P = 0.60). In the triple-negative *TP53* mutation positive group there was a higher ki-67 expression compared to triple-negative *TP53* mutation negative group, but this difference was not statistically significant (62.4% versus 54.7%, respectively; P = 0.325). There was no statistically significant difference between triple-negative *TP53* positive and negative groups in relation to T stage, lymph node status and stage of disease (Table 3.3.2.1.)

3.3.2.1. Table

The histopathological features of the triple-negative breast cancers according to *TP53* status

Characteristics	Triple-negative *TP53* positive n = 26 No. of patients (%)	Triple-negative *TP53* negative n = 40 No. of patients (%)	P-value*
T stage			
T1	6 (23.1%)	9 (22.5%)	0.95
T2	13 (50%)	23 (57.5%)	0.56
T3	6 (23.1%)	6 (15%)	0.43
T4	1 (3.8%)	2 (5%)	0.88
Nodal status			
N0	13 (50%)	18 (45%)	0.73
N1	6 (23.1%)	9 (22.5%)	0.95
N2	2 (7.7%)	11 (27.5%)	0.052
N3	5 (19.2%)	2 (5%)	0.09
Stage			
I	6 (23.1%)	5 (12.5%)	0.29
II	12 (46.1%)	21 (52.5%)	0.63
III	8 (13.8%)	13 (32.5%)	0.89
IV	0 (0%)	1 (2.5%)	0.87

* Chi-square analysis

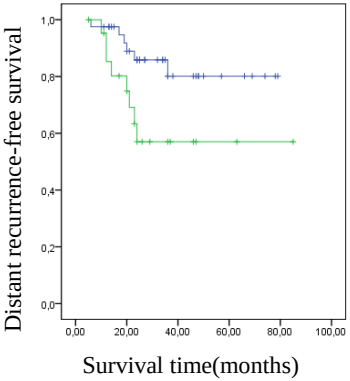
14 (53.8%) patients in the triple-negative *TP53* positive and 16 (40%) patients in the triple-negative *TP53* negative group underwent breast-conserving surgery (P = 0.29). 12 (46.2%) patients in the triple-negative *TP53* positive and 24 (60%) patients in the triple-negative *TP53* negative group underwent mastectomy (P = 0.29). There was no statistically significant difference in performed lymphadenectomy (19 (73.1% versus 32 (80%), respectively)) and sentinel node biopsy (7 (26.9%) versus 8 (20%), respectively) between triple-negative *TP53* positive and negative groups (P = 0.53). There was no statistically significant difference in received chemotherapy regimens between two groups. The vast majority of patients both in the triple-negative *TP53* positive and negative groups received anthracycline-based chemotherapy. There was no significant difference between triple-negative *TP53* positive and negative group in received radiation therapy (22 (84.6%) versus 32 (80%), respectively; P = 0.66).

3.3.3. The impact of the *TP53* sporadic mutations on survival outcomes in the triple-negative breast cancer group

There was no significant difference in the LRR rate between triple-negative *TP53* positive and negative group (1 (3.9%) versus 2 (5%), respectively; P = 0.87). 7 (26.9%) patients in the triple-negative *TP53* mutations positive group and 7 (17.5%) patients in the triple-negative *TP53* negative group experienced distant recurrences (P = 0.38). There was no statistically significant difference between two groups in incidence of sites of recurrence (P = 0.76). There was no statistically significant difference in DRFS between triple-negative *TP53* mutations positive and *TP53* mutations negative groups (P = 0.37). The DRFS was 28.1 months (range, 8–63 months) in the triple-negative *TP53* positive group compared to 33.5 months (range, 8–79 months) in the triple-negative *TP53* negative group. There was no statistically

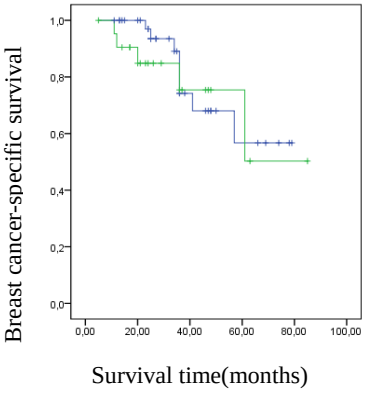
significant difference in the number of deaths between triple-negative *TP53* mutations positive and *TP53* mutations negative groups (7 (26.9%) versus 9 (22.5%), respectively (P = 0.68)).

Deleterious *TP53* mutations were associated with statistically significant negative impact on distant-recurrence-free survival (63.6% versus 85.0%, respectively; P < 0.036) (Figure 3.3.3.1.). *TP53* deleterious mutations showed no statistically significant prognostic impact on breast cancer-specific survival. However, there was a tendency towards worse breast cancer-specific survival in the triple-negative *TP53* deleterious mutations positive group compared to negative group (80% versus 77.3%; P = 0.65) (Figure 3.3.3.2.).



3.3.3.1.Figure. Distant recurrence-free survival (DRFS) in the triple-negative *TP53* sporadic deleterious mutations carriers (green line) and triple-negative *TP53* sporadic deleterious mutations non-carriers (blue line). P < 0.036

There was an insignificant tendency towards worse distant recurrence-free survival in the patients with deleterious mutations who were treated with anthracycline-based chemotherapy (61.5% versus 85.7%, respectively; P = 0.13).



3.3.3.2. Figure. Survival curves of triple-negative *TP53* sporadic deleterious mutations carriers (green line) and triple-negative *TP53* sporadic deleterious mutations non-carriers (blue line). P = 0.65

However, positive *TP53* deleterious mutations showed no significant impact on breast cancer-specific survival compared to negative group (69.2% versus 82.1%, respectively; P = 0.74).

4. DISCUSSION

4.1. The clinicopathological characteristics and survival outcomes of sporadic triple-negative, luminal A, luminal B HER2 negative breast cancers

According to our results the triple-negative breast cancer subtype is associated with significantly younger age at diagnosis compared to luminal A breast cancer subtype and a tend to younger age at diagnosis compared to luminal B breast cancer subtype. Similar results to our was published by *Dent et al*., where median age at diagnosis was 54.4 years for triple-negative breast cancer group compared to 57.7 years in the other group ($P < 0.0001$) [*Dent et al*., 2007]. *Liedtke et al*., analysed 1.732 patients with triple-negative breast cancer and showed that younger age at diagnosis (≤ 40 years) is associated with poor tumor differentiation and is an independent predictor of worse disease-free and overall survival despite more intense systemic treatment [*Liedtke et al*., 2013]. In contrast, our study showed no impact of patients' age at diagnosis on disease-free and breast cancer-specific survival both in the univariate and multivariate analysis.

The most frequent histological subtype in the triple-negative breast cancer group was ductal breast carcinoma (78.2%). This results are in agreement with *Carey et al*., study there the majority of triple-negative breast cancer patients had a ductal carcinoma of no special type [*Carey et al*., 2007]. *Vincent-Salmon et al*., demonstrated that medullary breast carcinoma is a specific entity within the basal-like breast cancer subtype that is characterized by higher immunohistochemical expression of CK5/6 and distinct genetic alterations [*Vincent-Salmon et al*., 2007]. In our study medullary breast carcinoma was siginificantly more common in the triple-negative breast cancer group ($P < 0.02$) than in the luminal A and luminal B breast cancer groups.

Several studies demostrated a more favorable prognosis for medullary breast carcinomas [*Marginean et al*., 2010]. In our study the histological type didn't have statistically significant impact on distant recurrence-free and breast cancer-specific survival in the univariate and multivariate analysis. However, no patients with medullary breast carcinoma in the triple-negative breast cancer group experienced local, distant recurrence or death due to breast cancer in the median follow-up period of 26 months. Triple-negative breast cancer patients were more likely to have poorly differentiated tumors ($P < 0.0001$) with higher Ki-67expression than in the luminal A and luminal B HER2 negative breast cancer subtype ($P < 0.0001$). 83.6% of triple-negative breast cancer patients were poorly differentiated (grade III) compare to 17.8% in the luminal A group and 47.9% in the lumunal B HER2 negative group. Similar results were published by several previous studies [*Dent et al*., 2007; *Bauer et al*., 2007; *Onitilo et al*., 2009]. *Bauer et al*., reported that 76% of triple-negative breast cancer patients have grade III tumors compared to only 28% in the other breast cancer group [*Bauer et al*., 2007]. In our study in the univariate analysis grade III failed to show to be a predictor of reduced distant recurrence-free and breast cancer-specific survival.

According to our study, the median tumor size is statistically significantly larger in the triple-negative breast cancer group than in the luminal A breast cancer group. This results are in concordance with previous studies [*Dent et al*., 2007; *Bauer et al*., 2007]. A statistically significantly lower proportion of patients had T1 and T2 breast cancer in the triple-negative breast cancer group (26.9% and 48.7%) compared to luminal A breast cancer group (60.5% and 26.7%). Similar results were published by *Dent et al*., there 36.5% of triple-negative breast cancer patients had T1 tumors compared to 62.7% in the other breast cancer group [*Dent et al*., 2007]. There is a contraversial data reported about the frequency of axillary lymph node metastases at the time of diagnosis in the triple-negative breast cancer group [*Reis-Filho et al*., 2008].

Several studies demonstrated no statistically significant difference in lymph node positivity between triple-negative breast cancer group and other breast cancer groups [*Rakha et al*., 2009]. In contrast, other studies published a higher proportion of positive lymph nodes at the time of diagnosis in the triple-negative breast cancer group compared to other breast cancer group [*Dent et al*., 2007]. Our study similary to *Tischkowitz et al*., demostrated a lower rate of lymph node positive breast cancer patients in the triple-negative group compared to luminal A breast cancer group [*Tischkowitz et al.,* 2007]. Furthermore, there was no significant correlation between tumor size and positive lymph node status in the triple-negative breast cancer patients with tumors smaller than 5 cm in diameter. Similar data were also reported by several previous studies [*Dent et al*., 2007; *Foulkes et al*., 2012]. In our study, in the univariate analysis T3/T4 stage versus T1/T2 and N2/N3 versus N0/N1 status showed weak positive predictive value of worse distant recurrence-free survival. However, T stage and lymph node status failed to show predictive value of breast cancer-specific survival in the univariate analysis and distant recurrence-free and breast cancer-specific survival in the multivariate analysis. Interestingly, *Dent et al*., reported no association of tumor size with distant recurrence and breast cancer-specific survival in the basal-like breast cancer group. However, there was a transient negative effect of size of the tumor on distant recurrence in the basal-like breast cancer group in a short period of time after the diagnosis. After 10 years survival rates were similar for patients with small and large basal-like tumors [*Dent et al.,* 2009]. Therefore, in our study weak correlation between increasing tumor size and worse distant recurrence-free survival could be explained with relatively short median follow-up period of 36 months in the *BRCA1* negative triple-negative breast cancer group. According to our data triple-negative and luminal B HER2 negative breast cancer patients were less likely to be diagnosed in stage I than luminal A breast cancer patients (38.5%, 41.9% and 70.9%, respectively; $P < 0.0001$). A

statistically significantly higher proportion of triple-negative and luminal B HER2 negative breast cancer patients were diagnosed in stage III compared to luminal A breast cancer patients (38.5%, 32.1% and 15.1%, respectively; $P < 0.0001$). Similar results were presented by *Bauer et al.*, there triple-negative breast cancer patients were significantly more likely to be diagnosed at more advanced stages [*Bauer et al.*, 2007].

According to our study results, there was a tendency of increased risk of LRR in the triple-negative breast cancer group compared to luminal A and luminal B HER2 negative breast cancer groups, but these difference didn't reach statistical significance. In our study LRR rate in the triple-negative breast cancer group is lower than reported in other previous studies (3.9% versus 8.8–21% in other studies) [*Dent et al*., 2007; *Voduc et al* ., 2010; *Ho et al*., 2012]. The median time to LRR was shoter in the triple-negative breast cancer group compared to luminal A breast cancer group (5.7 versus 27.5 months, respectively). *Dent et al*., reported similar results where was no statistically significant difference in the LRR rate between triple-negative and other breast cancer group with significantly shorter mean time to LRR in the triple-negative breast cancer group compared to other breast cancer group [*Dent et al*., 2007]. A study by *Lowery et al.*, performed a meta-analysis of 15 studies there a total of 12.592 patients who underwent either BCT (N = 7.174) or mastectomy (N = 5.418) were included. They concluded that triple-negative breast cancer patients have an increased risk of LRR regardless of the type of surgery (BCT (RR = 0.49; 95% Cl: 0.33–0.73) versus mastectomy (RR = 0.66; 95% Cl: 0.53–0.83)) compared to luminal breast cancer patients. In our study 36 (46%) triple-negative breast cancer patients underwent breast-conserving therapy and 42 (54%) patients underwent mastectomy. 2 (66.7%) triple-negative breast cancer patients in the mastectomy group and 1 (33.3%) patient in the breast-conserving therapy group experienced LRR. A number of studies reported a significant improvement of locoregional control after more aggressive systemic

treatment in the ER-negative and HER2-positive breast cancer patients [*Fisher et al*., 1996; *Romond et al*., 2005]. Therefore, in our study a relatively low rate of LRR in the triple-negative breast cancer group with no statistically significant difference compared to luminal A breast cancer group could be partially explained by high propotion of patients who received systemic therapy (69 (88.5%)). A higher proportion of triple-negative breast cancer patients experienced distant recurrence compared to luminal A and luminal B HER2 negative breast cancer patients (28.2% versus 1.2% versus 5.4%, respectively; $P < 0.0001$). The DRFS was shoter in the triple-negative breast cancer group compared to luminal A and luminal B HER2 negative breast cancer groups (32.2 months versus 45 months and versus 42 months, respectively). There was a tendency to visceral metastases in the triple-negative breast cancer group compared to luminal A and luminal B HER2 negative breast cancer groups. Similar results were published by number of previous studies, where triple-negative breast cancer group showed increased likelihood of distant recurrence and was associated with increased risk of visceral metastases [*Dent et al*., 2007; *Liedtke et al*., 2008]. In our study triple-negative breast cancer patients had a significantly lower breast cancer-specific survival compared to luminal A and luminal B HER2 negative breast cancer patients (76.9% versus 98.8% versus 94.6%, respectively; $P < 0.0001$). These results are in agreement with previously published data, where triple-negative breast cancer patients showed significantly lower overall and breast cancer –specific survival compared to luminal A and luminal B HER2 negative breast cancer patients [*Dent et al*., 2007; *Liedtke et al*., 2008].

Although, our median follow-up period of 3 years is relatively short, previous studies reported that the risk of any recurrence in the triple-negative breast cancer group is high in first 1–3 years after diagnosis with majorityof breast cancer-related events occuring within the first 5 years [*Dent et al*., 2007;

Liedtke et al., 2008]. Thus, our follow-up period is quite adequate to distinquish the majority of treatment outcomes.

4.2. Triple-negative germline founder *BRCA1* mutations positive and negative breast cancers

The evidence from this study suggests that triple-negative breast cancer patients with germline *BRCA1* founder mutations (4153delA and 5382insC) and no evidence of ovarian cancer or other cancers in advanced stage have statistically significantly improved prognosis relative to non-carriers. We showed that positive *BRCA1* mutation status statistically significantly reduce the risk of distant recurrence and breast cancer-specific death. After adjustment for age, T stage, nodal status, stage, surgery, radiation therapy and chemotherapy positive *BRCA1* mutation status was independent prognostic factor for lower distant recurrence risk.

So far there are only few studies published concerning the prognostic role of positive *BRCA1* mutation status in the triple-negative breast cancer subtype. Contrary to our work results, these studies showed no significant difference in survival outcomes between triple-negative *BRCA1* mutation carriers and non-carriers [*Lee et al*., 2010; *Bayraktar et al*., 2011; *Gonzalez-Angulo et al*., 2011].

Lee et al., reported similar 5-years breast cancer specific and overall survival rates in both triple-negative *BRCA1* mutation carriers and non-carriers treated with alkylating chemotherapy (HR = 0.64; P = 0.25) [*Lee et al*., 2010]. *Gonzalez-Angulo et al*., reported superior recurrence-free survival in the triple-negative *BRCA1* mutation positive patients compared to *BRCA1* mutation negative triple-negative breast cancer patients treated with surgery and anthracycline-taxane chemotherapy (P = 0.031), but failed to demonstrate significant diference in overall survival (P = 0.225) [*Gonzalez-Angulo et al*.,

2011]. Similarly, *Bayraktar et al*., showed no statistically significant difference in 5 year-overall survival rates between *BRCA1/2* mutation carriers and non-carriers [*Bayraktar et al*., 2011].

However, these studies have had some limitations: the cut-off levels for ER and PR negativity were not specified [*Lee et al*., 2010] or defined as nuclear staining ≤ 10% [*Bayraktar et al*., 2011], both groups were not homogenized by received chemotherapy regimens [*Gonzalez-Angulo et al*., 2011], missing information about accomponying cancers [*Gonzalez-Angulo et al*., 2011] or patients with previous ovarian cancer included in the study%[*Bayraktar et al*., 2011], no breast cancer-specific survival were evaluated [*Gonzalez-Angulo et al*., 2011] and prognostic significance of separate *BRCA1* mutations were not evaluated [*Lee et al*., 2010; *Bayraktar et al*., 2011; *Gonzalez-Angulo et al*., 2011].

In our study, the adoption of strict criteria of ASCO / CAP guideline recommendations for immunohistochemical testing of ER and PR (ER or PR are considered negative if < 1% of tumor cell nuclei are immunoreactive) to identify triple-negative breast cancer phenotype significantly diminished the number of triple-negative breast cancer cases included in the study [*Hammond et al*., 2010].

Although, our study data were based on relatively small number of cases, both groups were homogenious by tumor grade, the median tumor size, T stage, stage of the disease, received chemotherapy and only patients with two common germline founder *BRCA1* mutations (4153delA and 5382insC) were included in the study.

In previous studies, a different survival outcomes for various *BRCA1* germline mutations' variants were reported [*Plakhins et al*., 2011]. *Plakhins et al*., reported a worse overall survival for breast cancer patients with positive *BRCA1* 4153delA mutation compared with 5382insC [*Plakhins et al*., 2011].

One more principal advantage of our study was that patients with ovarian cancer and other cancers in advanced stages were not included in the study population. Inspite of significantly better prognosis for *BRCA1* mutation carriers with ovarian cancer reported by *Bolton et al*., 5-years overall survival for these patients was only 46% [*Bolton et al.*, 2012]. In all patients excluded from the study ovarian cancer was diagnosed in advanced stages (IIIC or IV) and all patients died from disseminated ovarian cancer within median period of 28.5 (range 6–45 months) months from the time of diagnosis. The risk of ovarian cancer is, approximately, 3 % by the age of 40 years and 54% by the age of 60 years [*Easton et al*., 1995; *Finch et al*., 2012]. Several studies have shown a significant heterogeneity of breast and/ ovarian cancer prevalence among different mutations of *BRCA1* gene [*Easton et al*., 1995; *Plakhins et al*., 2011]. The prophylactic salpingo-oophorectomy reduces the penetrance of ovarian/ fallopian tube cancer by 75–96% and breast cancer by 53–56 % [*Finch et al*., 2012] in patients with *BRCA1* mutation. In addition, *Bayraktar et al*., showed that bilateral prophylactic oophorectomy allow statistically significantly reduce the risk for death in patients with triple-negative breast cancer (HR = 0.01; 95% CI:0.01–0.69; P < 0.02) [*Bayraktar et al*., 2011].

A better breast-cancer specific survival in the triple-negative breast cancer *BRCA1* mutation carriers compared to non-carriers could be explained by biological differences and/ or higher sensitivity to chemotherapy. In our study *BRCA1* mutation carriers were statistically significantly younger than non-carriers (48.8 years versus 54.4 years, respectively; P < 0.034). Similar results to our study were published by number of studies [*Lee et al*., 2011; *Gonzalez-Angulo et al*., 2011]. *Lee et al*., reported a median age at diagnosis 39.9 (range, 28.1–73.4) years in the triple-negative *BRCA1* mutation carriers group compared to 51.3 (range, 28.1–75.6) years in the *BRCA1* mutation non-carriers group(P < 0.001) [*Lee et al.*, 2011]. *Gonzalez-Angulo et al*., showed a median age at diagnosis 45 (range, 27–61) years in the triple-negative *BRCA1*

mutation carriers compared to 53 (range, 28–83) years in the *BRCA1* mutation non-carriers group(P < 0.0051) [*Gonzalez-Angulo et al*., 2011]. In our study, there was no statistically significant difference in median age at diagnosis between triple-negative *BRCA1* mutation carriers and *BRCA1* mutation non-carriers younger than 50 years (40.1 years versus 40.2 years, respectively; P = 0.953). Similar to our study data, *Bayraktar et al*., showed no statistically significant difference in median age at diagnosis between triple-negative *BRCA1* mutation carriers and non-carriers younger than 50 years (41years (range, 22–71 years versus 40 years (range, 21–74 years), respectively; P = 0.74) [*Bayraktar et al*., 2011].

In the *BRCA1* carriers group compared to non-carriers group a higher proportion of node negative breast cancers were observed (65.8% versus 37.2%; P < 0.004) with no statistically significant difference in T stage between two groups. Number of studies reported a similar data about the prevailing node-negativity in *BRCA1* mutation carriers, even in those patients with large tumor size. These could be characterized as one of the main biological features of *BRCA1* carriers [*Eisinger et al*., 1998; *Chappuis et al.*, 2000; *Foulkes et al*., 2003; *Brekelmans et al*., 2005]. Tumor size and nodal status are independent prognostic factors for survival outcomes. In the univariate analysis T stage and nodal status as well as clinical stage were a strong predictors of outcomes. In the multivariate analysis this factors fail to predict outcomes in both triple-negative breast cancer *BRCA1* mutation carriers and non-carriers, may be due to relatively small study population. Similar to our study results, *Brekelmans et al*., showed that both tumor size and nodal status have a strong prognostic impact on survival outcomes in the *BRCA1* mutation carriers. However, positive lymph node status was a weak prognoctic factor and had a significant impact on survival outcomes only if more than four lymph nodes were positive [*Brekelmans et al*., 2006]. In our study, there was no correlation between increasing tumor size and lymph node status among patients with tumors of <5

cm both in the triple-negative breast cancer *BRCA1* mutation carriers and non-carriers. In contrast, *Brekelmans et al*., showed strong correlation between tumor size and lymph node status [*Brekelmans et al*., 2006]. However, *Foulkes et al*., demonstrated no association between increasing tumor size and lymph node positivity in *BRCA1* mutation positive breast cancers. In addition, tumor size and nodal status were also a weak predictors of outcomes in *BRCA1* mutation carriers. The author proposed that this phenomenon could be associated with hematoganeous spread of these tumors [*Foulkes et al.,* 2003; *Foulkes et al*., 2004].

A gene-expression signatures identified by *Hedenfalk et al*., allowed to differentiate between *BRCA1*-related and sporadic breast cancers. All of 7 *BRCA1*-related tumors and 14 of 15 sporadic breast tumors were precise identified. Interestingly, that one sporadic breast cancer misclassified as *BRCA1*-related had a low level of BRCA1 expression due to *BRCA1* gene hypermethylation [*Hedenfalk et al.,* 2001]. *Van't Veer et al*., identified 100 gene set that allowed to subclassify ER-negative breast tumors into *BRCA1*-related and sporadic breast cancers [*van't Veer et al*., 2001]. In contrast, gene expression profile analysis performed by *Sorlie et al*., showed that *BRCA1*-related tumors clustered together with basal-like breast cancers [*Sorlie et al*., 2003].

A higher chemosensitivity for *BRCA1* mutation positive breast cancer patients compared to sporadic breast cancer patients was proposed in previous studies [*Robson et al*., 2004; *Rennert et al*., 2007]. *Rennert et al*., reported a significantly better 10-year survival rates for *BRCA1* mutation carriers than for non-carriers, who were treated with chemotherapy and no difference in survival rates among patients who didn't receive chemotherapy [*Rennert et al*., 2007]. *Robson et al*., showed better survival outcomes for *BRCA1* mutation carriers, who received adjuvant chemotherapy compared to *BRCA1* mutation carriers, who received no adjuvant chemotherapy [*Robson et al*., 2004]. In our study

94.7% of patients in the *BRCA1* mutation carriers group and 85.9% of patients in the *BRCA1* mutation non-carriers group received chemotherapy (P = 0.30). Chemotherapy versus no chemotherapy both in the triple-negative *BRCA1* carriers and non-carriers failed to show statistically significant impact on distant recurrence-free and breast cancer-specific survival in the univariate and multivariate analyses. These results could be explained by a small number of patients in the triple-negative *BRCA1* carriers group (2(5.6%)) and *BRCA1* non-carriers group (9(11.5%)) who received no chemotherapy. Recently, similar results to our study was published by *Narod et al*., where 379 stage I breast cancer patients with *BRCA1* mutation carriers or patients with *BRCA1* mutation detected in a close blood relatives were included. 267 of 379 patients received chemotherapy. There was a statistically unsignificant tend towards a better 15-years survival in women, who received chemotherapy compared to those with no chemotherapy (89.4% versus 73.1%, respectively; P < 0.008). The difference in 15-years survival was statistically significant only in women with ER-negative breast tumors(P = 0.02) [*Narod et al*., 2013].

There is a lack of prospective randomized trials comparing different chemotherapy regimens among *BRCA1* mutation carriers. According to the last ESMO clinical practice quidelines for management of *BRCA* positive breast cancer patients, decisions about the chemotherapy in the *BRCA1* mutation carriers should be based on the same standard prognostic features as in the patients with wild-type and standard chemotherapy regimens are recommended [*Balmana et al*., 2010].

4.3. The frequency and prognostic significance of *TP53* sporadic mutations in the triple-negative breast cancer *BRCA1* carriers and non-carriers

The frequency of *TP53* sporadic mutations varies across the studies and is mainly dependent on the techniques used to detect the mutation, screened

coding region of the *TP53* gene, definitions and methods used to identify basal-like/triple-negative breast cancers, number of tumor samples analyzed and differences in quality of DNA extracted from formaline-fixed paraffin-embedded (FFPE) or fresh-frozen tissue. The differences in assay techniques and study designs in other researches embarrass the interpretation and analysis of our results.

The majority of studies used IHC to detect mutant p53 protein accumulation in the cancer cell nuclei, because it is an inexpensive and easy to use in routine practice. However, the lower sensitivity and specificity of this method has been reported compared to cDNA sequencing method with relatively high false positive and false negative results and lower prognostic value ot this method [*Sjorgen et al*., 1996; *Norberg et al*., 1998; *Manie et al*., 2009]. *Chaeng et al*., reported a 40.2% (13 of 32 cases) of p53 expression in the triple-negative breast cancer group defined by ER/PR and HER2 IHC statining. However, there was no difference in the p53 expression rate between triple-negative and non-triple-negative breast cancer groups (40.2% versus 42.7%) [*Chaeng et al*., 2009]. *Ryu et al*., showed similar results with 37.1% of triple-negative breast cancers overexpressing p53. The triple-negative breast cancers in this study was defined based on IHC assay with cut-off levels for ER and PR negativity < 10% of positive nuclear staining [*Ryu et al.,* 2012]. In contrast, *Ryu et al*., demonstrated a higher p53 expression rate (58.5%) in the triple-negative breast cancer group where 33 of 94 (35.1%) patients had a basal-like breast cancer (defined by IHC staining for ER, PR, HER, CK 5/6, EGFR) and 61 (64.9%) patients had a non-basal-like triple-negative breast cancer. However, there was no statistically significant difference in p53 overexpression between basal-like and non-basal-like triple-negative breast cancer patients (57.6% versus 59.0, respectively; P = 0.532) [*Ryu et al*., 2012].

Manie et al., where 89% (34 of 38 cases) *TP53* sporadic mutations were identified in the group of *BRCA1* germline negative basal-like breast

cancers and 83% (29 of 35 cases) *TP53* sporadic mutations were identified in the group of *BRCA1* germline positive basal-like breast cancers using direct sequencing of the exons 2-11 coding regions in each sample [*Manie et al*., 2009].

In contrast, in our study 40% (22 of 55) of triple-negative *BRCA1* gemline mutations negative breast cancers harboured at least one *TP53* alternation. Our results could be explained by lower proportion of true basal-like breast cancers in the group of triple-negative breast cancers defined by IHC assay. The previous studies demostrated that approximately 40–80% of all triple-negative breast cancers are basal-like [*Carey et al.*, 2007; *Rakha et al.*, 2009; *Cheang et al*., 2008].

Interestingly, that in our study there was also no statistically significant difference in the frequency of the *TP53* sporadic mutations in the triple-negative *BRCA1* germline mutations positive and negative groups (4 of 11(36.4%) cases versus 22 of 55(40%) cases, respectively; P = 0.84).

In addition, in our study only exons 5-8 were screened for sporadic *TP53* mutations. However, it has been proposed that approximately 90% of mutations occur this region [*Pharoah et al*., 1999].

In contrast, in our study we used real-time PCR with subsequent HRM and bidirectional direct DNA sequencing performed on RT-PCR-positive specimens. RT-PCR with subsequent HRM used as a scanning methodology diminishes the amount of sequencing required, therefore, optimizing the process of the *TP53* mutations detection and making the process less time-consuming and more cost-effective [*Krypuy et al*., 2007]. *Krypuy et al*., reported a 100% sensitivity and 100% positive predictive value for the RT-PCR with subsequent HRM [*Krypuy et al*., 2007].

There are no studies published so far where sporadic *TP53* mutations prognostic significance in the triple-negative/basal-like breast cancer have been evaluated. However, there are few studies that evaluated the prognostic role of

p53 overexpression in the triple-negative breast cancer [*Chae et al*., 2009; *Jung et al*., 2011; *Biganzoli et al*., 2011; *Ruy et al*., 2012]. *Ryu et al*., reported that p53 overexpression have no prognostic value in the triple-negative breast cancer group. However, in this study authors used a cut-off levels for ER/PR negativity of less than < 10% [*Ryu et al*., 2012]. In contrast, *Jung et al*., showed a statistically significant negative impact on disease-free survival in the lymph node negative triple-negative breast cancer group [*Jung et al*., 2011]. Others showed similar results with statistically significant difference in survival outcomes by p53 protein expression in the triple-negative breast cancer group, but not in the non-triple-negative breast cancer group [*Chae et al*., 2009]. In addition, it was reported that in the triple-negative breast cancer group p53 protein overexpression was associated with previolusly defined `basal-like` cluster and associated with worse overall and event-free survival [*Biganzoli et al*., 2011]. cDNA-based sequencing method provides a more precise prognostic information than IHC [*Sjorgen et al*., 1996; *Norberg et al*., 1998].

Our study showed that positive status for deleterious *TP53* mutations is associated with significantly worse distant recurrence-free survival (P < 0.036). There was an insiginificant tendency towards worse breast cancer-specific survival in the triple negative *TP53* deleterious mutations positive group compared to negative group (80% versus 77.3%; P = 0.65). Very similar findings with our study was published *by Fernandez-Cuesta et al*. Authors concluded that *TP53* positive status is not associated with worse survival outcomes in breast cancer patients. Only positive truncating *TP53* mutations status was a significant prognostic factor for increased recurrence risk in the patients group treated with anthracycline or/and taxane-based chemotherapy (HR = 3.21; 95% CI:1.740-5.935; P<0.0002) [*Fernandez-Cuesta et al*., 2012]. Number of studies demostrated that tumors positive for *TP53* mutations/ p53 overexpressing show worse survival outcomes compared to wild-type after treatment with anthracycline-based chemotherapy [*Aas et al.,* 1996; *Chae et al*.,

2009]. In our study 81.1% of triple-negative breast cancer patients received anthracycline-based chemotherapy. However, in this patients group positive *TP53* status or *TP53* truncating mutations showed no statistically significant impact on distant recurrence-free or breast cancer-specific survival. Interestingly, that Betheau et al., reported that positive *TP53* status and basal-like breast cancer was an independent predictors of a pCR. Patients, who achieved pCR had a favorable prognosis and those with residual disease positive *TP53* status predicted worse survival outcomes [*Bertheau et al*., 2007].

5. CONCLUSIONS

1. Sporadic triple-negative breast cancers are characterized by younger age at diagnosis, higher expression of ki-67, larger tumor size, higher proportion of poorly differentiated tumors, medullary breast cancers and tumors in an advanced stages, higher distant recurrence rate and worse breast cancer-specific survival compared to luminal A breast cancers.
2. Sporadic triple-negative breast cancer group is not associated with significantly higher LRR rate compared to luminal A sporadic breast cancer group and the type of surgery do not statistically significantly impact distant recurrence-free survival and breast cancer specific survival in the triple-negative sporadic breast cancer group .
3. Triple-negative germline *BRCA1* founder mutations carriers are associated with axillary lymph node negativity and have statistically significantly improved distant recurrence-free survival and breast cancer-specific survival compared to non-carriers.
4. Positive *BRCA1* mutation status is the independent prognostic factor for lower distant recurrence-free survival risk.
5. Sporadic mutations in the *TP53* gene are associated with worse distant recurrence-free survival in the triple-negative breast cancer

6. PRACTICAL RECOMMENDATIONS

1. Positive germline *BRCA1* founder mutations (4153delA and 5382insC) status could be used as an independent prognostic factor for more favourable prognosis in the triple-negative breast cancer group.
2. We recommend to test all triple-negative breast cancer patients for *BRCA1* founder mutations (4153delA and 5382insC)
3. Sporadic *TP53* mutations detection could be recommended to identify women with worse survival outcomes in the triple-negative breast cancer group

7. LIST OF PUBLICATIONS AND REPORTS ON THE STUDY THEME

SCIENTIFIC PUBLICATIONS-(4)

1. **Maksimenko J.**, Irmejs A., Nakazawa-Miklasevica M., Melbarde-Gorkusa I., Trofimovics G., Miklasevics E., Gardovslis J. The prognostic role of *BRCA1* mutation in patients with triple-negative breast cancer. Oncology letters 2014, 7(1):278–284.

2. **Maksimenko J.**, Irmejs A., Trofimovics G., Miklasevics E. Breast-conserving surgery in early-stage triple-negative breast cancer: is there a higher risk of locoregional recurrence? Acta Chirurgica Latviensis 2013, (1):11–14.

3. **Maksimenko J**., Liepniece-Karele I., Irmejs A., Trofimovics G. The clinicopathologic characteristics and prognostic significance of triple-negative breast cancer phenotype. Acta Chirurgica Latviensis 2011, 11(1):16–20.

4. Liepniece-Karele I., **Maksimenko J.**,Trofimovics G. Basal CK19 expression in triple-negative breast cancer. Collection of Scientific papers of Riga Stradins University 2011, P. 88–93.

INTERNATIONAL THESES AND REPORTS-(10)

1. Thesis. **Maksimenko J**., Irmejs A., Miklasevics E., Trofimovics G. *BRCA1* mutation in the Triple-negative Breast Cancer Group. Hereditary Cancer in Clinical Practice 2012, 10(Suppl 4): A15.

2. Thesis and poster presentation. **Maksimenko J**., Irmejs A., Trofimovics G., Miklasevics E. *BRCA1* mutation and its prognostic role in the triple-negative breast cancer(TNBC) subtype. 13th St.Gallen International Breast Conference. 13.–16. marts, 2013, St.Gallen, Šveice. Abstract book.

3. Thesis and poster presentation. **Maksimenko J**., Irmejs A., Trofimovičs G., Miklaševičs E. Breast-conserving surgery in early-stage triple-negative breast

cancer: is there a higher risk of locoregional recurrence? 32nd Congress of the European Society of Surgical Oncology. 19.–21. septembris, 2012, Valensija, Spanija. Abstract book, P. 262.

4. Thesis and poster presentation. **Maksimenko J**., Irmejs A.,Trofimovičs G., Miklaševičs E. Lobular Histology Shows Tendency of Higher Risk of Involved Margins After First Breast-Conserving Surgery. 8th European Breast Cancer Conference, Vīne, Austrija. Abstract book. P. 609.

5. Thesis and poster presentation. **Maksimenko J**., Reteris M., Irmejs A.,Trofimovičs G.Operable breast cancer in very young women, is there a difference? Breast cancer in young women conference. 8.–10. novembris, Dublina, Īrija. The Breast 2012, 21 (Suppl 1). P. 030.

6. Thesis and poster presentation. **Maksimenko J**., Irmejs A., Trofimovičs G., Miklaševičs E. Prophylactic mastectomy in patients with hereditary breast and ovarian cancer syndrome. 7th Congress of Baltic Association of Surgeons. 27.–29.septembris, 2012. Rīga, Latvija. Abstract book.

7. Thesis and poster presentation. Reteris M., **Maksimenko J**., Irmejs A., Trofimovičs G. Breast cancer in young women under 35 years of age. 7th Congress of Baltic Association of Surgeons. 27.–29.septembris, 2012. Rīga, Latvija. Abstract book.

8. Thesis and poster presentation. **Maksimenko J**., Liepniece-Karele I., Kudaba I. The analysis of triple-negative breast cancer in th Riga Eastern Clinical University Hospital. 5th Baltic Congress of Oncology. 13.–15.maijs, 2010. Rīga, Latvija. Abstract book, –44 lpp.

9. Thesis and oral presentation. **Maksimenko J**., Irmejs A., Trofimovičs G., Miklaševičs E. Is a triple-negative breast cancer a contraindication for breast-conserving surgery? 7th Congress of Baltic Association of Surgeons. 27.–29.septembris, 2012. Rīga, Latvija. Abstract book.

10. Oral presentation. **Maksimenko J**., Trofimovičs G., Irmejs A., Miklaševičs E., Melbarde-Gorkuša I. The prevalence of *BRCA1* mutation in triple-negative

breast cancer. Eurasian Forum on Breast Cancer. 3.–4. septembris, 2011, Maskava, Krievija.

LOCAL THESES AND PRESENTATIONS -(4)

1. Thesis and oral presentation. **Maksimenko J**., Irmejs A.,Trofimovičs G., Miklaševičs E. The prognostic role of *BRCA1* mutation in patients with triple-negative breast cancer. Zinātniskā konference, Rīgas Stradiņa universitāte. 21.–22.marts, 2013. Rīga, Latvija. Tēžu grāmata. – 244 lpp.
2. Thesis and oral presentation. **Maksimenko J**., Irmejs A.,Trofimovičs G., Miklaševics E. Better prognosis for *BRCA1* mutation carriers among triple-negative breast cancers. Zinātniskā konference, Rīgas Stradiņa Universitāte.29.-30.marts, 2012. Rīga, Latvija. Tēžu grāmata. – 250 lpp.
3. Thesis and poster presentation. **Maksimenko J**., Reteris M., Irmejs A.,Trofimovics G. Operable breast cancer in very young women (< 35 years), is there a difference? 9. Zinātniskā konference, Rīgas Stradiņa Universitāte. 29.–30.marts, 2012. Rīga, Latvija. Tēžu grāmata. – 251 lpp.
4. Thesis and poster presentation. Liepniece-Karale I., **Maksimenko J**. CK 19 imūnhistoķīmija trīskārši negatīva krūts vēža gadījumā. Zinātniskā konference, Rīgas Stradiņa universitāte. 18.–19.marts, 2010. Rīga, Latvija. Tēžu grāmata. – 265 lpp.

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