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

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ANNOTATION

Triple-negative breast cancer is a heterogeneous clinicopathological entity defined as an oestrogen (ER), progesterone (PR) and HER2/neu negative breast cancer that is characterized by agressive clinical behavior with high recurrence and deaths rate, especially in the first five years after diagnosis. In previous studies a strong relationship between *BRCA1* mutation-associated tumors and triple-negative breast cancers has been manifested, approximately 57–88% of all *BRCA1*-related tumours are triple-negative or/and basal-like. 60–88% of triple-negative / basal-like or *BRCA1*-related breast cancers have *TP53* mutations. However, inconsistent and limited data are available regardless the prognostic and predictive implication of *BRCA1* germline mutations and *TP53* sporadic mutations in the triple-negative breast cancer subgroup.

Therefore, the aim of our study was 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.

The study was designed as a combined prospective-retrospective cohort.

In the prospective part of the study invasive breast cancer patients were tested for germline *BRCA1* founder mutations and clinical data were prospectively obtained. In the retrospective part of the study an analysis of somatic *TP53* mutations was retrospectively performed in the triple-negative breast cancer group and correlation between somatic *TP53* mutations and clinical outcomes were retrospectively analysed.

The evidence from our study suggests that germline *BRCA1* founder mutations (4153delA and 5382insC) carriers 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 and is an independent prognostic factor for lower distant recurrence risk. In addition we showed that sporadic deleterious *TP53* mutations could be used as prognostic factor of worse distant recurrence-free survival in the triple-negative breast cancer group.

ANOTĀCIJA

Trīskārši negatīvs krūts vēzis ir heterogēna klīniskā patoloģija, kas definēta kā estrogēna (ER), progesterona (PR) un HER2/neu negatīva krūts vēža apakšgrupa. Trīskārši negatīvs krūts vēzis raksturojas ar agresīvu klīnisku gaitu ar augstu recidīvu un nāves gadījumu skaitu, īpaši pirmo piecu gadu laikā pēc diagnozes noteikšanas.

Iepriekš veiktajos pētījumos ir novērota izteikta sakarība starp *BRCA1* mutāciju saistītiem audzējiem un trīskārši negatīviem krūts vēžiem, apmērām 57–88% no visiem ar *BRCA1* saistītiem audzējiem ir trīskārši negatīvi un/vai bazāli krūts vēži.

60–88% trīskārši negatīvu / bazālu krūts vēžu tiek konstatētas *TP53* somatiskas mutācijas. Agrāk veiktajos pētījumos ir iegūti pretrunīgi un ierobežoti rezultāti par prognostisku un predikatīvu pārmantotu *BRCA1* mutāciju un *TP53* somatisku mutāciju nozīmi trīskārši negatīva krūts vēža grupā.

Tādējādi, mūsu pētījuma mērķis ir noskaidrot divu pārmantotu *BRCA1* ciltstēva (4153delA and 5382insC) mutāciju un *TP53* somatisku mutāciju prognostisku nozīmi pacientēm ar trīskārši negatīvu krūts vēzi.

Pētījums pēc uzbūves ir kombinēts (prospektīvs / retrospektīvs).

Pētījuma prospektīvā fāzē pacientes ar invazīvu krūts vēzi tika testētas uz pārmantotām *BRCA1* ciltstēva (4153delA and 5382insC) mutācijām un klīniskie dati tika prospektīvi apkopoti. Pētījuma retrospektīvā fāzē trīskārši negatīva krūts vēža pacientēm tika noteiktas *TP53* somatiskas mutācijas un novērtēta to saistība ar klīniskiem iznākumiem.

Mūsu petījuma rezultāti liecina par statistiski nozīmīgi labāku prognozi pārmantotu *BRCA1* ciltstēva mutāciju pozitīvām trīskārši negatīva krūts vēža pacientēm salīdzinot ar *BRCA1* ciltstēva mutāciju negatīvām trīskārši negatīva krūts vēža pacientēm. Pozitīvs *BRCA1* mutācijas statuss statistiski nozīmīgi samazina distāla recidīva risku un risku nomirt no krūts vēža un ir neatkarīgs labvēlīgs bez distāla recidīva dzīvildzes prognostisks faktors. Somatiskas *TP53* mutācijas (proteīnu funkciju ietekmējošas) ir nelabvēlīgs bez distāla recidīva dzīvildzes prognostisks faktors trīskārši negatīva krūts vēža grupā.

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LIST OF ABBREVIATIONS

AC – Doxorubicin, cyclophosphamide

ATM/ATR kinases – Ataxia telangiectasia mutated/ Ataxia telangiectasia mutated and Rad – 3 related kinases

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

IL-2 – Interleukin 2

IL-7 – Interleukin 7

LRR – Locoregional recurrence

LRFS – Locoregional recurrence- free survival

LSAB2 – Labelled Streptavidin-Biotin2 System

mTOR – Mammalian target of rapamycin

NCCN – The National Comprehensive Cancer Network

PARP – Poly (adenosine diphosphate) ribose polymerases

pCR – Pathologic complete response

PI3K – Phosphoinositide 3-kinase

PMRT – Postmastectomy radiation therapy

PR – Progesterone Receptor

SNP – The Single Nucleotide Polimorphism

TAC – Docetaxel, doxorubicin, cyclophosphamide

TNF – Tumor Necrosis Factor

TP53 – Tumor protein 53

FDG/PET – Flourine-18 fluorodeoxyglucose / Positron emission tomography

1. INTRODUCTION

Triple-negative breast cancer is a heterogeneous clinicopathological entity defined as an oestrogen (ER), progesterone (PR) and HER2/neu negative breast cancer [*Dent et al*., 2007; *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 [*Livasy et al*., 2006; *Bertucci et al*., 2008; *Rakha et al*., 2009]. Triple-negative breast cancer accounts for approximately 10–20% of all breast cancer subtypes [*Bauer et al*., 2007; *Kaplan et al*., 2008]. 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 [*Rouzier et al*., 2005; *Carey et al*., 2007; *Sirohi et al*., 2008; *Hugh et al*., 2009]. 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; *Lakhani et al*., 2005; *Atchley et al*., 2008; *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 [*Young et al*., 2009; *Gonzalez-Angulo et al*., 2010; *Evans et al*., 2011; *Hartman et al*., 2012]. *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 [*Sirohi et al*., 2008; *Rottenberg et al*., 2008; *Frasci et al*., 2009; *Silver et al*., 2010; NCT00532727; NCT00861705]. 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; *El-Tamer et al.*, 2004; *Kennedy et al.*, 2002, *Robson et al.*, 2004; *Brekelmans et al.*, 2006; *Bonadona et al.*, 2007; *Rennert et al.*, 2007; *Moller et al.*, 2007; *Lee et al.*, 2010; *Hagen et al.*, 2009; *Bordeleau et al.*, 2010; *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 [*Philips et al.*, 1999; *Greenblatt et al.*, 2001; *Sorlie et al.*, 2001; *Langerod et al.*, 2007; *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; *Olive 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; *Reed et al.*, 2000; *Ferrero et al.*, 2000; *Overgaard et al.*, 2000; *Cuny et al.*, 2000; *Linderholm et al.*, 2000; *Rudolph et al.*, 2001; *Kato et al.*, 2000; *Liu et al.*, 2001; *Joensuu et al.*, 2003; *Goffin et al.*, 2003; *Bull et al.*, 2004; *Olivier et al.*, 2006; *Nakagawa et al.*, 2011; *Lee et al.*, 2011]. 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.

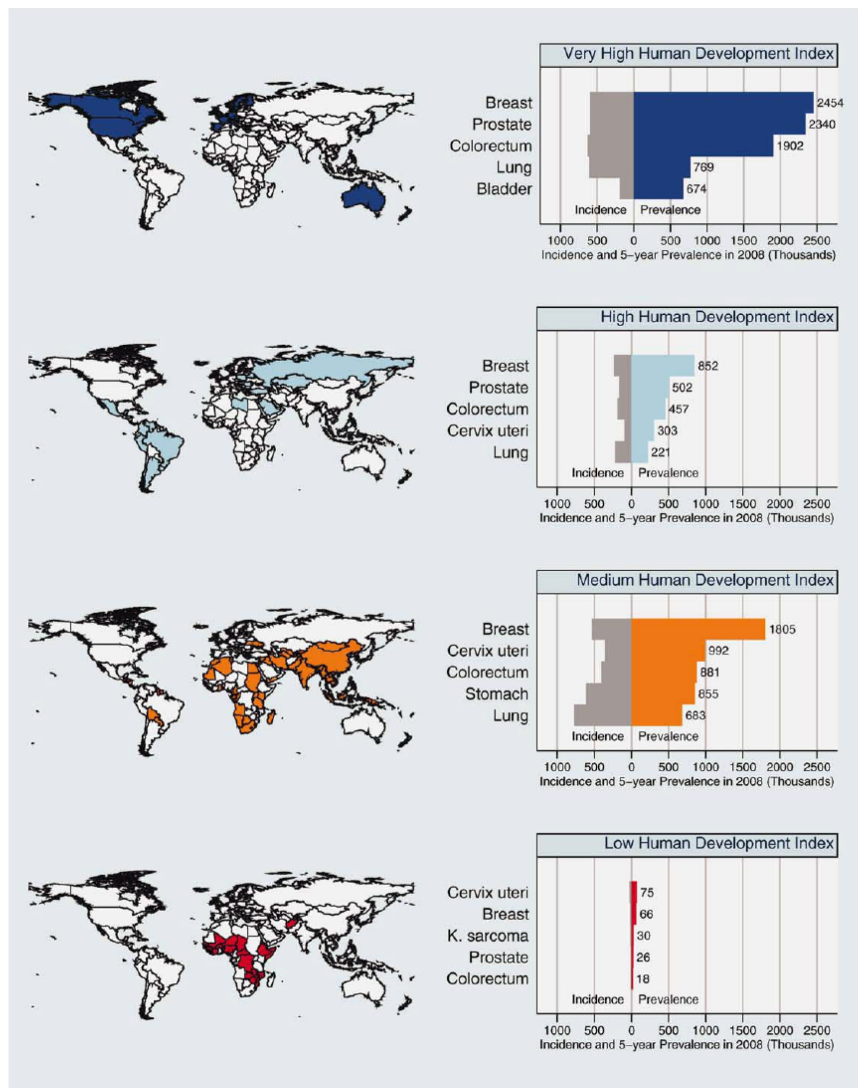
1.6. Ethics statement

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

2. LITERATURE REVIEW

2.1. Magnitude of the problem and trends over time worlwide and in Latvia

Breast cancer is the most common form of cancer and the leading cause of cancer mortality among women both in economically developed and developing countries worldwide (Figure 2.1.1.) [*Bray et al.*, 2008; *Jemal et al.*, 2011].

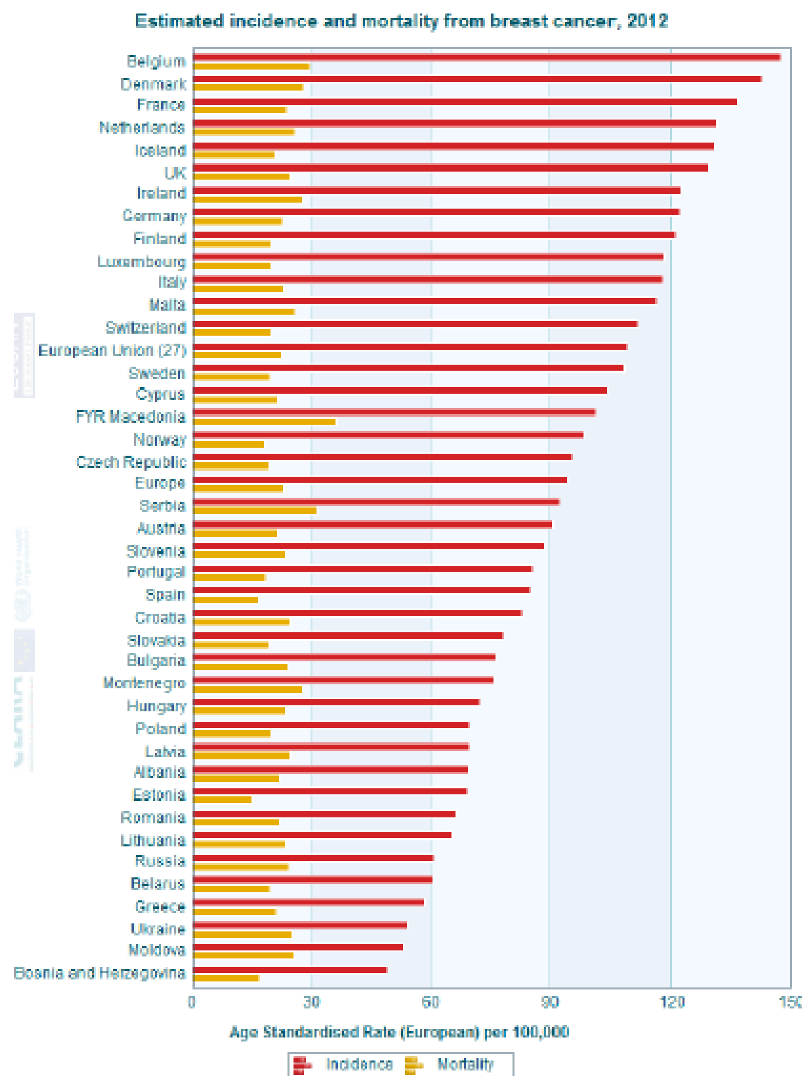


1.1.1. Figure. Incidence and 5-year prevalence (in thousands) of different sites of cancer in the adult population (both sexes) worldwide by the level of human development index (very high, high, medium, low) in 2008

Adapted from *Bray et al.*, 2013

It accounts for 23% (1.38 million) of all new cancer cases diagnosed in 2008 and 14% (458.400) of all cancer deaths worldwide [*Jemal et al.*, 2011; *World Health*

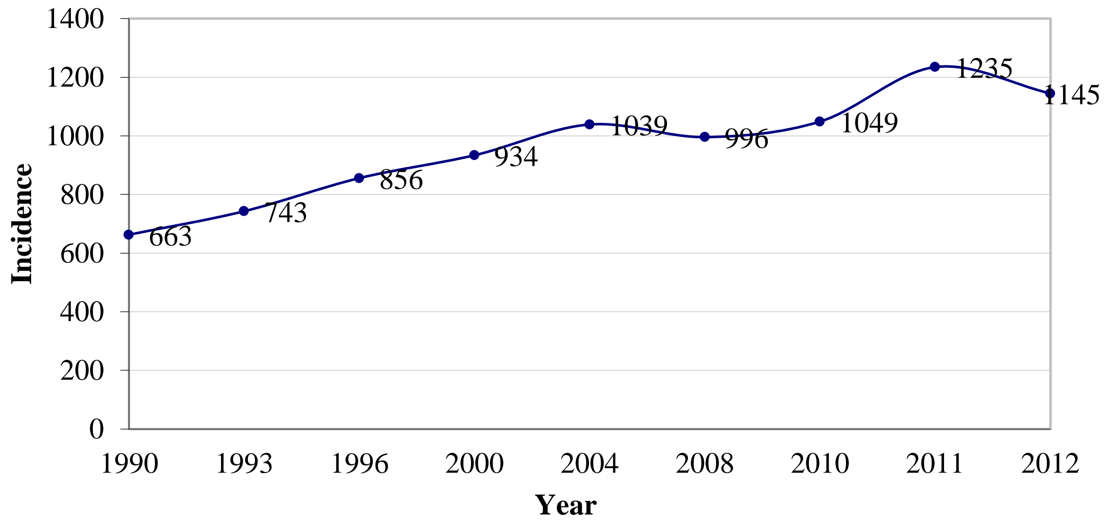
Organisation Databank]. 463.800 (94.2 per 100.000 female inhabitants (age standardised rate)) of new breast cancer cases in women were diagnosed and 131.200 (23.1 per 100.000 female inhabitants (age standardised rate)) women died from breast cancer in 2012 in Europe [*Ferlay et al.*, 2013]. In Latvia a 1145 (69.8 per 100.000 female inhabitants (age standardised rate(Europe)) women were diagnosed with breast cancer and 433 (24.5 per 100.000 female inhabitants (age standardised rate(Europe)) died from breast cancer in 2012 (Figure 2.1.2.) [*Bray et al.*, 2013; *Ferlay et al.*, 2013; *World Health Organisation Databank*].



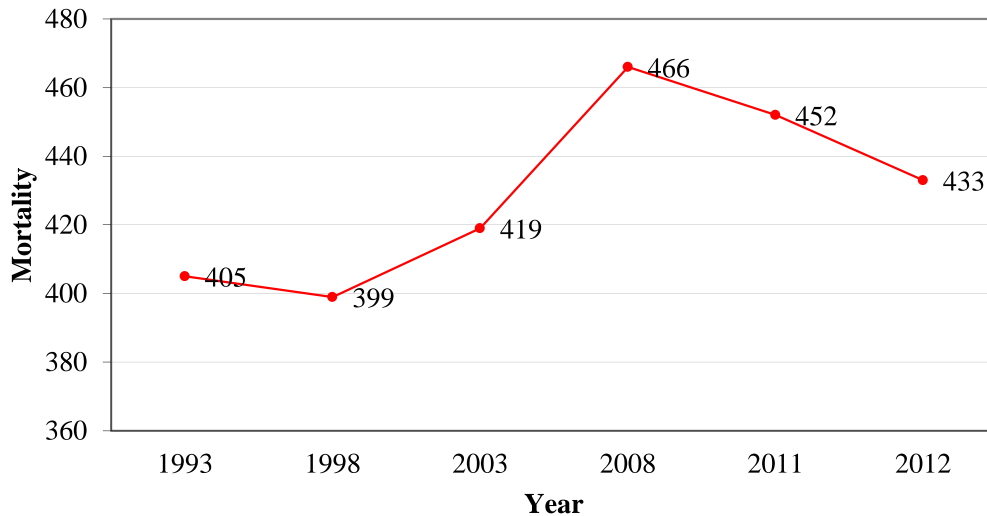
1.1.2. Figure. Incidence and mortality rate (age standardised rate(European) per 100.000 female inhabitants) of breast cancer in Europe in 2012

Adapted from http://eco.iarc.fr/eucan. [*World Health Organisation Databank*.]

During the period of 1990–2012, the incidence and mortality rates in Latvia have been rising (Figure 2.1.3.; Figure 2.1.4.) [World Health Organisation Databank; Central Statistical Bureau of Latvia].

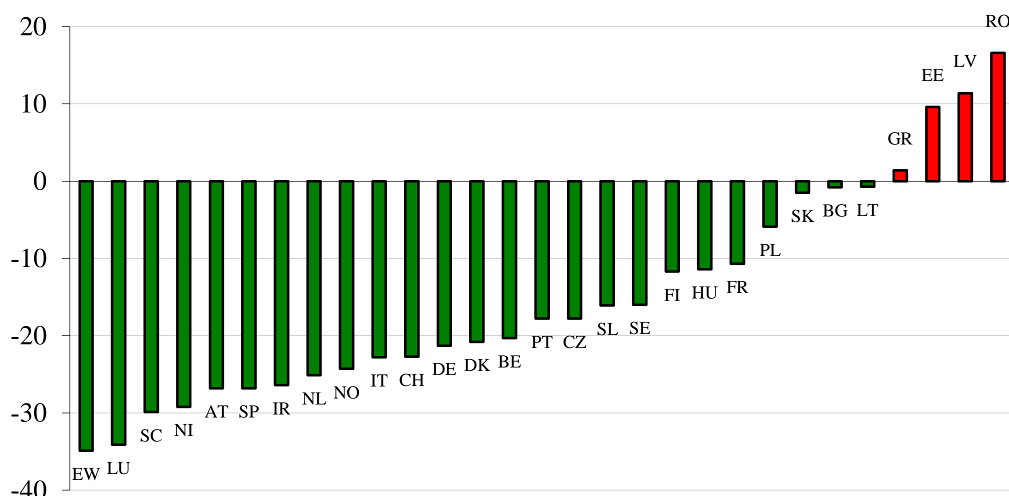


2.1.3.Figure. The incidence of female breast cancer in the adult population in the period 1990–2012 in Latvia



2.1.4. Figure. Mortality of female breast cancer patients in the adult population in the period 1990–2012 in Latvia

According to *Autier et al.*, study, breast cancer mortality in 30 European countries from 1989 to 2006 overall declined by 19%, ranging from a 45% decrease in mortality rate in Iceland to a 17% increase in Romania. Latvia was one of four European countries (Estonia, Romania, Greece, Latvia) where the breast cancer mortality continued to rise with overall mortality increase by 11.4% (Figure 2.1.5.) [*Autier et al.*, 2010].



2.1.5. Figure. Changes (%) in overall breast cancer mortality in 30 European countries during 1989–2006

AT – Austria, BE – Belgium, BG – Bulgaria, CH – Switzerland, CZ – Czech Republic, DE – Germany, DK – Denmark, GR – Greece, EE – Estonia, ES – Spain, EW – England and Wales, FI – Finland, FR – France, HU – Hungary, IC – Iceland, IR – Republic of Ireland, IT – Italy, LT – Lithuania, LU – Luxem-burg, LV – Latvia, NI – Northern Ireland, NL – Netherland, NO – Norway, PL – Poland, PT – Portugal, RO – Romania, SC – Scotland, SE – Sweden, SL – Slovenia, SK – Slovakia

2.2. Molecular classification of breast cancer

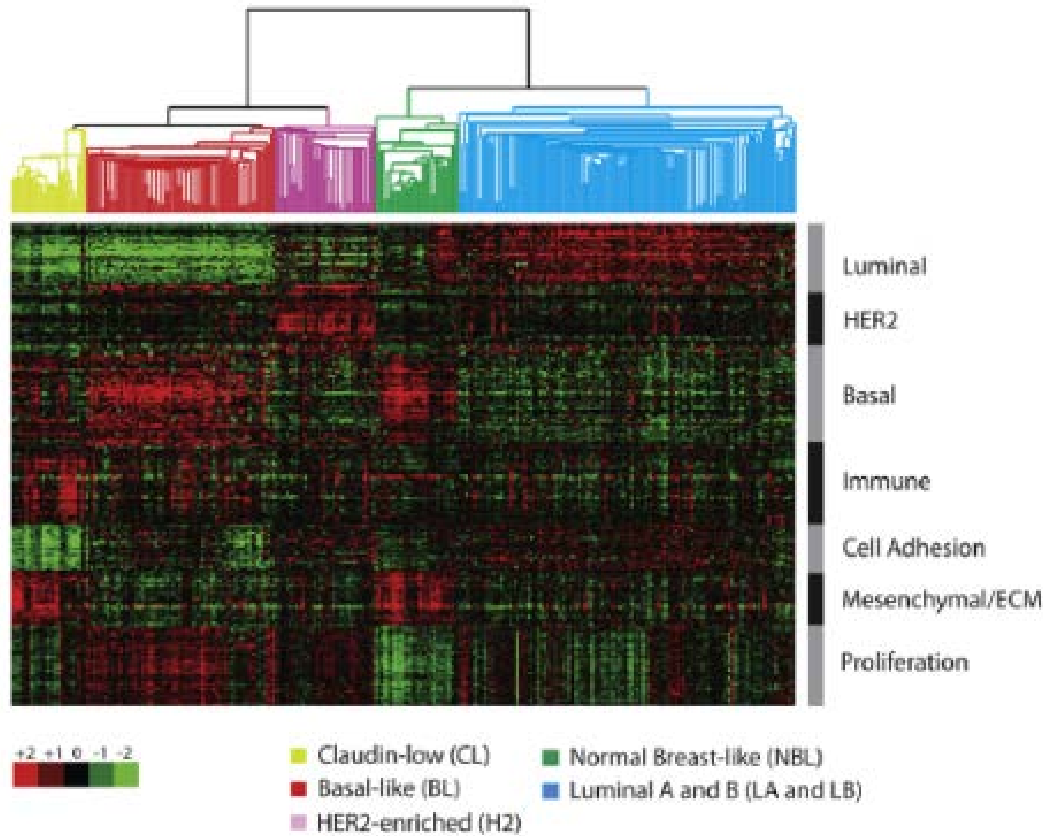
Breast cancer is an extremely biologically heterogeneous disease with different response to treatment, patterns of recurrence and clinical outcomes. During the last decades, gene expression studies using cDNA-microarray profiling and hierarchial clustering identified several breast cancer distinct subtypes [*Perou et al*., 2000; *Sorlie et al*., 2001; *Prat et al*., 2010; *Prat et al*., 2011]:

- Luminal A – high expression of the luminal-specific genes including ER-related cluster (Figure 2.2.1.). Clinically, a relatively favoruoble prognosis (Figure 2.2.2.).
- Luminal B – low to moderate expression of the luminal-specific genes including ER-related cluster [*Perou et al*., 2000], a higher expression of proliferation signatures [*Hu et al*., 2006] than in the luminal A subtype [*Cheang et al*., 2009; *Nielsen et al*., 2010] (Figure 2.2.1.). Clinically, poor prognosis, high risk of recurrence [*Sorlie et al*., 2001; *Fan et al*., 2006*; Prat et al*., 2010] (Figure 2.2.2.).
- HER2/neu enriched – high expression of HER2 and growth factor receptor-bound protein7 (GRB7) gene and low expression of luminal – specific genes.

Clinically associated with poor survival outcomes, high risk of recurrence [*Sorlie et al*., 2001; *Fan et al*., 2006; *Prat et al*., 2010] (Figure 2.2.2.).

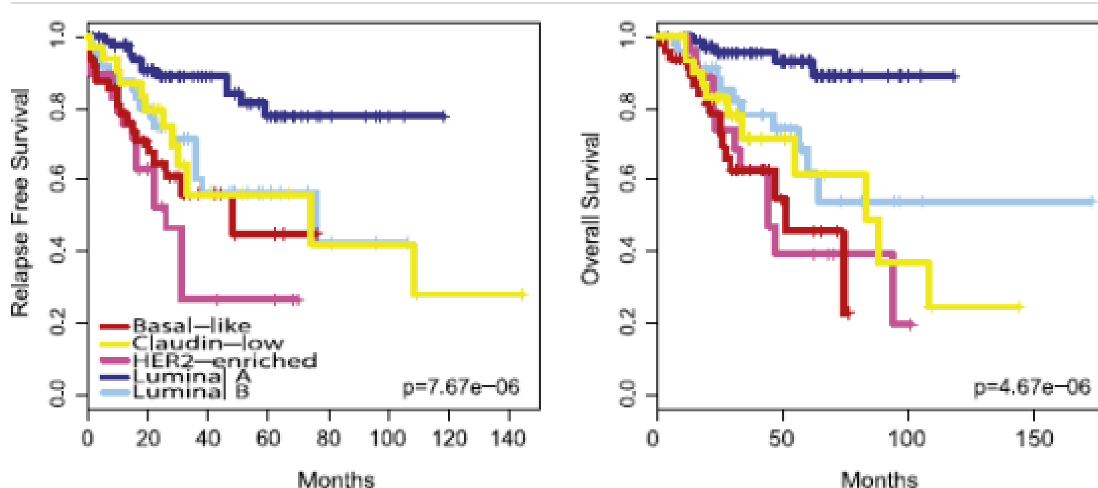
- Basal-like – high expression of basal cytokeratin (5, 17) genes and low expression of luminal-specific and HER2-related genes. Clinically associated with poor outcomes, high risk of recurrence [*Sorlie et al*., 2001; *Fan et al.,* 2006; *Prat et al*., 2010] (Figure 2.2.2.).
- Claudin-low – shows lack of expression of epithelial cell-cell adhesion genes (claudin 3, 4, 7, occludin, E-cadherin), basal keratins (5, 14, 17), HER2, luminal-specific gene cluster, proliferation genes [*Prat et al*., 2010] and high expression of epithelial- to-mesenchymal transition (EMT) [*Taube et al*., 2010], and cancer stem-cell-like features [*Creighton et al*., 2009; *Hennessy et al*., 2009; *Prat et al*., 2010] (Figure 2.2.1.). Clinically, are mostly high grade, invasive ductal carcinomas with a high rate of metaplastic and medullary differentiation ER/PR and HER2/neu negative (~15–20% are ER/PR positive), associated with increased invasiveness, high metastatic potential and worse prognosis compared to luminal A, and no difference in survival between luminal B, HER2/neu enriched and basal-like subtypes (Figure 2.2.2.).

Normal-breast-like – high expression of genes similar to normal breast-high expression of basal epithelial genes and genes expressed by adipose tissue, low expression of luminal epithelial genes. It may be explained by the fact that analysed samples contain predominantly normal breast tissue not tumor tissue. Therefore, the existence of this group is questionable, futher investigations are required [*Perou et al*., 2000; *Sorlie et al*., 2001; *Prat et al*., 2010].



2.2.1. Figure. Gene expression patterns of 320 human breast cancer samples and 17 normal breast samples analysed by hierarchial clustering using the 1900 gene intristic set [Parker et al., 2009]. The sample associated dendrogram colored according to tumor subtypes. Red squares represent high gene expression pattern, black squares represent moderate gene expression pattern and green squares represent low gene expression pattern

Adapted from *Prat et al*., 2010



2.2.2. Figure. Relapse-free survival and overall survival curves of different breast cancer intristic subtypes

Adapted from *Prat et al.*, 2010

The expensiveness of high-throughput sequencing technologies make its application in routine clinical practice and clinical trials impossible. Therefore, simplified classification, based on clinicopathological signs of intristic breast cancer subtypes had been developed and adopted in clinical practice. The biological subtyping using a four-marker surrogate immunohistochemistry panel (ER, PR, HER2, Ki-67) demostrates similar, but not identical prognostic value to gene expression profile-defined breast cancer subtypes [*Goldhirsch et al*., 2011] (Table 2.2.1.). Exact detection of ER, PR, HER2/neu and Ki-67 index plays an important role in the distinction between breast cancer subtypes. Guidelines for ER, PR, HER2/neu and Ki67- index determination have been subsequently published. The panel defined a negative ER/PR finding as < 1% of tumor nuclei that are immunoreactive, a positive HER2/neu test as either IHC result of 3+ cell surface protein expression or FISH result of amplified HER2 gene copy number or HER2/CEP17 ratio > 2.2, a negative HER2/neu test as IHC result of 0 or 1+ for cellular membrane protein expression, or a FISH result showing HER2/CEP17 ratio < 1.8 and equivocal HER2/neu IHC result as 2+ or FISH result as HER2/CEP17 ratio 1.8–2.2 [*Wolff et al*., 2007; *Hammond et al*., 2010; *Goldhirsch et al*., 2011].

2.2.1. Table

Surrogate definitions and treatment recommendations for intristic subtypes

Intristic Subtype	Clinicopathological definition	Treatment
Luminal A	**'Luminal A'** ER* and/or PR* positive HER2* negative Ki-67 low (<14%)	Endocrine therapy ± Cytotoxic treatment for some high risk patients (For example, positive lymph nodes or high score of the 21-gene signature (Oncotype DX) or 70-gene signature (Mammaprint))
Luminal B	**'Luminal B (HER2 negative)'** ER and/or PR positive HER2 negative Ki-67 high **'Luminal B(HER* positive)'** ER and/or PR positive Any Ki-67 HER2 over-expressed or amplified	Endocrine treatment± cytotoxic treatment Cytotoxic treatment+ anti-HER2+ endocrine treatment
HER2-enriched	**'HER2 positive'** HER2 overexpressed or amplified ER and PR absent	Cytotoxic treatment + anti-HER2
Basal-like	**'Triple-negative'** ER and PR absent HER2 negative	Cytotoxic treatment

ER – oestrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor

2.3. Triple-negative breast cancer

Triple-negative breast cancer is a heterogeneous clinicopathological entity defined as an oestrogen (ER), progesterone (PR) and HER2/ neu negative breast cancer [*Dent et al.*, 2007; *Bauer et al.*, 2007]. Triple-negative breast cancer accounts for approximately 10–20% of all breast cancer subtypes [*Bauer et al.*, 2007; *Kaplan et al.*, 2008].

2.3.1. The heterogeneity of triple-negative breast cancer

Using gene expression profiling triple-negative breast cancers are stratified into several different molecular subtypes: the vast majority are being basal-like (50–75%)

and the rest including HER2-enriched, luminal A, luminal B, claudin-low, normal breast-like [*Perou et al.*, 2010; *Prat et al.*, 2011].

Very recently, study group by *Lehmann et al.*, have subclassified 587 triple-negative breast cancer cases from 21 breast cancer datasets into six different subtypes [*Lehmann et al.*, 2011]:

- Basal-like 1(BL-1) – high expression of genes involved in cell cycle and cell division, proliferation and DNA damage response pathways.
- Basal-like 2 (BL-2) – high expression of genes involved in the growth factor signaling, cell-cycle, DNA damage response genes.

Both BL-1 and BL-2 have a high Ki-67 mRNA expression (MKI67) and IHC staining for Ki-67 than compared to other subtypes (BL1 + BL2 = 70% versus 42% other subtypes; $P < 0.05$). BL1 and BL2 subtypes after treatment with taxane-based regimens showed a significantly higher pCR(63%) compared with 31% in the mesenchymal-like subtype and 14% in the luminal androgen receptor subtype [*Bauer et al.*, 2010; *Juul et al.*, 2010]. Basal-like breast cancer cell lines showed higher sensitivity to cisplatin treatment.

- Immunomodulatory (IM) – high expression of genes involved in the cell immune processes: immune cell signaling, cytokine signaling (cytokine pathway, IL-2, IL7 pathways), antigen processing and presentation, immune transductional pathways. IM subtype gene signatures overlap with medullary breast cancer gene signatures [*Bertucci et al.*, 2006.].
- Mesenchymal (M) – high expression of genes involved in cell motility and extracelullar matrix.
- Mesenchimal stem-like (MSL) – high expression of genes involved in cell motility and extracelullar matrix, growth factor signaling (EGFR, PDGFR), low expression of claudins 3 ,4 ,7 and proliferation-associated genes M and MSL cell lines were more sensitive for the Src inhibitor dasatinib than LAR cell lines and dual PI3K/mTOR inhibitor than basal-like subtypes.
- Luminal androgen receptor (LAR) – high expression of genes involved in hormonally regulated pathways including steroid synthesis, porphyrin metabolism and androgen/estrogen metabolism. Because of the high expression of luminal cytokeratins LAR subtype belongs to either luminal A or B intristic subtype despite triple-negative status [*Lehmann et al.*, 2011; *Doane et al.*, 2006]. LAR cell lines showed high sensetivity to bicalutamide (androgen receptor inhibitor) and PI3K inhibitors.

Although, the molecular classification of triple-negative breast cancer is still controversial and its clinical implication requires further investigation.

2.3.2. Risk factors of triple-negative breast cancer

Triple-negative and basal-like breast cancer are associated with younger age at diagnosis [*Bauer et al*., 2007; *Morris et al*., 2007], occur more commonly in premenopausal women, *BRCA1* mutation carriers and African-American women [*Yang et al*., 2007; *Morris et al*., 2007; *Millikan et al*., 2008; *Phipps et al*., 2008; *Trivers et al*., 2009*; Lund et al*., 2009; *Shinde et al*., 2010]. Other lifestyle factors such as an increased number of parity combined with lack of breastfeeding, use of medications for lactation supression, early meanrche, younger age at first-term pregnancy, eleveted waist-hip ratio in pre- and postmenopausal women, adiposity since childhood increase the risk of triple- negative breast cancer [*Millikan et al.,* 2008; *Trivers et al*., 2009; *Shinde et al*., 2010]. A statistically significant association between triple-negative breast cancer and metabolic syndrome was observed in the study of *Maiti et al*., 58.1% of patients with triple-negative breast cancer had a metabolic syndrome compared to 36.7% in the non-triple-negative breast cancer group [*Maiti et al*., 2009]. According to *Dolle et al*., study results, women under age of 45 years who had used an oral contaceptives ≥ 1 year had a 2.5 times higher risk of triple-negative breast cancer compared to women who had never used oral contraceptives or used oral contraceptives less than 1 year [*Dolle et al*., 2009].

2.3.3. Histopathology of triple-negative breast cancer

The majority of triple-negative breast cancer are presented by ductal carcinomas [*Carey et al*., 2006], but several other histological breast cancer types also could express lack of ER/PR and HER2/neu immunohistochemical staining (medullary, apocrine, pleomorphic lobular, metaplastic, adenoid cystic carcinomas) [*Jacquemier et al.,* 2005*; Livasy et al.,* 2006*; Reis- Filho et al*., 2006]. An apocrine, adenoid cystic and classical medullary carcinomas showed favourable prognosis [*Azoulay et al., 2005; Vincent-Salomon et al*., 2007; *Marchio et al*., 2009]. In contrast, metaplastic triple-negative breast cancer showed a similary poor prognosis as high grade adenocarcinomas, but were less sensitive to conventional chemotherapy [*Hennessy et al*., 2009].

Triple-negative and basal-like breast cancers are characterized by large tumor size [*Dent et al*., 2007; *Carey et al*., 2006; *Bertucci et al.,* 2006*; Bauer et al*., 2007], high histological grade (only up to 10% of triple-negative breast cancers are grade I) [*Dent at al.*, 2007], elevated mitotic count, marked nuclear pleomorphism [*Carey et al*., 2006], central fibrosis and necrosis, pushing margins of invasion, stromal lymphocytic response [*Fulford et al*., 2004; *Livasy et al*., 2006].

2.3.4. Clinical presentation and imaging of triple-negative/ basal-like breast cancer

Triple-negative breast cancers are associated with advanced stage at diagnosis than non-triple-negative breast cancers [*Nielsen et al*., 2004; *Dent et al*., 2007; *Bauer et al*., 2007; *Liedke et al*., 2008]. There is no clear correlation between tumor size and positive lymph nodes in the triple-negative and basal-like breast cancers. Even small tumors in the triple-negative breast cancer group have a high rate of positive lymph nodes. *Foulkes et al*., speculated that small basal-like tumors may harbor a cells with cancer stem-like features and therefore be more clinically aggressive and more likely to metastasize. This phenomenon is also observed in *BRCA1*- related breast cancers [*Foulkes et al*., 2003; *Dent et al*., 2007; *Foulkes et al*., 2008; *Foulkes et al*., 2010; *Foulkes et al*., 2012].

As reported *Dent et al.*, triple-negative breast cancers were less often screen-detected by mammography or ultrasound than other breast cancers (19.6% versus 36%; $P < 0.0008$) in patients ≥ 50 years [*Dent et al*., 2007]. A case- control study by *Collett et*

al., showed that basal-like breast cancers were more likely to present in the 2-year interval between regular mammograms than non-basal-like breast cancers. In a logistic regression model, dense breast, younger age and positive p53 expression were positive predictors of interval cancers [*Collett et al.*, 2005]. This may be explained by a more rapid growth of triple-negative breast cancers or by the differences in breast density of women with triple-negative breast cancer [*Dent et al.*, 2007].

Triple-negative breast cancers are more likely to appear on mammograms as a mass (49–62.4%) with "pushing" margins – smooth or circumscribed lesions (20.8–22%), mostly without calcifications (49–100%) and / or spiculated margins [*Wang et al.*, 2008]. In 9–21% triple-negative breast cancers are presented as focal asymmetry [*Wang et al.*, 2008; *Dogan et al.*, 2010]. On ultrasound triple-negative breast cancers were more likely to present as a mass with well-circumscribed margins in 21–27% and more likely to show posterior acoustic enhancement and less likely to have echogenic halo [*Kim et al.*, 2008; *Au-Yong et al.*, 2009*; Ko et al.*, 2010; *Dogan et al*,. 2010; *Kojima et al.*, 2011]. Some ultrasonographic triple-negative breast cancer features, such as well-circumscribed mass and posterior acoustic enhancement are typical also for benign diseases (benign breast neoplasms, cysts, abscess), therefore triple-negative breast cancer may mimic non-malignant lesions. However, ultrasound show very high sensitivity for triple-negative breast cancer (92–100%). On MR images the majority of triple-negative breast cancers are presented as a mass lesions with smooth margins, rim enhancement and very high intratumoral intensity on T2-weighted images [*Uematsu et al.*, 2009]. Triple-negative breast cancer is detected with higher sensitivity and shows enchanced flourine- 18 fluorodeoxyglucose (FDG) uptake on FDG-PET imaging compare to ER/PR positive, HER2 negative tumors. The enchanced glycolitic rate of triple-negative breast cancers may be related to high proliferation and biological aggressiveness of this breast cancer subtype [*Basu et al.*, 2008]. FDG-PET is a potentially useful to detect distant recurrences and control response to chemotherapy in the triple-negative breast cancers [*Schwarz-Dose et al.*, 2009; *Groheux et al.*, 2010; *Basu et al.*, 2008].

2.3.5. Prognosis and patterns of distant recurrence of triple-negative / basal-like breast cancer

Multiple studies have shown an agressive clinical behavior of triple-negative breast cancer with high recurrence and death rate. Triple-negative breast cancer patients have a 4.2 times higher risk of event than other breast cancer subtypes [*Mersin et al.*, 2008]. In the cohort study of 1.601 patient, triple-negative breast cancer group had a distinct pattern of distant recurrence compare to other patients' group, with a tend to a higher risk of distant recurrence in the first 3 years after diagnosis and sharp decrease hereafter. The median time to recurrence was 2.6 years in the triple-negative patients group compared to 5.0 years in the other patients' group [*Dent et al.*, 2007]. Similar results showed *Rhee et al.*, 90% of recurrent triple-negative breast cancer patients had relapse within 3 years after diagnosis compared to 57.3% in the non-triple-negative breast cancer group. A shorter recurrence- free survival for triple-negative breast cancers were widely reported [*Sorlie et al.*, 2003; *Rakha et al.*, 2007; *Tian et al.*, 2008; *Rhee et al.*, 2008]. Triple-negative breast cancers are more likely to develop visceral metastases than bone or lymph-node relapse and have a higher risk of brain metastases [*Minn* et al., 2005; *Hicks et al.*, 2006; *Fulford et al.*, 2007; *Hines et al.*, 2008; *Luck et al.*, 2008; *Lin et al.*, 2008; *Liedtke et al.*, 2008] than other breast cancer patients. *Carey et al.,* population-based study showed statistically significantly lower breast cancer-specific survival in the basal-like and HER2-overexpressing breast cancer patients compare to other patients' groups (basal-like breast cancer – 75%, HER2-overexpressing – 52%, luminal A – 84%, luminal B – 87%, $P < 0.001$). A worse breast cancer-specific survival was observed both in lymph node-negative and positive basal-like breast cancers [*Carey et al.*, 2006.]. *Van de Rijn et al.*, reported that expression of CK17 and / or CK5/6 is associated with inferior survival in node negative patients. Shorter survival for triple-negative/basal-like breast cancer is widely reported [*Sorlie et al.*, 2003; *Nielsen et al.*, 2004; *Rakha et al.*, 2007; *Dent et al.*, 2007; *Tian et al.*,2008; *Rhee et al.*, 2008]. According to *Dent et al.*, study, 70% of deaths in the triple-negative breast cancer group occured in the first five years after diagnosis compared to 44% of deaths in the other patients group [*Dent et al.*, 2007]. Triple-negative breast cancers was also associated with shorter median time from recurrence to death compared to other breast cancers (9 months versus 20 months, $P < 0.02$) [*Dent et al.*, 2007]. All deaths in the triple-negative breast cancer group was observed within first 10 years after diagnosis compared to 18 years in the other patients' group [*Dent et al.*, 2007].

Several studies showed that despite initial chemosensitivity after neoadjuvant chemotherapy triple-negative breast cancer patients had significantly worse distant recurrence-free survival and overall survival than patients with luminal breast cancers. This paradox was explained by the high relapse rate in the triple-negative breast cancer patients with residual disease after chemotherapy, especially in the first 3 years after diagnosis. Triple-negative breast cancer patients, who achieved pCR had a favourable prognosis [*Carey et al*., 2007; *Liedtke et al*., 2008].

2.3.6. Local and regional recurrence (LRR) in the triple-negative / basal-like breast cancer group

Dent et al., in the study population of 1601 patients did not find a significant difference in locoregional recurrence (LRR) rate between triple-negative and non-triple-negative breast cancers (13% versus 12%, respectively; P = 0.77). Women in the triple-negative breast cancer group were less likely to experience LRR before distant recurrence compared to other breast cancer group (25% versus 44%, respectively; P < 0.02). The median time to LRR for triple-negative breast cancer patients was statistically significantly shorter than for other breast cancer patients (2.8 years versus 4.2 years, repectively; P < 0.02) [*Dent et al*., 2007]. Similar results showed *Haffty et al*., with no statistically significant difference in local recurrence rate between triple-negative and non-triple-negative breast cancers. Triple-negative breast cancers had a slightly (5-year nodal recurrence rate of 6% versus 1%, respectively), but statistically insignificant increase in nodal relapse compare to non-triple-negative breast cancer group [*Haffty et al*., 2006]. In contrast, *Wang et al*., reported higher likelihood of LRR in the triple-negative and HER2/neu overexpressing groups within first 3 years after treatment [*Wang et al*., 2011]. *Montagna et al.*, reported a higher risk of subsequent event and death for patients with LRR and triple-negative breast cancer subtype [*Montagna et al*., 2012].

Multiple studies basal-like and HER2/neu breast cancers demonstrated a significantly increased risk of LRR after breast-conserving therapy (BCT) [*Voduc et al*.,2010; *Millar et al*., 2009; *Arvold et al*., 2011]. *Kyndi et al*., showed higher LRR rate after mastectomy in the triple-negative and HER2/neu-overexpressing breast cancer subtypes compare to other subtypes. There was no obvious improvement found in overall survival in patients, who received postmastectomy radiation therapyn (PMRT)

in the group of triple-negative breast cancer compared to hormone receptor positive, HER2/neu negative group. However, PMRT significantly reduced LRR rate in the triple-negative, hormone receptor positive HER2/neu negative and hormone receptor positive HER2/neu positive breast cancer groups, but not in HER2/neu-overexpressing group. The effect of PMRT on LRR risk in the triple-negative group was significantly reduced compared to hormone positive/HER2/neu negative breast cancer group [*Kyndi et al*., 2008]. In the retrospective *Jagsi et al*., study 877 triple-negative node-negative breast cancer patients treated with mastectomy were included. The study demostrated an increased LRR rate for patients with risk factors (tumor size greater > 2 cm, margins < 2 mm, premenopausal status, lymphovascular invasion) and suggested that they may benefit form PMRT [*Jagsi et al*., 2005]. In a population of 1691 patients with small size (pT1mic/T1a/T1b) lymph node-negative tumors, triple-negative breast cancers and HER2/neu overexpressing had a significantly increased risk of LRR compared to other breast cancer subtypes [*Cancello et al*., 2011]. In a systematic review by *Lowery et al.,* a total of 12.592 patients (7.174 underwent BCT and 5.418 underwent mastectomy) from 15 studies were identified. Triple-negative breast cancer and HER2/neu overexpressing subtypes had a higher risk of LRR after BCT and after mastectomy compared to luminal A subtype [*Lowery et al*., 2012]. *Adkins et al*., reported better 5-year LRR-free survival (76% versus 71%; $P < 0.032$), distant metastasis-free survival (68% versus 54%; $P < 0.0001$) and overall survival (74% versus 63%; $P < 0.0011$) in triple-negative breast cancer patients after BCT compared to mastectomy. However, there was a significantly higher incidence of lymphovascular invasion, larger tumor size and higher nodal stage in the mastectomy group. Multivariate analysis showed no impact of the type of surgery on LRR risk [*Adkins et al*., 2011]. In the study by *Ho et al*., 129 patients after BCT and 65 patients after mastectomy without radiation therapy with T1a/T1b lymph node-negative triple-negative breast cancers were included, 58% of whom received adjuvant chemotherapy. There were excellent 5-year LRR and distant recurrence rates reported, irrespective of the type of surgery performed [*Ho et al*., 2012].

Most recent study by *Zumsteq et al*., analysed 646 triple-negative breast cancer patients with stage T1-2N0, who underwent breast-conserving surgery and 198 patients, who underwent mastectomy without postmastectomy radiation. There was no difference found in LRR, distant metastases and overall survival rate between two groups. High

tumor stage and absence of chemotherapy were independent predictors of inferior overall survival [*Zumsteq et al.*, 2013].

2.3.7. *BRCA1* mutation and breast cancer

Breast and ovarian cancer predisposing gene *BRCA1 (BReast Cancer susceptibility gene 1)* was identified in 1994 [*Miki et al.,* 1994]. *BRCA1* is a tumor supressor gene located on chromosome 17q12-21 region [*Hall et al*., 1990]. The gene is organized in 24 exons (22 protein-coding) which span an 81 kb of genomic DNA and encodes a protein of 1863 amionoacids [*Miki et al*., 1994; *Smith et al.,* 1996]. The BRCA1 protein contains several functional domains:

- N (amino)-terminal Ring (Really Interesting New Gene) finger domain – has a E3 ubiquitin-ligase function [*Lorick et al*., 1999; *Venkitaraman et al*., 2002; *Gudmundsdottir et al*., 2006]. The interaction of BRCA1 RING – domain with BARD1 (BRCA1 Associated RING Domain protein 1) and BRCA1/BARD1 heterodimeric complex formation leads to a dramatic increase in ubiquitin ligase enzymatic activity [*Wu et al*., 1996; *Hashizume et al*., 2001; *Kerr et al*., 2001]. The BRCA1/BARD1 heterodimer can conjugate mono- and polyubiquitin chains to the substrate proteins. Therefore, it has impact on DNA repair, can target proteins for degradation, is required for normal cell cycle progression from G2 to mitosis, may function as a transcriptional regulator, meiotic sex chromosome inactivation [*Hashizume et al*., 2001; *Mallery et al*., 2002; *Ohta et al*., 2011; *Roy et al*., 2011; *Clark et al*., 2012].
- two C (carboxyl)-terminal BRCT (BRCA1-C-terminal) – plays role in the DNA repair, transcriptional regulation [*Joo et al.,* 2002; *Leung et al*., 2011; *Roy et al*., 2011; *Clark et al*., 2012].
- serine cluster domain – has concentrated phosphorylation sites, that are phosphoryled by ATM/ATR kinases activated by DNA damage. Phosphorilation causes localisation of BRCA1 to the sites of double strand breaks *[Traven et al*., 2005; *Roy et al*., 2011; *Clark et al*., 2012].

Therefore, BRCA1 is a multifunctional protein, that plays a role in maintaining genome integrity through DNA damage repair, cell cycle checkpoints control, in apoptosis, prevention of global DNA hypomethylation [*Harkin et al*., 1999; *Xu et al*., 2001; *Jasin et al*., 2002; *Venkitaraman et al*., 2002; *Deng et al*., 2003; *Boulton et al*.,

2006; *Gudmundsdottir et al.,* 2005; *Shukla et al .,*2010*; Roy et al*., 2011; *Charita et al*., 2013].

Over 1900 unique *BRCA1* mutations are reported and approximately 900 of these mutations are clinically significant [*Breast Cancer Information Core Database*]. Mutations in the *BRCA1* results in genomic instability and predispose normal cells to higher risk of malignant transformation [*Deng et al*., 2001; *Deng et al*., 2006].

Approximately 3–5% of breast cancers and 10% of ovarian cancers are associated with *BRCA1* and *BRCA2* germline mutations [*Robson et al*., 2001; *Risch et al*., 2006; *Gardovskis et al*., 2008; *ACOG Committee on Practice Bulletins.*, 2009]. *BRCA1* mutation carriers have a 70–80% lifetime risk of developing breast cancer, and a 50% lifetime risk of developing ovarian cancer, significantly increased risk of fallopian tube cancer and peritoneal papillary serous carcinoma [*Brose et al*., 2002; *Levine et al*., 2003; *Antoniou et al*., 2003; *Olivier et al*., 2004; *Roy et al*., 2012]. A 40% 10-year risk of a contralateral breast cancer for *BRCA1*-positive breast cancer patients is reported [*Melcalfe et al*., 2004]. Among *BRCA1* carriers with primary breast cancer the 10-year acturial risk of developing subsequental ovarian cancer is 12.7% [*Melcalfe et al*., 2005].

A study by *Grann et al*., found that a 30 year-old *BRCA1/2* carrier could prolong her survival by 0.9 years (95% probability interval, 0.4–1.2 years) by having bilateral oophorectomy, 3.4 years (2.7–3.7 years) by having bilateral mastectomy, and 4.3 years (3.6–4.6 years) by having both bilateral oophorectomy and mastectomy compare to surveillence [*Grann et al.,* 2000]. The bilateral risk-reducing salpingooophorectomy decreased the risk of ovarian cancer by 96% and the risk of breast cancer by 53% [*Rebbeck et al*., 2002]. In the large multicenter, prospective study by *Kauff et al*., risk-reducing bilateral salpingo-oophorectomy decreased the risk of gynecological cancer by 85% in the *BRCA1*-carriers and the risk of breast cancer by 72% in the *BRCA2*-carriers. Although, there was no statistically significant risk reduction in *BRCA1*-associated breast cancer and *BRCA2*-associated gynecologic cancer. The authors concluded that protection effect of risk-reducing bilateral salpingo-oophorectomy may differ between *BRCA1* and *BRCA2*-mutation carriers [*Kauff et al*., 2008]. In contrast, the meta-analysis of ten studies performed by *Rebbeck et al*., showed that risk-reducing bilateral salpingo-oophorectomy significantly reduce the risk of breast and gynecological cancer in both *BRCA1* and *BRCA2* carriers [*Rebbeck et al*., 2009]. The bilateral risk-reducing mastectomy reduce the risk of subsequent breast cancer by 89.5–100% [*Hartmann et*

al., 2001; *Meijers-Heijboer et al.,* 2001]. *Rebbeck et al*., published that in the *BRCA1/2* mutation carriers bilateral prophylactic mastectomy reduced the risk of breast cancer by 95% in patients with prior or simultaneous bilateral prophylactic oophorectomy and by 90% in patients with no prior bilateral prophylactic oophorectomy at a median follow-up of 6.4 years [*Rebbeck et al*., 2004]. *Skytte et al*., reported a 0.8% annual incidence of breast cancer in the *BRCA1*-carriers after risk-reducing mastectomy compared to 1.7% in the control group, who underwent no surgery [*Skytte et al*., 2011]. During the past decade the rate of women undergoing contralateral prophylactic mastectomy both for in situ and invasive breast cancer more than doubled [*Tuttle et al*., 2007; *Tuttle et al*., 2009]. Contralateral prophylactic mastectomy significantly reduce the risk of contralateral breast cancer and improve disease-free and breast cancer survival in high-risk breast cancer patients [*McDonnell et al*., 2001; *Herrinton et al*., 2005]. The majority of *BRCA1/2* carriers, who elect prophylactic mastectomy were satisfied with their decision, despite the negative impact on body image perception, physical wellbeing and the intimate relationship [*Forst et al*., 2000; *Lodder et al*., 2002; *Geiger et al*., 2007]. Mastectomy with subsequent reconstruction was reported to have a lower impact on patients' self-esteem and sexual life compared to mastectomy [*Markopoulos et al*., 2009]. Approximately 69% of patients choose to have breast reconstruction after prophylactic mastectomy. However, younger age and absent personal history of breast cancer were associated with higher rate of breast reconstruction after prophylactic mastectomy [*Semple et al*., 2013]. It was shown that sparing of the nipple-areola complex improve patients aesthetic satisfaction after breast- reconstruction [*Shaikh-Naidu et al*., 2004]. Skin-sparing mastectomy with/ without removal of the nipple-areola complex with immediate breast reconstruction was reported to be oncologically safe with overall high satisfaction levels of cosmetic outcomes [*Gerber et al*., 2003; *Mustonen et al*., 2004; *Yiacoumettis et al.,* 2005*; Sacchini et al*., 2006; *Gahm et al.,* 2010].

Tamoxifen is not currently registered for primary chemoprevention of breast cancer in the *BRCA1* mutation carriers. There is a limited amount of information regarding the role of tamoxifen in reducing breast cancer risk in the unaffected *BRCA1* mutation carriers. As the majority of *BRCA1*-associated tumors are hormon-receptor negative and the majority of *BRCA2*-associated tumors are hormone-receptor positive, it was hypothesized that tamoxifen is less effective in the *BRCA1*-mutation carriers than in the *BRCA2*-mutation carriers [*Verhoog et al*., 1998; *Lakhani et al*., 2002]. This theory

was confirmed by *King et al.*, randomized double-blind study, there tamoxifen failed to reduce breast cancer incidence in healthy *BRCA1*-carriers (HR = 1.67; 95%Cl : 0.32–10.7), but significantly reduced the breast cancer risk in the *BRCA2*-carriers (HR = 0.32; 95%Cl : 0.06–1.56). However, there was a small number of *BRCA1* and *BRCA2* carriers (8 and 11, respectively) included in the study [*King et al.*, 2001]. Although, adjuvant tamoxifen demonstrated a reduction in a contralateral breast cancer risk. In the study by *Cronwald et al.*, the use of adjuvant tamoxifen was associated with decreased risk of contraleteral breast cancer both in the *BRCA1* (OR = 0.5; 95%Cl : 0.3–0.85) and *BRCA2*-mutation carriers (OR = 0.42; 95%Cl : 0.17–1.02) [*Cronwald et al.*, 2006]. Additionally, a retrospective multi-institutional study showed a significantly decreased risk of contralateral breast cancer in *BRCA1* mutation carriers treated with tamoxifen in the adjuvant setting (HR = 0.31; P = 0.05) [*Pierce et al.*, 2006].

Current breast cancer screening recommendations for *BRCA1* carriers are shown in Table 2.3.7.1.[*Balmana et al.*, 2011].

2.3.7.1. Table

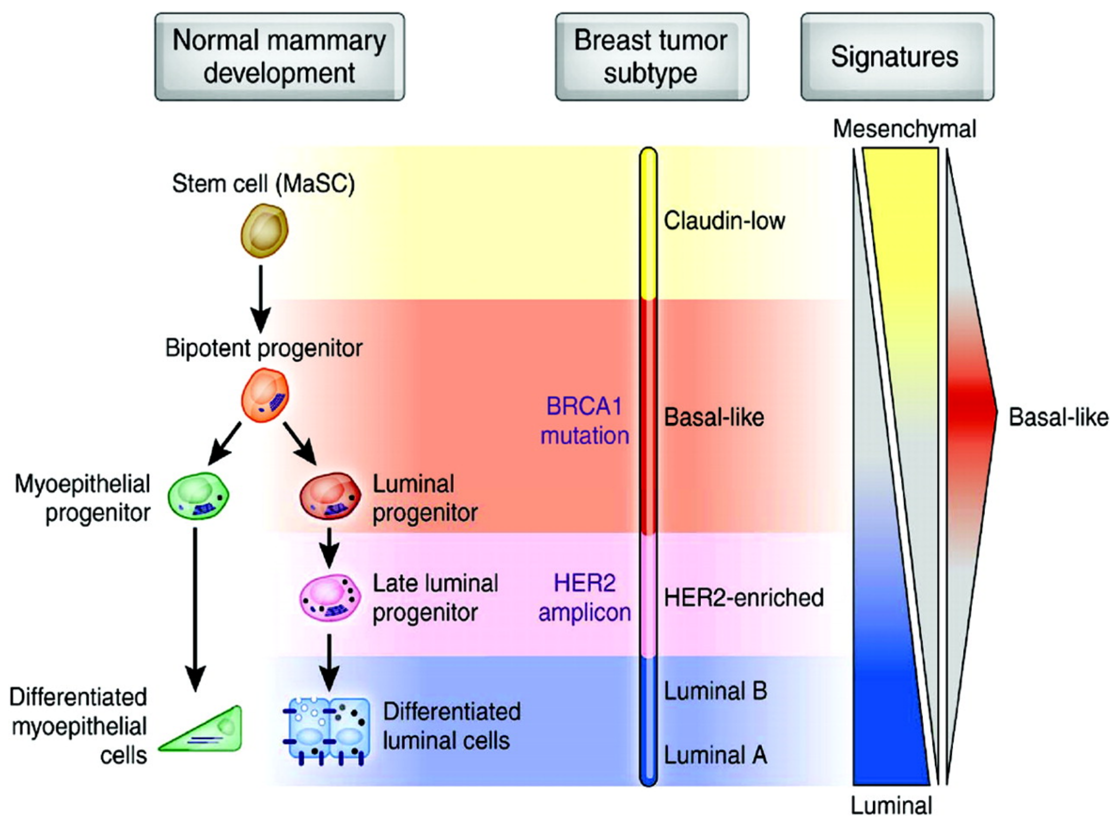
Breast cancer screening recommendations for *BRCA1* carriers

Self-examination	Monthly breast self-examination beginning at age 18
Clinical breast examination	Twice a year, beginning at age 18
Mammography and MRI	Annualy, beginning at age 25 or 5 years younger than the age at diagnosis of the youngest affected relative

2.3.8. Triple-negative / basal-like breast cancers and *BRCA1* mutation

Increasing evidence suggests a strong relationship between the BRCA1-pathway, basal-like and triple-negative breast cancer [*Turner et al.*, 2004; *Turner et al.*, 2006]. Approximately 50–88% of all *BRCA1*-related tumours have a triple-negative or/and basal-like phenotype [*Foulkes et al.*, 2003; *Lakhani et al.*, 2005; *Palacious et al.*, 2005; *Diaz et al.*, 2007; *Stefansson et al.*, 2009]. Like sporadic basal-like breast cancers *BRCA1*-related breast cancers are characterized by high tumor grade ductal carcinomas of no special type, high proliferation rate, presence of central necrosis and pushing borders, overexpression of EGFR, expression of basal cytokeratins (CK5/6, 14, 17), myoepithelial markers (caveolin 1 and 2, osteonectin, c-kit, P-cadherin, fascin) [*Foulkes et al.*, 2003; *Arnes et al.*, 2005; *Lakhani et al.*, 2005; *Palacious et al.*, 2005; *Pinilla et al.*,2006; *Turner et al.*, 2006; *Rodriquez-Pinilla et al.*, 2006; *Kreike et al.*, 2007; *Rakha*

et al., 2007; *Foulkes et al*., 2010]. Reanalyze of cDNA microarray data from *van't Vee*r showed that *BRCA1*-related tumors have a sporadic basal-like breast cancer gene expression profile [*Sorlie et al*., 2003]. Deletions in chromosome 5q described as one of the hallmarks of basal-like breast cancers are also associated with *BRCA1*-related tumors [*Johannsdottir et al*., 2006; *Hu et al*.,2009]. Like *BRCA1*-deficient tumors basal-like and triple-negative breast cancers have abnormalities in the inactive X chromosome (Xi) that results in activation of genes that are non-active in non-cancerous cells [*Ganesan et al*., 2002; *Richardson et al*., 2006; *Turner et al*., 2007]. This considerations suggests that loss of BRCA1 function could play a role in the development of basal-like breast cancers [*Richardson et al*., 2006]. Recent studies revealed that *BRCA1*-related and basal-like breast cancers have more similar gene expression profiles to luminal-progenitor cells than to stem- cells [*Lim et al*., 2009; *Molyneux et al*.,2010]. Therefore, researches believe that basal-like breast cancers arise from the luminal-progenitor cells. *Liu et al*., showed that BRCA1 plays a critical role for the differentiation of ER-negative stem/progenitor cells to ER-positive luminal cells. The loss of BRCA1 arrests cell further differentiation [Figure 2.3.8.1.]. [*Prat et al*., 2009; *Perou et al*., 2013]



2.3.8.1. Figure. Development of normal breast myoepithelial cell and its possible association with breast cancer intristic subtypes
Adapted from *Prat et al*., 2009

A number of studies have evaluated the prevalence of *BRCA1* mutation in especially selected triple-negative breast cancer patients. A study by *Young et al.*, in a population of 54 women, who were diagnosed with high grade, triple-negative breast cancer before age < 40 and without family history of breast or ovarian cancer, identified 5 (9%) *BRCA1* mutation carriers. In an isolated 63 cases of triple-negative breast cancer diagnosed before < 41 years 8 (12.7%) *BRCA1* mutations was detected [*Evans et al.*, 2011]. This results suggest that association of triple-negative breast cancer with young age at diagnosis can be used for identifying individuals for genetic testing regardless of family history. *BRCA1* mutation prevalence among selected triple-negative breast cancer patients are summarised in Table 2.3.8.1.

2.3.8.1. Table

Germline *BRCA1* mutation in the selected triple-negative breast cancer cases

Selection criteria	N	*BRCA1* mutation	%	References
Underwent *BRCA* testing in the Genetic clinics	93	32	34.4	*Atchley et al.,* 2008
Ashkenazi Jewish heritage, tested for founder mutations	64	19	30	*Comen et al.,* 2008
TN< 40 years and did not qualify for testing according to ASCO guidelines	54	5	9.2	*Young et al.,* 2009
Early age of onset and/or family history of breast cancer	149	30	20	*Robertson et al.,* 2012
Early age of onset/family history of breast/ovarian cancer	110	23	21	*Phuah et al.,* 2012
Total	*470*	*109*	*23*	-

Several other studies have evaluated the prevalence of *BRCA1* mutation in the unselected cohorts of triple-negative breast cancer patients. In a study population of 77 patients with triple-negative breast cancer and unknown family history *BRCA1* mutation was detected in the 14.3% of cases [*Gonzalez-Angulo et al.*, 2011]. In a cohort of 199 triple-negative breast cancer patients *BRCA1* mutation was found in 6.5%. These study demostrated that diagnosis of triple-negative breast cancer before age 50 years irrespective of family history increase the likelihood of carrying *BRCA1* mutation and in conjuction with positive family history increase the likelihood of carrying *BRCA1* mutation after > 50 years [*Hartman et al.*, 2013]. Similar results showed *Rummel et al.* in this study *BRCA1* mutation prevalence was 9%. Mutation frequency was higher in

patients diagnosed with triple-negative breast cancer before <50 years (11 of 78, 14.1%) compared to 5 of 104 (4.8%) in patients diagnosed after > 50 years [*Rummel et al.*, 2013]. *BRCA1* mutation prevalence among unselected triple-negative breast cancer patients are summarised in Table 2.3.8.2.

2.3.8.2. Table

Germline *BRCA1* mutation in the unselected triple-negative breast cancer cases

Number of cases	*BRCA1* mutation	%	References
144	20	14	*Collins et al.*, 2009
77	11	14	*Gonzalez-Angulo et al.,* 2011
159	15	9	*Robertson et al.,* 2012
182	16	9	*Rummel et al.,* 2013
199	13	6.5	*Hartman et al.,* 2012
761	75	10.5	Total

2.3.9. The prognostic role of carrying germline *BRCA1* mutation

Multiple of earlier retrospective studies showed contraversial data with lower or similar survival rate for *BRCA1* mutation carriers compare to sporadic cases [*Robson et al.*, 1999; *Stoppa-Lyonnet et al.*, 2000; *El-Tamer et al.*, 2004; *Kennedy et al.*, 2002, *Robson et al.*, 2004; *Brekelmans et al.*, 2006; *Bonadona et al.*, 2007; *Rennert et al.*, 2007; *Moller et al.*, 2007*; Hagen et al.*, 2009; *Lee et al.*, 2010; *Bordeleau et al.*, 2010]. In the study by *Hagen et al*, an inferior prognosis for *BRCA1* mutation carriers was reported. There was no association found between received chemotherapy or the type of surgery and survival rates. Node-negative *BRCA1*-carriers had significantly worse overall survival rates than node-negative *BRCA1* non-carriers. Analysis of survival curves showed initially better survival for *BRCA1*-carriers that later disappeared with worse overall survival for *BRCA1* carriers. More detailed analysis of specific causes of deaths showed that deaths from ovarian cancer have had a negative effect on survival curves in *BRCA1* mutation carriers. Of 167 *BRCA1* mutation carriers 104 underwent bilateral prophylactic salpingooophorectomy with occult ovarian cancer detected in 8%. 34 (20.4%) *BRCA1* mutation carriers underwent surgery for suspected ovarian cancer [*Hagen et al.*, 2009]. A prospective study by *Moller et al.*, also showed significantly

worse survival for *BRCA1* mutation carriers, even for carriers with early stage breast cancer, compared to *BRCA2*- carriers and non- carrier [*Moller et al*., 2007]. *BRCA1* mutation carriers, who underwent oophorectomy had a trend for better 5- year survival compared to other *BRCA1* mutation carriers [*Moller et al*., 2002; *Moller et al*., 2007].

In the recently published large Dutch study 5.518 patients diagnosed with breast cancer before 50 years were included, 3.6% of patients had *BRCA1* mutation and 1.2% had a *BRCA2* mutation. 29% of patients in the *BRCA1* mutation group was ER-positive compared to 86% in the non-carriers groups and 81% in the *BRCA2*-carriers group. *BRCA1* mutation carriers were 1.5 times more likely to have breast cancer recurrence and 1.2 times more likely to die from breast cancer than non-carriers and also confirmed that early stage *BRCA1* mutation carriers have a worse prognosis [*Schmidt et al*., 2013].

A large national population-based study of Israeli women found similar breast cancer-specific survival rates for *BRCA1* mutation carriers and non-carriers. A breast cancer- specific survival at 10 years was 67% for 76 *BRCA1* mutation carriers and 67% for non-carriers (P = 0.25). 88% of deaths in the *BRCA1* carriers group occured within first 5 years after diagnosis compared to 68% in the non-carriers group (P < 0.04). There was no statistically significant difference in hazard ratios for breast –specific death adjusted for age, tumor size, lymph node status, presense of distant metastasis between *BRCA1*- carriers and non-carriers. *BRCA1* mutation carriers were less likely ER-positive than non-carriers (24% versus 65%). Interestingly, that among women who didn’t receive adjuvant chemotherapy the 10-year survival was 76% for *BRCA1* carriers and 74% for non-carriers, compared to 71% for *BRCA1* mutation carriers and 46% for non-carriers, who received adjuvant chemotherapy [*Rennert et al*., 2007].

In the study by *Robson et al*., *BRCA1* mutation status was an independent predictor of worse breast cancer-specific survival among patients,who didn’t receive adjuvant chemotherapy (HR = 4.8; 95%CI : 2.0–11.7; P < 0.001), but not in patients who received chemotherapy [*Robson et al*., 2004].

However, *Veronesi et al*., reported an equal or better prognosis for *BRCA1/2* (9 *BRCA1* and 30 *BRCA2* patients) mutation carriers compared with wild-type. A 20 years, projected survival was 85% in the *BRCA1/2* mutation carriers group and 55% in the *BRCA1/2* mutation non-carriers group, but this difference didn’t reach a statistical significance (HR = 0.9; 95%CI : 0.2–5.3, P = 0.68). This data was supported by *Cortesi et al*., publication, there was a statistically significant overall survival advantage in

BRCA1 positive patients compared to *BRCA1* mutation negative and sporadic breast cancers (77% versus 77% versus 73%, respectively; P < 0.0001).

None of these studies evaluate the prognostic significance of *BRCA1* mutation in the context of breast cancer subtypes, histological types, tumor grade, received chemotherapy regimens. However, several recent studies have focused attention on the prognostic role of positive *BRCA1* mutation status in the triple-negative breast cancer subtype and have demonstrated similar outcomes in *BRCA* mutation carriers and non-carriers [*Lee et a1*., 2011; *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 *BRCA1* mutation carriers and non-carriers (HR = 0.64; P = 0.25). In this study both groups were good balanced, all patients received alkylating chemotherapy, but the definition of triple- negative breast cancer and positivity of ER and PR cut-off levels were not specified. Futhermore, 8% of patients received hormonal treatment.

Gonzalez-Angulo et al., showed better RFS for *BRCA1* mutation positive patients treated with surgery and anthracycline-taxane chemotherapy than *BRCA1* mutation non- carriers (P = 0.031), but failed to demonstrate significant diference in overall survival (P = 0.225). The main limitation of this study was that there was a statistically significant difference in received chemotherapy between two groups and there was a missing information about accomponing cancers and breast cancer-specific survival was not evaluated.

In the *Bayraktar et al*., study 227 patients with triple-negative breast cancer were included, from 114 *BRCA* mutation carriers 94 had *BRCA1* mutation and 20 had *BRCA2* mutation. Patients with bilateral and/or metastatic breast cancer and with previous breast cancer were not included in the study population. No statistically significant difference in 5 year-overall survival rates were found between *BRCA1/2* mutation carriers and non-carriers (93% versus 85%, respectively; P = 0.11). After adjusment for patients' age and disease stage no association with *BRCA1/2* mutation status and overall survival was found (HR = 0.51; 95%CI : 0.23–1.17; P = 0.11). In this study negative ER and PR status was defined as nuclear statining ≤ 10%, and patients with previous ovarian cancer were included in the study.

The prognostic significance of separate *BRCA1* mutations were not evaluated in previous studies [*Lee et al*., 2011; *Bayraktar et al.,* 2011; *Gonzalez-Angulo et al*., 2011]. A *BRCA1* germline mutations' variants cause different changes in the structure of the BRCA1 proteins that impact breast or/and ovarian cancer risk and clinical outcomes.

For example, the worse overall survival of breast cancer *BRCA1* 4153delA mutation carriers compared with 5382insC, has been reported [*Thompson et al.*, 2002; *Plakhins et al.,* 2011].

2.3.10. *BRCA1* pathway in sporadic triple-negative breast cancers

Multiple studies showed, that the majority of sporadic basal-like breast cancers have dysfunctional *BRCA1* pathway [*Foulkes et al.*, 2004; *Turner et al.*, 2006; *Rakha et al.*, 2008]. A low BRCA1 protein expression have been reported in the sporadic basal-like breast cancer [*Yoshikawa et al.*, 1999; *Abd El-Rehim, et al.*, 2005; *Rebeiro- Silva et al.*, 2005; *Bal et al.*, 2013]. Somatic *BRCA1* mutations are found only in a small proportion of sporadic breast cancers [*Futreal et al.*, 1994; *Zhang et al.*, 2010]. Reduced BRCA1 expression may be explained by *BRCA1* promoter hypermethylation in up to 30-40% of triple-negative breast cancers [*Esteller, et al.*, 2000, *Herman et al.,* 2003; *Birgisdottir et al.*, 2006; *Lips et al.*, 2013; *Hsu et al.*, 2013, *Bal et al.*, 2013] and up to 60% of medullary [*Esteller et al.*, 2000; *Osin et al.*, 2003] and metaplastic [*Turner et al.*, 2007] breast cancers of basal-like phenotype. High levels of inhibitor of DNA binding 4 (Id4) have been reported to downregulate BRCA1 expression [*Beger et al.*, 2001]. The expression levels of Id4 were reported to be 9.1-fold higher in basal-like breast cancers than in other breast cancers [*Turner et al.*, 2007].

Study by *Hsu et al.,* showed that *BRCA1* promoter hypermethylation was significantly associated with triple-negative breast cancer subtype and poor overall and disease-free survival [*Hsu et al.*, 2013]. Several studies reported a better response to anthracycline-based and cisplatin chemotherapy for triple-negative breast cancer patients with *BRCA1* promoter methylation [*Silver et al.*, 2010; *Lips et al.*, 2013]. The recent study showed, that patients with *BRCA1*-methylated triple-negative breast cancers, who received adjuvant chemotherapy have better 10-year disease- free survival (75% versus 55%, respectively; $P<0.009$) and breast cancer-specific survival (85% versus 69%, respectively; $P < 0.024$) than patients with unmethylated triple-negative breast cancers [*Xu et al.*, 2013].

2.3.11. *TP53* mutations and breast cancer

TP53 is a 20 kb tumor supressor gene located on chromosome 17p13.1 that encodes the p53 protein [*McBride et al.,* 1986; *Kern et al*.,1991]. After activation by oncogenic stressors wild-type p53 functions as a sequence-specific DNA binding transcription factor that regulates genes involved in cell cycle arrest, DNA repair or apoptotic cell death, inhibition of angiogenesis and invasion [*Gasco et al*., 2002; *Vousden et al*., 2009]. Mutation in *TP53* results in loss of these tumor supressor functions. The frequency of mutated *TP53* or overexpression of p53 protein in human breast cancer ranges from 20-30% [*Borresen-Dale et al*., 2003] and approximately 60–88% of triple-negative / basal-like breast cancers harbour *TP53* mutations or overexpression of p53 protein [*Sorlie et al*., 2001; *Langerod et al*., 2007; *Shah et al*., 2012; *Dumay et al*., 2013]. Study by *Shah et al.,* reported that *TP53* mutations play a key role in early tumorigenesis of the triple-negative breast cancers [*Shah et al*., 2012]. *TP53* mutations predominantly occur in exons 5–8, which encode the central DNA-binding domain of the protein and approximately 10% of mutations are found outside this region [*Pharoah et al*., 1999].

Various types of *TP53* somatic mutations (point mutation, insertion, deletion, stop codon) exert different effects on p53 protein synthesis and function, and can lead to complete inhibition of protein synthesis or synthesis of functionally altered proteins that lead to different biological effects and could impact tumor clinical behavior and outcomes [*Monti et al*., 2002; *Levine et al.,* 2009; *Jordan et al*., 2010; *Freed-Pastor et al*., 2012].

A multiple studies have reported conflicting data about the prognostic role of p53 protein overexpression or *TP53* mutations in prediction of worse outcomes in breast cancer patients [*Reed et al*., 2000; *Ferrero et al*., 2000; *Overgaard et al*., 2000; *Cuny et al*., 2000; *Linderholm et al*., 2000; *Rudolph et al*., 2001; *Kato et al*., 2000; *Liu et al*., 2001; *Joensuu et al*., 2003; *Goffin et al*., 2003; *Bull et al*., 2004; *Olivier et al*., 2006] (Table 2.3.11.1).

However, only few studies have evaluated the prognostic significance of the *TP53* mutations instead of the p53 protein expression [*Pharoach et al*., 1999; *Overgaard et al*., 2000; *Cuny et al*., 2000; *Olivier et al*., 2006]. According to previously published data immunohistochemistry(IHC) of p53 protein failed to provide sufficiently accurate results and, therefore, cannot be integrated into clinical practice. Several

studies compared a complementary DNA(cDNA)-based sequencing with immunohistochemical (IHC) methods of detection of p53 alternations and concluded that use of cDNA-based sequencing method provides a more precise prognostic information than IHC [*Sjorgen et al*., 1996; *Norberg et al*., 1998]. The use of IHC is based on the fact that the missense *TP53* mutations often lead to stable protein production, that accumulates in the nucleus [*Tsuda et al*., 1994; *Ozcelik et al*., 2004]. In approximately 20 % of *TP53* mutations result in truncated p53 protein, that is unstable and cannot be detected by IHC analysis. Thus, approximately 92.9% of missense *TP53* mutations stain positive by IHC and 88.5% of truncation mutations stain negative by IHC [*Chae et al*., 2009]. *Norberg et al*., reported that a sensitivity and a specificity of IHC method to detect p53 alternations was 72.2% and 92%, respectively compared with cDNA sequencing method [*Norberg et al*., 1998]. Published data suggest, that cDNA-based sequencing method of *TP53* mutation detection provides better prognostic information than detection of p53 protein expression by IHC [*Sjorgen et al*., 1996; *Norberg et al*., 1998].

The meta-analyses of 11 studies with a total of 2319 unselected cases investigated, concluded that *TP53* mutations are strongly associated with inferior survival outcomes [*Pharoach et al*., 1999]. In the large study with 1.794 primary breast cancers included, *TP53* mutations in the exons 5-8 were associated with approximately 2-3 higher relative risk of dying from breast cancer within 10 years after surgical treatment compared to patients without mutations. Missense and non- missense *TP53* mutations in the DNA-binding domain had a similar strong negative impact on survival rates compared to *TP53* mutations non-carriers. In contrast, missense mutations outside the DNA-binding domain were associated with sligthly better prognosis than missense mutations in the DNA-binding domain [*Olivier et al*., 2006].

However, American Society of Clinical Oncology do not recommend the use of p53 as a prognostic or predictive marker for management of breast cancer patients in the routine practice, because of insuficient data to change current guidelines[*Harris et al*., 2007].

Recently, several conflicting data have been published about the prognostic value of p53 protein overexpression in the triple-negative breast cancer subtype. Some reports suggest that p53 protein overexpression could be a meaningful prognostic marker in the triple-negative breast cancer [*Nakagawa et al*., 2011; *Lee et al.,* 2011]. Similar results were reported by *Jung et al*., there was a statistically significant

difference in disease-free survival by p53 protein expression in the triple-negative breast cancer group (94.1% versus 78.7%; P < 0.002) [*Jung et al*., 2012]. *Biganzoli et al.*, showed no difference in survival estimates in the overall and non-triple-negative breast cancers by p53 expression. In the triple-negative breast cancer group p53 protein overexpression was associated with previolusly defined `basal-like` cluster and showed worse overall and event-free survival [*Ambrogi et al*., 2006; *Soria et al*., 2010; *Biganzoli et al*., 2011]. The positive predictive value of p53 protein overexpression for higher pCR after neoadjuvant chemotherapy was reported by *Guarneri et al* [*Guarneri et al*., 2010]. *Bidard et al*., showed a tendency toward a higher pCR rates in the triple-negative breast cancer patients, who received anthracyclines/alkylating agent-based regimens compared to other breast cancer subtypes (22% versus 10%, respectively; P=0.08) [*Bidard et al*., 2008]. In the group of breast cancer patients treated with adjuvant anthracycline-based chemotherapy, triple-negative breast tumors overexpressing p53 protein were associated with worse relapse-free and overal survival [*Chae et al*., 2009]. In contrast, other studies concluded that p53 protein expression cannot be used as prognostic and predictive marker in the triple-negative breast cancer and additional studies are required toanalyze the impact of carrying *TP53* mutations on response to chemotherapy and survival outcomes in the triple-negative breast cancer subtype [*Keam et al*., 2007; *Ryu et al*., 2012]. However, reports showed conflicting data with not strong enough evidence about the value of *TP53* mutations to predict response to anthracyclines or/and taxane-based treatment regimens in the breast cancer not specified by intristic subtypes [*Wahl et al*., 1996; *Cimoli et al*., 2004; *Harris et al.*, 2006; *Di Leo et al*., 2007].

Somatic *TP53* mutations are significantly more frequently presented in the *BRCA1* mutation-carriers than in non-carriers [*Phillips et al*., 1999; *Greenblatt et al.,* 2001]. *Greenblat et al*., reported different spectrum of *TP53* mutations in the *BRCA1*-carriers compared to non-carriers [*Greenblatt et al.,* 2001]. In mice mammary epithelial cells with excised exon 11 of the *BRCA1* gene mammary gland tumor oocured after long latency and only in 25% of cases. Interestingly, that in mices bearing both defective *BRCA1* and *Trp53*-null allele mammary gland tumor developed more rapidly and in almost 100% of cases[*Xu et al*., 1999; *Brodie et al*., 2001]. Association of both *BRCA1* and *TP53* loss resulted in 2-fold increase in sensitivity to doxorubicin and epirubicin cancer cells in vitro [*Fedier et al*., 2003]. There was found a statistically significant difference between *BRCA1/2* mutation non-carriers/p53 protein

overexpressing and *BRCA1/2* mutation carriers/p53 protein overexpressing breast cancer patients in overall and disease-free survival, but multivariate analysis failed to show significant interactions between *BRCA1* mutation status and p53 IHC status [*Goffin et al*., 2003].

Table 2.3.11.1

The prognostic role of p53 protein overexpression or *TP53* mutations in prediction of survival outcomes in the breast cancer patients

References	No. of cases	Case selection	Follow-up (months)	Method	Prognostic value (Univariate analysis)		Prognostic value (Multivariate analysis)	
					Disease-free survival	Overall survival	Disease-free survival	Overall survival
Linderholm et al., 2000	833	Node-positive and node-negative breast cancers	56	cytosol p53	No	Yes	No	Yes
Reed et al., 2000	613	Node-negative breast cancers	307	IHC	No	No	No	No
Overgaard et al., 2000	294	Sporadic early breast cancer	294	Mutations	Yes	Yes	Yes	Yes
Goffin et al., 2003	278	Ashkenazi Jewish women younger than 65 years, invasive breast carcinoma	96	IHC	Yes	Yes	No	No
Bull et al., 2004	543	Node-negative Breast cancer	85	SSCP	Yes	Yes		

Table 2.3.11.1 (end)

References	No. of cases	Case selection	Follow-up (months)	Method	Prognostic value (Univariate analysis)		Prognostic value (Multivariate analysis)	
					Disease-free survival	Overall survival	Disease-free survival	Overall survival
Ferrero et al., 2000	297	Node-negative breast cancer	132	IHC	Yes	Yes	No	No
Liu et al., 2001	331	Node-negative breast cancer	190	IHC	Yes	No	No	No
Joensuu et al., 2003	852	Unilateral pT1N0M0	114	IHC	Yes	-	No	No
Olivier et al., 2006	1.794	Unilateral breast cancer	120	Mutations	No	Yes	No	Yes
Cuny et al., 2000	363	Node-negative breast cancer, no neoadjuvant chemotherapy	66	Mutations	Yes	Yes	Yes	Yes
Rudolph et al., 2001	261	Node-negative, invasive ductal breast cancer	96	IHC	Yes	Yes	No	No
Kato et al., 2001	260	Japanese patients with node-negative breast cancer	240	IHC	Yes	Yes	No	No

SSCP – single-stranded comformational polymorphism; IHC – immunohistochemistry

2.3.12. Systemic treatment

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 recommended [*NCCN*; *Goldhirsch et al.*, 2011; *Aebi et al.*, 2010]. According to *Scwentner et al.*, retrospective study of 3.659 patients there was a strong evidence that guideline violations negatively impact triple-negative breast cancer disease-free and overall survival [*Schwentner et al.*, 2011]. Several studies reported, that the absence of ER expression in breast tumors predicts better response to polychemotherapy compared to ER-positive breast tumors [*Berry et al.*, 2006; *Clarke et al.*, 2008]. A collaborative meta-analyses of clinical data for 6.000 ER- negative breast cancer cases enroled in 46 trials concluded that patients who received non-taxane-based polychemotherapy versus no chemotherapy have a significantly lower risk of recurrence, breast cancer mortality and death from any cause [*Clarke et al.*, 2008]. A retrospective analysis of MA5 adjuvant trial found that axillary lymph node positive premenopausal basal-like breast cancer (defined by five immunohistochemical markers- ER/PR negative, HER2/neu negative, EGFR and CK5/6 positive) patients treated with CMF (cyclophosphamide, methotrexate, 5-fluouracil) showed significantly better 5-year survival compared to CEF (cyclophosphamide, epirubicin, 5-fluorouracil) regimen (71% versus 51%, respectively) [*Cheang et al.*, 2009; *Joensuu et al.*, 2012]. Results of International Breast Cancer Study Group Trials VIII and XI there operable, lymph node negative 2.257 breast cancers were included showed, that triple-negative breast cancers have a statistically convincing higher benefit from three or six CMF cycles versus no chemotherapy than hormone- receptor negative breast cancers (HR = 0.46; 95%Cl : 0.29–0.73; P < 0.009) [*Colleoni et al.*, 2010]. A post-hoc analysis of a phase III trial performed by *Rocca et al.*, showed that triple-negative breast cancer patients with very high (> 40%) Ki-67 index had a significantly worse 5-year disease-free survival and overall survival than treated with CMF compared to CMF plus epirubicin. It was concluded, that very high Ki-67 index identify triple-negative breast cancer patients, who are likely to benefit from epirubicin [*Rocca et al*,. 2011].

Triple-negative breast cancers have a higher pCR rates after anthracycline-containing neoadjuvant treatment compared to non-triple- negative breast cancers (22% versus 11%, respectively; P < 0.034), with excellent survival estimates in those who

achieved pCR [*Liedtke et al*., 2008]. The large meta-analysis study, there 6.377 patients with operable or locally advanced, non-metastatic breast cancer, who received neoadjuvant anthracycline-taxane-based treatment were included, revealed, that only pCR defined as no residual invasive or in situ cancer both in the breast and lymphnodes (pT0N0) are suitable surrogate to predict a favourable prognosis in tirple-negative breast cancer group [*von Minckwitz et al*., 2012]. *Rastogi et al*., showed no statistically significant difference in disease-free and overall survival between triple-negative breast cancer patients, who received the same anthracycline-based regimen in neoadjuvant versus adjuvant settings. A study by *Di Leo et al*., showed that anthracycline-based therapy are superior than CMF regimen among triple-negative breast cancer patients [*Di Leo et al*., 2008]. Neoadjuvant docetaxel added to doxorubicin and cyclophosphamide (AC) significantly improved pCR rate compared to AC alone (26% versus 13%, respectively; $P < 0.0001$) [*Rastogi et al*., 2008]. In the GEPAR Trio study operable or locally advanced triple-negative breast cancer patients treated with TAC (docetaxel/doxorubicin/cyclophosphamide) in 39% achieved a pCR, defined as no invasive disease in both breast and axilla. Tumor grade and age at diagnosis was a favourable predictors of increased benefit from noadjuvanvt treatment among triple-negative breast cancer with pCR rate of 57% in this patients'subset [*Huober et al*., 2010]. The meta-analysis of 12 randomized phase III trials showed that adjuvant docetaxel-based chemotherapy improves disease-free and overall survival in triple-negative breast cancer [*Laporte et al*., 2009]. *Martin et al*., reported that adjuvant TAC (docetaxel, doxorubicin, cyclophosphamide) is superior than adjuvant FAC (5-fluouracil, doxorubicin, cyclophosphamide) in treatment of high-risk (at least one high-risk factor according to the St. Gallen criteria) axillary-lymph-node – negative breast cancer patients. TAC showed higher disease-free survival benefit than FAC in triple-negative breast cancer group (HR = 0.59; 95%Cl : 0.32–1.07; P = 0.08) [*Martin et al*., 2010]. In agreement, the BCIRG 001 trial demostrated that adjuvant TAC show a trend to higher 3-year disease-free benefit from TAC compared to FAC among patients with triple-negative breast cancer (74% versus 60%, respectively; HR = 0.50; 95%Cl : 0.29–1.00; P = 0.051) [*Hugh et al*., 2009]. *Sparano et al*., reported that weekly paclitaxel after adjuvant 4-cycles of intravenous doxorubicin and cyclophosphamide improved disease-free (HR = 1.37; 95%Cl : 0.98–1.93) and overall survival (HR = 1.33; 95% Cl : 0.91–1.94) in women with triple-negative breast cancer compared to paclitaxel every 3 weeks [*Sparano et al*., 2008]. In contrast, the TACT trial fail to show overall benefit

from addition of docetaxel to standart athracycline-based treatment for ER-negative, HER2-negative breast cancers [*Ellis et al*., 2009].

BRCA1-related, triple-negative, basal-like breast tumors 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].

In studies on an experimental cell system *BRCA1*-defective cell lines have shown higher sensitivity to platinum agents compared to *BRCA1* competent cell lines and resistance to taxanes [*Tassone et al*., 2003]. Platinum agents in the retrospective neoadjuvant setting showed higher pCR rates in *BRCA1* carriers in comparison to other agents [*Byrski et al*., 2009]. The significantly superior pCR(88%) after neoadjuvant platinum-based regimens in the triple-negative breast cancer have been reported in compare to pCR of 51% in the non- triple-negative breast cancer with a trend for worse 5-year overall survival in earlier triple-negative breast cancer. For patients with advanced disease pCR rates were also higher in the triple-negative breast cancer group (41% vs. 31%), along with a trend for improved survival outcomes [*Sirohi et al*., 2008]. A randomized clinical trials are now underway to clarify the efficacy of platinum-based regimens in compare with conventional regimens in the triple-negative breast cancer patients [NCT00532727; NCT00861705]. The evidence for use of taxanes in the *BRCA1*-related is limited and controversial. A number of small clinical trials have reported a lower sensitivity to taxane chemotherapy for hormone receptor negative metastatic *BRCA1/2*-carriers [*Kriege et al*., 2011] and for metastatic triple-negative breast cancer patients with low level of BRCA1 [*Kurebayashi et al.,* 2006].

Poly (adenosine diphosphate) ribose polymerases (PARP) are nuclear enzymes involved in the base excision repair pathway. Inhibition of PARP in *BRCA1/2* deficient cells results in accumulation of double-strand DNA break that leads to cell death. This phenomenon is called a "synthetic lethality" [*Bryant et al*., 2005; *Farmer et al*., 2005; *Ashworth et al*., 2008]. The intravenous PARP1 inhibitor iniparib(BSI 201) in phase II randomized trial in metastatic triple-negative breast cancer in combination with gemcitabine and carboplatine significantly improved the median overall survival (12.3 versus 7.7 months; P = 0.01) and progression-free survival (5.9 versus 3.6 months; P = 0.01) [*O'Shaughnessy et al*., 2011]. Although in phase III trial with identical chemotherapy regimens failed to meet the pre-specified criteria for primary endpoints for overall survival and progression-free survival [*O'Shaughnessy et al*., 2011]. In a

phase I trial oral PARP inhibitor olaparib showed in 63% clinical benefit rate (radiological or tumor marker response or stable disease for at least 4 months) in patients with *BRCA1*-related cancers with low toxicity rate [*Fong et al*., 2009]. A multicentre phase II study proof the efficacy and acceptable safety of olaparib in patients with *BRCA* mutation related breast cancers [*Tutt et al*., 2010].

3. MATERIALS AND METHODS

3.1. The study design

The study was designed as a combined prospective-retrospective cohort:

In the prospective part of the study invasive breast cancer patients, who have given written consent to have their blood and tissue samples used for DNA analysis were tested for germline *BRCA1* founder mutations and clinical data were prospectively obtained.

In the retrospective part of the study an analysis of somatic *TP53* mutations was retrospectively performed in the triple-negative breast cancer group and correlation between somatic *TP53* mutations and clinical outcomes were retrospectively analysed.

3.2. The study group

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 Stradinš 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 3.2.1.);

3.2.1. 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 3.2.2.) 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.

3.2.2. 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.

3.3. Biological sample banking

The prospective phase of the study.

Tumor pathology blocks from Riga Eastern Clinical University Hospital and Daugavpils regional hospital were collected and prospectively reviewed by dedicated breast pathologists. All breast pathologic specimens from Pauls Stradins Clinical University hospital were analyzed by dedicated breast pathologists.

The retrospective phase of the study.

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

3.4. Pathological examination and immunohistochemistry

3.4.1. Human breast tumor tissue collection and histopathology

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.

3.4.2. Immunohistochemistry

Estrogen (ER) and progesterone (PR) status and Ki-67 index were determined using standard immunohistochemistry (IHC). The 3-µm tissue sections were cut from paraffin-embedded blocks on a microtome and mounted on electrostatic Histobond slides (Marienfeld, Germany). Sections were allowed to dry at 60° for 1 hour. After that tissue section were deparaffinized and rehydrated using four changes of xylene (5 min each station) and decreasing concentrations of alcohols (99.9% 2 changes for 3 min, 96% 4 changes for 3 min, 70% 1 change for 5 min). The intristic peroxidase activity was blocked with methanol and 0.5% hydrogen peroxide for 10 min. The tissue sections were immersed in a TBS solution for 5 min and treated with heat in a microwave oven ($3 \times$ 5min) in a alkaline (TEG, pH 9.0, Tris base 10 mM/L, EGTA 0.5 mM/L) buffer and allowed to cool for 20 min at room temperature. The sections were encircled with a hydrophobic pen (*Dako*, Glostrup, Denmark) and placed in the magnetic containers (*CellPath plc)*. After the immersion in a TBS (pH 7.6 Tris buffered saline, THAM-HCI 50 mMl/L, NaCl 150 mM/L) buffer for 5 min, the sections were incubated with primary antibodies for 20 min at room temperature. The following primary antibodies were

used: Monoclonal Mouse Anti-Human Estrogen Receptor alpha, Clone 1D5, Code M7047(*DAKO Cytomation*, Clostrup, Denmark) (dilution 1:1), Monoclonal Mouse Anti-Human Progesterone Receptor, Clone PgR 636, Code M3569 (*DAKO Cytomation*, Clostrup, Denmark) (dilution 1:1) , Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1, Code M7240 (*DAKO Cytomation*, Clostrup, Denmark) (dilution 1:100). After the incubation sections were washed in phospate-buffered saline and incubated for 30 min with secondary biotinylated antibody, and incubated for 30 min with streptavidin peroxidase complex, and 3,3'-diaminobezidine (LSAB2 visualisation system). After the staining procedure was completed, the sections were dehydrated, cleared and mounted using permanent mounting medium. Negative and positive control slides were included in each assay. 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].

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].

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). The FISH results were expressed as a ratio of of HER-2/ neu signal to CEP 17 signal and were interpreted as positive (amplified) when the ratio was ≥ 2.2 and negative (unamplified) when the ratio was < 2.2 for gene amplification according to the manufacturer's recommended scoring system. In the absence of HER-2/neu gene amplification, tumors scored 2+ by IHC were considered as

negative for HER2/neu. 5 ER/PR negative cases with equivocal HER2/neu IHC (2+) test results were retested by FISH. In 4 cases FISH were negative and 1 case FISH was postitive. Patient with postitive FISH result was not included in the study and received Herceptine.

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.

3.5. Molecular diagnostics

3.5.1. *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.

3.5.2. Detection of sporadic *TP53* gene mutations

First, purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution was used.

The equipment:

Bio Vortex V1 (Biosan, Latvia)

Centrifuge 5415D (Eppendorf, Germany)

Thermoblock TDB-120 (Biosan, Latvia)

Spectrophotometer Nanodrop ND1000(Thermo Scientific, USA)

Automated micropipettes(Eppendorf, Germany)

Microcentrifuge with rotor for 2 ml tubes

The reagents:

QIAmp DNA FFPE Tissue Kit (Qiagen, Germany)

Buffer ATL

Buffer AL

Buffer AW1

Buffer AW2

Buffer ATE

Proteinase K

96% Ethanol

Deparaffinization Solution

Up to 8 sections each with a thickness of up to 10 µm tissue sections were cut from paraffin-embedded blocks on a microtome and immediately placed in a 1.5 ml microcentrifuge tube. 320 µl Deparaffinization Solution was added. The tube was subsequently vortexed vigorously for 10 seconds and briefly centrifuged to collect sample in the bottom of the tube. After that, the tube was incubated at 56 °C for 3 minutes, and then allowed to cool at room temperature(15–25 °C). Subsequently 180 µl Buffer ATL was added, and mixed by vortexing. The tube was centrifuged for 1 minute at 11.000 x g. After that, the 20 µl proteinase K to the lower, clear phase was added and mixed by pippeting. The tube was incubated at 56 °C until the complete lysation of the sample and then subsequently incubated at 90 °C for 1 hour. The 1.5 ml tube was briefly centrifuged to remove drops from inside the lid. The lower, clean phase was transfered into a new 1.5 ml microcentrifuge tube. 200 µl Buffer AL was added to the sample, and mixed throughly by vortexing. Then 200 µl 96% ethanol was added, and mixed throughly by vortexing. The 1.5 ml tube was briefly centrifuged to remove drops from the inside of the lid. The entire lysate was transfered to the QIAamp Min Elute column, and centrifuged at 8000 rpm for 1 minute. The QIAamp MinElute Column was placed in a clean 2 ml collection tube, and the collection tube containing the flow-through was discarded. The 500µl Buffer AW1 was added and the sample was centrifuged at 8000 rpm for 1 minute. After that, the QIAamp MinElute Column was placed in a clean 2 ml collection tube, and the collection tube containing the flow-through was discarded. The 500µl Buffer AW2 was added and the sample was centrifuged at 8000 rpm for 1 minute. After that, the QIAamp MinElute Column was placed in a clean 2 ml collection tube, and the collection tube containing the flow-through was discarded. The samples was centrifuged at full speed (13.000 rpm) for 3 minutes to dry the membrane completely. The QIAamp MinElute column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the flow-through discarded. 50µl Buffer ATE was applied to the centre of the membrane of the QIAamp MinElute column. The sample was incubated at room temperature for 5 minutes and centrifuged at full speed (13.000 rpm) for 1 minute.

NanoDrop® ND-1000 spectrophotometer is used to detect the average concentrations and purity of DNA present in the solution. Spectrophotometric analysis

for nucleic acids concentration is based on measurements of absorbtion intensity of electromagnetic radiation at specific wavelenghts. Result is calculated expressed as nanograms in microliter (ng/µl). The ratio of absorbance at 260nm / 280nm and 260nm/230nm is used to assess the purity of DNA. The 260/280 ratio of 1.7–1.9 is generally accepted as "pure" for DNA. If the 260nm / 280nm ratio is not in the range of 1.7–1.9, it may indicate the presence of a contaminating protein. The 260nm / 230nm ratio of 2.0–2.2 is generally accepted as "pure". If the 260nm / 230nm ratio is not in the range of 2.0–2.2, it may indicate the presence of a contaminating carbohydrates.
The NanoDrop software application module "Nucleic Acid" was used.

The equipment:

Spectrophotometer Nanodrop ND1000 (Thermo Scientific, USA)

Automated micropipettes (Eppendorf, Germany)

The reagents:

DNA in ATE buffer

Buffer ATE (Qiagen, Germany)

Nuclease-free water (Qiagen, Germany)

Somatic *TP53* mutations were analysed in exons 5, 6, 7 and 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). Before HRM analysis RT-PCR was used to amplify and quantify the targeted DNA region. At the end-point of each amplification cycle the amount of amplicons produced were measured by the use of fluorescent marker. Fluorescent reporter used in the RT-PCR was intercalating SYTO 9 dye, that binds specifically to double-stranded DNA. As amplicons accumulate, the dye generates a signal that is directly proportional to the amount of double-stranded DNA.

The equipment:

Rotor Gene™ 6000 (Qiagen, Germany)

Automated micropipettes (Eppendorf, Germany)

NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, USA)

The reagents:

2.5 mM dNTP mixture (Fermentas, Lithuania)

Taq DNA polymerase (Qiagen, Germany)

10x Taq polymerase buffer (Qiagen, Germany)

25mM $MgCl_2$ (Fermentas, Lithuania)

5 mM Syto 9 Green Fluorescence Nucleic Acid Stain

Nuclease-free water (Qiagen, Germany)

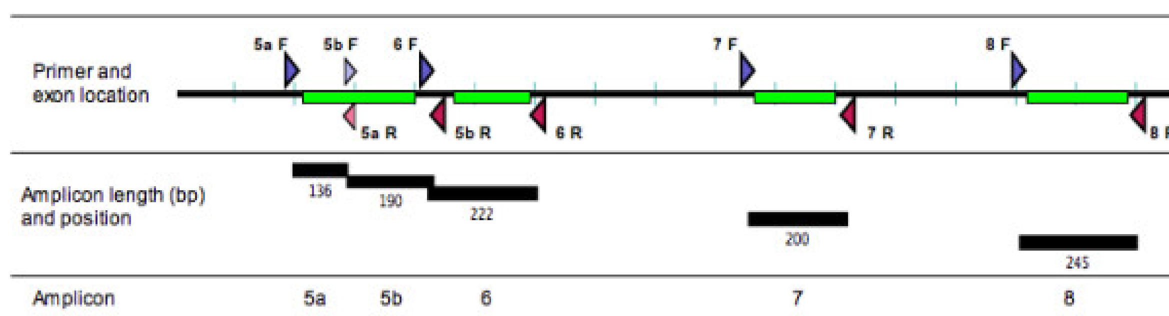
Primers (Table 3.5.2.1. and Figure 3.5.2.1.)

3.5.2.1. Table

***TP53* sequencing primers and annealing temperature conditions**

Exon	Primer name	Sequence	Annealing temperature
5a	*TP 53*_ Exon5a_ Forward primer	CAACTCTGTCTCCTTCCTCTTCCTAC	65–55 °C touchdown 0.5 °C/ cycle for 10 cycles
5a	*TP 53* _Exon5a_ Reverse primer	AGCCATGGCACGGACGCG	
5b	*TP 53* _Exon5b_ Forward primer	CTCCTGCCCGGCACCCGC	65–55 °C touchdown 0.5°C/ cycle for 10 cycles
5b	*TP 53* _Exon5b_ Reverse primer	5’-CTAAGAGCAATCAGTGAGGAATCAGA-3’	
6	*TP 53* _Exon6_ Forward primer	5’-CAACCACCCTTAACCCCTCCT-3’	68–58 °C touchdown 1.0 °C/ cycle for 10 cycles
6	*TP 53* _Exon6_ Reverse primer	5’-AGACGACAGGGCTGGTTGC-3’	
7	*TP 53*_ Exon7_ Forward primer	5’-AGGCGCACTGGCCTCATC-3’	68–58 °C touchdown 1.0 °C/ cycle for 10 cycles
7	*TP 53* _Exon7_ Reverse primer	5’-GAGGCTGGGGCACAGCA-3’	
8	*TP 53* _Exon8_ Forward primer	5’-GACCTGATTTCCTTACTGCCTCTTG-3’	63.5–58.5 °C touchdown 0.5 °C/ cycle for 10 cycles
8	*TP 53* _Exon8_ Reverse primer	5’-AATCTGAGGCATAACTGCACCCTT-3’	

Adapted from *Krypuy et al*., 2007 with slight modification



3.5.2.1. Figure. The *TP53* seguencing primers binding sites

F – forward primer; R – reverse primer

Adapted from *Krypuy et al*., 2007

The PCR reaction mixture was made using the components shown in Table 3.5.2.2.

3.5.2.2. Table

The standard RT-PCR reaction mixture (a total volume 20µl)

The reagents	Amount for 1 reaction
Nuclease-free water	11.9 µl
Syto 9 dye	1.0 µl
10 x Taq polymerase buffer	2.0 µl
25 mM MgCl2	0.5 µl
2.5 mM dNTP mixture	0.4 µl
Forward primer (10pmol)	1.0 µl
Reverse primer (10pmol)	1.0 µl
Taq DNA polymerase	0.2 µl
DNA (5 ng/ µL)	2.0 µl

The DNA samples were diluted with nuclease- free water to a concentration of 5 ng/µL. DNA concentration was measured using NanoDrop® ND-1000 Spectrophotometer. 2 µl of test DNA and 18 µl of the prepared PCR mixture were stirred with the pipette in the 0.2 ml microcentrifuge tube or in 100-well plates. All PCR reactions was performed in triplicate. The 100-well plates were sealed using a 100 µm polymer film on a hot plate. Next, the PCR tubes or 100-well plates were placed into Rotor Gene™ 6000 and subjected to the following program:

Initial DNA denaturation	95 °C	15 min	
DNA denaturation	95 °C	10 s	× 50
Primers binding	58 °C*	5 s	× 50
DNA synthesis	72 °C	20 s	× 50
Final DNA synthesis	95 °C	1 s	× 50
Pause	72 °C	90 s	× 50

*Primer annealing temperature are shown in Table 3.5.2.1.

After the final cycle of the RT-PCR reaction the HRM step was performed. HRM analysis was used to detect variations in nucleic acid sequences. In the study method described by *Krypuy et al.,* was used. The amplified products were reheated and denaturated raising the temperature by 0.1 °C per 1 second from 72 °C to 95 °C that resulted in decrease in fluorescent signal as the DNA became single-stranded. These data were reported as graphs-melting curves that showed the relation between the level

of fluorescence and temperature. DNA sequence alterations changed the shape of a HRM curves.

HRM curve analysis was performed with Rotor-Gene Q Series Software 1.7 and analysed by two scientists. Mutations detected by RT-PCR/HRM were confirmed by DNA sequencing.

Next, *TP53* gene sequencing was performed.

The equipment:

Centrifuge 5810R(Eppendorf, Germany)

Genetic Analyzer 3130(Applied Biosystems, USA)

Automated micropipettes(Eppendorf, Germany)

NanoDrop® ND-1000 Spectrophotometer(Thermo Scientific, USA)

The reagents:

2.5 mM dNTP mixture (Applied Biosystems, USA)

10x TaKaRa PCR buffer (TaKaRa, Japane)

Taq HS DNA polymerase(250U)(5 units/µl)(TaKaRa, Japane)

MinElute 96UF PCR Purification Kit(Qiagen,Germany)

BigDye Terminator v3.1 Cycle sequencing kit(Applied Biosystems, USA)

BigDye sequencing buffer 5x (Applied Biosystems,USA)

10x Genetic Analyzer Buffer w/EDTA (Applied Biosystems, USA)

5x Q-Solution (Qiagen, Germany)

HI-Di Formamide (Applied Biosystems, USA)

Polymer POP-7 (Applied Biosystems, USA)

3M Sodium acetate(Ambion, USA)

Nuclease-free water(Qiagen, Germany)

Ethanol 96%, 70%

The PCR mixture for 1 reaction was made using the following components(a total 50 µl):

10x TaKaRa PCR buffer	5 µl
2.5 mM dNTP mixture	0.5 µl
Forward primer (Table 2.6.1.)	1 µl
Reverse primer (Table 2.6.1.)	1 µl
TaKaRa Taq HS DNA polymerase(5 units/µl)	0.2 µl
Nuclease-free water	26.3 µl
5x Q-Solution	10 µl

6 µl (50 ng) of test DNA and 44 µl of the prepared PCR mixture were stirred with the pipette in the PCR 96-well plates. Next, the PCR plate was sealed with sealing tape and placed into PCR-thermocycler and subjected to the following program:

95 °C	5 min	
95 °C	30 s	
55 °C*	45 s	× 40
72 °C	60 s	
72 °C	3 min	
40 °C	Pause	

*Primer annealing temperature are shown in Table 3.5.2.1.

DNA fragments were purified with the MinElute 96UF PCR Purification Kit according to the manufacturer's instructions.

The concentration of the purified DNA samples was determined using NanoDrop® ND-1000 Spectrophotometer. The concentration of purified PCR product should be 5 ng/µl. For sequencing reaction a 7.5 ng of purified PCR product is required. If the concentration of PCR product after purification was to high, the sample was diluted with nuclease-free water. If the concentration of PCR product was to low, a greater volume of PCR product was used and the volume of nuclease-free water in the PCR mixture was reduced respectively.

Next, the sequencing reaction was performed. The mixture for 1 reaction was made using the following components(a total reaction volume 20 µl):

5x Sequencing dilution buffer	4 µl
Big Dye Terminator Mix v3.1.	2 µl (1:4)*
Primer (Table 2.6.1.)	1 µl
PCR product(concentration of 5 ng/µl)	1.5 µl
Nuclease-free water	11.5 µl

*BigDye v3.1. 0.5 µl nuclease-free water 1.5 µl

Sequencing reactions was performed in duplicate, using forward and reverse primers in order to correct for sequencing errors. 20 µl of sequencing reaction was transfered to the PCR 96-well plates. Next, the PCR plate was sealed with sealing tape and placed into PCR-thermocycler and subjected to the following program:

95 °C	1 min		
94 °C	25 s		
55 °C*	20 s		× 50
60 °C	30 s		
72 °C	40 s		
4 °C	5 min		

*Primer annealing temperature are shown in Table 3.5.2.3.

3.5.2.3.Table

***TP53* sequencing primers and annealing temperature conditions**

Exon	Primer name	Annealing temperature
5a	*TP 53*_ Exon5a_ Forward primer	55 °C
5a	*TP 53* _Exon5a_ Reverse primer	55 °C
5b	*TP 53* _Exon5b_ Forward primer	55 °C
5b	*TP 53* _Exon5b_ Reverse primer	55 °C
6	*TP 53* _Exon6_ Forward primer	60 °C
6	*TP 53* _Exon6_ Reverse primer	60 °C
7	*TP 53*_ Exon7_ Forward primer	60 °C
7	*TP 53* _Exon7_ Reverse primer	60 °C
8	*TP 53* _Exon8_ Forward primer	58.5 °C
8	*TP 53*_ Exon8_ Reverse primer	58.5 °C

2 µl 20 mM sodium acetate (freshly diluted) and 60 µl 96% ethanol was added to each PCR-plate well. The plate was spinned at 3200 rpm for 40 minutes 4 °C. Next, the plate was inverted and spinned briefly for 2–3 s to remove excess ethanol / sodium acetate. Then 70 µl 70% ethanol was added to each well and spinned at 3200 rpm for 15 minutes 4 °C. The plate was inverted, spinned briefly for 2–3 s to remove excess ethanol/sodium acetate and placed into PCR-thermocycler at 95 °C for 1 min.

After 20 µl Hi-Di formamide was added to each well. The PCR plate was covered with adhesive film, placed into PCR-thermocycler and subjected to the following program:

95 °C	2 min
4 °C	5 min

Plate was removed from the thermocycler block. Next, an adhesive sealing film was removed and reaction plate was sealed with rubber 96-well septa. The sample plate with septa was placed on the plate base and the plate retainer was snapped onto the plate and plate base. Than the sample plate was loaded into the Genetic Analyzer 3130. DNA sequencing by capillary electrophoresis was performed according to the standard protocol (Applied Biosystems) and using the 36 cm capillary array and 3130 POP-7 polymer.

Data analysis was performed using Applied Biosystems software for DNA sequencing, SeqScape and NCBI BLAST.

Initially Sequencing analysis software was used to evaluate the quality of DNA sequences – the quality value (QV). DNA sequences with low quality value (0–14) was considered unsuitable for analysis. For DNA sequences with medium quality value (15–19) manual DNA sequences analysis was performed. Whereas, high quality value (> 20) indicates that DNA sequence is high quality, pure and precise.

After that SeqScape software was used for reference based analysis, SNP and frameshift mutations discovery and validation. SNPs (single nucleotide polymorphisms) were confirmed using NCBI BLAST software, that aligns PCR product sequences against those in the sequence databases. For interpretation of the results several databases were used: SNP – NCBI (National Center for Biotechnology Information), COSMIC (Catalogue of Somatic Mutations In Cancer).

3.6. 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. The database contains information regarding patients' clinicopathological characteristics, received chemotherapy and surgical treatment, local and distant recurrence, survival, *BRCA1/2* genetic testing results and family history, accompaning cancers. Survival data were supplemented with Latvian cancer registry data- prospective database of Centre for Disease Control and Prevention.

3.7. Treatment

All patients underwent definitive surgery. The types of chemotherapy received and postoperative radiotherapy were at the discretion of the multidisciplinary treating team.

3.8. Follow-up

The routine follow-up was performed every 3–6 months for 3 years, every 6–12 months for 4–5 years and annually thereafter. 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.

3.9. 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.

3.10. Statistical methods

Clinicopathological characteristics and treatment modalities of *BRCA1* negative luminal A, luminal B HER2 negative and triple-negative *BRCA1* mutation carriers and

non-carriers were compared using a chi-square, Fisher's exact test, independent samples t-test and one-way analysis of variance (ANOVA).

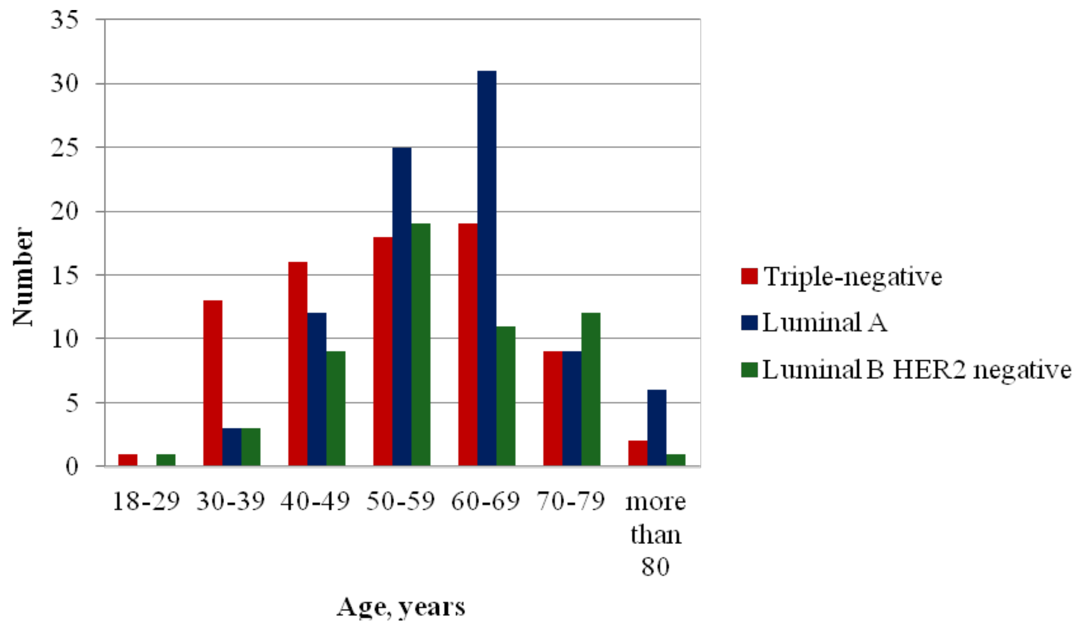
The univariate and multivariate Cox proportional hazards models were used to compute independent predictors of BCS and DRFS. The following prognostic variables were analyzed: age, T stage, nodal status, clinical stage, *BRCA1* status, *TP53* status, type of surgery performed, radiation and chemotherapy. 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. Statistical analysis was performed using the statistical software SPSS version 16.0.

4. RESULTS

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

4.1.1. The clinicopathological characteritics of 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 (range, 31–82 years), in the luminal A breast cancer group the mean age at diagnosis was 60.1 years (range, 30–84 years) and in the luminal B HER2 negative breast cancer group the mean patients' age was 57.2 (range, 25–80 years). Patients in the triple-negative breast cancer group was statistically significantly younger than in the luminal A group ($P < 0.004$). Patients in the triple-negative breast cancer group were more younger than in the luminal B HER2 negative group, but the difference didn't reach statistical significance ($P = 0.18$). 30 (38.5%) patients in the triple-negative breast cancer group were diagnosed before age 50 compared to 15 (17.4%) in the luminal A breast cancer group and 13 (23%) patients in the luminal B HER2 negative breast cancer group ($P < 0.008$). 48 (61.5%) patients in the triple-negative breast cancer group were diagnosed before age 60 compared to 40 (46.5%) patients in the luminal A breast cancer group and 32 (57.1%) patients in the luminal B HER2 negative breast cancer group ($P = 0.14$) (Figure 4.1.1.1.).



4.1.1.1. Figure. The age distribution in the triple-negative, luminal A and luminal B HER2 negative breast cancer groups

Patients' age under 50 years in the triple-negative breast cancer group didn't correlate with the histological type (P = 0.96), poor differentiation of the tumor (P = 0.56), advanced T stage (P = 0.87), positive nodal status (P = 0.15), stage of the disease (P = 0.54), the type of surgery (P = 0.17) and likelihood of getting chemotherapy (P = 0.29) or radiation therapy (P = 0.51). In the luminal A group breast cancer group there was a marginally significant correlation between age at diagnosis under < 50 years and sentinel node biopsy (P = 0.05), and a statistically significant correlation between age at diagnosis under < 50 years and likelihood to receive chemotherapy (P < 0.038). Patients' age under 50 years in the luminal A breast cancer group didn't correlate with the histological type (P = 0.83), poor differentiation of the tumor (P = 0.94), advanced T stage (P = 0.95), positive nodal status (P = 0.64), stage of the disease (P = 0.78), the type of surgery (P = 0.18) and likelihood of getting radiation therapy (P = 0.72). There was no correlation in the luminal B HER2 negative breast cancer group between age at diagnosis under < 50 years and the histological type (P = 0.43), poor differentiation of the tumor (P = 0.64), advanced T stage (P = 0.10), positive nodal status (P = 0.13), stage of the disease (P = 0.97), the type of surgery (P = 0.99) and likelihood of getting chemotherapy (P = 0.31) or radiation therapy (P = 0.48).

Histopathological features of breast cancer subtypes are shown in Table 4.1.1.1. The majority of triple-negative, luminal A and luminal B HER2 negative breast cancers

were classified as ductal carcinomas. No significant difference was found between breast cancer subgroups in percentage of cases of ductal and lobular breast cancers. Triple-negative subgroup was more likely to have medullary breast cancer. Luminal A breast cancers had significantly higher ratio of grade I tumors than triple-negative and luminal B HER 2 negative breast cancers. The majority of luminal B HER2 negative breast cancers were grade II and grade III breast cancers. 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)

4.1.1.1. Table

The distribution of histological subtypes and tumor differentiation grade in the *BRCA1* negative triple- negative, luminal A and luminal B HER 2 negative breast cancer groups

Characteristics	Triple negative breast cancer n=78 No. of patients (%)	Luminal A breast cancer n=86 No. of patients (%)	Luminal B Her2 negative breast cancer n=56 No. of patients (%)	P-value*
Histology				
Ductal carcinoma	61 (78.2%)	62 (72.1%)	48 (85.7%)	P = 0.16
Lobular carcinoma	12 (15.4%)	20 (23.2%)	6 (10.7%)	P = 0.13
Medullary carcinoma	4 (5.1%)	0 (0%)	0 (0%)	P < 0.02
Apocrine	1 (1.3%)	0 (0%)	0 (0%)	P = 0.40
Mucinous	0 (0%)	4 (4.7%)	2 (3.6%)	P = 0.17
Tumor grade				

4.1.1.1. Table (end)

Characteristics	Triple negative breast cancer n=78 No. of patients (%)	Luminal A breast cancer n=86 No. of patients (%)	Luminal B Her2 negative breast cancer n=56 No. of patients (%)	P-value*
Well-differentiated	0 (0%)	19 (30.6%)	2 (4.2%)	P < 0.0001
Moderately differentiated	12 (16.4%)	32 (51.6%)	23 (47.9%)	P < 0.0005
Poorly differentiated	49 (83.6%)	11 (17.8%)	23 (47.9%)	P < 0.0001

*Chi-square analysis

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), but difference didn’t reach statistical significance than compared with luminal B HER2 negative breast cancer group (32.9 mm versus 28.4 mm, respectively; P = 0.23). 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 (Table 4.1.1.2.).

4.1.1.2. Table

The differences in tumor size, T stage, nodal status and stage of disease between the *BRCA1* negative triple-negative, luminal A and luminal B HER 2 negative breast cancers

Characteristics	Triple negative breast cancer n=78 No. of patients (%)	Luminal A breast cancer n=86 No. of patients (%)	Luminal B HER2 negative breast cancer n=56 No. of patients (%)	P-value*
T stage				
T1	21 (26.9%)	52 (60.5%)	11 (19.6%)	P < 0.0001
T2	38 (48.7%)	23 (26.7%)	36 (64.3%)	P < 0.0001
T3	12 (15.4%)	4 (4.7%)	6 (10.7%)	P = 0.07
T4	7 (18.4%)	7 (8.1%)	3 (5.4%)	P = 0.72
Nodal status				
N0	30 (38.5%)	61 (70.9%)	24 (42.9%)	P < 0.0001
N1	24 (30.8%)	17 (19.8%)	18 (32.1%)	P = 0.23
N2	16 (20.5%)	6 (7.0%)	10 (17.9%)	P < 0.03
N3	8 (10.2%)	2 (2.3%)	4 (7.1%)	P = 0.11
Stage				
I	15 (19.2%)	42 (50%)	8 (14.3%)	P < 0.0001
II	33 (42.3%)	31 (34.9%)	30 (53.6%)	P = 0.12
III	30 (38.5%)	13 (15.1%)	18 (32.1%)	P = 0.003
IV	0 (0%)	0 (0%)	0 (0%)	

* Chi-square analysis

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 a statistically significantly higher rate of lymph node positivity in small tumors of ≤ 1 cm

in the triple-negative breast cancer, compare to luminal A and luminal B HER2 negative breast cancers (P < 0.006) (Table 4.1.1.3.).

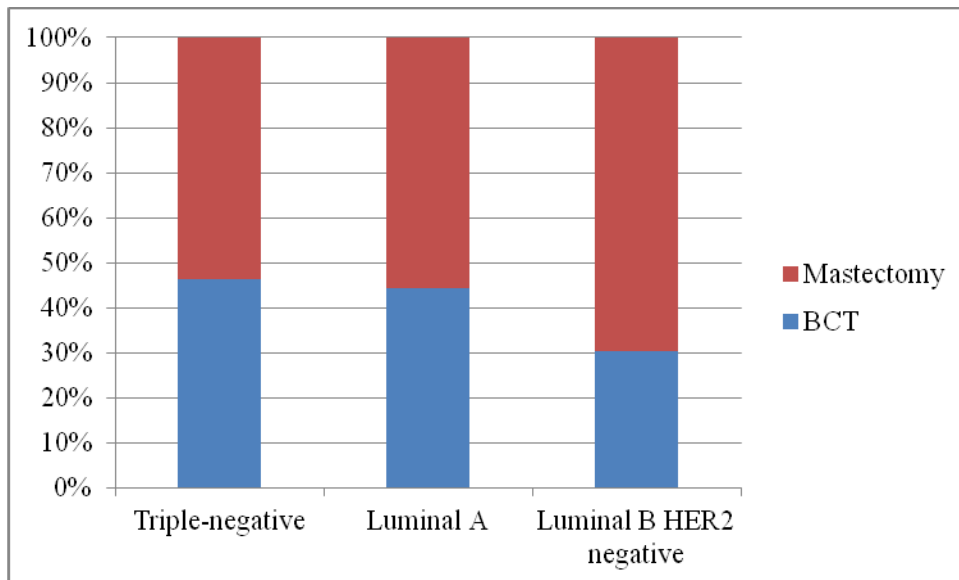
4.1.1.3. Table

Tumor size and lymph node status in the *BRCA1* negative triple-negative, luminal A and luminal B HER2 negative breast cancers

Tumor size	Positive lymph node status, n (%)		
	Triple-negative n=78	Luminal A n=86	Luminal B HER2 negative n=56
≤ 1 cm	2 (66.7%) of 3	0 (0%) of 15	1 (16.7%) of 6
1 to 2 cm	6 (33.3 %) of 18	10 (27%) of 37	2 (40%) of 5
2 to 5 cm	27 (65.9%) of 41	12 (44.4%) of 27	25 (62.5%) of 40
> 5 cm	13 (81.2%) of 16	3 (42.9%) of 7	4 (80%) of 5
P-value	P = 0.168	P < 0.002	P < 0.026

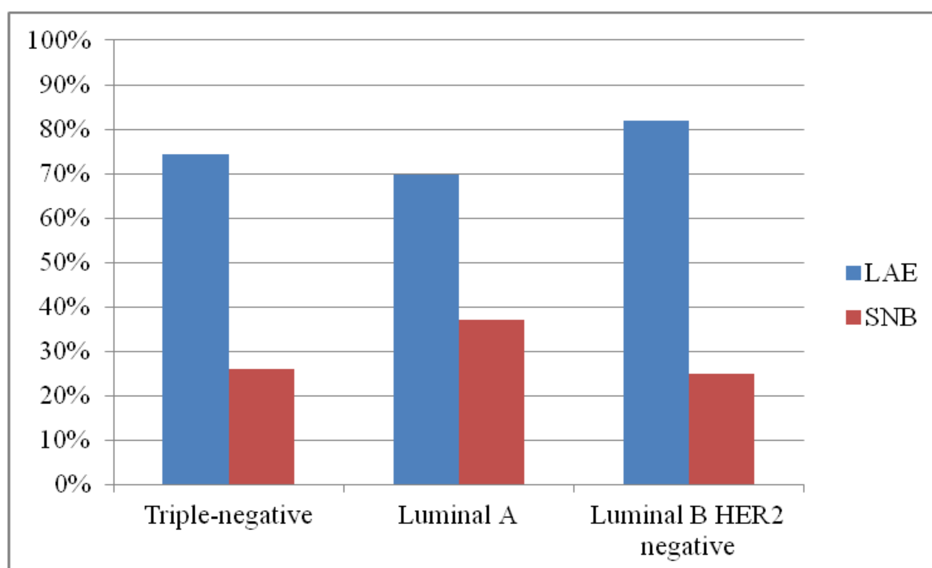
There was no statistically significant difference in performed type of surgery between breast cancer subtypes (P = 0.15) (Figure 4.1.1.4.).

2 of 78 cases (2.6%) in the triple-negative breast cancer group, 2 of 86 cases (2.3%) in the luminal A breast cancer and 1 of 56 cases (1.8%) in the luminal B HER2 negative breast cancer groups had positive margins at initial resection. 3 (60%) of patients underwent breast- conserving therapy and 2 (40%) patients underwent mastectomy. The mean tumor size in patients with positive margins was 2.1 cm. Of these patients 2 (40%) had involved deep margin and 3 (60%) had involved lateral margin. 3 (60%) had an invasive carcinoma at the margins and in other cases carcinoma in situ was detected. 1 (20%) underwent additional surgery-mastectomy, to achieve negative margins. Postoperative radiation was performed for 4 (80%) patients. 4 (80%) patients received adjuvant chemotherapy.



4.1.1.4. Figure. The type of surgery in the *BRCA1* negative triple-negative, luminal A and luminal B HER2 negative breast cancer groups
BCT – breast-conserving therapy

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 ($P < 0.02$). There was a statistically significantly higher proportion of patients, who underwent axillary lymphadenectomy in the luminal B HER2 negative and triple-negative breast cancer groups, compare to luminal A group ($P < 0.02$) (Figure 4.1.1.5.).



4.1.1.5. Figure. The rate of sentinel node biopsy and axillary lymphadenectomy in patients with *BRCA1* negative triple-negative, luminal A and luminal B HER2 negative breast cancers subtypes
SNB – sentinel node biopsy, LAE – lymphadenectomy

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. 5 of 22 (22.7%) patients in the triple-negative breast cancer group achieved a pathologic complete remission (pCR) after neoadjuvant systemic therapy, 11 of 22 (50%) achieved partial remission and 6 of 22 (27.3%) had stable disease. In the luminal A breast cancer group 2 of 3 (66.7%) achieved partial remission after neoadjuvant therapy and 1 (33.3%) patient showed no response to chemotherapy. In the luminal B HER2 negative breast cancer group 2 of 3 (66.7%) achieved partial remission after neoadjuvant therapy and 1 (33.3%) patient had stable disease. The distribution of chemotherapy regimens in the triple-negative, luminal A and luminal B HER2 negative breast cancer groups are summarised in Table 4.1.1.4.

4.1.1.4. Table

The distribution of chemotherapy regimens in the triple-negative, luminal A and luminal B HER2 negative breast cancer groups

Characteristics	Triple negative breast cancer n=78 No. of patients (%)	Luminal A breast cancer n=86 No. of patients (%)	Luminal B HER2 negative breast cancer n=56 No. of patients (%)	P-value*
Chemotherapy**				
Yes	69 (88.5%)	27 (31.4%)	31 (55.4%)	P < 0.0001
Anthracycline-based	47 (68.1%)	17 (63%)	28 (90.3%)	P < 0.03
CMF	6 (8.7%)	8 (29.6%)	1 (3.2%)	P < 0.004
Platine-based	3 (4.35%)	0 (0%)	0 (0%)	P = 0.28
Anthracycline+ taxane	12 (17.4%)	2 (7.4%)	2 (6.5%)	P = 0.20

4.1.1.4. Table (end)

Characteristics	Triple negative breast cancer n=78 No. of patients (%)	Luminal A breast cancer n=86 No. of patients (%)	Luminal B HER2 negative breast cancer n=56 No. of patients (%)	P-value*
Chemotherapy**				
Unknown chemotherapy regimen	1 (1.45%)	0 (0%)	0 (0%)	
No	9 (11.5%)	59 (68.6%)	25 (44.6%)	P < 0.0001
Neoadjuvant chemotherapy				P < 0.0001
Yes	22 (28.2%)	3 (3.5%)	3 (5.4%)	
No	56 (71.8%)	83 (96.5%)	53 (94.6%)	
Anthracycline-based	13 (59.1%)	3 (100%)	3 (100%)	P = 0.16
Anthracycline+ taxane	9 (40.9%)	0 (0%)	0 (0%)	
Adjuvant chemotherapy				P < 0.0001
Yes	57 (73.1%)	27 (31.4%)	31 (55.4%)	
No	21 (26.9%)	59 (68.6%)	25 (44.6%)	
Anthracycline-based	40 (70.2%)	18 (66.7%)	28 (90.3%)	P < 0.01
CMF	6 (10.5%)	8 (29.6%)	1 (3.2%)	P < 0.008
Platinum-based	3 (5.3%)	0 (0%)	0	P = 0.21
Anthracycline-taxane	8 (14%)	1 (3.7%)	2 (6.5%)	P = 0.25

* Chi-square analysis
** In total
CMF, cyclophosphamide, methotrexate, 5-fluorouracil

None of the patients in the triple-negative group received adjuvant endocrine therapy. There was no statistically significant difference in received endocrine therapy

regimens between luminal A and luminal B HER2 negative breast cancer groups. The distribution of endocrine therapy regimens in the luminal A and luminal B HER2 negative breast cancer groups are summarised in Table 4.1.1.5.

4.1.2. Estimates of survival outcomes in the 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). 1 (33.3%) patient in the triple-negative group had an isolated local recurrence and 2 (66.7%) patients experienced distant recurrence after local recurrence. All patients in the luminal A group experienced an isolated local recurrence, without distant recurrence during the follow-up period. 2 (66.7%) patients with local recurrences in the triple-negative group underwent mastectomy and 1 (33.3%) patient underwent breast-conserving surgery. 2 (66.7%) patients with local recurrences in the triple-negative group received radiation therapy, 1 (33.3%) patient received neoadjuvant chemotherapy and all patients received adjuvant chemotherapy. All patients who experienced LRR in the luminal A group underwent breast- conserving surgery followed by radiation therapy and 1 (50%) patient received adjuvant chemotherapy. 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, 6–85 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 (Table 4.1.2.1.).

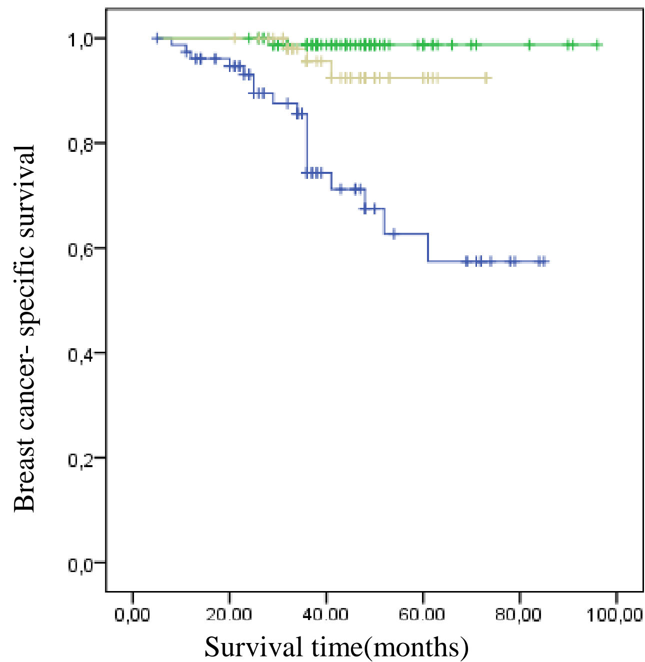
4.1.2.1. Table

Estimates of sites and incidence of distant recurrences in triple-negative, luminal A and luminal B HER2 negative breast cancer groups

Site of distant recurrence	Triple-negative n=22 (28.2%) No. of patients (%)	Luminal A n=1 (1.2%) No. of patients (%)	Luminal B HER2 negative n=3 (5.4%) No. of patients (%)	P-value*
Lung	9 (40.9%)	0 (0%)	0 (0%)	P = 0.30
Bone	8 (36.4%)	1 (100%)	2 (50%)	P = 0.30
Liver	4 (18.2%)	0 (0%)	2 (50%)	P = 0.15
Brain	4 (18.2%)	0 (0%)	0 (0%)	P = 0.65
Other nodal groups	4 (18.2%)	0 (0%)	0 (0%)	P = 0.65

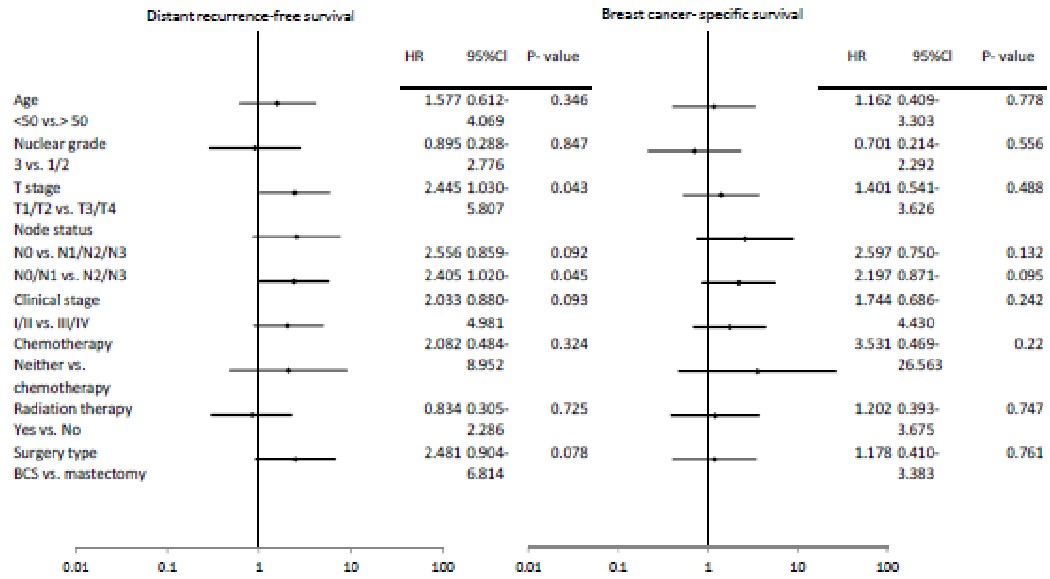
* Chi-square analysis

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 4.1.2.2.).



4.1.2.2. 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

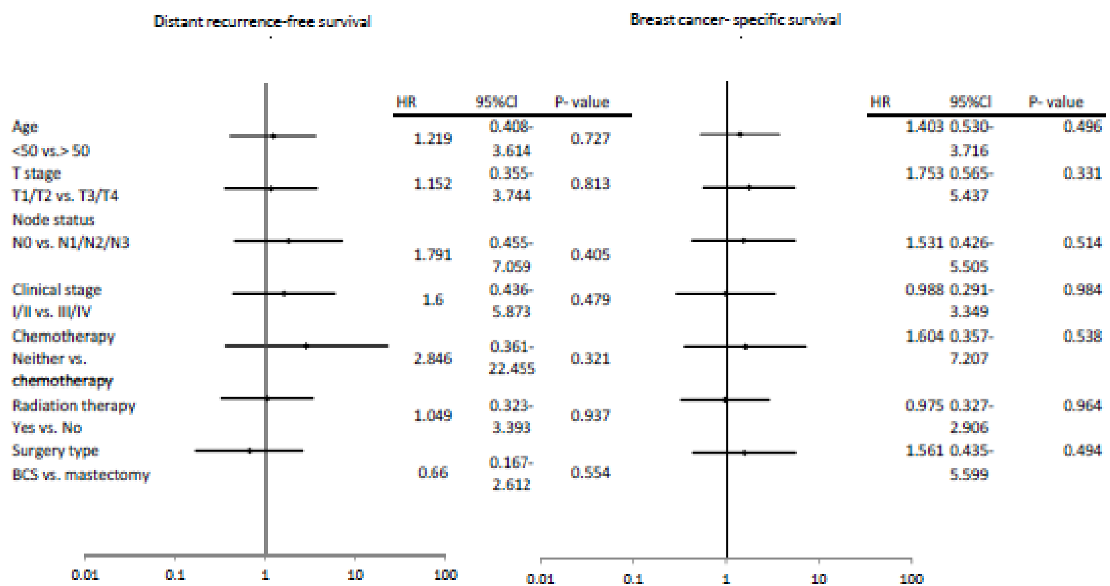
In the univariate analyses, clinical T stage 3 and 4 (HR = 2.445; 95%Cl : 1.030–5.807; P < 0.043) and positive lymph node status (HR = 2.405; 95%Cl : 1.020–5.670; P < 0.045) was associated with a higher risk of distant recurrence, no statistically significant effect of evaluated risk factors on breast cancer-specific survival was found (Figure 4.1.2.3.).



4.1.2.3. 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 4.1.2.4.).



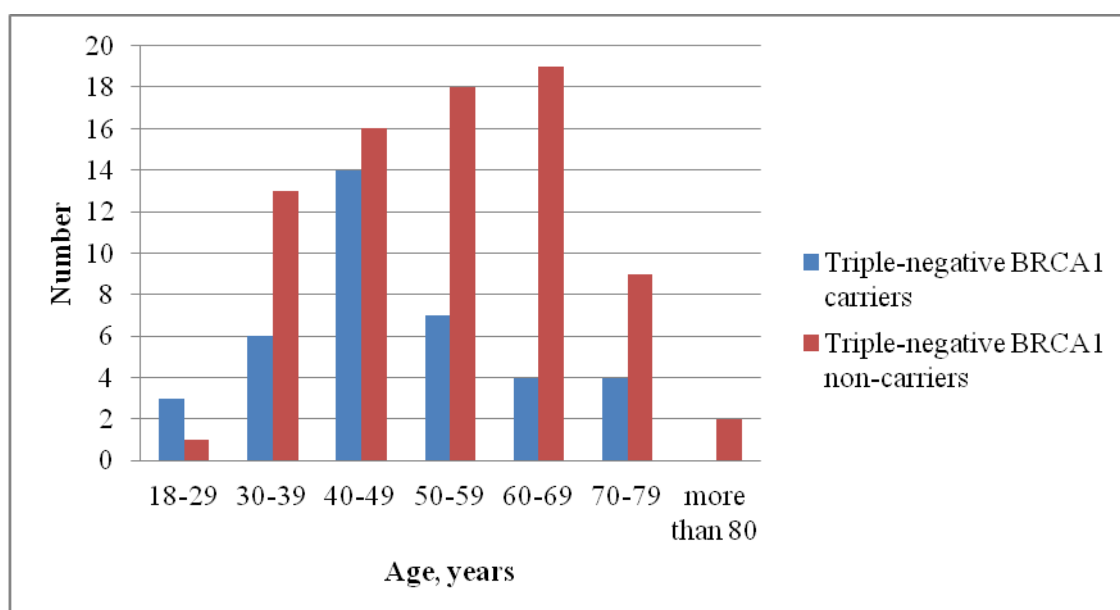
4.1.2.4. Figure. Multivariate Cox proportional hazards model for distant recurrence- free survival and breast cancer-specific survival

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

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

4.2.1. 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 (range, 27–75) years compared to 54.3 (range, 31–82) years in the triple-negative *BRCA1* mutation negative group. Triple-negative *BRCA1* mutation carriers were statistically significantly younger at diagnosis than non-carriers (P < 0.034). A statistically significantly higher proportion of patients in the triple-negative *BRCA1*-carriers group were diagnosed before age 50 compared to triple-negative *BRCA1*-non-carriers group (31 (39.8%) patients versus 23 (60.5%) patients, respectively; P < 0.039). Triple-negative *BRCA1*-carriers were more likely to be diagnosed before age 60 (30 (79%) patients versus 48 (61.5%) patients, respectively), but the difference did not reach statistical significance (P = 0.06) (Figure 4.2.1.1.). Interestingly, that 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 = 0.95) and younger than 60 years (43.5 years versus 46 years, respectively; P = 0.22).



4.2.1.1. Figure. The age distribution in the triple-negative *BRCA1* mutation carriers and *BRCA1* mutation non-carriers

Histopathological features of triple-negative *BRCA1* carriers and non-carriers are shown in Table 4.2.1.1. 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. No significant difference was found between triple-negative *BRCA1*-carriers and non-carriers in percentage of cases of medullary carcinoma. 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)

4.2.1.1.Table

The distribution of histological subtypes, tumor differentiation grade in the triple-negative *BRCA1* mutation carriers and non-carriers

Characteristics	Triple-negative *BRCA1* mutation carriers n=38 No. of patients (%)	Triple-negative *BRCA1* mutation non-carriers n=78 No. of patients (%)	P-value*
Histology			
Ductal carcinoma	26 (68.4%)	58 (74.4%)	P = 0.51
Lobular carcinoma	0 (0%)	12 (15.4%)	P < 0.006
Medullary carcinoma	5 (13.2%)	4 (5.1%)	P = 0.16
Tumor grade			P = 0.54
Well-differentiated	0 (0%)	0 (0%)	
Moderately differentiated	7 (26.9%)	12 (20.7%)	
Poorly differentiated	19 (73.1%)	49 (83.6%)	

* Chi-square analysis

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) (Table 4.2.1.2.)

4.2.1.2. Table

The differences T stage, nodal status and stage of the disease between triple-negative *BRCA1* mutation carriers and non-carriers

Characteristics	Triple-negative *BRCA1* mutation carriers n=38 No. of patients (%)	Triple-negative *BRCA1* mutation non-carriers n=78 No. of patients (%)	P-value*
T stage			
T1	7 (18.4%)	21 (26.9%)	P = 0.33
T2	24 (63.2%)	38 (48.7%)	P = 0.15
T3	3 (7.9%)	12 (15.4%)	P = 0.28
T4	4 (10.5%)	7 (18.4%)	P = 0.78
Nodal status			
N0	25 (65.8%)	29 (37.2%)	P < 0.004
N1	5 (13.2%)	23 (29.5%)	P = 0.11
N2	5 (13.2%)	15 (19.2%)	P = 0.25
N3	3 (7.9%)	8 (10.2%)	P = 0.88
Ki-67	59.8%	52.2%	P = 0.27
Stage			
I	7 (18.4%)	15 (19.2%)	P = 0.93
II	21 (55.3%)	33 (42.3%)	P = 0.19
III	8 (21%)	30 (38.5%)	P = 0.063
IV	1 (2.6%)	0 (0%)	P = 0.33

* Chi-square analysis

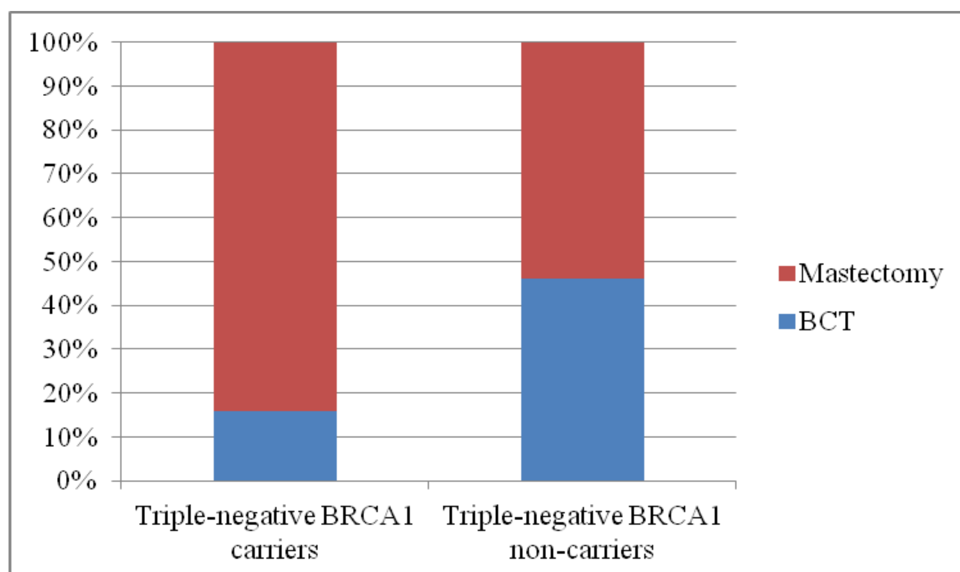
There was no statistically significant correlation between tumor size and positive lymph node status among patients with tumors of < 5 cm both in the triple-negative *BRCA1* positive (P = 0.079) and *BRCA1* negative groups (P = 0.17) (Table 4.2.1.3.).

4.2.1.3. Table

Tumor size and lymph node status in the triple-negative breast cancer *BRCA1* mutation carriers and non-carriers

Tumor size	Positive lymph node status, n (%)	
	Triple-negative *BRCA1* non-carriers n=78	Triple-negative *BRCA1* carriers n=38
≤ 1 cm	2 (66.7%) of 3	0 (0%) of 0
1 to 2 cm	6 (33.3 %) of 18	1 (11.1%) of 9
2 to 5 cm	27 (65.9%) of 40	10 (41.7%) of 24
> 5 cm	13 (81.2%) of 16	3 (60%) of 5
P- value	P = 0.17	P = 0.079

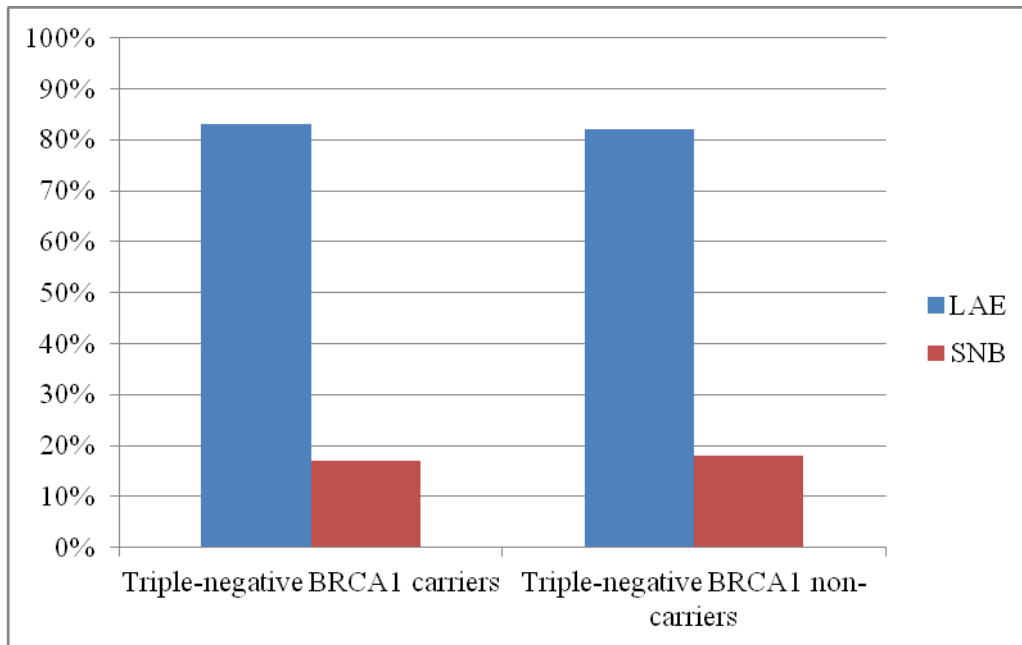
A higher proportion of triple-negative *BRCA1* mutation carriers compared to *BRCA1* mutation non-carriers underwent mastectomy (32 (84.2%) versus 42 (53.9%), respectively; P < 0.001) (Figure 4.2.1.2.).



4.2.1.2. Figure. The type of surgery in the triple-negative *BRCA1* mutation carriers and non-carriers

BCT-breast-conseving therapy

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 (Figure 4.2.1.3.).



4.2.1.3. Figure. The rate of sentinel node biopsy and axillary lymphadenectomy in patients with triple-negative *BRCA1* mutation positive and *BRCA1* mutation negative
SNB – sentinel node biopsy, LAE – lymphadenectomy

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) (Table 4.2.1.4.). 4 (44.4%) patients in the triple-negative *BRCA1* mutation positive group achieved a pCR compared to 5 (22.7%) patients in the triple-negative *BRCA1* mutation negative groupafter neoadjuvant chemotherapy(P = 0.27). 4 (44.4%) patients in the triple-negative *BRCA1* mutation positive group achieved a partial remission compared to 11 (50%) patients in the triple-negative *BRCA1* mutation negative groupafter neoadjuvant chemotherapy (P = 0.79). 1 (11.2%) patients in the triple-negative *BRCA1* mutation positive group had a stable disease compared to 6 (27.3%) patients in the triple-negative *BRCA1* mutation negative groupafter neoadjuvant chemotherapy (P = 0.39).

4.2.1.4. Table

The distribution of chemotherapy regimens in the triple-negative *BRCA1* carriers and non-carriers

Characteristics	Triple-negative *BRCA1* mutation carriers n=38 No. of patients (%)	Triple-negative *BRCA1* mutation non-carriers n=78 No. of patients (%)	P-value*
Chemotherapy**			
Yes	36 (94.7%)	69 (88.5%)	P = 0.3
Anthracycline-based	18 (50%)	45 (57.7%)	P = 0.14
CMF	4 (11.1%)	6 (7.7%)	P = 0.69
Platinum-based	4 (11.1%)	3 (3.8%)	P = 0.22
Anthracycline+taxane	8 (22.2%)	12 (15.4%)	P = 0.55
Unknown chemotherapy regimen	2 (5.6%)	3 (3.9%)	P = 0.78
No	2 (5.3%)	9 (11.5%)	P = 0.56
Neoadjuvant chemotherapy			P = 0.62
Yes	9 (23.7%)	22 (28.2%)	
No	29 (76.3%)	56 (71.8%)	
Anthracycline-based	3 (33.3%)	13 (59.1%)	P = 0.22
Anthracycline+taxane	4 (44.5%)	9 (40.9%)	P = 0.86
Platinum-based	2 (22.2%)	0 (0%)	P = 0.08
Adjuvant Chemotherapy			
Adjuvant Chemotherapy			
Yes	33 (86.9%)	57 (73.1%)	P = 0.098
No	5 (13.1%)	21 (26.9%)	
Anthracycline-based	20 (60.6%)	40 (70.2%)	P = 0.37

4.2.1.4. Table (end)

Characteristics	Triple-negative *BRCA1* mutation carriers n=38 No. of patients (%)	Triple-negative *BRCA1* mutation non-carriers n=78 No. of patients (%)	P-value*
Anthracycline+taxane	6 (18.2%)	8 (14%)	P = 0.61
CMF	4 (12.1%)	6 (10.5%)	P = 0.81
Platine-based	3 (9.1%)	3 (5.3%)	P = 0.51

* Chi-square analysis
** In total
CMF, cyclophosphamide, methotrexate, 5-fluorouracil

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).

4.2.2. 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). 2 patients with LRR in the *BRCA1* mutation non- carriers group underwent mastectomy and 1 patient underwent breast- conserving surgery, and in the *BRCA1* mutation group1 patient with LRR in the right axillary lymphnodes underwent breast- conserving surgery. 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). The DRFS was 32.2 months (range, 6–85 months) in the *BRCA1* mutation non-carriers group and 39 months (range, 9–85 months) in the *BRCA1* mutation carriers

group. The most common site of metastatic spread in the *BRCA1* mutation non- carriers was lung (40.9%), bone, liver, brain and other nodal groups. In the *BRCA1* mutation carriers group the first site of metastatic spread was lung (50%), followed by bone, liver and brain. There was no statistically significant difference between the two groups in incidence of sites of distant recurrence (Table 4.2.2.1.).

4.2.2.1. Table

Estimates of sites and incidence of distant recurrences in *BRCA1* mutation carriers and non-carriers

Site of distant recurrence	*BRCA1* non-carriers n= 22 No. of patients (%)	*BRCA1* carriers n= 4 No. of patients (%)	P-value
Lung	9 (40.9%)	2 (50%)	P = 0.76
Bone	8 (36.4%)	1 (25%)	P = 0.72
Liver	4 (18.2%)	1 (25%)	P = 0.75
Brain	4 (18.2%)	1 (25%)	P = 0.75
Other nodal groups	4 (18.2%)	0 (0%)	P = 0.49

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 4.2.2.1.). 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).

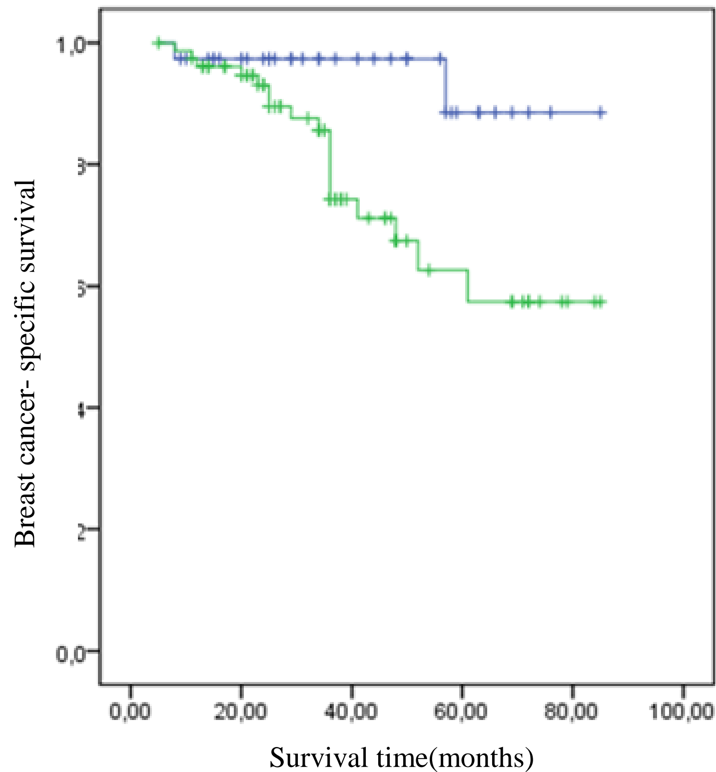
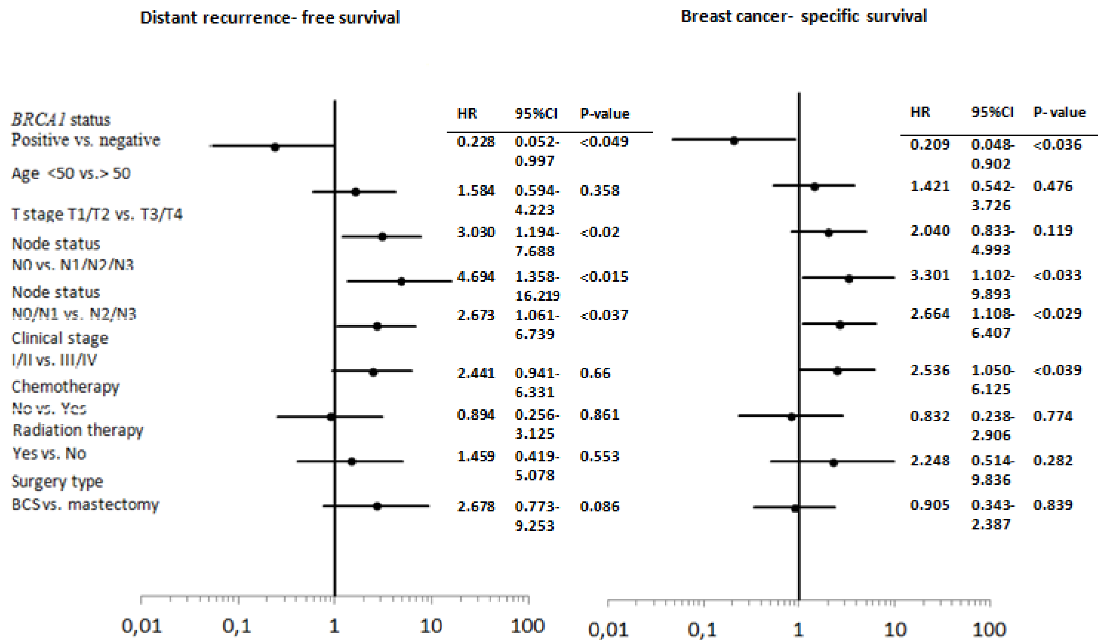


Figure 4.2.2.1. Survival curves of triple-negative *BRCA1* mutation carriers (blue line) and triple-negative *BRCA1* mutation non-carriers (green line). P < 0.02

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 4.2.2.2.).

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 4.2.2.3.).

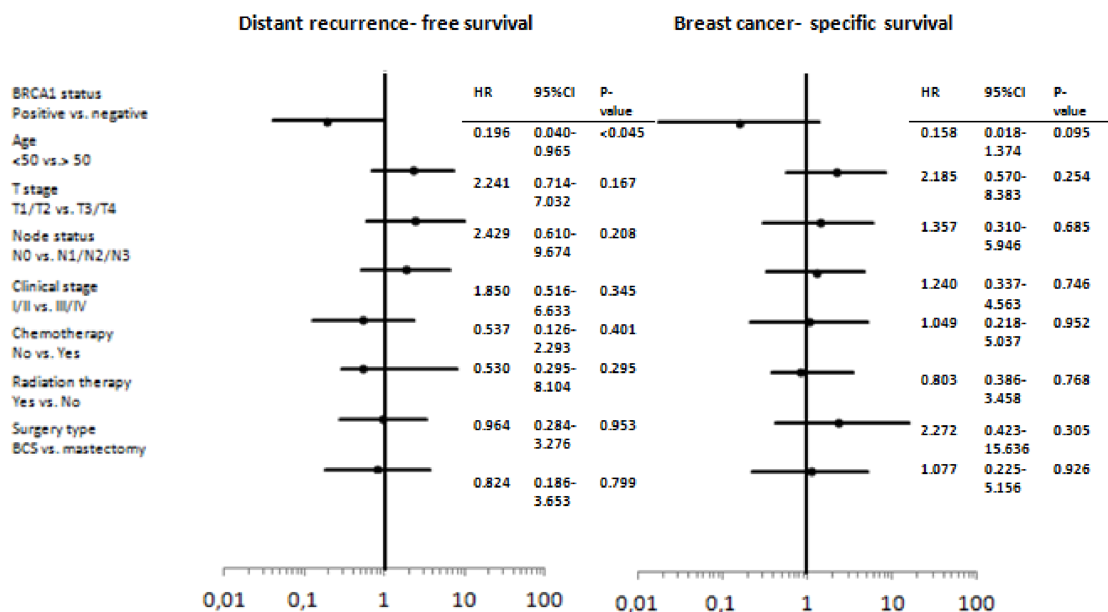


4.2.2.2. Univariate Cox proportional hazards model for distant recurrence-free survival and breast cancer-specific survival

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 4.2.2.2.).

In the multivariate analysis Cox proportional hazards model no statistically significant effect of evaluated risk factors on breast cancer-specific survival was found (Figure 4.2.2.3.).



4.2.2.3. 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

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

4.3.1. Clinicopathological and treatment characteristics of triple-negative breast cancer

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 (Supplement Table 1.). There was no statistically significant difference between triple negative *BRCA1* germline mutations non-carriers and carriers in relation to age at diagnosis, the type of histology, tumor grade, ki-67 status, tumor size, lymph node status, stage of the disease, the type of surgery, received chemotherapy regimens and radiation therapy.
TP53 sporadic mutations were found in 26 (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).

Detailed information about the clinicopathological characteristics of the triple-negative *BRCA1* non-carriers and carriers in a retrospective series are presented in the Table 4.3.1.1.

4.3.1.1.Table

The clinicopathological characteristics of the triple-negative breast cancer group

Characteristics	Triple-negative *BRCA1* non-carriers n=55 No. of patients (%)	Triple-negative *BRCA1* carriers n=11 No. of patients (%)	A total n=66 No. of patients (%)
Median age (years)	53.7 (range, 28–80)	49.4 (range, 39–72)	52.7 (range, 28–80)
Histology			
Ductal carcinoma	41 (74.5%)	10 (90.9%)	51 (77.3%)
Lobular carcinoma	9 (16.4%)	0 (0%)	9 (13.6%)
Medullary carcinoma	4 (7.3%)	0 (0%)	4 (6.1%)
Tumor grade			
Well-differentiated	0 (0%)	0 (0%)	0 (0%)
Moderately differentiated	4 (9.8%)	3 (27.3%)	7 (13.7%)
Poorly differentiated	37 (90.2%)	7 (63.6%)	44 (86.3%)
T stage			
T1	14 (25.4%)	1 (9.1%)	15 (22.7%)
T2	28 (50.9%)	8 (72.7%)	36 (54.5%)
T3	11 (20%)	1 (9.1%)	12 (18.2%)
T4	2 (3.6%)	1 (9.1%)	3 (4.6%)
Nodal status			
N0	24 (43.6%)	7 (63.6%)	31 (47%)
N1	14 (25.5%)	1 (9.1%)	15 (22.7%)
N2	10 (18.2%)	3 (27.3%)	13 (19.7%)

4.3.1.1.Table (continued)

Characteristics	Triple-negative *BRCA1* non-carriers n=55 No. of patients (%)	Triple-negative *BRCA1* carriers n=11 No. of patients (%)	A total n=66 No. of patients (%)
N3	7 (12.7%)	0 (0%)	7 (10.6%)
Stage			
I	10 (18.2%)	1 (9.1%)	11 (16.7%)
II	26 (47.3%)	7 (63.6%)	33 (50%)
III	19 (34.5%)	2 (18.2%)	21 (31.8%)
IV	0 (0%)	1 (9.1%)	1 (1.5%)
Surgery			
Breast-conserving therapy	27 (49.1%)	1 (9.1%)	30 (45.4%)
Mastectomy	28 (50.9%)	10 (90.9%)	36 (54.5%)
Chemotherapy			
Yes	43 (78.2%)	10 (90.9%)	53 (80.3%)
Anthracycline- based	37 (86%)	6 (60)	43 (81.1%)
CMF	0 (0%)	2 (20%)	2 (3.8%)
Anthracycline+taxane	3 (7%)	1 (10%)	4 (7.5%)
Platinum-based	2 (4.7%)	1 (10%)	3 (5.7%)
Unknown chemotherapy regimen	1 (2.3%)	0 (0%)	1 (1.9%)
No	12 (21.8%)	1 (9.1%)	13 (19.7%)
Radiation therapy			
Yes	45 (81.8%)	9 (81.8%)	54 (81.8%)
No	10 (18.2%)	2 (18.2%)	12 (18.2%)
Ki-67	56.5%	63.7%	57.3%

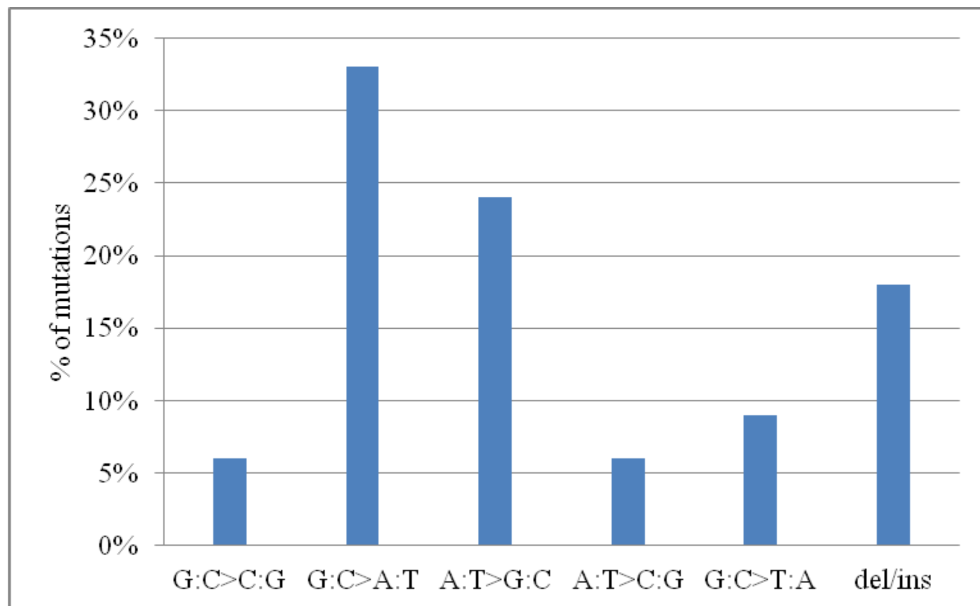
Characteristics	Triple-negative *BRCA1* non-carriers n=55 No. of patients (%)	Triple-negative *BRCA1* carriers n=11 No. of patients (%)	A total n=66 No. of patients (%)
TP53 mutant	22 (40%)	4 (36.4%)	26 (39.4%)
TP53 wild-type	33 (60%)	7 (63.6%)	40 (60.6%)

* Chi-square analysis

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

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 4.3.2.1. 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. 1 triple-negative *BRCA1* carrier and two non-carriers had a two *TP53* sporadic mutations simultaneously. 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. Detailed information about the types and location of *TP53* mutations in the triple-negative *BRCA1* carriers and non- carriers are summarised in Table 4.3.2.1.



4.3.2.1. Figure. The types of the *TP53* sporadic mutations in the triple-negative *BRCA1*carriers/non-carriers group

Del / ins – deletions / insertions

4.3.2.1. Table

The analysis of *TP53* sporadic mutations in the triple-negative *BRCA1* germline positive and negative breast tumors

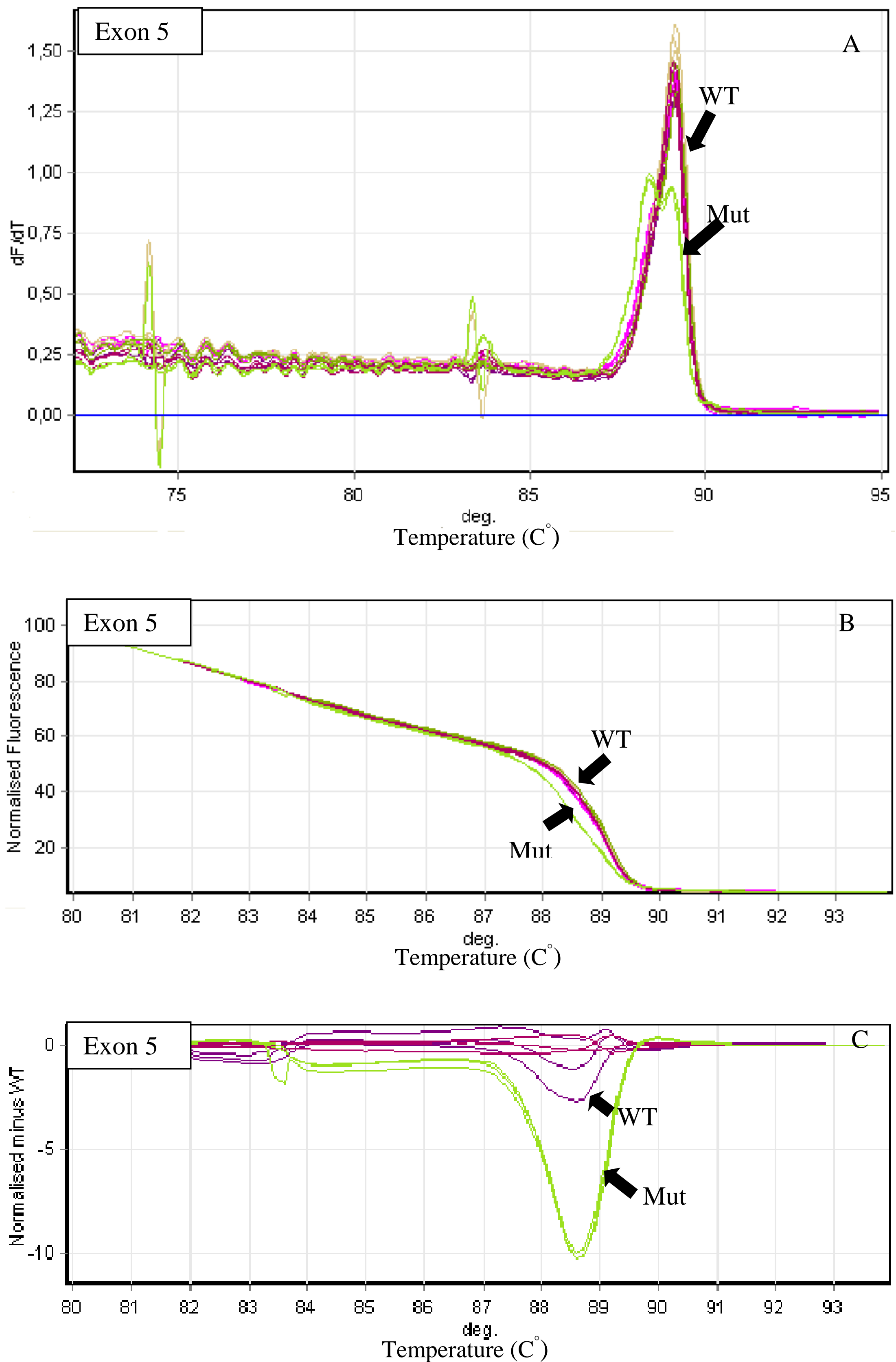
Triple-negative *BRCA1*positive	Mutation (cDNA sequence)	Protein description	Exon number	Effect of the mutation on protein sequence	Type of the mutation	IARC TP53 Database/Cosmic	Effect on protein function
TNB1-44	c.733G>A	p.G245S	Exon 7	Missense	Transition	Yes/Yes	Deleterious
	c.824G>A	p.C275Y	Exon 8	Missense	Transition	Yes/Yes	Deleterious
TNB1-49	c.770T>C	p.L257P	Exon 7	Missense	Transition	Yes/Yes	Deleterious
TNB1-53	c.844C>G	p.R282G	Exon 8	Missense	Transversion	Yes/Yes	Deleterious
TNB1-58	c.916C>T	p.R306X	Exon 8	Nonsense	Transition	Yes/Yes	Deleterious
Triple-negative *BRCA1* negative							
TN-1	c.804C>T	p.N268N	Exon 8	Silent	Transition	Yes/Yes	Neutral
TN-8	c.701A>G	p.Y234C	Exon 7	Missense	Transition	Yes/Yes	Deleterious
TN-9	c.746G>A	p.R249K	Exon 7	Missense	Transition	Yes/Yes	Deleterious
TN-10	c.844C>T	p.R282W	Exon 8	Missense	Transition	Yes/Yes	Deleterious
TN-12	c.476C>A	p.A159D	Exon 5	Missense	Transversion	Yes/Yes	Deleterious
TN-16	c.424delC C	Unknown	Exon 5	Frameshift	del	Absent/Absent	Deleterious
	c.608T>C	p.V803A	Exon 6	Missense	Transition	Yes/Yes	Neutral
	c.907A>C	p.S303R	Exon 8	Missense	Transversion	Yes/Yes	Deleterious
TN-20	c.639A>G	p.R213R	Exon 6	Silent	Transversion	Yes/Yes	Neutral
TN-29	c.818G>A	p.R273H	Exon 8	Missense	Transition	Yes/Yes	Deleterious
TN-31	c.723delC	p.C242fs*5	Exon 7	Frameshift	del	Absent/Yes	Deleterious
TN-35	c.655C>A	p.P219T	Exon 6	Missense	Transversion	Yes/Yes	Deleterious
	c.827C>T	p.A276V	Exon 8	Missense	Transition	Yes/Yes	Deleterious

4.3.2.1. Table (end)

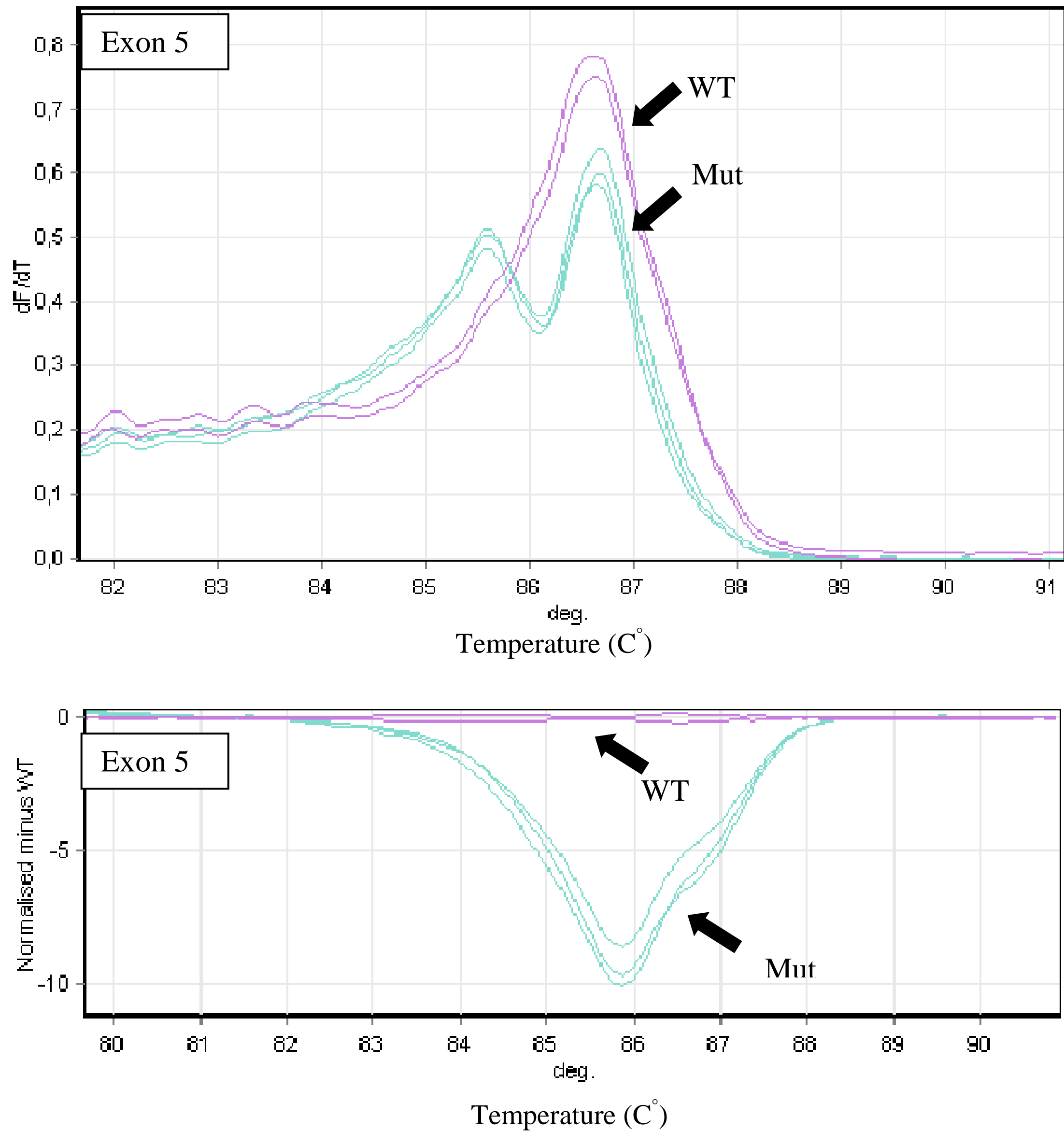
Triple-negative *BRCA1*positive	Mutation (cDNA sequence)	Protein description	Exon number	Effect of the mutation on protein sequence	Type of the mutation	IARC TP53 Database/Cosmic	Effect on protein function
TN-39	c.630C>T	p.N210N	Exon 6	Silent	Transition	Yes/Yes	Neutral
	c.864delT	Unknown	Exon 8	Frameshift	del	Absent/Absent	Deleterious
TN-41	c.510insTAG	Unknown	Exon 5	In- frame	ins	Absent/Absent	Deleterious
	c.853G>A	p.E285K	Exon 8	Missense	Transition	Yes/Yes	Deleterious
TN-42	c.856G>A	p.E286K	Exon 8	Missense	Transition	Yes/Yes	Deleterious
TN-43	c.844C>G	p.R282G	Exon 8	Missense	Transversion	Yes/Yes	Deleterious
TN-47	c.885T>C	p.P295P	Exon 8	Silent	Transition	Yes/Yes	Silent
TN-55	-	-	Exon 5	Unknown	del*	-	Deleterious
	c.747G>A	p.R249R	Exon 7	Silent	Transition	Yes/Yes	Neutral
TN-59	c.916C>T	p.R306*	Exon 8	Nonsense	Transition	Yes/Yes	Deleterious
TN-60	c.431A>C	p.Q144P	Exon 5	Missense	Transversion	Yes/Yes	Neutral
TN-61	c.824G>T	p.C275F	Exon 8	Missense	Transversion	Yes/Yes	Deleterious
TN-64	c.639A>G	p.R213R	Exon 6	Silent	Transition	Yes/Yes	Neutral
TN-68	c.722C>T	p.R241F	Exon 7	Missense	Transition	Yes/Yes	Deleterious
TN-69	c.446delC	-	Exon 5	Frameshift	del	Absent/Absent	Deleterious

del – deletion; ins – insertion; IARC TP53 – International agency of research on cancer; COSMIC – catalogue of somatic mutations in cancer

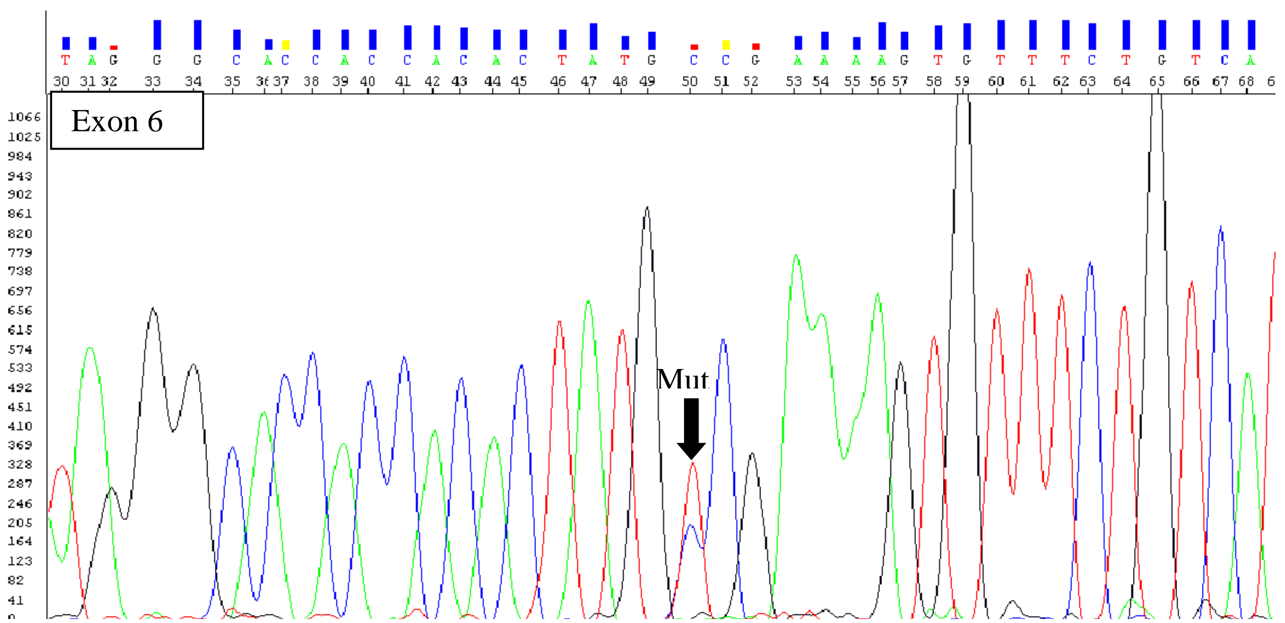
* Not interpretable



4.3.2.2. Figure. The melting plots: A. Derivate plots of wild-type (WT) samples (TN-4, 8, 29; TNB1-5) and positive control (Mut); B. Normalised plots of wild-type(WT) samples (TN-4, 8, 29; TNB1-5) and positive control (Mut); C. Difference plots of wild-type (WT) samples (TN-4, 8, 29; TNB1-5) and positive control (Mut)



4.3.2.3. Figure. The melting plots: A. Derivate plots of mutated (Mut) *TP53* sample (TN-71) and wild- type(WT) control sample; B. Difference plots of mutated (Mut) *TP53* sample (TN-71) and wild-type (WT) control sample



4.3.2.4. Figure. Sequence traces of *TP53* mutation (c.639A>G) in exon *6* (TN-64) sample

4.3.3. 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 ducta l (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 4.3.3.1.)

4.3.3.1. 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%)	P = 0.95
T2	13 (50%)	23 (57.5%)	P = 0.56

Characteristics	Triple-negative *TP53* positive n=26 No. of patients (%)	Triple-negative *TP53* negative n=40 No. of patients (%)	P-value*
T3	6 (23.1%)	6 (15%)	P = 0.43
T4	1 (3.8%)	2 (5%)	P = 0.88
Nodal status			
N0	13 (50%)	18 (45%)	P = 0.73
N1	6 (23.1%)	9 (22.5%)	P = 0.95
N2	2 (7.7%)	11 (27.5%)	P = 0.052
N3	5 (19.2%)	2 (5%)	P = 0.09
Stage			
I	6 (23.1%)	5 (12.5%)	P = 0.29
II	12 (46.1%)	21 (52.5%)	P = 0.63
III	8 (13.8%)	13 (32.5%)	P = 0.89
IV	0 (0%)	1 (2.5%)	P = 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 (Table 4.3.3.2.). 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).

4.3.3.2. Table

The distribution of chemotherapy regimens in 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*
Chemotherapy			
Yes	19 (73.1%)	34 (85%)	P = 0.25
Anthracycline-based	16 (84.2%)	27 (79.4%)	P = 0.7
Anthracycline+ taxane	2 (10.5%)	2 (5.9%)	P = 0.58
CMF	0 (0%)	2 (5.9%)	P = 0.41
Platinum- based	1 (5.3%)	2 (5.9%)	P = 0.97
Unknown chemotherapy regimen	0 (0%)	1 (2.9%)	P = 0.65
No	7 (26.9%)	6 (15%)	P = 0.25

* Chi-square analysis

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

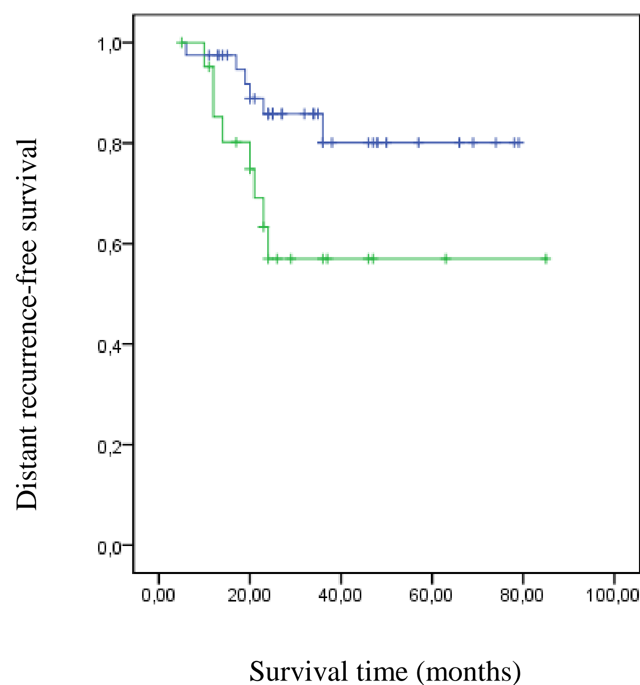
There was no significant difference in the LRR rate between triple-negative *TP53* positive and negative group (1 (3.9%) versus 2 (5%), respectively; P = 0.87). The LRFS in the triple-negative *TP53* mutations positive group was 5 months compared to 10.7 months (range, 4–20 months) in the *TP53* mutations negative group.

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 were 2 (22.2%) bone recurrences in the triple-negative *TP53* mutations positive group compared to 2 (15.4%)

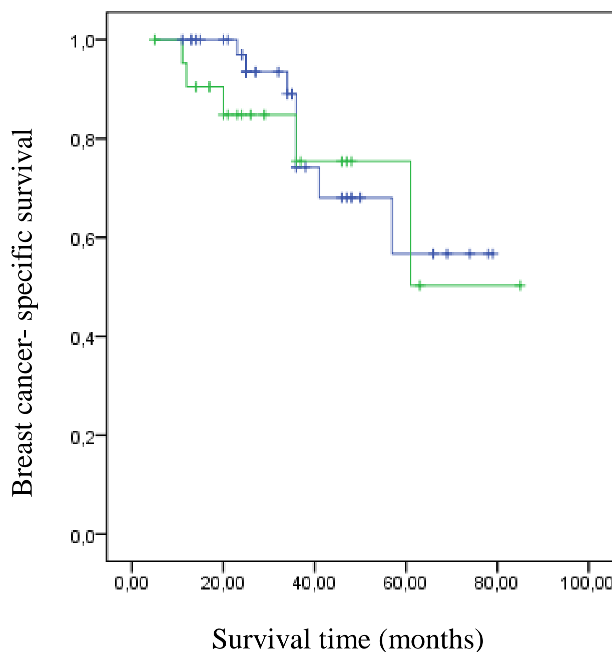
bone recurrences in the *TP53* mutations negative group and 9 (77.8%) visceral recurrences in the *TP53* mutations positive group compared to 11 (84.6%) visceral recurrences in the *TP53* mutations negative group.

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 4.3.4.1.). *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 4.3.4.2.).



4.3.4.1. 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



4.3.4.2. 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

TP53 deleterious mutations positive status showed no statistically significant impact on breast cancer-specific survival (P = 0.84) and distant recurrence-free survival (P = 0.80) in the group of triple-negative breast cancer patients under 50 years at diagnosis and in the group of triple-negative breast cancer patients older than 50 years at diagnosis (P = 0.80 versus P = 0.35, respectively). No correlation was found between TP53 deleterious mutations positive status and distant recurrence-free survival (P = 0.86) and breast cancer-specific survival (P = 0.15) in the triple-negative lymph node negative and lymph node positive groups (P = 0.51 and P = 0.60, respectively).

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).

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).

5. DISCUSSION

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

5.1.1. The age at diagnosis

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]. The *Bauer et al.*, reported that approximately 63% of triple-negative breast cancer patients are younger than 60 years [*Bauer et al.*, 2007], this results are similar to our study where 61.5% of patients are younger than 60 years at diagnosis. *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 and there was no statistically significant correlation between age under < 50 years with the histological type (P = 0.96), poor differentiation of the tumor (P = 0.56), advanced T stage (P = 0.87), positive nodal status(P = 0.15), stage of the disease (P = 0.54), the type of surgery (P = 0.17) and likelihood of getting chemotherapy (P = 0.29) or radiation therapy (P = 0.51).

5.1.2. The histological features of triple-negative, luminal A, luminal B HER2 negative breast cancer

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*., 2006]. There was no statistically significant difference in the frequency of ductal and lobular breast cancer subtypes between triple-negative, luminal A and luminal B breast cancer groups. Number of studies showed that medullary breast carcinoma are strongly associated with triple-negative / basal-like breast cancer subtype [*Jaquemier et al*., 2005; *Bertucci et al*., 2006; *Rodriquez- Pinilla 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 [*Vu-Nishino et al*., 2005; *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-67 expression 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 [*Haffty et al*., 2006; *Rakha et al*., 2006; *Dent et al*., 2007; *Bauer et al*., 2007; *Tischkowitz et al*., 2007; *Onitilo et al*., 2010]. *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.

5.1.3. The tumor size, T stage, lymph node status and correlation between tumor size and lymph node status

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 [*Rakha et al*., 2006; *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]. *Onitilo et al*., also reported statistically significant difference in the proportion of theT stage between triple-negative and other breast cancer groups [*Onitilo et al*., 2009]. 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*., 2006; *Haffty et al*., 2006]. 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*., 2008; *Dent al*., 2009; *Foulkes et al*., 2012]. Study by *Foulkes et al*., analysed correlation between increasing tumor size and number of positive lymph nodes in the 1.324 primary invasive breast cancer cases (1.324 (87.1%) non-basal-like and 196 (12.9%) basal-like breast cancer cases). According to study results there were positive correlation between increasing tumor size ($P<0.001$) and number of metastatic lymph nodes in the non-basal-like breast cancer group and no correlation in the basal-like breast cancer group ($P = 0.58$). The increasing size of the tumor was related to worse breast cancer-specific survival in the non-basal-like breast cancer, but failed to predict worse breast cancer-specific survival in the basal-like breast cancer group. Author is speculating that relationship between larger tumor size, positive lymph node status and worse survival is breast cancer subtype-dependent and is disrupted in the basal-like breast cancer subtype (defined by expression of basal cytokeratins or / and EGFR), but not in the triple-negative breast cancer group [*Foulkes et al*., 2009; *Foulkes et al*., 2012]. Based on that, our data may indicate, that our cohort of triple-negative breast cancer was highly enriched in basal-

like breast tumors. 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.

5.1.4. The surgical treatment and the prognostic role of type of surgery in the triple-negative breast cancer group

In our study there was no statistically significant difference in performed type of surgery between triple-negative, luminal A and luminal B HER2 negative breast cancer groups ($P = 0.15$). In the triple-negative breast cancer group 36 (46%) patients underwent breast- conserving therapy and 42 (54%) patients underwent mastectomy. Similar results were published by *Wiechmann et al.*, there in the total group of 7.906 patients with primary breast cancer who were treated in the Memorial Sloan-Kettering Cancer Center between 1998 and 2007 59% of patients underwent mastectomy and 41% of patients underwent breast-conserving surgery [*Wiechmann et al.*, 2009]. Patients in the breast-conseving treatment group compared to mastectomy group tend to be

younger (52 versus 56.6 years, respectively; P = 0.097), but these difference didn't achieve stastistical significance. Triple-negative breast cancer patients, who underwent breast-conserving surgery compared to mastectomy were statistically significantly associated with smaller tumor size (23 mm versus 42.8 mm, respectively; P < 0.003), T1/T2 stage (34 versus 26 cases, respectively; P < 0.0006). Triple-negative breast cancer patients in the breast-conserving group compared to mastectomy group were more likely to receive radiation therapy (35 versus 28 cases, respectively; P < 0.008). Triple-negative breast cancer patients in the mastectomy group were more likely to have multicentric/multifocal breast cancers compared to breast-conserving group (7 versus 1 patient, respectively; P < 0.04). Regardless of imbalance between breast-conserving and mastectomy groups, the type of surgery (breast-conserving therapy or mastectomy) showed no statistically significant impact on distant recurrence-free and breast cancer-specific survival in the univariate analysis. After adjusment for age, T stage, node status, clinical stage, chemotherapy and radiation therapy the type of surgery showed no statistically significant impact on distant recurrence-free and breast cancer-specific survival in the multivariate analysis in the triple-negative breast cancer group. However, there was no statistically significant difference between two groups in received chemotherapy (31 triple-negative breast cancer patients in the breast-conserving group versus 38 triple-negative breast cancer patients in the mastectomy group; P = 0.57). The similar findings with our study was published by *Parker et al*. A total of 220 triple-negative breast cancer patients' cases were retrospectively analysed. 61(30%) of patients underwent breast-conserving surgery and 141 (70%) of patients underwent mastectomy. In this study, the mastectomy group had a more advanced T stage (T3/T4 stage: 4% of cases in the breast-conserving surgery group versus 27% of cases in the mastectomy group; P < 0.0002), nodal disease (N2/3: 8% of cases in the breast-conserving surgery versus 25% of cases in the mastectomy group; P < 0.0003) and stage of disease (stage III: 8% of cases in the breast-conserving surgery versus 35% of cases in the mastectomy group; P < 0.0001). Therefore, the 5-year overall survival, in this study, was better for the breast-conserving group than for the mastectomy group (89% versus 69%; P < 0.018). However, there was no statistically significant impact of type of surgical treatment on disease-free and overall survival in the multivariate analysis [*Parker et al*., 2010].

5.1.5. The response to chemotherapy in the triple-negative, luminal A and luminal B HER2 negative *BRCA1* negative breast cancer groups

Because of the lack of targeted therapy a conventional chemotherapy remains the backbone of triple-negative breast cancer systemic treatment. A significantly higher proportion of patients in the triple-negative breast cancer group received chemotherapy compared to luminal A and luminal B HER2 negative breast cancer groups. According to the international guidelines chemotherapy should be considered for the triple-negative breast cancer [*NCCN*; *Goldhirsch et al*., 2011; *Aebi et al*., 2010]. Furthermore, the NCCN (National Comrehensive Cancer Network) guidelines recommend to consider chemotherapy for pT1b/c N0 triple-negative breast cancers and for the pT1a pNmic/1 [*NCCN*]. Several studies reported a higher risk of recurrence in pT1 N0 triple-negative breast cancer patients compared to luminal A breast cancer patients [*Kaplan et al*., 2009; *Cancello et al*., 2011]. In our study statistically significantly higher proportion of triple-negative breast cancer patients with pT1b/c pN0/+ (18 (85.7%) versus 7 (13.5%) versus 4 (36.4%), respectively; $P < 0.0001$) and pT2N0/+ (32 (84%) versus 10 (43.5%) versus 19 (52.8%), respectively; $P < 0.005$) received chemotherapy compared to luminal A and luminal B HER2 negative breast cancer groups. These results are in agreement with international guidelines [*NCCN*; *Goldhirsch et al*., 2011; *Aebi et al*., 2010] and study published by *Kaplan et al*., there 65% of T1b and 73% T1c women with triple-negative breast cancer received chemotherapy [*Kaplan et al*., 2009]. In our study a significantly higher proportion of triple-negative breast cancer patients received neoadjuvant chemotherapy compared to luminal A and luminal B HER2 negative breast cancer subtypes (22 (28.2%) versus 3 (3.5%) versus 3 (5.4%)), respectively; $P<0.0001$). This can be explained by significantly higher percentage of advanced T stage, positive lymph node status and advanced stage of the disease in the triple-negative breast cancer group. In addition, equal rates of breast-conserving therapy in the triple-negative and luminal A breast cancer groups were achieved due to higher proportion of neoadjuvant treatment in the triple-negative breast cancer subgroup compared to luminal A breast cancer subgroup.The most commonly used chemotherapy regimens in all breast cancer subtypes were anthracycline-based, anthracycline+taxane-based and CMF. These results are in agreement with international guidelines [*NCCN*; *Goldhirsch et al*., 2011; *Aebi et al*., 2010]. A higher proportion of triple-negative breast cancer patients in both the adjuvant and neoadjuvant settings received anthracycline-based chemotherapy

compared to other regimens. *Carey et al.*, reported a higher chemosensitivity to anthracycline-based neoadjuvant chemotherapy in the triple-negative breast cancer group with higher pathologic complete response rate (pCR) compared to luminal A breast cancer group (27% versus 7%, respectively; $P < 0.01$). Triple-negative breast cancer patients who achieved pCR had a favourable prognosis and those with residual disease experienced higher recurrence rate and had a worse overall survival [*Carey et al.*, 2007]. In our study 13 (59.1%) triple-negative breast cancer patients received athracycline-based neoadjuvant chemotherapy. 3 (23.1%) triple-negative breast cancer patients achieved a pCR after neoadjuvant anthracycline-based chemotherapy and experienced no breast cancer-related events in the median follow-up period of 20.3 months (range, 14–21 months). There were 5 (50%) distant recurrences and 3 (30%) deaths in the group of triple-negative breast cancer patients with residual disease after neoadjuvant anthracycline-based chemotherapy in the median follow-up period of 36 months (range, 17–61 months). This result are in agreement with several previous reports [*Carey et al.*, 2007; *Liedtke et al.*, 2008]. 9 (40.9%) triple-negative breast cancer patients in our study received neoadjuvant anthracycline-taxane-based chemotherapy, 2 (22.2%) of whom achieved pCR. There were no breast cancer-related events in the group of patients with pCR in the median follow-up period of 24 months (range, 14–34 months) and 1 distant recurrence with subsequent death in the group of patients with residual disease in the median follow-up period of 32.3 months after anthracycline-taxane-based neoadjuvant chemotherapy. *Liedtke et al.*, reported similar results to our study with pCR rate of 28% in the triple-negative breast cancer group after neoadjuvant anthracycline-taxane-based chemotherapy [*Liedtke et al.*, 2008]. Due to relatively small number of patients our data showed no statistically significant improvement in response rate and survival outcomes after addition of taxanes to athracycline-based neoadjuvant chemotherapy. In contrast, *Rastogi et al.*, showed significantly higher pCR rate after anthracycline-taxane chemotherapy compared to anthracycline-based chemotherapy with superior survival outcomes in those patients who achieved pCR [*Rastogi et al.*, 2008]. Therefore, there is a growing evidence supporting that pCR have a predictive value of long-term favorable outcomes in the triple-negative breast cancer group [*Carey et al.*, 2007; *Liedtke et al.*, 2008; *Rastogi et al.*, 2011; *von Minkowitz et al.*, 2012]. In our study 12 (17.4%) of triple-negative patients received adjuvant athracycline-taxane-based chemotherapy. Number of clinical trials demostrated superior outcomes in the triple-negative breast cancer patients after anthracycline+taxane combination compared

to anthracycline chemotherapy alone in the adjuvant setting [*Laporte et al*., 2009; *Hugh et al* ., 2009; *Martin et al*., 2010]. In our study 6 (8.7%) of patients in the triple-negative breast cancer group received CMF regimen compared to 8 (29.6%) patients in the luminal A group and 1 (3.2%) in the luminal B HER2 negative breast cancer groups (P < 0.004). The previously published data show conflicting results about the effectiveness of CMF regimen in the triple-negative breast cancer group. Retrospective analysis of NCIC-CTC trial showed that adjuvant CMF are superior to anthracycline- based regimens in basal-like breast cancer group[*Cheang, et al*., 2009]. In contrast, *Rocco et al*., reported that adjuvant CMF was inferior to anthracycline plus CMF in the triple-negative breast cancer [*Rocco et al*., 2011]. 3 (5.3%) patients in the triple-negative breast cancer group and no patients in the luminal A and luminal B HER2 negative breast cancer groups received adjuvant platine-based chemotherapy (P = 0.21). Loss of BRCA1 pathway function in triple-negative / basal-like breast cancers sensitizes tumor cells to DNA-damaging agents, such as platinum-based chemotherapy [*Tassone et al*., 2003; *Kennedy et al*., 2004]. A good response to platinum-based regimens in the triple-negative breast cancer group had been proposed in previous studies with conflicting data regarding the impact of survival [*Sirohi et al*., 2008; *Frasci et al*., 2009; *Staudacher et al*., 2011]. In our study, there were no breast cancer-related events in the triple-negative group after platine-based adjuvant chemotherapy in the follow-up period of 46 months (range, 14–78 months). *Silver et al*., reported that good response to cisplatine is associated with BRCA1 promoter methylation (P = 0.04), low BRCA1 mRNA expression (P = 0.03), p53 nonsense or frameshift mutations (P = 0.01). In our study chemotherapy in the triple-negative breast cancer group showed no statistically significant effect on distant recurrenc-free and breast cancer-specific survival in the univariate analysis. However, there was a relatively small number of triple-negative breast cancer patients, who received no chemotherapy (9 (11.5%)). In contrast, *Clarke et al*., in a large metaalysis of 46 randomised trials showed that polychemotherapy versus no chemotherapy in the group of ER-poor breast cancer patients statistically significantly reduces recurrence, breast cancer-specific and overall mortality [*Clarke et al*., 2008].

5.1.6. The survival outcomes in the triple-negative, luminal A and luminal B HER2 negative breast cancer groups

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; *Kyndi et al*., 2008; *Millar et al*., 2009; *Voduc et al* ., 2010; *Arvold et al*., 2011; *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). There was a tendency to isolated LRR in the luminal A group, without subsequent distant recurrence. In contrast, only 1 patient in the triple-negative breast cancer group had an isolated LRR and 2 patients experienced distant recurrence after LRR.

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. Contrary, to our results LRR was a risk factor for subsequent distant recurrence only in the other breast cancer group, but not in the triple-negative 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.

5.2. 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.

Several previous studies are in great contradiction to our results, where no difference or worse survival outcomes between *BRCA1* mutation carriers and non-carriers were reported [*Robson et al.,* 1999; *Stoppa- Lyonnet et al*., 2000; *El-Tamer et al*., 2004; *Kennedy et al*., 2002, *Robson et al*., 2004; *Brekelmans et al*., 2006; *Bonadona et al.*, 2007; *Rennert et al*., 2007; *Moller et al*., 2007; *Hagen et al*., 2009; *Bordeleau et al.*, 2010]. However, *Veronesi et al*., showed a trend toward better prognosis for BRCA mutation carriers compared with wild- type, but this difference didn't reach a statistical significance (HR = 0.9; 95%CI : 0.2–5.3; P = 0.68). In contrast to our study, in both groups the majority of patients were ER and PR positive with tend toward higher ER negativity among *BRCA* mutation carriers compared to wild- type (43% versus 29%, respectively; P = 0.18). Interestingly, that a higher proportion of long-term survivors were in the *BRCA* mutation carriers group. However, in contrast to our study 30 (76.9%) of patients in the *BRCA* carriers group were *BRCA2* mutation positive. According to previously published data *BRCA2* mutation carriers are more likely ER-positive than *BRCA1* mutation carriers and have a similar or slightly better prognosis than sporadic breast cancer patients [*Verhoog et al*., 2000; *Eerola et al*., 2001; *Budroni et al*., 2009; *Dutch study*., 2013]. Similar results to our findings were reported by *Cortesi et al*., where was a statistically significant overall survival advantage in *BRCA1* positive patients compared to *BRCA1* mutation negative and sporadic breast cancer patients (77% versus 77% versus 73%, respectively; p < 0.0001). In addition, similary to our study *Cortesi et al*., showed that protective effect of *BRCA1* positive status was attributable also in the multivariate analysis and was associated with chemotherapy [*Cortesi et al*., 2010]. In contrast to our study, none of these studies evaluated the prognostic significance of *BRCA1* mutation in the context of breast cancer subtypes, histological types, tumor grade, received chemotherapy regimens.

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 [*Thompson et al*., 2002; *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; *King et al.*, 2003; *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; *Thompson et al.*, 2002; *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 % [*Rebbeck et al.*, 2002; *Eisen et al.*, 2005; *Kauff et al.*, 2008; *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 40years (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].

BRCA1-mutated tumours carriers a dysfunctional DNA double-strand break repair mechanism and therefore is thought to be sensitive to DNA- damaging agents and to inhibitors of the poly (ADP-rybosil) – polymerase [*Kennedy et al*., 2004; *Farmer et al*., 2005].

In studies on an experimental cell system *BRCA1*-defective cell lines have shown higher sensitivity to platinum agents compared to *BRCA1* competent cell lines and resistance toathracyclines and taxanes [*Tassone et al*., 2003]. *Quinn et al*.,

demostrated that the presence of *BRCA1* mediates chemotherapy induced- apoptosis and induces a resistance to the DNA-damaging agents and sensitivity to the spindle poisons, such as paclitaxel [*Quinn et al*., 2003].

In the study by *Chappuis et al*., 38 patients (7 *BRCA1* carriers, 4 *BRCA2* carriers and 27 non-carriers) in stage I–III, who received neoadjuvant anthracycline-based chemotherapy were included. 4 patients (2 *BRCA1* and 2 *BRCA2* carriers) in the *BRCA* mutation positive group (44%) achieved pCR (defined as no tumor cells in the breast and axillary lymph nodes) compared to 1 (4%) patient in the *BRCA* mutation negative group (P < 0.009) [*Chappuis et al*., 2002]. In contrast, in our study 3 (33.3%) triple-negative *BRCA1* mutation carriers received neoadjuvant anthracycline-based chemotherapy, of whom no patients achieved pCR, 2 (66.7%) patients achieved a partial pathological remission and 1 (33.3%) patient had a stable disease. There was a statistically insignificant trend towards a better response to the athracycline-based neoadjuvant chemotherapy in the triple-negative *BRCA1* non-carriers compared to *BRCA1* carriers, where 3 (23.1%) patients achieved a pCR in the *BRCA1* non-carriers group compared to 0 (0%) patients in the *BRCA1*-carriers group. Similar to our study *Petit et al*., published an inferior pCR (defined as an absence of invasive cancer in breast and axillary lymoh nodes) rates after neoadjuvant anthracycline-based chemotherapy in the triple-negative *BRCA1* carriers compared to non-carriers (2 of 12 (17%) versus 23 of 55 (42%)) [*Petit et al.,* 2007]. Interestingly, that despite a trend toward inferior response to the neoadjuvant anthracycline chemotherapy in the triple-negative *BRCA1* carriers compared to non-carriers, there were no breast cancer-related events in the *BRCA1* carriers in the median follow-up period of 39 months (range, 14–69 months) compared to 5 (50%) distant recurrences and 3 (30%) deaths in the *BRCA1* non- carriers with residual disease after neoadjuvant anthracycline-based chemotherapy in the median follow-up period of 36 months (range, 17–61 months). In addition, *Foulkes et al*., reported that *BRCA1*-related cancers were more likely to recur early similar to basal-like breast cancers [*Foulkes et al*., 2006].

Arun et al., reported a higher pCR rates in the *BRCA1* carriers compared to non-carriers in the patients after anthracycline and/or taxane-based neoadjuvant chemotherapy (46% versus 22%, respectively; P < 0.001). There was no statistically significant difference in pCR rates between triple-negative *BRCA1* mutation carriers and non-carriers [*Arun et al*., 2011]. This data agree with our study, where 7 (77.8%) of triple-negative *BRCA1* carriers received neoadjuvant anthracycline and/or taxane

chemotherapy, of whom 3 (42.85%) patients achieved pCR, 3 (42.85%) patients achieved partial pathological remission and 1 (14.3%) patient had a stable disease. However, there was a trend for a higher pCR rates in the triple-negative *BRCA1* carriers compared to non-carriers, this difference didn't reach statistical significance (42.3% versus 22.7%, respectively; P = 0.35). In contrast, *Byrski et al.*, reported a worse response to the neoadjuvant docetaxel in combination with doxorubicin in the *BRCA1* carriers compared to non-carriers (P = 0.001) [*Byrski et al.*, 2008]. In addition, *Wysocki et al.*, showed a high frequency of *BRCA1* mutations in the group of metastatic docetaxel- refractory breast cancer patients (5 of 19 (26.3%) patients). Interestingly, that all 5 patients had a triple-negative breast cancer that accounts for 71% of triple-negative breast cancer patients included in the study [*Wysocki et al.*, 2008].

In our study, 6 triple-negative *BRCA1* carriers received platine-based chemotherapy (4 patients in the neoadjuvant setting and 2 patients in the adjuvant setting) compared to 3 patients in the triple-negative *BRCA1* non-carriers (P = 0.22). 1 (50%) triple-negative *BRCA1* carrier achieved a pCR and 1 (50%) *BRCA1* carrier achieved a partial pathological remission. There was no breast cancer-related events in the triple-negative *BRCA1* carriers and non-carriers in stage I–III in the median follow-up period of 18 months (range, 9–25 months) and 46 months (range, 14–78 months), respectively. Similar findings to our study was reported by *Byrski et al.*, where a high rate of pCR (83%) was observed in the *BRCA1* carriers after platinum-based neoadjuvant chemotherapy. There were a lower pCR rates observed in the *BRCA1* carriers treated with CMF and taxane-based chemotherapy (1 (7%) of 14 patients and 2(8%) of 25 patients, respectively) [*Byrski et al.*, 2008]. In the study by *Silver et al.*, where 28 triple-negative breast cancer patients in stage I–III were included 6 (28%) patients achieved pCR, of whom 2 patients were *BRCA1* carriers (100% of *BRCA1* carriers included in the study) [*Silver et al.*, 2010]. An ongoing randomized the *BRCA* trial (NCT00321633, NCT00532727) will clarify the role of platinum-based and taxane-based chemotherapy in the *BRCA* mutation carriers [*Balmana et al.*, 2010].

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

5.3.1.The frequency and spectrum of *TP53* sporadic mutations in the triple-negative breast cancer *BRCA1* carriers and non-carriers

The high frequency (40.6–88%) of *TP53* sporadic mutations or mutated p53 protein have been reported in the previous studies in the triple-negative / basal-like breast cancers [*Sorlie et al.*, 2001; *Langerod et al.*, 2007; *Chae et al.,* 2009; *Lee et al.*, 2011; *Shah et al.*, 2012; *Ryu et al.*, 2012; *Dumay et al.*, 2013]. However, 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, *Elledge et al.*, 1996; *Norberg et al.*, 1998; *Chae et al.*, 2009; *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 [*Lee et al.,* 2011]. 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].

Sorlie et al., reported a 82% (9 of 11) of *TP53* alternations in the basal-like breast cancer group identified by gene expression patterns using cDNA microarrays [*Sorlie et al*., 2001]. In the study by *Holstege et al*., 95%(20 of 21 cases) of the basal-like breast cancers (identified according to gene expression profile) and 90%(19 of 21cases) of *BRCA1*- mutated breast cancers had a *TP53* sporadic mutations. In all basal-like breast cancer samples *TP53* gene exons 2–11 were sequenced [*Holstege et al*., 2009]. Similar results was reported by *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*., 2006; *Rakha et al.,* 2007; *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 the study by *Manie et al*., 51(81%) *TP53* mutations in the basal-like *BRCA1* germline positive and negative breast cancers were detected in the exons 5–8 compared to 12 (19%) *TP53* mutations in the exons 4, 9 and 10 [*Manie et al*., 2009].

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]. In our study we observed a high number of samples that were positive by HRM and negative by sequencing. There were 7 abberant melt profiles detected by HRM in 5a exon, 11 abberant melt profiles detected in 5b exon, 24 abberant melt profiles detected in 6 exons, 43 abberant melt profiles detected in 7 exon and 26 abberant melt profiles detected in 8 exon. The subsequently performed bidirectional direct DNA sequencing confirmed the presense of TP53 sporadic mutations in 4 cases in 5a exon, in 2 cases in 5b exon, in 5 cases in 6 exon, in 7 exon and in 15 cases in 8 exon (Supplement Table 1.). The discordant results between HRM and sequencing is difficult to interpret. First, it could be explained by the low percentage of the *TP53* sporadic mutations positive cancer cells in the sample that made detection of mutation on HRM and DNA sequencing technically difficult [*Taniquchi et al*., 2008; *Do et al*., 2009]. In our study only for 32(48.5%) of 66 samples data about the percentage of cancer cells in the sample were available. In addition, previous studies reported that mutant DNA in dilution down to 5% could be detected using the RT-PCR/HRM [*Krypuy et al*., 2006; *Krypuy et al*., 2007]. In contrast, the proportion of the mutant allele at least 10-20% are detectable by sequencing. When the presense of mutant allele are at lower proportion, it is not reliably discriminable from the sequencing background. Therefore, discrepant results between HRM and sequencing may be associated with different sensitivity of these methods when a low proportion of mutant allele are present in the tissue sample [*Kobelt et al*., 1998; *Do et al*., 2009]. Second, HRM analysis requires a high quality DNA and carefull attention to the details in design of pre-HRM PCR, because the presense of a homogenious melting domain makes melting curve analysis more reliable [*Krupuy et al*., 2007; *Solassol et al*., 2011]. In contrast, in our study we used DNA extracted from FFPE. It has been previously reported that the mutation identification using DNA extracted from FFPE could be quite challenging due to DNA degradation and the presense of sequence artefacts. In our study the DNA integrity was evaluated using 2% agarose gel electrophoresis. The majority of the DNA samples were partially degraded. However, in our study the relative short amplicons were used for PCR (136–245pb). Therefore, the analysis of PCR amplification products using 2% agarose gel electophoresis showed an acceptable quality and quantity of the amplified DNA fragments. The cause of sequence artefacts are poorly understood. However, it may occur due to DNA modifications during tissue fixation and embedding procedures, spontaneous DNA hydrolysis and oxidative

damage, and deamination of cytosine bases [*Lindahl et al*., 1993; *Hofreiter et al*., 2001; *Solassol et al*., 2011; *Do et al.,* 2012].

Finally, the lower frequency of *TP53* sporadic mutations in our study could be explained by the limited sensitivity of both HRM and direct sequencing methods with higher false-negative results. *Solassol et al*., reported a lower rate of *KRAS* mutations among FFPE colorectal cancer tissue samples with higher false-negative results compared to the fresh-frozen samples. When DNA extracted from FFPE specimens were used HRM showed false-negative *KRAS* status in 2 (6%) of 33 cases and direct sequencing showed false- negative status in 6 (18.1%) of 33 cases [*Solassol et al*., 2011].

According to the data published by *Petitjean et al*., approximately 70% of all breast cancer *TP53* mutations reported in the IARC TP53 database are missense point mutations [*Soussi et al*., 2006; *Petitjean et al*., 2007].

In our study in triple-negative *BRCA1* carriers and non-carriers 27 (81.8%) point mutations, 5 (15.2%) deletions and 1 (3%) insertion were detected in the *TP53* gene.

There was no statistically significant difference in the types of *TP53* mutations between triple-negative *BRCA1* carriers and non-carriers. In contrast, *Manie et al*., reported a higher rate of complex (deletions/insertions) mutations in the basal-like *BRCA1* carriers group compared to basal-like non-carriers group (14 (42%) of 33 cases compared to 13 (9%) of 34 cases, respectively) [*Manie et al*,. 2009]. *Holstege et al*., demonstrated that 11 (52%) of 21 *BRCA1* positive and 12 (57%) of 21 *BRCA1* negative basal-like breast tumors harbour complex/truncating mutations (frameshift, splice, nonsense, in-frame insertions/deletions) compared to approximately 3 (7%) of 44 luminal breast cancers [*Holstege et al*., 2010]. In our study there was no complex (deletions/insertions) mutations in the triple-negative *BRCA1* carriers group compared to 6 (22.2%) complex (deletions / insertions) mutations in the non-carriers group (P = 0.27). However, there was 1 (16.7%) complex/truncating(nonsense) mutation in the triple-negative *BRCA1* carriers. *Dumay et al*., reported that 35% of basal-like and apocrine group harbour complex (insertions/deletions) mutations compared to 18% in the luminal breast cancer group (P = 0.02) [*Dumay et al*., 2013]. In contrast, in our study there was a lower rate of complex (insetions/deletions) mutations (18.2%) in the whole triple-negative(*BRCA1* positive/negative) breast cancers group. Similar to *Dumay et al*., published data in our study a high rates of C:G to T:A transitions were demonstrated in the triple-negative *BRCA1* positive/negative breast cancer group

[*Dumay et al*., 2013]. According, to previous reports C:G to T:A mutations are very common endogeneous mutations in human cancers caused by the spontaneous deamination of cytosines or 5-methylcytosines [*Greenblatt et al*., 1994; *Venitt et al*., 1996]. However, *Do et al*., reported that C:G > T:A transitions are the most common sequence artefacts in FFPE DNA that result due to cytosine deamination to uracil [*Do et al*., 2009; *Do et al*., 2012]. However, *Solassol et al*., observed no false-positive mutations in the DNA extracted from FFPE samples using HRM with subsequent direct sequencing [*Solassol et al*., 2011].

Manie et al., demonstrated no difference in the rate of transversions between basal-like *BRCA1* carriers and non-carriers (9 (27%) of 33 compared to 8 (24%) of 34, respectively) [*Manie et al*., 2009]. This agree with our study, where was also no significant difference in the rate of transversions between triple-negative *BRCA1* carriers and non-carriers (1 (16.7%) versus 6 (22.2%), respectively; P = 0.83). In our study, there were 15 (55.6%) transitions in the triple-negative *BRCA1* carriers group. Similar results to our findings was published by *Manie et al*., where in the 23 (68%) of 34 cases transitions were detected. However, in this study a significantly lower proportion of the *BRCA1* positive basal-like tumors harboured transitions compared to *BRCA1* negative basal-like tumors (P = 0.002) [*Manie et al*., 2007]. In contrast, in our study we observed no statistically significant difference in the rate of transitions between two groups (P = 0.66). *Holstege et al*., reported no statistically significant difference in the rate of deleterious missense mutations between basal-like *BRCA1* carriers and non-carriers (11 (52.4%) versus 8 (38.1%), respectively). In contrast, in our study there was an insignificant trend towards a higher rate of *TP53* missense deleterious mutations both in the triple-negative *BRCA1*carriers and non-carriers compared to *Holsetege et al*., results [*Holstege et al*., 2010].

In our study, a significantly higher proportion of *TP53* mutations were detected in 8 exon compared to 7, 6 and 5 exons (15 (45%) in 8 exon compared to 7 (21.2%) in 7 exon, 5 (15%) in 6 exon and 6 (18.2%) in 5 exon; P < 0.0017). All mutations in the triple-negative *BRCA1* carriers group was detected in exons 7 and 8. In contrast, *Manie et al*,. showed that the higher proportion of *BRCA1* positive tumors harboured *TP53* mutations in 5 exon (13 of 33) and a significantly less tumors had a *TP53* mutations in 6 exon (2 of 33). In the sporadic basal-like tumor group there was a 6 (17.6%) of 34 *TP53* mutations detected in 5 exon and 13 (38.2%) of 34 *TP53* mutations datected in 6 exon

[*Manie et al*., 2009]. In contrast, in our study there were 6 (18.2%) *TP53* mutations detected in 5 exon and 4 (12.1%) *TP53* mutations detected in 6 exon.

In our study we identified three non-canonical sporadic *TP53* mutations (c.510 ins TAG in exon5, c.446del C in exon 5 and c.864delT in exon 8) that were not found in the Cosmic and IARC TP53 databases. These results should be interpretted with caution due to multiple studies that reported artifactual nucleotide changes in DNA isolated from FFPE. However, the vast majority of these artifactual changes were C>T/G>A or A>G/T>C transitions [*Marchetti et al*., 2006]. In contrast, in our study all three novel *TP53* sporadic mutations were deletions or insertions. *Solassol et al*., reported discordant nucleotide changes in FFPE samples compared to fresh-frozen samples that did not significantly impact genotype interpretation [*Solassol et al*., 2011].

5.3.2.The prognostic significance of *TP53* sporadic mutations in the triple-negative breast cancer group

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; *Lee 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]. Other studies 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; *Lee et al*., 2011]. 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 [*Ambrogi et al*., 2006; *Soria et al*., 2010; *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.*, where 520 node-positive breast cancer patients treated with anthracycline or anthracycline/taxane-based adjuvant therapy were included. 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].

It is plausible that a number of limitations could have influenced the results obtained in the retrospective phase of the study. First, we failed to obtain retrospectively the parrafin blocks from all 116 triple-negative *BRCA1* positive/ negative brest cancer patients, and some analyses failed technically. Therefore, we loss approximately one-third of our cases. Another possible source of error is that we used the FFPE samples. *Solassol et al.*, demostrated that HRM and direct sequencing are less sensitive and could cause false-negative results than FFPE samples are used, especially, than percentage of tumor cells in the sample is low. In our study only for 32 (48.5%) of 66 samples data about the percentage of cancer cells in the sample were available.

Additionally, we can’t exclude the possibility of sequence artefacts in FFPE DNA [*Do et al.*, 2012]. However, Sollasol et al., reported no false-positive results in a series of 33 FFPE specimens compared to fresh-frozen tissues, but there were

discordant nucleic achanges discovered in 3 of 33 samples caused by conservation process [*Sollasol et al*., 2011].

6. CONCLUSIONS

1. Triple-negative sporadic 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. Triple-negative sporadic 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.

7. 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.

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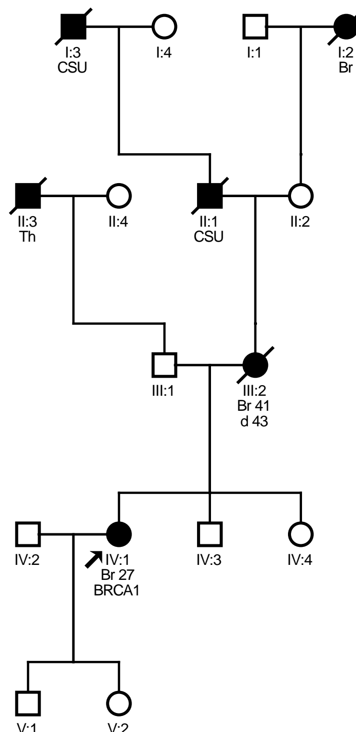
SUPPLEMENT

A case report No.1

A 28-years old woman in 1st trimester of her 3rd pregnancy with a rapidly increasing lump in the right breast presented at the Breast unit in Pauls Stradins Clinical University Hospital in 2008. A 1.5 cm mass was detected on clinical breast examination. Ultrasound examination revealed a suprareolarly located hypodense mass in the right breast measuring 1.3 × 1.2 cm that was interpreted as BIRADS 4 category. A core needle biopsy of the mass revealed an ER/PR negative and HER2 negative medullary breast cancer.

Given the early-onset of her breast cancer and a positive family history (Figure 4.1.1.), the patient was offered a genetic testing for two common founder mutations in *BRCA1* in Latvia. Genomic DNA was extracted from peripheral blood cells. The *BRCA1* (5328insC) mutation was detected using a multiplex-specific polymerase chain reaction (PCR) assay



1.1. Patient's family pedigree. Filled symbols show affected individuals. The proband is individual IV:1 and is noted by an arrow. Slashed symbols denoted deceased individuals. Age at diagnosis is shown beside diagnosis, age at death is shown beside abbreviation "d".

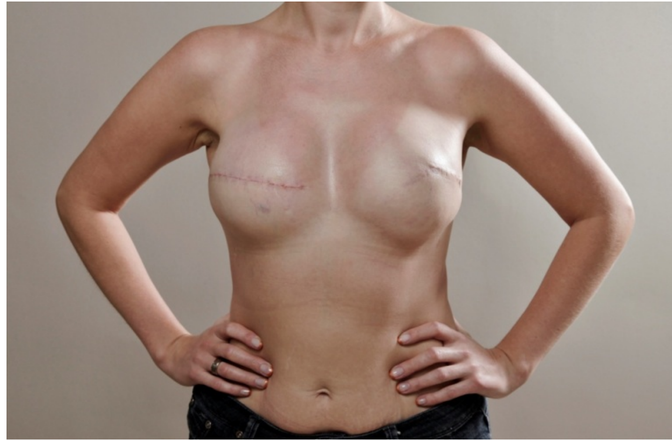
d – death, Br – breast cancer, BRCA1 – *BRCA1* mutation carrier, Th – thyroid cancer, CSU – cancer site unknown

Medical abortion was recommended by medical oncologist at first trimester of pregnancy.

Staging ultrasound of the abdomen and pelvis, chest X-ray and bone scan showed no evidence of distant metastasis (M0).

She underwent a right-sided modified radical mastectomy with sentinel node biopsy and level I axillary lymphadenectomy in 2008. Pathology analysis showed 1.5 cm medullary cancer (pT1) with lymphovascular invasion. There were tumor-free surgical margins (R0). None of eight lymph nodes were involved(pN0). Immunohistochemical analysis revealed ER/PR negative (0%), HER2 negative (1+) medullary cancer with high Ki-67 proliferation index (58%).

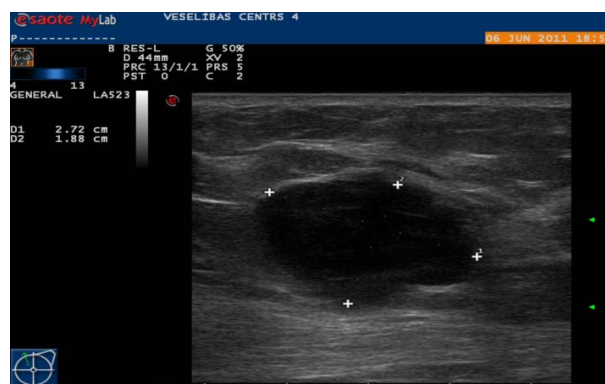
The patient received four cycles of adjuvant cisplatin (250 mg/m2 q3W) and doxorubicin (50 mg/m2 q3W). Patient underwent contralateral risk-reducing mastectomy with sentinel node biopsy and simultaneous two-stage bilateral breast reconstruction in 2009. There was no tumor revealed in a surgical specimen of contralateral breast. The sentinel node (1/0) was free of metastasis by hematoxyllin/eosin staining. Bilateral textured surface expanders were placed in a complete submuscular position at the time of contralateral risk-reducing mastectomy. Expansion was started 3 weeks after insertion and continued every 7–10 days until complete volume was achieved. 7 months later expanders were replaced with textured surface implants. After the operation patient was complaining of pain in her right side and there was a slight displacement of the implant in the left side. 22 months after breast implant placement, bilateral implants were removed, left-sided capsulotomy and bilateral capsulorrphaphy was performed and bilateral textured implants were placed in a complete submuscular position (Figure 1.1.). There was no futher complications after reconstruction. A good symmetry was achieved. Patient was completely satisfied with final aesthetic outcome. Given her high lifetime risk of ovarian cancer and right side ovarian cyst diagnosed, a bilateral laparoscopic adnexectomy was performed in 2011. Surgical specimen was free of ovarian cancer and revealed follicular, luteal ovarian cysts with multiple haemorrhagia. There is no evidence of recurrent disease at 57 months after diagnosis.



1.2. Patient after the right-sided therapeutic mastectomy with sentinel node biopsy and level I lymphadenectomy in 2008 and contralateral risk-reducing mastectomy with two-stage breast reconstruction after mastectomy with complete submuscular tissue implant position in 2009

A case report No. 2

A 28-years old woman was presented with complaints of a lump in the left breast of 1 week duration at the Breast unit in Pauls Stradins Clinical University Hospital in 2008. She was breastfeeding at 2.5 months after her 3rd delivery. The patient's mother had been diagnosed with breast cancer at age of 39 years. Physical examination showed 3–4 cm palpable mass in the left breast and no palpable axillary adenopathy. Ultrasound confirmed the presence of 3.1 cm suspicious mass(BIRADS 4) in the left breast (Figure 2.1.) and no pathologic axillary lymphnodes.

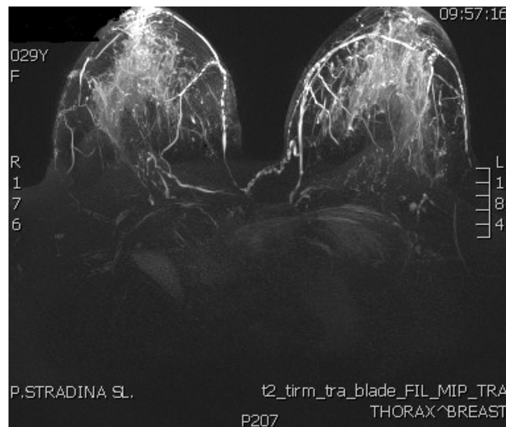


2.1. The ultrasound image of a hypodense lesion in the left breast located in the upper quadrant medial to the nipple measuring 3.1 cm

A core needle biopsy of the breast mass was performed. Pathological examination of the specimen revealed invasive medullary carcinoma with lymphovascular invasion. Immunohistochemical staining showed negative testing for

ER/PR (0%) and HER(0) with high Ki-67 proliferation index (72%). Staging ultrasound of the abdomen and pelvis, chest X-ray and bone scan showed no evidence of distant metastasis (T2N0M0, stage IIA). Given the early-onset of her breast cancer and a positive family history of breast cancer, the patient was offered a genetic testing for two common founder mutations in *BRCA1* in Latvia. Genomic DNA was extracted from peripheral blood cells. The *BRCA1* (5328insC) mutation was detected using a multiplex-specific polymerase chain reaction (PCR) assay. A neoadjuvant chemotherapy was considered at the multidisciplinary meeting. Four cycles of neoadjuvant chemotherapy was administered by cisplatin (150 mg/m^2) and doxorubicin(90 mg/m^2) in 21-day cycles. A rapid reduction of tumor volume was observed already after first cycle of chemotherapy.

The effect of treatment was classified as a complete clinical and radiological remission (Figure 2.2.).



2.2. The breast MR contrast-enhanced image shows no evidence of mass in the left breast

4 weeks later, the patient underwent sentinel lymph node biopsy. Pathological examination revealed negative lymph node of one biopsied. After 1 week a skin-sparing mastectomy with simultaneous breast reconstruction with textured surface expander placed in a complete submuscular position was performed.

Pathological examination of specimen revealed no evidence of invasive cancer and DCIS (complete pathological response-pCR).

2 months later patient underwent contralateral risk-reducing mastectomy with sentinel node biopsy and simultaneous reconstruction with textured surface expander placed in a complete submuscular position. There was no tumor revealed in a surgical specimen of contralateral breast. The sentinel node (1/0) was free of metastasis by hematoxyllin/eosin staining. Expansion was started 3 weeks after insertion and continued every 7–10 days. 7 months later expanders were replaced with textured surface implants. There were no complications in the postoperative period. Patient was satisfied with final aesthetic outcome. In 2012 a bilateral laparoscopic risk-reducing adnexectomy was performed. Surgical specimen was free of ovarian cancer. There is stable disease in the follow-up period of 24 months.

Table 1

Summary of HRM and sequencing results for the triple-negative *BRCA1* carriers and non-carriers

	Exon 5 5a		Exon 5 5b		Exon 6		Exon 7		Exon 8	
Sample ID	HRM results	Sequencing results	HRM results	Sequencing results	HRM results	Sequencing results	HRM results	Sequencing results	HRM results	Sequencing results
Triple-negative *BRCA1* positive										
TNB1-5	wt	-	wt	-	wt	-	wt	-	Mut	wt
TNB1-18	wt	-	Mut	wt	Mut	wt	wt	-	wt	-
TNB1-33	wt	-	wt	-	wt	-	Mut	wt	wt	-
TNB1-44	wt	-	wt	-	Mut	wt	Mut	c.733 G > A	Mut	c.824 G > A
TNB1-49	wt	-	wt	-	wt	-	Mut	c.770 T > C	Mut	-
TNB1-52	wt	-	Mut	wt	wt	-	Mut	wt	wt	-
TNB1-53	wt	-	wt	-	wt	-	Mut	wt	Mut	c.844 C > G
TNB1-54	wt	-	wt	-	Mut	wt	Mut	wt	Mut	wt
TNB1-56	wt	-	wt	-	Mut	wt	Mut	wt	Mut	wt
TNB1-58	wt	-	wt	-	wt	-	wt	-	Mut	c.916 C > T
TNB1-69	wt	-	wt	-	wt	-	Mut	wt	wt	-

Table 1 (continued)

	Exon 5 5a		Exon 5 5b		Exon 6		Exon 7		Exon 8	
Sample ID	HRM results	Sequencing results	HRM results	Sequencing results	HRM results	Sequencing results	HRM results	Sequencing results	HRM results	Sequencing results
Triple-negative *BRCA1* negative										
TN-1	wt	-	wt	-	wt	-	wt	-	Mut	c.804 C > T
TN-3	wt	-	Mut	wt	wt	-	wt	-	Mut	wt
TN-4	wt	-	wt	-	wt	-	Mut	wt	wt	-
TN-6	wt	-	wt	-	wt	-	wt	-	wt	-
TN-7	Mut	wt	wt	-	wt	-	wt	-	wt	-
TN-8	wt	-	wt	-	wt	-	Mut	c.701 A > G	Mut	wt
TN-9	Mut	wt	wt	-	wt	-	Mut	c.746 G > A	Mut	NA
TN-10	wt	-	wt	-	wt	-	wt	-	Mut	c.844 C > T
TN-12	wt	-	Mut	c. G > T	Mut	wt	Mut	wt	wt	-
TN-13	wt	-	wt	-	Mut	wt	Mut	wt	wt	-
TN-14	wt	-	wt	-	Mut	wt	wt	-	wt	-
TN-15	wt	-	wt	-	Mut	wt	wt	-	wt	-
TN-16	Mut	c.424del CC	Mut	NA	Mut	c.608 T > C	wt	-	Mut	wt
TN-17	Mut	-	Mut	-	wt	-	wt	-	wt	-
TN-19	Mut	wt	wt	-	wt	-	wt	-	wt	-
TN-20	wt	-	wt	-	Mut	c.639 A > G	Mut	wt	wt	-
TN-21	wt	-	wt	-	wt	-	wt	-	wt	-
TN-22	wt	-	wt	-	Mut	wt	Mut	wt	wt	-

TN-26	wt	-	wt	-	Mut	wt	Mut	wt	wt	-
TN-27	wt	-	wt	-	Mut	wt	Mut	wt	wt	-
TN-28	wt	-	wt	-	Mut	wt	Mut	wt	wt	-
TN-29	wt	-	wt	-	wt	-	Mut	wt	Mut	c.818 G > A
TN-30	wt	-	Mut	wt	wt	-	Mut	wt	wt	-
TN-31	wt	-	wt	-	wt	-	Mut	c.723delC	wt	-
TN-32	wt	-	wt	-	wt	-	wt	-	Mut	wt
TN-34	wt	-	wt	-	wt	-	Mut	wt	Mut	wt
TN-35	wt	-	wt	-	Mut	c.655 C > A	wt	-	Mut	c.827 C > T
TN-36	wt	-	wt	-	wt	-	wt	-	wt	-
TN-37	wt	-	wt	-	wt	-	wt	-	wt	-
TN-38	wt	-	Mut	wt	Mut	wt	Mut	wt	wt	-
TN-39	wt	-	wt	-	Mut	c.630C>T	Mut	wt	Mut	c.864delT
TN-40	Mut	wt	wt	-	wt	-	wt	-	wt	-
TN-41	wt	-	Mut	c.510insTAG	wt	-	Mut	wt	Mut	c.853 G > A
TN-42	wt	-	wt	-	Mut	wt	Mut	wt	Mut	c.856 G > A
TN-43	wt	-	wt	-	wt	-	wt	-	Mut	c.844 C > G
TN-45	wt	-	wt	-	wt	-	Mut	wt	Mut	wt
TN-46	wt	-	Mut	wt	wt	-	Mut	wt	wt	-
TN-47	Mut	wt	wt	-	wt	-	wt	-	Mut	c.885 T > C
TN-48	wt	-	wt	-	wt	-	Mut	wt	wt	-
TN-50	wt	-	wt	-	Mut	wt	Mut	wt	wt	-
TN-51	wt	-	Mut	wt	Mut	wt	Mut	wt	wt	-
TN-55	Mut	Del*	wt	-	wt	-	Mut	c.747 G > A	wt	-
TN-57	wt	-	wt	-	Mut	wt	wt	-	wt	-

Table 1 (end)

TN-59	wt	-	wt	-	wt	-	Mut	wt	Mut	c.916 C > T
TN-60	Mut	c.431 A > C	wt	-	wt	-	Mut	wt	wt	-
TN-61	Mut	wt	wt	-	wt	-	Mut	wt	Mut	wt
TN-62	wt	-	wt	-	Mut	wt	Mut	wt	wt	-
TN-63	wt	-	wt	-	wt	-	Mut	wt	wt	-
TN-64	wt	-	wt	-	Mut	c.639 A > G	Mut	wt	wt	-
TN-65	Mut	wt	wt	-	wt	-	wt	-	wt	-
TN-66	wt	-	wt	-	wt	-	Mut	wt	wt	-
TN-67	wt	-	wt	-	wt	-	Mut	wt	wt	-
TN-68	wt		wt		wt		Mut	c.722 C > T	Mut	wt
TN-70	wt	-	wt	-	Mut	wt	Mut	wt	wt	-
TN-71	Mut	c.446delC	wt	-	wt	-	Mut	wt	wt	-

Not interpretable

NA- Not amplified